Antibody Distance from the Cell Membrane Regulates Antibody Effector Mechanisms

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Immunotherapy using mAbs, such as rituximab, is an established means of treating hematological malignancies. Abs can elicit a number of mechanisms to delete target cells, including complement-dependent cytotoxicity, Ab-dependent cellular cytotoxicity, and Ab-dependent cellular phagocytosis. The inherent properties of the target molecule help to define which of these mechanisms are more important for efficacy. However, it is often unclear why mAb binding to different epitopes within the same target elicits different levels of therapeutic activity. To specifically address whether distance from the target cell membrane influences the aforementioned effector mechanisms, a panel of fusion proteins consisting of a CD20 or CD52 epitope attached to various CD137 scaffold molecules was generated. The CD137 scaffold was modified through the removal or addition of cysteine-rich extracellular domains to produce a panel of chimeric molecules that held the target epitope at different distances along the protein. It was shown that complement-dependent cytotoxicity and Ab-dependent cellular cytotoxicity favored a membrane-proximal epitope, whereas Ab-dependent cellular phagocytosis favored an epitope positioned further away. These findings were confirmed using reagents targeting the membrane-proximal or -distal domains of CD137 itself before investigating these properties in vivo, where a clear difference in the splenic clearance of transfected tumor cells was observed. Together, this work demonstrates how altering the position of the Ab epitope is able to change the effector mechanisms engaged and facilitates the selection of mAbs designed to delete target cells through specific effector mechanisms and provide more effective therapeutic agents. The Journal of Immunology, 2017, 198: 3999–4011.
are not a simple consequence of the Ab isotype (22); therefore, it is logical to conclude that the nature of the epitope bound influences the effector mechanism engaged.

As of July 2015, 22 mAbs were approved or pending approval for the treatment of cancer in the United States and the European Union (23). Nearly two thirds are direct-targeting mAbs against only eight Ags. Many more targets have been assessed in oncology, so it is unclear what makes these targets so effective as Ags for mAb-mediated immunotherapy. Various explanations for the relationship between the Ag and mAb effector mechanisms have been proposed that relate to Ag density, size, position on the target, and mobility. Ag-expression levels have been correlated with anti-CD20 mAb efficacy (21, 24), and work performed by Derer et al. (25) demonstrated that initiation of CDC was more dependent on the expression level of EGFR than was NK-mediated ADCC. However, it has long been known that Ag specificity (and not simply Ab isotype or expression level) is an important factor in determining the efficacy of CDC (26). Another observation is that the targets approved for cancer immunotherapy are generally small proteins (18, 27) or, if a larger protein (e.g., Her2), the Ab binds relatively close to the cell membrane (28). Accordingly, two of the most successful targets in oncology, and highly represented in the list of approved reagents, are CD20 and CD52, both of which are highly expressed surface receptors with small extracellular domains.

Targeting small Ags, or membrane proximal epitopes, has been shown to be beneficial for chimeric AgR T cells; a decrease in the T cell cytotoxicity was observed when binding domains were positioned further away from the target cell (22, 29). However, this property has not been investigated directly for mAb immunotherapy. Therefore, we hypothesized that the distance between an epitope and the target cell membrane was important for mAb immunotherapy, particularly for the engagement of the Fc-mediated mechanisms CDC, ADCP, and ADCC. To investigate this directly, while avoiding the potential caveats arising from using mAbs with different affinities and/or targeting subtly different mAb epitopes, we generated a panel of fusion proteins based upon a CD137 scaffold displaying the exact target epitope recognized by RTX or CAMPATH-1H at different distances from the target cell membrane. These proteins were then expressed in a variety of target cells and used as model Ags in deletion assays in vitro and in vivo. We found that the effector mechanisms engaged by anti-CD20 and anti-CD52 mAbs were dependent on the fusion protein targeted and its distance from the cell surface. This work has important implications for the development of new therapeutics that seek to exploit specific effector mechanisms and elicit more effective deletion.

Materials and Methods

Abs

F4/80–allophycocyanin was from Serotec (clone CL:A3-1), and B220–PerCP (clone RA3-6B2), anti-mouse Fc–Fab2–PE (clone Poly4053), DCSPE (clone RIKO-3), and CD59–PE (clone mCD59.3) were purchased from BioLegend. RTX–human (h)lgG1 and RTX–mouse (m) lgG2a were produced in-house from patented sequences (22). CAMPATH-1H was kindly provided with the UniProt reference sequence). These gene constructs were gifted of Prof. M. Pule and Dr. B. Philip, University College London (33, 34). Each fusion protein consisted of the CD52 leader sequence, Rps/ Cp11, and CD137 (with domains removed based on the annotations provided with the UniProt reference sequence). These gene constructs were cloned into the pcDNA3.1–(neo) expression vector (Invitrogen).

Cell transfections

A total of 10 × 10^6 CHO-S cells was transfected with 10 µg of plasmid DNA using lipofection with FreeStyle MAX Reagent (Invitrogen). A20 cells were nucleofected with 2 µg of plasmid DNA using an Amaxa Cell Line Nucleo-fector Kit T (Lonza). Following nucleofection, cells were cultured in the presence of 1 ng/ml Genetix (Life Technologies) to select for stable transfectants.

CDC assay

A total of 1 × 10^5 cells was labeled with diluting concentrations of Ab for 15 min at room temperature. Human serum (Sigma) was added at 15% final concentration (v/v) and incubated at 37°C for 30 min. Cell death was assessed with propidium iodide inclusion by flow cytometry using a FACSscan (BD). Nonopsonized cells were used to establish basal cell death, and the raw data were adjusted to the transfection efficiency using the following equation:

CDC-specific lysis = (%PI of sample − baseline/100 − %PI of baseline) × 100/transfected cells × 100

ADCP assay

Target cells were labeled with 5 µM CFSE for 10 min at room temperature before washing in complete media. CFSE-labeled targets were then opsonized with diluting concentrations of Ab before coculturing at a 1:5 E:T ratio with BMDMs in 96-well plates for 1 h at 37°C. BMDMs were then labeled with anti-F4/80–allophycocyanin for 15 min at room temperature and washed with PBS twice. Plates were kept on ice, wells were scraped to collect BMDMs, and phagocytosis was assessed by flow cytometry using a FACScalibur (BD) to determine the percentage of F4/80^+CFSE^+ cells within the F4/80^+^ cell population.

