Infliximab partially impairs the anti-Mycobacterium tuberculosis immune responses of severe psoriasis patients with positive tuberculin skin-test
ORIGINAL ARTICLE

Infliximab partially impairs the anti-Mycobacterium tuberculosis immune responses of severe psoriasis patients with positive tuberculin skin-test


†Laboratory of Dermatology and Immunodeficiencies, Medical School of the University of São Paulo, São Paulo, Brazil
‡Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands
§Division of Clinical Dermatology, Hospital das Clínicas, Medical School of the University of São Paulo, São Paulo, Brazil
*Laboratory of Medical Mycology (LIM-53), Tropical Medicine Institute, University of São Paulo, São Paulo, Brazil
*Correspondence: G. Benard, MD, PhD. E-mail: mahong@usp.br

Abstract

Background Infliximab and etanercept are now widely used for treating severe psoriasis. However, these drugs, especially infliximab, increased the risk of tuberculosis reactivation. Surprisingly, epidemiological data suggest that the tuberculosis rate in patients taking infliximab in São Paulo State, Brazil, is similar to that of some developed, non-endemic countries.

Objective The aim of this study was to better understand the effect of infliximab on Mycobacterium tuberculosis (Mtb) immune responses of psoriasis patients in an endemic setting (Brazil).

Methods We evaluated the tuberculosis-specific immune responses of severe psoriasis patients and healthy individuals, both tuberculin skin test (TST) positive, in the presence/absence of infliximab. Patients had untreated severe psoriasis, no co-morbidities affecting the immune responses and a TST >10 mm. Healthy TST+ (>10 mm) individuals were evaluated in parallel. PBMC cultures from both groups were stimulated with different Mycobacterium tuberculosis (Mtb) antigens (ESAT-6, 85B and Mtb lysate) and phytohemagglutinin, with or without infliximab (5 μg/mL). Parameters evaluated were TNF-α, IFN-γ and IL-10 secretion by ELISA, overnight IFN-γ ELISpot and lymphocyte proliferative response (LPR).

Results Infliximab almost abolished TNF-α detection in PBMC supernatants of both groups. It also significantly reduced the LPR to phytohemagglutinin and the Mtb antigens as well as the IFN-γ levels secreted into day 5 supernatants in both groups. There was no concomitant exaggerated IL-10 secretion that could account for the decreases in these responses. ELISpot showed that, contrasting with the central-memory responses above, infliximab did not affect effector-memory INF-γ-releasing T-cell numbers.

Conclusions Infliximab affected some, but not all aspects of the in vitro antituberculosis immune responses tested. The preserved effector-memory responses, putatively related to exposure to environmental mycobacteria, may help to explain the lower than expected susceptibility to tuberculosis reactivation in our setting.

Received: 29 December 2010; Accepted: 9 March 2011

Conflict of interest

All authors declare no conflicts of interest.

Financial support

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (contracts # 05/57761-7 and 05/60075-8), the Netherlands Leprosy Relief Foundation (NLR) and the European Commission. GB and AJSD are senior researchers of the Conselho Nacional para o Desenvolvimento Científico e Tecnológico.

