DISCUSSION

Pathogenic mycobacteria have developed several strategies to interfere with innate immune responses and manipulate defense mechanisms to enhance their survival within the human host cell and achieve long term persistence. Although several studies have demonstrated that mycobacteria are able to dampen or subvert the host innate immune response (Giacomini et al. 2001; Nau et al. 2002; Beltan et al., 2000), the molecular mechanism underlying this phenomenon is still largely unknown. Because of their importance to virulence and bacterial viability, the exported proteins of Mtb and their respective protein export systems plays an important role in the biology and pathogenicity of the etiological agent of human TB, and have been subject of numerous studies from various research groups.

The role of the ESX-5 system in the export of selected PPE and PE proteins in *M. marinum*, as well as the involvement of this system in modulation of cytokine responses by *M. marinum* infected human macrophages are well established (Abdallah et al., 2006; 2008; 2009; Daleke et al., 2011). In contrast, the function of the ESX-5 system in Mtb and its impact on virulence and host-pathogen interaction remains to be elucidated.

Therefore, in this study we have analyzed the ability of several ESX-5 mutant strains to modulate the immune response of human professional phagocytes. Furthermore, since our findings show that the Mtb*rv1794ko* mutant strain is able to induce a higher production of IL-1β and IL-18 in both Mφ1 and Mφ2, we have dissected the molecular pathway responsible for the inflammasome activation to achieve a better understanding of the mechanism(s) leading to the increased IL-1β and IL-18 production by Mtb*rv1794ko* infected cells.
Phagocytic cells play important roles in the initiation and maintenance of protective immune response following infection with Mtb. The ability of Mtb to infect and persist within these specialized cells is a result of the bacteria’s ability to adapt to a fluctuating host environment and to utilize the sources available to it within the host (Hestvick et al., 2005).

According to many studies aiming at deciphering the molecular cross-talk between pathogen and host, we performed our investigations in MoDCs and polarized type-1 and type-2 macrophages, which bear resemblance to in vivo infection. The availability of well-defined pro- and anti-inflammatory human macrophages subsets and DCs in vitro allows a comprehensive analysis of their functional profiles and their responses induced by mycobacteria.

In line with recent works (Verreck et al., 2004; 2006), we observed that, although both macrophage populations support the intracellular growth of mycobacteria, Mφ1 appear as mononuclear phagocytes, which are distinct from DCs but share a pro-inflammatory cytokine secretion profile with mature DCs, whereas Mφ2 exhibit a suppressive cytokine profile. However, we observed that, although Mφ2 showed a typical anti-inflammatory profile when stimulated with LPS from E. coli, upon infection with mycobacteria they were able to secrete pro-inflammatory cytokine such as IL-1β and IL-18 at high levels, emphasizing the capability of these polarized cells to adjust their response according to the changing environmental conditions.

As the maturation status of DCs is characterized by the upregulation of the expression of MHC molecules and costimulatory molecules, we first analysed the expression of MoDC cell surface markers. We found no difference among MoDCs infected with Mtbwt and the ESX-5 mutants for any of the maturation markers analyzed. Similarly, no differences among MoDCs infected with Mtbwt and the ESX-5 mutants for the profile of cytokines analysed were observed. Thus, inactivation or deletion of various
ESX-5 components do not appear to have an impact on the maturation of human MoDCs in vitro.

Although we found no difference among Mϕ1 and Mϕ2 infected with Mtbwt, MtbeccDsko or MtbΔppe25-pe19 for the profile of cytokines analysed, we observed that that disruption of rv1794 gene resulted in significant increase of secretion of pro-inflammatory cytokines IL-1β and IL-18 from both human macrophage populations. Moreover we observed that when the two macrophage subsets were infected with hk bacteria, this difference was abolished and the IL-1β and IL-18 levels were significantly reduced as compared to cells infected with live bacteria, suggesting that metabolically active bacteria might be essential to induce IL-1β and IL-18 secretion from human macrophage populations. These data are in keeping with a previous report in which hk Mtb results in an attenuated secretion of IL-1β, demonstrating that bacterial viability is important in signalling release of IL-1β during infection (Koo et al., 2008). The complemented strain Mtbrv1794ko-C induced release of IL-1β and IL-18 at levels comparable to those of Mtbwt, proving that the presence of rv1794 gene reduces production/secretion of these cytokines.

