EXTENDED REPORT

Effect of treatment of rheumatoid arthritis with infliximab on IFNγ, IL4, T-bet, and GATA-3 expression: link with improvement of systemic inflammation and disease activity

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Objective: To study interferon γ (IFNγ) production and the expression of T-bet and GATA-3, the transcription factors associated with Th1 and Th2, in peripheral blood mononuclear cells (PBMC) from patients with rheumatoid arthritis before and during infliximab treatment, so as to distinguish between a disease specific and a disease activity dependent defect.
Methods: Rheumatoid PBMC were obtained at weeks 0 and 6 of infliximab treatment and cultured for seven days with or without interleukin (IL)12 or the combination of IL12 and IL18. IFNγ concentrations in supernatants were determined by ELISA. mRNA expression of IFNγ, IL4, T-bet, and GATA-3 was determined by real time RT-PCR in whole blood at weeks 0 and 22.
Results: A reduction in spontaneous IFNγ production and in the response to Th1 inducing cytokines occurred in rheumatoid PBMC. Reduction of systemic inflammation with infliximab treatment increased IFNγ production in response to IL12 or IL12+IL18. The IFNγ/IL4 expression ratio of rheumatoid blood before treatment was lower than in healthy controls but was increased by infliximab treatment. T-bet expression or T-bet/GATA-3 ratio of rheumatoid blood was less than in controls. The T-bet/GATA-3 ratio was not influenced by infliximab treatment.
Conclusions: Regulation of T-bet and GATA-3 or IFNγ and IL4 expression appeared different. The IFNγ/IL4 ratio might express the blood Th1/Th2 balance better than the T-bet/GATA-3 ratio. Reduced IFNγ production by rheumatoid PBMC and levels of IFNγ and IL4 mRNA expression in blood were linked to disease improvement, indicating an association between this systemic Th1 feature and disease activity.

Anti-tumour necrosis α (TNFα) treatment has been shown to decrease disease activity in patients with rheumatoid arthritis, but cases of severe tuberculosis have been reported during the early stage of treatment.[1] However, an increased incidence of tuberculosis appears to be associated with rheumatoid arthritis even in the absence of anti-TNF treatment.[2] Although the role of TNFα in the human immune response to mycobacteria is incompletely understood, TNFα has a pivotal role in granuloma formation and in the control of the disease in animals.[3]

Considering the critical role of interferon γ (IFNγ)—the prototypic Th1 cytokine—in tubercular infection, an underlying defect of cell mediated immunity in rheumatoid blood may be related to the development of tuberculosis. Indeed, a disturbed balance between T helper (Th)1 and Th2 subsets has been reported in the pathogenesis of rheumatoid arthritis.[4] With others, we have shown the selective accumulation of Th1 and Th0 cells in the synovium by intracellular cytokine staining of peripheral blood and synovial tissue T cells from rheumatoid patients.[5] On the other hand, the Th1/Th2 balance in the peripheral circulation remains controversial in rheumatoid arthritis. At the protein level, rheumatoid blood cells produced less IFNγ and more interleukin (IL)4 in response to non-specific stimulation than controls.[6] We recently found that peripheral blood mononuclear cells (PBMC) from rheumatoid patients were less sensitive to the Th1 inducing cytokines IL12 and IL18 than those from controls.[7] Furthermore, mRNA expression of IFNγ and IL4, as well as T-bet and GATA-3—the transcription factors involved in Th1 and Th2 development, respectively—showed a decreased Th1 pattern in rheumatoid blood compared with control.[7]

As anti-TNF treatment for rheumatoid arthritis can often improve disease activity rather rapidly, we studied whether such treatment might affect the underlying systemic Th1 pattern. We looked at mRNA expression of IFNγ, IL4, T-bet, and GATA-3 in the circulation of rheumatoid patients at week 0 and week 22 of infliximab treatment. In addition, we studied IFNγ production by PBMC from rheumatoid patients in response to IL12 and IL18 at week 0 and 6. Responders, but not non-responders, augmented the IFNγ/IL4 ratio in whole blood. Infliximab treatment augmented the levels of IFNγ production by rheumatoid PBMC cultures with or without IL12 or IL12+IL18. The systemic Th1 immune pattern of rheumatoid arthritis indicated by the impaired IFNγ production by rheumatoid PBMC appears to be related to disease activity.

