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Role of crosslinking for agonistic CD40 monoclonal antibodies as immune therapy of cancer

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Abstract

Agonists of the TNF superfamily of receptors hold promise as novel therapy for cancer. Recent data on agonistic anti-murine TNF receptors (TNFR) such as CD40 suggest that the specific engagement of Fc-receptor (FcR) is required for optimal antitumor effects, prompting calls to engineer anti-human CD40 and other TNFR mAb accordingly. CP-870,893 is a fully human anti-CD40 mAb, selected in part because it is an IgG2 which is presumed to have poor reactivity with FcR; however, CP-870,893 has been evaluated in multiple clinical trials with beneficial activity in patients with melanoma, pancreatic and other cancers. Here, we confirmed that the activity of anti-murine CD40 mAb was dependent on FcγRIIB engagement, was decreased significantly in *FcγRIIB*$^{-/-}$ mice, and upon Fc-crosslinking anti-mouse CD40 mAb enhanced the activation of antigen presenting cells. In contrast, the CP-870,893-mediated activation of human B cells was not enhanced with anti-IgG-crosslinking nor abrogated when used as an F(ab)'$_2$ reagent. Crosslinking of CP-870,893 using the CD32-expressing K562 cells yielded an Fc-dependent modest increase in the expression of some activation markers relative to that of the soluble CP-870,893 mAb. Classic Fc-dependent functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC) were minimal for CP-870,893 as compared to the IgG1 anti-CD20 mAb rituximab, which mediated both ADCC and CMC in parallel assays. Anti-mouse CD40 mAb competed for the CD40 ligand binding site, but CP-870,893 did not. Thus, Fc-crosslinking is not an essential requirement for agonistic anti-human CD40 mAb, whose potency is more dependent on the CD40 epitope recognized and the strength of the signal achieved.

Keywords

CD40; Fc Receptor; tumor immunity

Introduction

Cell-surface receptors of the tumor necrosis factor (TNF) superfamily are important regulators of apoptosis and immunity, and in particular they play critical roles in the crosstalk between T cells and antigen presenting cells (APC). Agonistic monoclonal antibodies (mAb) specific for the TNF receptors superfamily (TNFR) have shown promise as potential cancer therapy in murine models, and several of these mAb have progressed to evaluation in human clinical trials. Based on experiments with agonistic anti-mouse TNFR mAb specific for CD40, death receptor 5 (DR5), or glucocorticoid-induced TNFR-related protein (GITR), it has been suggested that optimal biological and antitumor effects of

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agonistic anti-TNFR antibodies require Fc receptor (FcR) co-engagement (1–5). An inhibitory Fc receptor, FcγRIIB, appears particularly important for CD40 activity, as *FcγRIIB*$^{-/-}$ mice respond poorly to anti-CD40 therapy *in vivo* (2, 3). Other agonistic TNFR mAb (and non-TNFR immunomodulatory antibodies such as the anti-CTLA-4 mAb) depend on other FcγRs (1, 5–7). Strategies to enhance the interactions of FcR with mAb against TNFRs and other immunoregulatory molecules are being considered as important and even necessary next steps for successful clinical development.

CD40 is broadly expressed on APC and other cells; as a member of the TNFR, CD40 is a well-described mediator of T cell activation (8). The interactions between CD40 on APC and CD40-ligand (CD40L) on CD4 T cells contributes to "licensing" of APCs *in vivo* and drives antigen-specific CD8 T cell responses, including those against tumors (9,10). In some circumstances, agonist anti-CD40 mAb that mimic the action of CD40L can substitute fully for T cell help in mediating adaptive immune responses (11–13). Using rat anti-murine CD40 reagents, multiple laboratory groups have explored the role of FcR affinity in mediating the biological effects of CD40 antibodies (1–3). It has been demonstrated that improved Fc-FcR affinity increases the agonistic effect of anti-murine mAb and enhances the rates of rejection of implanted tumors; however, little data are available regarding the clinical grade anti-human CD40 mAb.

