

## Research Article

# Tumor-shed antigen CA125 blocks complement-mediated killing via suppression of C1q-antibody binding

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C1q-engagement with IgG and IgM type antibodies is the initiating step of classical complement-mediated immunity. The tumor shed antigen CA125 has been reported to have immunosuppressive effects on host tumor responses as well as commercially approved and experimental monoclonal antibody (mAb)-based therapeutic agents. To better understand this effect, molecular and cellular studies were carried out testing the ability of CA125 to perturb the classical complement pathway. Here, we show that patient-derived CA125 inhibits IgG1, IgG3, and IgM-mediated complement-dependent cytotoxicity (CDC) by perturbing antibody-Fc interaction with the C1q complement-initiating protein only in those mAbs that are directly bound by CA125. This mechanism was found to impact naturally generated IgM antibodies as well as experimental and clinically approved mAbs, such as farletuzumab and rituximab, respectively. These data support a role for CA125 in humoral immune suppression and as a potential mechanism by which tumors may possibly avoid host immune responses.

**Keywords:** CDC · C1q · Complement-dependent cytotoxicity · Humoral immune suppression · Tumor-shed antigen CA125



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Malignant cells employ a number of biological mechanisms to evade the host's immune responses. This has been most recently highlighted by the use of immune checkpoint inhibitors of the CTLA-4 and PD-1 pathways, whereby blockade of these pathways results in cellular immune activation and anti-tumor activity [1, 2]. Recently, emerging studies have suggested that tumors may also suppress humoral immune activities via tumor-cell surface and tumor-shed antigens [3, 4]. This effect was most recently demonstrated from findings of a Phase 3 clinical trial testing the investigational mAb farletuzumab that targets tumor-expressed folate

receptor alpha (FRA). A clinical trial testing farletuzumab plus standard-of-care chemotherapy in patients with recurrent ovarian cancer found that a subset of patients with low serum levels (less than or equal to three times the upper limit of normal) of the CA125 tumor-shed antigen had improved progression-free survival (HR 0.49,  $p = 0.0028$ ) and overall survival (HR 0.44,  $p = 0.0108$ ) as compared to placebo control [5]. Additional studies have also reported the effect of CA125 level to clinical outcome, and these effects were reported to be in part a result of direct CA125 binding to the (FAB')<sub>2</sub> domain of a subset of IgG1-type mAbs that in turn reduced engagement with the high and low affinity Fc- $\gamma$  activating receptors CD16a and CD32a [6, 7]. The pharmacologic activity of farletuzumab as well as that of a number of other therapeutic mAbs is mediated in part through antibody-dependent

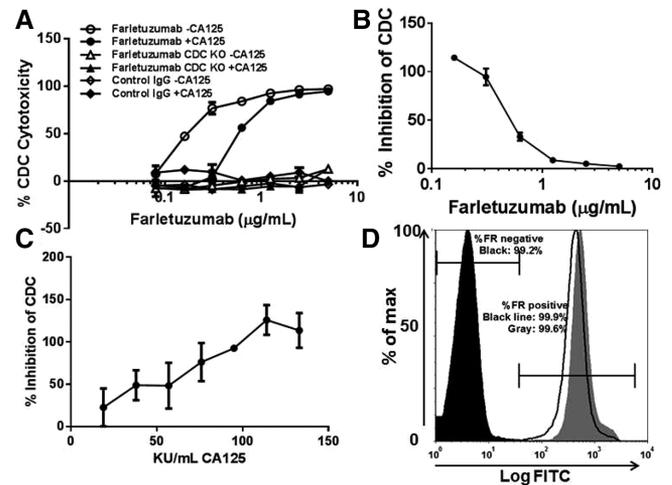
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cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [8]. While the effect of CA125 on suppressing ADCC and its clinical association with therapeutic outcome has been reported, and CA125 has been shown to suppress complement mediated lysis [9], the potential molecular effect(s) of CA125 on CDC-mediated immune-effector activity remain unknown. Here, we analyze the molecular and cellular effects of CA125 on the Fc engagement of the C1q classical complement-initiating protein. Our results show that CA125 can bind to a subset of IgG and IgM isotype antibodies and suppress their interaction with C1q that in turn results in suppressed antibody-mediated CDC. These data further support a role for the CA125 tumor-shed antigen in humoral immune suppression and suggest opportunities to improve therapeutic mAb response in cancers where CA125 protein is highly prominent.

## Results

### CA125 inhibits the CDC activity of farletuzumab

Immunomodulation by CA125 has been previously shown to inhibit effector cell activation and ADCC killing by physical blockade of CD4-CD8 target cell fusion; suppressed NK cell activation via Siglec-7 and -9 pathway activation; and reduced engagement of the Fc- $\gamma$  CD16a and CD32a activating receptors [6, 10, 11]. To determine if CA125 has a direct effect on the classical complement pathway, we tested the effects of CA125 on the CDC activity of mAbs known to utilize this function. We first tested the effect of CA125 on farletuzumab-mediated CDC against CHO-FRA target cells. Farletuzumab is a high affinity humanized IgG1-isotype mAb that binds to FRA. Its mechanism for killing FRA-expressing tumor cells includes both ADCC and CDC, as demonstrated in *in vitro* and *in vivo* assays [8, 12]. CHO-FRA is an engineered CHO cell line stably expressing cell surface human FRA. Cells were grown in 96-well plates and tested for farletuzumab CDC activity in the presence or absence of CA125 (Fig. 1). As shown in Fig. 1A and B, CA125 had a significant effect on decreasing CDC activity in a dose-dependent manner while irrelevant IgG1 controls and an engineered farletuzumab variant (farletuzumab CDC KO) that was previously demonstrated to be unable to engage C1q showed no killing [12]. A dose-dependent effect was also observed when a single concentration of farletuzumab was used in conjunction with increasing concentrations of CA125 (Fig. 1C). To rule out the possibility that the decrease of CDC activity was due to CA125 blocking farletuzumab binding to cell surface FRA, flow cytometry was performed in the presence or absence of CA125. As shown in Fig. 1D, farletuzumab retains similar binding to FRA in the presence or absence of CA125. To determine if the reduced CDC effect was due to CA125 binding and sequestering of C1q, ELISA analysis was conducted using immobilized CA125 and a C1q-biotinylated probe, which has been shown to be effective in measuring C1q binding (see below). Results found that C1q is not bound by CA125. Together, these data suggest that CA125

