

Characterization of the Anti-PD-1 Antibody REGN2810 and Its Antitumor Activity in Human *PD-1* Knock-In Mice



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Abstract

The Programmed Death-1 (PD-1) receptor delivers inhibitory checkpoint signals to activated T cells upon binding to its ligands PD-L1 and PD-L2 expressed on antigen-presenting cells and cancer cells, resulting in suppression of T-cell effector function and tumor immune evasion. Clinical antibodies blocking the interaction between PD-1 and PD-L1 restore the cytotoxic function of tumor antigen-specific T cells, yielding durable objective responses in multiple cancers. This report describes the preclinical characterization of REGN2810, a fully human hinge-stabilized IgG4 (S228P) high-affinity anti-PD-1 antibody that potently blocks PD-1 interactions with PD-L1 and PD-L2. REGN2810 was characterized in a series of binding, blocking, and functional cell-based assays, and preclinical *in vivo* studies in mice and monkeys. In cell-based assays, REGN2810 reverses PD-1-dependent attenuation of

T-cell receptor signaling in engineered T cells and enhances responses of human primary T cells. To test the *in vivo* activity of REGN2810, which does not cross-react with murine PD-1, knock-in mice were generated to express a hybrid protein containing the extracellular domain of human PD-1, and transmembrane and intracellular domains of mouse PD-1. In these mice, REGN2810 binds the humanized PD-1 receptor and inhibits growth of MC38 murine tumors. As REGN2810 binds to cynomolgus monkey PD-1 with high affinity, pharmacokinetic and toxicologic assessment of REGN2810 was performed in cynomolgus monkeys. High doses of REGN2810 were well tolerated, without adverse immune-related effects. These preclinical studies validate REGN2810 as a potent and promising candidate for cancer immunotherapy. *Mol Cancer Ther*; 16(5); 861–70. ©2017 AACR.

Introduction

Immune surveillance plays a critical role in controlling tumor progression. However, cancer cells engage multiple mechanisms to inhibit antitumor immune responses and promote immune evasion. Cancer cells undergo immunoediting, thereby allowing tumors to avoid recognition by immune effector cells (1, 2). In addition, tumors evade immune-mediated destruction by engaging immunosuppressive receptors on immune, stromal, and cancer cells. Substantial experimental evidence derived from cell-based assays, animal studies, and more recently from clinical trials indicates that PD-L1/PD-1 interaction is a key mediator of tumor immune evasion (3–5).

PD-1 is an inhibitory member of the CD28 receptor family expressed on activated B and T lymphocytes and myeloid cells

(6, 7). PD-1 receptor engagement by B7 family ligands PD-L1 and PD-L2 delivers inhibitory checkpoint signals that contribute to the establishment and maintenance of peripheral immune tolerance (8–10). PD-1 deficiency in mice results in heightened immune activation, especially in mouse strains susceptible to autoimmunity (11–13). PD-L1, the primary PD-1 ligand, is expressed in tumors, where its interaction with PD-1 results in decreased lymphocyte infiltration, reduced proliferation, effector function, and ultimately immune evasion (14, 15). In preclinical models, blockade of PD-1/PD-L1 interaction improved antitumor immunity (16, 17), and blocking both PD-L1 and PD-L2 signaling showed additive effects (18, 19). PD-L1 expression is observed in a variety of human tumors (20, 21), and increased PD-L1 expression adjacent to tumor-infiltrating T cells in baseline biopsies suggests pre-existing antitumor immunity and serves as a clinical response marker for anti-PD-1/PD-L1 therapy (22, 23).

Recent clinical trials showed that blockade of the PD-L1/PD-1 signaling with anti-PD-1 or anti-PD-L1 antibodies is effective in several malignancies and produces durable responses in a subset of patients (24, 25). Clinical efficacy of PD-1-blocking antibodies has been demonstrated in patients with advanced melanoma, lung, and renal cancer (26–30). Moreover, combination therapy with PD-1- and CTLA-4-blocking antibodies has been approved for therapy of advanced melanoma with

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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BRAF mutations (31–32) and is being tested in other malignancies, with many other PD-L1/PD-1-based combinations entering clinical development (33–34).

This article describes the preclinical characterization of REGN2810, a fully human hinge-stabilized IgG4 (S228P) high-affinity anti-PD-1 antibody that potently blocks PD-1/PD-L1 functional interaction. REGN2810 was generated using VelocImmune mice containing human immunoglobulin gene segments (35–36). REGN2810 enhances human primary T-cell responses *in vitro* and inhibits the growth of syngeneic colorectal carcinomas in mice genetically engineered to express a human/mouse PD-1 chimeric receptor from the mouse locus. Of note, human PD-1 is capable of interacting with mouse PD-L1 ligand, and cell type and tissue-specific expression of PD-1 is conserved between mouse and human (37). This strategy allowed for the first time to evaluate the preclinical activity of a human PD-1-blocking antibody, which does not bind mouse PD-1, *in vivo*. The comprehensive preclinical results presented here supported the initiation of REGN2810 clinical trials in cancer patients with solid tumors.

Materials and Methods

Antibody generation

VelocImmune knock-in mice, in which the mouse Ig heavy and kappa light variable germ-line gene segments are replaced with their human counterparts while leaving the mouse constant regions intact (35, 36), were used to generate human anti-human PD-1 antibodies. Mice were immunized with recombinant human PD-1-mFc protein (Regeneron), containing the extracellular domain of PD-1 (amino acids 1–167) and the Fc portion of mouse IgG2a. Splenocyte-derived hybridomas producing human mAb reactive to recombinant human PD-1-hFc (extracellular domain of human PD-1 fused to the Fc portion of human IgG1) were screened by binding to HEK293 cells expressing human PD-1 and by ELISA. The cloned human immunoglobulin variable regions from antibodies exhibiting the desired characteristics were joined to human IgG4 constant region genes, containing a S228P (serine to proline exchange) hinge mutation to minimize half-antibody formation (38), and antibodies were produced in Chinese hamster ovary (CHO) cells.

Kinetics of REGN2810 binding to human and monkey PD-1

Binding kinetics of REGN2810 to PD-1 were determined by capturing REGN2810 with an anti-human Fc antibody (GE Life Sciences) immobilized on a CM5 sensor chip (Biacore T200), over which PD-1 extracellular domains of human, monkey, rat, and mouse PD-1 in monomeric or dimeric (fused to mouse Fc) were applied. Serial dilutions of PD-1 proteins ranging from 100 nmol/L to 0.78 nmol/L (human and monkey) and 1 μ mol/L to 12.3 nmol/L (mouse and rat) were individually injected over surface-captured REGN2810 surface for 3 minutes, allowing 10- to 30-minute dissociation time. The binding kinetics of human and mouse PD-1/PD-L1 interactions was determined by capturing mouse or human PD-1 and PD-L1 proteins fused to human Fc on a sensor chip immobilized with goat anti-human Fc antibody (GE Healthcare). Human and mouse PD-1 and PD-L1 proteins fused to mouse Fc were individually injected over the chip. Kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using curve fitting software scrubber 2.0c and Biacore T200 Evaluation.

