Isotype Switching Converts Anti-CD40 Antagonism to Agonism to Elicit Potent Antitumor Activity

Highlights

- Antagonistic anti-CD40 mAbs can be converted into agonists by isotype switching to hlgG2
- Transformation is based upon the hlgG2 hinge
- Transforms an antagonist to an agonist four times more potent than existing anti-CD40 mAbs
- This converted antagonist exhibits antitumor synergy with cell therapy and vaccination

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In Brief

Yu et al. show that isotype switching can convert clinically relevant anti-CD40 antagonistic antibodies to potent FcγR-independent agonists. The converted antibodies can elicit strong antitumor responses in mouse models.
Isotype Switching Converts Anti-CD40 Antagonism to Agonism to Elicit Potent Antitumor Activity

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SUMMARY

Anti-CD40 monoclonal antibodies (mAbs) comprise agonists and antagonists, which display promising therapeutic activities in cancer and autoimmunity, respectively. We previously showed that epitope and isotype interact to deliver optimal agonistic anti-CD40 mAbs. The impact of Fc engineering on antagonists, however, remains largely unexplored. Here, we show that clinically relevant antagonists used for treating autoimmune conditions can be converted into potent FcγR-independent agonists with remarkable antitumor activity by isotype switching to hIgG2. One antagonist is converted to a super-agonist with greater potency than previously reported highly agonistic anti-CD40 mAbs. Such conversion is dependent on the unique disulfide bonding properties of the hlgG2 hinge. This investigation highlights the transformative capacity of the hlgG2 isotype for converting antagonists to agonists to treat cancer.

INTRODUCTION

CD40 is a costimulatory tumor necrosis factor (TNF) receptor widely expressed on immune and non-immune cell types (Elgueta et al., 2009; Lievens et al., 2009). The interaction between CD40 and its endogenous ligand CD40L is critical for mounting an effective immune response against exogenous pathogens and naturally arising tumors. Consequently, a breakdown in the homeostasis of the CD40/CD40L axis leads to both immunodeficiency and autoimmunity (Kamell et al., 2019; Senhaji et al., 2015). For example, patients with CD40 deficiency exhibit hyper IgM syndrome and are more susceptible to infections; while CD40 over-stimulation is implicated in various autoimmune syndromes, such as lupus and colitis (Banchereau et al., 1994; Peters et al., 2009). Moreover, CD40-mediated allogeneic T cell responses constitute a major mechanism of transplant rejection (Larsen and Pearson, 1997; Pinelli and Ford, 2015). These opposing immune pathologies have led to the development of two distinct classes of anti-CD40 antibodies that selectively modulate the CD40/CD40L axis.

Agonistic anti-CD40 mAbs mimic signals from CD40L-expressing helper CD4+ T cells to activate antigen-presenting cells, such as dendritic cells (DC), to provide signals for the licensing and expansion of CD8+ CTL (Bennett et al., 1998; Ridge et al., 2000; 2002).
Epitope Characterization of Antagonistic Anti-CD40 mAb 341G2

341G2 (bleselumab) is an hIgG4 antagonistic anti-CD40 mAb in clinical trials for plaque psoriasis and kidney transplant rejection (Anil Kumar et al., 2018; Harland et al., 2017). Extensive preclinical work in non-human primates demonstrates its safety and efficacy in prolonging survival of renal, pancreatic islet, and hepatic allografts (Imai et al., 2007; Oura et al., 2012; Watanabe et al., 2013). To characterize its binding epitope, we generated CHO-k1 cells expressing either the full-length CD40 molecule or truncated variants comprising one, two, or three of its CRDs. Consistent with previous reports, ChiLob 7/4, whose epitope was defined within CRD1 by X-ray crystallography (Yu et al., 2018), bound to cells expressing the full-length CD40 but not to variants lacking CRD1 (Figure 1A), while Lob 7/6 bound to all variants containing the CRD3 domain. Similar to ChiLob 7/4, 341G2 only bound to cells expressing the full-length CD40 molecule, indicating an epitope within CRD1 (Figure 1A). This was further supported by western blotting of similar soluble CD40 variants showing 341G2 only bound to the full-length CD40 protein (Figure 1B). As domain truncation might destabilize the protein structure, we performed alanine-scanning mutagenesis in which two consecutive residues were mutated to alanines to minimize structural disruption. Alanine-scanning mutagenesis confirmed that 341G2 binds to CRD1 but also indicated interaction with CRD2, with the majority of indicated contact residues located within CRD2 (Figures 1C and 1D). To better visualize the binding epitope, a 341G2 Fab-CD40 complex was generated, purified by size-exclusion chromatography (SEC) and crystallization trials performed alongside small-angle X-ray scattering (SAXS). No diffractable crystals were generated and so we performed homology modeling of 341G2 coupled to docking analysis, using the available knowledge of binding criteria as constraints: the surface residues of CD40 CRD1/2 identified through alanine-scanning mutagenesis to be in contact with 341G2 and the derived model indicates that 341G2 engages both CRD1 and CRD2, overlapping with the CD40L binding interface, and diametrically opposite the ChiLob 7/4 binding site (Figure 1E). Thus, 341G2 is distinct among previously characterized anti-CD40 mAbs in that binding is not limited to any single CRD but rather spreads between CRD1 and CRD2.

While epitope divides anti-CD40 mAbs into agonists and antagonists based on their intrinsic ability to block CD40/CD40L engagement, the effect of isotype and particularly the differential disulfide bonding pattern conferred by hlgG2 on antagonists remains unknown. Therefore, here we aimed to address these issues with respect to a series of clinically relevant antagonist mAbs.

RESULTS

Epitope Characterization of Antagonistic Anti-CD40 mAb 341G2

341G2 (bleselumab) is an hIgG4 antagonistic anti-CD40 mAb in clinical trials for plaque psoriasis and kidney transplant rejection (Anil Kumar et al., 2018; Harland et al., 2017). Extensive preclinical work in non-human primates demonstrates its safety and efficacy in prolonging survival of renal, pancreatic islet, and hepatic allografts (Imai et al., 2007; Oura et al., 2012; Watanabe et al., 2013). To characterize its binding epitope, we generated CHO-k1 cells expressing either the full-length CD40 molecule or truncated variants comprising one, two, or three of its CRDs. Consistent with previous reports, ChiLob 7/4, whose epitope was defined within CRD1 by X-ray crystallography (Yu et al., 2018), bound to cells expressing the full-length CD40 but not to variants lacking CRD1 (Figure 1A), while Lob 7/6 bound to all variants containing the CRD3 domain. Similar to ChiLob 7/4, 341G2 only bound to cells expressing the full-length CD40 molecule, indicating an epitope within CRD1 (Figure 1A). This was further supported by western blotting of similar soluble CD40 variants showing 341G2 only bound to the full-length CD40 protein (Figure 1B). As domain truncation might destabilize the protein structure, we performed alanine-scanning mutagenesis in which two consecutive residues were mutated to alanines to minimize structural disruption. Alanine-scanning mutagenesis confirmed that 341G2 binds to CRD1 but also indicated interaction with CRD2, with the majority of indicated contact residues located within CRD2 (Figures 1C and 1D). To better visualize the binding epitope, a 341G2 Fab-CD40 complex was generated, purified by size-exclusion chromatography (SEC) and crystallization trials performed alongside small-angle X-ray scattering (SAXS). No diffractable crystals were generated and so we performed homology modeling of 341G2 coupled to docking analysis, using the available knowledge of binding criteria as constraints: the surface residues of CD40 CRD1/2 identified through alanine-scanning mutagenesis to be in contact with 341G2 and the derived model indicates that 341G2 engages both CRD1 and CRD2, overlapping with the CD40L binding interface, and diametrically opposite the ChiLob 7/4 binding site (Figure 1E). Thus, 341G2 is distinct among previously characterized anti-CD40 mAbs in that binding is not limited to any single CRD but rather spreads between CRD1 and CRD2.
Antagonist Anti-CD40 mAbs Inhibit CD40L-Mediated Functions

To characterize the antagonistic nature of 341G2, we studied its ability to inhibit CD40L-mediated activities. Both the parental 341G2 hIgG4 and its hIgG1 variant inhibited the binding of CD40L to CD40-expressing Ramos cells in a dose-dependent but isotype-independent manner (Figure 2A), consistent with the fact that steric blockade of CD40/CD40L interaction by antagonists is independent of the Fc domain. Moreover, both 341G2 hIgG4 and 341G2 hIgG1 were able to inhibit CD40L-mediated hCD40Tg mouse splenic B cell and human B cell proliferation and homotypic cell-cell adhesion (Figures 2B and 2C).

To confirm the immunosuppressive effect of 341G2 in vivo, mice were immunized with ovalbumin (OVA) protein and the generation of anti-OVA IgG quantified. We found that both 341G2 hlgG4 and 341G2 hlgG1 significantly reduced the level of anti-OVA IgG in serum (Figure 2D); alongside a concomitant decrease in the level of circulating B cells (Figure 2E), a phenomenon observed in clinical trials of anti-CD40 mAbs possessing an intact Fc domain (Vonderheide, 2019). To confirm that the reduction in anti-OVA IgG in serum was not due to peripheral B cell depletion, we generated deglycosylated 341G2 hIgG1, lacking Fc effector functions (Lux et al., 2013; Nimmerjahn and Ravetch, 2008), which effectively suppressed the anti-OVA IgG response but did not result in B cell depletion (Figures 2D and 2E).