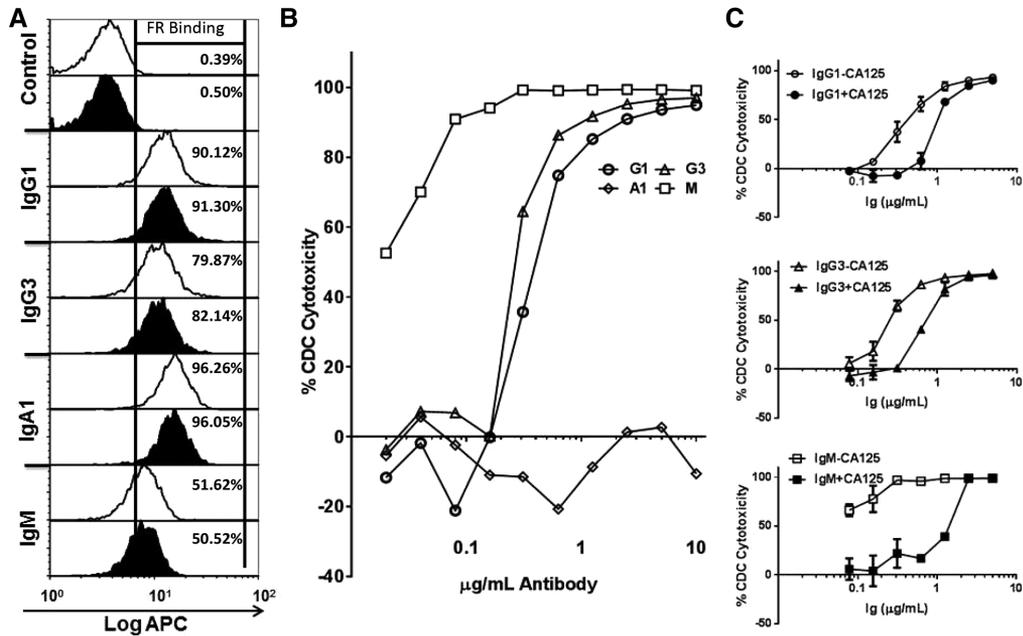


**Figure 1.** CA125 inhibits farletuzumab CDC activity, but not its ability to bind to its cell surface FRA target antigen. (A) CHO-FRA target cells were incubated with increasing concentrations of farletuzumab in the presence of 30 KU/mL of CA125 and human complement. Cytotoxicity was determined by quantitating remaining live cells. Open circles (farletuzumab only); solid circles (farletuzumab + CA125); open and solid triangles (CDC knockout mutant  $\pm$  CA125); open and solid diamonds (isotype control Ab  $\pm$  CA125). (B) A summary of the normalized dose-dependent inhibition of farletuzumab CDC in the presence of a fixed amount of CA125. Percent normalized inhibition of CDC cytotoxicity was calculated as  $(1 - (\text{CDC killing Ab} + \text{CA125}/\text{CDC killing Ab alone})) \times 100\%$  from data in A. (C) Dose-dependent normalized percent inhibition of farletuzumab CDC with an increasing concentration of CA125. CHO-FRA target cells were incubated with increasing concentrations of CA125 in the presence of farletuzumab and human complement. Cytotoxicity was determined by quantitating remaining live cells. Percent normalized inhibition of CDC cytotoxicity was calculated as  $(1 - (\text{CDC killing Ab} + \text{CA125}/\text{CDC killing Ab alone})) \times 100\%$ . (D) CHO-FRA cells were stained with farletuzumab and complement in the presence or absence of CA125 to test its effect on antibody binding to cell surface antigen. Farletuzumab binding was detected by staining with goat anti-huIgG-FITC and analysis by flow cytometry. Solid black (no farletuzumab); Gray (farletuzumab only); Black outline (+CA125). Data are shown as mean  $\pm$  SD and representative of three independent experiments each conducted in triplicate.

inhibits the activation of the classical complement cascade via a direct effect on the antibody-complement complex.

### CA125 inhibits the CDC activity of farletuzumab's IgG1, IgG3, and IgM isotypes

Farletuzumab is an IgG1 isotype antibody. Despite being more than 90% identical in amino acid sequence, IgG isotypes differ in key functional regions responsible for flexibility, Fc- $\gamma$  receptor binding and complement fixation [13]. CA125 was previously shown to bind to the (FAB')<sub>2</sub> domain of farletuzumab and rituximab [6, 14]. To investigate the effects of CA125 on other antibody isotypes, Fc isotypes of farletuzumab (IgA1, IgA2, IgD, IgE, IgG1, IgG3, IgG4, and IgM) were engineered linking the same (FAB')<sub>2</sub> domain to each human antibody Fc isotype domain. All mAbs were found to retain robust FRA binding via ELISA (data not shown) as well as via flow cytometry using CHO-FRA cells (Fig. 2A). Farletuzumab isotype binding to CHO-FRA cells in the presence of



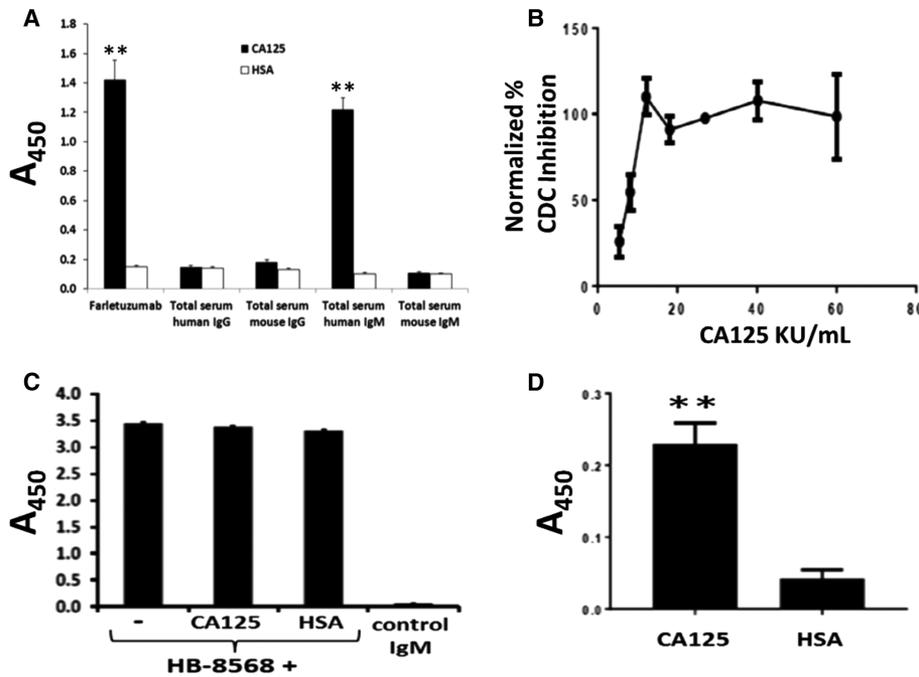
**Figure 2.** Farletuzumab isotypes with CDC activity are inhibited by CA125 but not cell surface antigen binding. (A) Farletuzumab engineered isotype variants were used to stain CHO-FRA cells in the presence or absence of CA125 to test for CA125 effect of antibody cell surface antigen binding. White histograms are antibody staining without CA125, while black histograms are isotype antibody staining in the presence of CA125. (B) In vitro CDC activity of farletuzumab isotypes. Wells with increasing concentrations of farletuzumab isotypes were used to test CDC-mediated killing of CHO-FRA cells in the presence of complement. Isotypes IgG1, IgG3, and IgM were the only antibodies showing CDC activity. (C) CA125 inhibition of CDC-active farletuzumab isotypes. Wells with increasing concentrations of IgG1, IgG3, and IgM farletuzumab isotypes were used to test CDC killing of CHO-FRA cells in the presence of CA125 and complement. Data are shown as mean  $\pm$  SD and are representative of three independent experiments each conducted in triplicate.

