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INVESTIGATIVE REPORT

Infliximab Does Not Lead to Reduction in the Interferon-gamma and Lymphoproliferative Responses of Patients with Moderate to Severe Psoriasis

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Treatment of patients with immune-mediated inflammatory diseases with anti-tumour necrosis factor (anti-TNF) agents increases the risk of tuberculosis reactivation, suggesting that it may affect their cellular immune responses. We evaluated cellular immune responses of 12 severe psoriasis patients before and during infliximab treatment. Peripheral blood mononuclear cells were stimulated with phytohaemagglutinin, the superantigen enterotoxin B (SEB), a cytomegalovirus lysate (CMV), and *Mycobacterium tuberculosis* (Mtb) antigens. The lymphocyte proliferative and IFN-γ responses were evaluated. Treatment with infliximab did not lead to reduction in the IFN-γ and lymphoproliferative responses: it rather increased the overnight release of IFN-γ in phytohaemagglutinin and SEB stimulated cultures. This effect was most noted at the peak of the anti-TNF clinical effect and less prominent at its nadir. Immunoreactivity to CMV was also either unaffected or slightly increased by the anti-TNF. Of note, the IFN-γ and proliferative responses to Mtb by the two tuberculin skin test-reactors were also increased at the peak of infliximab, declining at its nadir. The deleterious consequences of TNF blockade in severe psoriasis patients undergoing infliximab treatment are apparently attenuated by the abbreviation of the immunosuppressive effect of TNF overexpression.
Key words: TNF; infliximab; interferon-γ; cellular immune response; psoriasis.

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With the advent of the immunobiologicals, especially the tumour necrosis factor (TNF)-blocking agents, in the treatment of patients with moderate to severe psoriasis, the diagnosis of latent tuberculosis infection (LTBI) has become a crucial step in the management of these patients, since they were at high risk of tuberculosis (TB) reactivation (1). Classically, the tuberculin skin test (TST) is performed. However, due to limitations in this *in vivo* test, *in vitro* assays, based on the release of interferon (IFN)-γ upon overnight stimulation of peripheral blood mononuclear cells (PBMC) with specific *M. tuberculosis* antigens, have been used increasingly. Several studies have pointed to its better performance, compared with TST, particularly in *Bacillus Calmette–Guérin* (BCG)-vaccinated populations (2).

It is well known that immunosuppressive conditions (cancer, HIV infection) or treatments (chemotherapy, steroids, disease-modifying anti-rheumatic drugs) decrease the rate of positive responses to both the TST and IFN-γ release assays (3–5). However, there still is some controversy about whether treatment with anti-TNF agents in patients with immune-mediated inflammatory disease (IMID) alters the performance of the diagnostic tests of LTBI. It has been shown that patients with rheumatoid arthritis (RA) and ankylosing spondylitis (SA) undergoing anti-TNF treatment presented increased rates of TST responses, and that IFN-γ release assays also performed well in these patients (6, 7). Qumseya et al. (8) showed that anti-TNF treatment does not suppress IFN-γ responses, while Hatemi et al. (9) have shown that it did not suppress the TST and IFN-γ responses. However, other groups showed that anti-TNF treatment inhibited IFN-γ responses (10, 11). Differences in the types of IMID studied and/or IFN-γ release assays employed may partially explain such conflicting data. Of note, none of these studies addressed patients with psoriasis.

The aim of the present study was to determine the effect of the treatment with infliximab (IFX) on the cellular immune responses of patients with moderate to severe psoriasis.

