I. INTRODUCTION

1. Principles and rationale

Tuberculosis still remains a major public health problem throughout the world. By 1993 the World Health Organization (WHO) declared tuberculosis to be a global emergency infectious disease. It is estimated that one-third of world's population, 2 billion out of 6 billion, are infected by the tubercle bacilli and 8 million new cases of tuberculosis were occurring each year worldwide (Davies, 2001). A recent report by the WHO predicts that by the year 2005, this disease will kill 4 million people annually. This is a significant increase from an estimated 3 million deaths worldwide caused by TB in 1992. This resurgence has been linked to the lack of wide array of chemotherapeutic agents against its causative agent, *Mycobacterium tuberculosis*, and the deveolpment of the multidrug-resistant (MDR) strains (Fenton and Vermeulen, 1996). More than 50 million people are already infected with MDR strains of *M. tuberculosis*. A situation has been exacerbated by the spread of human immunodeficiency virus (HIV), which is the greatest known risk factor for tuberculosis, increasing the risk of infection resulting in disease by at least 100-fold. Half a million people died in the past year because of co-infected with *M. tuberculosis* and the HIV (Kaufman, 2002).

While TB is a preventable and largely curable disease, our understanding of the cellular and molecular interactions between mycobacteria and host immune cells is far from complete, and the topic presents significant research challenges. Understanding the pathology of mycobacterial diseases such as tuberculosis requires knowledge of the interaction of the mycobacterium with its host cell, the macrophages. The consequences of mycobacterial infection are dependent on two parameters: the virulence of the infecting mycobacteria as well as the immune response against the organisms of the host. During the initial phase of *M. tuberculosis* infection, bacteria that reach the distal airspace of the lung are phagocytosed by alveolar macrophages. *M. tuberculosis* enters the macrophage via receptor-assisted phagocytosis that is mediated by several distinct cell surface molecules. Following attachment and

subsequent phagocytosis of *M. tuberculosis*, sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enzymes, reactive oxygen intermediates (ROIs), and reactive nitrogen intermediates (RNIs) such as nitric oxide (NO). A principal mechanism by which *M. tuberculosis* avoids these dangers is the arrest of the maturation of its phagosome at a stage in which the phagosome interacts with early and late endosomes, but not with lysosome (Clemens et al., 2002). This arrest of normal phagosomal maturation prevents acidification of the vesicles, a process that has been associated with *M. tuberculosis*-dependent retention of the protein TACO (Ferrari et al., 1999). Macrophages produce reactive oxygen species and reactive nitrogen species that have potent antimicrobial activity, however, *M. tuberculosis* can produce a Cu, Zn superoxide dismutase, which has superoxide dismutase activity, to avoid from annihilation by oxidative burst products generated by activated macrophages (Piddington et al., 2001).

Additionally, it has been reported that when the surface capsule-like layer of the mycobacteria was removed by sonication, resulting in an enhanced propensity of the mycobacteria to bind to macrophages. Enhanced binding of mycobacteria to macrophages following sonication is significantly greater within members of *M. tuberculosis* family (pathogens) than within the *Mycobacterium avium* complex (opportunistic pathogens) or for *Mycobacterium smegmatis* (saprophyte). These data demonstrated that the surface capsule on members of the *M. tuberculosis* family, which is pathogens, may be an important virulence factor involved in the survival of *M. tuberculosis* in the mammalian host (Stokes et al., 2004).

Apoptosis of macrophages may be a pathogen-directed mechanism of immune escape or may represent appropriate host response to infection. As found in *Shigella flexneri*-infected macrophages, which directly activation of caspase-1 by the invasin protein antigen, resulting in apoptosis of infected macrophages (Zychlinsky et al., 1992). Similar mechanisms result in macrophage apoptosis associated with *Salmonella typhimurium*, in which host cell killing is associated with survival of intracellular bacteria (Monack et al., 1999). The immune response of macrophages to *Streptococcus pneumoniae* includes a novel form of apoptosis that is associated with successful phagocytosis and bacterial killing (Dockrell et al., 2001).