Isotype Switching to hlgG2 Converts Antagonist to Super-agonist

We previously showed that isotype switching to hlgG2 could significantly enhance the activity of agonistic anti-CD40 mAbs; however, the effect of hlgG2 on anti-CD40 antagonists has not been investigated. Therefore, we explored the impact of switching 341G2 to the hlgG2 isotype. In vitro functional assays showed that both 341G2 hlgG1 and 341G2 hlgG4 failed to
induce B cell proliferation at a range of concentrations, consistent with its antagonistic epitope; however, isotype switching to hlgG2 led to profound proliferation and homotypic cell-cell adhesion in hCD40Tg splenic B cells and purified human B cells (Figures 3A and 3B). A time course showed that 341G2 hlgG2-mediated proliferation was extremely rapid, with proliferation detectable as soon as 1 day after treatment and reaching a maximum on day 2 (Figure 3C). In contrast, CP870,893 (also hlgG2), reached maximal activity on day 4 and induced significantly less proliferation (Figure 3C). To enable the comparison of 341G2 hlgG2 activity with other clinically relevant anti-CD40 agonists, we generated the hlgG1 and hlgG2 variants of ADC1013, APX005M, CP870,893, ChiLob 7/4, and SGN40, and showed that 341G2 hlgG2 induced by far the most proliferation, similar to a trivalent CD40L (Figures 3D, S1A, and S1B). Its powerful agonism was further supported by its ability to trigger strong nuclear factor κB (NF-κB) signaling (Figure S1C) in the absence of any FcγR interactions, which are lacking in this system. To further probe the underlying molecular mechanism of such hlgG2-mediated, FcγR-independent agonism, we
Figure 3. 341G2 hIgG2 (h2) Is a Super-agonist In Vitro
(A) Purified hCD40Tg mouse splenic B cells were incubated with various concentrations of 341G2 h1, h2, and h4 for 2 days. Cell culture images were taken on day 2. Proliferation was measured by $^{3}H$-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments. Scale bar, 0.5 mm.
(B) Purified human B cell proliferation assay performed the same as in (A). Means ± SEM, n = 3, data representative of three donors. Scale bar, 0.5 mm.
(C) Purified hCD40Tg mouse splenic B cells were incubated with anti-CD40 mAbs for various periods of time as indicated above each plot. Proliferation was measured by $^{3}H$-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments.
(D) Purified hCD40Tg mouse splenic B cells were incubated with 2 µg/mL clinical anti-CD40 mAbs for 3 days as indicated above each plot. Proliferation was measured by $^{3}H$-thymidine incorporation. Means ± SEM, n = 4, data representative of three experiments.

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examined mAb-mediated CD40 clustering of a cell line expressing GFP-conjugated CD40. As shown in Figure 3E, the agonistic 341G2 hlgG1 caused no significant changes in CD40 clustering compared with the untreated control; in contrast, 341G2 hlglG2 induced significant clustering akin to that delivered by CD40L, indicating that hlglG2 converts antagonists to agonists by promoting receptor clustering. Furthermore, confocal analysis suggested that clusters remained proximal to the plasma membrane, even after extended periods of incubation (Figures S1D and S1E). The lack of apparent internalization was supported by in-vitro assays using both human and hCD40Tg mouse B cells, which demonstrate minimal differences in the level of CD40 detected at 37°C and 4°C, a condition known to reduce receptor internalization (Figure S1F).

To assess in vivo activity, we used an OTI CD8+ T cell expansion assay (White et al., 2011). Consistent with in vitro data, 341G2 hlglG1 was unable to expand OTI cells in vivo, whereas 341G2 hlglG2 led to significant expansion of the adoptively transferred cells, approximately 4-fold higher than CP870,893 (Figure 4A). Importantly, mice tolerated the delivery of this more active mAb. To assess potential toxicity, we assessed weight loss of mice after mAb treatment. We found that both 341G2 hlglG2 and CP870,893-hlgG1, the isotype which is the most agonistic in the mouse (Yu et al., 2018), induced similar levels of agonistic activity and toxicity (Figure S2A). To better recapitulate the human FcγR system, we also investigated the toxicity of 341G2 hlglG2 in hCD40Tg/mFcgr2b-/-/hFcgr2b+/+ mice that express both hCD40 and hFcγR1B. Using these mice, we compared the toxicity of 341G2 hlglG2 with APX005M, another strong anti-CD40 agonist observed in the clinic (O’Hara et al., 2015), 341G2 hlglG2 mediated stronger agonism than APX005M but induced no greater toxicity, demonstrating the possibility to separate agonism and toxicity and the potential therapeutic utility of 341G2 hlglG2 (Figure S2B). To evaluate potential cytokine release syndrome (CRS) effects we assessed for typical cytokine markers after anti-CD40 treatment. Consistent with clinical experience (Irenaeus et al., 2019; Vorderheide et al., 2007), agonistic anti-CD40 treatment transiently increased serum interleukin-6 (IL-6), TNF-α, and interferon-γ (IFN-γ) levels which returned to baseline after 48 h (Figure S2C). Interestingly, CP870,893-hlgG1 induced higher levels of inflammatory cytokines than 341G2 and CP870,893 hlglG2 at these times, demonstrating the impact of isotype on CRS-based toxicity.

To assess the requirement of FcγR for in vivo function, we generated hCD40Tg mice selectively deficient in FcγRIIB (hCD40Tg/Fcgr2b-/-) or lacking all FcγRs (hCD40Tg/FcγRnul) (Fransen et al., 2018). The levels of OTI expansion induced by 341G2 hlglG2 were similar between hCD40Tg, hCD40Tg/Fcgr2b-/-, and hCD40Tg/FcγRnul mice (Figure 4B), supporting the notion that the agonistic activity of 341G2 hlglG2 in vivo was independent of FcγR. Such FcγR-independent activity was further supported by the ability of 341G2 hlglG2-N297Q, an aglycosylated variant that exhibits significantly reduced affinity for all FcγR (Lux et al., 2013), and 341G2 hlglG2-V234A/G237A/P238S/H268A/V309L/A330S/P331S (c4d), an Fc mutant known to have almost no interaction for all FcγR (Vafa et al., 2014), to induce similar levels of B cell proliferation as the wild-type 341G2 hlglG2 in vitro (Figure 4C).

To further dissect the mechanism of the hlglG2-mediated, FcγR-independent, super-agonism, we examined the requirement for the hlglG2 hinge. The hlglG2 CH1 and hinge contain two additional cysteines that are absent in hlglG1 and crucial for the FcγR-independent activity of agonistic anti-CD40 mAbs via differential disulfide bonding (White et al., 2015). Consistent with previous reports, the ability of 341G2 hlglG2 to induce in vitro B cell proliferation and in vivo OTI expansion was lost when the CH1 and hinge domain of hlglG2 were replaced with those of hlglG1 (hinge 1/2) but not when the CH2 and CH3 domains in hlglG2 were replaced with those from hlglG1 (hinge 2/1) (Figures 4D and 4E). Differential disulfide bonding is also known to give rise to A and B isomers which differ in their conformation (White et al., 2015). We generated recombinant locked A (C232S/C233S) and B (C127S) forms of 341G2 hlglG2 via selective mutagenesis of key cysteine residues and found that, consistent with our previous findings, only the B form retained significant agonistic activity (Figures 4F and 4G).

As the hlglG2 isotype has previously been shown to induce cytotoxicity in vivo (Lux et al., 2014), we assessed the impact of 341G2 hlglG2 on the B cell compartment, a major CD40-expressing immune cell population prone to antibody-mediated depletion. Both 341G2 hlglG2 and its locked B form evoked a significant reduction in the proportion of circulating B cells in blood and concomitant increase in splenic B cells in both hCD40Tg and hCD40Tg/FcγRnull mice (Figures 4H and 4I), indicating FcγR-independent, CD40 activation-mediated B cell re-localization (as opposed to deletion), supported by the upregulation of CD23 on splenic B cells in vivo (Figure 4I).

