(Anti-)TNF alpha matters in rheumatoid arthritis
Wijbrandts, C.A.

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Chapter 13

General discussion and summary
This thesis comprehends a multitude of studies covering important aspects of TNF blocking therapy for rheumatoid arthritis (RA) and is divided into three lines of investigation. The first and main line of investigation, discussed in section I, is focused on exploring predictors of response to anti-TNF therapy. In addition, several other aspects of TNF blockade in RA such as immunogenicity of TNF blockers, the induction of apoptosis, and the effect of treatment on lipid profiles and osteoporosis were studied. The second line of investigation is discussed in section II of this thesis and is focused on distinguishing potential pathogenetic subtypes of RA. The third line of investigation is discussed in section III and explores two novel outcome measures in RA clinical research.
BACKGROUND

RA affects approximately 1% of the population and is characterized by chronic inflammation of the joints, leading to symmetrical joint swelling, pain, stiffness and loss of function (1). Ultimately, this chronic inflammation results in cartilage degradation and erosion of bone. RA is an immune-mediated disease associated with auto-antibody formation, including rheumatoid factor and antibodies against citrullinated peptides (ACPA antibodies). The presence of ACPA has been associated with a more progressive erosive disease (2). Besides joint inflammation extra-articular manifestations such as pericarditis, vasculitis and rheumatoid nodules may occur in RA. Furthermore, cardiovascular morbidity and mortality are increased in RA patients due to accelerated atherogenesis (3) but also systemic bone loss resulting in osteoporosis is more frequently seen (4).

Chronic inflammation within the joint leads to proliferation of the intimal lining layer and increased cellularity in the synovial sublining (5). This process is in part driven by tumor necrosis factor alpha (TNF), an important pro-inflammatory cytokine known to perpetuate the inflammation. TNF can promote neoangiogenesis and induce expression of vascular adhesion molecules, cytokines (for instance IL-6, IL-1) and chemokine (such as IL-8, MCP1) production, which together results in the influx of more T cells, macrophages, and other cells to the site of inflammation. Fibroblast-like synoviocytes (FLS) become activated and secrete matrix metalloproteinases (MMPs) causing cartilage degradation; activation of chondrocytes further enhances the production of MMPs. Furthermore, activation of osteoclasts causes erosion of bone (reviewed in (6;7)).

RA treatment is focused on inhibiting inflammation, hence halting progression of destructive disease. The standard RA treatment regimen consists of at least one disease modifying antirheumatic drug (DMARD) such as methotrexate, alone, or together with prednisolone and a non-steroidal anti-inflammatory drug (NSAID) to improve symptoms of pain and stiffness. When initial conventional DMARD therapy fails, TNF blocking therapy can be initiated combined with methotrexate. TNF blockers belong to a relatively new group of drugs introduced in the 1990’s (8-11), also referred to as “biologicals” or “biologics”. At present there are three registered TNF blockers: infliximab, adalimumab and etanercept. Infliximab is a chimeric IgG1 monoclonal antibody (mAb), adalimumab a fully human IgG1 mAb, and etanercept a TNF receptor (p75) Fc-fusion protein. Infliximab and adalimumab only bind TNF, while etanercept also binds LTα.

In chapter 1 the basics on the central role of TNF in inflammation are explained and the mechanism of action of TNF blocking therapy is highlighted. Most importantly, by blocking TNF, the binding of this cytokine to its receptor on effector cells is interrupted resulting in reduced expression of adhesion molecules, cytokines and chemokines (12-15). This leads to a decrease
in the number of cells infiltrating the synovial tissue and the subsequent inhibition of progressive joint destruction. Furthermore, side effects and the heterogeneous clinical response to TNF blocking therapy as well as co-morbidity associated with RA are discussed in this chapter.

