Restricted immune activation and internalisation of anti-idiotype complexes between drug and antidrug antibodies

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ABSTRACT

Objectives Therapeutic antibodies can provoke an antidrug antibody (ADA) response, which can form soluble immune complexes with the drug in potentially high amounts. Nevertheless, ADA-associated adverse events are usually rare, although with notable exceptions including infliximab. The immune activating effects and the eventual fate of these 'anti-idiotype' complexes are poorly studied, hampering assessment of ADA-associated risk of adverse events. We investigated the in vitro formation and biological activities of ADA-drug anti-idiotype immune complexes using patient-derived monoclonal anti-infliximab antibodies.

Methods Size distribution and conformation of ADA-drug complexes were characterised by size-exclusion chromatography and electron microscopy. Internalisation of and immune activation by complexes of defined size was visualised with flow imaging, whole blood cell assay and C4b/c ELISA.

Results Size and conformation of immune complexes depended on the concentrations and ratio of drug and ADA; large complexes (>6 IgGs) formed only with high ADA titres. Macrophages efficiently internalised tetrameric and bigger complexes in vitro, but not dimers. Corroborating these results, ex vivo analysis of patient sera demonstrated only dimeric complexes in circulation. No activation of immune cells by anti-idiotype complexes was observed, and only very large complexes activated complement. Unlike Fc-linked hexamers, anti-idiotype hexamers did not activate complement, demonstrating that besides size, conformation governs immune complex potential for triggering effector functions.

Conclusions Anti-idiotype ADA-drug complexes generally have restricted immune activation capacity. Large, irregularly shaped complexes only form at high concentrations of both drug and ADA, as may be achieved during intravenous infusion of infliximab, explaining the rarity of serious ADA-associated adverse events.

INTRODUCTION

Millions of patients are nowadays treated with therapeutic monoclonal antibodies that can be immunogenic. Antidrug antibody (ADA) formation is a concern during the development of any biopharmaceutical; the potential impact of immunogenicity is highlighted by the recent discontinuation of the PCSK9 inhibitor bococizumab, which was partly due to immunogenicity issues, despite promising clinical results.[1]

For efficacy and safety reasons, immunogenicity assessment of therapeutic protein products, including monoclonal antibodies, is presently scrutinised by regulatory agencies such as the Food and Drug Administration. High ADA titres towards therapeutic proteins are associated with lower efficacy and reduced clinical response, although low amounts of ADA have little apparent consequences.[2–6] Concerning safety, serious ADA-induced adverse events have been reported to various therapeutic proteins; erythropoietin (EPO) likely being the most notorious example, with cases of ADA formation cross-reacting to endogenous EPO leading to pure red cell aplasia.[7]

For therapeutic antibodies, ADA formation may also positively correlate with incidence of adverse events. Particularly for infliximab and natalizumab, the risk of acute hypersensitivity reactions is increased in ADA-positive patients.[8 9] However, for the majority of monoclonal antibodies, ADA positivity does not increase the occurrence of (severe) adverse events even in cases where ADA formation is clearly associated with diminished clinical response (eg, adalimumab[2 10]). A better understanding on the possible biological consequences of ADA would aid in assessment of immunogenicity-related risk and guidance of strategies for immunogenicity testing.

In ADA-positive patients, immune complexes between ADA and drug will form in vivo on treatment. The contribution of these complexes to drug clearance and immune-mediated adverse events is not clear, but is expected to depend—among others—on size and shape. Therapeutic antibodies like adalimumab, infliximab or natalizumab predominantly induce ADA responses towards the antigen binding site (or idiotype),[9 11 12] thereby limiting the number of ADA molecules that may bind each Fab arm to just one.[13] Consequently, therapeutic antibodies may be expected to bind to ADA—of which IgG is the major isotype[14–16]—in alternating fashion, forming circular or string-shaped immune complexes such as dimers, tetramers and hexamers (see lower panels of figure 1B).

Several studies investigated the size of these anti-idiotype complexes. Dimer-sized complexes were found in the majority of ADA-positive patients
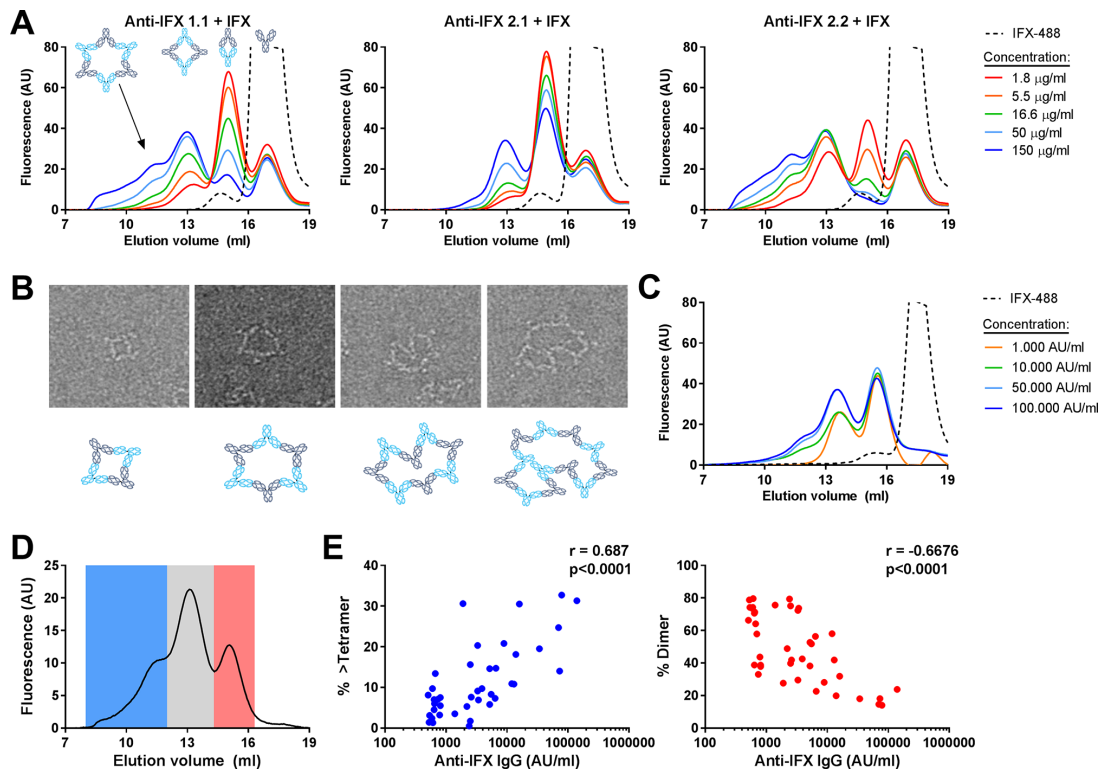
Figure 1 Immune complex size is highly dependent on concentration. (A) Infliximab (IFX)-488 and different monoclonal anti-IFX antibodies were mixed at increasing concentrations in a 1:1 ratio. Afterwards, samples were diluted so that each sample contained an equal amount of labelled drug, and analysed by high-performance size-exclusion chromatography (HP-SEC). (B) Transmission electron microscopic (TEM) analysis of complexes between monoclonal anti-IFX 2.2 and IFX at 150 μg/mL, and schematic representation of their conformation. See also online supplementary figure S5. (C) A titration of polyclonal anti-IFX antibodies from a single patient mixed with an equimolar amount of IFX-488, analysed by HP-SEC. n=3 separate experiments. (D, E) Serum of anti-IFX-positive patients was mixed with an equimolar amount of IFX-488 and analysed using HP-SEC. (D) Example of the elution pattern of one patient serum. Percentage of dimers (red box) and complexes bigger than tetramers (blue box) was determined by dividing the area under the curve (AUC) of these complexes by the total AUC. (E) Percentage of specified complexes was correlated to the anti-IFX IgG titre for each patient (n=41). Spearman's r for >tetramers: r=0.687, p<0.0001 and for dimers: r=−0.6676, p<0.0001.

