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ORIGINAL ARTICLE

Development of CDX-1140, an agonist CD40 antibody for cancer immunotherapy

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Abstract

Limitations of immunotherapy include poorly functioning events early in the immune response cycle, such as efficient antigen presentation and T cell priming. CD40 signaling in dendritic cells leads to upregulation of cell surface costimulatory and MHC molecules and the generation of cytokines, which promotes effective priming of $CD8^+$ effector T cells while minimizing T cell anergy and the generation of regulatory T cells. This naturally occurs through interaction with CD40 ligand (CD40L) expressed on $CD4^+$ T-helper cells. CD40 signaling can also be achieved using specific antibodies, leading to several agonist CD40 antibodies entering clinical development. Our approach to select a CD40 agonist antibody was to define a balanced profile between sufficiently strong immune stimulation and the untoward effects of systemic immune activation. CDX-1140 is a human IgG2 antibody that activates DCs and B cells and drives NFkB stimulation in a CD40-expressing reporter cell line. These activities are Fc-independent and are maintained using an F(ab′)2 fragment of the antibody. CDX-1140 binds outside of the CD40L binding site, and addition of recombinant CD40L greatly enhances DC and B activation by CDX-1140, suggesting that CDX-1140 may act synergistically with naturally expressed CD40L. CDX-1140 also has both direct and immune-mediated anti-tumor activity in xenograft models. CDX-1140 does not promote cytokine production in whole blood assays and has good pharmacodynamic and safety profiles in cynomolgus macaques. These data support the potential of CDX-1140 as part of a cancer therapy regimen, and a phase 1 trial has recently commenced.

Keywords CD40 · Agonist antibody · Immunotherapy · Antigen presenting cells

Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
CD40L	CD40 ligand also referred to as CD154
CHO	Chinese hamster ovary
CRD1	Cysteine-rich domain 1
CRD2	Cysteine-rich domain 2
CRD3	Cysteine-rich domain 3
CRD4	Cysteine-rich domain 4
Cyno	Cynomolgus macaque
ECD	Extracellular domain
Hu	Human
MSD	Meso Scale Discovery
MTD	Maximum tolerated dose
NOAEL	No observable adverse effect level
rCD40L	Recombinant CD40L
TMB	3,3′,5,5′-tetramethylbenzidine
TRAF	TNF receptor associated factors

Introduction

CD40 is a TNFR superfamily member expressed on APCs including DCs [1], B cells [2], and monocytes [3]; on a variety of non-immune cells including platelets [4] and endothelial cells [5]; and on a wide range of tumor cells [6]. An important function of the interaction of CD40 with its ligand CD40L (CD154) expressed on $CD4^+$ T-helper cells [7, 8]

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is to activate and "license" DCs to prime $CD8^+$ effector T cells (cytotoxic T lymphocytes, CTL). This is accomplished through the upregulation of cell surface costimulatory and MHC molecules and the generation of cytokines by the DCs leading to effective T-cell activation. In the absence of CD40 signaling, activation of CTLs by "unlicensed" DCs leads to T-cell anergy or deletion and the generation of Tregs [9]. Similarly, ligation of CD40 on B cells leads to their activation, proliferation, and enhanced antigen presentation [7, 8, 10]. Further, CD40-activated macrophages can be tumoricidal and in some cases, deplete tumor stroma [11]. Thus, CD40 on APCs plays a critical role in the induction of effective immune responses.

In contrast to the CD40-mediated activating and proliferative effects observed for normal human cells, the ligation of CD40 on certain malignant cells using CD40L or anti-CD40 antibodies inhibits proliferation or triggers apoptotic cell death, for example, of colon cancer cells [12], ovarian carcinoma cells [13], or B cell lymphoma [14, 15]. Thus, two independent mechanisms provide opportunities for the use of agonist anti-CD40 mAb in cancer therapy: the enhancement of anti-tumor immunity, and the direct growth inhibition or killing of tumors.

As with many TNFR/ligand interactions, CD40 signaling through the receptor appears optimal when the CD40L is constrained in a trimeric arrangement, thereby enforcing a clustering of the CD40 itself, and the subsequent recruitment and interaction with TNF receptor-associated factors (TRAFs) [16]. Two research groups concluded that FcγRIIb engagement by the CD40 mAb was required for anti-CD40 agonist activity [17, 18]; however, it was cross-linking and not the intracellular signaling that was required, and the cellular distribution as much as the affinity of the FcγR was important for in vivo activity. Subsequently, it was shown that human IgG2 isotypes of certain anti-CD40 mAbs had potent agonist activity independent of FcR interactions due to a structural consequence of the IgG2 hinge region [19]. In general, the capacity of a specific mAb to promote clustering of CD40, whether through FcγR interactions or independent of such interactions, favors downstream signaling and agonistic activities.

Several candidates targeting CD40 mAbs have entered clinical development as cancer therapies and have been recently reviewed [20]. Except for CP-870,893, the other anti-CD40 mAbs are human IgG1 isotype (in some cases with modified Fc for enhancing specific FcR interactions) and generally require FcR interactions for their activity. The CP-870,893 mAb (currently called RO7009789 or selicrelumab) is a fully human IgG2 with potent agonist activity that has shown promise in early clinical trials [21–25]. However, CP-870,893 has a limited maximum tolerated dose (MTD) of 0.2 mg/kg with the most common adverse events being cytokine release syndrome (grade 1 and 2). Such a low dose (≤ 0.2 mg/kg) may not be ideal for systemic use because saturation of CD40 on tissue-resident DCs or macrophages may not be achieved at these dose levels due to the wide-spread expression of CD40 on non-immune cells and some tumors [6]. An alternative anti-CD40 mAb better suited for systemic use would have a somewhat lower level of agonist activity than CP-870,893 and thus could be used at higher doses, improving the likelihood of fully engaging macrophages and DCs in these tissues. It was this generalized goal that guided our generation and selection of an agonist CD40 mAb for use in cancer therapy.

This report describes the generation and characterization of such an agonist CD40 mAb and the IND-enabling studies that have allowed this mAb, designated CDX-1140, to initiate phase 1 clinical development. The selection of the IgG2 clone 3C3 for clinical development as CDX-1140 was based on a balanced set of desired characteristics including agonist activity with a linear dose–response curve, independent of FcR binding, and synergy with the natural CD40L signaling.

Materials and methods

Generation of CDX-1140

Human anti-CD40 mAbs were generated by immunizing the H2L2 strain of Harbour® transgenic mice (Harbour Antibodies BV, US Patent 9,131,669) with a human CD40-Ig fusion protein. Splenocytes were used for hybridoma preparation by standard polyethylene glycol fusion techniques. Hybridomas were screened by ELISA using CD40-Ig fusion proteins based on either the human or cynomolgus macaque CD40 extracellular domain sequences. The V_H and V_L coding regions were sequenced and inserted into an expression vector containing human IgG1κ and IgG2κ antibody-constant domains and transfected into Chinese hamster ovary (CHO) cells for production and purification. The anti-CD40 mAb, 21.4.1 (US patent 8,388,971, also known as CP-870,893), was similarly prepared by introducing the V_H and V_L coding regions of 21.4.1 into human IgG2κ antibody-constant domains and expressed from transfected HEK-293 cells. Antibodies were purified by protein A and determined to contain < 0.5 endotoxin units/mg.

