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ORIGINAL ARTICLE

Lung granulomas from *Mycobacterium tuberculosis*/HIV-1 co-infected patients display decreased *in situ* TNF production

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Abstract

Tuberculosis/HIV-1 co-infection is responsible for thousands of deaths each year, and previous studies have reported that co-infected individuals display major morphological alterations in tissue granulomas. The purpose of this study was to evaluate immunohistopathological characteristics in lung tissues from pulmonary TB/HIV-1-co-infected individuals. Following autopsy, tuberculosis-positive HIV-1-negative cases displayed granulomas with normal architecture, mainly composed of a mononuclear infiltrate with typical epithelioid, as well as giant cells, and exhibiting caseous necrosis. In contrast, lesions from the TB/HIV-1-co-infected group showed extensive necrosis, poorly formed granulomas, and a marked presence of polymorphonuclear cells. More importantly, TNF staining was greatly reduced in the TB/HIV-1-co-infected individuals. Our data suggest that HIV-1 infection alters the organization of pulmonary granulomas by modulating TNF and, possibly, cell trafficking, leading to an impaired anti-tuberculosis response.

Keywords: *Mycobacterium tuberculosis*; Autopsy; Granuloma; HIV; TNF

Introduction

Mycobaterium tuberculosis infection is endemic throughout the world and causes many deaths. The World Health Organization (WHO) estimates that the occurrence of tuberculosis (TB) exceeds 14 million cases per year, with a mortality of 2 million people per year [33]. The most common form is pulmonary TB, which presents a high prevalence and incidence in the developing world due to poor sanitary conditions and malnutrition. In addition, HIV-1 infection is an important factor that increases the incidence and severity of tuberculosis. While AIDS clearly leads to heightened susceptibility to mycobacterial infection, evidence also suggests that *M. tuberculosis* may accelerate the progression of HIV disease through a process of immune activation of viral expression [5,9,14,32]. However, the interaction between HIV-1 and *M. tuberculosis* in different tissues, particularly in lungs, is not fully understood.

The hallmark of *M. tuberculosis*-infected tissues is the granuloma, a cellular accumulation around the bacilli that is comprised mainly of macrophages, epithelioid

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cells, multinucleated giant cells, and surrounded by a rim of T lymphocytes [23,25]. The main functions of granulomas are thought to be the containment of the infection preventing bacterial spread to surrounding tissues and organs, and to concentrate the immune response to a limited infectious area [23]. A number of reports have shown that a well-structured granuloma is critical to control *M. tuberculosis* infection, and a role for cytokines, such as TNF to restrict mycobacteria growth, has been demonstrated in both human and experimental tuberculosis [4,7,13]. For example, it has been shown that in the absence of TNF, the initial recruitment of monocytes into the site of infection is impaired with influx of inflammatory cells (e.g. neutrophils) and development of necrotic lesions [4,12], thus suggesting that TNF is involved in granuloma development. In contrast, it has been recently reported by Iliopoulus et al. that three patients, receiving anti-TNF therapy, did develop normal granulomas but failed to control disease [16]. Whether TNF plays a direct role in mycobacterial-induced granuloma in humans remains to be determined.

HIV-1 has been shown to influence the clinical course of *M. tuberculosis* infection [6]. These pathogens are co-endemic in many areas of the world, and patients simultaneously infected with these two agents exhibit greater morbidity and mortality than individuals harboring either pathogen alone [5,9]. However, immunopathological features for the breakdown of host resistance in dually infected individuals are unclear. During TB, the production of pro-inflammatory cytokines, such as TNF, may be important to control disease, and a change in this profile in dually infected patients has been reported [8,15,31]. In contrast, *M. tuberculosis* can induce HIV-1 expression in both TNF-dependent and -independent pathways *in vitro*, as well as experimental models [2,3,26,31], suggesting that while important in restricting bacterial proliferation, TNF could stimulate HIV-1 expression in infected tissues. Elevated HIV-1 replication may lead to decreased CD4 + T cell counts and could affect granuloma formation and IFN-γ synthesis [18]. These findings indicate that complex *in situ* responses to both pathogens take place in co-infected individuals. Despite its importance, *in situ* granuloma responses that are present in the lungs in TB/HIV-1-co-infected individuals are not well characterized.

