In Vitro Observations of T Cell Responsiveness to Recall Antigens During Tumor Necrosis Factor-α-Blocking Therapy in Patients with Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** Anti-tumor necrosis factor-α (TNF-α) therapy can induce reactivation of tuberculosis and an increase of other infections in patients with ankylosing spondylitis (AS). This raises the question if an alteration of T cell function can be detected by *in vitro* analysis to identify patients who might be more at risk of acquiring such infectious diseases.

Methods. We examined peripheral blood from AS patients without history of tuberculosis before and after 10–14 and 24–36 weeks of therapy with adalimumab (n = 8) or infliximab (n = 10). Fresh peripheral blood mononuclear cells were stimulated with cytomegalovirus antigens and with the *Mycobacterium tuberculosis* antigen purified protein derivative and early secretory antigen target 6. Interferon-γ production of CD4+ T cells was assessed after *in vitro* antigen-specific stimulation by intracellular cytokine staining and flow cytometry.

Results. There was no significant change, either decrease or increase, of the T cell response to recall antigens during therapy compared to controls without treatment, if the mean values of all patients treated with adalimumab or infliximab were compared at the given timepoints. However, analysis on the individual patient level of such T cell responses revealed 1 adalimumab-treated patient and 2 infliximab-treated patients with a clear decrease of T cell response during therapy. Longterm analysis indicated that such a decrease of T cell responsiveness is generally transient and reconstituted at the latest after 52 weeks.

Conclusion. Some patients treated with adalimumab or infliximab showed a decrease of T cell responsiveness, which seems to be transient. These patients in particular might be at risk for intracellular infections. (First Release Oct 1 2007; J Rheumatol 2007;34:2264–70)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS T CELL RESPONSE RECALL ANTIGENS
TUMOR NECROSIS FACTOR-α BLOCKING THERAPY

Ankylosing spondylitis (AS) is the most frequent subtype of spondyloarthritides, with a prevalence between 0.2% and 1.1%[1-4]. There is evidence that therapy with the tumor necrosis factor-α (TNF-α) blockers adalimumab, infliximab, and etanercept is highly effective in AS[5]. However, these drugs have been associated with an increase of infections[6]. In particular, reactivation of latent tuberculosis during treatment with the anti-TNF-α monoclonal antibodies has become a major concern[7].

Possible mechanisms of TNF-α-blocking agents have been discussed: (1) infliximab, adalimumab, and etancercept all neutralize soluble TNF-α; (2) infliximab and adalimumab effectively bind to membrane-anchored TNF-α[8-11]; and (3) infliximab can induce apoptosis in activated monocytes and lamina propria T cells as shown in Crohn's disease[9,12,13]. It has been suggested that especially the last 2 mechanisms are relevant for the reactivation of tuberculosis seen in patients treated with antibodies against TNF-α[9].

Before treatment with TNF-α-blocking agents is started latent tuberculosis is normally assessed by chest radiographs and tuberculin skin test (TST). However, patients previously vaccinated with Bacillus Calmette-Guerín (BCG) might also reveal a positive TST result[14,15]. Recently, *Mycobacterium tuberculosis* (Mtb)-derived specific proteins have been described that are encoded in the genome of Mtb but are different from tuberculin purified protein derivative (PPD). One

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of these is the early secretory antigen target 6 (ESAT-6), which allows differentiation between Mtb-specific T cell responses in BCG-vaccinated patients and patients with latent tuberculosis[16-19].

We investigated the T cell response to Mtb-derived and to other recall antigens derived from cytomegalovirus (CMV), and also to staphylococcus enterotoxin B (SEB) in patients with AS before and during TNF-blocking therapy for up to 1 year in order to investigate whether depletion of T helper-1 cells occurs and whether a change in T cell response correlates with clinical improvement. These results were compared with AS controls not treated with TNF-α blockers.

MATERIALS AND METHODS

Patients. We obtained fresh peripheral blood from patients with AS who received infliximab (intravenously 3–5 mg kg body weight at 0, 2, and 6 weeks, and then every 6 weeks) or adalimumab (subcutaneous 1 × 40 mg every 2 weeks) before therapy, after 10–14 weeks (adalimumab n = 16, infliximab n = 10), and after 24–36 weeks of therapy (adalimumab n = 8, 2 wks after last subcutaneous injection; infliximab n = 10, 6 wks after last infusion). The mean age in the infliximab-treated patients with AS was 38.7 years (range 25–51), 9/9 were HLA-B27+, and the mean disease duration was 6.5 years (range 2–18). The adalimumab-treated patients with AS (n = 8) had a mean age of 33.5 years (range 25–46), 7/8 were HLA-B27+, and the mean disease duration was 5.7 years (range 2–16). Both groups of patients were responders to treatment with TNF-α-blocking agents and reached a 50% improvement of Bath AS Disease Activity Index (BASDAI) or an improvement of at least 2 in a BASDAI scale of 0 to 10, except for one infliximab-treated patient, who had a clear improvement of acute inflammation as shown by magnetic resonance imaging (Figure 1). A second group of adalimumab-treated patients included 8 nonresponders who were investigated at 10–14 weeks. The mean age in the nonresponder group was 44.4 years (range 35–57), 6/8 were HLA-B27+, and they had a mean disease duration of 12.3 years (range 1–24). Those nonresponders were defined as patients who did not reach a 50% improvement of BASDAI or an improvement of at least 2 in a BASDAI scale of 0 to 10. None of the above mentioned patients was treated with disease modifying antirheumatic drugs (DMARD) or steroids.