CDX-1140 Binding studies

ELISA and flow cytometry

Recombinant CD40 proteins were generated by purification from transient transfections. In addition to the full-length extracellular domain (ECD) spanning amino acid residues 21–193 of human CD40, three overlapping fragments coding for amino acids 21–114, 56–150, and 104–193 were

synthesized and inserted in-frame into an expression vector with an N-terminal human kappa light chain and a C-terminal Flag tag. The binding of anti-CD40 mAbs was detected using a goat anti-human IgG-HRP and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. The binding of CDX-1140 to related TNFR superfamily members was assessed by plates coated with commercially available recombinant proteins (R&D Systems) and detected with goat anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch) developed with TMB substrate. For flow cytometry, the HEK-293-CD40 cells, human lymphoma cell lines (ATCC), or human B cells in PBMCs (Biological Specialty) were incubated with anti-CD40 mAbs and detected with PE-labeled goat anti-human IgG probe (Jackson ImmunoResearch). The cell-associated fluorescence was determined by analysis using a FACSCanto II™ instrument (BD Biosciences).

Affinity determination using bio-layer interferometry

CDX-1140 was captured on Anti-Human Fc Capture biosensors (Pall ForteBio). Binding was determined by exposing the antibody-loaded biosensor to human CD40-Ig on an Octet instrument. Affinity measurements were determined using twofold serial dilutions of analyte ranging from 3.13 to 0.098 nM. Fortebio's Data Analysis Software was used to derive kinetic parameters from the serial concentrations of analyte in dilution buffer binding to captured antibody. The association and dissociation curves were fitted to a 1:1 binding model using the data analysis software according to the manufacturer's guidelines.

CD40L competition

ELISA assays were performed using plates coated with 2 µg/mL human rCD40-Ig (R&D Systems) and purified recombinant CD40L (rCD40L) expressed by CHO cells as a truncated CD40L-isoleucine zipper peptide fusion protein that exists as a non-covalently linked 66 kDa homotrimer [26]. CDX-1140 at various concentrations was added, followed by the addition of human rCD40L-biotin (0.5 µg/mL). The bound rCD40L was detected with streptavidin-HRP and TMB substrate. Similarly, in flow cytometry experiments, HEK-293-CD40 cells were incubated with varying amounts of CDX-1140 and rCD40L-biotin. The cell-bound rCD40L was detected with streptavidin-PE and analyzed using a FACSCanto II™ instrument (BD Biosciences).

CD40 activation studies

NFκB reporter assay

An NFκB luciferase reporter HEK-293 cell line was transfected to express full-length human CD40. The HEK-293-CD40 cells were incubated for 6 h at 37 °C, 6% CO_2 with CDX-1140, isotype controls, or rCD40L at 0.1 µg/mL. Luciferase expression was detected with the Luciferase Assay System by Promega according to the manufacturer's directions

B-cell activation

B cells were isolated from PBMCs by magnetic selection using CD19 beads (Miltenyi). The cells were labeled with 0.5 µM CFSE and then cultured in the presence of anti-CD40 mAbs with or without sCD40L for 6 days before analysis using a FACSCanto II™ instrument. B cell activation in whole blood was measured after incubation with anti-CD40 mAbs overnight at 37 °C, 6% CO_2. The cells were stained with anti-CD19 PE, anti-HLA-DR V450, anti-CD69 PerCP-Cy5.5 and anti-CD86 allophycocyanin (BD Biosciences), washed, and red blood cells were lysed prior to analysis on a FACSCanto II™ instrument. Upregulation of CD95 was assessed using Ramos cells incubated overnight at 37 °C, 6% CO_2 with anti-CD40 mAbs. Cells were stained with PE-conjugated anti-CD95 antibody (BD Biosciences) and then analyzed on a FACSCanto II™ instrument.

PBMC activation

PBMCs were obtained from whole blood by Ficoll isolation and incubated for 6 days at 37 °C, 6% CO_2 with anti-CD40 mAbs before harvesting the supernatant for cytokine analysis either by multiplexing bead technology (Eve Technologies) or ELISA (R&D Systems). Some experiments used PBMCs depleted of B cells or monocytes with anti-CD19 or anti-CD14 magnetic beads (Miltenyi).

Dendritic cell activation

Following Ficoll isolation of human PBMCs, monocytes were selected by adhering to plastic and then cultured for 7 days in RPMI containing 10% FBS, 10 ng/mL IL-4 (R&D Systems) and 100 ng/mL GM-CSF (R&D Systems). Cells were harvested and confirmed to be dendritic cells by expression of CD11c (not shown). Cells were incubated with anti-CD40 mAbs with or without rCD40L for 48 h at 37 °C, 6% CO_2. Supernatant was collected and IL-12p40 was evaluated by ELISA (R&D Systems).

Xenograft models

Xenograft tumor studies were performed using the human B cell lymphoma cell lines Ramos and Raji and a bladder cancer cell line EJ138 (also known by the name MGH-U1). For these studies, tumors were implanted subcutaneously into the flanks of immunodeficient SCID mice (Ramos,

0.5×10^6; Raji, 1×10^6; EJ138, 3×10^6) followed by intraperitoneal administration of CDX-1140 (0.3 mg) or controls as indicated in figure legends. For some studies, Ramos and Raji cells were mixed with 3×10^6 human PBMCs before implanting into mice. Tumors were measured 2–3 times per week and mice were euthanized according to pre-defined health criteria.

Pilot non-human primate study

A small study was performed in naïve cynomolgus macaques (Charles River Laboratories, Shrewsbury, MA) with CDX-1140, mAb 21.4.1, or saline control. The antibodies or saline were administered to three males per group by intravenous injection in a saphenous vein on Day 1 (0.2 mg/kg or saline) and again on Day 29 (2 mg/kg or saline). Animals were followed through to Day 45. Evaluations included clinical signs, body temperature, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), cytokines, flow cytometry, and toxicokinetic parameters. Body weights were recorded once prior to antibody administration and weekly thereafter. This was designed as a survival study with no planned necropsy.

Safety assessment studies

Whole blood cytokine release

The mAbs were added to plates at 50 µL/well at 0.1 µg/mL, 1 µg/mL or 10 µg/mL and either left uncovered (dry coated) or covered (wet coating) overnight in a biosafety cabinet. Uncoated plates with antibody added directly to whole blood were used as well. Whole blood was dispensed into the wells and positive controls, PHA (Sigma) and LPS (Invivogen), were added at 10 µg/mL. The plates were incubated for 24 h at 37 °C, 6% CO_2. Plasma was harvested and assessed for cytokine and chemokine production by multiplexing bead technology (Eve Technologies).

Tissue-cross-reactivity

A tissue-cross-reactivity study was performed of CDX-1140 with cryosections of human and non-human primate (cynomolgus macaque) tissues (Charles River Laboratories, Frederick, MD). CDX-1140 was applied to cryosections (3 donors per tissue, where available) at two concentrations (10 and 2 µg/mL). As a control, the CDX-1140 was substituted with isotype control.