Introduction

Two TNF-α antagonists, infliximab (IFX) and etanercept (ETA), have now been used for approximately 10 years for treating patients with severe psoriasis with good results. In 2008, another antagonist, adalimumab, has also been approved for treating psoriasis. These drugs, however, were associated with an increased incidence of intracellular infections, especially pulmonary and extra pulmonary tuberculosis (TB).1 In fact tumour necrosis factor
alpha (TNF-α) plays a critical role in the control of TB immune responses. It was soon noticed that the rate of TB was much higher in association with IFX and adalimumab than with ETA, probably reflecting their immunological effects due to differences in their structures, pharmacokinetics and mechanisms of action, adalimumab and IFX having similar structures and mechanisms of action. As a consequence, several medical societies published guidelines recommending screening and, upon evidence of a latent infection, chemoprophylaxis for TB before starting treatment with these drugs. Upon implementation of these recommendations, the rate of TB among patients taking TNF-α antagonists for treating immunomediated inflammatory diseases (IMID) decreased significantly. This was well documented in Spain where the rate of TB among TNF-α exposed patients fell from 522 to 117 per 100 000 patients-years. Nevertheless, it still remained several folds higher than prevalence among the general population. In France, in the period of 2004–2006, the rate of TB was 187.5 for patients with IMID taking IFX (and 215 for those taking adalimumab), which is twenty-fold higher than the rate of TB in the general population (9/100 000 persons-year). Many reasons can account for these persistently abnormal rates. Among them are failure to comply with chemoprophylaxis, limited efficacy of the actual chemoprophylaxis regimens (able to reduce by 60% only the risk of TB reactivation), and perhaps most importantly, false negative results with the TST. In TB endemic areas, the TST misses up to 50% of the patients with latent TB probably due to the immunological imbalance underlying the IMIDs (EA Lima and G Benard, unpublished data). In the French study, 43.5% of the IMID patients who developed TB escaped the criteria for, and did not receive, chemoprophylaxis. In Greece, a TB rate of 449 per 100 000 patients was estimated despite the implementation of the TB screening recommendations. This raised serious concern with regard to the use of TNF antagonists in TB endemic areas, such as Brazil. High cost of the TNF antagonists has limited their use in poor countries. However, in Brazil, the State Health Department included the TNF-α antagonists in its high-cost medication programme, facilitating their access to low-income patients. Therefore, e.g., in 2009 a total of 6037 patients with IMID had access to TNF-α antagonists in São Paulo State. Surprisingly, preliminary data suggest that the rate of TB in patients with IMID taking IFX was 279.9/100 000 for the year 2009, i.e. 7.4 times higher than that of the general population in the state for that year (37.9/100 000) (TB Division, Center for Epidemiological Surveillance, Health Department, São Paulo State Government). This proportion is not different (or even lower) than that observed in developed, countries with low TB endemicity, as discussed above. This number is particularly intriguing because of the limitations of the TST as a screening tool, and in addition, because it is estimated that at least 5% of the patients prescribed TNF antagonists do not undergo TB screening (JS Carvalho, unpublished data).

To better understand the effect of TNF-α blockade with IFX on the susceptibility to TB reactivation in severe psoriasis patients in a TB endemic setting, we evaluated the in vitro M. tuberculosis (Mtbt)-specific immune responses of severe psoriasis patients and healthy donors, both with a positive TST, in the presence or absence of IFX.

Materials and methods

Patients and healthy donors

Patients were selected from a cohort of moderate to severe psoriasis patients admitted at our outpatient unit as described previously. Briefly, patients were >18 years of age, had fully active lesions compromising >10% of the body surface, lack of co-morbidities or treatments with agents known to interfere with the immune system. All eleven patients selected for the study were without systemic treatment for psoriasis for at least the last 3 months and were strong TST responders (>10 mm induration, 2 UT/units PPD, Serum State Institute, Copenhagen, Denmark). None of the TST responders had clinical or radiological evidence of active TB. In parallel, 13 healthy adults, strong responders to TST (>10 mm induration), were studied. All patients and healthy individuals gave written informed consent. The study was approved by the ethics committee of the Hospital das Clínicas da Universidade de São Paulo (protocol # 830/05).

**Mycobacterium tuberculosis antigens preparation**

Three different Mtbt antigen preparations, one consisting of a crude mixture of Mtbt antigens and two purified, recombinant Mtbt proteins (Ag85 and ESAT-6), were produced as described previously. The sonicated lysate (SAG) of M. tuberculosis H37Rv was kindly donated by M. Palaci (Universidade Federal do Espírito Santo, Brazil). The recombinant antigen 85B was obtained from the Colorado State University through contract # BR0300; the recombinant antigen ESAT-6 was overexpressed and purified as described elsewhere.

