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Research Article

Cancer Immunology Research

In Vitro Characterization of the Anti-PD-1 Antibody Nivolumab, BMS-936558, and *In Vivo* Toxicology in Non-Human Primates

Changyu Wang[1], Kent B. Thudium[1], Minhua Han[1], Xi-Tao Wang[1], Haichun Huang[1], Diane Feingersh[1], Candy Garcia[1], Yi Wu[1], Michelle Kuhne[1], Mohan Srinivasan[1], Sujata Singh[1], Susan Wong[1], Neysa Garner[1], Heidi Leblanc[1], R. Todd Bunch[2], Diann Blanset[3], Mark J. Selby[1], and Alan J. Korman[1]

Abstract

The programmed death-1 (PD-1) receptor serves as an immunologic checkpoint, limiting bystander tissue damage and preventing the development of autoimmunity during inflammatory responses. PD-1 is expressed by activated T cells and downmodulates T-cell effector functions upon binding to its ligands, PD-L1 and PD-L2, on antigen-presenting cells. In patients with cancer, the expression of PD-1 on tumor-infiltrating lymphocytes and its interaction with the ligands on tumor and immune cells in the tumor microenvironment undermine antitumor immunity and support its rationale for PD-1 blockade in cancer immunotherapy. This report details the development and characterization of nivolumab, a fully human IgG4 (S228P) anti-PD-1 receptor-blocking monoclonal antibody. Nivolumab binds to PD-1 with high affinity and specificity, and effectively inhibits the interaction between PD-1 and its ligands. *In vitro* assays demonstrated the ability of nivolumab to potently enhance T-cell responses and cytokine production in the mixed lymphocyte reaction and superantigen or cytomegalovirus stimulation assays. No *in vitro* antibody-dependent cell-mediated or complement-dependent cytotoxicity was observed with the use of nivolumab and activated T cells as targets. Nivolumab treatment did not induce adverse immune-related events when given to cynomolgus macaques at high concentrations, independent of circulating anti-nivolumab antibodies where observed. These data provide a comprehensive preclinical characterization of nivolumab, for which antitumor activity and safety have been demonstrated in human clinical trials in various solid tumors. *Cancer Immunol Res; 2(9); 1–11. ©2014 AACR.*

Introduction

Cancer can be considered as an inability of the host to eliminate transformed cells. Although the immune system is the principal mechanism of cancer prevention, transformed cells counteract immunosurveillance. Natural control mechanisms that limit T-cell activation, thereby preventing collateral damage from unrestrained T-cell activity, may be exploited by tumors to evade immune responses (1). Restoring the capacity of immune effector cells—especially T cells—to recognize and eliminate cancer is the goal of immunotherapy. The concept of inhibitory receptor blockade, also known as checkpoint blockade, has been validated in humans with the approval of the anti-CTLA-4 antibody ipilimumab for metastatic melanoma (2, 3).

Authors' Affiliations: [1]Biologics Discovery California, Bristol-Myers Squibb Company, Redwood City, California; [2]Bristol-Myers Squibb Company, Mount Vernon, Indiana; and [3]Medarex, Princeton, New Jersey

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Corresponding Author: Alan J. Korman, Bristol-Myers Squibb, 700 Bay Road, Redwood City, CA 94063. Phone: 650-260-9586; Fax: 650-260-9898; E-mail: alan.korman@bms.com

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Programmed death-1 (PD-1) is an additional inhibitory receptor expressed by T cells. Engagement of PD-1 by its ligands, PD-L1 and PD-L2, induces an inhibitory signal resulting in reduced T-cell proliferation, cytokine production, and cytotoxic activity (4, 5). PD-1 deletion in mice can lead to autoimmunity (6, 7), most notably when bred onto backgrounds of autoimmune-susceptible mouse strains (8). Elevated PD-1 expression on T cells, observed during chronic viral infections in humans and mice, is associated with reduced T-cell functionality or "exhaustion." T cells become progressively more nonresponsive as they express additional inhibitory receptors (9). Tumor-infiltrating T cells may also be functionally inert, due in part to the expression of PD-1 along with other inhibitory receptors (10, 11). In multiple syngeneic mouse tumor models, blockade of PD-1 or its ligands promotes antitumor activity (12–14); anti-PD-1 activity *in vivo* can be enhanced by combination with antibodies to other T-cell negative regulators, such as CTLA-4 and LAG-3 (15–17).

PD-L1 is expressed by many human tumors, including melanoma, lung, and kidney (10, 18, 19). PD-L1 engagement of PD-1 may be one mechanism whereby tumors evade immunosurveillance by directly limiting effector T-cell activity. Several studies support the notion that PD-L1 expression and, in some cases, PD-L2 expression is associated with tumor aggressiveness and adverse patient outcome (14, 20–22).

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Alternatively, PD-L1 expression in metastatic melanoma, upregulated by the expression of IFNγ through locally activated T cells, may indicate preexisting antitumor activity. Accordingly, patients with PD-L1$^+$ tumors had improved survival relative to those with PD-L1$^-$ tumors (23). Although greater responses to PD-1 blockade in humans are associated with PD-L1 expression on 5% or more of melanoma tumor cells, responses have also been seen in PD-L1$^-$ patients (24).

Here, we describe the selection and characterization of the anti-PD-1 antibody nivolumab (BMS-936558, MDX-1106, and ONO-4538). Nivolumab was generated in transgenic mice, which contain a human immunoglobulin minilocus for the heavy chain together with the human immunoglobulin light chain kappa locus along with mutations that prevent the production of murine antibodies. Antibodies arising from immunization of these mice are fully human and have low immunogenicity in human patients. Nivolumab has shown promising early results in patients with advanced malignancies, including melanoma, lung, and renal cancer, with generally manageable side effects (25–27).