treated with adalimumab 2 weeks after adalimumab administration.[12 17 18] In cynomolgus monkeys treated with infliximab, dimers as well as complexes larger than 670 kDa (>4 antibodies) could be demonstrated.[19] Infusion of (radiolabeled) infliximab into three ADA-positive patients led to dimer-sized complexes in two cases, without adverse events. However, complexes larger than 1000 kDa (>6 antibodies) were detected in a third patient that developed a severe infusion reaction, tentatively relating immune complex size to the adverse event.[20]

The differential effects of ADA on the occurrence of adverse events indicate that ADA-drug complexes are not invariably benign or harmful. However, for intravenously administered antibodies such as infliximab and natalizumab, high peak concentrations in serum of about 100–150 μg/mL are achieved, corresponding to roughly 1% of total serum IgG.[21 22] If ADAs are present in serum, administered drug will quickly be bound upon infusion, leading to rapid formation of soluble IgG complexes, potentially reaching high concentrations in case of high ADA titres.

In this study, patient-derived human monoclonal anti-infliximab antibodies are used to investigate the interplay between concentration and binding characteristics of ADA and drug, and the formation of different types of anti-idiotype immune complexes. Furthermore, the immune activating potential and clearance of ADA/drug complexes is investigated. The results of this study may advance the immunogenicity-related risk assessment of (new) therapeutic antibodies.

METHODS
See online supplementary materials and methods.

RESULTS
A panel of patient-derived recombinant monoclonal anti-infliximab antibodies was produced.[23] All monoclonal antibodies competed with tumour necrosis factor (TNF) for binding infliximab, as determined with a TNF competition assay (online supplementary table S1).[24] This indicates that, similar to polyclonal anti-infliximab antibodies,[24] they bind (close to) the drug's idiotype.

In vitro, size of monoclonal and polyclonal complexes depends on concentration and ratio
We sought to investigate which factors influence the size of immune complexes. High-performance size-exclusion chromatography (HP-SEC) analysis of monoclonal anti-infliximab clones combined with infliximab in a 1:1 molar ratio demonstrated different propensities of each anti-infliximab clone to form dimers, tetramers, hexamers and bigger complexes (online supplementary figure S1). Furthermore, for all ADA clones, the size of immune complexes depended highly on the concentrations in which drug and ADA were mixed. At a 1:1 ratio, higher concentrations resulted in larger complexes, whereas dimers eventually became the predominant type of complex at very low concentrations, regardless of the clone (figure 1A). This

concentration dependency was corroborated by a similar titration of polyclonal anti-infliximab antibodies from a patient's serum (figure 1C). Additionally, sera of 41 patients with anti-infliximab levels ranging from 510 to 140 000 AU/mL (median 1900 AU/mL) were mixed with an equimolar amount of infliximab and the size distribution of complexes was analysed (figure 1D). A strong correlation was found between the titre and the percentage of complexes larger than tetramers, whereas an inverse correlation was found for dimers (figure 1E).

During infusion, ADA and drug are not immediately present in equimolar concentrations. We therefore tested the influence of different antibody/drug ratios. As expected,[25] deviation of the 1:1 molar ratio caused a reduction of complex size (online supplementary figure S2A,B). A similar concentration and ratio dependency of immune complex size was also observed by combining monoclonal or polyclonal anti-adalimumab antibodies with adalimumab (online supplementary figure S3A,B), indicating that these observations are a general phenomenon for anti-idiotype complex formation.

Since HP-SEC analysis could not accurately resolve complex sizes larger than hexamers, monoclonal antibody complexes were additionally analysed with asymmetric flow field flow fractionation (AF4). Again, no distinct peaks of complexes larger than hexamers were observed (online supplementary figure S4),

suggesting that they vary highly in their size and conformation. Transmission electron microscopic (TEM) analysis of these complexes supported this observation. Importantly, the conformation of complexes as shown by TEM indicated Fab-Fab interactions (figure 1B, online supplementary figure S5), in line with the anti-idiotypic nature of the interactions formed between ADA and drug.[9 11 12] Thus, ADAs towards infliximab and adalimumab form circular or string-shaped anti-idiotype immune complexes, the size of which is influenced by both the concentration and ratio of ADA and drug.