In the present study, we analyzed lung samples obtained from autopsies of TB/HIV-1-co-infected and TB-positive but HIV-1-negative individuals to investigate the local immune response and histopathological patterns of *M. tuberculosis*-induced granulomas in dually infected patients. We found that, in addition to major alterations in the TB granuloma aspects, TB/HIV-1 co-infected individuals displayed an impaired TNF production in the granuloma. This result suggests that HIV-1 infection modulates immunological responses in the lung granulomas, which may influence bacterial growth restriction, as well as virus replication *in situ*.

Material and methods

Necropsy study

We performed a retrospective necropsy study of adults who had died from pulmonary tuberculosis between June 1995 and June 2001 at the Prof. Edgar Santos University Hospital, Bahia, Brazil. To prevent autolysis and tissue disintegration, the time between death and autopsy was approximately 4 h (minimum legal interval between death and autopsy required by Brazilian legislation), and the samples were immediately fixed in 10% formalin. Cases had a mean age of 34 years (range: 18–40) with a 1.5:1 male:female ratio. HIV-1-negative and positive cases (as determined by serology) were selected for the study, including a total of five lung samples from TB/HIV-1-positive cases and four from TB/HIV-1-negative cases. Only TB-positive cases were included in the study, following detection of the *M. tuberculosis* DNA fragment IS6110 by PCR [19,29] in deparaffinized lung sections as described in the literature [19].

Mycobacterial staining and histophatological analysis

Three pathologists, blinded from the patients' clinical diagnoses and HIV status, analyzed the necropsy lung samples. Hematoxylin and eosin, silver methenamine (fungi detection), and Ziehl-Nielsen (acid-fast bacilli detection) stains were performed in lung sections.

Cytokine staining of lung sections

Cytokine staining of lung samples was detected as described in the literature [28]. Briefly, paraffin-embedded sections were incubated with trypsin and 0.1% of calcium chloride ($CaCl_2$) at 37 °C for 30 min. After extensive washing with TBSS (Tris-buffered-saline and 0.1% saponin), possible unspecific epitopes at the sections were neutralized (5% milk, 0.1% saponin in Tris-buffered-saline) for 30 min at 25 °C and then incubated with biotin-conjugated antibodies against TNF (Genzyme, MA) or TGF-β (Santa Cruz Biotechnology, CA) at 37 °C for 45 min. After incubation with conjugated antibodies, sections were washed with TBSS and followed by neutralization of endogenous peroxidase (methanol and 6% hydrogenous peroxide) at 25 °C for 30 min. Positive and negative non-isotype antibody

controls to all cytokines were used in the reactions. After washing, slides were incubated with streptavidin-conjugated horseradish peroxidase (DAKO, CA), and reaction was developed using 3,3′,5,5′-tetramethylbenzidine (DAKO), followed by counterstaining with Harris' hematoxylin. Sections were examined microscopically, and images were recorded with a digital camera.

Results

Morphological features of TB/HIV-1-positive lung granulomas

To investigate whether HIV-1 co-infection influences granuloma composition in TB patients, we compared several morpho-immunological aspects of TB/HIV-negative and TB/HIV-1-positive lung samples. The number of granulomas per slide varied from 4 to 10, and all lesions displayed a concentric aspect as observed in mature granulomas. As expected, lung collected at autopsy from the TB/HIV-1-negative group displayed well-organized typical granulomas with three defined zones: (1) a central area of caseous necrosis; (2) an intermediary region mainly consisting of epithelioid and giant cells; and (3) a rim of lymphocytes present in the external zone (Fig. 1A and C). In contrast, postmortem lung tissue from TB/HIV-1-co-infected individuals showed much less structured granuloma morphology with areas of necrosis and a heterogeneous cellular population composed mainly of polymorphonuclear cells and eosinophils (Fig. 1B and D). In addition, only

