

Identification and Characterization of MEDI4736, an Antagonistic Anti-PD-L1 Monoclonal Antibody

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Abstract

Programmed cell-death 1 ligand 1 (PD-L1) is a member of the B7/CD28 family of proteins that control T-cell activation. Many tumors can upregulate expression of PD-L1, inhibiting antitumor T-cell responses and avoiding immune surveillance and elimination. We have identified and characterized MEDI4736, a human IgG1 monoclonal antibody that binds with high affinity and specificity to PD-L1 and is uniquely engineered to prevent antibody-dependent cell-mediated cytotoxicity. *In vitro* assays demonstrate that MEDI4736 is a potent antagonist of PD-L1 function, blocking interaction with PD-1 and CD80 to overcome inhibition of primary human T-cell activation. *In vivo* MEDI4736 significantly inhibits the growth of human tumors in a novel xenograft model containing coimplanted human T cells. This activity is entirely dependent on the presence of transplanted T cells, supporting the immunological mechanism of action for

MEDI4736. To further determine the utility of PD-L1 blockade, an anti-mouse PD-L1 antibody was investigated in immunocompetent mice. Here, anti-mouse PD-L1 significantly improved survival of mice implanted with CT26 colorectal cancer cells. The antitumor activity of anti-PD-L1 was enhanced by combination with oxaliplatin, which resulted in increased release of HMGB1 within CT26 tumors. Taken together, our results demonstrate that inhibition of PD-L1 function can have potent antitumor activity when used as monotherapy or in combination in preclinical models, and suggest it may be a promising therapeutic approach for the treatment of cancer. MEDI4736 is currently in several clinical trials both alone and in combination with other agents, including anti-CTLA-4, anti-PD-1, and inhibitors of IDO, MEK, BRAF, and EGFR. *Cancer Immunol Res*; 3(9); 1052–62. ©2015 AACR.

Introduction

Immune surveillance of emerging cancer cells is one of the body's defenses against the growth of tumors (1, 2). However, over time and in response to selective pressure, cancers exploit strategies to evade the immune system, allowing them to develop unchecked, a process termed immune escape (1, 3). One such strategy involves upregulation of surface proteins, such as PD-L1 (B7-H1, CD274), which modulate the immune response by delivering inhibitory signals to T cells.

PD-L1 is a type I transmembrane protein belonging to the B7 family (4). It is normally expressed on antigen-presenting cells

(APC) and binds either the programmed-death 1 (PD-1; CD279) receptor expressed on activated T cells or CD80 expressed on both activated T cells and APCs. The result of either interaction is delivery of an inhibitory signal that acts to limit T-cell activation and expansion (5, 6). PD-L1 is also expressed on non-immune cells (including the islets of the pancreas, Kupffer cells of the liver and vascular endothelium) and is upregulated on selected epithelia during inflammatory episodes (7). In this context, PD-L1 is believed to regulate inflammatory responses and maintain both peripheral tolerance and immune privilege in specific tissues (8).

PD-L1 expression is detected frequently across a broad range of cancers and is believed to result in inhibitory signals to tumor-specific T cells, which protects the tumor from immune elimination (9). Indeed, the expression of PD-L1 is associated with reduced survival and an unfavorable prognosis in a number of cancers, including lung (10), renal (11–13), pancreatic (14–16), and ovarian (17). In contrast, the levels of tumor-infiltrating lymphocytes, and more specifically cytotoxic T cells, have been correlated with improved prognosis in a number of cancers (18). Such observations suggest that, even in the face of active immunosuppressive mechanisms, antitumor immune responses could be beneficial and support the hypothesis that enhancing the immune response could provide further benefit to patients.

Antibodies that block the interaction between PD-L1 and its cognate receptors can relieve PD-L1-dependent immunosuppressive effects *in vitro*, enhancing the cytotoxic activity of antitumor

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T cells (19). Based in part on these observations, anti-PD-L1 antibodies could be used therapeutically to enhance antitumor immune responses in patients with cancer. In fact, multiple preclinical studies have demonstrated antitumor activity for anti-PD-L1 or anti-PD-1 antibodies in mouse models (15, 20–25), and two recently completed phase I clinical trials have similarly reported encouraging activity signals for anti-PD-L1 (26, 27) and anti-PD-1 (28–31) in patients.

Here, we describe the identification and characterization of a human mAb, MEDI4736, which binds specifically to human PD-L1. Results from ligand inhibition assays, functional assays in primary human immune cells, and studies in models of cancer in mice demonstrate that MEDI4736 is a potent antagonist of PD-L1 function with significant antitumor activity in mouse models, and support clinical development of the antibody for the treatment of cancer.

Materials and Methods

Antibody generation

IgG2 and IgG4 XenoMouse animals (32) were immunized with human PD-L1-Ig or CHO cells expressing human PD-L1. Hybridomas were established, and supernatants screened for binding to human PD-L1-transfected HEK 293 cells and inhibition of PD-1 binding to PD-L1 expressing CHO cells. MEDI4736 was selected based on a favorable affinity, activity, and specificity profile in these screens. The constant domain of the antibody was then exchanged for a human IgG1 triple-mutant domain. This constant domain contains three point mutations that reduce binding to C1q and the Fc gamma receptors, resulting in reduced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC; ref. 33).

Cell lines

The A375, HPAC, SKBR3, and NK-92 MI cell lines were obtained from the American Type Culture Collection (ATCC). A375 was maintained by culture in DMEM media, HPAC by culture in 50% v/v Ham's F12 and SKBR3, and NK-92 MI by culture in advanced RPMI 1640 (Gibco). All media were supplemented with 10% FBS (Gibco). NK-92 MI media were additionally supplemented with 2.5 µg/mL blasticidin and 1 µg/mL puromycin. The CT26 cell line was obtained from LGC Standards and maintained in RPMI media containing 10% FBS. All cell lines were cultured in humidified incubators at 37°C and 5% CO₂. A375 and HPAC cell lines were characterized for PD-L1 expression by flow cytometry using anti-PD-L1 (BD Pharmingen) as per the manufacturer's instructions.

Specificity ELISA

Recombinant human PD-L1, B7-H3 and B7-DC, and mouse PD-L1 (R & D Systems) were coated overnight at 5 µg/mL in PBS onto 96-well plates (Nunc). Plates were washed with PBS, blocked for 1 hour at room temperature with PBS containing 3% (w/v) milk powder, and washed again. Fifty microliters of 15 µg/mL biotinylated MEDI4736 was added to wells, and plates were incubated for 2 hours at room temperature. After 3 washes in PBS, 50 µL of 0.5 µg/mL europium-labeled streptavidin (Perkin Elmer) was added to each well in DELFIA assay buffer (Perkin Elmer), and plates were incubated for 3 minutes. After incubation, plates were washed 7 times in DELFIA wash buffer (Perkin Elmer), and time-resolved fluorescence was measured on an EnVision

plate reader (Perkin Elmer) using 340-nm excitation and 615-nm emission wavelengths.

