Dynamics of TNF during TNF inhibitor treatment

Berkhout, L.C.

Publication date
2021

The effect of certolizumab drug concentration and anti-drug antibodies on TNF neutralization

Clinical and Experimental Rheumatology 2020

L.C. Berkhout
E.H. Vogelzang
M.H. Hart
F.C. Loeff
L. Dijk
N.I.L. Derksen
R. Wieringa
W.A. van Leeuwen
C.L.M. Krieckaert
A. de Vries
M.T. Nurmohamed
G.J. Wolbink
T. Rispens

6

Abstract

Objective Tumor necrosis factor (TNF) inhibitors like certolizumab, elicit an immunogenic response leading to the formation of anti-drug antibodies (ADAs). We sought to mechanistically investigate the relationship between certolizumab concentrations, ADAs, and the effective TNF neutralizing capacity in sera of rheumatoid arthritis (RA) patients. TNF neutralizing capacity of certolizumab was compared to the neutralizing capacity of adalimumab.

Methods Serum samples were collected from 40 consecutive certolizumab-treated RA patients at baseline and 4, 16, 28 and 52 weeks after treatment initiation [Dutch Trial Register NTR (Nederlands Trial Register) Trial NL2824 no. 2965]. Certolizumab concentration and ADA titre were measured with a certolizumab bridging enzyme-linked immunosorbent assay (ELISA) and a drug-tolerant radioimmunoassay (RIA), respectively. TNF neutralization by certolizumab and adalimumab, in presence or absence of ADAs, was analyzed with the TNF-sensitive WEHI bioassay.

Results Despite a high incidence of ADAs during one year of follow-up (65%; 26/40 patients), certolizumab levels of >10 μg/mL were measured in most patients. The capacity for TNF neutralization highly correlated with certolizumab serum concentration, whereas no association with ADAs was observed. Similar results were obtained for adalimumab. The relative *in vitro* neutralizing potency was higher for certolizumab compared to adalimumab.

Conclusion Anti-certolizumab antibodies were detected in a large proportion of patients, but in most cases where ADAs were detected, certolizumab was also present in high concentrations, directly correlating with *in vitro* neutralizing capacity. These results indicate that measurement of certolizumab drug levels, rather than ADAs, have direct clinical significance.

Introduction

Biological disease-modifying anti-rheumatic drugs (bDMARDs) have been developed to inhibit the activity of inflammatory cytokines such as tumor necrosis factor (TNF). These TNF inhibitors have proven to be a successful treatment option for patients with rheumatoid arthritis (RA) and other inflammatory disorders.[1,2] Therapeutic antibodies such as certolizumab have been shown to elicit an immunogenic response leading to the formation of anti-drug antibodies (ADAs). However, the reported incidence, and levels of anti-certolizumab antibodies varies highly between different studies (~5-37% of the patients).[2–4] In a recent study by Jani *et al.* detection of ADAs in certolizumab-treated RA patients was not associated with the 12 months European League Against Rheumatism (EULAR) response.[4] In contrast, in certolizumab-treated Crohn's disease patients persistent ADAs were correlated with reduced efficacy, while transient ADAs were not.[5] These mixed results are seemingly in contrast with many studies demonstrating clear correlations between ADA formation to adalimumab or infliximab and a lower likelihood of minimal disease activity or clinical remission.[6–10]

The detection of ADAs in patients varies widely between studies, depending on duration of follow-up, concomitant medication, the type of TNF inhibitor and the type of assay that is used to detect ADAs; a golden standard for the quantification of ADAs is missing.[11] In particular, drug concentration may profoundly affect the detection of ADAs, depending on how drug-tolerant the ADA assay is. Importantly, ADAs and drug levels will affect each other mutually.

ADAs are generally expected to only affect treatment efficacy by lowering exposure to free active drug, via neutralization and/or enhanced clearance. Hence, ADAs might only influence clinical response when they affect pharmacokinetics (PK) to a noticeable degree.[12] When enough free active drug is left to bind to its target, despite the presence of ADAs, ADAs are unlikely to impair clinical response. Previously, we have shown that the antibody response to a range of therapeutic antibodies, including certolizumab and adalimumab, is highly restricted to the antigen binding site, thereby predominantly neutralizing.[13,14] In a number of studies, increasing serum concentrations of TNF inhibitors, including certolizumab, were associated with better clinical outcome.[4,7,15–18] Furthermore, the amount of TNF inhibition will depend on the strength of binding between TNF inhibitor and ADAs on the one hand and TNF inhibitor and TNF on the other hand. In other words, the balance between TNF inhibitor concentration, ADA titre and TNF concentration plays a role in determining the TNF neutralizing efficacy of the TNF inhibitor.

In the present study we describe the incidence of anti-certolizumab antibodies, as well as the relationship between serum certolizumab concentrations and the TNF neutralizing capacity in presence and absence of ADAs. We compared the certolizumab neutralizing capacity with the neutralizing capacity of adalimumab, since these drugs are structurally different and have a different binding strength for TNF.[19]

Materials and Methods

Details about the methodology can be found in the Supplemental Materials. Briefly, certolizumab concentration and anti-certolizumab antibody titre were measured with a rabbit anti-certolizumab bridging ELISA and a one-tiered or two-tiered certolizumab RIA, respectively, in 40 consecutive RA patients starting certolizumab treatment [Dutch Trial Register NTR (Nederlands Trial Register) Trial NL2824 no. 2965]. The study was approved by the medical ethics committee of the Slotervaart Hospital and Reade Medical Research Ethics Committee, Amsterdam, the Netherlands (CCMO NL35209.048.11). All patients gave written informed consent.

To determine the TNF neutralizing activity of certolizumab in patient sera, in presence or absence of ADAs, the TNF-sensitive WEHI bioassay was used. The TNF neutralizing capacity of certolizumab was compared with the neutralizing capacity of adalimumab. A selection of samples was made, to represent a range of serum certolizumab (4.8–60 µg/mL) or adalimumab concentrations (0.52–32.5 µg/mL and ADA titres (20–830 AU/mL anti-certolizumab antibodies and 30–113 AU/mL or 30–1380 AU/mL anti-adalimumab antibodies, detected with the drug-sensitive RIA or drug-tolerant ARIA, respectively). This selection was irrespective of any other (clinical) parameter.