**Lymphocyte stimulation tests**

Lymphocyte proliferative response assays were carried out as described previously. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis, MO, USA), resuspended in RPMI-1640 (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 20 mmol/L HEPES (GIBCO-BRL), 2 mmol/L glutamine (GIBCO-BRL), 0.1 mmol/L sodium pyruvate (GIBCO-BRL) and 10% heat-inactivated human AB serum (Sigma-Aldrich) and plated in 96-wells plates in triplicates (2.5 x 10⁵ cells per well). PBMCs were cultured in triplicate wells in 5% CO₂ at 37 °C for 3 days with phytohemagglutinin (PHA, 2.5 μg/mL, Sigma-Aldrich) and for 6 days with ESAT-6 (5 μg/mL), Ag85B (2.5 μg/mL) and SAG (5 μg/mL), as described elsewhere. IFX (kindly donated by Schering-Plough, São Paulo, Brazil) was added at a final concentration of 5 μg/mL. PBMCs were pulsed with [³²P]thymidine (Amersham International, Buckinghamshire, UK) 18 h before
harvest; radioactivity incorporation was measured using a beta-counter (Betaplate 1205, Perkin-Elmer, Boston, MA, USA). A triplicate of wells with unstimulated cells served as background proliferation. Results were analysed both as stimulation index (SI) [ratio between the mean count (cpm) of the stimulated wells and the mean count of the unstimulated wells], and Δcpm (cpm of the stimulated wells minus cpm of the unstimulated wells), which yielded similar results. Only results in SI are shown.

Quantification of IFN-γ and IL-10 secretion in supernatants using ELISA
Peripheral blood mononuclear cells cultures were set up as above; supernatants were harvested at days 2 and 5 and stored in aliquots at −80 °C until use. TNF-α, IL-10 and IFN-γ levels were quantified by ELISA (Pierce-Endogen, Rockford, IL, USA) according to the manufacturer. The lower limit of detection of the assays was 10 pg/mL.

ELISpot assay for IFN-γ
The assay was performed and analysed as described previously.9 Briefly, Multiscreen-IP plates (Millipore Corporation, Bedford, MA, USA) were coated with antihuman IFN-γ monoclonal antibody (MAb) (4 μg/ml, Pierce-Endogen) overnight at 4 °C. PBMCs were plated (2 × 10^5 cells/well) in triplicates in the presence of PHA or Mtb antigens at the concentrations described above, with or without IFX (5 μg/ml), and left overnight at 37 °C in 5% CO₂. Plates were then washed and further incubated for 2 h at 37 °C with human IFN-γ MAb biotin labelled (1 μg/ml, Pierce-Endogen). The reaction was revealed using streptavidin-alkaline phosphatase (Pierce-Endogen) and BCIP/NBT substrate. The number of spot forming cells (SFC) = 1 × 10^6 for each stimulus was counted using the software ImmunoSpot 3.2 (CTL ImmuNoSpot®, S4 Analyzer, C.T.L., Cleveland, OH, USA). Values were expressed after subtracting the background. The assay was validated if the PHA wells gave >20 spots/well.

Statistical analysis
The Wilcoxon matched pairs test (Graphpad Prisma 5, Microsoft, USA) was used to compare the data with and without TNF-α antagonist. Significance level was set at 0.05.

Results
Cultures of PBMC from TST⁺ psoriasis patients and TST⁺ healthy individuals were stimulated with Mtb antigens and PHA, in the presence or absence of therapeutic concentration of IFX (5 μg/ml) as indicated in Materials and methods. Mtb antigens were a sonicated lysate (SAg) of M. tuberculosis; the recombinant protein 85B, which has been used to elicit in vitro recall responses and as a vaccine candidate, and whose homolog is present in all mycobacteria; and the recombinant protein ESAT-6, which, in contrast, is more specific because its encoding region is absent in most environmental mycobacteria, including the BCG vaccine strain.

