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

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Abstract

Therapeutic antibodies can elicit an anti-drug antibody (ADA) response resulting in anti-idiotype immune complexes. Nevertheless, ADA-associated adverse events are usually rare, albeit with some exceptions, including infliximab. We investigated formation, clearance, and biological activity of these ADA-drug complexes using patient-derived monoclonal anti-infliximab antibodies.

Ratio and concentrations of drug and ADA determined immune complex size, with large, irregularly shaped complexes (>6 IgG molecules) formed only in case of high ADA titers. Macrophages efficiently phagocytosed tetrameric and bigger complexes *in vitro*, but not dimers. Corroborating these results, *ex vivo* analysis of patient sera taken at trough demonstrated only dimeric complexes in circulation. Strikingly, none of the anti-idiotype complexes induced immune cell activation in a whole-blood assay, and only very large complexes activated the complement system. Notably, anti-idiotype hexamers did not activate complement, unlike Fc-linked hexamers that may form upon opsonizing a cellular target, demonstrating that besides size, conformation governs immune complex potential for triggering effector functions.

In conclusion, we demonstrate predominant formation of small, soluble anti-idiotype immune complexes with restricted capacity for immune activation. Large immune complexes will only form at high concentrations of both drug and ADA, as may be achieved during intravenous infusion of infliximab, explaining the rarity of severe ADA-associated adverse events.

Introduction

Millions of patients are nowadays treated with therapeutic monoclonal antibodies that are found to be immunogenic. Unwanted anti-drug antibody (ADA) responses against the drug may develop to widely different degrees in different patients, with different clinical consequences. Immunogenicity 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 nowadays scrutinized by regulatory agencies such as the FDA. High antibody titers towards therapeutic proteins and monoclonal antibodies 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 monoclonal antibodies, however, ADA-induced adverse events are much less frequent. There are examples of therapeutic antibodies where ADA formation positively correlates with incidence of adverse events, particularly infliximab and natalizumab. However, for most 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, such as adalimumab.[2] A better understanding on the possible biological consequences of ADA would provide more insight into the effects of immunogenicity on the safety of monoclonal antibodies, which might aid in assessment of immunogenicity-related risk and guidance of strategies for immunogenicity testing.

A poorly investigated aspect of ADA towards monoclonal antibodies is *in vivo* immune complex formation between ADA and drug, which inevitably occurs in all ADA-positive patients upon treatment. The extent to which these complexes are contributing to drug clearance as well as immune-mediated adverse events is not clear. Some predictions on immune complex formation can be made based on ADA characteristics. We have recently shown that ADA responses towards the TNF inhibitors infliximab, adalimumab, golimumab, certolizumab as well as to the α4-integrin blocking antibody natalizumab, have a predominance for targeting the idiotype (or antigen binding site) of the drug.[8–10] Consequently, each Fab arm of the therapeutic antibody may bind to just one ADA molecule.[11] Therefore, therapeutic antibodies are expected to bind to ADA – of which IgG is the major isotype[12–14] – in alternating fashion, forming circular or string-shaped immune complexes such as dimers, tetramers, and hexamers (see lower panels of Figure 1B).

A few studies investigated the size of these anti-idiotype complexes. Small complexes the size of dimers were found in the majority of ADA positive patients treated with adalimumab two weeks after adalimumab administration.[10,15,16] A study by Rojas et al. showed the formation of dimers as well as complexes larger than 670 kDa (>four antibodies) in cynomolgus monkeys treated with infliximab. Finally, van der Laken et al. investigated serum samples of three ADA+ patients taken right after infusion of radio-labelled infliximab. Small complexes the size of dimers were found in two patients that did not show adverse events. However, complexes larger than 1000 kDa (>six antibodies) were detected in another patient that developed a severe infusion reaction, tentatively relating immune complex size to the adverse event.[17]

As mentioned above, for some drugs, notably infliximab[18] and natalizumab[8], ADA positivity is clearly associated with an increased chance on adverse events such as infusion reactions. For many other drugs (e.g. adalimumab[19] and vedolizumab[20]) these associations are not observed, indicating that ADA-drug immune complexes are not invariably benign or harmful. Since infliximab and natalizumab are intravenously administered, high peak concentrations in serum of around 100-150 µg/ml are achieved for these drugs, 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 immune complexes, potentially reaching high concentrations in case of high ADA titers.

The interplay between concentrations and binding characteristics of ADA and drug, and formation of different types of anti-idiotype immune complexes, as well as their clearance and biological effects remain largely unexplored. In this study, we used patient-derived human monoclonal anti-infliximab to investigate formation of immune complexes in a controlled manner. In addition, we examined the clearance of small and large complexes by macrophages and assessed their immune activating potential. Our data imply a restricted potential of anti-idiotype antibodies for immune activation, with only high concentrations of drug and ADA resulting in large complexes that could lead to adverse reactions.

Results

To investigate the characteristics of immune complexes in a controlled manner, several patient-derived recombinant monoclonal anti-infliximab antibodies were produced.[23] All monoclonal antibodies were neutralizing as determined with a TNF competition assay, meaning that they compete with TNF for binding the drug and thus bind (close to) the drug's idiotype (Table 1).

In vitro, size of monoclonal and polyclonal complexes depends on concentration and ratio.
We sought to investigate which factors influenced the size of immune complexes. HP-SEC analysis of monoclonal anti-infliximab clones combined with infliximab in a 1:1 ratio demonstrated different propensities of each monoclonal antibody to form dimers, tetramers, hexamers and bigger complexes (Supplemental Figure 1). 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 titer 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, and we therefore tested the influence of different antibody ratios. As expected,[24] deviation of the 1:1 ratio caused a reduction of complex size (Supplemental Figure 2A,B). A similar concentration and ratio dependency of immune complex size was also observed by combining monoclonal or polyclonal anti-adalimumab antibodies with adalimumab (supplemental Figure 3A,B).
Since HP-SEC analysis could not accurately resolve complex sizes larger than hexamers, monoclonal antibody complexes were additionally analyzed with asymmetric flow field flow fractionation (AF4). Again, no distinct peaks of complexes larger than hexamers were observed (Supplemental Figure 4), suggesting that they vary highly in their size and conformation. Transmission electron microscopy (TEM) analysis of these complexes supported this observation. Importantly, the conformation of complexes as shown by TEM indicated Fab-Fab interactions (Figure 1B, supplemental Figure 5), in line with the anti-idiotypic nature of the interactions formed between ADA and drug.[8][9][10] Thus, ADA towards infliximab and adalimumab form circular or string-shaped anti-idiotype immune complexes of which the size 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 whether immune complexes are still present *ex vivo* at trough. Sera of patients with varying anti-infliximab titers (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),

although they could be observed in monoclonal antibody complexes subjected to the same fractionation procedures (Figure 2B and supplemental Figure 6). However, small complexes resembling dimers were found in patients with low ADA titers.

Large immune complexes are phagocytosed by macrophages
The discrepancy between the formation of large complexes *in vitro* and their absence in *ex vivo* suggests that these complexes are preferentially cleared *in vivo*. Since clearance is likely to occur via FcγRs, we first tested the binding of fractionated immune complexes containing only dimers, tetramers, hexamers or larger complexes (Figure 3A) to different FcγRs immobilized on a biosensor chip using surface plasmon resonance (SPR). As expected, a dimeric complex resulted in enhanced binding compared to 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. Of note, the exact immune complex size required for phagocytosis has not been identified so far. We made use of the distinct characteristics of different ADA clones to form dimers, tetramers, and larger complexes. We generated pools only consisting of dimers and tetramers using anti-infliximab 2.1, pools containing significant amounts of complexes >tetramers using anti-infliximab 2.4, and virtually pure dimeric complexes using the anti-adalimumab clone 2.7, which was found to have a very strong preference for making dimers with adalimumab (Figure 4A).[11] We visualized phagocytosis of immune complexes by monocyte derived GM-CSF or M-CSF macrophages using flow imaging (Figure 4B), and quantified internalization as the intensity concentration ratio (ICR) that calculates the logit-transformed ratio between fluorescent intensity inside the cell and the fluorescent intensity of the entire cell (Figure 4C).
Monomeric infliximab and adalimumab were found not to be phagocytosed by GM-CSF macrophages and only slightly by M-CSF macrophages. Interestingly, also dimers are not efficiently internalized, whereas using pools of dimers and tetramers (anti-infliximab 2.1+infliximab) efficient internalization is observed, similar to pools of tetramers and bigger complexes (anti-infliximab 2.4+infliximab; see also Supplemental Figure 7 for time-lapse confocal imaging). Blocking of all FcγRs strongly inhibited binding and/or phagocytosis of infliximab and complexes (Supplemental Figure 8A). 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 (Supplemental Figure 8B). Together, these results show that tetramers and larger complexes are phagocytosed in a FcγR-mediated fashion, but that internalization is much less efficient for dimers, consistent with the serological analysis shown in Figure 2B.

Immune complexes do not activate immune cells
During intravenous administration of drug, complex formation occurs in the blood and thus their primary effects are likely to happen within the blood circulation. We therefore investigated a possible pro-inflammatory effect of immune complexes in a whole blood cell activation assay, using 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 (Figure 5). In contrast, biotinylated beads opsonized with anti-biotin antibodies strongly induced IL-6 production, which was completely suppressed after addition of EDTA, indicating the involvement of the complement system in cellular activation by these immune complexes.

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 of small and large anti-idiotype immune complexes for their capacity to activate complement. As shown in Figure 6A, pools of (predominantly) dimers and tetramers (anti-infliximab 2.1+infliximab) did not activate complement. 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 observed for the antibody coated beads used in the whole blood cell assay (Supplemental Figure 9).

To more specifically pinpoint the size of anti-idiotype complexes required for 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. As shown in Figure 6B, no activation was seen for hexamers and smaller complexes, but a (non-significant) trend towards complement activation was found for the isolated fractions with the highest molecular weight, suggesting that anti-idiotype immune complexes should be (substantially) larger than hexamers to activate the complement system.

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 upon opsonising a cellular target. These hexameric complexes potently activate the complement system via C1q.[25,26] 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 demonstrate that the conformation of immune complexes is crucial for complement activation.

Discussion

Immunogenicity of therapeutic monoclonal antibodies is a recognized 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.[8–10]

Concentration and ratio were found to greatly influence complex size in patient sera. Although large immune complexes were formed *in vitro*, *ex vivo* these complexes were not detected in trough serum samples of infliximab treated patients. Further experiments with macrophages showed rapid phagocytosis of tetramers and larger complexes, suggesting that these complexes are cleared *in vivo*. Clearance by macrophages was already proposed in studies in humans[17] and cynomolgus monkeys,[27] where accumulation of radiolabeled ADA-infliximab immune complexes was detected in the liver and spleen.