Results

Development of a rabbit anti-certolizumab bridging ELISA

In order to measure certolizumab levels in certolizumab-treated RA patients, we designed a bridging ELISA using polyclonal rabbit anti-idiotype antibodies for both capture and detection (Figure 1A). This approach resulted in a highly sensitive assay with a quantifiable range between 0.1 and 120 µg/mL (Figure 1B). The same principle was previously demonstrated to be valid by using rabbit anti-natalizumab antibodies and natalizumab Fab.[20]

An advantage of this certolizumab bridging ELISA format is that it allows specific detection of certolizumab. To test whether this assay only detects functional certolizumab, we conducted an inhibition experiment with TNF. Almost complete inhibition of assay response was observed when excess TNF was titrated into a sample containing 5 ng/mL certolizumab (Figure 1C). In other words, if the TNF binding site of certolizumab is blocked with TNF it is no longer detected in this assay. Therefore, we anticipate that if the TNF binding site would be blocked by anti-idiotype antibodies formed in a patient, this fraction - that will no longer be active - will also not be measured. This assures that only functional, free certolizumab is measured in this assay.

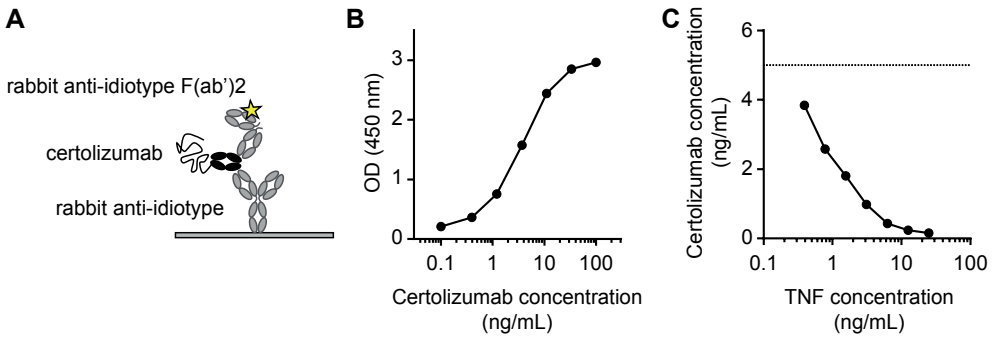


Figure 1. Development of bridging ELISA for certolizumab. A cross-linking assay in which for both capture and detection polyclonal rabbit anti-idiotype antibodies were used, resulted in a sensitive assay to quantify certolizumab serum concentrations. (**A**) Schematic overview of the certolizumab concentration assay. Certolizumab in serum is captured by polyclonal rabbit-anti certolizumab antibodies. Subsequently, biotinylated F(ab')2 fragments of the same polyclonal rabbit anti-certolizumab antibodies are added for detection of certolizumab. (**B**) Representative calibration curve of the certolizumab concentration assay. (**C**) Inhibition of certolizumab specific signal by increasing concentration of TNF. The concentration assay does not detect certolizumab bound to its target, assuring that only functional certolizumab is measured in this assay.

Patients

Over one year, 147 serum samples from 40 consecutive RA patients who started treatment with certolizumab were included. The median age was 56 years and 75% was female. The majority of patients was concomitantly treated with MTX (70%) and half of the patients was previously treated with a different biologic. Patients' baseline characteristics are shown in Table 1. 18 patients completed the study follow-up of one year. Twenty-two patients dropped out of the study: twelve due to treatment failure, three due to treatment failure and side effects, five due to adverse events (including recurrent respiratory infections, allergic reactions and psoriasis pustulosis) and two due to withdrawal of their informed consent. This high drop-out rate could probably be explained by the fact that approximately half of the patients were not TNF inhibitor naïve, and thus previously failed

treatment with other TNF inhibitors. We observed 6 cases with a drug hypersensitivity reaction. However, there was no significant difference between patients with or without detectable ADAs (respectively 2/26 [7.7%] and 4/14 [28.6%], P = 0.214).

Table 1. Demographics, previous and concomitant therapies, and disease status at baseline.

	Patients (n = 40)
Demographics	
Age, median (IQR) (years)	56 (47-63)
Female, no. (%)	30 (75)
BMI, median (IQR)	24.4 (22.0-27.8)
DMARD therapy	
Previous biologic, no. (%)	20 (50)
Previous DMARDs, median (IQR)	1 (0-2)
DMARD use, no. (%)	34 (85)
MTX use, no. (%)	28 (70)
MTX dose, median (IQR) (mg/week)	22.5 (15-25)
Prednisone use, no. (%)	11 (28)
Prednisone dose, median (IQR) (mg/day)	5 (5-10)
Disease Status	
Disease duration, median (IQR) (years)	8 (3-16)
ACPA positive, no. (%)	30 (75)
IgM-RF positive, no. (%)	23 (58)
Erosive, no. (%)	16 (40)
DAS28, median (IQR)	4.6 (3.2-5.3)
ESR, median (IQR) (mm/hour)	20 (9-37)
CRP, median (IQR) (mg/liter)	6 (2-17)
Tender joint count, median (IQR)	6 (4-9)
Swollen joint count (IQR)	3 (2-6)

IQR, inter quartile range; no., number; BMI, body mass index; DMARD, disease-modifying anti-rheumatic drug; MTX, methotrexate; ACPA, anti-citrullinated protein antibody; IgM-RF, immunoglobulin M rheumatoid factor; DAS28, disease activity score in 28-joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Longitudinal certolizumab and anti-certolizumab antibody concentrations

An initial loading dose resulted in certolizumab trough levels reaching maximum concentration at week four after treatment initiation. Substantial variation in certolizumab trough levels was observed between patients, with concentrations of 45.0 (3.00–136) µg/mL, 29.5 (4.00–67.0) µg/mL, 24.5 (0.1–78.0) µg/mL and 20.4 (0.6–59) µg/mL (median

(inter quartile range (IQR)) at week 4, 16, 28 and 52, respectively. Despite inter-patient variation in certolizumab concentration, longitudinal intra-patient variation was minimal; in 58% of the patients certolizumab concentrations remained stable in time within a factor three (Figure 2A).

Next, we investigated anti-certolizumab antibody formation with the drug-tolerant one-step RIA.[21] Although in two patients (5%) ADAs could already be detected after four weeks of treatment (green and orange line, Figure 2B), in most patients with detectable antibody formation, ADAs could be detected from week 16 onwards. Overall, the incidence of ADA positivity during one year of follow-up was 65% (26/40 patients). ADA titres of the ADA positive patients were 166 and 1120 AU/mL (n = 2) at week four and 44 (21-517) AU/mL, 112 (22-3120) AU/mL and 160 (22-830) AU/mL (median (IQR)), at week 16, 28 and 52, respectively. Of all patients who completed the one-year follow-up, two patients had an apparent transient ADA response.

