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Induction of an Altered CD40 Signaling Complex by an Antagonistic Human Monoclonal Antibody to CD40

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Blocking the interaction of CD40 with its ligand CD154 is a desirable goal of therapies for preventing and/or ameliorating autoimmune diseases and transplant rejection. CD154-blocking mAbs used in human clinical trials resulted in unanticipated vascular complications, leading to heightened interest in the therapeutic potential of antagonist mAbs specific for human CD40. Abs that do not require physical competition with CD154 to inhibit CD40 signaling have particular therapeutic promise. In this study, we demonstrate that the antagonist anti-human CD40 mAb PG102 fails to trigger CD40-mediated activation, as well as impairs CD154-mediated CD40 activation, via a distinct nonstimulatory CD40 signaling mechanism. PG102 did not induce early CD40-induced signaling events, and it inhibited early kinase and transcription factor activation by CD154 or agonist anti-CD40 mAbs. However, PG102 stimulated normal CD40-mediated TNFR-associated factor (TRAF)2 and TRAF3 degradation. PG102 induced the formation of a CD40 signaling complex that contained decreased amounts of both TRAF2 and TRAF3 and TRAF2-associated signaling proteins. Additionally, PG102-induced CD40 signaling complexes failed to recruit TRAF6 to detergent-insoluble membrane fractions. Fab fragments of PG102, while retaining CD40 binding, did not induce TRAF degradation, nor could they inhibit CD154-stimulated B cell signaling, indicating that CD40 aggregation is required for the signaling inhibition induced by PG102. The antagonistic impact of PG102 on CD40 signaling reveals that the manner of CD40 ligation can determine sharply different outcomes for CD40 signaling and suggests that such information can be used to therapeutically manipulate these outcomes. The Journal of Immunology, 2015, 194: 4319-4327.

C D40, a member of the TNFR superfamily, is expressed by multiple immune and nonimmune cell types and plays key roles in stimulating effective Ag presentation and humoral immunity (1). CD40 signaling is implicated in the pathogenesis of both autoimmunity (2, 3) and transplant rejection (4, 5), and disruption of CD40-mediated activation has been of considerable therapeutic interest (reviewed in Ref. 5). The first interventions tested used mAbs specific for the CD40L, CD154, to block the receptor–ligand interaction. This approach showed great promise in preclinical mouse models of arthritis (6) and allograft transplant (7). However, the first human clinical trial using anti-CD154–blocking mAb was halted as the result of several fatal thromboembolic events (8), which may have been due to the effect of immune complexes upon activated platelets expressing CD154. Although a variety of approaches to blocking CD40–CD154 interactions have since been explored and may prove successful (5), an attractive alternative is direct inhibition of the CD40-mediated activation pathway.

The mAb PG102 was derived from the previously described anti-human CD40 (hCD40) mAb SD12 (9), which inhibits the proliferation of human B cells stimulated with activated T cells or agonistic CD40-specific mAbs (10, 11). Subsequently, a chimeric form of SD12 (chSD12) containing human IgG4 constant regions was produced. The chSD12 mAb prevents development of experimental autoimmune encephalomyelitis in marmosets (12), as well as prolongs the survival of kidney allografts and allows repeated systemic administration of adenoviral gene therapy vectors in rhesus monkeys (13, 14). In an open-label dose-escalation phase II/III study of Crohn’s disease patients, chSD12 was well tolerated and showed promising clinical benefit (15). Several amino acids in both H and L chains of the chSD12 mAb were subsequently changed to remove potential MHC-binding epitopes and thus reduce possible immunogenicity (M. de Boer, personal communication); the resultant mAb is PG102. Previous studies showed that chSD12 inhibits CD40-induced production of proinflammatory cytokines (16), and we observed this as well with PG102 in B cell cultures (K.L. Oxley and G.A. Bishop, unpublished observations).