Our results were compared to 9 patients with AS (mean BASDAI 5.9 at 0 wks and 5.7 at 12 wks) with a mean age of 38.3 years, 7/9 HLA-B27+, and a mean disease duration of 10.2 years who did not receive TNF-blocking agents, glucocorticoids, or any DMARD.

All blood samples from these patients were freshly analyzed before, after 10–14 weeks (including the control group), and after 24–36 weeks of therapy.

In order to analyze the longterm effect of TNF-α-blocking therapy over 1 year, an additional group of patients with AS treated with infliximab or adalimumab was investigated. For this, peripheral blood mononuclear cells (PBMNC) from patients with AS that were frozen before therapy and after 3, 6, and 12 months of therapy (adalimumab, n = 9; infliximab, n = 8) were analyzed. Different from the analysis of MNC from fresh whole blood, all samples from the same patient were analyzed on the same day. All these patients with AS had a BASDAI of ≥ 4 before therapy and responded well to therapy.

All patients gave consent to the study.

Staining for T cell surface markers, intracellular cytokines and analysis by flow cytometry. T cells were stained after *in vitro* stimulation as described[20]. Briefly, cells from whole PB were resuspended in fluorescence-activated cell sorting (FACS) lysing solution including less than 50% diethylene glycol and less than 15% formaldehyde (diluted 1:10 with reagent-grade water, Becton Dickinson, Heidelberg, Germany), washed with PBS/BSA, centrifuged (300 g, 10 min, 4°C), and stained for the CD4 and CD69 surface markers and for the intracellular cytokine interferon-γ (IFN-γ). All stainings were performed in FACS™ Permeabilizing Solution (Becton Dickinson). To avoid nonspecific binding of antibodies to Fc-receptors, staining was done in the presence of Beriglobin (1 mg/ml, Centeon pharma, Berlin, Germany). The following antibodies were used: anti-human CD4 PerCP (clone SK3), anti-CD69 PE (clone L78), and anti-IFN-γ coupled to allophycocyanin (APC) (clone B27; all Becton Dickinson). Positive cells were subsequently quantified by flow cytometry using a FACS Calibur from Becton Dickinson (San Jose, CA, USA) with Cellquest Software. After gating on CD4+ T cells, only cytokine-positive T cells that were also positive for the early activation surface antigen CD69 were counted.