ADCC assay

SKBR3 cells, in Advanced RPMI 1640 (Gibco) containing 10% low IgG FBS (Gibco), were added to each well of a 96-well tissue culture-treated plate (Corning) at a density of 30,000 cells per well and incubated overnight at 37°C. Culture media were aspirated, and test reagents were added at the indicated concentrations. Following incubation for 30 minutes at 37°C, NK-92 MI cells (ATCC), stably transfected with the V158 allotype of human CD16a and a luciferase reporter gene driven by a nuclear factor of activated T cells (NFAT)-responsive promoter, were added at a density of 80,000 cells per well, and incubation was allowed to continue for a further 5 hours. At this time, the level of luciferase activity in each well was measured using the Steady Glo assay system (Promega) and an Envision plate reader (Perkin Elmer).

Ligand inhibition assay

PD-1 and CD80 (R & D Systems) were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher) as per the manufacturer's instructions. PD-L1 (R & D systems) was labeled using europium cryptate (Cisbio) as per the manufacturer's instructions. Biotinylated PD-1 or CD80 were mixed with streptavidin XL665 (Cisbio) and europium cryptate-labeled PD-L1 in a 384 shallow well assay plate (Corning). MEDI4736, or an isotype-matched control antibody, was added to wells at the indicated concentrations. The maximal binding between PD-L1 and either PD-1 or CD80 was determined by omitting MEDI4736. The signal resulting from nonspecific binding (NSB) between PD-L1 and either PD-1 or CD80 was determined by adding a saturating concentration of an alternative anti-PD-L1 antibody. Plates were incubated at room temperature for 3 to 5 hours and left overnight at 4°C. Time-resolved fluorescence was then measured on an EnVision plate reader (PerkinElmer). Raw data were corrected for well-to-well variability and signal quenching from assay components, and the values expressed as a percentage of DELTA F, where

$$\% \text{ DELTA F} = \left(\frac{[\text{sample ratio} - \text{NSB-control ratio}]}{\div \text{NSB-control ratio}} \right) \times 100.$$

The percentage-specific binding was then calculated from the percentage of DELTA F values using the following equation:

$$\text{Percentage-specific binding} = \left(\frac{[\text{sample} - \text{average NSB}]}{\div [\text{average total} - \text{average NSB}]} \right) \times 100.$$

T-cell activation assay

Mouse Ig capture beads were coated with anti-CD3 (Clone HIT3a; BD Pharmingen), anti-CD28 (Clone CD28.2; BD Pharmingen), and PD-L1 Fc fusion (MedImmune) by incubation under rotation at 4°C for 1 hour. Control beads were coated with either mouse IgG2a isotype control (BD Pharmingen) alone or with anti-CD3, anti-CD28, and a mouse IgG1 isotype control (MedImmune) using the same methodology.

Human peripheral blood mononuclear cells (PBMC) were isolated from blood buffy coats (National Health Service Blood Transfusion Service, UK) by layering over Ficoll-Paque Plus (GE Healthcare) as per the manufacturer's instructions. CD4⁺

T cells were isolated using a Robosep and the Easysep CD4 enrichment Kit (Stem Cell) as per the manufacturer's instructions. CD4 T cells were cultured in 96-well plates (Corning) together with coated beads, with or without MEDI4736 at the concentrations indicated, for 3 days at 37°C in RPMI1640 Glutamax I (Invitrogen) supplemented with 4% human AB serum (Invitrogen). Culture supernatant was removed for quantitation of IFN γ after which plates were pulsed with tritiated thymidine and returned to culture for a further 18 hours. Incorporation of thymidine was measured by harvesting onto glass fiber plates (Perkin Elmer), addition of Microscint 20 (Perkin Elmer), and reading on a Topcount (Perkin Elmer). Levels of IFN γ in supernatants were measured by DELFIA as follows. 96-well plates (Nunc) were coated with anti-IFN γ antibody (BD Pharmingen) overnight and washed. Plates were blocked with PBS containing 3% (w/v) milk powder and supernatants were added. Following incubation for 2 hours at room temperature, plates were washed and bound IFN γ was detected by addition of biotinylated anti-IFN γ (BD Pharmingen), washing, and addition of europium-labeled streptavidin (Perkin Elmer). After a final series of washes in DELFIA wash buffer, time-resolved fluorescence was measured using an EnVision plate reader (PerkinElmer). The amount of IFN γ was determined by comparison with a standard curve of known amounts of human IFN γ (R & D Systems).

Mixed lymphocyte reaction

Human PBMCs were isolated from leukapheresis packs using Ficoll-Paque Plus as per the manufacturer's instructions. Cells were cultured in serum-free RPMI 1640 for 1 hour at 37°C, nonadherent cells were removed, and remaining monocytes were cultured in RPMI 1640 supplemented with 5% human AB serum, 2 ng/mL GM-CSF, and 10 ng/mL IL4 (BD Biosciences). Fresh media with cytokine supplements were added every 2 to 3 days. Mature dendritic cells were induced by addition of 20 ng/mL TNF α (BD Biosciences) on day 6 and culture for 24 hours. Dendritic cells were harvested, phenotyped, and frozen for later use. CD4⁺ T cells were isolated from PBMCs using magnetic beads (Dyna) as per the manufacturer's instructions.

CD4⁺ T cells were cultured in 96 well-flat bottom plates (Costar) together with allogeneic dendritic cells at a ratio of 1:2.5, using RPMI 1640 supplemented with 10% human AB serum. Dendritic cells were treated with 100 μ g/mL of mitomycin C (Sigma) before addition. MEDI4736 or controls were added as indicated. Thymidine incorporation was measured on day 5 by a 16-hour pulse with tritiated thymidine (Perkin-Elmer). Supernatants were harvested before radioactive labeling and analyzed for IFN γ release by Luminex assay (BioRad) as per the manufacturer's instructions.

In vivo studies

Xenograft studies used 5- to 9-week-old female NOD.CB17-Prkdc^{scid}/NCrHsd (NOD/SCID) mice (Taconic Farms and Harlan Laboratories). Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited and United States Department of Agriculture (USDA)-licensed facility under sterile and standardized environmental conditions. Mice received autoclaved food and bedding, and acidified drinking water *ad libitum*.

Human PBMCs were obtained as described above and were enriched for CD4⁺ or CD8⁺ T cells using RosetteSep (Stem Cell) T-cell enrichment as per the manufacturer's instructions. Tumor

reactive T cells were expanded by culture with mitomycin C-treated A375 or HPAC cells and for 7 to 10 days, in RPMI 1640 supplemented with 10% FBS and IL2.