Non-human primate toxicology

CDX-1140 was administered by slow bolus intravenous injection, on days 1, 15 and 29 to cynomolgus macaques (Citoxlab, Laval, QC, Canada). Euthanasia occurred on day 31 or day 45. Standard in-life and terminal endpoints, including full histopathology, were performed. Identification of lymphocyte cell populations in peripheral blood was performed by direct staining of samples using a combination of fluorochrome-conjugated antibodies on a BD Biosciences FACSVerse flow cytometer. Serum was tested for IL-1β, IL-6 and IL-12/IL-23p40 by multiplex assay using Meso Scale Discovery (MSD) instrument with biotinylated capture antibodies and SULFO-TAG™ conjugated detection antibodies specific for each of the cytokines. The serum was also tested for CDX-1140 concentration by ELISA using human CD40-Ig coated plates and detection with biotinylated goat anti-human κ-chain antibody followed by streptavidin-HRP. After addition of TMB substrate the plate was read at an absorbance of 450 and 650 nm.

Statistical analysis

Statistical significance was evaluated using paired or unpaired Student's *t* test as appropriate.

Results

Development and binding characteristics of CDX-1140

CDX-1140 was selected from a panel of CD40-specific human antibodies generated by immunization of H2L2 mice transgenic for human heavy and light chain antibody variable regions with human CD40-Ig fusion protein. The variable domains of the mAbs in this panel were cloned and expressed on human IgG1 and IgG2 backbones and tested in a variety of in vitro and in vivo assays. As expected, the IgG2 isotype antibodies had greater agonist activity (Supplementary Table 1) from which a lead candidate with good, but notably not the highest agonist activity, was chosen and named CDX-1140.

CDX-1140 binds human and cynomolgus macaque CD40 with similar kinetics, and its epitope is in the cysteine-rich domain 1 (CRD1) of the molecule as shown using overlapping fragments of CD40 (Fig. 1a, b). CDX-1140 does not bind to murine CD40 (data not shown). The binding affinity of CDX-1140 as determined by bio-layer interferometry has an equilibrium dissociation constant K_D of 10 pM using human CD40-Ig as the analyte. CDX-1140 does not cross-react with other TNFR superfamily members that share homology with CD40 (Supplementary Fig. 1). CDX-1140 binds to cell-surface expressed CD40 as demonstrated by flow cytometry using HEK-293 cells transfected with CD40, normal human B cells, and the lymphoblastoma cell line Ramos (Fig. 1c). CDX-1140 does not significantly compete

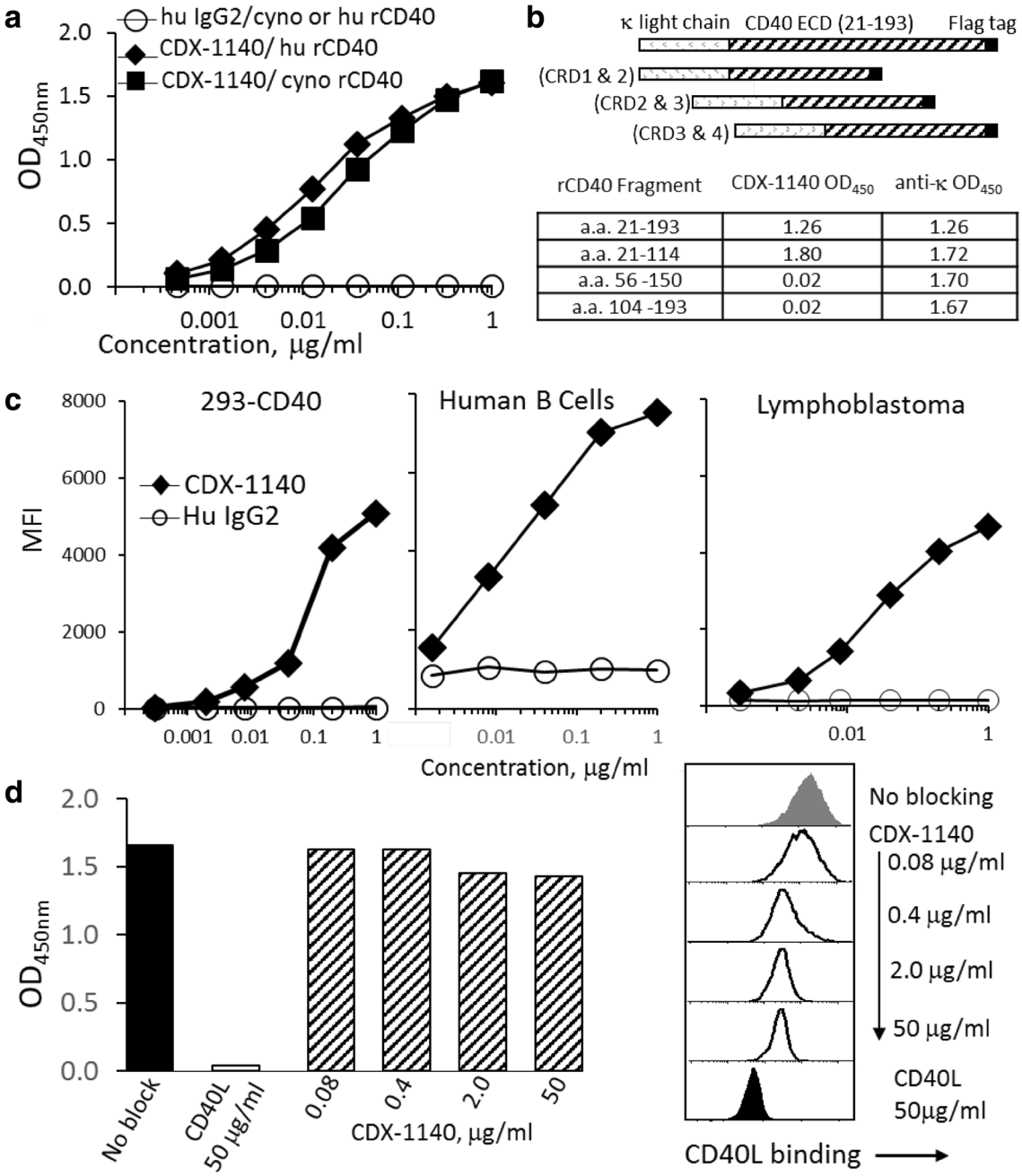

Fig. 1 Binding properties of CDX-1140. **a** CDX-1140 binding to human and cynomolgus rCD40. The rCD40 proteins were bound on a plate and mAb binding was detected with goat anti-human IgG-HRP. Controls included human (hu) or cynomolgus macaque (cyno) IgG2 or rCD40 as indicated. **b** CDX-1140 binds within CRD1. Overlapping fragments of CD40 ECD incorporated into fusion proteins were captured with an anti-FLAG antibody. Anti-CD40 binding was detected with goat anti-human IgG-HRP. Presence of the captured rCD40 fragments was verified using goat anti-human κ–HRP. **c** Binding of CDX-1140 to HEK-293 cells transfected with CD40, human B cells, or the Ramos lymphoblastoma cell line as measured by flow cytometry. CDX-1140 binding was detected with goat anti-human IgG-PE. Human B cells were detected with anti-CD20-allophycocyanin. **d** Impact of CDX-1140 on recombinant CD40L binding. CDX-1140 was added to recombinant CD40-coated plate or CD40-expressing HEK-293 cells at varying concentrations. Biotin-labeled recombinant CD40L was added and detected with either HRP-labeled streptavidin (ELISA OD at 450 nm) or PE-labeled streptavidin (flow cytometry histograms)

with rCD40L when using recombinant CD40 in an ELISA format, yet higher concentrations of the antibody reduce the level of rCD40L binding to cell-surface expressed CD40 by flow cytometry (Fig. 1d).

