The Human Agonistic CD40 Antibody ADC-1013 Eradicates Bladder Tumors and Generates T-cell-Dependent Tumor Immunity

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Abstract

Purpose: Local administration of immune-activating antibodies may increase the efficacy and reduce the immune-related adverse events associated with systemic immunotherapy of cancer. Here, we report the development and affinity maturation of a fully human agonistic CD40 antibody (IgG1), ADC-1013.

Experimental Design: We have used molecular engineering to generate an agonistic antibody with high affinity for CD40. The functional activity of ADC-1013 was investigated in human and murine in vitro models. The in vivo effect was investigated in two separate bladder cancer models, both using human xenograft tumors in immune deficient NSG mice and using a syngeneic bladder cancer model in a novel human CD40 transgenic mouse.

Results: Activation of dendritic cells (DC) by ADC-1013 results in upregulation of the costimulatory molecules CD80 and CD86, and secretion of IL12. ADC-1013 also activates DCs from human CD40 transgenic mice, and peptide-pulsed and ADC-1013-stimulated DCs induce antigen-specific T-cell proliferation in vitro. In vivo, treatment with ADC-1013 in a syngeneic bladder cancer model, negative for hCD40, induces significant antitumor effects and long-term tumor-specific immunity. Furthermore, ADC-1013 demonstrates significant antitumor effects in a human bladder cancer transplanted into immunodeficient NSG mice.


Introduction

CD40 is a cell surface–expressed glycoprotein that belongs to the TNF receptor (TNFR) superfamily and plays a central role in the immune system (1). It is expressed on a variety of immune cells, such as B cells, dendritic cells (DC), monocytes, and macrophages, but also on several tumors, such as B cell lymphomas and carcinomas (1). CD40 agonists trigger effective antitumor responses in preclinical models (2, 3). These responses occur via two distinct mechanisms: (i) tumor-specific immune responses, and (ii) direct tumoricidal effects such as apoptosis and antibody-dependent cellular cytotoxicity (ADCC; ref. 2). The indirect antitumor immune effect, which is independent of the CD40 status of the tumor, is mediated by tumor-specific cytotoxic T lymphocytes (4–6), and possibly macrophages in certain tumor types (7). The direct tumoricidal effects, on the other hand, depend highly on the CD40 expression of the tumor.

Several clinical trials have been reported for agonistic CD40 antibodies (2, 8), including CP-870,893 (IgG2; ref. 9), Chilob 7/4 (10), and SGN-40 (IgG1), the latter being a weak CD40 agonist (11). Systemic administration of CD40 agonists may result in dose-limiting immune-related adverse effects, such as cytokine release syndrome (12, 13). Recently, it was demonstrated that local administration of agonistic CD40 antibodies into tumor-bearing mice resulted in improved antitumor effect, and caused less adverse immune-related events than systemic treatment (4, 14, 15). The concept of intratumoral CD40 stimulation has also been validated using local injection of adenoviral vectors expressing CD40L, demonstrating antitumor effects in human and murine bladder cancer as well as in dog melanoma, with minimal side effects (16, 17).

To translate these findings into the clinic, we set out to engineer a CD40 agonistic antibody (18, 19). ADC-1013, using Fragment INduced Diversity (FIND; ref. 20). The affinity and potency of the antibody were increased because these properties are likely to be of importance for local administration but also advantageous for systemic administration. Initially, the antitumor effect of the optimized antibody was evaluated on an EJ human bladder cancer cell line in immunodeficient NSG mice, with or without transfer of human monocyte-derived DCs (moDC) and T cells (21, 22). To further delineate the mode of action, we used a human CD40 (hCD40) transgenic mouse (hCD40tg) on a C57BL/6 background to demonstrate immune-related antitumor effects of ADC-1013 in a fully immune-competent host.
Materials and Methods