Confocal imaging of ADCP

BMDMs were plated overnight at 1 × 10^5 cells per milliliter onto poly-t-lysine (Sigma)-coated coverslips. Target cells were labeled with CFSE and 10 µg/ml of Ab, as described above (ADCP assay). Following 1-h coculture, coverslips were washed in PBS before fixation in 2% paraformaldehyde for 15 min at room temperature. A total of 100 µg/ml of rhodamine wheat germ agglutinin was added for 30 min at room temperature before washing in PBS. Lastly, nuclei were stained with DAPI before mounting. Images were collected using a Leica TCS SP5 with Leica software (LAS-AF v2; both from Leica Microsystems) and processed using Adobe Photoshop CC (version 7 SP1).

ADCC assay

Target cells were labeled with calcine AM, followed by the addition of diluting concentrations of Ab. Target cells were cocultured with human PBMCs at a 50:1 E:T ratio for 4 h at 37°C. The plate was centrifuged at 400 × g for 5 min to pellet the cells, and the supernatant was transferred to a white 96-well plate. Calcine release was measured using a Varioskan (Thermo Scientific) at 455 nm, and the percentage of maximal release was calculated as follows:

%max release = (sample/triton treated) × 100

In vivo experiments

All animal experiments were performed in accordance with the Animal (Scientific Procedures) Act 1986 within a Home Office–approved facility
under project license 302964. WT female BALB/c mice younger than 4 mo of age were used. The actual severity of all animal experiments was recorded as mild in compliance with the project license. A total of $1 \times 10^6$ A20 cells was administered i.v. on day 0. On day 3, 100 $\mu$g of RTX-mIgG2a (22) or PBS was given by i.v. injection. The spleen, liver, and inguinal lymph nodes were collected from all mice when the control group developed terminal tumor burden (defined as visible signs of ill health, including expansion of the liver). Organs were homogenized into a single-cell suspension in 3 ml of sterile PBS and passed through a 100-$\mu$m cell strainer before analysis by flow cytometry.

**Western blotting of cell lysates**

A total of $6 \times 10^6$ cells was lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS containing protease inhibitor mixture [Sigma], 50 mM NaF, and 0.2 mM Na3VO4). A total of 25 $\mu$g of lysate was loaded onto 10% Bis-Tris gels, and proteins were separated under constant voltage. The proteins were transferred onto a nitrocellulose membrane, blocked using a 5% milk solution (Marvel), and probed with a polyclonal rabbit anti-hCD137 Ab overnight at 4°C. The membrane was washed, and bound Ab was detected using an HRP-conjugated anti-Rabbit secondary Ab (NA9340; GE Healthcare) and ECL substrate (Thermo Scientific) before chemiluminescence film was applied and developed using a Xograph Imaging Systems Compact X4.

**Statistics**

Data were analyzed using one- or two-way ANOVA, where appropriate. All statistical analyses were performed using GraphPad Prism (version 7.01).

**Results**

**Generation of a model Ag system**

A panel of engineered molecules was designed to assess the relationship between the epitope targeted along a target protein (from its membrane proximal to distal domains) and the effector mechanisms engaged. These first constructs contained a small peptide [previously identified to be bound by the anti-CD20 mAb RTX (34)] attached to the N-terminal domain of hCD137. hCD137 contains four fairly equivalently sized extracellular cysteine-rich domains (Fig. 1A) and so is ideal for modular manipulation (35). Through the use of overlap-extension PCR, extracellular domains were removed or replicated, resulting in a panel of fusion proteins designed to display the RTX epitope at varying distances from the cell surface, estimated to range from 0.3 to 16 nm away (Fig. 1A). These constructs were transiently transfected into CHO-S cells, with protein expression confirmed by flow cytometry using Abs directed against the RTX epitope and individual domains of CD137 (Fig. 1B). Importantly, the position of the epitope did not appear to affect binding, because RTX bound equally well at all positions along CD137. Western blotting was also performed, in particular to confirm the size difference between the R-4d and R-8d constructs, which was not possible by flow cytometry because of the duplication of the same extracellular domains in CD137 (Fig. 1C).

**Assessment of Ab effector engagement with RTX**

CHO-S cells expressing each of the fusion proteins were used as targets to assess whether the ability of RTX to initiate CDC, ADCC, or ADCP was altered depending on the distance that the epitope was held from the cell membrane. The first mechanism investigated was CDC; cells were cultured with RTX in the presence of human serum, and cell death was recorded by flow cytometry (Fig. 2A). In preliminary experiments, using CHO-S cells expressing the R-4d construct as targets, a serum titration was performed and found that maximal killing could be achieved at 15% serum, with no further improvement, even when the serum concentration was increased to 45% (Supplemental Fig. 1). As such, 15% serum was used in all subsequent CDC assays. To adjust for any differences in the protein expression of the different constructs, the degree of target cell lysis was corrected according to the level of expression, as described in the Materials and Methods. These experiments clearly showed that the ability of RTX to initiate CDC was diminished when targeted furthest away from the cell surface with the R-8d construct (Fig. 2B). Less than half of the cells were lysed under saturating (10 $\mu$g/ml) concentrations of Ab, with little-to-no lysis observed when the Ab concentration was $\leq 0.4 $g/ml. Although the three other constructs were able to initiate CDC similarly at the higher mAb concentrations, R-4d showed a trend (although not statistically significant) toward being less effective as a target at lower concentrations of Ab (<0.4 $\mu$g/ml) compared with constructs targeting the mAb closer to the cell surface (R-1d and R-flush).

