Recombinant H22(scFv) blocks CD64 and prevents the capture of anti-TNF monoclonal antibody
A potential strategy to enhance anti-TNF therapy

Dmitrij Hristodorov1, Radoslav Mladenov1, Hannes Brehm1, Rainer Fischer2,3, Stefan Barth1,2,1, Theo Thepen2,1,1

1Department of Experimental Medicine and Immunotherapy; Institute of Applied Medical Engineering; University Hospital RWTH Aachen; Aachen, Germany; 2Department of Pharmaceutical Product Development; Fraunhofer Institute for Molecular Biology and Applied Ecology IME; Aachen, Germany; 3Institute of Molecular Biotechnology (Biology VII); RWTH Aachen University; Aachen, Germany

y These authors contributed equally to this work.

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Abbreviations: TNF, tumor necrosis factor; mAb(s), monoclonal antibodie(s); IFN-γ, interferon gamma; scFv, single chain fragment variable; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; AD, atopic dermatitis; mTNF, transmembrane tumor necrosis factor; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cellular cytotoxicity; aglycoIgG1, aglycosylated IgG1; Fcγ, fragment crystallizable gamma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that plays a critical role in many inflammatory diseases. Soluble TNF can be neutralized by monoclonal antibodies (mAbs), and this is a widely-used therapeutic approach. However, some patients do not respond to anti-TNF therapy due to the increased expression of CD64 on monocytes and macrophages. A recent study has shown that CD64 captures anti-TNF mAbs via their Fcγ domain, which induces the transcription of pro-inflammatory genes. Specific blocking of CD64 could therefore be a promising strategy to improve the response to anti-TNF therapy. We used the CD64-specific antibody fragment H22(scFv) and tested its activity against the human CD64+ cell line HL-60. When stimulated with interferon gamma (IFN-γ), these cells represent a pro-inflammatory phenotype of the monocyte/macrophage lineage. We found that H22(scFv) binds selectively to and blocks CD64, preventing the capture of anti-TNF mAb. Importantly, H22(scFv) itself does not induce CD64 activation. We also found that transmembrane TNF on HL-60 cells stimulated with IFN-γ also contributes to the capture of anti-TNF mAb, although via their Fab domain. In conclusion, the specific blocking of CD64 by H22(scFv) could be used a possible anti-inflammatory mechanism for potentiating the effect of anti-TNF antibodies.

Introduction

Tumor necrosis factor (TNF) is a key cytokine involved in the pathogenesis of several inflammatory diseases, including inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and atopic dermatitis (AD).1-3 It is initially produced as a transmembrane protein (mTNF), which is cleaved off by the membrane-resident protease ADAM17 to yield its soluble form.4 Both forms can bind to either of the two known TNF receptors (TNFR1 or TNFR2), thus activating NF-κB and AP-1,5 which are regulators of pro-inflammatory genes including those encoding cytokines such as IL-1β, IL-6, IL-8, GM-CSF and TNF itself.6

The development of monoclonal antibodies (mAbs) that neutralize soluble TNF has led to a number of successful therapies that interrupt the downstream cascade of pro-inflammatory events.7 However, some patients do not respond to anti-TNF therapy, and recently this was shown to correlate with the increased expression of CD64 on monocytes and macrophages in non-responding IBD patients.8 The lower response was dependent on interactions between the Fcγ domain of the full-length anti-TNF mAbs and CD64. For example, when the two anti-TNF mAbs infliximab (Remicade®) and adalimumab (Humira®) are captured by CD64 via the Fcγ domain, this induces a pro-inflammatory downstream signaling cascade, which counteracts the positive effects from neutralizing TNF. Pre-blocking the Fcγ receptors with purified IgG1 Fcγ domains improved the efficacy of these mAbs under laboratory conditions.8 However, this approach is unsuitable in the clinic because the low-affinity Fcγ receptors CD32 and CD16 will also be blocked, thus inducing immunosuppression by inhibiting