Our study furthermore shows that dimers can persist in circulation for prolonged times, in line with previous observations from our group for adalimumab[10], which can be attributed to impaired internalization by macrophages, implying that dimers are too small to be efficiently cleared.

The valency required for inhibiting FcγR mediated activation was also investigated by Ortiz et al.[28] A Fc-trimer was proposed to have the optimal inhibiting effect. Notably, these Fc-trimers were not phagocytosed by FcγR expressing THP-1 cells, in contrast to Fc-pentamers. Apparently, as shown in our study, four Fc moieties are required for efficient phagocytosis. Dimers were not able to inhibit phagocytosis of these tetramers, illustrating the delicate line between FcγR mediated phagocytosis and its inhibition. In contrast to their findings, our results suggest a clear dependency on immune complex conformation, in addition to mere Fc tail valency, as an important factor which determines the immune activating properties of an immune complex (see below).

In this study, complement activation was only seen 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

multimerization of antibodies on a surface or in fluid phase, but the detailed molecular requirements for this multimerization process are only now being fully elucidated. In older studies, chemically crosslinked IgG forming dimers, trimers and tetramers were found to increasingly activate complement.[29,30] 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[25,26]. 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 to 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 disorganized, as also suggested by our AF4 and TEM results, which allows some of the Fc-tails into closer contact and serving as a more optimal C1q docking platform. Large disorganized complexes may therefore activate complement to a certain extent, but this is expected to be restricted to situations of high ADA titers. Therefore, the restricted anti-idiotypic nature of the antibody response to many antibody drugs appears to be an important factor that limits the potential of the immune complexes to induce detrimental systemic fluid phase complement activation.

Clinical studies on infliximab[18,21,31] and natalizumab[8,32] nevertheless show that ADA positive patients are more prone to develop side effects such as infusion reactions. An infusion reaction encompasses all symptoms that occur during or short after the infusion of drug, and may be mild (e.g. dizziness, nausea), moderate (e.g. chest tightening, urticaria) or severe (e.g. significant hyper/hypotension, stridor).[33] While these symptoms resemble those of an IgE-mediated Type I hypersensitivity, we have recently shown that the vast majority of infliximab-induced infusion reactions is negative for IgE anti-infliximab.[23] Others also found no IgE anti-infliximab, elevated total IgE or elevated tryptase levels in IR+ patients, suggesting that the majority of infusion reactions are not IgE-mediated.[33,34] The association of infusion reactions with ADA positivity[21] and high ADA titers[35] indicate that IgG ADA play a crucial role in these adverse events. Our study shows, for the first time, the mechanistic link between high ADA titers 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 are more likely to be a Type III hypersensitivity reaction.

During infusion of drug in ADA positive patients, drug is effectively *in vivo* titrated to ADA. The concentration of ADA at the site of infusion and the infusion rate will influence the type of immune complexes that are formed. At first, ADA will theoretically be in excess, probably resulting predominantly in smaller, trimeric complexes (i.e. 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 IV 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 resulting immune complexes are likely small and are distributed slowly (as is the drug[36]), preventing systemic reactions in a dual manner.

As shown in this study, immune complexes larger then dimers are *in vitro* efficiently taken up by macrophages. ADA formation thus may reduce the *in vivo* drug concentration,[27] but then inherently also reduces its own concentration. This complicates the assessment of immunogenicity, since one cannot measure ADA that is already cleared. Due to immune complex clearance, the immunogenicity of antibody therapeutics may therefore be underestimated. In any case, these

results warrant caution against interpretation of quantitative aspects of ADA formation, and show that even multi-tiered testing approaches may not reveal the full extent of an anti-drug antibody response.

Taken together, although immune complex formation between ADA and drug is an inevitable event, consequences vary. The size of immune complexes considerably increases when the concentration of both antibodies is high, but the anti-idiotype conformation largely prevents the formation of complexes capable of complement activation. Furthermore, no immune cell activation was observed in vitro and complexes larger than a dimer are likely rapidly cleared, whereas dimeric complexes may circulate for extended periods of time. 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 ug/mL), typically only during iv administration. For the vast majority of therapeutic monoclonal antibodies, however, the immune complexes that may be formed will generally have little impact on the safety of the drug.

Materials and Methods

Human samples
To determine the correlation between ADA titer and immune complex size, sera (n=41) with a wide range in ADA titer were selected. For the ex vivo analysis of immune complexes, 8 sera were selected for ADA+ in the antigen binding test (ABT), and 2 sera were selected because they tested negative in the ABT but positive in the drug tolerant acid-dissociation radioimmunoassay (ARIA). All samples (n=51) were sent to Sanquin Diagnostic Services for evaluation of infliximab and ADA concentration using the ABT. For these samples no ethical approval was obtained, since materials used for this study were leftovers from samples taken for routine diagnostic purposes. Materials were used anonymously without any connection to clinical data.
Phagocytosis and FcγR expression was investigated using monocyte derived macrophages. Monocytes were isolated from buffy coats of healthy volunteers (Sanquin) using a CD14 MACS isolation kit (Miltenyi Biotec) or from fresh apheresis material (Sanquin) of healthy volunteers upon informed consent using ELUTRA™ cell separation system (Gambro, Lakewood, CO, USA). For the whole blood cell assays, heparinized blood was used of healthy volunteers (Sanquin). Complement activation was determined using sera of healthy volunteers (Sanquin). Serum was directly frozen at -80°C and only used once after thawing.

Therapeutic antibodies and monoclonal anti-drug antibodies
Adalimumab (Humira, Abbvie) and infliximab (Remicade, Centocor) were used throughout this study. The recombinant human monoclonal anti-infliximab antibodies were described before by van Schie et al.[23] and the monoclonal anti-adalimumab antibodies were described by van Schouwenburg et al.[11] The neutralization status was determined using the TNF competition assay.[37]

Determining affinity of anti-infliximab monoclonal antibodies for infliximab
Surface Plasmon Resonance measurements were performed using a Biacore T200 instrument (GE Healthcare) at 25°C. Monoclonal mouse anti-human IgG (MH16-1, Sanquin Reagents) was immobilized at a concentration of 5 µg/mL in 10 mM sodium acetate, pH 5.0, on a CM5 sensor chip using N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (NHS/EDC) at a flow rate of 10 µL/min. Anti-infliximab monoclonal antibodies were dissolved at 5 µg/mL in PBS (Fresenius Kabi, The Netherlands), pH 7.4, containing 0.05% Tween 20 (PBS-T, Merck), and passed through the cells at 15 µL/min yielding ca. 15-75 response units (RU) of bound antibody. Subsequently, binding of infliximab Fab' (0.42 – 1.25 µg/mL, generated using pepsin, followed by reduction and alkylation with dithiothreitol and iodoacetamide respectively, essentially as described.[15]) dissolved in PBS-T was measured at a flow rate of 15 µL/min. After each run,

immobilized ligand was regenerated by removing bound analyte with 5 µL of 0.1 M phosphoric acid. Adsorptions obtained in the reference channel without bound anti-infliximab antibody were subtracted from those in the other cells. Association and dissociation kinetics were fitted using the models provided with the Biacore analysis software; K_D and SEM were calculated using the same software.

Formation of monoclonal antibody complexes
Complexes between monoclonal anti-drug antibodies and drug were made by separately diluting the antibodies in PBS in a concentration twice the final concentration, after which both antibodies were combined and mixed thoroughly by pipetting up and down. The mixture was incubated for at least 1 hour at room temperature. The (diluted) complexes remained stable for at least 48 hours at 4°C. Diluting the complexes after 1 hour incubation did not significantly alter the complex size; concentrating the complexes resulted in a slight increase in complex size.
To determine the effect of concentration on immune complex size, drug (infliximab or adalimumab labelled with DyLight488 [Invitrogen, labelled according to the manufacturer's instructions], further called IFX-488 or ADL-488) was mixed with an equal amount of monoclonal anti-drug antibodies at a final concentration ranging between 1.8 – 225 µg/ml of both antibodies. After 1 hour incubation at RT, samples were diluted in PBS containing 0.02% Tween-20 so that each sample contained an equal amount of labelled drug, and samples were analysed using HP-SEC.
Essentially the same setup was used to determine the effect of ratio on immune complex size, except that labelled drug was mixed with monoclonal anti-drug antibodies in different ratios.

Analysis of immune complex size using HP-SEC
Samples were analysed by applying 100-250 µl to a Superdex 200 or Superose 6, 10/300 GL column (GE Healthcare, Uppsala Sweden), which was connected to an ÄKTAexplorer HPLC system (GE Healthcare, Uppsala Sweden). Unless stated otherwise, PBS was used as running buffer at a flow speed of 0.5 ml/min, with a total of 30 ml for each run. Elution profiles were monitored by measuring absorbance at 280 nm or by measuring fluorescence in case of IFX-488 or ADL-488 (excitation/emission 488/525 nm) using a Prominence RF-20Axs on-line fluorescence detector (Shimadzu, Kyoto, Japan).

Electron microscopy
A droplet of 3 µl of protein solution (100x diluted to 3 µg/ml in 10 mM Hepes, 140 mM NaCl, pH 7) was applied to a glow-discharged, copper grid supported, carbon film. After incubation for 1 minute the grid was blotted with filter paper (Whatman #1) and stained for 1 minute with 2,3% uranyl acetate in water. Staining solution was blotted with filter paper after which the grid was air-dried. Transmission electron microscopy was done using a Tecnai F20 (Thermo Fisher, formerly FEI company) operating at 120 kV. Images were recorded with a Gatan Ultrascan 4000 digital camera (Gatan).

Formation of polyclonal antibody complexes
To determine the effect of concentration on complex formation, anti-infliximab positive sera were mixed with an equimolar concentration of IFX-488. For each anti-infliximab positive serum, the equimolar concentration of infliximab was determined by adding a titration of IFX-488. Generally, 1 AU/ml anti-infliximab equalled 1-8 ng/ml IFX-488. The highest concentration in which no monomeric IFX-488 was present (as determined by HP-SEC) was used as equimolar concentration.
IFX-488 was diluted to the equimolar concentration in PBS-I (PBS containing 0.1 mg/ml IVIG (Nanogam, Sanquin)) and mixed with an equal volume of anti-infliximab positive serum. After 1 hour incubation at 37°C, samples were diluted with PBS-I to 4 ng/ml IFX-488, 0.2 µm filtered (Pall corporation, USA) and analyzed using HP-SEC.
For each serum, the total area under the curve between the elution volume of 8 and 16.8 ml was set at 100%, after which the percentage of monomers, dimers, tetramers and >tetramers was calculated.

To investigate the effect of ratio on complex formation, essentially the same setup was used, except that anti-infliximab positive serum was incubated with IFX-488 in a 0.25:1, 1:1 or 4:1 ratio (ng/ml IFX-488: AU/ml anti-infliximab). Serum without IFX-488 was used as control to determine the elution pattern of autofluorescent albumin.