341G2 Human IgG2 Activates Dendritic Cells

Agonistic anti-CD40 mAbs are thought to enhance antigen-specific T cell responses via DC stimulation (Ma and Clark, 2009). To confirm that the 341G2 hlglG2-induced CD8+ T cell expansion in vivo was driven by DC activation, we analyzed the splenic CD11c+CD8+DEC205+ DC subset known to be crucial for antigen cross-presentation (den Haan et al., 2000; Pooley et al., 2001) (Figure S3A). Consistent with their ability to drive CD8+ T cell expansion, both 341G2 hlglG2 and its locked B form induced significant upregulation of costimulatory molecules CD80 and CD86 in both hCD40Tg and hCD40Tg/FcγRnull mice, while hlglG1 and the locked hlglG2 A form were inert (Figures 5A and 5B). Moreover, anti-CD40 treatment did not significantly alter the frequency of this DC population (Figure 5B), indicating that they were not deleted. To evaluate the agonistic potential of 341G2 hlglG2 on human DCs, we generated monocyte-derived immature DCs and showed that treatment with 341G2 hlglG2 or 341G2 hlglG2-N297Q significantly upregulated costimulatory molecules CD86, CD70, and CD80, whereas 341G2 hlglG1 was inert (Figures 5C and S3B). Moreover,
Figure 4. 341G2 h2 Mediates Super-agonistic Activity In Vivo
(A) OTI cells (1 × 10^5) were adoptively transferred into hCD40Tg mice 1 day before treatment with 30 μg anti-CD40 mAbs as indicated. Mice were bled on day 5 and SIINFEKL^+^ cells were expressed as a percentage of total CD8^+^ T cells. Means ± SEM, n = 5, data representative of three experiments. Each dot represents one mouse. Two-tailed, non-paired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.
(B) OTI expansion assay performed the same as in (A) in hCD40Tg, hCD40Tg/Fcgr2b^−/−^, and hCD40Tg/FcγRnull mice. Means ± SEM, n = 5, data representative of two experiments. Each dot represents one mouse.
(C) Purified hCD40Tg mouse splenic B cells were incubated with various concentrations of 341G2 h2 Fc mutants as indicated for 2 days. Proliferation was measured by ^3^H-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments.
(D) Purified hCD40Tg mouse splenic B cells were incubated with various concentrations of 341G2 Fc hinge swapped variants as indicated for 2 days. Proliferation was measured by ^3^H-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments.
(E) OTI expansion assay performed the same as in (A); mice were treated with 341G2 Fc hinge swapped variants. Means ± SEM, n = 5, data representative of two experiments. Each dot represents one mouse.
(F) Purified hCD40Tg mouse splenic B cells were incubated with various concentrations of locked 341G2 h2 A and B forms for 2 days. Proliferation was measured by ^3^H-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments.
(G) OTI expansion assay performed the same as in (A); mice were treated with locked 341G2 h2 A and B forms. Means ± SEM, n = 5–7, data representative of two experiments. Each dot represents one mouse. Two-tailed, non-paired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.
(H) hCD40Tg and hCD40Tg/FcγRnull mice received 30 μg anti-CD40 mAbs on day 0 and were bled on day 2. The level of circulating CD19^+^ B cells in peripheral blood on day 2 was quantified by anti-mouse CD19-APC and expressed as the percentage of CD45.2^+^ cells. Means ± SEM, n = 6–7, data pooled from two experiments. Each dot represents one mouse. Two-tailed, non-paired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.

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341G2 hlgG2 induced a significant production of pro-inflammatory TNF-α and IFN-γ (Figure S3C). Interestingly, a lack of Fc effector function conferred by N297Q mutation led to higher levels of TNF-α, IL-12p70, and IFN-γ (Figure S3C). Furthermore, mixed leukocyte reaction assays showed that DCs treated with 341G2 hlgG2 mediated robust allogeneic T cell proliferation akin to the TLR4 agonist LPS (Figure 5D).

**Antagonist-Turned Super-agonist Exhibits Antitumor Activity**

Next, we examined the antitumor activity of the antagonist-turned super-agonist in three different solid tumor models: MC38 colon carcinoma, EG7 thymoma, and TC1 lung carcinoma. Mice with established MC38 tumors were treated with either the antagonistic 341G2 hlgG1 or agonistic 341G2 hlgG2. Consistent with its antagonistic nature, 341G2 hlgG1 conferred no therapeutic benefit compared with the control, whereas 341G2 hlgG2 significantly improved tumor control (Figure 6A), although it did not lead to appreciable numbers of long-term survivors (Figure 6A).

Given the limited efficacy of single-agent anti-CD40 mAbs in both preclinical models and clinical trials (Hoves et al., 2018; Perry et al., 2018; Vonderheide, 2019; Wiehagen et al., 2017), we explored different therapeutic strategies in an attempt to improve the long-term survival benefit. 341G2 hlgG2 was combined with anti-PD-L1 to remove inhibitory T cell signaling or with the immune-stimulatory mAb anti-CD27 to augmentulatory T cell signaling. Expectedly, anti-PD-L1 monotherapy imparted some modest antitumor activity; however, combination with 341G2 hlgG2 did not result in any synergy (Figure 6B). More encouragingly, while anti-CD27 mAb monotherapy conferred modest survival benefit, combination with 341G2 hlgG2 demonstrated a trend toward improved long-term survival (Figure 6C).

To further explore anti-CD40 mAb therapy for effective cancer treatment, we investigated the potentiating effect of 341G2 hlgG2 on adoptive T cell transfer that has demonstrated clinical efficacy in some difficult-to-treat cancers (Maude et al., 2018; Neelapu et al., 2017). Mice with established OVA-expressing EG7 tumors received adoptively transferred OTI cells and were then treated with anti-CD40 mAbs and OVA. The robust OTI expansion in response to 341G2 hlgG2 treatment was retained in those tumor-bearing mice (Figure 6D). Consistently, while 341G2 hlgG1 did not impart increased therapeutic efficacy to this modality, 341G2 hlgG2 significantly delayed tumor growth and led to long-term survival in almost 100% of mice (Figure 6E).

As adoptive cell transfer may not be amenable for large-scale treatment, we also explored the efficacy of 341G2 hlgG2 in a vaccination setting. The TC1 tumor cell line expresses the HPV-16 E6 and E7 oncoproteins (Lin et al., 1996), and a long HPV-16 peptide vaccine has led some vulvar intraepithelial neoplasia patients to exhibit complete responses in the clinic (Kenter et al., 2009). Mice were inoculated with TC1 tumor cells and then vaccinated with 341G2 hlgG2 and the peptide on day 5. Similar to the EG7 model, 341G2 hlgG2 led to a significant reduction in tumor size and 100% long-term survival, superior to CP870,893 (Figure 7A). Importantly, the same potent antitumor activity was recapitulated in hCD40Tg/FcγRnull mice, supporting the Fc-independent activity of 341G2 hlgG2 (Figure 7B).

The potent antitumor activity was supported by the induction of a robust endogenous tumor-specific CD8 T cell response (Figure 7C). The same therapeutic efficacy was recapitulated when a lower dose of the peptide vaccine was used (Figure S4).

**hlgG2-Mediated Antagonist-to-Agonist Conversion Is Generally Applicable**

As all clinical anti-CD40 antagonists are either hlgG1 or hlgG4, we investigated whether the ability to convert antagonists into agonists by isotype switching to hlgG2 is a general phenomenon. We chose CFZ533 (iscilimab, HCD122), currently being investigated clinically as an Fc-silent hlgG1 for Graves’ hyperthyroidism, primary Sjögren’s syndrome, and prolonging kidney allograft survival. Alanine-scanning mutagenesis mapped the epitope of CFZ533 toward the N terminus of CRD2, overlapping with the CD40L binding site (Figures 8A and 8B), consistent with its antagonistic properties. Similar to 341G2, CFZ533 hlgG2, but not CFZ533 hlgG1, was able to induce CD40 clustering (Figure 8C) and significantly activate the NF-κB signaling pathway (Figure 8D). Both CFZ533 hlgG2 and its aglycosylated variant CFZ533 hlgG2-N297Q comprised A and B isoforms (Figures S5A and S5B) and induced robust proliferation in hCD40Tg and hCD40Tg/Fcgr2b−/− mouse splenic B cells, whereas CFZ533 hlgG1 was inactive (Figures 8E and 8F). Moreover, CFZ533 hlgG2 significantly expanded OTI cells in vivo (Figure 8G). In addition, we investigated another anti-CD40 antagonist Abbv-323 (ravagailmab) that has undergone clinical testing in Crohn’s disease as an Fc-silent hlgG1. Similar to CFZ533, isotype switching to hlgG2 transformed Abbv-323 to an agonist able to trigger CD40 clustering, activate the NF-κB signaling pathway, induce hCD40Tg B cell proliferation, and expand OTI cells in vivo (Figures S6A–S6D).

**DISCUSSION**

The CD40/CD40L axis remains a prime target for immunotherapy. The CD40/CD40L pathway is central to both cellular and humoral adaptive immunity with implications for effective tumor surveillance/control, immune homeostasis, and autoimmune. Anti-CD40 mAbs that either potentiate or inhibit this pathway therefore possess powerful immune effects and are being explored in clinical trials to treat cancer and autoimmune diseases, respectively. The molecular requirements for effective agonism and antagonism, especially in relation to clinically relevant mAbs, have not been addressed equally. We previously showed that for agonistic anti-CD40 mAbs, both epitope and isotype interplay to govern the level of agonism (Yu et al., 2018). mAbs that target the membrane distal CDR1 display agonistic activity while those that target CRD2 to CRD4 exhibit antagonism (Yu et al., 2018).
et al., 2018). This activity is further modulated by both the fine epitope and isotype. Accordingly, binding of certain isotypes to the inhibitory FcγRIIB, expressed on B cells and various myeloid cell populations, was found to be indispensable for the activity of many agonistic anti-CD40 mAbs (Li and Ravetch, 2011; White et al., 2011). It is hypothesized that this activity is delivered through the trans engagement between the Fc domain and FcγRIIB stabilizing the Fab-CD40 complex to mimic the interaction between membrane-bound CD40L and CD40 (Beers et al., 2016; Li and Ravetch, 2013). More recently, however, we found that the requirement of the Fc domain for agonism could be obviated by isotype switching to hIgG2 (Yu et al., 2018). hIgG2 uniquely exhibits disulfide bond shuffling at its hinge region giving rise to isoforms that differ in their structural properties (Dillon et al., 2008; Martinez et al., 2008; Wypych et al., 2008). When engrafted onto agonistic anti-CD40 mAbs, hlgG2 was shown to impart enhanced agonistic activity independent of the Fc domain.