*Correspondence to: Theo Thepen; Email: theophilus.thepen@ime.fraunhofer.de
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antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). This is of particular concern because anti-TNF therapy can already induce moderate immunosuppression, so further compromising the immune system would be highly undesirable. We therefore aimed to develop a proof of concept for an intervention strategy that targets CD64 but does not affect CD16 or CD32. We used the human pro-myelocytic cell line HL-60 as a model because these cells represent the pro-inflammatory phenotype of the monocyte/macrophage lineage, and their basal CD64 expression can be induced by stimulation with, for example, interferon gamma (IFN-\(\gamma\)). Initial experiments with human IgG4, whose affinity toward CD16 and CD32 is 100-fold lower than CD64, showed that the capture of anti-TNF mAb can be reduced. In contrast to our expectations however, this effect was mediated by blocking CD16 rather than CD64. Human aglycosylated mAbs (aglycoIgG1) can be used as an alternative because their affinity toward all Fc\(\gamma\) receptors is substantially reduced compared with antibodies with correct glycan structures, but they still retain a residual affinity toward CD64, allowing them to be used as CD64-selective inhibitors. Notably, human aglycoIgG1 blocked CD32 but not CD64, and did not reduce the capture of anti-TNF mAb, indicating that CD32 is not involved in this process.

CD64-selective blocking was facilitated by the heterologous expression of an Fc-free, anti-CD64, single chain antibody fragment, called H22(scFv). This was shown to bind specifically to CD64 cells and to substantially reduce the capture of anti-TNF mAb by pro-inflammatory (stimulated with IFN-\(\gamma\)) HL-60 cells. In contrast to adalimumab, H22(scFv) did not activate CD64 upon binding. We also found that the expression of mTNF on the surface of IFN-\(\gamma\)-stimulated pro-inflammatory HL-60 cells also contributes to capture of anti-TNF mAb.

**Results**

**Generation and analysis of the CD64-specific antibody fragment H22(scFv)**

We used a single chain antibody fragment, H22(scFv), which binds specifically to CD64. The H22(scFv) coding sequence was fused in frame with an N-terminal peB leader peptide and a His\(_{10}\) tag (Fig. 1A). The protein was successfully expressed in *Escherichia coli* (E. coli), using the periplasmic stress expression protocol. We obtained a yield of 0.14 mg purified protein/g bacterial pellet. The identity of purified H22(scFv) was confirmed by SDS-PAGE followed by staining the gel with Coomassie brilliant blue, and by western blot using polyhistidine-specific antibodies (Fig. 1B). In addition, flow cytometry showed that H22(scFv) specifically binds to CD64\(^+\) target cells (Fig. 1C), with no binding to CD64\(^-\) L540cy cells (data not shown). Bound H22(scFv) was detected with anti-His\(_{10}\)-Alexa Fluor 488.

**Figure 1.** Generation and analysis of CD64-specific H22(scFv). (A) Schematic representation of the expression cassette for H22(scFv). The open-reading frame is under the control of a T7 promoter and contains the following elements: peB leader peptide, signal peptide for periplasmic secretion; His\(_{10}\), polyhistidine sequence used for purification and detection of the recombinant protein; ECS, enterokinase cleavage site; V\(_{H}\), heavy chain variable fragment; V\(_{L}\), light chain variable fragment. (B) Analysis of purified H22(scFv). The recombinant protein was purified by immobilized metal ion affinity chromatography and size exclusion chromatography. Enrichment was confirmed by discontinuous SDS-PAGE followed by staining with Coomassie brilliant blue and western blotting using a mouse-anti-His\(_{5}\) primary mAb (1:5000) detected using an alkaline phosphatase-conjugated anti-mouse-IgG mAb (1:5000) followed by staining with NBT/BCIP substrate (Life Technologies). The theoretical molecular weight is ~27 kDa. (C) Cell-binding analysis. Purified H22(scFv) was tested for its ability to bind CD64\(^+\) HL-60 cells (CD64\(^-\) L540cy cells were used as a negative control; data not shown). Bound H22(scFv) was detected with anti-His\(_{10}\)-Alexa Fluor 488.

The reduced capture of anti-TNF mAb correlates quantitatively with CD64-specific blocking by H22(scFv)

Pre-blocking of CD64 with recombinant H22(scFv) resulted in decreased capacity of the FITC-labeled mouse-anti-human CD64 (clone 10.1) mAb to bind to CD64 (Fig. 2A). In addition, we could show that as a consequence of CD64 blocking, H22(scFv) strongly reduced the capacity of HL-60 cells to capture anti-TNF mAb molecules (Fig. 2B).