Ex vivo analysis of immune complexes.
Anti-infliximab positive sera (600 ul) were 0.1µm filtered (Whatman) and brought onto a Superose 6 column. The eluate was collected in fractions of 250 µl from 4 ml to 22 ml in PBS containing HSA (Albuman, Sanquin; final concentration 0.1 mg/ml) to avoid loss of protein through sticking. As a control, monomeric anti-infliximab 2.1 and complexes between anti-infliximab 2.4 and infliximab were fractionated as well. Fractions were analysed for anti-infliximab positivity using a temperature-shift radioimmunoassay (TRIA) described previously.[16] In short, dissociation and reassociation of immune complexes present in the fractions was promoted by incubation at 37°C in presence of an excess of infliximab F(ab')2-biotin. This was followed by overnight incubation with protein A sepharose, and anti-infliximab was detected using ^{125}I labelled streptavidin. Sample measurements were normalized to the percentage of total radioactively labelled input (% binding).

Association of drug-ADA complexes with Fc-receptors
Biotinylated human FcγRIIa H131, FcγRIIb and FcγRIIIa V158 were purchased from SinoBiologicals (Bejing, China). Fusion FcγRIIIb-IgG2-Fc constructs composed of the extracellular domain of the FcγRIIIb of NA2 allotype and a Fc domain were cloned, produced and site specifically biotinylated as described by Dekkers et al. [manuscript in preparation]. All receptors were simultaneously spotted using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a single SensEye G-streptavidin sensor (Senss, The Netherlands) allowing binding of each antibody to all FcγRs simultaneously. The biotinylated FcγR were spotted in three-fold dilutions, ranging from 30 nM to 1 nM in PBS containing 0.075% Tween-80 (Amresco, USA), pH 7.4.
The immune complexes were then injected over the sensor, determining binding by SPR using the IBIS MX96 (IBIS Technologies). Injection of monomer and complexes took place at 1.5-fold dilution series in PBS 0.075% tween-80 ranging from 2.6 to 20 µg/ml. Regeneration after each sample was carried out with acid buffer (10 mM Gly-HCl pH2.5 + 0.075% Tween-80). Data analysis was done using SPRINT 1.9.4.4 software (IBIS technologies).

Culture of monocyte-derived macrophages
Monocytes were isolated from buffy coats of healthy volunteers (Sanquin) using a CD14 MACS isolation kit (Miltenyi Biotec). Monocytes were cultured in 6-well plates ($1*10^6$/well, Nunc™, Thermo Scientific), 96-well plates ($3.3*10^4$/well, Nunc™, Thermo Scientific), or 8-wells chambered coverglasses ($8.3*10^4$/well, Nunc™, Thermo Scientific) in macrophage culture medium (IMDM (Lonza) supplemented with 10% fetal calf serum (FCS, Bodinco, The Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin (both from Gibco)) at 37°C and 5% CO_2. Cells were incubated for 9 days with GM-CSF (10 ng/ml, CellGenix) or M-CSF (50 ng/ml, eBioscience). Culture medium was changed once.

Imaging flow cytometry analysis of macrophages
Complexes of IFX-488 and either anti-infliximab 2.1 or 2.4 were made in macrophage culture medium at a final concentration of 150 µg/ml of each antibody. Complexes of ADL-488 and anti-adalimumab 2.7 were made at a final concentration of 25 µg/ml of each antibody.
Complexes, IFX-488 or ADL-488 was added to the macrophages at a final concentration of 15 µg/ml of each antibody and incubated together for 15 minutes at 37°C. Culture medium was refreshed and macrophages were incubated for another 60 minutes to allow phagocytosis. Plates were then put on ice, washed twice with cold PBS and cells were harvested using accutase solution (Sigma). Cells were fixed with 4% paraformaldehyde (Sigma Aldrich) in PBS, washed with PBS and analysed with ImagestreamX (Merck Millipore) either unstained or stained with anti-HLA-DR APC (BD Biosciences) prior to analysis. Data was further processed using IDEAS software version 6.1 (Merck

Millipore). Gating strategy involved gating for single cells (Aspect Ratio vs. Area of the brightfield and the channel 2 image) followed by gating on focussed cells (Gradient RMS of the brightfield and channel 2 image). The intensity concentration ratio (ICR, used on channel 2) feature was used to determine internalisation.

Confocal microscopy
Complexes of IFX-488 and anti-infliximab 2.4 or ADL-488 and anti-adalimumab 2.7 were made as described above. Complexes or IFX-488 were added to GM-CSF macrophages at a final concentration of 15 µg/ml of each antibody. After 2 minutes of incubation, culture medium was changed and samples were immediately imaged. Z-stacks of 2 µm were taken every 3 minutes for a total of 60 minutes using a HCX PL 63x 1.32 oil objective of a Leica SP8 confocal microscope adapted with a climate control chamber set at 37°C. LAS AF Lite software (Leica) was used for data analysis.

Blocking of FcγRs and FcγR expression
To block FcγRs, anti-CD16 F(ab')2 (FcγRIII, Ancell), anti-CD32 F(ab')2 (FcγRII, clone AT10, AbD Serotech) and purified human IgG-Fc fragments (FcγRI, Bethyl) were used; the latter because no anti-CD64 antibody gave complete blocking of FcγRI. All antibodies were used at 10 µg/ml and incubated for 20 minutes at 37°C and 5% CO_2. IFX-488 or complexes (IFX-488 + anti-infliximab 2.4, made at a final concentration 150 µg/ml of each antibody) were added to the macrophages at a final concentration of 15 µg/ml of each antibody and incubated together for 15 minutes at 37°C. Macrophages were subsequently harvested as described above.
FcγR expression was determined using the same F(ab')2 fragments for FcγRII and FcγRIII, but for FcγRI anti-CD64 F(ab')2 (clone 10.1, Ancell) was used. F(ab')2 fragments were subsequently stained with goat-anti-mouse IgG APC (H+L; Invitrogen). Binding/phagocytosis of IFX-488 or complexes to macrophages and FcγR expression were determined using the FACSCanto II (BD Biosciences) and analysed using Flowjo V10.

Whole blood cell cultures
Samples were made in WBC culture medium (IMDM containing 0.1% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin) in twice the final concentration. Heparinized blood was diluted five times in WBC culture medium with addition of 30 U/ml heparin (LEO), 0.2% poloxamer and 50 µg/ml polymyxine B (Sigma). Samples and diluted blood were mixed 1:1 in a flat bottom 96-well plate and incubated overnight at 37°C and 5% CO_2. Positive controls consisted of 10 pg/ml LPS, biotin coated fluorescent yellow polystyrene particles (0.05-0.15 µm; Spherotech) or biotin coated fluorescent pink polystyrene particles (0.7-0.9 µm; Spherotech); beads were incubated with anti-biotin IgG1 antibodies (in-house development) in a 10-times molar excess compared to the amount of binding sites on the beads. To obtain uncoated beads, a similar concentration of IVIG instead of anti-biotin was used. Macrophages were incubated overnight at 37°C and 5% CO_2. Supernatant was harvested and stored at -20°C until further analysis. IL-6 concentrations were determined with a human IL-6 ELISA kit (PeliKine, Sanquin) according to the manufacturer's instructions.

Activation of the complement system
All antibodies were dialyzed to Veronal buffer containing 2mM $MgCl_2$, 10 mM $CaCl_2$ and 0.02% tween-20 (Veronal3+). Complexes were made between anti-infliximab 2.1 or 2.4 and infliximab with a final concentration of 150 µg/ml infliximab in Veronal3+ with an additional 0.3% bovine serum albumin solution (BSA, Sigma-Aldrich [Veronal4+]), after which 10 µl of sample was mixed with 20 µl healthy donor serum and incubated for 1 hour at 37°C. After incubation, 20 µl of 0.5M EDTA in H_2O (Titriplex III, Merck) was added and the samples were immediately put on ice. Complement system activation was determined by C4b/c release using an ELISA described previously.[38] In short, monoclonal anti-C4-1 that recognizes a neo-epitope on C4b/c was used to catch C4/bc, after which anti-human C4 was used as detection antibody. Aged human serum containing a known amount of activated C4 was used for the calibration curve.

The buffer control consisted of Veronal4+ as sample; the positive control consisted of aggregated human gammaglobulin (AHG, IVIG incubated at 63°C for 60 minutes). Monomeric anti-infliximab 2.1, anti-infliximab 2.4 and infliximab were tested at 150 µg/ml. IgG1-b12-RGY was a kind gift of Frank Beurskens, Genmab, and tested at 45 µg/ml.

Fractionation of monoclonal antibody complexes for complement analysis.
Complexes were made between anti-infliximab 2.1 or 2.4 and infliximab with a final concentration of 150 µg/ml of each antibody. The complexes were concentrated twice using a 10 kDa Amicon Ultra Centrifugal Filter (Merck Millipore) and brought onto a Superose 6 column. Veronal buffer containing 2mM $MgCl_2$ and 10 mM $CaCl_2$ was used as running buffer. The eluate was collected from 6.5 ml to 16 ml in fractions of 250 µl in 30% BSA (final concentration 0.3% BSA) to avoid loss of protein through sticking. Antibody concentration in each fraction was determined using the area under the curve in mAU*ml, measured at 280 nm. The antibody concentration was calculated und the assumption that 1.4 mAU = 1 µg/ml. Fractions were diluted in Veronal4+ to 45 µg/ml. To determine complement activation, 10 ul of each fraction was mixed with 20 ul of serum, and tested as described above.

Statistics
For correlation analysis, Spearman's rank test was performed. Comparison between more than two groups was performed using a one-way ANOVA, with Sidak's or Dunnett's multiple comparison test. p-values of ≤0.05 were considered significant.

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 Rand. *Circ J*. 2017. doi:10.1253/circj.CJ-16-1310.

2. Bartelds GM, Krieckaert CLM, Nurmohamed MT, et al. Development of Antidrug Antibodies Against Adalimumab and Association With Disease Activity and Treatment Failure During Long-term Follow-up. *J Am Med Assoc*. 2011;305(14):1460-1468.

3. Vennegoor A, Rispens T, Strijbis EMM, et al. Clinical relevance of serum natalizumab concentration and anti-natalizumab antibodies in multiple sclerosis. *Mult Scler J*. 2013;19(5):593-600. doi:10.1177/1352458512460604.

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 (Oxford)*. 2013;52(7):1245-1253. doi:10.1093/rheumatology/ket017.

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. *Jt Bone Spine*. 2016;83(2):167-171. doi:10.1016/j.jbspin.2015.04.020.

6. Bender NK, Heilig CE, Dröll B, Wohlgemuth J, Armbruster F-P. Immunogenicity , efficacy and adverse events of adalimumab in RA patients. *Rheumatol Int*. 2007;27:269-274. doi:10.1007/s00296-006-0183-7.