MATERIALS AND METHODS

Patients

A total of 12 patients with untreated severe psoriasis (5 women and 7 men, age range 19–66 years), were studied. All patients had active lesions, compromising > 10% of the body surface (moderate to severe psoriasis, mean Psoriasis Area and Severity

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Index (PASI) 35.6), lacked co-morbidities or treatments with agents known to interfere with the immune system, and have not received any systemic treatment for psoriasis 3 months prior to admission to the study. All patients underwent a chest X-ray and a tuberculin skin test (TST) using 2 tuberculin units purified protein derivative (PPD) (Serum State Institute, Copenhagen, Denmark). All chest X-rays were normal, and 2 out of 12 patients had positive TST (> 5 mm induration). These patients started a 6-month prophylaxis with isoniazid 1 month prior to IFX treatment. The patients had no evidence of current or past tuberculosis (TB). IFX (5 mg/kg) was infused at weeks 0, 2, 6 and 14, and thereafter every 8 weeks; blood samples were collected just before starting IFX (week 0 or baseline) and at week 7, at the peak of the anti-TNF effect, week 10, when its levels already started to decline, and at week 14, just before the next IFX infusion, when the drug reached its nadir. The PASI was also scored at each blood collection.

All patients provided written informed consent. The study was approved by the ethics committee of the Hospital das Clínicas da Universidade de São Paulo (#0467/08).

Peripheral blood mononuclear cells isolation and storage

PBMCs were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich, St Louis, MO, USA) and then stored frozen at –120°C in liquid nitrogen in 10% dimethyl sulphoxide (DMSO) (Merck, Darmstad, Germany) until use. Aliquots of the cells collected at the different time-points from each patient were thawed and resuspended at the same time and tested in parallel for all laboratory determinations. Only aliquots with > 80% viability by trypan blue exclusion done after 4 h rest were used. PBMC were stimulated with the mitogen phytohaemagglutinin (PHA), the superantigen enterotoxin B (SEB), from *Staphylococcus aureus*, a cytomegalovirus (CMV) lysate, and *M. tuberculosis* (Mtb) antigens, and the lymphocyte activation was evaluated by enzyme-linked immunosorbent spot assay (ELISpot) for enumeration of IFN-γ-secreting cells, enzyme-linked immunoassay (ELISA) for detection of secreted IFN-γ, and by [^{3}H] thymidine incorporation for lymphocyte proliferation measurement. These assays were selected as in previous studies (12) because the ELISpot detects T cells that, after a few hours (overnight) exposure to antigens or other stimuli, secrete IFN-γ, a feature of effector memory T cells, while the IFN-γ ELISA and lymphocyte proliferation assay preferentially measure functions of central memory T cells, which need several days after activation to undergo clonal expansion or secrete large amounts of cytokines.

Enumeration of IFN-γ-secreting cells by ELISpot

Enumeration of the IFN-γ-secreting cells elicited with the Mtb antigens early secreted antigenic target 6 kDa protein (ESAT-6) and culture filtrate protein-10 (CFP-10) was done using the T-SPOT.TB test (Oxford Immunotec, Abingdon, UK) according to manufacturer's instructions (13). Briefly, 250,000 PBMC/well in complete medium (Roswell Park Memorial Institute medium (RPMI) supplemented with 20 mM HEPES, 2 mM glutamine, 0, 1 mM sodium pyruvate (all from GIBCO-BRL, Gaithersburg, MD, USA) and 10% heat-inactivated human AB serum (Sigma-Aldrich) were stimulated overnight with Mtb antigens. The test was considered positive when the spots count with either ESAT-6 or CFP-10 minus negative control spots count was ≥ 6. The assay was considered valid when the positive control well gave > 20 spots. All tests were considered valid. ELISpot with PHA, SEB and PPD was performed as previously standardized in our laboratory (12). Briefly, Multiscreen-IP plates (Millipore Corporation, Bedford, MA, USA) were coated with anti-human IFN-γ monoclonal antibody (MAb) (4 mg/ml, Pierce-Endogen, Rockford, IL, USA) overnight at 4°C. PBMCs were plated (2×10^5 cells/well) in triplicates in the presence of PPD, SEB or PHA and left overnight at 37°C in 5% carbon dioxide (CO_2). Plates were then washed and further incubated for 2 h at 37°C with human IFN-γ MAb biotin labelled (1 mg/ml, Pierce-Endogen). The reaction was revealed using streptavidin-alkaline phosphatase (Pierce-Endogen) and and 5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium 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, CTL, Cleveland, OH, USA). Values were expressed after subtracting the background.