The present study was undertaken to test the hypothesis that PG102 inhibits CD40 signaling by inducing a signaling cascade that is distinct from that initiated by binding of CD154 or agonistic anti-CD40 Abs to CD40. Results reveal that engagement of CD40...
by PG102-induced formation of a dysfunctional CD40 signaling complex characterized by altered TNFR-associated factor (TRAF) recruitment and association, with resultant ineffective induction of kinase and transcription factor activation. Importantly for potential therapeutic applications, although PG102 did not inhibit CD154–CD40 binding, it inhibited and prevented CD154-induced CD40 activation.

Materials and Methods

Cells

The human B cell line T5-1 was described previously (17). PBMCs from human blood donors were isolated from discarded Leukocyte Reduction System cones obtained from the DeGowin Blood Center (University of Iowa). The Blood Center has University of Iowa Institutional Review Board approval to provide these deidentified samples to investigators. Cells were cultured in RPMI 1640 medium with 10% heat-inactivated FCS (Atlanta Biotechs, Atlanta, GA), 10 μM 2-ME (Inviron, Grand Island, NY), 2 mM l-glutamine, penicillin, and streptomycin (B cell medium [BCM]). Human primary monocyte-derived dendritic cells (DCs) were obtained from the above-described human blood samples, as follows. Monocytes were isolated from these PBMCs by culturing for 30 min and washing away nonadherent cells. A cell scraper was used to dislodge monocytes, which were washed and resuspended in RPMI 1640 medium with 10% FCS, 1% HEPES, 1% l-glutamine, 1% penicillin/streptomycin, and 1% essential amino acids) at 1 x 10^6 cells/ml with GM-CSF (100 ng/ml) and recombinant human IL-4 (10 ng/ml). Cells were cultured in six-well tissue culture plates at 3 ml/well. At days 2 and 5, 900 μl culture medium was renewed and replenished with 1 ml fresh RPMI containing 300 ng/ml GM-CSF and 30 ng/ml IL-4. To generate mature DCs, immature DCs were harvested on day 5, washed with PBS, seeded (5 x 10^6 cells/well) in fresh RPMI supplemented with GM-CSF (100 ng/ml) and recombinant human IL-4 (10 ng/ml), and cultured with LPS (1 μg/ml) for 48 h.

mAbs and CD154

The agonistic anti-hCD40 mouse IgG1 mAb G28.5 (18) was produced by saturated AmSulf precipitation of supernatant from a hybridoma obtained from the American Type Culture Collection (Manassas, VA). The antagonistic humanized anti-hCD40 mAb PG102 is a less immunogenic form of the humanized anti-hCD40 mAb ch5D12 (12) and was provided by Fast Forward Pharmaceuticals. The humanized anti-hCD40 control mAb 4D11 was used in Fig. 1 (19). The isotype-control Abs used were mlgG1 (Southern Biotech, Birmingham, AL) and IgG4s (Sigma-Aldrich, St. Louis, MO) for G28.5 and PG102, respectively. The source of human CD154 was membranes of CD154-expressing Hi5 insect cells produced in the Bishop laboratory, as previously described (20). Hi5 cells grow at 25˚C; they die at 37˚C. Cells were then pelleted and lysed, as described previously (22). Bead-bound proteins were resuspended in 2 x SDS-PAGE loading buffer and boiled for 3–5 min at 95˚C prior to loading samples for PAGE. For Western blotting, 10–15 μl sample was resolved on 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore), Membranes were blocked with 10% dry milk in TBST for 1 h, washed in TBST, incubated with primary Abs overnight, washed in 10% dry milk in TBST, and incubated with secondary Abs for 1–2 h or overnight. Protein bands were visualized using a chemiluminescent detection reagent (Pierce, Rockford, IL). Images of blots were recorded with a low-light imaging system (LAS3000; Fujifilm Medical Systems Stamford, CT). Immunoblots were reprobed with appropriate Abs specific for control proteins, such as actin, to verify equal protein loading in each lane.