Ex vivo, large immune complexes are absent in serum

Upon infusion of drug, ADA-positive patients will form immune complexes in vivo. We sought to determine which complexes are still present ex vivo just before the next infusion (ie, trough level). Trough sera of patients with varying anti-infliximab titres (range 12–70 000 AU/mL, median 40 AU/mL) were fractionated and tested for presence of anti-infliximab. No large complexes were detected in any of the sera (figure 2A and online supplementary figure S6), although they could be observed in monoclonal antibody complexes subjected to the same fractionation procedures (figure 2B). However, small complexes resembling dimers were found in patients with low anti-infliximab titres.
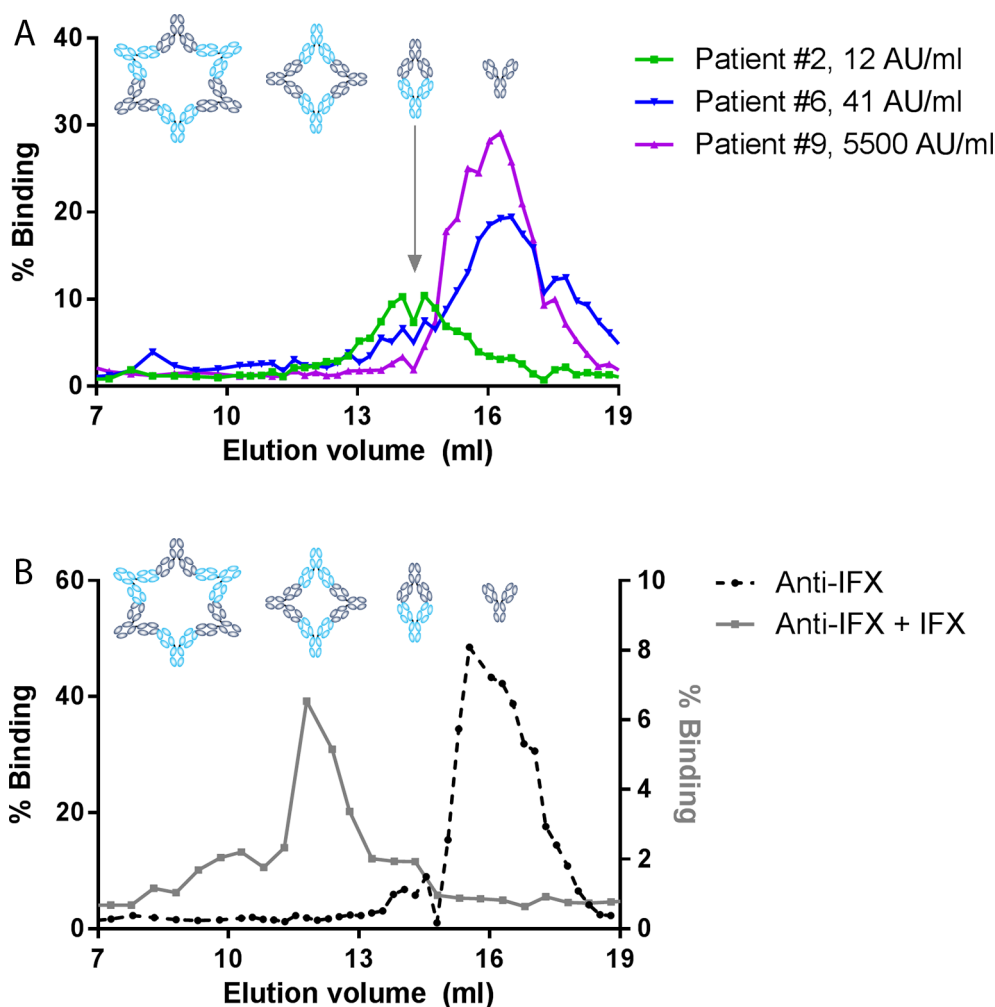
Figure 2 Only small complexes and monomeric anti-infliximab antibodies are detected in patients at trough. Anti-infliximab antibody presence was determined with a drug-tolerant assay in (A) fractionated anti-infliximab positive sera and (B) fractionated monomeric anti-infliximab 2.1 and complexes of anti-infliximab 2.4 and infliximab. Representative plots are shown for 3 out of 10 patients (A) and duplicate measurements of monoclonal complexes (B). Percentage binding is the sample measurement normalised to the total radioactively labelled input. IFX, infliximab.
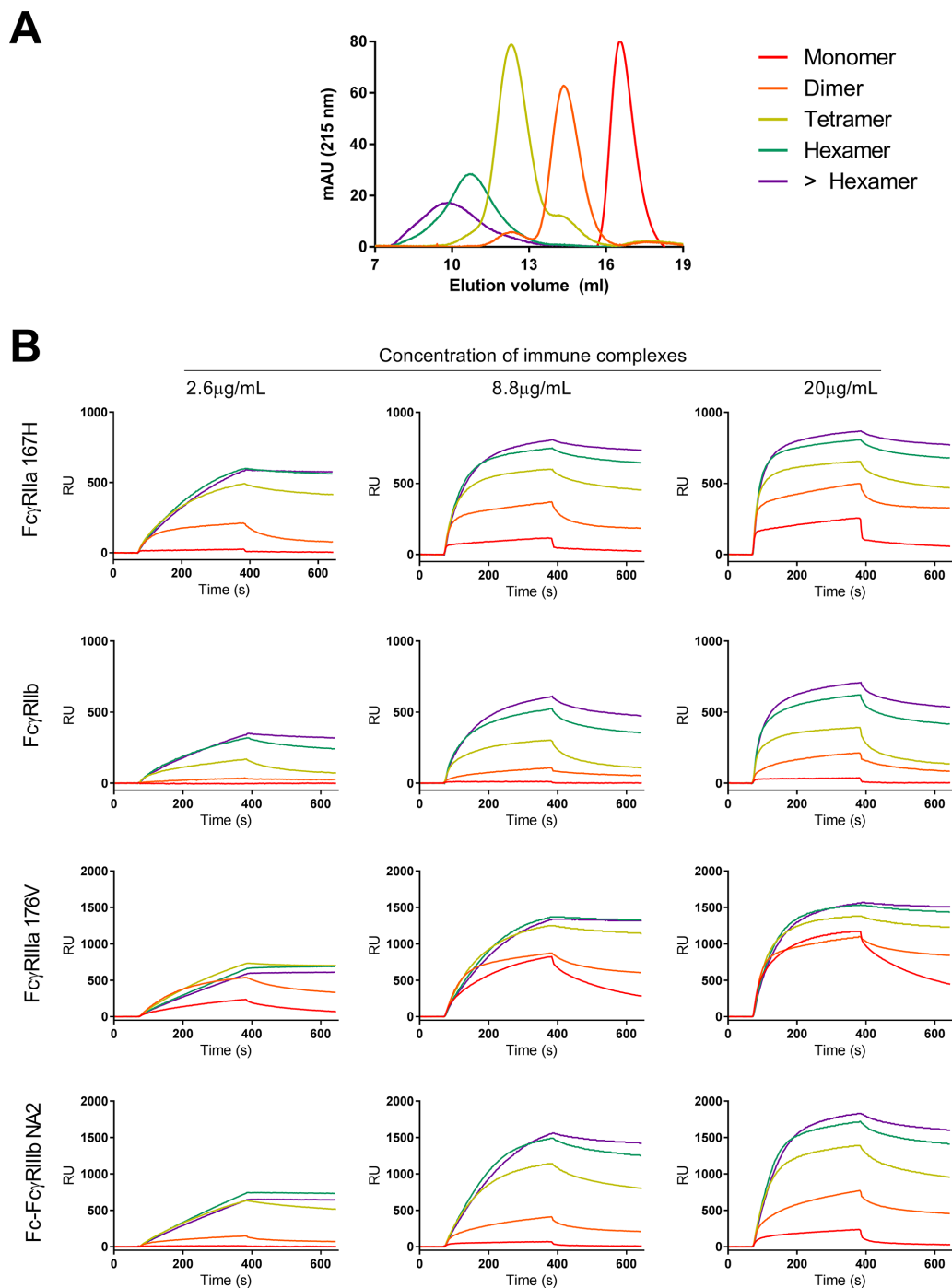
Figure 3 Binding to FcγRs increases with complex size. (A) Example of reanalysed fractions of immune complexes, done with high-performance size-exclusion chromatography (HP-SEC). (B) Representative sensograms of immune complexes binding to FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb on the chip. Receptor densities of shown sensograms are 10 nM for FcγRIIa and FcγRIIb, 3 nM for FcγRIIIa and 30 nM for FcγRIIIb, n=3 individual experiments. RU, response unit.