Next, we assessed the ability of the constructs to function as targets for phagocytosis using mouse BMDMs as effectors (Fig. 2C). In these ADCC assays, targeting all of the constructs, with the exception of R-flush, resulted in effective phagocytosis of RTX-opsonized targets (Fig. 2D). Although R-8d was less sensitive to ADCC compared with the R-1d and R-4d at lower concentrations of Ab (<1 $\mu$g/ml), this was not statistically significant. Importantly, expression of the R-flush domain was equivalent to the other constructs (Fig. 1B) and was sufficient for effective CDC (Fig. 2B), indicating that displaying the Ag too close to the cell surface was specifically detrimental to phagocytosis, as opposed to simply being inaccessible to mAb binding.

The ability of the various constructs to mediate ADCC activity was also evaluated. Human PBMCs were used as the source of effector cells in these assays, with the resident NK cells previously shown to be the main mediators of cytotoxic activity (36). Targeting R-flush resulted in the greatest level of ADCC, whereas targeting R-8d elicited the lowest level of killing (Fig. 2E), similar to the trend seen with CDC.

**Assessment of Ab effector engagement with CAMPATH-1H**

To confirm that the results generated above were related to Ab distance and were not a unique property of the RTX epitope, a second panel of fusion proteins was generated. These contained the same CD137 backbone as before (Fig. 1A), but the RTX epitope was replaced by one recognized by the anti-CD52 Ab, CAMPATH-1H (alemtuzumab) (33). These proteins were expressed in CHO-S cells and were recognized specifically by CAMPATH-1H (Fig. 3A) and not RTX (data not shown). When used as targets in the three in vitro assays (Fig. 3B, 3C), the same trends in terms of distance and the effector mechanism engaged were identified, such that CDC and ADCC were ineffective when targeted furthest away from the target cell membrane (C-8d), and ADCC was suboptimal when targeted too close to the cell membrane (C-flush).

**Stable expression compared with transient expression systems**

The engagement of Ab effector mechanisms, such as CDC, can be influenced by the expression levels of the targeted Ag (21, 24, 25). The transient transfection approach presented previously resulted in a heterogeneous population containing nontransfected cells and cells with a range of expression levels for each fusion protein, including a proportion of cells expressing extremely high levels of target (Figs. 1B, 3A). Although this approach is useful for avoiding potential selection artifacts, it may obscure more subtle observations at lower expression levels that occur as a consequence of distance. To determine whether engagement of the FcγR-dependent effector mechanisms was affected by the Ag expression level, the murine cell line A20 was transfected with R-4d, and stable clones of different expression levels were isolated.
These clones (referred to as R-4d–Low, R-4d–Med, and R-4d–High) were assessed in the FcγR-mediated assays: ADCP (Fig. 4B) and ADCC (Fig. 4C). In both cases, there was a clear decrease in the ability to engage these mechanisms when targeting Ag was expressed at lower levels, although the difference between the high and medium expressing clones is not statistically significant.

Having established this clear relationship, to exclude this variable when comparing between the panel of proteins, the A20 cell line was transfected with the RTX-binding fusion constructs (R-flush, R-4d, and R-8d) to produce a panel of stable cell lines with equivalent expression levels (Fig. 5A). These cells were used as targets in the three effector assays. As seen in Fig. 5B and 5D, the results confirmed the observations reported for the transient CHO-S system (i.e., CDC and ADCC were diminished when targeting the cells displaying the R-8d construct, and ADCP was poor when targeting the R-flush–transfected cells). These data provide further evidence that the effector mechanisms engaged by an Ab are directly influenced by the epitope distance from the target cell membrane.

Having established these principles in a controlled system, we then sought to validate them using a more typical scenario in which Abs are available to different epitopes on the same target molecule. This approach has the added advantage that the expression profile of the target molecule, and the target cell itself, is exactly the same for both of the mAbs used. Using a recently generated pair of anti-CD137 mAbs with similar affinities that are known to bind the most proximal (SAP3–6) or distal...
(SAP1–3) domains of CD137 (Fig. 1A), we explored their relative efficacy in CDC, ADCP, and ADCC. In the CDC and ADCC assays, the most membrane-proximal mAb, SAP3–6, was more effective than the distal mAb (Fig. 6A, 6C), particularly in ADCC. In contrast, in our ADCP assay (Fig. 6B), there was no clear difference between the two Abs; both were able to elicit significant phagocytosis compared with the isotype control. The similarity observed between SAP1–3 and SAP3–6 in the ADCP assay was comparable to that reported earlier between the four-domain and one-domain constructs (Figs. 2D, 3C). This indicates that, although SAP3–6 binds the bottom domain of CD137, it is at a similar distance from the cell membrane as the R-1d construct (rather than R-flush) and so is ideal for ADCP. These data further corroborate the distance hypothesis that binding close to the target cell membrane is preferable for ADCC and CDC.

Resolving the conflict between Cp11-flush and WT CD52 in ADCP assay

The work presented thus far demonstrated that engagement of the mAb effector mechanisms was influenced by the distance that an Ab binds from the target cell membrane, initially with RTX and subsequently with CAMPATH-1H. hCD52, the Ag for CAMPATH-1H, consists of a 12-aa extracellular peptide tethered to the cell membrane via a GPI anchor (37, 38). This is similar to the C-flush construct generated within this study, which contained an 11-aa extracellular peptide tethered via a protein transmembrane domain (Fig. 1A). In vitro, CAMPATH-1H is highly effective at initiating CDC to delete target cells (39), whereas in vivo, human and murine studies indicate that the effectiveness and prolonged clearance observed are primarily mediated by FcγR (40, 41).

