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Caracterização da resposta *in vitro* de fagócitos a micobactérias

Tese de Mestrado
Escola de Ciências

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Assim é círculo da vida... Nada se perde tudo se transforma...

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ABBREVIATIONS

APC Antigen presenting cells

AraLam Arabinosylated lipoarabinomannan

BCG Bacille Calmette-Guérin

CFU Colony forming units

DCs Dendritic cells

DC-SIGN Dendritic cell-Specific intercellular adhesion molecule-3-grabbing non-integrin

DNA Deoxyribonucleic acid

ERK Extracellular signal-regulated kinase

GM-CSF Granulocyte macrophage colony stimulating factor

HIV Human immunodeficiency virus

IFN Interferon

IL Interleukin

IFN-γ Interferon-γ

iNOS inducible NOS

IRF Interferon regulatory factor

KDa Kilodalton

KO Knockout

LAM Lipoarabinomannan

LAMP-1 Lysosomal-associated membrane protein – 1

ManLam Mannose-capped lipoarabinomannan

MAPK Mitogen-activated protein kinase

MDR-TB Multidrug-resistant tuberculosis

MHC Major histocompatibility complex

MyD88 Myeloid differentiation protein 88

NF-kB Nuclear factor-kB

NK Natural killer

NO Nitric oxide

NODs Nucleotide-binding oligomerization domain

NOS2 Nitric oxide synthase 2

ORF Open reading frame

PGLI Phenolic glycolipid I

PIM Phosphatidylinositol mannoside

PRR pattern recognition receptors

R Receptor

RD Region of deletion

RNI Reactive nitrogen intermediates

ROI Reactive oxygen intermediates

TACO Tryptophan-aspartate containing coat protein

TB Tuberculosis

TGF-β Transforming growth factor β

Th T helper

TLRs Toll-like receptors

TNF Tumour necrosis factor

WHO World health organization

ABSTRACT

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the main threats to mankind. Despite the intense research on the immune response to tuberculosis, major questions remain unsolved, one of which relates to the fact that the efficacy of the current vaccine, *Mycobacterium Bovis* BCG, is variable. However, no other experimental vaccines against tuberculosis developed in the last 100 years were proven to be better than BCG. Therefore BCG remains the only vaccine available to date to prevent tuberculosis. A possible explanation for the variability of BCG efficacy relies on the fact that the immune response triggered by BCG and *M. tuberculosis* is different. Nevertheless, *M. tuberculosis*-based experimental vaccines tend not to protect better than BCG. We therefore hypothesized that the basic protective response triggered by BCG must be appropriate, although it needs to be improved in order to confer higher and longer lasting protection.

In this work, we performed a comparative study of *M. tuberculosis versus* BCG infection on dendritic cells (DCs) and macrophages to better understand how these pathogens interact with cells of the innate immune system and how that might translate into an effective, or not, T helper (Th) cell response. We found that macrophages and DCs respond differently to *M. tuberculosis* or BCG stimulation in what concerns cytokine expression. The expression of IL-12p40, TNF and IL-6 is significantly higher in DCs than in macrophages stimulated with either mycobacteria. Nevertheless, the expression of IL-12p35 and IL-10 was similar in both cell types. We also found that the same cell type respond differently to *M. tuberculosis* or BCG. *M. tuberculosis*-stimulated DCs induced higher levels of p40 and p19 monomers and of the bioactive cytokines IL-12 and IL-23, respectively. BCG-stimulated DCs produced higher amounts of TNF. The amounts of IL-6 and IL-10 secreted upon stimulation of DCs with *M. tuberculosis* or BCG were similar. The differential expression of IL-12 and IL-23 might be correlated in part to a higher activation of the MAP kinase ERK1/2 in DCs in response to *M. tuberculosis* than to BCG. Although macrophages were in general poorly induced to produce cytokines, when compared to DCs, the threshold of ERK1/2 activation was higher in stimulated macrophages. A consequence of the differential activation of DCs was reflected on the distinct type of Th responses developed when *M. tuberculosis*- or BCG-infected DCs presented OVA peptide to TCR-transgenic $CD4^+$ T cells. *M. tuberculosis*-infected DCs were able to induce the development of both Th1 and Th17 responses, whereas BCG-infected DCs presented a shift towards Th17 responses. These differences are of interest considering the importance of Th1/Th17 balance during vaccination. Further understanding the molecular mechanisms dictating this differential Th response will be used for the development of new vaccines.

RESUMO

A tuberculose é causada pelo *Mycobacterium tuberculosis* e constitui um grave problema a nível mundial. Apesar de todo o esforço dedicado ao estudo desta infecção, questões como a variabilidade da eficácia da corrente vacina em uso, *M. bovis* BCG, permanecem inexplicáveis. No entanto, nos últimos 100 anos, não foi experimentalmente desenhada outra vacina mais eficiente do que o BCG. Sendo assim, a única vacina disponível até ao momento para combater a tuberculose é o BCG. Uma possivel explicação para a variabilidade da vacina consiste no facto de o *M. tuberculosis* e o *M. bovis* BCG desencadearem uma resposta imunológica diferente.Contudo, vacinas experimentais baseadas no *M. tuberculosis* não conferem maior protecção do que o BCG. A nossa hipótese, é que a resposta desencadeada pelo BCG deve ser adequada, embora necessite de ser melhorada de modo a conferir uma protecção maior e mais prolongada.

Neste trabalho foram comparadas as infecções por *M. tuberculosis versus* BCG em células dendríticas (DCs) e em macrófagos, com os objectivos de compreender melhor a interacção entre estes agentes patogénicos e o sistema imunológico inato, e como é que isso se traduz, ou não, numa reposta T de ajuda (Th) eficaz. Os nossos resultados mostram que as respostas induzidas pelos macrófagos e pelas DCs estimulados por *M. tuberculosis* ou por BCG, em termos de expressão de citocinas, são diferentes. A expressão de IL-12p40, TNF e IL-6 induzida por ambas as micobactérias foi significativamente maior em DCs do que em macrófagos. Contudo, a expressão de IL-12p35 e IL-10 foi semelhante em ambos os tipos celulares. Observamos, também, que o mesmo tipo celular responde de modo diferente ao *M. tuberculosis* ou ao BCG. As DCs estimuladas pelo *M. tuberculosis* induziram níveis elevados de p40 e p19, bem como das respectivas citocinas bioactivas IL-12 e IL-23. As DCs estimuladas pelo BCG produziram elevados níveis de TNF. A produção de IL-6 e IL-10 foi semelhante quer nas DCs estimuladas pelo *M. tuberculosis* quer pelo BCG. A expressão diferencial de IL-12 e de IL-23 poderá estar correlacionada, em parte, com uma maior activação da MAP cinase ERK1/2 pelas DCs em resposta ao *M. tuberculosis* relactivamente ao BCG. Embora a indução de citocinas pelos macrófagos fosse menor do que pelas DCs, o *threshold* de activação do ERK1/2 induzido pelos macrófagos foi maior. A diferente activação induzida pelo *M. tuberculosis* ou BCG em DCs reflectiu-se no tipo de resposta Th diferenciada. Em DCs estimuladas com *M. tuberculosis* ou BCG, que apresentam o péptido OVA a células T $CD4^+$ cujo TCR é transgénico, observamos que as DCs estimuladas com *M. tuberculosis* induziram o desenvolvimento de respostas Th1 e Th17, enquanto que DCs estimuladas com BCG induziram uma resposta Th17 superior à observada com *M. tuberculosis.* Considerando a importância do balanço Th1/Th17 durante a

vaccinação, as diferenças observadas são de extrema relevância, dado que a compreensão dos mecanismos moleculares que ditam esta resposta diferencial consiste numa estratégia para o desenvolvimento de novas vacinas contra a tuberculose.

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I. INTRODUCTION

1.1. EPIDEMIOLOGY

In 1993, the World Health Organization (WHO) declared tuberculosis (TB) a global emergency. Since then, TB remains one of the main threats to mankind. Nowadays, TB is the world's second commonest cause of mortality and morbidity from infectious diseases, despite the improvements in the health care services and all the efforts devoted to the understanding of this disease in the last decades (Corbett et al., 2003).

The WHO has estimated that one-third of the world's population (~2 billion people) is infected with *Mycobacterium tuberculosis*, the etiologic agent of TB. Of these, 1 in 10 people will become sick with active TB in their lifetime. The latest estimates of the global burden of TB show that there were ~9.27 million new cases of TB in 2007 (including ~1.37 million cases among human immunodeficiency virus (HIV)-positive people). There were also approximately 0.5 million new cases of multidrugresistent-TB (MDR-TB), of which around 0.3 million were among people not previously treated for TB. South-East Asia and Western Pacific regions account for 55% of global cases and the African Region for 31%; the other three regions (the Americas, European and Eastern Mediterranean regions) account for small fractions of the global cases. Among the 15 countries with the highest estimated TB incidence rates, 13 are in Africa, a phenomenon linked to high rates of HIV coinfection (WHO, 2009). The estimates of cases and deaths in HIV-positive individuals in 2007, as well as in previous years are substantially higher than those published previously by WHO (1.32 million deaths from TB in HIV-negative people with an additional 0.46 million TB deaths in HIV-positive people) (WHO, 2009).

Regarding the incidence rate of TB in Western Europe, the situation in Portugal is considered one of the most severe (DGS, 2006; EuroTB, 2006). Nearly 3 000 of new cases of all forms of TB were notified in 2007, being 20% of all TB cases in HIV-positive people, and 0.9% of all new cases MDR-TB (Hollo et al., 2009; WHO, 2009).

Collectively, these statistics show that TB remains a major global health problem and that there is an urgent need for more effective control and prophylactic resources to fight TB worldwide.

1.2. PREVENTION AND THERAPY OF TUBERCULOSIS

Mycobacterium bovis BCG is the only vaccine currently available against TB. BCG is an attenuated strain of *M. bovis* (the etiological agent of cattle TB), derived from a virulent strain at the start of the last century, after more than 13 years of continuous *in vitro* passage (Andersen and Doherty, 2005). After almost a century from its discovery and more than 3 billion administrations, BCG is still in use today (Fine, 1995). However, BCG vaccination did not match all the expectations it evoked, because although it prevents disseminated TB in newborns (Colditz et al., 1995; Lanckriet et al., 1995; Murhekar et al., 1995; Trunz et al., 2006; Zodpey et al., 2005; Zodpey et al., 1998) its protection against the most common form of the disease, pulmonary TB in adults, can range anywhere from below zero to over 80% (Fine, 1995; Kaufmann, 2000; Sterne et al., 1998). This difference in protection is not well understood, however some aspects might account for it, such as the interference with the immune response to BCG vaccination by previous exposure to environmental mycobacteria (Brandt et al., 2002; Demangel et al., 2005; Roche et al., 1995); differences in BCG sub-strains (Behr, 2001a, b; Fine, 1995; Fine et al., 1994); deletion of protective antigens from BCG; failure of BCG to stimulate adequate immunity (Aagaard et al., 2009); differences in the route of administration (Skeiky and Sadoff, 2006), age of administration (Skeiky and Sadoff, 2006). Furthermore, the protection afforded by BCG is not life-long lasting, and it is believed that BCG is protective for only 10-20 years, which implies that protection wanes just as the risk of getting pulmonary TB increases (Sterne et al., 1998). The need to develop a new vaccine against TB is urgent and efforts are being made to do so. The most promising strategies for the generation of vaccine candidates are subunit protein vaccines, attenuated live vaccines or the combination of both. While attenuated live vaccines provide prolonged exposure of the host immune system to newly synthesized antigens, the advantage of subunit vaccines is the possibility that their efficacy may not be compromised by exposure to environmental mycobacteria or by prior BCG vaccination (Andersen, 2007; Andersen and Doherty, 2005).

Although it is clear that BCG is limited to confer protection against TB, the search for a better vaccine has so far failed. The efforts underlying the search of a new vaccine against TB should perhaps not only focus on the understanding of *M. tuberculosis* infection, but also on the understanding of BCG immunobiology, the way it activates innate immunity and further induces T cell responses. Additionally, the way that *M. tuberculosis* and BCG interfere with the host immunity should be a target of investigation and an issue to be taken into account in the

development of a new vaccine against TB. Therefore, a comprehensive analysis of the interaction of both *M. tuberculosis* and BCG with the immune system is important and pertinent.