**MAIN FINDINGS**

Although TNF blocking therapy has brought dramatic improvement in disease activity for the majority (60-70%) of patients, a subset (30-40%) does not respond to treatment (8-11). At present there is still no explanation for this heterogeneous response. Therefore, the main focus of this thesis was to identify predictors of response to TNF-blocking therapy. We hypothesized that patients with increased synovial TNF expression before start of treatment would respond better to TNF blockade than patients expressing little TNF in the inflamed joint. Previous studies demonstrated large heterogeneity in synovial cytokine expression between patients (16;17), and an in vitro bioassay study suggested that patients with high TNF bioactivity responded better to TNF blocking therapy than patients with low TNF bioactivity (18). Some genetic studies on the other hand suggested that low TNF production is found in responders to TNF blocking therapy (19). **Chapter 2** describes a study analyzing synovial tissue obtained prior to initiation of infliximab, with the aim of identifying synovial predictors of clinical response to TNF blocking therapy. Detailed immunohistochemical analysis of the synovial cell infiltrate, cytokine, chemokine, growth factor and adhesion molecule expression confirmed the hypothesis that increased TNF expression levels are present in both the intimal lining layer and synovial sublining in responding compared to non-responding patients.

Furthermore, in responders increased synovial macrophage and T-cell infiltration was observed compared to non-responders. Of note, these cells are known to be the main producers of TNF (20). Multivariate logistic regression analysis of synovial markers showed that TNFα expression in the sublining could explain about 10% of the variance in response to therapy, and after adjusting for disease activity at baseline this further increased to 17%. Hence, the predictive value of synovial TNF expression is statistically significant, but overall limited. This clearly indicates that variables other than synovial TNFα expression are involved as well, and at present synovial TNF expression in the pre-treatment biopsy can not be used as a biomarker for clinical response of the individual patient.

To expand the search for predictors of response to TNF blocking therapy a microarray study was performed. This study, discussed in **chapter 3**, was aimed at identifying synovial gene expression profiles reflecting biological processes related to clinical response to TNF blockade. Previous work had shown large heterogeneity in synovial gene expression profiles between RA patients (21). The results of this study extend and corroborate the findings shown in chapter...
1. 2, demonstrating that increased baseline transcription of genes involved in inflammatory pro-
2. cesses, such as T-cell mediated immunity, and cytokine and chemokine mediated signaling
3. pathways, are associated with clinical response to anti-TNF treatment.
4.
5. In summary, chapters 2 and 3 demonstrate that primary clinical response is associated with
6. increased TNF expression, increased numbers of T cells and macrophages, and enhanced
7. expression of genes associated with inflammatory processes. Both studies identified predictors
8. of response to TNF blocking therapy, but no factors could be found that specifically predict a
9. lack of response (non-response) to treatment in the individual patient.
10.
11. It can be hypothesized that in non-responders to TNF blocking therapy, TNF may not be the main
12. cytokine driving the inflammatory process. Another potential explanation for non-response
13. could be the formation of antibodies against the therapeutic drug, since all therapeutic antibod-
14. ies are potentially immunogenic (22). In chapter 4 we specifically studied the incidence of anti-
15. adalimumab antibodies as a potential explanation of secondary non-response after 28 weeks
16. of treatment. Secondary non-response refers to patients who lose clinical efficacy over time. As
17. an antibody response develops over time after repeated antigen exposure, this phenomenon
18. is unlikely to explain a complete lack of clinical response from the start of treatment (primary
19. non-response). The formation of antibodies against infliximab, which is a chimeric (part mouse,
20. part human) anti-TNF antibody, was previously demonstrated in 43% of the patients (23;24).
21. Immunogenicity of fully human therapeutic antibodies, such as adalimumab, is expected to be
22. lower but can still result in an immune response (25). For this reason the incidence and clinical
23. relevance of antibodies against adalimumab were studied.
24.
25. The results showed that 17% of RA patients formed anti-adalimumab antibodies within 28
26. weeks after initiation of treatment. Furthermore, the presence of especially high anti-adali-
27. mumab antibody titers was associated with less clinical improvement and low trough serum
28. adalimumab titers. The low to undetectable serum adalimumab titers that were observed espe-
29. cially in the presence of high anti-adalimumab antibody titres might be due to the increased
30. clearance of the immune complexes (adalimumab – anti-adalimumab antibody) by the liver
31. as was observed in a study with 99technetium-labeled infliximab (26). Of note, the presence of
32. anti-adalimumab antibody titers could not be detected in the presence of high serum adalim-
33. umab levels.
34.
35. Less antibody formation (12%) was observed with concomitant methotrexate use compared
36. to adalimumab mono-therapy (38%), which was in line with previous observations (25). Hence,
37. immunogenicity can be a cause of secondary non-response to anti-TNF antibody therapy in
38. some patients. However, non-response was also observed in the absence of anti-drug anti-
bodies, possibly reflecting the “true” non-responders to TNF blocking therapy due to a TNF independent mechanism driving inflammation.