Given the key role for macrophages in Ab immunotherapy (17, 42),

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**FIGURE 2.** Comparison of cytotoxic activity engaged by RTX when targeting CHO-S cells displaying the RTX epitope at different distances from the plasma membrane. CHO-S cells transfected with each of the fusion proteins (Fig. 1) were used as targets to investigate Ab effector mechanisms in vitro. (A and B) Transfected CHO-S cells were labeled with RTX or an isotype control (ISO) and incubated with 15% human serum for 30 min. CDC was measured by flow cytometry using propidium iodide inclusion as a measure of cell lysis. Representative data (A) and results from n = 3 independent experiments (B). (C and D) CFSE-labeled targets were opsonized with RTX or ISO before use as targets in an ADCP assay with murine BMDMs. The percentage of BMDMs that phagocytosed the target cells (defined as the F4/80+CFSE+ population) was recorded and plotted. (E) CFSE-labeled targets are green, wheat germ agglutinin (WGA)-stained surface membranes are red, and DAPI-stained nuclei are blue (original magnification ×63). (F) Results from n = 3 independent experiments. *p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001, two-way ANOVA.
it is logical to assume that CAMPATH-1H is also effective in ADCP. However, we demonstrated that CAMPATH-1H was unable to effectively elicit phagocytosis when targeting the C-flush construct (Fig. 3C). Knowing that the engagement of CDC and ADCC by CAMPATH-1H when binding the C-flush construct fitted the expected mechanism of action for this mAb (Fig. 3B, 3D), the poor phagocytosis when binding an Ag similar to its natural target warranted further investigation. This was not the consequence of the CHO-S transient expression system used, because transfecting these cells with WT CD52, followed by CAMPATH-1H, resulted in highly effective ADCP (Supplemental Fig. 2A), indicating that the C-flush construct must differ in some other key property.

Both proteins consist of small extracellular domains; however, CD52 is glycosylated just outside of the CAMPATH-1H epitope, whereas the C-flush construct is not (37, 43). Therefore, it was postulated that the presence of the glycan on CD52 may stabilize the interaction of CAMPATH-1H, leading to preferential ADCP compared with the C-flush fusion protein. A mutant form of CD52 (referred to as CD52-NQ), in which the Asn was mutated to a Gln, was generated, expressed on CHO-S cells (Supplemental Fig. 2B), and assessed in the ADCP assay (Fig. 7A). The nonglycosylated mutant was still able to elicit ADCP following CAMPATH-1H treatment, demonstrating that glycosylation of CD52 was not important for the effective induction of ADCP by CAMPATH-1H.

We next considered whether the method of membrane attachment was influencing the efficiency of ADCP. To test this, a GPI-anchored version of the CAMPATH-1H epitope was generated. The C-GPI construct contained the same leader and C-terminal sequences as hCD52, as well as the Pro-Ser of the CD52 GPI-attachment site (Fig. 7B). This was based on previous work that proposed that 2 aa prior to the GPI attachment site and the carboxy sequence were important for mediating the posttranslational attachment of the protein to the GPI anchor (44, 45). The C-GPI construct was expressed in CHO-S cells (Supplemental Fig. 2C), bound by CAMPATH-1H, and compared in ADCP assays alongside C-flush and WT CD52 (Fig. 7C). These experiments clearly demonstrated a rescue in ADCP function when the CAMPATH-1H epitope was tethered through the GPI format (C-GPI), leading to a construct that was at least as efficient as WT CD52 and far more effective than C-flush. These data indicate that the GPI anchor likely overcomes the restriction on ADCP for targets that are extremely close to the cell surface.

In vivo function of RTX targeting different distances

Investigating the effector mechanisms in individual effector assays in vitro clearly identified differences between the most proximal and distal epitope constructs, R-flush and R-8d, respectively. To explore the relevance of these findings for immunotherapy in vivo where all mechanisms might be in play, we used A20 cells stably transfected with R-flush or R-8d (Fig. 5A). These were injected i.v. into WT female BALB/c mice, and the resulting tumors were detected and distinguished from endogenous B cells using flow cytometry with an A20-specific anti-idiotypic mAb (Fig. 8A). At terminal tumor burden, the A20 cells were located predominantly in the spleen and liver.

To compare the capacity of these two Ags to facilitate tumor deletion, a single dose of PBS or RTX-mlgG2a was administered 3 d following tumor inoculation (Fig. 8B), and the presence of A20 cells expressing each construct was determined in the spleen and liver. Although all groups had high tumor burden in the liver, resulting in them becoming terminal, when comparing the percentage of tumor cells present in the spleens, mice that received tumor expressing R-flush followed by RTX-mlgG2a were
were measured by flow cytometry. The three cell lines (R-4d–Low, R-4d–Med, and R-4d–High) were used as targets for ADCP (stable clones expressing different levels at the cell surface were isolated. The same target can elicit striking differences in therapeutic effectiveness by engaging Fc-mediated effector functions, such as CDC, ADCP, epitope from the cell surface. For two different specificities (anti-CD20 and anti-CD52), CDC was ineffective when targeting the epitope furthest away (R-8d, C-8d) from the membrane and was less sensitive at lower mAb concentrations when binding cells expressing the four-domain constructs (R-4d and C-4d, Figs. 2B, 3B). A similar trend was observed for ADCC (Figs. 2E, 3D). ADCP displayed different requirements; a minimum distance of a single extracellular domain was required before phagocytosis of the target cell was achieved, and membrane-distal epitopes (R-8d, C-8d) were still relatively effective as targets (Figs. 2D, 3C). Importantly, the ineffective phagocytosis of the membrane-flush epitopes (R-flush and C-flush) was not due to an inability of the mAb to bind these targets, as demonstrated by flow cytometry (Figs. 1B, 3A) and their efficacy in the other effector assays (ADCC and CDC).

The effect of membrane distance on CDC is intuitively logical. The classical pathway of complement is known to be self-regulated by the short half-life of the active components at all stages of the cascade (53). Being activated far away from the target cell membrane means that the activated complement components C2a and C4b have a reduced chance of stabilizing on the cell surface as a result of the extended diffusion through the extracellular matrix. Therefore, the greater the distance from the cell surface, the lower the efficiency in the establishment of the C3 convertase complex, a key determinant in the cascade that leads to cell lysis via the formation of the membrane attack complex. This same reasoning was used to explain ofatumumab’s superior initiation of CDC

**FIGURE 4.** Importance of expression level for engagement of FcyR-mediated mechanisms. A20 cells were transfected with the R-4d construct, and stable clones expressing different levels at the cell surface were isolated. (A) The clones were labeled with RTX-FITC or ISO-FITC, and expression levels were measured by flow cytometry. The three cell lines (R-4d–Low, R-4d–Med, and R-4d–High) were used as targets for ADCP (B) and ADCC (C). The mean and SD of three independent experiments are presented. *p < 0.05, one-way ANOVA.

effectively cleared compared with those receiving PBS (Fig. 8C). In contrast, the tumors expressing the R-8d construct were not cleared in either location, indicating inefficient targeting of the relevant effector mechanisms.