Antibodies and cell lines
ADC-1013 was optimized as described in Supplementary Methods (see also Supplementary Table S1), S2C6 (chimeric, human γ1/κ; ref. 23) and B44 (19) were produced by transient expression in HEK cells and subsequent purification on protein A columns. An aglycosylated variant of ADC-1013, referred to as ADC-1013-N297Q, was produced by introducing a single N297Q mutation in the Fc-region. Endotoxin levels were analyzed using the Quantitative LAL test using Endosafe–Portable Test System. Agonistic rat-anti-mouse CD40 (clone: FGK4.5) and rat IgG2a (clone: 2A3) were purchased from Bio X Cell. The hCD40⁺ Raji cell line was cultured in R10 medium (RPMI-1640 supplemented with l-glutamine and 10% FBS), at 37°C and 5% CO₂. The human EJ bladder cancer cell line (21) was cultured in R10 medium supplemented with 100 U/mL penicillin-streptomycin at 37°C and 5% CO₂. EJ cells were confirmed CD40⁺ and HLA-A2⁺. The murine bladder transitional cell carcinoma cell line Mouse Bladder-49 (MB49; a kind gift from Dr. K. Esuvaranathan, National University of Singapore, Singapore in 1996) and Lewis lung carcinoma-1 (LLC-1) (ATCC; CRL-1642) were cultured in DMEM GlutaMax supplemented with 10% FBS, 0.1 mmol/L sodium pyruvate, 100 U/mL penicillin-streptomycin at 37°C and 5% CO₂. Human CD40⁺ MB49 was transfected with human CD40-expressing vector under the control of the CMV promoter. For cross-linking of ADC-1013 in human moDC assays, goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) was used. The structure models of B44 and ADC-1013 were generated by Saromics Biostuctures (Lund, Sweden) (Fig. 1D).

Translational Relevance
Local administration of immune-activating antibodies has the potential to increase the efficacy and reduce the immune-related adverse events associated with systemic immunotherapy of cancer. We have generated a human CD40 agonistic antibody (ADC-1013) that represents the first immunomodulatory human antibody optimized for local immunotherapy of cancer. Local treatment with ADC-1013 results in potent antitumor effects and generation of tumor-specific immunity in a human CD40 transgenic mouse model. The preclinical data strongly support the initiation of clinical investigation of ADC-1013. ADC-1013 is currently in late preclinical development.
Human cells obtained from peripheral blood mononuclear cells

Buffy coats from peripheral blood were obtained from healthy blood donors at Uppsala University Hospital Blood Center and Lund University Hospital in Sweden. Human peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. CD14+ monocytes, CD19+ B cells, and CD56+ NK (natural killer) cells were isolated using magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotec). Monocytes were cultured for 6 to 7 days in R10 with recombinant human IL4 and GM-CSF with medium changed every second day and moDCs were confirmed to be CD1a<sup>hi</sup> and CD14<sup>-</sup> by flow cytometry. T cells for the in vivo experiments were isolated using the Dynabeads Untouched Human T cells Kit according to the manufacturer's protocol (Invitrogen Life Technologies).

Flow cytometry

The flow cytometry protocol used to analyze the human cells in vitro has been described previously (24). Briefly, cells were blocked with ChromPure Mouse IgG (Jackson Immunoresearch Laboratories) and incubated with anti-human Abs for 15 minutes at 4°C: anti-CD1a, anti-CD14, anti-CD19, anti-CD86 (Dako), anti-CD80 (BD Biosciences), and anti-CD19 (ImmunoTools) antibodies. Cells in live gate were acquired on a FACS Canto II (BD Biosciences).

For analysis of murine cells by flow cytometry, the spleens were cut into small pieces and incubated in serum-free media with Liberase TL (Roche Applied Science). Subsequently, cells were stained with antibodies binding to CD11b, F4/80, CD11c, B220, CD3, NK1.1, CD8a, and to CD40 or to murine CD40 antibodies. Cells from a CD40<sup>-/-</sup> mouse strain (25) were used to define the expression level. Conventional DCs were gated as CD11c<sup>hi</sup>, B220<sup>-</sup> and subsequently as CD8a<sup>+</sup> CD11b<sup>low</sup> or CD8a<sup>-</sup> CD11b<sup>+</sup>. Macrophages were gated as CD11b<sup>+</sup>, F4/80<sup>+</sup>, CD11c<sup>-</sup>, and B220<sup>-</sup>. B cells were gated as CD20<sup>+</sup>, CD11b<sup>-</sup>, and CD11c<sup>+</sup>. T cells were gated as CD3<sup>+</sup>, NK1.1<sup>-</sup>. NK cells were gated as CD3<sup>+</sup>, NK1.1<sup>+</sup>. NK cells were stained with antibodies binding to CD11b, F4/80, CD11c, B220, CD3, NK1.1. Anti-LFA1 and anti-CD11b antibodies were obtained from BioLegend.