1.3. *Mycobacterium tuberculosis* COMPLEX - *M. tuberculosis* AND *M. bovis* BCG FEATURES

TB, in humans and in animals, results from exposure to bacilli within the *M. tuberculosis* complex (i.e*., Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium pinnipedi, Mycobacterium microti, Mycobacterium caprae,* or *Mycobacterium canettii* (Cousins et al., 2003). Mycobacteria from *M. tuberculosis* complex share more than 99% homology for some loci (Brosch et al., 2002; Mostowy and Behr, 2005; Smith et al., 2006). The *Mycobacterium* genus comprises several bacteria, including non-pathogenic environmental mycobacteria (Dormans et al., 2004; Kremer et al., 1998; Mostrom et al., 2002; van Soolingen et al., 1998); but also virulent mycobacteria, in addition to *M.* tuberculosis, such as *M. leprae* and *M. ulcerans*, the causative agents of leprosy and Buruli ulcer, respectively (Debacker et al., 2004; WHO, 2000). Another pathogenic mycobacteria is *M. avium*, although it only causes disease in immunocompromised individuals, such as HIV-positive patients (Pozniak, 2002; Primm et al., 2004). *M. tuberculosis* and *M. bovis* cause a similar course of infection and pathology in humans (Cosma et al., 2003). Although *M. tuberculosis* has no natural reservoir outside humans, several TB experimental animal models exist (Boshoff and Barry, 2005; Cosma et al., 2003; Flynn, 2006; Kaufmann, 2003; North and Jung, 2004; Young, 2009). In contrast, *M. bovis* has a broad range of natural hosts, from humans to cattle (O'Reilly and Daborn, 1995). Mycobacteria share a characteristic cell wall, composed by mycolic acids, that makes up more than 50% of its dry weight. The lipid content of this cell wall enables the retention of basic dyes in the presence of acid alcohol, a hallmark characteristic of mycobacteria (Brennan, 2003; Cosma et al., 2003; Kaufmann, 2006).

The genome of *M. tuberculosis* has been sequenced and is 4.41 Mb in size. It contains near 4000 protein-coding genes of which 52% have known function (Cole et al., 1998). Only 376 putative proteins share no homology with known proteins and presumably are unique to *M. tuberculosis* (Camus et al., 2002; North and Jung, 2004).

Whole genome DNA microarray techniques have identified 129 *M. tuberculosis* specific open reading frames (ORFs) that are absent in the genome of BCG vaccine strains (Behr et al., 1999). These ORFs are clustered in 16 regions of deletion (RDs). A total of 61 ORFs (clustered in

9 RDs) are missing in all *M. bovis* strains, including BCG, and 29 ORFs are missing in some BCG strains only. There are 39 ORFs (clustered in 3 RDs) that are missing in all BCG strains. Clearly, the *M. tuberculosis* ORFs that are absent in BCG represent candidates not only for virulence factors, but also for protective antigens. For instance, three genomic RDs were identified to be present in virulent *M. bovis*, *M. tuberculosis* and BCG - RD1, RD2 and RD3 (Mahairas et al., 1996). Among these, the RD1 region seems to be most interesting for the specific diagnosis of TB because the genes predicted in this genomic DNA segment are deleted from all the vaccine strains of BCG, while they are conserved in all of the tested virulent laboratory and clinical isolates of *M. bovis* and *M. tuberculosis* (Mahairas et al., 1996). The RD1 region of *M. tuberculosis* encodes two low molecular weight secretory proteins, the 10-kDa culture filtrate protein - CFP-10, and the 6-kDa early-secreted target antigen - ESAT-6, two-major T-cell antigens of *M. tuberculosis* (Arend et al., 2000a; Arend et al., 2000b; Mustafa et al., 1998). Not only the deletion of the RD1 locus attenuates the virulence of *M. bovis*, but also, conversely, re-introduction of the *M. tuberculosis*-RD1 in the genome of *M. bovis* BCG increases the latter's virulence and immunogenicity (Behr, 2002; Demangel et al., 2005; Pym et al., 2002).

1.4. THE INNATE IMMUNE RESPONSE TO *M. tuberculosis*

1.4.1. *M. tuberculosis* AND HOST PHAGOCYTE INTERPLAY

Infection of a host with *M. tuberculosis* follows the inhalation of droplets (aerosols) containing a small number of bacilli (Kaufmann, 2001b). Since the main route of entry of the causative agent is the respiratory route, the resident macrophages of the lung (alveolar macrophages) are the primary cell type involved in the initial uptake of mycobacteria (Orme and Cooper, 1999). After inhalation of tubercle bacilli, alveolar macrophages ingest the bacilli through phagocytosis and often destroy them. Phagocytosis of mycobacteria was shown to be an active process, mediated by an array of different receptors expressed on the surface of phagocytes (Brightbill et al., 1999; Cambi et al., 2005; DesJardin et al., 2002; Ernst, 1998; Greenberg, 1999; Kang et al., 2005; Swanson and Hoppe, 2004).

Both in natural and experimental infections mycobacteria are found and proliferate essentially inside macrophages and dendritic cells (DCs), even though neutrophils and eosinophils have also been shown to phagocytose mycobacteria (Castro et al., 1991; Kisich et

al., 2002). Macrophages and DCs are the main cells harbouring mycobacteria, functioning simultaneously as host and effector cells.

Following phagocytosis, mycobacteria are contained inside phagosomes, membrane-bound intracellular vesicles in which microorganisms can be killed and digested. The phagosome-containing ingested bacterium is then fused to lysosomes that contain numerous hydrolytic enzymes and are very acidic organelles. Phagosome-lysosome fusion is a highly regulated event and constitutes a significant antimicrobial mechanism of phagocytes (Flynn and Chan, 2001). Mechanisms involved in killing of *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen intermediates (ROI) and nitrogen oxides (Flynn and Chan, 2001). Hydrogen peroxide (H_2O_2), one of the ROI generated by macrophages via the oxidative burst, was the first identified effector molecule that mediated mycobactericidal effects of mononuclear phagocytes (Walker L, 1981). The ability of ROI to kill *M. tuberculosis*, although well demonstrated in mice, remains to be confirmed in humans. Several studies have demonstrated that *M. tuberculosis* infection induces the accumulation of macrophages in the lung, accompanied by H_2O_2 production (North and Medina, 1998). Moreover, different strains of *M. tuberculosis* induce the production of different amounts of ROI, which was suggested to be associated with the virulence of the strains (Firmani and Riley, 2002; Laochumroonvorapong et al., 1997).

Upon activation, phagocytes also generate nitric oxide (NO) and related reactive nitrogen intermediates (RNI) via inducible nitric oxide synthase (iNOS) using L-arginine as substrate. The role of RNI in defence against mycobacteria has been demonstrated following the observation that in genetically altered NOS gene knock-out mice, *M. tuberculosis* replicates much faster than in wild type animals (MacMicking et al., 1997). High levels of NOS2 expression have been detected in macrophages from broncho alveolar lavage of patients with active pulmonary TB (Nicholson et al., 1996).

Following *M. tuberculosis* infection, programmed cell death also constitutes an effector mechanism of the macrophage (Lee et al., 2009b). Apoptosis contributes to host defence by eliminating a protected intracellular environment favourable to bacterial replication, forcing the infecting pathogen to re-establish residence in a naive host cell, and by packaging *M. tuberculosis* bacilli and specific molecules in apoptotic bodies. The subsequent engulfment of these apoptotic bodies by newly recruited macrophages and DCs promotes the control of infection and the induction of the adaptive immune response (Lee et al., 2009b). Phagocytosis of apoptotic bodies

derived from *M. tuberculosis*-infected macrophages by DCs could lead to the presentation of mycobacterial lipid and peptide antigens and subsequent activation of specific T-cells (Schaible et al., 2003), a process defined as "crosspriming" (Guermonprez and Amigorena, 2005). Remarkably, apoptotic bodies containing mycobacterial antigens have the capacity to protect mice from challenge by virulent *M. tuberculosis* (Winau et al., 2006). The importance of apoptosis in the host's innate immune response was underlined by a report showing that apoptotic cell death reduced mycobacterial viability, whereas necrotic cell death had no effect on bacterial viability (Fratazzi et al., 1997; Keane et al., 2002; Molloy et al., 1994). In line with these findings is a report demonstrating that the susceptibility of different mouse strains to mycobacterial infections could be linked to the capacity of infected macrophages to either undergo necrotic or apoptotic cell death upon infection, with the former imparting a susceptible phenotype and the latter a resistant phenotype (Pan et al., 2005).

In order to survive in its host, *M. tuberculosis* has evolved several mechanisms to overcome the host macrophage defence mechanisms (Flynn and Chan, 2001; North and Jung, 2004). These immune evasion strategies allow *M. tuberculosis* to survive inside the host cells, thus contributing to the virulence of this pathogen. *M. tuberculosis* interferes with host trafficking pathways by modulating events based mainly on the arrest of phagosome maturation (Houben et al., 2006). The non-fusogenicity of mycobacterial phagosomes is believed to be a major factor in the capacity of pathogenic mycobacteria to survive within the potentially hostile environment of the macrophages (Nguyen and Pieters, 2005; Vergne et al., 2004). By blocking its delivery to lysosomes, *M. tuberculosis* is able to avoid the acidic proteases of the lysosomes, avoid exposure to the bactericidal mechanisms that operate within lysosomes, prevent degradation and hence processing and presentation of mycobacterial antigens to the adaptative immune system (Pancholi et al., 1993; Pieters, 2001). Another strategy apparently used by mycobacteria to modulate host immune responses in order to avoid the bactericidal activity of phagocytes (Chan J., 1994) is the production of superoxide dismutase and catalase that detoxify ROI (Andersen et al., 1991). In addition, mycobacterial components such as sulphatides, lipoarabinonannan (LAM) and phenolic-glycolipid I (PGLI) are potent oxygen radical scavengers (Chan et al., 1991; Chan et al., 1989).

M. tuberculosis was also shown to inhibit host cell apoptosis (Balcewicz-Sablinska et al., 1998; Fratazzi et al., 1999; Sly et al., 2003; Spira et al., 2003). Upon infection, *M. tuberculosis* was demonstrated to induce the up-regulation of anti-apoptotic genes that encode for Bcl-2-like

proteins (Spira et al., 2003). Other studies have shown that the expression of anti-apoptotic proteins is upregulated in cells infected with virulent strain of *M. tuberculosis* H37Rv, but not with the avirulent strain *M. tuberculosis* H37Ra (Spira et al., 2003), while pro-apoptotic proteins are inactivated following *M. tuberculosis*-H37Rv infection (Maiti et al., 2001). In order to inhibit tumor necrosis factor (TNF)-induced apoptosis, *M. tuberculosis*-infected macrophages have been reported to exhibit increased secretion of soluble TNFR2 (sTNFR2). The sTNFR2 binds to TNF in the extracellular milieu and thus inhibits its binding to the TNFR1 (Balcewicz-Sablinska et al., 1998; Fratazzi et al., 1999).

The interaction of DCs with the infectious agents plays a vital role in the initiation of the immune response against the pathogens (Lopez-Bravo and Ardavin, 2008). Although *M. tuberculosis* is able to grow equally well within DCs and macrophages, and activated DCs and macrophages were equivalent in their ability to inhibit replication of *M. tuberculosis* in an NOS2-dependent manner (Bodnar et al., 2001), DCs interact with live *M. tuberculosis* bacilli in a manner different from that of macrophages. DCs are considered to be the professional antigen presenting cells (APC), due to their ability to endocytose antigens and express abundant quantities of MHC class II, co-stimulatory molecules, and cytokines (Giacomini et al., 2001), that all together drive T helper (Th) cell differentiation. Previous studies have revealed that human and murine DCs can ingest *M. tuberculosis*, and that DCs exposed to *M. tuberculosis in vitro* undergo a typical maturation program and upregulate their antigen-presenting activities (Bodnar et al., 2001; Demangel et al., 1999; Demangel and Britton, 2000; Giacomini et al., 2001; Gonzalez-Juarrero and Orme, 2001; Tascon et al., 2000). In addition, it was shown that DCs, but not macrophages, infected with *M. tuberculosis* are capable of driving Th1 polarization of naive $CD4^+$ T cells (Hickman et al., 2002). Therefore, it is likely that DCs play an important role in initiating the acquired immune response to *M. tuberculosis*. An additional property of DCs that contributes to their effectiveness in initiating immune responses *in vivo* is their ability to migrate from peripheral tissues to secondary lymphoid tissues after acquiring antigens and in the presence of proinflammatory stimuli (Alvarez et al., 2008). Infection of DCs by *M. tuberculosis* results in the expression of CCR7 and subsequent migration of these cells to the lymph node (Bhatt et al., 2004). Maturation and migration of DCs from the lung to the draining lymph nodes is a key step for the initiation of naive T cell activation (Bhatt et al., 2004; Chackerian et al., 2002; Demangel et al., 2002; Humphreys et al., 2006; Khader et al., 2006; Skold and Behar, 2008; Winslow et al., 2008; Wolf et al., 2008).

In summary, upon infection of the host, alveolar macrophages become activated and initiate several effector mechanisms that aim at eliminating *M. tuberculosis*. Soon after, DCs become exposed to *M. tuberculosis* in the lung, maturate and migrate to the draining lymph nodes where the initiation of the T cell response takes place. The understanding of the interaction of the pathogen with both macrophages and DCs is therefore important to provide clues on possible ways to modulate both the innate and the acquired immune responses. These steps of pathogen recognition, phagocytosis by macrophages and presentation by DCs are of particular importance for the understanding of the immune response to *M. tuberculosis versus* BCG.