TRAF-degradation assay

Cells were placed in a 24-well plate at 1 x 10^6 cells/well. Anti-CD40 mAbs were added to appropriate wells at 10 μg/ml, and cells were incubated at 37˚C for 5 h. Cells were then transferred to 1.5 ml Eppendorf tubes and pelleted (7000 rpm, 1 min, 4˚C). Cells were resuspended in 2 x SDS sample dye and sonicated (Branson Sonifier 450, 1.5 output, 90% duty cycle), and samples were boiled at 95˚C for 3 min.

Analysis of detergent-soluble and insoluble membrane signaling complexes

Cells (1 x 10^6/condition) were stimulated with mAbs or insect cell membranes, as described above, or left unstimulated and then pelleted (400 x g, 1 min). The cell pellets were resuspended in lysis buffer (1% Brij, 150 mM NaCl, 20 mM Tris [pH 7.5], 50 mM β-glycerophosphate, 2 mM complete EDTA-free tablets, and sodium vanadate) and incubated for 30 min on ice. After incubation, cell lysates were centrifuged (30 min, 4˚C, 400 x g), and supernatants were transferred to a new tube. Lysates were then 5% SDS and 1% 2-ME were added. Pellets were sonicated (1.5 output, 90% duty cycle), and 2 x SDS- PAGE buffer was added to both pellet and supernatant. Both fractions were boiled (3 min, 95˚C).

Generation of PG102 Fab fragments

Fab fragments from PG102-antibody IgGs were generated using the Pierce Fab Preparation Kit (Thermo Fisher Scientific, Boston, MA), according to the manufacturer’s instructions. After this procedure, only PG102 Fab fragments were found by standard SDS-PAGE and Coomassie Blue staining (data not shown). Subsequently, the binding of these PG102 Fab fragments to CD154-expressing JY B cells was compared with PG102-antibody IgGs using flow cytometry. Results are presented in Supplementary Fig. 1B and confirm effective binding of Fabs to hCD40, indistinguishable from the binding of intact PG102.
Statistical analysis

The statistical significance of the results from multiple experiments was determined by the Student t test. The ranges of p values are indicated in the figure legends.

Results

Independent CD40 binding by PG102 and CD154

Most reagents designed to inhibit CD40 signaling for therapeutic purposes block the binding of CD40 to CD154 (reviewed in Refs. 2, 5). Thus, it was of interest to determine whether the previously reported inhibitory impact of the hCD40-specific mAb PG102 on CD40-mediated immune cell activation results from blocking of the CD40–CD154 interaction. This was addressed using two complementary approaches, described in detail in Materials and Methods. An ELISA approach used plates coated with soluble hCD40, incubated with various combinations of soluble hCD154, PG102, or control Abs. Fig. 1A shows that, regardless of whether hCD154 or PG102 was added first, neither inhibited the binding of the other. The same results were obtained using intact human B cells, analyzed by flow cytometry (Fig. 1B). Fig. 1B also presents, for comparison, results with the 4D11 anti-hCD40 mAb, which blocked hCD154 binding to CD40. Thus, PG102 does not impair CD154 binding to CD40 and vice versa. We also found that PG102 binding to hCD40 on human B cells did not result in rapid internalization of the CD40 complex; after 2 h of incubation with PG102 at 37˚C, no detectable differences in CD40 surface levels were observed (Supplemental Fig. 1A).

Lack of effective induction of early CD40 signaling by PG102

Engagement of CD40 by agonistic anti-CD40 Abs in B lymphocytes and myeloid cells results in rapid induction of activation of MAPKs, including JNK, ERK, and p38, as well as activation of the canonical NF-kB transcriptional pathway (23, 24). Because the 5D12/PG102 mAb inhibits human B cell proliferation and Ig production induced by either activated T cells or agonistic anti-hCD40 Ab (10, 11), we investigated whether this corresponds to a lack of CD40-mediated early signals induced by PG102. Supplemental Fig. 2A demonstrates that the agonistic anti-hCD40 mAb G28.5, as expected, stimulated robust activation of MAPK and NF-kB1 in human primary DCs. In contrast, PG102 stimulated detectable, but quite modest, MAPK activation and little to no NF-kB1 activation.