In vitro cell assays in human cells

Dendritic cell assay. DCs were derived from human peripheral blood monocytes (24). The moDCs and dilution series of stimulating antibodies or isotopic control antibody (IgG1) were cultured for 48 hours in R10 medium with IL4 and GM-CSF. Upregulation of the activation markers CD86 and CD40 was analyzed by flow cytometry. Secretion of IL12p40 was measured by ELISA (BioLegend). IL12p70 was measured using ELISA (eBioscience and R&D Systems). For cross-linking experiments, the antibodies were cross-linked using anti-human goat antibody (Jackson ImmunoResearch Laboratories, Inc.).

B-cell proliferation assay. CD19<sup>+</sup> cells (5 x 10<sup>5</sup>/well, >95% purity) were cultured in R10 medium with 10 ng/mL of IL4 and dilution series of antibodies. After 48 to 72 hours, the metabolic activity was measured with CellTiter-Glo (Promega).

ADCC assay. CD56<sup>+</sup> NK cells were mixed with Raji cells in a 5:1 ratio in ADCC-medium (RPMI-1640 + 10% Ultra low IgFCS + 10 mmol/L Hepes + 1 mmol/L Na-pyruvate). The cytotoxicity was measured after 2 hours of incubation using aCella-TOX Kit (Cell Signaling Technology, Inc.) according to the manufacturer's instructions.

In vivo experiments

Female NSG NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>m1Wjl</sup>/J.Svl mice were obtained from the Jackson Laboratory. Cryopreserved human T cells were thawed and grown with human recombinant IL2 1 day before. EJ cells (3 x 10<sup>6</sup>), alone or mixed with 1 x 10<sup>7</sup> moDCs and 5 x 10<sup>5</sup> T-cells were injected s.c. Antibodies were administered together with the cell solution on day 0 and thereafter peritumorally on days 7 and 14. Tumor size was measured with caliper and weighed at the end-point day (day 23). The treatment groups with moDC and T-cells (DC/T) consisted of 8 mice per group, for which T-cells and DCs from 2 donors were used (4 mice/group/human donor). The experiment using EJ cells alone consisted of 6 mice per group.

The human CD40 transgene mice contains the hCD40 sequence downstream of the murine CD40 promoter and regulatory elements (26) and do not express murine CD40 (Supplementary Methods; ref. 27). The mice were analyzed for human CD40 expression by IHC using tissue cryo sections from spleen, lymph node, pancreas, CNS, liver, lung, and kidney from one female and one male hCD40tg mouse. Human and mouse spleen were used as positive and negative controls. Briefly, the sections were blocked in 5% normal mouse serum (Jackson ImmunoResearch Laboratories) and stained with mouse anti-hCD40-FITC (BD Biosciences). The sections were subsequently stained with rabbit anti-FITC (Abcam) and anti-rabbit-HRP (Brightvision poly-HRP rabbit, Immunologic Immunologic BV). The slides were analyzed in a Leica DMRX-e microscope and representative photos were taken from all tissues. CD40<sup>+</sup> C57Bl6/mice were obtained from the Jackson Laboratory B6.129P2-Cd40<sup>tm1Bgr</sup>/J (002928).

For in vivo pharmacokinetic experiments, female scid mice C.B-17/IgH<sup>1</sup>/Jcrtac-Prkdc<sup>scid</sup> were used. 10<sup>6</sup> Raji cells were injected s.c. in the left flank. Tumors at approximately 500 mm<sup>3</sup> were injected with antibodies (30 µg in 60 µL) in the tumor mass and blood was collected upon termination 4 hours after injection. For intratumoral retention studies, hCD40<sup>+</sup> MB49 cells were inoculated s.c. in male hCD40tg mice and antibodies were injected intratumorally in 20 µL when the tumor reached approximately 150 mm<sup>3</sup>. The tumors were homogenized in T-PER (Thermo Scientific). The antibody levels were measured by sandwich ELISA.

In vivo immune activation profiling in cells from hCD40tg mice

Bone marrow–derived DCs (BMDC) were generated by extraction of cells from the bone marrow and subsequent culturing with mGM-CSF for 8 days. Immature BMDCs were stimulated with a concentration range of ADC-1013 or isotype control antibody (IgG1), or LPS (lipopolysaccharide; Sigma Aldrich). Activation was measured by IL12p40 production or CD86 expression after 48 hours of stimulation using an in-house ELISA assay or flow cytometry (see Supplementary Methods).