1.4.2. ROLE OF TLR SIGNALLING FOR *M. tuberculosis* RECOGNITION

Early recognition of *M. tuberculosis* or mycobacterial products is a crucial step for the initiation of an effective host response. Recognition of infectious agents depends on a variety of pattern recognition receptors (PRRs) (Akira et al., 2006; Bhatt and Salgame, 2007; Geijtenbeek et al., 2003; Rothfuchs et al., 2007). Several PRRs have been involved in the recognition of *M. tuberculosis* by macrophages and DCs. This is the case of toll-like receptors (TLRs) 2, 4 and 9 (Bafica et al., 2005; Pai et al., 2004; Quesniaux et al., 2004); dectin-1 (Lee et al., 2009a; Rothfuchs et al., 2007; Yadav and Schorey, 2006); the mannose receptor (Desjardins et al., 1994; Kang et al., 2005); DC-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Maeda et al., 2003; Tailleux et al., 2003) and nucleotide-binding oligomerization domain (NODs) (Ferwerda et al., 2005). Of these, the better characterised interaction is the one mediated by TLRs. Accumulating data indicate that *M. tuberculosis* expresses a large repertoire of TLR2 ligands. The 19-kDa lipoprotein (LpqH), a secreted antigen of *M. tuberculosis,* was the first *M. tuberculosis* ligand shown to interact specifically with TLR2 and to induce TNF and nitric oxide production from both murine and human macrophages (Brightbill et al., 1999). In addition, the 19-kDa lipoprotein is a major inducer of IL-12 production in human monocytes (Brightbill et al., 1999). Abel *et al.* demonstrated that phosphatidylinositol mannoside (PIM) structures can also elicit cellular activation via TLR4 (Abel et al., 2002), with the induction of nuclear factor-kB (NF-kB) activation in a dose dependent manner. Interestingly, mannose-capped lipoarabinomannan (ManLam) derived from virulent *M. tuberculosis* fails to activate either TLR2- or TLR4-transfected cells (Means et al., 1999). In contrast, arabinosylated lipoarabinomannan (AraLAM) purified from fast-growing mycobacteria is capable of TLR2-mediated cellular activation (Means et al., 1999).

Binding of TLR ligands to TLRs activate downstream signalling cascades through the adaptor protein myeloid differentiation protein 88 (MyD88), which links to IL-1R-associated kinase (IRAK), a serine kinase that activates transcription factors like NF-kB to signal the production of inflammatory cytokines and chemokines (O'Neill and Bowie, 2007), needed to promote the attraction of innate immune cells and the initiation and polarization of adaptive immune responses (Akira et al., 2006). The mitogen-activated protein kinases (MAPK) family, composed of the ERK1/2, p38 and SAPK/JNK pathways, has have been implicated in the mediation of TLRs signalling and activation of cytokine gene transcription (Liu et al., 2007). Several reports have shown that mycobacteria in general activate the MAPK pathway (Chan et al., 2001; Cobb, 1999; Jones et al., 2001a; Jones et al., 2001b). For instance, *Jones and colleagues* demonstrated that AraLAM, isolated from avirulent mycobacteria, and PIM, isolated from *M. tuberculosis*, stimulated ERK1/2 phosphorylation and activated the transcription factors NF-kB and AP-1 in a murine macrophage cell line, in a TLR2-dependent manner (Jones et al., 2001a; Jones et al., 2001b). In addition, *Chan and colleagues* shown that, although ERK1/2 and p38 were phosphorylated, activation of ERK1/2 was sufficient for the induction of the NOS2 gene following the stimulation of a macrophage cell line with both ManLAM and interferon (IFN)-γ (Chan et al., 2001). Mycobacteria are able to modulate MAPK signalling to promote their survival in the host cell. Several studies show that virulent strains of mycobacteria caused greater inhibition of MAPK, particularly the ERK1/2 pathway, as compared to avirulent strain (Florido et al., 1999; Hasan et al., 2003; Roach and Schorey, 2002).

The relevance of TLR signalling for the development of the immune response to *M. tuberculosis* has been addressed in several studies *in vitro* and *in vivo*. MyD88 was found to be essential for *M. tuberculosis*-induced macrophage activation (Fremond et al., 2004; Scanga et al., 2004; Shi et al., 2003). In addition, *M. tuberculosis*-infected MyD88-deficient mice have increased numbers of bacteria in the lung in comparison to wild type controls (100 to 1000-fold) (Fremond et al., 2004; Scanga et al., 2004; Sugawara et al., 2003b). As for TLR2, its role in the infection by *M. tuberculosis* remains controversial. In a model of low-dose aerosol infection, TLR2 deficiency did not affect host defence against *M. tuberculosis* infection (Reiling et al., 2002; Sugawara et al., 2003a). However, with high-dose aerosol infection, a role for TLR2 in host resistance was revealed (Reiling et al., 2002; Sugawara et al., 2003a). TLR2-deficient mice were not compromised in their ability to induce Th1 immunity, but on the contrary, exhibited exaggerated immunopathology (Reiling et al., 2002). *In vitro* studies have shown that

engagement of TLR2 with *M. tuberculosis* ligands induces inhibition of macrophage MHC class II antigen presentation (Noss et al., 2001) and also blocks macrophage responsiveness to IFN-γ (Banaiee et al., 2006; Fortune et al., 2004). Together with the *in vivo* studies, these *in vitro* findings suggest that TLR2 signalling negatively modulates macrophage functions. Recent data indicate that TLR9 cooperates with TLR2 to recognize *M. tuberculosis* in macrophages as well as splenic DCs (Bafica et al., 2005). When murine TLR9$^{-/-}$ splenic DCs were stimulated with live *M. tuberculosis*, there was a partial reduction in IL-12p40 (Bafica et al., 2005). However, in TLR2/9 double deficient cells, there was further inhibition of cytokine production to background levels, suggesting that the majority of TLRs-mediated mycobacterial signalling is through these two receptors (Bafica et al., 2005). Moreover, TLR2/9 double deficient mice displayed markedly enhanced susceptibility to infection (Bafica et al., 2005). Interestingly, TLR2/4 double deficient mice have been found to display unimpaired resistance to *M. tuberculosis* (Shi et al., 2005) as well as to BCG infection (Nicolle et al., 2004). In what regards BCG infection, in some studies TLR2, TLR4 and TLR6 were shown to be redundant for the control of infection (Fremond et al., 2003; Heldwein et al., 2003; Nicolle et al., 2004). However, in another study TLR2 appears to be necessary for the expansion of effector T cells and for the induction of IFN-γ secretion by these cells, while, TLR4 was shown to be necessary for the development of a normal Th1 response against BCG, however only when larger bacterial numbers are encountered by the host (Heldwein et al., 2003).

1.5. ROLE OF CD4$^+$ T CELLS AND CYTOKINES IN THE INFECTION BY *M. tuberculosis*

The protective response against TB requires cell-mediated immunity (Boom, 1996; Cooper, 2009a; Flynn and Chan, 2001; Kaufmann, 2001a). Among T lymphocytes, the CD4$^+$ T-cell subset is of primary importance in the protection against *M. tuberculosis* (Saunders et al., 2002). Studies in mouse models deficient in CD4$^+$ T cells clearly demonstrated that these cells are required for the control of infection (Saunders et al., 2002). In addition, other studies demonstrated that adoptive transfer of CD4$^+$ T cells enhanced protection against TB (Orme and Collins, 1984). Moreover, the high numbers of individuals co-infected with HIV and *M. tuberculosis* strongly suggest that the loss of CD4$^+$ T cells greatly increases the susceptibility of human hosts to both acute TB and to reactivation of TB (Jones et al., 1993; Lawn et al., 2002).

Upon aerosol infection with *M. tuberculosis,* the acquired cellular response is show to be induced in the lung, and dissemination of the mycobacteria from the lung to the draining lymph node has been suggested to be required for the activation of antigen-specific T cells and the induction of effector function (Bhatt et al., 2004; Chackerian et al., 2002; Demangel et al., 2002; Humphreys et al., 2006; Khader et al., 2006; Skold and Behar, 2008; Winslow et al., 2008; Wolf et al., 2008). This migration of DCs to the lymph nodes during mycobacterial infection appears to be promoted by IL-12p40 homodimers (Khader et al., 2006) and limited by IL-10 (Demangel et al., 2002). Following infection by mycobacteria, two subsets of $CD4^+$ Th cells have been shown to differentiate in the lymph node and to subsequently migrate to the infected tissue to exert their effector activities. These subsets are Th1 (Cooper et al., 1995; Flynn et al., 1995) and Th17 (Khader et al., 2005) cells. The differentiation of Th1 cells is determined mainly by the presence of IL-12, and results in the production of high levels of IFN-γ (O'Garra and Robinson, 2004). In the presence of transforming growth factor (TGF)-β and IL-6, naive T cells differentiate into a Th17 phenotype that produces high levels of IL-17 and requires IL-23 for survival (Veldhoen and Stockinger, 2006).

The expression of IL-12, IL-6 and IL-23 is mainly associated with cells of the innate immune response. IL-12 is a heterodimeric cytokine consisting of the two subunits IL-12p40 (p40) and IL-12p35 which are covalently linked (Trinchieri, 2003). IL-12 expression is induced following phagocytosis of *M. tuberculosis* bacilli in macrophages and DC (Henderson et al., 1997; Ladel et al., 1997). Two studies comparing murine macrophages and DC demonstrated that DC release significantly higher amounts of IL-12 than did macrophages in response to live *M. tuberculosis* (Giacomini et al., 2001; Hickman et al., 2002). *In vitro*, *M. tuberculosis*-infected DC also primed naive T cells toward Th1 development, while macrophages did not (Verreck et al., 2004). IL-23 is another cytokine of the IL-12 family (Hunter, 2005) and, as IL-12, is a heterodimeric cytokine composed by the p40 subunit covalently linked to a p19 subunit . IL-23 secretion in response to TLRs activation appears to be more pronounced in DC than in macrophages (Gerosa et al., 2008; Jang et al., 2008; Lyakh et al., 2008). Interestingly, secretion of IL-12 and IL-23 by *M. tuberculosis* stimulation of DC can, in addition to TLRs, be also dependent on signals mediated by Dectin-1 (Gerosa et al., 2008; Rothfuchs et al., 2007; Zenaro et al., 2009).

Several studies highlight the role of IL-12, IL-23, IL-6 and of IFN-γ and IL-17 during the course of infection. A role for IL-12 and cytokines of the Th1 axis, predominantly IFN-γ, is

established in protection against mycobacterial infections in both mouse models (Cooper, 2009a; Flynn et al., 1993) and human disease (Casanova and Abel, 2002; Flynn and Chan, 2001) (Cooper, 2009b). In particular, IFN-γ that in addition to Th1 cells can also be produced by natural killer (NK) cells (Scharton and Scott, 1993) and $CD8^+$ T cells (Barnes et al., 1993; Lalvani et al., 1998; Orme et al., 1992) is also the hallmark molecule for protection against TB (Chackerian et al., 2002; Cooper et al., 1993; Ellner et al., 2000; Flynn et al., 1993; Ottenhoff et al., 1998). The role of IL-23 and Th17 responses in the infection by *M. tuberculosis* is yet not fully understood. Studies performed in mice lacking p40, p35, p19 or combinations of these genes, showed that IL-23 is less critical than IL-12 for protection against *M. tuberculosis*, and only provides a moderate level of protection to the host in the absence of biologically active IL-12 (Khader et al., 2005). IL-17, secreted not only by $CD4^+$ Th17 cells (Khader et al., 2007), but also by γδ T cells (Lockhart et al., 2006), has been shown to have a limited role in host defense against *M. tuberculosis* during primary infection (Aujla et al., 2007). However, vaccination has been shown to trigger an IL-17-dependent accelerated IFN-γ response by $CD4^+$ T cells in the lung during subsequent *M. tuberculosis* infection (Khader et al., 2007). Importantly, IL-17 promotes neutrophil recruitment to the site of infection (Fossiez et al., 1996; Jones and Chan, 2002; Ye et al., 2001), but this ability of IL-17 to induce chemokine production and cell recruitment to the infected tissue can in certain situations be associated with the development of immune pathology (Cooper, 2009a) (Cruz et al, umpublished data).

Another important cytokine with a key role in the immune response to *M. tuberculosis* is TNF. *M. tuberculosis* induces TNF secretion by macrophages, DC and T cells (Barnes et al., 1993; Henderson et al., 1997; Ladel et al., 1997; Serbina and Flynn, 1999). The requirement for TNF for the control of *M. tuberculosis* infection is complex, but it clearly is an important component for macrophage activation, as TNF, in synergy with IFN-γ, induces NOS2 expression (Chan et al., 1992; Liew et al., 1990). The importance of this cytokine in granuloma formation in TB and other mycobacterial diseases has been significantly documented (Flynn and Chan, 2001; Miller and Ernst, 2008).