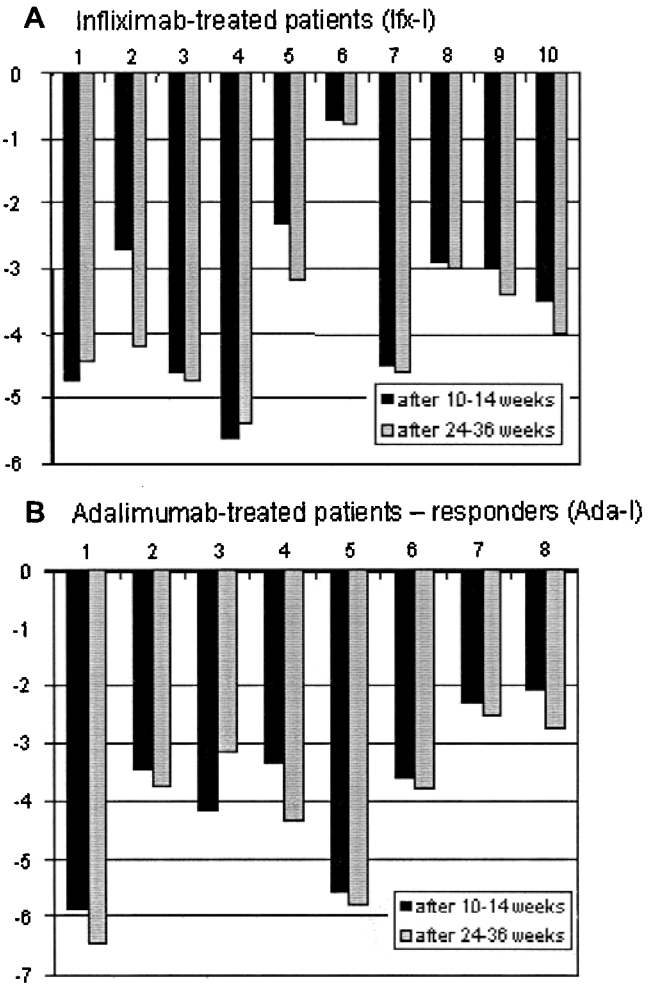

Figure 1. Changes in BASDAI scores during therapy with TNF-α-blocking agents. A. Infliximab at 10–14 weeks and 24–36 weeks. B. Adalimumab responders at 10–14 weeks and at 24–36 weeks.

CD4+ T cells were regarded as positive after *in vitro* antigen-specific stimulation as judged by the percentage of CD69/cytokine double-positive cells if the gated CD4+ T cells were positive in comparison to background staining (stimulation with anti-CD28 without antigen only).

T cell stimulation with recall antigens and Staphylococcus enterotoxin B. Fresh MNC were stimulated for 6 hours in the presence of anti-CD28 alone (1 µg/ml; clone B27.2, Becton Dickinson), with Staphylococcus enterotoxin B (SEB; 1 µg/ml, used as a positive control to indicate that T cells can be stimulated; Sigma-Aldrich, Deisenhofen, Germany), or with anti-CD28 plus one of the following antigens: human CMV pp65 antigen (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) was used at a concentration of 2 µl per 1×10^6 cells, according to the manufacturer's recommendations; a mixture of ESAT-6 peptides was obtained from JPT Peptide Technologies GmbH (Berlin, Germany) and was used at a final concentration of 1 µg/ml; recom-

binant ESAT-6 protein (provided by Statens Serum Institute, Copenhagen, Denmark) was used at a concentration of 5 µg/ml; tuberculin PPD was obtained from Chiron Behring GmbH (Marburg, Germany) and used at a concentration of 6 tuberculin units/ml.

After 2 hours of stimulation, brefeldin A (BFA, 10 µg/ml, Sigma Aldrich, Taufkrichen, Germany) was added to inhibit cytokine release from cells[21]. Frozen MNC from different timepoints of therapy were thawed at the same time for each patient. T cell responsiveness was analyzed to SEB and CMV antigen. Further analysis to other recall antigens was not possible due to limited amounts of MNC. If fresh cells were analyzed, responders to (1) CMV were defined as those with an initial T cell response > 0.1% CD4+/IFN-γ+ T cells, to (2) PPD, and (3) ESAT-6 antigen preparations > 0.02% CD4+/IFN-γ+ T cells in comparison to background staining with anti-CD28 alone.

The use of frozen MNC for intracellular cytokine staining might result in a lower T cell response. Therefore, the threshold for defining initial responders was reduced by 50%: responders to CMV were defined as those with an initial T cell response of > 0.05% CD4+/IFN-γ+ T cells.

We defined a clear decrease of T cell responsiveness to a specific recall antigen and SEB as a reduction of ≥ 25% CD4+, IFN-γ+ T cells after treatment was started. A general reduction of T cell responsiveness was defined as a condition in which the T cell response to SEB, CMV antigen, or PPD plus one of the ESAT-6 samples showed a reduction of at least 25% for all of them.

A change of response to recall antigens could only be counted if there was T cell reactivity before treatment.

Statistics. For group analysis of data before therapy and at the given timepoints we used the Wilcoxon test and SPSS for Windows.

RESULTS

T cell response to recall antigens using fresh MNC — group analysis of infliximab (Ifx-I) or adalimumab (Ada-I)-treated patients and controls. We first analyzed the T cell response to SEB and recall antigens using fresh MNC from patients with AS before and 10–14 and 24–36 weeks after therapy with infliximab or adalimumab was initiated. The mean values of all patients were analyzed at the given timepoints and results were compared to baseline. While no differences in the adalimumab-treated patients with AS were seen, we found a decrease of T cell response in infliximab-treated patients after 10–14 weeks and 24–36 weeks if compared to baseline; this was not statistically significant, however (Table 1).

We also compared the differences between baseline and Week 10–14 in TNF-α-treated patients and untreated AS controls, and found no significant differences.

In order to define the longterm effect of TNF-α-blocking therapy in patients treated with adalimumab or infliximab, we analyzed frozen samples of patients with AS treated with these drugs, and investigated the T cell responsiveness to SEB and CMV antigen. If the mean values of T cell responses were compared, no significant changes during treatment were observed (data not shown).