The two patients with detectable ADAs at week four also had the lowest certolizumab concentrations at that point (green and orange line, Figure 2, A and B). Moreover, the patient with the highest antibody concentrations overall had undetectable certolizumab at week 28 and discontinued treatment at that time, due to skin abnormalities (blue line, Figure 2, A and B). Another patient had undetectable certolizumab at week 28, but ADAs could only be detected at week 52. In the majority of patients, levels of >10 µg/mL certolizumab were detected despite the simultaneous detection of ADAs (Spearman's $\rho = -0.688$, $P < 0.0001$; Figure 2C). Therefore, we hypothesized that in the majority of ADA-positive patients, certolizumab can still exert its TNF neutralizing function.

TNF neutralization by certolizumab correlates with drug concentration

To further examine the relationship between anti-TNF, ADAs, and neutralization capacity, we tested TNF neutralizing activity of patient sera with a TNF-sensitive WEHI bioassay. This assay quantifies killing of WEHI cells by biologically active TNF (Figure 3A). Killing of WEHI cells can be rescued by neutralization of TNF by certolizumab or adalimumab in serum. However, the presence of ADAs can abolish this protective effect. A selection of patient samples was made (see Materials and Methods), to represent a range of serum certolizumab concentrations and ADA titres, irrespective of any other (clinical) parameter. These samples were serially diluted and 100 pg/mL TNF was added to determine the neutralizing capacity. Subsequently, these samples were added to the WEHI cells and after 24 hours TNF neutralization was determined with the MTT-reduction method. The EC50 of all samples was determined (as described in Materials and Methods), with higher EC50 values representing larger TNF neutralizing capacity.

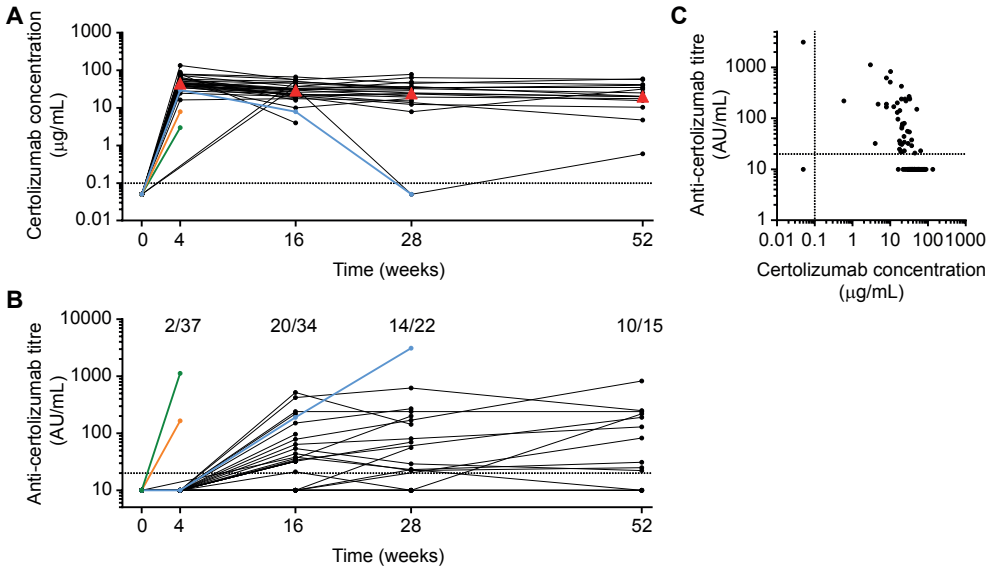


Figure 2. Longitudinal certolizumab and anti-certolizumab antibody concentration. (**A**) Longitudinal certolizumab trough serum concentrations (µg/mL) were measured in 40 consecutive certolizumab-treated RA patients during one year follow-up. Red triangles indicate median certolizumab concentration of all patients at the individual time points. (**B**) Longitudinal anti-certolizumab antibody titres (AU/mL) measured with a drug-tolerant one-step RIA. Numbers above the graph indicate anti-certolizumab antibody positive samples / total sample number. (A-B) Solid lines connect measurements at different time points within a patient. Certolizumab concentrations and anti-certolizumab antibodies in orange, green and blue are from the same individual, respectively. (**C**) Correlation between certolizumab concentration and anti-certolizumab antibody titre (Spearman's $\rho = -0.688$, $P < 0.0001$ ($n = 108$)). Each dot represents the combined certolizumab trough level and ADA titre from a single individual at one time point. Baseline samples were excluded. (A-C) Dashed lines indicate LLOQ of 0.1 µg/mL certolizumab or LOD of 20 AU/mL anti-certolizumab antibody.

Healthy donor serum and baseline patient samples were tested as negative controls and were indeed not able to neutralize TNF (EC50 <40; serum #1 in Figure 3B). TNF could be dose-dependently neutralized by certolizumab, as shown by a representative certolizumab serum sample titration in Figure 3B (serum #2). Next, we analyzed TNF neutralizing activity in the abovementioned selected serum samples, with different certolizumab concentrations and ADA titres. The TNF neutralizing activity is expressed by an EC50 value. TNF neutralization was highly correlated with certolizumab serum concentration (Pearson $r = 0.909$, $P < 0.0001$; Figure 3C). By contrast, no correlation was observed between anti-certolizumab antibodies and TNF neutralization (Pearson $r = -0.471$, $P = 0.122$; Figure 3D). Despite the presence of ADAs, certolizumab could still exert its TNF neutralizing activity.

