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Therapeutic activity of agonistic, human anti-CD40 monoclonal Abs requires selective FcγR-engagement

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Summary

While engagement of the inhibitory Fcγ-Receptor (FcγR) IIB is an absolute requirement for in vivo antitumor activity of agonistic mouse anti-CD40 monoclonal antibodies (mAbs), a similar requirement for human mAbs has been disputed. By using a mouse model humanized for its FcγRs and CD40, we revealed that FcγRIIB-engagement is essential for the activity of the human CD40 mAbs, while engagement of the activating FcγRIIA inhibits this activity. By engineering Fc variants with selective enhanced binding to FcγRIIB, but not to FcγRIIA, significantly improved antitumor immunity was observed. These findings highlight the necessity of optimizing the Fc domain for this class of therapeutic antibodies by using appropriate pre-clinical models that accurately reflect the unique affinities and cellular expression of human FcγR.

Graphical abstract

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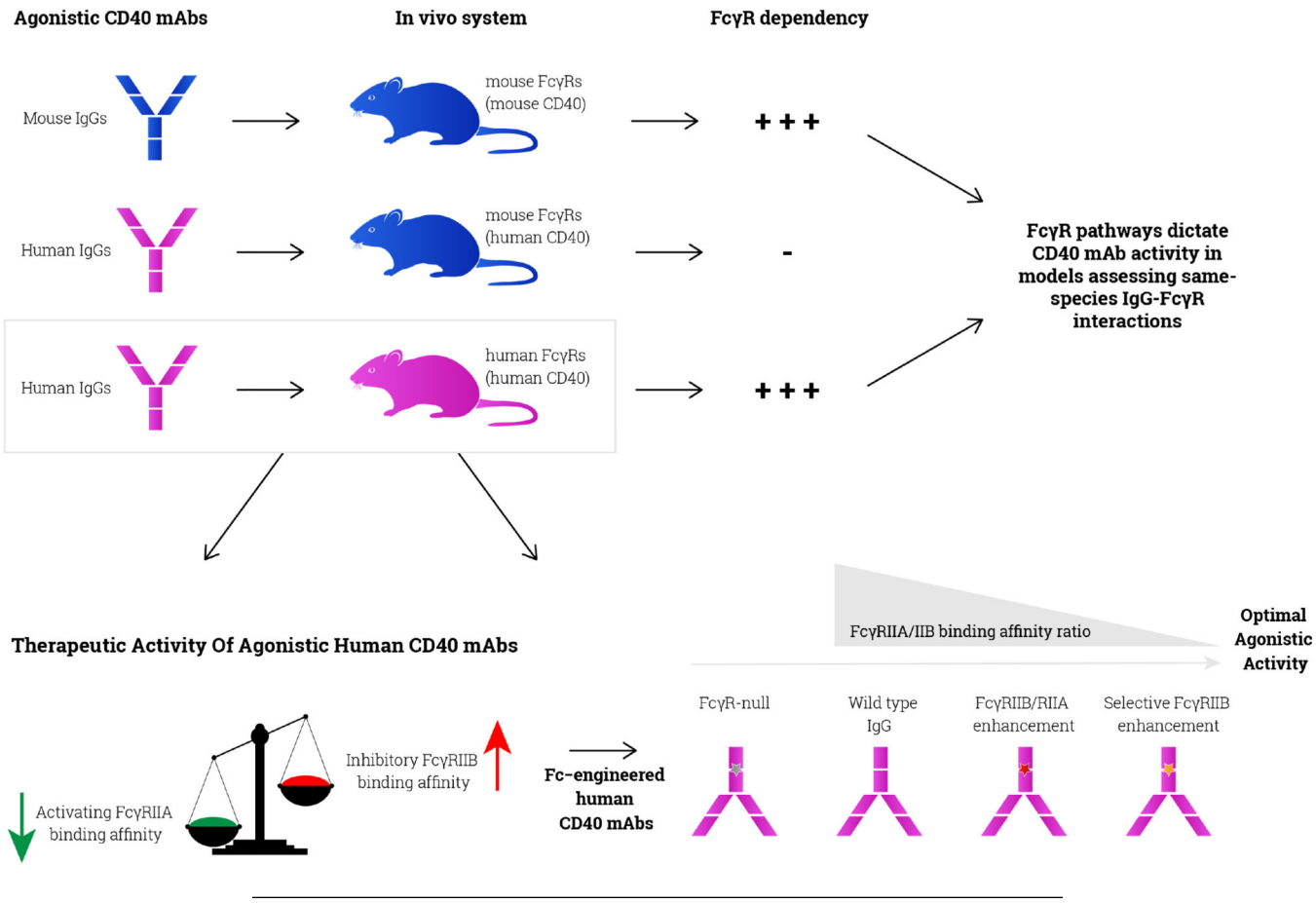

Introduction

Therapeutic targeting of CD40 by agonistic mAbs aims to activate $CD40^+$ APCs to effectively boost tumor specific cytotoxic T cells to eliminate tumor cells. In principle it has the advantage of greater tumor specificity over other T cell activation approaches, such as checkpoint inhibition or indiscriminate T cell activation. While agonistic CD40 mAbs have been developed for immunotherapy (Melero et al., 2007; Vonderheide and Glennie, 2013), their clinical efficacy has been limited (Chowdhury et al., 2014; Furman et al., 2010), suggesting that optimization is required. The in vivo activity of agonistic mAbs targeting mouse CD40 was shown to be dependent on FcγR-crosslinking by the inhibitory FcγRIIB (Li and Ravetch, 2011; Li and Ravetch, 2012; White et al., 2011). FcγRIIB functions in trans, i.e. located on neighboring cells to antibody-ligated $CD40^+$ cells, and independently of FcγRIIB signaling components (Li and Ravetch, 2013). FcγRIIB acts as a scaffold to enhance the clustering of CD40 on the membrane, thereby mimicking the effect of their endogenous multimeric ligands engaging these multimeric receptors.

There are two conserved classes of Type I FcγRs that can be distinguished functionally: the activating receptors (FcγRI, FcγRIII, and FcγRIV in mice; FcγRIA, FcγRIIA/C, and FcγRIIIA in humans) and the inhibitory receptor (FcγRIIB in both mice and humans). A third class of Type I FcγRs is uniquely found in humans: a GPI-linked receptor expressed on

neutrophils, FcγRIIIB, that functions to concentrate immune complexes on neutrophils. (For detailed reviews see (Nimmerjahn and Ravetch, 2008; Pincetic et al., 2014). FcγR-dependent responses mediated by different IgG subclasses are dependent on their differential binding affinities to activating vs. inhibitory FcγRs (Nimmerjahn and Ravetch, 2005). Mouse IgG1 has preferential binding to the inhibitory FcγRIIB and is the only subclass of mouse CD40 agonistic mAbs that is active in vivo (Li and Ravetch, 2011; White et al., 2011). The relatively high homology between mouse and human FcγR systems accounts for many of the general aspects of conserved FcγR-mediated mechanisms between the species. However, several aspects that differentiate human from mouse need to be considered when translating the FcγR-mediated mechanisms observed in the mouse system into human IgG-based therapeutics. First, there are both structural diversity and different cellular expression patterns of FcγRs between the species. Second, mouse and human IgG subclasses differ in their affinities to their cognate FcγRs.

CP-870,893, a fully human IgG2, is the most potent agonistic CD40 mAb among those tested in clinical trials to date and has demonstrated immune activation in vitro and evidence of objective tumor responses in patients (Bajor et al., 2014; Vonderheide et al., 2007). Based on previous studies using mouse antibodies in wild-type and FcγR deficient mice it was proposed that human, agonistic anti-CD40 antibodies would show a similar dependence on FcγRIIB engagement to optimize CD40 crosslinking (Li and Ravetch, 2011). However, the low affinity interactions of the IgG2 isotype of CP-870,893 with human FcγRs, including FcγRIIB (see Figure 1), raised the question of whether FcγRIIB-engagement was an absolute requirement for the activity of this mAb, and whether Fc optimization would be necessary to result in improved potency. The notion that Fc engagement might be unnecessary for this antibody was first suggested in an in vitro study demonstrating FcγRIIB-independent activity of CP-870,893 (Richman and Vonderheide, 2014). Subsequently, a recent in vivo study further supported an FcγR-independent activity of the human IgG2 subclass mediated by the unique hinge properties of this subclass (White et al., 2015). These studies, however, were limited in their ability to recapitulate the activity of CP-870,893 and other human CD40 mAbs in the model systems used. Attempts to model human IgG interactions with human FcγR-expressing cells in vitro may fail to mimic the diversity of cellular populations that may be required for an in vivo response. Thus, while in vivo assays are required, the studies reported to date were performed in either wild-type or mouse FcγR knockout strains and thus fail to address the unique binding affinities and tissue distribution of human FcγRs as compared to their mouse homologues.

