## Dynamics of TNF during TNF inhibitor treatment

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# Summarizing discussion

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The introduction of tumor necrosis factor inhibitors (TNFi) revolutionary improved the treatment of inflammatory autoimmune diseases. With five TNFi presently used in the clinic there is no need for more TNFi options, but there is a need for more insights into how to optimize the use of the existing TNFi. At present, many studies focus on evaluation of the TNFi during therapy. Insights in the target TNF itself, however, may contribute to optimization and personalization of TNFi treatment as well. The studies in this thesis focus on TNF during TNFi treatment. New assays have been developed in order to accurately measure TNF in the presence of large amounts of TNFi. These TNF concentrations have been studied in relation with clinical response.

**Identification of TNF as a therapeutic target**

In the early 1980s an imbalance between pro- and anti-inflammatory cytokines was observed in rheumatoid arthritis (RA) biopsies, and TNF was identified as a key mediator driving inflammatory autoimmune diseases.[1–5] Also in mice studies, TNF is a major player. Intraarticular administration of TNF in an arthritis mice model accelerated arthritis,[6] and transgenic mice stably overexpressing a human TNF transgene spontaneously developed chronic inflammatory arthritis.[7] The development of arthritis in these mice could be suppressed with anti-human TNF antibodies.[8,9] Therapeutic administration of anti-TNF in an arthritis mice model revealed a significant reduction in clinical score, degree of joint inflammation and paw swelling.[10–13] This was the first hint that TNF could potentially become a therapeutic target.

**Elevated TNF associates with inflammation**

The importance of research into TNF was recognized before, and TNF concentrations were investigated in health and disease. TNF concentrations were found to be higher in autoimmune disease patients compared to healthy individuals, although often not more than ca. twofold, and in a concentration range that is not easily quantifiable (see section “Challenges in TNF measurements”).[14–21] The amount of TNF associated with clinical disease: the more TNF the higher the disease activity.[14,18,19,21–23] These results fit with the idea that too much TNF contributes to inflammation in chronic autoimmune diseases. TNFi are very efficient in neutralizing TNF, and outcompete the binding to TNF receptors (TNFRs). Blocking TNF with TNFi will eliminate the surplus of TNF and therefore ameliorates the symptoms. A schematic overview of this concept is shown in Figure 1A.

Since the clinical application of TNFi, numerous studies also investigated TNF

concentrations during TNFi treatment. A paradoxical increase in circulating TNF was observed shortly after initiation of TNFi treatment.[16,24–30] TNF has a short half-life, in the order of minutes, and TNF is therefore rapidly cleared from the circulation.[31,32] However, TNF bound to a TNFi has a prolonged half-life due to its tight binding to the TNFi, which themselves have a very long half-life. The prolonged TNF half-life most likely explains the observed increase in TNF. Of note, despite the measurement of high TNF concentrations during TNFi treatment, these high amounts of circulating TNF were in complex with the TNFi and thus biologically inactive.[24,33]

A study by Charles and colleagues indicated that the increase in TNF shortly after treatment initiation was followed by a gradual decline in TNF.[24] This decline was associated with a significant reduction in C-reactive protein (CRP).[34] Moreover, TNFi treatment could be that effective that once in remission a proportion of patients can successfully discontinue treatment (reported range, 20 to 79% of the patients in remission).[35–37] This indicates that in these patients blocking TNF is no longer required for disease control.

Together, these observations suggest a mechanism where successful treatment discontinuation hinges on a decline in TNF production, thereby alleviating the need for blocking TNF (Figure 1A). In this thesis we quantified TNF in large cohorts of adalimumab- and etanercept-treated patients and investigated the relation between TNF concentrations and clinical response. At the start of the research described in this thesis, we expected an overall increase in circulating TNF in the first phase of treatment, followed by a decrease in TNF in patients who are in clinical remission. As such, monitoring TNF during TNFi treatment could be a potential biomarker to identify those patients that can successfully discontinue TNFi treatment.

**Challenges in TNF measurements**

Quantification of TNF is challenging for a number of reasons. First, the TNF half-life is in the order of minutes and TNF is therefore rapidly cleared from the circulation.[31,32] Second, continuous monomeric subunit exchange makes TNF unstable in biological samples.[38–41] Last, the trimeric TNF structure is easily compromised, e.g., during freezing and thawing of serum samples, with direct impact on TNF quantification.[42] Consequently, the amount of TNF that can be measured in circulation is low, with reported TNF concentrations in health and disease in the low picogram range.

