

## Role of TNF- $\alpha$ in host immune response in tuberculous pleuritis

C. Prabha\*, Kripa V. Jalapathy\*, Ram Prasad Matsa<sup>†</sup> and Sulochana D. Das<sup>\*‡</sup>

\*Tuberculosis Research Centre (Indian Council of Medical Research), Mayor V.R. Ramanathan Road, Chennai 600 031, India

<sup>†</sup>Madras Medical College, Chennai 600 003, India

**Tumour Necrosis Factor (TNF)- $\alpha$ , a pro-inflammatory cytokine has a dual role in host immunity and immunopathology of tuberculosis and is considered to be pivotal for determining the clinical course of the disease, either beneficial or detrimental. The assessment of TNF- $\alpha$  in pleural tuberculosis will help us to understand its role in host defence mechanism against *Mycobacterium tuberculosis* (MTB) infection. In this study, TNF- $\alpha$  and IFN- $\gamma$  levels were measured in plasma and pleural fluid of both tuberculosis (TB) and non-TB patients and in the supernatants of blood and pleural fluid mononuclear cells (PBMCs and PFCs) stimulated *in vitro* with PPD, culture filtrate and heat-killed (MTB). In addition, apoptosis induced by PPD and MTB was also studied. TNF- $\alpha$  and IFN- $\gamma$  were significantly elevated in pleural fluid than in plasma of pleural tuberculosis patients, suggesting the compartmentalization of Th1 cytokine-secreting cells at the site of disease. *In vitro* stimulation of PFCs with PPD and MTB showed a significant increase in these cytokine levels and also enhanced apoptosis of these cells. This increase in TNF- $\alpha$  levels may contribute to the containment of infection by synergizing with IFN- $\gamma$  to activate infected macrophages or by the regulation of T-cell apoptosis.**

TUBERCULOUS pleuritis occurs in about 30% of patients with tuberculosis (TB). This percentage increases with HIV-positive conditions. HIV-positive individuals tend to develop tuberculous pleuritis during their early stage of immunosuppression<sup>1</sup>. The emergence of multi drug-resistant tuberculosis (MDR-TB) has added burden to TB therapy. Currently, immunotherapeutic studies are being carried out to reduce the duration of therapy and to find an alternate treatment for MDR-TB. Tuberculous pleuritis serves as the best model to understand the immunity at the site of infection. The local cell-mediated immune (CMI) response generated by macrophages in cooperation with T-lymphocytes in tuberculous pleuritis resolves the disease without chemotherapy. The response is mediated by the secretion of complex cytokine pattern by the antigen-specific T-cells present in the pleural space. The cytokine pattern in tuberculous pleuritis is suggestive of Th1 type<sup>2,3</sup>. Cytokines like gamma interferon (IFN- $\gamma$ ), Tumour Necrosis Factor (TNF)- $\alpha$ , IL-2, IL-12 and IL-18 are the ones that play a major role in bringing about the

reactions like granuloma formation, intramacrophage elimination of the bacillary antigens and fibrosis that lead to the resolving of the disease<sup>4</sup>.

TNF- $\alpha$  is produced by macrophages, monocytes and dendritic cells in response to Mycobacteria or its products, or to other microorganisms like *Listeria monocytogenes*. It has immuno-regulatory properties and brings about the activation of macrophages, granuloma formation, T-cell stimulation and regulation of chemokine induction, thus controlling infection<sup>5</sup>. High levels of TNF- $\alpha$  were reported in chronic TB accompanied by the elevated release of TNF- $\alpha$  receptors resulting in fever, necrosis and weight loss. This implies that TNF- $\alpha$  has both protective and immunopathologic effects<sup>6</sup>. IFN- $\gamma$ , a Th1-type cytokine is essential in TB immunity, which is the single most important factor for macrophage activation and TNF- $\alpha$  induction<sup>7</sup>. Individuals who lack either IFN- $\gamma$  or IFN- $\gamma$ R are highly susceptible to severe systemic infections which are important in the control of TB<sup>8,9</sup>.