Tumor reactive T cells were mixed with HPAC cells at a 1:6 ratio or with A375 cells at a 1:4.5 ratio. Cell mixtures were implanted by subcutaneous injection of 2.5×10^6 cells into the left flank of NOD/SCID mice (Taconic Farms/Harlan Laboratories). Antibodies were administered i.p. 1 hour after implantation and on days 3, 5, 8, and 10 at the doses indicated. Tumor growth was measured with calipers in two dimensions, and tumor volume (mm³) was calculated using the formula (width² \times length)/2. Tumor growth inhibition was calculated by comparison of the mean change in tumor volume for control and MEDI4736-treated groups. A comparison between MEDI4736-treated and isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a Mann-Whitney rank sum test.

Experiments using C57BL/6J or Balb/c mice were conducted under a U.K. Home Office Project Licence in accordance with the U.K. Animal (Scientific Procedures) Act 1986 and in accordance with EU Directive EU 86/609.

Eight-week-old female C57BL/6J mice (Charles River) were immunized on day 0 by subcutaneous injection of keyhole limpet hemocyanin (KLH; Pierce) in Complete Freund's Adjuvant at the concentrations indicated. Anti-mouse PD-L1 Clone 10F.9G2 (Biolegend) or isotype control antibodies (MedImmune) were administered i.p. at the concentrations indicated on days 1 and 4. Spleens were harvested on day 7, and 1×10^6 cells were cultured in 96-well plates using DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin and streptomycin. KLH or ovalbumin (Thermo Scientific) were added to cultures at a concentration of 300 μ g/mL. IFN γ release into supernatants was measured after 3 days by MesoScale Discovery (MSD). One-way ANOVA was used to assess statistical significance of any intergroup differences.

CT26 tumors were established in 8- to 10-week-old, female BALB/c mice (Charles River) by subcutaneous implantation of 5×10^5 CT26 cells (ATCC). Following 11 days of tumor growth, mice with measurable tumor were randomized using spiral design to treatment groups of 8 animals. Oxaliplatin (Hospira) was administered i.p. once on the day of randomization at the dose indicated. Antibodies were administered i.p. on the day of randomization and then twice a week for 3 weeks, at the dose indicated. Two-way ANOVA was used to assess the statistical significance of differences observed between groups.

Assessment of HMGB1

Tumors were removed by dissection and placed in buffer containing protease inhibitors. Tissue was dissociated and a protein lysate produced using the GentleMACS (Miltenyi Biotec) as per the manufacturer's instructions. Levels of HMGB1 within lysates were assessed using ELISA (IBL International) as per the manufacturer's instructions.

Flow cytometric assessment of splenic T-cell activation

Spleens were removed by dissection and placed into 15 mL of RPMI1640 Glutamax I (Invitrogen). Tissue was disaggregated by passing through a 40- μ m nylon cell strainer, and cells were pelleted by centrifugation and resuspended in red blood cell lysis buffer (Sigma). Following incubation for 3 minutes at room temperature, cells were washed and resuspended in flow

cytometry buffer (Ebioscience). Cells were stained using fixable live/dead aqua (Invitrogen), anti-CD4 (Ebioscience), anti-CD8 (Ebioscience), anti-CD69 (Ebioscience), and anti-CD25 (Biolegend). Stained cells were analyzed using a FACSCanto II (Becton Dickinson).

Results

MEDI4736 binds specifically to PD-L1, inhibits its interaction with PD-1 and CD80, and does not trigger ADCC

A sequence homology BLAST search identified human programmed death ligand 2 (PD-L2, B7-DC) and B7-H3 as the only proteins that shared 30% or more amino acid identity with human PD-L1. An ELISA confirmed that MEDI4736 bound specifically to human PD-L1, with no cross-reactivity to these two related proteins (Fig. 1A). In addition, no cross-reactivity to mouse PD-L1 was detected.

A competitive binding assay, based on homogeneous time-resolved fluorescence (HTRF), showed that MEDI4736 completely blocked the binding of PD-L1 to both PD-1 (Fig. 1B) and CD80 (Fig. 1C), with an IC₅₀ of 0.1 and 0.04 nmol/L, respectively.

Given the reported expression of PD-L1 on select normal epithelium and on several key cells of the immune system, the potential for Fc-mediated effector function was removed from

MEDI4736 by engineering the constant domain to include 3 point mutations (33). The ADCC potential of MEDI4736 was assessed in a reporter gene assay. SKBR3 cells were coated with MEDI4736, or a version of the same antibody with a wild-type (WT) IgG1 isotype, and incubated with a derivative of the NK-92 MI cell line stably transfected with the V158 allotype of CD16a and a luciferase reporter gene linked to an NFAT-sensitive promoter. When sufficient clustering of an appropriate IgG-Fc is achieved in this assay, a signal is delivered through CD16a, as occurs during ADCC. This signal results in increased NFAT activation, which is measured as luciferase activity, and can be considered a surrogate for ADCC activity. The WT IgG1 MEDI4736 resulted in a concentration-dependent increase in luciferase activity within this assay, as expected of an IgG1 in the presence of its cognate antigen. In contrast, MEDI4736 itself did not result in any luciferase activity at any of the concentrations tested (Fig. 1D).

MEDI4736 overcomes PD-L1-mediated inhibition of primary human T cells *in vitro*

We developed an *in vitro* T-cell activation assay to investigate the ability of MEDI4736 to impact the function of PD-L1. In this assay, freshly isolated primary human T cells cultured together