Materials and Methods

Antibody generation

Transgenic mice comprising germline configuration human immunoglobulin miniloci in an endogenous IgH and Igκ knockout background (28, 29) were used to generate human anti-PD-1 monoclonal antibodies (mAb). The transgenic mice were immunized with recombinant human PD-1-Fc protein consisting of the extracellular domain of PD-1 (amino acids 1–167) and the Fc portion of human IgG1, and Chinese hamster ovary (CHO) cells expressing human PD-1 (CHO-PD-1 cells). Spleen cells from immunized mice were fused with SP2/0 myeloma cells and screened for hybridomas producing human mAbs reactive to PD-1-Fc by enzyme-linked immunosorbent assay (ELISA). The CHO cell line was provided by Dr. Lawrence Chasin (Columbia University, New York, NY). The SP2/0 and SK-MEL-3 cell lines were purchased from the ATCC. All cell lines were confirmed to be *Mycoplasma*-free by RT-PCR analysis. No other authentication assays were performed.

Nivolumab binding to human and cynomolgus PD-1

CD4$^+$ T cells purified from human peripheral blood mononuclear cells (PBMC) using a CD4$^+$ T-cell positive selection kit (Dynal) were activated with plate-coated anti-CD3 antibody (clone UCHT-1; BD Biosciences) for 4 days and tested for nivolumab binding in a fluorescence-activated cell sorting (FACS)–based assay using a fluorescein isothiocyanate (FITC)–conjugated anti-human kappa antibody (Jackson ImmunoResearch). Binding kinetics of nivolumab to PD-1 were determined using recombinant human PD-1-Fc (R&D Systems) or FLAG-tagged cynomolgus PD-1 protein (containing amino acids 1–169 of the cynomolgus PD-1 extracellular domain) coated on a CM5 (Biacore) sensor chip or captured on a CM5 sensor chip precoated with anti-FLAG mAb M2 (Sigma-Aldrich) with low antigen density, respectively. Nivolumab was flowed over the antigen-coated chip, and avidity was determined using surface plasmon resonance (Biacore). Alternatively, affinity was determined by capture of nivolumab on an anti-CH1 antibody precoated CM5 chip over which human PD-1-Fc protein was applied.

Immunohistochemistry

The nivolumab tissue-binding profile was assessed in a small panel of normal human tissues, including tonsil (hyperplasia, three samples), spleen, cerebellum, heart, kidney, liver, lung, and pituitary (five samples). Snap-frozen, optimal cutting temperature compound–embedded, unfixed tissues were purchased from Analytical Biological Services Inc., Asterand Inc., Cooperative Human Tissue Network, and National Disease Research Interchange (Philadelphia, PA). FITC-conjugated nivolumab (0.2–10 μg/mL) was applied to acetone-fixed sections, followed by anti-FITC as a bridging antibody, and visualized using the EnVision+ System (Dako).

In vitro functional assays

Mixed lymphocyte reaction. Dendritic cells (DC) were generated by culturing monocytes isolated from PBMCs using a monocyte purification kit (Miltenyi Biotec) *in vitro* for 7 days with 500 U/mL interleukin-4 (IL-4) and 250 U/mL GM-CSF (R&D Systems). CD4$^+$ T cells (1×10^5) and allogeneic DCs (1×10^4) were cocultured with or without dose titrations of nivolumab added at the initiation of the assay. After 5 days, IFNγ secretion in culture supernatants was analyzed by ELISA (BD Biosciences), and cells were labeled with ^{3}H-thymidine for an additional 18 hours to measure T-cell proliferation.

Staphylococcal enterotoxin B stimulation of PBMCs. PBMCs from healthy human donors ($N = 18$) were cultured for 3 days with nivolumab or an isotype control antibody (20 μg/mL) at the initiation of the assay together with serial dilutions of staphylococcal enterotoxin B (SEB; Toxin Technology). IL-2 levels in culture supernatants were measured by ELISA analysis (BD Biosciences).

Antigen-specific recall response in vitro. In a cytomegalovirus (CMV)-restimulation assay, 2×10^5 PBMCs from a CMV-positive donor (Astarte) were stimulated using lysate of CMV-infected cells (Astarte), with serial dilutions of nivolumab added at the initiation of the assay. After 4 days, supernatants were assayed for IFNγ.

Suppression assay with regulatory T cells. CD4$^+$CD25$^+$ regulatory T cells (Tregs) and CD4$^+$CD25$^-$ responder T cells were purified from PBMCs (CD4$^+$CD25$^+$ Treg isolation kit; Miltenyi Biotec). In an allogeneic mixed lymphocyte reaction (MLR) assay, Tregs (5×10^4) were cocultured with 1×10^5 responder T cells and 2×10^4 monocyte-derived DCs, with 20 μg/mL nivolumab. After 5 days, IFNγ production was assessed in supernatants, and cells were labeled with ^{3}H-thymidine for an additional 18 hours for proliferation analysis.

Antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) was assayed using the DELFIA Cell Cytotoxicity Kit (PerkinElmer). PBMCs were incubated overnight with 50 U/mL IL-2 (R&D Systems) and used as effector cells. Activated CD4$^+$ T cells labeled with BATDA reagent were used as target cells at an effector-to-target cell ratio of 50:1. Serial dilutions of nivolumab or positive control [anti-major histocompatibility

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complex (MHC) class I antibody; Bristol-Myers Squibb] were added; the cells were incubated for 3 hours at 37°C. To measure cytotoxicity, supernatant was mixed with Europium solution and read using a RUBYstar Model 460 microplate reader (BMG LABTECH).

