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An Fcγ Receptor-Dependent Mechanism Drives Antibody-Mediated Target-Receptor Signaling in Cancer Cells

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SUMMARY

Antibodies to cell-surface antigens trigger activatory Fcγ receptor (FcγR)-mediated retrograde signals in leukocytes to control immune effector functions. Here, we uncover an FcγR mechanism that drives antibody-dependent forward signaling in target cells. Agonistic antibodies to death receptor 5 (DR5) induce cancer-cell apoptosis and are in clinical trials; however, their mechanism of action in vivo is not fully defined. Interaction of the DR5-agonistic antibody drozitumab with leukocyte FcγRs promoted DR5-mediated tumor-cell apoptosis. Whereas the anti-CD20 antibody rituximab required activatory FcγRs for tumoricidal function, drozitumab was effective in the context of either activatory or inhibitory FcγRs. A CD40-agonistic antibody required similar FcγR interactions to stimulate nuclear factor-κB activity in B cells. Thus, FcγRs can drive antibody-mediated receptor signaling in target cells.

INTRODUCTION

The interaction of antigen-bound antibodies with FcγRs on the surface of leukocytes triggers retrograde signals into these FcγR-bearing cells. FcγRs fall into two signaling subclasses: activatory or inhibitory (Nimmerjahn and Ravetch, 2008). With the exception of human glycophosphatidylinositol-anchored FcγRIIIB, all mouse and human activatory FcγRs signal either through a cytoplasmic immunoreceptor tyrosine-based activating motif (ITAM), or via the ITAM-containing common γ chain. The engagement of leukocyte activatory FcγRs by antibody-antigen complexes may lead to various biologic effects, including cytokine secretion, oxidative burst, increased phagocytosis, and enhanced antibody-dependent, cell-mediated cytotoxicity (ADCC). ADCC is mediated by release of perforin and cytotoxic granules containing granzyme B, which, upon entry into target cells, activates apoptotic executioner caspases such as caspase-3 (Bolitho et al., 2007; Martin et al., 1996; Wilson et al., 2009). In addition to expressing activatory FcγRs, innate immune cells such as macrophages, monocytes, dendritic cells (DCs), mast cells, and granulocytes, express an inhibitory FcγR (FcγRIIB) (Amigorena et al., 1992; Nimmerjahn and Ravetch, 2008). FcγRIIB

Significance

Therapeutic antibodies targeting antigens on cancer cells often rely on antibody-dependent, cell-mediated cytotoxicity (ADCC) for efficacy: ADCC is controlled by antibody interaction with activatory versus inhibitory Fcγ receptors (FcγRs) on immune cells. A class of antibodies aims to trigger tumor-cell death via the proapoptotic receptors DR4 and DR5; how these agents work in vivo is not completely defined. We discovered that FcγRs on tumor-associated leukocytes provide a crosslinking scaffold that promotes antibody-dependent, DR5-mediated apoptosis in cancer cells. Both activatory and inhibitory FcγRs can support DR5 activation, unlike ADCC. A similar FcγR-based mechanism mediates antibody-dependent apoptosis activation through DR4, and NF-κB stimulation via a more distant receptor, CD40. These results have implications for clinical testing and optimization of agonistic antibodies.

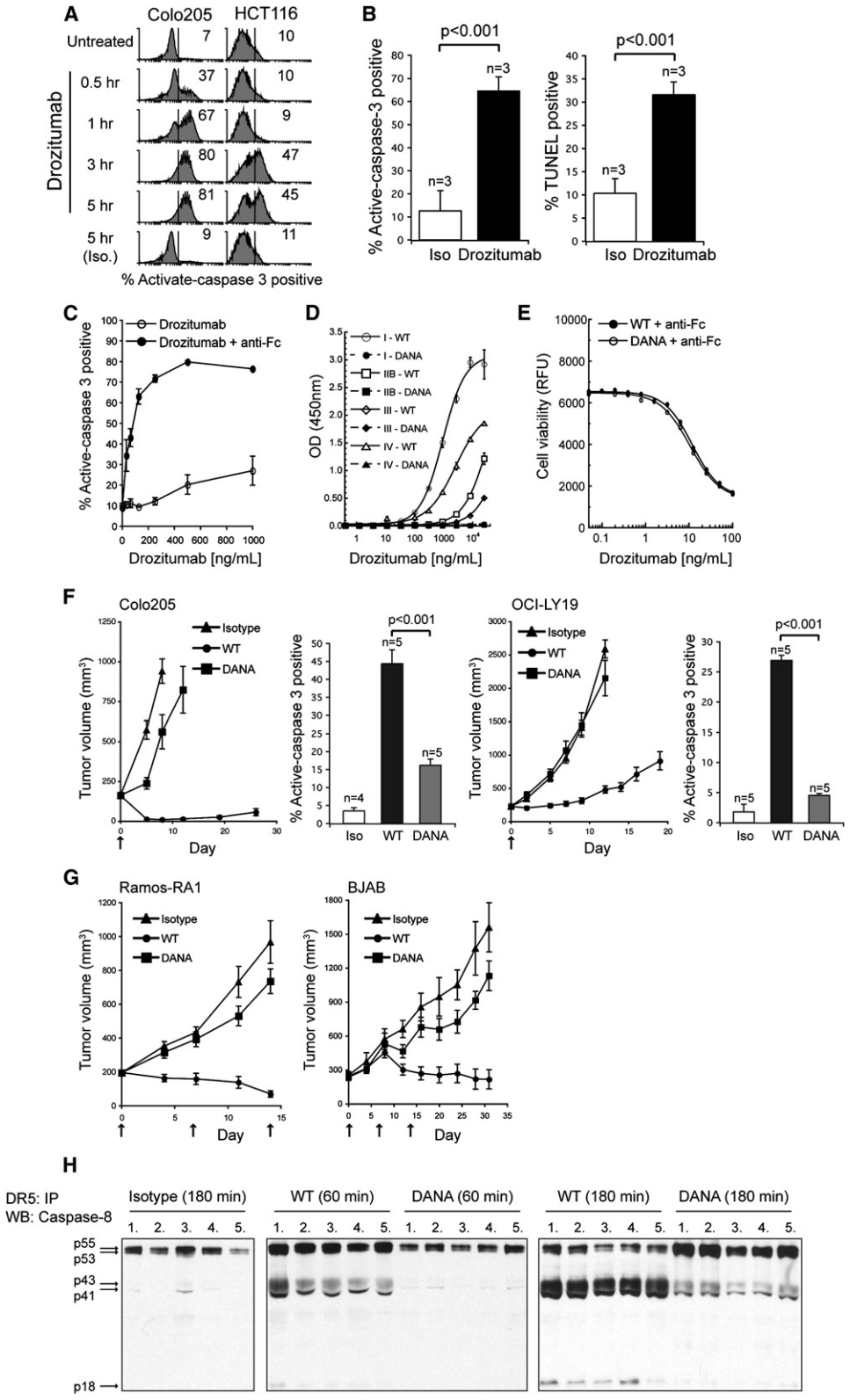

Figure 1. Drozitumab's Antitumor Activity Requires Interaction with FcγRs

(A) Colo205 and HCT116 colon tumors grown in Rag2$^{-/-}$ mice were treated for the indicated time with drozitumab. The percent of tumor cells containing cleaved caspase-3 was quantified by flow cytometry and compared to cells isolated from untreated or isotype (Iso) injected mice. Representative profiles are shown from n = 3–4 mice per group.

contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which negatively regulates the magnitude of signaling by ITAM-containing activatory receptors (Takai et al., 1996). Different IgG subclasses have varying affinity and specificity for distinct FcγRs (Nimmerjahn et al., 2005; Nimmerjahn and Ravetch, 2006). In humans, IgG1 and IgG3 function as proinflammatory IgG subclasses, based on their higher affinity for activatory versus inhibitory FcγRs. In mice, this latter feature characterizes the IgG2a and IgG2b subclasses (Dijstelbloem et al., 2001; Nimmerjahn and Ravetch, 2006).