CD64 blocking was quantified by measuring the minimal concentration of anti-CD64 mAb required to saturate all CD64 molecules on the cell surface, revealing a minimum saturating concentration of \(\geq 50\) nM (Fig. 3A). Because H22(scFv) is monovalent, whereas the full-length anti-CD64 mAb is bivalent, 100 nM of H22(scFv) should theoretically be sufficient to block all CD64 molecules. This was confirmed by directly titrating H22(scFv) against cultured HL-60 cells (Fig. 3B), and we found that blocking CD64 with H22(scFv) quantitatively correlated with the reduced capture of anti-TNF mAb (Fig. 3C).
H22(scFv) binding does not induce pro-inflammatory downstream signaling

The Fcγ part of anti-TNF mAbs (e.g., infliximab or adalimumab) was shown to activate the Fc receptor.8 To elucidate whether H22(scFv), which lacks an Fc part, would also lead to increased receptor activation, IFN-γ-stimulated HL-60 cells were incubated with H22(scFv) alone, adalimumab alone, or pre-incubated with H22(scFv) followed by addition of adalimumab. While adalimumab strongly induced phosphorylation of the receptor, no significant CD64 activation could be detected for H22(scFv). Moreover, H22(scFv) prevented adalimumab-mediated activation, if used in combination (Fig. 4). This inert binding of H22(scFv) to CD64 was also in line with gene expression results, where H22(scFv) did not induce the expression of pro-inflammatory cytokines (Fig. S1). Poor responses to anti-TNF therapy in some patients are associated with the overexpression of CD64, which leads to the capture of anti-TNF mAbs and the induction of a pro-inflammatory response.8 Blocking CD64 with H22(scFv) would reduce the capture of anti-TNF mAb, thus also limiting the downstream pro-inflammatory effects.

The expression of mTNF is upregulated on IFN-γ-stimulated HL-60 cells

TNF exists as both a soluble cytokine and as a transmembrane protein, the latter lacking the systemic inflammatory effects of its soluble counterpart. Because inflammatory CD64high monocytes/macrophages are likely to express high levels of mTNF, the capture of anti-TNF mAbs would be increased via their antigen binding Fab domain. We therefore tested IFN-γ-stimulated (pro-inflammatory) and non-stimulated HL-60 cells for the expression of mTNF using a mAb that selectively recognizes the transmembrane form.19 We found that only the stimulated HL-
60 cells expressed mTNF (Fig. 5), indicating the existence of an additional mechanism to capture anti-TNF mAbs.

The capture of anti-TNF mAb is mediated by CD64 and mTNF

To determine whether mTNF is involved in the capture of anti-TNF mAb, we pre-blocked mTNF on the surface of stimulated HL-60 cells using the mTNF-specific antibody described above, before measuring the capacity of the cells to capture anti-TNF mAbs (Fig. S2 illustrates the experimental set-up). Blocking mTNF reduced the ability of the cells to capture anti-TNF mAbs as expected (Fig. 6A–C), and the additive effect of blocking by H22(scFv) and anti-mTNF was found to match the total capacity of stimulated HL-60 cells to capture anti-TNF mAb (Fig. 6D). This result confirms that the capture of anti-TNF mAb is dependent on the expression of both CD64 and mTNF.

Discussion

TNF is a key component of the inflammation cascade and an attractive target for therapeutic intervention in inflammatory diseases. Several anti-TNF mAbs are already widely used in the clinic, but despite their success, not all patients respond to treatment. In a small study of IBD patients, this phenomenon has been associated with the increased expression of CD64, which binds anti-TNF IgG1 mAbs via their Fcγ domain and induces a downstream signaling cascade that ultimately leads to the expression of several pro-inflammatory cytokines including TNF itself, and thus partly neutralizes the anti-inflammatory effect of anti-TNF therapy. Interestingly, the inhibitory effect of anti-TNF mAb was restored by pre-treating cells with the purified Fcγ domains of human IgG1 molecules, thereby blocking CD64. However, human IgG1-Fcγ domains cannot be used in the clinic as they not only affect CD64, but also other Fcγ receptors like CD16 and CD32. The latter are necessary to mediate effector functions, including ADCC and CDC, thus providing essential protection against invading pathogens. Blocking all Fcγ receptors would severely compromise the immune system and leave patients susceptible to opportunistic infections. We therefore aimed to use a CD64-specific therapeutic protein to prevent the capture of the Fcγ domain.