**Discussion**

Although mAbs are being used increasingly in the clinic to treat an ever-expanding array of diseases, there is still much that we do not know about this class of therapeutics. The diversity of molecules targeted by mAbs has increased exponentially over the last decade (23), but, surprisingly, we are still ignorant of some of the most basic rules for their behavior. Perhaps the simplest class of therapeutic mAbs to understand are the direct-targeting mAbs. These reagents bind directly to the tumor cell target and elicit anticancer effects by inducing changes to signaling (direct cell death) or by engaging Fe-mediated effector functions, such as CDC, ADCP, and ADCC. Although it is accepted that different mAb binding to the same target can elicit striking differences in therapeutic efficacy, evidence for why this occurs is often scarce.

mAb properties, such as Fe-yR affinity (46), isotype (47), and binding level (24, 25), have well-established effects on mAb effector systems and can certainly help to explain why some mAbs are more active than others. However, for other mAbs, more subtle differences can also have a major impact. For example, in the case of the anti-CD20 mAb, type I and II engage different effector functions (4, 22) while binding to an overlapping, cross-blocking epitope (48). Given that several successful and approved antitumor mAbs bind to surface-proximal Ags, such as CD20 and CD52, in this study we examined the effect of position along a target molecule, from membrane proximal to distal, on the effector mechanisms engaged by direct-targeting Abs.

The target molecule chosen, CD137, has four extracellular cysteine-rich domains (35). Its atomic structure has not been resolved, but, modeled upon the TNFR1 crystal structure (49), the four domains would appear to gravitate away from the plasma membrane in a relatively linear fashion (Fig. 1A); therefore, it is likely that the positions along CD137 correspond to increasing distances from the target cell surface. However, it is important to note that some of our findings may have been influenced by our choice of receptor. For example, our previous data highlighted the particular potency of CD20 as a target molecule as a result of its ability to cluster and redistribute into lipid raft areas of the plasma membrane (19). CD137 is an integral membrane protein expressed on T cells, and it is a member of the TNFR superfamily (50), forming trimers with its ligand for activation (51). Cross-linking of CD137 was subsequently shown to cause its redistribution to lipid raft regions at the contact site with ligand-expressing cells (52); therefore, like CD20 and CD52, CD137 may reflect a particularly potent target molecule that can become redistributed and concentrated within the plasma membrane to augment mAb effector mechanisms.

Using a combination of approaches involving transient and stable expression systems, both in vitro and in vivo, we were able to reveal differences in the ability to engage the three Fe-mediated effector mechanisms depending upon the position of the mAb epitope from the cell surface. For two different specificities (anti-CD20 and anti-CD52), CDC was ineffective when targeting the epitope furthest away (R-8d, C-8d) from the membrane and was less sensitive at lower mAb concentrations when binding cells expressing the four-domain constructs (R-4d and C-4d, Figs. 2B, 3B). A similar trend was observed for ADCC (Figs. 2E, 3D). ADCP displayed different requirements; a minimum distance of a single extracellular domain was required before phagocytosis of the target cell was achieved, and membrane-distal epitopes (R-8d and C-8d) were still relatively effective as targets (Figs. 2D, 3C). Importantly, the ineffective phagocytosis of the membrane-flush epitopes (R-flush and C-flush) was not due to an inability of the Ab to bind these targets, as demonstrated by flow cytometry (Figs. 1B, 3A) and their efficacy in the other effector assays (ADCC and CDC).
compared with RTX: it binds a more membrane-proximal loop of hCD20 (5, 21).

More recent work has demonstrated that activation of the classical complement pathway is better facilitated through a hexameric association of Ab Fc regions. Although the formation of hexamers can be engineered via Fc modification (54), they can form naturally upon the surface of the target cell via lateral movement of the Ag:Ab complexes (7). All of the constructs generated in this study contained identical transmembrane and intracellular domains; therefore, it was assumed that they would have equivalent ability to redistribute across the cell surface and form hexamers. However, the extracellular domains of each construct vary in size. Therefore, differing levels of steric hindrance and interactions with other endogenous cell surface proteins could

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**FIGURE 5.** Comparison of cytotoxic activity engaged by RTX when targeting A20 cells stably displaying the RTX epitope at different distances from the plasma membrane. (A) A20 cells were stably transfected to express the fusion proteins R-flush, R-4d, and R-8d and then clones with equivalent levels of expression were selected. The flow cytometry data show that the three cell lines express similar levels of the fusion protein when detected with FITC-conjugated RTX. The filled line graphs reflect staining with the ISO control. These cells were then used to assess CDC (B), ADCP (C), and ADCC (D) in vitro. The mean and SD of three independent experiments are presented. *p < 0.05, **p < 0.001, two-way ANOVA.
influence membrane redistribution and restrict the hexameric complexes from forming.

All of the work presented in this article used the hIgG1 isotype (or mIgG2a in vivo as the closest mouse parallel) (10), because this reflects the isotype typically adopted for direct-targeting mAbs in the clinic (23). Whether other isotypes will follow the same rules remains to be determined, because it is well established that they differ in their ability to engage the various effector mechanisms. Recently, hIgG3 has been investigated as an alternative to hIgG1 because of its superior engagement of FcγR (55) and binding of C1q (47). Brüggemann et al. (47) used a panel of chimeric Abs containing the same specificity but class-switched H chains to demonstrate that hIgG1 and hIgG3 were best at engaging CDC and ADCC. One interesting point is that, although hIgG3 is better at binding C1q, this binding does not translate directly to improved CDC (26, 47). Other work reported that a chimeric Ab, containing the CH1 and hinge region of hIgG1 attached to a hIgG3 Fc domain, was better able to initiate CDC compared with WT hIgG3 (56). One main difference between the hIgG1 and hIgG3 classes is the length of the hinge region, with hIgG3 having a hinge region approximately four times that of hIgG1 (55). In light of our work presented in this article, the improved lytic ability of the hIgG1/3 hybrid may be due to the far shorter hinge region, in turn bringing the complement activation closer to the cell surface for the initiation of CDC. It would be interesting to determine whether combining this isotype chimera with epitopes directed to the membrane-proximal region of Ags would result in even more potent reagents.