In contrast to our results, it has been demonstrated that macrophages infected with a M. marinum mutant strain for the MMAR_2676 gene (the rv1794 orthologue in Mtb) triggers no IL-1β production as compared to the wild type strain (Abdallah et al., 2008; 2011). However the rv1794, in contrast to its orthologue MMAR_2676 which in M. marinum was reported to affect EsxN secretion and export of various PE and PPE proteins (Abdallah et al., 2009), is not implicated in the secretion of EsxN in Mtb H37Rv (Bottai et al., 2012). These findings suggest that, in spite of high amino acid similarity (96% identity), Rv1794 and MMAR_2676 might have different functions in the ESX-5 secretion machinery that have evolved as a consequence of the adaptation of these two mycobacterial species to
different hosts. Substantial differences in function of proteins encoded by ESX clusters from different mycobacterial species have been previously described for the ESX-1 secretion associated protein EspG1 (Bottai et al., 2011), which shows weak similarity with Rv1794. While EspG1Mma is required for EsxA secretion and virulence in M. marinum (Gao et al., 2006), the EspG1Mtb is not directly involved in EsxA secretion, although it plays a relevant role in virulence of Mtb (Bottai et al., 2011).

The roles of IL-18 and IL-1β in the pathogenesis of TB still remain controversial. These cytokines, in concert with other cytokines or chemokines, may exert both beneficial and detrimental effects to the host, resulting in a complex pathology.

A study comparing patients with pulmonary TB with control subjects demonstrated that both IL-1β and IL-18 are necessary for the formation of the granuloma, in which bacteria can establish long-lived latent infection (Pechkovsky et al., 2006). Thus, IL-1β and IL-18 appear to be involved in the creation of a niche for the bacteria protected from a sterilizing immune response. On the contrary Master et al. have demonstrated that IL-1β is an effective anti-TB agent when induced or exogenously supplied (Master et al., 2008). Complementary to these findings a recent report has indicated that IL-1 receptor may play a role in the control of Mtb infection (Fremond et al., 2007).

In order to get insights about the possible mechanism(s) of the augmented IL-1β and IL-18 secretion by the Mtb rv1794ko infected macrophages, we tried to investigate the cellular pathways underlying the synthesis and secretion of these cytokines.

Production and release of IL-1β and IL-18 are regulated at several levels: they are first synthesized as biologically inactive pro-form, then processed into mature, active cytokines by caspase-1, and subsequently released into the extracellular milieu. Hence, we first determined whether Mtb rv1794ko triggers a major mRNA expression of IL-1β and IL-18 in human
macrophages at 4h and/or 24h post infection. We determined that the mRNA expression of pro-IL-1β was equally induced in all the experimental conditions. Consistent with the IL-1β mRNA expression pattern, Western blot analyses of cell lysates showed no differences in the protein levels of pro-IL-1β, whereas higher levels of the active IL-1β were detected in culture supernatants of macrophages infected with Mtb_{rv1794ko} as compared to Mtb_{wt} infected cells. It has been shown that IL-1β and IL-18 are secreted immediately after conversion into mature forms (Mariathasan et al., 2006). Concordantly, we detected IL-1β in the culture supernatant but not in the cell lysate, suggesting that IL-1β is secreted after conversion from pro-IL-1β to activated IL-1β in this experimental system. These data suggest that the significantly higher IL-1β and IL-18 levels observed in the supernatants of Mtb_{rv1794ko} infected macrophages as compared to Mtb_{wt} might be due to a higher rate of processing rather than a higher production of such cytokines.

The processing of IL-1β is reported to be mediated by caspase-1 activation (Fantuzzi and Dinarello 1999; Burns et al., 2003). Indeed in our experimental conditions, IL-1β and IL-18 secretion upon infection with Mtb was dependent on caspase-1 activation, as caspase-1 inhibitor Ac-YVAD-fmk strongly reduced the secretion of these cytokines. When we analysed both the mRNA expression and the activation status of caspase-1 the results indicated that the caspase-1 activity was increased in macrophages infected with Mtb_{rv1794ko} as compared to cells infected with Mtb_{wt}, although no significant differences in caspase-1 mRNA levels were observed between macrophages infected with Mtb_{rv1794ko} and Mtb_{wt} or Mtb_{rv1794ko-C} infected cells.

