Engineered Protease-resistant Antibodies with Selectable Cell-killing Functions*\textsuperscript{5}

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\textbf{Background:} Proteases can cleave human IgG1 antibodies, resulting in loss of cell-killing functions.

\textbf{Results:} Mutation of the lower hinge of IgG1 confers protease resistance but disrupts Fc effector functions.

\textbf{Conclusion:} Compensating mutations in the C\textsubscript{H}2 domain can selectively restore Fc effector functions on a protease-resistant backbone.

\textbf{Significance:} Protease-resistant antibodies may be desirable for microenvironments with high protease content and/or when selected cell-killing functions are needed.

Molecularly engineered antibodies with fit-for-purpose properties will differentiate next generation antibody therapeutics from traditional IgG1 scaffolds. One requirement for engineering the most appropriate properties for a particular therapeutic area is an understanding of the intricacies of the target microenvironment in which the antibody is expected to function. Our group and others have demonstrated that proteases secreted by invasive tumors and pathological microorganisms are capable of cleaving human IgG1, the most commonly adopted isotype among monoclonal antibody therapeutics. Specific cleavage in the lower hinge of IgG1 results in a loss of Fc-mediated cell-killing functions without a concomitant loss of antigen binding capability or circulating antibody half-life. Proteolytic cleavage in the hinge region by tumor-associated or microbial proteases is postulated as a means of evading host immune responses, and antibodies engineered with potent cell-killing functions that are also resistant to hinge proteolysis are of interest. Mutation of the lower hinge region of an IgG1 resulted in protease resistance but also resulted in a profound loss of Fc-mediated cell-killing functions. In the present study, we demonstrate that specific mutations of the C\textsubscript{H}2 domain in conjunction with lower hinge mutations can restore and sometimes enhance cell-killing functions while still retaining protease resistance. By identifying mutations that can restore either complement- or Fc\textgamma receptor-mediated functions on a protease-resistant scaffold, we were able to generate a novel protease-resistant platform with selective cell-killing functionality.

The mechanism of action for several therapeutic monoclonal antibodies (mAbs) is thought to be due to Fc-mediated effector functions (1, 2). The most common human immunoglobulin isotype used for therapeutic intervention is IgG1 (3), which contains two Fab arms linked to a single Fc domain by a flexible hinge region. The Fc domain of IgG1 can bind to Fc\gammaRs\textsuperscript{2} expressed on immune effector cells and mediate target cell destruction by cellular means, including antibody-dependent cellular cytotoxicity (ADCC) and/or antibody-dependent cellular phagocytosis (ADCP) (4). Antigen-engaged mAbs can also recruit serum components of the immune system and mediate target cell destruction by initiating the complement cascade (5–7). The interactions of both immune cell Fc\gammaRs and the C1q component of complement require key amino acid recognition motifs in the lower hinge and proximal C\textsubscript{H}2 region of human IgG (2, 8–21). Our group and others have shown that the human IgG1 subclass is susceptible to limited proteolysis in the hinge region by a number of physiologically relevant proteases associated with microbial infections (e.g. GluV8 of Staphylococcus aureus and IdeS of Streptococcus pyogenes) and invasive cancers (e.g. the matrix metalloproteinases (MMPs)) (22–24). Cleavage within the lower hinge of IgG1 occurs in a two-step process where first one heavy chain is cleaved, resulting in a singly cleaved intermediate (8, 22, 23, 25). Cleavage of the second heavy chain separates the Fc from the Fab arms, resulting in an Fc fragment and an F(ab\textsuperscript{'})\textsubscript{2} fragment. Previous studies have indicated that the singly cleaved intermediate is the dominant cleavage product generated on the cell surface (8) and that single cleavage results in abrogated binding to Fc\gammaRs (8, 26) and a loss of complement-dependent cytotoxicity (CDC) (8). Accordingly, the singly cleaved intermediate displays a profound loss of function in terms of cell killing both \textit{in vitro} and \textit{in vivo} (8, 26).

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\textsuperscript{1} This article contains supplemental Fig. S1 and Tables S1–S3.

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However, the singly cleaved intermediate retains antigen binding capabilities as well as the long circulating half-life of the intact IgG1 counterpart (8). For these reasons, our group and others have hypothesized that antibody cleavage by tumor-associated and microbial proteases can potentially function as an immune evasion mechanism (for reviews, see Refs. 27 and 28).

