RESEARCH ARTICLE

Cytokine production from stimulated whole blood cultures in rheumatoid arthritis patients treated with various TNF blocking agents

Calin Popa[1,2], Pilar Barrera[2], Leo A.B. Joosten[1,2,3], Piet L.C.M. van Riel[2], Bart-Jan Kullberg[1,3], Jos W.M. van der Meer[1,3], Mihai G. Netea[1,3]

[1] Department of General Internal Medicine
[2] Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[3] Nijmegen Institute for Infection, Inflammation, and Immunity (N4i), Radboud University Nijmegen, Nijmegen, The Netherlands

Correspondence: C. Popa, Radboud University Nijmegen Medical Centre, Department of Rheumatology (470), P.O.Box 9101 6500 HB Nijmegen, The Netherlands
<c.popa@reuma.umcn.nl>

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ABSTRACT. Infectious complications are not rare in rheumatoid arthritis (RA), and the susceptibility to infections is increased during treatment with TNF blocking agents. As a possible mechanism contributing to that, we assessed the modulation of cytokine production induced by TNF neutralization. *Methods*. Whole blood cultures from six healthy volunteers and 13 RA patients starting therapy with either adalimumab (n = 7) or etanercept (n = 6) were stimulated with heat-killed *Salmonella typhimurium*, *Staphylococcus aureus* or with *S. typhimurium* lipopolysaccharide (LPS). The production of interleukin (IL)-1β, IL-6, IL10, IL-17, TNF, IL-8 and IFN-γ was measured by specific immunoassays. *Results*. Stimulation with *Salmonella* LPS resulted in a significantly lower production of IL-1β, TNF and a trend towards lower IL-6 and IFN-γ production in RA patients compared to healthy volunteers. Therapy with either of the agents did not significantly alter cytokine production capacity, with the exception of a lower IFN-γ and IL-8 production in patients treated with adalimumab and stimulated with heat-killed *S. aureus*. *Conclusion*. The results of our study suggest that the detrimental effects of anti-TNF agents on the immune response can vary quite widely, from very serious to limited effects, as reported here for etanercept and adalimumab. Because anti-TNF therapy can affect the cellular integrity of tuberculous granuloma, recruitment of new cells at the granuloma site becomes crucial. In line with this, an impaired chemokine production induced by anti-TNF agents may ultimately result in the reactivation of tuberculosis, as previously reported. Therefore, caution should be constantly exercised in order to prevent the development of severe infections and reactivation of tuberculosis whenever therapy with anti-TNF is initiated.

Keywords: anti-TNF therapy, rheumatoid arthritis, infections, immune response

Treatment strategies that modulate pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-1 (IL-1)-β constitute a breakthrough in the treatment of rheumatoid arthritis (RA) and other inflammatory diseases including juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and Crohn's disease. Agents including anakinra, adalimumab, etanercept, and infliximab yield substantial improvement in symptoms, disability, and quality of life and prevent joint damage in early and long-standing RA. However, safety issues of increased susceptibility to infections in individuals receiving these treatments, particularly with intracellular pathogens such as *Mycobacterium* species [1], represent a serious concern.

Interestingly, the rate of severe infections is lower in patients treated with the soluble receptor etanercept than in those treated with monoclonal anti-TNF agents, such as adalimumab and infliximab [2-5]. This may be due to differences in the capacity of these drugs to interact with soluble and membrane-bound TNF, to activate complement and to induce cytolysis [6, 7], but the exact mechanisms are not completely understood.

Cellular recognition of pathogens involves binding to pattern recognition receptors (PRRs), including toll-like receptors (TLR), which ultimately leads to the release of proinflammatory cytokines, such as TNF, IL-1β and IFN-γ, and activation of host defense. *Salmonella typhimurium* and *Staphylococcus aureus* are two microorganisms that have been previously reported to be able to cause severe infections in RA patients receiving anti-TNF drugs [8-11]. TNF neutralisation in RA results in a marked decrease of circulating, acute phase reactants, IL-6, IL-8 and soluble adhesion molecules, but does not affect white blood cells (WBC) counts and differentiation

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[12]. With the exception of infliximab, which seems to have no effect on the capacity of blood cells to produce IL-10 and IFN-γ after challenged with microbial agents [10, 13], little is known about the effect of the other anti-TNF agents on cytokine production capacity and the latter may be crucial for preventing infections. In the present study, we assessed the effect of adalimumab and etanercept on cytokine production capacity after microbial challenge.