In the clinic, agonistic anti-CD40 mAbs possess either wild-type or engineered Fc for enhanced FcγR engagement, whereas antagonistic anti-CD40 mAbs are either hlgG4 or hlgG1 mutants with abrogated Fc effector function (Karnell et al., 2019; Vonderheide, 2019). Antagonists exhibit favorable safety profiles devoid of the CRS typically associated with their agonistic counterparts (Vonderheide, 2019). These clinical experiences affirm the notion that CD40 antagonism is predominantly epitope driven, while antibody-mediated CD40 agonism has both epitope and Fc requirements.

The effect of hlgG2, however, remained unexplored for clinically relevant CD40 antagonists. In this study, we found that isotype switching to hlgG2 converted three antagonists, 341G2, Abbv-323 and CFZ533, all in clinical trials for autoimmunity and transplant rejection, into potent Fc-independent agonists. Such conversion demonstrates that hlgG2 can override the inhibitory effect of antagonistic epitope. Interestingly, APX005M, an agonist in the clinic, was
reported to bind an epitope overlapping with the CD40L binding site and require FcγR for activity (Björck et al., 2016), demonstrating that epitopes that block CD40/CD40L interaction do not necessarily lead to antagonism in the presence of FcγR engagement. Indeed, enhanced crosslinking delivered by FcγRIIB-expressing accessory cells can overcome antagonistic epitopes (Yu et al., 2018) and Fc mutations conferring higher FcγRIIB binding led to improved antitumor activity (Dahan et al., 2016).

Figure 6. 341G2 h2 Exhibits Antitumor Efficacy and Potentiates Adoptive T Cell Therapy
(A) hCD40Tg mice were inoculated with $5 \times 10^5$ MC38 tumor cells subcutaneously. On day 6, when the tumor became palpable, mice were treated with 30 μg anti-CD40 mAbs and again 3 days later. Tumor size and survival were assessed, n = 11–14, data pooled from two experiments. The fractions in parentheses indicate the number of tumor-free mice (numerator) out of the total number of mice (denominator) in that group at the end of the study. Survival curves were compared by log rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) hCD40Tg mice with established MC38 tumors were treated with 30 μg anti-CD40 mAbs in combination with 100 μg anti-PD-L1 mAbs on day 6 and again 3 days later. Tumor size and survival were assessed, n = 11–14, data pooled from two experiments. The fractions in parentheses indicate the number of tumor-free mice (numerator) out of total mice (denominator) in that group at the end of the study. Survival curves were compared by log rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

(C) hCD40Tg mice with established MC38 tumors were treated with 30 μg anti-CD40 in combination with 100 μg anti-CD27 mAbs on day 6 and again 3 days later. Tumor size and survival were assessed, n = 11–14, data pooled from two experiments. The fractions in parentheses indicate the number of tumor-free mice (numerator) out of total number of mice (denominator) in that group at the end of the study. Survival curves were compared by log rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

(D) hCD40Tg mice were inoculated with $5 \times 10^5$ EG7 cells subcutaneously. On day 7, when the tumor became palpable, mice received $1 \times 10^5$ OTI cells via tail vein injection and 1 day later were treated with 30 μg anti-CD40 mAbs as indicated. Mice were bled 5 days after mAb treatment and SIINFEKL$^*$ cells quantified as a percentage of total CD8$^+$ T cells. Means ± SEM, n = 6, each dot represents one mouse. Data representative of two experiments. Two-tailed, non-paired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.

(E) hCD40Tg mice were inoculated with $5 \times 10^5$ EG7 cells subcutaneously. On day 7 when the tumor became established, mice received $1 \times 10^5$ OTI cells via tail vein injection and 1 day later were treated with 30 μg anti-CD40 mAbs as indicated. Tumor size and survival were assessed, Means ± SEM, n = 6, data representative of two experiments. The fractions in parentheses indicate the number of tumor-free mice (numerator) out of the total number of mice (denominator) in that group at the end of the study. Survival curves were compared by log rank test. *p < 0.05, **p < 0.01, ***p < 0.001.
variants also significantly reduced the number of circulating B cells. Such reduction was likely dependent on Fc effector function since deglycosylation of the hlgG1 variant restored the B cell number. hlgG1 was shown previously to interact with all murine FcγRs, including FcγRI and FcγRIIIA (Dekkers et al., 2017), contributing to IgG-mediated cellular cytotoxicity in certain models (Bevaart et al., 2006; Biburger et al., 2011; Bruhns and Jonsson, 2015; Minard-Colin et al., 2008). Accordingly, 341G2 hlgG4 caused a smaller reduction in B cell number compared with hlgG1, presumably due to its interaction with fewer activatory FcγRs. The pharmacodynamic effect of 341G2 hlgG4 on B cells in the clinic was not disclosed. In the context of human FcγRIIa known to mediate cellular depletion, hlgG4 engages with the activatory FcγRIIa (131R allelic variant) expressed abundantly on phagocytes (Bruhns and Jonsson, 2015; Dekkers et al., 2017); thus, the parental 341G2 hlgG4 may have a depleting effect on B cells.

Remarkably, 341G2 hlgG2 was found to exhibit four times higher potency than CP870,893 which remains the most agonistic anti-CD40 mAb to be tested clinically to date, capable of delivering some objective partial responses in cancer patients (Vonderheide et al., 2007). However, 341G2 hlgG2 induced no
more weight loss than CP870,893 or APX005M, both strong clinically relevant agonists. Moreover, 341G2 hIgG2 induced similar levels of transient inflammatory cytokines as CP870,893-hIgG2 but significantly less than CP870,893-mIgG1, indicating the impact of isotype on toxicity. Our data thus demonstrate the possibility to separate agonism and toxicity and highlight the potential therapeutic utility of 341G2 hIgG2. In addition to CRS, clinical anti-CD40 agonists induce transient B cell depletion from circulation (Irenaeus et al., 2019; Johnson et al., 2015; Vonderheide et al., 2007). Our observation that 341G2 hIgG2 significantly reduced circulating B cells but increased splenic B cell activation and accumulation in both hCD40Tg and hCD40Tg/FcγRnull mice suggests the possibility that these CD40-activated B cells re-localize from systemic circulation to other tissues in an FcγR-independent manner.

While the mechanism behind the super-agonistic 341G2 hIgG2 remains unclear, alanine-scanning mutagenesis and SEC-SAXS followed by docking analysis revealed that 341G2 engages both CRD1 and CRD2 domains, which, to our knowledge is unique among anti-CD40 mAbs characterized to date that only engage a single CRD. It is possible that a rigid hIgG2 hinge conferred by differential disulfide bonding combined with this dual-CRD targeting induces greater CD40 conformational changes amenable to receptor clustering and activation. Indeed, our finding that 341G2 hIgG2 induced significant CD40 clustering in a system that lacks all FcγR supports this theory. Moreover, orthogonal viewing of our confocal images and quenching assays indicate that the majority of those clusters remained proximal to the cell surface as opposed to being internalized in those cells we studied. These data are consistent with the previous observation that antibody-mediated CD40 internalization is less efficient than other receptors, including DEC205 and the mannose receptor (Chatterjee et al., 2012) as well as CD20 (Vaughan et al., 2014). Such correlation between receptor clustering and agonistic activity is in accordance with the paradigm of TNF receptor activation and its prerequisite for downstream

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**Figure 8.** Antagonist CFZ533 Is Converted into an Agonist by Isotype Switching to hIgG2

(A) CHO-k1 cells expressing different hCD40 mutants were probed with anti-CD40 mAbs. Bound mAbs were detected by anti-mouse IgG-FITC.

(B) Deduced epitope of CFZ533 is shown in red on a color-coded CD40 molecular scaffold (left) and displayed relative to the CD40/CD40L binding interface (right). The structure model is based on PDB: 3QD6.

(C) Jurkat cells stably transfected with human CD40EC-GFP were treated with 10 μg/mL CFZ533 h1 or CFZ533 h2 for 1 h at 37°C. Cells were then fixed, nuclear-stained using DAPI, and imaged using a Leica SP8 confocal microscope. z stack images shown. Blue, nucleus; green, human CD40-GFP. Scale bar, 4 μm. Image representative of at least ten images taken.

(D) Jurkat NF-κB GFP reporter cells stably transfected with hCD40 were incubated with various concentrations of anti-CD40 mAbs for 8 h and the level of NF-κB activation was assessed by GFP expression using flow cytometry. Means ± SEM, n = 3, data representative of two experiments.

(E) Purified splenic B cells from hCD40Tg mice were incubated with various concentrations of anti-CD40 mAbs for 4 days. Proliferation was measured by 3H-thymidine incorporation. Means ± SEM, n = 3, data representative of three experiments.

(F) Experiments the same as (E), splenic B cells from hCD40Tg/FcγRnull mice were used. Means ± SEM, n = 3, data representative of three experiments.