Large immune complexes are phagocytosed by macrophages

The discrepancy between the formation of large complexes in vitro and their absence ex vivo suggests that these complexes are preferentially and rapidly cleared in vivo. Since clearance is likely to occur via FcγRs, we first tested the binding of fractionated complexes (dimers, tetramers, hexamers or larger complexes; figure 3A) to different FcγRs immobilised on a biosensor chip using surface plasmon resonance. As expected, dimeric complexes showed enhanced binding compared with monomeric IgG for all receptors tested, and larger complexes

bound substantially stronger, largely due to a further reduction of the dissociation rate (figure 3B).

Next, we evaluated FcγR-mediated phagocytosis by monocyte-derived macrophages in vitro. Of note, the exact immune complex size required for phagocytosis has not been identified so far. We generated three types of complexes (figure 4A): (A) virtually pure dimeric complexes using the anti-adalimumab clone 2.7, which has a strong preference for making dimers with adalimumab[13]; (B) pools of dimers and tetramers using anti-infliximab 2.1; and (C) pools containing significant amounts of
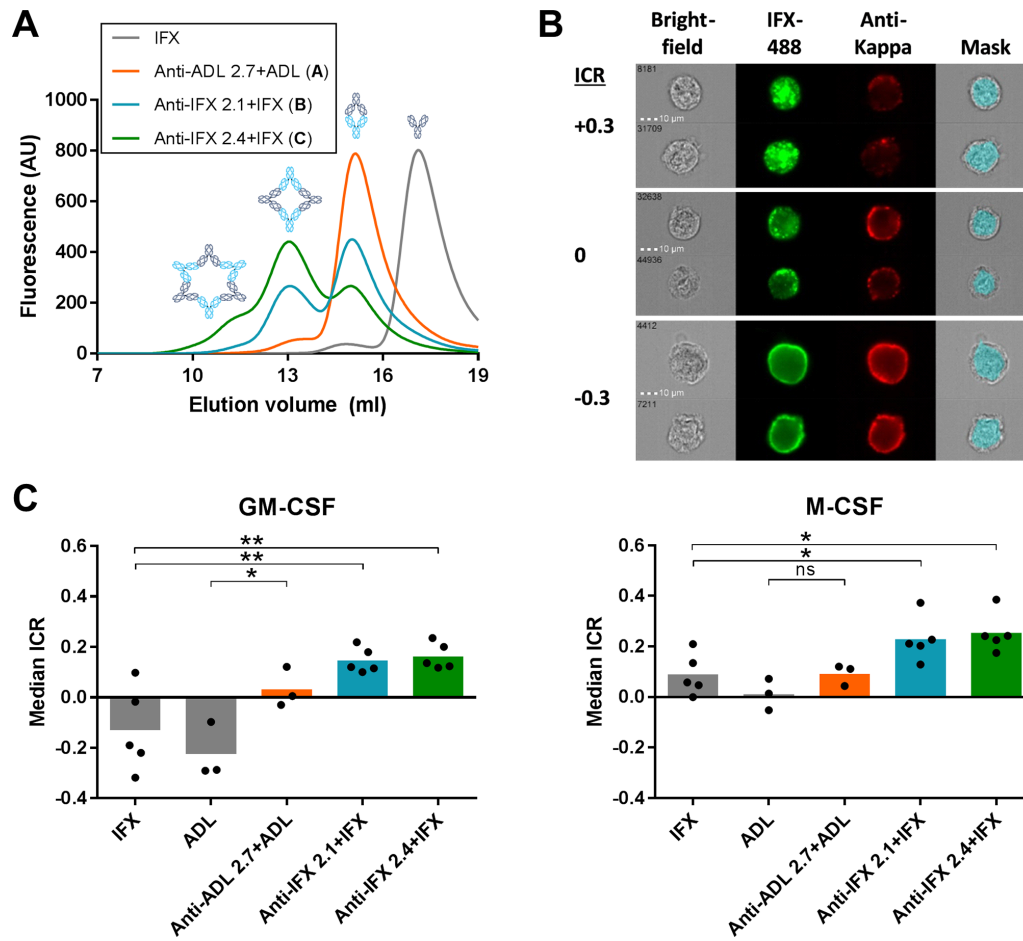
Figure 4 Complexes larger than dimers are efficiently internalised by macrophages. (A) Example of the fluorescent complexes used. (B) Representative examples of three different intensity concentration ratio (ICR) values. The ICR calculates the logit-transformed ratio between the fluorescent intensity inside the cell and the fluorescent intensity of the entire cell. From left to right: bright-field image, green fluorescence of infliximab (IFX)-488 (in complex with anti-IFX), red fluorescence of secondary staining with anti-kappa antibodies (not used in analysis), mask (blue) to determine the inside of a cell. (C) Median ICR of monomers, dimers or pools of larger immune complexes, determined for GM-CSF (left) or M-CSF (right) macrophages after 75 min. n≥3 individual donors measured in duplicate. Statistical differences were calculated by one-way analysis of variance (ANOVA), with Sidak's multiple comparison test; * and ** denote a statistical significance of p≤0.05 and p≤0.01, respectively. ADL, adalimumab; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; ns, not significant.