The agonistic anti-human CD40 mAb CP-870,893 is a fully human IgG2 immunoglobulin, selected for clinical development in part because of a presumed low affinity for FcR (14) that is a typical feature of IgG2 molecules. In more than 150 patients treated, CP-870,893 has been found to mediate the activation of APCs and often accompanied by a moderate but transient cytokine release syndrome on the day of infusion (10). Treatment with CP-870,893 alone or in combination with chemotherapy has resulted in tumor-regression in patients with a variety of malignancies, including melanoma and pancreatic cancer (15–19), with a RECIST-defined objective response rate of 20%-25%. In this study, we evaluated the function of the CP-870,893 Fc domain in an attempt to resolve the conundrum between the requirement of FcR engagement of agonistic anti-CD40 mAb in mice and the demonstrated clinical and immunological activity of CP-870,893 in patients. We examined and compared the Fc-dependence of agonistic anti-mouse CD40 mAb FGK45 and anti-human CD40 mAb CP-870,893.

Materials and Methods

Mice and reagents

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. C57BL/6 and *FcγRIIB*$^{-/-}$ C57BL/6 mice (~8–12 weeks of age) were purchased from Jackson Laboratory. Flow cytometry reagents are described in the Supplementary information.

In vitro stimulation of murine B cells

Magnetic column purification was used to purify splenic B cells (>95%). B cells were incubated for 48 hr at 37C/5% CO_2 in RPMI complete media (RPMI containing 10% FCS, 2 mM glutamine, 10 mM HEPES, 100 μg/ml gentamicin, and 50 μM 2-mercaptoethanol) in the presence of 1 μg/ml (or equimolar concentrations) of purified rat IgG2a, FGK45, FGK45 F(ab)'$_2$, or FGK45 crosslinked using goat anti-rat IgG (Jackson ImmunoResearch), incubated for 30 minutes at room temperature at a 2:1 molar ratio (crosslinking reagent to FGK45) before being added to the culture media. After 48 hr, $CD45^+$ $CD19^+$ $7AAD^{lo}$ cells were analyzed by flow cytometry for surface expression of CD80, CD86, CD70, MHC class I, and MHC class II, compared to isotype control IgG.

To study the fine specificity of the anti-CD40 antibody, splenic B cells were preincubated for 30 min at 4C with either buffer only, rat IgG2a, soluble CD40L, FGK45 (CD40), 3/23 (CD40), 1C10 (CD40), or FGK45 F(ab)'$_2$ (1 ug/ml for intact antibodies or equimolar concentrations of the other reagents) and then stained with PE-conjugated FGK45 and measured by flow cytometry.

Murine *in vivo* treatment with CD40 mAb

Wild-type and *Fc*γ*RIIB*$^{-/-}$ mice were injected intraperitoneally (i.p.) with 100μg FGK45 or rat IgG2a isotype control. We have previously shown that this dose of FGK45 produces the same pharmacodynamic effect on B cells in mice as the maximum tolerated dose of CP-870,893 does in patients (17). After 48 hr, mice were sacrificed, and the peripheral blood and splenocytes were harvested and processed for flow cytometry. Macrophages and B cells in the spleen and peripheral blood were analyzed for the expression of MHC class II, MHC class I, CD86, and CD80. Viable cells were identified by forward and side scatter, CD45 positivity and 7AAD^{lo} cells; CD19 positivity was used to identify B cells, and F4/80 to identify macrophages.

Human antibodies and study reagents

Clinical grade, endotoxin-free anti-human CD40 mAb CP-870,893 (fully human IgG2) was obtained from Pfizer (14, 20). Clinical grade, endotoxin-free anti-human CD20 rituximab (chimeric IgG1) was purchased from Genentech. F(ab)'$_2$ fragments were generated by enzymatic digestion with a pepsin kit (G Biosciences) according to the manufacturer's instructions, and products validated using 10% polyacrylamide bis-tris-HCl buffered precast gels (Invitrogen). SR, Ramos, and Daudi lymphoblastoid cell lines were purchased from ATCC. K562 cells expressing human CD32 (K32) were a gift from Dr. Carl June at the University of Pennsylvania and were generated as previously described (21).