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(7):469-475.

8. Calabresi PA, Giovannoni G, Confavreux C, et al. The incidence and significance of anti-natalizumab antibodies: Results from AFFIRM and SENTINEL. *Neurology*. 2007;69:1391-1403. doi:10.1212/01.wnl.0000277457.17420.b5.

9. 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 Clincial Immunol*. 2016;3(2):125-126. doi:10.1016/j.nurpra.2012.12.024.

10. 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(1):104-109. doi:10.1136/annrheumdis-2012-201445.

11. 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(50):34482-34488. doi:10.1074/jbc.M114.615500.

12. Lundkvist M, Engdahl E, Holmen C, et al. Characterization of anti-natalizumab antibodies in multiple sclerosis patients. *Mult Scler J*. 2012. doi:10.1177/1352458512462920.

13. Kosmač M, Avčin T, Toplak N, Simonini G, Cimaz R, Šerbec VC. Exploring the binding sites of anti-infliximab antibodies in pediatric patients with rheumatic diseases treated with infliximab. *Pediatr Res*. 2011;69(3):243-248.

14. 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(5):1000-1006. doi:10.1007/s10875-012-9705-0.

15. van Schouwenburg PA, Bartelds GM, Hart MH, Aarden L, Wolbink GJ, 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. doi:10.1016/j.jim.2010.09.005.

16. 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. doi:10.1016/j.jim.2015.01.007.

17. 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;66:253-256. doi:10.1136/ard.2006.057406.

18. 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):1-6. doi:10.1097/01.MIB.0000436951.80898.6d.

19. 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(2):323-333. doi:10.1053/j.gastro.2005.11.030.

20. Colombel J-F, Sands BE, Rutgeerts P, et al. The safety of vedolizumab for ulcerative colitis and Crohn's disease. *Gut*. 2016;(Cd):1-13. doi:10.1136/gutjnl-2015-311079.

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. doi:10.1186/s13075-016-0930-4.

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(1):164. doi:10.1186/s12974-016-0635-2.

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(7):1285-1288. doi:10.1136/annrheumdis-2016-211035.

24. Heidelberger BYM, Kendall FE. The precipitin reaction between type III pneumococcus polysaccharide and homologous antibody. *J Exp Med*. 1935;61(4):563-591.

25. Diebolder C a, Beurskens FJ, de Jong RN, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science (80-)*. 2014;343(6176):1260-1263. doi:10.1126/science.1248943.

26. Wang G, de Jong RN, van den Bremer ETJ, et al. Molecular Basis of Assembly and Activation of Complement Component C1 in Complex with Immunoglobulin G1 and Antigen. *Mol Cell*. 2016;63(1):135-145. doi:10.1016/j.molcel.2016.05.016.

27. 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(2):578-585. doi:10.1124/jpet.104.079277.coincident.

28. 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(365):365ra158. doi:10.1126/scitranslmed.aaf9418.

29. Wright JK, Tschopp J, Jaton JC, Engel J. Dimeric, trimeric and tetrameric complexes of immunoglobulin G fix complement. *Biochem J*. 1980;187:775-780.

30. Doekes G, Vanes L a, 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(4):705-713.

31. Steenholdt C, Svenson M, Bendtzen K, Thomsen OØ, Brynskov J, Ainsworth MA. Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease. *Aliment Pharmacol Ther*. 2011;34:51-58.

32. 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 neuroinflammation*. 2014;1(4):e47. doi:10.1212/NXI.0000000000000047.

33. 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(6):1315-1324. doi:10.1016/S0002-9270(03)00231-4.

34. 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(April):1172-1183. doi:10.1111/apt.12330.

35. 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 (Oxford)*. 2011;50(8):1445-1452. doi:10.1093/rheumatology/ker124.

36. Kaur P, Chow V, Zhang N, Moxness M, Kaliyaperumal A, Markus R. A randomised, single-blind, single-dose, three-arm, parallel-group study in healthy subjects to demonstrate pharmacokinetic equivalence of ABP 501 and adalimumab. *Ann Rheum Dis*. 2016:annrheumdis-2015-208914. doi:10.1136/annrheumdis-2015-208914.

37. 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;74:311-314. doi:10.1136/annrheumdis-2014-206237.

38. Wolbink GJ, Bollen J, Baars JW, et al. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the

classical pathway. *J Immunol Methods*. 1993;163(1):67-76. doi:10.1016/0022-1759(93)90240-8.

Table 1: *Characteristics of the eight monoclonal anti-infliximab antibodies.* All monoclonal antibodies were neutralizing, as determined with the TNF competition assay.[37] K_D and SEM were determined using SPR (n=4).