Quantification of IFN-γ in culture supernatants by ELISA

PBMCs (250,000 cells/well in complete medium) were cultured in triplicate in 5% CO_2 at 37°C for 3 days with phytohaemagglutinin (PHA, 2.5 µg/ml, Sigma-Aldrich) and for 6 days with PPD (5 µg/ml). The supernatants were harvested at days 2 (PHA) and 5 (PPD), and stored in aliquots at –80°C until use. IFN-γ levels were quantified by ELISA (Pierce-Endogen) according to the manufacturer (12). Lower limit of detection of the assay was 10 pg/ml.

Lymphocyte proliferative response assay

Lymphocyte proliferative response (LPR) assays were carried out as described previously (13). PBMC cultures were set up as above, then pulsed with 0.5 µCi/well [^{3}H]thymidine (Amersham International, Amersham, 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.

Statistical analysis

The Mann–Whitney test (Graphpad Prisma 5, Microsoft, USA) was applied. Significance level was set at $p < 0.05$.

RESULTS

At baseline, the patients presented significant numbers of IFN-γ secreting cells in response to the non-specific stimuli PHA and SEB. At week 7, at the peak of IFX clinical effect (patients achieved a reduction in the PASI score of 72%, from 35.60 to 9.85), these responses were significantly increased compared with baseline (Fig. 1A). Antigenic-specific responses to CMV (>5 spots/well) also increased from baseline to week 7, with all patients becoming CMV-responders. Nine of the 12 patients were diagnosed as not having LTBI according to their negative response to TST; this was confirmed by their negative responses on the ELISpot assays with Mtb antigens (data not shown). Data from 2 TST-responders of the 3 are shown in Fig. 2A: one patient had negative ELISpot responses to all 3 Mtb antigens, while the other patient had positive response only to CFP-10. At week 7 there was a marked increase in the responses to the Mtb antigens in both patients. In addition, we sought to determine if the increase in the number of IFN-γ secreting cells persisted even when the levels of the anti-TNF agent decreased (weeks 10