Macrophage apoptosis has also been suggested as an important mechanism for killing of intracellular mycobacteria. Macrophage apoptosis occurs within the granuloma, which is essential for successful immunity to tuberculosis. In vitro macrophage apoptosis is associated with the killing of intracellular *Mycobacterium tuberculosis*. A greater understanding of these observations will lead to new immunotherapies and improved vaccine design (Fairbairn, 2004). From the studied of Molloy and colleagues demonstrated that monocytes infected with bacillus Calmette-Guerin (BCG), which the monocytes were killed by exposure to toxic mediator including an inducer of cell necrosis (hydrogen peroxide, H_2O_2) and an inducer of cell apoptosis (adenosine triphosphate, ATP^{4-}), had a different in viability of BCG. They found that H_2O_2-induced killing had no effect on BCG viability. In contrast, ATP^{4-}-induced killing was associated with a reduction of BCG viability as enumerated by colony-forming units (Molloy et al., 1994).

Furthermore, an inverse correlation between *M. tuberculosis* virulence and ability to induce alveolar macrophage apoptosis has been observed. An attenuated strain of *M. tuberculosis* (H37Ra) was shown to induce more apoptosis of infected human alveolar macrophges than a virulent strain of *M. tuberculosis* (H37Rv) (Keane et al., 1997). Increased apoptosis was associated with decreased growth of intracellular *M. tuberculosis*. Specifically, virulent *M. tuberculosis* grew more rapidly in alveolar macrophages, compared to an attenuated strain, while causing less macrophage apoptosis. These data indicated that increased intracellular bacillary burden is not responsible for the observed high alveolar macrophage apoptosis rates with these strains and suggests that host macrophage apoptosis might contribute to mycobacterial growth restriction (Keane et al., 2000 ; Silver et al., 1998). It has been reported that incubation of uninfected autologous macrophages with apoptotic macrophages infected with *M. avium*, an opportunistic pathogen in AIDS patients, results in 90% inhibition of bacterial growth. The uninfected macrophages adhere to *M. avium*-infected apoptotic, but not to nonapoptotic *M. avium*-infected macrophages. Apoptosis of the host macrophages also prevents the release of the intracellular components and the spread of the mycobacterial infection. These findings indicate that apoptosis of *M. avium*-infected macrophages is an important defense mechanism (Fratazzi et al., 1997). In macrophages infected either with an attenuated (H37Ra) or

with a virulent (H37Rv) strain of *M. tuberculosis*, the apoptotic death of macrophages was associated with a substantial reduction in bacillary viability. Infected macrophages also showed a reduce susceptibility to FasL-induced apoptosis correlating with a reduce level of Fas expression. This finding suggested that interference by *M. tuberculosis* with the FasL system might represent an escape mechanism of the bacteria attempting to evade the effect of apoptosis (Oddo et al., 1998). Furthermore, the 19-kDa *M. tuberculosis* glycoprotein (p19) is both cell wall-associated and secreted and is a candidate virulence factor showed to induce apoptosis in differentiate THP-1 cells and monocyte-derived macrophages and that this effect is both dose- and time dependent. Moreover, this effect of p19 is mediated through Toll-like receptor 2 (TLR2) (Lopez et al., 2003).

The study to define the microbial factors that cause apoptosis demonstrated that some components of *M. tuberculosis*, such as cord factor or mycolic acid or whole bacteria can prolong cell survival compared to control. Conversely, tuberculostearic acid induced early cell death. The result also showed that prolonged viability of the treated cells with mycolic acid or cord factor was correlated with a significant increase in Bcl-2, which is an inhibitor of apoptosis, expression (Nuzzo et al., 2002). Interestingly, Klingler and colleagues have demonstrated that the Bcl-2 protein, an inhibitor of apoptosis, was downregulated in peripheral blood monocytes (PBM) between 2 and 6 h following infection with *M. bovis* BCG or induction with heat-killed *M. tuberculosis* H37Ra. At the same time points, the expression of Bax or Bcl-xs, an inducer of apoptosis, did not change. They also observed significantly more apoptosis in alveolar macrophages from involved sites in patients with active pulmonary tuberculosis than in those of normal control or in uninvolved segments (Klingler et al., 1997). In summary, The mechanisms of apoptosis in mycobacterial infection benefits the host or the pathogen in vivo remains to be definitively determined.

2. Objective of the study

To study the apoptosis of macrophages from tuberculosis and normal persons after infected with virulent and non-virulent *Mycobacterium tuberculosis*.