Initial studies of PG102 examined CD40-mediated B cell activation, so we focused upon this cell type for our subsequent experiments. Fig. 2 shows that PG102 did not induce detectable phosphorylation of either JNK or IkB in the human B cell line T5-1, which was previously reported as a robust model of CD40 signaling to human B cells (20, 25). Fig. 2A compares the phosphorylated forms of JNK and IkB in a representative Western

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FIGURE 1. Lack of CD154–PG102 binding competition. (A) PG102 and hCD154 binding to plate-bound CD40. Binding assays were performed as described in Materials and Methods, using binding of HRP-labeled secondary reagents as a detection method. Cells were incubated first with PG102 or control mAb, followed by CD154, and CD154 was detected (upper panel). The initial incubations were with CD154, followed by PG102 or control mAbs; PG102 binding was detected (lower panel). Results represent the mean ± SD of triplicate cultures from a representative of three experiments. (B) PG102 or 4D11 mAb and hCD154 binding to cell-expressed hCD40. hCD40-expressing human B lymphoblastoid cells (JY) were incubated with FITC-labeled hCD154, followed by PE-labeled PG102 or vice versa, as described in Materials and Methods (left panel). Cells were incubated first with PE-4D11 mAb and then FITC-hCD154 (right panel). Flow cytometry of stained cells from a representative of three experiments is shown. BG, background staining.
FIGURE 2. Failure of PG102 to effectively activate early CD40-mediated signaling events. (A) Cells of the human B cell line T5-1 were incubated with the hCD40-specific mAbs G28.5 or PG102 for the indicated minutes (Min), as described in Materials and Methods. Cell lysates were subjected to SDS-PAGE, and Western blotting was performed for total or p-JNK, p-IkBα, and actin (as a loading control; total IkBα decreases with activation so cannot serve this function). A representative of three experiments is shown. (B) Relative quantification, as in Materials and Methods, of CD40-induced phosphorylation of JNK and IkBα, normalized to total JNK or actin, respectively. Data are mean ± SD of three experiments. **p < 0.01. Iso, cells treated for 30 min with isotype-control Abs for PG102 or G28.5.

Inhibition of CD154-mediated B cell activation by PG102

PG102 did not bind to or block binding of CD154 (Fig. 1). Thus, we wished to test the hypothesis that binding of PG102 induces an altered CD40 signaling complex that is refractory to agonistic stimuli. Therefore, we treated human B cells with membrane-bound CD154 alone, together with PG102, or subsequent to PG102 addition.

FIGURE 3. PG102-mediated inhibition of CD154 signaling. (A) T5-1 human B cells were stimulated with hCD154 alone, CD154+PG102, or PG102 alone for 30 min, followed by CD154 for the indicated minutes. Phosphorylation of JNK and IkBα was assessed as in Fig. 2. A representative of three experiments is shown. (B) Quantification, as in Materials and Methods, for three replicates of the experiment shown in (A), expressed as fold increase over values at the zero time point. Data are mean ± SD. (C) T5-1 human B cells were stimulated with hCD154 alone, CD154+PG102, PG102 for 30 min + CD154, or CD154 for 30 min + PG102 for the indicated number of minutes. Phosphorylation of JNK and IkBα was assessed as in (A); line graphs represent fold increase over values at the zero time point. **p < 0.01. Iso, cells treated for 30 min with isotype-control Ab for PG102.
Results presented in Fig. 3 indicate that either simultaneous or pretreatment of B cells with PG102 abrogated CD154-mediated JNK and IκB phosphorylation. Fig. 3A (representative Western blot) and Fig. 3B (quantification of results from three experiments) show that either PG102 pretreatment or simultaneous addition of PG102 and hCD154 to B cells resulted in marked inhibition of early signaling events. Fig. 3C further demonstrates that this inhibition lasted throughout the course of JNK or NF-κB activation and could be mediated by PG102, even when this mAb was added to cells subsequent to an hCD154 stimulus. Similar findings were obtained in myeloid cells (M. Thewissen and M. de Boer, unpublished observations). These data suggest several potential mechanisms, which are not mutually exclusive. One explanation is that a distinct, negative signal delivered via PG102 to the CD40 signaling complex counteracted and prevented normal agonist-mediated activating signals. Another possibility is that PG102 induced formation of a defective CD40 signaling complex, which competed with the functional signaling complex induced by CD154. We next examined the nature of CD40 signals induced by PG102.