For T-cell activation, transgenic pmel T-cells (TCR specific for human gp100 in H-2D<sup>b</sup>, obtained from The Jackson Laboratory 005023), were isolated from splenocytes by CD8-negative selection (Dynal invitrogen). BMDCs were generated as described above and loaded with suboptimal concentration (1 pg/mL).
of human gp100 (KVPRNQDWL) and stimulated with ADC-1013 or an isotype control antibody. After 24 hours, BMDCs and CFSE-labeled (Invitrogen) CD8+ pmel cells were mixed and T-cell proliferation was assessed by flow cytometry after 3 days.

BMDCs were additionally stimulated with or without LPS (1 μg/mL) in vitro and pulsed with hgp100 (1 μg/mL) before injection i.v. into naïve wt C57BL/6 mice (1 × 10⁶ cells/mouse), which were injected i.v. the day before with CFSE-labeled splenocytes (11 × 10⁶ cells/mouse) isolated from pmel mice. The in vivo proliferation of pmel CD8+ cells was analyzed using flow cytometry 4 days after BMDC injection.

**In vivo experiments in the syngeneic MB49 bladder cancer model**

ADC-1013 was evaluated in hCD40tg mice and CD40−/− mice. Tumors were inoculated by injection of 2.5 × 10⁶ cells in the flank. Antibodies were administrated peritumorally with indicated doses on days 7 and 10 after inoculation, and tumor measurements were made with caliper. Blood was taken 4 hours after every injection and serum or plasma was obtained. Free antibody levels in serum were measured by a sandwich ELISA specific for human γ1 and λ chains or human CD40.

**Immunologic memory**

To characterize the immunologic memory, T-cells were depleted in naïve and tumor-experienced mice, in which ADC-1013 had induced complete tumor regression, by intraperitoneal injection of anti-CD4 (clone GK1.5) and anti-CD8 (clone, 53–6.72) at a dose of 10 μg/g in body weight, and subsequently mice were given a MB49 rechallenge (antibodies from Bio X Cell). Antibodies were administrated on days −2, −1, 0, 4, 8, and 12 related to tumor challenge. T-cell depletion (or T-cell inactivation by complete target occupancy) was confirmed by flow cytometry. Tumor-specific immunity was also characterized by a rechallenge using MB49 cells or the irrelevant syngeneic LLC-1 cell line.

**Ethical considerations and statistical analyses**

Animals were housed according to regional regulations. All animal experiments were approved by the regional Ethics Committees in Lund/Malmö and Uppsala, Sweden. Statistical analyses were performed using GraphPad prism software (GraphPad Software, Inc.).

**Results**

**Selection of ADC-1013, a CD40 agonist optimized for local immunotherapy of cancer**

B44 (18), a CD40 agonist with moderate affinity (Supplementary Table S2), was used as template for four focused libraries designed to generate functional diversity for FIND recombination and lead optimization (Fig. 1; ref. 20). The starting phage libraries were subjected to a first round of selection and the outputs were subsequently recombined using FIND, creating a new library combining functional diversity from all the sublibraries. The FIND-recombined library was subjected to four additional rounds of selection with increasing stringency and the output from each selection was expressed in scFv format, and 2000 clones from each selection round were analyzed in a concentration-independent HTS assay.

The 95 unique clones with the highest affinity were reexpressed in IgG format, and the selected clones were further analyzed in Biacore, demonstrating affinities for most of the variants in the order of 10 to 40 pmol/L (Fig. 1). The agonistic properties of the selected variants were confirmed using a B-cell proliferation assay (data not shown). As the tumor milieu is acidic in many tumors (28), the kinetic constants were measured at a low pH (5.4) at 37°C. This allowed selection of a lead candidate (ADC-1013) with 100-fold increased affinity (Kd = 1 × 10−11 mol/L at pH 7.4, 37°C) compared with B44 at both physiologic and acidic pH (Supplementary Table S2). The EC50 value for binding to CD40-expressing cells was increased approximately 30-fold (Supplementary Table S3). Analysis of the structure model indicates that one of the three mutated positions directly changes the surface charge of the paratope, whereas the other two mutations may induce conformational changes (Fig. 1D). Moreover, ADC-1013 had 3- to 5-fold increased potency compared with B44, as measured by B-cell proliferation assay or activation of moDCs (Supplementary Table S3), whereas the efficacy was similar. The selectivity of ADC-1013 was assessed by flow cytometry of human peripheral blood and IHC (data not shown). ADC-1013 does not cross-react with murine, rat or canine CD40, but has similar affinity to cynomolgus monkey CD40 and hCD40, evaluated by binding to peripheral B cells by flow cytometry and B-cell proliferation assay (data not shown).