In vitro stimulation of human B cells and other *in vitro* assays

Using magnetic column purification, healthy donor human B cells were freshly isolated (>95%) (Miltenyi Biotech) and incubated at 37C/5% CO_2 for 48 hr in X-VIVO complete media (X-VIVO 20 from Lonza containing 10% fetal calf serum, 2 mM glutamine, 10 mM HEPES, and 100 μg/ml gentamicin) at 1 μg/ml (or equimolar concentrations) of purified human IgG2 (Sigma-Aldrich), CP-870,893, CP-870,893 F(ab)'$_2$, or CP-870,893 crosslinked using goat anti-human IgG Fc polyclonal antibody, or CP-870,893 F(ab)'$_2$ crosslinked with goat anti-human IgG F(ab)'$_2$ polyclonal antibody (Jackson Immunoresearch), incubated for 30 minutes at room temperature at a 2:1 molar ratio (crosslinking reagent to CP-870,893) before being added to the culture media. Binding for each polyclonal antibody crosslinking reagents to CP-870,893 or CP-870,893 F(ab)'$_2$ was confirmed by flow cytometry (Supplementary Fig. 1). In other experiments, B cells were incubated with soluble CP-870,893 (1 μg/ml) or CP-870,893 F(ab)'$_2$ for 30 minutes at 37 C and then added at a ratio of 1:1 to K32 cells. After 48 hr at 37C/5% CO_2, CD45$^+$ CD19$^+$ 7AAD^{lo} cells were analyzed by flow cytometry for surface expression of CD70, CD86, MHC class II, MHC class I, and CD40, compared to isotype control.

Antibody-dependent cellular cytotoxicity assay and Complement-mediated cytotoxicity assay were performed as previously described (22) and noted in the Supplementary information.

Statistical analysis

Data are shown as mean values +/− standard deviation (SD) or standard error (SE), as appropriate. Comparisons between experimental groups were made using Student's t test,

with significance at $p < 0.05$. Prism software (GraphPad Software, Inc.) was used for analysis.

Results and Discussion

Activation with anti-mouse CD40 mAb *in vitro* requires crosslinking

Dependency on Fc-crosslinking for the bioactivity of agonistic anti-mouse CD40 mAb was investigated *in vitro* using the CD40 mAb FGK45 in B-cell activation assays. We found that neither intact FGK45 nor purified $F(ab)'_2$ fragments of FGK45 induced the upregulation of CD86, CD70, CD80, and MHC class II (I-A,I-E) on purified mouse splenic B cells (>99% $CD40^+$) after incubation for 48 hr at 1 μg/ml, compared to isotype control immunoglobulin (Fig. 1). A small B cell activation signal was observed only at markedly excessive mAb concentrations of FGK45 (>300 μg/ml) (data not shown). In contrast, artificial crosslinking of FGK45 or FGK45 $F(ab)'_2$ using a goat-anti-rat IgG (H+L) reagent produced marked and statistically significant upregulation of these markers at FGK45 concentrations of 1 μg/ml or equimolar concentrations of FGK45 $F(ab)'_2$ (Fig. 1), suggesting that the activation with anti-mouse CD40 mAb FGK45 *in vitro* requires crosslinking.