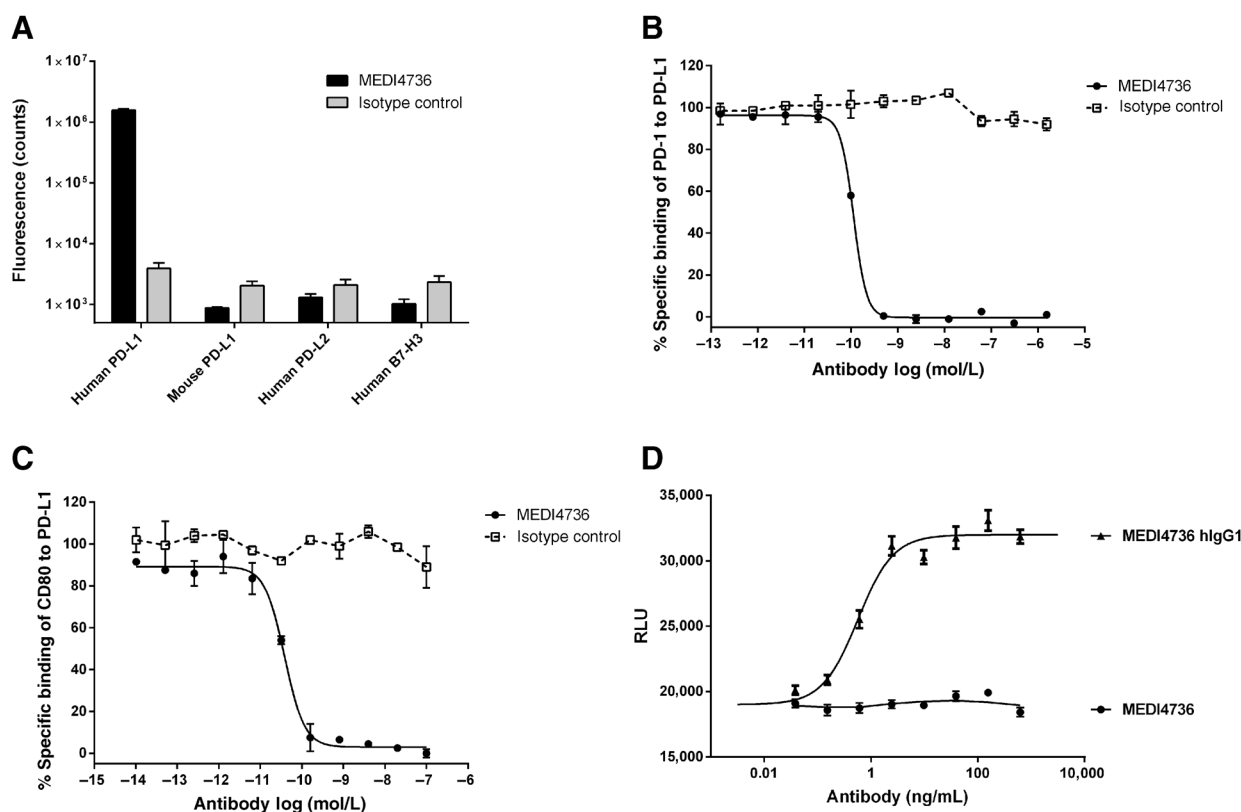


Figure 1. MEDI4736 binds specifically to PD-L1, inhibits its interactions with PD-1 and CD80, and does not trigger ADCC. A, the signal derived in an ELISA from MEDI4736 binding to human PD-L1 or the related proteins B7-H3 and B7-DC. B, the % specific binding observed between PD-1 and PD-L1 in an HTRF-based ligand inhibition assay in the presence of increasing concentrations of MEDI4736 or an isotype control antibody. C, the % specific binding observed between CD80 and PD-L1 in an HTRF-based ligand inhibition assay in the presence of increasing concentrations of MEDI4736 or an isotype control antibody. D, the level of NFAT-driven luciferase activity in a CD16a-transfected NK-92 MI cell line following incubation with SKBR3 cells coated in MEDI4736 or a version of MEDI4736 bearing a wild-type IgG1 Fc domain.

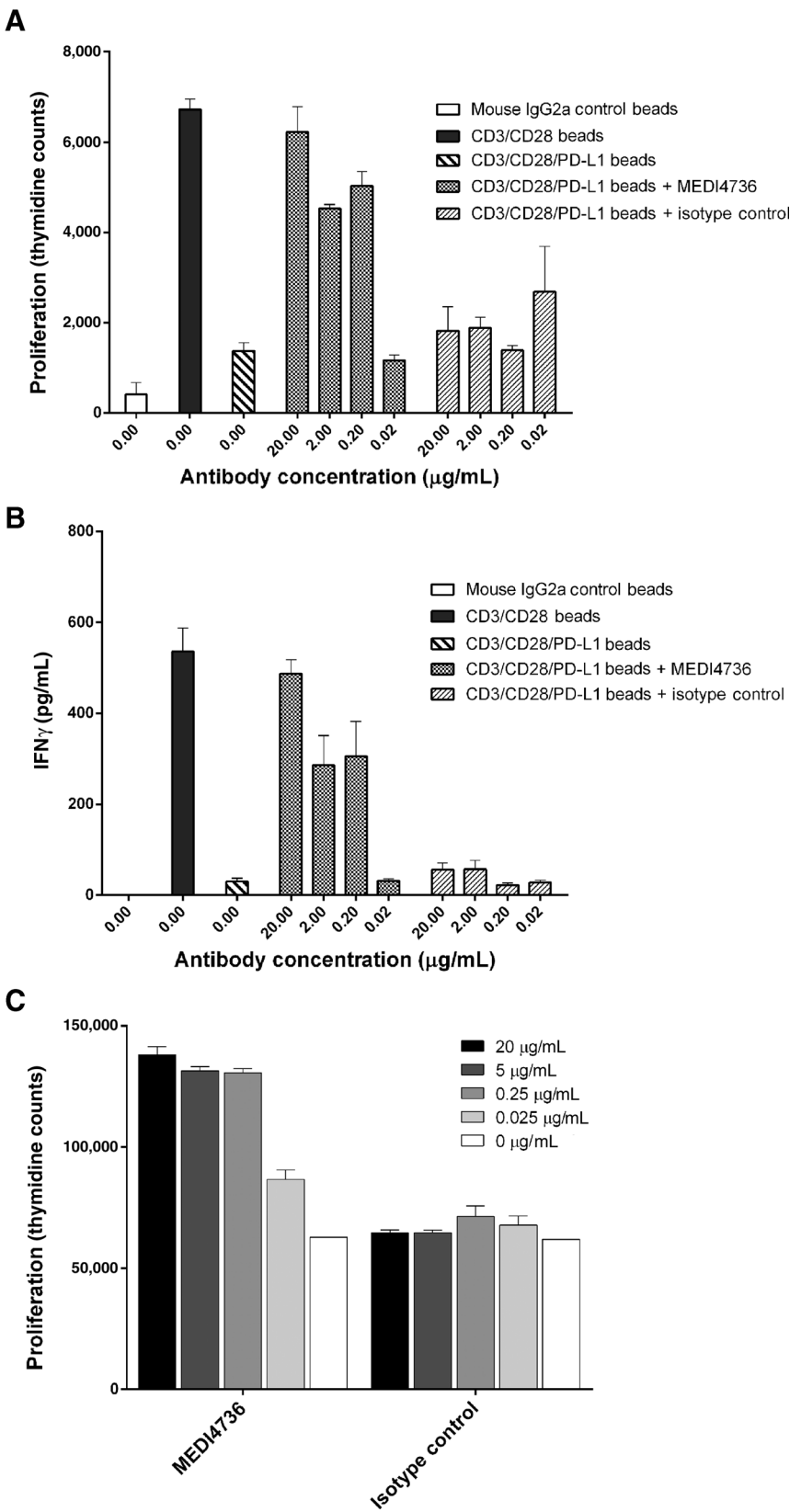


Figure 2. MEDI4736 overcomes PD-L1-mediated inhibition of T-cell activation. The effect of increasing concentrations of MEDI4736 on (A) thymidine incorporation and (B) IFN_γ release in primary T cells stimulated with beads coated in anti-CD3, anti-CD28, and either PD-L1 or irrelevant isotype control IgG. C, thymidine incorporation in a MLR in the presence of increasing concentrations of MEDI4736 or an isotype control antibody.