Although various TNF assays have been reported, significant drawbacks of these assays prompted us to develop new assays for the quantification of TNF. For example, bioassays using cell lines susceptible to TNF are often relative insensitive, especially for

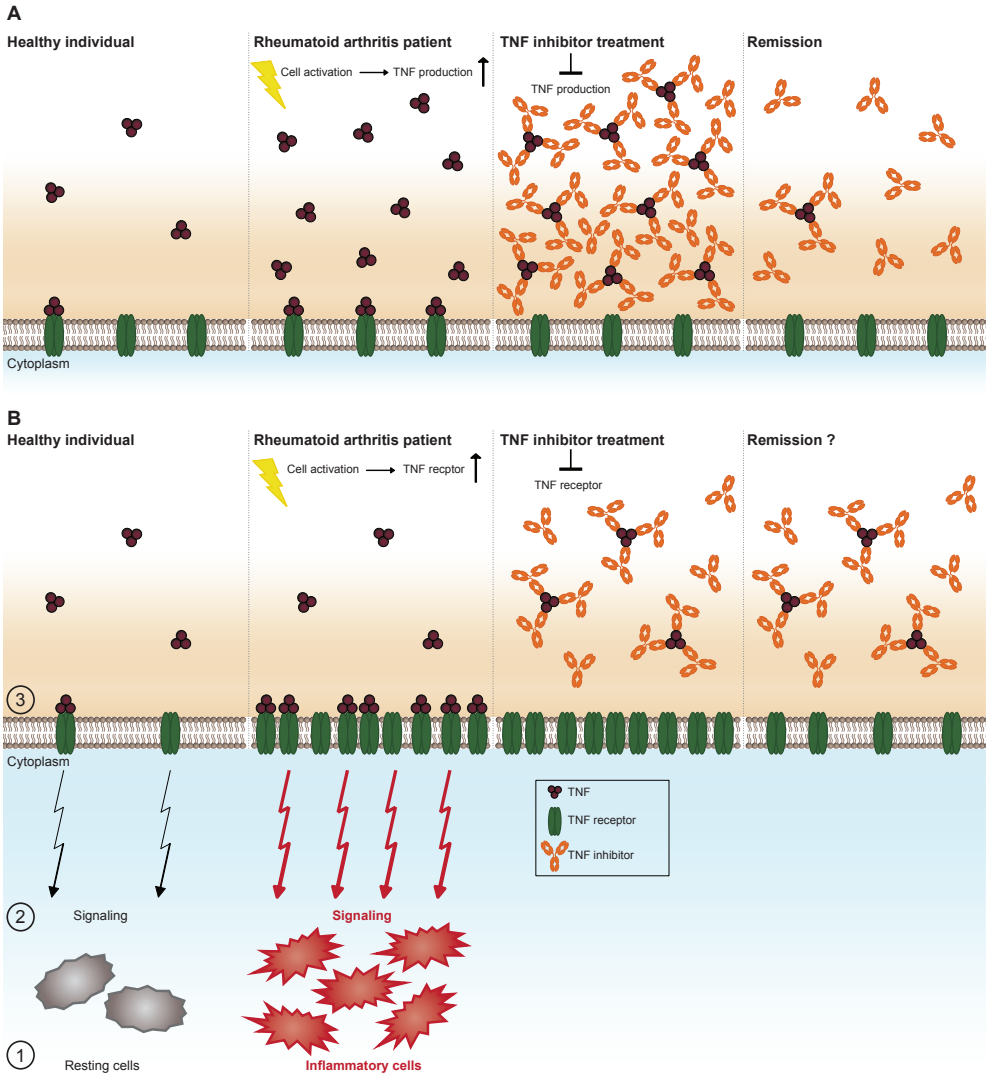


**Figure 1. Schematic representation of the local site of inflammation. Panel A** (discussed in section "Elevated TNF associates with inflammation") illustrates the idea that too much TNF contributes to inflammation in chronic autoimmune diseases. A certain (unknown) trigger activates inflammatory cells, which start to produce high amounts of TNF. Significantly higher TNF concentrations have previously been measured in autoimmune disease patients, compared to healthy controls. However, based on our recent findings we propose that it is not the amount of circulating TNF perse that defines a patient, but that it is the local environment, together with TNF, that defines disease severity in a patient, as is illustrated in **Panel B** (discussed in the section "What is the pathogenic role of TNF in autoimmune diseases?").

the measurement of low concentrations of TNF.[4] More important, biological samples from patients (i.e. serum or synovial fluid) consist of many uncharacterized factors, which might affect the target cells in these bioassays. Later, more sensitive enzyme-linked immunosorbent assays (ELISAs) were developed. However, immunoassays are prone to interference due to the high amounts of cytokine-binding proteins, auto-antibodies and soluble receptors in biological samples. Especially when TNF is measured in the low concentration range which has been reported in health and disease. A firmly consistent outcome of those studies is that TNF concentrations are mostly very low, around the detection limit of most immunoassays, even during active disease. This makes quantitative interpretation difficult. In addition, when studies compared the results from TNF measurements with different commercially available ELISA kits, relevant differences in TNF concentrations were reported.[43–46] It is therefore complicated to compare TNF concentrations between studies.