CA125 exhibited no loss of binding with IgG1, IgG3, IgM, or IgA1 as compared to binding in the absence of CA125. Similar results were found for IgG2, IgG4, IgGA2, IgD, and IgE isotypes (data not shown). Therefore, like IgG1 farletuzumab, there does not seem to be an inhibitory effect by CA125 on antigen binding of any of the engineered isotypes. Next, to determine the effect of CA125 on the complement-mediated killing activity of these isotypes, CDC assays were performed (Fig. 2B). As expected, significant cytotoxicity was observed only with isotypes IgG1, IgG3, and IgM, all of which are known to have strong CDC activity via binding of the C1q, with the IgM isotype having the most robust CDC activity most likely due to its efficient IgM-complement fixation through multiple site interactions [15]. We then tested if CA125 inhibited the CDC activity of these three isotypes. As shown in Fig. 2C, CA125 inhibited the CDC activity of all three isotypes in a dose-dependent manner, with the IgM-isotype being the most sensitive to the CA125 inhibitory effect.

### CA125 inhibits the CDC activity of polyclonal circulating IgM

In light of our observed farletuzumab-mediated CDC suppression and the previous reports that CA125 can affect ADCC by direct antibody binding [6, 7], we determined if other circulating antibodies were capable of binding CA125. First, total purified human

IgM and IgG1 from serum of healthy volunteers were tested for their ability to bind CA125. As shown in Fig. 3A, along with the monoclonal farletuzumab antibody, polyclonal human serum IgM showed binding to immobilized CA125 in contrast to total human-derived serum IgG1 or total mouse immunoglobulins. Based on the finding that CA125 can bind total serum polyclonal IgM, we next investigated if CA125 could inhibit CDC activity of a naturally produced monoclonal IgM previously shown to mediate CDC on target cells. HB-8568 is a monoclonal IgM against the ganglioside marker GD2, which was isolated from B cells of a patient with melanoma [16]. The CDC activity of HB-8568 against the GD2-positive melanoma cell line M14 was tested in the presence or absence of CA125 (Fig. 3B). As shown, CA125 was able to suppress the CDC-mediated effects of HB-8568 on M14 cells. ELISA analysis showed that this effect was not due to interference of HB-8568 binding to cell surface antigen as it could still bind its target in the presence of CA125 (Fig. 3C) and was found to directly bind to CA125 (Fig. 3D). Taken together, these data show that CA125 directly binds to serum IgM, as well as a patient-generated monoclonal IgM and that this interaction inhibits CDC activity via a direct effect on antibody and the complement cascade. Interestingly, while CA125 is able to bind farletuzumab and a subset of other monoclonal IgG1s, it was not able to bind total serum IgG1 (Fig. 3A), suggesting that the presence of a sequence specific CA125-binding motif on serum IgG1s is less frequent as compared to IgMs.

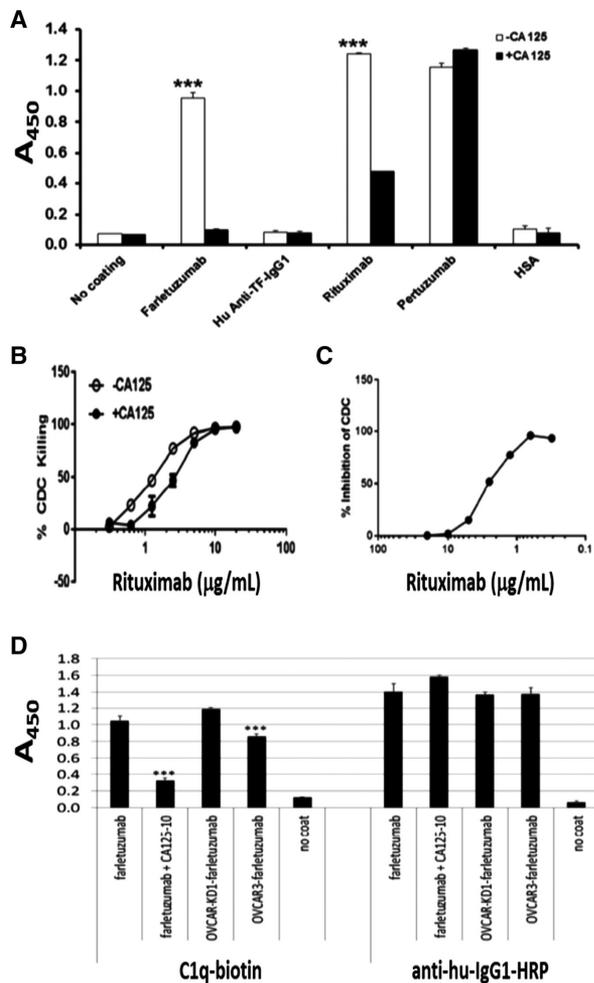


**Figure 3.** Pooled human serum-derived IgM and the monoclonal IgM HB-8568 bind CA125 and CA125 inhibits the CDC activity of HB-8568. (A) ELISA plates were coated with CA125 or human serum albumin (HSA) and probed with biotinylated antibodies. Binding was detected with streptavidin-HRP. (B) Inhibition of HB-8568 CDC by CA125. M14 (GD2-positive) target cells were incubated with increasing concentrations of CA125 in the presence of 1  $\mu$ g/mL human anti-GD2 IgM (HB-8568) and human complement. Cytotoxicity was determined by quantitating remaining live cells. Percent normalized inhibition of CDC cytotoxicity was calculated as  $[1 - (\text{CDC killing Ab} + \text{CA125/CDC killing Ab alone})] \times 100\%$ . (C) ELISA plates were coated with GD2 and probed with HB-8568 or isotype control in the presence or absence of CA125 and HSA to determine the CA125 effect on antigen binding. (D) CA125 and HSA were used to coat ELISA plates and probed with biotinylated HB-8568 to test antibody binding to these soluble factors. Data are shown as mean  $\pm$  SD. All experiments are representative of at least three independent experiments and each conducted in triplicate. \*\* $p < 0.005$  using the two tailed t-test

### CA125 inhibits the binding of C1q to a subset of IgGs

Previous studies found that CA125 can bind to a subset of IgG1 antibodies and consequently inhibit their interaction with CD16a and CD32a Fc- $\gamma$  receptors [6, 7, 14]. Cancer cells are known to develop multiple mechanisms to avoid CDC and impair the efficacy of therapeutic mAbs by producing membrane complement regulatory proteins including CD59, decay accelerating factor and membrane cofactor protein [17–19]. These mechanisms complicate the relative quantitation of different antibody-mediated CDC activity due to the varying endogenous expression levels of these proteins on tumor cell lines that may naturally express an antibody-specific target. In order to standardize the potential inhibitory effects of CA125 on the CDC of various antibodies, we utilized a cell-free assay to test the potential effects of CA125 on the binding of complement proteins to antibody. The first step in the classical complement cascade is the oligomerization of the C1q complex on the antibody. An *in vitro* ELISA assay was developed that is capable of quantitating the level of C1q binding to immobilized antibody by utilizing biotinylated human C1q protein. Using this assay, we quantitated the deposition of C1q on a variety of experimental and clinically relevant mAbs in the presence or absence of CA125 (Fig. 4A). As expected due to its observed CDC activity, farletuzumab was capable of binding to C1q; however, in the presence of CA125, C1q binding was significantly inhibited in a dose-dependent manner. Hu-anti-TF-IgG1, an experimental antibody against the cell surface CD142 target, was used as a negative control for C1q as it has been previously found not to have CDC activity. Of the commercial therapeutic antibodies (rituximab, cetuximab, pertuzumab, and panitumumab) tested, all showed robust