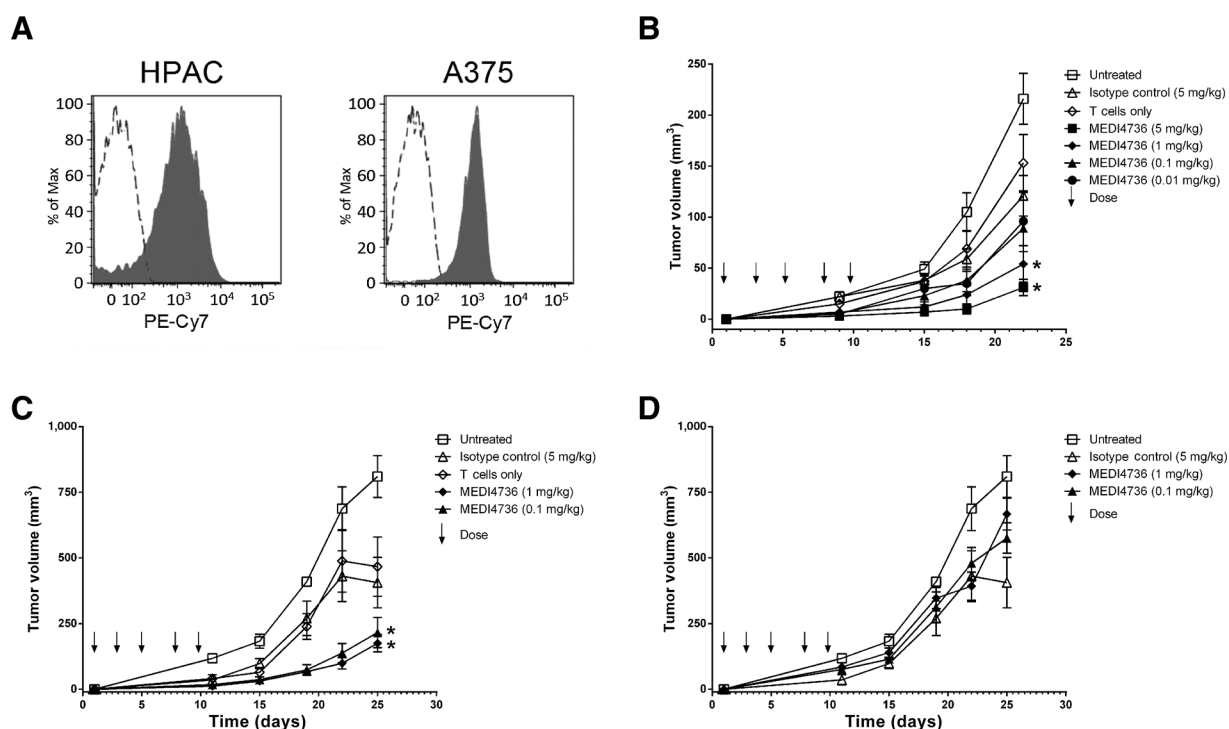


Figure 3. MEDI4736 shows antitumor activity in xenograft mouse models of human cancer. A, fluorescence observed on HPAC and A375 cell lines by flow cytometry following staining with an anti-PD-L1 (filled) antibody or an isotype control antibody (dotted). B, HPAC tumor volumes (mm^3) in NOD/SCID mice following coimplantation of primary human T cells and administration of MEDI4736 i.p. at 5, 1, 0.1, and 0.01 mg/kg twice per week for 3 weeks as indicated. C, A375 tumor volumes (mm^3) in NOD/SCID mice following coimplantation of primary human T cells and administration of MEDI4736 i.p. at 5, 1, and 0.1 mg/kg twice per week for 3 weeks as indicated. D, A375 tumor volumes (mm^3) in NOD/SCID mice without coimplantation of primary human T cells and administration of MEDI4736 i.p. at 1 and 0.1 mg/kg twice per week for 3 weeks as indicated. *, $P \leq 0.05$ as determined by a Mann-Whitney rank sum test.

with anti-CD3 and anti-CD28-coated beads demonstrated increased proliferation and IFN γ release. Both proliferation and IFN γ release were significantly reduced, by 5-fold and 18-fold, respectively, when PD-L1 was present on the beads (Fig. 2A and B), confirming that PD-L1 provided an inhibitory signal in the context of the assay. MEDI4736 was able to inhibit this activity of PD-L1 in a concentration-dependent manner, and at 20 $\mu\text{g}/\text{mL}$ MEDI4736, IFN γ release was comparable in magnitude with that in the absence of PD-L1. No effect was observed when an isotype control antibody was added (Fig. 2B). Comparable results were obtained when T-cell proliferation, measured by thymidine incorporation, was used as an assay endpoint (Fig. 2A).

To confirm the activity of MEDI4736 in a more physiologic setting, mixed lymphocyte reaction (MLR) were performed using dendritic cells and T cells, isolated from different healthy human donors. Addition of MEDI4736 to such MLRs resulted in a concentration-dependent increase in thymidine incorporation, suggesting an increase in the proliferation of responding T cells in the cultures (Fig. 2C).

MEDI4736 inhibits the growth of human tumors in a mouse model via a T-cell-dependent mechanism

The antitumor activity of MEDI4736 was investigated *in vivo* using xenografts of human melanoma (A375) or pancreatic (HPAC) tumor cell lines. These lines were confirmed to express PD-L1 by flow cytometry (Fig. 3A). Extended culture with primary human T cells was used to generate allogeneic T-cell lines, with

specificity to each tumor cell line, which were implanted subcutaneously in NOD/SCID mice together with tumor cells. MEDI4736 was given 1 hour after implantation, with subsequent doses administered as indicated.

MEDI4736 significantly inhibited the tumor growth of both HPAC and A375 xenografts compared with an isotype-matched control antibody. Tumor growth inhibition of the HPAC cells reached 74% (Fig. 3B), whereas inhibition of the A375 cells reached 77% (Fig. 3C). When administered in the absence of T cells, MEDI4736 had no effect on the growth of the A375 tumor xenograft (Fig. 3D).

An anti-mouse PD-L1 antibody enhances the immune response to KLH

Because MEDI4736 does not cross-react to mouse PD-L1 (mPD-L1), an anti-mPD-L1 antibody, 10F.9G2, was used to study the effects of PD-L1 blockade in immunocompetent mouse models. The affinity of 10F.9G2 to mPD-L1 was determined to be within 3-fold of the affinity measured for MEDI4736 binding to human PD-L1 (data not shown). In addition, the ability of 10F.9G2 to inhibit the binding of mPD-L1 to both mPD-1 and mCD80 was confirmed (Fig. 4A). 10F9.G2 was around 10-fold less potent than MEDI4736 in this regard, demonstrating an IC_{50} of 1 and 0.3 nmol/L for blockade of mPD-1 and mCD80, respectively. In addition to this difference in potency, 10F.9G2 is a rat IgG2b antibody and so, in contrast with MEDI4736, has the potential to mediate Fc receptor-dependent effector functions.