CDX-1140 agonist properties

A variety of assays were used to quantify the agonist activity of CDX-1140. To directly demonstrate CD40 activation, a luciferase reporter cell line was developed by transfecting a commercially available HEK-293 NFκB−luciferase reporter cell line with human CD40. CDX-1140 induced a concentration-dependent activation of NFκB, though at a somewhat lower magnitude than rCD40L (Fig. 2a). Additional hallmarks of CD40 activation include B-cell activation and proliferation, DC activation, and induction of CD95 (FAS) expression. CDX-1140 exhibited similar patterns of a near linear dose-dependent increase in B cell proliferation, IL-12p40 production by monocyte-derived DCs, and CD95 expression on a lymphoma cell line (Fig. 2b–d). As expected since IgG2 antibodies have minimal FcR engagement, the $F(ab')_2$ form of CDX-1140 retains the agonistic properties of the whole mAb (Fig. 2e-f).

The activation of B cells was also evidenced by upregulation of activation markers such as CD86, CD69, and HLA-DR, and was observed when CDX-1140 was added directly to whole blood samples (Supplementary Fig. 2). When incubated with human PBMCs for 6 days, CDX-1140 induced high levels of TNF-α and other cytokines and chemokines

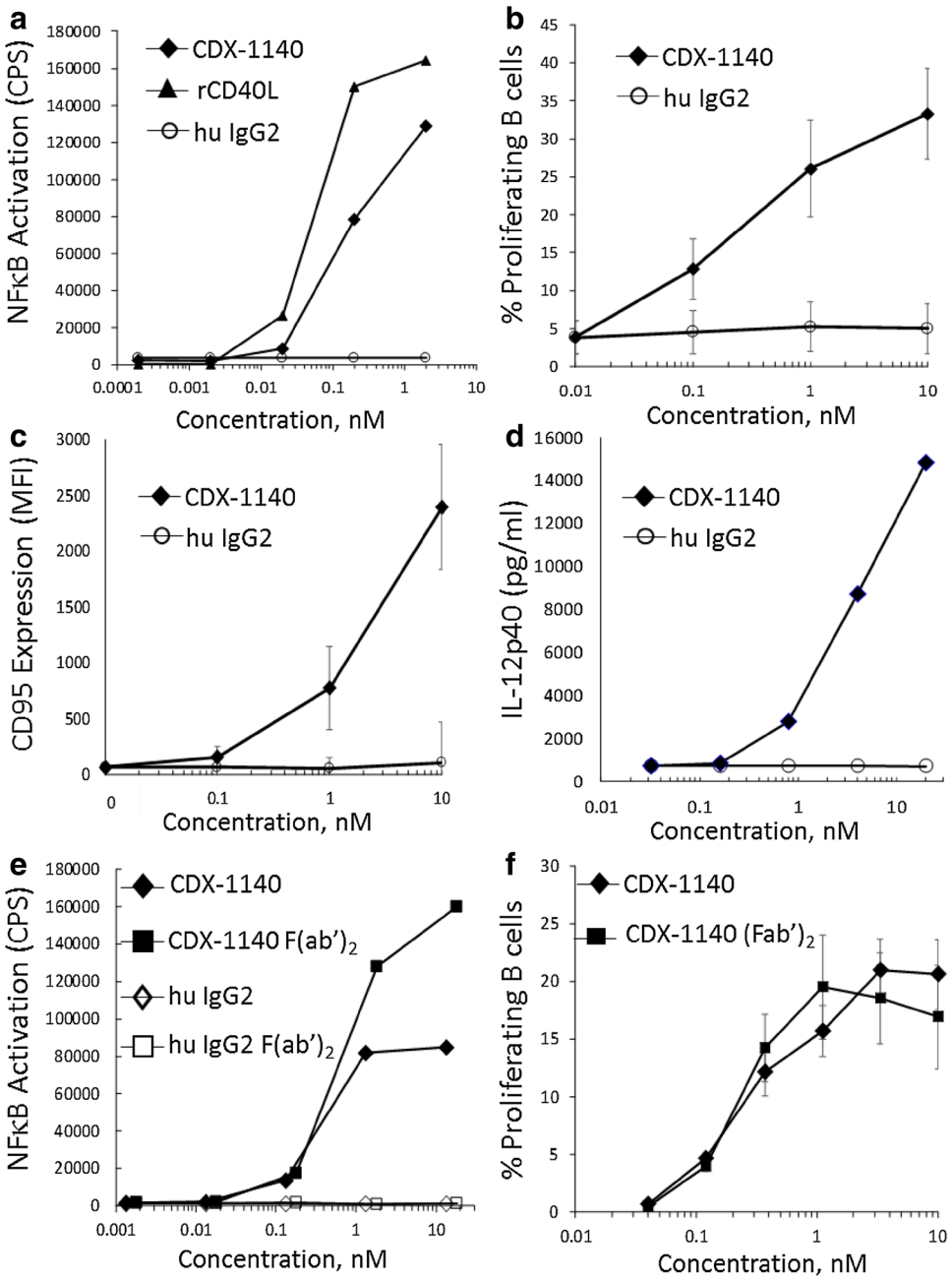

Fig. 2 CDX-1140 has dose-dependent agonist activity that is independent of Fc receptor engagement. **a** HEK-293-CD40 cell line with an NFκB-luciferase reporter system was incubated with antibodies for 6 h at 37 °C (5% CO_2). NFκB signaling was detected using the Luciferase Assay System (Promega). **b** Purified B cells were CFSE-labeled and incubated with antibody at the indicated concentration for 6 days at 37 °C (5% CO_2). Proliferation was assessed by flow cytometry by determining the number of cells with reduced CFSE fluorescence due to cell division. Values represent the mean from 5 donors ± SEM. **c** Ramos cells were incubated overnight with CDX-1140 and then stained for CD95 expression and analyzed by flow cytometry. **d** Monocyte-derived dendritic cells were incubated for 48 h with antibodies at 37 °C (5% CO_2). Cell-free supernatant was assessed for IL-12p40 production by ELISA. Values represent the mean from 5 donors ± SEM. **e** CDX-1140 and its F(ab′)2 fragments were tested for NFkB signaling (as in **a**). **f** Effects on B cell proliferation was assessed following incubation with CDX-1140 or its F(ab′)2 fragments (as in **b**). Values represent the mean ± SEM ($n = 3$)

(Supplementary Fig. 3a). Interestingly, the cytokine production was found to be entirely dependent on $CD14^+$ cells, as removal of these cells (and not $CD19^+$ cells) resulted in no upregulation of TNF-α production (Supplementary Fig. 4b).

Comparison of CDX-1140 to CD40 agonist mAb 21.4.1

The CD40 agonist mAb clone 21.4.1 was used for comparison to the agonist activity of CDX-1140. The two mAbs have very similar binding to recombinant CD40 (rCD40) protein (Fig. 3a) and to cells expressing CD40 (data not shown), but have different profiles regarding CD40-mediated activation. The 21.4.1 mAb consistently stimulated more IL-12p40 from monocyte-derived dendritic cells than CDX-1140, though this was not statistically significant due to high variability among donors (Fig. 3b). In B cell proliferation assays, mAb 21.4.1 was more potent at lower concentrations, but CDX-1140 induced greater proliferation of B cells at high concentrations (Fig. 3c). Similarly, in whole blood assays, B-cell activation measured by CD86 expression was generally greater with mAb 21.4.1 relative to CDX-1140 (Fig. 3d). Overall, we found mAb 21.4.1 to have potent agonist activity that showed less concentration dependence (across the concentrations used in the study) compared to CDX-1140.