Previously, we have shown that the human IgG2 subclass is resistant to cleavage by a number of physiologically relevant proteases, including MMP-3, MMP-7, MMP-12, and MMP-13 as well as GluV8 (29). Sequence alignments comparing the lower hinge of IgG1 and IgG2 suggested that the resistance to cleavage may be due to amino acid differences between IgG1 and IgG2 (29). However, the IgG2 subclass has very weak binding to FcγRIIIα (30) and thus low to undetectable ADCC capacity. The IgG2 subclass also has greatly reduced CDC activity compared with IgG1 (31). Several groups have exchanged the lower hinge/proximal C_{H2} of IgG1 and IgG2 (18, 32) and characterized the loss of function associated with this domain exchange. Armour et al. (32) demonstrated that introduction of the lower hinge/proximal C_{H2} of IgG2 into IgG1 resulted in a profound loss of function and proposed that these substitutions could serve as a silent Fc platform. Shields et al. (18) introduced the lower hinge/proximal C_{H2} of IgG2 into IgG1 and showed a greater than 20-fold loss of binding to FcγRs. Therefore, additional efforts would be required to generate protease-resistant variants containing an IgG2 lower hinge/proximal C_{H2} region that retain Fc-dependent cell-killing functions.

Antibody engineering has long been recognized as a means to improve mAb-based therapies (2, 33), particularly with regard to antibodies that target tumor antigens (4, 34). Efforts to engineer anti-tumor mAbs in the Fc domain are often directed toward increasing the cell killing capacity of the mAb by augmenting binding to FcγRs (18, 35–37) or the C1q component of complement (5, 6). One common method to augment cell-killing functions is to mutate amino acids in the C_{H2} region and screen for variants with increased binding to C1q or FcγRs (5, 6, 18, 35, 36). A number of published reports have also documented efforts to engineer the hinge region to improve effector function or increase antibody stability (38, 39). In this study, we demonstrate that mutation of the lower hinge of IgG1 confers protease resistance but also results in the loss of Fc-mediated cell-killing functions. We show that specific mutations incorporated into the C_{H2} region of engineered mAbs with a protease-resistant lower hinge cannot only restore functional activities to IgG1 but in some cases substantially enhance Fc effector functions. Furthermore, we show that cleaved IgGs are detected within the tumor microenvironment of human head and neck squamous cell carcinoma, highlighting the need for a protease-resistant platform for diseases characterized by the presence of IgG-cleaving proteases.

EXPERIMENTAL PROCEDURES

Antibodies—The V-region cDNA sequences of the anti-CD20 variants were the same as those used in rituximab (VL GenBank™ accession number AR015962 and VH GenBank accession number AR000013), and the heavy and light chains were engineered onto IgG subclasses and variants by molecular cloning. Transient transfection and expression in 293T and/or CHO cells were performed with standard procedures at Janssen Research and Development, LLC. mAbs were purified using protein A columns and underwent in-house quality controls for >95% purity prior to further experimental analyses. The complementarity-determining region sequences of the humanized, complementarity-determining region-grafted anti-CD142 mAb were derived from the murine anti-human CD142 mAb TF8-5G9, which originated at the Scripps Research Institute and has been described previously (40, 41).

Protease Digestions—Protease digestions were performed at pH 7.5 at 37 °C for 24 h in PBS for Ides and GluV8 or in Tris-buffered saline with 5 mM CaCl₂ for the MMP reactions. Anti-CD142 antibodies were used at a concentration of 0.5 mg/ml, and protease concentrations of 10% molar ratio for MMP-3 (Janssen Research and Development, LLC) and MMP-12 (Enzo Life Sciences), 20% molar ratio for GluV8 (Biocentrum), and 1% (w/w) for IdeS (Genovis) were used. Kinetic digests were performed with a 10% molar ratio for MMP-3 and 0.1% (w/w) IdeS and were quenched at the indicated time points using a final concentration of 10 mM EDTA for the MMP-3 digest and 10 mM iodoacetamide for the IdeS digest. The percentage of intact IgG remaining was calculated as done previously (29).

Immunohistochemistry—All immunohistochemistry was performed by QualTek Molecular Laboratories (Newtown, PA). Four-micrometer sections were dewaxed through four changes of xylene (5 min each) followed by a graded alcohol series to distilled water. Steam heat-induced epitope recovery was used for 20 min in the capillary gap in the upper chamber of a Black and Decker steamer. Sections were incubated with primary rabbit polyclonal anti-hinge detection antibodies (125 ng/ml) directed against three cleavage sites in the hinge that were described previously (23). An anti-rabbit biotinylated secondary antibody was applied followed by avidin-biotin complex-HRP. Secondary antibodies were detected with 3,3'-diaminobenzidine chromogen. Positive staining was indicated by the presence of a brown chromogen reaction product. Sections were counterstained with hematoxylin for 1 min. Slides were analyzed under a microscope using a 40× objective.