A possible explanation for this observation is that the rv1794 gene product is, directly or indirectly, involved in the induction of IL-1β activation possibly through modulating the activation of caspase-1. The exact
mechanism by which caspase-1 activation occurs remains unclear and different possible pathways of caspase-1 activation via microbial components in the host cytosol through NLR proteins, has been previously described. For instance, *S. typhimurium* uses a type III secretion system to secrete SipB into the cytosol of macrophages, which binds and activates caspase-1 in infected cells (Cook *et al.*, 2007). Similarly, *Helicobacter pylori* uses a type IV secretion system for the delivery in the cytosol of infected host cells of peptidoglycan-derived molecules, which activates NOD1, another NLR family member (Viala *et al.*, 2004). Thus, it would not be surprising that mycobacterial ESX effector molecules may interact, either directly or through another host factor in the cytosol, with inflammasome adaptors to promote the activation of caspase-1. As the mycobacterial peptidoglycan fragment MDP can activate NLRP3 inflammasomes (Martinon *et al.*, 2004) and ESX-1 enables phagosomal translocation, Koo *et al.* (2008) have previously suggested that ESX-1 allows entry of MDP or other bacterial products into cytosol to induce inflammasome activation. The ESX-1 substrate ESAT-6 was subsequently reported to have a direct activation effect on inflammasomes (Mishra *et al.*, 2010). This activation will lead to the concomitant release of the pro-inflammatory cytokine IL-1β. Nevertheless, negative regulation of caspase-1 processing has been identified as well. The cowpox virus inhibits inflammasome activation via a direct block of caspase-1 activation (Johnston *et al.*, 2005). Pneumolysin-deficient *Streptococcus pneumoniae* induce elevated caspase-1 activation and IL-1β secretion in human DCs (Littmann 2009). This is surprising since other pore-forming toxins, by contrast, are known to activate caspase-1 (Cordoba-Rodriguez 2004, Craven 2009). Master *et al.* have demonstrated that Mtb prevents the activation of the inflammasome in caspase-1 dependent manner, suggesting that it may dampens the early host response by limiting the IL-1β activation/secretion.
Considering the growing body of evidence suggesting the strong potential of IL-1β action against mycobacteria (Juffermans et al., 2000; Yamada et al., 2000; Fremond et al., 2007), our data might be considered in agreement with the observations of Master et al. stressing the hypothesis that certain pathogenic microorganisms modulate inflammasome activation in ways best suited to their infectious cycles. Since IL-1β is critical in the effort to eradicate foreign microbial infection, inflammasome-mediated signaling pathways provide an obvious target for achieving pathogenic stealth by minimizing the host immune response.

Pro-IL-1β is processed mainly through caspase-1-dependent inflammasome complexes to generate mature, bioactive IL-1β, although caspase-1 independent processing via noncanonical inflammasomes has also been reported (Netea et al., 2010; Kayagaki et al., 2011). Interestingly, it has been recently demonstrated that dectin-1, a C-type lectin receptor mainly expressed on DCs, functions as an extracellular sensor for fungal and mycobacterial PAMPs that directly activates caspase-8 for the processing of pro-IL-1β (Gringhuis et al., 2012). Since we hypothesized that the rv1794 gene product might directly or indirectly interfere with caspase-1, the emergence of noncanonical inflammasomes dependent on caspase-8 for the processing of pro-IL-1β in DCs, might represent a possible explanation to the fact that we observed no difference in IL-1β production in DCs infected with Mtbwt or Mtbrv1794ko.