In contrast to the fully human (adalimumab) or the chimeric (infliximab) anti-TNF mAbs used in the clinics, we used a mouse-derived anti-human TNF mAb due to availability issues. Mouse IgG1-Fcγ has been reported to have a low affinity toward human CD64, which would significantly affect our experimental set-up. However, in contrast to the data found in literature, the mouse-anti-human TNF mAb used here could be strongly captured by human CD64 (and also CD16 and CD32) in our experimental cell-based in vitro system, allowing us to study the effects of CD64-blocking molecules on the capture of the anti-TNF mAb. We initially considered replacing the Fcγ domain of human IgG1 with its IgG4 counterpart, which binds CD64 with > 100-fold greater affinity than CD32 or CD16.

We indeed found IgG4 to block the binding of the anti-TNF mAb. However, IgG4 was also captured by CD16 (Fig. S3). As we want to specifically block anti-TNF mAb binding by CD64, this renders IgG4 unsuitable for our intended purpose. Whether produced genetically or enzymatically, aglycosylated forms of the IgG1-Fcγ domain are known to lose their ability to bind CD16 and CD32, but they do retain residual binding affinity toward CD64. This could make them an ideal candidate of CD64 blocking. However, we found that aglycosylated IgG1 antibodies do not prevent binding of anti-TNF mAb to CD64 (Fig. S3). Since both IgG4 and aglycosylated IgG1 proved to be unsuitable for specific CD64 blocking, we examined CD64 blocking with specific antibody.
reduce the cell surface level of CD64, even under serum conditions. On top of its ability to prevent CD64 from capturing the anti-TNF mAb, binding of H22(scFv) to CD64 had no ill effects. Infliximab and adalimumab were described to activate Fc receptors and thus induce the expression of pro-inflammatory cytokines, including TNF itself, upon binding to CD64 via its Fcγ domain.\textsuperscript{8} In contrast, CD64 binding of H22(scFv) induced neither receptor activation nor the expression of pro-inflammatory cytokines. Together, these features make H22(scFv) or molecules containing H22(scFv) interesting therapeutic candidates for treatment of chronic inflammatory diseases.

We next addressed the possibility that the efficacy of anti-TNF mAbs in some patients might be reduced by mechanisms other than capture by CD64. Notably, the cytokine TNF exists in both soluble and membrane-bound forms. Although the soluble form is pathological under chronic inflammatory conditions and is the therapeutic target of anti-TNF mAbs, the role of mTNF is not well understood. However, the current generation of anti-TNF mAbs does not discriminate between the two forms, suggesting that the capture of anti-TNF mAbs via their Fab domain could also be mediated by the increased expression of mTNF on the cell surface, thus reducing the effective therapeutic concentration of antibodies in circulation. Accordingly, we detected the surface expression of mTNF solely on stimulated (pro-inflammatory) HL-60 cells. Because chronic inflammatory diseases are characterized by the predominance of pro-inflammatory monocytes and macrophages, this result emphasizes the potential inhibitory effect of mTNF on the efficacy of anti-TNF mAbs. We confirmed that blocking both CD64 and mTNF abolished the ability of stimulated HL-60 cells to capture anti-TNF mAb.

In conclusion, the CD64-blocking antibody fragment H22(scFv) prevents the capture of anti-TNF mAb by CD64, whose overexpression on the surface of inflammatory monocytes and macrophages is associated with the poor response of some patients toward anti-TNF therapy. H22(scFv) can therefore be employed to avoid this. Unlike the binding of anti-TNF mAbs to CD64, H22(scFv) binding does not activate CD64 nor induce expression of pro-inflammatory cytokines, making H22(scFv) or molecules containing H22(scFv) in this respect safe for use. We also demonstrated that the increased expression of mTNF contributes to the capture of anti-TNF mAb via their Fab domain. However, therapeutic targeting of mTNF with full-length mAbs could lead to opsonization of mTNF expressing immune cells (i.e., B cells, T cells, pro-inflammatory monocytes/ macrophages) that could finally result in the induction of ADCC. Although the
relationship between anti-TNF therapy and the occurrence of lymphoma is still a debate, several case studies have reported that mTNF-mediated death of these cells can increase the risk for the development of lymphomas and serious infections.28-30 Indeed, the use of an mTNF targeting molecule, which lacks an Fc part (e.g., Fab fragment or scFv), would still be an opportunity to address this drawback.