Apoptosis is a crucial phenomenon occurring at the site of infection in TB. Previous studies indicate that the microenvironment of a *Mycobacterium tuberculosis* (MTB)-infected focus is favourable for apoptosis of mononuclear cells<sup>10,11</sup>. Infected macrophages secrete TNF- $\alpha$ , which acts as an important apoptotic factor. Assessment of TNF- $\alpha$ , together with its inducer IFN- $\gamma$ , will help us formulate its functional role in apoptosis and to understand the local CMI response that results in the clearance of the bacilli. Moreover, most reports on TNF- $\alpha$  and IFN- $\gamma$  are from either *in vivo* levels or about *in vitro* studies, but not from both<sup>12-14</sup>. Hence we assessed these cytokine levels simultaneously, both *in vivo* and *in vitro*, with an idea to confirm previous reports from *in vivo* studies and extend them to *in vitro* results.

We included 75 patients from the Government General hospital, Chennai with pleural effusion. These patients were broadly divided into two groups according to the diagnosis.

- (i) A TB group consisting of 46 patients with tuberculous pleural effusion. The effusion was unilateral. Diagnosis was confirmed by the clinical picture, AFB positivity for the smear, culture positivity or positive by PCR.
- (ii) A non-TB (NTB) group of 29 patients with transudative effusion due to causes other than tuberculous pleuritis. This included patients with congestive cardiac failure and liver diseases.

The pleural fluid and blood collected for therapeutic and diagnostic purposes respectively, were utilized for this study according to the ethical guidelines of the Government General Hospital, Chennai. A written, informed consent for participation in the study was obtained from each patient. Blood and pleural fluid were collected before the start of the treatment.

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood (10 U/ml) by Ficoll-

<sup>‡</sup>For correspondence. (e-mail: sulochanadas@rediffmail.com)

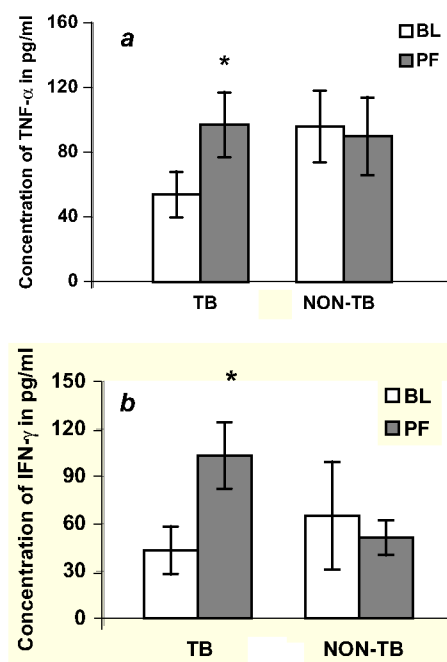
Hypaque (Pharmacia) density gradient centrifugation, as described elsewhere<sup>15</sup>. To obtain pleural fluid mononuclear cells (PFMCs), the heparinized pleural fluid (1 U/ml) was layered over Ficoll-Hypaque and centrifuged at 1800 rpm for 30 min. The interphase was collected and washed twice with HBSS (Whittaker) at 1800 rpm for 10 min. PBMCs and PFMCs were suspended at the concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% autologous serum. Viability of the cells was assessed by trypan blue exclusion method. The cell-free plasma and pleural fluid were stored at  $-70^\circ\text{C}$  for assessment of cytokines.

Only cells from those patients who were positive by all the diagnostic criteria (clinical, smear, culture and PCR) and for whom the cells were adequate were taken for *in vitro* studies. Accordingly, 18 out of 46 TB samples of tuberculous pleuritis were included in this study. Next,  $1 \times 10^6$  PBMCs and PFMCs were distributed in 48 well round-bottom plates (Nunc) and incubated with different mycobacterial antigens like PPD (Weybridge, UK), culture filtrate (CF) and heat-killed *MTB* at  $10 \mu\text{g/ml}$  concentration. These cultures were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The CF antigen and heat-killed *MTB* were prepared in our laboratory. Mononuclear cells incubated with medium alone served as control. Cell-free supernatants were collected after 48 h at which the cytokine concentration were found to be maximum, and stored at  $-70^\circ\text{C}$  for cytokine estimation. TNF- $\alpha$  and IFN- $\gamma$  levels were measured in plasma and pleural fluid and in supernatants of *in vitro* experiments using a commercial sandwich ELISA kit (R&D Systems, Minneapolis, USA) and following the manufacturer's instruction. Diagnostic PCR using IS6110 primers (Bangalore Genei Private Limited, India) was performed to find the positivity for the presence of *MTB* bacilli.