The proinflammatory response which is initiated by *M. tuberculosis* is antagonized by anti-inflammatory cytokines that contribute to the control of the magnitude of the inflammatory responses. TGF-β was found to be present in granulomatous lesions of TB patients and is produced by human monocytes after stimulation with *M. tuberculosis* (Toossi et al., 1995) or *M. tuberculosis* lipoarabinomannan (Lam) (Dahl et al., 1996). TGF-β has important anti-

inflammatory effects, including deactivation of macrophage production of ROI and RNI (Ding et al., 1990), inhibition of T cell proliferation (Toossi and Ellner, 1998), interference with NK and CTL function and downregulation of IFN-γ (Ruscetti et al., 1993), TNF and IL-1 release (Ruscetti et al., 1993). TGF-β is also an important mediator of immune-suppression by regulatory T cells (Gorelik and Flavell, 2002). However, together in the presence of IL-6, TGF-β induces Th17 differentiation (Veldhoen et al., 2006).

The anti-inflammatory cytokine IL-10 is also expressed during *M. tuberculosis* infection (Barnes et al., 1993; Boussiotis et al., 2000; Gerosa et al., 1999; Shaw et al., 2000). IL-10 is needed to limit tissue damage by controlling the immune response (Moore et al., 2001). However, an excess of IL-10 most likely prevents pathogen clearance. Indeed, transgenic mice constitutively expressing IL-10 were less capable of clearing a BCG infection, although T cell responses including IFN-γ production were unimpaired (Murray et al., 1997), thus suggesting that IL-10 may counter the macrophage activating properties of IFN-γ . Interestingly, IL-10$^{-/-}$ mice were not more resistant to acute *M. tuberculosis*, compared to wild type mice (North, 1998). However, lack of IL-10 was recently link to a decreased control over the inflammatory response that eventually resulted in progression of disease, bacterial multiplication and morbidity (Higgins et al., 2009).

An appropriate immune response to *M. tuberculosis* is the result of a balance between inflammation and regulation. In what extent does the vaccination with BCG change the parameters of this response is still not fully known. This knowledge is however important in order to improve the efficacy of BCG vaccination or to develop new vaccination strategies.

II. AIMS

BCG is the only approved vaccine used for TB prevention. Although BCG is used worldwide, the cellular and molecular mechanisms by which this vaccine acts are still poorly understood. Although BCG is protective against tuberculous meningitis, it shows great variability in the prevention of pulmonary disease.

Several experimental vaccines are being developed against TB. Some are able to induce the same level of protection as BCG, although none were demonstrated to induce better protection than BCG. Therefore it is fundamental to understand how BCG modulates the innate and cellular immunity and where it fails, in order to improve vaccination strategies to TB.

The main goal of this work was to perform a comparative study of BCG *versus M. tuberculosis* infection on DCs and macrophages to better understand how these pathogens interact with cells of the innate immune system, what molecular pathways are triggered, and how that interaction translates into an effective, or not, Th cell responses

Specifically, the following aims were addressed:

1. Investigation of the magnitude of macrophage and DCs responses to *M. tuberculosis* or BCG, in terms of cytokine production by these phagocytes;
2. Elucidation of the molecular events that might explain differences in the cytokine response of macrophages and DCs to *M. tuberculosis* or BCG;
3. Understanding the type of Th cell responses developed in the presence of *M. tuberculosis*- or BCG- infected DCs;
4. Clarification of the effector activity of macrophages in what regards the control of *M. tuberculosis* or BCG growth.

Dissecting the cellular and molecular events that occur upon mycobacterial challenge will help to reveal possible weak points of both the host and the bacteria that can be targeted and will subsequently provide clues for the design of new and better vaccines.

3.1. Animals

Eight-week-old female C57BL/6 and Balb/c mice were obtainned from Charles River Laboratory (Barcelona, Spain). Mice transgenic for the DO11.10 α/β TCR were backcrossed on a RAG-deficient (RAG 2/2) BALB/c background and were kindly provided by Dr. Anne O'Garra (NMIR, London, England).

3.2. Bacteria

The H37Rv strain of *M. tuberculosis* and *M. bovis* BCG Pasteur were grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 1-ml aliquots at -80°C. Bacterial viability was determined by counting the number of CFU (colony forming units) on Middlebrook 7H11 agar plates. *M. bovis* BCG Pasteur were obtained from Trudeu Institute *Mycobacterium* Collection (TMC 1011).

3.3. Culture of Bone Marrow Derived Macrophages

Primary mouse bone marrow derived macrophages were generated from WT C57BL/6 animals as described elsewhere(Saraiva et al., 2009). Petri dishses were initially seeded with $1x10^5$ cells in complete medium with 20% of L-929 conditioned medium (LCCM) and incubated at 37°C in 5% CO_2 humidified air chamber. On day 4, the medium was renewed and cultures were used at day 7. Macrophages were stimulated with *M. tuberculosis or* BCG at a multiplicity of infection (MOI) 2:1 (bacteria/macrophage) for different periods of time. Some cultures received 100 U/ml of mouse IFN-γ (R&D Systems).

3.4. Culture of Bone Marrow Derived Dendritic Cells

Primary mouse bone marrow derived dendritic cells (DCs) were differentiated from WT C57BL/6 mice as described previously (Saraiva et al., 2009). Cells were culture in 6-well plates, containing $5x10^6$ cells in complete medium with 20% of granulocyte-macrophage colony-stimulating factor (GM-CSF) and incubated at 37°C in 5% CO_2 humidified air chamber. On day 2, 4 and 6, the medium was renewed and cultures were used at day 7. DCs were exposed to live *M. tuberculosis* or BCG at a MOI of 2 for different periods of time.

3.5. Quantitative Real Time-PCR (RT-PCR) Analysis

Total RNA from cultured macrophages was extracted with TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reverse transcription was done with whole RNA in a final volume of 20 µl using SuperScript II (Invitrogen) and Oligo(dT) (Roche) according to the manufacturer's instructions. The cDNA was then subjected to real-time PCR for quantification of IL-12p40, IL-12p35, IL23p19, TNF, IL-6, IL-10 and Ubiquitin (used as house-keeping gene) in 10 µl with SYBR Green Supermix (Bio-Rad) in an CFX 96 Real-Time (Bio-Rad) system. The specific conditions of each PCR are listed in the table 1. All reaction were performed using the following cycling parameters: 1 cycle of 95°C for 15 min, followed by 30 cycles of 95°C for 15 min, 58°C for 20 min and 72°C for 15 min, and 2 amplification cycles of 65°C for 1.3 min and 95°C for 15min; and 1 cooling cycle of 35°C for 1.3 min. Relative mRNA expression was calculated accordingly with the following equation: 1.8 ^ (ubiquitin mRNA expression – specific cytokine gene mRNA expression) x 100000.

Table 1 - **Sequence of the primers specific for genes and conditions used in RT-PCR reaction.**

Gene	Primer	Probe Sequence
Ubiquitin	**Sense**	5'- TGG CTA TTA ATT ATT CGG TCT GCA -3'
	Anti-sense	5'- GCA AGT GGC TAG AGT GCA GAG TAA -3'
IL-12p40	**Sense**	5'- CAA ATT ACT CCG GAC GGT TCA -3'
	Anti-sense	5'- AGA GAC GCC ATT CCA CAT GTC -3'
IL-12p35	**Sense**	5'- TGC TGG TGG CCA TCG AT -3'
	Anti-sense	5'- GCA GAG TCT CGC CAT TAT GAT TC-3'
IL-12p19	**Sense**	5'- CGT ATC CAG TGT GAA GAT GGT TGT -3'
	Anti-sense	5'- GCT CCC CTT TGA AGA TGT CAG A-3'
TNF	**Sense**	5'- GCC ACC ACG CTC TTC TGT CT -3'
	Anti-sense	5'- TGA GGG TCT GGG CCA TAG AAC -3'
IL-6	**Sense**	5'- ACA CAT GTT CTC TGG GAA ATC GT -3'
	Anti-sense	5'- AAG TGC ATC ATC GTT GTT CAT ACA -3'
IL-10	**Sense**	5'- TTT GAA TTC CCT GGG TGA GAA -3'
	Anti-sense	5'- GCT CCA CTG CCT TGC TCT TAT T -3'

3.6. ELISA Assay

Supernatants from *M. tuberculosis*- or BCG-infected DCs were collected at 24h post-infection and screened for IL-12p70, IL-12p40, IL-23p19, TNF, IL-6 or IL-10 Ready Set-Go ELISA kit (eBioscience) by sandwich ELISA.

3.7. Protein Analysis

Macrophages and DCs were cultured in medium containing 1% FBS overnight before stimulation with *M.tuberculosis* or BCG and washed in PBS before lysis (1% NP-40, 0.1% SDS, 0.5% deoxycholate acid, 50 mM Tris HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 2 mM sodium-pyrophosphate, 50 mM sodium fluoride, 100 mM vanadate [all from Sigma-Aldrich], and complete EDTA-free protease inhibitor cocktail (Roche). Immunoblotting of proteins was performed as previously described (Saraiva et al., 2005) and visualized with ECL (GE Healthcare) or SuperSignal West Femto Substrate (Thermo Fisher Scientific). Using specific antibodies that exclusively recognize the bi-phosphorylated forms (activated) of ERK1/2 or the total form of the same enzyme, the ratio between the Western Blot signals obtained for the phosphorylated versus the total form, allow the quantification of the amount of ERK1/ERK2 activated within the cells upon *M. tuberculosis* or BCG stimulation. Antibodies used: rabbit (polyclonal) anti-ERK1/2 [pTpY$^{185/187}$) phosphospecific (Biosource), rabbit (polyclonal anti-ERK1/2 pan (Biosource).

3.8. *In vitro* CD4 T Cell Activation

Naive CD4$^+$ T cells were generated from OVA-TCR transgenic DO.11.10 mice as described (Cruz et al., 2006) and cultured (1x10^6 cells/ml) with *M. tuberculosis*- or BCG- infected DCs (1x10^6 cells/ml) for 72h at 37°C in 5% CO_2, and in 10 ng/ml IL-2 and 5 μM $OVA_{323-339}$ peptide. Stimulated T cells were washed and counted, and the frequency of IFN-γ- and IL-17-producing CD4$^+$ T cells determined by ELISPOT.

3.9. *In vivo* Infection

C57BL/6 animals were infected by the intravenous route with *M. tuberculosis* strain H37Rv or with BCG (1x10^6 CFU) and at different time points the animals were sacrificed and the spleens removed. The ability of the splenocytes to produce IFN-γ or IL-17 in response to Antigen 85 was assessed by ELISPOT.

3.10. ELISPOT Assay

ELISPOT was performed as described previously (Cruz et al., 2006). Briefly, a total of 1x10^5 cells was added to Ab-coated wells, 2-fold dilutions were made, and irradiated splenocytes from Balb/6 or C57/BL6 mice were added at 1x10^6 cells per well. A peptide representing an I-A^b-restricted epitope of antigen85A (Cole et al., 1998) were used to stimulate cells from infected

mice, whereas $Ova_{323-339}$ stimulated DO.11.10 T cells (Camus et al., 2002); all wells contained 10 ng/ml IL-2. After 24 h, plates were washed and the number of IFN-γ- or IL-17-producing $CD4^+$ T cells determined as described (Cruz et al., 2006). Cells from mice infected with *M. tuberculosis* or BCG, but cultured in the absence of antigen, did not produce spots.

3.11. Bacterial Load Determination

To determined the number of viable bacteria, macrophage monolayers were lysed with 0.1% (final concentration) saponin and the bacterial suspensions were serially diluted and plated onto Middlebrook 7H11 agar medium. Bacterial colony formation was counted after 3 weaks of incubation at 37°C.

3.12. Nitrites Quantification

Nitrite production by macrophage monolayers was determined by the colorimetric Griess assay as described elsewhere (Turner et al., 2001). Briefly, supernatants from macrophage cultures were placed into 96-well enzyme-linked immunosorbent assay plate in duplicates, and equal volume of Griess reagent (1% sulfanilamine, 0.1% nathylethylenediamine, 2,5% H_3PO_4) was added. The absorbance was measured at 550nm on a spectrophotometer, and the concentrationof nitrite was calculated by comparing optimal density values to a standard curve of $NaNO_2$.

3.13. Immunofluorescence

Macrophages were cultivated as described above except that lamellae were placed on the bottom of the wells of the 24-wells incubation plaques. At specific time points after infection, the lamellae were collect and fixed in 2% PFA. Next, the lamellae were incubated with the primary antibody affinity-purified rabbit anti-human/mouse caspase 3 active (R&B Systems) and was detected with goat anti-rabbit IgG (H+L) Alexa Fluor 568 (Molecular Probes A11011). DAPI was used to counter stained and to detect nuclei. Pictures were observed with Olympus BX61 microscope and images were recorded with Olympus DP70 camera.

3.14. Stastistical Analysis

The results are given as means ± SE of the mean. Statistical significance was performed by using Studant's *t* test. Values of $p < 0.05$ were considered significant.