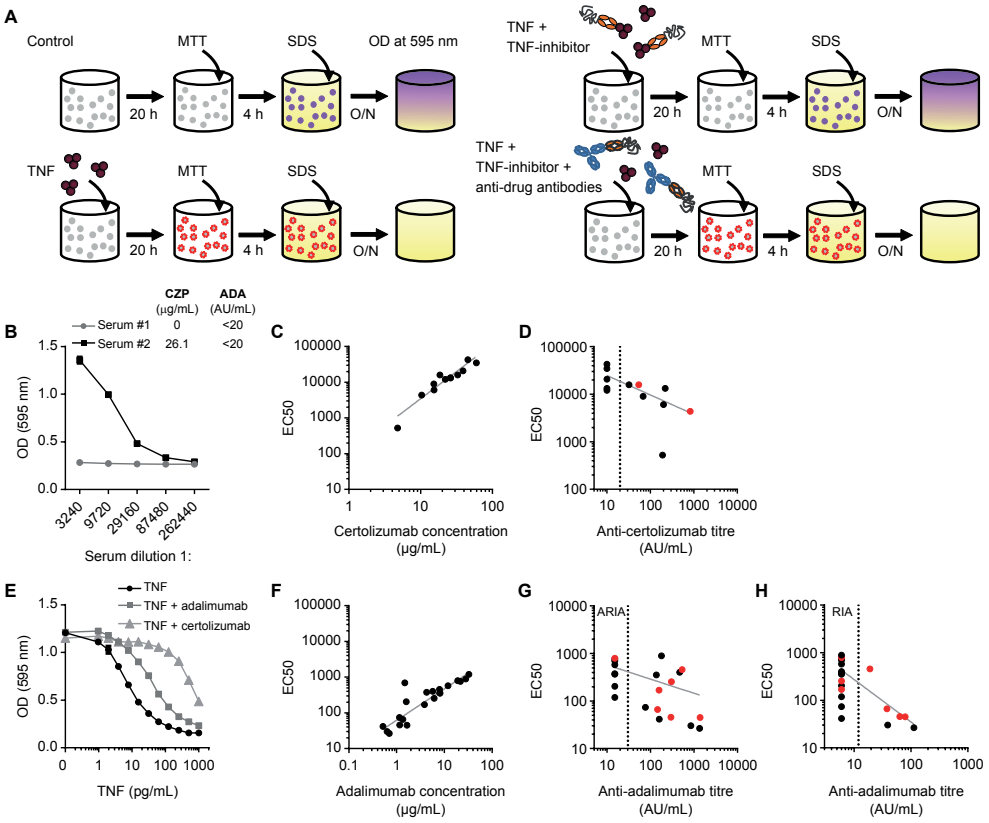


Figure 3. TNF neutralization by certolizumab and adalimumab serum samples, in presence or absence of ADAs. (**A**) Schematic representation of the TNF-sensitive WEHI bioassay, used to analyze TNF neutralizing activity. The intensity of the purple color, measured at 595 nm in a microtitre plate reader, is used as a readout for cell viability. TNF neutralization by certolizumab or adalimumab in serum samples rescues WEHI cells (right upper panel), whereas presence of ADAs abolishes this protective effect (right lower panel). (**B**) Serum samples were serially diluted and incubated with 100 pg/mL TNF to determine TNF neutralization. Decreasing drug concentration resulted in reduced TNF neutralization, as represented by decreasing OD. A representative certolizumab serum sample is shown (Serum #2), and a baseline certolizumab sample was included as negative control (Serum #1). Data expressed as mean ± SD of duplicate measurements. (**C-D**) Correlation between TNF neutralization, as expressed by EC50 values, and (C) certolizumab concentration (Pearson r = 0.909, P < 0.0001 (n = 12)) or (D) anti-certolizumab antibody titre (Pearson r = -0.471, P = 0.122 (n = 12)). (**E**) Bioactivity of a titration of TNF incubated in presence or absence of 5 ng/mL certolizumab or adalimumab. Data expressed as mean ± SD of duplicate measurements. Graph depicts representative data of three independent experiments. (**F-H**) Correlation between TNF neutralization, as expressed by EC50 values, and (F) adalimumab concentration (Pearson r = 0.900, P < 0.0001 (n = 21) or (G) anti-adalimumab antibody titre, measured with the drug-tolerant ARIA (Pearson r = -0.434, P = 0.049 (n = 21)) (H) or measured with the drug-sensitive RIA (Pearson r = -0.500, P = 0.021 (n = 21)). (D, G-H) Red dots represent samples derived from patients that did not use methotrexate, black dots represent samples derived from patients concomitantly treated with methotrexate. Stratification by methotrexate did not change the results. (C-D, F-H) Grey lines indicate log-log linear fit, weight by $1/Y^2$.

Since certolizumab is a Fab domain and thus structurally different from IgG1 antibodies, we compared TNF neutralization by certolizumab to the TNF neutralizing activity of adalimumab. To investigate whether there is any difference in TNF neutralization by certolizumab and adalimumab itself, we first analyzed TNF bioactivity in the presence or absence of 5 ng/mL certolizumab or adalimumab (Figure 3E). Although both drugs neutralized TNF, the relative neutralizing potency of certolizumab was higher compared to adalimumab. Next, we analyzed TNF neutralization by serum samples from adalimumab-treated RA patients.[6] Like certolizumab, adalimumab serum levels were highly correlated with TNF neutralizing activity (Pearson r = 0.900, P < 0.0001; Figure 3F). A weak correlation was observed between the anti-adalimumab antibody titre, measured with a drug-tolerant ARIA, and TNF neutralization (Pearson r = -0.434, P = 0.049; Figure 3G). Anti-adalimumab antibodies measured with a drug-sensitive RIA assay showed a slightly stronger correlation with TNF neutralization (Pearson r = -0.500, P = 0.021; Figure 3H). However, this correlation was still weak compared to the correlation between adalimumab serum levels and TNF neutralization. Together, we showed that TNF neutralization by certolizumab relates directly to drug concentration, but not anti-certolizumab antibodies. The same applied for adalimumab.

Discussion

The present study describes the relationship between certolizumab concentrations and TNF neutralizing capacity, in presence and absence of anti-certolizumab antibodies, using sera derived from certolizumab-treated RA patients. We showed that certolizumab trough concentrations were highest four weeks after initiation of treatment, and in 58% of the patients certolizumab concentrations remained stable in time within a factor three. The minor drop in certolizumab concentration after week four was not associated with the formation of anti-certolizumab antibodies as shown in this study, but could rather be explained by the initial loading dose of certolizumab.

We demonstrated an inverse correlation between anti-certolizumab antibodies and drug concentrations (Figure 2C). Only in a minority of patients (35%) no anti-certolizumab antibodies could be detected during one year of follow-up. However, in most samples in which anti-certolizumab antibodies were detected, certolizumab serum concentrations remained well above 10 µg/mL, which is within the therapeutic range according to the certolizumab concentration-effect curve,[17] irrespective of the presence of detectable ADAs.

We demonstrated that both certolizumab and adalimumab neutralized TNF, although the relative *in vitro* neutralizing potency was higher for certolizumab compared to the neutralizing potency of adalimumab. Certolizumab binds TNF with higher affinity compared to adalimumab, and can therefore more efficiently bind and neutralize TNF.[19] Of note, certolizumab is a monovalent Fab fragment, whereas adalimumab is a bivalent antibody. However, when the concentrations would have been corrected for equal amounts of binding sites, the difference in neutralizing efficacy would have been even more pronounced.