Limitations of the assays used for the quantification of TNF become apparent, especially when measuring TNF in these low ranges as have been reported. Abovementioned studies tend to show higher TNF concentrations in patients, but it will be hard to conclude whether TNF concentrations are truly increased in patients compared to healthy individuals. However, we think it will be unlikely that pre-treatment TNF can be used as reliable biomarker for baseline disease activity.

In contrast, TNF bound to a TNFi has a prolonged half-life due to its tight binding to the TNFi. As a result, TNF concentrations during TNFi treatment are in a higher range. This makes these measurements more reliable, compared to pre-treatment TNF concentrations. Nonetheless, the measurement of TNF during TNFi treatment is still challenging. Due to complex formation the TNFi interferes with the quantification of TNF. This results most likely in a severe underestimation of the TNF concentration,[16] which affects all of the above mentioned studies. To overcome this limitation of drug-interference, the development of a new drug-tolerant assay is described in **Chapter 2** for adalimumab, in **Chapter 3** for etanercept and in **Chapter 5** for the remaining three TNFi golimumab, infliximab and certolizumab.

**Drug-tolerant assay development**

All five TNFi neutralize TNF, but they bind a different TNF epitope. The first approach for development of a drug-tolerant assay aimed to identify a pair of anti-TNF antibody clones, that were both able to bind TNF. However, none of the tested antibody combinations were able to bind TNF simultaneously with the TNFi, and did therefore not result in a useful assay (data not shown). Helped by the data of Votsmeier and colleagues, describing

adalimumab variants with amino acid substitutions that resulted in increased affinity to TNF,[47] we could develop a drug-tolerant assay. Although the final assay is somewhat different for the different TNFi, the overall principle is similar: a high affinity adalimumab mutant antibody can efficiently displace the TNFi from TNF. In the resulting assays, quantitative recovery of TNF in the presence of large amounts of TNFi was achieved.

**Longitudinal TNF concentrations during TNFi treatment**

In **Chapter 2** and **Chapter 3** we used our new drug-tolerant assays to investigate the dynamics of circulating TNF in large cohorts of adalimumab- and etanercept-treated RA patients, respectively. As expected, TNF concentrations were very low at baseline, before TNFi administration, but significantly increased upon adalimumab and etanercept treatment. Although both the number of patients and duration of follow-up were lower or shorter, respectively, similar findings were observed for golimumab, infliximab and certolizumab in **Chapter 5**. After the initial increase in the early phase of treatment, TNF concentrations remained remarkably stable in the majority of patients for at least two years. So, in contrast to what was expected did TNF not decrease in patients in remission. However, TNF did rapidly decrease in some patients, but this was only observed in patients in whom adalimumab became undetectable, coinciding with the appearance of anti-drug antibodies (ADAs). In fact, these patients failed to respond to TNFi treatment.

Surprisingly, a similar increase in TNF was found in healthy volunteers after one dose of adalimumab. Altogether, these results imply that circulating TNF during TNFi treatment is not primarily associated with (suppressed) inflammation or disease activity and that circulating TNF does not associate with clinical response. TNF can thus not be used as a biomarker for treatment response.

Tanaka and colleagues recently used baseline TNF as a biomarker for treatment response.[48] They adjusted infliximab dose based on TNF concentrations at baseline, under the assumptions that baseline TNF concentrations can be accurately measured, and that high disease activity (i.e. more inflammation) is associated with higher TNF concentrations. Our findings argue against these assumptions. It is therefore not unexpected that there was no difference in the proportion of sustained remission after one year of infliximab discontinuation between the standard treatment arm (standard infliximab dosing) compared to the programmed treatment arm (infliximab dose adjusted based on baseline TNF). In **Chapter 4** we comment on this study.

**Impact of ADAs on circulating TNF concentrations**

Although TNF cannot be used as a biomarker for treatment discontinuation, we did

find that TNF can be used to predict ADA formation in the early phase of treatment. In **Chapter 2** we showed that TNF concentrations at week four were significantly lower in patients who became ADA positive over 52 weeks of treatment. The association between early low TNF and subsequent ADA formation was validated and confirmed in a second independent cohort of RA patients. Previously, it has been shown that ADA formation is strongly associated with lower drug levels.[49] Drug level reduction is a consequence of immune complex formation between drug and ADA, which results in enhanced clearance of te drug.[50] However, the association between low TNF at week four and ADA formation over 52 weeks is different from the observation that TNF concentrations substantially dropped over time because of ADA formation in some patients. In case of the latter, ADA formation led to undetectable amounts of adalimumab and, consequently, the disappearance of TNF. In contrast, at week four, at the time TNF concentrations were significantly low, all patients had adalimumab serum concentrations >0.1 µg/mL. We demonstrated that this is sufficient for near quantitative capture of TNF *in vivo*. In other words, the low TNF concentration in patients who became ADA positive is not due to undetectable adalimumab.