PD-1 competition binding ELISA

REGN2810 or an isotype control antibody was incubated with human or monkey PD-1-mFc proteins for 1 hour at room temperature and then transferred to 96-well plates coated with human PD-L1-hFc or human PD-L2-hFc (R&D Systems). After 1 hour, plate-captured PD-1-mFc was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse Fc-specific polyclonal antibody (Jackson ImmunoResearch) and developed with TMB colorimetric substrates (BD Biosciences). Absorbance at 450 nm was detected on a Victor X5 plate reader and plotted as a function of the anti-PD-1 antibody concentrations. IC₅₀ values were used as a measure of blocking potency.

T-cell activation bioassays

The ability of REGN2810 to antagonize PD-L1-mediated PD-1 inhibition was determined in cell-based assays using either engineered T cell lines or primary human T cells. Jurkat/AP-1-Luc/hPD-1 T cells expressing full-length human PD-1 protein and an AP-1-driven luciferase reporter (Qiagen) were engineered by lentiviral transduction. Antigen-presenting cell (APC)-like HEK293 cells were generated by lentiviral transduction of human CD20 and human PD-L1. T-cell receptor (TCR) activation was achieved by an anti-CD3 x anti-CD20 bispecific antibody (Regeneron). To generate a dose response curve for anti-CD3 x anti-CD20, the bispecific molecule was serially diluted and tested with 50,000/well Jurkat/AP-1-Luc/hPD-1 and 10,000/well HEK293/hCD20 or HEK293/hCD20/hPD-L1 cells in a 96-well plate. Serially diluted REGN2810 was tested under similar conditions in the presence of a fixed concentration of anti-CD3 x anti-CD20 (100 pmol/L). Plates were incubated at 37°C for 4 to 6 hours. ONE-Glo (Promega) luciferase substrate was added to each well and Relative Luminescence Units (RLU) were captured on a Victor X5 multilabel plate reader. The EC₅₀ of REGN2810 was determined by fitting the RLU-concentration data to a four-parameter logistic equation (GraphPad Prism).

In the primary T-cell assay, human CD4⁺ T cells were isolated from healthy donor leukopacks using Human CD4⁺ T-cell Enrichment Cocktail (STEMCELL Technologies). Purified human CD4⁺ T cells were activated with Human T-activator CD3/CD28 beads (Dynabeads®; Invitrogen) for 48 hours to induce PD-1 expression and were then "rested" for 24 hours after bead removal. HEK293/hCD20/hPD-L1 cells were treated with 50 mg/mL mitomycin C (Sigma) for 30 minutes at 37°C to inhibit proliferation. Serially diluted REGN2810 was incubated with 50,000/well preactivated CD4⁺ T cells and 25,000/well of HEK293/hCD20/hPD-L1 cells in the presence of 2 nmol/L anti-CD3 x anti-CD20 bispecific antibody in a 96-well plate for 72 hours. ³H-thymidine was added for an additional 6 hours to measure T-cell proliferation.

Generation of human PD-1 knock-in mice

VelociGene technology was used to generate human PD-1 knock-in mice as described previously (39). Briefly, a targeting vector was engineered that replaced 898 bp of the extracellular portion of the mouse *Pdcd1* gene (including exon 2 and part of exon 3) with the corresponding 883 bp region of the human gene (exon 2 and part of exon 3). Correct gene targeting in F1H4 (C57BL/6 \times 129 hybrid) embryonic stem (ES) cell clones was identified by a loss of allele assay as described previously (40). Targeted ES cells were injected into uncompact 8-cell stage Swiss Webster embryos to produce fully ES cell-derived F0

generation heterozygous mice for breeding with C57BL/6N[TAC] to homozygosity. The resulting genetically modified mice express a hybrid PD-1 protein comprising the extracellular portion of human *PDCD1* and the transmembrane and intracellular portion of mouse *Pdcd1*. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH. The protocol was approved by the Regeneron Pharmaceuticals Institutional Animal Care and Use Committee.

In vivo studies

MC38 mouse colon carcinoma cells were obtained from NIH repository in 2012 and were authenticated by short tandem repeat profiling in 2016 (IDEXX BioResearch). MC38 cells were engineered to express full-length soluble chicken ovalbumin (Ova, amino acids 1–386). All experiments were conducted with low-passage cell cultures (< passage 10). For tumor studies, adult human *PD-1* knock-in mice were injected subcutaneously with 5×10^5 MC38.Ova cells into the flank on day 0. In a minimal disease model, REGN2810 or isotype control antibody was injected i.p. on day 3, and then twice a week for 2 weeks. In the established disease model, mice were randomized 11 to 14 days following tumor inoculation when tumors reached 100 mm³. Antibodies were administered i.p. on the randomization day and then twice a week for 2 weeks, at indicated doses. Mice were euthanized when the tumor volumes reached 1,500 mm³.

At the end of the study, spleens were collected, dissociated into single-cell suspension, and stained with anti-mouse CD3e (clone 145-2C11; Biolegend), anti-mouse CD4 (clone GK1.5; Biolegend), anti-mouse CD8 α (clone 53-6.7; Biolegend), and biotinylated REGN2810 (Regeneron) followed by streptavidin-PE.

Pharmacokinetics, toxicity, and immunogenicity of REGN2810 in cynomolgus monkeys

In a single-dose pharmacokinetic (PK) study, cynomolgus monkeys (*Macaca fascicularis*) received REGN2810 at 1, 5, or 15 mg/kg (5 females/group) by i.v. administration (30-minute infusion). Blood samples were collected before dose and at various times for 56 days following infusion. REGN2810 concentrations in serum were measured by ELISA. REGN2810, captured on the ELISA plate coated with the extracellular domain of human PD-1, was detected with a biotinylated mouse anti-human IgG4-specific monoclonal antibody, which in turn was detected with NeutrAvidin conjugated to HRP. Anti-REGN2810 antibodies (anti-drug antibodies, ADA) were measured using an electrochemiluminescence-based bridging immunoassay, in which a mouse-anti-human IgG, Fc-specific, monoclonal antibody (REGN2567) served as a positive control, and biotinylated REGN2810 was used for detection.

In a 1-month toxicity study, cynomolgus monkey groups (5/sex/group) received 4 weekly i.v. infusions of 0 (vehicle), 2, 10, or 50 mg/kg REGN2810 over 30 minutes, administered at a constant volume of 4 mL/kg, followed by an 8-week recovery period. Assessment of toxicity was based on mortality, morbidity, body weight, safety pharmacology evaluations, and clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis). Safety pharmacology evaluations included cardiovascular, respiratory, neurological, and hemodynamics analysis before dose, toward the end of the dosing period (3rd to 4th week), and the end of the recovery period (12 weeks). Gross necropsy examinations, measurement of organ weights, and histopathology were also conducted.

Results

REGN2810 binds to PD-1 with high affinity and specificity, inhibits PD-1 binding to PD-L1 and PD-L2 ligands, and does not induce ADCC or CDC

Human antibodies reactive to human PD-1-Fc protein were generated by immunizing VelocImmune mice transgenic for human Ig variable regions (35, 36). Clone REGN2810 was selected based on its ability to bind human PD-1 with high affinity and specificity, block PD-1 interactions with PD-L1 and PD-L2, and enhance T-cell function.