Results

Generation of CD40/FcγR humanized mice

To be able to accurately and efficiently evaluate the activity of human anti-CD40 Fc variants and to enable the selection of an optimized clinical candidate, we generated an isogenic mouse model humanized for CD40 and FcγRs on a background lacking the mouse homologues of these receptors (described in detail in Experimental Procedures). First, we generated humanized CD40 mice on a mouse CD40-deficient background. We evaluated the expression pattern of the human CD40 on different immune cell populations in these mice

and found that, similar to the pattern found on human cells, human CD40 expressed on blood B cells, dendritic cells, monocytes and macrophages, but not on T cell, neutrophils, or NK cells (Figure 1A). The expression pattern of CD40 in these mice is similar to the expression of the mouse homolog in wild type mice (Figure S1A).

To verify the functionality of the human CD40 (hCD40) transgene in these mice, we evaluated the formation of germinal centers (GC), a process that requires CD40 signaling (Basso et al., 2004). While *Cd40−/−* mice lost the ability to form a GC, this phenotype is restored upon the introduction of the huCD40 transgene (Figure 1B), mediated by the interaction of the huCD40 with the mouse CD40 ligand, which have similar binding kinetics and affinity to human CD40 ligand (Figure S1B). *Cd40−/−* mice have a deficient antigen-specific IgG response upon immunization, which is restored upon introduction of the huCD40 transgene (Figure 1C), confirming that hCD40 transgene functionally complements the mouse CD40 deficiency. Together, these data demonstrate that the humanized CD40 mice recapitulate the expression pattern and function of the human CD40 gene in human. To generate a mouse model in which fully human agonistic IgGs against human CD40 can be evaluated, we crossed these CD40 humanized mice to our previously described humanized FcγR mice (DiLillo and Ravetch, 2015; Smith et al., 2012) resulting in a strain of mice expressing the human *CD40* and *FCGR1A*, *FCGR2A*^{R131}, *FCGR2B*^{I232}, *FCGR3A*^{F158}, and *FCGR3B* under the control of their endogenous human regulatory elements on an isogenic background deleted for the homologous mouse genes. These mice recapitulate hCD40 and hFcγR expression patterns and expression levels in human, and the human receptors are fully competent to signal appropriately in the mouse background.

Generation of human anti-CD40-Fc-variant clinical candidates

To test whether the in vivo activity of human IgGs targeting human CD40 requires interaction with hFcγRIIB and determine if such interactions can be further engineered to optimize the activity of the parent antibody, we cloned the variable regions of anti-CD40 CP-870,893 Ab (originally an IgG2 isotype) into Fc-modified Abs with differential capacity to engage human FcγRs. These include wild type human IgG1 and a series of mutated IgG1s with increased binding affinities to hFcγRIIB. We verified by ELISA (Figure 1D) and Surface Plasmon Resonance (SPR) (Table S1) that the different Fc domains introduced into CP-870,893 Abs did not alter either their binding specificity or their affinity to human CD40. Table S2 summarizes the affinities of different Fc variants of CP-870,893 to recombinant hFcγRI, hFcγRIIA, hFcγRIIB, and hFcγRIIIA, as evaluated by SPR.

FcγR-engagement is required for the in vivo activity of human anti-CD40 mAbs

While the IgG1 isotype has relatively high affinity interactions with all human FcγRs, the IgG2 isotype of CP-870,893 has very low binding affinities to human FcγRs, with the exception of the FcγRIIA^{H131} (Bruhns et al., 2009)(Figure 2A and Table S2). We wished to compare the efficacy of the IgG1 and IgG2 isotypes of anti-CD40 in vivo in the context of human FcγRs. Therefore, we tested their ability to activate and expand T cells in the humanized CD40/FcγR model. We delivered ovalbumin (OVA) to dendritic cells by the chimeric anti-DEC205 Ab conjugated to OVA (“DEC-OVA” (Li and Ravetch, 2011)) together with either human IgG1-Fc, IgG2-Fc, or N297A Fc-null subclasses of CP-870,893

anti-CD40 mAb, and monitored for the presence of OVA-specific T cells in the circulation after 7 days (Figure 2B). While both IgG1 and IgG2 isotypes had adjuvant effects on T cell activation, IgG1 resulted in a significantly higher T cell response as compared to the IgG2 isotype of the same anti-CD40 clone. The activity of IgG1 was completely abolished by introducing the N297A mutation, which prevents binding to FcγRs, implying that FcγR-engagement is required for the agonistic activity of the anti-CD40 IgG1. The significant activity of IgG2 was lost when tested as deglycosylated form that has reduced binding affinity to FcγRIIb compared to wild type IgG2 (Figure S2A, S2B). This implies on FcγR-dependent activity of anti-CD40 IgG2 as well and suggesting that the relatively reduced activity of IgG2 compared to IgG1 isotype may be explained by its low binding affinities to human FcγRs. In contrast to that conclusion, the agonistic activity of an anti-CD40 antibody of the IgG2 subclass has been proposed to be FcγR-independent and the result of the unique configuration of the IgG2 hinge (White et al., 2015), mediated by shuffled disulfide bonds between the IgG2 hinge and CH1 regions. To test that possibility, we mutated specific cysteines of CP-870,893 that resulted in locked conformational forms (Figure S2C)- the classical Y conformation “IgG2-A” and the more compact conformation “IgG2-B” obtained by C232S and C127S mutations, respectively (Allen et al., 2009). Both forms of IgG2 resulted in activity similar to wild type IgG2, implying that the hinge region conformation does not dictate the in vivo agonistic activity of the IgG2 isotype of this Ab clone in the context of human FcγRs (Figure 2B). Moreover, we compared the in vivo agonist activity of the IgG2-A and IgG2-B forms of CP-870,893 in human CD40 transgenic mice on either a mouse or a human FcγR background and found that in the mouse FcγR background only the IgG2-B form is active while in the human FcγR background both forms have significant and similar activity (Figure 2C). The data obtain from the CP-870,893 Abs is supported further by the similar hierarchy in agonistic activity observed for the IgG1 and the 2A and 2B forms of IgG2 subclass of ChiLob 7/4, another agonistic human CD40 Ab clone that recognizes an epitope distinct from CP-870,893. While the IgG2 subclass of ChiLob 7/4 was reported to have superior potency compared to its IgG1 form in the absence or presence of mouse FcγRs, we also observed that for this clone, in the presence of human FcγRs, the IgG1 subclass is superior to IgG2, and that both IgG2 conformational forms have similar activity (Figure 2D). As observed for CP-870,893, when tested in huCD40/mFcγR mice, only the IgG2-B form is active and results in significant enhanced activity as compared to IgG2-A (Figure 2E).

These data imply that in the physiological context of human FcγRs, agonistic, human anti-CD40 IgGs depend on FcγR-engagement but not on their hinge-conformation for their in vivo activity. Importantly, the data highlights the importance of using the humanized FcγR mouse model in order to appropriately study human IgGs activity. By considering the interaction of human IgG with human FcγRs, these mice avoid the confounding results that can be generated by using human IgGs in models carrying mouse FcγRs.

Optimized activity of anti-CD40 human IgGs achieved by Fc variants enhanced for FcγRIIB- but not FcγRIIA-binding

We next determined whether increasing the binding interactions between human anti-CD40 antibodies and hFcγRIIB will result in increased in vivo efficacy. The binding affinity and

selectivity of human IgGs to hFcγRIIB can be increased by mutagenesis of their Fc domain. Fc variants of CP-870,893 carrying the mutations S267E (SE) and S267E/L328F (SELF) (Chu et al., 2008) result in 30- and 70-fold increased binding affinity to hFcγRIIB, respectively (Figure 3A and Table S2). When administrated to the humanized FcγR/CD40 mice, these Fc variants resulted in small but significant increases in their ability to activate T cells as compared to both the wild type IgG1 and IgG2 variants of CP-870,893 (Figure 3B).

Due to the sequence and structural similarity between hFcγRIIA and hFcγRIIB, the SE and SELF mutations also result in increased binding affinity to the activating hFcγRIIA. Therefore, despite an increase in their binding affinity to the inhibitory hFcγRIIB, the FcγRIIA/FcγRIIB binding affinity ratio of these mutated IgGs is similar to that of wild type IgG1 and were thus predicted to result in limited increased activity of this subclass as was observed (Figure 3B). To optimize the Fc-engagement of FcγRIIB over FcγRIIA, we generated Fc variants of CP-870,893 with the recently described mutations, G237D/P238D/P271G/A330R (V9) and G237D/P238D/H268D/P271G/A330R (V11), which enhance binding affinity specifically to hFcγRIIB but not to hFcγRIIA (Mimoto et al., 2013). V9-CP-870,893 and V11-CP-870,893 Fc variants result in 32- and 97-fold increased binding affinity to hFcγRIIB, respectively, and about 3-fold decreased binding affinity to $hFc\gamma RIIA^{R131}$ (Figure 3A and Table S2). Both V9 and V11 Fc variants have significantly improved in vivo activity compared to the IgG2 subclass of CP-870,893 (IgG2), and its SE-, and SELF-Fc variants enhanced for binding of both hFcγRIIB and hFcγRIIA. The CP-870,893-V11 variant results in 25-fold increase in T cell activation compared to CP-870,893-IgG2, and 5-fold increase compared to the activity obtained by the SELF variant (Figure 3B). A similar hierarchy was observed for CP-870,893 Fc variants when change in body weight was determined after antibody administration (Figure S3A). While all Fc variants tested resulted in statistically significant decreases in body weight after a single injection of CP-870,893, the group injected with the V11-Fc had the most significant reduction.