FIGURE 6. Comparison of cytotoxic activity initiated by mAb binding different domains of CD137. CHO-S cells expressing R-4d were labeled with different concentrations of SAP1–3 (binds membrane-distal domain of CD137) or SAP3–6 (binds membrane-proximal domain of CD137) and used as targets in CDC (A), ADCP (B), and ADCC (C) assays. In (A), the mean and SD of three samples in a single experiment are plotted. In (B), the mean and SD of three independent experiments are plotted. Data in (C) are representative of three independent experiments. *p < 0.05, ****p < 0.0001, two-way ANOVA. n.s., not significant.

FIGURE 7. Importance of the GPI anchor in WT CD52 for ADCP ability. (A) A nonglycosylated mutant of hCD52 (CD52-NQ) was generated and expressed in CHO-S cells. Its ability to facilitate ADCP of target cells when opsonized with CAMPATH-1H was compared with CHO-S cells expressing C-flush or WT CD52. (B) Gene schematics for the generation of C-GPI consisting of the peptide CAMPATH-1H epitope attached to the same GPI anchor as CD52. (C) The C-GPI construct was generated and expressed in CHO-S cells. Its ability to facilitate ADCP of target cells when opsonized with CAMPATH-1H was compared with CHO-S cells expressing C-flush or WT CD52. The mean and SD of three independent experiments are shown. *p < 0.05, **p < 0.005, ***p < 0.001, two-way ANOVA. n.s., not significant.
Perhaps more surprising was the contrasting dependence on distance seen with the FcγR-mediated mechanisms ADCP and ADCC. ADCP was significantly reduced when binding the membrane-flush constructs compared with those that were held at least one extracellular domain away (Figs. 2D, 3C). ADCC was effective for these constructs, but activity was lost when targeting an epitope positioned distal to the target cell membrane (Figs. 2E, 3D). The approach undertaken within this study used the same epitope and mAb pairing; as such, the binding affinities remained consistent among all of the constructs investigated. In addition, the same Ab was used, with no changes to the isotype or Fc region; therefore, the kinetics behind Fc:FcγR interactions would also be equivalent among systems. These observations reveal fundamental differences in the requirements of activation for these mechanisms. Although ADCC and ADCP require the engagement of activating FcγR on the cell surface, the downstream effects and outcomes are clearly very different, although the reasons for this remain unanswered.

Release of cytotoxic granules by NK cells following FcγR activation has been demonstrated to use relatively small immune synapses, analogous to those induced during T cell–mediated cytotoxicity (11). There is evidence that a T cell synapse needs to be \( \sim 2 \) nm, according to the kinetic-segregation model (57, 58). Assuming that the requirement for NK cell killing is similar, the fact that there is poor activation of ADCC when targeting the epitope held furthest away would fit this model, because the eight-domain fusion protein exceeds the maximum synapse size by \( \sim 2 \) nm (58).

For phagocytosis, whether FcγR-mediated or via other means, a high level of intracellular reorganization is required to form the phagosome and provide the means to engulf the targeted cell (12). This process is likely to last longer/require more continuous signaling compared with that for cytotoxic release; therefore, the multimeric complex among the Ag, mAb, and FcγR would need to be more stable and to mediate the sustained signaling required. Therefore, it is likely that, when targeting the membrane-flush constructs, although the binding kinetics remain similar to those of the more distant epitopes, the endogenous proteins present within the cell membrane (not including the fusion protein itself) may provide steric resistance and pressures on the binding, resulting in an overall less stable multimeric complex and leading to less efficient ADCP. Phagocytosis is proposed to proceed through a zippering mechanism whereby integrins promote the efficient binding of FcγR:Fc complexes and serve to integrate signals from multiple, potentially disparate receptors (12). Because zippering requires the formation of sequential FcγR:Fc complexes for continuing phagocytic cup generation and closure, it could be possible that, within this process, engagement of surface-proximal “flush” constructs do not facilitate appropriate integrin distribution or efficient zippering for the reasons listed above.

Ag density has long been correlated with the effectiveness of CDC engagement in vitro and in clinical studies (21, 24). High

**FIGURE 8.** In vivo clearance of A20 cells expressing different RTX constructs following RTX therapy. (A) WT female BALB/c mice were inoculated with \( 1 \times 10^6 \) A20 cells i.v. and screened for tumor development within the liver, spleen, and lymph nodes in terminal animals by flow cytometry, as idiootype‘B220’ cells. (B) WT BALB/c mice were inoculated with A20 cells expressing R-flush or R-8d on day 0, with 100 μg Ritm2a or PBS given on day 3. (C) Once mice had become terminal, the liver and spleen were harvested, and the percentage of tumor cells was identified by flow cytometry (idiootype‘B220’). The percentage of tumor present for each individual mouse was normalized against the mean tumor burden within the control group in each experiment. The data show the results from six mice from two independent experiments. \(* * * * p < 0.0001, \) one-way ANOVA. n.s., not significant.
expression levels allow for a better chance of juxtaposed Ab Fc regions being present in the correct orientation for favorable engagement of C1 (6, 26). However, the relationship between Ag expression level and engagement of the FcγR-mediated mechanisms is less defined. Derer et al. (25) indicated that NK and polymorphonuclear leukocyte–mediated killing was less dependent on Ag expression compared with CDC; however, there seemed to be a minimum level required before cell death was observed. It is worth noting that, in that study, different clones of Ab were used that were potentially directed to different epitopes and with different affinities, rather than a single epitope and Ab targeted in this study, making it difficult to directly compare the efficacy of each epitope location. Nonetheless, by using a range of cell lines stably expressing different levels of the R-4d fusion protein, it was clear that the percentage of ADCP and ADCC that occurred in response to RTX treatment positively correlated with the expression level (Fig. 4). This finding agrees with the conclusions made by Derer et al. (25) with regard to CDC and ADCC and supports other work from our group in which the Ag expression level correlates positively with the amount of ADCC observed (L. Dou, S.L. Buchan, S.N. Dunn, S.G. Booth, C. Lai, M. Semmrich, I. Teige, H.J. Kim, J.E. Willoughby, L.N. Dahal, K.L. Cleary, P. Kannisto, M. Jernetz, E.L. Williams, E. Healy, J.S. Verbeek, B. Frende´us, M.S. Cragg, M.J. Glenmie, J.C. Gray, A. Al-Shamkhani, and S.A. Beers, manuscript in preparation).