4.1. DC responses to *M. tuberculosis* and to BCG are stronger than macrophage responses, and *M. tuberculosis* is a stronger stimulus than BCG to DCs

To understand the early steps of the immune response to *M. tuberculosis* and BCG and since DCs and macrophages are the central sensory components of the immune system, being within the first cells to recognize the presence of pathogens and to respond to it, we decided to compare the response of both cells types to either *M. tuberculosis* or BCG in a systematic and extensive fashion.

The first point to be addressed in our comparison was the response of macrophages and DCs to *M. tuberculosis* or BCG in terms of cytokine production. To do this, we prepared primary cultures of macrophages and DCs derived from mouse bone marrow and stimulated with live *M. tuberculosis* or BCG at a MOI of 2. At different time points post-infection, we extracted RNA from the stimulated cells, prepared cDNA and measured the expression of several cytokines by RT-PCR. We were particularly interested in studying whether the expression of cytokines with a role in T cell differentiation induced by *M. tuberculosis* or BCG was different. Since IL-12p70 and IL-23 are important for Th1 and Th17 responses, respectively, we started by measuring the transcription of the monomers that compose IL-12 and IL-23 (p40-p35 and p40-p19, respectively). We also measured the expression of other immune mediator cytokines such as TNF, which contributes to the initial control of the infection, for example by activating the infected macrophages in an autocrine way (Chan et al., 1992; Liew et al., 1990); IL-6 described to be required for the development of Th17 cells (Veldhoen and Stockinger, 2006); and IL-10, an anti-inflammatory cytokine known to inhibit macrophage and DCs functions (Demangel et al., 2002; Madura Larsen et al., 2007).

As shown in Fig.1, we observed that both macrophages and DCs express various cytokines in response to *M. tuberculosis* or BCG. Both cell types showed similar patterns of expression for p35, TNF, IL-6 and IL-10 in response to either mycobacterium. However, major differences were identified in terms of the IL-12 subunits p40 and p19. Indeed, whereas macrophages stimulated with *M. tuberculosis* or with BCG did not express detectable amounts of IL-12p19 monomer, DCs did express this molecule, but the levels obtained in response to *M. tuberculosis* stimulation were higher than those obtained with BCG stimulation. In what regards the expression of IL-12p40, the pattern was similar to the one observed for p19. Again, maximum p40 expression was induced

by *M. tuberculosis* in DCs. BCG induced only modest amounts of p40 in DCs and the expression of this molecule by macrophages was low, but detectable. Interestingly, *M. tuberculosis*-stimulated DCs, always expressed higher levels of the tested cytokines, suggesting that *M. tuberculosis* is stronger than BCG as a stimulus to DCs. As for macrophages, the two agents yielded similar responses, thus suggesting that different regulatory pathways are in place in macrophages *versus* DCs. Overall, our data show a differential cytokine expression by macrophages and DCs when stimulated with *M. tuberculosis* or BCG.

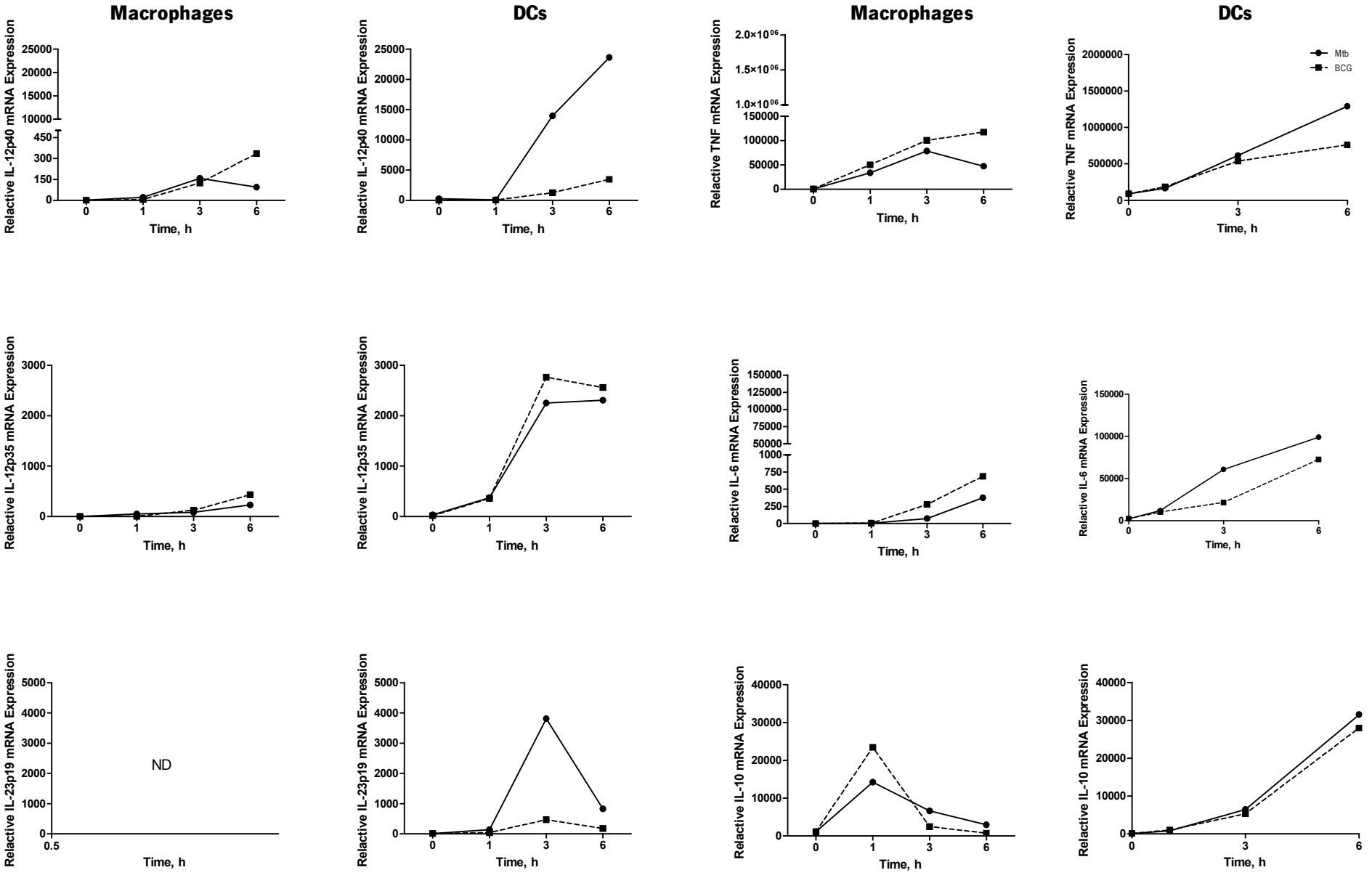

Figure 1 - M. tuberculosis and BCG are stronger stimuli to DCs than to macrophages, and M. tuberculosis is a stronger stimulus than BCG to DCs. Macrophages and DCs were generated from BL/6 mice and exposed to M. tuberculosis or BCG at an MOI of 2. At different time points post infection, total RNA was extracted. The expression of IL-12p40, IL-12p35, IL-23p19, TNF, IL-6 and IL-10 was assessed by RT-PCR and normalised to the expression of ubiquitin. Points of the graphs represent pools of cells in each time point (n=3). Results are representative of three independent experiments with similar results. Mtb (M. tuberculosis); ND (not detected).

4.2. *M. tuberculosis*-stimulated DCs are stronger producers of IL-12 and IL-23 than BCG-stimulated DCs.

From our mRNA analysis, we concluded that the transcription of p40 and p19 by DCs was only induced in relevant levels by *M. tuberculosis* stimulation. Taking into consideration the role of IL-12 (p40-p35) and IL-23 (p40-p19) molecules in Th cell differentiation, we decided to assess the secretion of IL-12p70 and IL-23 by *M. tuberculosis* or BCG stimulated DCs. For that, we collected DCs culture supernatants 24 hours post-stimulation and measured by immunoassay the amounts of cytokines secreted. Consistently with the observed gene expression pattern, the analysis of supernatants from stimulated DCs showed that both IL-12p70 and IL-12p40 release was higher in DCs stimulated with *M. tuberculosis* comparing to stimulation with BCG (Fig. 2). IL-23 production was only detectable in *M. tuberculosis*-stimulated DCs. We also measured the secretion of TNF, IL-6 and IL-10. We found that IL-6 and IL-10 production was similar in both *M. tuberculosis*- and BCG-stimulated DCs, but TNF production was higher in DCs stimulated with BCG (Fig.2). Thus our data suggest that, in terms of protein expression, *M.* tuberculosis-stimulated DCs are potent producers of IL-12 and IL-23 whereas BCG-stimulated DCs are not. Importantly, our data also show that, despite the difference observed for IL-12 and IL-23, BCG is able to induce the production of certain cytokines by DC, thus suggesting that activation signals can be generated by BCG. We are currently performing the ELISA assays for macrophages supernatants to further validate the results obtained by PCR.

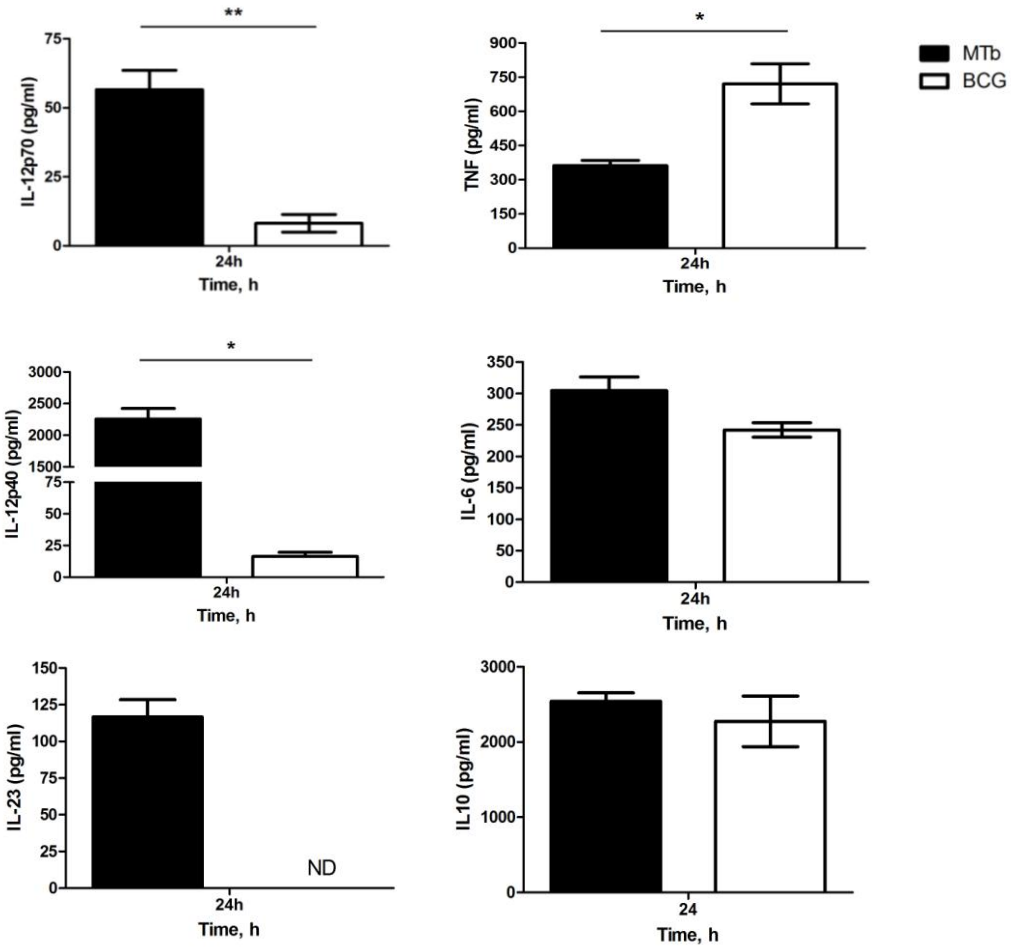

Figure 2 - *M. tuberculosis* infected DCs produce higher amounts of IL-12 and IL-23 than BCG-infected DCs. DCs were differentiated from WT mice and exposed to *M. tuberculosis* or BCG at an MOI of 2. DCs culture supernatants were harvested 24 hours post stimulation and analyzed by ELISA for IL-12p70, IL-12p40, IL-23, TNF, IL-6 and IL-10 concentration. Each time point represents the mean of three wells. Results are representative of three independent experiments with similar results and show mean ± SD; *, **, $p<0.05$ and 0.01, respectively. Mtb (*M. tuberculosis*). ND (not detected)

4.3. *M. tuberculosis* induces earlier and stronger activation of ERK phosphorylation in macrophages as compared to DCs

From the results shown in the previous sections, macrophages and DCs respond differently to *M. tuberculosis* or BCG stimulation in terms of cytokine expression, particularly in what regards the expression of IL-12 and IL-23 (Fig.1 and Fig.2). We hypothesised that these differences might be a consequence of a differential activation of intracellular signalling cascades. Previous studies have suggested the activation of various signalling cascades following mycobacterial infection, such as the MAPK pathway (Cobb, 1999) that includes three main

cellular kinases: ERK, p38 and JNK. We have started by addressing the activation of the MAPK cascade induced by *M. tuberculosis* or BCG by monitoring the ERK pathway. Phosphorylation of the two isoforms, ERK1 and ERK2, induces their translocation to the nucleus, followed by activation of several targets and culminating with the expression of cytokine genes. Thus, to further investigate if ERK activation is differently induced by *M.* tuberculosis and BCG in macrophages and DCs, we measured the phosphorylation of this MAPK by Western Blot, using specific antibodies that exclusively recognize the bi-phosphorylated forms (activated) of ERK1/ERK2 or the total form of the same enzyme. To control the amount of total protein used, we detected the amount of actin present in each condition, in parallel to phosphor-ERK1/2 or total-ERK1/2. The ratio between the Western Blot signals obtained for the phosphorylated *versus* the total form, allowed the quantification of the amount of ERK1/ERK2 activated within the cells upon *M. tuberculosis* or BCG stimulation. As shown in Fig.3, *M. tuberculosis* stimulation of macrophages induced an earlier and stronger ERK phosphorylation than that induced in DCs. Although the kinetics of ERK phosphorylation was similar for *M. tuberculosis*- or BCG-stimulated macrophages, this activation was weaker when BCG was used as stimulus. The lowest induction of ERK phosphorylation was observed in DCs stimulated with BCG. Therefore our results suggest that *M. tuberculosis* and BCG trigger the ERK pathway with distinct intensities in macrophages and DCs. Despite the higher levels of ERK phosphorylation observed for macrophages, these cells do not respond to *M. tuberculosis* in a stronger way than DCs, suggesting that tight regulatory mechanisms must be in place.