Immune activation with FGK45 *in vivo* is FcγRIIB-dependent

A potential explanation for the *in vitro* finding is that agonistic anti-mouse CD40 mAb FGK45 requires the engagement of the FcγRIIB receptor for optimal activity (2, 3). We therefore measured the *in vivo* bioactivity of endotoxin-free FGK45 administered to wild-type (WT) vs *FcγRIIB*$^{-/-}$ mice. We had previously determined that 100 μg of FGK45 delivered i.p. yields optimal B cell and macrophage activation in WT mice (17). 48 hr after the i.p. administration of 100 μg of either FGK45 or isotype-control mAb, splenocytes and peripheral blood from WT mice were harvested and examined by flow cytometry to determine the expression of activation markers on B cells and macrophages. As expected, purified splenic B cells and peripheral blood B cells from WT mice treated with FGK45 exhibited statistically significant upregulation of MHC class II, MHC class I ($H\text{-}2D^b$), CD86, and CD80 compared to those from WT mice treated with isotype-control mAb (Fig. 2A). In *FcγRIIB*$^{-/-}$ mice, FGK45 treatment upregulated MHC class II expression on both splenic and peripheral blood B cells, but not the MHC class I and CD80 expression. CD86 upregulation on splenic B cells was modest and less than the effect of FGK45 in WT mice; this difference is statistically significantly. For peripheral blood B cells, FGK45-treated *FcγRIIB*$^{-/-}$ mice demonstrated no statistically different upregulation of CD86 compared to that in control mAb-treated *FcγRIIB*$^{-/-}$ mice (Fig. 2A). FGK45 injection also induced the upregulation of MHC class II, MHC class I, CD86, and CD80 on splenic macrophages from WT mice, but no statistically significant upregulation of these activation markers was seen on splenic macrophages from FcγRIIB$^{-/-}$ mice (Fig. 2B), further highlighting the Fc-dependence of agonistic anti-CD40-induced immune activation *in vivo* in mice.

Activity of CP-870,893 *in vitro* is Fc-independent

To determine if the activity of CP-870,893 also requires Fc-FcR interaction, we studied the ability of CP-870,893 vs. CP-870,893 $F(ab)'_2$ to activate purified human B cells *in vitro*. Compared to isotype control, CP-870,893 and CP-870,893 $F(ab)'_2$ equally upregulated the cell-surface expression of CD70, CD86, HLA-DR (MHC Class II), and HLA-ABC (MHC class I) (Fig. 3A). Although the extent of cell surface upregulation increased with increasing concentrations of CP-870,893 or CP-870,893 $F(ab)'_2$ (maximizing at 1 ug/ml), the effects were equal for CP-870,893 or CP-870,893 $F(ab)'_2$ across four log titrations (Supplementary Fig. 2). Moreover, there was no additional upregulation of these markers when CP-870,893 or CP-870,893 $F(ab)'_2$ was artificially crosslinked using polyclonal goat anti-human IgG crosslinking antibodies (Fig. 3A, C). One exception was a slight further increase in HLA-

DR expression with crosslinking that varied depending on the donor studied. Binding of the crosslinking reagents to CP-870,893 and CP-870,893 F(ab)'$_2$ was demonstrated by flow cytometry using a fluorescent conjugated form of each polyclonal crosslinking antibody (Supplementary Fig. 1). Since CP-870,893 blocks the CD40 epitope recognized by anti-CD40 mAb clone HB14, we were able to confirm CD40-binding by CP-870,893, CP-870,893 F(ab)'$_2$, and crosslinked CP-870,893 by the loss of reactivity to HB14 after incubation of B cells with each of these reagents (Fig. 3B). We further explored a role for FcR-crosslinking by incubating CP-870,893-labeled B cells with K562 cells transfected with the FcR CD32 (K32). Binding of CP-870,893 to CD32 on K32 cells was confirmed by flow cytometry (Supplementary Fig. 1). We found that the K32-mediated crosslinking upregulated CD70, CD86, HLA-ABC (but not HLA-DR) more than CP-870,893 alone, and this difference is statistically significant (Fig. 3C); for each of these three markers, the additional upregulation was modest. Nevertheless, Fc-FcR dependency in the K32 assay was demonstrated by the loss of additional upregulation when CP-870,893 F(ab)'$_2$ instead of CP-870,893 was used to label B cells prior to incubation with K32 (Fig. 3C). Results from this latter experiment argue against a contribution from a constitutively expressed soluble or cell surface factor from K562 cells in this assay.