For the detection of apoptosis  $1 \times 10^6$  cells (PBMCs and PFMCs) of 10 out of the 18 TB patients chosen for *in vitro* studies, were treated with known apoptosis-inducing agent like Dexamethasone ( $100 \mu\text{g/ml}$ ) and mycobacterial antigens (PPD and *MTB*- $50 \mu\text{g/ml}$ ) for three days. The cells were harvested, permeabilized and stained with  $500 \mu\text{l}$  of fluorochrome solution ( $200 \mu\text{g}$  RNAase A (Amersham Corporation) and  $100 \mu\text{g}$  propidium iodide (Sigma Chemical Co.) in  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free PBS and were incubated at  $37^\circ\text{C}$  for 1 h. Acquisition was done with FACSORT (Becton Dickinson, CA) and analysis was done using CellQuest software. The PI staining intensity of cells was determined by measuring the red fluorescence. The percentage of cells in the less than 2N region (hypodiploidy) was taken as the quantitative measure of the apoptotic cells, as described previously<sup>16</sup>.

Comparison between groups was done using paired or unpaired Student's *t* test as appropriate for normally distributed data. Wilcoxon's rank sum test was performed for the data that were not normally distributed. The values were expressed as mean  $\pm$  standard error of the mean, both in the text and in the figures.

With a view to understand the local *in vivo* role of TNF- $\alpha$  in relation with IFN- $\gamma$ , the mean TNF- $\alpha$  and IFN- $\gamma$  concentrations in the plasma and pleural fluid of TB and NTB groups were assessed, the results are shown in Figure 1. The mean levels of TNF- $\alpha$  and IFN- $\gamma$  in pleural fluid were significantly higher ( $P < 0.05$ ) than the plasma levels in patients with tuberculous pleuritis. There was no significant difference between the plasma and pleural fluid levels in transudative effusions from the NTB patients. A number of studies have been reported on the protective role of IFN- $\gamma$  and TNF- $\alpha$  in TB<sup>8,9,17</sup>, and higher levels of these cytokines in pleural fluid than in plasma of tuberculous pleuritis patients<sup>14,18</sup>. The selective increase observed in these cytokine levels in pleural fluid supports these studies. The high concentration of these cytokines in pleural fluid reflects the stimulation of local, immuno-reactive cells by mycobacterial components that facilitate bacillary elimination and granuloma formation. Moreover, it also suggests that there is a compartmentalization of TH1 cytokine-secreting cells, as reported earlier<sup>19</sup>. This compartmentalization occurs because of the migration of TNF- $\alpha$  and IFN- $\gamma$  secreting cells to the site of the disease and hence not accessible through the sampling of plasma of peripheral blood, thus attributing to the lesser plasma levels of these cytokines in tuberculous pleuritis patients than in NTB patients. The comparable *in vivo* levels of TNF- $\alpha$  in pleural fluid of NTB patients with that of TB patients may be due to the generalized TNF- $\alpha$  secretory response to any inflammatory conditions, but not due to compartmentalization. This is evident from the



**Figure 1.** *In vivo* levels of TNF- $\alpha$  (a) and IFN- $\gamma$  (b) in plasma (BL) and pleural fluid (PF) of patients with tuberculous pleurisy (TB) and patients with effusions other than tuberculosis (Non-TB); \* $P < 0.05$ .

similar levels of TNF- $\alpha$  noticed in the plasma and pleural fluid of NTB patients.

Observing the enhanced response of TNF- $\alpha$  *in vivo*, we further decided to investigate its *in vitro* response to the widely used crude mycobacterial antigens. The levels of TNF- $\alpha$  and IFN- $\gamma$  in the supernatants of PBMCs stimulated with PPD, CF and *MTB* did not show any difference when compared with their control levels. This implies that the TNF- $\alpha$  and IFN- $\gamma$  secreting cells present in the PBMCs population are not specific for *MTB* antigens. However, PFMCS stimulated with PPD and *MTB* but not with CF, showed significant increase when compared with their control levels ( $P < 0.05$ ; Figure 2). This suggests that these cells are specific for mycobacterial components. The increase in the cytokine response may be due to the memory response of the cells towards the mycobacterial antigens. Overall, the *in vitro* levels of TNF- $\alpha$  in the supernatants of PBMCs were higher than those found in the supernatants of PFMCS under all stimulated conditions.