Pharmacokinetics, toxicity, and immunogenicity of nivolumab in cynomolgus macaques

In a single-dose pharmacokinetic (PK) study, cynomolgus monkeys (*Macaca fascicularis*) received i.v. nivolumab, 1 mg/kg (3 males and 3 females) or 10 mg/kg (3 males). The optical densities (OD) of a set of nivolumab concentration standards were determined and used to plot an OD versus concentration standard curve that was analyzed by four-parameter curve fit. Nivolumab serum concentrations were determined from the standard curve using SOFTmax Pro version 4.3 software. Anti-nivolumab antibodies were measured using a bridge ELISA and were detected with biotinylated nivolumab. Post-dose samples with mean OD $> 1.5 \times$ predose mean OD were reported as positive for anti-nivolumab antibody response. Each positive sample at day 28 was further characterized by dilutional titration and recovery of spiked nivolumab.

In a 3-month toxicity study, cynomolgus monkeys (6 males and 6 females per dose group) were injected i.v. with 0 (vehicle), 10, or 50 mg/kg nivolumab twice weekly for a total of 27 doses. Dosing levels were based on results from a 1-month toxicity study, in which doses up to 50 mg/kg weekly were well tolerated (results not shown). Twenty-four monkeys (4/gender/group) were euthanatized 1 day following the last dose for primary necropsy. The remaining 12 monkeys (2/gender/group) were euthanatized 28 days after the last dose for recovery necropsy. Analyses included body weight, cardiovascular, neurologic, and respiratory assessments; urinalysis; clinical pathology [hematologic assessments and analysis of plasma hormones: triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), growth hormone, ACTH, and α-MSH]; organ weights; and macroscopic and microscopic pathology.

Immunization of SK-MEL-3 melanoma cells and hepatitis B virus surface antigen in cynomolgus macaques

Vaccination studies were undertaken to examine the effects of nivolumab on activation of immune responses. Groups of 6 cynomolgus monkeys were dosed monthly with 10 mg/kg i.v. nivolumab, ipilimumab, or saline control (three doses total). In addition, all groups simultaneously received mitomycin C–inactivated SK-MEL-3 melanoma cells (5×10^6 cells) and hepatitis B virus surface antigen (Engerix-B; GlaxoSmithKline) injected subcutaneously at three independent sites. Peripheral blood samples from all animals were drawn immediately before and 2 weeks following each immunization. Antibodies to HBsAg were quantified using a commercially available kit (DiaSorin).

Antibody responses to the SK-MEL-3 cellular vaccine were measured with SK-MEL-3 cells incubated with monkey plasma samples at 4°C for 30 minutes. After washing, bound antibodies were detected with a phycoerythrin (PE)-conjugated F(ab′)2 goat anti-human IgG, Fcγ-specific antibody (Jackson ImmunoResearch), and analyzed by FACS. Anti-human leukocyte antigen (HLA)-A2404 titer was measured in a 96-well plate coated with A2404 monomer (Baylor College of Medicine, Houston, TX) at 2 μg/mL.

Results

Binding analysis of nivolumab and inhibition of ligand binding

One clone (PD1.5) was selected from a panel of human antibodies generated by immunization of human immunoglobulin transgenic mice based on its ability to bind PD-1 with high affinity and specificity, to inhibit PD-1 ligand binding to PD-1, and to promote T-cell function. The variable regions of this antibody were sequenced and grafted onto human kappa and IgG4 constant region sequences containing an S228P mutation, and the resulting antibody (nivolumab) was expressed and purified from a transfected CHO cell line. The complete characterization of nivolumab is described below; preliminary data have been reported previously (25, 30).

Nivolumab bound to CHO cells expressing PD-1 with an EC_{50} of 1.66 nmol/L, but did not bind to the parental CHO cell line (data not shown). To confirm that nivolumab recognized native PD-1, binding of nivolumab to activated human $CD4^+$ T cells was assessed (Supplementary Fig. S1A). Nivolumab bound to PD-1 on activated T cells with an EC_{50} of 0.64 nmol/L. Additional flow cytometric analysis of human T-cell subsets revealed that nivolumab stained memory and effector, but not naïve $CD4^+$ or $CD8^+$ T cells from human peripheral blood (Supplementary Fig. S1B). $CD4^+CD25^{hi}$ Tregs were also bound by nivolumab (Supplementary Fig. S1C). By Scatchard analysis, nivolumab bound to PD-1 on activated human $CD4^+$ T cells, which expressed approximately 10,000 PD-1 receptors per activated T cell, with an affinity of 2.6 nmol/L (data not shown). Nivolumab demonstrated a similar affinity for cynomolgus PD-1 (1.7 nmol/L) by assessing binding to activated T cells by Scatchard analysis (data not shown). Cynomolgus PD-1 has a 96% identity with human PD-1 in the extracellular domain (Genbank NP_001271065.1). Using surface plasmon resonance, the affinity of nivolumab for recombinant human PD-1 protein was 3.06 nmol/L when the chip was coated with low antigen density, and 2.64 nmol/L when antibody was captured on the chip using anti-IgG, in good agreement with the Scatchard analysis. The affinity for cynomolgus PD-1 was 3.92 nmol/L. Using Bio-layer Interferometry (ForteBio), the affinity of nivolumab for PD-1-Fc protein was substantially higher, at 2.7 pmol/L (data not shown). The reason for this difference is unclear.