We first determined the effect of IFX on the TNF-α concentration released in the supernatants. Release of TNF-α by unstimulated cells from patients and healthy individuals was undetectable in the presence or absence of IFX (not shown). Figure 1 shows that IFX almost abolished the detection of the cytokine released by stimulated cells in both groups. Because lower amounts of TNF-α were released by PBMCs of healthy individuals when stimulated with ESAT-6 and 85Ag, their further reduction by IFX did not reach statistical significance.

We then assessed the effect of IFX on the proliferative responses of PBMCs. As shown in Fig. 2, patient and healthy individual groups showed positive proliferative responses to the stimuli. IFX significantly reduced the responses to all stimuli in both groups.

Interferon-γ, together with TNF-α, is a key cytokine in the development and stability of well-formed granulomas, the main host mechanism for the containment of mycobacteria. Evaluation of the IFN-γ release by PBMC was carried out in two ways, reflecting either the total amount of IFN-γ produced or the number of IFN-γ producing cells. First, we used the ELISpot assay to

Figure 1 Quantification of TNF-α production. TNF-α production was quantified using ELISA in day 2 PBMC culture supernatants from untreated TST⁺ severe psoriasis patients (n = 9) and TST⁺ healthy individuals (n = 9), stimulated with M. tuberculosis SAg, Ag85B, ESAT-6 and PHA in the presence (white columns) or absence (grey columns) of infliximab (IFX, 5 μg/ml). Results are shown as mean ± SE. *, P < 0.05; **, P < 0.01, Wilcoxon matched pairs test. Non-stimulated wells had undetectable levels of the cytokine.
determine the number of cells releasing the cytokine rapidly early (overnight) after being challenged with the respective stimuli, a feature of effector memory T-cells (Tem). In both groups, as expected for TST+ subjects, the Mtb antigens induced high frequencies of IFN-γ-secreting cells; these frequencies remained unaltered in the presence of IFX for most subjects tested (Fig. 3). The same held true for the positive control, PHA. Secondly, the IFN-γ released in supernatants after 6 days was measured using ELISA; in this case, it is expected that the cytokine is produced mostly by central memory T cells (Tcm), which require several days after challenge to reach their maximum in cytokine secretion. IFX reduced the total IFN-γ secretion by the TST+ psoriasis patients’ cells; this reduction was statistically significant for stimulation with PHA, Ag85B and SAg but not with ESAT-6. The reduction in total IFN-γ secretion was less consistent in the healthy individuals and was statistically significant only with Ag85B (Fig. 4).

To determine whether the inhibitory effects on the IFN-γ secretion and LPR were due to exaggerated IL-10 secretion, we measured the IL-10 released on day-2 supernatants. Except for PHA, the amounts of IL-10 induced by the Mtb antigens were ~1 log lower compared with IFN-γ; these levels were either further decreased or unaffected by the presence of IFX in both groups (data not shown). Thus, these results do not corroborate the hypothesis that IL-10 plays a role in the decreased responses induced in the presence of IFX.

**Discussion**

The mechanisms by which neutralizing anti-TNF-α antibodies, such as IFX, reactivate latent TB are not yet fully known. In addition, there are no studies available regarding the effects of these agents on the anti-Mtb responses of patients with moderate/severe psoriasis.
Infliximab and immune responses to mycobacteria in psoriasis