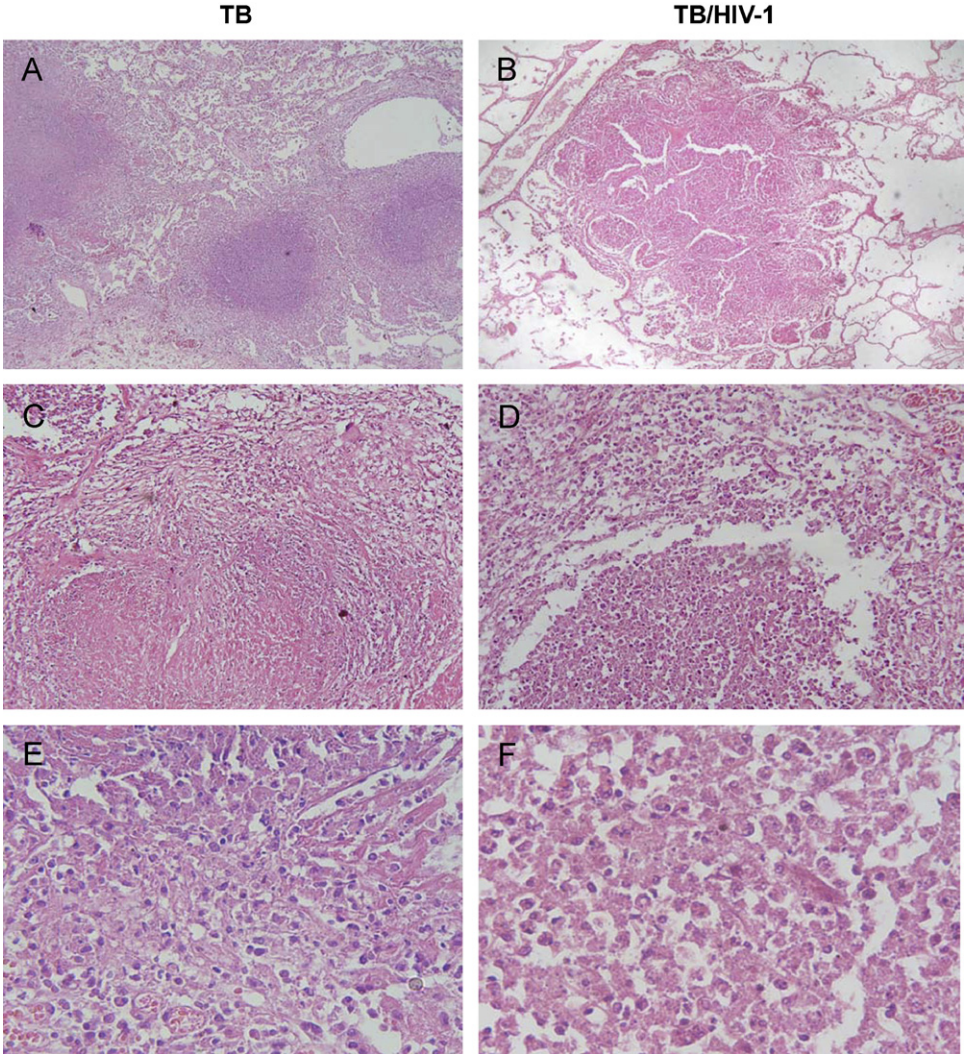

Fig. 1. Histopathological aspects of pulmonary lesions from TB and TB/HIV-1-positive cases. (A) A typical tuberculous granuloma (magnification × 40). (B) Poorly formed granuloma in TB/HIV-1 dually infected patients (magnification × 40). (C) Transitional zone and central caseous necrosis (magnification × 100). (D) Prominent area of necrosis with high cellularity (magnification × 100). (E) Mononuclear inflammatory infiltrate (magnification × 400). (F) Predominance of polymorphonuclear neutrophils inflammatory cells (magnification × 400). Slides shown are representative of five HIV + and four HIV- patients.