T cell response to recall antigens using fresh MNC — infliximab-treated patients with AS (Ifx-I). Next, we asked whether there would be single patients with a decrease of T cell response to recall antigens that were not identified on the group level. We found a general reduction of T cell response to SEB, CMV plus PPD plus one of the ESAT-6 antigen preparations in 2 infliximab-treated patients (Ifx-I.2 and 6). The analysis of patient Ifx-I.2 is shown in Figure 2. Patient Ifx-I.9 was a borderline case, who showed a clear reduction to ESAT-6 antigen preparations but did not fulfill our criteria (Figure 3).

Inconsistently with this, there was a transient increase of T cell responses to some of the recall antigens. However, we did not observe a general increase of T cell responses to CMV, PPD, and one of the ESAT-6 preparations after 10–14 and 24–36 weeks in our cohort of patients.

T cell response to recall antigens using fresh MNC — adalimumab-treated AS patients (Ada-I), responders. We next analyzed the T cell response in individual AS patients to SEB and recall antigens in freshly drawn MNC before and 10–14 and 24–36 weeks after therapy with adalimumab (Ada-I) was initiated. Only one patient (Ada-I.2) showed a sustained general reduction of T cell responsiveness to all recall antigens (Figure 4).

Inconsistently, there was a transient increase of T cell responses to some of the recall antigens. In patients Ada-I.5, 6, and 7 the T cell response to both ESAT-6 antigen preparations rose during therapy. However, we did not identify an adalimumab-treated patient who showed a general induction of T cell responsiveness to CMV, PPD, and one of the ESAT-6 antigen preparations.

Table 1. Group analysis of AS patients treated with infliximab or adalimumab and untreated AS controls.

	Infliximab-Treated Patients, n = 10					Adalimumab-Treated Patients, n = 8					Controls, n = 9				
	SEB	CMV	PPD	ESAT-6 protein	ESAT-6 peptides	SEB	CMV	PPD	ESAT-6 protein	ESAT-6 peptides	SEB	CMV	PPD	ESAT-6 protein	ESAT-6 peptides
Baseline	5.79 ± 3.01	0.37 ± 0.47	0.19 ± 0.32	0.02 ± 0.03	0.03 ± 0.03	3.18 ± 2.09	0.19 ± 0.24	0.04 ± 0.04	< 0.01	< 0.01 ± 0.03	2.99 ± 1.76	0.24 ± 0.38	0.04 ± 0.03	< 0.01	0.01 ± < 0.01
10–14 weeks	5.13*† ± 3.41	0.38*† ± 0.47	0.15*† ± 0.17	0.02*† ± 0.03	0.02*† ± 0.02	3.02*† ± 2.19	0.28*† ± 0.32	0.01*† ± 0.01	< 0.01*†	0.01*† ± 0.01	3.41* ± 1.68	0.27* ± 0.36	0.05* ± 0.04	< 0.01*	0.01* ± < 0.01
24–36 weeks	4.43* ± 2.66	0.23* ± 0.22	0.1* ± 0.17	0.02* ± 0.03	0.02* ± 0.02	3.94* ± 2.59	0.2* ± 0.22	0.03* ± 0.01	0.01* ± < 0.01	0.02* ± 0.02	—	—	—	—	—

* p > 0.05 compared to baseline, † p > 0.05 if difference to baseline was compared to untreated AS controls. AS: ankylosing spondylitis; SEB: staphylococcus enterotoxin B; CMV: cytomegalovirus antigen pp65; PPD: tuberculin purified protein derivative; ESAT-6: early secretory antigen target 6.