For both certolizumab and adalimumab, there was a strong correlation between drug levels and TNF neutralizing capacity as measured in the WEHI bioassay, although we observed higher EC50 values for certolizumab than for adalimumab. However, the direct comparison of EC50 values should be done with caution, since certolizumab serum concentrations are much higher compared to adalimumab concentrations. The high dosing regimen of certolizumab is remarkable, as we demonstrated efficient TNF neutralization by certolizumab. Golimumab for example, binds TNF with higher affinity compared to adalimumab,[19] and has a lower dosing regimen compared to adalimumab. In striking contrast, certolizumab binds TNF with even higher affinity compared to golimumab, while the dosing regimen of certolizumab is the highest. Nonetheless, dose-finding studies demonstrated a dose-dependent improvement in clinical response with increasing certolizumab doses.[22–24] Recently, it was shown that higher certolizumab plasma levels were associated with larger DAS28 improvement from baseline,[4,18] although a plateau effect in ΔDAS with 20 µg/mL certolizumab has been described.[17] Since certolizumab efficiently neutralizes TNF, it is noteworthy that high certolizumab doses are required for clinical response. There appears to be a discrepancy between *in vitro* efficacy of TNF neutralization and *in vivo* clinically effective certolizumab concentrations. One might wonder whether local certolizumab concentrations, at the site of inflammation, are lower compared to systemic concentrations. However, as certolizumab effectively entered the inflamed tissue,[25,26] a lack of distribution to the local site of inflammation does not explain this controversy. Furthermore, differences in FcRn-mediated recycling and Fc-mediated clearance between certolizumab, adalimumab and golimumab might contribute to the difference in dosing regimen, given that certolizumab is a Fab domain while adalimumab and golimumab are IgG1 antibodies. These structural differences might also result in different Fc-mediated effector functions, including apoptosis and complement activation.[27–29] Finally, it is possible that high certolizumab dosing contributes to the induction of tolerance, thereby suppressing immunogenicity. This would suggest that after the initial certolizumab loading dose, lower doses of certolizumab might be sufficient to maintain clinical effectiveness.

However, the main effect of antibody formation is expected to be lowering the effective drug exposure. In a concentration-effect curve the impact of immunogenicity on effective drug levels c.q. drug exposure is taken into account, and this analysis still suggests added benefit of certolizumab concentrations up to almost 20 µg/mL for RA.[17]

In this study, antibodies were detected in 65% of the patients using a previously developed ADA assay.[21] This is much higher compared to three previously published certolizumab trials.[2–4] Two of these studies did not report which assay had been used, making it difficult to compare results from one study to another. The high percentage of antibody positive patients in our cohort, could potentially be explained by the drug-tolerant assay we used.[21]

Jani *et al*. used the same drug-tolerant one-step RIA for the detection of anti-certolizumab antibodies, as used in this study.[4] The difference in the percentage (37% in the study by Jani *et al*. vs. 65% in our study) of patients in whom ADAs are detected might, partly, be due to differences in sampling strategy. In this study samples were taken at trough, as opposed to random sampling in Jani *et al*.. Even with a drug-tolerant assay, drug levels will still influence detection of ADAs to certain degree. Furthermore, in the study by Jani *et al*. relatively more bDMARD naïve patients started certolizumab compared to our study (92% in the study by Jani *et al*. vs. 50% in our study). It might be that in our study some patients failed their previous bDMARD, due to immunogenicity. Therefore, these patients might be prone to develop ADAs against other bDMARDs,[11,30] further explaining the difference in the percentage of ADA positive patients.

The small sample size is a limitation of the present study. Consequently, a direct analysis of the relationships between certolizumab concentrations, anti-certolizumab antibodies and clinical response i.e. EULAR response or drug adherence, could not be performed. Instead, we used TNF neutralizing capacity as a functional outcome measurement. For a direct assessment of an association between certolizumab concentrations and clinical response, larger studies should be performed.

Of note, anti-certolizumab antibody titres were also determined using a two-tiered screening and confirmation approach, conform the FDA guidelines,[31] but this appeared to have little consequences for the overall number of samples identified as positive (Supplemental Figure S1).

In conclusion, we demonstrated that although a large proportion of patients has detectable anti-certolizumab antibodies high concentrations of certolizumab were found in most patients. We showed that drug concentrations, but not the presence of anti-certolizumab

antibodies, was highly correlated with the capacity to neutralize TNF. So, serum drug concentrations reflect clinical effectiveness, which indicates that drug measurements can be used for the identification of under treatment. Overall, we advocate not to measure ADAs in a clinical setting, unless certolizumab concentrations are very low.

Acknowledgements
The authors would like to thank Karin van Schie for assistance with the WEHI assay.

Funding
This work was supported by ZonMw, the Netherlands Organisation for Health Research and Development, in the program 2Treat (Grant 436001001). The certolizumab and adalimumab cohort studies were partially supported by UCB (IIS-2014-101540) and AbbVie (ACA-NETH-05-012), respectively. UCB and AbbVie had no involvement in the study design; in the collection, analysis, and interpretation of data.

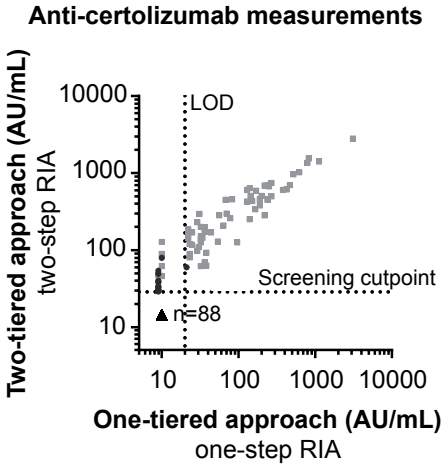
Competing interests
M.T. Nurmohamed has received consultancy fees from Abbott, Roche, Pfizer, MSD, UCB, SOBI and BMS, and payment for lectures from Abbott, Roche and Pfizer. G.J. Wolbink has received a research grant from Pfizer (paid to the institution) and honoraria for lectures from UCB, Pfizer, AbbVie, Biogen and BMS. T. Rispens has received honoraria for lectures from Pfizer, AbbVie and Regeneron, and a research grant from Genmab. The other co-authors have declared no competing interests.