Table 1. Major immuno-histopathological observations found in TB/HIV-1-co-infected and TB/HIV-negative pulmonary lesions

H&E	TB/HIV-1 co-infected	TB alone
Typical granuloma	–	+ + +
Disorganized cell accumulation	+ + +	–
Necrosis	+ + +	+ +
Granuloma architecture	Disorganized, 3 zones unapparent	Organized, 3 zones well-defined
Cellular composition	Polymorphonuclear neutophils, eosinophils, plasmocytes and mononuclear cells	Macrophages, lymphocytes, epithelioid cells and giant cells
Bacilli presence	+ + +	+
Cytokine staining	TNF-α negative TGF-β positive	TNF-α positive TGF-β positive

a few mononuclear cells were observed, and neither giant nor epithelioid cells were seen in granulomas from TB/HIV-1-positive cases (compare Fig. 1E and F). Moreover, AFB staining revealed a higher number of bacilli in the pulmonary granulomas from cases of TB/HIV-1-positive compared to lung from TB/HIV-1-negative individuals (Table 1). Because a wide range of bacilli quantification in lung sections from TB/HIV-positive cases was observed, we used a logarithmic scale to quantify bacilli numbers in the lesions as shown in Table 1. One possible explanation for the observed variability could be different stages of HIV-disease existing in the TB/HIV-1-co-infected patients analyzed.

Impaired *in situ* TNF production in lung granulomas from TB/HIV-1-positive cases

To further analyze immunological markers within the lung granulomas, we performed cytokine immunohistochemistry of paraffin-embedded sections from both TB/HIV-1-negative and TB/HIV-1-positive cases. The TB/HIV-1-positive group displayed lower TNF expression with both surface and intra-cytoplasmatic patterns of staining (Fig. 2B inset). In contrast, lung specimens from the TB/HIV-1-negative group revealed TNF staining that was mainly detected in both intermediary and periphery of the granuloma (Fig. 2A). In sections from the TB/HIV-1-positive group, faint staining could be observed mostly in the granuloma periphery (Fig. 2B). Interestingly, TGF-β, a cytokine known to be involved in the regulation of tissue damage associated with human tuberculosis [30], was found to be weakly positive in both groups (Fig. 2C and D). This cytokine was found in granulomatous areas of lung lesions, and only a few cells were positive in the cytoplasm, whereas there was no staining in adjacent areas of the granulomas. Our major findings are summarized in Table 1.

Discussion

The present study investigates the possible effect of HIV on morpho-immunological features of *M. tuberculosis*-infected lungs. Our results showed that lungs from TB/HIV-1-co-infected cases display a major change in the cellular composition of the tuberculous granuloma associated with differences in acid-fast bacilli burden. Indeed, the morphology of these lesions is completely distinct from the typical granuloma observed in tissues from pulmonary TB (HIV-negative) cases. These findings suggest that the cellular architecture of the granuloma appears to be critically important to control bacilli replication in the onset of pulmonary tuberculosis [23,25]. Although not investigated in the present study, our data suggested that HIV-1 infection alters cell dynamics in the pulmonary TB granuloma, perhaps due to decreased numbers of CD4+T cells in AIDS patients [18]. In addition, it seems that the pathogenic process of HIV acute infection is, in fact, initiated by a massive viral replication, which will set up the disease outcome, and mucosal tissue CD4+T cells appear to be key players in this process [21]. In the necropsy cases studied here, no correlation between immuno-pathological findings and clinical parameters, such as circulating CD4+T cell numbers, was made due to lack of clinical information in the patients' reports. Nevertheless, we have detected decreased numbers of lymphocytes in the granulomas from HIV/TB-individuals (Fig. 1). Whether HIV-1 infection regulates specific patterns of cell migration and recruitment to the granuloma remains to be determined.

The immuno-modulation within the tuberculous granuloma appears to be different in dually infected individuals. We have shown here that TNF production is greatly reduced in pulmonary lesions from *M. tuberculosis*/HIV-1-co-infected cases. The TNF deficiency in TB/HIV-1-positive group may be a consequence of altered immune cell trafficking within