In infliximab-treated patients we observed a similar trend, in that TNF concentrations gradually increased. However, the cohort was insufficiently sized to demonstrate a significant association between low TNF concentrations and ADA formation. We initially supposed that the drop in TNF upon infliximab administration, as described by Charles and colleagues, associated with reduced disease activity.[24] However, it seems that this decrease in TNF most likely reflects ADA-mediated clearance of TNF. In contrast, during golimumab and etanercept treatment were steady-state TNF concentrations reached already at week four. This difference can be explained by the lack of clinically relevant ADA formation in etanercept- and golimumab-treated patients.[51,52]

The association between early low TNF and ADA formation was even more pronounced in healthy volunteers who had received a single dose of adalimumab biosimilar. We identified that the drop in TNF was well before the decrease in adalimumab and even before the detection of ADAs. We hypothesized that in the early phase of an immunogenic response low-affinity anti-adalimumab antibodies (probably of the IgM isotype) are present that might preferentially bind multimeric TNF-adalimumab complexes. At this stage low levels of low-affinity antibodies are not (yet) detectable and do not affect monomeric adalimumab (serum) drug concentration. The presence of antigen-specific B-cells might also have an important effect, in addition to antibodies. TNF-adalimumab complexes have three adalimumab Fab domains available (compared to two Fab domains in free adalimumab), which can be bound by antigen-specific B-cells. In **Chapter 5** we

demonstrated that three Fc domains is the minimum for Fc-gamma receptor (FcγR)-mediated clearance and a subtle difference between two and three Fab domains can make a difference as well. This could result in selective cell-mediated clearance of TNF-adalimumab complexes, leading to a severe decrease in TNF. Of note, the number of B-cells peak around 1-2 weeks, exactly at the time we observe the decrease in TNF.

**Impact of clearance on steady-state TNF concentrations**

We observed a significant increase in TNF upon treatment with all five TNFi. However, steady-state TNF concentrations half a year after treatment initiation were significantly different. TNF concentrations were significantly higher during etanercept and certolizumab treatment, compared to patients treated with adalimumab, golimumab or infliximab. We demonstrated that the amount of TNF was not associated with serum drug concentration (explained in more detail below), nor with disease activity. One might therefore wonder what determines this difference in steady-state TNF concentration. In **Chapter 5** we provide mechanistic insights in the factors contributing to the amount of circulating drug-bound TNF during treatment.

FcγR-mediated clearance appeared to be an important factor. Etanercept and certolizumab, the two TNFi with the highest steady-state TNF concentrations, were not internalized by macrophages. In contrast to TNF in complex with adalimumab, golimumab or infliximab, which were efficiently cleared by macrophages. The lack of clearance could be attributed to the structure and configuration of TNF-etanercept and TNF-certolizumab complexes. Fc-mediated internalization depends on FcγR cross-linking, and the valency of a complex is thus important.[50,53] It has previously been shown that tetramers and larger IgG immune complexes are efficiently internalized by macrophages, while monomers and dimers are not.[50] We demonstrated that TNF-etanercept is the only TNF-TNFi complex that is formed in a 1:1 ratio. The 1:1 ratio explains the lack of internalization by macrophages. Although certolizumab is formed in 1:3 TNF-TNFi ratio, the lack of Fc tails in these complexes explains the absence of internalization. Variation in TNF concentrations can thus most likely be explained by differences in clearance of TNF-TNFi complexes. However, we cannot exclude that TNFi differentially affect TNF production, which might result in differences in steady-state TNF concentrations as well. Overall, we demonstrated that TNFi did not noticeably impact TNF production.

TNF in complex with etanercept and certolizumab are both not cleared from circulation, and TNF production is not different for the two TNFi. Still, we observed significantly higher TNF concentrations in certolizumab-treated patients. This can most likely be explained by the fact that the half-life of etanercept is with 3 days[54,55] much shorter compared to 14 days

for certolizumab.[56,57]

**Proposed model**

The data from healthy volunteers revealed that TNF rapidly increased within one week and that inter-individual variability in TNF was minimal at this time. After a peak TNF concentration at week one, TNF concentrations differentially started to decrease. In the large cohort of adalimumab-treated RA patients we observed a gradual increase in TNF from week four onwards. This might suggest that the TNF concentration in this cohort would initially have been higher than that we now measure at week four.