Our further study of membrane-proximal targets revealed an initial contradiction when targeting native CD52, as opposed to the C-flush construct. We identified that attaching the CAMPATH-1H epitope mimic to a GPI anchor (C-GPI construct) restored sensitivity of the target cell toward phagocytosis (Fig. 7C). GPI anchors are a commonly found posttranslational modification; however, there are still unknowns in terms of their structure, size, and function. Resolved structures of GPI anchors are lacking; however, computer modeling indicates that they may be far larger than anticipated (∼1.5 nm in size) (59). Assuming that CD52 uses a similar anchor as modeled for CD59 (27, 59), then it is more equivalent in distance from the membrane to the C-1d construct, which is able to engage efficient ADCP, compared with the C-flush molecule, in support of our original hypothesis.

In vivo experiments in syngeneic immune-competent mice revealed a clear depletion of A20 tumor cells expressing the R-flush, but not R-8d, construct from the spleen of BALB/c mice following treatment with RTX-m2a (Fig. 8C). This indicates that the smaller, more membrane-proximal epitope was more effective in depleting tumor cells and that ADCC and/or CDC were principally responsible. Although at odds with our recent findings identifying ADCP as the main effector mechanism (17, 60), it is reminiscent of our data using xenograft models (20) and likely reflects the use of a highly selected in vitro cell line that has undergone loss of complement defense and/or NK control. The lack of complement defense through CD55 and CD59 was subsequently confirmed (Supplemental Fig. 3). In contrast, no depletion was observed in the liver for either of the two tumor cell lines generated.

The differences in the clearance of R-flush–expressing cells between the spleen and the liver reflect another factor that is not clearly understood: the role of the tumor microenvironment in regulating mAb efficacy. It is now evident that the tumor microenvironment can promote infiltrating immune cells to adopt a more protumor/immune-suppressive phenotype (61); however, its direct effect on mAb therapy once again is unclear. It is possible that altering the epitope targeted within an Ag may allow for more efficacious Ab therapies to be developed that can overcome the specific suppressive elements present. In addition to direct anti-tumor targets, this may benefit reagents seeking to deplete immune-suppressive cells themselves (e.g., regulatory T cells [Tregs]) (62). Indeed, the two anti-CD137 mAbs used in this study (SAP1–3 and SAP3–6) demonstrated an application of our findings (Fig. 6). CD137 is expressed on resting Tregs and is upregulated following activation (63, 64); therefore, it may serve as a useful target for Treg deletion. SAP1–3 and SAP3–6 have the same isotype and bind at equivalent levels and with similar affinities toward CD137 (data not shown). Although they were broadly equivalent with regard to their induction of ADCP, differences in the engagement of the effector mechanisms ADCC and CDC were apparent, particularly in relation to the minimum concentration required to elicit cell death. Because both mAbs are equivalent in all other measurable ways, the difference likely lies in the bound epitope and its relation to the target cell membrane.

As detailed above, the tumor can manipulate its local microenvironment and has the capacity to impair the various effector mechanisms to circumvent mAb-mediated killing. For example, a highly suppressive macrophage environment might exist with a low activatory/inhibitory FcγR ratio but where complement components are plentiful. Similarly, if the environment is driving high levels of FcγRIIB, NK cell–mediated ADCC may remain functional, because they, unlike macrophages, monocytes, and dendritic cells, do not express this inhibitory receptor. Using the knowledge gained in this study and switching the fine specificity of an Ab (i.e., the distance it binds from the cell surface), the most effective effector mechanisms can be selected in each situation, potentially leading to more potent Ab therapeutics.

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References
1. Molina, A. 2008. A decade of rituximab: improving survival outcomes in non-Hodgkin’s lymphoma. Annu. Rev. Med. 59: 237–250.
2. Weiner, L. M., J. C. Murray, and C. W. Shuptrine. 2012. Antibody-based immunotherapy of cancer. Cell 148: 1081–1084.
3. Ivanov, A., S. A. Beers, C. A. Walshe, J. Honeychurch, W. Aldaujai, K. L. Cox, K. N. Potter, S. Murray, C. H. Chan, T. Klymenko, et al. 2009. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. J. Clin. Invest. 119: 2143–2159.
4. Aldaujai, W., A. Ivanov, J. Honeychurch, E. J. Checkle, S. Potlari, S. H. Lim, K. Shimada, C. H. Chan, A. Tuitt, S. A. Beers, et al. 2011. Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. Blood 117: 4519–4529.
5. Oldham, R. J., K. L. Cleary, and M. S. Cragg. 2014. CD20 and its antibodies: past, present, and future. Eur. Immunopathol. Dis. Therap. 5: 7–23.
6. Borsos, T., and H. J. Rapp. 1965. Complement fixation on cell surfaces by 19S and 7S antibodies. Science 150: 505–506.
7. Diebold, C. A., P. J. Beurskens, R. N. de Jong, R. I. Koning, K. Strumane, M. A. Linderfer, M. Voorhorst, D. Ugarlar, S. Rosati, A. J. Heck, et al. 2014. Complement is activated by IgG hexamers assembled at the cell surface. Science 343: 1260–1263.
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8. van Lookeren Campagne, M., C. Wiesmann, and E. J. Brown. 2007. Macrophage complement receptors and pathogen clearance. Cell. Microbiol. 9: 2095–2102.