METHODS AND MATERIALS

Patients and controls

Six healthy controls and 13 RA patients attending our outpatient clinic, and who were about to start anti-TNF treatment with either adalimumab (n = 7) or etanercept (n = 6) were enrolled in the study, after giving written informed consent. Adalimumab was given as subcutaneous injections at a dose of 40 mg, every other week, while etanercept was delivered in doses of 25 mg, twice weekly. Stable dosages of disease-modifying anti-rheumatic drugs (DMARDs) and oral corticosteroids (CS, prednisone < 10 mg/day) were allowed during the study. Patient characteristics are presented in more detail in *table 1*. Patients received anti-TNF drugs for a period of at least three months. The regional medical ethics committee approved the study.

Whole blood cytokine production

Cytokine production in whole blood cultures has been investigated as previously described [13]. In short, venous blood was collected from the cubital vein in 4-ml lithium-heparin tubes. Whole blood was diluted 1:5 with RPMI 1640 in 24-well plates and incubated at 37°C with heat-killed *Salmonella typhimurium* (10^7 microorganisms/ml), *Staphylococcus aureus* (10^7 microorganisms/ml), and *S. typhimurium* LPS (μg/mL). Incubation with RPMI was used as a negative control. TNF, IL-6, IFN-γ, IL-10, IL-1β, IL-8, and IL-17 production was measured in the supernatants using commercially available kits (Bio-Rad) according to the manufacturer's instructions. Cytokine levels were measured and analyzed with the Bio-Plex system (Bio-Rad). The sensitivity of the cytokine assay was < 5 pg/mL for all cytokines measured.

Statistical analysis

Differences between groups were assessed using Mann-Whitney U-test. Differences within groups were assessed using the paired Wilcoxon test. Unless otherwise stated, results are expressed as means ± standard error of the mean (SEM).

RESULTS

Cytokine production capacity in RA at baseline

Stimulation of whole blood cultures with *Salmonella* LPS resulted in a significant lower production of IL-1β (567 ± 119 pg/mL *versus* 289 ± 49 pg/mL, p = 0.042) and TNF (417 ± 77 pg/mL *versus* 198 ± 36 pg/mL, p = 0.015) *(figure 1A)* in RA patients compared to healthy controls. The production of IL-6 and IFN-γ was also lower in RA patients although this did not reach statistical significance: 4 992 ± 596 pg/mL *versus* 3 609 ± 492 pg/mL (p = 0.06) for IL-6 and 382 ± 53 pg/mL *versus* 264 ± 38 pg/mL (p = 0.06) for IFN-γ, respectively *(figure 1A, D)*. No difference in IL-8 production could be observed between RA patients and healthy controls under the same conditions *(figure 1A)*. Interestingly, cytokine production in RA and controls did not differ after stimulation with the whole *S. typhimurium* micro-organisms *(figure 1B*, *E)* or with heat-killed *S. aureus (figure 1C*, *F)*.

Table 1
Characteristics of patients.

	Controls	RA patients (total)	RA patients (adalimumab)	RA patients (etanercept)
Age (years)	51 ± 5	61 ± 13	61 ± 15	60 ± 10
Gender ratio (F:M)	3:3	8:5	5:2	3:3
WBC (x 10^9/L)				
Baseline	7.14 ± 1.75	6.98 ± 2.35	8.10 ± 1.46	5.68 ± 2.62
Three months	-	6.48 ± 2.14	7.57 ± 1.56	5.22 ± 2.13
DAS28				
Baseline	-	4.29 ± 1.79	4.29 ± 1.79	4.61 ± 1.16
Three months	-	2.65 ± 1.75*	2.65 ± 1.75*	3.61 ± 0.58*
ESR (mm/h)				
Baseline	7 ± 5	22 ± 24	16 ± 13	28 ± 32
Three months	-	15 ± 21*	9 ± 6*	22 ± 31*
CRP (mg/L)				
Baseline	< 5	21 ± 27	18 ± 17	24 ± 38
Three months	-	9 ± 12*	6 ± 3*	12 ± 18*
Medication (N)				
Methotrexate	-	3	2	1
Corticosteroids	-	2	2	

WBC: white blood cell count; DAS28: disease activity score; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein. Results are expressed as mean ± SD; * p < 0.05.