ADCC—The ADCC assays were performed as described previously with several modifications (42). ADCC assays were performed with increasing anti-CD20 IgG1 antibody variant concentrations. Briefly, human PBMCs purified from leukopaks were used as effector cells, and WIL2-S cells were used as targets in a 50:1 ratio. The WIL2-S cells were labeled with 2,2′-6′,2′-terpyridine-6,6′-dicarboxylate reagent (PerkinElmer Life Sciences) for 30 min, washed twice, and resuspended in RPMI 1640 medium supplemented with GlutaMAX, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen). 0.5 × 10⁶ PBMCs, 1 × 10⁶ labeled WIL2-S, and antibody at the indicated concentration were combined in 200 μl total in U-shaped 96-well plates, centrifuged for 2 min at 200 × g, and incubated at 37 °C for 2 h. At the end of the assay, plates were centrifuged again at 200 × g for 5 min, and 20 μl of supernatant were mixed with 200 μl of DELPHIA europium-based reagent. The fluorescence signal was measured using an Envision 2101 Multilabel Reader (PerkinElmer Life Sciences). The percentage of lysis was calculated as (Experimental release − Spontaneous release)/(Maxi-
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mal release — Spontaneous release) × 100. Data were log-transformed and fit to a sigmoidal dose-response curve using GraphPad Prism v5.

CDC—CDC assays were performed as described previously (8) with WI-2-S cells as the target. A total of 50 μl of 0.05 × 10^6 cells was added to the wells of 96-well U-bottom plates in RPMI 1640 medium, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. An additional 50 μl of medium was added with and without mAbs, and plates were incubated at room temperature for 1 h. After incubation, a total of 50 μl of a 10% rabbit complement (Invitrogen) solution was added, and plates were incubated at 37 °C for 20 min. Plates were centrifuged again at 200 × g for 5 min, and 50 μl of supernatant were mixed with 50 μl of the lactate dehydrogenase cytotoxicity detection kit (Roche Applied Science). Plates were incubated for 15 min at room temperature and then analyzed on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) at 490 nm. Data were normalized to maximal cytotoxicity with Triton X-100 (Sigma) and minimal control containing only cells and complement in the absence of mAb. Data were log-transformed and fit to a sigmoidal dose-response model using GraphPad Prism v5.

Twenty-four-hour ADCP—Human PBMCs were isolated from leukopaks (Biologics Specialty) using Ficoll gradient centrifugation. CD14<sup>pos</sup> monocytes were purified from PBMCs by negative depletion using a CD14 isolation kit that did not deplete CD16<sup>pos</sup> monocytes (Stem Cell Technologies). Purified monocytes were plated at 0.1 × 10<sup>6</sup> cells/cm<sup>2</sup> in X-VIVO-10 medium (Lonza) containing 10% FBS. Macrophages were differentiated from monocytes by the addition of 25 ng/ml macrophage colony-stimulating factor (R&D Systems) for 7 days. IFNγ (50 ng/ml; R&D Systems) was added for the final 24 h of differentiation. The target cells for the assay were GFP-expressing MDA-MB-231 cells (40). Isolated macrophages were incubated in a 37 °C incubator with GFP-expressing MDA-MB-231 cells at a ratio of four macrophages (0.1 × 10<sup>6</sup> cells/well) to one MDA-MB-231 cell (25,000 cells/well) for 24 h with wild-type anti-CD142 or protease-resistant variants of anti-CD142 in 96-well U-bottom plates. The final volume of medium (DMEM + 10% FBS) used for the assay was 200 μl. At the end of 24 h, plates were centrifuged at 300 × g for 5 min, and the cells were removed from the 96-well plates using Accutase (Sigma). Macrophages were identified with anti-CD11b (clone ICRF44) and anti-CD14 (clone M5E2) antibodies (both from BD Biosciences) coupled to Alexa Fluor 647 (Invitrogen), and then cells were acquired on an LSRFortessa flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (Tree Star). The percentage (%) of cell killing was determined by measuring fluorescence using GraphPad Prism v5.

ELISA for Antigen Binding—Nunc-Immuno MaxiSorp plates were coated overnight with 10 μg/ml streptavidin (Invitrogen) at 4 °C. At room temperature, plates were blocked for 1 h with 3% BSA, PBS and then coated with biotinylated CD142 in 3% BSA, PBS for an additional hour. Antibody variants were added at the indicated concentration for 1 h. The plate was washed three times with 0.15 M NaCl, 0.02% Tween 20 and then treated with anti-CD142 (Millipore) at a 1:5000 dilution for 1 h. Plates were washed three times, 3',3',5',5'-tetramethylbenzidine reagent (Sigma) was added for 5 min, and the reaction was stopped with 3 M HCl. The absorbance was read at 450 nm. Data were log-transformed and fit to a sigmoidal dose-response curve using GraphPad Prism v5.