We analyzed the pharmacokinetic (PK) properties of these Fc variants to test whether their differential FcγR binding leads to differential PK clearance rates that can account for their differential agonistic activities. SELF and V11 Fc variants of CP-870,893 were found to have a faster clearance rate than the IgG2 subclass (Figure S3B), presumably a result of their enhanced FcγRIIB binding activity. However, despite the fact that SELF and V11 have faster clearance rates, they display superior agonistic activity compared to IgG2. Similarly, despite the similar PK properties of SELF and V11, V11 is a superior agonist compared to SELF. We can therefore exclude the possibility that different PK properties of these Fc variants account for their agonistic activity.

To further evaluate the influence of hFcγRIIA-engagement on the activity of CP-870,893, we compared its activity in mice transgenic for human CD40 and FcγRIIB, but not for FcγRIIA or mice transgenic to human CD40, FcγRIIB, and FcγRIIA (Figure 3C). The T cell activation potency of CP-870,893-IgG2 is significantly enhanced in the absence of FcγRIIA, indicating the negative role of FcγRIIA-engagement on the agonistic activity of this anti-CD40 mAb.

Taken together, our data demonstrate that engineering CP-890,873 for enhanced hFcγRIIB-engagement while keeping a low FcγRIIA/FcγRIIB binding ratio results in optimized in vivo agonistic activity of human anti-CD40 IgGs.

Fc-optimized anti-CD40 IgGs can increase agonistic activity without enhancing platelet activation related toxicity

Human platelets express both CD40 and FcγRIIA (the only FcγR expressed on platelets). Both CD40 ligation (Inwald et al., 2003) and FcγRIIA crosslinking (Meyer et al., 2009; Robles-Carrillo et al., 2010) on platelets can result in their activation. Since a transient decrease in platelet count was reported in clinical trials after administration of CP-870,893-IgG2 (Ruter et al., 2010; Vonderheide et al., 2013), we sought to compare platelet counts in response to administration of the different Fc variants in the humanized CD40/FcγR mice (Figure 3D). We observed a statistically significant decrease in platelet count 24 hours after injection for all the tested CP-870,893 Fc variants, while the magnitude of the decrease for each Fc variant directly correlated with its agonistic potency. Thus, the decrease of platelet count is dependent on CD40- and not FcγRIIA- expression on platelets and requires FcγRIIB-crosslinking. An exception was observed for the IgG1 subclass, which results in the greatest degree of thrombocytopenia, similar to the levels observed for V9 and V11 variants. This is despite the relatively low agonistic activity of this clone compared to the other tested Fc variants. IgG1 is the only tested subclass that significantly engages activating FcγRIIIA and therefore presumably mediates direct platelet depletion through phagocytosis and not through FcγRIIB-dependent agonism. Similar to what was reported in the clinical studies, the decrease in platelet count observed in our humanized mouse model was also transient with a return to baseline 7 days after the mAb injections (Figure S3C).

To test whether increasing the agonistic activity mediated by the V11-Fc will be accompanied by enhanced thrombocytopenia, we reduced the dose of the injected CP-870,893-V11 to a level that resulted in similar platelet counts for that Fc variant as to that of the unmodified IgG2 version (Figure S3D). We found that reducing the dose of V11 by 2.5-fold resulted in a comparable level of thrombocytopenia to that of IgG2 and resulted in similar levels of platelet depletion for the two antibodies. However, at this reduced dose of the V11 variant we still observe significantly improved agonistic activity over IgG2 in vivo. At a five-fold reduced dose of V11 no decrease in platelet counts were observed while agonistic activity was maintained at a level comparable to that of human IgG2 (Figure S3D). Altogether, these data imply that modifying CP-870,893 into V11-Fc can result in improved therapeutic efficacy without increasing its related toxicity.

Multiple agonistic, anti-CD40 antibodies demonstrate FcγRIIB-mediated enhancement, regardless of the epitopes they recognize

We sought to test if this hierarchy in the in vivo agonistic activity between the FcγRIIB-enhanced Fc variants can be applied to additional agonistic anti-CD40 mAbs. Previous studies have suggested that certain epitopes recognized by agonistic anti-CD40 antibodies may display FcγR-independent activity and that such epitope-dependent distinction of their FcγR-requirement is determined by their ability to compete with CD40L binding (Richman and Vondeheide, 2014). We therefore evaluated two recently developed human anti-CD40

mAbs, clones CD40.1 and CD40.2 that are distinguished in their ability to block CD40L binding to hCD40. Antibodies CD40.1 and CD40.2 were developed from standard immunization methods in mice by immunization with recombinant CD40 or CD40 expressing cells followed by screening, resulting in antibodies binding to CD40, and functional activation of B and DC cells. CD40.1 and CD40.2 bind to a monomer of CD40 with a KD of 4.8 nM and 9.1 nM in SPR experiments, respectively. CD40.2 recognizes epitopes overlapping with the CD40L binding site whereas CD40.1 binds hCD40 at an epitope distinct from its CD40L binding site, similar to CP-870,893 (Figures 4A and 4B).

In vitro DC activation assays demonstrated significant activity for the SE-, and V11-Fc variants of CD40.1 clone while CD40.1 Fc variants exhibit enhanced activity compared to CD40.2 variants (Figure S4A, S4B). Although the in vitro activity of the CD40.1-Fc variants is greater than the wild type IgG1 (Figure S4B), the in vitro studies do not distinguish between the activity of the different Fc variants. We therefore evaluated the activity of these antibodies and their Fc variants in the humanized CD40/FcγR mice. As was observed for clone CP-870,893, the selective FcγRIIB-enhanced V11 Fc variant of these mAbs displayed superior activity in vivo compared to its FcγRIIA/IIB-enhanced SE variant and the wild type IgG1 subclass (Figure 4C). Moreover, the magnitude of the in vivo activity of the CD40.1-V11 is about 20-fold higher than the activity of CP-870,893 (IgG2) (Figure S4C). Similar to CP-870,893 derivatives, CD40.1 Fc variants exhibit a direct correlation between their agonistic potency and the magnitude of the decrease in platelet count (Figure 4D), demonstrating the generality of these observations.

These data demonstrate that selective enhancement of FcγRIIB-binding increases the in vivo activity of multiple anti-CD40 mAbs and that in vitro assays are not predictive of the in vivo activity of these Fc variants. Moreover, increased immunostimulatory activity by selective enhancement of FcγRIIB binding was demonstrated for CP-870,893 and CD40.1 mAbs that do not block the binding of hCD40 to CD40L, and for the CD40L blocking anti-CD40 mAb CD40.2, demonstrating that agonist human anti-CD40 mAbs can be optimized by selective enhancement of FcγRIIB-binding through Fc engineering independent of their binding epitopes and their ability to cross block CD40L binding to CD40.

V11 Fc variant of anti-CD40 Abs has superior anti-tumor activity

We next evaluated whether the increased agonistic activity observed for the FcγRIIB-enhanced mutated Fc variants can be translated into increased anti-tumor activity of anti-CD40 mAbs. Humanized CD40/FcγR mice were inoculated with the syngeneic MC38 colon adenocarcinoma tumors and treated with IgG2-, SELF-, and V11- Fc variants of CP-870,893 (Figure 5A). Treatments with both IgG2 and SELF result in similar antitumor effects (about 65% reduction in tumor volume compared to untreated control and 20% to 33% of tumor free mice, respectively). However, treatment with the V11 Fc variant results in a dramatically improved antitumor response and complete recovery from tumors of all the mice. A similar trend was observed using the B16 metastatic melanoma model in which a statistically significant reduction in the number of lung metastases was observed only in mice treated with V11, but not with SELF or IgG2 Fc variants (Figures 5B, S5). Next we evaluated the anti tumor activity of CD40.1 and CD40.2. Wild type IgG1, SE and V11

mutants of each clone were tested for their activity in the MC38 tumor model (Figure 5C). The FcγRIIB-selectively enhanced V11 variants of these Abs have superior anti tumor activity compared to the other tested Fc variants, consistent with their in vivo T cell stimulation activity and with the anti-tumor hierarchy observed for CP-870,893 derivatives. We did not observe the clearance of circulating dendritic cells after treatment with CD40.1 IgG1 (data not shown), indicating that its diminished activity relative to V11 or SE variants is due to its reduced binding affinity to FcγRIIB and not a result of activating FcγR-mediated elimination of $CD40^+$ cells. Nevertheless, introduction of agonistic CD40 Abs as an IgG1 subclass may result in depletion of dendritic cells and other $CD40^+$ target cells, depending on the dosing and the epitope-specificity of each Ab clone.