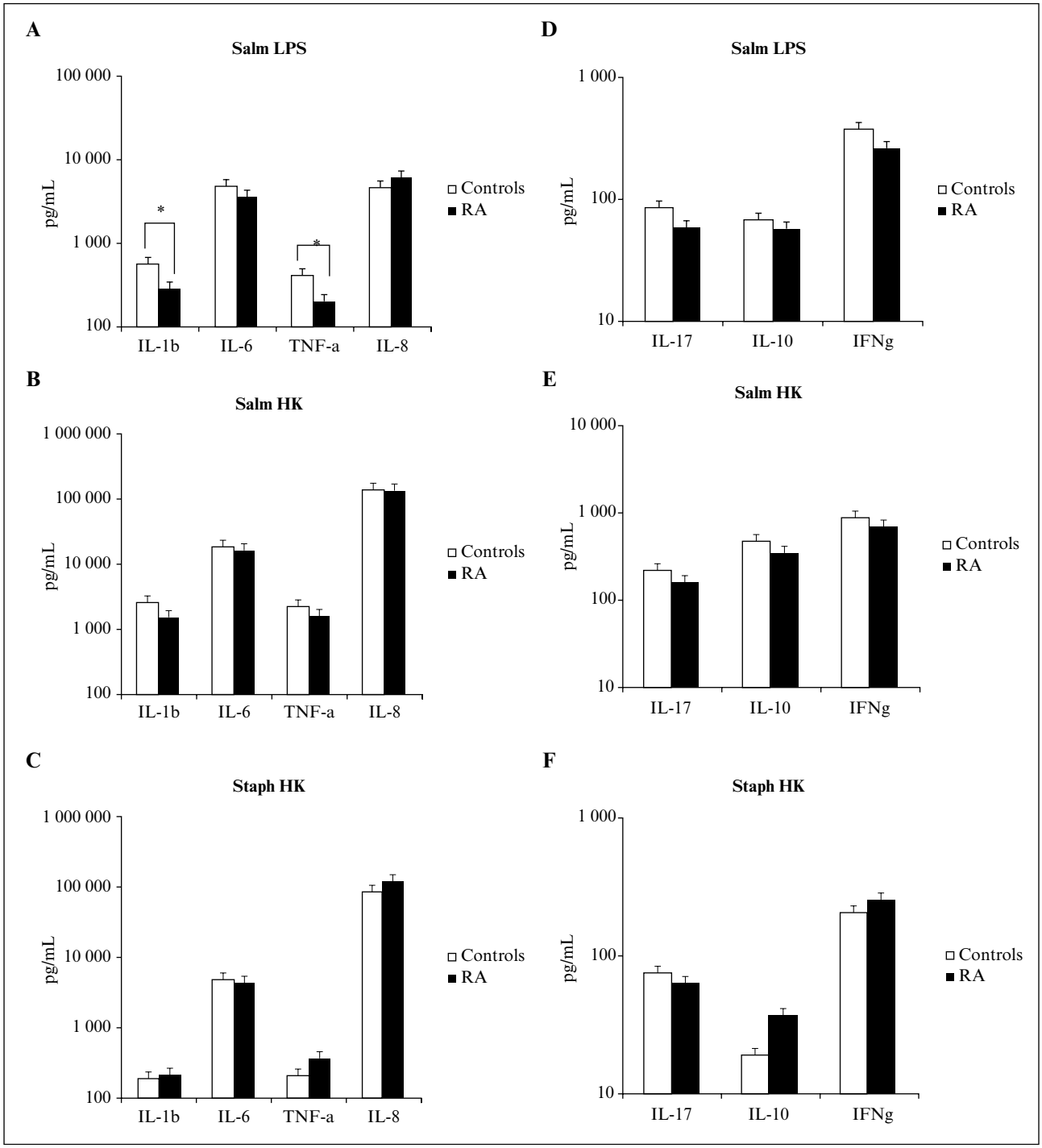

Figure 1

Production of cytokines in healthy controls (white bars) and RA patients, prior to receiving anti-TNF therapy (black bars). Whole blood cultures were stimulated with *Salmonella typhimurium* lipopolysaccharide (Salm LPS) (**A, D**), heat-killed *Salmonella typhimurium* (Salm HK) (**B, E**) and heat-killed *Staphyloccocus aureus* (Staph HK) (**C, F**). Values are expressed as means ± SEM. P value calculated using Mann-Whitney *U*-test. * $p < 0.05$.