Using surface plasmon resonance (SPR), equilibrium dissociation constants (K_D) of REGN2810 were determined to be 6.11 nmol/L for monomeric human PD-1-mmH and 628 pmol/L for dimeric human PD-1-mFc proteins, respectively (Table 1). REGN2810 demonstrated similarly potent binding for monomeric and dimeric forms of cynomolgus monkey PD-1 recombinant proteins, with a K_D of 7.43 nmol/L and 520 pmol/L, respectively. The 10-fold tighter binding of REGN2810 to dimeric versus monomeric human or monkey PD-1 proteins likely reflects avidity-driven interactions. REGN2810 did not bind to monomeric rat and mouse PD-1, as expected from the low amino acid sequence homology between the extracellular domains of human versus rat or mouse PD-1 (65% and 61% identity, respectively).

REGN2810 bound to human Jurkat cells engineered to overexpress human or cynomolgus monkey PD-1 protein with a similar EC_{50} values of approximately 0.8 nmol/L and 1 nmol/L, respectively (Supplementary Fig. S1A). Parental Jurkat cells showed minimal REGN2810 binding consistent with low levels of endogenous surface PD-1 expression (41).

Flow cytometric analysis confirmed REGN2810 binding to PD-1 on activated primary human CD3⁺ T cells (Supplementary Fig. S1B). Isolation and activation of cynomolgus monkey T cells are described in Supplementary Materials and Methods. REGN2810 bound PD-1 on activated cynomolgus monkey CD4⁺ and CD8⁺ T cells expressing either low or high level of the early activation marker CD69 with similar EC_{50} values ranging from 1.3 to 1.8 nmol/L (Supplementary Materials and Methods; Supplementary Fig. S1C).

Table 1. REGN2810 binds with high affinity to human and cynomolgus monkey PD-1

Test ligand	Biacore kinetic parameters for REGN2810 binding to soluble PD-1 ectodomain at 25°C			
	k_a (M ⁻¹ s ⁻¹) ^a	k_d (s ⁻¹) ^b	K_D (M) ^c	$T_{1/2}$ (min) ^d
Human PD-1-mmH	1.59×10^5	9.72×10^{-4}	6.11×10^{-9}	11.9
Human PD-1-mFc	3.17×10^5	1.99×10^{-4}	6.28×10^{-10}	58.0
Monkey PD-1-mmH	1.36×10^5	1.01×10^{-3}	7.43×10^{-9}	11.4
Monkey PD-1-mFc	3.14×10^5	1.64×10^{-4}	5.20×10^{-10}	70.6
Rat PD-1-mmH	—	—	NB	NB
Mouse PD-1-mmH	—	—	NB	NB

NOTE: Human or cynomolgus monkey monomeric PD-1-mmH (myc-myc-hexahistidine tag) or dimeric PD-1-mFc proteins, as well as rat or mouse monomeric PD-1-mmH, were injected across a low-density anti-hFc-captured REGN2810 chip surface.

Abbreviation: NB, No detectable binding under the assay conditions tested.

^aAssociation rate constant.

^bDissociation rate constant.

^cEquilibrium dissociation constant.

^dDissociation half-life $T_{1/2}$.

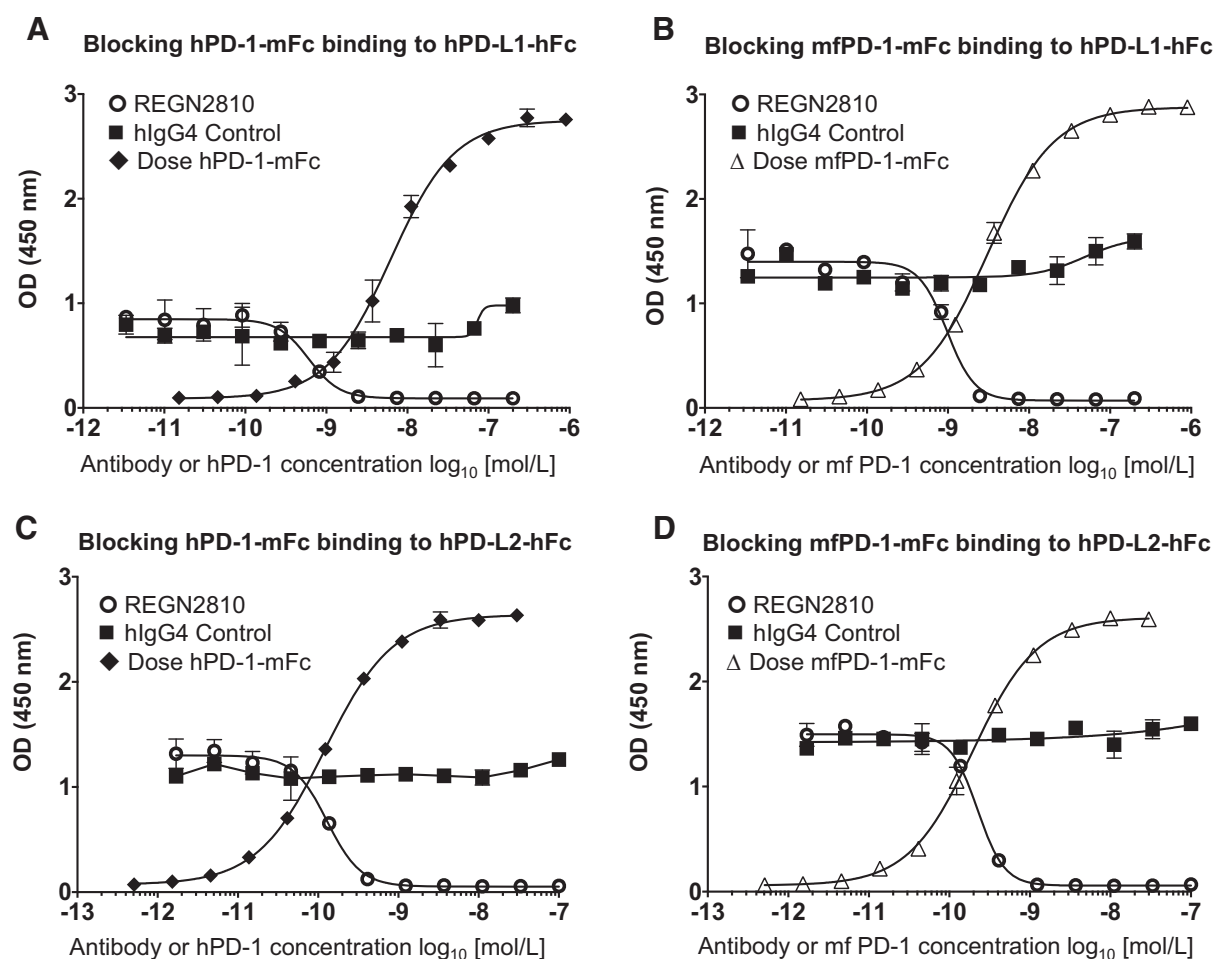


Figure 1.

