Supporting Information

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SI Text

IgG Digestion Conditions. Protease digestions of purified IgGs were carried out at pH 7.5 in PBS or, for the MMPs, in Tris-buffered saline buffer at 37 °C. $CaCl_2$ was included in the MMP reactions at 1 mM for MMP-12 and 10 mM for other MMPs. Antibody concentrations were 1–2 mg/mL, and reactions were initiated by addition of enzyme to a 1% (wt/wt) ratio to IgG unless otherwise specified. IgG cleavage was assessed by SDS-PAGE and Agilent Biosizing microcapillary electrophoresis as previously described (1).

For purification of single-cleaved IgG intermediates, the protease digests were followed until intact IgG was no longer detectable. Reactions were terminated (20 mM EDTA for MMPs or 1 mM iodoacetamide for IdeS). $F(ab')_2$ was separated from single-cleaved IgG and free Fc as the unbound fraction on protein A affinity chromatography. The latter two components (≈160 and ≈50 kDa, respectively) were separated by size exclusion chromatography on Superdex 200 (GE Healthcare), and the single-cleaved IgGs were free of detectable intact IgG and free Fc.

Proteolysis of cell-bound mAb was as follows: MDA-MB-231 cells were seeded in 24-well plates at a concentration of 0.1×10^6 cells/mL (1 mL/well) in DMEM, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine overnight at 37 °C. Cells were washed with serum-free DMEM, and then 1 mL of 1 μg/mL intact mAb4 was added in serum-free DMEM. Cells were washed after 1 h with serum-free DMEM, then 1 mL of 10 μg/mL MMP-3 (in serum-free DMEM) was added at −24, −8, −4, and −2 h. At 0 h (24 h after the first addition of MMP-3), cells were removed from the 24-well plates for flow cytometry. Cleaved mAb4 was detected with a rabbit anti-hinge polyclonal antibody (2) coupled to AlexaFlour 647 (Invitrogen). The Fcγ portion of mAb4 was detected with a FITC anti-Fc gamma fragment specific antibody (Jackson Immunoresearch). Cells were acquired on a FACS Calibur (Becton Dickinson), and the results were analyzed with FloJo Software (Tree Star).

AlphaScreen Competition Binding Assays. AlphaScreen (PerkinElmer) was used to assess competitive binding of mAb5 and its derivatives to FcγRs and FcRn. Human FcγRI, IIa(H131), IIb were purchased from R&D Systems. Human FcγRIIIa(V158) and FcRn were cloned, expressed, and purified at Centocor R&D. Competition binding studies were carried out in half-well volume 96-well opaque plates (Corning) in assay buffer (PBS, 0.05% BSA, 0.01% Tween-20) at pH 7.4 (with the exception of FcRn binding studies that were performed at pH 6.2). All competition studies were carried out against biotinylated IgG1 (1 IgG: 2 biotin, using EZ Link NHS-LC-biotin; Pierce) at a set concentration of 1 μg/mL in the final assay concentration and varying levels of competing mAb5 and its derivatives in serial ½ log step dilutions. FcR concentrations ranged from 0.5 to 2 μg/mL in final concentration of the assays. Biotinylated IgG1 (10 μL) and various concentrations of mAb5 and its derivatives (10 μL) were sequentially added to each row of a 96-well plate in duplicates. Thereafter designated FcγRs or FcR was added followed by the sequential addition of 10 μL each of 1/50 diluted nickel chelate (Ni)-acceptor beads and streptavidin (SA)-donor beads. The opaque plate was covered with an aluminum seal to maintain light-safe conditions while shaking for 45 min on an orbital shaker. Thereafter the seal was removed and the fluorescence was read on an Envision plate reader (PerkinElmer) equipped with appropriate filter set of AlphaScreen (PerkinElmer) excitation/emission spectra. Raw data were transferred to GraphPad Prism software and normalized for maximal signal, and competition curves were plotted using nonlinear regression curve-fitting software.

Pharmacokinetic Studies. PK studies were conducted in female BALB/c mice. Treatment antibodies and proteolytic derivatives were free of aggregates and were within the acceptance guidelines for endotoxin contamination (<40 endotoxin units/kg). Agents were injected i.p. at the dose of 2 mg/kg. Three animals were sampled at each time using a staggered schedule in six animals to minimize the number of samples from any single animal. Blood samples (≈60 μL) were collected from CO_2-anesthesized mice by retro-orbital bleeds. Blood samples were allowed to clot at ambient temperature before centrifugation at 3,500 rpm for 15 min to obtain serum. ELISA measurements of the human antibodies in mouse serum used goat anti-human IgG, $F(ab')_2$-specific as the capture, and peroxidase-conjugated goat anti-human IgG (heavy and light chain specific) for detection (Jackson). A standard curve of each antibody version was used for quantification of intact IgGs, single-cleaved IgGs, and $F(ab')_2$s, respectively. Linear regression analyses were performed using GraphPad Prism v 4.

Detection of IgG in Synovial Fluid from Rheumatoid Arthritis Patients and Breast Tumor Extracts. Synovial fluid (SF) samples from patients with rheumatoid arthritis were obtained from Bioreclamation. and stored at −80 °C. The SF was thawed, diluted 1:5 in PBS, and centrifuged for 20 min at 2,000 rpm, and the supernatant was collected. Flash frozen breast tumors were obtained from BioServe and stored at −80 °C. The frozen tumor sections were dissociated and then incubated in Tissue Protein Extraction Reagent (T-PER; Thermo Scientific), pH 3.0, containing protease inhibitor mixture (Roche) on ice for 20 min. The pH was neutralized, and the extracts were then centrifuged for 20 min at 13,000 rpm, and the supernatant was collected. The buffer of the tumor extracts supernatants was then exchanged to PBS. The SF and tumor extract supernatants were individually applied to a protein A column (GE Healthcare). Bound antibody was eluted with 0.1 M glycine (pH 2.5), and the pH was neutralized. The buffer of the eluted antibodies was exchanged to PBS. Western blots were performed by running 100 ng antibody per lane on a NuPAGE 3%–8% Tris-acetate SDS-PAGE gel (Invitrogen). After electrophoresis, proteins were transferred to PVDF with the iBlot transfer system (Invitrogen) and then blocked in 5% BSA in 100 mM Tris-buffered saline, 0.1% Tween 20 (TBS-T) overnight at 4 °C. Membranes were incubated for 30 min at room temperature with 1:20,000 dilution of peroxidase-conjugated mouse anti-human IgG Fc gamma domain specific (Jackson Immunoresearch) in blocking buffer. The PVDF membrane wash washed in TBS-T, treated with Lumigen PS-3 detection reagent (GE Healthcare) and exposed to X-ray film (Kodak).

Human Materials. Human donor blood cells for use in ADCC assays were collected from employee volunteers with all necessary permissions and approvals. The protocol and informed consent form, and relevant study-related documentation were approved by a third party Institutional Review Board. Human SF samples were commercially obtained from Bioreclamation.

Animal Studies. Animal study protocols were approved by the Institutional Animal Care and Use Committees, and all animals were housed and maintained according to conditions described in the *Guide for the Care and Use of Laboratory Animals*.

1. Diemel RV, ter Hart HGJ, Derksen GJA, Koenderman AHL, Aalberse RC (2005) Characterization of immunoglobulin G fragments in liquid intravenous immunoglobulin products. *Transfusion* 45:1601–1609.
2. Ryan MH et al. (2008) Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid. *Mol Immunol* 45:1837–1846.