Supplementary
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 Bartels 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 immunised 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 humanised variable domains derived from a mouse monoclonal antibody against TNF, an IgG1 CH1 domain, and a kappa CL domain. Since it is humanised, 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 (Fig. 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-polymerised horseradish peroxidase (poly-HRP) (1:10000 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 (10 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 H2SO4. 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 calibration 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.
Anti-certolizumab measurements

Supplementary Fig. 1. 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 confirmed 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 28.5 AU/ml for the one-tiered and two-tiered approach, respectively.

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 I^125-labeled 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 I^125-labeled 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 I^125-labeled 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 I^125-labeled F(ab)2 adalimumab. Unbound label was removed by washing five times and Sepharose-bound radioactivity was measured. Adalimumab specific antibodies were detected with I^125-labeled 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 neutralised 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 neutralising activity of certolizumab and adalimumab in patient sera, in presence or absence of ADAs, was analysed 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 NuncIon 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 (two-fold 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 0-2,000 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% CO₂. 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, Bio Tek). All conditions were analysed in duplicate. TNF neutralisation 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 analysed with a Spearman or Pearson test using GraphPad Prism software version 7.04.

References