Many cancer-therapeutic antibodies that bind cancer cell-surface antigens require ADCC to mediate tumor-cell elimination in vivo. Indeed, evidence from preclinical animal models suggests that ADCC is an important component of the therapeutic activity of human IgG1 antibodies that target the CD20 B cell-differentiation antigen (e.g., rituximab) (Maloney et al., 1997; de Haij et al., 2010), human epidermal growth factor receptor 2 (HER2) (trastuzumab) (Barok et al., 2007; Clynes et al., 2000; Musolino et al., 2008), epidermal growth factor receptor (cetuximab) (Hara et al., 2008), fibroblast growth factor receptor 3 (R3Mab) (Qing et al., 2009), CD52 (alemtuzumab) (Golay et al., 2006), and EphA2 receptor (Bruckheimer et al., 2009).

Proapoptotic receptor agonists (PARAs) activate the extrinsic apoptotic pathway by engaging death-domain-containing tumor necrosis factor receptor superfamily (TNFRSF) members on tumor cells (Ashkenazi, 2008a; Wilson et al., 2009). PARAs include recombinant human Apo2L/TRAIL (dulanermin), and agonistic antibodies that target the human receptors DR4 or DR5 (Ashkenazi, 2008a, 2008b; Ashkenazi and Herbst, 2008; Johnstone et al., 2008). Several DR4 and DR5 agonistic antibodies have demonstrated potent antitumor efficacy in preclinical cancer models and are now in clinical trials. Drozitumab is a human DR5-specific IgG1 antibody (Adams et al., 2008; Camidge et al., 2010). Drozitumab mediates DR5 clustering and binding of the apical signaling adaptor FADD, which in turn recruits the apoptosis-initiating protease, caspase-8, to assemble a death-inducing signaling complex (DISC) (Wilson et al., 2009). Once stimulated, caspase-8 activates executioner caspases such as caspase-3 and -7, either directly, or via the cell-intrinsic apoptotic pathway. Caspase-3 and -7 cleave multiple cellular substrates, inducing apoptotic cell death.

In the present study, we investigated the in vivo mechanism of action of agonistic antibodies targeting DR5 and DR4.

RESULTS

Drozitumab Requires Interaction with FcγRs to Exert Antitumor Activity

We quantified the extent and kinetics of drozitumab-induced apoptosis in xenografted tumor cells by measuring caspase-3 cleavage and DNA fragmentation ex vivo after in vivo antibody administration. Drozitumab induced marked activation of caspase-3 in the CD45-negative ($CD45^{neg}$) tumor-cell fraction of Colo205 or HCT116 colon carcinoma xenografts, peaking at ~3 hr (Figure 1A). A significant increase in caspase-3 activity correlated with DNA fragmentation after drozitumab treatment (Figure 1B), confirming apoptotic tumor-cell death.

Various agonistic human DR4 and DR5 antibodies display maximal proapoptotic signaling upon artificial Fc crosslinking (Ichikawa et al., 2001; Li et al., 2008; Natoni et al., 2007; Pukac et al., 2005; Yada et al., 2008; Zhang et al., 2007a). Indeed, drozitumab-induced caspase-3 activation and cell death in diverse cancer cell lines were augmented by crosslinking with an antihuman Fc-specific F(ab')2 reagent (Figure 1C; see Figure S1A available online) (Adams et al., 2008). In vivo, however, drozitumab displayed potent antitumor activity in the absence of any exogenous crosslinking reagent (Figure 1A). We hypothesized that FcγRs, expressed on immune cells in the tumor microenvironment, may facilitate drozitumab's in vivo activity by mediating ADCC or through some other mechanism. To explore this, we generated a drozitumab variant with two mutations in the IgG1 Fc region (D265A and N297A, dubbed DANA), which eliminate FcγR binding (Shields et al., 2001). Wild-type (WT) drozitumab bound to murine FcγRs with the following rank order of affinity: I > IV > (IIB, III), whereas drozitumab-DANA did not bind to any of these receptors (Figure 1D). WT drozitumab bound also to human FcγRs, with the following rank order of affinity: I > IIIA-V158 > (IIIA-F158,IIA) > IIB, whereas drozitumab-DANA did not exhibit detectable binding (data not shown). The DANA mutations did not affect drozitumab binding to the salvaging neonatal FcγR (FcγRn), which is important for maintaining proper antibody half-life in plasma (Roopenian et al., 2003); accordingly, the WT and DANA variants displayed similar pharmacokinetic properties in mice (data not shown). Furthermore, the two variants were equally potent at inducing death of Colo205 cells in vitro in presence of a F(ab')2 crosslinking reagent (Figure 1E), confirming that the DANA mutations do not compromise drozitumab's ability to interact with DR5.

(B) Caspase-3 activity was correlated with an increase in TUNEL-positive Colo205 cells 4 hr following drozitumab or isotype (Iso) antibody treatment.
(C) Caspase-3 activation in Colo205 tumor cells cultured with drozitumab alone or combined with 1 μg/ml of anti-human Fc-specific (Fab')2 (anti-Fc) reagent.
(D) Binding of the wild-type (WT) or DANA drozitumab to recombinant murine FcγR (I, IIB, III, or IV).
(E) Colo205 cells treated with a dose titration of WT or DANA drozitumab in combination with the anti-Fc crosslinking reagent) (1 μg/ml). Cell survival was analyzed with the AlamarBlue cell viability assay after 48 hr.
(F) $Rag2^{-/-}$ mice bearing Colo205 or OCI-LY19 tumors were treated with WT or DANA drozitumab or an isotype control antibody on day 0 (n = 10/group). Histograms: Caspase-3 activation was quantified ex vivo in established Colo205 or OCI-LY19 tumors 4 hr after WT or DANA drozitumab treatment.
(G) $Rag2^{-/-}$ mice bearing Ramos-RA1 or OCI-LY19 tumors received the following treatment; wild-type anti-CD20 (WT), the anti-CD20-DANA mutant (DANA), or an isotype IgG control antibody. Treatment regimens were administered on days 0, 7, and 14 following after group-out (n = 8–10/group).
(H) $Rag2^{-/-}$ mice with pre-established (approximately 300 mm^3) Colo205 tumors were treated with WT or DANA drozitumab. Tumors were dissected 60 or 180 min after treatment and homogenized in lysis buffer. DR5 was immunoprecipitated from equal tumor weights and caspase-8 recruitment and processing in the DISC was evaluated by immunoprecipitation of DR5 and immunoblotting for caspase-8 (n = 5 individual tumors/group). Where indicated, a Student's t test was used to calculate statistical significance.
Error bars in (B)–(D) indicate the standard deviation (SD). Error bars in (F) and (G) indicate the standard error of the mean (SEM). Data shown in Figure 1 are representative of two or more independent experiments. See also Figure S1.

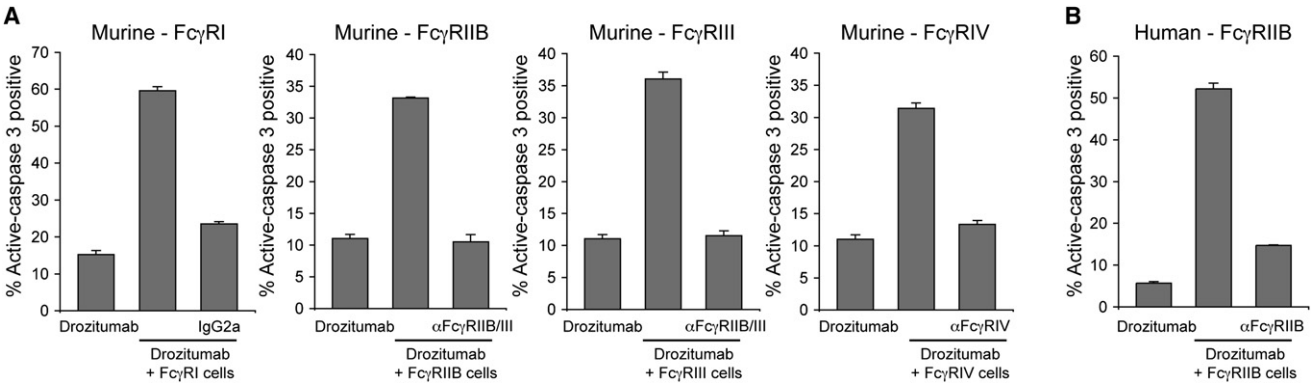

Figure 2. Cell Lines Expressing Either Activatory or Inhibitory FcγRs Similarly Support Drozitumab-Induced Caspase-3 Activation in Tumor Cells

(A) CFSE-labeled Colo205 target cells were cocultured with HEK293 cell lines stably expressing murine FcγRIIB, III, or IV or Jurkat cells stably expressing FcγRI (at a 1:5 ratio). Caspase-3 activation was assessed by flow cytometry 4 hr after drozitumab (250 ng/ml) addition. Drozitumab binding to murine FcγRs was inhibited by preblocking FcγR expressing cells for 30 min at 4°C with 1 μg/ml of murine IgG2a isotype antibody (FcγRI), anti-FcγRIIB/III (anti-FcγRIIB/III), or anti-FcγRIV (anti-FcγRIV).