Clone	Neutralizing	Affinity	
		K_D (pM)	SEM
1.1	Yes	76	4
1.2	Yes	280	85
1.3	Yes	1220	165
1.4	Yes	259	20
2.1	Yes	1670	171
2.2	Yes	134	4,5
2.3	Yes	460	35
2.4	Yes	143	10
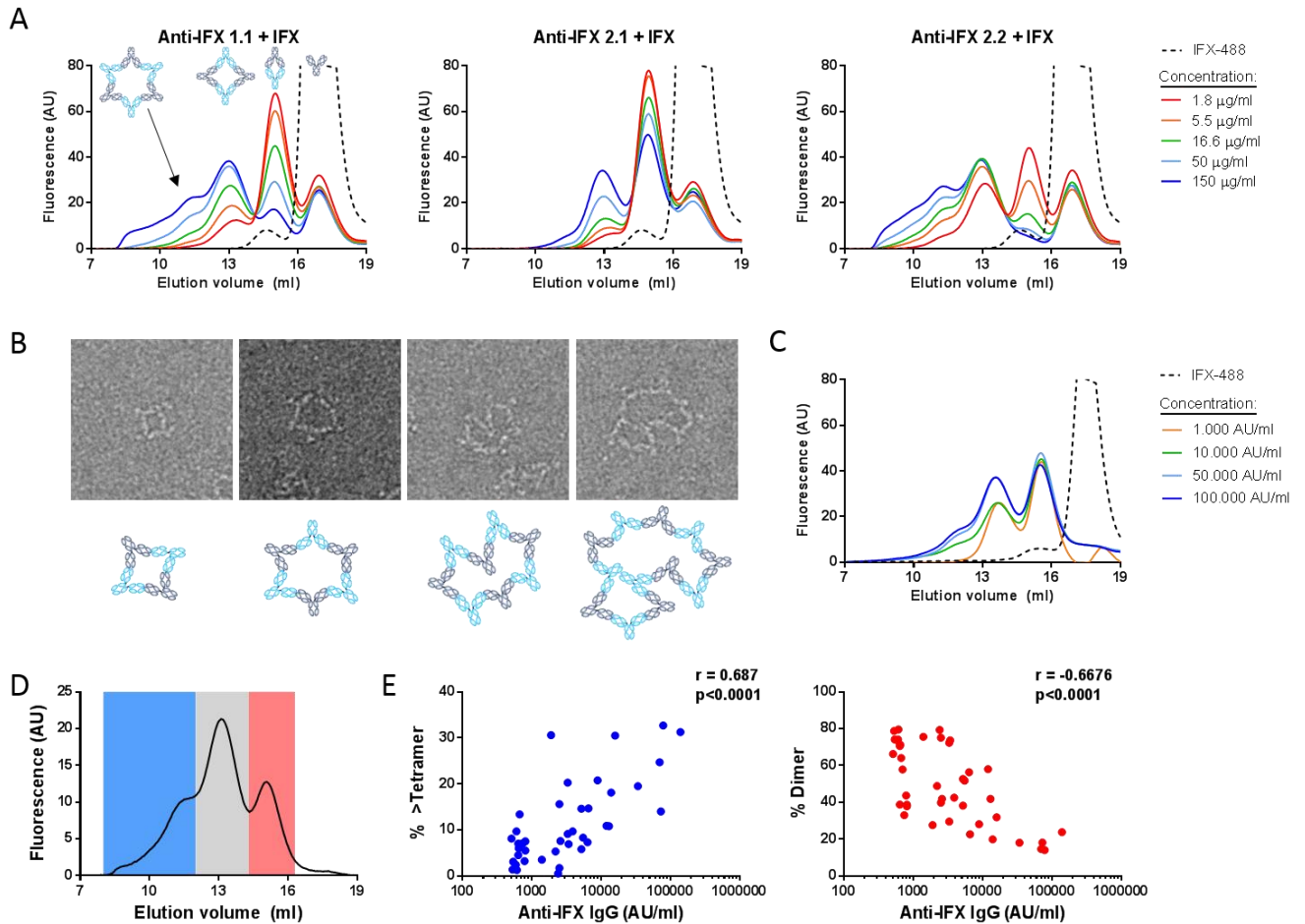
Figure 1: *Immune complex size is highly dependent on concentration.* **A)** IFX-488 and different monoclonal anti-infliximab antibodies are mixed at increasing concentrations. Afterwards, samples were diluted so that each sample contained an equal amount of labelled drug, and analysed by HP-SEC. **B)** TEM analysis of complexes between monoclonal anti-infliximab 2.2 and infliximab at 150 µg/ml, and schematic representation of their conformation. See also Supplemental Figure 5 **C)** A titration of polyclonal ADA from a single patient mixed with an equimolar amount of IFX-488, analysed by HP-SEC. n=3 separate experiments **D,E)** Serum of ADA positive patients is 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) are 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-infliximab IgG titer for each patient (n=41). Spearman's ρ for >Tetramers: r=0.687, p<0.0001 and for dimers: r=-0.6676, p<0.0001.
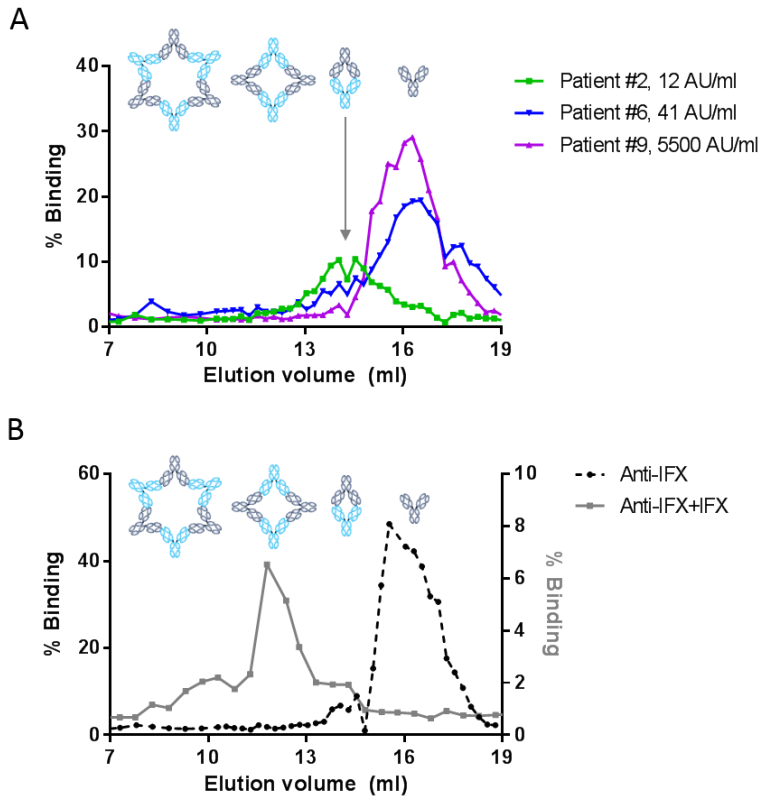
Figure 2: O*nly small complexes and monomeric ADA are detected in patients at trough.* ADA presence was determined with a drug tolerant assay in **A)** fractionated ADA+ sera and **B)** fractionated monomeric anti-infliximab 2.1 and complexes of anti-infliximab 2.4 and infliximab. Representative plots are shown for 3 out of 10 patients (A) and duplicate measurements of monoclonal complexes (B). Percentage binding is the sample measurement normalized to the total radioactively labelled input.
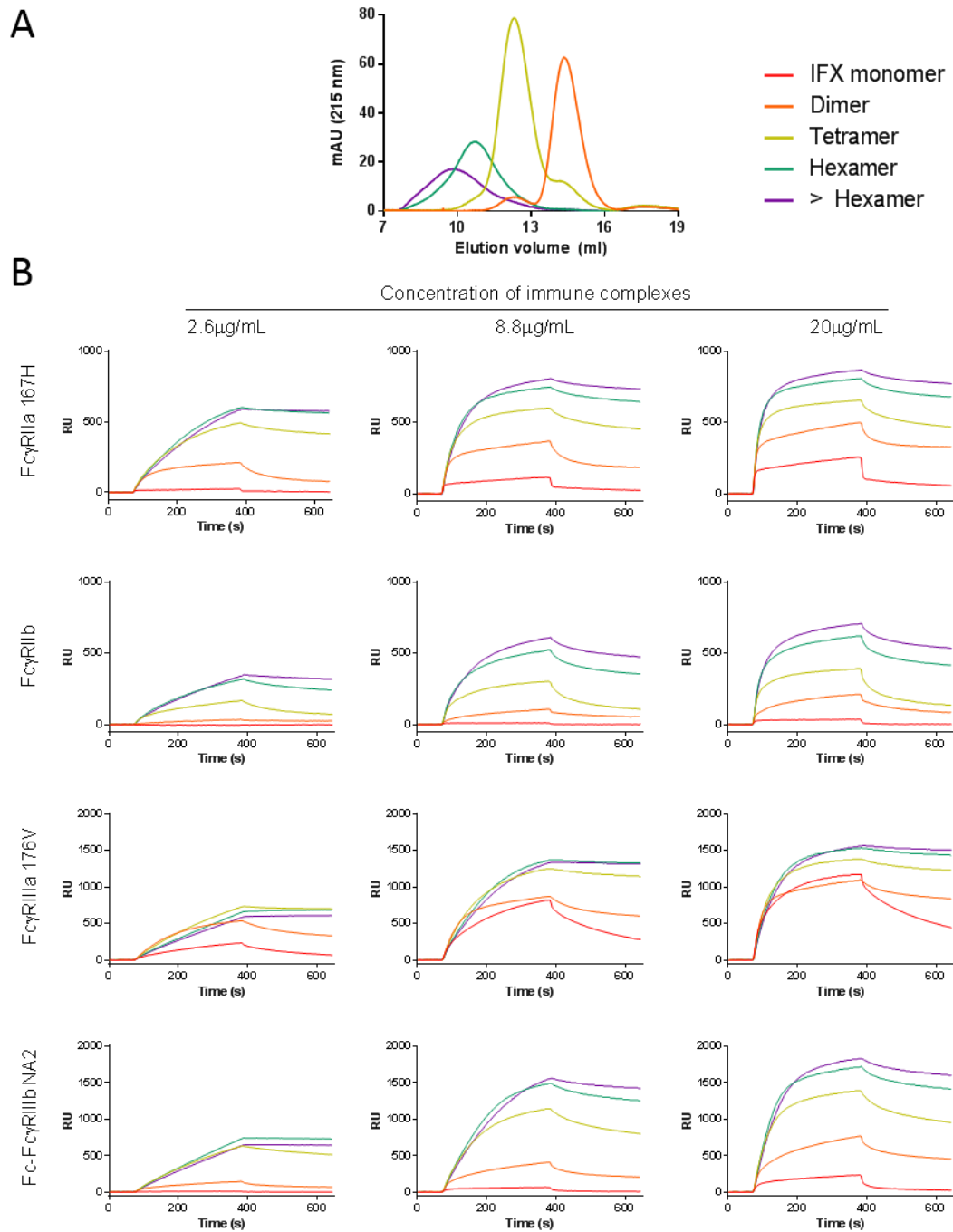