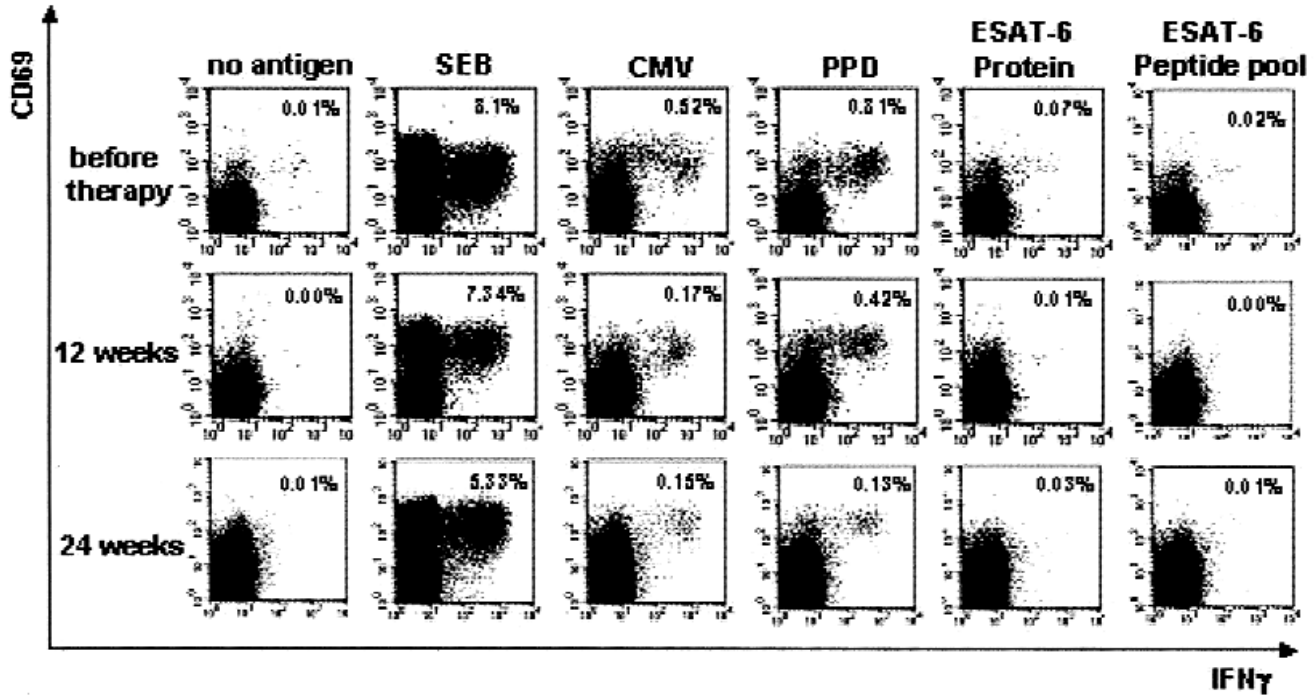

Figure 2. Example of an AS patient treated with infliximab: dot blot analysis of intracellular IFN-γ staining of CD4+ T cells from fresh mononuclear cells of an AS patient after stimulation with Staphylococcus enterotoxin B (SEB) and different recall antigens before and after 10–14 weeks and 24–36 weeks of therapy with infliximab. A clear decrease of T cell responsiveness was observed after 12 weeks after stimulation with SEB, cytomegalovirus antigen (CMV) pp65, and antigens from *M. tuberculosis* (PPD and at least one of the ESAT-6 antigens).