In Vitro Competition FcRn Binding Analysis—A competitive binding assay was used to assess relative affinities of different antibody samples to in-house recombinant human FcRn-H<sub>56</sub> (transmembrane and cytoplasmic domains of FcRn were replaced with a polystyrene affinity tag). Ninety-six-well copper-coated plates (Thermo Scientific) were used to capture FcRn-H<sub>56</sub> at 5 μg/ml in PBS after which plates were washed with 0.15 M NaCl, 0.02% Tween 20, pH 7.5 and then incubated with blocking reagent (0.05 M MES, 0.025% BSA, 0.001% Tween 20, pH 6.0, 10% ChemiBLOCKER (Millipore)). Plates were washed as above, and then serial dilutions of competitor test antibody in blocking reagent were added to the plate in the presence of a fixed 1 μg/ml concentration of an indicator antibody (a biotinylated human IgG1 monoclonal antibody). Plates were incubated at room temperature for 1 h, washed three times as above, and then incubated with a 1:10,000 dilution of streptavidin-HRP (Jackson ImmunoResearch Laboratories) at room temperature for 30 min. Plates were washed five times as above, and bound streptavidin-HRP was detected by adding 3,3',5',5'-tetramethylbenzidine peroxidase substrate with Stable Stop (Fitzgerald Industries International) and incubating for 4 min. Color development was stopped by addition of 0.5 M HCl. Optical densities were determined with a SpectraMax Plus384 plate reader (Molecular Devices) at 450-nm wavelength. Data were fitted to a sigmoidal dose-response curve using GraphPad Prism v5.

B Cell Depletion in Cynomolgus Monkeys—The B cell depletion studies were performed by Huntingdon Life Sciences (East Millstone, NJ). Four cynomolgus monkeys per group were injected with saline or rituximab variants at 1 mg/kg. At the indicated time points, blood was collected and analyzed by flow cytometry. Cell events within a lymphocyte scatter were further subdivided into T lymphocytes (CD3<sup>pos</sup>, CD20<sup>neg</sup>), natural killer lymphocytes (CD3<sup>neg</sup>, CD159a<sup>pos</sup>), or B lymphocytes (CD3<sup>neg</sup>, CD19<sup>pos</sup>). Antibodies to CD3 (SP34), CD20 (2H7), CD16 (3G8), and CD40 (5C3) and corresponding isotype control antibodies were purchased from BD Biosciences. Antibodies to CD159a (Z199), CD14 (RMO52), and CD19 (J3-119) were
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purchased from Beckman Coulter. Individual antibodies or appropriate antibody mixtures were added to 12 × 75-mm FACS tubes. Blood samples were mixed before use (e.g. gentle inversion or rolling). Fifty microliters of blood and 50 μl of heat-inactivated FBS (Sigma) were added to each of the tubes. Sample tubes were briefly vortexed after all additions, protected from light, and incubated at ambient temperature for a minimum of 10 min. Red blood cells were then lysed, and samples were fixed using the TQ Prep Workstation™ (Coulter). Samples were stored at 2–8 °C protected from light until analysis. All sample acquisition took place on an FC500 flow cytometer (Coulter). FlowCount beads (Coulter) were utilized to determine cell concentrations. Data analysis was performed using FCS Express (v3.0).

RESULTS

Detection of Cleaved IgGs in Human Squamous Cell Carcinoma—Based on the observation that a number of physiologically relevant proteases associated with invasive cancers can cleave human IgGs in vitro, we assessed the presence of cleaved IgGs in human tumor tissue. We had previously generated anti-hinge antibodies that bind to cleaved IgGs but do not react with the intact IgG counterpart (8, 23). The anti-hinge antibodies were used for immunohistochemical detection of cleaved IgGs in human head and neck squamous cell carcinoma (HNSCC). As shown in Fig. 1A (left panel), cleaved IgGs were detected in HNSCC with an enrichment of detection at the tumor/stromal interface. To demonstrate that the staining was specific for cleaved IgGs, we added a pool of hinge-cleaved F(ab')2 fragments (generated with MMP-3, GluV8, and IdeS) to block the antigen-binding arm of the anti-hinge antibodies (Fig. 1A, right panel). Inclusion of pooled F(ab')2 fragments effectively blocked detection of cleaved IgGs, confirming the specificity of the detection reagent. Positive staining for cleaved IgGs was observed in 19 of 51 (37%) individual HNSCC cases (Fig. 1B). It was not unexpected that cleavage was not seen in all of the sections due to the heterogeneity of human tumor samples (e.g. total levels of EGF receptor vary widely in human tumor samples and are not uniform among different individuals (43)). This finding indicated that human IgGs were subject to proteolytic cleavage in the lower hinge region within the tumor microenvironment, especially at the expanding edge of the tumor where protease expression is known to be heightened (44).