(G) 1 x 10^5 OT1 cells were adoptively transferred into hCD40Tg mice 1 day before treatment with 100 μg anti-CD40 mAbs as indicated. Mice were bled on day 5 and SIINFEKL+ cells were quantified as a percentage of total CD8+ T cells. Means ± SEM, n = 5, each dot represents one mouse, data representative of two experiments. Two-tailed, non-paired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figures S5 and S6.
receptor signaling (Ward-Kavanagh et al., 2016). Powerful epitope-driven agonism was previously reported for CP870,893, which binds to residues on the CRD1 domain facing away from the CD40L binding site and exhibits similarly high levels of agonistic activity independent of isotype (Yu et al., 2018). Interestingly, 341G2 hlgG2 was previously described to provide synergy with the TLR3 agonist poly(IC:LC) in a viral vaccination setting, although neither the rationale for using hlgG2 isotype nor the antagonistic nature of 341G2 were discussed (Thompson et al., 2015).

The agonistic activity of 341G2 hlgG2 was recapitulated in mice lacking FcγRIIb or all FcγRs, demonstrated by both in vivo DC activation and CD8+ T cell expansion, consistent with the previous finding that hlgG2 imparts agonism in an FcγR-independent manner (White et al., 2015). Such FcγR-independent agonism may overcome the challenge of heterogeneous FcγR expression among different tumor microenvironment and inter-patient variability to deliver more consistent agonistic activity. Accordingly, the aglycosylated variant 341G2 hlgG2-N297Q also potently activated B cells and human DCs. Interestingly, at the cytokine level, the absence of Fc effector function conferred by the N297Q mutation induced significantly higher levels of TNF-α, IL-12p70, and IFN-γ in human DCs, which suggests that Fc-FcγR engagement on DCs may negatively regulate CD40-mediated activation. Human monocye-derived DCs express predominantly FcγRIIA and FcγRIIB; moreover, FcγRIIB-mediated DC suppression has been described before (Boruchov et al., 2005; Dhodapkar et al., 2007). FcγRIIB present on DCs (in cis or in trans) could potentially influence CD40 activation through the ITIM signaling motif (Pauls and Marshall, 2017). Although hlgG2 does not engage FcγRIIB in solution, interactions could occur when the FcγRIIB density reaches a critical level, such as when FcγRIIB-expressing cells are used in assays to provide Fc crosslinking (Dudek et al., 2019; White et al., 2011).

In addition to the direct immunomodulatory effect, we also investigated the antitumor activity of the antagonist-turned agonist. 341G2 hlgG2 monotherapy significantly improved the survival rate of tumor-bearing mice compared with its agonistic hlgG2 and an agonistic anti-CD27 mAbs in improving long-term survival, and future experiments will focus on mechanistic dissection of this synergy to inform on better clinical strategy (Buchan et al., 2018). In addition to combination therapy, the route of administration could influence the clinical utility of anti-CD40 mAbs. For example, it was demonstrated that intratumoral, rather than systemic, delivery of anti-CD40 mAbs led to enhanced antitumor activity with reduced toxicity (Knorr et al., 2018).

Besides antibody therapy, the CD40/CD40L axis has been exploited to improve the response rate of chimeric antigen receptor (CAR) T cell therapy in solid tumors. The ectopic expression of CD40L in CAR T cells has been shown to potentiate endogenous tumor-specific T cell responses (Kuhn et al., 2019). Moreover, the introduction of the CD40 signaling domain into CAR T cells led to superior T cell effector function (Mata et al., 2017). Consistent with these reports, our data show that 341G2 hlgG2 enabled adoptively transferred T cells to achieve long-term survival in a solid tumor model. A similarly impressive synergistic therapeutic effect was achieved in a peptide vaccination setting—with 341G2 hlgG2 exhibiting greater efficacy than CP870,893.

In summary, we demonstrate that antagonistic anti-CD40 mAbs can be converted into potent FcγR-independent agonists by isotype switching to hlgG2, in a manner that is dependent upon the hlgG2 hinge. The success of agonistic anti-CD40 mAbs in oncology will likely involve combination with other agents and we provide data in support of possible approaches for subsequent development. Further investigation into immunological function and dysfunction associated with anti-CD40 mAbs will help inform on the rational combinations to advance to the clinic.
TNF receptors, such as 41BB, as well as members of the B7-CD28 superfamily (White et al., 2015), and so similar antagonism to agonism conversion remains a possibility for these other specificities. The hlgG2 isotype has previously been used as a more Fc-“inert” IgG molecule with reduced effector functions due to its restricted C1q and FcγR-binding properties (Vidrunsson et al., 2014). However, the transformative property of the hlgG2 isotype described in this study, converting antagonists to potential super-agonists, heeds caution for the use of this isotype in the development of these reagents where pure blocking and antagonism are desired.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.ccell.2020.04.013.

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**AUTHOR CONTRIBUTIONS**

X.Y. designed and performed the experiments, analyzed and interpreted data, and wrote the manuscript. H.T.C.C., C.A.P., J.K., H.F., T.I., C.I.M., R.R.F., P.J.D., L.R.D., V.E., J.S.V., A.L.W., and I.T. generated or provided key reagents or performed and analyzed the research. M.J.G. designed the study, supervised data collection, discussed and interpreted data, and edited the manuscript. M.S.C. designed the study, supervised data collection, discussed and interpreted data, and wrote the manuscript with X.Y. M.J.G. and M.S.C. were co-senior authors on the study.

**DECLARATION OF INTERESTS**

M.S.C. acts as a consultant for a number of biotech companies, being retained as a consultant for Biolinvent and has received research funding from BioInvent, GSK, UCB, Tieso, and Roche. M.J.G. acts as a consultant to a number of biotech companies and receives institutional payments and royalties from antibody patents and licenses. This work is related to patent Family WO 2015/145360 protecting antibodies containing modified hlgG2 domains which elicit agonist or antagonistic properties.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**       |        |            |
| Goat F(ab’)2 Anti-Human IgG - Fc (PE) | Abcam | Cat# ab986596; RRID:AB_10673825 |
| 341G2 and isotype variants | In house | Published patent: US8776451B2 |
| CP870,893 and isotype variants | In house | Published patent: US20090130715A1 |
| ADC1013 and isotype variants | In house | Published patent: WO2016023960A1 |
| APX005M and isotype variants | In house | Published patent: WO2014070934A1 |
| SGN40 and isotype variants | In house | Published patent: WO2007075326A2 |
| HCD122 and isotype variants | In house | Published patent: WO2012075111A1 |
| Abbv-323 and isotype variants | In house | Published patent: WO2016196314A1 |
| Goat Anti-Human IgG Fc (HRP) | Abcam | Cat# ab98624; RRID:AB_10673832 |
| Goat F(ab’)2 Anti-Mouse IgG Fc (PE) | Abcam | Cat# ab98649; RRID:AB_10674947 |
| Mouse monoclonal anti-OVA IgG (clone KB4) | In house | N/A |
| Anti-mouse CD19-APC | Biolegend | Cat# 152410; Clone 1D3; RRID:AB_2629839 |
| Anti-mouse CD45.2-FITC | Biolegend | Cat# 109806; clone 104; RRID:AB_313443 |
| Anti-mouse CD8-APC | eBioscience | Cat# 17-0081-82; Clone 53-6.7; RRID:AB_469335 |
| Anti-mouse CD23-PE | eBioscience | Cat# 12-0232-83; Clone B3B4; RRID:AB_465594 |
| Anti-mouse CD11c-Pacific Blue | Biolegend | Cat# 117322; Clone N41B; RRID:AB_755988 |
| Anti-mouse DEC205-PE-Cy7 | Biolegend | Cat# 138210; Clone NLDC-145; RRID:AB_10643581 |
| Anti-mouse CD86-APC | Biolegend | Cat# 105012; Clone GL-1; RRID:AB_493342 |
| Anti-mouse CD80-APC/Fire™ 750 | Biolegend | Cat# 104740; Clone 16-10A1; RRID:AB_2687095 |
| Anti-human CD86-Alexa Fluor® 488 | Biolegend | Cat# 305414; Clone IT2.2; RRID:AB_528881 |
| Anti-human CD70-PE | Biolegend | Cat# 355104; Clone 113-16; RRID:AB_2561431 |
| Anti-human CD80-PerCP/Cy5.5 | Biolegend | Cat# 305232; Clone 2D10; RRID:AB_2566491 |
| Anti-mouse PD-L1 (B7-H1) | BioXCell | Cat# BE0101; Clone 10F.9G2; RRID:AB_10949073 |
| Anti-mouse CD27 (clone AT124-1, mouse IgG1) | In house | N/A |
| Anti-mouse CD8-Brilliant Violet 510™ | Biolegend | Cat# 100752; Clone 53-6.7; RRID:AB_2563057 |
| Anti-mouse FcγR2B-FITC (clone AT150-2) | In house | N/A |
| **Biological Samples** | | |
| Healthy human PBMC | Southampton General Hospital National Blood Service | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| PE-labeled SIINFEKL/H-2Kb tetramer | In house | N/A |
| Ovalbumin | Sigma-Aldrich | Cat# A2512 |
| Lymphoprep | Axis-Shield | Cat# 07861 |
| Erytholyse Red Cell Lysis Buffer | AbD Serotec | Cat# BUF04B |
| N-Glycosidase F (PNGaseF) | Promega | Cat#V483A |
| DAPI | Life Technologies | Cat# D1306 |
| Recombinant human CD40 and domain truncated variants | In house | N/A |
| Recombinant human CD40 alanine-scanning mutants | In house | N/A |
| Tritium thymidine (Methyl-3H) | PerkinElmer | Cat# NET027250UC |
| PE-labeled RAHYNIVTF/H-2Db tetramer | In house | N/A |
| HPV-16 peptide GQAEPDRAHYNIVTFCCKCDSTLRCSTLRLCVQSTHVDIR | GL Biochem | Customized |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Professor Mark Cragg (msc@soton.ac.uk)

**Materials Availability**
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

**Data and Code Availability**
The original SAXS data for CD40 - 341G2 F(ab) complex have been deposited into the small angle scattering biological data bank.
SASBDB: SASDHN8
Data supporting the current study are available from the corresponding author upon request.