Tumor-free mice from the CD40.1-V11 treated group were re-challenged with MC38 tumor cells 30 days after their CD40 Ab treatment. All mice in this group rejected the tumors within two weeks of the re-challenge, implying a long-term protection that is mediated by single injection of CD40-V11 (Figure 5D).

These data indicate that the antitumor activity of agonistic human CD40 Abs can be enhanced by Fc engineering that provides selective enhancement of FcγRIIB-engagement and highlight the V11 Fc variant as the optimal clinical platform of this class of Abs.

Discussion

The requirements for FcγR interactions for the therapeutic activity of an increasing number of immunomodulatory mAbs (Bulliard et al., 2013; Bulliard et al., 2014; Dahan et al., 2015; Selby et al., 2013; Simpson et al., 2013) have emphasized the need to optimize these reagents by proper selection of the Fc domain. The importance of FcγRIIB-engagement for the activity of the CD40 mAb has been characterized in details using mouse FcγR model systems (Li and Ravetch, 2011; Li and Ravetch, 2012; Li and Ravetch, 2013; White et al., 2011), but their translational relevance into human therapeutics has been disputed. Here we demonstrate the importance of FcγR interactions for human CD40 mAb and how selective manipulation of FcγR-interactions by Fc-engineering enhances the potency of these mAbs. These conclusions were made possible through the use of a representative in vivo model that faithfully recapitulates the diversity and cell type specificity of the human FcγR system. Such in vivo models have recently been demonstrated to be essential to evaluating the FcγR-mediated activities of human IgGs in other applications (DiLillo and Ravetch, 2015; Schwab et al., 2015; Stylianos et al., 2014).

The gap in our knowledge regarding FcγR-requirements for the activity of human CD40 mAbs in clinical development was due to the lack of an appreciation of the species differences between mice and humans and the use of inappropriate pre-clinical models to evaluate interactions between human Abs and FcγRs. By using CP-870,893 and its derivatives in the humanized CD40/FcγR mice, we observed not only a lack of activity of the Fc null variant but also an increased potency of selective FcγRIIB-enhanced Fc variants. These findings were confirmed using additional CD40 agonistic mAbs, demonstrating that mice humanized for FcγRs are an important model to evaluate Fc-FcγR interactions and their effect on the activity of human IgGs. In vitro studies have failed to reflect the

complexity and diversity of hFcγRs in vivo and led to inappropriate conclusions regarding FcγR-requirement for CP-870,893 activity (Richman and Vonderheide, 2014). Our study also refutes the notion that the epitope specificity of agonistic CD40 mAb determines its FcγR-requirements (FcγR-independent vs, -dependent, respectively) for activity. CP-870,893 and CD40.1 do not compete with CD40L binding but proved to be FcγR-dependent for their optimal activity in vivo. Moreover, we generated a human mAb, CD40.2, which is similar to the murine Abs that block CD40L binding to CD40, and demonstrated that the activity of this mAb is also optimized by FcγRIIB-engagement.

Different modes-of-action can be observed between different mAbs although they share the same target molecule. For example, we have recently observed that antagonistic PD-1 mAbs have the potential to deliver FcγRIIB-dependent agonism based on their epitope specificity (Dahan et al., 2015b). Although mouse models can be very informative for evaluating mAb activity, translating mAb activity in the mouse to human therapeutic is not straightforward and therapeutic mechanisms observed for a mouse mAb can be altered while developing the homologous human IgGs. By humanizing both CD40 and FcγRs, we generated a mouse model that enables in vivo evaluation of clinical products by considering the activity mediated by both their Fab and Fc domains. These mice allow for “lead” selection among a panel of human CD40 mAbs based on their in vivo agonistic potency. A similar approach should be used for the generation of mice humanized for other therapeutic targets on the huFcγR background for optimal selection of additional immunomodulatory mAbs.

The humanized CD40/FcγR mice used in this study are a significant improvement over previous models used to characterize the activity of human CD40 Abs. Nevertheless, the presence of murine IgG in the serum of these mice may lead to unnecessary complications mediated by the interaction of human FcγRs with the endogenous murine IgGs. However, the binding of human FcγRs to mouse IgGs are significantly lower than to their cognate human IgGs (Ravetch and Nimmerjahn, 2008) and are not expected to confound the interpretation. To address this potential complication we are working on incorporating human IgG Fc in place of the mouse locus in order to evaluate the activity of human Abs in the context of human serum IgG antibodies. In addition, this model will allow to test long term chronic administration of hAbs by providing tolerance to human IgGs.

Although improved agonistic activity is mediated by Fc variants enhanced for both FcγRIIA and FcγRIIB binding, their potency is limited by their enhanced binding to the activating FcγRIIA. Therefore, Fc variants with selective enhancement in binding solely to the inhibitory FcγRIIB have been indicated by this study as the most potent CD40 mAb derivatives. Lack of activity of mouse CD40 mAbs carrying the IgG2a subclass that preferentially binds activating FcγRs is associated with depletion of CD40 expressing cells (Li and Ravetch, 2011). Since human FcγRIIIA, but not FcγRIIA, mediates in vivo depletion by mAbs (DiLillo and Ravetch, 2015), and the S267E or S267E/L328F mutants are enhanced for FcγRIIA but not FcγRIIIA affinity, we speculate that the reduced potency of the CD40 mAb mediated by FcγRIIA-engagement is not the result of depletion of CD40 expressing cells. The mechanism that accounts for this inhibitory effect by FcγRIIA is the subject of our ongoing studies to determine if the signaling activity of FcγRIIA is required

for this inhibitory effect or if the reduced agonistic activity results from a signaling independent mechanism.

Histidine (H)/arginine (R) polymorphism at position 131 of FcγRIIA dictates its binding affinity to IgG2, FcγRIIa^{H131} has about 5-times higher affinity to IgG2 than FcγRIIa^{R131} (van Sorge et al., 2003). Due to the inhibitory effect of FcγRIIA on the activity of CP-870,893, patients carrying the FcγRIIA$^{131H/H}$ genotype may have a reduced response to CP-870,893 treatment. Our humanized mice carry the FcγRIIA$^{131R/R}$ genotype but an FcγRIIA$^{131H/H}$ strain is being generated so that the contribution of FcγRIIA allele polymorphism to the activity of anti-CD40 mAbs can be elucidated.

The unique hinge conformation of the human IgG2 isotype has recently reported to enhance the agonistic activity of CD40 mAbs in an FcγR-independent manner. It was therefore suggested that the superior agonistic activity observed for CP-870,893 is due to its IgG2 isotype compared the IgG1 isotype of the other agonistic CD40 Abs in clinical evaluations, ChiLob 7/4 and SGN40. When ChiLob 7/4 and SGN40 were generated as IgG2, they resulted in enhanced potency compared to their original IgG1 isotype (White et al., 2015). A drawback of that study is that the mAbs were evaluated only in the presence (or absence) of mouse FcγRs and their isotype-dependent potency in the correct context of human FcγRs was not evaluated. Here we demonstrated that using ChiLob 7/4 as IgG2 results in reduced activity compared to IgG1 in the context of human FcγRs. We further evaluated the activity of IgG1 vs IgG2 subclasses, including the 2A and 2B forms of IgG2, of both CP-870,893 and ChiLob 7/4 and found that IgG1 is more potent than IgG2 and its activity is FcγR-dependent. We therefore conclude that the superior agonistic activity of anti-CD40 human IgG2 observed in mice is not relevant to its clinical activity in humans. Moreover, the relatively high potency of CP-870,893 compared to the other CD40 mAbs is not a result to the IgG2 isotype and is likely the result of the mAb recognition of a unique specific agonistic epitope. Finally, selective enhancement for FcγRIIb-binding is by far the most efficient strategy to enhance the potency of CD40 mAb agonism.

Experimental Procedures

See also in Extended Experimental Procedures.