(B) Caspase-3 activation was monitored by flow cytometry in CFSE-labeled Colo205 cells cocultured for 4 hr with HEK293 cells expressing human FcγRIIB and drozitumab (250 ng/ml). HEK293-FcγRIIB cells were preblocked for 30 min at 4°C with anti-FcγRIIB (anti-FcγRIIB).

Error bars indicate the SD. Data shown are representative of three independent experiments.

To examine whether FcγRs are important for the antitumor efficacy of drozitumab, we treated Rag2$^{-/-}$ mice (Shinkai et al., 1992) bearing various human tumor xenografts with the two antibody variants. WT drozitumab caused tumor regression or significantly restricted tumor progression in a number of models (Figure 1F; Figure S1B); in contrast, the DANA variant displayed little to no antitumor activity. As compared with WT, the DANA variant induced ~65%–80% less caspase-3 activity in Colo205 and OCI-LY19 B cell lymphoma tumors (Figure 1F, histograms), with similar reductions in apoptosis as measured by the TUNEL or hypodiploid DNA assays (data not shown). A DANA derivative of anti-CD20 antibody also displayed markedly less antitumor activity as compared to WT anti-CD20 in two models of B cell lymphoma (Figure 1G). Hence, both the anti-DR5 and anti-CD20 antibodies require FcγR interactions for optimal efficacy.

To assess the activation of DR5 at the biochemical level, we performed ex vivo analysis of the formation of a DR5-associated DISC in tumors. We treated Rag2$^{-/-}$ mice harboring Colo205 tumors with the WT or DANA drozitumab variants, immunoprecipitated DR5 from tumor-cell lysates, and used immunoblot analysis to detect caspase-8 recruitment into the DISC; as well as caspase-8 activation, as indicated by its proteolytic processing (Figure 1H). Caspase-8 recruitment was evident within 60 min of WT drozitumab treatment, with substantial processing and appearance of the p18 catalytic subunit by 180 min. These events were markedly less pronounced upon treatment with the DANA variant. Similar caspase-8 processing occurred in HCT116 tumor xenografts after 180 min of WT drozitumab treatment (Figure S1C).

Various Mouse and Human FcγRs Can Support Drozitumab-Mediated Apoptosis In Vitro

Two key FcγR-dependent mechanisms may contribute to drozitumab's efficacy: immune cell-mediated ADCC and/or DR5 signaling. Importantly, each could account for an apoptotic phenotype, as both involve caspase-3 activation (Bolitho et al., 2007; Martin et al., 1996; Wilson et al., 2009). We therefore examined the capacity of individual activatory and inhibitory FcγRs to support apoptosis stimulation by drozitumab. We cocultured transfected cell lines stably expressing individual FcγRs with CFSE-labeled Colo205 target cells, added drozitumab, and measured apoptosis after 4 hr by quantifying caspase-3 cleavage in the CFSE^{high} tumor-cell population. All cell lines expressing murine or human FcγRs, including those bearing the inhibitory receptor FcγRIIB, were capable of supporting drozitumab-mediated caspase-3 activation (Figures 2A and 2B; data not shown); preincubation of the cell lines with FcγR-blocking antibodies inhibited caspase-3 activation. Hence, both activatory and inhibitory FcγRs can support drozitumab-mediated DR5 activation in vitro.

FcγR-Expressing Cells in the Tumor Microenvironment Support Drozitumab-Mediated Apoptosis in Tumor Cells

To better define the interaction between drozitumab and FcγRs within the tumor microenvironment, we profiled the FcγRs expressed by enriched CD45^{high} leukocytes extracted from Colo205 tumors (Figure S2A). The largest tumor-associated leukocyte population consisted of myelomonocytic cells, identified as F4/80^{high}CD11c$^{int\text{-}high}$CD11b^{high} (CD11b^{high}F4/80^{high}) (Figure S2B). Other, less abundant leukocytic populations also were detected in the tumors using specific cell-surface lineage markers (Figures S2C and S2D).

Next, we analyzed expression of FcγRIIB, III and IV on tumor-associated leukocytes, as compared with phenotypically similar immune-cell populations in the spleen of Rag2$^{-/-}$ mice (Figure 3A). Myeloid immune cells reportedly express both activatory and inhibitory FcγRs, whereas NK cells only express activatory FcγRIII (Nimmerjahn and Ravetch, 2006). One caveat with distinguishing between mouse FcγRIIB and FcγRIII is that the available 2.4G2 antibody recognizes a common polymorphic

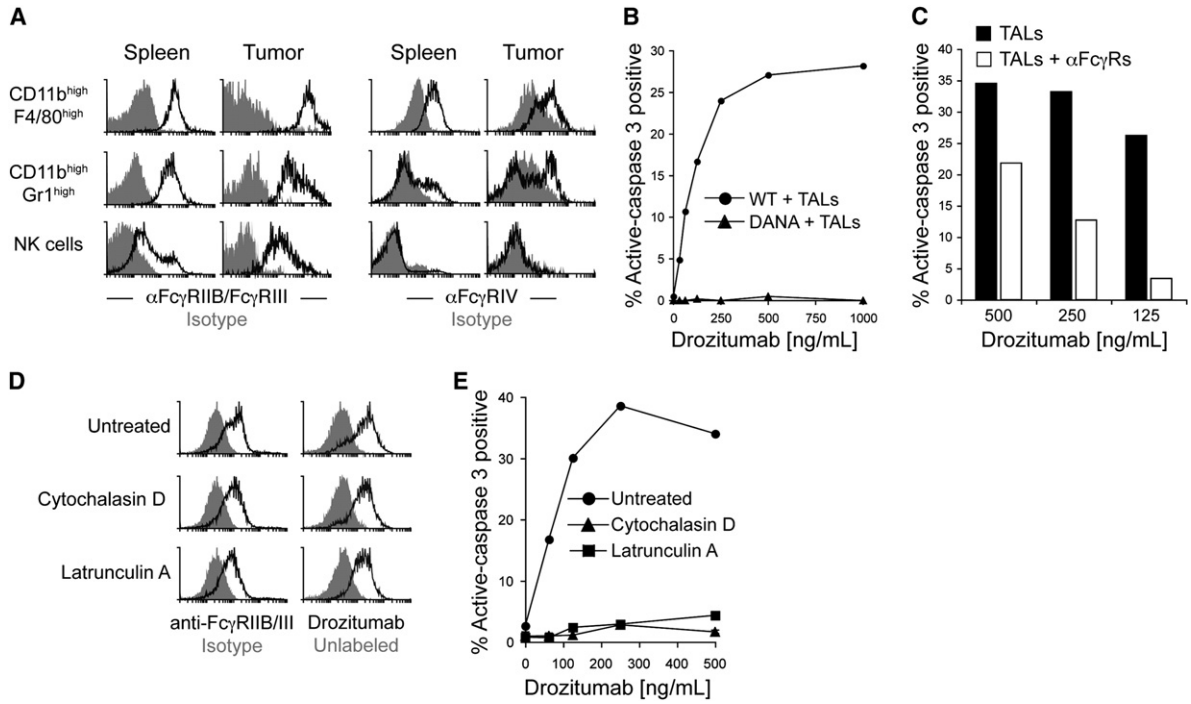

Figure 3. FcγRs Expressed by Tumor-Associated Leukocytes Support Drozitumab-Mediated Apoptosis in Tumor Cells

(A) Histograms showing cell surface expression of FcγRIIB/IIII and FcγRIV by tumor-associated leukocytes (TALs), as compared with phenotypically similar immune cell populations in the spleen. Leukocytes were blocked with a 250 μg/ml cocktail of rat IgG2b, armenian hamster IgG, and rat IgG1 (250 μg/ml) isotype antibodies 30 min prior to FcγR expression analysis. To evaluate FcγRIIB/III, anti-FcγRIV was added to the preblocking antibody cocktail. Conversely, anti-FcγRIIB/III was included prior to staining for FcγRIV.