Several inflammasomes activate IL-1β during bacterial infections, such as the NLRP3 inflammasome that recognizes bacterial ligands including MDP (Martinon et al., 2004), the NLRP1 inflammasome that recognizes anthrax lethal toxin (Boyden and Dietrich, 2006), and the Salmonella induced Ipaf inflammasome (Franchi et al., 2006; Miao et al., 2006). It has been found that NLRP3 and ASC are involved in the activation of caspase-1 in Mtb-infected macrophages (Koo et al., 2008). In line with these findings, we observed that the mRNA expression of NLRP3 and ASC was upregulated
following infection with mycobacterial strains, whereas no significant
differences in the mRNA levels were observed between Mtbw and
Mtbrv1794ko.
Various danger signals activate the NLRP3 inflammasome. The
mechanisms by which these structurally distinct molecules trigger NLRP3
oligomerization and inflammasome activation are currently unclear
(Tschopp and Schroder, 2010; Bryant and Fitzgerald, 2009). However, all
the proposed models agree that [K+] crucially affects inflammasome
activation. Bacterial components, such as LPS or peptidoglycan, have been
proposed to be delivered into the cytosol via pannexin-1, a plasma
membrane protein that is responsible for the large change in intracellular
[K+] and subsequent caspase-1 activation (Kanneganti et al., 2007;
Marina-Garcia et al., 2008). IL-1β processing and release by human
macrophages infected with M. abscessus were found to be dependent on
K+ efflux (Lee et al., 2011). Moreover, a recent evidence suggested that the
RD1 locus in the Mtb genome is implicated in the activation of caspase-1
via induction of the K+ efflux in infected macrophages (Kurenuma et al.,
2009). In agreement with recent studies, we have observed that the
presence in culture medium of KCl, to inhibit the potential K+ efflux,
reduced the IL-1β and IL-18 production by macrophages infected with all
the mycobacterial strains used in our experiments. A possible explanation
to these observations might be given by a recent study in which it has
been demonstrated that the ESX-1 secretory system of Mtb is capable of
delivering several effector proteins to the host cytosol (Koo et al., 2008).
These mycobacterial factors may cause changes in the membrane
integrity, leading to a decrease in the intracellular potassium level, which,
in turn, contributes to the formation of the inflammasome and
consequently IL-1β and IL-18 production and secretion.
In addition to K+ efflux, other mechanisms might control the assembly of
the NLRP3 inflammasome. Inflammasome activation has recently been
reported to be triggered by cathepsin B in the context of infection (Meixenberger et al., 2010; Duncan et al., 2009; Willingham et al., 2007). Moreover, sterile stimuli such as MSU, beta-amyloid and various environmental insults (e.g. silica, asbestos), induce inflammasome activation through the release of cathepsin B due to phagosomal rupture (Hornung et al., 2008; Rock et al., 2010), a process which has been described to be a determining factor for mycobacterial pathogenicity (Abdallah et al., 2008).

Abdallah et al. have suggested that mycobacteria trigger the disruption of lysosomes leading to the release of the cathepsin B, which represents a novel inflammasome activation pathway. In agreement with that study, we observed that inhibition of cathepsin B, but not other proteases such as cathepsin D or cathepsin L, strongly reduced the secretion of IL-1β and IL-18 as compared to untreated cells, suggesting a specific role for cathepsin B in inflammasome activation.

As a mechanism of spreading, intracellular microorganisms must leave the infected host cells. Killing of macrophages may allow bacteria to escape, infect neighboring cells and disseminate to other tissues. Mtb infected macrophages can undergo two general models of cell death: apoptosis and necrosis. These two forms of cell death appear to have drastically different outcomes for the course of infection. Apoptosis (programmed cell death) is an energy-dependent process mediated by the caspase cascade, which results in the ordered degradation of cellular contents and the formation of apoptotic vesicles. It has been demonstrated that apoptotic cell death of Mtb infected macrophages is directly associated with mycobacterial killing (Oddo et al., 1998; Lopez et al., 2003; Lee et al., 2009).

On the other hand, necrotic cell death is associated with the disordered, energy independent death of the cell. In Mtb infection, a necrosis-like form of death has been observed and demonstrated to allow the release of viable mycobacteria for subsequent re-infection (Lee et al., 2011). Necrotic cell
death may be an important factor in granuloma formation, inflammatory tissue damage and, ultimately, transmission of the bacterium. Several studies have suggested that pathogenic Mtb strains use inhibition of apoptosis and induction of necrotic death as a virulence mechanism (Zang et al., 2005; Park et al., 2006; Chen et al., 2006; Velmurugan et al., 2007; Sohn et al., 2009).

Consistent with this idea, we observed that the percentage of dead cells upon infection with all the mycobacterial strains tested was higher than that of apoptosis, thus supporting the hypothesis that virulent Mtb can actively promote necrosis over apoptosis.

**CONCLUSION**

Type VII Secretion Systems play important roles in the biology and pathogenicity of Mtb, which makes them interesting and important targets for potential new prevention and control strategies. In this study we investigated the ability of several ESX-5 mutant strains to modulate the immune response of human phagocytes. We found that the Rv1794 protein seems to modulate the production of IL-1β and IL-18 from differentiated human macrophage populations. Our results suggest that the augmented production of IL-1β and IL-18 in macrophages infected with Mtbrv1794ko as compared to Mtbwt infected cells might be due to a higher rate of processing rather than a higher production of such cytokines. Thus we hypothesize that Rv1794 protein may directly or indirectly interfere with the caspase-1 activation, and, in turn, impair the production/secretion of two key pro-inflammatory cytokines that contribute to host defence against infection.