Figure 3: *Binding to FcγRs increases with complex size.* **A)** Example of reanalysed fractions of immune complexes, done with HP-SEC. **B)** Representative sensograms of immune complexes binding to FcγRIIa, FcγRIIb, FcγRIIIa, and FcγRIIIb on the chip. Receptor densities of shown sensograms are 10 nM for FcγRIIa and FcγRIIb, 3nM for FcγRIIIa and 30 nM for FcγRIIIb, n=3 individual experiments, RU = response unit.
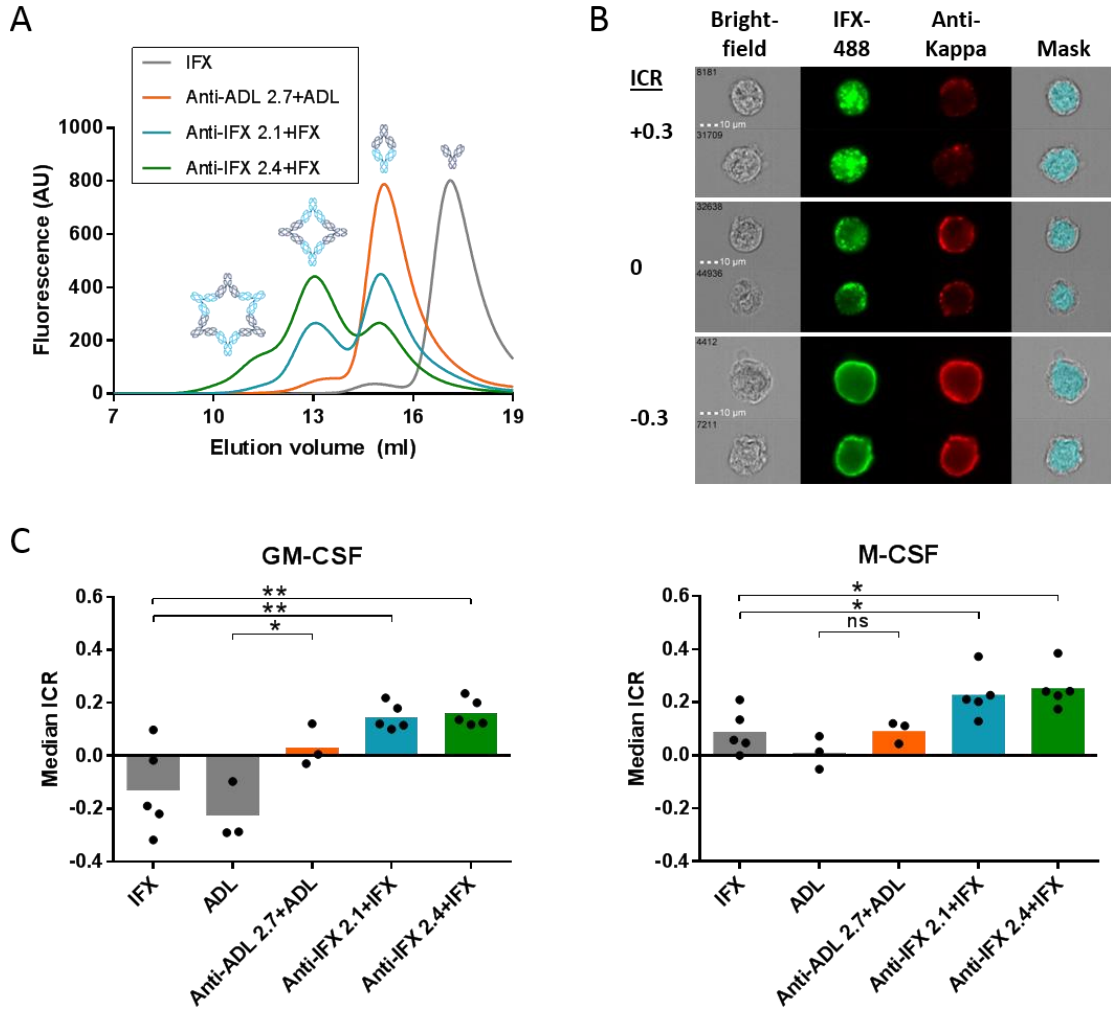
Figure 4: *Complexes larger than dimers are efficiently internalized by macrophages.* **A)** Example of the fluorescent complexes used. **B)** Representative examples of three different intensity concentration ratio (ICR) values. The ICR calculates the logit-transformed ratio between the fluorescent intensity inside the cell and the fluorescent intensity of the entire cell. From left to right: bright-field image, green fluorescence of 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. n≥3 individual donors measured in duplicate. Statistical differences were calculated by one-way ANOVA, with Sidak's multiple comparison test.
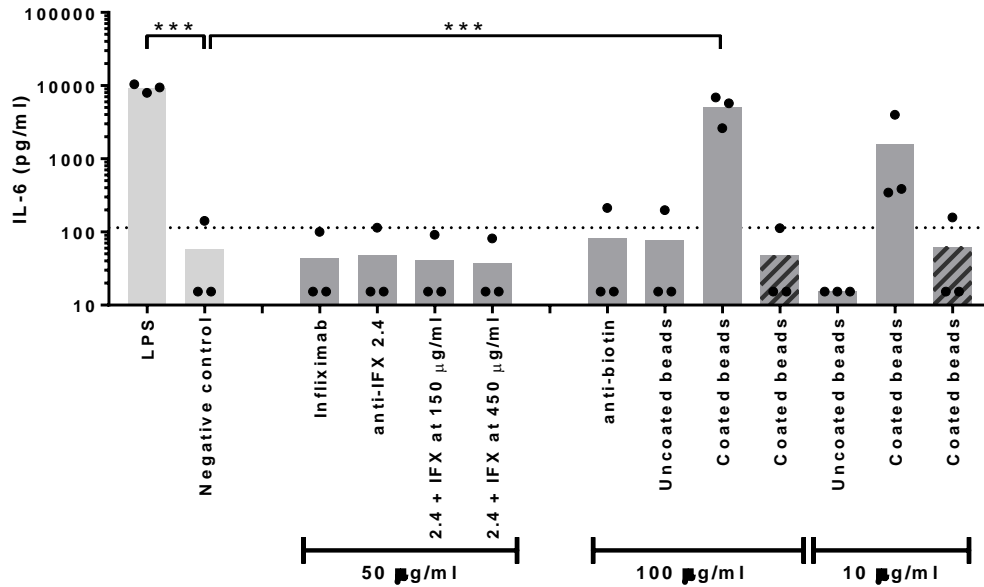
Figure 5: Large immune complexes do not have a pro-inflammatory effect on whole blood cell cultures. IL-6 production by whole blood cell cultures was determined after addition of monomeric antibodies or large immune complexes (infliximab + anti-infliximab 2.4 mixed at 150 and 450 µg/ml of both antibodies), all tested at 50 µg/ml of each antibody. Anti-biotin coated beads (100 or 10 µg/ml) and LPS were used as positive control, WBC medium was used as negative control. To inhibit the complement system EDTA was added (striped bars). Statistical differences were calculated by one-way ANOVA, and further compared with the negative control using Dunnett's multiple comparison test. Unless indicated, no significant differences were found.