Effects of anti-TNF therapy on cytokine production

Cytokines were measured in the whole blood cultures, where the presence of the therapeutic anti-TNF drugs prevented us from reliably measuring endogenous TNF production. Thus, the capacity to produce TNF under microbial stimulation during the therapy could not be assessed. In RA patients treated with adalimumab, the production of IFN-γ and IL-8 decreased after three months of treatment when blood was stimulated with *S. aureus* (240 ± 39 pg/mL *versus* 194 ± 44 pg/mL, $p < 0.05$ and 162 ± 29 ng/mL *versus* 47 ± 16 ng/mL, $p < 0.01$ respectively) *(figure 2)*, whereas their production remained unchanged in etanercept users *(figure 3)*. IL-6 production after stimulation with *S. typhimurium* decreased in etanercept users (15 049 ± 5 791 pg/mL 8 966 ± 1 640 pg/mL, $p < 0.05$) *(figure 3)*, but showed a trend to increase in adalimumab users (15 859 ± 3 321 pg/mL *versus*

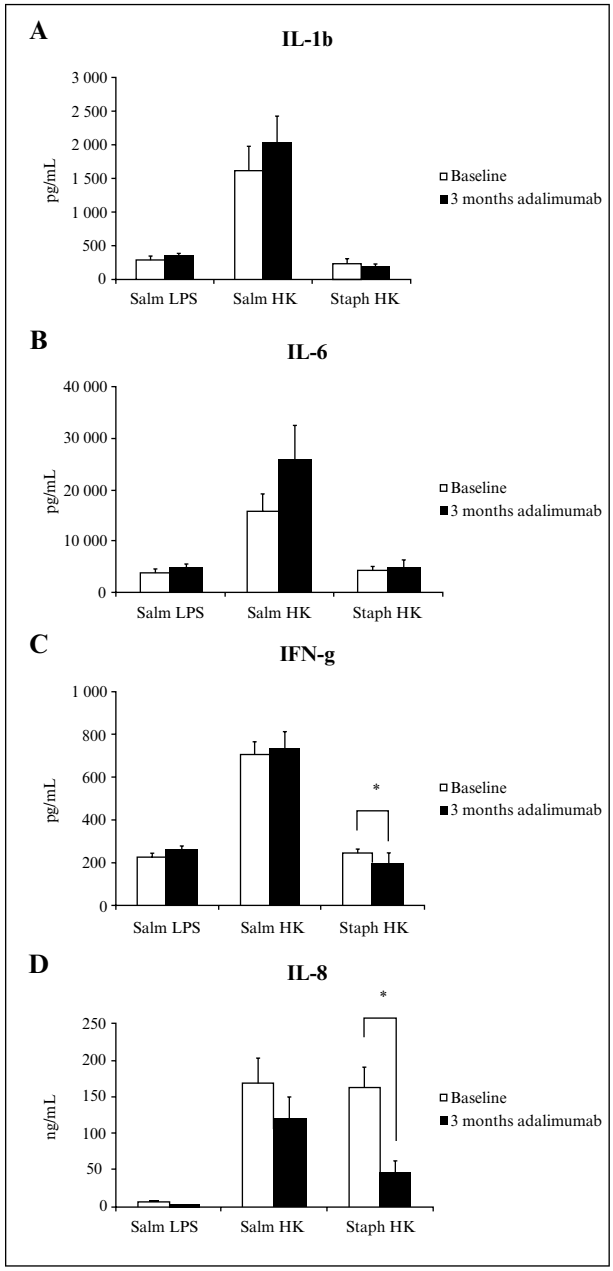

Figure 2
IL-1β (**A**), IL-6 (**B**), IFN-γ (**C**) and IL-8 (**D**) production by stimulated whole blood cultures in RA patients treated with adalimumab. The following stimuli were used: *Salmonella typhimurium* lipopolysaccharide (Salm LPS), heat-killed *Salmonella typhimurium* (Salm HK) and heat-killed *Staphyloccocus aureus* (Staph HK). Values are expressed as means ± SEM. P value calculated using Wilcoxon paired *t*-test. * $p < 0.05$.