A pilot study was performed in naïve cynomolgus macaques in which CDX-1140, 21.4.1 mAb, or saline control were administered at a low dose on Day 1 (0.2 mg/kg or saline) and a high dose on day 29 (2 mg/kg or saline). Hematology assays and flow cytometry on blood samples demonstrated transient but significant decreases in white blood cells, lymphocytes, platelets, and particularly B cells in animals dosed with CDX-1140 and tended to be of greater magnitude in animals dosed with 21.4.1 (Fig. 3e). The antibody administrations were well tolerated, with only minor changes in serum chemistry (Fig. 3f). IL-12p40 was the most significantly increased cytokine and 21.4.1-dosed monkeys showed higher levels of IL-12p40 in serum relative to CDX-1140-dosed animals (Fig. 3g). These data are consistent with the in vitro data above suggesting that CDX-1140 agonist

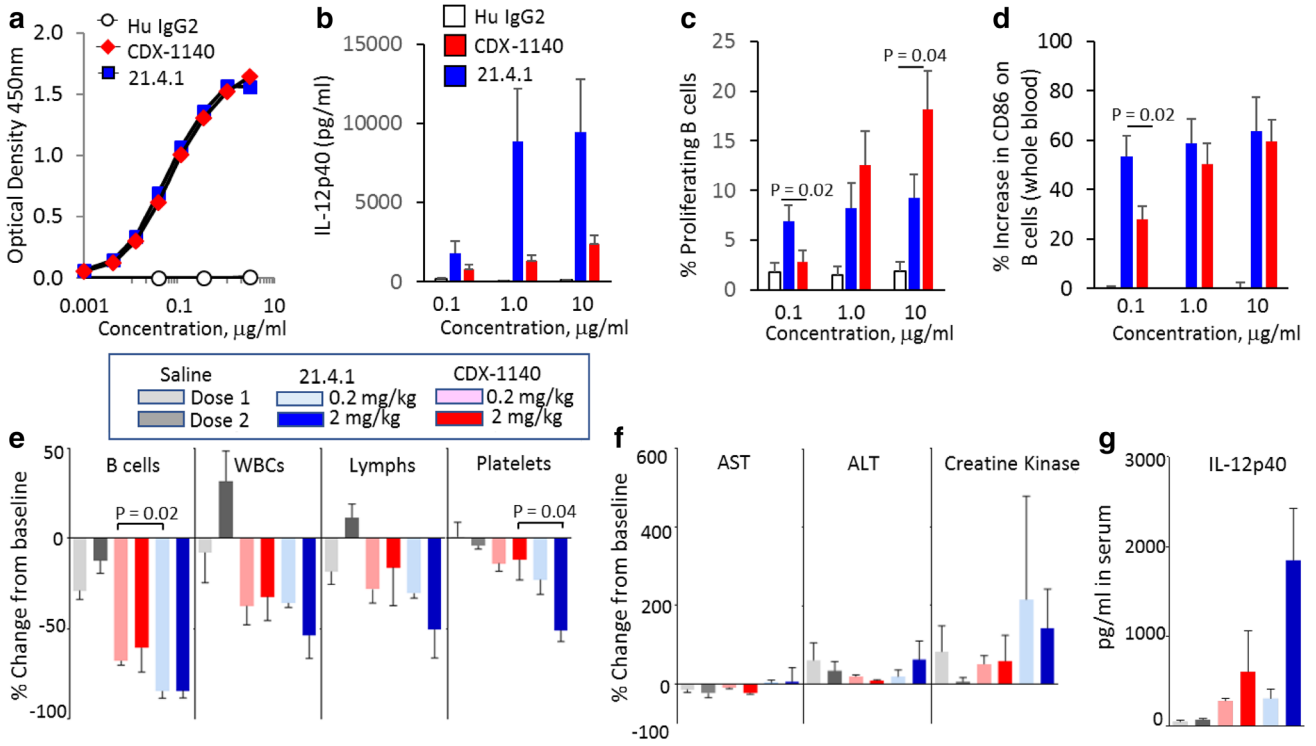

Fig. 3 Comparison of CDX-1140 with agonist anti-CD40 Ab 21.4.1. **a** Binding of CDX-1140 or 21.4.1 to human rCD40 by ELISA. Antibodies were added to microtiter plates coated with rCD40 and detected with goat anti-human IgG-HRP. **b** IL-12p40 induction by CDX-1140 or 21.4.1 was assessed using monocyte-derived DCs as in Fig. 2d. Values represent the mean ± SEM ($n = 6$). **c** B cell proliferation by CDX-1140 or 21.4.1 was assessed as in Fig. 2b. Values represent the mean ± SEM ($n = 5$). **d** Activation of B cells from whole blood with CDX-1140 or 21.4.1. Whole blood was incubated overnight with antibodies at 37 °C (5% CO_2). B cells were stained for CD19 and CD86 and expression was analyzed by flow cytometry. Values represent the mean ± SEM ($n = 9$). **e** Peak change in the levels of circulating B cells, WBCs, lymphocytes, and platelets in cynomolgus macaques (n = 3 ± SEM) after low or high-dose administration of antibodies. **f** Peak change in serum chemistry parameters. **g** Peak serum level of IL-12p40. Statistical significance for CDX-1140 versus 21.4.1 was evaluated by paired *t* test (**a**–**d**) or unpaired *t* test (**e**–**g**) with p values < 0.05 indicated

activity is somewhat attenuated compared to 21.4.1 at the antibody concentrations tested.

Low doses of CDX-1140 synergize with CD40L

Since CDX-1140 does not block CD40L binding, we sought to determine if there may be synergistic agonist activity when combined at suboptimal concentrations. In fact, the combination of rCD40L with CDX-1140 at concentrations that have minimal impact individually, had very potent effects on B cell proliferation and DC IL-12p40 production (Fig. 4a-b). Similar synergy was observed on upregulation of activation markers on DCs, and B cells (data not shown).

Activity against CD40-expressing tumors

The Ramos and Raji human lymphoblastoma cell lines express CD40 and have been shown to be sensitive to CD40 agonist antibodies [27, 28]. CDX-1140 up-regulated CD95 (FAS) on the lymphoma cell lines in vitro (Fig. 2d and data not shown) and showed direct anti-tumor activity in the xenograft models (Fig. 5a). However, animals generally succumbed to the tumors, particularly in the Ramos model. In subsequent studies, we supplemented the immune-deficient mice with PBMCs during the tumor cell inoculation to determine if CDX-1140 activation of immune cells could improve long-term survival in these models. Figure 5b shows that the addition of PBMCs on its own did not improve long-term survival, but the combination of CDX-1140 and PBMCs was highly effective at promoting complete rejection of both Ramos and Raji tumors.

The activity of CDX-1140 was also demonstrated in a xenograft model of the epithelial EJ138 bladder carcinoma cell line known to express CD40 [29] (Fig. 5c).