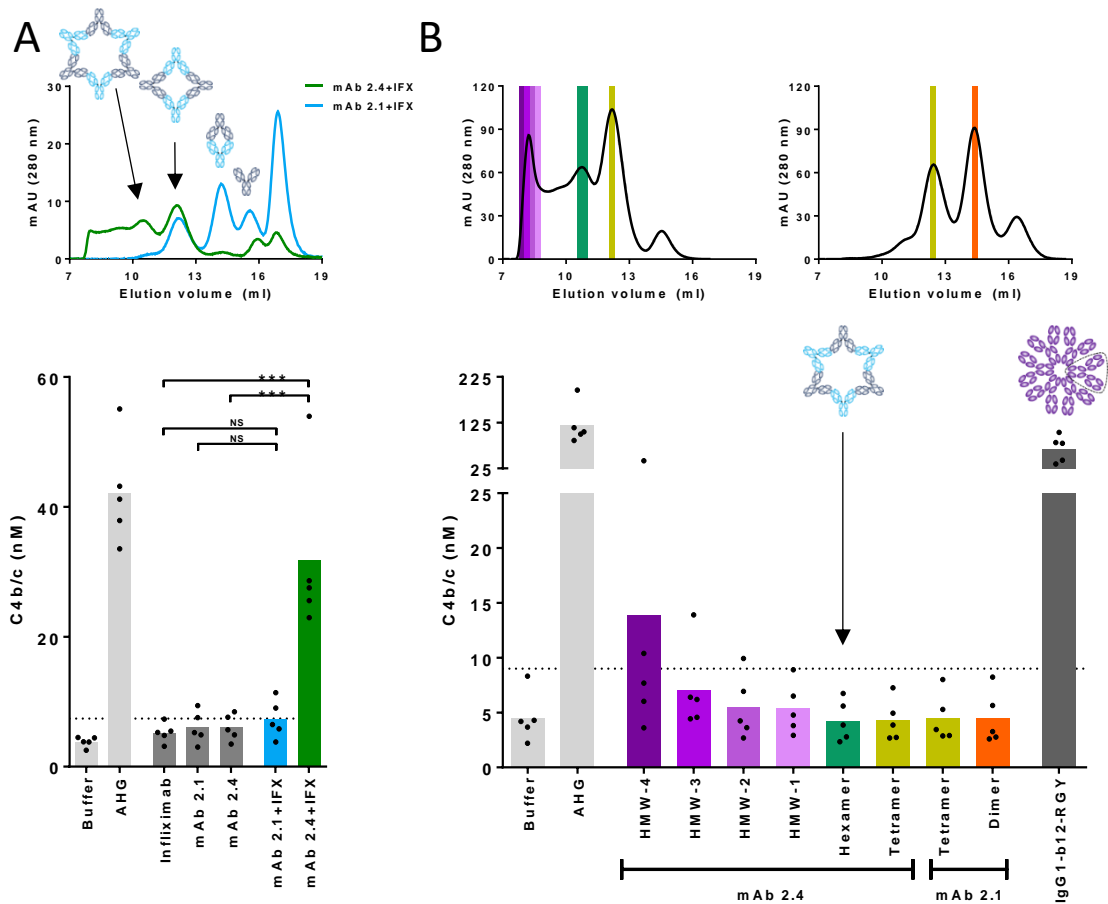
Figure 6: *Anti-idiotype complexes larger than hexamers are required for activation of the complement system.* **A)** Representative elution patterns of complexes between infliximab with either anti-infliximab 2.1 or 2.4 made at 150 µg/ml of both antibodies. Bottom: Complexes (final antibody concentration 100 µg/ml in serum) were tested in the C4b/c ELISA. n= 5 serum donors, each dot represents the mean of 2-5 experiments per donor. Significant differences were calculated by one-way ANOVA, with Sidak's multiple comparison test. **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 colored boxes. Bottom: All fractions and IgG1-b12-RGY were separately tested in the C4b/c ELISA (final antibody concentration 15 µg/ml in serum). HMW = high molecular weight, n=5 serum donors, representative of ≥4 individual experiments. Buffer was used as negative and AHG (aggregated human gammaglobulin) as positive control. Dotted line is twice the mean buffer value. Similar colors in top and bottom figures show corresponding samples.
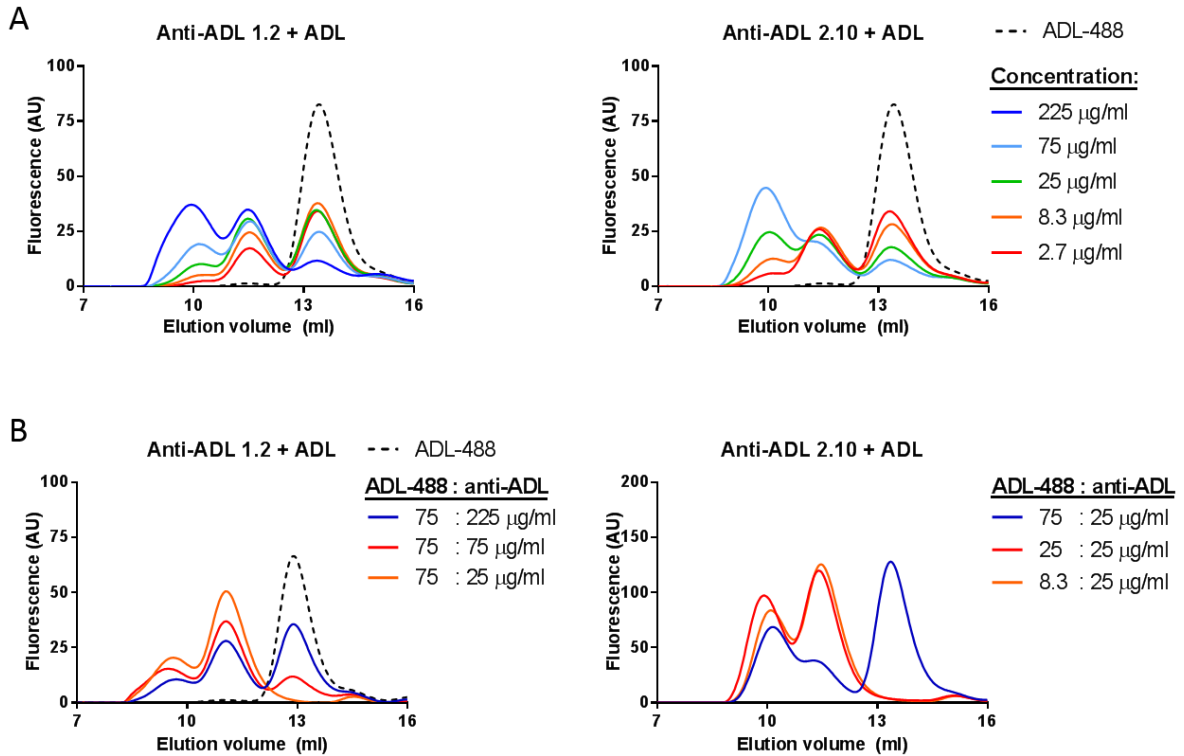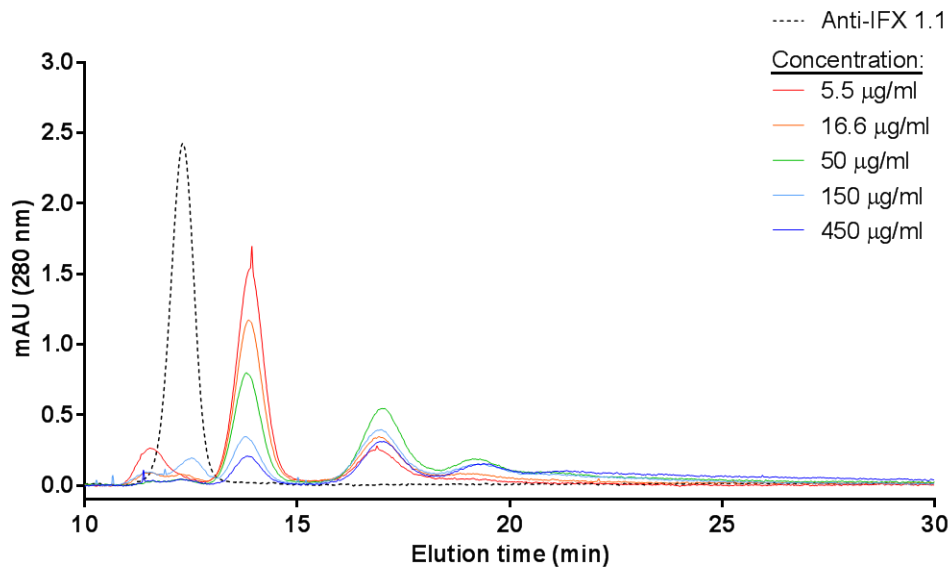
Supplemental Figures
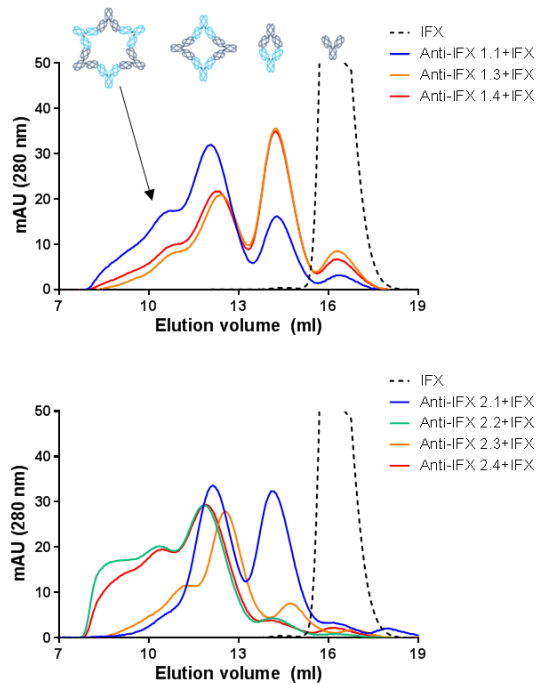
Supplemental figure 1: *Distinct sizes of complexes are formed by each monoclonal anti-infliximab antibody.* Infliximab (IFX) and anti-infliximab (mAb) were mixed at 150 µg/ml of each antibody. Immune complex size was analyzed using HP-SEC. Dotted line is infliximab in monomeric form.
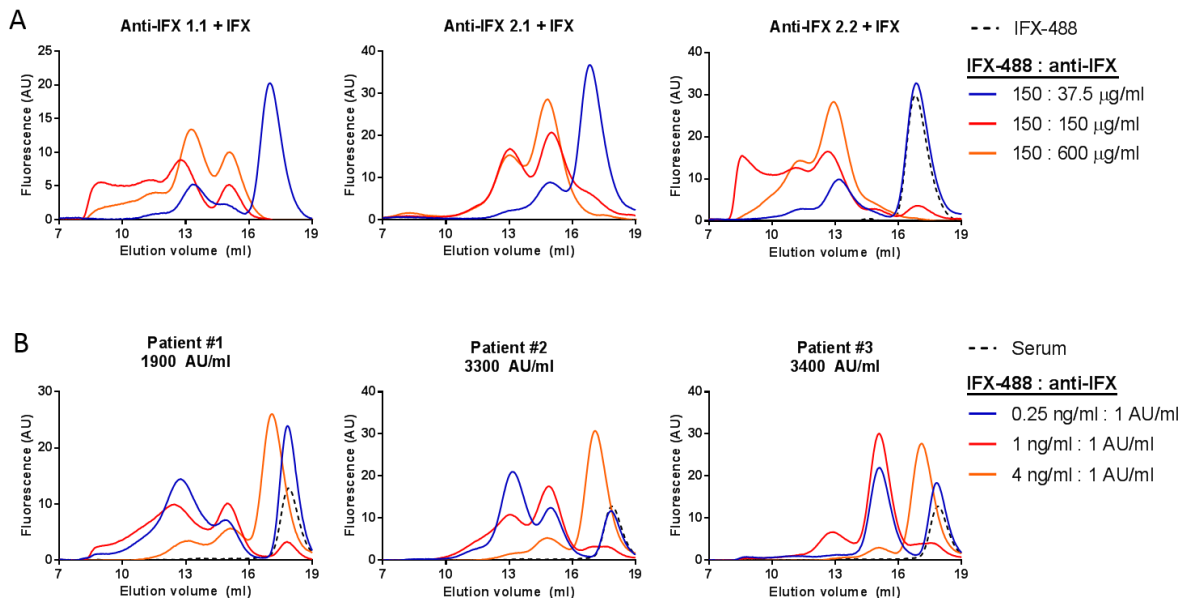