large complexes (>tetramers) using anti-infliximab 2.4. Uptake of complexes by monocyte-derived macrophages polarised with granulocyte-macrophage colony stimulating factor (GM-CSF) (proinflammatory phenotype) or macrophage colony stimulating factor (M-CSF) (anti-inflammatory phenotype) was analysed using flow imaging (figure 4B,C). Monomeric infliximab and adalimumab were not phagocytosed by GM-CSF macrophages and only slightly by M-CSF macrophages. Interestingly, also dimers (pool A) were not efficiently internalised. However, efficient internalisation was observed for tetramers (pool B) and bigger complexes (pool C; see also online supplementary figure S7 for time-lapse confocal imaging). Blocking of all FcγRs strongly inhibited binding and/or phagocytosis of infliximab and complexes (online supplementary figure S8A). Immune complex phagocytosis was predominantly mediated by FcγRI in GM-CSF and by FcγRII in M-CSF macrophages, likely due to differential FcγR expression on these cells (online supplementary figure S8B). Together, these results show that tetramers and larger complexes are phagocytosed in an FcγR-mediated fashion, but internalisation is much less efficient for dimers, consistent with the serological analysis shown in figure 2A.

Immune complexes do not activate immune cells

During intravenous administration of drug, complex formation occurs in the blood and the primary effects of antibody complexes are thus likely to happen within the blood circulation. We therefore investigated a possible proinflammatory effect of immune complexes in a whole blood cell activation assay, using interleukin (IL)-6 production as read-out for activation. However, even very large complexes (anti-infliximab 2.4+infliximab made at 450 μg/mL, concentrations exceeding those achieved in patients) were not able to induce IL-6 production at 20 μg/mL (data not shown) nor at 50 μg/mL (figure 5). In contrast, biotinylated beads opsonised with anti-biotin antibodies strongly induced IL-6 production. These results demonstrate that anti-idiotype antibodies have little proinflammatory capacity, possibly due to their size and/or conformation.

Complement activation requires anti-idiotype complexes larger than hexamers

The lack of IL-6 production in whole blood cell cultures does not exclude the possibility that circulating immune complexes can activate the complement system. We therefore also tested pools
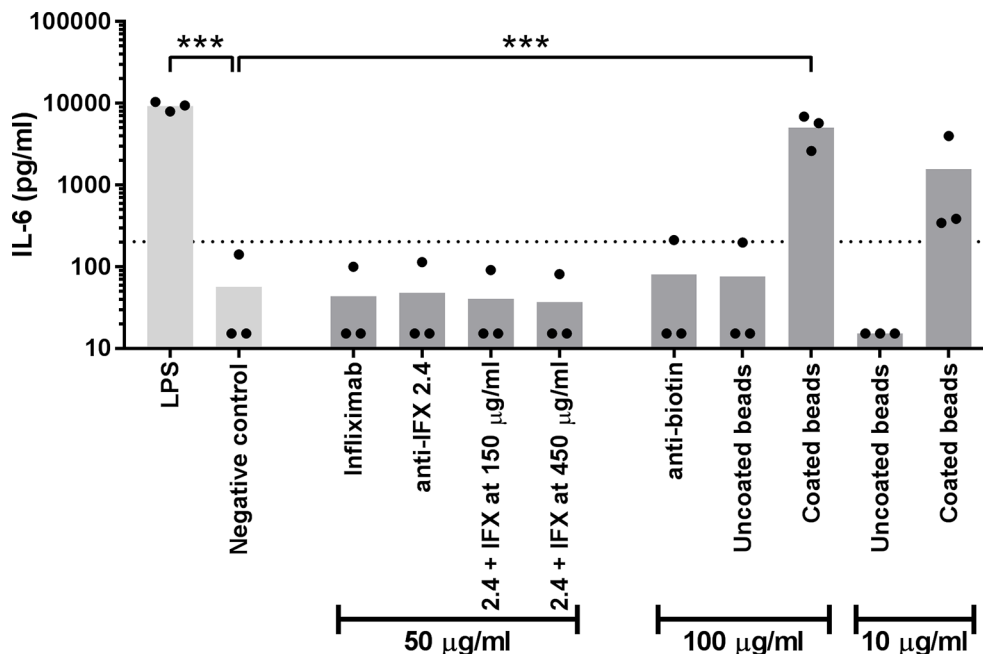
Figure 5 Large immune complexes do not have a proinflammatory effect on whole blood cell (WBC) cultures. Interleukin (IL)-6 production by WBC cultures was determined after addition of monomeric antibodies or large immune complexes (infliximab+anti-infliximab 2.4 mixed at 150 and 450 μg/mL of both antibodies). All samples were tested at 50 μg/mL of each antibody in 10-fold diluted blood, giving a supratherapeutic concentration. Anti-biotin-coated beads (100 or 10 μg/mL) and lipopolysaccharide (LPS) were used as positive control, WBC medium was used as negative control. Statistical differences were calculated by one-way analysis of variance (ANOVA), and further compared with the negative control by using Dunnett's multiple comparison test; *** denotes a statistical significance of p≤0.001. Unless indicated, no significant differences were found. Cut-off (dotted line) was set at the mean plus twice the SD of the negative control. IFX, infliximab.

of small and large anti-idiotype complexes for their capacity to activate complement. Pools of (predominantly) dimers and tetramers (anti-infliximab 2.1+infliximab) did not activate complement (figure 6A). However, pools containing—in addition to tetramers—hexamers and a variety of bigger complexes (anti-infliximab 2.4+infliximab) gave significant activation of the complement system. As expected, strong complement activation was also observed for the antibody-coated beads used in the whole blood cell assay (online supplementary figure S9).