References

1. Keystone EC, Kavanaugh AF, Sharp JT, et al. Radiographic, clinical, and functional outcomes of treatment with adalimumab (a human anti-tumor necrosis factor monoclonal antibody) in patients with active rheumatoid arthritis receiving concomitant methotrexate therapy: a randomized, placebo-controlled, 52-week trial. Arthritis Rheum. 2004;50(5):1400-1411.
2. Smolen J, Landewé R, Mease P, et al. Efficacy and safety of certolizumab pegol plus methotrexate in active rheumatoid arthritis: the RAPID 2 study. A randomised controlled trial. Ann Rheum Dis. 2009;68(6):797-804.
3. Keystone E, Heijde V Der, Mason D, et al. Certolizumab pegol plus methotrexate is significantly more effective than placebo plus methotrexate in active rheumatoid arthritis: findings of a fifty-two-week, phase III, multicenter, randomized, double-blind, placebo-controlled, parallel-group study. Arthritis Rheum. 2008;58(11):3319-3329.
4. Jani M, Isaacs JD, Morgan AW, et al. High frequency of antidrug antibodies and association of random drug levels with efficacy in certolizumab pegol-treated patients with rheumatoid arthritis: results from the BRAGGSS cohort. Ann Rheum Dis. 2016;76(1):208-2013.
5. Sandborn WJ, Wolf DC, Kosutic G, et al. Effects of transient and persistent anti-drug antibodies to certolizumab pegol: Longitudinal data from a 7-year study in Crohn's disease. Inflamm Bowel Dis. 2017;23(7):1047-1056.
6. Bartelds GM, Krieckaert CL, Nurmohamed MT, et al. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. JAMA. 2011;305(14):1460-1468.
7. Chen D, Chen Y, Tsai W, et al. Significant associations of antidrug antibody levels with serum drug trough levels and therapeutic response of adalimumab and etanercept treatment in rheumatoid arthritis. Ann Rheum Dis. 2015;74(3).
8. Wolbink GJ, Vis M, Lems W, et al. Development of antiinfliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis. Arthritis Rheum. 2006;54(3):711-715.
9. Pascual-Salcedo D, Plasencia C, Ramiro S, et al. Influence of immunogenicity on the efficacy of long-term treatment with infliximab in rheumatoid arthritis. Rheumatology. 2011;50:1445-1452.
10. Radstake TRDJ, Svenson M, Eijsbouts AM, et al. Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. Ann Rheum Dis. 2009;68(11):1739-1745.
11. van Schouwenburg PA, Rispens T, Wolbink GJ. Immunogenicity of anti-TNF biologic therapies for rheumatoid arthritis. Nat Rev Rheumatol. 2013;9(3):164-172.
12. Bloem K, Hernández-breijo B, Martínez-feito A, Rispens T. Immunogenicity of therapeutic antibodies: Monitoring antidrug antibodies in a clinical context. Ther Drug Monit. 2017;39(4):327-332.
13. van Schie KA, Hart MH, de Groot ER, et al. The antibody response against human and chimeric anti-TNF therapeutic antibodies primarily targets the TNF binding region. Ann Rheum Dis. 2015;74(1):311-314.
14. van Schie K, Kruithof S, van Schouwenburg P, et al. Neutralizing capacity of monoclonal and polyclonal anti-natalizumab antibodies: The immune response to antibody therapeutics preferentially targets the antigen-binding site. J Allergy Clin Immunol. 2016;3(2):125-126.
15. Kneepkens EL, Krieckaert CLM, Kleij D Van Der, Nurmohamed MT, Rispens T, Wolbink GJ. Lower etanercept levels are associated with high disease activity in ankylosing spondylitis patients at 24 weeks of follow-up. Ann Rheum Dis. 2015;74:1825-1829.
16. Pouw MF, Krieckaert CL, Nurmohamed MT, et al. Key findings towards optimising adalimumab treatment: The concentration-effect curve. Ann Rheum Dis. 2015;74(3):513-518.
17. Wolbink G, Goupille P, Sandborn W, et al. Association between plasma certolizumab pegol concentration and improvement in disease activity in rheumatoid arthritis and Crohn's disease [abstract]. Arthritis Rheumatol. 2016;68.
18. Gehin JE, Syversen SW, Warren DJ, et al. Certolizumab pegol serum levels ≥20 mg/l are associated with improvement in das28 in rheumatoid arthritis patients. Data from the nor-dmard study [abstract]. Ann Rheum Dis. 2018;77(Supplement):A953.
19. van Schie KA, Ooijevaar-de Heer P, Dijk L, Kruithof S, Wolbink G, Rispens T. Therapeutic TNF inhibitors can differentially stabilize trimeric TNF by inhibiting monomer exchange. Sci Rep. 2016;6(32747).
20. Rispens T, Leeuwen A van, Vennegoor A, et al. Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody. Anal Biochem. 2011;411(2):271-276.

21. Rispens T, Hart MH, Ooijevaar-de Heer P, et al. Drug interference in immunogenicity assays depends on valency. J Pharm Biomed Anal. 2013;85:179-185.
22. Keystone E, Choy E, Kalden J, et al. CDP870, a novel, PEGylated humanised TNF-inhibitor is effective in treating the signs and symptoms of rheumatoid arthritis. Arthritis Rheum. 2001;44(12):2946.
23. Choy EHS, Hazleman B, Smith M, et al. Efficacy of a novel PEGylated humanized anti-TNF fragment (CDP870) in patients with rheumatoid arthritis: a phase II double-blinded, randomized, dose-escalating trial. Rheumatology. 2002;41:1133-1137.
24. Goel N, Stephens S. Certolizumab pegol. MAbs. 2010;2(2):137-147.
25. Palframan R, Airey M, Moore A, Vugler A, Nesbitt A. Use of biofluorescence imaging to compare the distribution of certolizumab pegol, adalimumab, and infliximab in the inflamed paws of mice with collagen-induced arthritis. J Immunol Methods. 2009;348:36-41.
26. Carron P, Lambert B, Van Praet L, et al. Scintigraphic detection of TNF-driven inflammation by radiolabelled certolizumab pegol in patients with rheumatoid arthritis and spondyloarthritis. RMD Open. 2016;2(1).
27. Arora T, Padaki R, Liu L, et al. Differences in binding and effector functions between classes of TNF antagonists. Cytokine. 2009;45(2):124-131.
28. Albanesi M, Daëron M. The interactions of therapeutic antibodies with Fc receptors. Immunol Lett. 2012;143(1):20-27.
29. Armuzzi A, Lionetti P, Blandizzi C, et al. Anti-TNF agents as therapeutic choice in immune-mediated inflammatory diseases: focus on adalimumab. Int J Immunopathol Pharmacol. 2014;27(I):11-32.
30. Bartelds GM, Groot E De, Nurmohamed MT, et al. Surprising negative association between IgG1 allotype disparity and anti-adalimumab formation: a cohort study. Arthritis Res Ther. 2010;12(6):R221.
31. Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products Guidance for Industry.