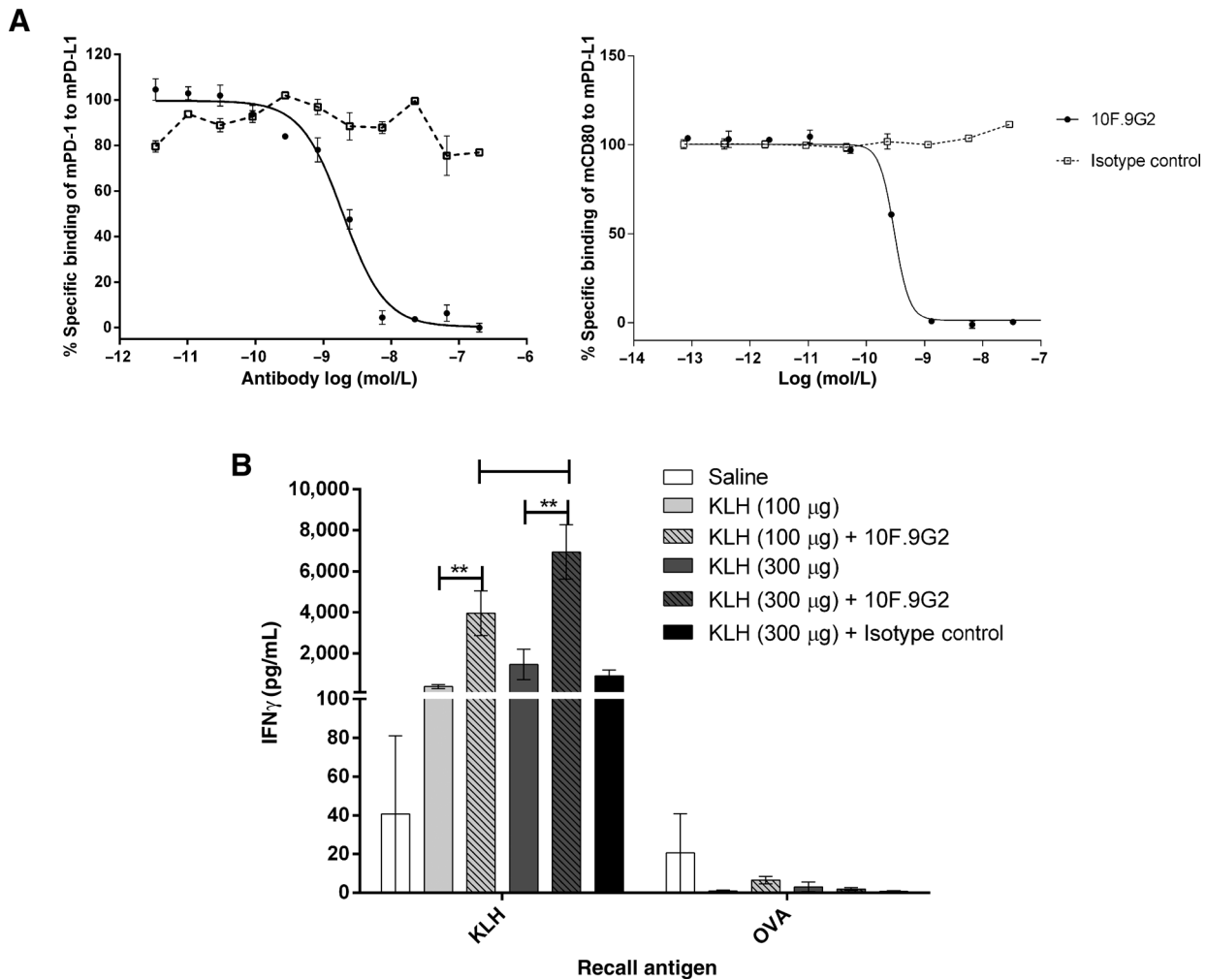


Figure 4. The anti-mouse PD-L1 antibody, 10F.9G2, blocks the interaction between mouse PD-L1 and its ligands, resulting in enhanced responses to foreign antigen. A, the % specific binding observed between mouse (m)PD-1 (left) or mCD80 (right) and mPD-L1 in an HTRF-based ligand inhibition assay in the presence of increasing concentrations of 10F.9G2 or an isotype control antibody. B, the anti-mouse PD-L1 antibody, 10F.9G2, enhances the recall response to foreign antigen. IFN γ release by splenocytes after 72 hours of culture in the presence of KLH or ovalbumin (OVA). Seven days before culture mice were immunized with 100 or 300 mg of KLH in complete Freund's adjuvant alone, or with coadministration of 10F.9G2 or an isotype control antibody. Data shown are mean \pm SEM. **, $P < 0.05$ as determined by a Student t test between indicated groups.

Given these differences in potency and isotype, 10F.9G2 is not a perfect surrogate reagent for MEDI4736, but was considered to represent an adequate reagent with which to explore the biology of PD-L1 in immunocompetent mouse models.

Having established the activity profile of 10F.9G2 *in vitro*, we confirmed its ability to modulate immune responses *in vivo* (Fig. 4B). C57BL/6 mice were immunized with KLH at the doses indicated. Antibodies were administered i.p. at 10 mg/kg on days 1 and 4 after immunization. On day 7, mice were sacrificed and recovered splenocytes were placed into culture with 300 μ g/mL KLH. The IFN γ release into culture supernatant was measured by MSD after 72 hours. Relative to splenocytes from mice that received mock immunization, splenocytes from mice immunized with KLH demonstrated increased IFN γ release when cultured with KLH, but not ovalbumin, an irrelevant control antigen. The extent of IFN γ release was not significantly different between splenocytes from mice that were immunized with 100 μ g/mL or

300 μ g/mL KLH (Fig. 4B). Splenocytes from mice that received anti-PD-L1 following immunization demonstrated significant ($P \leq 0.05$) further increases in IFN γ release when compared with splenocytes from mice that received only KLH or KLH and an isotype control antibody. The level of IFN γ release was greater in splenocytes from mice receiving 300 μ g/mL KLH together with anti-PD-L1 than in those receiving 100 μ g/mL KLH together with anti-PD-L1, but this difference did not reach statistical significance ($P = 0.1$; Fig. 4B).