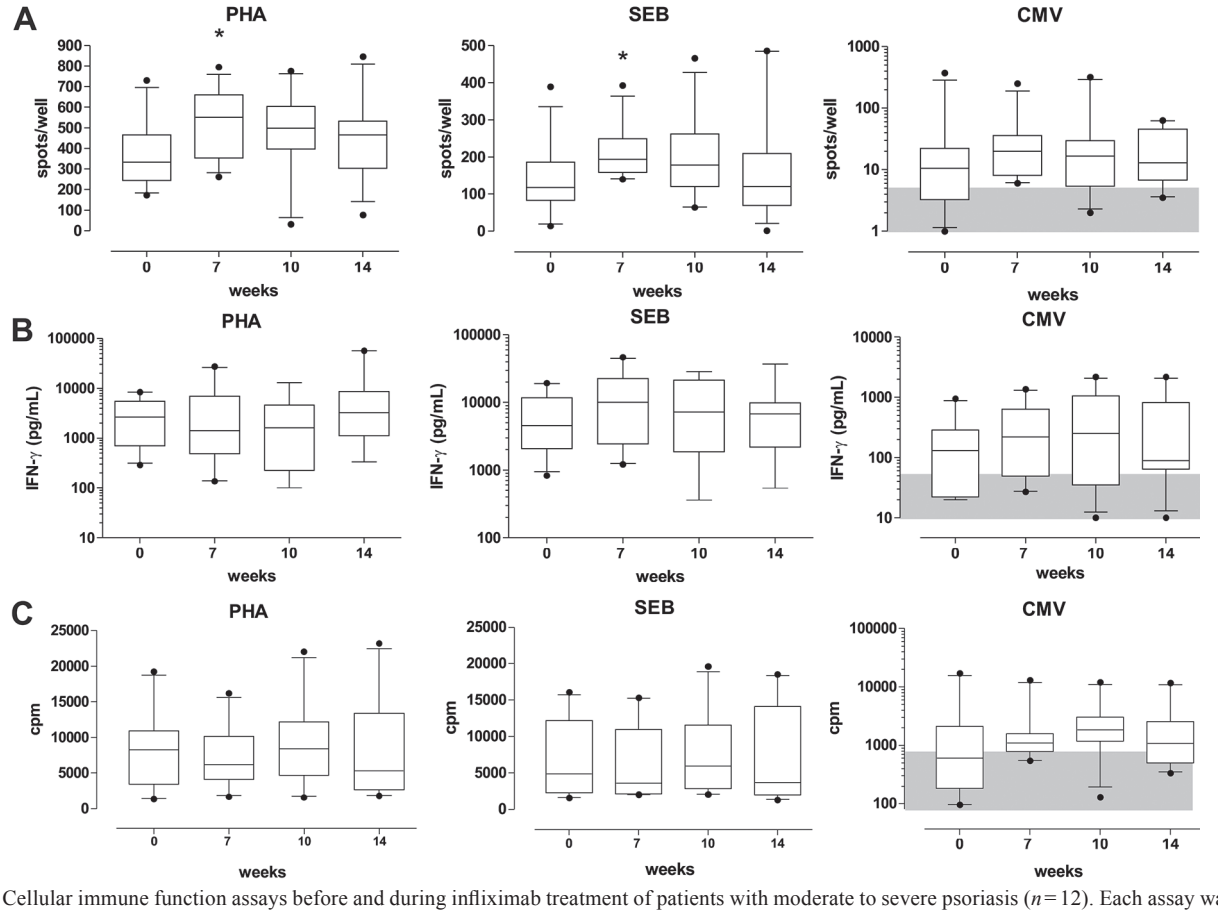

Fig. 1. Cellular immune function assays before and during infliximab treatment of patients with moderate to severe psoriasis ($n = 12$). Each assay was run in triplicate and data shown are box and whiskers and outliers. (A) Interferon-γ (IFN-γ)-releasing cells were quantified by enzyme-linked immunosorbent spot assay (ELISpot) after overnight stimulation of peripheral blood mononuclear cells (PBMC) with phytohaemaglutinin (PHA), superantigen enterotoxin B (SEB) and cytomegalovirus (CMV) lysate. *$p < 0.05$ vs. week 0. Median counts of spots in non-stimulated wells were not significantly different ($p > 0.05$): 0.25, 1.5, 1.5 and 0.25, weeks 0–14, respectively). (B) IFN-γ production was quantified by ELISA in day 5 PBMC culture supernatants stimulated with PHA, SEB and CMV lysate. Median levels of IFN-γ in non-stimulated wells were not significantly different ($p > 0.05$): 5.0, 8.0, 13.0 and 11.5 µg/ml, for weeks 0–14, respectively). (C) Lymphocyte proliferative responses to PHA, SEB, and CMV lysate as determined by [^{3}H]thymidine incorporation. Results are shown in counts per minute (cpm). Median counts of non-stimulated wells were not significantly different ($p > 0.05$): 232, 469, 454, and 199 cpm, for weeks 0–14, respectively). The grey area in the CMV figures represents the negative responses of the assays.

and 14). In fact, there was a trend for a reduction in the Mtb responses compared with week 7, although remaining slightly higher or equal to those at baseline. The third TST-responder had negative ELISpot responses at baseline, a positive response only to PPD at week 7, and again negative responses at weeks 10 and 14 (data not shown).