(B) Caspase-3 activation in CFSE-labeled Colo205 cells incubated with enriched TALs, and a dose titration of WT or DANA drozitumab variants. Caspase-3 activation in the absence of TALs was subtracted for each antibody.

(C) Caspase-3 activation in Colo205 cells incubated with drozitumab and TALs, either untreated or pretreated with anti-FcγRIIB/III plus anti-FcγRIV antibodies (murine IgG2a isotype).

(D) Surface levels of FcγRIIB/III on untreated, or cytochalasin D or latrunculin A pretreated splenocytes from Rag2$^{-/-}$ mice (left), and cell surface binding of a fluorochrome-conjugated drozitumab (right).

(E) Caspase-3 activation in CFSE-labeled Colo205 cells incubated with untreated, cytochalasin D, or Latrunculin A pretreated splenocytes (preincubated for 30 min and then washed two times) and a dose titration of drozitumab.

Data in Figure 3 are representative of two or more independent experiments. See also Figure S2.

epitope on the extracellular domain of both receptors (Unkeless, 1979). Therefore, to verify expression of FcγRIIB, we isolated leukocytes from tumors grown in Rag2$^{-/-}$ mice intercrossed with FcγRIII$^{-/-}$ mice (Rag2$^{-/-}$FcγRIII$^{-/-}$) (Hazenbos et al., 1996). All the myeloid cell populations we identified expressed FcγRIIB (Figure 3A; Figure S2D and data not shown), whereas no FcγR expression could be detected in the nonhematopoietic tumor-cell compartment (human MHC class I^{neg}CD45^{neg}) (Figure S2E).

To interrogate whether FcγRs on leukocytes could support drozitumab-mediated caspase-3 activation in tumor cells, we cocultured the CD45-enriched cell fraction with CFSE-labeled Colo205 targets in the presence of antibody (Figure 3B). Tumor-associated leukocytes supported caspase-3 activation by WT drozitumab but not the DANA variant; preincubation of the leukocytes with FcγR-blocking antibodies inhibited caspase-3 activation (Figure 3C). To probe the functional requirement for FcγR interaction further, we treated FcγR-expressing splenocytes for 30 min with the actin polymerization inhibitors latrunculin A or cytochalasin D (de Oliveira and Mantovani, 1988; Mimura and Asano, 1976); we then washed the inhibitors out and added drozitumab and tumor-cell targets. Inhibitor pretreatment did not affect the expression of FcγRIIB/III on the splenocytes or the binding of fluorochrome-conjugated drozitumab to these cells (Figure 3D); however, it substantially diminished the ability of the FcγR-expressing splenocytes to support drozitumab-mediated caspase-3 activation in tumor cells (Figure 3E). Hence, the interface provided by leukocyte FcγRs to support drozitumab-dependent DR5 activation is dynamic, requiring actin poymerization. Taken together, these results indicate that FcγRs on tumor-associated leukocytes provide a platform for drozitumab-mediated clustering of DR5 to drive apoptotic signaling. However, these findings do not rule out an additional potential contribution of ADCC to tumor-cell apoptosis.

Drozitumab Induces Apoptosis and Tumor Regression Independently of NK Cells and CD11b-Expressing Myeloid Cells

To define the contribution of specific immune-effector cells to drozitumab's tumoricidal activity in vivo, we used a combination

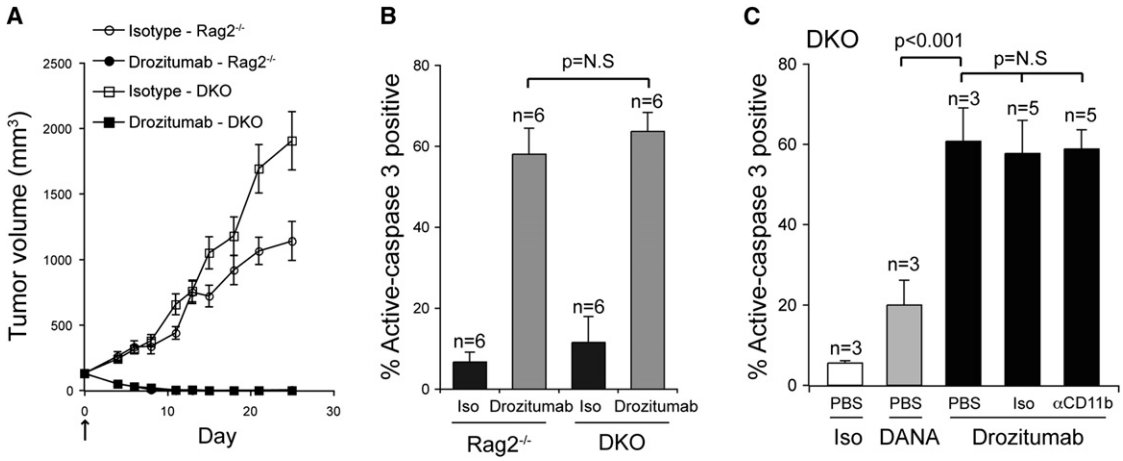

Figure 4. The Antitumor Activity of Drozitumab Is Independent of Tumor-Associated NK or Myeloid Effector Cell Activity

(A) Colo205 tumors were grown in NK cell containing Rag2$^{-/-}$ or NK cell deficient (Rag2$^{-/-}$IL2Rγ$^{-/-}$, DKO) mice. Mice were assigned into cohorts and treated with drozitumab or an isotype IgG control antibody (n = 10/group).

(B) Caspase-3 activation was assessed in established (~500 mm^3) Colo205 tumors grown in Rag2$^{-/-}$ or DKO mice 4 hr after drozitumab or isotype (Iso) control antibody treatment.

(C) Beginning 1 day prior to Colo205 tumor cell inoculation, DKO mice were dosed daily with PBS, mouse αCD11b or isotype control antibody. Fourteen days after tumor cell inoculation, tumor-bearing mice (~300 mm^3) were treated with WT or DANA drozitumab, or an isotype control antibody. Caspase-3 activation in tumor cells was evaluated ex vivo 4 hr after antibody treatment by flow cytometry.