## Table:

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant human CD40 ligand | In house | N/A |
| Critical Commercial Assays |
| ELISA assay to detect mouse TNF-α | Biolegend | Cat# 430901 |
| ELISA assay to detect mouse IL-6 | Biolegend | Cat# 431301 |
| ELISA assay to detect mouse IFN-γ | Thermofisher | Cat# BMS606TWO |
| Luminex bioplex to detect human TNF-α, IP-10, MCP-1, IL-12p70 and IFN-γ | Biorad | Customized |
| Mouse B cell isolation set | Stemcell Technologies | Cat# 19854 |
| Human monocyte isolation set | Miltenyi Biotec | Cat# 130-096-537 |
| Human CD4+ T cell isolation set | Stemcell Technologies | Cat# 19852 |
| Deposited Data |
| SAXS data for CD40 - 341G2 F(ab) complex | Small angle scattering biological data bank | Accession Code: SASDHN8 |
| Experimental Models: Cell Lines |
| Mouse tumor: EG7 | (White et al., 2015) | N/A |
| Mouse tumor: TC1 | (Lin et al., 1996) | N/A |
| Mouse tumor: MC38 | Dr Rienk Offringa (German Cancer Research Center) | N/A |
| Jurkat | ATCC | TIB-152 |
| Ramos | ATCC | CRL-1596 |
| CHO-k1 | ATCC | CCL-61 |
| ExplicHO | Thermofisher | A29127 |
| FreeStyle 293F | Thermofisher | R79007 |
| Experimental Models: Organisms/Strains |
| OT-I TCR transgenic on C57BL/6 background | Charles River Laboratories | Strain code: 642 |
| hCD40Tg on C57BL/6 background | (White et al., 2015) | N/A |
| hCD40Tg/Fce1g-/-/FcγR2b-/- on C57BL/6 background | This paper | N/A |
| hCD40Tg/mFcγR2b-/-/hFcγR2b-/- on C57BL/6 background | (Roghanian et al., 2015) | N/A |
| Software and Algorithms |
| Prism | GraphPad | N/A |
| Flowjo | BD Biosciences | N/A |
| FCS Express | De Novo Software | N/A |
| Leica Application Suite X | Leica | N/A |
| ScAtter v3.2h - R. Rambo | Dr. Robert Rambo (Diamond Light Source) | http://www.bioisis.net/ |
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**

hCD40 transgenic mice (hCD40Tg) were kindly provided by Randolph Noelle (King’s College, London) and were described before (Ahonen et al., 2002). Fcer1g−/−, Fcgr2b−/− mice (C57BL/6 background) were generated by Dr J. Sjef Verbeek (Toin University of Yokohama, Japan), and FcγRII null mice (Fcer1g−/− x Fcgr2b−/−) were generated through breeding Fcer1g−/− and Fcgr2b−/− mice to generate homozygous FcγRII null mice (Fransen et al., 2018). The hCD40Tg/Fcgr2b−/− mice were generated by crossing hCD40Tg mice with Fcgr2b−/− mice, and hCD40Tg/FcγRII null mice were generated by crossing hCD40Tg mice with homozygous FcγRII null mice. The hCD40Tg/mFcgr2b−/−/hFcgr2b−/− mice were generated by crossing hCD40Tg mice with mFcgr2b−/−/hFcgr2b−/− mice described previously (Roghanian et al., 2015). The presence of hCD40 and lack of FcγRs were confirmed by flow cytometry. The lack of FcγRIIB, the only FcγR known to be expressed by murine B cells, is shown in Figure S7A. OTI TCR transgenic mice were from Charles River Laboratories (Kent, UK). All animals, including wild-type C57BL/6 mice, were maintained and bred in house. For all experiments, age and sex-matched animals were randomized and assigned to experimental groups. All experiments were conducted under UK Home Office licence numbers PB24EEE31, P4D9C89EA, P540CBA98, and P39FE2AA7 and following approval by local ethical committees, reporting to the Home Office Animal Welfare Ethical Review Board (AWERB) at the University of Southampton.

**Human Samples**

Human B cells, T cells and monocytes were purified from human PBMCs obtained from healthy donor leukocyte cones through Southampton National Blood Services with prior informed consent and Ethical approval from the East of Scotland Research Ethics Service, Tayside, UK.

**Cell Lines**

MC38 and EG7 (Moore et al., 1988) (ATCC) cell lines were maintained in a humidified incubator at 37°C and 5% CO2 and cultured in RPMI supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1 mM pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. The MC38 cell line was kindly provided by Dr Rienk Offringa (German Cancer Research Center). EG7 cells were cultured in the additional presence of 0.4mg/mL genetin and 50 µM β-Mercaptoethanol. The TC1 cell line (Lin et al., 1996) transduced with the HPV E6 and E7 oncogenes was originally from Dr Bjarni Bogen (University of Oslo), and cultured in IMDM supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 1mM pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.4 mg/mL genetin and 50 µM β-Mercaptoethanol.

**METHOD DETAILS**

**Antibodies and Reagents**

ChiLob 7/4 and Lob 7/6 were generated in-house, their variable domain sequences were amplified by PCR from the hybridoma cells to allow isotype switching. The variable domain sequences of 341G2 (US8716451B2), CP870,893 (US20090130715A1), ADC1013 (WO2014070934A1), APX005M (WO2014070934A1), SGN40 (WO2007075326A2), HCD122 (WO2012075111A1) and Abbv-323 (WO2016196314A1) were derived from published patents and genes containing these sequences were synthesized by GeneArt. DNA fragments encoding the light and heavy chain variable domain sequences were then cloned into the pEE12.4 and pEE6.4 expression vectors (Lonza, UK), respectively at the HindIII (N-terminus end) and EcoRI (C-terminus end) restriction sites. pEE6.4 vectors encoding different IgG isotypes were generated to allow isotype switching. Fc mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Agilent, UK). Antibodies were produced either from hybridoma cells or by transient expression in CHO cells and subsequently purified on protein A columns (GE healthcare, UK). All antibodies were checked to contain < 1% aggregate determined by HPLC and tested to contain < 5EU endotoxin per 1 mg antibody assessed by the Endosafe-PTS portable test system (Charles River Laboratories, L’Arbresle, France). Antibody deglycosylation was achieved by treatment with PNGase F (Promega, UK) for 48 hours at 37°C, and deglycosylation was confirmed by a heavy chain band shift in reducing SDS-PAGE. Capillary electrophoresis (CE-SDS) was used to distinguish the A and B forms of hIgG2 using a Beckman PA800 Plus analyser. The CE-SDS profiles of 341G2 and CFZ533 are shown in Figure S5.

SIINFEKL peptide was from Peptide Protein Research Ltd. The HPV-16 peptides RAHYNIVTF (E749-57, UniProt numbering) and GQAEPRAHYNIVTFCKCDSTLRCLVQSTHVDIR (E743-77, UniProt numbering) were purchased from GL Biochem (Shanghai, China). PE-conjugated SIINFEKL/H-2Kb tetramer and PE-conjugated RAHYNIVTF/H-2Db tetramer were both produced in-house. Chicken ovalbumin was purchased from Sigma. Recombinant trimeric CD40L was produced in house. DNA construct encoding human CD40L (Met113-Leu261) fused with a FLAG tag and GCN4 leucine zipper motif via a (G3S)3 linker at the N-terminus was synthesized by GeneArt and then subcloned into pDSG104 vector (IBA Life Sciences, Germany). The plasmid was transfected into MEXI293E cells (IBA Lifesciences) for 7 days before purification by anti-FLAG Affinity Gel (Sigma, UK).

Flow cytometry experiments were conducted using FACSCalibur or FACSCanto II (both from BD Biosciences).
Generation of CD40 Constructs and Epitope Mapping

DNA sequences encoding the wild-type full-length extracellular (EC) domain of CD40 (i.e. CRD1-4), or truncated variants CRD2-4, CRD3-4 and CRD4 were amplified by PCR and cloned into pcDNA3.1 for recombinant soluble protein expression. For cell surface expression, the same constructs were cloned into the pcIpuro vector (Promega) that contains the CD40 transmembrane domain at the N-terminus for membrane anchoring. The full-length human CD40EC-GFP fusion construct was cloned into pcIpuro for cell surface expression. For recombinant soluble protein expression of truncated CD40 variants, FreeStyle 293F cells (Thermo Fisher Scientific) were transfected with the DNA/PEI complex and cultured for 7 days before supernatant was harvested for purification using Ni-NTA column (GE Healthcare). For cell surface expression, CHO-k1 cells were transfected with various CD40 constructs using Gen-ePORTER Transfection reagent (Amsbio, UK) and stable clones were selected using puromycin (Invivogen, UK).