CP-870,893 does not mediate Fc-dependent effector functions

We studied whether CP-870,893, a fully human IgG2 molecule, can interact with FcR by evaluating its ability to trigger ADCC effector functions *in vitro*. Human IgG2 molecules typically interact poorly with FcR and classically are not known to be potent mediators of Fc-dependent effector functions such as ADCC and CMC, although the common H131R polymorphism has improved FcγRIIB affinity for IgG2 and has been shown to mediate ADCC by myeloid cells and neutrophils through the IgG2 molecules (23–25). Because some tumor cells express CD40, it has remained an open question as to whether CP-870,893-mediated ADCC is a potential therapeutic mechanism of action. As a positive control, we evaluated the IgG1 anti-CD20 mAb rituximab, which is known to exert its therapeutic effect through the induction of potent ADCC and CMC. As single agents, compared to isotype controls, CP-870,893 and rituximab triggered minimal to no cytotoxicity of lymphoblastoid cell lines expressing CD20 and CD40 when incubated in media containing heat-inactivated complement (Fig. 4). Neither CP-870,893 nor rituximab mediated ADCC of $CD40^{neg}CD20^{neg}$ SR cells; rituximab (but not rituximab F(ab)'$_2$) triggered ADCC against Ramos and Daudi cells, both of which are $CD20^{high}$ (Fig. 4). In the same experiments, however, no ADCC activity was observed with CP-870,893 (nor with CP-870,893 F(ab)'$_2$), even though both Ramos and Daudi are $CD40^{high}$. Similarly, rituximab (but not rituximab F(ab)'$_2$) mediated CMC of Ramos and Daudi, but not SR cells, whereas CP-873,893 did not trigger CMC of any target (Fig. 4). Thus, Fc-mediated functions of CP-870,893 are weak, likely because as an IgG2 molecule, it does not interact well with FcR under these conditions. Moreover, ADCC and CMC are unlikely the primary mechanisms of action for CP-870,893 in cancer patients.

Fine specificity of mouse vs. human CD40 mAb

Our data raise the question regarding potential differences in the fine specificity of anti-mouse vs. anti-human CD40 mAb. A key point is whether the anti-CD40 antibodies (both mouse and human) compete with the CD40L for binding, as it has been previously published that CP-870,893 binds to a site on CD40 distinct from the CD40L binding site (14). Using flow cytometry, we observed that pre-incubation with recombinant soluble murine CD40L blocks the binding of FGK45 to CD40 on murine B cells *in vitro* (Supplementary Fig. 3). Pre-incubation with two additional murine agonist anti-CD40 antibodies (1C10 and 3/23, each previously shown to be FcR crosslinking-dependent (1–3)) also blocked binding of FGK45 to CD40, but as a control, there was no effect on binding of an unrelated antibody to

a different target (Supplementary Fig. 3). Thus, at the CD40L binding site, the fine specificity of CP-870,893 appears to differ from these three anti-mouse CD40 antibodies.

In summary, our studies were aimed at determining the potential role that the Fc domain of CP-870,893 plays in its agonistic properties including the cytokine release syndromes and the clinical responses observed in patients with solid tumors (10). By examining CP-870,893 activation of B cells *in vitro* and the ability of the antibody to mediate ADCC or CMC, we found that the Fc domain of the human anti-CD40 mAb CP-870,893 plays a minimal role in the agonistic potency of the antibody. Crosslinking CP-870,893 or its $F(ab)'_2$ fragment using a soluble agent did not lead to increased potency of the antibody in our assays whereas crosslinking with CD32-expressing cells led to modest additional upregulation of some but not all activation markers in an Fc-dependent manner. It is well-known that the strength of CD40 signaling via recombinant forms of its natural ligand, CD40L, increases *in vitro* with increasing crosslinking (26), and thus our results with K32 cells are not particularly surprising. Nevertheless, our results emphasize that CP-870,893 is an active, soluble compound independent of its Fc domain. Thus, our results stand in contrast to those for agonistic anti-murine CD40 mAb in which crosslinking via the Fc domain is important, if not required, for optimal biological activity. Indeed, using the agonistic anti-murine CD40 mAb FGK45 we demonstrated that Fc-crosslinking was necessary for B cell activation *in vitro* and that the activity of FGK45 was limited in $Fc\gamma RIIB^{-/-}$ mice, consistent with previous published findings for other anti-mouse CD40 mAb such as 1C10 and 3/23 (2, 3). On the one hand, these findings may reflect a fundamental difference in the nature of the human versus the mouse systems, but on the other hand, they also suggest that CP-870,893 may bind to an epitope on human CD40 with a uniquely strong signaling capability that is either without a murine homologue or such a homologue is not recognized by anti-mouse CD40 mAb. We found that FGK45 (as well as the two other anti-mouse CD40 mAb, 1C10 and 3/23, each of which depends on FcR-crosslinking) competes with sCD40L for binding to CD40; in contrast, CP-870,893 binds to a site distinct from the CD40L binding site (14), suggesting a potential underlying mechanism for the functional differences we observed. The consequence is that experimentally, FGK45 is considerably less potent in mice than CP-870,893 is in humans: a 25-times higher dose of FGK45 (5 mg/kg) is needed to achieve the same pharmacodynamic effect on peripheral B cells in mice as CP-870,893 achieves at its maximum tolerated dose (0.2 mg/kg) in patients (15). Similar findings of crosslinking-dependence in other anti-TNFR mAb may also reflect epitope-specificity, a subject for future studies.