Mice

FcγRα^{null} mice have FcγR α chain deletion of *Fcgr2b*, *Fcgr3*, and *Fcgr4*, and are crossed to FcγRI$^{-/-}$ mice (Barnes et al., 2002). They were generated in a C57BL/6 background and characterized as previously described (Smith et al., 2012). FcγR humanized mice (FcγRα^{null}, hFcγRI^{+}, FcγRIIa^{R131+}, FcγRIIb^{+}, FcγRIIIa^{F158+}, and FcγRIIIb^{+}) generated and extensively characterized as previously described (Smith et al., 2012). Human CD40 transgenic mice were generated on a C57BL/6 genetic background by pronuclear injection of linearized RP11-177B15 bacterial artificial chromosome DNA (Osoegawa et al., 2001) and were mated with CD40-knockout ("*Cd40*$^{-/-}$") mice (The Jackson Laboratory) to obtain *Cd40*$^{-/-}$huCD40$^{+/+}$ mice. *Cd40*$^{-/-}$huCD40$^{+/+}$ mice were mated with FcγR humanized mice to obtain the humanized CD40 and FcγR mice (referred "hCD40/FcγR"). hCD40/hFcRIIB^{+}/

hFcRIIA$^+$ and hCD40/hFcRIIB$^+$/hFcRIIA$^-$ mice described in Figure 3D were obtained during the mating described for the generation of hCD40/FcγR mice.

All mice were maintained in The Rockefeller University Comparative Bioscience Center. All experiments were performed in compliance with federal laws and institutional guidelines and had been approved by the Rockefeller University IACUC.

OVA-specific T cell response

Mice were immunized through i.p. injection with 2 μg of DEC-OVA(mIgG1-D265A) (produced as previously described by (Li and Ravetch, 2011) in the presence or absence of 10 μg of anti-CD40 IgGs (except of ChiLob IgGs that were used at 40 μg/mice) with one of the various Fc's. Seven days later peripheral blood was collected and stained with FITC-conjugated anti-CD4, APC-conjugated anti-CD8α and PE-conjugated OVA peptide SIINFEKL H-2^b tetramer (tet-OVA, Beckman Coulter) and analyzed on BD LSRForttesa.

Flow cytometry

Cell populations were defined by the following markers; DC (human: HLA-DR$^+$BDCA1$^+$CD209$^+$CD3$^-$CD14$^-$CD19$^-$CD59$^-$; mouse: CD11b$^+$CD11c$^+$MHC II$^+$F4/80$^-$), monocytes (human: CD14$^+$HLA-DR$^+$CD15$^-$; mouse: CD11b$^+$Ly6C$^+$F4/80$^-$CD11c$^-$), macrophages (human: CD14$^+$CD68$^+$; mouse: CD11b$^+$F4/80$^+$Ly6C$^-$Ly6G$^-$), B cells (human: CD19$^+$; mouse: B220$^+$), T cells (human: CD3$^+$CD56$^-$; mouse: CD3), NK cells (human: CD16$^+$CD56$^+$CD3$^-$; mouse: NK1.1), neutrophils (human: CD15$^+$CD16$^+$CD49d$^-$; mouse: CD11b$^+$Ly6G$^+$Ly6C^{int}F4/80$^-$)

H1N1 immunization

Mice were immunized with recombinant influenza H1N1 (Sino Biological Inc.) in the presence of Alum as adjuvant. After 11 days blood was collected and analyzed for anti-influenza H1N1-specific IgG using standard ELISA protocol.

SPR

All experiments were performed with a Biacore T100 surface plasmon resonance (SPR) system (Biacore, GE Healthcare), as previously described(Bournazos et al., 2014). Briefly, experiments were performed at 25 °C in HBS-EP buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 3.4 mM EDTA; 0.005% (v/v) surfactant P20). For the measurement of the affinity of IgG subclass variants for FcγRs and CD40 recombinant IgGs were immobilized on Series S CM5 chips by amine coupling and soluble ectodomains of FcγRs or CD40 samples were injected through flow cells at different concentrations. For some FcγRs, the measurements were repeated in a reversed orientation while immobilizing the FcγR and injecting soluble IgGs. Background binding to blank immobilized flow cells was subtracted and affinity constants were calculated using BIAcore T100 evaluation software (GE Healthcare) using the 1:1 Langmuir binding model.

SPR-based competition assay

SPR competition experiments were performed on a Biacore T100 instrument using a running buffer of 10 mM sodium phosphate, 130 mM sodium chloride, 0.05% Tween 20, pH

7.1 at 25°C, on a surface consisting of hCD40-Fc immobilized on a CM5 sensor chip using standard amine coupling chemistry. Competition for binding to hCD40L-Fc was assessed using the “dual injection” function in the T100 control software, by injecting molecule 1 (parental antibody or CD40L), immediately followed by the same concentration of molecule 1, or a mixture of molecule 1 plus molecule 2. Binding responses were compared to a control injection of molecule 2 alone. All experiments were performed using 180 s association and dissociation times at 30 μl/min. The surface was successfully regenerated between cycles using two 15s pulses of 10 mM glycine pH 1.5 at a flow rate of 30 μl/min.

CD40 binding ELISA

Binding specificity and affinity of IgG subclasses were determined by ELISA using recombinant CD40 (Sino Biological Inc.). ELISA plates (Nunc) were coated overnight at 4C with recombinant extracellular domain of human CD40 (1 μg/well). All sequential steps were performed at room temperature. After being washed, the plates were blocked for 1 hr with PBS/2% skim milk and were subsequently incubated for 1 h with serially diluted IgGs (1:3 consecutive dilutions in PBS/2% skim milk). After washing, plates were incubated for 1 hr with HRP-conjugated anti-human IgG (Jackson ImmunoResearch). Detection was performed using two-component peroxidase substrate kit (KPL) and reactions stopped with the addition of 1 M phosphoric acid. Absorbance at 405nm was immediately recorded using a SpectraMax Plus spectrophotometer (Molecular Devices) and background absorbance from negative control samples was subtracted.

Generation of anti-CD40 Fc variants

Anti human CD40 Ab CP-870,893 is clone 21.4.1 mentioned in patent US7338660 (ATCC accession number PTA-3605). The variable heavy and light regions of CP-870,893 were synthesized (Genewiz) and cloned into mammalian expression vectors with human IgG1, human IgG2, or human kappa Fc backbones, as previously described (Li and Ravetch, 2011). For the generation of Fc domain variants of human IgG1 (N297A, S267E, S267E/L328F, G237D/P238D/P271G /A330R, G237D/P238D/H268D/P271G/A330R) and human IgG2 (C127S, C232S), site-directed mutagenesis using specific primers was performed based on the QuikChange site-directed mutagenesis Kit II (Agilent Technologies) according to manufacturer’s instructions. Mutated plasmid sequences were validated by direct sequencing (Genewiz).

Antibodies were generated by transient transfection of HEK293T cells (ATCC), purified using Protein G Sepharose 4 Fast Flow (GE Healthcare), dialyzed in PBS, and sterile filtered (0.22 mm), as previously described (Nimmerjahn et al., 2005). Purity was assessed by SDS-PAGE and Coomassie staining, and was estimated to be >90%. Abs used for in vivo experiments were quantified for endotoxin (LPS) contamination by the Limulus Amebocyte Lysate (LAL) assay and verified to have levels < 0.1 EU/μg. Polyclonal human IgG was were purchased from Bio X Cell.

Tumor challenge and treatment

MC38 cells (2×10^6) were implanted subcutaneously and tumor volumes were measured every 2–3 days with an electronic caliper and reported as volume using the formula (L_1^2 x

L_2)/2, whereas L_1 is the shortest diameter and L_2 is the longest diameter. 7 days after tumor inoculation, mice were randomized by tumor size (day 0) and received intraperitoneal (i.p) injection of 200μg (CP-870,893) or 50μg (CD40.1 and CD40.2) anti-CD40 or control IgGs. For the experiment shown for CP-870,893 Abs, mice received an additional 200 μg of IgG treatment at days 3. Mice were followed for 20–24 days after treatment onset until the majority of the untreated control group had to be sacrificed due to Rockefeller University IACUC limitation for tumor size. For the B16 lung metastasis model, mice were injected intravenously with 1×10^6 B16-F10 cells and treated with 40μg of the indicated Abs on days 1 and 4 after tumor cell injection. On day 14 lungs were harvested and analyzed for the presence of surface metastasis foci by using a dissecting microscope.

Statistical Analysis

One-way ANOVA with Tukey post-test was used to compare all groups to control groups in tumor growth experiments. Unpaired two-tailed t test was used when two groups were compared and to compare groups in experiments assessing cells types percentages. Data were analyzed with Graphpad Prism software (Graphpad) and p values of < 0.05 were considered to be statistically significant and indicated as *p≤0.05; **p≤0.01; ***p≤0.001; ns, non-significant. Asterisks indicate statistical comparison to the control group unless indicated otherwise on the graphs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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White AL, Chan HT, Roghanian A, French RR, Mockridge CI, Tutt AL, Dixon SV, Ajona D, Verbeek JS, Al-Shamkhani A, et al. Interaction with FcgammaRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. Journal of immunology. 2011; 187:1754–1763.