Supplemental figure 2: *Complex size is determined by the ratio between infliximab and anti-infliximab.* **A)** Monoclonal antibody complexes consisting of a fixed concentration of IFX-488 mixed with varying concentrations of anti-infliximab. **B)** Polyclonal antibody complexes made with a fixed amount of serum and varying concentrations of IFX-488. Analysis was performed with HP-SEC. Elution patterns were selected from at least 5 (A) or 12 (B) separate measurements of different ratios.

Supplemental figure 3: *Concentration and ratio determine the size of complexes between adalimumab and monoclonal anti-adalimumab antibodies.* **A)** ADL-488 and different monoclonal anti-adalimumab antibodies are mixed at increasing concentrations and analysed by HP-SEC. **B)** Complexes between ADL-488 and monoclonal anti-adalimumab when mixed in different ratios and analysed with HP-SEC. Elution patterns were selected from 5 separate measurements of different ratios.


Supplemental figure 4: *Immune complex size is highly dependent on concentration.* IFX-488 and anti-infliximab 1.1 are mixed at increasing concentrations and analysed by AF4. Complexes larger than hexamers do not form distinct peaks likely due to their highly varying conformations. Representative of n=2 experiments.
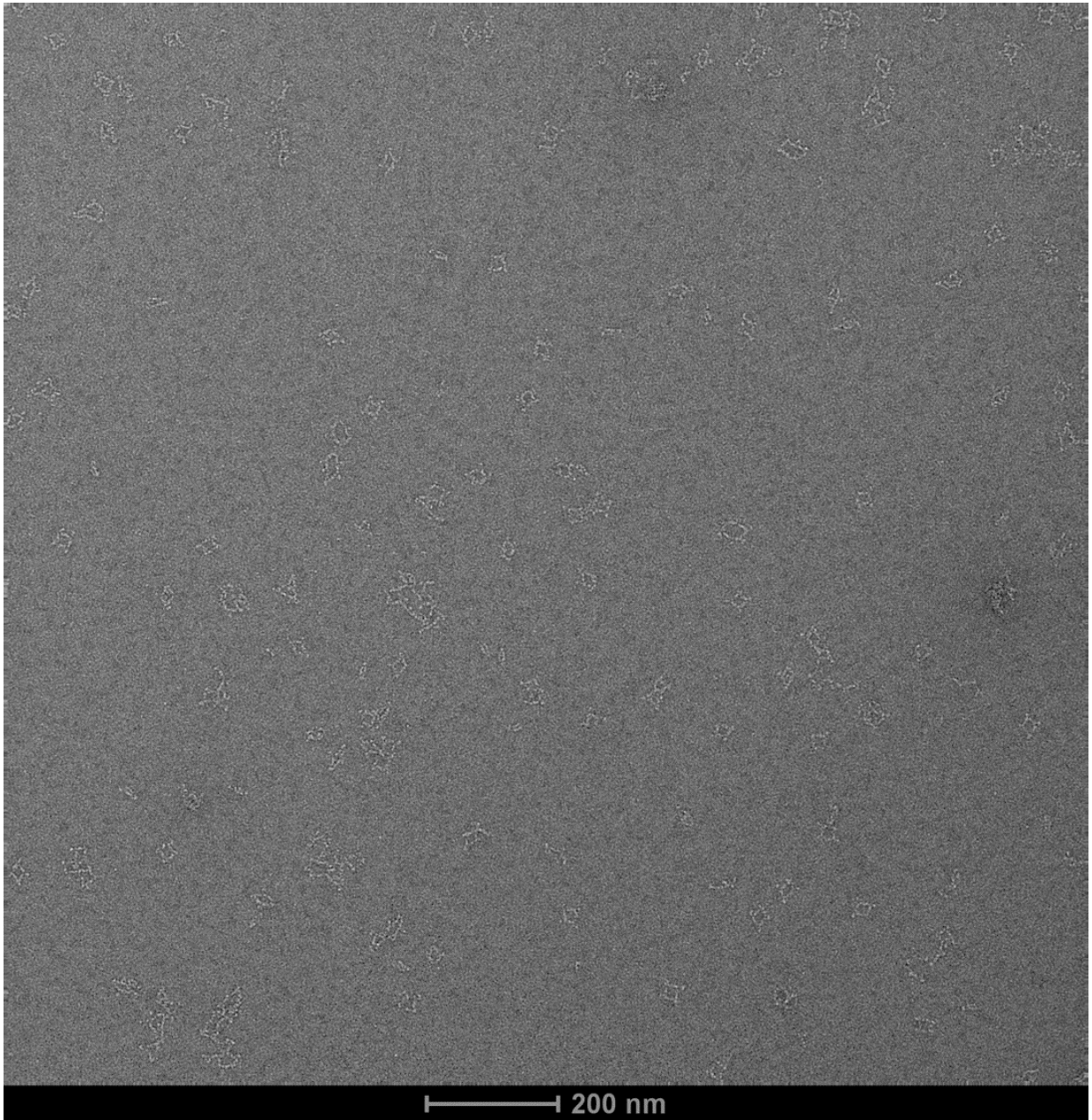
Supplemental figure 5: *Many different sizes of immune complexes are formed.* TEM analysis of complexes between monoclonal anti-infliximab 2.2 and infliximab at 150 µg/ml.
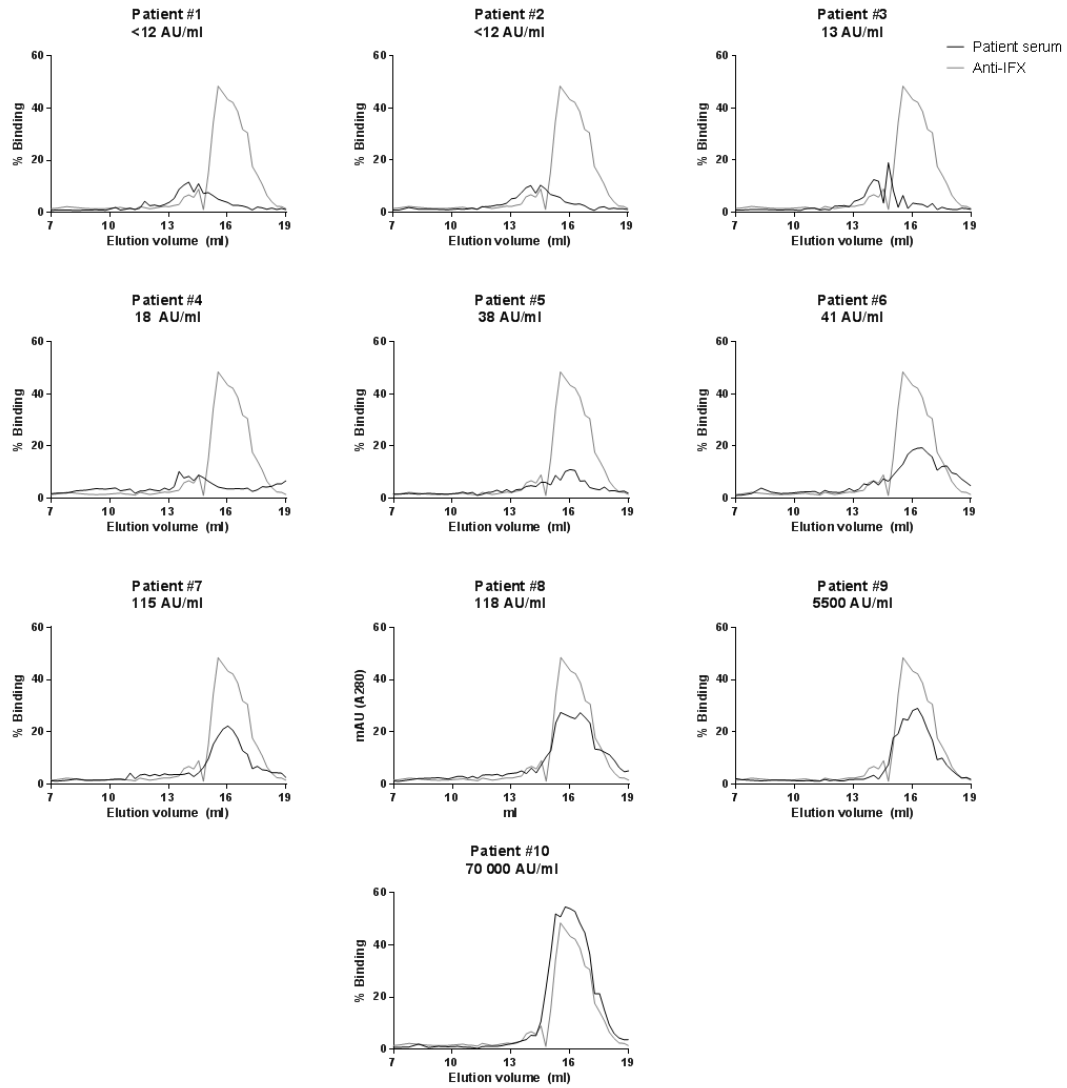
Supplemental figure 6: *Only small complexes and monomeric ADA are detected in patients at trough level.* ADA presence was determined with a drug tolerant assay in fractionated patient sera containing various ADA titers (range <12 - 70 000 AU/ml). Fractionated monomeric anti-infliximab 2.1 was used to determine the elution volume of monomeric ADA. Percentage binding is the sample measurement normalized to the total radioactively labelled input.
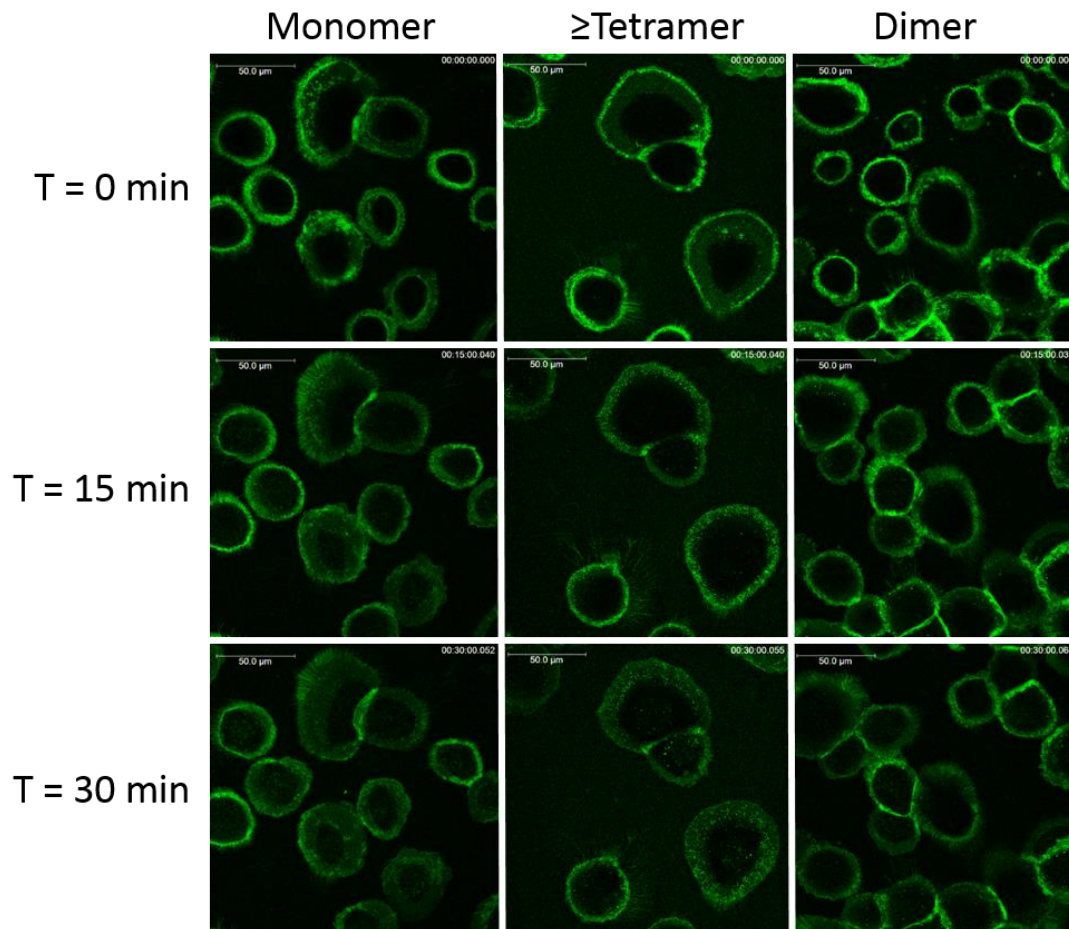
Supplemental figure 7: *Complexes larger than dimers are efficiently internalized.* Stills of a time-lapse of GM-CSF macrophages incubated with green fluorescent monomers (IFX-488), dimers (ADL-488+anti-ADL 2.7) or a pool of complexes larger than dimers (IFX-488+anti-IFX 2.4). Combined stacks, scale bars indicate 50 μm, representative images shown of n=3 individual donors.
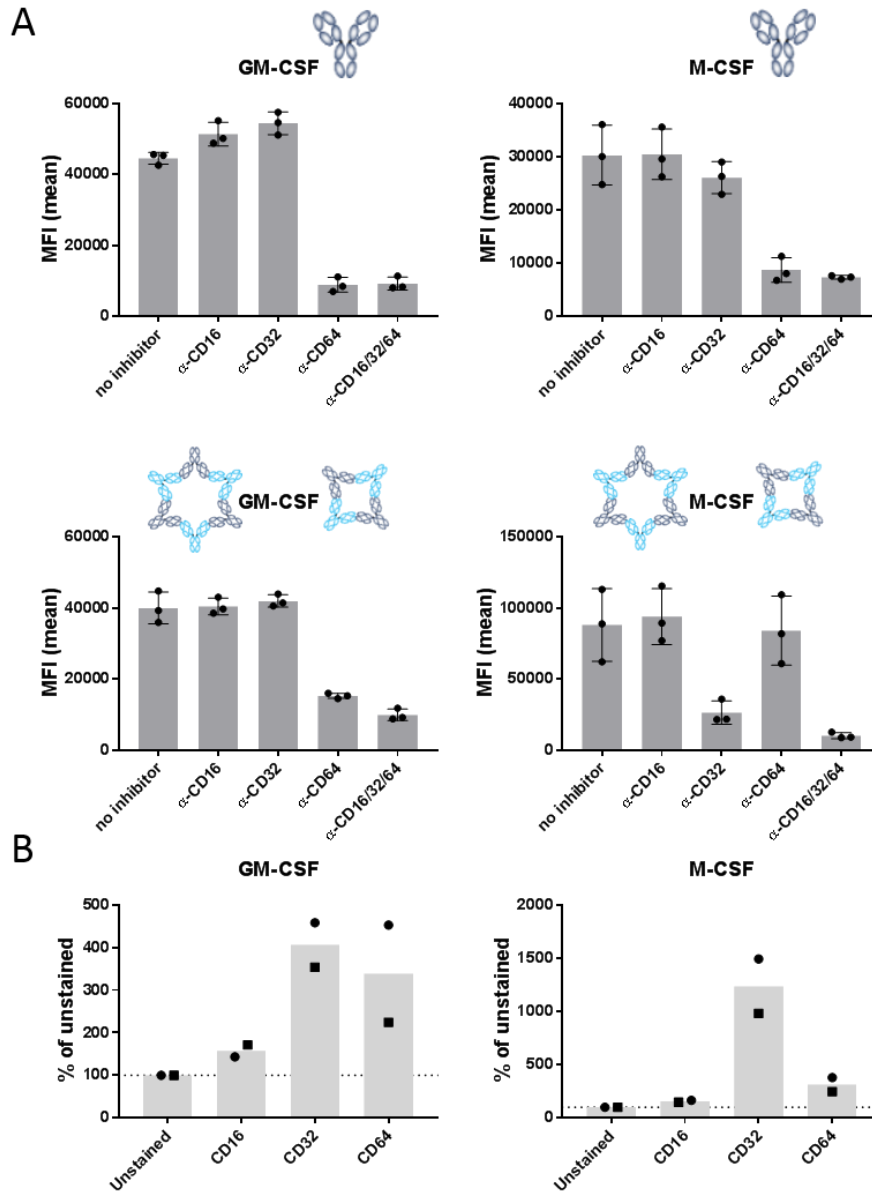
Supplemental figure 8: *Blocking of FcγRs abrogates binding and/or phagocytosis of infliximab and immune complexes.* A) FcγRs were blocked individually or combined, after which IFX-488 (top graphs) or complexes (IFX-488 + anti-infliximab 2.4 at 150 µg/ml, bottom graphs) were added to GM-CSF (left graphs) or M-CSF (right graphs) macrophages. Binding/phagocytosis of antibodies was assessed using FACS. Duplicate measurements of n=3 healthy donors, MFI = mean fluorescence intensity. B) FcγR expression on GM-CSF (left) or M-CSF (right) macrophages, determined using FACS. Duplicate measurements of n=2 healthy donors, MFI of unstained samples is set at 100%.
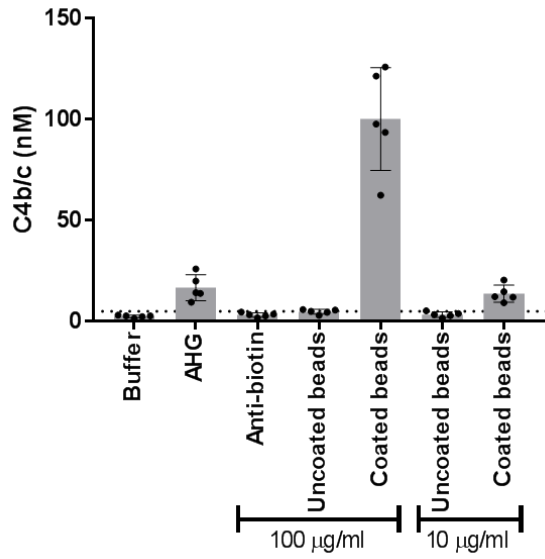
Supplemental figure 9: *Antibody coated beads activate complement.* Complement activation was determined for beads coated with anti-biotin antibodies at 100 or 10 µg/ml. The monomeric anti-biotin antibodies or uncoated beads were tested as well. Buffer and AHG (aggregated human gammaglobulin) were used as negative and positive control, respectively. Dotted line is twice the mean buffer value, n=5 healthy serum donors.