The mechanism by which anti-TNF therapy reduces local synovial inflammation (13) is still not completely understood. One hypothesis could be that the reduction of inflammation is entirely explained by neutralization of TNF through binding to the TNF blocker. An alternative hypothesis could be that local cell death at the site of inflammation within the first days after TNF blocking therapy is in part responsible for the observed reduction of the synovial infiltrate. In light of the latter hypothesis several mechanisms have been suggested, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), as demonstrated by in vitro cell death after binding of infliximab to murine myeloma cells, which expressed uncleavable transmembrane TNFα (tmTNF) (27). Alternatively, binding of a TNF blocking monoclonal antibody to tmTNF could induce caspase-mediated apoptosis through reverse signaling (28). To investigate the role of apoptosis in more detail we performed a study in the peripheral blood and synovial tissue of RA patients before, and within 1 hour or 24 hours after the first infusion of infliximab. In this study discussed in Chapter 5 we observed no early apoptotic monocytes or lymphocytes in peripheral blood before, 1 hour, or 24 hours after the first infusion of infliximab. Synovial tissue was studied in 5 patients before and 1 hour after infliximab and in five other patients before and 24 hours after the first infliximab infusion. A few TUNEL and active caspase 3 positive cells could be detected in the tissue, but on the group level no marked increase was observed after treatment; we detected an increase in both TUNEL and active caspase 3 positive cells in only one patient 24 hours after infliximab, which could be confirmed by electron microscopic detection of apoptotic cells. Furthermore, complement activation and nucleosome levels were measured in peripheral blood at the above mentioned time points, which showed no increase over time within these first 24 hours. Hence, this study did support the notion that apoptosis induction is not important directly after initiation of infliximab in RA patients. However, increased apoptosis has been suggested after a more prolonged treatment period (29), though this may be a secondary phenomenon rather than a direct anti-apoptotic effect (30).

The risk for cardiovascular morbidity is increased in RA patients with active disease (reviewed in (31)). In addition to traditional risk factors for atherogenesis such as, smoking, diabetes, obesity and dyslipidemia, chronic systemic inflammation is thought to enhance the atherosclerotic process. Hence, a reduction of disease activity should reduce atherogenesis. In line with this notion previous studies observed a decrease in cardiovascular morbidity after reducing RA disease activity by DMARD therapy (32,33). Since TNF blockade was shown to have a beneficial effect on lipid levels (34), insulin resistance (35), vascular adhesion molecule expression (13), and endothelial function (14) a beneficial effect on atherogenesis and cardiovascular disease could be expected. Recent studies suggested that cardiovascular morbidity and mortality may indeed be reduced after TNF blocking therapy (36,37). In light of the potential beneficial effect
of TNF blockade on atherogenesis we studied the effect of the TNF blocker adalimumab on lipid profiles at week 16 and week 52 after start of treatment compared to baseline. In addition, we measured serum levels of macrophage migration inhibitory factor (MIF), as this cytokine has come to light as a potential link between RA and atherogenesis (38). In addition, MIF is overexpressed in the inflamed synovium of RA patients (Figure 1).