CDX-1140 safety studies to support human clinical trials

To satisfy current regulatory expectations, we assessed the cytokine release profile of CDX-1140 in soluble and solid-phase formats using whole blood from healthy individuals [30, 31]. CDX-1140 was tested for cytokine production in whole human blood assays in plate-bound and solution formats. Although B-cell activation is clearly observed under these conditions (Supplementary Fig. 2), no significant increases of cytokines (TNF-α, IL-6, IL-1β, IFNγ, IL-8, IL-12p40, and IL-2) were measured above an isotype control (data not shown).

A tissue-cross-reactivity study of CDX-1140 was performed on full panels of human and cynomolgus macaque tissues. As expected, membrane-staining was present in epithelium of several tissues and on mononuclear cells. The staining of human and monkey tissues with CDX-1140 showed similar patterns.

The pharmacological activity and safety profile of CDX-1140 was assessed in cynomolgus macaques, which have similar binding to CDX-1140 as human CD40 (Fig. 1a). Animals were dosed by intravenous injection on days 1, 15, and 29 with either saline, 0.01, 0.1, 1, or 10 mg/kg

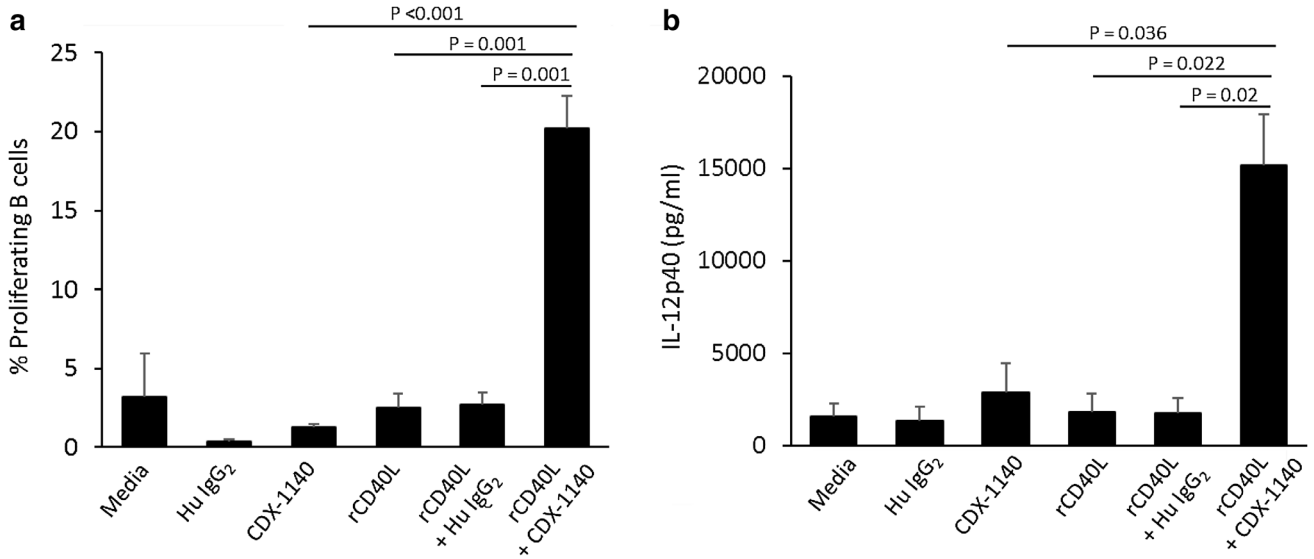

Fig. 4 Synergistic activation of B cells and DCs with CDX-1140 and rCD40L. **a** B cell proliferation with suboptimal concentrations of CDX-1140 (0.1 µg/mL) and soluble rCD40L (0.1 µg/mL) alone or in combination. Proliferation was assessed as in Fig. 2b. Values represent the mean ± SEM ($n = 3$). **b** IL-12p40 production from DCs with suboptimal concentrations of CDX-1140 (0.1 µg/mL) and rCD40L (0.1 µg/mL) alone or in combination. Induction of IL-12p40 was assessed as in Fig. 2d. Values represent the mean ± SEM ($n = 3$).

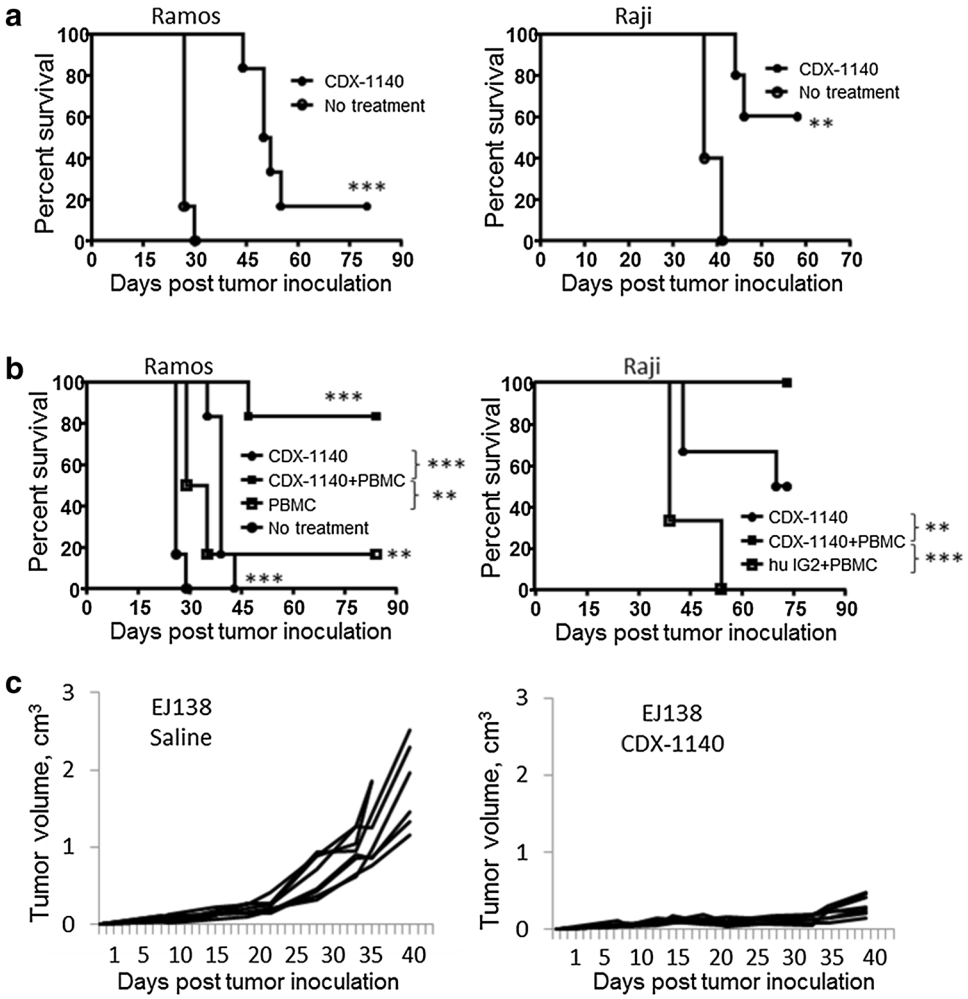

Fig. 5 CDX-1140 activity in xenograft models. **a** Ramos tumor cells (0.5×10^6) or Raji tumor cells (1×10^6 cells) were injected subcutaneously in the flanks of SCID mice. Mice were split into groups of 5 (Raji) or 6 (Ramos), and treated mice received CDX-1140 (0.3 mg) by intraperitoneal injection on days 1, 6 and 13 after tumor cell inoculation. **b** Ramos and Raji cells were mixed with 3×10^6 human PBMCs and implanted into SCID mice as above and treated with CDX-1140 (0.3 mg) or controls on days 1, 8, and 15 after tumor inoculation ($n=6$ per group). **c** Mice received EJ138 cells subcutaneously and were administered CDX-1140 (0.3 mg) or saline intraperitoneally, once a week for three weeks once the mean tumor volume reached approximately 0.1 to 0.2 cm^3. Data are representative of at least 2 separate studies including different donors for the PBMC experiments. Statistical significance is indicated by **($p<0.01$) or ***($p<0.001$)