Materials and Methods

Antibodies and cytokines

Mouse-anti-human CD16 (Cat. No. 16–0167–82), mouse-anti-human CD32 (Cat. No. 16–0329–81), mouse-anti-human CD64 (Cat. No. 16–0649–81), and mouse-anti-human TNF (Cat. No. 16–7348–81) were purchased from eBioscience. Human anti-IgG4 (Cat. No. HCA050A) was obtained from AbD Serotech. Human aglycoIgG1 was produced in-house and kindly provided by Stefanie Kapelski (Fraunhofer IME, Aachen, Germany). Mouse-anti-human mTNF mAb 52B83 (Cat. No. HM2010) was purchased from Hycultec GmbH. Mouse-anti-His (Cat. No. 34660) and mouse-anti-Hisγ-Alexa Fluor 488 (Cat. No. 35310) were obtained from Qiagen. Alkaline phosphatase-conjugated anti-mouse-IgG (Cat. No. 300–02) and mouse-anti-IgG4 (Cat. No. HCA050A) were obtained from AbD Serotec. Human-anti-human mTNF mAb 52B83 (Cat. No. HM2010) was purchased from Hycultec GmbH. Mouse-anti-Hisγ-Alexa Fluor 488 (Cat. No. 35310) were obtained from Qiagen. Alkaline phosphatase-conjugated anti-mouse-IgG (Cat. No. A3652) was obtained from Sigma. Goat-anti-mouse-phycocerythrin (Cat- No. 115–115–164; GAM-PE) was from Dianova. Human IFN-γ (Cat. No. 300–02) and human TNF (Cat. No. 34–8329–85) were obtained from Peprotech and eBioscience, respectively. Mouse-anti-human CD16 (Cat. No. 16–0167–82), mouse-anti-human CD32 (Cat. No. 16–0329–81), mouse-anti-human CD64 (Cat. No. 16–0649–81), and mouse-anti-human TNF (Cat. No. 16–7348–81) were purchased from eBioscience. Human anti-IgG4 (Cat. No. HCA050A) was obtained from AbD Serotech. Human aglycoIgG1 was produced in-house and kindly provided by Stefanie Kapelski (Fraunhofer IME, Aachen, Germany). Mouse-anti-human mTNF mAb 52B83 (Cat. No. HM2010) was purchased from Hycultec GmbH. Mouse-anti-Hisγ-Alexa Fluor 488 (Cat. No. 35310) were obtained from Qiagen. Alkaline phosphatase-conjugated anti-mouse-IgG (Cat. No. A3652) was obtained from Sigma. Goat-anti-mouse-phycocerythrin (Cat- No. 115–115–164; GAM-PE) was from Dianova. Human IFN-γ (Cat. No. 300–02) and human TNF (Cat. No. 34–8329–85) were obtained from Peprotech and eBioscience, respectively. Adalimumab (Humira®) was kindly provided by Prof. Dr. Harald Wajant, Head of Department for Molecular Internal Medicine at the University Clinic in Wuerzburg, Germany. Mouse anti-phosphotyrosine antibody (Cat. No. 309301) was obtained from Biolegend. Horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Cat. No. W4031) and horseradish peroxidase-conjugated goat anti-β actin (Cat. No. MA5–15739-HRP) were purchased from Promega and Thermo Scientific, respectively.