Since MIF is able to induce TNF secretion, and conversely TNF can augment MIF secretion (38;39), we hypothesized that TNF blocking therapy might decrease MIF levels with a potential beneficial effect on atherogenesis. The results of this study, discussed in chapter 6, showed that after 16 weeks of adalimumab therapy, both DAS28, lipoprotein (a) (Lp(a)), and MIF levels were significantly decreased at week 16, which was sustained up to week 52. HDL cholesterol levels were significantly increased at week 16, but returned to baseline at week 52, while Apo A-I levels

Figure 1. MIF expression in synovial tissue A) 3 examples of immunofluorescent staining of MIF (ALEXA-red) in the endothelium. B) CD68+ macrophages (FITC-green), MIF (ALEXA-red), and double staining of CD68 and MIF. C) CD3+ T cells (FITC-green), MIF (ALEXA-red), and double staining of CD3 and MIF. DAPI was used to stained the nucleus blue (unpublished Figure).

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remained significantly elevated up to 52 weeks after the start of adalimumab therapy. This resulted in an improved apo B/ A-I ratio. Furthermore, MIF levels were not associated with serum CRP levels as was shown previously (40), but MIF may be regulated by TNF as levels decreased after TNF blocking therapy independent of clinical response. Hence, apart from reducing systemic inflammation as was shown by reduced CRP and ESR levels, the sustained decrease in apo B/A-I ratio together with a sustained increase in Lp(a) levels and a decrease in MIF levels, suggest a favorable effect of adalimumab therapy on markers associated with atherogenesis.

Besides at risk for cardiovascular disease, RA patients are also at risk for developing osteoporosis (4), with major clinical implications such as fractures after falling and subsequent disability. Of interest, the cytokine TNF does not only play a role in synovial inflammation of RA patients, but is also involved in postmenopausal bone loss (41;42). Therefore, blockade of TNF may inhibit systemic bone loss under the influence of TNF. In Chapter 7 data are presented from a study on the effect of the anti-TNF antibody adalimumab on bone mineral density (BMD) one year after treatment. As expected, baseline femoral neck BMD was higher in men than in women. Furthermore, disease activity and disease duration were negatively correlated with baseline femoral neck BMD. Instead of the annual decrease in bone mineral density, an arrest of further bone loss was observed on the group level. Of interest, the arrest of bone loss was most prominent in patients using concomitant low dose prednisone. This was a somewhat unexpected finding, as prednisone is known to induce osteoporosis. However, the anti-inflammatory effect of this drug and the low dosage may have resulted in the inhibition of bone loss. This positive effect of prednisone has been described previously (43).

In chapter 4 the influence of antibody formation against the TNF blocking antibody adalimumab on clinical response is discussed. In case of non-response or loss of response, a choice needs to be made for an alternative anti-rheumatic drug. Possible choices are: to switch to an alternative TNF blocker or to choose a biological with an alternative mode of action such as B-cell depletion with rituximab (anti-CD20 antibody) or abatacept (CTLA4-Ig) treatment. We asked the question whether the clinical response after switching from infliximab to adalimumab is similar to the clinical response in anti-TNF therapy naïve patients using adalimumab as a first TNF blocker. Furthermore, we investigated whether antibody formation to infliximab is related to the clinical response and the formation of antibodies to the second TNF blocking antibody adalimumab. The study discussed in Chapter 8 was performed in light of these research questions and included a total of 235 RA patients. The data showed that the mean decrease in DAS28 after start of adalimumab treatment was significantly greater for anti-TNF naïve patients compared to switchers. Twenty percent of all 235 patients formed anti-adalimumab antibodies within 28 weeks after start of treatment which was associated with significantly less improvement in DAS28 compared to patients without antibody formation. An interesting finding was the fact that patients who formed anti-infliximab antibodies significantly more often formed anti-adalimumab antibodies.
(27%) than anti-TNF naive patients (18%). Furthermore, response to adalimumab was limited in switchers without anti-infliximab antibodies, potentially reflecting the true anti-TNF non-responders. These data give an insight into mechanisms behind non-response and the influence of antibody formation on switching TNF blocking antibodies. Larger prospective studies are needed to find out whether clinical decision making can be based on these findings.