Highlights

- Wild type mice fail to model the activity of human CD40 IgG subclasses
- Humanized FcγR/CD40 mice enabled assessment of hFcR contribution to CD40 mAb activity
- Binding affinity to FcγRIIB dictates the agonistic activity of human CD40 IgGs
- CD40 mAbs enhanced for FcγRIIB, but not FcγRIIA, have optimal anti tumor activity

Dahan et al. generate a mouse model fully humanized for FcγRs and CD40. Using this model, they discover that FcγRIIB-engagement is essential for the antitumor activity of human CD40 agonistic monoclonal antibodies (mAbs), whereas FcγRIIA-engagement is inhibitory. They use this knowledge to improve these mAbs.

Significance

The use of immunomodulatory mAbs to stimulate effective anticancer immunity has shown clinical successes for treatment of various cancer types. An approach currently under clinical development uses agonistic anti-CD40 mAbs to induce antigen presenting cells (APC) activation, thereby harnessing the potency of the immune response to eradicate tumors. However, the current mAbs under development have not been optimized for FcγR-engagement to implement the full potential of these mAbs. Here we describe a humanized mouse model that evaluates human anti-CD40 mAbs and enables the selection of optimized next-generation Fc-engineered human mAbs that can be readily translatable to clinical trials. Our studies also provide a broad rationale for the development path of therapeutic human antibodies based on their requirement for selective FcγR-interactions.

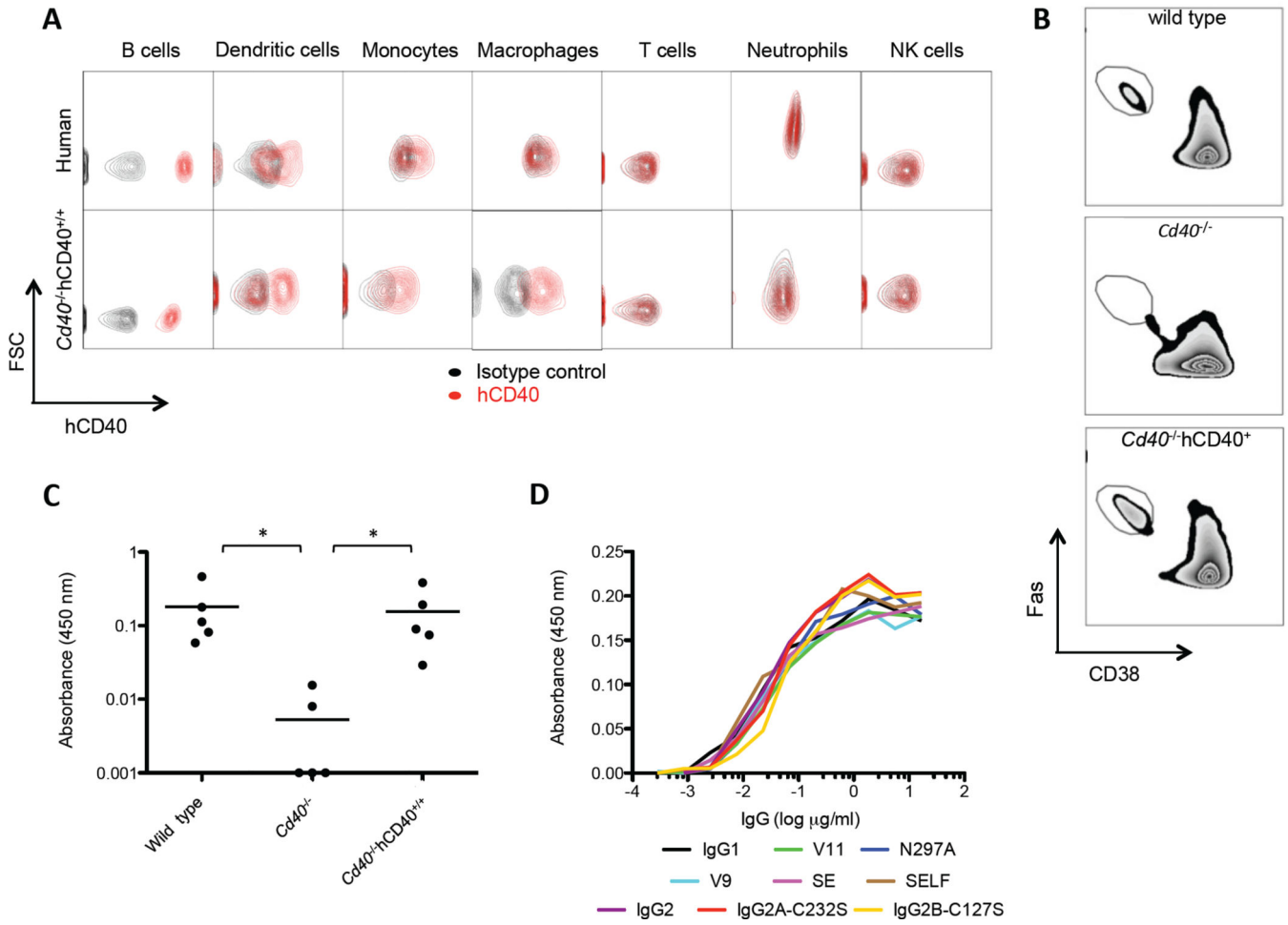

Figure 1. Characterization of CD40/FcγR humanized mice and anti-CD40 Fc variants

(A) Representative flow cytometry staining of hCD40 expression on the indicated cell populations in blood from human and *Cd40*−/−hu*CD40*$^{+/+}$ mice.

(B) Representative flow cytometry staining of GC B cells from mesenteric lymph nodes of the indicated mice. Percentages of GC B cells (CD38^{-}Fas^{hi}) from total live B220^{+} cells in wild type (1.61%), *Cd40*−/− (0.36%), and *Cd40*−/−h*CD40*$^{+/+}$ (2.19%) mice.

(C) ELISA for detection of serum levels of influenza H1N1-specific IgG from mice immunized with recombinant influenza H1N1. Each dot represents individual mouse. Horizontal lines indicate mean.

(D) Binding specificity and affinity of the indicated Fc variants of CP-870,893 anti-CD40 Ab assessed by ELISA using recombinant hCD40. Data are represented as means.

See also Figure S1 and Tables S1 and S2.

A

Subclass	Inhibitory	Activating		
	FcγRIIB	FcγRI	FcγRIIA	FcγRIIIA
IgG1	++	+++++	++	++
N297A	-	-	-	-
IgG2	+	-	+	+

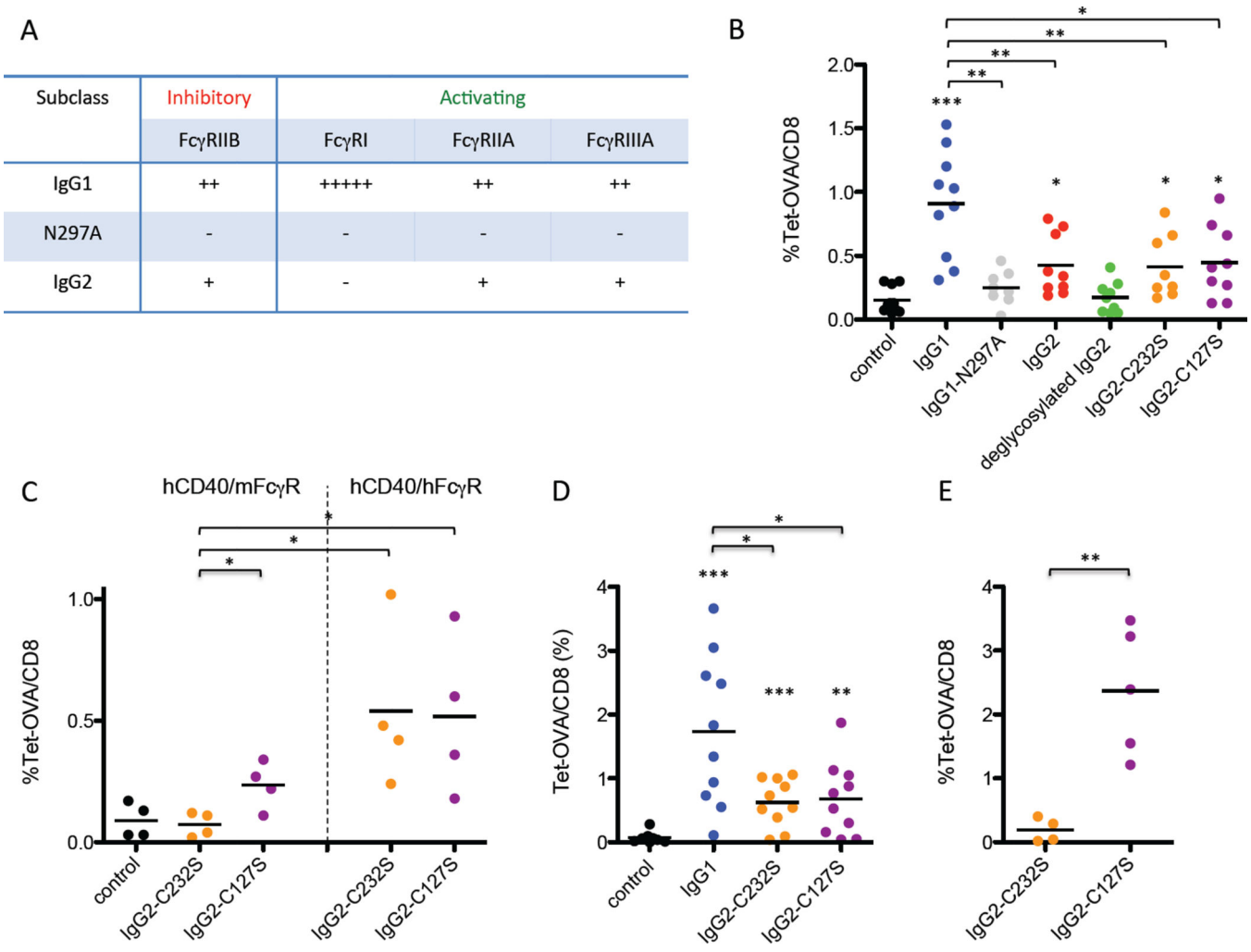

Figure 2. Human CD40 mAbs requires FcγR-engagment for in vivo activity

(A) FcγRs binding profile of CP-870,893 anti-CD40 Fc variants. Based on SPR measurements.