To more specifically pinpoint the size of anti-idiotype complexes required for complement activation, we fractionated the pools of complexes to obtain samples with only dimers, tetramers, hexamers or complexes larger than hexamers (high molecular weight, HMW), and tested their individual activation capacity. No activation was seen for hexamers and smaller complexes, but a (non-significant) trend towards complement activation was found for the complexes with the highest molecular weight, suggesting that anti-idiotype complexes should be (substantially) larger than hexamers to activate complement (figure 6B). To exclude the possibility that abnormal Fc glycosylation of recombinant antibodies reduced the complement activating potential, Fc glycopeptides of all antibodies were analysed (online supplementary figure S10). However, the slight differences that were found compared with IVIg could not explain the lack of complement activation.[26]

Recently it was shown that RGY-mutant antibodies, which have a triple-mutated Fc, form fluid phase Fc-Fc interacting hexamers mimicking similar hexameric structures formed by IgG on opsonising a cellular target. These hexameric complexes potently activate the complement system via C1q.[27 28] Since our anti-idiotype hexamers do not activate complement, we investigated the potency of Fc-Fc interacting hexamers of IgG1-b12-RGY in our system, and indeed found strong complement

activation (figure 6B). The contrasting results between anti-idiotype and Fc-Fc interacting hexamers strongly suggest that the conformation of immune complexes is crucial for complement activation.

DISCUSSION

Immunogenicity of therapeutic monoclonal antibodies is a recognised problem, and the relation between ADA formation and reduced efficacy has been demonstrated in numerous studies. Nevertheless, few studies addressed the pathophysiological effects of these immune responses, and therefore the impact of ADA on safety remains largely unknown. In this study, we investigated the factors influencing ADA-drug complex formation and determined the biological activities of these complexes. Data presented in this paper show for the first time that anti-idiotype complexes with distinct conformations are formed between drug and ADA, and that the majority of these complexes have a restricted capacity to cause immune activation. This would imply that for many therapeutic antibodies an ADA response will not ordinarily result in adverse events, given that such responses tend to predominantly consist of anti-idiotype antibodies.[9 11 12]

Antibody concentration and ADA/drug ratio were found to greatly influence complex size in patient sera. Although large complexes were formed in vitro, these were not detected ex vivo in trough serum samples of infliximab-treated patients. The rapid phagocytosis of tetramers and larger complexes by macrophages suggests that these complexes are cleared in vivo. On the other hand, our study shows that dimers can persist in circulation for prolonged times, in line with previous observations from our group for adalimumab.[12] Their impaired internalisation by macrophages suggests that dimers are too small to be efficiently cleared.
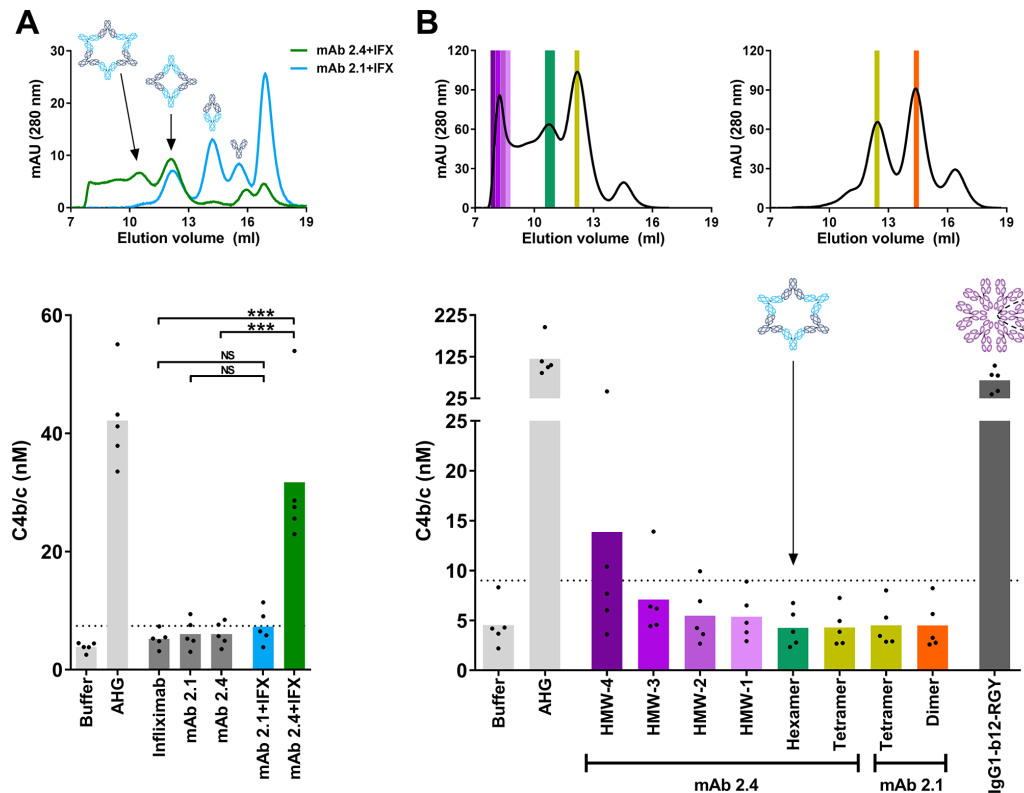
Figure 6 Anti-idiotype complexes larger than hexamers are required for activation of the complement system. (A) Representative elution patterns of complexes between infliximab with either anti-infliximab 2.1 or 2.4 made at 150 and 450 μg/mL of both antibodies, respectively. Bottom: complexes (final antibody concentration 100 μg/mL in serum) were tested in the C4b/c ELISA. n=5 serum donors, each dot represents the mean of two to five experiments per donor. Significant differences were calculated by one-way analysis of variance (ANOVA), with Sidak's multiple comparison test; *** denotes a statistical significance of p≤0.001. (B) Representative elution patterns of immune complexes consisting of infliximab with either anti-infliximab 2.4 (upper left) or 2.1 (upper right). Selected fractions are shown in coloured boxes. Bottom: all fractions and IgG1-b12-RGY were separately tested in the C4b/c ELISA (final antibody concentration 15 μg/mL in serum). n=5 serum donors, representative of ≥4 individual experiments. Buffer was used as negative control and aggregated human gammaglobulin (AHG) as positive control. Dotted line is twice the mean buffer value. Similar colours in top and bottom figures show corresponding samples. HMW, high molecular weight; IFX, infliximab; ns, not significant.