Our results show that IFX indeed affected some, but not all aspects of the in vitro immune responses tested. T-cell activation and function were impaired since LPR and IFN-γ production to mycobacterial antigens of both healthy individuals and psoriasis patients were significantly decreased in the presence of IFX. Previous studies reported that IFX inhibited the in vitro IFN-γ secretion by activated intestinal T-lymphocytes from Crohn’s disease patients. Hamdi et al. studied patients with other IMIDs (mainly rheumatoid arthritis and ankylosing spondylitis) and also observed that IFX decreased the in vitro T-cell activation induced with mycobacterial (and other microbial) recall antigens. Saliu et al. examined the anti-Mtb immune responses of healthy TST+ individuals and showed that IFX decreased T-cell activation and the IFN-γ responses to mycobacteria antigens. Interestingly, our results showed that the effects of IFX were similar between healthy individuals and psoriasis patients. Thus, our data corroborate previous reports showing the down modulatory effects on immunity to Mtb of IFX and extend these to psoriasis patients. Similarly to the findings of Saliu et al., we could not detect any exacerbation of IL-10 in the presence of IFX that might account for the down regulatory effect of the antagonist. Thus, the combined effect of TNF-α blockade and inhibition of IFN-γ production seen in our latently infected psoriasis patients could explain the higher risk to TB reactivation, as pointed out earlier for the other IMIDs. In fact, experimental studies provided evidence that the antimycobacterial activity of human monocytes is affected by TNF-α antagonists independent of IFN-γ signalling, suggesting that simultaneous deprivation of both cytokines may have an additive or even synergistic effect. Another mechanism would be a temporary decrease in the frequency of CD8+ Tem cells with antimycobacterial activity, as shown in patients with RA undergoing IFX therapy. However, Saliu et al. also observed that the ability to control mycobacterial growth by 24 h whole blood cultures was unaffected by IFX, indicating that innate or other unknown immune bactericidal mechanisms may escape the anti-TNF effect.

To address this, we performed in vitro studies in which IFX (5 μg/mL, within the therapeutic range concentration observed in patients) was added to PBMC cultures from TST+ severe psoriasis patients and healthy individuals stimulated with M. tuberculosis SAg, Ag85B, ESAT-6 ad PHA in the presence (filled dots) or absence (empty dots) of infliximab (IFX, 5 μg/mL). Results are shown as mean ± SEM. *, P < 0.05; **, P < 0.01, Wilcoxon matched pairs test. Control wells with non-stimulated cells had undetectable levels of the cytokine.

Figure 4: Quantification of IFN-γ production. IFN-γ production was quantified using ELISA in day 5 PBMC culture supernatants from untreated TST+ severe psoriasis patients (n = 10) and TST+ healthy individuals (n = 12), stimulated with M. tuberculosis SAg, Ag85B, ESAT-6 and PHA in the presence (filled dots) or absence (empty dots) of infliximab (IFX, 5 μg/mL). Results are shown as mean ± SEM. *, P < 0.05; **, P < 0.01, Wilcoxon matched pairs test. Control wells with non-stimulated cells had undetectable levels of the cytokine.
patients in a similar fashion to their healthy TST-non-responders counterparts, while both the LFP response and IFN-γ release into supernatants, probably more related to central memory T-cell responses, were strongly decreased.9

The rate of patients with IMID exposed to TNF-α antagonists with reactivated TB in our setting was probably lower than expected and may otherwise suggest that they still preserve some degree of immunoprotection against bacillus reactivation. Our present in vivo data are corroborated by our in vitro studies, where the frequency of IFN-γ-releasing Tem cells elicited by recall antigens, including Mb antigens, is measured by overnight ELISpot in the peripheral blood of 13 severe psoriasis patients undergoing TNF-α antagonists therapy. The preliminary data show that the frequencies were augmented in a subset of these patients (8 of the 13 patients) after 7 weeks on therapy (LCM Ortigosa, LCR Silva & G Benard, unpublished data). We thus suggest that in TB endemic or tropical areas the recurrent exposure to environmental mycobacteria, either pathogenic or not, would result in sufficient levels of IFN-γ-releasing Tem cells to keep in check, at least partially, TB reactivation in a proportion of patients even in a TNF-α deficient microenvironment.

Acknowledgements

We would like to acknowledge Moisés Palaci for the assistance with the antigens used in the study.

References