Finally, from a clinical standpoint, modification of CP-870,893 to improve FcR binding is not necessary for its biological activity. Indeed, even if increased systemic potency were to be achieved by this or other means, there may be a clinical downside as worsening toxicity or increased activation-induced immune suppression may result. Future directions also include the evaluation of tumor tissue from CP-870,893-treated patients, and the potential correlation of the CD32 H131R polymorphism to toxicity or degree of tumor response to CP-870,893. Current efforts focus on the combination regimens of anti-CD40 mAb with other therapies or alternative dosing strategies, such as subcutaneous delivery, to enhance the agonistic effect of anti-CD40 mAb as anti-cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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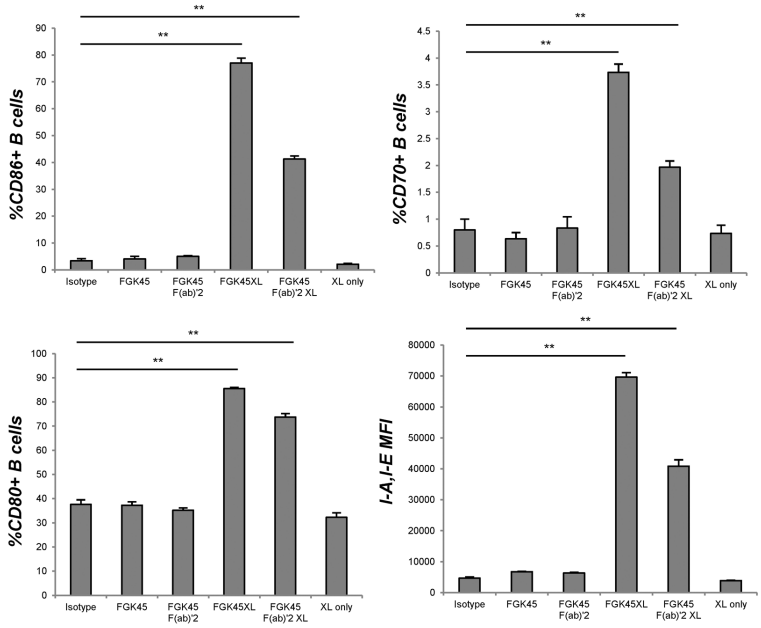

Figure 1.

Anti-mouse CD40 mAb B cell activation *in vitro* is Fc-dependent. Purified mouse splenic B cells were analyzed for activation markers after incubation for 48 hr with either the isotype control, the agonistic anti-CD40 mAb FGK45, its $F(ab)'_2$ fragment, artificially crosslinked FGK45, artificially crosslinked FGK45 $F(ab)'_2$, or crosslinking reagent (XL) alone *in vitro*. Cells were then analyzed by flow cytometry for the upregulation of CD86, CD70, CD80, or MHC class II. Error bars represent SD; ** indicates $p<0.001$. Data shown are from one of four independent experiments conducted in triplicate.