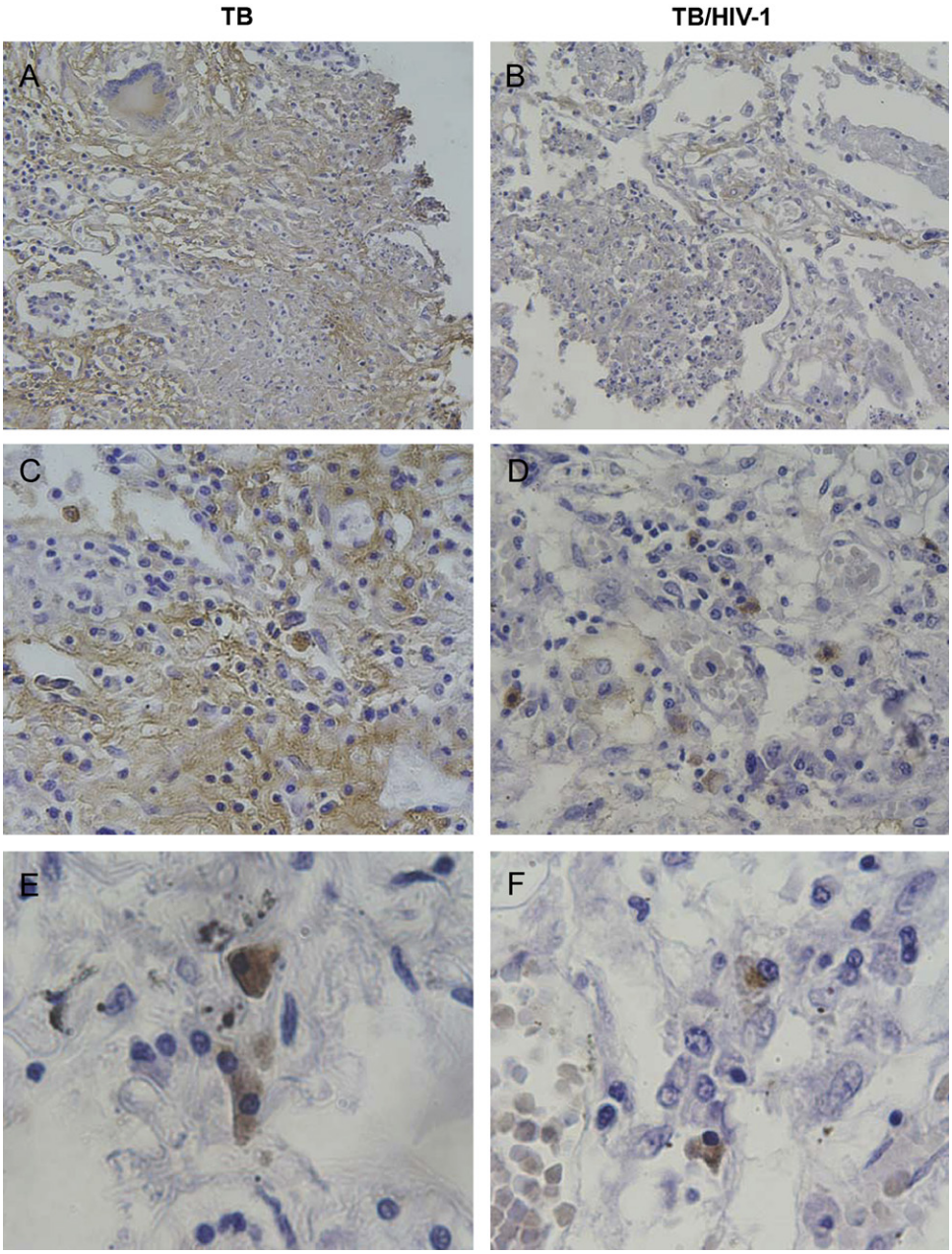

Fig. 2. Lung lesions from TB-infected patients display a higher TNF staining compared to TB/HIV-1-co-infected individuals. TNF immunohistochemical staining from (A) a TB-infected patient (magnification × 100) and (C) (magnification × 400). Note TNF staining on cell surface and in intracellular space. (B) A TB/HIV-1-positive patient (magnification × 100) and (D) (magnification × 400). (E and F) TGF-β staining from (E) a TB/HIV-negative and (F) a TB/HIV-positive patient (magnification × 800). Slides shown are representative of five HIV + and four HIV-negative patients.

the lesions and/or impairment of macrophage cytokine production regulated by the virus. However, it is more likely to be a factor contributing to the disorganized granuloma morphology given the important role for TNF in establishing and maintaining granuloma integrity, in addition to being critical in the control of mycobacterial [4,12,13]. Nevertheless, we could not exclude the role of others cytokines that might be involved in this process of granuloma formation, such as IL-10 and IL-13, since it can undermine Th1-mediated immunity [11,24].