High-resolution epitope mapping of 341G2 and HCD122 was achieved by alanine scanning mutagenesis of CD40 CRD1 and CRD2 domains as described previously (Yu et al., 2018). Briefly, two consecutive residues were mutated to alanines throughout CRD1 and CRD2; each construct was expressed on the CHO-k1 cell surface and stable clones were selected using 10 μg/mL puromycin (Invivogen, USA). For recombinant soluble protein expression of CD40 alanine scanning mutants, DNA was cloned into the pcIpuro vector with a C-terminal His-tag, expressed in ExpiCHO cells and purified using a Ni-NTA column.

Confocal Microscopy

Jurkat cells were transfected with pcIpuro plasmid encoding a human CD40EC-GFP construct using Nucleofector Kit V (Lonza) and stable clones were selected using 1 μg/mL puromycin. To assess the effect of anti-CD40 mAb on CD40 receptor clustering, cells were incubated with 10 μg/mL anti-CD40 mAb for one hour at 37°C and then fixed with methanol before DAPI staining for the nucleus. Confocal images were acquired using a Leica SP8 confocal microscope and analysed using Leica Application Suite X (both from Leica).

In Vitro Receptor Internalization Assay

The level of CD40 internalization was quantified using a fluorescence quenching assay as previously described (Austin et al., 2004). Briefly, anti-CD40 mAbs were labelled with AF488 using AF488 Antibody Labeling Kit (Thermo Fisher Scientific) and then added to various purified B cell cultures as indicated for 10, 30, 60, 120 or 180 minutes at 4°C or 37°C. Cells were then washed and half the cells were treated with anti-AF488 antibody (Thermo Fisher Scientific) at 4°C that quenches AF488 fluorescence. The remaining AF488 fluorescence analysed by flow cytometry correlates to internalized CD40. % Total Expression quantifies remaining cell surface-bound CD40 and was calculated as % (unquenched fluorescence – quenched fluorescence)/ (unquenched fluorescence).

Surface Plasmon Resonance

The affinity of various anti-CD40 mAbs for hCD40 was analysed using a Biacore T100, all reagents used were from GE Healthcare Life Sciences, UK. hCD40 was immobilized onto a CM5 sensor chip via amine coupling according to manufacturer’s recommendations. Anti-CD40 mAbs were injected through the flow cells at 250, 50, 10, 2, 0.4, and 0 nM in HBS-EP+ running buffer at a flow rate of 30 μL/min, allowing 300 seconds for association and 300 seconds for dissociation. The sensorgrams were fitted with 1:1 Binding model, and the ka, kd, and KD were calculated using Biacore Bioevaluation software. The parental 341G2 and its Fc variants bound CD40 with similar affinities (Figure S7B, Table S1). To analyse the affinity of anti-CD40 for hCD40 mutants, His-tagged soluble hCD40 variants were captured for 30 seconds using anti-His mAb immobilized on CM5 chip, and Lob 7/6 and 341G2 (both hIgG1 isotype) were injected at 1000, 333, 111, 37, 12.4, and 4.1 nM using the Biacore T100 instrument. The association and dissociation phases lasted 180 and 300 seconds, respectively.

Western Blot

His-tagged recombinant CD40 proteins were run on 18% polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked in PBS 5% non-fat dried milk 0.05% Tween 20 and then incubated with various anti-CD40 mAb at 4°C overnight before detection using secondary polyclonal goat anti-human IgG-HRP (Abcam, UK). The membranes were washed with PBS before the addition of ECL and the signals were captured on a UVP Biospectrum Imaging System.

Size Exclusion Chromatography Coupled with Small-Angle X-Ray Scattering (SEC-SAXS)

A F(ab) fragment of 341G2 hlgG1 was generated by digestion with papain-agarose (Thermo Fisher Scientific) followed by incubation with protein A to deplete intact IgG1 and Fc fragments, before further purification by SEC. Purified 341G2 F(ab) and hCD40 EC domain were mixed at a 1:1 molar ratio for 1 hour and the F(ab)-CD40 EC complex was subsequently purified by SEC. SEC-SAXS data were collected at the B21 beamline at Diamond Light Source, UK. 45 μL of F(ab)-CD40 EC complex at 7.7 mg/mL was injected in a Superdex 200 increase 3.2/200 column using PBS as the SEC-buffer at a flow rate of 0.075 mL/minute. Measurements were performed at 20°C. Data were recorded from 620 second continuous exposures using a Pilatus 2M detector. Data were processed and analysed using the Scatter software (V3.2h, R.Rambo, DLS). Frames for analysis were selected using the subtract tab, allowing for identification of buffer frames from the SEC-buffer flow through and scattering frames from the elution peak. The final curve was generated after buffer subtraction. A list of SAXS-derived parameters are shown below:
Homology Modelling and Docking
341G2- the sequence of 341G2 Fab was submitted to the SWISS-MODEL server to generate a homology model (Waterhouse et al., 2018). The model was generated based on the 56.a.09 antibody (PDB: 5K9J) with which 341G2 Fab shares 90.4% sequence identity and 0.57 sequence similarity score. The Global model quality estimation (GMQE) score for the resulting model was 0.97 with the QMEAN score being 0.62 indicating good confidence in the quality of the generated model.

CD40- Phyre2 (Kelley et al., 2015) was used to generate a model of CD40EC. The CD40EC sequence (Uniprot: P25942) was submitted for modelling, returning a model based on PDB: 5DMJ that had a confidence score of 100% with 97% coverage indicating a suitable model for further analysis.

Docking of 341G2 F(ab) and CD40 was performed using the HADDOCK 2.2 webserver (van Zundert et al., 2016). Residues on CD40 known to affect binding (identified through alanine scanning mutagenesis experiments), and the CDR loop residues of 341G2 were provided to the docking server as restraints in the docking following the HV-Epi9 protocol detailed in (Ambrosetti et al., 2020). For CD40 these residues were S49, D50, F67, L68, T75, H76, H78 and Q79. For the 341G2 model, the CDR loop residues were 26 to 34, 53 to 59, 104 to 112, 260 to 268, 284 to 288 and 325 to 331. The CDR loops were defined as active residues, while the CD40 residues were defined as active with a 9Å radius of passive residues. All HADDOCK settings were default apart from rigid-body refinement sampling which was increased to 5000 models and the flexible and water refinement sampling were increased to 400 models. Docking produced 10 clusters comprising 40 representative structures. Based on the knowledge that 341G2 and ChiLob 7/4 do not cross-block, a large number of the representative models were able to be excluded due to clashing with the Chilob 7/4 Fab from the crystal structure PDB: 6FAX when aligned on CD40 (Yu et al., 2018). This left a total of 11 models from 3 clusters that were taken

### Sample Details

| Sample Details                                           | CD40EC-341G2 Fab |
|----------------------------------------------------------|------------------|
| Organism                                                 | Homo sapiens     |
| Uniprot sequence ID (residues in construct)              | –                |
| Extinction coefficient                                    | 101760           |
| Protein mass from chemical composition                   | 67637            |
| SEC-SAXS, Superdex 200 increase 3.2/200                  |                  |
| Loading concentration (mg/ml)                            | 7.7              |
| Injection volume                                         | 45 μL            |
| Flow rate (mL/min⁻¹)                                     | 0.0075           |
| SEC-buffer                                               | PBS              |

### SAXS Data-Collection Parameters

| Instrument/data-processing                              | B21, Diamond Light Source (DLS), Harwell (UK) |
|----------------------------------------------------------|-----------------------------------------------|
| Wavelength (Å)                                           | 1.0                                           |
| Beamsize (μm)                                            | 1 x 1                                         |
| Camera length (m)                                        | 4.0                                           |
| q measurement range (Å⁻¹)                                | 0.004 – 0.408                                 |
| Absolute scaling method                                  | Comparison with BSA                           |
| Normalisation                                            | To integrated intensity from beam-stop diode  |
| Monitoring for radiation damage                          | Frame comparison                              |
| Exposure time                                            | 1860 (620 x 3 s)                              |
| Sample temperature (°C)                                  | 20                                            |

### Software Employed for SAXS Data Reduction, Analysis and Interpretation

| SAXS data reduction                                      | DAWN pipeline (DLS, Harwell, UK)               |
|----------------------------------------------------------|-----------------------------------------------|
| Extinction coefficient estimate                           | ProtParam                                     |
| Basic analyses: Guinier, P(r), Vp                        | PRIMUSqt from ATSAS 2.8.2                     |
| Atomic structure modelling                               | CRYSOL from PRIMUSqt in ATSAS 2.8.2          |
| Sequence modelling                                       | SWISS-MODEL, Phyre2                          |
| Molecular graphics                                       | PyMol 2.3.0                                   |

**Homology Modelling and Docking**

341G2- the sequence of 341G2 Fab was submitted to the SWISS-MODEL server to generate a homology model (Waterhouse et al., 2018). The model was generated based on the 56.a.09 antibody (PDB: 5K9J) with which 341G2 Fab shares 90.4% sequence identity and 0.57 sequence similarity score. The Global model quality estimation (GMQE) score for the resulting model was 0.97 with the QMEAN score being 0.62 indicating good confidence in the quality of the generated model.