Generation of H22(scFv)

The open reading frame for H22(scFv) was inserted into the bacterial expression vector pMT28 in frame with a pelB leader peptide sequence and the purification and detection tag His10. Successful cloning was confirmed by test digestion and sequencing. Expression, purification, and protein analysis were performed as previously described.18,29

Cell culture

The human pro-myelocytic HL-60 cell line (ATCC: CCL-240) and the human Hodgkin lymphoma cell line L540cy (DSMZ: ACC 72) were cultivated in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 50 μg/ml penicillin and 100 μg/ml streptomycin at 37°C, 100% humidity, and 5% CO2. HL-60 cells were stimulated with 50 U/ml IFN-γ 24 h before each experiment.

Cell-binding analysis by flow cytometry

The cell-binding activity of each antibody was analyzed by flow cytometry. We incubated 4 × 105 cells with known amounts of antibody in PBS (pH 7.4) containing 2 mM EDTA and 0.5% (w/v) BSA for 30 min on ice followed by washing with PBS. Unconjugated primary antibodies were detected using an anti-Hisγ-Alexa Fluor 488 (1:100) or GAM-PE (1:100) for 30 min on ice in the dark. The cells were then washed twice with PBS and analyzed on a FACS Calibur flow cytometer (Becton Dickinson).

Preparation of cell lysates

HL-60 cells (1×106 cell/ml) were stimulated with 50 U/ml IFN-γ for 24 h. The cells were then incubated for 24 h with either 100 nM H22(scFv) alone, 50 μg/ml adalimumab alone, or pre-incubated with 100 nM H22(scFv) for 30 min followed by the addition of 50 μg/ml adalimumab. Finally, the cells were washed once with TBS buffer (pH 7.6), lysed with 100 μl ice-cold cell lysis buffer (10%(w/v) SDS, 10mM β-mercaptoethanol, 1 mM DTT, 10mM sodium fluoride, 20 mM levamisole, in TBS-T (TBS + 0.05% Tween-20) buffer pH 7.6), shortly vortexed and left on ice for 20 min. Afterwards, cell were vigorously vortexed for 5 s and centrifuged (13,200 × g, 20 min, 4°C). Supernatants were collected and used for Western Blot analysis.

SDS-PAGE and Western Blotting analysis

Sodium dodecylsulfate PAGE (SDS-PAGE) followed by western blotting was performed as previously described.30 Membranes were blocked with 2.5% (w/v) BSA in TBS-T for 2 h at room temperature with gentle shaking. Primary antibodies (anti-phospho-tyrosine and anti-β actin) were diluted to a final concentration of 200ng/ml in TBS containing 1% (w/v) BSA and incubated with the membranes overnight at 4°C. Secondary antibodies were diluted to a final concentration of 100 ng/ml in TBS buffer containing 1% (w/v) BSA and incubated for 1 h at room temperature. Bands were detected by using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, USA) and chemiluminescence was visualized by Stella 3200 machine (Raytest, Straubenhardt, Germany).

RNA isolation, cDNA synthesis, and real-time PCR analysis

RNA was isolated using SV Total RNA Isolation System (Promega). CDNA (cDNA) was produced using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using SYBR Green PCR Kit (Qiagen). The cycle conditions were: stage 1 (50°C, 2 min); stage 2 (95°C, 10 min); stage 3: 35 repeats (95°C, 15 s; 60°C, 1 min). The expression levels of the target genes were normalized to the expression levels of GAPDH using the comparative threshold cycle method. The following primers were used: IL-1β_f: ACAGATGAAAG TGCCTCTTCT A, IL-1β_r: GTCC GAGATT CGTAGCTGGA T, IL-8_f: ACTGAGAGTG ATTGAGAGTG GAC, IL-8_r: AACCCCTCAGCC ACCAGTTT T C, TNF-α_f: GGGAGAAGGTT GACCCGACTA, TNF-α_r: CTG CCCAGAC TCGGCCAA, GAPDH_f: TGCACCCACA ACTGC TTAGC, and GAPDH_r: GGCATGGACT GTGGTCATGA G. Data were analyzed using GraphPad Prism 5 software (GraphPad Software). All experiments were performed in triplicate.
Disclosure of Potential Conflicts of Interest

D.H., S.B., and T.T. are co-inventors on two corresponding patents assigned to Fraunhofer. The authors otherwise disclose no conflicts of interest.

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Supplementary Material

Supplemental data for this article can be accessed on the publisher’s website.

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Supplementary Material

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mAbs