**In vitro characterization of ADC-1013**

The primary mode of action of ADC-1013 is the activation of immune cells and specifically DCs. Stimulation of human moDCs with ADC-1013 resulted in upregulated expression of the cell surface activation markers, CD86 (Fig. 2A) and CD80 (data not shown), and induction of IL12p40 (Fig. 2B). IL12p70 was also significantly induced compared with isotype control, but at a lower level 145 pg/mL ± 80 (mean ± SEM, see Supplementary methods), similar to previous observations with CD40 agonists (29). As a control, the DCs were stained with Annexin V, demonstrating that viability of DCs was not significantly affected by the treatment (Supplementary Fig. S1). The secondary mode of action of ADC-1013 is direct killing of hCD40+ cells, for example, by ADCC. To evaluate this, we used hCD40+ Raji cells as target cells and human primary NK cells as effector cells. ADC-1013 induces ADCC in Raji cells in a dose-dependent manner (Fig. 2C). The ability of ADC-1013 to induce ADCC was also confirmed in hCD40+ Ramos cells (data not shown). We used the weak CD40 agonist S2C6-hlgG1 (23, 30), as a control (Fig. 2A and C). S2C6-hlgG1 only weakly stimulated moDC (Fig. 2A), but induced a similar level of target-dependent ADCC (Fig. 2C). Furthermore, ADC-1013 with an N297Q mutation (ADC-1013N297Q), which inhibits glycosylation, and thereby binding to FcγR (it binds, however, with similar affinity to CD40, Kd = 1.5 × 10−11 mol/L at pH 7.4, 37°C), did not induce ADCC (data not shown).

The binding to FcγR affects the agonistic properties of CD40-directed antibodies as recently described by several groups (31–33). The FcγR dependency of ADC-1013 was assessed using ADC-1013N297Q, in the DC assay. CD86 was significantly less upregulated by ADC-1013N297Q [CD86 MFI (mean fluorescent intensity) not above background, Fig. 2D] than by ADC-1013. The ability to upregulate CD86 was restored upon cross-
linking with an anti-human IgG (background-subtracted MFI 130, Fig. 2D).

**Inhibition of growth of human bladder cancer in NSG mice repopulated with human DC and T-cells**

To evaluate ADC-1013 in vivo, we initially used a xenograft model. Immunodeficient NSG mice (22) were repopulated with human moDCs and T-cells and the antitumor effect on the EJ human bladder cancer cell line was investigated (21). Human T cells and moDCs were obtained from the same donor, similar to the setup used by Gladue and colleagues (29). T-cells and moDCs (DC/T) were injected together with the EJ cells on day 0 and ADC-1013 (30 μg/dose) was administered peritumorally on days 0, 7, and 14. The treatment resulted in significant antitumor responses in mice repopulated with human moDC/T-cells (n = 6/group), as compared with the isotype control (\( P < 0.05 \), two-way ANOVA comparing the tumor growth over time). Bladder tumor data display representative data from one of two similar experiments. Data, with ±SEM.

A novel hCD40\(^{+}\) transgenic mouse expressing functional hCD40 on monocytes and DCs

To further study the antitumor effects related to the immune-activating properties of ADC-1013, an hCD40tg mouse was used. The transgenic mouse expresses the hCD40 gene under the control of the murine CD40 promoter and regulatory elements (26) on a murine CD40-negative background (hCD40tg). Immunohistochemical analysis of the hCD40 expression in one female and one male hCD40tg mouse was performed on kidney, spleen, liver, lung, lymph node, brain, and pancreas. The staining pattern demonstrated strong, widespread expression of hCD40 in spleen and in lymph nodes (Fig. 3A). Human CD40 was also highly expressed in macrophages in the liver of hCD40tg mice (Supplementary Fig. S2).

The expression pattern of hCD40 on immune cells was further studied using flow cytometry. Spleen and blood were obtained from wild-type (wt) mice, hCD40tg mice and mCD40\(^{-/-}\) mice (see Supplementary Table S4). Conventional DC populations (Fig. 2B) as well as blood monocytes (Supplementary Table S4) obtained from the hCD40tg mouse display an hCD40 expression pattern that is similar to the mCD40 expression pattern on the same cell types obtained from wt mice. The endogenous CD40 expression on B-cells in wt mice is greater compared to the hCD40 expression on B-cells in hCD40tg mice in both spleen and blood (Fig. 3B). Both NK and NKT cells expressed CD40 in both the C57BL/6 wt and the hCD40tg strain. As expected, the bulk T-cell pool from both hCD40tg mice and wt mice expressed very low levels of mCD40 and hCD40, respectively (Supplementary Table S4).
Anti-CD40 (ADC-1013) activates hCD40tg-derived DCs resulting in improved T-cell responses