Supplemental Materials



Supplemental Figure S1. Anti-certolizumab antibodies measured with a one-tiered and two-tiered screening and confirmation approach. Correlation between the one-tiered and two-tiered RIA approach, used for the measurement of anti-certolizumab antibodies (AU/mL). Black triangles represent samples that are negative confirm both the one- and two-tiered approach (n = 88). Black dots represent samples that are above the screening cutpoint, but which could not be confirmed (n = 15). As expected, the majority of these samples (n = 14) had ADA titres below the LOD in the on-tiered approach. Grey squares represent samples that are above the screening cutpoint and which could be confirmed (n = 69). Only a minority of these samples (n = 6) had ADA titres below the LOD in the one-tiered approach. Dashed lines indicate LOD of 20 AU/ mL and screening cutpoint of 28.5 AU/mL for the one-tiered and two-tiered approach, respectively.

Materials and Methods

Patients

40 consecutive RA patients starting certolizumab treatment were enrolled in a prospective observational cohort study between 2011 and 2016 [Dutch Trial Register NTR (Nederlands Trial Register) Trial NL2824 no. 2965]. Patients were treated with 400 mg certolizumab subcutaneously at week 0, 2 and 4 (loading dose), followed by 200 mg once every two weeks. Serum samples were drawn at baseline (week 0) and 4, 16, 28 and 52 weeks after initiation of certolizumab treatment.

To investigate the TNF neutralising capacity of adalimumab, a set of serum samples was selected from consecutive adalimumab-treated RA patients from a prospective observational cohort study, as previously described by Bartelds *et al*. (2011).[1] These patients were enrolled between 2004 and 2007 and treated with a standard-dose adalimumab of 40 mg subcutaneously every other week. Serum samples were drawn at baseline and 4, 16, 28, 40, 52, 78 and 104 weeks after initiation of adalimumab treatment.

Both studies were approved by the medical ethics committee of the Slotervaart Hospital and Reade Medical Research Ethics Committee, Amsterdam, the Netherlands (CCMO NL35209.048.11). All patients gave written informed consent. At baseline, all patients fulfilled the American College of Rheumatology 1987 revised criteria for RA[2] and had active disease indicated with the 28-joint Disease Activity Score (DAS28) >3.2.

Generation of polyclonal rabbit antibodies directed against the idiotype of certolizumab

Polyclonal antibodies to certolizumab were raised essentially as described before.[3] Briefly, two female New Zealand white rabbits were immunized and boosted with 100 µg/mL certolizumab pegol, using Montanide ISA-50 as an adjuvant. Serum was collected by apheresis and total IgG was purified by protein A affinity chromatography. Antibodies recognising constant regions in certolizumab were removed with an IVIg-Sepharose column. Rabbit anti-idiotype antibodies were digested with pepsin to prepare F(ab')2 fragments, which were subsequently biotinylated.

Measurement of certolizumab and adalimumab concentration

Rabbit anti-certolizumab bridging ELISA

Certolizumab is a pegylated Fab fragment of an antibody that binds human TNF. It consists of humanized variable domains derived from a mouse monoclonal antibody against TNF, an IgG1 CH1 domain, and a kappa CL domain. Since it is humanized, the number of unique

determinants in certolizumab will be limited. Furthermore, because it is a Fab fragment and not a regular bivalent IgG antibody, each determinant will be present only once. This puts severe limitations to the possibilities for selective and specific detection of certolizumab in a human serum matrix. Several assay formats were explored based on capture using TNF and detection using an anti-kappa antibody, but none of these was found to yield a satisfactory assay format due to substantial background signals, especially in sera of RA patients. Therefore, an alternative assay format was designed using polyclonal rabbit anti-certolizumab antibodies. The (polyclonal) rabbit antibodies will contain a collection of anti-idiotype antibodies directed against certolizumab that can bind to multiple unique determinants. A subset of these antibodies will be able to bind simultaneously to different epitopes of certolizumab. Therefore, it is possible to set up a cross-linking assay in which for both capture and detection the polyclonal rabbit anti-idiotype antibodies will be used (Figure 1A).

Rabbit anti-certolizumab antibodies were diluted to 1 µg/mL in phosphate buffered saline (PBS) and used for coating 96-well ELISA plates (Greiner bio-one, Frickenhausen, Germany) with 100 µL/well overnight. Plates were washed with PBS containing 0.02% Tween (PBS-T). Samples were diluted (50- to 3200-fold in four steps) in high-performance ELISA (HPE) buffer (Sanquin Reagents, Amsterdam, The Netherlands) containing 1% IMIg (GammaQuin, Sanquin Plasma Products, Amsterdam, The Netherlands), and 1% normal rabbit serum (HPE++) followed by incubation for 1 hour at 37°C. Plates were washed five times with PBS-T. Subsequently, 100 µL of 0.125 µg/mL biotinylated rabbit anti-certolizumab F(ab')2 fragments in HPE++ buffer was added to each well followed by incubation for 1 hour at 37°C. Plates were washed five times with PBS-T, and 100 µL of streptavidin-polymerized horse radish peroxidase (poly-HRP) (1:10,000 dilution in HPE buffer) was added to each well and incubated for 30 minutes at 37°C. The plates were washed five times with PBS-T, and 100 µL of tetramethylbenzidine (TMB) substrate (100 µg/mL) and 0.003% (v/v) hydrogen peroxide in 0.11 M sodium acetate buffer (pH 5.5) was added to each well. After 30 minutes the reaction was stopped by the addition of 2 M H_2SO_4. Absorbance was measured at 450 nm. Concentrations were calculated with a 7-point, serially 2-fold diluted calibration curve. All pipetting steps were automated and performed with a Tecan EVO platform. The Lower Limit of Quantification (LLOQ) for this assay was 0.1 µg/mL. Accuracy and precision of calibrator were between 98 and 104%, and <8%, respectively; accuracy of quality controls were between 84 and 121% in the range between 1 and 120 µg/mL and 134% at 0.1 µg/mL; precision was between 3 and 9%. No interference was observed for adalimumab or infliximab, nor methotrexate (MTX) or prednisolone.

Adalimumab ELISA

Adalimumab levels were measured by ELISA, as previously described.[4,5] Briefly, microtitre plates were coated with mouse monoclonal anti-TNF clone 7 and then incubated with recombinant TNF-α. Bound adalimumab from patient serum was detected with a biotinylated adalimumab specific anti-idiotype antibody. Adalimumab concentrations were calculated with an adalimumab titration curve. The LLOQ for this assay was 0.01 µg/mL.

Measurement of anti-drug antibody concentration

One-tiered certolizumab RIA

Anti-certolizumab antibodies were measured with a (one-step) RIA, that was previously described and shown to be drug-tolerant.[6] Briefly, serum samples were incubated overnight with protein A Sepharose beads, in the presence of a fixed amount of ^{125}I-labelled certolizumab. Unbound label was removed by washing five times and Sepharose-bound radioactivity was measured using a gamma counter (2480 WIZARD2, PerkinElmer, Waltham, MA). Antibody titres were calculated with a titration curve of a pool of rabbit-sera, containing anti-certolizumab antibodies and expressed in arbitrary units (AU/mL). Limit of detection (LOD) of this assay was 20 AU/mL.