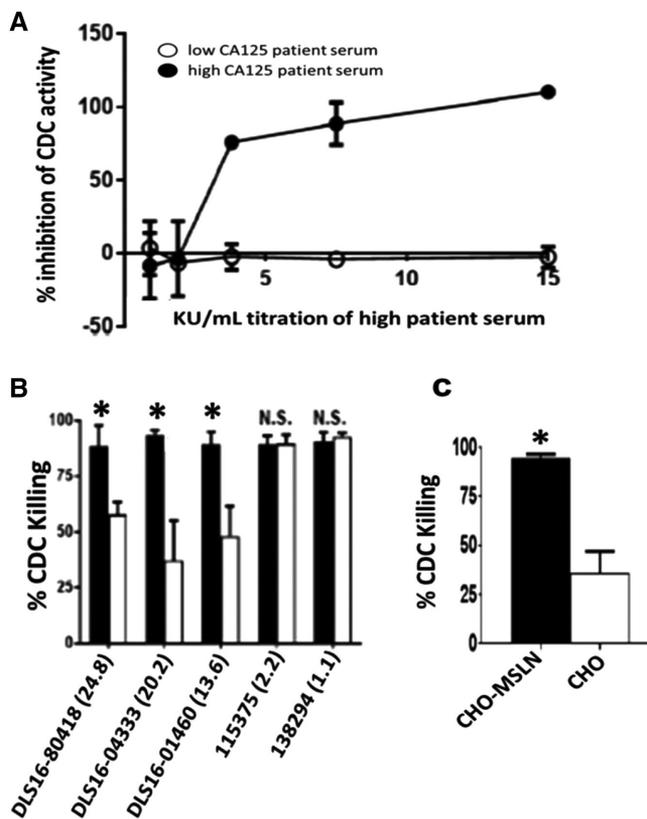
C1q binding except for panitumumab, which exhibited low level C1q binding (not shown). This was expected, as all of the mAbs are IgG1 isotype, except for panitumumab, which is an IgG2, an isotype known to have very poor CDC activity due to low complement fixation [20]. Of the commercial IgG1-type antibodies, rituximab showed significant inhibition of C1q binding in the presence of CA125, while pertuzumab did not. Previous studies have shown that CA125 does not bind pertuzumab nor does it affect its ability to engage with CD16a, therefore further supporting the finding that CA125 direct antibody binding perturbs the ability of C1q engagement and subsequent CDC activity [6, 7]. Daudi cells have been reported to be susceptible to the CDC-mediated killing by rituximab *in vitro* [21]. We employed these cells in CDC assays to determine if CA125 affected rituximab-mediated CDC killing. Similar to the CDC effect seen by CA125 on CHO-FRA cells by farletuzumab (Fig. 1), CA125 was found to inhibit the rituximab-mediated CDC killing of Daudi cells in a dose-dependent manner (Fig. 4B and C). Taken together, these data show that CA125 is able to inhibit antibody-mediated CDC activity by blocking the first step of the classical complement cascade (C1q binding) via direct binding of CA125 to a subset of therapeutic antibodies. The effect of CA125-C1q inhibition appears to involve soluble CA125 as well as membrane bound CA125 as C1q binding to isogenic tumor cell lines expressing the FRA antigen in which CA125 was knocked down by shRNA bound less C1q as compared to its parental wild type (Fig. 4D). These results are similar to what was reported using the same cell lines measuring the effect of CA125 on CD16a and CD32a Fc- $\gamma$ -receptor binding [6] as well as complement-mediated cell lysis studies of OVCAR3 previously reported by Murdoch et al. [9].



**Figure 4.** CA125 inhibits CDC-mediated killing of a subset of antibodies via direct antibody binding and suppressed antibody-C1q interaction. (A) A subset of IgG isotype antibodies bind C1q. ELISA plates were coated with a variety of experimental (farletuzumab and hu-anti-TF-IgG1) and commercial (rituximab and pertuzumab) human IgGs. Biotinylated C1q was added to wells in the presence (black bars) or absence (white bars) of CA125, and after washing, the amount of C1q bound was quantitated. Percent C1q binding inhibition was calculated as  $(1 - [A_{450} + CA125] / [A_{450} - CA125]) \times 100\%$ . N.S. refers to no significant inhibition ( $p > 0.16$ ). Asterisks indicate statistically significant inhibition ( $p < 0.01$  for antibodies). (B) CA125 inhibits rituximab-mediated CDC killing. Daudi cells were incubated with increasing concentrations of rituximab in the presence or absence of CA125 and human complement. Cytotoxicity was determined by quantitating remaining live cells. Percent CDC cytotoxicity was calculated as  $(1 - (\text{experimental well/cells} + \text{complement})) \times 100\%$ . (C) Dose-dependent inhibition of rituximab CDC occurs by a fixed concentration of CA125. Percent inhibition of CDC cytotoxicity was calculated as  $(1 - (\text{CDC killing Ab} + \text{CA125/CDC killing Ab alone})) \times 100\%$  from data in B). (D) C1q binding to farletuzumab is perturbed by soluble and membrane-bound CA125. Biotinylated C1q was used to probe farletuzumab incubated with or without soluble CA125 alone (left columns 1 and 2) or in the presence of membrane preparations of the isogenic cells OVCAR3 parental (CA125-positive) or OVCAR3-KD1 shRNA CA125 knockdown (CA125 negative) (left columns 3 and 4). Replica wells (right side columns) were probed for farletuzumab concentrations using an anti-human-IgG1 antibody to ensure the reduction of C1q binding was not due to less farletuzumab being retained in each well after various treatment. Data are shown as mean  $\pm$  SD. All experiments are representative of at least three independent experiments and each conducted in triplicate. \*\*\* $p < 0.005$  using the two tailed t-test.