CD40- Phyre2 (Kelley et al., 2015) was used to generate a model of CD40EC. The CD40EC sequence (Uniprot: P25942) was submitted for modelling, returning a model based on PDB: 5DMJ that had a confidence score of 100% with 97% coverage indicating a suitable model for further analysis.

Docking of 341G2 F(ab) and CD40 was performed using the HADDOCK 2.2 webserver (van Zundert et al., 2016). Residues on CD40 known to affect binding (identified through alanine scanning mutagenesis experiments), and the CDR loop residues of 341G2 were provided to the docking server as restraints in the docking following the HV-Epi9 protocol detailed in (Ambrosetti et al., 2020). For CD40 these residues were S49, D50, F67, L68, T75, H76, H78 and Q79. For the 341G2 model, the CDR loop residues were 26 to 34, 53 to 59, 104 to 112, 260 to 268, 284 to 288 and 325 to 331. The CDR loops were defined as active residues, while the CD40 residues were defined as active with a 9Å radius of passive residues. All HADDOCK settings were default apart from rigid-body refinement sampling which was increased to 5000 models and the flexible and water refinement sampling were increased to 400 models. Docking produced 10 clusters comprising 40 representative structures. Based on the knowledge that 341G2 and ChiLob 7/4 do not cross-block, a large number of the representative models were able to be excluded due to clashing with the Chilob 7/4 Fab from the crystal structure PDB: 6FAX when aligned on CD40 (Yu et al., 2018). This left a total of 11 models from 3 clusters that were taken.
forward for further analysis. The remaining structures were validated against the SAXS data using the WAXSIS server to compare the structures to the experimental SAXS data. The models that best fitted the SAXS data were selected by their $\chi^2$ score. The best fitting model gave a $\chi^2$ score of 2.21 +/- 0.37, with a clear trend seen between better and worse fitting models based on relative binding orientation to CD40. This model also came from the cluster with the best HADDOCK score (-102.0 +/- 10.9). The flowchart for SEC-SAXS and subsequent homology modelling and docking is shown in Figure S8.

**NFκB Assay**

The Jurkat NF-κB GFP reporter cell line was purchased from System Biosciences, USA and then transfected with the pCIpuro vector encoding the full-length hCD40 using Lipofectamine2000 (Thermo Fisher Scientific). Stable clones were selected using 1 μg/mL puromycin. To study NFκB activation, cells were incubated with various anti-CD40 mAb for 8 hours at 37°C and the level of NFκB activation was measured by GFP production by flow cytometry.

**B Cell Activation and Proliferation**

The hCD40Tg B cells were purified from hCD40Tg mouse splenocytes, and human B cells were purified from healthy donor PBMC, both by magnetic negative selection kits (StemCell Technologies, UK). For B cell proliferation, 1x10^5 B cells per well were incubated with different treatments in 96-well round bottom plates in a total of 200 μL media for various periods of time as indicated for individual experiments. Proliferation was assessed by adding 1 μCi of ^3H thymidine (PerkinElmer) to each well for the last 18 hours of incubation before cells were harvested and analysed for ^3H thymidine incorporation by TopCount. To assess B cell activation, cells were imaged 48 hours after the initial treatment using conventional light microscope (Olympus CKX41), and then stained for surface expression of CD80, CD86 and CD70 using flow cytometry. To assess the ability of 341G2 hlgG1 and 341G2 hlgG4 to block CD40L-mediated B cell activation, B cells were co-incubated with 2 μg/mL CD40L and 5 μg/mL anti-CD40 mAb.

**Human Dendritic Cell Activation and Mixed Leukocyte Reaction**

Human immature DCs were derived from CD14^+ monocytes as described before (Sallusto and Lanzavecchia, 1994). Briefly, CD14^+ monocytes were isolated from human PBMC by magnetic negative selection kit (Miltenyi Biotech, UK) and then cultured in the presence of 500 IU/mL IL-4 and 1000 IU/mL GM-CSF (both recombinant, produced in-house) for 6 days before phenotyping for the expression of CD11c and DC-SIGN by flow cytometry. To study the direct effect of anti-CD40 mAb, DCs were treated with 5 μg/mL anti-CD40 mAb for 48 hours and then the surface expression of CD86, CD80 and CD70 were analysed by flow cytometry. Moreover, the levels of TNF-α, IP-10, MCP-1, IL-12p70 and IFN-γ in the cell culture supernatant were quantified by Luminex Bioplex 200 instrument (Biorad, UK). For mixed leukocyte reaction, DCs were pre-treated with various anti-CD40 mAb for 24 hours before being washed and subsequently incubated with purified allogeneic CD4^+ T cells (Stemcell Technologies) at various ratios for 5 days. ^3H thymidine was added at 1 μCi per well for the last 18 hours to assess T cell proliferation.

**In Vivo Assessment of Agonistic and Antagonistic Activity**

To assess the antagonistic activity of anti-CD40 mAb in vivo, hCD40Tg mice were administered 500 μg OVA and 100 μg anti-CD40 mAb intraperitoneally on day 0, and 100 μg anti-CD40 mAb intraperitoneally on day 3. Mice were bled on day 2 to quantify CD19^+ B cells in peripheral blood by flow cytometry and on day 18 to quantify the level anti-OVA IgG in serum. To detect anti-OVA IgG in serum, ELISA plates (Thermo Fisher Scientific) were coated with 5 μg/mL OVA in PBS overnight. The next day, plates were blocked with 1% BSA and then serially diluted serum was added to each well, the mouse anti-OVA IgG mAb KB4 (in-house) was used to create a standard curve. Bound anti-OVA IgG was detected by secondary goat anti-mouse IgG-HPR (Abcam). To assess the agonistic activity of anti-CD40 mAb, 1x10^5 OTI cells were transferred into hCD40Tg mice via tail vein injection, the next day, 100 μg OVA and 30 μg anti-CD40 mAb were injected intravenously. The level of OTI expansion in peripheral blood was assessed 5 days after mAb treatment by staining for CD8 and SIINFEKL tetramer positive cells by flow cytometry.

To assess the effect of agonistic anti-CD40 mAb on B cells and DCs in vivo, hCD40Tg mice were administered 30 μg anti-CD40 mAb intravenously on day 0 and then bled and spleens harvested on day 2 to quantify CD19^+ B cells and CD23 expression by flow cytometry. Splenic DCs were gated as CD11c^+CD8^+DEC205^+ and analysed for the level of CD80 and CD86 by flow cytometry.

**Serum Cytokine Analysis**

hCD40Tg mice were administered 30 μg anti-CD40 mAb intravenously and bled 6 hours and 48 hours later to collect serum. Levels of IL-6, TNF-α and IFN-γ were quantified using ELISA MAX Standard Set kits according to manufacturer’s protocols (Biolegend).

**Tumor Models and Immunotherapy Treatment**

All mice were monitored for tumor growth by digital caliper measurements three times a week. Mice were culled when the sum of the tumor length and width reached 30 mm or when the general health of animals fell below the criteria set out in the corresponding Home Office project license. The agonistic anti-CD40 mAb 341G2 hlgG2 was injected at 30 μg per dose. Tumor volume was calculated using the formula: $V = (W^2 \times L)/2$ where $W$ is tumor width and $L$ is tumor Length. All tumor cells were resuspended in PBS and 100 μL was injected on the right flank of mice.
MC38 colon carcinoma model- 5x10⁵ cells were injected subcutaneously and treatment started on day 6 when the sum of tumor length and width reached approximately 10mm. 30 µg anti-CD40 mAb alone or in combination with 100 µg anti-PD-L1 (clone 10F.9G2, BioXcell) or 100 µg anti-CD27 (AT124-1 mouse IgG1, in house) were injected intraperitoneally every 3 days for 2 doses.

EG7 thymoma model- 5x10⁵ cells were injected subcutaneously. On day 7, when the sum of tumor length and width reached ~10mm, 1x10⁵ OTI cells resuspended in PBS were adoptively transferred via tail vein injection; the following day, 30 µg anti-CD40 mAb was injected intraperitoneally.

TC1 lung carcinoma model- 1x10⁵ TC1 cells were injected subcutaneously. On day 5, mice were vaccinated with either 150 µg or 3 µg long peptide (GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR) in combination with 30 µg anti-CD40 mAb intravenously. For monotherapy, mice were treated with 30 µg anti-CD40 mAb starting on day 5 every 3 days for 3 doses.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Flow cytometry data analysis was performed using either FCS Express software Version 3 (De Novo Software) or Flowjo Version 10.6 (BD Biosciences). All other data analysis were performed using GraphPad Prism 7.05 (GraphPad Software). Two-tailed, non-paired Student t test was used for most pairwise comparisons. For assays that investigate the effect of anti-CD40 mAb on human DCs, pairwise comparisons were made using two-tailed, paired Student t test. Statistical comparisons of survival curves were performed by Log-rank test. Throughout "p < 0.05, ""p < 0.01, """"p < 0.001.