Macrophages are central cells in *M. tuberculosis* killing, and it has been shown that HIV-1 inhibits the *in vitro* production of cytokines, such as TNF by macrophages [20]. In contrast, TB and HIV co-infection was demonstrated to induce a higher production of TNF in the serum [17]. Although TB patients (and HIV-1-negative) display an increased TNF staining in lung

granuloma as reported earlier [10], our findings demonstrated that TNF expression is greatly impaired in lesions from TB/HIV-1-positive cases. Nevertheless, because the cellular composition of the granulomas in TB/HIV-1-positive lung samples was altered, this may reflect the *in situ* TNF production in the lesions. Possible explanations could be the stage of HIV-associated disease in the TB/HIV-1 cases used in our study or a dissociation of TNF production within granulomas and systemic TNF production as reflected in serum TNF levels [17]. Of interest, differences between regional and systemic responses have been described in pleural TB [1]. We had no opportunity to evaluate plasma TNF levels in the present cases as this was a retrospective, autopsy-based study, and sera were not stored. Interestingly, TGF-β was present in the sections with no differences between the two groups. Additionally, we were unable to detect IFN-γ by immunohistochemistry in the paraffin-embedded sections in either group. IFN-γ is thought to be a major player in the immune response against TB and displays opposite effects compared to TGF-β [13]. These results also argue against TGF-β being the major cytokine involved in the maintenance of tuberculous granuloma, as previously suggested [30].

We found high numbers of neutrophils in the granulomas of co-infected cases. Whether or not these cells participate in controlling human *M. tuberculosis* infection is still unknown [27]. A higher number of bacilli were observed in these neutrophilic areas, suggesting that these cells may not play a major role in *M. tuberculosis* resistance in humans. Nevertheless, the higher bacterial replication could lead to an increased recruitment of neutrophils to the granuloma. In association with the marked increase in neutrophils, we observed prominent necrosis and apoptotic cells in the TB/HIV-1-positive tissues. Of interest, this process was not the typical caseous necrosis observed in the HIV-1-negative TB cases. These observations suggested differential regulation of granuloma formation and necrosis in TB/HIV-1-positive lungs. In support of this hypothesis, regulators of granuloma development, such as matrix metalloproteinase (MMP)-9, have been shown to be associated with tissue destruction within tuberculous granuloma [22], and TNF, a critical cytokine for antimycobacterial granuloma formation, has been shown to be a key autocrine and paracrine regulator of MMP-9 secretion [22]. Additional studies are planned to elucidate the impact of TNF on the induction of HIV-1 replication in pulmonary tuberculosis patients and the contribution of regulators, such as MMP-9 and chemokines, to the antimycobacterial control.

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References

[1] S. Arruda, Cell-mediated immune responses and cytotoxicity to mycobacterial antigens in patients with tuberculous pleurisy in Brazil, Acta Trop. 71 (1998) 1–15.
[2] A. Bafica, Cutting edge: *In vivo* induction of integrated HIV-1 expression by mycobacteria is critically dependent on Toll-like receptor 2, J. Immunol. 171 (2003) 1123–1127.
[3] A. Bafica, Influence of coinfecting pathogens on HIV expression: evidence for a role of Toll-like receptors, J. Immunol. 172 (2004) 7229–7234.
[4] A.G. Bean, Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection which is not compensated for by lymphotoxin, J. Immunol. 162 (1999) 3504–3511.
[5] K.R. Collins, Impact of tuberculosis on HIV-1 replication, diversity, and disease progression, AIDS Rev. 4 (2002) 165–176.
[6] E.L. Corbett, HIV-1/AIDS and the control of other infectious diseases in Africa, Lancet 359 (2002) 2177–2187.
[7] P. Costamagna, Tuberculosis associated with blocking agents against tumor necrosis factor-alpha – California, 2002–2003, Morb. Mortal Weekly Rep. 53 (2004) 683–686.
[8] R.M. de Castro Cunha, Interferon-gamma and tumour necrosis factor-alpha production by CD4+ T and CD8+ T lymphocytes in AIDS patients with tuberculosis, Clin. Exp. Immunol. 140 (2005) 491–497.
[9] J. Del Amo, Does tuberculosis accelerate the progression of HIV disease? Evidence from basic science and epidemiology, AIDS 13 (1999) 1151–1158.
[10] G. Fenhalls, Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8T cells producing IL-12p40 mRNA in human lung tuberculous granulomas, Immunology 105 (2002) 325–335.
[11] I.E. Flesch, Role of cytokines in tuberculosis, Immunobiology 189 (1993) 316–339.
[12] J.L. Flynn, Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice, Immunity 2 (1995) 561–572.
[13] J.L. Flynn, Immunology of tuberculosis, Annu. Rev. Immunol. 19 (2001) 93–129.
[14] D. Goletti, Effect of *Mycobacterium tuberculosis* on HIV replication. Role of immune activation, J. Immunol. 157 (1996) 1271–1278.
[15] T. Hertoghe, T cell activation, apoptosis and cytokine dysregulation in the (co)pathogenesis of HIV and pulmonary tuberculosis (TB), Clin. Exp. Immunol. 122 (2000) 350–357.