Based on the data shown in this thesis, we propose a model where the dynamics in TNF can be divided in three phases (Figure 2). 1) TNF concentrations rapidly increase after TNFi administration, which is explained by a prolonged TNF half-life due to TNF-TNFi complex formation. TNF concentrations peak within a few days. During this phase inter-individual variation in TNF is minimal. From the first TNFi dose on, all TNF will be bound to TNFi, since there is a large excess of TNFi over TNF. One might therefore wonder why TNF concentrations do not peak right after first TNFi administration. We think this can be explained by the constitutive production of TNF. Directly after the first dose, all circulating TNF will be bound to the TNFi. Meanwhile, new TNF is produced, which will also become bound, etc. This will lead to a buildup in TNF until a balance between TNF production and clearance has been established. Similar increases have previously been observed for other anti-cytokine antibodies, for instance IL-6.[58] 2) During the second phase, once a balance between TNF production and clearance has been established, TNF concentrations remain stable over time, in most of the patients. This has been demonstrated in etanercept-treated patients. However, in case of an immunogenic TNFi, we hypothesize that low-affinity ADAs and B-cells start to play a role. As explained before, these low-affinity ADAs and B-cells will preferentially bind, and subsequently clear, TNF-drug complexes. This results in a differential decrease in TNF concentrations. Of note, the clearance of complexes in this phase is independent of the adalimumab serum concentration, and ADAs are not yet detectable in serum. This decrease in TNF is less pronounced in patients concomitantly treated with methotrexate (explained in more detail below). The humoral immune response can be of transient nature, which in time results in a new balance between clearance and production, and a steady-state TNF concentration. This suggests that in fact we are looking at a re-increase in TNF. 3) Once steady-state has been reached, TNF concentrations remain remarkably stable over time in most patients, irrespective of disease status. However, in some patients TNF concentrations rapidly dropped, after an initial increase from baseline. This decrease in TNF is dependent on the serum adalimumab concentration, as in these patients adalimumab became undetectable,

coinciding with the detection of ADAs. Therefore, opposite from what was expected, did we observe a drop in TNF in patients who fail to respond to TNFi treatment. Overall, low TNF concentrations in the early phase of treatment associate with future detection of ADAs, that impact efficacy. This suggests a potential role for TNF measurements in the early phase of treatment as predictive biomarker for immunogenicity.

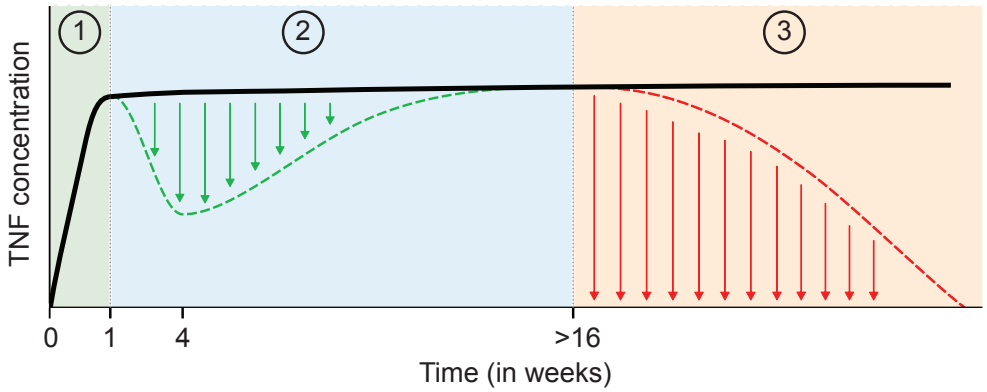


**Figure 2. Proposed model of the dynamics in TNF during TNF inhibitor treatment over time.** The dynamics in TNF during TNF inhibitor (TNFi) treatment can be divided in three phases. Black lines shows the amount of TNF in time. Green and red dashed lines represent diminished TNF due to ADA formation in the early and late phase of treatment, respectively.

### Methotrexate and TNF concentrations

Early low TNF concentrations in the early phase of treatment not only associated with future ADA formation but also with less frequent baseline methotrexate use. Methotrexate is dose-dependently associated with less ADA formation,[59,60] and is suggested to inhibit pro-inflammatory cytokine production as well.[61,62] It is therefore remarkable that patients concomitantly treated with methotrexate had significantly higher TNF concentrations at week four, compared to patients without methotrexate treatment. Although a direct effect of methotrexate on TNF production cannot be excluded, we hypothesized that methotrexate affects ADA formation, which in turn is associated with early low TNF concentrations and less frequent remission. The effect of methotrexate on TNF concentrations, independent of ADAs, was studied in more detail in **Chapter 3**. We made use of etanercept-treated RA patients, since it is assumed that immunogenicity and ADA formation does not play a clinically relevant role in etanercept treatment.[51] We demonstrated that week four TNF concentrations were not directly associated with baseline methotrexate use, thereby confirming our hypothesis. This contrasts the findings reported for adalimumab, and implies that the association between early low TNF and

methotrexate in adalimumab-treated patients is indeed most likely due to antibody formation.