The molecular epitope of nivolumab on human PD-1 was determined using mass spectrometry. Two peptides from protease-treated human PD-1, ^{29}SFVLNWYR-MSPS<u>N</u>QTDKLAAF-PEDR^{53} (putative glycosylation site underlined) and ^{85}SGTYLC-GAISLAPKAQIKE^{103}, bound to nivolumab (Supplementary Fig. S2A). Previous studies identified several human PD-1 residues as critical for PD-L1 and PD-L2 binding (31–34), and these residues are contained within the two sequences (Supplementary Fig. S2B). The amino acid sequence of the nivolumab epitope is identical between cynomolgus and human species.

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Nivolumab inhibits the interaction between PD-1 and its ligands, PD-L1 and PD-L2, with IC_{50} values of 2.52 and 2.59 nmol/L, respectively, as shown by surface plasmon resonance (Supplementary Fig. S3). In a previous study using FACS to evaluate ligand binding to PD-1 expressed on CHO cells, the IC_{50} values for nivolumab-mediated inhibition of PD-1 binding to PD-L1 or PD-L2 were similar (1.04 and 0.97 nmol/L, respectively; ref. 25).

Binding specificity and immunohistochemistry of nivolumab in normal human tissues

Nivolumab binds specifically to PD-1 and not to other immunoglobulin superfamily proteins, such as CD28, CTLA-4, ICOS, and BTLA (25). The specificity and tissue-binding properties of nivolumab were assessed by immunohistochemistry using a panel of normal human tissues. In tonsil, there was strong, specific staining by nivolumab in a subset of small- to medium-sized lymphocytes (Fig. 1A and B). These PD-1-positive cells were primarily in the periphery of reactive germinal centers (centrocytes), with a few scattered PD-1-positive cells in the mantle zone and the interfollicular region. When follicle zonation was observed, positive cells were primarily in the light zone. These results are consistent with $PD\text{-}1^{+}$ expression on T_{FH} cells (35, 36).

In four of five pituitary samples, immunoreactivity was detectable in a very small number of scattered endocrine cells (Fig. 1G–I); staining was primarily cytoplasmic and observed at higher antibody concentrations (5 or 10 μg/mL). In two samples, staining was localized mainly in large cytoplasmic spherical organelles, likely enigmatic bodies (large lysosomes), which are characteristic structures of corticotroph cells. Pituitary immunoreactivity required a 20-fold increase in antibody concentration, suggesting that PD-1 expression in the pituitary is very low or that nivolumab binds a cross-reactive pituitary tissue antigen with low affinity. However, PD-1 expression has not been reported in this cell type, and RT-PCR analysis for PD-1 mRNA in pituitary cells was also negative (data not shown). There was no specific staining in the other tissues examined (Fig. 1C–F), including cerebellum, heart, liver, lung, kidney, and spleen.

In vitro activity of nivolumab

The ability of nivolumab to promote T-cell responses was evaluated *in vitro* using human T cells; these assays include an allogeneic MLR, stimulation of human PBMCs by the superantigen SEB, and antigen-specific stimulation of T cells from CMV-responsive donors. In an allogeneic MLR, PD-1 blockade with nivolumab systematically resulted in a titratable