(B) T cell activation assay determined by flow cytometry analysis for OVA-specific CD8+ T cells in the blood of humanized CD40/FcγR mice immunized with DEC-OVA in the presence or absence of the indicated CP-890,873 anti-CD40 Fc variants. Each dot represents individual mouse. Horizontal lines indicate mean.

(c) T cell activation assay as described in (B) was performed for the indicated IgG2 mutants of CP-870,893 Ab in hCD40/mFcγR or hCD40/hFcγR mice. Each dot represents individual mouse. Horizontal lines indicate mean.

(D) T cell activation assay as described in (B) was performed for the indicated ChiLob 7/4-IgGs in hCD40/hFcγR mice. Each dot represents individual mouse. Horizontal lines indicate mean.

(E) T cell activation assay as described in (B) was performed for the indicated IgG2 mutants of ChiLob 7/4 Ab in hCD40/mFcγR mice. Each dot represents individual mouse. Horizontal lines indicate mean.

See also figure S2

A

Variant	FcγRIIB -fold	IIA/IIB
IgG1	1	2.7
SELF	70	2.4
SE	30	0.9
V9	32	0.02
V11	96	0.01

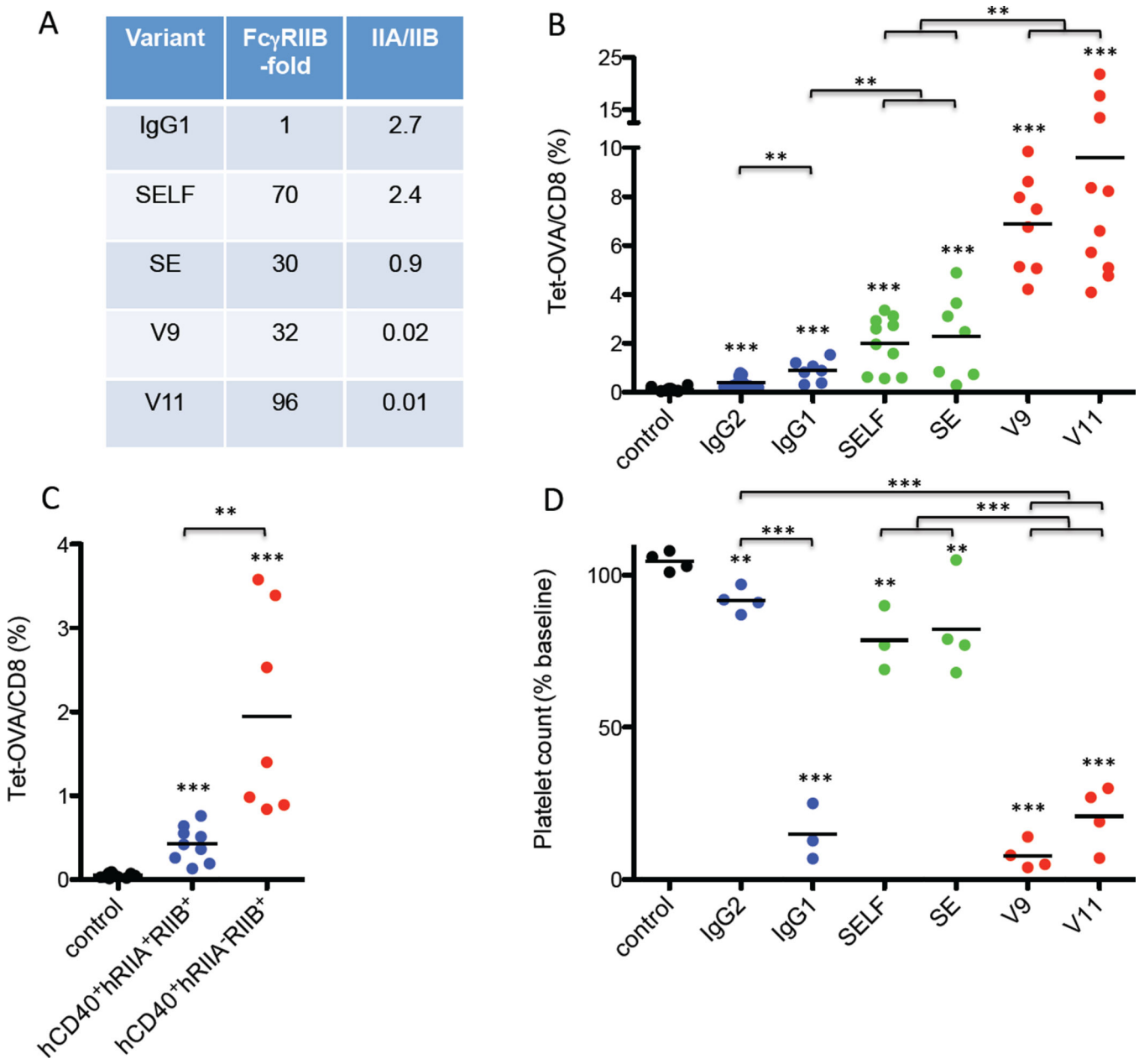

Figure 3. Increased activity of CP-870,893 by Fc-engineering for FcγRIIB specific enhancement

(A) Fold-change in hFcγRIIB and hFcγRIIB/hFcγRIIA^{R131} binding affinities of CP-870,893 anti-CD40 Fc variants. Based on SPR measurements.

(B) Flow cytometry analysis for OVA-specific CD8$^+$ T cells in the blood of huCD40/FcγR mice immunized with DEC-OVA in the presence or absence of the indicated CP-870,893 anti-CD40 Fc variants. Each dot represents individual mouse. Horizontal lines indicate mean. Wild type IgGs are in blue, FcγRIIA/B-enhanced IgGs are in green, selective FcγRIIB-enhaced IgGs are in red.

(c) hCD40$^+$/hFcγRIIA$^+$/hFcγRIIB$^+$ or hCD40$^+$/hFcγRIIA$^-$/hFcγRIIB$^+$ mice were immunized with DEC-OVA in the presence of CP-870,893-IgG2 and analyzed for the

percentages of OVA-specific $CD8^+$ T cells in the blood at day 7. Each dot represents individual mouse. Horizontal lines indicate mean.

(D) Platelet counts in blood 24 hours after administration of CP-870,893 anti-CD40 Fc variants into humanized CD40/FcγR mice. Each dot represents individual mouse. Horizontal lines indicate mean. Wild type IgGs are in blue, FcγRIIA/B-enhanced IgGs are in green, selective FcγRIIB-enhaced IgGs are in red.

See also Figure S3.