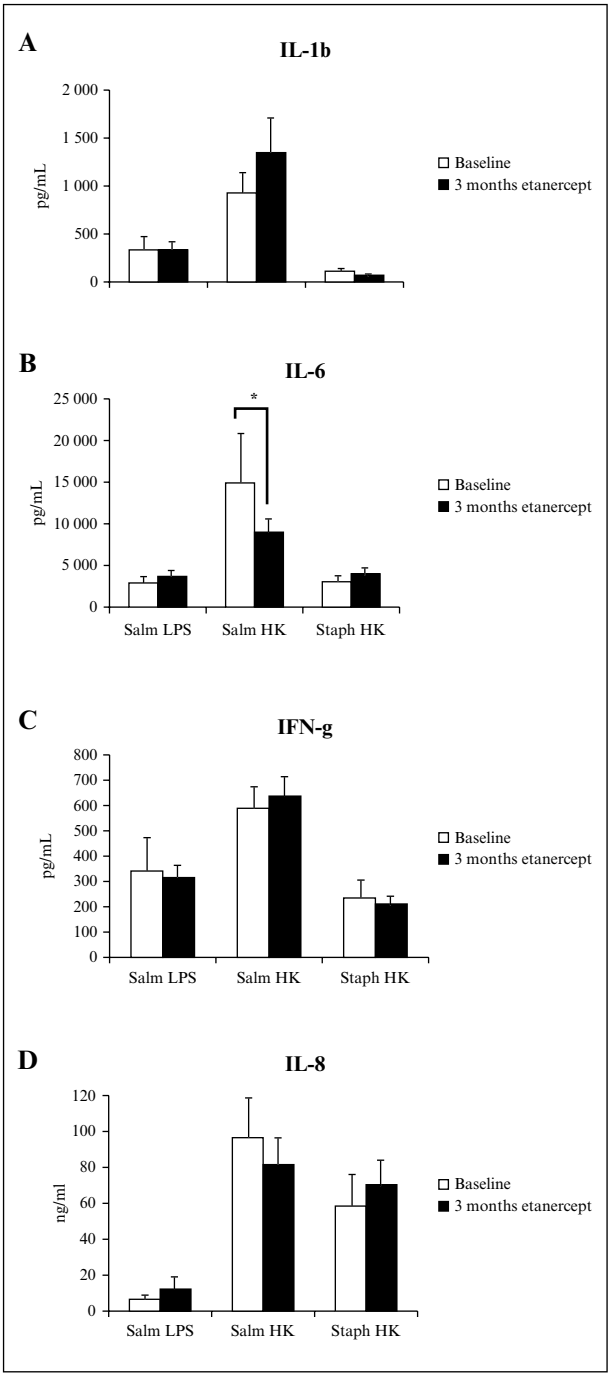

Figure 3
IL-1β (**A**), IL-6 (**B**), IFN-γ (**C**) and IL-8 (**D**) production by stimulated whole blood cultures in RA patients treated with etanercept. The following stimuli were used: *Salmonella typhimurium* lipopolysaccharide (Salm LPS), heat-killed *Salmonella typhimurium* (Salm HK) and heat-killed *Staphyloccocus aureus* (Staph HK). Values are expressed as means ± SEM. P value calculated using Wilcoxon paired *t*-test. * $p < 0.05$.

25 984 ± 6 472 pg/mL, $p = 0.06$) *(figure 2)*. IL-1β production did not change within the three months of therapy with any of the agents tested *(figures 2, 3)*. These observed changes in cytokine production were not explained by changes in WBC count since these remained stable during the study *(table 1)*.