The second line of investigation in this thesis focused on distinguishing potential pathogenic subtypes of RA. Because RA is a heterogeneous and systemic disease, we investigated whether the heterogeneity and pathogenic events in the host are reflected in peripheral blood (PB) cells. Chapter 9 discusses the findings of this study in 35 patients with RA and 15 healthy controls aimed at identifying gene expression profiles in PB that distinguish patients with RA from healthy controls. Using microarray analysis a spectrum of genes was found to be elevated in patients with RA compared to healthy individuals. Statistical analysis revealed significantly upregulated expression of interferon (IFN) inducible genes. Subsequent real-time PCR analysis showed a high correlation with the microarray data and confirmed the expression of key genes (RSAD2 and GIP2) of the IFN pathway in all samples. More detailed analysis using type I (genes responding to IFN α and IFN β) and type II (genes responding to IFNγ) IFN response gene sets, revealed that the type I IFN gene set was significantly higher in patients with RA, while the type II IFN gene set was similar between patients and controls.

A more detailed quantitative analysis demonstrated that IFN response genes were increased in approximately half of the patients (IFN$^{\text{high}}$). Additional pathway analysis suggested upregulation of pathways involved in coagulation, the complement cascade, and fatty acid metabolism in the IFN$^{\text{high}}$ group while RA patients in the IFN$^{\text{low}}$ group were more similar to the healthy controls. Of interest, a subsequent study (44) (not discussed in this thesis) demonstrated the clustering of a subgroup of RA patients with smallpox virus-infected macaques both expressing genes involved in innate immunity defense system, while other RA patients and healthy individuals co-clustered with non-infected macaques. A recent study found antiviral gene expression to be significantly increased in RA synovium compared to osteoarthritis synoviumm, especially after Toll like receptor ligands and TNFα stimulation (45). In summary, a subgroup of RA patients shows several characteristics of a viral infection with an increased type I IFN signature. This subgroup of patients also had significantly higher serum ACPA titers. Infectious agents may be potential triggers for immune-mediated responses in RA (46;47), although endogenous danger signals (such as DNA release from damaged cells, heat shock proteins) may equally induce a pathogen response signature.

In light of the ambition to identify both clinical and pathogenic disease subtypes, Chapter 10 discusses a study focused on the detection of lymphocyte aggregates in the synovial tissue of 103 patients with RA. The study was aimed at determining whether the presence of synovial
lymphoid neogenesis is related to characteristics of inflammation and disease severity. Lymphocyte aggregates were graded by size (0 = absent or 1-3) (48), where grade 2 and 3 aggregates were referred to as lymphoid neogenesis. In 32% of the RA synovial tissues, lymphoid neogenesis was present whereas only small grade I aggregates were present in an additional 25% of synovial tissues. Follicular dendritic cells were present in 28% of the samples with lymphoid neogenesis (8% of the total cohort). Immunohistologic examination of synovial tissue revealed increased infiltration by T and B cells, plasma cells and macrophages as well as increased expression of TNFα and lymphotoxin β (LTβ) in patients who exhibited lymphoid neogenesis. RA patients with lymphoid neogenesis also had higher C-reactive (CRP) protein levels and erythrocyte sedimentations rates (ESR) as well as elevated leukocyte and thrombocyte counts. Of interest, there was no relationship between the presence of lymphoid neogenesis and increased clinical signs and symptoms, as shown by the DAS28, tender and swollen joint count, and the presence of rheumatoid nodules or erosions. Finally, the presence of auto-antibodies (IgM-rheumatoid factor and ACPA) was not associated with the presence of synovial lymphoid neogenesis, which might suggest that the responsible B cells are found outside the joints, for instance in the lymphnodes or spleen (49). In summary, lymphoid neogenesis does not seem to define a specific disease phenotype, and accumulating data support the notion that lymphoid neogenesis is a consequence rather than the cause of chronic synovial inflammation in RA (50;51).