REGN2810 inhibits binding of human and monkey PD-1 to human PD-L1 and PD-L2. **A** and **B**, Binding of 1.5 nmol/L hPD-1-mFc (**A**) or 2.0 nmol/L mfPD-1-mFc (**B**) to plate-coated hPD-L1-hFc in the presence of increasing concentration of REGN2810 or an isotype control antibody. **C** and **D**, Binding of 0.1 nmol/L hPD-1-mFc (**C**) or 0.25 nmol/L mfPD-1-mFc (**D**) to plate-coated hPD-L2-hFc in the presence of increasing concentration of REGN2810 or an isotype control antibody. hPD-1, human PD-1; mfPD-1, cynomolgus monkey PD-1.

Competition sandwich ELISA demonstrated that REGN2810 inhibited both hPD-1-mFc and mfPD-1-mFc from binding to plate-bound hPD-L1-hFc with IC_{50} values of 0.60 nmol/L and 0.97 nmol/L, respectively (Fig. 1A and B). Similarly, REGN2810 prevented hPD-1-mFc and mfPD-1-mFc binding to hPD-L2-hFc with IC_{50} values of 0.13 nmol/L and 0.22 nmol/L, respectively (Fig. 1C and D).

REGN2810 did not mediate antibody-dependent cell-mediated cytotoxicity (ADCC) activity of NK92/CD16^{176V} effectors against unstimulated or anti-CD3/anti-CD28 antibody-activated Jurkat targets expressing high level of PD-1 (Supplementary Materials and Methods; Supplementary Fig. S2A and S2B). In contrast, REGN3696, the positive control anti-HLA class I mIgG2a antibody with an amino acid sequence identical to clone W6/32 (42), exhibited dose-dependent ADCC activity against both unstimulated and stimulated targets with EC_{50} values of 0.1 nmol/L and 0.3 nmol/L, respectively (Supplementary Fig. S2B). In addition, REGN2810 did not mediate complement-dependent cytotoxicity (CDC) in activated Jurkat cells incubated with human serum complement, whereas the positive control anti-hCD20 antibody

rituximab caused a marked CDC response against Raji cells (Supplementary Fig. S2C, ref. 43).

The tissue-binding properties of REGN2810 were determined by immunohistochemistry using cryosections of a panel of human tissues stained with biotinylated REGN2810 (REGN2810-Bio). REGN2810-specific staining of rare mononuclear cells located within the germinal centers in the human tonsil tissue was consistent with reported PD-1 expression in human tonsils (44) and served as a positive control. Low-grade and infrequent immunoreactivity with REGN2810 was evident in the cytoplasm of mononuclear leukocytes in human lymphoid tissues, including lymph node, spleen, thymus, and tonsil, as well as in an interstitial aggregate in the kidney. In addition, plasma membrane staining of mononuclear leukocytes was evident in lymph nodes, spleen, and tonsil, consistent with PD-1 expression in lymphocytes and other myeloid cells (3, 5). There was no specific immunoreactivity in other human tissues analyzed (bladder, breast, colon, gastrointestinal tract, heart, liver, lung, ovary, pancreas, prostate, striated muscle, testis, thyroid, and uterus).

REGN2810 blocks PD-1/PD-L1 inhibitory signals and promotes T-cell activation *in vitro*

The ability of REGN2810 to enhance T-cell function was investigated using either engineered Jurkat T cells or preactivated primary human T cells (Fig. 2A), in combination with HEK293 APC-like cells engineered to express human CD20 with or without human PD-L1 (Fig. 2A). In both systems, TCR engagement was accomplished by an anti-CD3 x anti-CD20 bispecific antibody binding CD20 on HEK293 cells and CD3 on T cells. Jurkat T cells were engineered to express a luciferase reporter driven by the AP-1 promoter and to overexpress human PD-1 (Jurkat/AP-1-Luc/PD-1). In the presence of HEK293/hCD20 cells, addition of the bispecific anti-CD3 x anti-CD20 antibody resulted in dose-dependent luciferase activity in Jurkat/AP-1-Luc/PD-1 cells with an EC₅₀ of 35 pmol/L and 47-fold maxi-

imum increase in activity (Fig. 2B, black circles). When HEK293/hCD20/hPD-L1 cells were used as APC, luciferase activity was attenuated, with an EC₅₀ of 77 pmol/L and an only 9-fold maximum increase in activity (Fig. 2B, white circles). This inhibition was overcome by the addition of REGN2810 with an EC₅₀ of 3.9 nmol/L and a 4.3-fold increase in T-cell activity in the presence of 100 pmol/L anti-CD3 x anti-CD20 (Fig. 2D). As expected, REGN2810 had no effect in the presence of HEK293/hCD20 that did not express PD-L1 (Fig. 2C). A similar experimental design was used with primary human T cells that were preactivated to induce PD-1 expression (Fig. 2E, representative donor). Serially diluted REGN2810 or isotype control antibody was added to preactivated primary CD4⁺ T cells that were further stimulated with 2 nmol/L of anti-CD3 x anti-CD20 antibody in the presence of HEK293/hCD20/hPD-L1 cells.