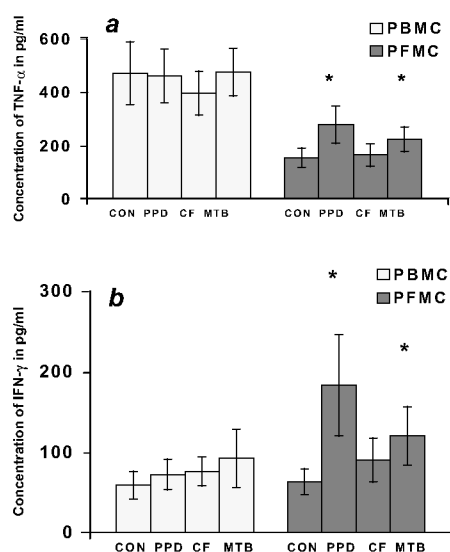
Apoptosis is a feature of tuberculous granulomata in pulmonary TB. Previous *in vitro* study from TB patients has reported that there occurs T-cell hyporesponsiveness linked to spontaneous or *MTB*-induced apoptosis of T-cells<sup>20</sup>. Thus PFMCS at the site of infection constitute the mycobacterial antigen-sensitized population and hence are more prone to apoptosis. Our results showed the induction of apoptosis of PFMCS by mycobacterial antigens (Figure 3). As expected, we observed maximum spontaneous apoptosis (21%) occurring in PFMCS compared to PBMCs (5%) when there is no stimulation. Also, a known apoptotic agent like Dexamethasone induced maximum apoptosis (43%) in PFMCS. With mycobacterial antigens, marginal apoptosis (12–18%) was seen in PBMCs while PFMCS showed significantly high (34–45%) levels of apoptosis. We have also observed that it is the T-cell

population of PFMCS that undergoes enhanced apoptosis (S. Das unpublished data). Such spontaneous or *MTB*-induced apoptosis of T-cells has been previously documented<sup>21,22</sup>. In another study based on pleural tuberculosis, the loss of IFN- $\gamma$ -producing cells was found to be limited specifically to *MTB*-responsive cells<sup>23</sup>. These studies provide evidence that T-cell apoptosis is biologically significant in the disease process.

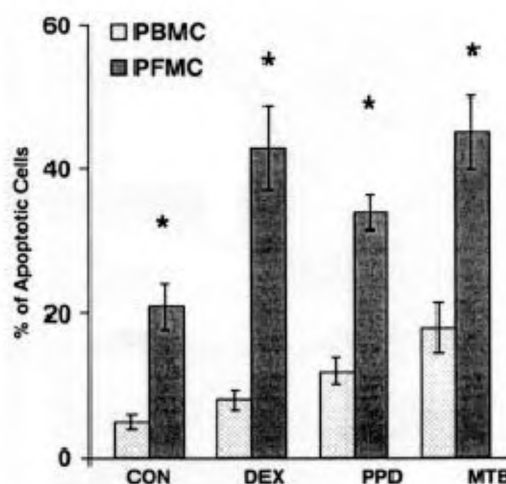
Most studies indicate a critical role for TNF- $\alpha$  and IFN- $\gamma$  in the apoptosis mediated by *MTB*<sup>11,20,24</sup>. Association between the high levels of these cytokines and enhanced apoptosis of PFMCS in our study indicates that these cytokines have a dual role at the site of infection. As known, IFN- $\gamma$  induces TNF- $\alpha$ , which plays an immunoprotective role by containing *MTB* infection and facilitating granuloma formation. On the other hand, it can also bring about the apoptosis of sensitized T-cells, that has immunopathologic outcomes. The immunopathologic role of IFN- $\gamma$  is also supported by the recent observation of its role in upregulating the transcription of genes involved in apoptosis (*FasL*, *Fas*, *Bak*)<sup>25–27</sup>.