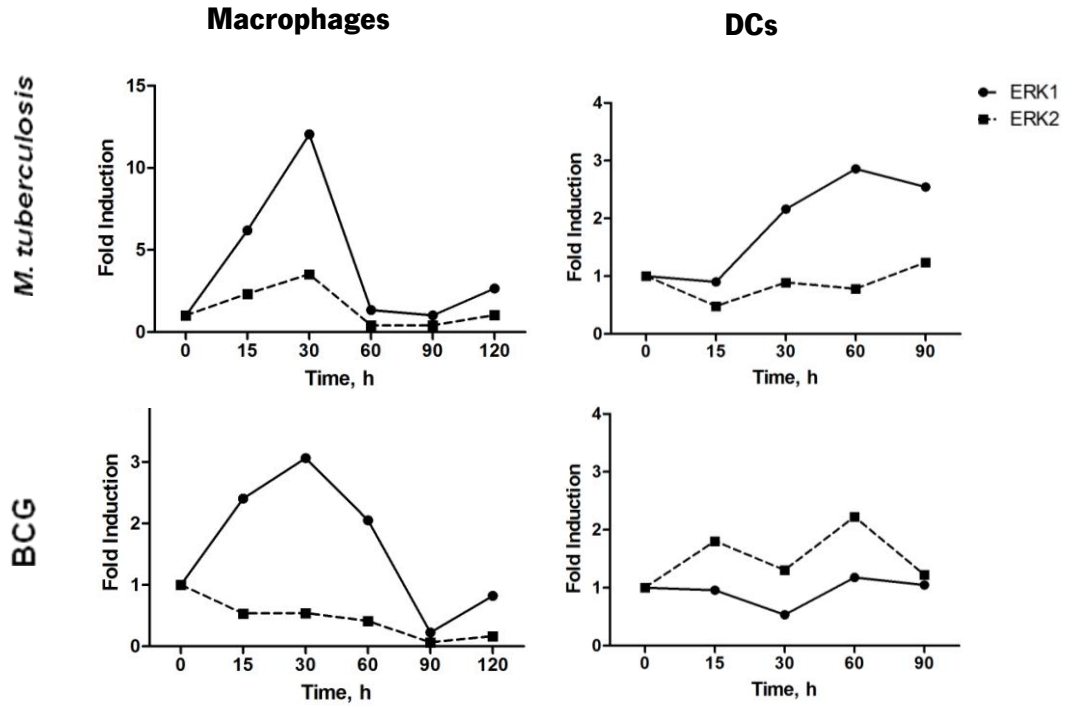

Figure 3 – *M. tuberculosis*-stimulated macrophages earlier and stronger activate ERK phosphorylation as compared to DCs. Mouse DCs and macrophages were differentiated in culture and stimulated with *M. tuberculosis* or BCG at a MOI of 2. At the indicated time points, cellular extracts were prepared, proteins separated by SDS-PAGE and specific phosphorylation of ERK detected by Western Blot. Total ERK and actin proteins were detected as loading controls. Points of the graphs represent pools of triplicate cell cultures in each time point and are from one experiment.

4.4. Differential Th responses are induced *in vitro* by *M. tuberculosis*- or BCG-infected DCs

The presence of IL-12 (p40-p35) and IL-6 or TGF-β and IL-23 (p40-p19) in the cytokine milieu dictates the differentiation of Th1 or the differentiation and survival of Th17 responses, respectively (Veldhoen and Stockinger, 2006). Since *M. tuberculosis* or BCG stimulation of DCs induced a differential expression pattern of IL-12 and IL-23, but similar levels of IL-6 (Fig.1 and Fig.2), we decided to investigate if this difference resulted in differential Th responses developed in the presence of *M. tuberculosis* or BCG. A difficulty in studying early T cell differentiation is the low number of antigen-specific T cells present during immune initiation. Thus, we used a well stabilised model, the DO11.10 TCR-transgenic mice that express a TCR specific for $OVA_{323-339}$ peptide (OVA peptide), to evaluate the impact of *M. tuberculosis* or BCG infection on DCs-driven T cell differentiation. Bone marrow DCs were infected with *M. tuberculosis* or BCG (MOI of 2).

Twenty four hours later, the infected DCs were recovered and co-cultured with $CD4^+$ T cells isolated from the spleens of DO11.10 TCR transgenic mice in the presence of OVA peptide and IL-2, following a previously reported method. Three days later, the $CD4^+$ T cells were recovered and their cytokine profile, in terms of IFN-γ or IL-17 expression, determined by ELISPOT, using OVA peptide as antigen.

We observed that *M. tuberculosis*-infected DCs were able to induce the development of both Th1 and Th17 responses, whereas BCG-infected DCs induced preferentially Th17 responses (Fig. 4). As a control, non-infected DCs induced undetectable Th1 responses and very few IL-17-producing $CD4^+$ T cells (Fig.4). All together, our data strongly suggest that the differential activation of DCs responses by *M. tuberculosis* or BCG, observed in terms of signaling cascades activated and of differential expression of certain cytokines, does have an impact in terms of Th cell differentiation.

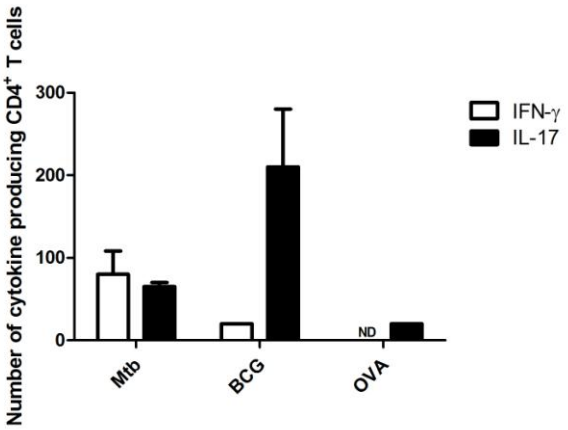

Figure 4 - Differential Th responses are induced *in vitro* by *M. tuberculosis*- or BCG-infected DCs. Purified DO11.10 transgenic $CD4^+$ T cells were cultured for 3 days with DCs from Balb/c mice. The DCs were either previously infected with *M. tuberculosis* or BCG (MOI of 2) or left uninfected. All cultures contained OVA peptide and IL-2. After 3 days the cells were washed and the ability of the differentiated $CD4^+$ T cells to produce IFN-γ or IL-17 in response to OVA peptide was determined by ELISPOT. Points of the graph represent pools of cells for each condition. Results show mean ± SD for ELISPOT dilutions from one experiment. Mtb (*M. tuberculosis*).

4.5. *M. tuberculosis* and BCG induce a differential kinetics of Th responses kinetics *in vivo*

Having observed *in vitro* that *M. tuberculosis*-infected DCs were able to induce the development of both Th1 and Th17 responses, whereas BCG-infected DCs preferentially induced Th17 responses *in vitro*, we wanted to explore if this observation had an *in vivo* parallel. To

investigate this, we infected mice intravenously with *M. tuberculosis* or BCG and at certain time points post-infection, the animals were sacrificed and their spleens harvested. Splenocyte suspensions from infected animals were prepared and restimulated *ex vivo* for 18 hours with a class I-A^b-restricted epitope of the dominant mycobacterial antigen, antigen85A, present in both BCG and *M. tuberculosis*. The splenocytes ability to produce IL-17 or IFN-γ was determined by ELISPOT. As shown in Fig.5, we observed a higher number of IFN-γ-producing $CD4^+$ T cells in *M. tuberculosis*-infected animals than in BCG-infected ones, for the tested time points. In contrast, BCG infection induced a higher number of IL-17-producing $CD4^+$ T cells as compared with the *M. tuberculosis* infection. Our *in vivo* data are therefore in line with the *in vitro* findings, thus suggesting that the differential Th responses observed for *M. tuberculosis* and BCG might be an interesting point to address and explore in the future in terms of vaccine development.

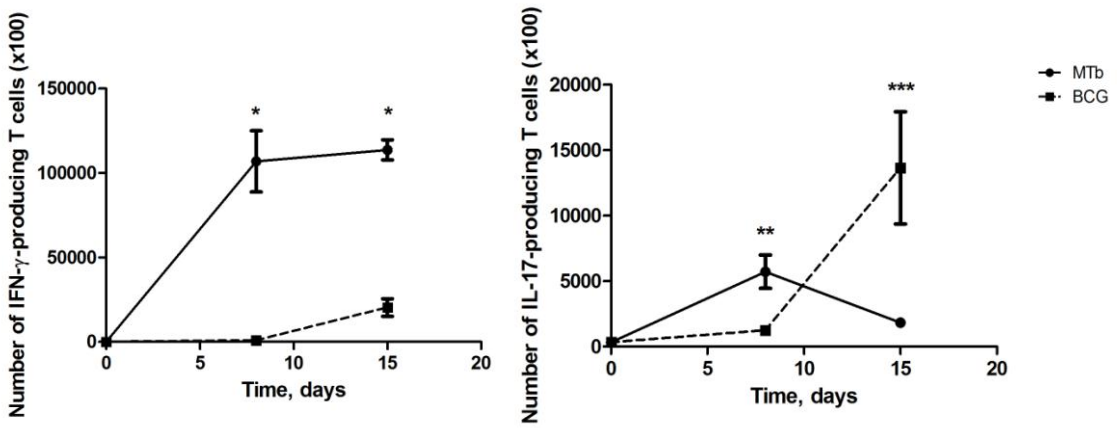

Figure 5 - Differential Th responses are induced by *M. tuberculosis* or BCG during an *in vivo* infection. BL/6 mice were infected intravenously with $1x10^6$ CFU of *M. tuberculosis* or BCG and at the indicated time points splenocytes were isolated and assessed for their ability to produce IL-17 or IFN-γ in response to antigen85 by ELISPOT. Results show mean ± SD from one experiment, *, **, ***, $p<0.05$, 0.01 and 0.001, respectively. Mtb (*M. tuberculosis*).

4.6. The growth of *M. tuberculosis* in infected macrophages is faster than the growth of BCG

In the previous sections, we showed that the ability of BCG- or *M. tuberculosis*- infected DCs to differentiate $CD4^+$ T cells into Th1 or Th17 cell responses was different, which was related to the differential response of these cells to each of the bacteria. We next questioned if the effector activity of macrophages in what regards the control of *M. tuberculosis* or BCG growth

was also different. To test this, macrophages were infected with these two mycobacteria (MOI of 2) and on specific time points post-infection, the number of viable bacteria was determined. We observed an increase of bacterial burden on *M. tuberculosis*-infected macrophages, 2 days after infection. At this time point, macrophages infected with BCG showed significantly less bacterial burden. However, 4 days post-infection, the bacterial load observed for both *M. tuberculosis*- and BCG-infected macrophages was similar (Fig.6). Our results thus suggest that the effector activity of macrophages, able to control BCG during the initial time points of macrophage infection, might be compromised in infections by *M. tuberculosis*.