**CD40-mediated TRAF degradation induced by PG102**

CD40 signals to B cells rapidly induce recruitment of TRAF2 and TRAF3 to the CD40 cytoplasmic domain, followed by TRAF2-dependent K48-linked polyubiquitination and proteasome-mediated degradation of both of these TRAFs (21, 26–28). Because PG102 could not induce CD40-mediated activating signals, we examined whether the mAb induced this regulatory event. Data presented in Fig. 4 indicate that PG102 induced CD40-mediated degradation of both TRAF2 and TRAF3 in human B cells, to an extent that was not detectably different from degradation induced by the agonistic mAb G28.5. This was also true in primary human DCs (Supplemental Fig. 2B). Consistent with these findings, relative levels of ubiquitination of CD40-associated TRAF2 induced by PG102 versus G28.5 in human B cells were indistinguishable (K.C. Bankert and G.A. Bishop, unpublished observations).

**CD40 signaling complex formation induced by PG102**

Data shown in Fig. 4 indicate that PG102 showed selective induction of CD40-mediated signaling events, with normal degradation of TRAF2 and TRAF3. We next examined recruitment of signaling proteins known to play major roles in CD40-mediated signals to B cells (24, 29–31). Following stimulation with either G28.5 or PG102 mAbs, CD40 was immunoprecipitated from human B cells, and associated proteins were normalized to CD40 to determine their relative amounts. Although amounts of TRAF6 associated with the signaling complex were not statistically different, CD40 from PG102-stimulated B cells had significantly less associated TRAF2 and TRAF3 (Fig. 5). Representative Western blots in Fig. 5A show that this was not due to the decreased ability of PG102 to bind or precipitate hCD40. In addition, the important TRAF2-associated signaling proteins cIAP1, IKKγ, and HOIP showed significantly reduced amounts in the PG102-induced CD40 signaling complex.

Although T5-1 cells are a well-validated model for CD40 signaling to human B cells, this is a transformed cell line, and CD40 is also biologically important in a variety of immune cells, particularly myeloid cells. Thus, we examined CD40 signaling complex formation in primary human PBMCs. Fig. 5C presents a representative Western blot demonstrating that the marked decrease in TRAF2 and with CD40 in PG102- versus G28.5-stimulated cells is also seen in primary human immune cells. The decrease in TRAF3 was more modest and did not reach statistical significance, as it did in the T5-1 cells (Fig. 5B). However, the trend of reduced TRAF2/3 binding was consistent, and Fig. 5C indicates that this is seen for PBMCs from multiple healthy human blood donors.

Another feature of CD40-induced signaling complex formation is the localization of the complex to cholesterol-rich plasma membrane fractions (“rafts”), separated by their relative detergent insolubility in cell lysates (21, 32). Stimulation with either CD154 or PG102 induced movement of TRAF2 and TRAF3 to these membrane rafts; however, because their overall recruitment to CD40 was decreased (Fig. 5), the amounts in the rafts were also lower (data not shown). Fig. 5 shows that there was not a significant decrease in total TRAF6–CD40 binding induced by PG102 versus CD154. However, although CD40 translocates to the detergent-insoluble fraction following PG102 stimulation, PG102, when used alone to stimulate B cells, was unable to induce TRAF6 localization to this fraction (Fig. 6). Notably, PG102 treatment for 30 min or 2 h prior to the addition of hCD154 also inhibited hCD154-induced TRAF6 translocation (Fig. 6). Thus, the CD40 signaling complex induced by PG102 displayed altered associations of membrane CD40 with TRAF2, TRAF3, and TRAF6.