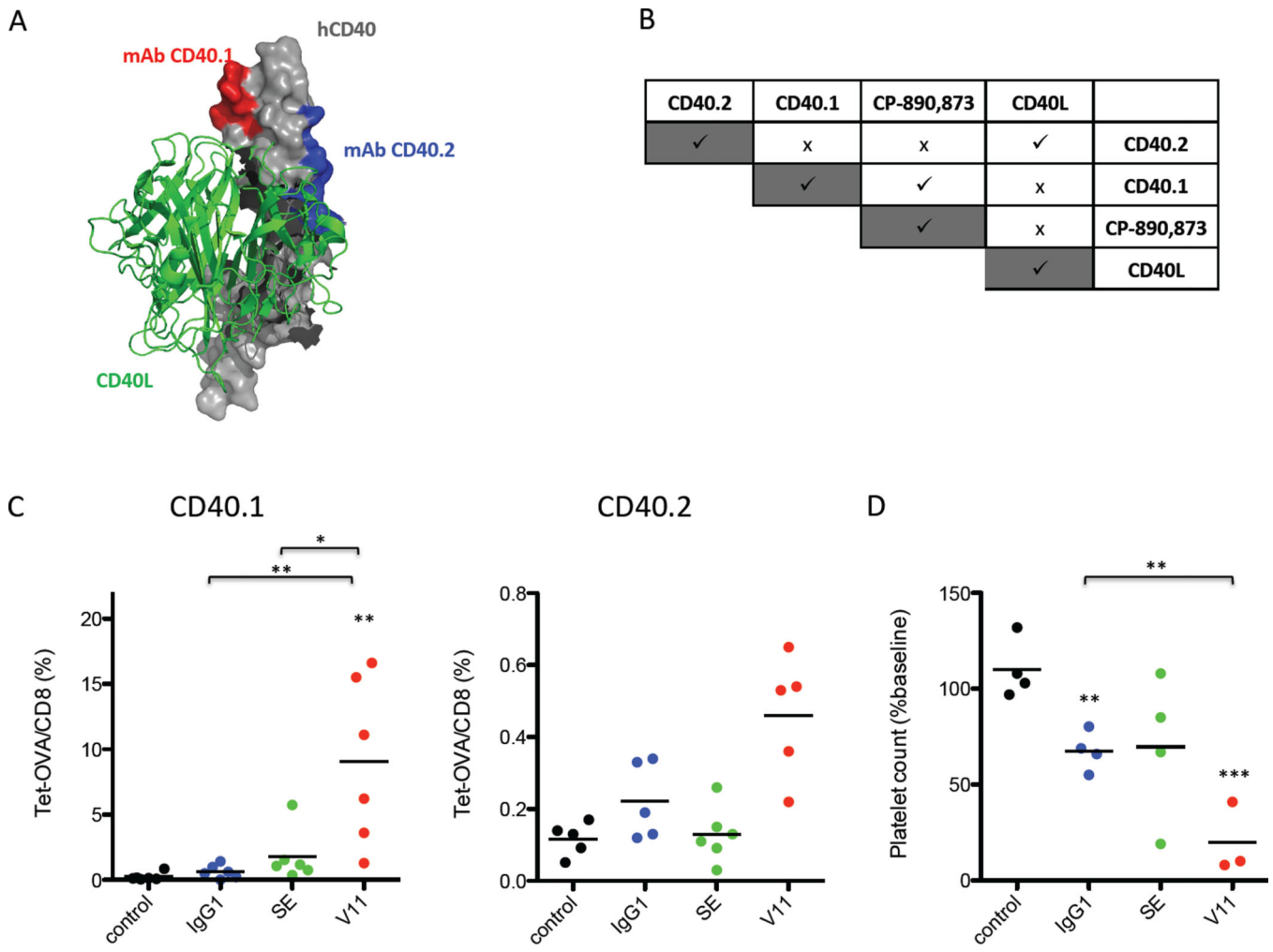

Figure 4. Multiple agonistic, anti-CD40 antibodies demonstrate FcγRIIB-mediated enhancement, regardless of the epitopes they recognize

(A) The binding epitope of mAb CD40.1 (red) and CD40.2 (blue) are highlighted on the structure of human CD40/CD40L complex. hCD40 is shown in gray, hCD40L is shown in green.

(B) Summary of SPR-based competitions assay between the anti CD40 Ab clones CP-870,893, CD40.1, and CD40.2 and between each Ab clone and recombinant human CD40L. denotes blocking, X denotes non-blocking.

(C) Flow cytometry analysis for OVA-specific CD8+ T cells in the blood of humanized CD40/FcγR mice immunized with DEC-OVA alone (control) or in the presence of the CD40.1 or CD40.2 Fc variants. Each dot represents individual mouse. Horizontal lines indicate mean.

(D) Platelet counts in blood 24 hours after administration of the indicated CD40.1 anti-CD40 Fc variants into humanized CD40/FcγR mice. Each dot represents individual mouse. Horizontal lines indicate mean.

See also Figure S4.

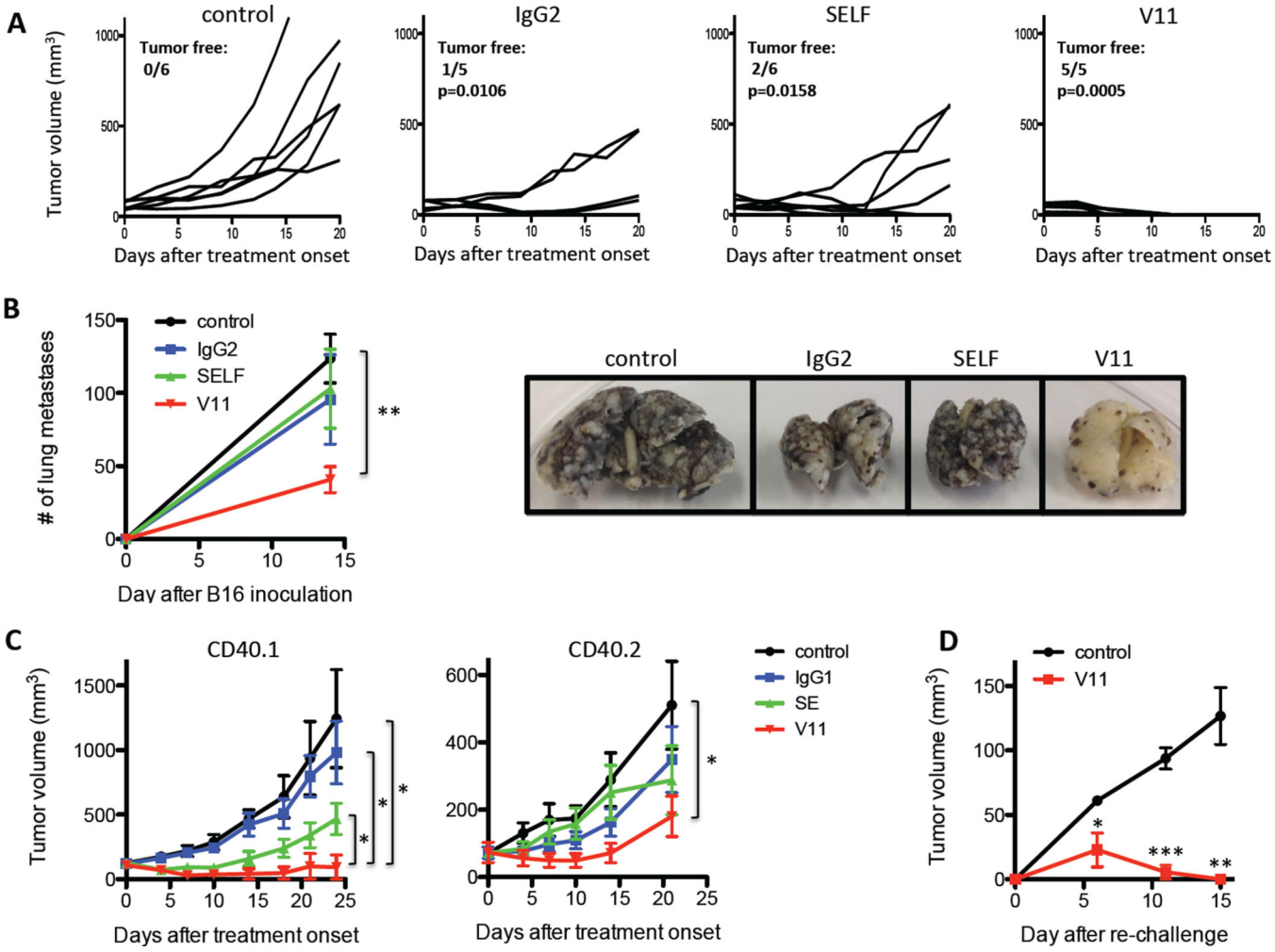

Figure 5. Superior anti-tumor activity of VI1 Fc variant of anti-CD40 mAbs

(A) Antitumor response of humanized CD40/FcγR mice that were inoculated with MC38 tumor cells and treated with Fc variants of anti-CD40 CP-870,893 clone. Results outline tumor growth curves of individual mice.

(B) Effect of CP-870,893 anti-CD40 Fc variants on the frequencies of lung metastases. Humanized CD40/FcγR mice were injected intravenously with B16 melanoma cells and treated with the indicated anti-CD40 Abs. At day 13 after injection lungs were harvested for counting the number of metastases. Results presented as means +/− SEM. n=4. Representative lung image from each group is presented.

(c) Antitumor response of humanized CD40/FcγR mice that were inoculated with MC38 tumor cells and treated with Fc variants of anti-CD40 CD40.1 and CD40.2 mAbs. Results presented as means +/− SEM. n=7 (CD40.1) or 6 (CD40.2).

(D) Tumor free mice from the CD40.1 group in the experiment described in C were re-challenged with MC38 cells subcutaneously and followed for tumor growth. Control group consists of naïve mice. Results presented as means +/− SEM. n=4.

See also Figure S5.