METHODS

Whole blood sample collection and storage

Blood samples were obtained from 12 healthy controls (three men, nine women) and from 15 patients with rheumatoid arthritis (four men, 11 women), who fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR),[8] before the first (week 0) and the fifth (week 22) infusion of infliximab (given in a dose of 3 mg/kg according

Abbreviations: ACR, American College of Rheumatology; IFNγ, interferon γ; IL, interleukin; PBMC, peripheral blood mononuclear cells; Th, T helper cell; TNFα, tumour necrosis factor α

to the ATTRACT protocol). The mean age of the healthy controls was 45.4 years (range 31 to 64), and of the rheumatoid patients, 48.2 years (range 28 to 68). Mean disease duration was 8.2 years (range 3 to 18). The majority of patients were being treated with non-steroidal anti-inflammatory drugs and methotrexate, alone or combined with prednisone. Patient response was quantified using the ACR response criteria.[9] Patients with an ACR score of 20 or more were defined as responders and those with a score of less than 20 as non-responders. Peripheral blood samples were collected in PAXgene tubes containing a cationic detergent and additive salts (PreAnalytiX/QIAGEN GmbH, Hilden, Germany), and then stored at −20℃.

RNA isolation from whole blood sample and RT-PCR

PAXgene blood RNA kit (PreAnalytiX) was used for purification of intracellular RNA from whole blood samples. The ThermoScript™ reverse transcriptase polymerase chain reaction (RT-PCR) system (Invitrogen SARL, Cergy Pontoise, France) was used to synthesise cDNA.

Parameter specific primer sets for IFNγ, IL4, T-bet, GATA-3, and β actin optimised for the LightCycler instrument (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, Indiana, USA) were developed and purchased from Search-LC (Heidelberg, Germany). PCR was carried out as recently described,[10] using the LightCycler FastStart DNA Sybr Green I kit (Roche Applied Science) according to the protocol provided with the parameter specific kits (45 amplification cycles, denaturation at 96℃, primer annealing at 68℃ with touchdown to 58℃, amplicon extension at 72℃). The copy number of IFNγ, IL4, T-bet, and GATA-3 was normalised by the housekeeping gene β actin and is presented as the number of transcripts per copy of β actin.

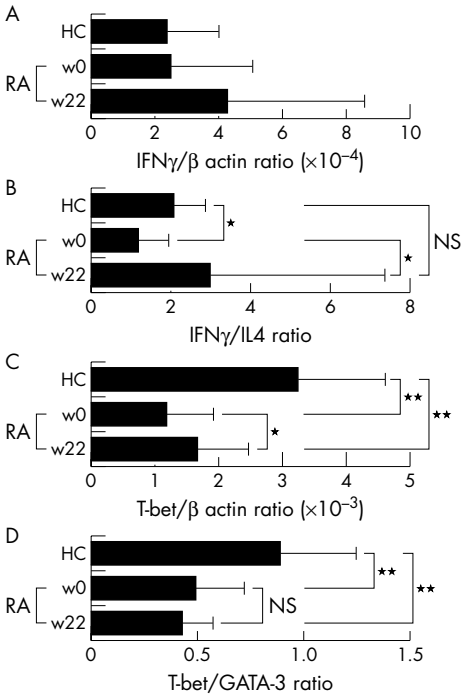

Figure 1 Increased type 1 response in whole blood from patients with rheumatoid arthritis following anti-TNFα treatment. Whole blood samples were obtained from 12 healthy controls (HC) and from 15 patients with rheumatoid arthritis (RA) at week 0 (w0) and 22 (w22) of infliximab treatment. IFNγ, IL4, T-bet, GATA-3, and β actin mRNA expression levels were determined by quantitative reverse transcriptase polymerase chain reaction. *p<0.05, **p<0.01

Cytokines and reagents

Recombinant human IL12 was purchased from R&D systems, Abingdon, Berkshire, United Kingdom. Recombinant human IL18 was from MBL, Nagoya, Japan. RPMI 1640 culture media was purchased from Invitrogen SARL, Cergy Pontoise, France and supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Invitrogen).