**Requirement for CD40 cross-linking to induce PG102-mediated signaling**

The results described above show that PG102-mediated CD40 engagement induced normal degradation of TRAF2 and TRAF3 but inhibited CD154-mediated CD40 signaling, without interfering with CD154 binding to CD40. Taken together, these data support the hypothesis that PG102-mediated inhibition of CD40 signaling is not passive, but instead induces formation of an abnormal signaling complex that inhibits CD154-induced CD40 signaling. Normal CD40 signaling requires cross-linking of membrane CD40. To determine whether aggregation of CD40 by PG102 is required for PG102-induced effects, we prepared Fab fragments of PG102, which bound normally to CD40 (Supplemental Fig. 1B). Fig. 7 demonstrates that, similar to intact mAb, PG102 Fabs did not induce CD40-mediated JNK or NF-κB1 activation, but the Fabs were ineffective in impairing agonist-induced activation of these events. Additionally, in contrast to intact PG102, Fab fragments did not induce TRAF degradation in human B cells. Thus, PG102 must induce CD40 aggregation to inhibit CD40 signaling by alteration of the signaling pathway.
Discussion

Initial discovery of the CD40 receptor preceded that of its ligand. Although agonistic mAbs to CD40 initially suggested its role as an immune cell activator (18, 33), the discovery of its key physiological roles in immune responses followed identification of the ligand for CD40 (initially called gp39 or CD40L, now designated CD154) (34–36). This, in turn, led to elucidation of the critical role for defective CD40 signaling in the human immunodeficiency disease X-linked hyper-IgM syndrome (37–40). Soon after the appreciation of CD40’s important functions in host immune defenses, studies implicated an important contribution of CD40-mediated activation in pathogenic processes, including transplant rejection, autoimmune diseases, and B cell malignancies (reviewed in Refs. 2–4, 41–43). Thus, CD40, and its activation by CD154 became attractive targets for these clinical problems.

The earliest and most frequent approach to inhibiting CD40-mediated signaling in vivo is to interfere with CD40–CD154 interactions. Blocking Abs to CD154 showed efficacy in the suppression of transplant rejection and graft-versus-host disease in both mouse and preclinical primate models (4, 7, 46–48). However, initial human clinical trials of the anti-hCD154 mAb hu5c8/BG9588/raplizumab in systemic lupus erythematosus patients, based upon these promising preclinical results, resulted in unexpected and serious thromboembolic complications in some patients. This problem also emerged in trials of other anti-CD154 mAbs (reviewed in Ref. 5). The cause of these complications is not entirely clear, but it may be related to the expression of CD154 on human platelets, together with FcRs that can bind the therapeutic mAbs.

An approach that circumvents the limitations of both of the prior strategies is the use of nonagonistic CD40 mAbs. A number of such reagents act by competing directly with CD154 for CD40 binding to CD154 showed efficacy in the suppression of transplant rejection and graft-versus-host disease in both mouse and preclinical primate models (4, 7, 46–48). However, initial human clinical trials of the anti-hCD154 mAb hu5c8/BG9588/raplizumab in systemic lupus erythematosus patients, based upon these promising preclinical results, resulted in unexpected and serious thromboembolic complications in some patients. This problem also emerged in trials of other anti-CD154 mAbs (reviewed in Ref. 5). The cause of these complications is not entirely clear, but it may be related to the expression of CD154 on human platelets, together with FcRs that can bind the therapeutic mAbs.