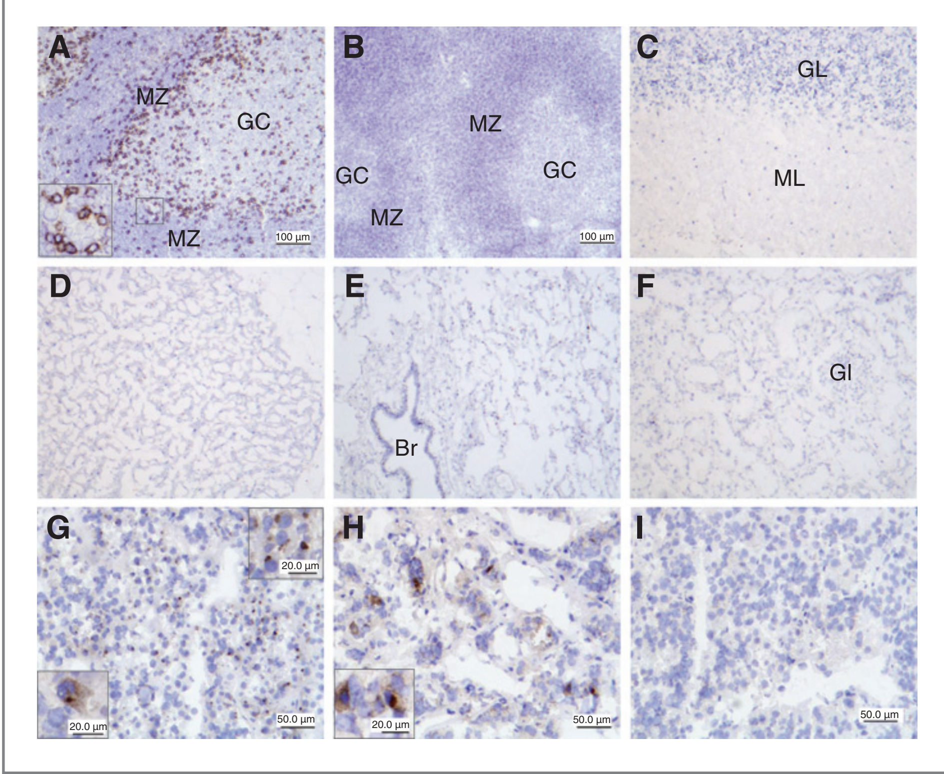

Figure 1. Limited PD-1 expression in normal human tissues. Immunohistochemistry in positive control tissue, hyperplastic tonsil (A), using FITC-conjugated nivolumab. Strong immunoreactivity was distributed in subsets of lymphocytes primarily in germinal center of the tonsil; FITC-conjugated human IgG4 was used as an isotype control in tonsil (B). No specific staining was observed in cerebellum (C), heart (D), lung (E), or kidney (F). Positive staining was revealed in a very small number of scattered endocrine cells (G–I) in four of five pituitary samples. G and H represent two positive samples, and I represents negative pituitary tissue. Insets in G and H are high-power views showing strong immunoreactivity in large cytoplasmic spherical organelles, enigmatic body-like structures, with weak cytoplasmic staining. Br, bronchiole of the lung; GC, germinal center of the tonsil; Gl, glomerulus of the kidney; GL, granular layer of the cerebellum cortex; ML, molecular layer of the cerebellum cortex; MZ, mantle zone of the tonsil.

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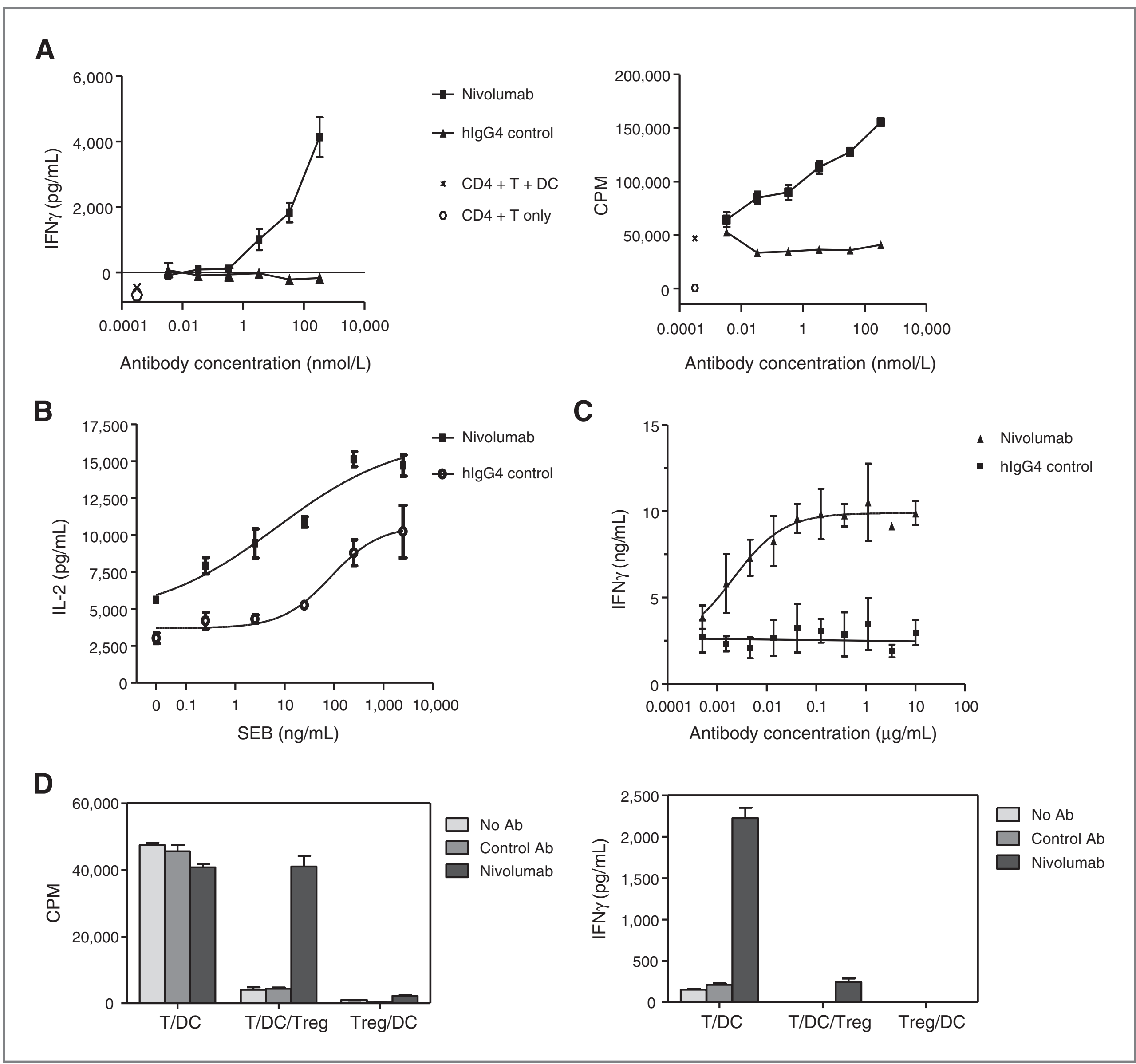