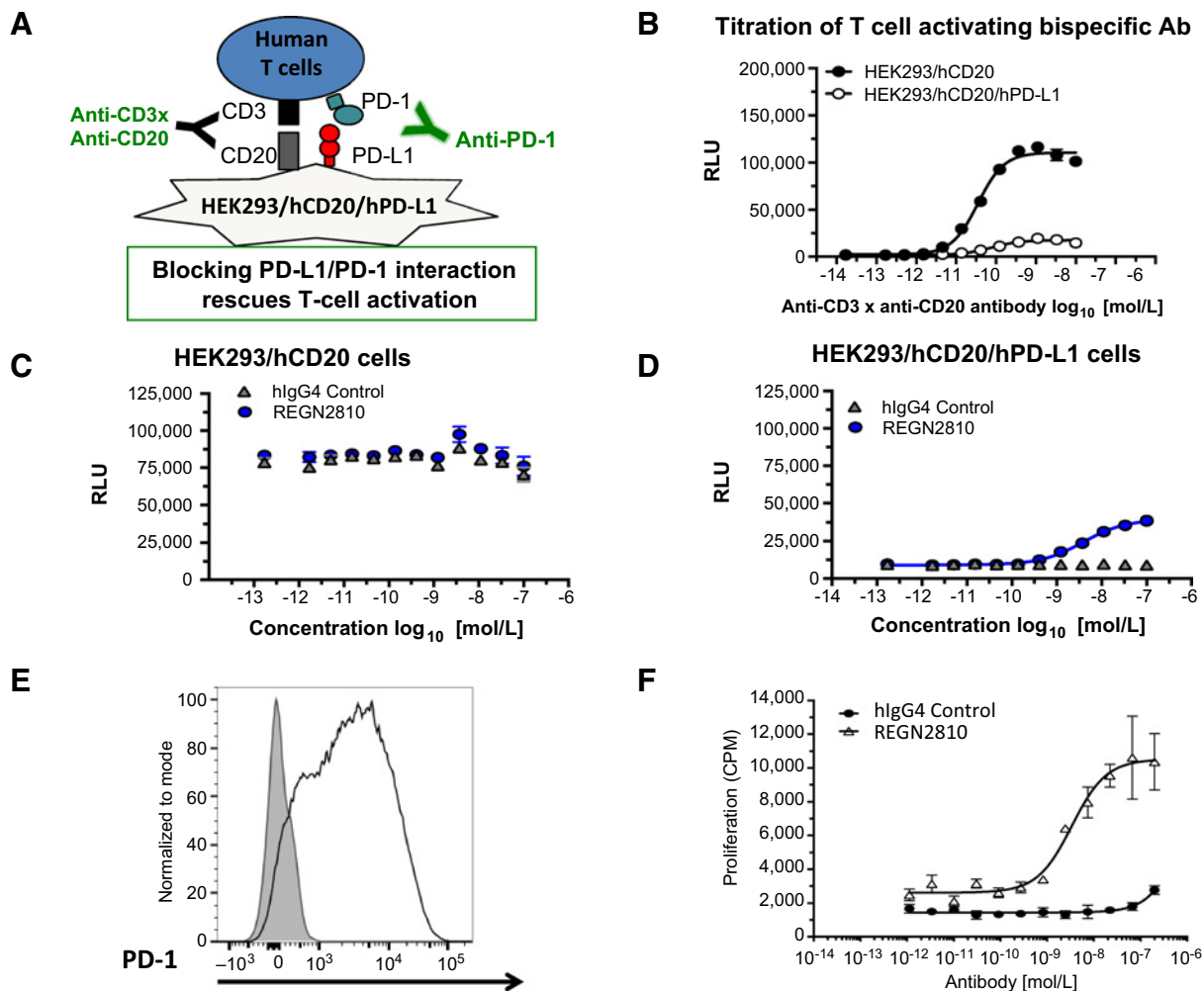


Figure 2. REGN2810 blocks PD-1/PD-L1 inhibitory signaling in a T cells/engineered APC bioassay. **A**, Bioassay schematic: To evaluate PD-1/PD-L1 inhibition, engineered Jurkat/AP-1-Luc/hPD-1 cells or preactivated primary human CD4⁺ T cells were incubated with HEK293/hCD20/hPD-L1-engineered APC in the presence of an anti-CD3 x anti-CD20 bispecific antibody. **B**, Jurkat /AP-1-Luc/PD-1 cells activation by anti-CD3 x anti-CD20 bispecific antibody in the presence of HEK293/hCD20 or HEK293/hCD20/hPD-L1 cells. **C** and **D**, REGN2810 rescues PD-1 inhibition in Jurkat/AP-1-Luc/hPD-1 cells in the presence of 100 pmol/L anti-CD3 x anti-CD20 bispecific Ab and HEK293/hCD20/hPD-L1 (**D**), but not HEK293/hCD20 cells (**C**). The x axis indicates concentration of antibodies (Log₁₀), and the y axis indicates the emitted light by the luciferase reaction expressed in RLU. **E**, staining for PD-1 expression on preactivated primary CD4⁺ T cells with anti-PD-1 APC (clone EH12.247, black line) or isotype control antibody APC (gray filled line). **F**, REGN2810 rescues CD4⁺ T-cell proliferation inhibited by HEK293/hCD20/hPD-L1 cells.

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REGN2810 induced a dose-dependent increase in T-cell proliferation (Fig. 2F, representative donor), with similar average and median EC₅₀ (1.87 nmol/L and 2.5 nmol/L, respectively) in 8 tested donors. Taken together, these assays demonstrate that REGN2810 suppresses inhibitory PD-1/PD-L1 engagement and enhances T-cell activity in the presence of TCR activation.

To determine whether REGN2810 by itself stimulates T cells in the absence of TCR activation, we employed a cytokine release assay (45). The superagonist anti-CD28 antibody, TeGenero TGN1412, known to stimulate a robust cytokine release in the absence of TCR activation, was generated in-house based on the publicly available amino acid sequence as a hinge-stabilized hIgG4 antibody (REGN2329). Both REGN2329- and anti-CD3 (clone OKT3)-positive control antibodies potentially increased cytokine production by human peripheral blood mononuclear cells (PBMCs). Consistent with published data for the anti-PD-1 antibody nivolumab (46), there was no significant release of inflammatory cytokines by human PBMCs cultured in plates with immobilized REGN2810 (Supplementary Fig. S3).

Functional replacement of mouse PD-1 with human homologue

To explore the *in vivo* effects of REGN2810, which does not recognize mouse PD-1, we generated human *PD-1* knock-in mice expressing the human PD-1 ectodomain using VelociGene technology (39). These mice express a chimeric protein comprised of the human PD-1 extracellular domain fused to the mouse PD-1 transmembrane and cytoplasmic domains from the endogenous *Pdcd1* locus (Supplementary Fig. S4A). Anti-human PD-1 stained the surface of activated splenocytes from human *PD-1* knock-in mice but not wild-type controls, whereas anti-mouse PD-1 stained wild-type controls but not human *PD-1* knock-ins (Supplementary Fig. S4B and S4C). Staining for human and mouse PD-1 in unstimulated splenic CD8⁺ and CD4⁺ T cells was indistinguishable from isotype control staining, indicating the absence of PD-1 from the surface of nonactivated cells. Upon stimulation, PD-1 was upregulated on CD8⁺ and CD4⁺ T cells derived from human *PD-1* knock-in mice, wild-type mice, and human PBMCs, indicating that human and mouse PD-1 protein expression is similarly regulated upon stimulation. Homozygous human *PD-1* knock-in mice displayed a normal life span and no overt signs of autoimmunity for at least 1 year, whereas *PD-1* knockout mice develop strain and tissue-specific autoimmunity (11, 12). Collectively, the chimeric PD-1 protein containing a human ectodomain is functional and human *PD-1* knock-in mice can be used to evaluate REGN2810 *in vivo*.

REGN2810 binds PD-1 in human *PD-1* knock-in mice and inhibits tumor growth

To confirm PD-1 binding of REGN2810 *in vivo*, we analyzed PD-1 occupancy on splenocytes from human *PD-1* knock-in mice engrafted with MC38.Ova tumors after injection with three doses of REGN2810 or isotype control antibody over 10 days (Supplementary Fig. S5A, representative panel, and S5B). In the isotype control-treated group, 31.5% of CD4⁺ T cells and 17.2% of CD8⁺ T cells stained with biotinylated REGN2810, followed by streptavidin-PE. In contrast, in mice that received REGN2810 at 25 mg/kg or 10 mg/kg, respectively, the frequency of PD-1⁺ cells was reduced to 7.0% and 4.6% for CD4⁺ T cells, and 12.4% and 5.7% for CD8⁺ T cells, suggesting that human PD-1 binding sites were occupied by REGN2810 antibodies *in vivo*. Because the human

PD-1 occupancy was not increased with the higher dose (25 mg/kg), a dose of 10 mg/kg appears to be sufficient to occupy human PD-1 in humanized *PD-1* mice. SPR-Biacore studies (Supplementary Table S1) determined that the dimeric human PD-1 protein hPD-1-mFc binds to human or mouse PD-L1-hFc with similar K_D values of 79 nmol/L and 92 nmol/L, respectively. Thus, human *PD-1* knock-in mice can be used to study the impact of REGN2810 on PD-1/PD-L1 interactions *in vivo*. MC38.Ova carcinoma cells express murine PD-L1, but not murine PD-L2, as determined by flow cytometry (Supplementary Fig. S4D).