DISCUSSION

In the present study, we have shown that rheumatoid arthritis patients react differently to microbial stimuli compared to healthy individuals in terms of cytokine production capacity in a whole-blood stimulation model. Therapeutic blockade of TNF with adalimumab or etanercept had a limited influence on the cytokine production capacity of these patients, with only subtle differences between the anti-TNF agents used by the patient.

Previous studies by our group have indicated that the capacity of immune cells to produce IFN-γ in response to bacterial stimuli is particularly impaired in patients

with RA compared to healthy volunteers [10, 13]. In one study, anti-TNF therapy with infliximab was suggested as the main factor responsible for this phenomenon, since the capacity of cells to produce IFN-γ was restored three weeks after the drug was discontinued [10]. Alternatively, long-lasting and relatively severe disease, together with the failure of other DMARDs to control this process, were likely to determine a lower IFN-γ production capacity in RA patients prior to infliximab therapy [13]. In contrast to infliximab, adalimumab at the lowest recommended doses and etanercept have been previously indicated to have a lower risk for inducing the development of severe infections or the reactivation of tuberculosis [2-4]. In the present study, a small but reproducible inhibitory effect of adalimumab on IFN-γ production capacity was observed after three months of treatment, whereas etanercept did not influence IFN-γ release. In addition, adalimumab decreased the production of IL-8, especially when blood was challenged with heat-killed *S. aureus*. Nevertheless, the extent of these changes makes it unlikely that this inhibition has major effects on susceptibility to infections. However, it cannot be excluded that higher doses of adalimumab may lead to a greater decrease in IFN-γ production at levels similar to those induced by infliximab [10], in line with the dose-dependency of the infectious side-effects associated with adalimumab reported by epidemiological studies [3]. This effect would then add to the chemotactic deficiency triggered by lower IL-8 levels and might thereafter impair the immune response, increasing the susceptibility to develop severe infections. Interestingly, therapy with either of the drugs did not affect the capacity of immune cells to produce IL-1β, while the only difference in IL-6 release was observed in the blood of the RA patients treated with etanercept. Therefore, the similar capacity of immune cells to respond to bacterial products during therapeutic TNF blockade with these agents may be of clinical relevance and explain the lower incidence of infections in patients treated with these agents.

Understanding the mechanisms underlying the establishment of latent infection and subsequent reactivation is critical to the control of *M. tuberculosis*. The tuberculous granuloma is a highly structured and yet dynamic entity comprised of a large variety of immune cells. TNF plays a crucial role in the control of acute and chronic tuberculosis. Recently, using a murine model of chronic tuberculosis, investigators have shown that upon TNF depletion, structural integrity of the tuberculous granuloma is disrupted [14]. This effect had been observed early after TNF neutralisation was started. Moreover, in another study investigating the capacity of human whole blood cells to produce cytokines and chemokines in response to *Mycobacterium bovis* [15], Newton *et al.* found an impaired production of chemokines thirty minutes and seven days respectively, after a 2-hour infliximab infusion. In line with these data, the results from our present study suggest that the decrease in IL-8 production in anti-TNF treated patients might be able to disrupt the cellular migration necessary to maintain granuloma integrity [14, 16], ultimately leading to the reactivation of tuberculosis in these patients.

In the present study, we observed that RA patients treated with etanercept or adalimumab preserved the capacity to release cytokines when stimulated with whole bacteria, despite a tendency to produce less IL-8 after three months of therapy with adalimumab. Recent systematic and vigorous screening for latent tuberculosis infection before starting anti-TNF therapy is likely to account for a different immunological background of the RA patients in this study compared with previous investigations, rendering them more immuno-competent compared to patients in previous studies [3]. Alternatively, the limited number of patients investigated might contribute to our results, reflecting a wide variation of the immune response to infectious agents, which occurs in the RA population.

The results of our study suggest that the detrimental effects of anti-TNF agents on the immune response can vary quite largely, from very serious to quite limited effects, as reported here for etanercept and adalimumab. Because anti-TNF therapy can affect cellular integrity of the tuberculous granuloma, recruitment of new cells at the granuloma site becomes crucial. In line with this, impaired chemokine production induced by anti-TNF agents may ultimately result in the reactivation of tuberculosis, as previously reported. Therefore, caution should be exercised in order to prevent the development of severe infections and reactivation of tuberculosis whenever therapy with anti-TNF is initiated.

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