As outcome measures are critical in the evaluation of new therapies, the final section focuses on two investigations describing new clinical and molecular outcome measures in RA research.

Previous research demonstrated that on the group level the mean improvement in disease activity is associated with the mean decrease in the number of synovial macrophages (52). More specifically, the same study suggested that the number of macrophages decreased in patients treated with effective anti-rheumatic therapy, but not in placebo-treated patients. The analysis described in Chapter 11 is an extension of the previous study now aimed at answering the question whether the change in sublining macrophages could also be used as a biomarker to distinguish clinically effective from ineffective anti-rheumatic therapies at an early stage of drug development (Phase I trials).

For a biomarker to pass the “discrimination criterion” of the so-called OMERACT filter, it should not only exhibit a high sensitivity to change, but it should also distinguish between effective and ineffective treatment (53). The sensitivity to detect change was determined by the standardized response mean (SRM), which was calculated by dividing the mean change by the standard deviation of the mean change. An SRM of >0.8 is considered to have high potential to detect change, an SRM of 0.5 as having moderate potential and an SRM of 0.2 as low potential to detect changes. The weighted mean of the SRM for the CD68 sublining macrophages was −0.89 (± SE 0.12) for the effective, 0.20 (± SE 0.18) for the ineffective, and 0.11 (± SE 0.18) for the placebo group. The difference between the effective and ineffective group was significant for
CD68 sublining macrophages as well as the DAS28. Hence, a clear distinction between effective and ineffective treatment was possible. These findings add to the validity of the use of the synovial biomarker in relatively small, high density of data, proof of concept studies to screen for potential efficacy in an early stage of drug development.

In Chapter 12 we introduce a new questionnaire in the rheumatology field, the Academic Medical Center Linear Disability Scale (ALDS) (54). The scale was designed to measure disability in patients with various conditions. The present study in 129 patients with RA describes the psychometric properties of this new instrument and sensitivity to change after effective treatment in comparison to the extensively used and validated Health Assessment Questionnaire-Disability Index (HAQ-DI). Both questionnaires were sensitive to detect change in disability in relationship to change in disease activity after treatment with the TNF blocker infliximab. A decrease in disease activity was associated with a decrease in disability reflected by the ability to perform more difficult tasks. The ALDS item bank demonstrated less susceptibility to ceiling effects. Another positive element of the ALDS is its linear scale (0-10) instead of ordinal scale such as the HAQ-DI making it easier to interpret changes. Furthermore, an item response theory (IRT) item bank can be used adaptively and will form a good foundation for computer adaptive testing, where the difficulty level is automatically adapted per question depending on the individual patient’s ability to perform the requested activity. The ALDS is an interesting new instrument that is simple to use for assessing the level of disability in patients with RA.

CONCLUDING REMARKS

Taken together, TNF blocking therapy was the beginning of a significant change in the rheumatology field. TNF blockers were the first “targeted” anti-rheumatic drugs, and since their introduction many new targeted anti-rheumatic drugs are either in use or under development. Although TNF blockade was already introduced in the 1990’s, many aspects of its mechanism of action and effects on co-morbidity became subject of study after their registration. This thesis contains several studies revolving around TNF blocking therapy varying from the prediction of clinical response to the effects on osteoporosis, lipid profiles, anti-drug antibody formation and the relevance of apoptosis induction by TNF blocking antibodies.

Data are presented on analysis of different pathogenetic subsets in RA and initial proof of concept is shown for the concept of personalized medicine. In addition, data were provided on new outcome measures that may facilitate future research in rheumatology. These studies may not only ultimately contribute to the improvement of patient care, but also add to an understanding of disease pathogenesis and the impact of treatment on the different processes involved in inflammation in RA.
REFERENCE LIST