## Clinical sera with endogenous CA125 inhibits C1q binding to farletuzumab and CDC on target cells

While maximal efficacy of farletuzumab in in vivo models depends in part on ADCC immune-effector function [12], the antibody also exhibits CDC-mediated cell killing activity that may also contribute to its therapeutic efficacy [8]. This dual effect has been reported for several other therapeutic antibodies that utilize ADCC, whereby a subset also demonstrate CDC activity that appears to contribute to their overall anti-tumor activity [22–24]. Post hoc analysis of the farletuzumab Ph3 clinical study found that of the commonly monitored tumor-shed antigens (i.e. MUC1, alpha fetoprotein, CA125, CA19-9, CEA, etc.) only CA125 appeared to correlate with clinical outcome. Patients with low CA125 serum levels showed a 7.7 month improvement in PFS as compared to placebo control ( $p < 0.0005$ ) suggesting that high concentrations of soluble CA125 in the tumor microenvironment may be sufficient to suppress immune-effector activity, including CDC [6, 7]. To address this possibility, we used patient serum containing varying endogenous levels of CA125 as a surrogate for soluble CA125 in the tumor microenvironment of these patients. Samples were grouped based on low ( $\leq 2.5$  KU/mL) or high ( $\geq 13$  KU/mL) CA125 serum levels and were tested for their potential ability to inhibit farletuzumab-mediated CDC killing of CHO-FRA cells. Patient sera were serially diluted and used in CDC assays as conducted above (Fig. 1). As shown in Fig. 5A, farletuzumab-mediated CDC activity was inhibited when sera containing endogenously high CA125 levels (i.e. sample DLS16-08777) were incubated with farletuzumab and human complement in contrast to cells exposed to sera with low CA125 levels (i.e. sample 431569). The observed inhibition by the high CA125 containing sera was shown to be intrasample dose dependent. To confirm that the CDC inhibitory effect was indeed due to the CA125 component within the sera, samples from patients with high or low CA125 levels were affinity-depleted for CA125 using methods previously described [6]. Briefly, the assay employs the CHO-MSLN cell affinity method that is able to effectively and specifically deplete soluble CA125 (sCA125) from sample preparations by exploiting the natural ability of engineered CHO-MSLN cells to absorb sCA125 via high affinity binding to the ectopically expressed cell surface-bound mesothelin (MSLN) protein [6, 25]. To monitor for assay specificity, serum samples were either CA125-depleted using CHO-MSLN or nondepleted using the isogenic CHO-K1 parental control cells. As shown in Fig. 5B, nondepleted high CA125-containing sera still retained their CDC inhibitory effects (open bars) while CA125 depletion of the same sera by CHO-MSLN ablated the CDC inhibition thereby restoring the antibody's CDC activity (solid bars). Removal of CA125 from the serum sample was confirmed via a quantifiable FRET-based assay and as previously shown [6]. As an inverse control, serum with low CA125 (sample 115375) was spiked with purified CA125, then repurified via the CHO-K1 and CHO-MSLN cell affinity and tested in CDC assays (Fig. 5C). In this case, CA125 spiked serum purified by CHO-K1 (CA125 is not removed) exhibited suppressed CDC killing (open bar), while CA125 spiked serum purified with CHO-MSLN cells (CA125 was absorbed to



**Figure 5.** Serum from patients with high CA125 levels inhibit farletuzumab-mediated CDC activity. Serum CA125 suppresses CDC activity. (A) Triplicate wells of CHO-FRA target cells were incubated with twofold dilutions of sera containing high or low CA125 levels in the presence of 2.5  $\mu$ g/mL farletuzumab and human serum complement. Cytotoxicity was determined by quantitating remaining live cells. Percent inhibition of CDC cytotoxicity was calculated as  $(1 - (\text{CDC killing Ab+serum} / \text{CDC killing Ab alone})) \times 100\%$ . Open circles, low CA125 (<1 KU/mL) sample ID 431569; Solid circles, high CA125 (13.7 KU/mL) sample ID DLS16-08777. (B) High CA125 serum sample inhibition of CDC can be reversed by CA125 depletion. Independent sera from ovarian cancer patients with high CA125 and from patients with low CA125 were preincubated with CHO-MSLN cells (solid bars) or CHO-K1 cells (open bars) as described in the Methods. The steady-state CA125 levels for each sample are shown in parenthesis. Samples were incubated with cells, then pelleted, and the soluble serum fractions were harvested and tested in farletuzumab CDC assays. Statistically significant differences in CDC inhibition are indicated by asterisks ( $p < 0.05$ ). N.S., no significant difference. (C) Serum controls show effect of CA125 on CDC suppression. Patient serum with low CA125 (sample 115375) was spiked with purified CA125 and then incubated with CHO-MSLN cells (solid bar) or CHO-K1 cells (open bar). CDC activity was calculated as  $(1 - (\text{CDC killing test sample} / \text{CDC killing serum + complement alone})) \times 100\%$ . Data are shown as mean  $\pm$  SD. Experiments shown in (A) and (B) are representative of four independent experiments and each conducted in triplicate. Data in (C) are representative of five independent experiments with single representative experiments shown. Asterisk indicates statistically significant difference in CDC,  $p < 0.05$  using the two-tailed t-test.

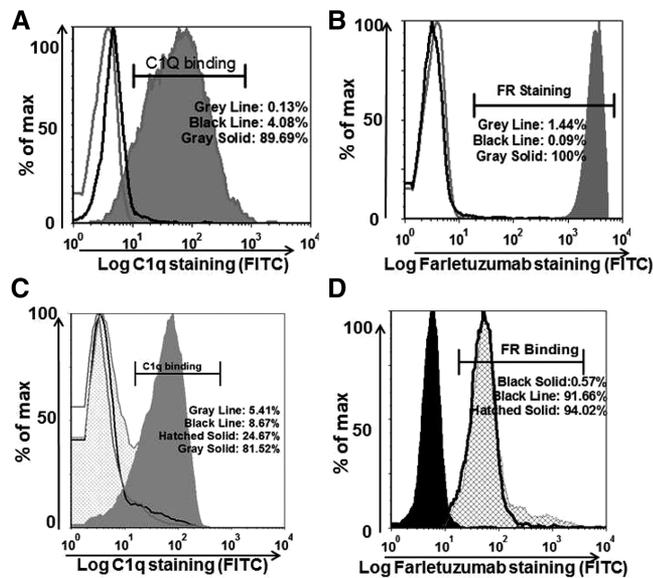
cells and removed from the soluble fraction) demonstrated robust CDC activity (solid bar). Taken together, these data suggest that patients with high levels of tumor-shed CA125 are susceptible to suppressed humoral immune response to antibody-mediated CDC killing by those antibodies that are bound by CA125.

## Purified and serum-derived CA125 inhibits C1q binding to cell surface bound farletuzumab

Figure 4A demonstrated that CA125 inhibits C1q-antibody interactions in an immobilized ELISA format. In this assay, each mAb is bound to the ELISA plate mimicking its binding to antigen, with oligomerization of the complement components reliant on the orientation and correct conformation of the antibody on the plate surface. To confirm CA125 inhibits CDC by blocking C1q in a more natural cellular setting, farletuzumab, and CHO-FRA cells were used in flow cytometry to measure the effect of CA125 on farletuzumab-mediated C1q binding. The C1q protein contained within complement active human serum was measured using a C1q-specific detection antibody. As shown in Fig. 6A, C1q from serum binds to farletuzumab bound to CHO-FRA cells in the absence of CA125 (shaded peak). When purified CA125 was added to the same culture system, C1q was no longer engaged with the cell-bound farletuzumab (black line peak). This observation appears to be a direct effect of CA125 blocking C1q binding to farletuzumab as flow cytometry showed that CA125 does not affect farletuzumab binding to cell surface FRA (Fig. 6B) similar to that shown in Fig. 1D. These findings rule out the possibility that the inhibitory effect of CA125 on decreased C1q-farletuzumab cell binding is a result of reduced farletuzumab-antigen cell surface binding. Finally, similar experiments were conducted in which patient sera with endogenous high or low CA125 levels were used to measure C1q binding to farletuzumab (Fig. 6C). Once again, C1q was blocked from binding farletuzumab when coincubated with serum containing high CA125 (sample DLS16-08777) levels, but not with serum containing low CA125 (sample 431569). Neither serum was able to block farletuzumab binding to target (Fig. 6d). These data support the effect of CA125 on suppressing humoral immune activation in part by perturbing complement-mediated immunity via blockade of C1q-mediated complement initiation.