Figure 2. PD-1 blockade enhances T-cell function. A, 10^5 purified $CD4^+$ T cells were cocultured with 10^4 allogeneic monocyte-derived DCs in the presence of a titration of nivolumab or isotype control antibody in triplicates for 6 days. Supernatants were collected at day 5 and measured for IFNγ production by ELISA. The cultured cells were labeled with 1 μCi ^{3}H-thymidine for another 18 hours before being analyzed for proliferation. Representative data from multiple donor DC/T-cell pairs are shown. CPM, counts per minute. B, 10^5 PBMCs were stimulated with serial dilutions of SEB in the presence of a fixed amount of nivolumab or isotype control antibody in solution (20 μg/mL). Supernatants were collected after 3 days for measurement of IL-2 by ELISA. Representative data from multiple healthy donors ($n = 18$) are shown. C, 2×10^5 PBMCs from a CMV-positive donor were stimulated with lysate from CMV-infected cells in the presence of nivolumab or isotype control. Supernatants were collected after 4 days and assayed for IFNγ secretion by ELISA. D, 5×10^4 $CD4^+CD25^+$ Tregs were cocultured with 10^5 $CD4^+CD25^-$ responder T cells and 2×10^4 DCs in the presence of 20 μg/mL of nivolumab or isotype control antibody in an allogeneic MLR for 6 days. IFNγ was analyzed from the supernatants collected at day 5 and proliferation was measured at day 6 after 18 hours of ^{3}H-thymidine labeling.

enhancement of IFNγ release, and in some donor T-cell/DC pairs, enhanced T-cell proliferation was observed (Fig. 2A). Nivolumab also enhanced IL-2 secretion over isotype control in response to SEB using PBMCs (Fig. 2B). Addition of nivolumab increased IL-2 secretion by a mean of 97% to 139% over control (Supplementary Table S1). Using a CMV-restimulation assay, nivolumab, compared with an isotype control, resulted in a concentration-dependent augmentation of IFNγ secretion from CMV-responsive donors (Fig. 2C). Although PD-1 expression can be observed in T cells before stimulation by allogeneic DCs, SEB, or CMV antigen, PD-1 expression is upregulated after T-cell activation in all of these assays (Supplementary Fig. S4A and S4B and data not shown). In addition, PD-L1 expression and upregulation can be observed in multiple cell subsets in these assays (Supplementary Fig. S4B and data not shown).

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As Tregs also express PD-1, nivolumab was assessed in an allogeneic MLR in which Tregs suppressed the proliferation and cytokine secretion of purified $CD4^+CD25^-$ responder T cells, which were stimulated by allogeneic DCs. In this assay, nivolumab completely restored proliferation and partially restored IFNγ release by the alloreactive T cells (Fig. 2D).

Taken together, these data show that nivolumab can, at very low concentrations (~1.5 ng/mL), enhance T-cell reactivity in the presence of a T-cell receptor stimulus. However, nivolumab had no stimulatory effect in the absence of antigen or T-cell receptor stimulus. Specifically, there was no significant release of inflammatory cytokines, including IFNγ, TNFα, IL-2, IL-4, IL-6, or IL-10, from unstimulated whole blood after coincubation with nivolumab. In contrast, positive control anti-CD3 antibody potently increased cytokine release (Supplementary Table S2). These results demonstrate that nivolumab does not cause nonspecific lymphocyte activation.

Finally, the ability of nivolumab (tested from 0.003 to 50 μg/mL) to mediate ADCC activity *in vitro* was tested. Using IL-2-activated PBMCs as a source of natural killer (NK) cells and activated human $CD4^+$ T cells expressing high levels of cell-surface PD-1 as target cells, nivolumab [IgG4 (S228P)] did not mediate ADCC (Fig. 3). Limited ADCC activity was observed at high concentrations with the parental form of nivolumab, an IgG1 antibody purified from hybridoma supernatant, whereas positive control anti-MHC class I antibody was able to mediate ADCC of T cells at low antibody concentrations. In addition, nivolumab did not mediate complement-mediated cytotoxicity (CDC) of activated human $CD4^+$ T cells in the presence of human complement (data not shown).

Pharmacokinetics, immunogenicity, and toxicity of nivolumab in cynomolgus monkeys

Single, i.v. administration of nivolumab to cynomolgus monkeys at 1 and 10 mg/kg was well tolerated with no effects on body weight or clinical observations. Mean concentration–time profiles for serum nivolumab were qualitatively similar for males and females at 1 mg/kg and for males at 10 mg/kg. Mean concentrations declined in a multi-phasic manner from C_{max}, observed within 0.5 hour at both doses. Serum PK parameter estimates are shown in Table 1. Mean apparent terminal elimination half-life estimates for males and females at 1 mg/kg were similar (124 and 139 hours, respectively), and the mean half-life estimate for males at 10 mg/kg was 261 hours. Anti-nivolumab antibodies were detected on day 28, but seemed to have no substantial impact on PK assessment [i.e., mean retention time (MRT), total clearance (CLT), and steady-state volume of distribution (V_{ss})]. In general, serum nivolumab had a relatively slow clearance with limited extra vascular distribution, as demonstrated by a V_{ss} value consistent with plasma volume. Five of the 6 animals in group 1 (1 mg/kg nivolumab), and 2 of 3 in group 2 (10 mg/kg nivolumab) were positive for an anti-nivolumab antibody response (including neutralizing antibodies) at day 28 (data not shown), but with no observable adverse effects.

In a 3-month toxicity study in cynomolgus monkeys, twice weekly i.v. administration of nivolumab at doses of 10 mg/kg and 50 mg/kg was also well tolerated, with no effect on body weight, and no other clinical findings. Serum chemistry changes were limited to a reversible 28% decrease in T3 at week 13 in females treated with 50 mg/kg. T4 and TSH levels were unchanged. In males treated with 50 mg/kg, there were no changes in T3, T4, or TSH levels. Nivolumab exposure increased in an approximately dose-proportional manner between 10 and 50 mg/kg, with no substantial sex differences noted (data not shown). Anti-nivolumab antibodies were detected in only 1 of 24 animals, although high nivolumab concentrations could have interfered with the assay. The highest well-tolerated dose in this study, 50 mg/kg twice weekly, is at least 20 times greater than doses reported to demonstrate antitumor activity in humans (≤10 mg/kg, every other week; ref. 26).