An anti-mouse PD-L1 antibody improves survival in a mouse syngeneic model of colorectal cancer, as monotherapy and in combination with oxaliplatin

10F.9G2 was tested in a syngeneic model of colorectal cancer to examine the antitumor effect of PD-L1 blockade as monotherapy and in combination with the chemotherapeutic oxaliplatin. Balb/c mice were implanted subcutaneously with CT26

cells and randomized to treatment groups after 11 days. Groups treated with vehicle or an isotype control antibody showed a rapid decline in survival, demonstrating a median survival of 19 and 23 days, respectively, with sacrifice of all animals by day 37. Groups treated with either oxaliplatin, at a suboptimal dose, or anti-PD-L1 alone demonstrated an increase in overall survival, resulting in a median survival of 30 and 26.5 days, respectively. It is noteworthy that we observed complete elimination of tumors in 25% of animals in these groups. The rate of complete tumor elimination increased dramatically, to 62.5%, in the group treated with the combination of anti-PD-L1 and oxaliplatin; no median survival could be calculated for this group, because greater than half the animals survived until the end of the study (Fig. 5A). In a subsequent study, tumor homogenates were collected from CT26 tumors at various time points following treatment with oxaliplatin and assessed, by ELISA, for the presence of high mobility group box 1 (HMGB1), a marker of immunogenic cell death (Fig. 5B). The level of HMGB1 present in tumors increased significantly as early as 1 hour after administration of oxaliplatin and was sustained at levels above that in untreated tumors for as long as

18 hours. In addition, spleens were harvested from several CT26-bearing mice following 9 days of treatment with either anti-PD-L1 or an isotype control antibody and examined by flow cytometry for potential markers of T-cell activation and differentiation. Significant ($P \leq 0.05$) upregulation of the surface markers CD69 and ICOS was observed on both CD4 and CD8 T cells (Fig. 5C).

Discussion

Immune enhancement for the treatment of cancer has a long history (34) that has culminated, recently, in successful phase III clinical trials (35–37). This success has encouraged greater exploration of how multiple immune pathways can be harnessed for therapeutic benefit. PD-L1 has been identified as a key regulator of the immune response, with potential utility for the treatment of cancer, a role supported by a range of preclinical studies in mice (15, 20–23, 25). More recently, the blockade of PD-L1 signaling has received even greater attention due to encouraging data from clinical trials assessing the activity of anti-PD-L1 and anti-PD-1 antibodies in late stage cancer patients, and the

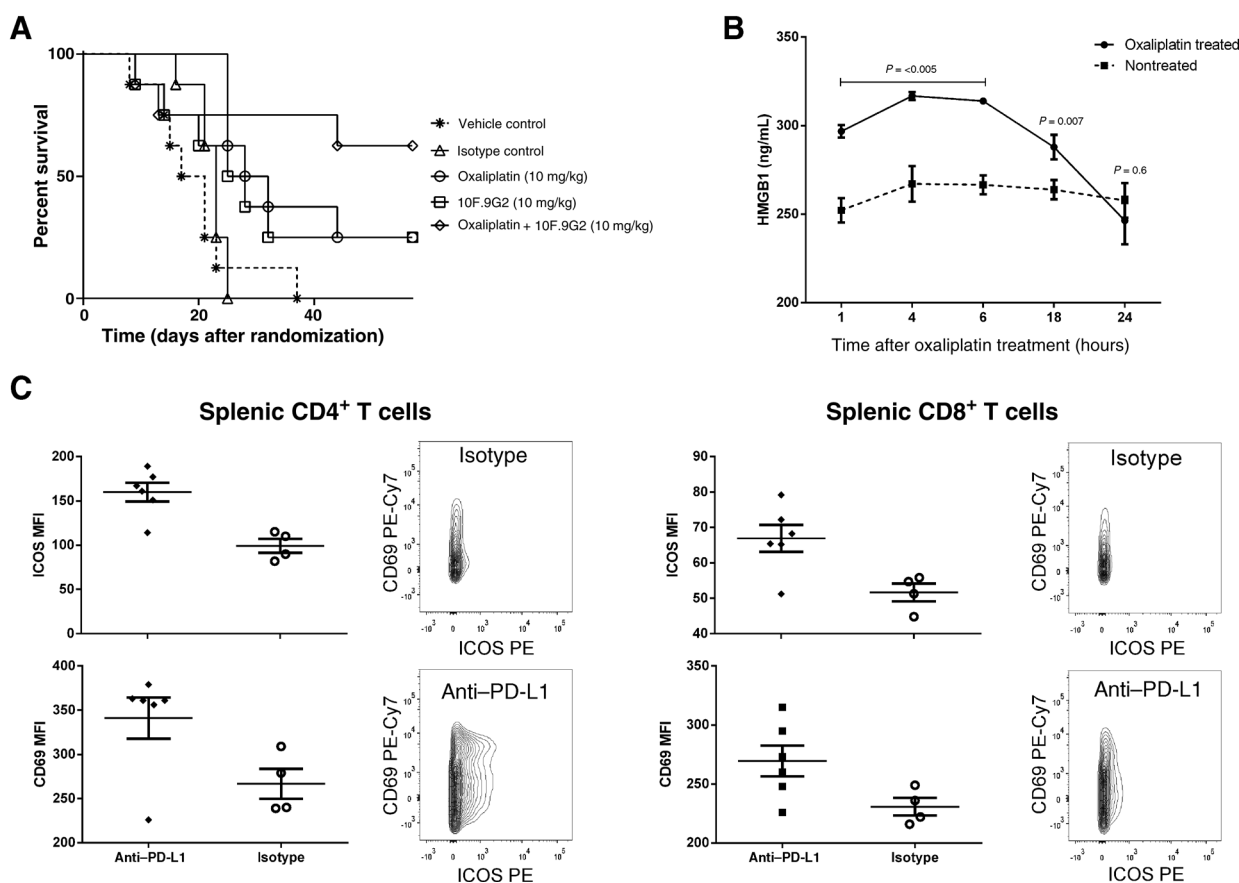


Figure 5. 10F.9G2 increases survival in the CT26 model of colorectal cancer alone and in combination with chemotherapy. A, Kaplan-Meier survival curve for mice bearing CT26 colorectal tumors. Oxaliplatin was administered once on day 11 at the dose indicated. Antibodies were administered on day 11 and then twice a week for 5 further doses. B, ELISA for HMGB1 carried out on tumor homogenates from mice treated once with either 10 mg/kg oxaliplatin or vehicle control. Data shown are mean \pm SEM. P values were determined by a Student t test between treated versus control groups at the indicated timepoints. C, flow cytometric analysis of CD69 and ICOS expression on CD4 (left) and CD8 (right)-positive T cells in the spleens of mice treated twice a week with 10 mg/kg anti-PD-L1 or an isotype-matched control antibody. P values were determined by a Student t test between treated versus control groups.

successful registration of the anti-PD-1 antibodies nivolumab and pembrolizumab for the treatment of melanoma and non-small cell lung cancer (NSCLC; refs. 27–31).

To selectively target PD-L1, we generated MEDI4736, a human mAb by immunization of the XenoMouse and subsequent screening of hybridomas. The constant domain of MEDI4736 was subsequently altered to include 3 point mutations in the constant domain (33) to reduce the potential for effector function triggering that could lead to cytotoxicity against of PD-L1 expressing healthy cells. A reporter gene-based ADCC assay confirmed that the introduction of these mutations had indeed eliminated the potential for MEDI4736 to mediate ADCC. An ELISA demonstrated that MEDI4736 binds specifically to human PD-L1 with no detectable cross-reactivity to the two most closely related human proteins, B7-H3 and PD-L2. Subsequent studies showed that the binding of MEDI4736 to PD-L1 completely inhibited the interaction of PD-L1 with CD80 and with PD-1, in a ligand inhibition assay. As such, MEDI4736 is able to block the interaction between PD-L1 and both of the known cognate receptors.