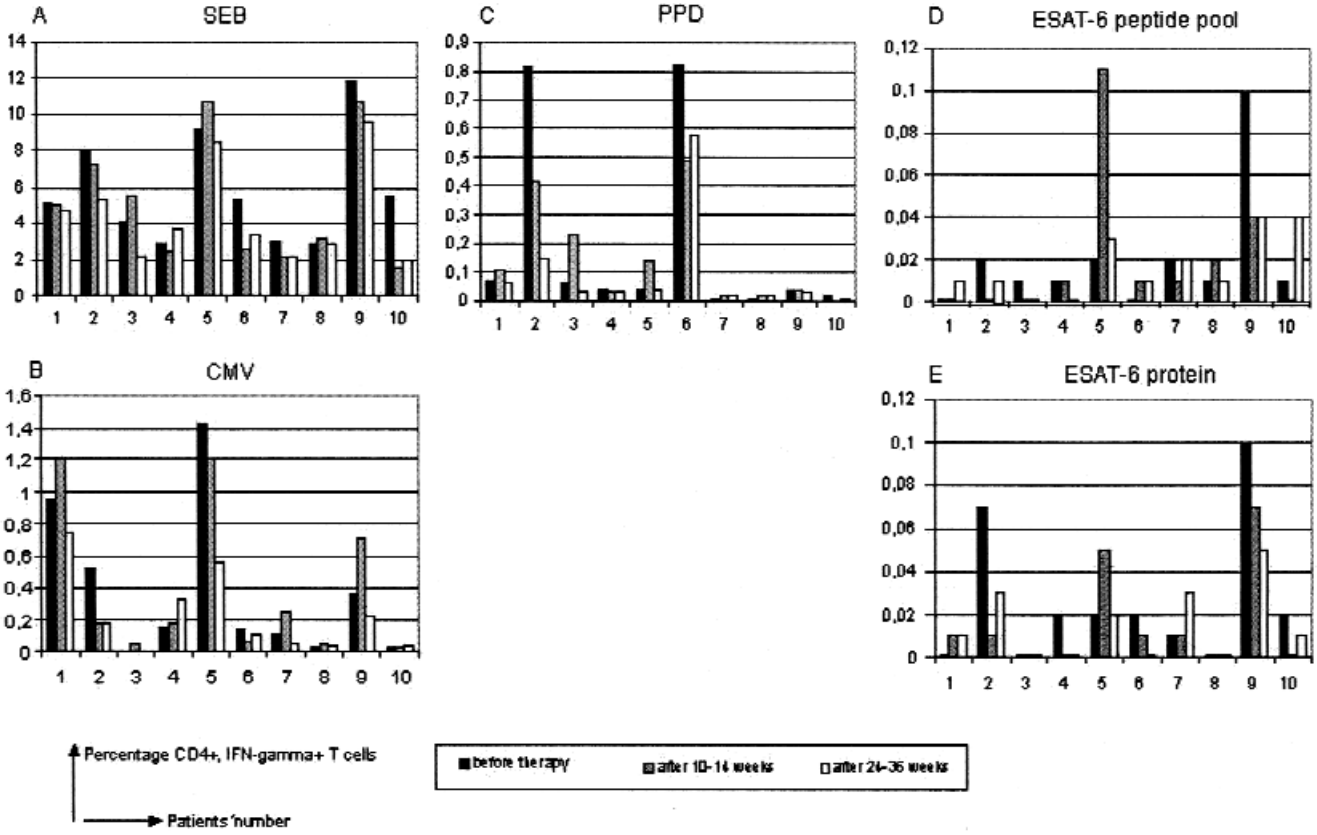

Figure 3. Summary of T cell responsiveness of all infliximab-treated patients with AS (Ifx-I) against SEB and recall antigens before and during therapy; A. against SEB, B. CMV pp65 antigen, C. PPD, D. early secretory antigen target-6 (ESAT-6) pooled peptides, and E. ESAT-6 proteins. A clear reduction of T cell responses to SEB and recall antigens during therapy was seen in patients Ifx-I.2 and 6.

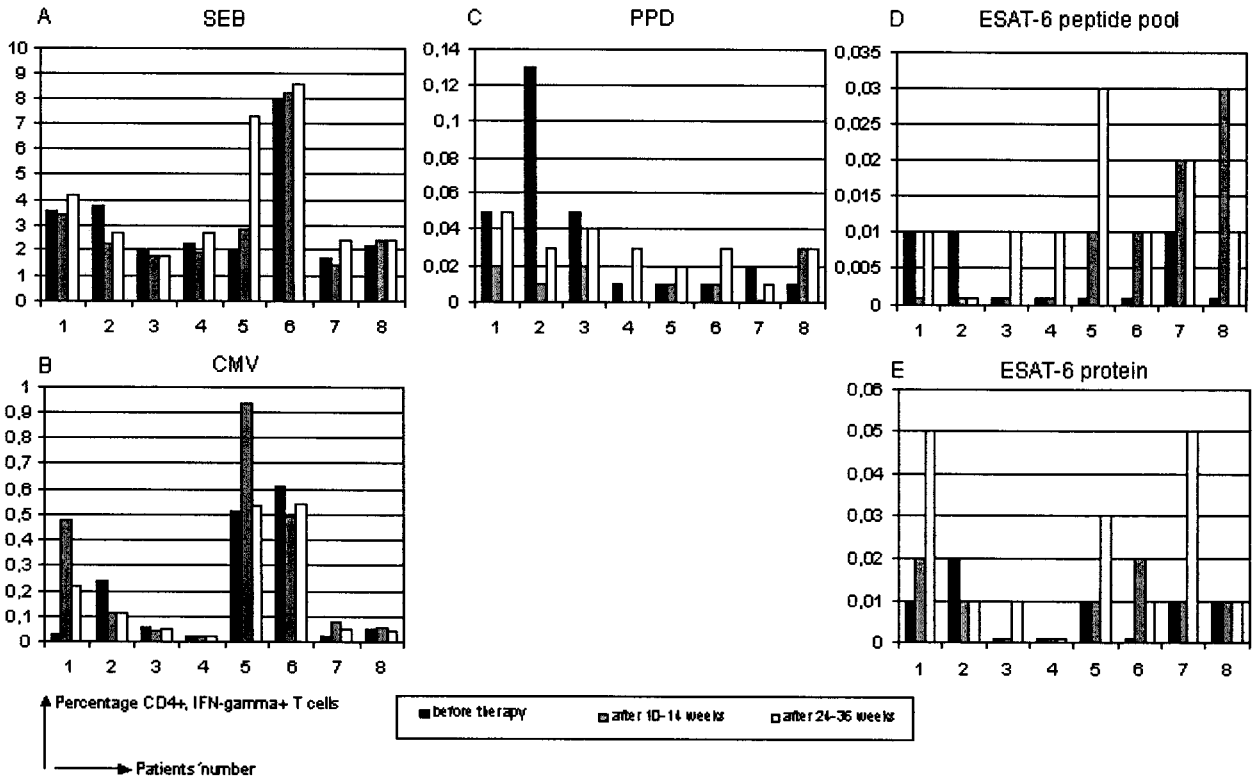

Figure 4. Summary of T cell responsiveness of all AS patients treated with adalimumab (Ada-I), against SEB and recall antigens before and during therapy; A. against SEB, B. CMV pp65 antigen, C. PPD, D. early secretory antigen target-6 (ESAT-6) pooled peptides, and E. ESAT-6 proteins. Only patient Ada-I.2 showed a clear reduction of T cell response to SEB, CMV, PPD, and one of the ESAT-6 antigen preparations.