Error bars shown in panel (A)–(C) indicate the SEM Student's t test was used to calculate statistical significance. See also Figure S3.

of genetically deficient mice and antibody-based cell depletion regimens. We examined dependence on NK-cell-mediated ADCC by inoculating Colo205 tumor cells into Rag2$^{-/-}$ mice, or into mice deficient in both Rag2 and the interleukin 2 receptor common γ-chain (Rag2$^{-/-}$IL2Rγ$^{-/-}$, dubbed DKO), which lack NK cells (Colucci et al., 1999). Drozitumab induced equally potent Colo205 tumor regression and caspase-3 activation in both mouse strains (Figures 4A and 4B). We obtained similar results with H2122 nonsmall cell lung cancer xenografts, or using antibody-based depletion of NK cells in Colo205 tumor-bearing Rag2$^{-/-}$ mice (data not shown). To further assess the contribution of myeloid effector cells, we blocked their recruitment into tumors by treating NK cell-deficient mice daily with anti-CD11b antibody, starting one day before tumor-cell inoculation (Smyth et al., 2006; Zhang et al., 1997). Anti-CD11b treatment itself did not affect tumor growth (Figure S3A), while gating on the remaining CD45^{high} cell fraction confirmed myeloid-cell exclusion from the tumor (Figure S3B). Tumor-associated CD45^{high}CD11b^{neg} cells isolated from NK cell-deficient mice coexpressed the DC lineage marker CD11c and MHC class II, but not for the macrophage marker F4/80 (Figure S3C), suggesting a DC-like phenotype. These cells expressed both activatory FcγRIII and inhibitory FcγRIIB (Figure S3D). WT drozitumab induced indistinguishable tumor-cell caspase-3 activation in mice treated with either an isotype control or an anti-CD11b antibody, while the DANA variant was markedly less effective (Figure 4C). These results indicate that drozitumab can induce apoptosis in cancer cells independently of tumor-associated NK or CD11b-expressing myeloid cells. It remains possible nonetheless that in some tumor types ADCC may also contribute to drozitumab's antitumor activity. Although not formally demonstrated, these results suggest that FcγR-expressing DC-like cells are sufficient to support drozitumab's activity in vivo.

Drozitumab Exerts Intact Antitumor Activity in Absence of Activatory FcγRs

To further dissect the contribution FcγRs to drozitumab's antitumor activity, we devised an in vivo strategy that eliminates interaction with activatory FcγRs. First, we intercrossed Rag2$^{-/-}$ mice with FcγRI$^{-/-}$FcγRIII$^{-/-}$ mice. To ablate interaction with the remaining activatory FcγRIV, we took advantage of the failure of this FcγR to bind to muIgG1 (Nimmerjahn et al., 2005), by generating a muIgG1 variant of drozitumab (Figure 5A). To confirm that FcγRIV cannot support activity of murine (mu) IgG1-drozitumab, we used cell lines expressing either FcγRIIB or FcγRIV. FcγRIIB-expressing cells facilitated caspase-3 activation in Colo205 cells by either human (hu) IgG1- or muIgG1-drozitumab (Figure 5B). In contrast, FcγRIV-expressing cells supported proapoptotic activity of the huIgG1 but not the muIgG1 drozitumab variant (Figure 5C). Specific FcγR-blocking antibodies attenuated caspase-3 activation by both drozitumab isotypes (data not shown). In Rag2$^{-/-}$FcγRI/III$^{-/-}$ mice, the mIgG1 isotype can interact only with the inhibitory FcγRIIB. Nonetheless, the tumoricidal activity of muIgG1-drozitumab was comparable in Rag2$^{-/-}$ and Rag2$^{-/-}$FcγRI/III$^{-/-}$ mice, and consistent with that of huIgG1-drozitumab in Rag2$^{-/-}$ mice (Figure 5D). We obtained similar results with a muIgG1 variant of an agonistic anti-DR4 antibody (Chuntharapai et al., 2001) (Figure 5E). These findings indicate that both activatory and inhibitory FcγRs on leukocytes are capable of driving antibody-mediated proapoptotic DR5 or DR4 signaling in cancer cells.

The tumoricidal activity of the anti-CD20 antibody rituximab is significantly impaired in mice lacking activatory FcγRs (Clynes et al., 2000). To confirm a similar phenotype in Rag2$^{-/-}$ FcγRI/III$^{-/-}$ mice, we used isotypic huIgG1 and muIgG1 rituximab versions. As compared with the huIgG1 isotype, muIgG1-rituximab displayed significantly less activity against BJAB

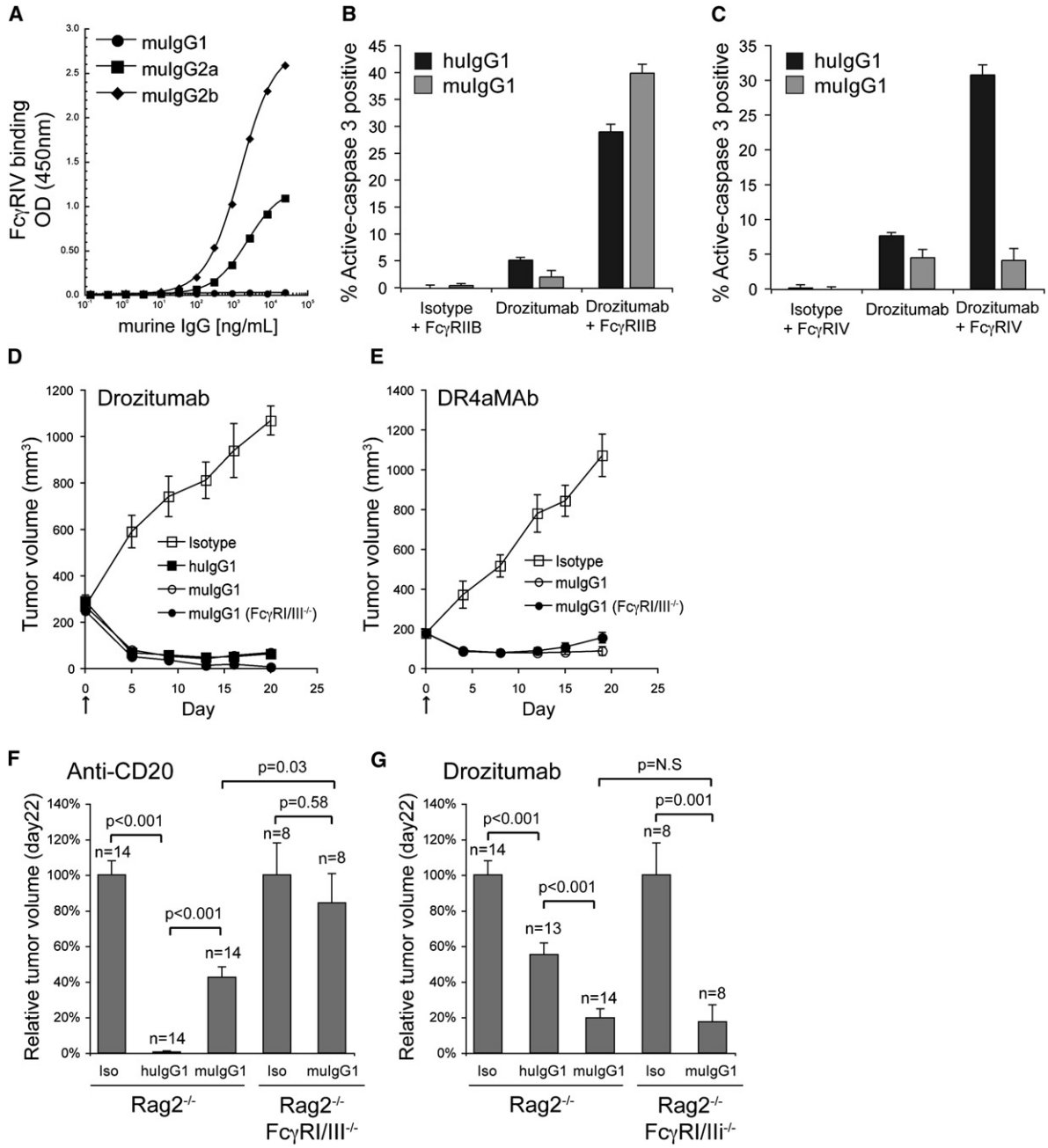

Figure 5. Drozitumab Exerts Antitumor Activity in the Absence of Activatory FcγRs

(A–C) (A) Recombinant FcγRIV binding to a dose titration of noncomplexed murine IgG1, IgG2a, or IgG2b. CFSE-labeled Colo205 target cells cocultured with HEK293 cells expressing murine FcγRIIB (B) or FcγRIV (C) in the presence of 250 ng/ml of muIgG1 or huIgG1 versions of drozitumab, compared with isotype control antibodies. Caspase-3 activity in CFSE-labeled tumor cells was quantified by flow cytometry 4 hr after antibody addition.