It is reported that the TNF- $\alpha$ -mediated pathology occurs only in the presence of IL-4 in certain inflammatory conditions, including TB, at the systemic level<sup>28</sup>. We extend this finding to the localized response in TB. Supporting this, we found an increase in *in vitro* levels of IL-4 (data communicated) in these patients, suggesting the tilt of immune response towards TH2/Th0 type. This increase in IL-4 production in response to *MTB* possibly influences the sensitization of T-lymphocytes to apoptosis by a TNF- $\alpha$ -mediated pathway, as suggested by Seah and Rook<sup>29</sup>. Cytokine profile-dependent action of TNF- $\alpha$  also suggests that the tissue damage was caused by TNF- $\alpha$  in the mixed TH1 + TH2/Th0 response<sup>30</sup>.

In conclusion, the threshold levels of TNF- $\alpha$  may contribute to the containment of the infection by synergizing



**Figure 2.** *In vitro* production of TNF- $\alpha$  (a) and IFN- $\gamma$  (b) by PBMCs and PFMCS of TB patients stimulated with PPD, culture filtrate (CF) and heat-killed *Mycobacterium tuberculosis* (MTB); \* $P < 0.05$ .



**Figure 3.** Apoptosis of PBMCs and PFMCS in 10 TB patients detected by flow cytometry. Cells were treated with known apoptosis-inducing agent – Dexamethasone (DEX) and with mycobacterial antigens PPD and heat-killed *MTB*.

with IFN- $\gamma$  to activate infected macrophages, or by the regulation of T-cell apoptosis; whereas the selective depletion of mycobacterium-reactive cells that secrete TNF- $\alpha$  may lead to enhanced susceptibility to *MTB* infection and result in the dissemination of the bacilli. Thus, TNF- $\alpha$  plays an immunoregulatory role in TB. In addition, we speculate that the high levels of IL-4 observed in our studies may also contribute to apoptosis in a TNF- $\alpha$ -dependent manner. It is interesting to note from our studies that the two antagonistic cytokines, viz. IFN- $\gamma$  and IL-4 bring about the same effect of apoptosis mediated through TNF- $\alpha$ . However, further experiments should focus on finding the conditions and mechanisms by which these cytokines bring about apoptosis to elucidate the functional role of TNF- $\alpha$  in apoptosis. Understanding such basic mechanisms would help us explore new therapeutic strategies for TB.