The valency required for FcγR-mediated inhibition of phagocytosis was also investigated by Ortiz et al,[29] showing that Fc trimers were not internalised by FcγR expressing THP-1 cells, in contrast to Fc pentamers. Our study now suggests that at least four Fc moieties are required for efficient internalisation.

Contrary to their findings, our results suggest a clear dependency on immune complex conformation, in addition to mere Fc-tail valency, as an important factor determining the immune-activating properties of an immune complex (see below).

We only observed complement activation for the very large—irregularly shaped—anti-idiotype complexes, whereas hexamers and smaller complexes did not activate complement. It has been known for decades that the classical pathway of the complement cascade is activated by multimerisation of antibodies on a surface or in fluid phase, but the detailed molecular mechanisms are only now being fully elucidated. In older studies, chemically cross-linked IgG forming dimers, trimers and tetramers were found to increasingly activate complement.[30 31] However, it is unclear to which extent these artificial complexes resemble complexes formed during an actual immune response. More recently, six Fc-Fc interacting antibodies were found to be the optimal amount for C1q docking and activation with the Fc tails pointing inward forming a hexameric structure also sometimes observed in crystal structures of human IgG.[27 28] This provides a platform for C1q to dock onto. Interestingly, the anti-idiotype complexes generated by ADA and drug have a completely opposite conformation compared with the Fc-Fc interacting hexamers: since ADA and drug interact through their Fab arms, the Fc tails will point outward. Therefore, ADA-drug-induced hexamers, but also dimers and tetramers, do not form a structurally optimal C1q platform like Fc-Fc interacting hexamers, probably explaining their lack of complement activity. Upon generation of larger complexes than hexamers the conformation is likely to become more disorganised (also suggested by our AF4 and TEM results), allowing some Fc tails to come into closer contact, thereby serving as a more optimal C1q docking platform. Large disorganised complexes may therefore activate complement to a certain extent, but this is expected to be restricted to situations of high ADA titres.

For infliximab[8 21 32] and natalizumab,[9 33] clinical studies show that ADA positivity and high ADA titres[34] are associated with infusion reactions. These encompass all symptoms that occur during or shortly after infusion, and may be mild (eg, dizziness, nausea), moderate (eg, chest tightening, urticaria) or severe (eg, significant hyper/hypotension, stridor).[35] While these symptoms resemble those of an IgE-mediated type I hypersensitivity, the vast majority of patients experiencing an infliximab-induced infusion reaction are negative for IgE anti-infliximab,[23 36 37] and no elevated tryptase levels are found in infusion reaction positive+ patients.[35 38] By contrast, our study provides a mechanistic link between high IgG ADA titres and the formation of

large, irregularly shaped immune complexes that—under privileged circumstances—can activate complement. Our results thus suggest that, instead of an IgE-mediated response, infusion reactions may represent type III hypersensitivity.

Anti-infliximab/adalimumab antibodies are largely (>90%)[24] anti-idiotype antibodies. We here demonstrate that ADA-drug complexes formed with anti-idiotypic antibodies have restricted immune activating capacities. It however remains unknown to what extent the small fraction of non-anti-idiotypic ADA influences this restricted activation.

During infusion of drug in ADA-positive patients, drug is effectively titrated to ADA in vivo. The concentration of ADA at the site of infusion and the infusion rate will influence the type of complexes that are formed. At first, ADA will theoretically be in excess, probably resulting predominantly in smaller, trimeric complexes (ie, one drug and two ADA molecules). However, when a ratio approaching equimolarity is reached, larger complexes will be formed. The concentration of both antibodies at equimolarity will determine the size of the complexes and their (clinical) effects. The current management to ameliorate infusion reactions by reducing the infusion speed may impact the effective (local) peak drug concentrations, thereby reducing the risk of a severe infusion reaction. In contrast to intravenous administered therapeutics, subcutaneous administration leads to a local high concentration of drug, thereby taking the ADA-drug ratio far from equimolarity. Although little is known about their actual in vivo formation, the formed complexes are expected to be small, and are likely even more slowly distributed than monomeric drug molecules.[39] These properties may prevent systemic reactions in a dual manner.

As shown in this study, immune complexes larger than dimers are efficiently taken up by macrophages in vitro. ADA formation thus may reduce the drug concentration in vivo,[19] but this inherently also reduces the ADA concentration. This complicates the assessment of immunogenicity, since one cannot measure ADA that is already cleared. So, immune complex clearance can lead to underestimation of the immunogenicity of antibody therapeutics. In any case, these results warrant caution against interpretation of quantitative aspects of ADA formation, and show that even multitiered testing approaches may not reveal the full extent of an ADA response.

Taken together, immune complex formation between ADA and drug results in large immune complexes when the concentration of both antibodies is high, but the anti-idiotype conformation limits the formation of complexes capable of complement or immune cell activation. Very large immune complexes may be formed and adverse events may occur in those cases where ADA levels are very high and the administered amounts of drug as well (upward of ca 50–100 µg/mL), typically only during intravenous administration.