We found similar results when we quantified the IFN-γ released in day 5 PBMC culture supernatants by ELISA (Fig. 1B). At baseline, patients' PBMC released significant amounts of the cytokine upon stimulation with PHA and SEB, without significant modifications at either the peak or nadir of the anti-TNF agent (Fig. 1B). Responses to CMV were variable. However, compared with baseline, there was a trend for higher responses at the peak of the anti-TNF and also at week 10, followed by a decrease at its nadir on week 14 (Fig. 1B). While TST non-responders patients did not produce IFN-γ in response to PPD at any time tested (not shown), 2 of the 3 TST-responders had a small baseline production (Fig. 2B) that increased ~15-fold at week 7, persisting elevated in one patient, but decreasing to become negative at week 14 in the other patient (Fig. 2B). The third TST-responder did not produce IFN-γ at any time.

Data from the proliferative responses are shown in Figs 1C and 2B. Responses to PHA and SEB were present at all weeks in a similar fashion. Responses to CMV increased from baseline to the peak of anti-TNF therapy, although it did not reach statistical significance. Two of the 3 TST-responders showed a lymphoproliferative response to PPD, one already at baseline, which increased further during IFX therapy, and the other showed a positive response only at the peak of IFX treatment, reverting to negative at the nadir of the drug. Neither the third TST-responder, nor the TST non-responders patients showed a proliferative response to PPD at any time (not shown). None of the 12 patients had developed TB after 2 years of follow-up.

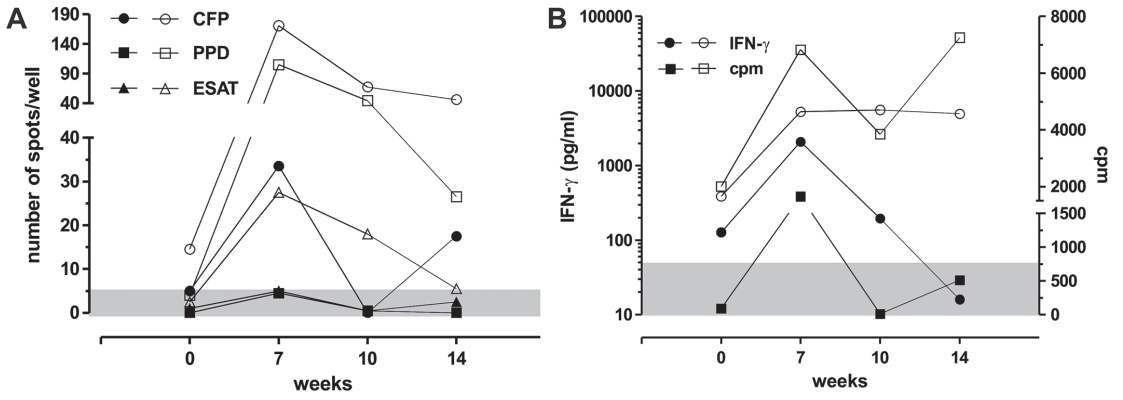

Fig. 2. Cellular immune responses to *Mycobacterium tuberculosis* antigens of the 2 tuberculin skin test-responders patients (#9 and #10) before and during infliximab treatment. (A) Interferon-(IFN)-γ-releasing cells quantified by enzyme-linked immunosorbent spot assay (ELISpot) after overnight culture of peripheral blood mononuclear cells (PBMC) stimulated with ESAT-6, CFP-10 and PPD. (B) IFN-γ response in day 5 PBMC culture supernatants (quantified by enzyme-linked immunoassay (ELISA), left y-axis) and lymphocyte proliferative response (as determined by [^{3}H]thymidine incorporation, right y-axis), elicited with PPD. The grey area represents the negative responses of the assays. Filled symbols denote patient #9 and empty symbols denote patient #10.

DISCUSSION

This study demonstrated that treatment with the TNF-blocking agent IFX does not lead to reduction in the IFN-γ and lymphoproliferative responses of patients with severe psoriasis. It rather increased the overnight release of this cytokine in PBMC cultures stimulated with the mitogen PHA and the superantigen SEB. This effect was most noted at the peak of the clinical effect of IFX (week 7 of treatment) and less prominent at its nadir (just before infusion of the next dose). Immune reactivity to a CMV lysate antigen was also either unaffected or slightly increased by IFX. Of note, the IFN-γ and proliferative responses to Mtb from 2 TST-responder patients were also remarkably increased at week 7, declining when IFX reached its nadir. A third TST-responder presented a transient ELISpot response to PPD at week 7 and lack of responses to the Mtb-specific ESAT-6 and CFP-10 antigens, indicating cross-reaction with non-tuberculous *Mycobacteria*.