Thus, alternative approaches to inhibit CD40 signaling are needed. This could include development of CD154-blocking Abs or Ab fragments that cannot bind FcRs but retain high affinity for CD154 and show normal, unaccelerated serum clearance. However, if direct ligation of platelet CD154, not just FcR binding, is responsible for certain complications, this drawback will remain. Peptidomimetics can also provide a way to block CD40–CD154 interactions (49). However, peptide-binding affinities can frequently be modest, requiring large and potentially impractical quantities to effectively compete with CD154 for CD40 binding.

An approach that circumvents the limitations of both of the prior strategies is the use of nonagonistic CD40 mAbs. A number of such reagents act by competing directly with CD154 for CD40 binding
Relative quantification of amounts of TRAFs was measured in T5-1 human B cells, as in Fig. 5. Cell fractions were blotted for TRAF6 and hCD40. A representative of three total experiments is shown.

There is precedent for the concept that ligation of different epitopes on the CD40 molecule can induce distinct signaling cascades (50, 51), and even agonistic anti-CD40 Abs are not equivalent to CD40 stimulation with CD154. This raises the attractive possibility that smaller amounts of PG102 can be therapeutically effective in inhibiting CD40-mediated immune activation in vivo, while also avoiding any complications arising from ligation of CD154.

To gain further insight into how possible allosteric effects of PG102 binding may alter formation of the CD40 signaling complex, we examined two key features of this process. CD40 ligation results in immediate redistribution of CD40 into cholesterol-rich detergent-insoluble fractions of the plasma membrane (31, 32), to which it rapidly recruits TRAF2 and TRAF3, followed by a somewhat slower recruitment of TRAF6 (21, 54). The TRAF2/3 recruitment facilitates TRAF2-dependent K48-mediated polyubiquitination of these TRAFs, leading to their subsequent proteasomal degradation (26–28). This degradation limits and prevents prolonged CD40 signaling (31, 55, 56).

Thus, we examined each of these events in response to PG102 compared with agonistic stimuli. The results revealed that PG102 induced levels of TRAF2 and TRAF3 degradation that were indistinguishable from those induced by an agonistic CD40 stimulus in both human B cells and DCs (Fig. 4, Supplemental Fig. 2). However, the amounts of TRAF2 and TRAF3 recruited to total cellular CD40 were decreased significantly in both a human B cell line and freshly isolated PBMCs from normal donors. This resulted in a concomitant decrease in PG102-induced CD40 binding of TRAF2-associated signaling proteins, including cIAP1, IKKγ, and the ubiquitination regulator HOIP.

The latter findings are complex and intriguing. Reduced HOIP and IKKγ recruitment is consistent with the inability of PG102 to stimulate CD40-mediated NF-κB activation. However, although cIAP1 was reported to participate in CD40-mediated ubiquitination and subsequent degradation of TRAF2 and TRAF3, we found no detectable impairment of this regulatory event following PG102 ligation. Thus, although PG102 effectively stimulated events involved in normal restraint of CD40 signaling, this mAb did not stimulate activation events. This suggests that the reduced recruitment of TRAF2, cIAP1, and HOIP was nonetheless sufficient to mediate TRAF2 and TRAF3 degradation downstream of PG102 ligation and/or that additional unidentified CD40-binding molecules can also mediate these events. Recruitment of CD40 and associated TRAF molecules to detergent-insoluble membrane fractions was also altered when PG102 was used to engage hCD40.
compared with CD154. Membrane subdomain redistribution of all of these molecules was reduced in PG102-stimulated B cells, despite robust binding of PG102 to CD40, as demonstrated by CD40 staining and immunoprecipitation by PG102. This suggests that membrane recruitment to lipid-rich subdomains is of particular importance to initiate CD40-mediated activation signals. It is again possible that this involves facilitation of access of CD40 to signaling proteins localized in these subdomains that have not yet been identified. Although it was shown previously that CD40-mediated TRAF6 recruitment to detergent-insoluble membrane fractions of fibroblast cell lines impacts TRAF2-dependent CD40 signals to these cells (57), in B cells, CD40 molecules that cannot bind TRAF6 still induce TRAF2-dependent signals (30), suggesting cell-type specificity in some aspects of CD40 signaling. PG102-induced TRAF2 degradation was not greater than that induced by agonistic CD40 signals (Fig. 4), also indicating that reduced PG102-mediated TRAF6 recruitment to lipid rafts was not due to increased TRAF2 degradation.