(D) Colo205 tumor-bearing Rag2$^{-/-}$ or Rag2$^{-/-}$FcγRI$^{-/-}$FcγRIII$^{-/-}$ (FcγRI/III$^{-/-}$) mice were treated with a single dose of human IgG1 (huIgG1) or murine IgG1 (muIgG1) drozitumab (n = 8–12/group).

(E) Same as in (D) except tumor-bearing Rag2$^{-/-}$ or FcγRI$^{-/-}$FcγRIII$^{-/-}$ mice were treated with vehicle or muIgG1-DR4aMAb (n = 8–12/group).

(F) BJAB tumor-bearing mice were treated with pooled isotype control antibodies (Iso), or human IgG1 (huIgG1) or murine IgG1 (muIgG1) anti-CD20 on days 0, 7 and 14 after group out (n = 8–14/group). Tumor volume relative to isotype control treated mice is indicated for day 22 posttreatment. Data are representative of two or more independent experiments.

(G) Same as in (F), except BJAB tumor-bearing Rag2$^{-/-}$ or Rag2$^{-/-}$FcγRI$^{-/-}$FcγRIII$^{-/-}$ (FcγRI/III$^{-/-}$) mice were treated with a single dose of pooled isotype control antibodies or human IgG1 (huIgG1) or murine IgG1 (muIgG1) drozitumab (n = 8–14/group). Tumor volume relative to isotype control treated mice is indicated for day 22 posttreatment.

Student's t test was used to calculate statistical significance in (F) and (G). Error bars in (B) and (C) indicate the standard deviation (SD). Error bars in (D)–(G) indicate the standard error of the mean (SEM). Data are representative of two or more independent experiments. See also Figure S4.

Burkitt's B cell lymphoma xeongrafts in Rag2$^{-/-}$ mice (Figure 5F). Antitumor efficacy of muIgG1-rituximab was further impaired in Rag2$^{-/-}$FcγRI/III$^{-/-}$ mice, consistent with previous evidence that FcγRIIB interactions inhibit effector-cell activity (Clynes et al., 2000; Nimmerjahn and Ravetch, 2008). In contrast, FcγRIIB sufficiently supported activity of either the murine or human IgG1 variants of drozitumab against BJAB tumors (Figure 5G). A suboptimal dose of huIgG1 drozitumab (0.1 mg/kg) was less active in Rag2$^{-/-}$FcγRIIB$^{-/-}$ mice as compared with Rag2$^{-/-}$ mice (Figures S4A and S4B), suggesting that FcγRIIB is not only sufficient but indeed contributes significantly to drozitumab-mediated DR5 activation.

In keeping with the in vivo results, FcγR-expressing splenocytes isolated from Rag2$^{-/-}$, or Rag2$^{-/-}$FcγRI/III$^{-/-}$, or Rag2$^{-/-}$FcγRIIB$^{-/-}$ mice supported tumor-cell caspase-3 activation ex vivo by either human or murine IgG1-drozitumab (Figure S4C). Moreover, muIgG1-drozitumab induced tumor-cell caspase-8 activation in xenografted Rag2$^{-/-}$FcγRI/III$^{-/-}$ mice, while infusion with an FcγRIIB-blocking antibody before mIgG1-drozitumab treatment attenuated this effect (Figure S4D). Thus, FcγRIIB is sufficient to support drozitumab-induced DISC activation in vivo.

Polymorphisms in Human FcγRIIA and FcγRIIIA Affect Drozitumab Binding and Proapoptotic Activity

Two human FcγR polymorphisms have been associated with improved progression-free survival in lymphoma patients treated with rituximab (Cartron et al., 2002; Weng et al., 2004; Weng and Levy, 2003). A similar correlation was reported for breast cancer therapy with trastuzumab and colorectal cancer treatment with cetuximab (Bibeau et al., 2009; Musolino et al., 2008; Zhang et al., 2007b). The most characterized human FcγR polymorphism with improved binding to human IgG1 is in activatory FcγRIIIA, wherein valine versus phenylalanine at position 158 (FcγRIIIA^{158V} or FcγRIIIA^{158F}) confers a 3- to 5-fold higher affinity for human IgG1 (Koene et al., 1997; Presta, 2008; Shields et al., 2001). A polymorphism in human FcγRIIA, with histidine versus arginine at position 131 (FcγRIIA^{131H} or FcγRIIA^{131R}), also improves binding to human IgG1 (Bruhns et al., 2009).

Consistent with other antibodies (Bruhns et al., 2009), huIgG1-drozitumab exhibited approximately 2- to 3-fold higher affinity for recombinant FcγRIIA^{131H} versus FcγRIIA^{131R} (Figure 6A). This increase was seen with noncomplexed and complexed drozitumab, using anti-κ antibody to form complexes and enhance FcγR-binding avidity. Avidity may increase upon simultaneous binding of multiple drozitumab molecules to DR5-expressing tumor cells and FcγR-bearing leukocytes. To examine this possibility, we generated transfected HEK293 cell lines stably expressing FcγRIIA^{131H} or FcγRIIA^{131R} and measured binding to huIgG subclasses (Figure S5A). Cells expressing FcγRIIA^{131H} as compared with FcγRIIA^{131R} showed markedly improved binding to huIgG1 and huIgG2, with similar binding to huIgG3 and inferior binding to huIgG4 (Figure S5B). Furthermore, cells expressing FcγRIIA^{131H} as compared to FcγRIIA^{131R} were superior at facilitating drozitumab-induced caspase-3 activation (Figure 6B). Human IgG1 binds with higher affinity to FcγRIIIA^{158V} versus FcγRIIIA^{158F} (Figure 6C) (Bruhns et al., 2009; Koene et al., 1997; Presta, 2008; Shields et al., 2001). Indeed, cells expressing FcγRIIIA^{158V} also were superior to those expressing FcγRIIIA^{158F} at supporting drozitumab-mediated caspase-3 activation in Colo205 cells (Figure 6D). Hence, stronger FcγR interactions can further augment drozitumab's proapoptotic activity.

NF-κB Activation by Anti-CD40 Agonistic Antibody Requires FcγRs

We next evaluated whether a similar FcγR-dependent mechanism extended to antibody-based activation of another TNFRSF member, CD40. To this end, we took advantage of the selective coexpression of FcγRIIB with CD40 (TNFRSF5) on B cells. We isolated splenic B cells from WT or FcγRIIB$^{-/-}$ mice and examined the ability of the agonistic anti-CD40 antibody FGK-45 (Elgueta et al., 2009; Saijo et al., 2002) to stimulate NF-κB signaling and cytokine production. Despite equivalent levels of CD40 expression, FGK-45 induced substantially less phosphorylation of IκBα, indicating weaker NF-κB activation, in FcγRIIB$^{-/-}$ versus WT B cells (Figure 6E; Figure S5C). Crosslinking of FGK-45 with an anti-rat Fc-specific F(ab')2 reagent reconstituted the antibody's activity on FcγRIIB$^{-/-}$ B cells (Figure 6F). Further analysis based on phosphorylation of the p65 subunit of NF-κB confirmed that activation by FGK-45 was severely impaired in FcγRIIB$^{-/-}$ versus WT B cells yet rescued by crosslinking (Figure S5D). Consistent with these results, FGK-45 induced markedly less interleukin (IL)-6 and IL-12/23 p40 cytokine production in FcγRIIB$^{-/-}$ as compared to WT B cells (Figure S5E). In contrast, IL-6 production in response to lipopolysaccharide (LPS) was indistinguishable (Figure S5F). These results suggest that a similar FcγR-dependent mechanism applies to antibody-mediated activation of diverse signaling functions by structurally distinct TNFRSF members.

DISCUSSION

FcγRs play important roles in modulating antibody function and host immunity, by transmitting differential retrogade signals into leukocytes through activatory or inhibitory sequence motifs (Nimmerjahn and Ravetch, 2008). ADCC is a key mechanism of innate immunity, allowing effector cells to attack and eliminate antibody-decorated target cells upon activatory FcγR stimulation; in contrast, signaling through inhibitory FcγRs negatively regulates ADCC (Nimmerjahn and Ravetch, 2008). ADCC is implicated also as a critical component of the anticancer action of several antibodies that target tumor-associated antigens (Barok et al., 2007; Bruckheimer et al., 2009; Clynes et al., 2000; Kimura et al., 2007; Kurai et al., 2007; Musolino et al., 2008; Peipp et al., 2008; Qing et al., 2009; Schneider-Merck et al., 2010). Elimination of activatory-FcγR interactions significantly attenuates the efficacy of the therapeutic agents rituximab and trastuzumab in preclinical models (Clynes et al., 2000), consistent with the importance of ADCC for antitumor activity.