To assess whether ADC-1013 activates BMDCs obtained from the hCD40tg mice in vitro, we cultured immature BMDCs with ADC-1013 or an isotype control antibody and measured IL12p40 in the supernatant after 48 hours. A dose-dependent increase of IL12p40 secretion was detected (Fig. 4A) in an hCD40-specific manner. Induction of IL12 in BMDCs from wt mice following stimulation with a mouse CD40-specific antibody, FGK4.5, exhibited a similar pattern (Fig. 4B). Furthermore, CD11c⁺ cells purified from hCD40tg mouse spleen could be stimulated by ADC-1013 in a target-dependent manner. Induction of IL12 in BMDCs from wt mice following stimulation with a mouse CD40-specific antibody, FGK4.5, exhibited a similar pattern (Fig. 4B). Furthermore, CD11c⁺ cells purified from hCD40tg mouse spleen could be stimulated by ADC-1013 in a target-dependent manner. Induction of IL12 in BMDCs from wt mice following stimulation with a mouse CD40-specific antibody, FGK4.5, exhibited a similar pattern (Fig. 4B). Furthermore, CD11c⁺ cells purified from hCD40tg mouse spleen could be stimulated by ADC-1013 in a target-dependent manner. Induction of IL12 in BMDCs from wt mice following stimulation with a mouse CD40-specific antibody, FGK4.5, exhibited a similar pattern (Fig. 4B). Further...

ADC-1013 eradicates hCD40⁻ bladder tumors and generates T-cell-dependent tumor immunity

The hCD40tg mice enabled the evaluation of in vivo antitumor effects of ADC-1013 in an immunocompetent host using a syngenic tumor model. Established MB49 tumors (negative for hCD40) were treated peritumorally with ADC-1013 (0, 10, 30, and 100 μg) on days 7 and 10 after tumor inoculation. A dose-related antitumor effect was observed (Fig. 5A) with a near maximal effect obtained at the 30-μg dose (1.2 mg/kg). ADC-1013 did not demonstrate any antitumor effects in CD40⁻/⁻ mice (Fig. 5B). Furthermore,
peritumoral treatment (100 μg at days 7 and 10) of an established tumor on one flank cured mice with a second tumor on the distal, untreated flank, similar to previous studies showing that local CD40 treatment generates a systemic immune response (Fig. 5C; refs. 14, 15).

To evaluate whether the immunologic memory induced by ADC-1013 treatment is T-cell dependent, mice cured of MB49 tumors by local ADC-1013 administration were rechallenged with MB49 cells while depleted of T-cells. Intact complete responders remained tumor free after MB49 rechallenge, while all other mice (complete responders depleted of T-cells or naïve mice with or without T-cells) succumbed to tumor growth (Fig. 5D), indicating that the tumor immunity is T-cell–dependent. Isotype control–treated hCD40tg mice also exhibited more tumor growth arrest than CD40−/− mice (Fig. 5B), which appears to depend on an endogenous T-cell response unrelated to the isotype antibody (Fig. 5D, non-treated naïve animals with and without T-cells). In addition, the antitumor memory was specific, as complete responders rechallenged with an irrelevant LLC-1 tumor succumbed to tumor growth (Fig. 5E).

Target-dependent decrease of free ADC-1013 in serum 4 hours after local administration

Serum collected from ADC-1013 or isotype-treated hCD40tg mice demonstrates that the antibody levels in serum 4 hours after injection of ADC-1013 were more than 100-fold lower than those of the isotype control antibody (Fig. 6A). To confirm that the effect was target dependent, ADC-1013 was measured in CD40−/− mice, where serum levels were similar to the isotype control antibody (Fig. 6B).