Two-tiered certolizumab RIA

For comparison sake, anti-certolizumab antibodies were also measured with a two-tiered screening and confirmation approach, according to Food and Drug Administration (FDA) guidelines.[7] The screening step was performed analogously to the one-tiered RIA but with addition of a wash step between overnight sample incubation and overnight incubation with the ^{125}I-labelled certolizumab. This two-step approach further limited the effect of drug-interference in the assay, by washing away unbound material – including certolizumab not in complex with anti-certolizumab antibodies. For the confirmation step, samples were re-run in similar fashion in the absence (non-inhibited condition, NIC) and presence (inhibited condition, IC) of 3.0 µg unlabelled certolizumab/ test (3.0 mg/mL serum equivalent) during overnight ^{125}I-labelled certolizumab incubation. The percentage difference in signal between the inhibited and the non-inhibited condition were calculated by the formula: (NIC-IC)/IC*100%. Screening and confirmation cutpoints were calculated using the assay results from the 40 pre-treatment samples from the RA patients in this cohort and were determined at 28.5 AU/mL and 59% inhibition, respectively.

Adalimumab RIA

Anti-adalimumab antibodies were measured using the drug-sensitive RIA, as reported previously.[8] Antibodies present in serum were captured overnight on protein A Sepharose

beads, in presence of ^{125}I-labelled F(ab)2 adalimumab. Unbound label was removed by washing five times and Sepharose-bound radioactivity was measured. Adalimumab specific antibodies were detected with ^{125}I-labelled F(ab)2 adalimumab. Antibody levels were calculated with a reference serum and expressed in AU/mL. LOD was 12 AU/mL.

ARIA

Anti-adalimumab antibodies were also measured using the drug-tolerant acid-dissociation radioimmunoassay (ARIA).[9] Serum was acidified, so antibody-drug complexes would dissociate. Subsequently, the serum was neutralized and incubated with biotinylated adalimumab F(ab')2 fragments. Then, antibodies were captured by protein A Sepharose and anti-adalimumab specific antibodies were quantified with radiolabelled streptavidin. The LOD was 30 AU/mL.

WEHI bioassay

The TNF neutralizing activity of certolizumab and adalimumab in patient sera, in presence or absence of ADAs, was analyzed with the TNF-sensitive WEHI bioassay. Serum samples with low, intermediate and high serum drug concentrations and ADA titres were randomly selected (covering a range of 4.8–60 µg/mL certolizumab and 0.52–32.5 µg/mL adalimumab and a range of 20–830 AU/mL anti-certolizumab antibodies and 30–113 AU/mL or 30–1380 AU/mL anti-adalimumab antibodies, detected with the drug-sensitive RIA or drug-tolerant ARIA, respectively). This selection was irrespective of any other (clinical) parameter.

Nunc MicroWell Plates with Nunclon Delta Surface (Thermo Scientific) were plated with 40,000 WEHI-164 cells/well in 50 µL IMDM (Bio Whittaker) supplemented with 5% fetal calf serum (FCS) (Bodinco), 100 U/mL penicillin, 100 µg/mL streptomycin (both from Gibco), 1 µg/mL Actinomycin D and 50 µM β-Mercapto-ethanol (both from Sigma) (assay medium). Patient samples were 1:3 serially diluted in assay medium and incubated 1:1 with 400 pg/mL TNF for 30 minutes (concentration TNF is 200 pg/mL). Subsequently, these diluted patient sera were added 1:1 to the WEHI-164 cells, resulting in 100 pg/mL TNF as final concentration in the assay. Baseline patient sera and healthy donor sera were used as negative controls.

As a reference, a titration of TNF (twofold dilution, 0-40,000 pg/mL TNF, Active Bioscience) was incubated 1:1 in presence or absence of 20 ng/mL certolizumab or adalimumab (concentrations TNF is 0-20,000 pg/mL). After 30 minutes, TNF, TNF-certolizumab or TNF-adalimumab titrations were added 1:1 to the WEHI-164 cells, resulting in 0-10,000 pg/mL TNF and 5 ng/mL certolizumab and adalimumab as final concentrations in the

assay. WEHI-164 cells were incubated at 37°C and 5% CO_2. After 24 hours, cell viability was determined with the MTT-reduction method. MTT (Sigma, diluted in 0.14 M NaCl and 0.01 M HEPES) was added in a final concentration of 0.83 mg/mL. After 4 hours, 5% SDS (Gibco, diluted in 0.01 M HCL) was added and incubated overnight. Absorbance was measured at 595 nm and as reference 670 nm with a plate reader (Synergy 2, BioTek). All conditions were analyzed in duplicate. TNF neutralization was expressed as EC50 values. A nonlinear regression curve with dilution factors on logarithmic x-axis and OD values on the y-axis was used to determine the EC50 of all samples.

Statistical analysis

Correlations were analyzed with a Spearman or Pearson test using GraphPad Prism software version 7.04.

References

1. Bartelds GM, Krieckaert CL, Nurmohamed MT, et al. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. JAMA. 2011;305(14):1460-1468.
2. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31(3):315-324.
3. Rispens T, Leeuwen A van, Vennegoor A, et al. Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody. Anal Biochem. 2011;411(2):271-276.
4. van Schouwenburg PA, Bartelds GM, Hart MH, Aarden L, Jan G, Wouters D. A novel method for the detection of antibodies to adalimumab in the presence of drug reveals “hidden” immunogenicity in rheumatoid arthritis patients. J Immunol Methods. 2010;362(1-2):82-88.
5. Pouw MF, Krieckaert CL, Nurmohamed MT, et al. Key findings towards optimising adalimumab treatment: The concentration-effect curve. Ann Rheum Dis. 2015;74(3):513-518.
6. Rispens T, Hart MH, Ooijevaar-de Heer P, et al. Drug interference in immunogenicity assays depends on valency. J Pharm Biomed Anal. 2013;85:179-185.
7. Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products Guidance for Industry.
8. Bartelds GM, Wijbrandts CA, Nurmohamed MT, et al. Clinical response to adalimumab: Relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. Ann Rheum Dis. 2007;66(7):921-926.
9. Bloem K, van Leeuwen A, Verbeek G, et al. Systematic comparison of drug-tolerant assays for anti-drug antibodies in a cohort of adalimumab-treated rheumatoid arthritis patients. J Immunol Methods. 2015;418:29-38.