Various anti-DR5 and anti-DR4 antibodies have been under investigation, with more than 20 human cancer clinical trials (Ashkenazi, 2002, 2008b; Ashkenazi and Herbst, 2008; Johnstone et al., 2008; Yang et al., 2010). Drozitumab was well tolerated in a Phase Ia study in patients with advanced malignancy (Camidge et al., 2010). While drozitumab displays much stronger proapoptotic activity in vitro upon artificial Fc crosslinking, it exhibits potent antitumor activity in vivo in various cancer models

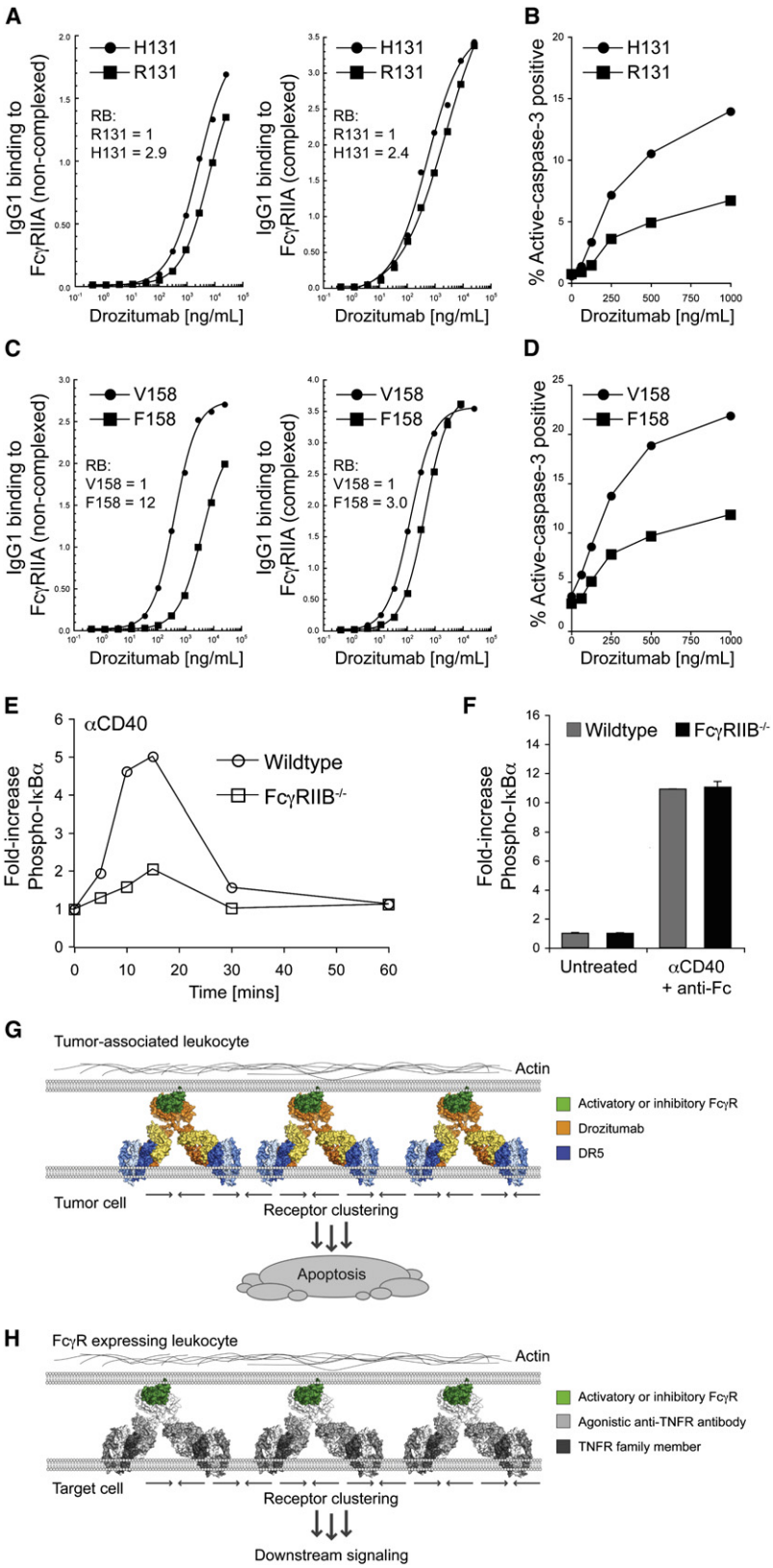

Figure 6. Differential Interaction of FcγR Polymorphs with Drozitumab

(A) Relative binding (RB) of recombinant human $Fc\gamma RIIA^{H131}$ versus $Fc\gamma RIIA^{R131}$ to drozitumab: noncomplexed (left) and complexed (right). The optical density was measured at 450 nm.

(B) Cell lines expressing $Fc\gamma RIIA^{H131}$ (H131) or $Fc\gamma RIIA^{R131}$ (R131) variants were incubated with CFSE-labeled Colo205 tumor cells and a dose titration of drozitumab. Caspase-3 activation in Colo205 cells was assessed after 4 hr by flow cytometry.

(C) Relative binding (RB) of recombinant human $Fc\gamma RIIIA^{F158}$ (F158) versus $Fc\gamma RIIIA^{V158}$ (V158) to drozitumab: noncomplexed (left) and complexed (right).

(D) HEK293 cells expressing $Fc\gamma RIIIA^{V158}$ or $Fc\gamma RIIIA^{F158}$ were incubated with CFSE-labeled Colo205 tumor cells and treated with a dose titration of drozitumab. Caspase-3 activation in Colo205 cells was assessed after 4 hr by flow cytometry.

(E) Kinetics of anti-CD40 antibody (10 μg/ml) induced IκBα phosphorylation in WT wild-type and $Fc\gamma RIIB^{-/-}$ B cells.

(F) IκBα phosphorylation in WT or $Fc\gamma RIIB^{-/-}$ B cells 15 min after the addition of 10 μg/ml of anti-CD40 antibody in the presence of 10 μg/ml of the anti-rat Fc-specific F (ab')2 reagent (anti-Fc). Error bars reflect ±SD.

(G) Schematic showing the proposed interface between FcγR-expressing tumor-associated leukocytes and DR5 positive tumor cells targeted by drozitumab. Drozitumab-mediated DR5 clustering is driven by an actin-dependent FcγR interaction that culminates in DISC activation and apoptotic tumor cell death.

(H) A proposed model for agonistic antibodies targeting CD40 or other TNFR family members, where either activatory or inhibitory FcγRs may facilitate downstream signal transduction.

Data are representative of two or more independent experiments. See also Figure S5.

in the absence of exogenous crosslinkers (Adams et al., 2008; Edgington et al., 2009; Zinonos et al., 2009). This intriguing observation prompted us to interrogate how drozitumab operates in vivo. We found that interaction with FcγRs expressed by tumor-associated leukocytes is necessary, while either activatory or inhibitory FcγRs are sufficient for optimal proapoptotic signaling by drozitumab. FcγRs on tumor-associated leukocytes appear to provide a dynamic platform that facilitates drozitumab-mediated DR5 engagement, DISC assembly, and apoptosis activation in tumor cells (see model in Figure 6G). Thus, leukocyte FcγRs can promote antibody-mediated signaling through a cognate receptor on target cells. This may have implications not only for DR5 antibodies but also more broadly for antibodies directed to other cell-surface receptors within and perhaps even outside the TNFR superfamily (Figure 6H).