- Fry, M. D., Pozik, C. J. and Saher, S. A., Tuberculous pleurisy is more common in AIDS than in non-AIDS patients with tuberculosis. *Chest*, 1997, **112**, 393–397.
- Barnes, P. F. and Wikel, B., Type 1 cytokines and the pathogenesis of tuberculosis. *Am. J. Respir. Crit. Care Med.*, 2000, **161**, 1773–1774.
- Flynn, J. L. and Chann, J., Immunology of tuberculosis. *Annu. Rev. Immunol.*, 2001, **19**, 93–129.
- Infante-Duarte, C. and Kamradt, T., Th1/Th2 balance in infection. *Springer Semin. Immunopathol.*, 1999, **21**, 317–338.
- Roach, D. R., Bean, A. G. D., Demangel, C., France, M. P., Briscoe, H. and Britton, W. J., TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J. Immunol.*, 2002, **168**, 4620–4627.
- Bekker, L. G., Maartens, L., Steyn and Kaplan, G., Selective increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. *J. Infect. Dis.*, 1998, **178**, 580–584.
- Boehm, U., Klamp, T., Groot, M. and Howard, J. C., Cellular responses to interferon-gamma. *Annu. Rev. Immunol.*, 1997, **15**, 749–795.
- Frucht, D. M. and Holland, S. M., Defective monocyte costimulation for IFN-gamma production in familial disseminated *Mycobacterium avium* complex infection: abnormal IL-12 regulation. *J. Immunol.*, 1996, **157**, 411–416.
- Dorman, S. E. and Holland, S. M., Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. *J. Clin. Invest.*, 1998, **101**, 2364–2369.
- Li, B. *et al.*, Involvement of the Fas/Fas ligand pathway in activation-induced cell death of mycobacteria-reactive human gamma delta T cells: a mechanism for the loss of gamma delta T cells in patients with pulmonary tuberculosis. *J. Immunol.*, 1998, **161**, 1558–1567.
- Keane, J. *et al.*, Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. *Infect. Immunol.*, 1997, **65**, 298–304.
- Barnes, P. F., Abrams, J. S., Lu, S., Sieling, P. A., Rea, T. H. and Modlin, R. L., Cytokine production at the site of disease in human tuberculosis. *Infect. Immunol.*, 1993, **61**, 197–203.
- Boom, W. H., Wallis, R. S. and Chervenak, K. A., Human *Mycobacterium tuberculosis*-reactive CD4+ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. *Infect. Immunol.*, 1991, **59**, 2737–2743.
- Ogawa, K., Koga, H., Hirakata, Y., Tomono, K., Tashiro, T. and Kohno, S., Differential diagnosis of tuberculous pleurisy by measurement of cytokine concentrations in pleural effusion. *Tuberc. Lung Dis.*, 1997, **78**, 29–34.
- Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D. and Britton, W. J., 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.*, 1999, **162**, 3504–3511.
- Norvell, A., Mandik, L. and Monroe, J. G., Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J. Immunol.*, 1995, **154**, 4404–4413.
- Kindler, V., Sappino, A. P., Grau, G. E., Piguet, P. and Fand Vassalli, P., The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*, 1989, **56**, 731–740.
- Olobo, J. O., Geletu, M., Demissie, A., Eggale, T., Hiwot, K., Aderaye, G. and Britton, S., Circulating TNF-alpha, TGF-beta, and IL-10 in tuberculosis patients and healthy contacts. *Scand. J. Immunol.*, 2001, **53**, 85–91.
- Manca, F. *et al.*, Limited clonal heterogeneity of antigen-specific T cells localizing in the pleural space during mycobacterial infection. *Infect. Immunol.*, 1991, **59**, 503–513.
- Hirsch, C. S. *et al.*, Augmentation of apoptosis and interferon-gamma production at sites of active *Mycobacterium tuberculosis* infection in human tuberculosis. *J. Infect. Dis.*, 2001, **183**, 779–788.
- Vanham, G., Edmonds, K. L., Quing, L., Hom, D., Toossi, Z. and Ellner, J. J., Generalized immune activation in pulmonary tuberculosis: co-activation with HIV infection. *Clin. Exp. Immunol.*, 1996, **103**, 30–36.
- Hirsch, C. S. *et al.*, Apoptosis and T cell hyporesponsiveness in pulmonary tuberculosis. *J. Infect. Dis.*, 1999, **179**, 945–953.
- Marchat, A. *et al.*, Polarization of PPD-specific T-cell response of patients with tuberculosis from Th0 to Th1 profile after successful antimycobacterial therapy or *in vitro* conditioning with interferon-alpha or interleukin-12. *Am. J. Respir. Cell. Mol. Biol.*, 2001, **24**, 187–194.
- Cooper, A. M., Adams, L. B., Dalton, D. K., Appelberg, R. and Ehlers, S., IFN-gamma and NO in mycobacterial disease: new jobs for old hands. *Trends Microbiol.*, 2002, **10**, 221–226.
- Martins, G. A., Vieira, L. Q., Cuncha, F. Q. and Silva, J. S., Gamma interferon modulates CD95 (Fas) and CD95 ligand (Fas-L) expression and nitric oxide-induced apoptosis during the acute phase of *Trypanosoma cruzi* infection: a possible role in immune response control. *Infect. Immunol.*, 1999, **67**, 3864–3871.
- Spets, H., Georgii-Hemming, S., Siliason, P., Nilsson, K. and Jernberg-Wiklund, H., Fas/APO-1 (CD95)-mediated apoptosis is activated by interferon-gamma and interferon in interleukin-6 (IL-6)-dependent and IL-6-independent multiple myeloma cell lines. *Blood*, 1998, **92**, 2914–2923.
- Der, S. D., Zhou, A., Williams, B. R. and Silverman, R. H., Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 15623–15628.
- Hernandez-Pando, R., Pavon, L., Arriaga, K., Orozco, H., Madrid Marina, V. and Rook, G., Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental mycobacterial saprophyte before infection. *Infect. Immunol.*, 1997, **65**, 3317–3327.
- Seah, G. T. and Rook, G. A. W., IL-4 influences apoptosis of mycobacterium-reactive lymphocytes in the presence of TNF-alpha. *J. Immunol.*, 2001, **167**, 1230–1237.
- Hernandez-Pando, R. and Rook, G. A. W., The role of TNF-alpha in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology*, 1994, **82**, 591–595.

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