[16] A. Iliopoulos, Tuberculosis and granuloma formation in patients receiving anti-TNF therapy, Int. J. Tuberc. Lung Dis. 10 (2006) 588–590.
[17] J.D. Klausner, The effect of thalidomide on the pathogenesis of human immunodeficiency virus type 1 and *M. tuberculosis* infection, J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 11 (1996) 247–257.
[18] J. Laurence, Viral cofactors in the pathogenesis of HIV disease, in: G.P. Wormser (Ed.), AIDS and other Manifestations of HIV Infection, second ed, Raven Press, New York, 1992, pp. 77–83.
[19] S. Loeschke, Improved detection of mycobacterial DNA by PCR in formalin-fixed, paraffin-embedded tissues using thin sections, Pathol. Res. Pract. 201 (2005) 37–40.
[20] H. Mayanja-Kizza, Macrophage-activating cytokines in human immununodeficiency virus type 1-infected and -uninfected patients with pulmonary tuberculosis, J. Infect. Dis. 183 (2001) 1805–1809.
[21] L.J. Picker, Immunopathogenesis of acute AIDS virus infection, Curr. Opin. Immunol. 18 (2006) 399–405.
[22] N.M. Price, Unopposed matrix metalloproteinase-9 expression in human tuberculous granuloma and the role of TNF-alpha-dependent monocyte networks, J. Immunol. 171 (2003) 5579–5586.
[23] M.P. Puissegur, An *in vitro* dual model of mycobacterial granulomas to investigate the molecular interactions between mycobacteria and human host cells, Cell Microbiol. 6 (2004) 423–433.
[24] G.A. Rook, Th2 cytokines in susceptibility to tuberculosis, Curr. Mol. Med. 7 (2007) 327–337.
[25] B.M. Saunders, Restraining mycobacteria: role of granulomas in mycobacterial infections, Immunol. Cell Biol. 78 (2000) 334–341.
[26] C.A. Scanga, Viral gene expression in HIV transgenic mice is activated by *Mycobacterium tuberculosis* and suppressed after antimycobacterial chemotherapy, J. Infect. Dis. 195 (2007) 246–254.
[27] P. Seiler, Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating *Mycobacterium tuberculosis*, J. Infect. Dis. 181 (2000) 671–680.
[28] T.M. Silva, *In situ* inflammatory immune response in human tegumentary leishmaniasis: morphologic evidence for a pathogenic role of TNF-alpha, Mem. Inst. Oswaldo Cruz 93 (1998) 49–50.
[29] D. Thierry, Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis, J. Clin. Microbiol. 28 (1990) 2668–2673.
[30] Z. Toossi, The role of TGF beta in the pathogenesis of human tuberculosis, Clin. Immunol. Immunopathol. 87 (1998) 107–114.
[31] Z. Toossi, Virological and immunological impact of tuberculosis on human immunodeficiency virus type 1 disease, J. Infect. Dis. 188 (2003) 1146–1155.
[32] C. Whalen, Accelerated course of human immunodeficiency virus infection after tuberculosis, Am. J. Respir. Crit. Care Med. 151 (1995) 129–135.
[33] WHO, The World Health Report 2006. WHO, Geneva, 2006.