In phenotypic analyses of PBMCs 1 day after the last dose, no significant difference in total T- and B-cell numbers was observed between groups (data not shown). There were significantly more $CD8^+$ effector memory T cells in the 50 mg/kg group than in the 10 mg/kg and untreated groups (Supplementary Fig. S5A), and a nonsignificant trend toward more $CD8^+$ central memory T cells in the nivolumab group, especially in the 50 mg/kg group. Naïve T-cell populations were decreased in the 50 mg/kg group, suggesting that PD-1 blockade may facilitate activation and differentiation of naïve T cells. Finally, there were more $CD11c^+$ DCs in the 50 mg/kg group than in the untreated group ($P = 0.014$; Supplementary Fig. S5B), suggesting a possible role for PD-1 blockade in promoting DC differentiation.

Immune responses in cynomolgus monkeys to cellular and particulate virus-like particle vaccines coadministered with nivolumab or ipilimumab

Enhancement of vaccine responses can demonstrate the activity or potency of immunomodulatory antibodies in non-human primates. Previous studies demonstrated that CTLA-4 blockade potentiated immune responses to an HBsAg vaccine or SK-MEL-3 melanoma cell vaccine (37). A similar experiment was conducted to examine the ability of PD-1 blockade to potentiate vaccine responses. As previously observed, ipilimumab strongly enhanced humoral immune responses to HBsAg as compared with control, whereas nivolumab showed no effect on HBsAg titers over control treatment (Fig. 4A). All groups had normal anamnestic responses with measurable antibody titers following the second vaccine dose, which peaked after the third dose and declined thereafter.

Responses to the SK-MEL-3 vaccine were elevated in both nivolumab- and ipilimumab-treated groups compared with control (Fig. 4B). There was a modest increase in the humoral vaccine response in the nivolumab group, with a greater increase in the ipilimumab group. Titers increased markedly following the second vaccine dose, but did not change substantially after the third dose, followed by a rapid decline in all groups. Antibody titers to HLA-A2404 (an allele of HLA expressed by SK-MEL-3 cells) were also increased in animals treated with ipilimumab (4.3-fold) or nivolumab (2.4-fold) on day 71 (Fig. 4C).

Discussion

Nivolumab is a fully human IgG4 PD-1 antibody that binds to human and cynomolgus PD-1 with high affinity and blocks the

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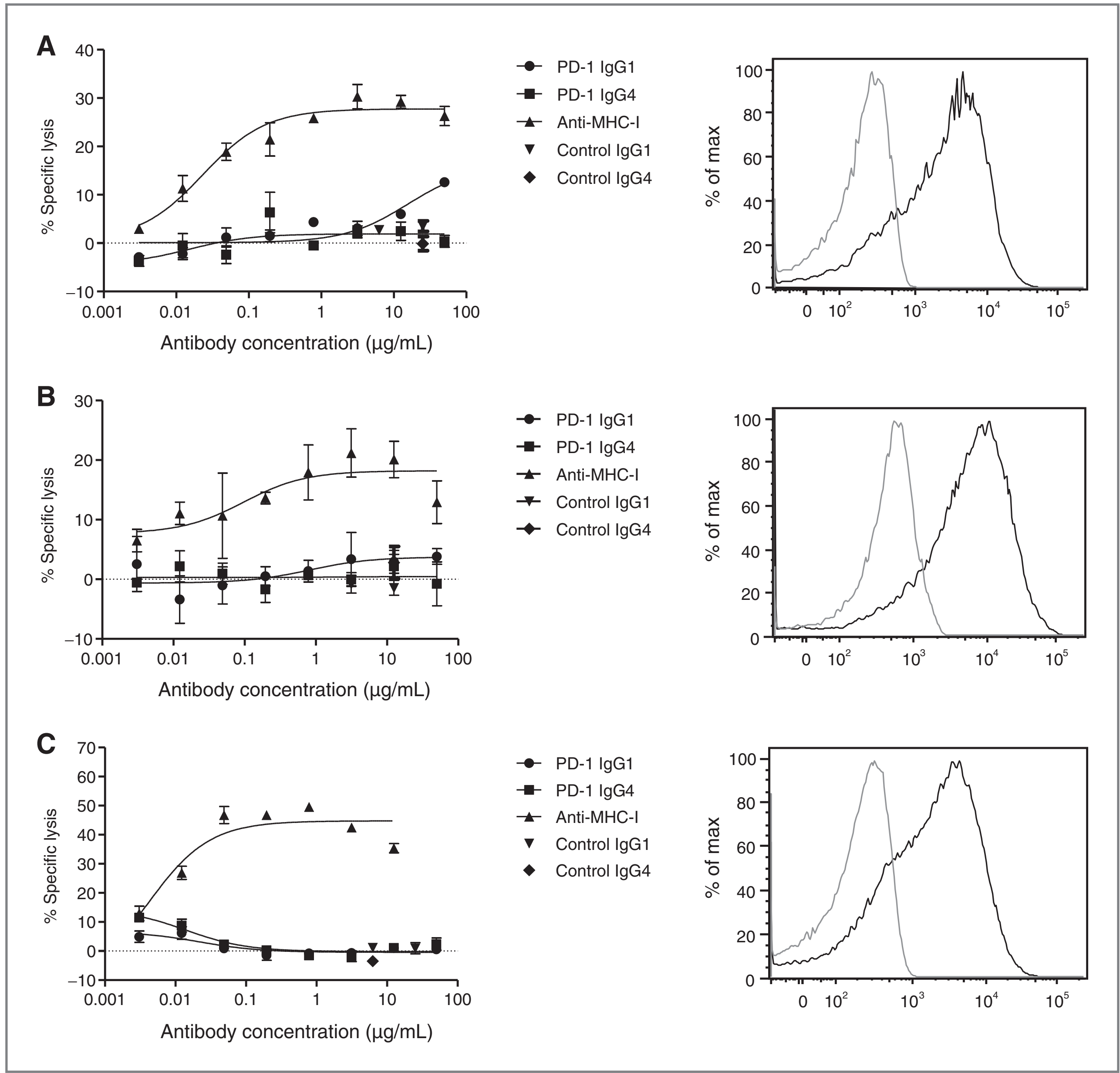