T cell response to recall antigens — adalimumab-treated patients with AS, nonresponders. All AS patients without clear clinical response to adalimumab showed a T cell response to SEB with a range of 2.0% to 9.8% CD4+/IFNγ+ T cells before therapy was started. A reduction of T cell responses in response to CMV, PPD, or ESAT-6 antigens was not detected in any of these patients (data not shown).

T cell response to recall antigens using fresh MNC — AS controls. As a control we analyzed the T cell response to SEB and recall antigens in patients with AS who did not receive TNF-blocking agents at Week 0 and after 12 weeks. All patients with AS responded well to SEB. A reduction of the T cell response to these antigens could be found over time in none of the 4 patients responding initially to CMV and in the 6 patients responding to PPD (data not shown). T cell responses of ≥ 0.02% CD4+, IFNγ+ T cells to the ESAT-6 peptide pool or ESAT-6 protein were observed in case of control Patients 5 and 8. Control Patient 8 showed a clear reduction to both ESAT-6 antigens but not to PPD, CMV, or SEB.

Thus, a general decline of T cell responsiveness to recall antigens was not observed in any of the AS controls.

T cell responsiveness to SEB and CMV antigen after 1 year of TNF-α-blocking therapy with adalimumab-treated patients (Ada-II) and infliximab-treated patients (Ifx-II). To analyze the longterm effect of TNF-α-blocking therapy we used frozen samples of patients with AS treated with adalimumab (n = 9) treated for 1 year. We identified a transient reduction of T cell responses to stimulation with CMV and SEB in 2 of 3 responders (Ada-II.6 and 7) to both SEB and CMV, which was, however, reconstituted after 28 weeks. In patients treated with infliximab (n = 8), in one patient (Ifx-II.4) out of 3 responders to SEB and CMV, a transient reduction of T cell responsiveness to SEB and CMV was detected that was also reconstituted after 28 weeks of therapy (Figure 5).

DISCUSSION

In our study 15 of 18 patients with AS who were successfully treated with either infliximab or adalimumab showed no significant changes in T cell responsiveness to recall antigens if fresh MNC were used. However, there was a minority of 3 patients (2 treated with infliximab and 1 with adalimumab) who showed a clear reduction of T cell responsiveness to SEB, CMV, PPD, and at least one of both ESAT-6 antigen preparations after 10–14 weeks of treatment without restoration during 6–9 months of therapy. A similar observation was made in another 3 patients (1 treated with infliximab and 2 treated with adalimumab) when we used frozen MNC from 17 infliximab or adalimumab-treated patients. However, in these patients the response was restored after 12 months while treatment was ongoing.