The present study identified key characteristics of the mechanism by which PG102 interacts with CD40 and its signaling complex. PG102 did not exert its inhibitory functions by competing with CD154; instead, it induced a CD154-refractory state of early signaling pathways in B cells, features that enhance its potential to inhibit CD40 signaling effectively without requiring high concentrations and without the risk for inducing complications related to binding to CD154. The altered signaling complex formation and distribution induced by PG102 can provide clues and predictive value as biomarkers for further development of effective therapeutic reagents to block CD40 signaling.

There are numerous clinical conditions for which inhibiting CD40 signaling has considerable potential benefit, as discussed above. In addition, agonist binding to CD40 inhibits the survival and growth of malignant cells of various types (reviewed in Ref. 43). Recently, evidence was presented for an important role for T cell CD40 as an inhibitor of pathogenic T cell function in visceral adipose tissue in diet-induced obesity (58). The ability of PG102 to induce negative regulatory events downstream of CD40, such as TRAF degradation, may allow this reagent to trigger negative regulatory events downstream of CD40, CD40 signaling has considerable potential benefit, as discussed above.

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Disclosures
MdB. and M.T. are employees of Fast Forward Pharmaceuticals, which owns the PG102 mAb. G.A.B. has consultant arrangements with, and has received payments for travel expenses from, Fast Forward Pharmaceuticals. The other authors have no financial conflicts of interest.

References


**Suppl. Fig. 1.** Characteristics of PG102 reagents. 

**A.** Intact PG102 mAb does not induce CD40 internalization. T5-1 human B cells were treated as follows: (thin black lines), incubated with isotype control mAbs (human IgG4 or mouse IgG1) for 2h at 37°C, then washed and incubated with FITC-labeled secondary Abs to human IgG4 or mouse IgG1; (heavy black lines), incubated with PG102 (left panel) or G28.5 (right panel) for 2h at 37°C, then washed and incubated with secondary Abs as indicated above; (gray lines), incubated with PG102 (left panel) or G28.5 (right panel) for 30 min on ice, then washed and incubated with secondary Abs as indicated above. Cells were analyzed by flow cytometry.

**B.** Binding of PG102 intact IgG (left panel) vs. PG102 Fab fragments (right panel) to human CD40. Fab fragments were prepared as described in Methods (main text). Human JY B cells were incubated with saturating (20 μg/mL) PG102 intact IgGs or PG102 Fabs. Then, these PG102 intact IgGs and Fabs were detected with a combination of biotin-conjugated F(ab')2 fragment goat anti-human IgG F(ab')2 fragment-specific antibodies (Jackson Labs) and PE-labeled streptavidin.
Suppl. Fig. 2. Activation of early signaling pathways and TRAF2/3 degradation in human primary monocyte-derived DC. DC from normal human blood donors, matured in vitro with LPS, were stimulated with mAbs G28.5 or PG102 at 10 mg/ml, as described in Methods of the main text. A. Phosphorylation of the MAPKs indicated and IkBa were detected in cell lysates stimulated for the indicated number of minutes, as described in Methods, main text. B. Stimulation of DC for 5h with isotype control or PG102 mAb, followed by preparation of lysates as above, and Western blotting for TRAFs 2 and 3, with actin as a lane loading control. Results in both panels are representative of 3 similar experiments with different human blood donors.