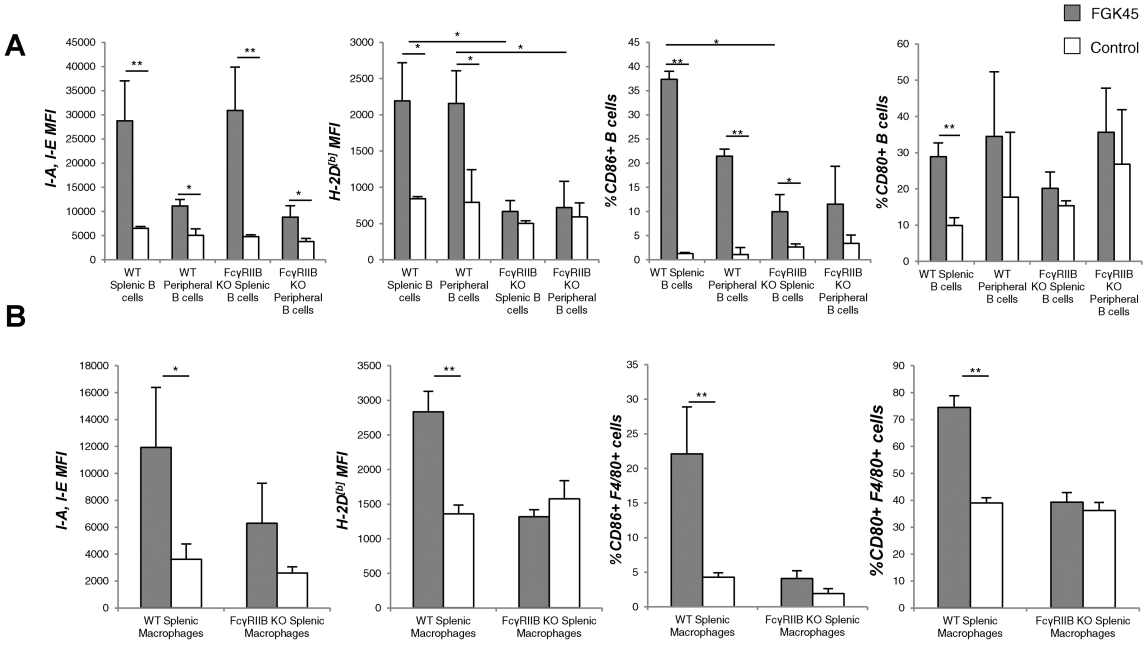

Figure 2.

Activation effects of FGK45 mAb *in vivo* are decreased in *FcγRIIB*$^{-/-}$ mice. Mice (n=4 per group) were injected intraperitoneally with FGK45 or rat IgG2a isotype control and sacrificed 48 hr later; splenocytes and peripheral blood were harvested. Tissues were processed and analyzed by flow cytometry for the activation markers on (A) B cells or (B) F4/80^{+} macrophages; markers analyzed include MHC class II (I–A, I–E), MHC class I (H-2D^{b}), CD86, and CD80. Error bars represent SD; * indicates $p<0.05$, ** $p<0.001$.