Preparation of PBMC culture

Peripheral blood samples were obtained from six controls (two men, four women), and from six rheumatoid patients (two men, four women) before the first (week 0) and the third (week 6) infusion of infliximab. The mean age of the controls was 48.3 years (range 44 to 61) and of the rheumatoid patients, 51.7 years (range 44 to 65), and mean disease duration was 9.6 years (range 4 to 38). PBMC were isolated from heparinised blood by Ficoll density gradient centrifugation, washed twice with phosphate buffered saline, and resuspended in RPMI1640 medium. PBMC were cultured in 96-well plates (Nunc, Roskilde, Denmark) at a density of 1×10^6 cells/ml in 200 µl of RPMI medium.

Determination of levels of IFNγ and serum C reactive protein

IFNγ levels were measured by quantitative sandwich enzyme linked immunosorbent assay (ELISA), using a commercially available ELISA kit (DuoSet ELISA Development System human IFNγ, R&D Systems). The detection limit of the assay was 20 pg/ml. C reactive protein concentrations were measured by the hospital laboratory, and the limit of assay was 1.5 mg/l. Undetectable levels were regarded as 0.

Statistical analysis

Results were expressed as mean (SD) of the indicated number of experiments. The statistical significance of differences between two groups was determined by the Mann–Whitney U test. Wilcoxon's signed rank test was used to analyse matched pairs.

RESULTS

Improvement of Th1 defect in rheumatoid blood by infliximab treatment

Paired peripheral blood samples from 15 rheumatoid patients at week 0 and 22 of infliximab treatment and from the healthy controls were collected directly in PAXgene tubes. IFNγ and IL4 mRNA expression levels were then determined

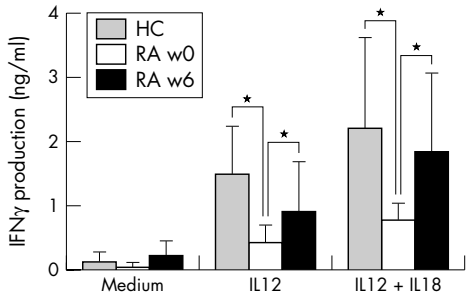

Figure 2 Improvement in impaired interferon γ (IFNγ) production of peripheral blood mononuclear cells (PBMC) from patients with rheumatoid arthritis resulting from anti-tumour necrosis α (TNFα) treatment. PBMC from healthy controls (HC) (n = 6) and rheumatoid patients (RA) (n = 6) obtained at weeks 0 and 6 of infliximab treatment were cultured with or without interleukin (IL)12 (1 ng/ml) or IL12+IL18 (5 ng/ml) for seven days. IFNγ levels in the supernatants were determined by a specific sandwich enzyme linked immunosorbent assay. *p<0.05

by quantitative RT-PCR. As shown in fig 1A, IFNγ expression level in rheumatoid blood at week 0 was almost identical to that of the controls, but the IFNγ/IL4 expression ratio was significantly lower in the rheumatoid samples (fig 1B). However, infliximab augmented IFNγ expression and the IFNγ/IL4 ratio in rheumatoid blood following 22 weeks of treatment. Eleven responders of 15 patients who had an ACR response score of ⩾20 at week 22 had an increased IFNγ/IL4 ratio in their blood (from 1.26 (0.76) to 3.78 (2.59), $p = 0.003$). However, four non-responders who had an ACR response score of less than 20 did not show an increased IFNγ/IL4 ratio (from 1.30 (0.83) to 0.82 (0.22), $p = 0.61$). Thus rheumatoid blood expressed markers of a decreased Th1/Th2 balance. This defect was modified by infliximab treatment only in the responders.

Impaired T-bet/GATA-3 ratio in rheumatoid blood

We next extended the results to the transcription factors T-bet and GATA-3, which have been described as Th1 and Th2 markers, respectively. Rheumatoid blood showed a decreased T-bet expression or T-bet/GATA-3 ratio compared with the controls at both week 0 and week 22 of infliximab treatment (fig 1C). Infliximab upregulated not only T-bet but also GATA-3 expression; therefore the T-bet/GATA-3 ratio was not influenced by infliximab treatment (fig 1D). Accordingly, the expression of IFNγ and T-bet mRNA in rheumatoid blood appeared to be regulated differently. The IFNγ/IL4 ratio could be more sensitive than the T-bet/GATA-3 ratio in evaluating the Th1/Th2 balance in blood samples.