**Lowest effective TNFi concentration**

TNFi are approved in a standard dose, but recent studies demonstrated that a large proportion of TNFi-treated patients are overexposed.[63,64] In view of potential adverse events and drug costs it is important to avoid overexposure. Data in **Chapter 2** and **Chapter 3** demonstrated that there was only a weak overall association between TNF and serum drug concentrations. Only low TNFi concentrations clearly associated with the amount of TNF, but above ~1 µg/mL no clear correlation was apparent. For adalimumab the data suggest that a concentration around as low as 0.1 µg/mL is sufficient for near quantitative capture of all TNF *in vivo.* Therefore, a minimal critical amount of TNFi is required to keep the majority of TNF in complex with the drug. Above this concentration no correlation was observed between TNF and serum drug concentration. Furthermore, in patients who discontinued adalimumab treatment we found that TNF concentrations remained stable up to even half a year after treatment discontinuation, as long as adalimumab concentrations remained above 0.1 µg/mL. A different picture was obtained from etanercept (unpublished data). Already within 12 weeks after treatment discontinuation TNF could no longer be detected in any patient. This coincided with a drop in etanercept below the detection limit. This difference between adalimumab and etanercept could probably be explained by a difference in the amount of serum adalimumab or etanercept at the time patients discontinued treatment. TNFi concentrations were around six times higher in adalimumab-treated patients compared to patients treated with etanercept (mean (SD) concentration of 5.5 (2.9) and 0.94 (0.47) µg/mL, respectively). Moreover, a difference in half-life between adalimumab and etanercept (around 2 weeks[65,66] and 3 days,[54,55] respectively) is another important factor contributing to undetectable TNF in etanercept-treated patients.

Since sufficient amounts of TNFi remained in circulation to bind all TNF up to half a year after adalimumab treatment discontinuation, the question is at which point in time a patient can truly be classified as having discontinued treatment. Since golimumab, infliximab and certolizumab have a comparable long half-life, this probably accounts for these TNFi as well. This is relevant when evaluating switch studies, studies aimed at evaluating the success of treatment with a second TNFi, after failure to a first TNFi. Efficacy of a second TNFi after switching might for instance be overestimated, since at that time TNF can be blocked by two TNFi simultaneously. The effect of switching to a second TNFi can in theory thus best be evaluated in patients switching from etanercept to a second TNFi. Moreover, these results raise the question whether a temporary discontinuation of

treatment in case of an infection or surgery is effective.

The number of patients who discontinued treatment was too small to investigate the relationship between TNF concentrations and clinical response, but it raises questions about the minimal amount of TNFi that is critical for maintaining clinical response. Calculations in **Chapter 5** also imply that for all TNFi a concentration of 0.1 µg/mL is sufficient for quantitative *in vivo* capture of TNF. However, one should keep in mind that there might be a discrepancy between *in vivo* capture of TNF and the amount of drug which is sufficient to remain in clinical remission. TNFi bound to TNF is a dynamic interaction, and when a TNFi molecule dissociates from TNF, there should be enough drug present in serum to favor re-association over binding to its receptors. We suggest, however, that stable high TNF concentrations reflect maximal clinical efficacy. Since stable, high TNF concentrations are maintained with TNFi concentrations as low as 0.1 µg/mL, this indicates that TNFi are usually massively overdosed. It is of interest to further investigate the critical amount of TNFi needed for clinical effectiveness, however, data regarding the minimal effective drug concentration for TNFi is not available, or limited. To obtain more insight into such a cut-off value, a large number of patients with low serum drug concentrations is required. However, the number of these patients is scarce in clinical settings. Retrospective analyses of dose-finding studies might provide more insight.

Numerous studies investigated the relationship between serum drug concentrations and clinical response, and demonstrated that higher drug concentrations associated with better clinical outcome.[67–71] However, the concentration-effect curve for adalimumab plateaus at 5 µg/mL, and concentrations between 5-8 µg/mL are sufficient for maintaining an adequate clinical response in RA, six months after treatment initiation.[70] Recent studies by l'Ami and colleagues,[63,64] in combination with results from **Chapter 2**, **Chapter 3** and **Chapter 5** suggest that concentrations of 0.1 - 0.5 µg/mL might be sufficient for maintaining clinical response. This contrasts the previous reported therapeutic range of 5-8 µg/mL. Important to mention, however, is that a distinction between the initial early treatment phase, and later treatment phases should be made. The concentration-effect relationship investigated clinical efficacy six months after start of TNFi treatment. During this initial phase, disease suppression is important, which might require high TNFi concentrations. Moreover, a high TNFi concentration in the first six months of treatment may be important to induce a state of tolerance through exhaustion of the immune response. In contrast, much lower TNFi concentrations might be sufficient during later treatment phases, once patients reach sustained remission. Half a year after start of TNFi treatment the dosing interval can indeed safely be prolonged.[63,64] Since up to 70% of adalimumab-treated RA patients in remission has a serum concentration >5 µg/mL and even 40% a concentration

>8 µg/mL, it would have an enormous impact to prolong the dosing interval regarding potential adverse events and drug costs.

In particular for certolizumab the high dosing regimen is remarkable, as we demonstrated efficient TNF neutralization by certolizumab in **Chapter 6**. The *in vitro* neutralizing capacity of certolizumab directly correlated with the certolizumab serum concentration, and was not affected by ADAs. Similar results were obtained for adalimumab, although the TNF neutralizing potency was higher for certolizumab compared to adalimumab. This is in striking contrast with the much higher dosing regimen of certolizumab compared to adalimumab (loading dose of 400 mg certolizumab at week 0, 2 and 4, followed by 200 mg once every two weeks, compared to 40 mg adalimumab once every other week).