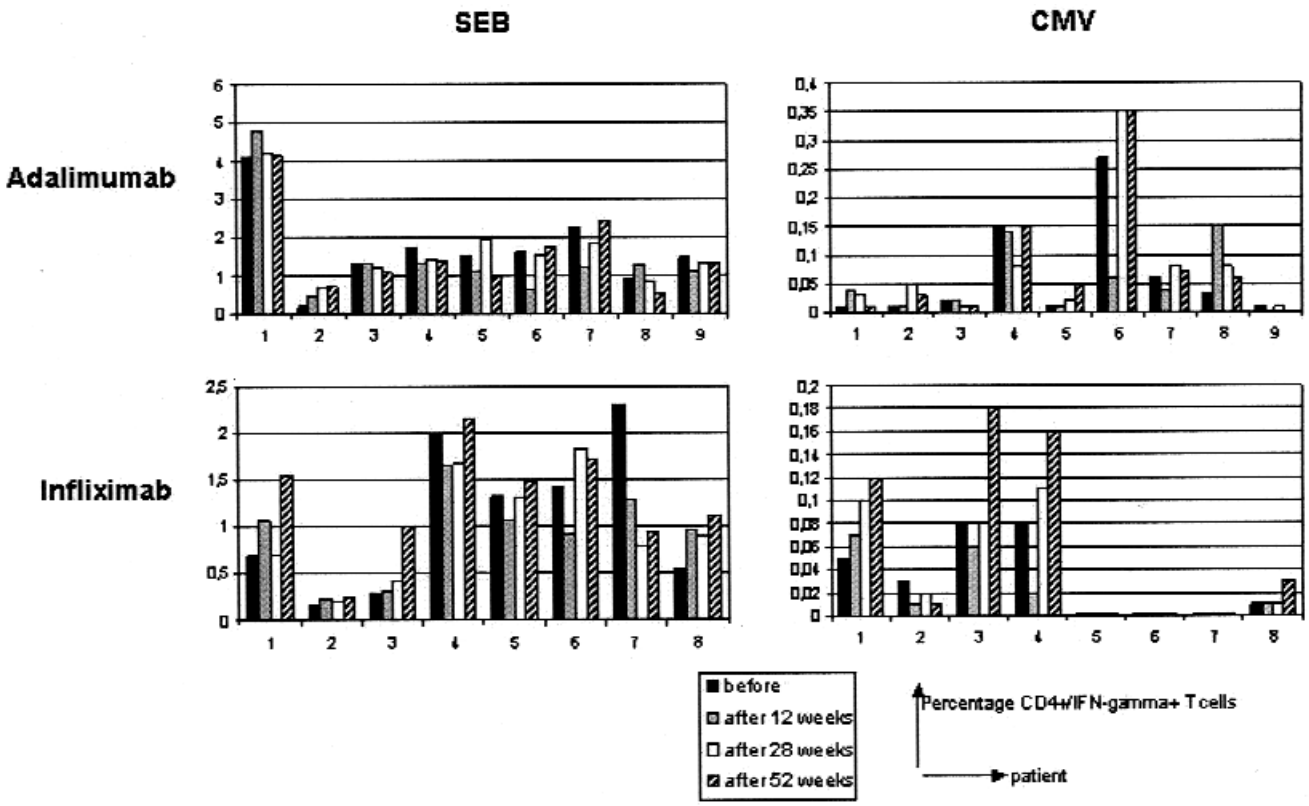

Figure 5. T cell responsiveness to SEB and CMV antigen. Longterm effect of TNF-α-blocking therapy in patients treated with adalimumab (Ada-II) or infliximab (Ifx-II) after 12, 28, and 52 weeks. In AS patients treated with adalimumab a clear transient reduction of T cell responsiveness to CMV and SEB was seen in 2 patients (Ada-II.6 and 7), which, however, was reconstituted after 6 months of therapy. In infliximab-treated patients only patient Ifx-II.4 showed a reduction of T cell responsiveness (reconstituted after 28 weeks). No patient with AS had a persistent reduction of T cell responsiveness in both analyses.

The observation of a decline in T cell response to recall antigens in some of our patients fits well with the observation that in patients with latent tuberculosis a reactivation of tuberculosis can occur that peaks 12 weeks after infliximab therapy was started[22]. In particular, patients with a decreased T cell response might be at risk and T cell monitoring might be a way to identify these patients. However, this assumption has to be confirmed in future studies by correlating T cell responses with manifestation of tuberculosis. On the other hand, the lack of a decrease in the T cell response at the group level, as shown in our study, would be in line with growing evidence that in patients treated with TNF-blockers there seems to be only a small increase in infections compared to conventional therapies[6,23].

Our analysis had some limitations: using frozen MNC samples might result in lower T cell responses, although the approach has the advantage that all samples were investigated at the same time; the number of patients was small; and in the analysis of longterm-treated patients we analyzed only the response to SEB and CMV antigens, also due to the limited numbers of cells available.

It was recently discussed that patients with AS have an impaired Th1 cytokine profile compared with healthy controls and that TNF-blocking agents might even reconstitute Th1 cytokines[24]. In a previous study we have reported a decline of Th1 responses during infliximab therapy over 12 weeks[25] if MNC were stimulated with aggrecan-derived antigens or phorbol myristate acetate/ionomycin. In our group analysis we could also identify a decrease of T cell responsiveness to recall antigens in infliximab-treated patients with AS after 10–14 and 24–36 weeks; this, however, was not statistically significant. We did not find such a decrease in the adalimumab-treated group of patients with AS.

We observed an increase of T cell responses during treatment with infliximab or adalimumab in a few patients, but this was not a constant finding. It is well known that TNF exerts immunosuppressive effects on T cells *in vivo*. Analysis of cell-mediated immunity in patients with active rheumatoid arthritis, before and after treatment with TNF-α-blocking reagents, revealed that treatment with anti-TNF restored the diminished proliferative responses of PBMC to mitogens and recall antigens towards normal in all patients tested[26], a finding that we could not confirm in this AS study.

We have recently reported an increase of Th1 responses in patients with AS treated with etanercept over 12 weeks of therapy[27], which might explain, at least partly, why tuberculosis reactivation is seen less often in patients treated with etancercept[28]. We are currently investigating the T cell

response to recall antigens in patients with AS treated with etanercept by using fresh MNC.

Our *in vitro* analysis gives evidence that the majority of patients with AS treated with adalimumab or infliximab do not have a constant alteration of T cell function. A decrease of T cell responsiveness in single patients might help to identify patients who are at risk for getting infections such as tuberculosis.

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