Increasing the affinity to CD40 should also enable the antibody to be released more slowly from the injection site, thereby decreasing the $C_{\text{max}}$ and minimize systemic side effects. To demonstrate this, the serum levels 4 hours after local injection of B44 and ADC-1013 into hCD40−/− Raji tumors...
on SCID mice was measured, indicating that the higher affinity of ADC-1013 resulted in slower systemic release of the antibody (Fig. 6C). A similar trend was seen in hCD40tg mice where the serum levels were measured 4 hours post treatment at days 7 and 10. The serum levels of ADC-1013 were low compared with levels of B44 (0.011 mg/mL for ADC-1013 compared with 0.078 mg/mL for B44 at day 10, Fig. 6D). The antitumor effect of B44 is similar to ADC-1013 (Fig. 6E). To further demonstrate that the increased affinity of ADC-1013 delays the release from the tumor, hCD40+ MB49 tumors with an average size of 150 mm³ were injected with ADC-1013 and B44. The tumors were homogenized and the antibody levels determined, showing an increased tumor to blood ratio of ADC-1013 at early time points (Fig. 6F).

**Discussion**

Local administration of immunomodulatory antibodies has the potential to increase the efficacy and reduce the immune-related adverse events associated with immunotherapy. The concept of generating a systemic immune response with local administration of immunomodulating antibodies has been demonstrated by us and others for CD40 antibodies but also for other targets such as CTLA-4 and CD137 (14, 15, 34–37). In this study, we describe the generation of an optimized CD40 agonistic antibody. The resulting antibody had a high affinity (Supplementary Table S2) and a very fast on-rate ($k_{on}$). The on-rate may be of particular importance for local administration. When an antibody is administered locally, it will immediately start to leak out of the tumor area, and the on-rate for the binding reaction will be a limiting factor (38). Furthermore, the increased affinity and potency of ADC-1013 would also be advantageous when administered systemically. The comparison of ADC-1013 and the starting clone (B44) shows that the affinity maturation delayed the release from the injection site. We showed this using two mouse models, the xenograft model with hCD40-negative MB49 cells in hCD40tg mice and in hCD40tg mice.
using hCD40+ syngeneic tumors. The resulting slower release from the injection is important for local immunotherapy, but may also be of clinical interest when administering the antibody systemically, for example, subcutaneously. However, further studies on the distribution in tumor, tissue, and blood using radiolabeled antibody, may be warranted to confirm these findings.

Several groups have demonstrated that agonistic effects of anti-CD40 antibodies depend on FcγR binding (31, 32). Herein, we demonstrate that the agonistic effect of ADC-1013 is abrogated using an aglycosylated variant of ADC-1013. The agonistic effect of the aglycosylated form can be restored by a secondary cross-linking antibody, suggesting that the main role of the FcγR binding is cross-linking of the CD40 receptor. The positive effect of FcγRII binding in trans was first described by Pound and colleagues (39), and the mechanisms behind this have recently been highlighted. FcγRIIB has been suggested to play an important role in mice (31, 32). However, the results by White and colleagues (32) suggest that cellular distribution and bioavailability of the FcγRs may also be of importance for the agonistic effect in vivo. However, the human CD40 agonistic antibody CP-870,893 does not rely on FcγR binding for its functional activity (40).

ADC-1013 has a dual mode of action in CD40+ tumors. It may induce direct tumor killing of CD40+ tumor cells, generating antigens readily taken up by DCs. Importantly, CD40 stimulation also activates DCs, licensing them to cross-present tumor antigens to T-cells. Herein, ADC-1013-induced DC activation results in the upregulation of costimulatory molecules (e.g., CD80 and CD86) and induction of IL12, allowing for an efficient activation of tumor-specific cytotoxic T-cells. Thus, there is a potential synergistic effect of the dual mode of action of ADC-1013.

ADC-1013 is effective against EJ human bladder cancer cells in immunodeficient NSG mice both with and without human immune cells, supporting both the immune-activating and direct tumor-killing modes of action of ADC-1013. However, only limited information concerning adaptive immune activation can be achieved using a xenograft model (29). Therefore, we used an hCD40 transgenic mouse model to further assess the mode of action of ADC-1013.