Mutation of the Lower Hinge of IgG1 Confers Protease Resistance—We next sought to determine whether we could mutate the lower hinge proximal C1,2 region of human IgG1 to confer protease resistance. Initially, three variants were generated for this purpose. The first variant contained the IgG1 to IgG2 domain exchange E233P/L234V/L235A with Gly236 deleted (EU numbering (45)); this substitution was designated 2h. To compensate for the loss of function associated with such a domain swap (18, 32), additional variants were generated with select mutations in the C1,2 region. The variant 2h-DE contained the IgG2 lower hinge and the C1,2 mutations S239D/I332E, which were previously shown to enhance FcγR binding to IgG1 (35) (supplemental Fig. S1). The variant 2h-AA contained the IgG2 lower hinge and the C1,2 mutations K326A/I332E, which were previously shown to enhance FcγR binding to IgG1 (35) (supplemental Fig. S1). The variant 2h-AA contained the IgG2 lower hinge and the C1,2 mutations K326A/I332E, which were previously shown to enhance FcγR binding to IgG1 (35) (supplemental Fig. S1). The variant 2h-DE did not restore CDC activity.

Enhanced ADCC—We probed the ability of the protease-resistant variants to mediate Fc-dependent immune effector functions using cell-based assays. For CDC activity, we opsonized WIL2-S lymphoma cells with anti-CD20 antibody variants containing the V-region of rituximab. IgG1 mediated CDC activity, whereas the IgG2 did not, and as expected, the 2h mutation containing the S239D/I332E mutations (variant 2h-DE) was all resistant to cleavage by MMP-3 and MMP-12. The variant 2h-AA were all resistant to cleavage by MMP-3 and MMP-12. The variant 2h-DE was uniquely resistant to the Group A streptococcal protease IdeS but had increased susceptibility to S. aureus protease GluV8 compared with IgG2, 2h, and 2h-AA. These results indicated that lower hinge mutations augmented protease resistance and that incorporation of mutations into the C1,2 region to influence protease resistance as well.

Select Protease-resistant Variants Have Restored CDC or Enhanced ADCC—We probed the ability of the protease-resistant variants to mediate Fc-dependent immune effector functions using cell-based assays. For CDC activity, we opsonized WIL2-S lymphoma cells with anti-CD20 antibody variants containing the V-region of rituximab. IgG1 mediated CDC activity, whereas the IgG2 did not, and as expected, the 2h mutation containing the S239D/I332E mutations (variant 2h-DE) did not restore CDC activity.

FIGURE 1. IgG hinge cleavage is detected at the tumor/stroma interface. A, representative 40× images of human head and neck squamous cell carcinoma tissue sections were assessed for the presence of cleaved IgGs using anti-hinge antibodies specific for lower hinge-cleaved IgGs but not the intact IgG counterpart (left panels). Representative adjacent sections were treated with anti-hinge antibodies in the presence of excess cleaved IgGs to determine the specificity of staining (right panels). B, bar graph depicting the number of cases with or without detection of IgG cleavage from a total of 51 sections from individual patients.

E333A, which were previously shown to enhance IgG1 CDC activity (5) (supplemental Fig. S1). To assess protease susceptibility of the newly generated variants, mAbs were incubated with proteases capable of cleaving human IgG1 (29). Similar to previous studies, IgG1 was cleaved by MMP-3, MMP-12, GluV8, and IdeS to varying degrees after a 24-h incubation (Fig. 2, A and B). IgG2, 2h, 2h-DE, and 2h-AA were all resistant to cleavage by MMP-3 and MMP-12. The variant 2h-DE was uniquely resistant to the Group A streptococcal protease IdeS but had increased susceptibility to S. aureus protease GluV8 compared with IgG2, 2h, and 2h-AA. These results indicated that lower hinge mutations augmented protease resistance and that incorporation of mutations into the C1,2 region influenced protease resistance as well.

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ADCC was assessed with anti-CD20 variants using peripheral blood mononuclear effector cells allotyped to be heterozygous for the *FcyRIIa 158V/V* polymorphism and WL2-S target cells. IgG1 mediated ADCC activity, whereas IgG2 and the variants 2h and 2h-AA did not (Fig. 3, right). The 2h-DE variant had enhanced ADCC activity with an ~11-fold average increase in potency as compared with IgG1 (supplemental Table S1). Thus, this engineering strategy resulted in a novel methodology to select for specific cell-killing functions that are at times enhanced.

Protease-resistant Variants That Elicit Both CDC and Enhanced ADCC—To develop a protease-resistant platform that could mediate Fc effector functions more broadly (i.e. both ADCC and CDC), we combined the CDC-restoring K326A/E333A mutations with either of the ADCC-restoring mutations, S239D or I332E, resulting in variants that were designated 2h S239D/K326A/E333A (2h-DAA) or 2h K326A/I332E/E333A (2h-AEA). Twenty-four-hour proteolytic digests confirmed that both 2h-DAA and 2h-AEA maintained protease resistance to MMP-3, MMP-12, and GluV8 compared with IgG1 (Fig. 4A). The 2h-AEA variant showed greater protease resistance to IdeS compared with IgG1 and 2h-DAA, suggesting that the I332E mutation on the 2h backbone influenced protease resistance to IdeS.