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REFERENCES
1 Yokote K, Kanada S, Matsuoka O, et al. Efficacy and safety of bococizumab (rn316/pf-04950615), a monoclonal antibody against proprotein convertase subtilisin/kexin type 9, in hypercholesterolemic Japanese subjects receiving a stable dose of atorvastatin or treatment-naive - results from a randomized, placebo-controlled, dose-ranging study. *Circ J* 2017;81:1496–505.
2 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;305:1460–8.
3 Vennegoor A, Rispens T, Strijbis EM, et al. Clinical relevance of serum natalizumab concentration and anti-natalizumab antibodies in multiple sclerosis. *Mult Scler* 2013;19:593–600.
4 Krintel SB, Grunert VP, Hetland ML, et al. The frequency of anti-infliximab antibodies in patients with rheumatoid arthritis treated in routine care and the associations with adverse drug reactions and treatment failure. *Rheumatology* 2013;52:1245–53.
5 Hoxha A, Calligaro A, Tonello M, et al. The clinical relevance of early anti-adalimumab antibodies detection in rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis: a prospective multicentre study. *Joint Bone Spine* 2016;83:167–71.
6 Bender NK, Heilig CE, Dröll B, et al. Immunogenicity, efficacy and adverse events of adalimumab in RA patients. *Rheumatol Int* 2007;27:269–74.
7 Casadevall N, Nataf J, Viron B, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med* 2002;346:469–75.
8 O'Meara S, Nanda KS, Moss AC. Antibodies to infliximab and risk of infusion reactions in patients with inflammatory bowel disease: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2014;20:1–6.
9 Calabresi PA, Giovannoni G, Confavreux C, et al. The incidence and significance of anti-natalizumab antibodies: results from AFFIRM and SENTINEL. *Neurology* 2007;69:1391–403.
10 Hanauer SB, Sandborn WJ, Rutgeerts P, et al. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology* 2006;130:323–33.
11 van Schie KA, Kruithof S, van Schouwenburg PA, 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* 2017;139:125–6.
12 van Schouwenburg PA, van de Stadt LA, de Jong RN, et al. Adalimumab elicits a restricted anti-idiotypic antibody response in autoimmune patients resulting in functional neutralisation. *Ann Rheum Dis* 2013;72:104–9.
13 van Schouwenburg PA, Kruithof S, Votsmeier C, et al. Functional analysis of the anti-adalimumab response using patient-derived monoclonal antibodies. *J Biol Chem* 2014;289:34482–8.
14 Lundkvist M, Engdahl E, Holmén C, et al. Characterization of anti-natalizumab antibodies in multiple sclerosis patients. *Mult Scler* 2013;19.
15 Kosmač M, Avčin T, Toplak N, et al. Exploring the binding sites of anti-infliximab antibodies in pediatric patients with rheumatic diseases treated with infliximab. *Pediatr Res* 2011;69:243–8.
16 van Schouwenburg PA, Krieckaert CL, Nurmohamed M, et al. IgG4 production against adalimumab during long term treatment of RA patients. *J Clin Immunol* 2012;32:1000–6.

17 van Schouwenburg PA, Bartelds GM, Hart MH, *et al*. 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;362:82–8.

18 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.

19 Rojas JR, Taylor RP, Cunningham MR, *et al*. Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomolgus monkeys. *J Pharmacol Exp Ther* 2005;313:578–85.

20 van der Laken CJ, Voskuyl AE, Roos JC, *et al*. Imaging and serum analysis of immune complex formation of radiolabelled infliximab and anti-infliximab in responders and non-responders to therapy for rheumatoid arthritis. *Ann Rheum Dis* 2007;66:253–6.

21 Park W, Yoo DH, Jaworski J, *et al*. Comparable long-term efficacy, as assessed by patient-reported outcomes, safety and pharmacokinetics, of CT-P13 and reference infliximab in patients with ankylosing spondylitis: 54-week results from the randomized, parallel-group PLANETAS study. *Arthritis Res Ther* 2016;18:25.

22 Sehr T, Proschmann U, Thomas K, *et al*. New insights into the pharmacokinetics and pharmacodynamics of natalizumab treatment for patients with multiple sclerosis, obtained from clinical and in vitro studies. *J Neuroinflammation* 2016;13:164.

23 van Schie KA, Ooijevaar-De Heer P, Kruithof S, *et al*. Infusion reactions during infliximab treatment are not associated with IgE anti-infliximab antibodies. *Ann Rheum Dis* 2017;76:1285–8.

24 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;74:311–4.

25 Heidelberger M, Kendall FE. The precipitin reaction between type iii pneumococcus polysaccharide and homologous antibody: iii. a quantitative study and a theory of the reaction mechanism. *J Exp Med* 1935;61:563–91.

26 Dekkers G, Treffers L, Plomp R, *et al*. Decoding the human immunoglobulin G-Glycan repertoire reveals a spectrum of Fc-receptor- and complement-mediated-effector activities. *Front Immunol* 2017;8:877.

27 Diebolder CA, Beurskens FJ, de Jong RN, *et al*. Complement is activated by IgG hexamers assembled at the cell surface. *Science* 2014;343:1260–3.

28 Wang G, de Jong RN, van den Bremer ET, *et al*. Molecular basis of assembly and activation of complement component C1 in complex with immunoglobulin G1 and antigen. *Mol Cell* 2016;63:135–45.

29 Ortiz DF, Lansing JC, Rutitzky L, *et al*. Elucidating the interplay between IgG-Fc valency and FcγR activation for the design of immune complex inhibitors. *Sci Transl Med* 2016;8:365ra158.

30 Wright JK, Tschopp J, Jaton JC, *et al*. Dimeric, trimeric and tetrameric complexes of immunoglobulin G fix complement. *Biochem J* 1980;187:775–80.

31 Doekes G, Vanes LA, Daha MR. Influence of aggregate size on the binding and activation of the first component of human complement by soluble IgG aggregates. *Immunology* 1982;45:705–13.

32 Steenholdt C, Svenson M, Bendtzen K, *et al*. Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2011;34:51–8.

33 de la Hera B, Urcelay E, Brassat D, *et al*. Natalizumab-related anaphylactoid reactions in MS patients are associated with HLA class II alleles. *Neurol Neuroimmunol Neuroinflamm* 2014;1:e47.

34 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;50:1445–52.

35 Cheifetz A, Smedley M, Martin S, *et al*. The incidence and management of infusion reactions to infliximab: a large center experience. *Am J Gastroenterol* 2003;98:1315–24.

36 Fréling E, Peyrin-Biroulet L, Poreaux C, *et al*. IgE antibodies and skin tests in immediate hypersensitivity reactions to infliximab in inflammatory bowel disease: impact on infliximab retreatment. *Eur J Gastroenterol Hepatol* 2015;27:1200–8.

37 Matucci A, Pratesi S, Petroni G, *et al*. Allergological in vitro and in vivo evaluation of patients with hypersensitivity reactions to infliximab. *Clin Exp Allergy* 2013;43:659–723.

38 Steenholdt C, Palarasah Y, Bendtzen K, *et al*. Pre-existing IgG antibodies cross-reacting with the Fab region of infliximab predict efficacy and safety of infliximab therapy in inflammatory bowel disease. *Aliment Pharmacol Ther* 2013;37:1172–83.

39 Kijanka G, Bee JS, Bishop SM, *et al*. Fate of multimeric oligomers, submicron, and micron size aggregates of monoclonal antibodies upon subcutaneous injection in mice. *J Pharm Sci* 2016;105:1693–704.