Reports exist on hCD40-expressing transgenic mice, in which hCD40 expression has either been restricted to B cells (27), or included DCs under the control of the murine I-E-alpha promoter (41). However, functional analysis of CD40-targeted therapy has not been published. The hCD40tg mice...
were mapped for CD40 expression in the immune cell compartment (Fig. 3 and Supplementary Table S4) revealing that hCD40 expression in hCD40tg mice was comparable to mCD40 expression in wt mice on endogenous DCs (CD8+ and CD8−). Expression was confirmed on macrophages and, at a lower level, the B cells. Furthermore, ADC-1013 could effectively, and in a dose- and target-dependent manner, activate DCs derived from hCD40tg mice in vitro, demonstrating the functionality of the DC population in these mice. To investigate the ability to induce T-cell activation, DCs were loaded with suboptimal hgp100 concentration in combination with ADC-1013 or irrelevant antibody. The added pmel-derived T-cells responded with vigorous proliferation to peptide-loaded and ADC-1013–activated DCs and demonstrated the potential of ADC-1013 to improve immune responses via T-cells. Furthermore, stimulation with CD40 agonist in the hCD40tg mice induced cytokines such as IL6 and TNFα (data not shown), in line with clinical findings of CD40 agonists (9). Notably, hCD40tg mice appear to have an improved endogenous T-cell-mediated antitumor response compared with CD40−/− mice. The response to LPS stimuli by DCs and the following T-cell priming response were similar between the two strains. The phenotypic and functional characterization of the hCD40tg mice supports the translational relevance of this strain for studying CD40-targeted therapy.

Our pharmacokinetic measurements demonstrate that the distribution of ADC-1013 is highly target dependent, which is in line with previous work on murine CD40 agonistic antibodies (4). This strong target-mediated effect on the serum levels of ADC-1013 is likely a result of the target distribution, redistribution, and antibody internalization following administration (4, 42). Antidrug antibodies are not likely to affect the measurements, since samples were taken within 3 days after the first treatment (4). These findings correlate well with clinical studies carried out with CD40 agonistic antibodies (9, 12), and further support the translational relevance of the hCD40tg mice.

We used MB49 bladder cancer model to investigate ADC-1013 therapy in hCD40tg mice. Importantly, this syngeneic tumor lacks hCD40, ensuring that only the immunologic antitumor effect was assessed. Our data demonstrate that local ADC-1013 therapy effectively cures bladder cancer tumors in a dose- and host CD40−/−-dependent manner. The mice that were cured by ADC-1013 treatment were immune to rechallenge more than 5 months after the cure. This immunity was eliminated following depletion of CD4 and CD8 cells using depleting antibodies, indicating that T-cells are important for the tumor immunity in concordance with previous studies (14, 15). Furthermore, the specificity of the tumor immunity suggests T-cell involvement in this process. No apparent toxicities were observed during the depletion study; however, it cannot be excluded that other cells may play a role in this respect. Furthermore, splenocytes obtained from cured mice produce significantly more IFNγ than corresponding splenocytes obtained from naive donors when stimulated by MB49 cells in vitro (data not shown), supporting a role of tumor-specific T-cells. Finally, the tumor immunity generated by ADC-1013 can be transferred to naive mice by splenocyte transfer from cured mice, generating a significantly improved effect compared with splenocytes obtained from naive mice (Supplementary Fig. S4).

We and others have previously demonstrated that local CD40 treatment generates a systemic immune response (14, 15). These studies were corroborated in this study showing effect also on distal, untreated tumors. The systemic Cmax in mice treated peritumorally with 100 μg ADC-1013 is approximately 30 ng/ml (data not shown), which is three times lower than the EC50 for cell activation (Figs. 2, 4 and Supplementary Table S3), indicating that local immune activation plays a role in the antitumor effect on the distal tumor, although systemic effects of free antibody cannot be excluded.

In conclusion, we have developed and optimized a human agonistic CD40 antibody for immunotherapy of cancer by improving the affinity, and thereby prolonging the tumor retention time. Our data demonstrate that ADC-1013 induces long-lasting antitumor responses and immunologic memory in a relevant experimental tumor model. Moreover, we have assessed the immunological properties of ADC-1013 in hCD40tg mice appropriate for the study of hCD40-targeting therapies. To the best of our knowledge, ADC-1013 represents the first immunomodulatory antibody optimized for directed immunotherapy of cancer. ADC-1013 is currently in late preclinical development.

Disclosure of Potential Conflicts of Interest
S.M. Mangsbo reports receiving a commercial research grant from Alligator Bioscience and has a royalty agreement with Alligator Bioscience on patent US2013004483A1, for which she is a co-inventor. E. Fletcher reports receiving a commercial research grant from Alligator Bioscience AB. N. Veitonmäki is an employee of Alligator Bioscience AB. E. Dahlén is an inventor of a patent application on CD40 antibodies, which is owned by Alligator Bioscience. T.H. Tötterman reports receiving a commercial research grant from and is a consultant/advisory board member for Alligator Bioscience AB. P. Ellmark is an employee of Alligator Bioscience and is an inventor of a patent application on CD40 antibodies, which is owned by Alligator Bioscience. No potential conflicts of interest were disclosed by the other authors.

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References


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