9. Müller-Eberhard, H. J. 1968. The membrane attack complex of complement. Annu. Rev. Immunol. 4: 503–528.

10. Bruns, P. 2012. Properties of mouse and human IgG receptors and their contribution to disulfide-bond formation. Blood 119: 5640–5649.

11. Cullen, S. P., and J. J. Martin. 2008. Mechanisms of granule-dependent killing. Cell Death Differ. 15: 251–262.

12. Freeman, S. A., J. Goyette, W. Furnay, E. C. Woods, C. R. Bertozzi, W. H. Bergermeier, B. A. van der Graaf, and S. Grinstein. 2006. Integrins form an expanding diffusion barrier that coordinates phagocytosis. Cell 164: 128–140.

13. Beron, W., C. Alvarez-Dominguez, L. Mayorga, and P. D. Stahl. 1995. Membrane trafficking and the phagocytic pathway. Trends Cell Biol. 5: 100–104.

14. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat. Med. 6: 443–446.

15. López-Albaiero, A. C., S. L. Lee, S. Morgan, J. R. Grandis, W. E. Gooding, S. Komorowski, and R. C. Schifer. 2009. Role of phosphofucic Fc gamma receptor IIa and EGFR expression level in cetuximab mediated, NK cell dependent in vitro cytotoxicity of head and neck squamous cell carcinoma cells. Cancer Immunol. Immunother. 58: 1853–1864.

16. Gil, N., L. Babes, K. Siegmund, R. Korthouwer, M. Bögels, R. Braster, G. Vidalzar, T. L. ten Hagen, P. Kubes, and M. van Emmongen. 2014. Macrophages eliminate circulating tumor cells after monoclonal antibody therapy. J. Clin. Invest. 124: 812–823.

17. Tipton, T. R., A. M. Rahman, R. J. Oldham, M. J. Carter, K. L. Cox, C. I. Mockridge, R. R. French, L. N. Dabal, P. J. Durie, P. G. Hargreaves, et al. 2015. Antigenic modulation limits the effector cell mechanisms employed by type I anti-CD20 monoclonal antibodies. Blood 125: 1901–1909.

18. Shim, H. 2011. One target, different effects: a comparison of distinct therapeutic monoclonal antibodies approved or in review in the European Union or the United States.

19. Cragg, M. S., S. M. Morgan, H. T. Chan, B. P. Morgan, A. V. Filatov, P. W. Johnson, C. H. Chan, S. James, R. R. French, T. van Meerten, S. Ebeling, T. Vink, J. W. Slootstra, et al. 2006. Biological activity of mouse and human IgG receptors and their contribution to disulfide-bond formation. Biochem. J. 401: 515–523.

20. Cragg, M. S., and M. J. Glennie. 2004. Antibody specificity controls in vivo antibody immunotherapy. J. Exp. Med. 199: 1659–1669.

21. James, L. C., G. Hale, and A. C. Bloomer. 1999. 1.9˚A structure of the therapeutic antibody CAMPATH-1H fab in complex with a synthetic peptide antigen. [Published erratum appears in 1999 J. Mol. Biol. 292: 1161–1163.]

22. Bruhns, P. 2012. Properties of mouse and human IgG receptors and their contribution to disulfide-bond formation. Immunoassays for monoclonal antibodies.

23. Varghese, B., A. Widman, J. Do, B. Taidi, D. K. Czerwinski, J. Timmerman, and H. Waldmann. 1993. Structure of the CAMPATH-1H antigen, a glycoprophosphatidylinositol-anchored glycoprotein which is an exceptionally good target for complement lysis. Biochem. J. 293: 633–640.

24. Haso, W., D. W. Lee, N. N. Shah, M. Stetler-Stevenson, C. M. Yuan, I. H. Pastan, J. A. Oliver, J. V. Ravetch, J. C. Poe, K. M. Haas, and T. F. Tedder. 2008. Direct and complement dependent cytotoxicity of rituximab, an anti-CD20 reagent, and antibody-dependent cellular cytotoxicity of anti-CD20 antibodies. J. Exp. Med. 199: 1659–1669.

25. Zent, C. S., C. R. Sorensen, B. R. LaPlant, N. D. Bone, T. G. Call, T. D. Shafof, D. F. Jensen, R. C. Tscharner, and N. E. Kay. 2008. Direct and complement dependent cytotoxicity of rituximab, an anti-CD20 reagent, and antibody-dependent cellular cytotoxicity of anti-CD20 antibodies. J. Immunol. 182: 3123–3135.
58. Choudhuri, K., D. Wiseman, M. H. Brown, K. Gould, and P. A. van der Merwe. 2005. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature* 436: 578–582.

59. Rudd, P. M., B. P. Morgan, M. R. Wormald, D. J. Harvey, C. W. van den Berg, S. J. Davis, M. A. Ferguson, and R. A. Dwek. 1997. The glycosylation of the complement regulatory protein, human erythrocyte CD59. *J. Biol. Chem.* 272: 7229–7244.

60. Beers, S. A., R. R. French, H. T. Chan, S. H. Lim, T. C. Jarrett, R. M. Vidal, S. S. Wijayaweera, S. V. Dixon, H. Kim, K. L. Cox, et al. 2010. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood* 115: 5191–5201.

61. Ruffell, B., N. I. Affara, and L. M. Coussens. 2012. Differential macrophage programming in the tumor macroenvironment. *Trends Immunol.* 33: 119–126.

62. Simpson, T. R., F. Li, W. Montalvo-Ortiz, M. A. Sepulveda, K. Bergerhoff, F. Are, C. Roddie, J. Y. Henry, H. Yagita, J. D. Wolchok, et al. 2013. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* 210: 1695–1710.

63. Marson, A., K. Kretschmer, G. M. Frampton, E. S. Jacobsen, J. K. Polansky, K. D. MacIsaac, S. S. Levine, E. Fraenkel, H. von Boehmer, and R. A. Young. 2007. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 445: 931–935.

64. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.