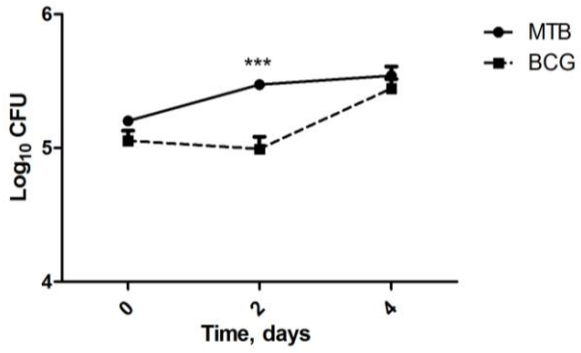

Figure 6 – The growth of *M. tuberculosis* in infected macrophages is faster than the growth of BCG. Macrophages from BL/6 mice were differentiated and exposed to *M. tuberculosis* or BCG at a MOI of 2. At the indicated time points, cells were lysed and the number of viable bacteria assessed by CFU counting. Each point is the mean of 6wells. Results show mean ± SD from two experiments; ***, $p<0.001$. MTB (*M. tuberculosis*).

4.7. The induction of NO by *M. tuberculosis* or BCG in infected macrophages is similar

Having observed a significant increase in the bacterial burden of *M. tuberculosis*-infected macrophages, 2 days after infection, in contrast to macrophages infected with BCG, we wanted to determine if the bactericidal activity of macrophages was more effective against BCG than against *M. tuberculosis* at that specific time point. Since the anti-mycobacterial function of macrophages has been associated, among other mechanisms, to the production of NO (Flynn and Chan, 2001), we analysed the generation of NO by *M. tuberculosis*- or BCG- infected macrophages. Macrophages were infected with *M. tuberculosis* or BCG. As the microbicidal activity of macrophages is highly potentiated by IFN-γ, we performed the infections in the presence or absence of this cytokine. At specific time points after infection, culture supernatants

were harvested and mixed with Griess reagent and NO release measured by spectrophotometry, using dilutions of $NaNO_2$ to obtain the standard curve. Infection by *M. tuberculosis* or BCG alone did result in detectable but very low levels of NO production. As expected, IFN-γ significantly increased the production of NO induced by both infections (Fig.7). However, the amount of NO released by macrophages upon infection by *M. tuberculosis* or BCG was similar. These results suggest that NO production most likely was not the effector mechanisms behind the increased protection observed on BCG-infected macrophages, at 2 days post-infection (Fig.6). Indeed, other bactericidal mechanisms of macrophages might account for the differential protection observed, as will be discussed later.

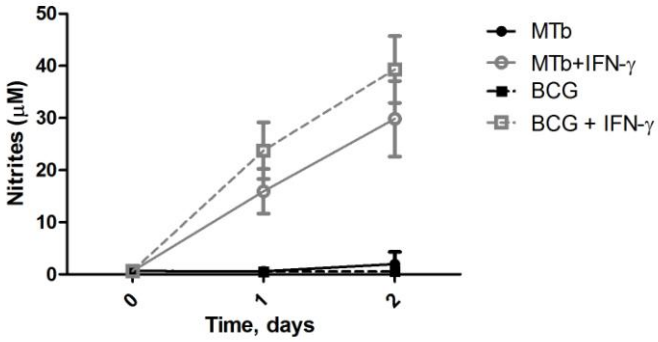

Figure 7- *M. tuberculosis*- or BCG-infected macrophages produce similar amounts of NO along the first 48 hours post-infection. Macrophages were differentiated from BL/6 mice and infected with *M. tuberculosis* or BCG (MOI of 2) in the presence or absence of IFN-γ. On day 0 and on days 1 and 2 post-infection, culture supernatants were recovered and the amount of nitrites quantified by the Griess assay (Turner et al., 2001). Each time point represents the mean of three wells. Results show mean ± SD from two experiments. MTb (*M. tuberculosis*).

4.8. *M. tuberculosis* induces high rates of caspase-3-mediated apoptosis at early time points post-infection

As the levels of NO production by macrophages following infection with *M. tuberculosis* or BCG were similar, we decided to investigate if other bactericidal mechanism were more effective during BCG than *M. tuberculosis* infection. During mycobacterial infections, macrophage apoptosis is associated with protection (Lee et al., 2009b). Since caspase-3 has been identified as being a key mediator of apoptosis, we decided to measure caspase-3 activation on macrophages infected with *M. tuberculosis* or BCG, by immunocytochemistry. As observed in Fig.8, the percentage of caspase-3 positive macrophages increased with both infections during

the time of infection. However, 2 days post-infection, *M. tuberculosis* induced higher percentage of caspase-3 activation in macrophages. At 4 days post-infection, this percentage was similar in both *M. tuberculosis* and BCG infections. In contrast to what happened with BCG infection, where a significantly increase of caspase-3 positive cells was observed over-time, during *M. tuberculosis* infection the number of caspase-3 positive cells was at its maximum on day 2 post-infection. Therefore, despite the existence of more cells undergoing apoptosis at early time points during the infection by *M. tuberculosis*, the bacterial burden was higher for this bacterium than for BCG. Our data strongly suggest that apoptosis induced by *M. tuberculosis* early on post-infection is not associated with a protective response.

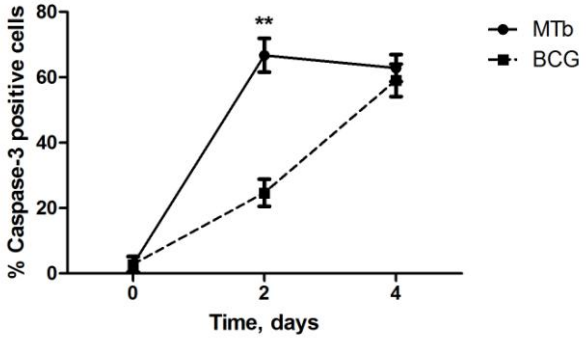

Figure 8 –*M. tuberculosis* induces high rates of caspase-3-mediated apoptosis at early time points post-infection. Macrophages were differentiated and placed under lamellae on the bottom of the wells of 24-wells incubation plaques. At specific time points after infection, the lamellae were collect and fixed in 2% PFA. Subsequently, the lamellae were incubated with the primary antibody against caspase-3 and detected with anti-rabbit IgG (H+L). DAPI was used to counter stained and to detect nuclei by fluorescence microscopy. Results show mean ± SD from two experiments, **, $p<0.01$. MTb (*M. tuberculosis*).

V. DISCUSSION

With one-third of the world's population infected with *M.tuberculosis*, TB remains an important disease not only in terms of public health implications but also because it is a disease that persists in the host.

The only vaccine currently available to prevent TB is BCG. It prevents disseminated TB in newborns, however fails to protect against the most common form of the disease, pulmonary TB in adults (Kaufmann, 2000). The reasons why the efficiency of BCG is variable are not fully understood, yet one possibility is that the immune responses triggered by BCG and *M.tuberculosis* might be different and so full protection is not achieved by vaccination. However, various experimental vaccines developed in recent years and tested in animal models proved to be less effective than BCG (Aagaard et al., 2009). This raises the issue that vaccine improvement needs to take into consideration the mechanism(s) of protection conferred by BCG and not only the immune response triggered by *M. tuberculosis*. Based on this rational, we proposed to compar the responses triggered by *M. tuberculosis* and BCG at various levels.

We started by comparing the cytokine responses of bone marrow derived macrophages and DCs to live *M. tuberculosis* or BCG. Our data showed that *M. tuberculosis* is a stronger trigger to DCs than BCG, while the macrophage responses to these two mycobacteria were similar (Fig.1 and 2).

Since cytokine production by DCs is central in the context of Th cell responses, we became particullary interested in the induction of cytokines that dictate Th cell differentiation and survival. In this this regard, we showed that DCs exposed to *M. tuberculosis* were induced to expressed high amounts of p40 and p19 monomers and of the respective bioactive molecules- IL-12 and IL-23, whereas BCG appeared to induce a "sub-optimal" stimulation of DCs with very little production of these cytokines. The observation that *M. tuberculosis*-infected DCs produce more IL-12p70 than BCG-infected DCs is in agreement with a recent study performed with human DCs (Giacomini et al., 2009). In contrast, IL-6 production by *M. tuberculosis*- or BCG-stimulated DCs was similar, and TNF secretion by DCs was higher with the BCG stimulus. Our data therefore suggest that distinct molecular pathways must be activated by *M. tuberculosis* and BCG in DCs (Fig.1 and Fig.2). Furthermore, we provide experimental evidence that *M. tuberculosis* and BCG stimulate macrophages and DCs differently, with distinct outcomes. Thus, why are macrophages and DCs responding differently to *M. tuberculosis* or BCG? Macrophages and DCs respond to

pathogen-derived products with the induction and production of effector molecules that regulate innate and adaptive immune responses (Akira, 2006; Beutler, 2004; Medzhitov, 2001). TLRs are within the PPRs that sense mycobacteria (Akira et al., 2006), leading to cytokine production. Data from our laboratory suggest that the main TLR involved in the recognition of *M. tuberculosis* and BCG by macrophages and DCs is TLR2. Therefore, the differences observed in terms of cytokine expression by DCs and macrophages in response to these mycobacteria is most likely not associated to a differential TLR recognition. TLR stimulation induces activation of MAPK such as ERK1/2 (Akira and Takeda, 2004). The signalling cascade involving ERK1/2 activation has been described before to be involved in the regulation of p40 and p19 gene expression (Goodridge et al., 2003; Jang et al., 2009; Kaiser et al., 2009; Saito et al., 2006). Since the differences we observed were precisely on the expression of these molecules, we decided to investigate a possible difference in terms of ERK1/2 activation. We report here that a differential activation of ERK1/2 is in place macrophages *versus* DCs stimulated with *M. tuberculosis* or BCG (Fig.3). Our data show that an earlier and stronger ERK phosphorylation occurs in macrophages stimulated with *M. tuberculosis*. In macrophages stimulated with BCG, the kinetics of ERK phosphorylation was similar to that observed in *M. tuberculosis*-stimulated macrophages, yet this activation was weaker. Additionally, a poor induction of ERK phosphorylation in DCs stimulated with BCG was observed in contrast to the other conditions. These results support our hypothesis that BCG induces a "sub-optimal" stimulation of macrophages and DCs and suggests the ERK1/2 pathway to be involved in this differential activation of DCs by *M. tuberculosis versus* BCG. Furthermore, we also found that ERK1/2 activation was different in macrophages *versus* DCs, stimulated with *M. tuberculosis*, being this stimulus in macrophages the strongest inducer of this cascade. Below, we propose a model (Fig.9) where the threshold of ERK1/2 appears to be an important regulatory mechanism for IL-12 and IL-23 induction when macrophages and DCs sense *M. tuberculosis* or BCG, although other mechanisms certainly also exist. Recently *Kaiser et al.* reported that TLR4 and TLR9 activation of ERK1/2 positively regulates IL-10 induction in myeloid macrophages and myeloid DCs, in contrast to its negative effects on IL-12 and IFN-β production (Kaiser et al., 2009). This work is in line with ours, suggesting that ERK1/2 signalling is interpreted differently by macrophages and DCs, and this might explain the different amounts of cytokine expression.

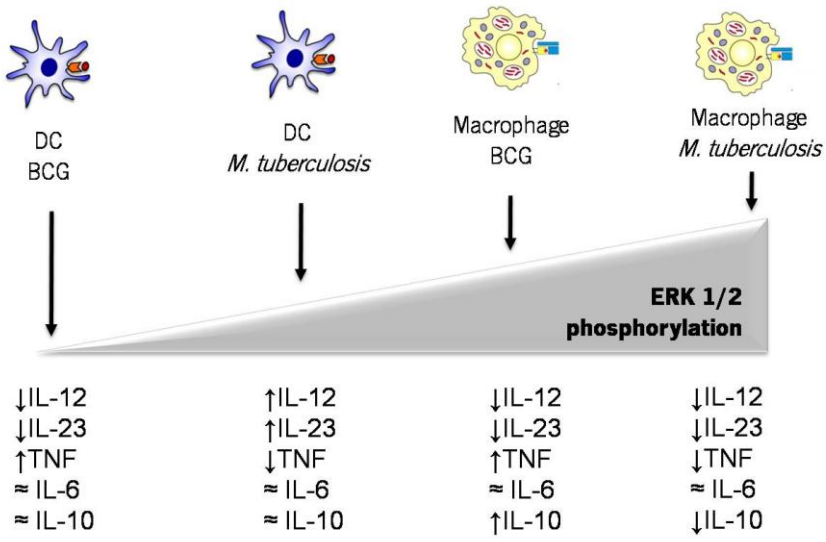

Figure 9 – Levels of ERK 1/2 phosphorylation induced by *M. tuberculosis* or BCG dictate the differential cytokines production by macrophages and DCs.