## Discussion

Several therapeutic antibodies have been reported to exhibit their anti-tumor effects via ADCC and CDC immune-effector activity. This humoral response is governed by the coordination of antibody-cell surface antigen engagement, positioning of the antibody on the antigen epitope and proximity to the cell surface, as well as binding and activation of NK or myeloid cells to initiate ADCC or C1q to initiate CDC [26]. These effects have been observed by the use of several therapeutic antibodies such as rituximab, trastuzumab, cetuximab as well as a number of experimental-staged antibodies including farletuzumab and amatuximab [8, 22–24, 27]. Similarly, endogenous humoral responses have shown to occur within patient's immune response to abnormal and/or aberrantly growing cells in response to vaccines and those with indolent disease yielding predominantly antibodies of the IgM class with antiproliferative as well as immune-mediated killing activity(s) [28, 29]. The findings presented here



**Figure 6.** CA125 inhibits the ability of C1q to bind to cell surface-bound farletuzumab. (A) CHO-FRA cells were stained with farletuzumab and complement in the presence or absence of purified CA125. C1q binding to farletuzumab was detected by staining with a goat anti-huC1q-FITC and analyzed by flow cytometry. After staining with appropriate antibodies as described in material and methods, a small aliquot of cells were tested for viability before flow cytometry and confirmed to be greater than 95% viable by Trypan blue dye exclusion. Gating strategy consisted of gating on viable cells using forward/side scatter in conjunction with FITC intensity for C1q or Farletuzumab binding using histogram plots. All cytometry experiments were performed using a Guava Easy Cyt 8HT Guava line and data analyzed using FCS Express 5 (De Novo software). Gray line, no Ab; solid gray shaded peak, farletuzumab only; black line, farletuzumab + CA125. Results show that CA125 blocks the ability of C1q to bind farletuzumab. (B) CA125 does not inhibit farletuzumab binding to cell surface FRA. Cells were incubated with farletuzumab and CA125 and assayed for farletuzumab binding by flow cytometry. Solid gray shaded peak, farletuzumab binding to target using detected with goat anti-huIgG-FITC secondary antibody; black line, isotype control; gray line, no antibody. (C) Patient serum containing high levels of CA125 inhibits C1q binding to farletuzumab on target cells. CHO-FRA cells were treated with farletuzumab and complement in the presence of sera containing high or low CA125 concentrations. C1q binding to farletuzumab was detected by staining with a goat anti-huC1q-FITC and analyzed by flow cytometry. Gray and black lines, low and high CA125 sera with no farletuzumab, respectively; solid gray peak, farletuzumab + serum with low CA125; hatched gray peak, farletuzumab + serum with high CA125. (D) Patient sera do not inhibit farletuzumab binding to cell surface FRA. Solid black peak, secondary antibody only; black line, farletuzumab binding to cell surface in the presence of serum with low CA125; Gray hatched peak, farletuzumab binding to cell surface in the presence of serum with high CA125. Data shown are representative of three independent experiments.

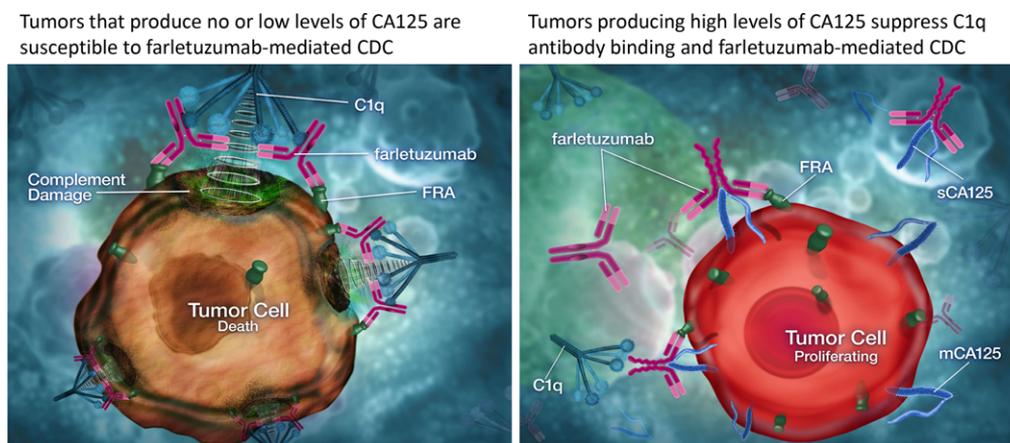
suggest these responses may be suppressed by the generation of tumor-shed antigens such as CA125 via a direct mechanism by which the tumor-shed antigens or other factors bind antibody and suppress their ability to engage with humoral response-mediating factors, including Fc- $\gamma$  activating receptors CD16a or CD32a, on immune cells and/or complement-mediating proteins, including C1q. A schematic overview of the CA125-mediated CDC-immune suppression is depicted in Fig. 7.

Interestingly, serum CA125 levels have been found to correlate with clinical outcome in a number of reported cases. These include

antibodies that rely on immune-effector agents as reported for farletuzumab in ovarian cancer and/or combines the effect of a therapeutic mAb along with chemotherapy-induced patient humoral responses as in the case of rituximab plus CHOP [5, 30, 31]. Several reports have also shown the correlation of serum CA125 levels and progression-free survival (PFS) in patients with Hodgkin's and Non-Hodgkin's lymphoma. Prochazka et al. showed that patients with follicular lymphoma treated with rituximab + CHOP had a 31.4% improvement in 5-yr PFS ( $p = 0.004$ ) when CA125 levels were no greater than the normal level ( $<35$  U/mL) [31], while Bairy et al. showed that CA125 was a prognostic factor in patients treated with standard chemotherapy who had a 26.3% improvement in 3-year survival when their CA125 levels were within the normal range as compared to above the normal range [30]. These effects are similar to the findings reported by Vergote et al. for farletuzumab in relapsed ovarian cancer [5] and the anti-mesothelin antibody amatuximab in first-line mesothelioma [32]. While CA125 has been identified as a biomarker for tumor presence, its steady-state levels have been reported by several independent groups not to simply reflect tumor bulk or burden, whereby small tumor lesions have been shown to produce high levels of CA125 while in other instances large bulky tumor expressed little to no CA125 [6, 32].

While the underlying biological role(s) of MUC16/CA125 have been hampered by its large molecular weight ( $\sim 1\text{--}2$  mDa) making it difficult to study in recombinant formats or refining protein-protein interactions, its pleiotropic role in cellular and humoral immune suppression has been documented by a number of independent studies. These include its ability to directly impact NK cell biology through the physical blockade of synapse formation with target cells as well as its ability to downregulate intracellular signaling for lymphocyte activation through the engagement of the Siglec-7 and -9 regulatory receptors [11]. Moreover, the recent findings that CA125 can directly bind to the (FAB')<sub>2</sub> region of antibodies and elicit an immunosuppressive effect by perturbing (i) the interaction of the CH2 domain of IgG1 and IgG3 for Fc- $\gamma$ -activating receptor engagement and (ii) C1q binding to the Fc domain within IgG1, IgG3, and IgM antibodies, has important biological and pharmacological implications.