**What is the pathogenic role of TNF in autoimmune diseases?**

To our knowledge, we are the first to show detailed dynamics of longitudinal TNF during TNFi treatment with all five TNFi. We provide new insights that the dynamics in TNF in the early phase of treatment are of significant importance to predict the immunogenic response of a TNFi. Once steady-state TNF concentrations have been established, TNF concentrations remain extremely stable during long-term follow-up, irrespective of disease activity. Surprisingly, we also demonstrated that TNF similarly increases in healthy volunteers. Altogether, these results appear to be in sharp contrast with the idea that that too much TNF contributes to inflammation in chronic autoimmune diseases. This paradigm shift raises questions about the role of TNF in pathophysiology and the mechanism of action of current TNFi treatments. Why are TNFi so efficient while the amount of TNF does not seem to be different in health and disease?

We cannot exclude the possibility that more TNF is produced at the local site of inflammation, but based on our recent data this does not seem very likely. It would imply immediate suppression of excessive TNF production, directly after TNFi administration. Otherwise, we would have observed higher amounts of TNF in patients compared to healthy individuals. Though we should be cautious, we think that measuring peripheral drug and target concentrations can be used as surrogate for levels at the site of inflammation. The synovium is in direct contact with the circulation, in particular during inflammation. Recent preliminary data from our group indeed confirmed that TNF, adalimumab and ADA concentrations in serum were not different from concentrations measured in synovial fluid.

Another important aspect to take into account is that we quantified soluble TNF and did not investigate TNF in its transmembrane conformation. Initially, TNF is

produced as a transmembrane protein, which is cleaved by metalloproteinases. Many researchers therefore wonder whether transmembrane TNF is an important factor in the pathophysiology of inflammatory autoimmune diseases. Previous studies show various effects of TNFi binding to transmembrane TNF.[72–74] However, these studies used target cells overexpressing transmembrane TNF, which does not reflect the *in vivo* situation. Immunohistochemistry of rheumatoid synovium indicated that only very low percentages of cells express transmembrane TNF.[75] In **Chapter 5** we also demonstrated that there is not much TNF expressed on cellular membranes. We therefore assume that transmembrane TNF is not the main driver of disease.

Finally, it might be argued that it is not the amount of circulating TNF perse that defines a patient, but that it is the local environment, together with TNF, that defines disease severity in a patient. Figure 1B shows a schematic overview of different factors in the local environment of which we propose that they might potentially be important in driving chronic inflammation in autoimmune diseases. TNF is a pleiotropic cytokine that signals via TNF receptor type 1 (TNFRI) and TNF receptor type 2 (TNFRII), eliciting a wide range of cellular responses. One of these responses is the activation of vascular endothelium, leading to increased expression of adhesion molecules. TNF-mediated induction of adhesion molecules is dependent on TNFRI.[76] Higher serum concentrations of the adhesion molecules ICAM, VCAM and E-selectin have indeed been reported in RA patients.[77,78] This results in subsequent recruitment and activation of immune cells to the site of inflammation. The number and type of inflammatory cells recruited to the site of inflammation might be important (illustrated as option 1 in Figure 1B). Furthermore, one could imagine that cells of autoimmune disease patients have different responsiveness to TNF (illustrated as option 2 in Figure 1B). Ultimately, a combination of excessive infiltration of activated inflammatory cells, which have different responsiveness to TNF might be important in driving inflammation in autoimmune disease.

Also, circulating TNFR concentrations were increased in RA patients, which associated with disease activity.[16,81–83] It is supposed that circulating soluble TNFR can function as natural TNFi. The association between soluble TNFR concentration and disease activity might therefore seem controversial. However, the interaction between soluble TNFR and TNF is weak, so despite high concentrations of soluble TNFR, levels are insufficient to neutralize TNF activity. Furthermore, increased concentrations of soluble TNFR imply increased shedding, and thus ultimately enhanced transmembrane surface expression of TNFRs. Enhanced mRNA and surface expression of TNFRs on synovial membrane from RA patients was indeed observed.[84,85] Enhanced sensitivity of cells to TNF by overexpression of TNFR has been illustrated as option 3 in Figure 1B.

Treatment with TNFi can induce various changes at abovementioned cellular levels. First of all, TNFi induce a reduction in adhesion molecule expression.[86–88] Consequently, inflammatory cell migration (including granulocytes, B-cells, T-cells and macrophages) diminishes.[86,89] Moreover, circulating amounts of TNFRI and II in RA patients decrease upon TNFi treatment.[16,24] When administration of recombinant TNF was followed by TNFi, a significant reduction in TNFR was observed in chimpanzees and healthy volunteers.[79] However, the reduction in TNFR concentration was not complete, and remained higher in RA patients compared to controls.[24,79]

Altogether, this suggests that the responsiveness of inflammatory cells to TNF at the local site of inflammation is crucial. Low amounts of locally produced TNF can mediate strong disastrous pro-inflammatory effects. It has previously been indicated that the percentage of cells expressing TNF was greatly outnumbered by the percentage of cells expressing TNFR, but the cells co-localized.[75] Overall, this might suggest that TNFRs, rather than TNF, might primarily reflect inflammation status.