We next assessed IgG proteolysis in kinetic digest assays comparing IgG1 with the protease-resistant variants 2h-DAA and 2h-AEA. MMP-3 cleavage of IgG1 was detected within 15 min, and no intact IgG1 was detected at the 24-h time point, whereas both 2h-DAA and 2h-AEA were resistant to MMP-3 cleavage throughout the 24-h assay (Fig. 4B). Because IdeS rapidly cleaves human IgG1 (8, 24), kinetic IdeS digests were performed with shorter time points at an enzyme concentration of 0.1% (w/w). Approximately 60% intact IgG1 was detected after 1 min, and complete loss of intact IgG occurred by 30 min. In contrast, the variant 2h-AEA maintained nearly complete resistance to IdeS throughout the assay. The 2h-DAA variant displayed some loss of intact IgG after 2 h with ~90% intact mAb remaining (Fig. 4C). These results confirmed that the combination of lower hinge mutations with I332E imparted the greatest level of resistance to IdeS.

Next, we characterized the Fc effector functions of the all the protease-resistant variants. The protease-resistant variants 2h-DAA and 2h-AEA displayed CDC activity at potencies comparable with the 2h-AA variant (Fig. 5A and supplemental Table S1). Either the S239D or the I332E mutation alone was capable of restoring ADCC activity to the 2h backbone with each variant demonstrating higher potency than IgG1 although to levels lesser than the 2h-DE variant (Fig. 5B and supplemental Table S1). The ADCC activities for both the 2h-DAA and 2h-AEA variants were ~4-fold higher than IgG1 with heterozygous *FcyRIIa 158V/V* polymorphism displayed similar relative differences in ADCC enhancements between the different Fc variants (Fig. 5C). ADCC was further enhanced by the protease-resistant variants compared with IgG1 when human PBMC donors that were homozygous for the high affinity *FcyRIIa 158V/V* polymorphism were used as effector cells (Fig. 5D). The 2h-DE variant displayed an ~30-fold average increase in potency compared with IgG1, whereas the 2h-DAA and 2h-AEA variants displayed average increases of ~23- and ~10-fold, respectively, among the two different donors tested. Therefore, three protease-resistant variants demonstrated increased ADCC potency compared with IgG1, and the increased potency was most apparent using low affinity *FcyRIIa 158V/V* polymorphism PBMC donors.
Antibody-dependent Macrophage Killing of Human Tumor Cell Lines—We recently demonstrated that tumor-associated macrophages display potent antitumor activity in the presence of an anti-tumor mAb (40). Therefore, we wanted to assess the function of the protease-resistant variants against mAb-opsonized tumor cells using macrophage effector cells. Fc-dependent effector functions of macrophages against mAb-opsonized tumor cells are often assessed by ADCP. This is typically accomplished by co-incubating macrophages with mAb-opsonized tumor cells and assessing internalization of the tumor cells within macrophages over a time frame typically no longer than 4 h (40, 46–48). Our group previously developed a flow cytometry and microscopy-based ADCP assay where the target tumor cells (MDA-MB-231) expressed GFP to obviate the need to label the tumor cells with a dye (29, 46). Macrophage internalization of MDA-MB-231 cells opsonized with a tumor targeting anti-CD142 mAb was assessed by either flow cytometry or fluorescence microscopy (29, 40). We had previously noted that the GFP fluorescence within macrophages was punctate, whereas the GFP fluorescence of non-internalized tumor cells was uniform (29, 40). Because the process of ADCP results in internalization and the subsequent lysosomal destruction of the tumor cell (4) and GFP fluorescence is dependent on the structural integrity of the GFP protein, we hypothesized that the punctate GFP signal was due to breakdown of the tumor target cell within the macrophage. To test the ability of macrophages to kill anti-CD142 mAb-opsonized, GFP-expressing MDA-MB-231 tumor cells, we extended a traditional ADCP assay to 24 h. In this assay, we could observe complete destruction of the tumor cell both by flow cytometry and immunofluorescence. This was accomplished by incubating effector macrophages in the presence of anti-CD142-opsonized, GFP-expressing MDA-MB-231 target cells and assessing the loss of GFP detection after 24 h (Fig. 6, A and B). The variants 2h-DE, 2h-DAA, and 2h-AA had the lowest level of tumor cell killing (Fig. 6C). These results demonstrated that the variants with ADCC-restoring mutations were capable of killing MDA-MB-231 tumor cells in the 24-h ADCP assay.