In the minimal tumor model, human *PD-1* knock-in mice engrafted with MC38.Ova cells were treated with REGN2810 doses ranging from 0.3 mg/kg to 10 mg/kg, starting on day 3, before the predicted appearance of measurable tumors. REGN2810 showed potent dose-dependent tumor growth inhibition, and at 10 mg/kg, 5 of 8 mice were tumor free, whereas none of the isotype control-treated animals were tumor-free (Fig. 3A). At 3 mg/kg and 1 mg/kg, REGN2810 was slightly less efficacious, with 3 of 8 tumor-free mice at the end of the study on day 38. Moreover, all REGN2810-treated groups showed prolonged survival ($P < 0.00001$), most evident at 10 mg/kg dose (Fig. 3B). Flow cytometric analysis of draining lymph nodes revealed an increased frequency of CD4⁺ and CD8⁺ T cells in mice treated with REGN2810 (Supplementary Fig. S6A). Taqman analysis of spleens revealed increased transcript levels for CD8, CD3, IFN γ , and TNF α in REGN2810-treated mice, suggesting an increase in CD8⁺ effector T cells and effector function (Supplementary Fig. S6B). These results further validate that PD-1 signaling is intact in human *PD-1* knock-in mice and confirm the immune-enhancing function of REGN2810 *in vivo*. REGN2810 activity was also examined in a therapeutic MC38.Ova tumor model, where treatment with REGN2810 or an isotype control antibody was initiated in individual animals when the tumor volume reached 100 mm³, 10 to 14 days after tumor cell engraftment. In this setting, REGN2810 caused a dose-dependent growth delay of established tumors (Fig. 4).

Pharmacokinetics and toxicity of REGN2810 in cynomolgus monkeys

In a PK study, the concentration–time profiles of REGN2810 were characterized by an initial brief distribution phase, followed by a linear beta elimination phase and a terminal target-mediated elimination phase. Following IV infusion, the terminal target-mediated elimination phase of the concentration–time profile of REGN2810 was evident at REGN2810 serum concentrations below approximately 5 to 20 μ g/mL in the 1 and 5 mg/kg groups (Supplementary Fig. S7). However, the target-mediated elimination phase was not observed in the 15 mg/kg group that led to REGN2810 serum concentrations greater than 20 μ g/mL throughout the 56-day study duration (Supplementary Fig. S7). REGN2810 PK parameters are shown in Table 2. Mean beta phase half-lives ($t_{1/2 \text{ beta}}$) were comparable across the 1, 5, and 15 mg/kg groups. The mean AUC_{last} values were 168, 1,100, and 3,950 day \cdot μ g/mL following i.v. infusion of 1, 5, and 15 mg/kg REGN2810, respectively. The corresponding dose-normalized mean AUC_{last} values (AUC_{last}/dose) of 168, 220, and 263 day \cdot μ g/mL per mg/kg indicated a greater than dose-proportional increase across the dose levels. Consistent with this finding, mean total body clearance (CL) was dose-dependent and decreased with increasing dose. Mean terminal half-lives ($t_{1/2 \text{ terminal}}$) of 1.19 and 2.02 days in the 1 and 5 mg/kg dose groups, respectively, were shorter relative to 9.85 days in the 15 mg/kg group. Anti-

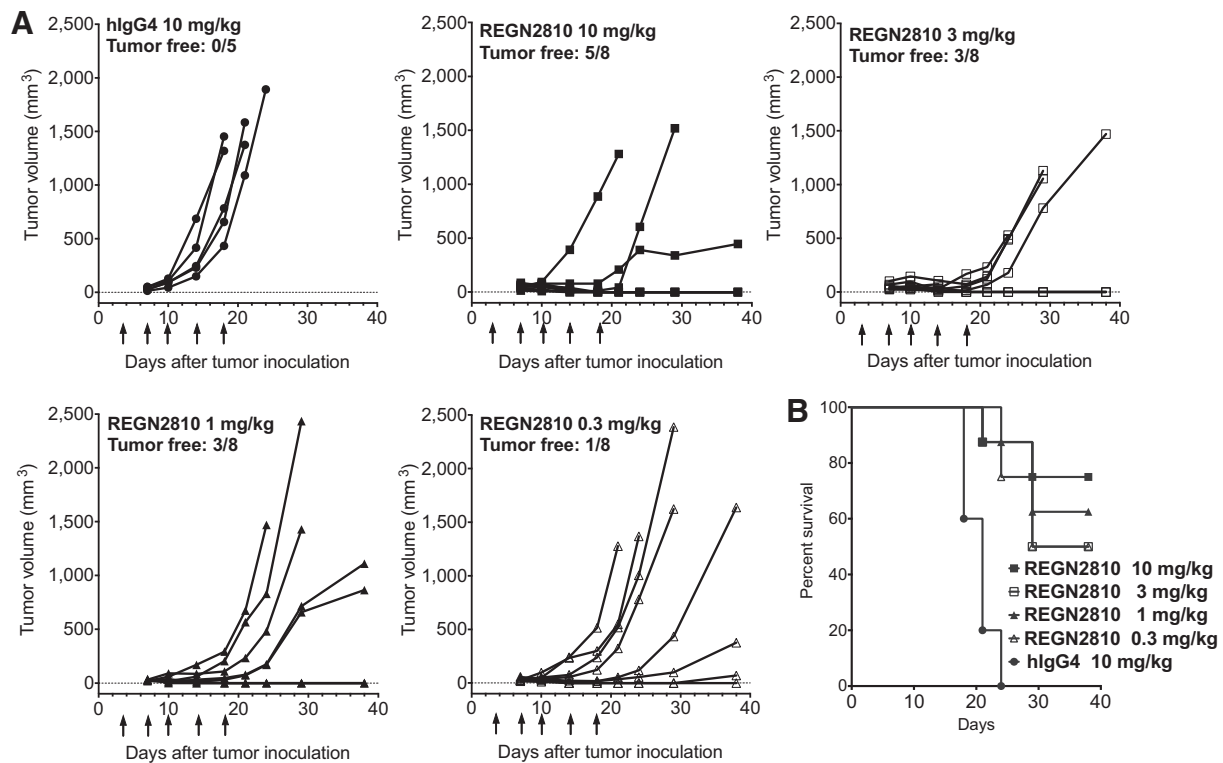


Figure 3.

REGN2810 therapy inhibits tumor growth and improves survival of tumor-bearing human *PD-1* knock-in mice. **A**, growth kinetics of MC38.Ova tumors in a minimal disease model. Mice were engrafted s.c. into the flank with MC38.Ova cells (5×10^5 cells/mouse) on day 0. Mice were treated i.p. with REGN2810 (10 mg/kg, 3 mg/kg, 1 mg/kg, or 0.3 mg/kg; $n = 8$ /group) or isotype control antibody (10 mg/kg; $n = 5$) on days 3, 7, 10, 14, and 18, and tumor volumes were monitored until day 38. Mice were euthanized at maximum allowed tumor burden. The number of tumor-free animals on day 38 is shown for each treatment group. **B**, Kaplan-Meier survival curves of mice treated with REGN2810 or control antibody. A log-rank (Mantel-Cox) test revealed that REGN2810 antibodies significantly prolonged mouse survival ($P < 0.00001$).

REGN2810 antibodies were observed in all animals by 28 days after dose, which resulted in accelerated elimination of REGN2810 from the serum of 67% of the animals across all of the dose levels (3/5, 4/5, and 3/5 animals in the 1, 5, and 15 mg/kg groups, respectively). These REGN2810 concentrations affected by ADA were excluded from the PK analysis.