Limitations of our study were the small number of patients evaluated and lack of long-term evaluation. However, TB reactivation occurs most frequently in the first 3 months of anti-TNF treatment (2).

Psoriasis is associated with a systemic immunodysregulation characterized by high expression of pro-inflammatory cytokines, but T-cell hypo-responsiveness, including decreased IFN-γ production (12, 14–18). Among them, TNF plays an early and key role in the pro-inflammatory cascade. Although anti-TNF agents have shown efficacy against most IMID, they increased the risk of reactivation of TB or other diseases caused by intracellular parasites, since TNF is also crucial for the development and maintenance of compact granulomas. Although anti-TNF treatment has been reported to further immunosuppress patients with IMID, our results showing increase in some cellular immune responses are not unexpected, since TNF blockade can, instead, overcome the adverse effects of the immunodysregulation caused by excess TNF. In fact, similar findings of restoration of Th-1 responses were observed in patients undergoing anti-TNF treatment for other IMID, such as RA and SA (19, 20).

A possible mechanism for the observed enhancing effects of anti-TNF treatment relies on the observation that chronic exposure to TNF leads to T-cell hypo-responsiveness (21, 22), and that monocytes/macrophages are a major source of the increased levels of TNF in patients with psoriasis. Monocytes are one of the major producers of TNF (23) and they are found in high numbers in pre-psoriatic and psoriatic skin lesions (24), suggesting that they are one of the triggering elements of the skin inflammatory process. A murine model of psoriasis has shown that activated macrophages are essential for the development of the chronic inflammatory process (25). Moreover, peripheral blood monocytes and macrophages isolated from lesions of psoriasis patients exhibit an activated phenotype and actively secrete TNF, among other pro-inflammatory cytokines (26–29). In fact, the dual, activating and deactivating, role of macrophages in inflammation has been discussed recently (30). Thus, one likely mechanism by which TNF blockade restores T-cell responsiveness would be the abbreviation of the chronic monocyte/macrophage-derived TNF down-modulatory effect on T cells. This hypothesis is further supported by a sequential study of biopsies of patients on anti-TNF treatment, showing marked decrease in the density of macrophages and dendritic cells in psoriatic plaques, which was accompanied by restoration of epidermal keratinocyte differentiation (31).

It was previously reported that an increase in incidence of TB in IMID patients taking IFX in São Paulo state, Brazil (32), was not much different from the increase observed in IMID patients in some low TB burden countries after the introduction of anti-TNF therapies (33–35). The partial restoration of the IFN-γ and proliferative responses to Mtb antigens by the anti-TNF treatment may help to explain this lower than expected

rise. Although TNF and IFN-γ act synergistically, TNF alone has been shown to be a key factor in activating monocytes and inducing granuloma formation (36). Experiments with animal models of immunodeficiency demonstrated that reactivation of TB occurred in presence of normal levels of IFN-γ expression (37). Although TNF blockade alone can account for the higher risk of TB reactivation, this risk is further increased when there is association with deficiency of Th-1 responses, such as IFN-γ production (38). However, the partial restoration of cellular immune responses resulting from the anti-TNF treatment may have decreased this risk and have contributed to the lower than expected rise in the TB incidence in IMID patients in our setting.

In conclusion, the deleterious consequences of the TNF blockade in patients with severe psoriasis undergoing anti-TNF treatment may be partially attenuated by an enhancing effect on the cell-mediated immunity of the patients due to the decrease in the immunosuppressive effect of TNF overexpression. Further studies with larger numbers of patients are warranted to elucidate this mechanism.

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The authors declare no conflicts of interest.

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