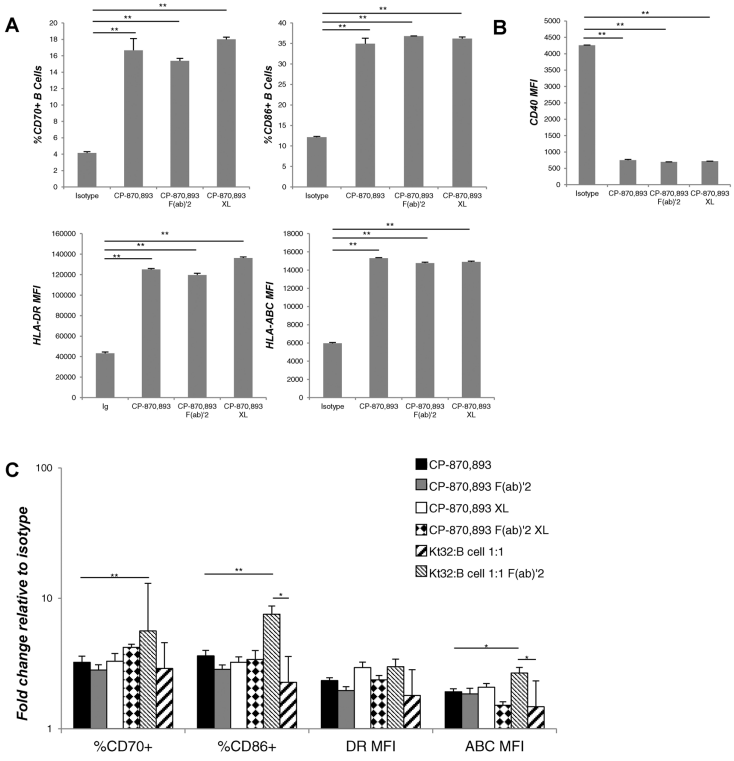

Figure 3.

Role of crosslinking for CP-870,893-mediated B cell activation *in vitro*. A and B, Purified human peripheral B cells were analyzed by flow cytometry for the expression of activation markers after 48 hr incubation with either the isotype control, CP-870,893, its $F(ab)'_2$ fragment, or CP-870,893 crosslinked (XL) as described in Materials and Methods. Error bars represent SD; ** indicates $p<0.001$. Data shown are from one of 4 to 17 independent experiments, each conducted in triplicate. C, Same experimental design as in (A,B) except the additional use of (i) K32 cells as a means of crosslinking CP-870,893 (or CP-870,893 $F(ab)'_2$ as a control) as described in Materials and Methods, and (ii) artificial crosslinking of CP-870,893 $F(ab)'_2$ (XL) as described in Materials and Methods. Flow cytometric data are expressed as fold-change in expression relative to incubation with isotype control, with error bars representing SE. * indicates $p<0.05$, ** $p<0.001$. Data shown are pooled from at least 4 independent experiments per condition, each performed in triplicate.

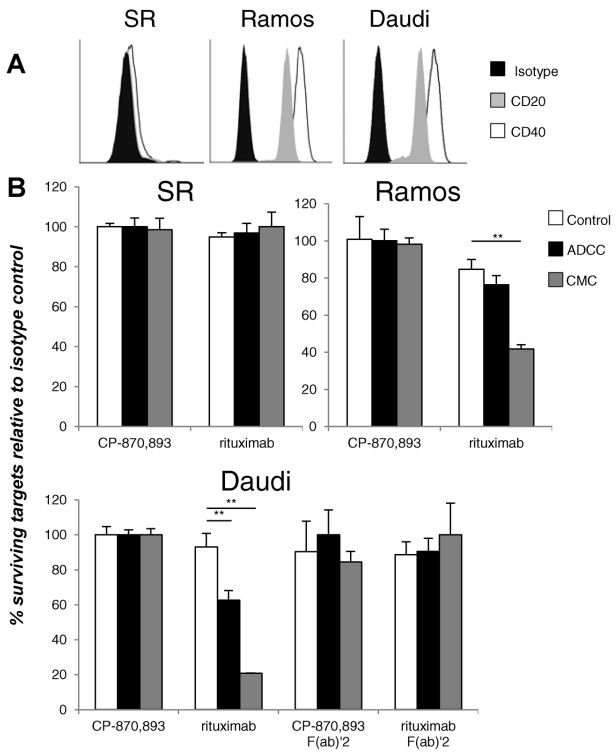

Figure 4.

CP-870,893 does not mediate Fc-dependent effector functions. A, Expression of CD40 and CD20 on the lymphoblastoid cell lines SR, Daudi, and Ramos, as determined by flow cytometry. Data are representative of at least 5 independent experiments. B, Cell lines were incubated with either CP-870,893 or ritixumab or their $F(ab)'_2$ *in vitro* for 4 hr and the percentage of cells surviving was calculated by flow cytometry relative to incubation with isotype control (purified human IgG2 for CP-870,893 and purified human IgG1 for rituximab) under conditions of complete media (white bars), ADCC (black bars), or CMC (gray bars), as described in Materials and Methods. Error bars represent SE; ** indicates $p<0.001$. Data shown are pooled from at least 6 independent experiments per condition, each performed in triplicate.