In a 1-month toxicity study, REGN2810 was well tolerated when administered weekly to both male and female cynomolgus monkeys via i.v. infusion at doses of 2, 10, or 50 mg/kg. There were no unexpected deaths during the study, no drug-related clinical signs were observed, and there was no effect on body weight. There were no gender differences related to the toxicokinetics or immunogenicity of REGN2810. Although during the course of the study, 77% of animals showed positive ADA response, continuous exposure to REGN2810 was maintained throughout the 4-week treatment phase in 80% of drug-treated animals.

Discussion

Targeting immune checkpoint receptors on activated T cells has rapidly emerged as a cornerstone of cancer immunotherapy. Antibodies blocking the interaction of PD-1 with PD-L1 have been shown to enhance antitumor immune responses in diverse cancers. Here, we report the biochemical, pharmacologic, and functional properties of REGN2810, a hinge-stabilized fully

human IgG4 anti-PD-1 antibody that potently blocks the PD-1/PD-L1 pathway.

REGN2810 is a fully human monoclonal antibody generated in VelocImmune mice (36) that contains a human light chain variable domain fused to human kappa constant and a heavy chain variable regions based on IgG4 Fc format. The human IgG4 constant region of REGN2810 contains a hinge S228P substitution, which precludes Fab arm exchanges known to occur for wild-type human IgG4 antibodies (38) while conserving the characteristically low affinity for activating Fcγ receptors (47). Indeed, we did not observe REGN2810-mediated ADCC or CDC activity, indicating that REGN2810 is unlikely to cause the depletion of PD-1-expressing cells.

In cell-based bioassays performed in the presence of sub-optimal TCR engagement, nanomolar concentrations of REGN2810 effectively blocked PD-1/PD-L1 interactions and thereby increased TCR signaling in engineered Jurkat T cells and increased proliferation of primary activated human T cells. To assess whether REGN2810 may cause the TCR-independent activation of T cells, we employed a human PBMC cytokine release assay. Super-agonist anti-CD28 (TeGenero TGN1412) or anti-CD3 antibodies served as positive controls. There was no significant cytokine release when human PBMCs were cultured in the presence of immobilized REGN2810, indicating that REGN2810 does not result in TCR-independent T-cell activation.

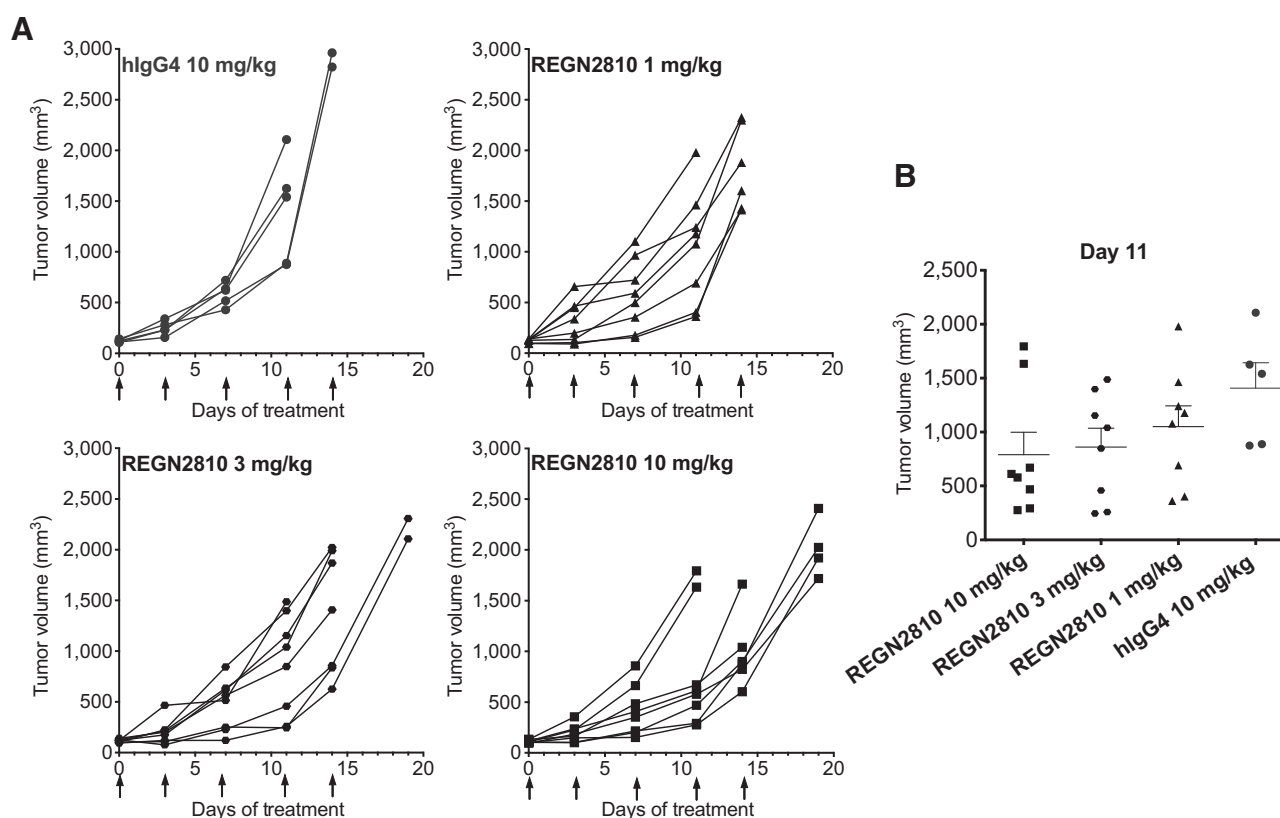


Figure 4.

REGN2810 delays growth of established tumors in human *PD-1* knock-in mice. Mice were engrafted s.c. into the flank with MC38.Ova cells (5×10^5 cells/mouse). Mice were randomized into treatment groups 10 to 14 days after engraftment when tumor volumes reached 100 mm³ (day 0). REGN2810 (10 mg/kg, 3 mg/kg, or 1 mg/kg, $n = 8$ /group) or the isotype control antibody (10 mg/kg, $n = 5$) was administered on days 0, 3, 7, 11, and 14, and tumor volumes were monitored until day 20. **A**, Individual tumor growth curves in each treatment group. **B**, Individual tumor volumes in each treatment groups were measured on day 11, the last time point when all animals in the study were alive.

REGN2810 does not cross-react with mouse PD-1. Thus, to study REGN2810 *in vivo*, we created human *PD-1* knock-in mice expressing the human PD-1 ectodomain. Of note, we and others (8) have demonstrated that human PD-1 functionally binds both human and mouse PD-L1 with similar affinity. We did not observe any signs of inflammation in human *PD-1* knock-in mice, indicating that the humanization of PD-1 did not result in spontaneous autoimmunity. As expected, T cells from human *PD-1* knock-in mice upregulated humanized PD-1 upon activation with anti-CD3/anti-CD28 antibodies. The human PD-1 knock-in was functional since the treatment of tumor-bearing mice with REGN2810 resulted in increased IFN γ and TNF α expression in the spleen. Taken together, expression data and functional studies have validated PD-1-humanized mice as a powerful model to test REGN2810 activity.