CDX-1140. CDX-1140 was well tolerated in the study and no CDX-1140-related effects were noted in clinical signs, body weights and body weight changes, body temperature, electrocardiography, ophthalmology, neurological assessments, urinalysis parameters, or bone marrow evaluations during the dosing period. The no observable adverse effect level (NOAEL) was determined to be the high dose level of 10 mg/kg. Pharmacological effects were observed that are consistent with CD40 engagement and activation and were similar to those reported in the Phase 1 study of CP-870,893 [21]. The most significant of these were observed at day 2 (24 h after the first dose) and included decreases in overall lymphocyte counts (Fig. 6a), decreases in circulating B cells (Fig. 6b), and increases in serum levels of IL-12p40 (Fig. 6c). These pharmacodynamic effects were minimal at the lowest dose of 0.01 mg/kg but were clearly observed at doses of 0.1 mg/kg and higher. The highest dose of CDX-1140 (10 mg/kg) maintained some of these pharmacological effects 2 days after the final dose (day 31). Lower dose groups were likely impacted by the development of anti-CDX-1140 antibodies which substantially impacted the pharmacokinetics of lower doses compared to the high-dose group (data not shown). Importantly, only small changes in platelet levels were observed including at the highest dose (median decrease day 2 = -11%, day 31 = -12%) (Fig. 6d).

Discussion

Over the past decade, great advances have been made in harnessing patients' immune response against their cancer. Thus far, the main successes have been limited to disruption of the checkpoint pathways of PD-1 and CTLA-4, for which efficacy has been linked to high mutational load, and active immune responses [32]. Enhancing the development and maintenance of de novo anti-tumor immune responses is needed to extend the progress of immunotherapy to a greater number of patients. CD40 is particularly promising in this respect, due to its critical role in driving immune responses, which was clearly demonstrated using anti-CD40L antibody treatment or CD40-deficient mice, both of which abrogated the ability to develop vaccine-induced immunity [33]. The

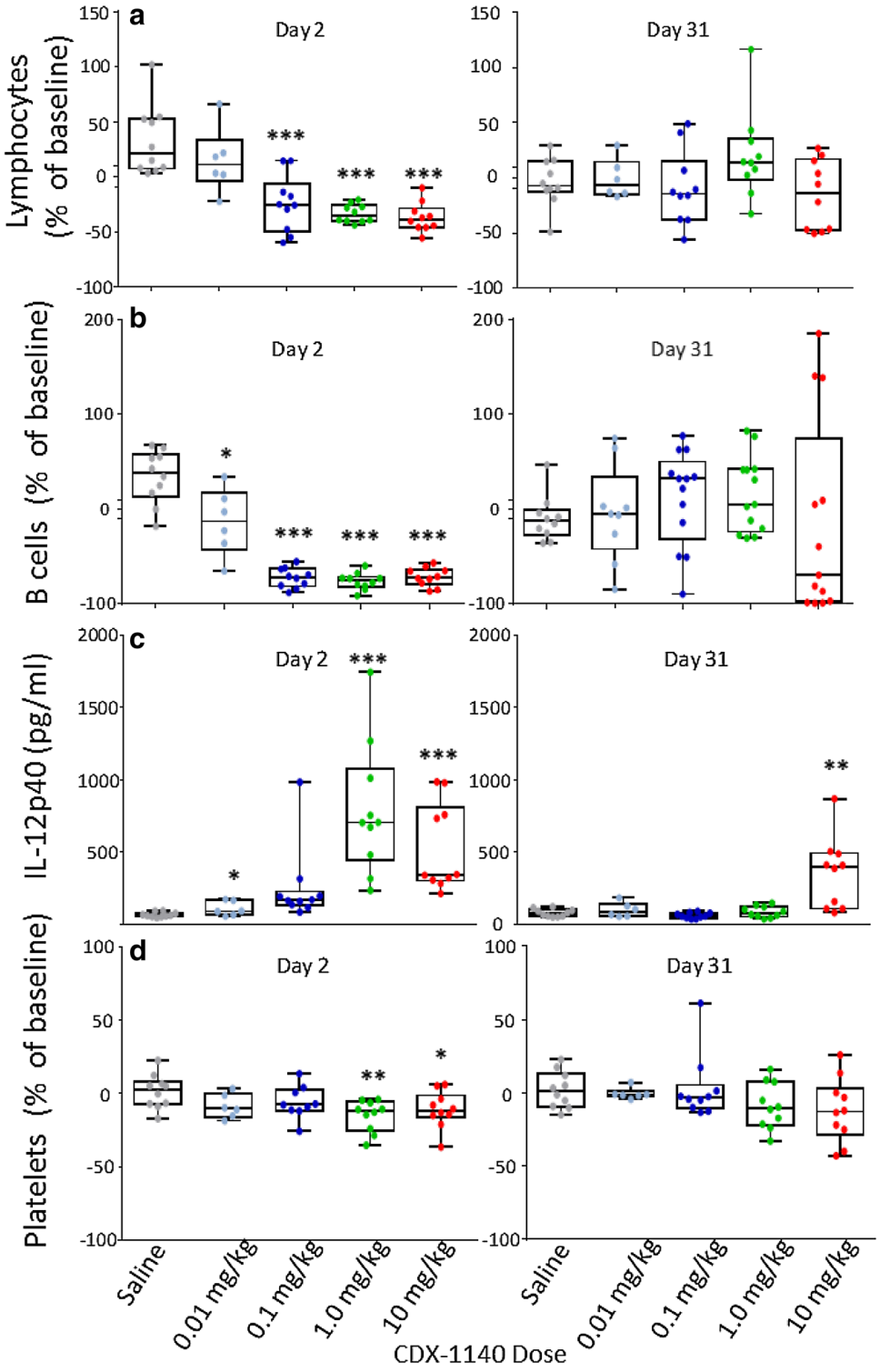

Fig. 6 CDX-1140 effects on lymphocytes and IL-12p40 in cynomolgus macaques. Groups of 10 animals (composed of males and females, only 6 animals in 0.01 mg/kg dose level) were administered with the indicated doses levels of CDX-1140 by intravenous injection. Shown are the analyses of samples taken 24 h after the first dose (day 2) and 24 h after the third dose (day 31). **a** Percent of lymphocytes are plotted from the absolute lymphocyte counts. **b** Percent of B cells were determined by flow cytometry. **c** Serum levels of IL-12p40 were determined by multiplex assay using an MSD instrument. Platelet counts are shown in **d**. Statistical significance versus saline control is indicated by *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$)

potential for CD40 as a target for immunotherapy is further supported by the efficacy of agonist anti-CD40 antibodies in preclinical models [34, 35] and in early clinical trials [21, 22].