Protease-resistant mAbs with Enhanced Effector Functions—To test that the protease-resistant mutations did not affect Fab arm function, we assessed the ability of the variants to bind to antigen in both plate-based and cell-based assays. The results indicated that variants engineered onto two different V-regions bound identically to their respective targets (Fig. 7, A and B). We next assessed the ability of the variants to bind to the neonatal Fc receptor, FcRn, which is thought to contribute to the long circulating half-life of IgGs. Fc binding to FcRn occurs proximal to the junction of the CH2 and CH3 regions (49), a region that is distal from the mutations present in all of the protease-resistant variants. All of the variants displayed FcRn binding comparable with IgG1 (Fig. 7C). These results demonstrate that the pro-
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FIGURE 5. Protease-resistant variants differ in the ability to mediate Fc-dependent cell killing. A, the K326A/E333A mutations restore CDC activity to the 2h backbone. CDC activity was measured using rituximab variants against WIL2-S target cells. Shown is a representative experiment of two independent experiments, each performed in duplicate. B–D, either the S239D or I332E mutation is sufficient to restore ADCC activity to the 2h protease-resistant backbone. ADCC activity was determined using anti-CD20 variants, WIL2-S targets, and PBMC donor cells that were homozygous (IgG1 (64.9 and 72.0 °C, respectively). Finally, protease-resistant mutations do not affect the ability of the mAbs to engage either antigen or FcRn.

We next assessed several biophysical properties of select variants (IgG1, IgG2, 2h-DE, 2h-DAA, and 2h-AEA). Cross-interaction chromatography was used to assess protein–protein interactions, and the results indicated that all of the assessed variants displayed minimal protein–protein interactions (data not shown). Differential scanning calorimetry was performed to assess the thermal stability of the variants (supplemental Table S2). The results indicated varying degrees of thermal stability in the Cα2 region with variants containing the I332E mutation having the lowest Tm (55.6 °C for 2h-DE and 60.7 °C for 2h-AEA). Of the variants tested, 2h-DAA decreased ~7 °C compared with IgG1 (64.9 and 72.0 °C, respectively). Finally, the expression titers for each of the variants were comparable with the parent IgG1 mAb (data not shown).

Protease-resistant Variants Mediate B Cell Depletion in Cynomolgus Monkeys—Because of sequence differences between mouse and human FcγRs, the in vivo function of mAbs engineered for binding to human FcγRs are often assessed in non-human primates (35, 50, 51) whose FcγRs are more homologous to the human counterparts. Because many anti-human CD20 mAbs are cross-reactive with cynomolgus CD20 expressed on B cells, the cytotoxic potential of engineered anti-CD20 variants is often assessed in cynomolgus monkey B cell depletion studies (52). A previous B cell depletion study in cynomolgus monkeys using an Fc silent anti-CD20 variant did not result in depletion of B cells in vivo (53), further supporting that this model is appropriate for the assessment of Fc-dependent effector functions of anti-CD20 mAbs. An anti-CD20 mAb was not used for B cell detection because murine-derived anti-CD20 mAbs, including rituximab, a murine/human chimeric antibody, often bind to a common epitope on the large extracellular loop of CD20 and can either fully or partially mask CD20 (54). Therefore, we used an anti-CD19 antibody (clone J3-119) to detect cynomolgus monkey B cells. A single dose intravenous injection of a 1.0 mg/kg concentration of anti-CD20 IgG1 or the variants 2h-DE or 2h-DAA resulted in nearly complete loss of detection of B cells by the 6-h time point (Fig. 8 and supplemental Table S3). Depletion was sustained through day 7 of the study with B cell recovery starting at day 14 of the study. These results indicated that the protease-resistant vari-
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FIGURE 7. Protease-resistant variant binding to antigen and FcRn is not affected. A, the variants 2h, 2h-AA, 2h-DE, 2h-DAA, and 2h-AEA containing the anti-CD142 V-region bound similarly to IgG1 on a plate-bound ELISA assay using CD142 antigen. Shown is a representative plot of two independent experiments with increasing concentrations of anti-CD142 IgG variants. B, antigen binding was also unaffected with variants containing the anti-CD20 V-region using WIL2-S target cells. Opsonized WIL2-S cells were detected with an IgG1 isotype control (gray bars) and anti-CD20 protease-resistant variants. FIGURE 8. B cell depletion in cynomolgus monkeys using anti-CD20 IgG1 and anti-CD20 protease-resistant variants. Each mAb (1.0 mg/kg) was administered on day 0. Bar charts indicate the frequency of CD19<sup>pos</sup> B cells present in the blood after the indicated time points. The symbols designate the following: saline control (open bars), 2h-DE (light gray bars), 2h-DAA (dark gray bars), and IgG1 (solid black bars). Bar heights correspond to the mean ± S.D. of four animals per group. Two asterisks indicates p < 0.01, and one asterisk indicates p < 0.05 as determined by unpaired, two-tailed Student’s t test. Error bars represent S.D.