We used the syngeneic MC38.Ova murine carcinoma model in human *PD-1* knock-in mice to explore the *in vivo* efficacy of REGN2810. The administration of REGN2810 inhibited the growth of MC38.Ova tumors, which express PD-L1, and provided a measurable survival benefit. *In vivo* binding of REGN2810 to humanized PD-1 on T cells in tumor-bearing mice was confirmed following REGN2810 administration of 25 mg/kg and 10 mg/kg.

The potential mechanism of REGN2810 activity was investigated by examining T-cell function in spleens of tumor-bearing

human *PD-1* knock-in mice. Increased expression of CD3, CD8, IFN γ , and TNF α was observed following REGN2810 treatment. Consistent with this finding, administration of anti-mouse PD-1 or PD-L1-blocking antibodies in preclinical mouse models resulted in increased proliferation of antigen-specific effector

Table 2. Mean PK parameters of REGN2810 in serum of cynomolgus monkeys following a single i.v. infusion

Parameter	Units	Dose of REGN2810		
		1 mg/kg	5 mg/kg	15 mg/kg
C_{max}	$\mu\text{g/mL}$	33.3 ± 1.91	121 ± 10.2	355 ± 64.7
AUC_{last}	day $\times \mu\text{g/mL}$	168	1,100	3,950
AUC_{last}/dose	day $\times \text{kg} \times \mu\text{g/mL/mg}$	168	220	263
$t_{1/2 \text{ terminal}}$	day	1.19	2.02	9.85
$t_{1/2 \text{ beta}}$	day	9.84 ± 1.13	10.9 ± 3.82	12.4 ± 1.67
CL	mL/day/kg	5.99	4.56	3.68

NOTE: $N = 5$ /group. AUC_{last} , AUC_{last}/dose , $t_{1/2 \text{ terminal}}$, and CL were estimated based on PK profiles of animals not affected by ADA; $n = 2, 1$, and 2 for the 1, 5, and 15 mg/kg dose groups, respectively. Concentrations considered to be outliers were excluded for one animal in each of the 1 and 15 mg/kg dose groups (≤ 2 timepoints/animal). Values for C_{max} and $t_{1/2 \text{ beta}}$ are mean \pm SD. Abbreviations: C_{max} , maximum drug concentration observed in serum; AUC_{last} , area under the concentration-time curve from time zero to the last measurable concentration; $t_{1/2 \text{ terminal}}$, half-life estimated by the observed terminal phase of the concentration-time curve; $t_{1/2 \text{ beta}}$, half-life estimated by the observed beta phase of the concentration-time curve.

CD8⁺ and CD4⁺ T cells and expression of key signaling molecules critical for T-cell function (48, 49).

A single-dose PK study in cynomolgus monkeys provided a REGN2810 PK profile that can support clinical testing. In addition, REGN2810 toxicokinetics were assessed in a 4-week cynomolgus monkey study. Cynomolgus monkeys were deemed a relevant species based on similar binding affinities of REGN2810 to monkey and human PD-1. The concentration–time profiles in monkey demonstrated the predominance of target-mediated clearance at low REGN2810 concentrations. Target-mediated elimination was not observed at REGN2810 concentrations ≥ 5 to 20 $\mu\text{g/mL}$, indicating target saturation and linear kinetics at these higher concentrations. Safety pharmacology endpoints were integrated into the repeat dose monkey toxicology study. Following i.v. dosing of REGN2810 at 2, 10, or 50 mg/kg/week for 4 weeks, no drug-related effects were observed in cardiovascular, respiratory, or central nervous system (CNS) function, nor were any deleterious microscopic changes observed in tissues associated with these systems. Although ADA against REGN2810 were detected in monkeys following single or repeat administration, this was not associated with adverse effects. Importantly, the immunogenicity in animals is not considered to be a good predictor of the immunogenicity in humans (50). Fully human biologics, such as REGN2810, which demonstrate immunogenicity in animals used in toxicology studies are not expected to be immunogenic in humans.

Numerous clinical trials of anti-PD-1 monoclonal antibodies have been conducted to treat human cancers, leading to FDA approval of nivolumab (Bristol-Myers Squibb) and pembrolizumab (Merck) for several indications, as well as many ongoing trials (22–32) for these and other clinical agents. Comparison of nivolumab and REGN2810, based on preclinical nivolumab data reported by Wang and colleagues (46), indicates that both antibodies show similar binding affinity to recombinant human and monkey PD-1 protein in nanomolar concentrations, as determined by SPR (Table 1 and ref. 46). In *in vitro* allogeneic MLR assays with human T cells, PD-1 blockade with nivolumab or REGN2810 in the presence of PD-L1–expressing cells and TCR stimulation resulted in the concentration-dependent enhancement of T-cell proliferation (ref. 46 and Fig 2F). Neither nivolumab nor REGN2810 caused nonspecific lymphocyte activation in an *ex vivo* cytokine-release assay, and both antibodies were shown to be well tolerated when administered to cynomolgus monkeys. Comparisons of pembrolizumab with REGN2810 are more difficult, because, to the best of our knowledge, there is no published preclinical information on pembrolizumab.

In conclusion, the studies presented in this report demonstrate that REGN2810 is a potent PD-1 inhibitor both *in vitro* and *in vivo*. Clinical efficacy of PD-1–blocking antibodies for cancer immunotherapy (26–29) has reaffirmed the ability of PD-1/PD-L1 axis blockade to yield significant benefits in patients by unleashing the cytotoxic function of tumor-specific T cells. Preclinical and clinical evidence for the enhanced benefit of PD-1 inhibitors in combi-

nation with other agents continues to grow (31–34). Taken together, the preclinical data support PD-1 blockade with REGN2810 to be a promising foundation for combination cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

E. Burova has ownership interest (including patents) in a patent (patent applications pending), assigned to Regeneron Pharmaceuticals. A. Hermann has ownership interest (including patents) in Regeneron Pharmaceuticals as a shareholder. J. Waite has ownership interest (including patents) in Regeneron Pharmaceuticals as a shareholder. T. Potocky has ownership interest (including patents) in Regeneron Pharmaceuticals as a shareholder. V. Lai has ownership interest (including patents) as an inventor of patent (patent applications pending), assigned to Regeneron Pharmaceuticals, Inc. and as a shareholder. A. Woodruff has ownership interest (including patents) in Regeneron Pharmaceuticals as a shareholder. A. D'Orvilliers has ownership interest (including patents) in stock. T. Huang is a senior director. E. Ioffe has ownership interest (including patents) in a patent (patent applications pending), assigned to Regeneron Pharmaceuticals, Inc. and as a shareholder. I. Lowy is a vice president and has ownership interest (including patents) in Regeneron Pharmaceuticals. A. Murphy has ownership interest (including patents) in Regeneron Pharmaceuticals as a shareholder and in a patent (patent applications pending), assigned to Regeneron Pharmaceuticals, Inc. G. Thurston has ownership interest (including patents) in a patent (patent applications pending), assigned to Regeneron Pharmaceuticals, Inc., and is a shareholder. No potential conflicts of interest were disclosed by the other authors.

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