First, the application of antibody-based therapies should be considered prior to advancing such agents into clinical development if a particular antibody is found to be bound by CA125. The finding that CA125 does not bind [6, 7] or affect the ability of C1q binding to the humanized pertuzumab mAb, which shares significant amino acid homology to other humanized mAbs within the framework and Fc regions, including farletuzumab, suggests that the critical region(s) for CA125 binding may lie within the CDRs. This is supported by the reports that (FAB')<sub>2</sub> fragments from farletuzumab and amatuximab were able to bind CA125 but not a fragment containing the Fc domain [6, 32]. These data were consistent with the ability of CA125 to bind all farletuzumab isotype chimeras shown in Fig. 2C. Moreover, the finding that polyclonal serum IgM but not the more processed serum-derived IgG binds CA125 also supports the hypothesis that CDR sequences may define those mAbs susceptible to CA125 binding.



**Figure 7.** Model showing inhibitory effect of CA125 on C1q-antibody binding and CDC activity. Left panel shows antibody binding to tumor antigen followed by C1q fixation that initiates the classical complement pathway leading to cell death. Right panel shows soluble (sCA125) or membrane bound (mCA125) CA125 binding to the (FAB)<sub>2</sub> domain of antibody that in turn perturbs Fc domain structure resulting in reduced C1q binding to antigen-bound antibody. The antibody depicted in this example is farletuzumab and its folate receptor alpha (FRA) cell surface antigen.

Interestingly, CA125 binding to mAbs tested here do not have any impact on antigen binding as shown in Fig. 6. Elucidation of these structures may offer insights into how this interaction may affect structural changes within the Fc region. Previous reports have demonstrated allosteric effects within the Fc region upon hapten binding to V region that may be a similar effect caused by CA125 engagement [33].

Second, the finding that CA125 can bind to total polyclonal serum IgM suggests that it may have an impact on malignant cell protection against one of the first humoral responses against aberrant cell phenotypes in the host [28, 29] and to cellular-based vaccines [34, 35]. These effects also occur in response to vaccine and cell-based immunization approaches and may offer opportunities to screen responders early to determine if CA125 is binding or negatively affecting humoral immune factors.

Finally, while the exact residues by which CA125 binds to immunoglobulin is still being elucidated, the generation of non-CA125 binding antibodies may offer a means to improve antibody-based, anti-cancer strategies to the CA125 antigen that has been reported to be upregulated by a number of different cancer types [36].

## Materials and methods

### Reagents

CA125 preparations were obtained from primary patient sera or independent vendors and purified as described [6]. Variant antibody isotypes were generated by Morphotek Inc. The CDC defective farletuzumab mutant was described previously [12]. Gangliosides were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-human C1q-FITC was purchased from Abcam (Cambridge, MA). Human purified complement was purchased from Quidel (San Diego, CA). Goat anti-human IgG+M-FITC were purchased

from Southern Biotech (Birmingham, AL). Cell Titer Glo was purchased from Promega Life Sciences (Madison, WI).

### Cell culture conditions

Daudi, CHO-K1, and M14 cell lines were grown in complete RPMI 1640 (cRPMI, Gibco) with 10% heat inactivated fetal bovine serum (Gibco). CHO-FRA and CHO-MSLN cells [6] were grown in cRPMI with 200  $\mu$ g/mL zeocin selection.

### Dose-dependent farletuzumab CDC activity in presence of CA125 assays

Triplicate wells containing  $5 \times 10^3$  CHO-FRA cells were seeded overnight in opaque 96-well plates in assay buffer (RPMI with L-glutamine + 2% ultralow Ig serum) and grown at 37°C in 5% CO<sub>2</sub>. The following day, increasing concentrations of antibodies were added in combination with a final concentration of 30 KU/mL CA125. Next, 25  $\mu$ Ls of human serum complement was added to wells for a total volume of 100  $\mu$ L. Plates were incubated 1.5 h at 37°C in 5% CO<sub>2</sub>, removed from the incubator and allowed to equilibrate to room temp for 20 min. An equal volume of Cell Titer Glo (Promega) was added then plates were incubated 10 min at room temp and luminescences were quantitated. Percent CDC cytotoxicity was calculated as  $(1 - (\text{experimental well}/\text{cells} + \text{complement})) \times 100\%$ .

### Titration of CA125 effects on CDC inhibition

Replicate wells of  $5 \times 10^3$  CHO-FRA or M14 cells were seeded overnight in opaque 96-well plates in assay buffer and grown at 37°C in 5% CO<sub>2</sub>. The following day, farletuzumab was added to CHO-FRA cells at a final concentration of 1  $\mu$ g/mL and the

HB-8568 anti-ganglioside IgM was added to M14 cells at a final concentration of 10  $\mu\text{g}/\text{mL}$  along with increasing concentrations of CA125 and complement. Plates were quantified for live cells as described above.

#### *Serum CA125 inhibition of CDC assays*

Triplicate wells of  $5 \times 10^3$  CHO-FRA cells were seeded overnight in opaque 96-well plates in assay buffer and grown as above. The following day, serial dilutions of patient sera or purified CA125 plus 2.5  $\mu\text{g}/\text{mL}$  farletuzumab were added to wells followed by 25  $\mu\text{L}$  of human serum complement. Plates were quantified for live cells as described above.

#### *Clearance of CA125 from sera for CDC assays*

Replicate wells of  $5 \times 10^3$  CHO-FRA cells were seeded overnight in opaque 96-well plates and grown as above. The following day, 100  $\mu\text{L}$  of patient-derived sera were added to microtubes containing  $1.7 \times 10^5$  CHO-K1 or CHO-MSLN cells and incubated 30 min at 37°C in 5%  $\text{CO}_2$ . Mesothelin has been reported to bind CA125 with high affinity [37]. As control, patient serum containing low CA125 levels (sample115375) were spiked with purified CA125 (76 KU/mL) and incubated in a similar manner. Next, cells were pelleted at 1,000 RPM for 2 min at room temp and supernatants transferred to fresh tubes. Plates containing CHO-FRA cells were washed of growth media and plated in 50  $\mu\text{L}$  assay media containing 10  $\mu\text{L}$  human complement, 5  $\mu\text{g}/\text{mL}$  farletuzumab and treated or untreated serum. Plates were quantified for live cells as described above.

#### *Dose-dependent rituximab CDC activity in the presence of CA125*

Triplicate wells containing of  $2 \times 10^4$  Daudi cells were seeded in assay buffer in opaque 96-well plates and grown at 37°C in 5%  $\text{CO}_2$  for 1 h. Increasing concentrations of antibodies mixed with CA125 or PBS were added in combination with 30 KU/mL of purified CA125. Twenty-five microliters of human serum complement were added to wells for a total volume of 100  $\mu\text{L}$ . Plates were quantified for live cells as described above.

#### *Flow cytometry quantitation of farletuzumab binding to cell surface FRA*

For all flow cytometry experiments, the “Guidelines for the use of flow cytometry and cell sorting in immunological studies” were followed [38]. The 96-well microplates were seeded with  $2.5 \times 10^5$  CHO-FRA cells in 100  $\mu\text{L}$ s PBS containing 10  $\mu\text{L}$  of complement, 2.5  $\mu\text{g}/\text{mL}$  farletuzumab with or without 30 KU/mL CA125 for 20 min on ice. Cells were then washed with 5 mL ice cold PBS, pelleted at 1,000 RPM for 3 min and resuspended in 0.5 mL PBS.