Several CD40 agonist antibodies have been developed, and there are currently five in active clinical trials including CDX-1140 for which the preclinical activity is described here. Different approaches have been undertaken to shape the quality and activity of each candidate therapeutic. In three cases, the agonist activity was conferred by interaction with specific Fc receptors either by the native IgG sequence or through engineered changes in the Fc domain

of the antibody (SEA-CD40 [36], APX005M [37], and JNJ-64457107 [38]). In contrast, CP-870-893 (clone 21.4.1) is a highly potent agonist that is independent of Fc receptor crosslinking, but is associated with toxicities that has limited its systemic exposure [22, 23]. We sought to develop a novel CD40 agonist antibody that was not dependent on Fc receptors for its agonist activity and could be systemically administered at doses that provide good biodistribution into tumors. To this end, we probed our mAb panel for agonist antibodies with a linear dose–response that achieve maximum potency at relatively high concentrations of antibody.

Characterization of the agonist activity of CDX-1140 indeed demonstrated a near linear dose–response over the range of 0.1 to 10 µg/mL using in vitro assays and was equally active when the Fc domain was removed. In comparison with CP-870,893, CDX-1140 had generally lower in vitro agonist activity, particularly at low mAb concentration, despite similar binding affinities. These observations were further supported in a small ($n = 3$ per group) study using cynomolgus macaques in which CDX-1140 and CP-870,893 were administered at low (0.2 mg/kg) or higher dose (2 mg/kg). In general, the effect of CDX-1140 on immunological and toxicological parameters was less than that observed with CP-870,893; however, the variability among animals and small numbers ($n = 3$ per group) made most of these differences not statistically significant.

The activity of CDX-1140 was further characterized in macaques as part of a toxicology study. In this study we observed a clear dose-dependent effect on the expected hematologic and cytokine (IL-12p40) changes, with maximum and prolonged effects at the highest dose of 10 mg/kg. Importantly, all doses including the highest dose of 10 mg/kg were well tolerated in cynomolgus macaques, without evidence of clinically meaningful toxicities including decreases in platelet counts as seen with CP-870,893.

We determined that the binding epitope for CDX-1140 resided in CRD1 of CD40, and thus outside of the CD40L-binding site that primarily involves interactions with amino acid residues within the cysteine-rich domain 2 (CRD2) and cysteine-rich domain 3 (CRD3) of CD40 [39, 40]. There is, however, a reported hydrophilic contact between residue K46 within CRD1 of CD40 and residue Y146 of CD40L [40], and it is possible that interference with this hydrophilic contact may account for the incomplete but measurable blocking of rCD40L binding at higher CDX-1140 concentrations. At lower concentrations, however, CDX-1140 does not substantially impact rCD40L binding, and when combined at these sub-optimal doses we observed strong synergistic activity with regards to B-cell and dendritic-cell activation. Although our studies used a soluble form of CD40L, it is tempting to speculate that CD40L-expressing T-helper cells may cooperate with CDX-1140 to provide enhanced activation of antigen-presenting cells at local sites of inflammation such as lymph nodes or tumors. This synergy may provide locally enhanced agonist activity without adding to systemic toxicities.

As has been shown for other CD40 agonist antibodies, CDX-1140 demonstrated significant anti-tumor activity in xenograft models of CD40 expressing lymphomas and a bladder cancer-derived cell line. CDX-1140, which does not bind murine CD40, was effective in delaying tumor growth when administered alone to immunodeficient mice bearing either Ramos or Raji subcutaneous lymphomas. Importantly, the addition of human PBMCs with the tumor cells made the CDX-1140 treatment more effective, leading to long-term survival in most animals. We believe the enhanced activity is likely due to the activation of monocytes, as our study demonstrated that CD14-positive cells were responsible for the production of cytokines by human PBMCs treated with CDX-1140.

The collective data support that CDX-1140 represents a CD40 agonist mAb with unique characteristics that may allow for higher systemic exposure than previously achieved with other potent CD40 agonist antibodies. A human clinical trial was recently initiated with CDX-1140 enrolling multiple solid tumor indications (clinicaltrial.gov identifier: NCT03329950). The study includes an initial dose escalation in which CDX-1140 is administered on a monthly schedule followed by expansion cohorts. We anticipate initiating combination strategies during this Phase 1 trial, in particular with CDX-301, a soluble form of Flt3 ligand that uniquely increases the number of dendritic cells and may augment the ability to initiate anti-tumor immune responses [41].

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Author contributions Laura A. Vitale: study design, methodology, data analysis, and manuscript preparation. Lawrence J. Thomas: study design, methodology, data analysis, and manuscript preparation. Li-Zhen He: study design, methodology, data analysis, and manuscript preparation. Thomas O'Neill: experimental design, methodology, and performance; data analysis. Jenifer Widger: recombinant DNA design, construction and characterization. Andrea Crocker: experimental design, methodology, and performance; data analysis. Karuna Sundarapandiyan: experimental design, methodology, and performance; data analysis. James R. Storey: recombinant DNA design, construction and characterization. Eric M. Forsberg: experimental design, methodology, and performance; data analysis. Jeffrey Weidlick: experimental design, methodology, and performance; data analysis. April R. Baronas: experimental design, methodology, and performance; data analysis. Lauren E. Gergel: experimental design, methodology, and performance; data analysis. James M. Boyer: experimental design, methodology, and performance; data analysis. Crystal Sisson: experimental design, methodology, and performance; data analysis. Joel Goldstein: study design, data analysis, and manuscript preparation. Henry C. Marsh, Jr.: study design, data analysis, and manuscript preparation. Tibor Keler: study design, data analysis, and manuscript preparation.

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Compliance with ethical standards

Conflict of interest All authors are employees of, and own stock or stock option in Celldex Therapeutics, Inc.

Ethical approval and ethical standards and animal sources.

Animals were sourced from IACUC-approved commercial sources. Murine xenograft studies (animal source: Taconic Biosciences) were approved by the Celldex IACUC of Hampton, NJ (AUP CDX-002) or the Celldex IACUC of Needham, MA (AUP 08-2017). Animal care followed the *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (National Research Council. 2011. Washington, DC: The National Academies Press). The pilot primate study (animal source: Charles River Laboratories) was approved by the Charles River Laboratories IACUC of Shrewsbury, MA (AUP 20097548). Those animals were handled according to *Guide for the Care and Use of Laboratory Animals: Eighth Edition* and AAALAC rules (Association for Assessment and Accreditation of Laboratory Animal Care International). The primate toxicology study (animal source: Kunming Biomed International LTD) was approved by the Citoxlab IACUC of Montreal, QC (AUP 1016–3273). Those animals were handled according to The Canadian Council on Animal Care and AAALAC rules.

Cell line authentication.

Cell lines were sourced directly from vendors that provide authentication. CHO and EJ138 cells were purchased from Millipore-Sigma, HEK-293 cells were purchased from InvivoGen, NFkB luciferase reporter HEK293 stable cell line was purchased from Signosis, and the Ramos and Raji cell lines were purchased from ATCC.

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