DISCUSSION

Both tumors and pathologic microorganisms use multiple mechanisms to invade host tissues and evade immune responses. In the case of tumors, proteases can promote invasion, receptor shedding, and breakdown of the basement membrane (55). Because of high interstitial tumor pressure, antibodies often concentrate in the perivascular regions (56) and at the invasive front of the tumor, presumably in close proximity to proteases (44). In this study, we detected cleaved IgG in the tumor microenvironment with an enrichment of cleaved IgG at the invasive front of HNSCC tumors. Fan et al. (26) recently detected cleaved trastuzumab in human breast cancer tumor tissue, demonstrating that mAb proteolysis also occurs in vivo. Although the quantitative extent of cleavage remains to be determined, these observations coupled with the profound loss of function associated with IgG cleavage (8, 26, 27) suggest that proteolytic disablement of anti-tumor antibodies within human tumors could impair the ability of IgGs to facilitate tumor cell killing. This would be particularly problematic for the sustained efficacy of tumor-targeting mAbs (8, 26, 27). Given that many anti-tumor mAbs have very high affinities for their respective tumor antigens and that several groups have argued that high affinities restrict mAb localization within the tumor microenvironment (56), the accumulation of cleaved IgGs bound to tumor cells could both abrogate cell-killing functions and mask the tumor antigen from Fc functional mAbs. It has been well documented that HNSCC tumors can become resistant to anti-EGF receptor mAb therapy (57), and these data suggest that mAb proteolysis could also be a contributing mechanism of resistance. Therefore, we believe that the generation of a protease-resistant antibody platform could provide significant improvements to current IgG-based cytotoxic therapeutics.

A key challenge faced in the generation of a protease-resistant platform was associated with the observation that cleavage by physiologically relevant proteases mapped to the lower hinge/proximal C<sub>H2</sub> region spanning amino acids Pro<sup>232</sup>-Gly<sup>237</sup> (27), a region otherwise considered critical for human IgG interactions with both complement and FcγRs (8–21). We had previously demonstrated that the lower hinge of IgG2 was resistant to many proteases (29); however, numerous studies have documented that exchanging the lower hinge/proximal C<sub>H2</sub> sequence of IgG1 with corresponding amino acids in IgG2 resulted in a profound decrease in binding to FcγRs (16, 18, 32). In this study, we demonstrate that discrete mutations in the C<sub>H2</sub> region can compensate for the loss of function associated with mutating the lower hinge of IgG1, challenging the long-standing observation that the lower hinge sequence is critical for initiating the complement cascade and facilitating potent ADCC relative to an IgG1. Furthermore, by identifying which mutations were capable of restoring either ADCC activity or CDC activity, we were capable of generating combinatorial variants with both ADCC and CDC capacity while still maintaining protease resistance. Others have demonstrated that mutations in either the C<sub>H1</sub> or C<sub>H3</sub> regions can restore effector functions to Fc modifications typically associated with a loss of function, particularly with regard to removal of the Fc glycan at Asn<sup>297</sup>, a traditional Fc silencing approach (58). Wittrup and co-workers (59) demonstrated that aglycosylated IgGs generated in yeast could selectively bind to FcγRs by introducing compensating mutations proximal to the Asn-X-Ser/Thr N-linked glycosylation motif. Georgiou and co-workers (60) demonstrated that point mutations in the C<sub>H3</sub> region could
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In this study, we have engineered variants that can selectively restore ADCC and/or CDC on a protease-resistant backbone, resulting in a versatile platform amenable to the fine-tuning of specific Fc-mediated effector functions. Furthermore, this study demonstrates that FcγRI and C1q-binding motifs localized in the lower hinge of IgG deemed critical for effector function are in fact dispensable but only in the context of compensating mutations engineered into the C_{1\gamma}^2 region. Because many cancers and invasive microorganisms are associated with proteases and FcγR-dependent functions are often considered a critical mechanism of action for mAbs against invasive diseases, our protease-resistant, Fc-functional antibodies present...
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a novel fit-for-purpose platform for particular disease microenvironments. Additionally, the improved ADCC activity may be useful for indications where IgG1 WT is insufficient in eliciting potent ADCC independent of protease expression. The utility of protease-resistant mAbs as a therapeutic platform will be further assessed for stability, pharmacodynamic properties when targeting antigens in invasive diseases, immunogenicity, and biodistribution. The results of this study suggest novel directions for enhanced functionality for monoclonal antibodies for cancer and infectious diseases.

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