A 10  $\mu\text{g}/\text{mL}$  goat anti-huIgG-FITC (Southern Biotech) was added for 20 min on ice. Cells were washed twice with 5 mL ice cold PBS and resuspended in 1 mL PBS and analyzed for cell binding via flow cytometry (Guava Instruments) using FSC gating and FITC mean fluorescence.

#### *Flow cytometry quantitation of variant farletuzumab isotype binding to FRA in the presence of CA125*

The 96-well plates containing  $2.5 \times 10^5$  CHO-FRA cells were stained with 10  $\mu\text{g}/\text{mL}$  of the various engineered farletuzumab isotypes with or without 30 KU/mL CA125 for 30 min on ice. Cells were then washed twice with 5 mL PBS, resuspended in 0.5 mL PBS and stained with 10  $\mu\text{g}/\text{mL}$  APC-labeled mouse anti-human kappa chain secondary antibody (ThermoFisher) for 30 min on ice. Cells were washed with 5 mL ice cold PBS, resuspended in 300  $\mu\text{L}$  PBS and analyzed for cell binding via flow cytometry (Guava Instruments) using FSC gating and APC mean fluorescence. Flow cytometry quantitation of C1q bound to farletuzumab-FRA complex

Farletuzumab was preincubated with CA125 (30 KU/mL) or PBS for 1 h at room temp. Mixtures were added to  $2.5 \times 10^5$  CHO-FRA cells with 10  $\mu\text{L}$  complement and 2.5  $\mu\text{g}/\text{mL}$  of farletuzumab in a total volume of 100  $\mu\text{L}$  PBS and incubated on ice for 1 h. Cells were then washed with 5 mL ice cold PBS, pelleted at 1,000 RPM for 3 min and resuspended in 0.5 mL PBS. A 10  $\mu\text{g}/\text{mL}$  goat anti-human IgG-FITC or goat anti-human C1q-FITC were added and samples incubated on ice for 30 min. Cells were washed twice with 5 mL ice cold PBS and resuspended in 0.5 mL PBS for analysis by flow cytometry (Guava Instruments) using FSC gating and FITC mean fluorescence.

#### *Flow cytometry detection of C1q binding in the presence of farletuzumab and human serum samples*

Patient-derived sera were incubated with 2.5  $\mu\text{g}/\text{mL}$  of farletuzumab for 1 hr at room temp. Samples were then added to plates containing  $2.5 \times 10^5$  CHO-FRA cells and 10  $\mu\text{L}$  of complement in a total volume of 100  $\mu\text{L}$  PBS and incubated for 1 h on ice. Cells were washed with 5 mL ice cold PBS, pelleted at 1,000 RPM for 3 min and resuspended in 0.5 mL ice cold PBS. A 10  $\mu\text{g}/\text{mL}$  goat anti-human C1q-FITC was added and samples were incubated for 30 min on ice. Cells were washed twice with 5 mL ice cold PBS and resuspended in 0.5 mL PBS and analyzed for cell binding via flow cytometry as described above.

#### *Biotinylation of proteins for ELISA*

All proteins used in assays were biotinylated using EZ-link Sulfo-NHS-LC-Biotin No Weight Format (Thermo Scientific) as per included instructions.

### Quantification of CA125 in patient sera

CA125 is quantitated in human serum using an immuno-based FRET assay as previously described [6]. Briefly, serum samples and CA125 standards were serially diluted 1:1 in cRPMI and tested for CA125 concentrations using Europium cryptate and d2-labeled anti-CA125 antibodies 151-30 and 151-29 (Lee Biosolutions) in opaque white 384 well microplates (Greiner) in the presence of diluted samples and control wells for 3.5 hours at room temperature. FRET values were measured using a Wall-E microplate reader (Paradigm). This method enables us to accurately and reproducibly monitor CA125 levels in very small volumes.

### Detection of antibody-C1q interactions in the presence or absence of CA125 by ELISA

The 96-well ELISA plates were coated with 5  $\mu\text{g}/\text{mL}$  antibodies or human serum albumin (HSA) in 50 mM carbonate buffer, pH 9.5 overnight at 4°C. For membrane binding studies, cellular preparations were extracted and quantified as described [6]. Next, equal amounts of membrane preps were used to coat wells overnight in carbonate buffer as above. Wells were washed twice with 50 mM PBS, pH 7.2 and blocked in same buffer with 5% BSA for 1 hr at room temp. Wells were washed with PBS and probed with 5  $\mu\text{g}/\text{mL}$  of C1q-biotin in PBS + 0.5% BSA in the presence or absence of CA125 (5–30 KU/mL) for 1 hr at room temp. Next, wells were washed with PBS and probed with a 1:30,000 dilution of streptavidin-HRP in PBS for 1 hr at room temp. Plates were washed as above and exposed for C1q binding by the addition of TMB substrate for 5 min, followed by the addition of 0.1N  $\text{H}_2\text{SO}_4$  stop solution. Binding was quantitated by spectrophotometer reading at  $A_{450}$ . To test the effect of CA125 on antibody binding to antigen, antibodies were biotinylated and used as primary probes (0.1–5  $\mu\text{g}/\text{mL}$ ) to assay plates coated with 1  $\mu\text{g}/\text{mL}$  of target specific antigen or control protein. To test the effects of CA125 on the binding of HB-8568 to GD2 antigen, assays were performed in a similar manner except 2  $\mu\text{g}/\text{mL}$  of ganglioside was used to coat wells overnight in ethanol at room temp and wells air dried overnight. Biotinylated HB-8568 was added at 10  $\mu\text{g}/\text{mL}$  in PBS, pH 7.2 in the presence or absence of 10–30KU/mL CA125 for 1 hr at room temp. Wells were washed 3 times with PBS and probed with streptavidin-HRP (Jackson labs) for 30 min at room temp. Next, wells were washed as above and exposed to TMB substrate to measure antibody binding as above. All assays were done in at least triplicate.

### Detection of antibody-CA125 interactions

The 96-well ELISA plates were coated overnight at 4°C with 1–15 KU/mL CA125 in 0.05M carbonate, pH9.5. Plates were blocked with 0.05M PBS, pH7.2 + 5% BSA for 1 hr at room temp then washed twice with 0.05M PBS, pH7.2. Biotinylated antibodies or HSA were added to the plate in 0.05M PBS, pH 7.2 at 1–5  $\mu\text{g}/\text{mL}$

for 1 hr at room temp. Wells were washed 3 times with 0.05M PBS, pH 7.2 followed by addition of streptavidin-HRP in 0.05M PBS, pH7.2 + 0.5% BSA for 1 hr at room temp, washed and quantified as described above.

### Experimental stats

All statistical analysis were conducted using two-tailed *t*-test.

**Acknowledgments:** We want to thank D. Martens for editing the manuscript. N.C.N., J.B.K, and L.G. designed, conducted experimental studies, and drafted the manuscript. E.N.R. and S.F. performed experiments. All authors contributed to manuscript preparation. This work was supported by internal funding by Morphotek Inc.

**Conflict of interest:** All authors are employees of Morphotek and all have no ownership interests.

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**Abbreviations:** ADCC: antibody-dependent cellular cytotoxicity · CDC: complement-dependent cytotoxicity · FRA: folate receptor alpha · HSA: human serum albumin · MSLN: mesothelin · PFS: progression-free survival

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Received: 20/5/2018  
Revised: 9/7/2018  
Accepted: 22/8/2018  
Accepted article online: 24/8/2018