To test whether FcγRs are important for drozitumab's proapoptotic activity, we generated a mutant variant of drozitumab, which lacked FcγR interaction while retaining DR5 binding. The DANA mutant displayed significantly impaired antitumor activity, demonstrating that FcγR interactions are critical for drozitumab's efficacy. We obtained similar results with a DANA variant of anti-CD20 antibody, which exerts much of its antitumor activity via an activatory FcγR-dependent ADCC. Since ADCC and DR5 signaling can trigger effector-caspase activation (Bolitho et al., 2007; Chowdhury and Lieberman, 2008; Cullen and Martin, 2008; Martin et al., 1996; Wilson et al., 2009), both mechanisms might account for the apoptotic phenotype in drozitumab-treated tumors. To address this, we tested the dependence of drozitumab's efficacy on activatory versus inhibitory FcγRs.

In vitro, both types of FcγR were capable of supporting drozitumab-induced caspase activation in tumor-cell targets. Activity was also facilitated ex vivo by isolated tumor-associated leukocytes, which expressed a spectrum of activatory and inhibitory FcγRs. Using genetic models in conjunction with a murine IgG1 version of drozitumab, we found that the inhibitory FcγRIIB was sufficient, if not more important, to support drozitumab's antitumor activity. We obtained a similar result with a DR4-agonistic antibody, suggesting a common mechanism for antibody-driven proapoptotic receptor activation. This contrasted with rituximab, which showed significantly impaired tumoricidal activity in context of inhibitory FcγRs. Hence, anti-DR5 or -DR4 agonistic antibodies can rely on interaction with either activatory and/or inhibitory FcγRs in the tumor microenvironment to drive DR5 or DR4 activation (Figure 6E). Moreover, an anti-CD40 agonistic antibody relied on FcγRIIB on B cells for stimulation of NF-κB and cytokine production. Given that CD40 lacks a death domain, this finding illustrates a common FcγR-dependent mechanism for antibody-mediated activation of diverse TNFRSF members to induce distinct signaling outcomes.

An antibody specific to murine DR5, clone MD5.1, has been used as a tumoricidal agent in several mouse models of cancer (Frew et al., 2008; Shanker et al., 2008; Smyth et al., 2006; Stagg et al., 2008; Takeda et al., 2004; Teng et al., 2007; Uno et al., 2006; van der Most et al., 2009). In contrast to drozitumab, MD5.1 appears to require aspects of innate and adaptive immunity to elicit optimal antitumor effects (Takeda et al., 2004; Uno et al., 2006). Further, the inhibitory FcγRIIB was not sufficient to support MD5.1-mediated antitumor activity in mice, and MD5.1's efficacy was dependent on immune effector function (Takeda et al., 2004). The differences between MD5.1 and drozitumab may be related to unique features of the DR5 signaling pathway in mice versus humans. A potential advantage of drozitumab's independence of adaptive immunity stems from the fact that the tumor microenvironment is often immunosuppressive (Rabinovich et al., 2007), a feature that can be exacerbated by chemotherapy (Zitvogel et al., 2008). Other agonistic antibodies to human DR5 may operate more similarly to drozitumab, given that their activity is augmented by Fc crosslinking and that they exhibit antitumor efficacy in immunodeficient mice without exogenous crosslinkers (Natoni et al., 2007; Yada et al., 2008).

Cells expressing the FcγRIIA^{131H} and FcγRIIIA^{158V} variants, which displayed higher affinity for drozitumab than the respective FcγRIIA^{131R} and FcγRIIIA^{158F} polymorphs, supported substantially greater proapoptotic activity. The FcγRIIA^{131H} and FcγRIIIA^{158V} variants have been correlated with improved effectiveness in cancer patients treated with rituximab, trastuzamab, or cetuximab (Bibeau et al., 2009; Cartron et al., 2002; Musolino et al., 2008; Weng et al., 2004; Weng and Levy, 2003; Zhang et al., 2007b). It will be important to search for similar correlations in clinical studies with DR5- and DR4-agonisic antibodies to assess whether FcγR polymorphisms affect efficacy. Furthermore, it may be possible to improve the potency of agonistic antibodies by modifying the amino acid or carbohydrate components of the Fc region to enhance affinity for FcγRs (Carter, 2006; Jefferis, 2009; Lazar et al., 2006; Presta et al., 2002; Satoh et al., 2006).

In conclusion, our studies identify an FcγR-based mechanism that drives antibody-mediated forward signaling in target cells. These findings may have implications for agonistic antibodies directed to various TNFRSF members, and perhaps more broadly, for antibody action in vivo (Andreu et al., 2010; Chan and Carter, 2010; Smith and Clatworthy, 2010; Weiner et al., 2010). Our findings may aid in the optimization of agonistic antibodies and help guide their clinical investigation.

EXPERIMENTAL PROCEDURES

Cell Lines

All cell lines were maintained in RPMI medium supplemented with L-glutamine and 10% fetal bovine serum (FBS) under conditions of 5% CO_2 at 37°C. Jurkat cells expressing full-length murine FcγRI, and HEK293 and expressing full-length mouse FcγRIIB, III, and IV, and human FcγRIIB, FcγRIIA-H131, or FcγRIIA-R131 and FcγRIIIA-V158 or FcγRIIIA-F158 variants have been previously described. In brief, cells were transfected with full-length FcγRs in pCMV.PD vector and puromycin selected. Flow cytometric sorting was used to generate single cell clones, and FcγR expression was confirmed by cell surface staining. The common γ chain was coexpressed, with the exception of cells expressing FcγRIIB.

In Vitro Cell Viability and Caspase-3 Assays

Cell viability following drozitumab treatment alone or in combination with an antihuman (Fab')2 reagent was determined using the AlamarBlue cell viability assay. Caspase-3 processing in tumor cells was monitored by flow cytometry using the cleaved caspase-3-specific antibody (clone C92-605). Colo205 tumor cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), as per the manufacturer's instructions (Invitrogen). Caspase-3 cleavage in all coculture experiments was assessed after 4 hr by gating on CFSE^{high} tumor cells. Anti-CD45 was included as an additional parameter where tumor cells were cocultured with CD45-enriched leukocytes. Where indicated, the

background level of caspase-3 activation induced by WT or DANA drozitumab alone was subtracted for clarity.

Mouse Models

Female mice (6- to 12-weeks-old) were used as indicated: C57Bl6.Rag2$^{-/-}$ and Balb/c.Rag2$^{-/-}$ mice were obtained from Taconic, Inc. Balb/c.Rag2$^{-/-}$ FcγRIII$^{-/-}$, Balb/B6 (mix).Rag2$^{-/-}$FcγRI$^{-/-}$FcγRIII$^{-/-}$, C57Bl6.Rag2$^{-/-}$FcγRIIB$^{-/-}$, Balb/c.Rag2$^{-/-}$IL2γ$^{-/-}$, and C57Bl6.Rag2$^{-/-}$IL2γ$^{-/-}$ were bred and maintained at Genentech, Inc. under specific pathogen-free conditions. Procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee, Genentech, Inc., and conform to the relevant regulatory standards.

Tumor Xenograft Models

Mice were injected subcutaneously with 5 × 10^6 cancer cells. Tumors were measured in two dimensions using a caliper. Tumor volume was calculated using the formula: V = 0.5axb^2, where a and b are the long and the short diameters of the tumor, respectively. For antitumor efficacy studies, mice bearing ~200 mm^3 tumors were randomly assigned into groups and injected intraperitoneally with drozitumab (human IgG1 (WT) or DANA IgG1 (DANA), or mouse IgG1 variant), DR4aMAb (clone 4H6, mouse IgG1), rituximab (human or mouse IgG1), or an isotype- control antibody. The DANA mutations in human IgG1 have been previously described (Shields et al., 2001).

Death-Inducing Signaling Complex (DISC), Cell Lysate, and Immunoblot Analyses

Colo205 tumors (<500 mm^3) were treated with 10 mg/kg of WT or DANA drozitumab, or an isotype control antibody. Caspase-8 recruitment and processing from total lysates or DR5 immunoprecipitated fractions were monitored by western blotting, as previously described (Adams et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.ccr.2010.11.012.

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