We now intend to investigate the molecular determinants that might explain the differential ERK activation by *M. tuberculosis* and BCG. We are also interested in determining whether the induction of other MAPKs is also differently activated in DCs, by *M. tuberculosis* or by BCG, and whether it contributes to explain differences in terms of cytokine expression. We will also use specific MAPK inhibitors to target the putatively differential signalling pathways. The differences in p40 and p19 expression will be further investigated at the molecular level, by including studies on cytokine gene regulation. The expression of transcription factors involved in the regulation of p40 and p19 genes, as well as their nuclear localization and function, will be addressed to find out whether *M. tuberculosis* or BCG lead to differential expression and/or function of transcription factors that ultimately explain the differences observed in cytokine responses. One transcription factor that has been proposed to be involved in differential stimulation of human DCs by *M. tuberculosis* or BCG is IRF-3 (Giacomini et al., 2009). This transcription factor was recently described to be activated upon *M. tuberculosis* infection, but not upon BCG infection of human DCs (Giacomini et al., 2009). It will be interesting to investigate if this is also happening in our system. Importantly, the molecular determinants that dictate the differential response of DCs to *M. tuberculosis* or to BCG might be associated with intrinsic properties of these mycobacteria. *Zenero and* collegues, have recently proposed that *M. tuberculosis* interacts with

selected receptors to subvert DCs maturation or change the pattern of DCs cytokine secretion (Zenaro et al., 2009). This issue will also be addressed in future experiments.

Since *M. tuberculosis* or BCG stimulation of DCs induced a differential expression pattern of IL-12 and IL-23 (Fig.1 and Fig.2), and taking into consideration the role of IL-12 (p40-p35) and IL-23 (p40-p19) molecules in Th1 differentiation and Th17 survival, respectively, our next goal was to determine the consequences of the differential activation of DCs by *M. tuberculosis* and BCG in $CD4^+$ T cell differentiation. We found that this differential activation of DCs was reflected on the distinct balance of Th responses developed when *M.tuberculosis*- or BCG-infected DCs presented OVA peptide to TCR-transgenic $CD4^+$ T cells (Fig.4). *M.tuberculosis*–infected DCs were able to induce the development of both Th1 and Th17 responses, which is in agreement with the secretion of IL-12, IL-23 and IL-6 by *M. tuberculosis*-stimulated DCs. On the other hand, we observed an unbalanced Th1/Th17 response differentiated in the presence of BCG-infected DCs. Indeed, BCG-infected DCs were able to induce a strong Th17 response, but only a limited Th1 response. We propose that the fact that BCG stimulation of DCs induced an amount of IL-6 similar to that induced by *M. tuberculosis* allows for the initiation of Th17 differentiation (Fig.1 and 2). It is surprising however that the strong Th17 response induced by BCG-infected DCs was not accompanied by the secretion of high levels of IL-23. However we only measured the production of this cytokine 24 hours post-infection and it is possible that IL-23 accumulates over time. Thus it is in our plans to measure IL-23 in supernatants of BCG-infected DCs during an extended period of time. It is also possible that the initial diminished IL-12 levels observed in BCG-infected DC, compromise Th1 differentiation, allowing for Th17 development. To test this hypothesis, we will perform the same assay, but we will exogenously provide IL-12 to the BCG-infected cultures, in order to see if the Th1/Th17 imbalance can be reverted. Additionally, several studies have shown that other cytokines play important roles in Th17 differentiation. This is the case of IL-21 (Fantini et al., 2007; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007) and IL-1β (Weaver et al., 2007). *In vitro* culture of naive $CD4^+$ T cells in the presence of IL-21 and TGF-β has shown to induced IL-17 production at levels similar to that induced by TGF-β and IL-6 (Fantini et al., 2007; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007). Moreover, it was demonstrated that Th17 development was impaired in the absence of IL-21 signalling (Fantini et al., 2007; Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007), and that in IL-21-deficient splenocytes, Th17 development *in vitro* and *in vivo* was almost completely abolished (Nurieva et al., 2007).

Similarly, a 2- to 3-fold reduction in IL-17 production was observed when purified $CD4^+$ T cells were cultured under Th17-inducing conditions in the presence of neutralizing anti-IL-21 antibody (Wei et al., 2007). It was also reported that IL-1β and IL-6 in conjunction with TGF-β were efficient inducers of Th17 differentiation from naive $CD4^+$ T cell precursors (Weaver et al., 2007). Therefore, monitoring the expression of IL-21 and IL-1β induced by *M. tuberculosis* and BCG will be also addressed in our future experiments. As for TGF-β, our preliminary data suggest that very low levels of this cytokine are being produced by DCs in response to *M. tuberculosis* and BCG. It is also important to refer that it is unlikely that suppressive effects mediated by IL-10 account for the differences observed, as the levels of this cytokine detected upon stimulation of DCs with *M. tuberculosis* or BCG were similar. Finally, another possible explanation for the Th17 shift observed in BCG-stimulated DCs relies on a differential induction of co-stimulatory molecules expressed by DCs. This will be addressed by measuring the presence of the main co-stimulatory molecules in *M. tuberculosis- versus* BCG-infected DC by flow cytometry. This hypothesis is in line with a recent study showing that BCG is less efficient in inducing human DCs maturation than *M. tuberculosis* (Giacomini et al., 2009).

Our *in vitro* studies on Th cell differentiation in response to *M. tuberculosis* or BCG were further explored *in vivo* (Fig4. and Fig.5). We showed that, for the time points tested, a higher number of IFN-γ-producing $CD4^+$ T cells developed in *M. tuberculosis*-infected animals than in BCG-infected ones. The peak of IL-17-producing $CD4^+$ T cells for *M. tuberculosis*-infected animals (day7) appeared to occur earlier than for BCG-infected animals. However soon after that peak, Th17 induced by BCG rose sharply. We are now interested in addressing weather these differences are maintained over the time and whether they might be related to different bacterial load in BCG- or *M. tuberculosis*-infected animals. These questions will be addressed in future experiments. Below, we propose a model (Fig.10) for IFN-γ and IL-17 -producing $CD4^+$ T cells developed by *M. tuberculosis* and BCG infections, with possible factors that might explain the observed differences.

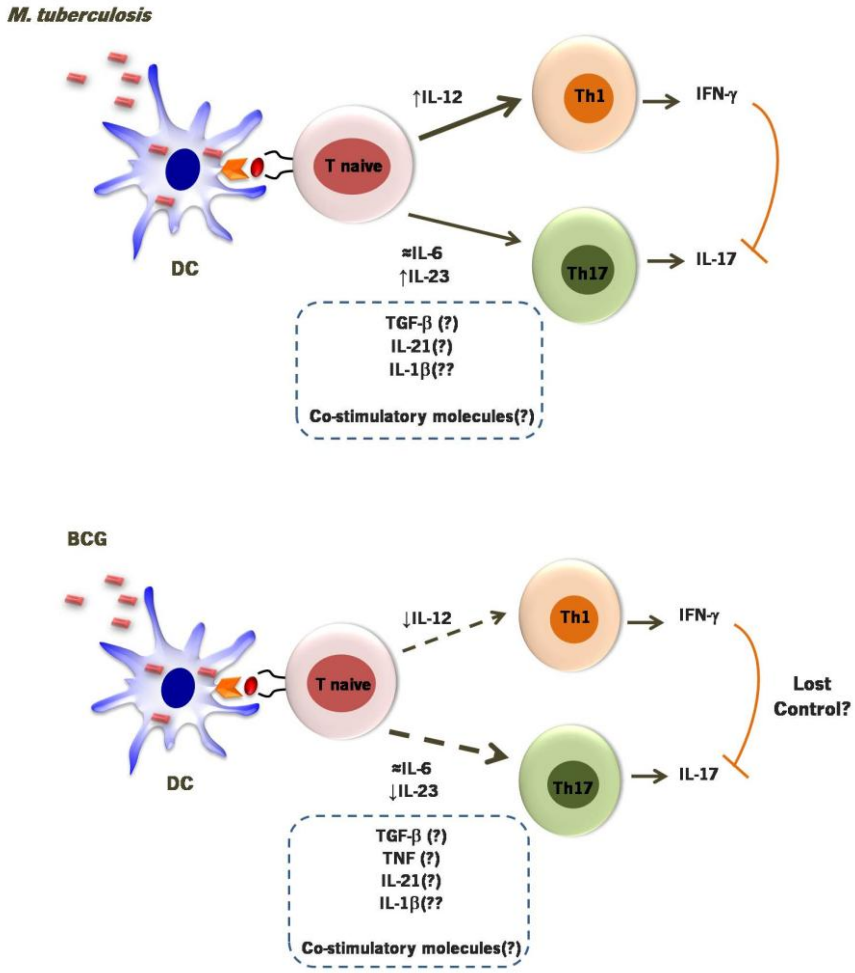

Figure 10 – Balance of Th cell differentiation in response to *M. tuberculosis* or BCG.

Our findings might be important for the understanding of the development of the immune response to BCG as compared to that of *M. tuberculosis*. Our findings suggest that BCG induces the development of an effective Th17 response that does not appear to be fully controlled by Th1 cells, which might be important for the protection achieved by this vaccine. This is in line with a previous report showing that Th17 responses are needed to achieve protection against TB, due to their role in recruiting Th1 cells to the site of infection (Khader et al., 2007). On the other hand, it has been suggested that increased Th1 responses or IFN-γ production are not necessarily needed to achieve high protection against TB (Jeevan et al., 2009). Therefore, our data is in agreement with the idea that the development of new vaccines to TB should consider the improvement of Th17 responses in an initial phase, and not only a very potent IFN-γ production.

As part of this work, we have also compared the macrophage response to *M. tuberculosis* or BCG. Although the cytokine expression pattern was similar in macrophages stimulated with *M. tuberculosis* or BCG (Fig.1), it appeared that BCG presented a delayed growth (day 2 post infection) in macrophages when compared to *M. tuberculosis*. To further clarify if the anti-mycobacterial activity of macrophages was more effective, at this time point, against BCG than against *M. tuberculosis,* we started by measuring NO production (a fast and earlier bactericidal mechanism) by BCG- or *M. tuberculosis*-infected macrophages. Here we showed that NO production most likely was not the effector mechanism behind the protection observed on BCG-infected macrophages at 2 days post-infection (Fig.6). Nonetheless, other bactericidal mechanisms of macrophages might account for the differential protection observed, such as H_2O_2/O^-_2, defensines, lysosomal hydrolases, chemokines, and phagosome-lysosoma fusion. The expression of several markers that follows phagosome maturation and phagosome-lysosome fusion, including tryptophan-aspartate containing coat protein (TACO), lysosomal-associated membrane protein (LAMP-1) and LRG-47 (Fol et al., 2006; Pieters, 2001) will be targets of further studies.

We also questioned whether the observed differences could be related to a distinct induction a apoptosis. Apoptosis of cells infected with intracellular pathogens may benefit the host by eliminating a supportive environment for bacterial growth. Members of the caspase family are central to initiation and execution of apoptosis and caspase-3 is one of the main forms activated during this pathway (Lee et al., 2009b). Our results show that *M. tuberculosis* induced high rates of caspase-3-mediated apoptosis in macrophages at early time points post-infection, reaching its maximum 2 days post-infection, while in BCG infection a significantly increase of caspase-3 positive cells was observed over the time of infection. Despite the existence of more cells undergoing apoptosis at 2 days post infection in the infection by *M. tuberculosis*, the bacterial burden was higher for this bacterium. This might mean that at this time point, *M. tuberculosis* is already able to survive and replicate within macrophages, thus suggesting that the type of apoptosis induced by *M. tuberculosis* early on infection is not associated with a protective response. However, *Keane et al* reported that the attenuated *M. tuberculosis* strain H37Ra was a more potent inducer of apoptosis than the virulent strain H37Rv (Keane et al., 1997). These authors proposed that bacillary control of the host cell apoptosis is a virulence-associated phenotype of *M. tuberculosis* strains and suggested that alveolar macrophages apoptosis contributes to innate immunity in TB (Keane et al., 1997). In a way, our results point to the

opposite idea, since in our study, the avirulent strain (BCG) was a slower inducer of apoptosis. We nonetheless must take into account the technical differences between the two works, such as the type of cells (alveolar *versus* bone marrow-derived macrophages), the bacterial strains (H37Ra *versus* BCG)and the doses of mycobacteria used (MOI of 5 *versus* MOI of 2), as well as the assessment of apoptosis (DNA fragmentation *versus* caspase-3). Future studies are required to clarify the differences observed in terms of *M. tuberculosis* or BCG growth within the infected macrophage.

VI. CONCLUSIONS

In this work we were able to show that macrophages and DC respond differently to *M. tuberculosis* or BCG stimulation, and this might be due in part to a differential activation of ERK 1/2 induced by *M. tuberculosis* or BCG. A consequence of this differential activation of DC was reflected on the distinct type of Th responses developed, both in vitro and in vivo. The effector activity of macrophages in what regards the control of *M. tuberculosis* or BCG growth was also different, although the differences found were not due to differential NO production or apoptosis.

A detailed comparative study of BCG *versus M. tuberculosis* infection on macrophages and DC might provide insights on how these mycobacteria interact with the immune system and how that translates into an effective, or not, Th cell response and also the mechanisms used by the pathogen to modulate the immune response. Furthermore, dissecting the cellular and molecular events that occur upon mycobacterial challenge will help to reveal possible weak points of both the host and the bacteria that can be targeted from a prophylactic point of view.

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