To test whether this inhibition resulted in antagonism of PD-L1 function, two primary cell assays were conducted. In the first, MEDI4736 was able to overcome the inhibitory effects of recombinant PD-L1 on CD4 T-cell activity in a CD4 T-cell activation assay. In the second, MEDI4736 was able to enhance the response of CD4 T cells to allogenic dendritic cells in an MLR, presumably through blockade of native PD-L1 expressed on the surface of the dendritic cells. Our results from these assays confirmed that MEDI4736 could overcome the inhibitory effects of PD-L1 on T-cell activation; however, they did not address the ability of MEDI4736 to enhance target cell killing mediated by cytotoxic T cells.

To examine the ability of MEDI4736 to enhance T-cell-mediated tumor cell killing, a novel xenograft model system was established. In this model, tumor reactive T-cell lines were generated by *in vitro* coculture with HPAC or A375 cell lines. The resulting T cells were then mixed together with the corresponding cancer cells before implantation in mice. This system enabled testing of the T-cell-enhancing effects of MEDI4736 *in vivo*. Using this model, significant ($P \leq 0.05$) tumor growth inhibition of both A375 and HPAC xenografts was observed following administration of MEDI4736. Critically, this activity was entirely dependent upon the presence of the tumor reactive human T cells. The data generated using this model, therefore, confirmed the ability of MEDI4736 to inhibit the immunosuppressive effects of PD-L1, resulting in increased tumor cell elimination by T cells, and supported the further development of MEDI4736 for the treatment of cancer.

It was not possible to explore the *in vivo* effects of MEDI4736 in a fully immunocompetent mouse model, because it is not mouse cross-reactive. Therefore, an anti-mouse PD-L1 antibody, 10F.9G2, was identified and characterized to determine relative functional characteristics to MEDI4736. To confirm the immune-enhancing potential of 10F.9G2, the antibody was administered to mice following immunization with KLH. Splenocytes taken from mice treated in this way demonstrated increased IFN γ release during *in vitro* culture with KLH, relative to those taken from mice treated with an isotype control antibody, which confirmed the potential of 10F.9G2 to enhance an on-going immune response. This result is consistent with other published studies looking at the effect of the PD-1/PD-L1 axis on response to

foreign antigens (38, 39), and supported the role of PD-L1 as a repressor of T-cell responses.

Having confirmed the immunostimulatory properties of 10F.9G2, CT26, a mouse model of colorectal cancer, was used to study the effects of PD-L1 blockade in a more therapeutically relevant system. As expected, 10F.9G2 provided a measurable survival benefit in this model, resulting in regressions in 25% of mice when compared with an isotype control. The potential mechanism of 10F.9G2 was further explored by examining T-cell activation in the spleens of mice following treatment. A significant upregulation of the surface markers CD69 and ICOS was observed on CD4 and CD8 T cells in the spleen following treatment with 10F.9G2. These data further support the conclusion that anti-PD-L1 acts through T-cell activation and indicate that this activation occurs in both major T cell subsets. The impact of this peripheral T-cell activation on T cells within the tumor was not examined in this particular study; however, a recent study by Duraiswamy and colleagues (40) showed that treatment of CT26-bearing mice with 10F.9G2 resulted in an increase in T-bet expression in intratumoral CD8 T cells and an overall increase in T-cell infiltration within the tumor. These data fit with our findings and suggest that the peripheral T-cell activation we observed results in favorable T-cell activation within the tumor.

Recent reports have indicated that chemotherapeutic agents, such as oxaliplatin, can have immune-enhancing properties through their ability to induce immunogenic cell death (41), leading to enhanced antigen processing and cross-presentation of tumor antigens. Based on these reports, we used the CT26 model to explore the combination of an anti-PD-L1 antibody with oxaliplatin. Oxaliplatin, when administered alone, demonstrated similar activity to 10F.9G2, resulting in 25% regressions. However, the combination of 10F.9G2 and oxaliplatin resulted in a significant ($P \leq 0.01$) improvement in survival, with greater than 60% of mice demonstrating complete regression of tumors. Although the potential additive nature of chemotherapy and checkpoint blockade has been demonstrated previously (42, 43) in mouse models, this represents the first time such data have been published in combination with an anti-PD-L1 antibody. To better understand the mechanistic basis for the additive activity observed with the combination, tumor homogenates were collected from oxaliplatin-treated tumors and assessed for levels of HMGB1, one of a number of damage-associated molecular pathogens implicated in the induction of immunogenic cell death (44). The level of HMGB1 was elevated above that seen in untreated tumors as early as 1 hour following a single dose of oxaliplatin. These significantly ($P = 0.005$ – 0.007) elevated levels of HMGB1 were sustained as late as 18 hours following oxaliplatin treatment, and declined back to levels observed in untreated tumors by 24 hours. Given the well-established role of HMGB1 in immunogenic cell death, and the existing evidence for the immunogenic potential of oxaliplatin (41), it is likely that enhanced antigen release and immune priming are occurring following oxaliplatin treatment in this model. Furthermore, this enhanced priming appears to be working cooperatively with the T-cell activation observed following administration of anti-PD-L1, and driving a more effective antitumor immune response, resulting in the increased antitumor activity observed in this study.

In 2007, The NCI Immunotherapy Agent Workshop ranked the PD-1/PD-L1 axis as the second most important immunotherapy target for pharmaceutical development (45). Confidence in the

promise of PD-1/PD-L1-directed therapy has grown further with the registration of nivolumab and pembrolizumab, and following a number of reports of early stage clinical trials (27–31), in which responses to treatment were observed in patients with a number of other cancers, including renal cancer and NSCLC.

Our preclinical data support the value of targeting PD-L1 for the treatment of cancer and expand on existing preclinical data by demonstrating the potential for further increases in therapeutic benefit through combination with other modalities, such as chemotherapy. MEDI4736 is a high potency PD-L1 antagonistic antibody currently being studied in a range of clinical trials. Initial data from the phase I clinical study, evaluating the dose/schedule, safety, pharmacokinetics, and pharmacodynamics of MEDI4736, was reported at the 2014 meeting of the American Society for Clinical Oncology (46–48). A pivotal development program in NSCLC and head and neck cancer, designed to investigate MEDI4736 both as monotherapy and in combination is currently under way.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Stewart, M. Morrow, S.A. Hammond, M. Alimzhanov, J.S. Babcook, I.N. Foltz, V. Bedian, R.W. Wilkinson, M. McCourt, A. Buchanan

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