**Consequences of the data in this thesis for next generation TNFi**

TNFi have significantly improved the treatment of chronic autoimmune diseases. However, despite its success almost one third of patients does not respond to TNFi treatment, and only few achieve complete remission. There is still no cure. Moreover, immunogenicity of TNFi remains an important issue. Hence, next generation TNFi are in development,[90–92] aiming to improve TNF blockade.

As hypothesized above, not the amount of TNF, but the responsiveness to TNF might have an important pathogenic role. The distinct biologic effects of TNF are mediated via TNFRI and TNFRII. It is proposed that signaling via TNFRI primarily promotes inflammatory responses, whereas TNFRII is important for immune regulation and maintaining tissue homeostasis.[93] An attractive alternative strategy to improve TNFi treatment could therefore be to shift from global TNF inhibition, to selective blocking of TNFRI.[90,94] Blocking of TNFRI allows selective targeting of the pathogenic effects of TNF, while maintaining the advantageous immunological responses. This might improve the therapeutic efficacy by minimizing adverse events, including reactivation of tuberculosis or increased risk of malignancies. Selective blocking of TNFRI is effective in collagen type II-induced arthritis (CIA) mice.[95] Next-generation selective TNFRI inhibitors are currently in development.[91,92,96] Ultimately, selective blocking of TNFRI can be combined with the selective stimulation of TNFRII.[97,98]

**Concluding remarks and outlook**

This thesis described research into TNF during TNFi treatment in context of rheumatic diseases. The development of drug-tolerant assays allowed the quantification of TNF in the presence of large amounts of TNFi. Interestingly, more than 20 years after the introduction of the first TNFi, our results show a paradigm shift in the field of TNFi treatment. Most importantly, we demonstrated that the increase in TNF concentrations was similar in healthy volunteers after one dose of adalimumab, compared to adalimumab-treated RA patients. Moreover, after an initial increase from baseline, TNF concentrations remained remarkably stable for two years of follow-up in RA patients. This was irrespective of disease activity. Overall, our results indicate that the amount of TNF is not a reflection of (suppressed) inflammation or disease activity.

We provide mechanistic insights in factors influencing steady-state TNF concentrations. The most important factor is Fc-mediated clearance of TNF-TNFi complexes, in combination with TNFi (or TNF-TNFi complex) half-life. Furthermore, we showed that immunogenicity impacts on the dynamics in TNF, especially in the early phase of treatment. Patients who became ADA positive during 52 weeks, had significantly lower TNF concentrations at week four. Previous studies already demonstrated the strong association between ADAs and non-response. Therefore, the results suggest that TNF measurements can potentially be used as predictive biomarker for ADA formation that have an impact on efficacy.

TNF was quantified from week four onwards, but in model Figure 2 we propose that TNF concentrations would initially have been higher. Unfortunately, at present there is a lack of samples in this window. Based on our recent data the following steps are important in order to elucidate the role of TNF in autoimmune diseases in more details. We currently work on the quantification of TNF in the first week after adalimumab administration in psoriasis patients, to provide more insight in the dynamics of TNF in the early phase of treatment. Furthermore, it is of interest to investigate the cellular compartment (in patients with and without concomitant methotrexate treatment) over time, as this might provide more details how the immunogenic response evolves in time. The current data namely implies that the association with, and thus the type of ADAs, change in time. Finally, in light of overexposure, our data suggests that TNFi concentrations around as low as 0.1 µg/mL in circulation are sufficient for near-quantitative *in vivo* capture of TNF. This raises questions about the amount of TNFi that is critical for maintaining clinical response. It would be of interest to further investigate what critical amount of TNFi is required for clinical effectiveness.

Together, the results question the role of TNF in pathophysiology and the mechanism of

action of current TNFi treatments. As we demonstrated that it is very unlikely that the amount of TNF is the most important factor driving inflammation, we propose a model were the responsiveness to TNF might be of significant importance.

**Key points**

- TNF concentrations remain stable during long-term TNFi treatment
- TNF concentrations do not decrease in patients in remission
- TNF concentrations – similarly as in patients – increase in healthy volunteers after one dose of adalimumab
- TNF concentrations are not a reflection of inflammation or disease activity, which is in contrast with the idea that too high TNF concentrations contribute to inflammation in chronic autoimmune diseases
- Early low TNF concentrations strongly associate with anti-drug antibody formation: early low TNF can be used as timely predictor of nonresponse to TNFi treatment
- TNFi concentrations of ~1 µg/mL might be sufficient for clinical efficacy

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