Improvement in impaired IFNγ production by rheumatoid PBMC following infliximab

PBMC samples from six rheumatoid patients at week 0 and week 6 of infliximab treatment and from six healthy controls were cultured for seven days with or without IL12 (1 ng/ml) or the combination of IL12 and IL18 (5 ng/ml). IFNγ levels in the supernatants were determined by ELISA. All patients had a decrease in C reactive protein concentrations (from 72.2 (47.9) to 19.4 (20.5) mg/l, $p = 0.02$) following six weeks of infliximab treatment. Rheumatoid PBMC at week 0 produced significantly lower levels of IFNγ than control. However, rheumatoid PBMC at week 6 produced higher levels of IFNγ than the week 0 samples, either in response to IL12 alone ($p = 0.04$) or to IL12+IL18 ($p = 0.04$) (fig 2), indicating improvement in the impaired IFNγ production by rheumatoid PBMC following to anti-TNF treatment in parallel with amelioration of the disease.

DISCUSSION

Several lines of evidence have shown the predominance of IFNγ producing Th1 cells at the site of chronic inflammation in rheumatoid arthritis. An increased IFNγ/IL4 ratio in synovial fluid compared with peripheral blood has also been reported. However, the Th1/Th2 balance in the peripheral circulation in rheumatoid arthritis remains to be clarified. We and others have shown that rheumatoid blood cells produce less IFNγ and more IL4 than cells from healthy controls,[6] indicating a decreased Th1 response in the peripheral circulation in rheumatoid arthritis. Recently we found that PBMC from rheumatoid patients produced lower levels of IFNγ in response to IL12 and IL18 than controls,[11] suggesting that rheumatoid patients have a systemic Th1 defect.

In the present study, we looked at the systemic Th1/Th2 balance in patients with rheumatoid arthritis before and after anti-TNFα treatment. Responders to anti-TNFα clearly upregulated their blood expression of IFNγ, but non-responders did not. This suggests that the decreased systemic Th1 pattern seen in rheumatoid arthritis may be related to disease activity rather than being disease specific. After anti-TNFα treatment PBMC from rheumatoid patients also produced higher levels of IFNγ than before treatment in response to IL12 and IL18. Our results showed that patients with active rheumatoid arthritis may have decreased systemic Th1, and improvement of this defect appears to be associated with amelioration of rheumatoid activity. The T-bet/GATA-3 ratio was also lower in rheumatoid blood than in controls, and this remained unchanged by infliximab treatment. T-bet is expressed by various blood cells such as T cells, B cells, and monocytes. Thus the T-bet/GATA-3 ratio may not be a sensitive Th1/Th2 marker in blood samples.

Accumulation of Th2 cells rather than Th1 cells in the blood may result from changes in T cell migration. Indeed, the numbers of IFNγ producing T cells were significantly increased in the peripheral blood of rheumatoid patients shortly after anti-TNFα treatment, resulting in a shift of the Th1:Th2 ratio towards Th1 in the blood.[12] Adhesion molecules such as P- and E-selectin or VCAM-I are considered to be important for the selective homing of Th1 cells but not of Th2 cells.[13] Expression of E-selectin and VCAM-I was significantly reduced by anti-TNF treatment.[14] Thus anti-TNF treatment may suppress the selective migration of Th1 cells into rheumatoid synovium through the rapid downregulation of adhesion molecules. It remains to be determined whether this is associated with an increased migration of Th2 cells with anti-inflammatory properties.

The contrast between the improvement described above and the reactivation of tuberculosis at an early stage of infliximab treatment needs to be explored. Our understanding of the increased risk based on these results is that at an early stage of treatment rheumatoid patients express a systemic immune defect related to disease activity. This defect is increased when anti-TNF is used because it affects cell interactions and granuloma formation. This has a beneficial effect on joint inflammation, leading to reduced cellularity of the synovium. However, the effect could also be deleterious, as tubercular granulomas may dissociate leading to massive spread of the disease. Later the risk is reduced following the improvement in disease activity and the associated immune defect.

In conclusion, our data show that successful anti-TNFα treatment not only improves systemic inflammation and rheumatoid disease activity but also improves the impairment of systemic Th1 immune function.

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