Figure 3. The absence of ADCC by nivolumab *in vitro*. IL-2–activated human PBMCs (effector cells) were incubated with activated human CD4$^+$ T cells (target cells) in an effector-to-target cell ratio of 50:1 in the presence of serial dilutions of nivolumab or a positive control anti-MHC class I antibody for 3 hour at 37°C. A–C, data from three individual ADCC assays using cells from different donors are shown. Purified CD4$^+$ T cells were activated by coated anti-CD3 antibody (4 µg/mL) plus soluble anti-CD28 antibody (1 µg/mL) and IL-2 (100 U/mL) for 3 days. PD-1 expression on activated CD4$^+$ T cells in each of the ADCC assays is shown in the right (solid line for PD-1, gray line for isotype control).

interaction of PD-1 with both PD-L1 and PD-L2 ligands. In functional *in vitro* assays, nivolumab enhanced cytokine production in human T-cell/DC MLR, SEB, and CMV recall response assays. In addition, antigen-specific CD8$^+$ T-cell responses from patients with melanoma increased after incubation with nivolumab and peptide antigen, and not by stimulation with an irrelevant peptide (30). Importantly, while anti-PD-1 antibody enhanced antigen-specific T-cell responses, it did not stimulate nonspecific responses by human blood cells, as determined by cytokine release upon incubation with antibody alone. In a Treg suppression assay, nivolumab completely restored CD4$^+$ T-responder cell proliferation and partially restored IFNγ production. Although it is unclear whether nivolumab acts directly on CD4$^+$ T-responder or Tregs, previous data have demonstrated that nivolumab could overcome Treg suppression of CD8$^+$ T cells by increasing resistance to Treg suppression, and also by directly limiting Treg-suppressive capacity (38).

The heavy chain constant region of nivolumab is a human IgG4 isotype with an S228P mutation, which replaces a serine

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Table 1. Mean and SD for serum PK parameter estimates for nivolumab following single i.v. administration to monkeys

Gender	Dose, mg/kg	C_{max}, μg/mL	T_{max}, h	$AUC_{(0-T)}$, μg × h/mL	$AUC_{(INF)}$, μg × h/mL	$T_{(1/2)}$, h	MRT, h	CLTs, mL/h/kg	V_{ss}, L/kg
Male	1	33.8 ± 2.36	0.25–0.5	4,010 ± 645[a]	4,470 ± 423	124 ± 20.3	200 ± 5.2	0.224 ± 0.021	0.046 ± 0.005
Female	1	28.6 ± 0.681	0.25–0.25	3,570 ± 573[a]	4,050 ± 616	139 ± 12.7	210 ± 27	0.250 ± 0.039	0.052 ± 0.002
Male	10	330 ± 34.2	0.25–0.5	47,100 ± 12,400[b]	64,200 ± 27,400	261 ± 226	400 ± 250	0.172 ± 0.059	0.060 ± 0.016

NOTE: Values are mean ± SD except for T_{max}, which is the range.

Abbreviations: CLT, total clearance; MRT, mean retention time; V_{ss}, steady state volume of distribution.

[a] $T = 384$ hours.

[b] $T = 648$ hours.

residue in the hinge region with the proline residue found at the corresponding position in IgG1 isotype antibodies. This mutation prevents Fab arm exchange with endogenous IgG4 antibodies, while retaining the low affinity for activating Fc receptors associated with wild-type IgG4 antibodies. Engagement of activating Fc receptors by a PD-1-blocking antibody could conceivably deplete antitumor effector T cells. However, no *in vitro* ADCC or CDC activity was observed with nivolumab in assays using PD-1-expressing activated T cells as target cells, suggesting that nivolumab is unlikely to deplete PD-1-positive cells. Lack of nivolumab-mediated ADCC or CDC activity is consistent with the expected lack of effector function of IgG4 Fc region, as observed by others (39, 40). Moreover, an IgG1 isotype of nivolumab resulted in limited ADCC activity at high antibody concentrations, indicating that the epitope recognized by nivolumab may not lead to potent ADCC activity. Although IgG4 isotype antibodies show low affinity for FcγRI *in vitro* as compared with IgG1 (41), it is unclear whether this translates into ADCC or phagocytic activity by FcγRI-expressing monocytes or macrophages *in vivo*. Normal levels of human immunoglobulin in sera have been reported to strongly inhibit IgG1-mediated ADCC (42). Although phase I trial data showed a transient decrease of peripheral blood T cells after nivolumab treatment, this was probably related to T-cell extravasation (25). In other clinical studies, nivolumab monotherapy did not change the median absolute lymphocyte count (ALC), or the number of activated $CD4^+$, $CD8^+$, or Tregs, suggesting that nivolumab does not mediate overt changes in T-cell percentages (24).

Immunohistochemical studies evaluated nivolumab reactivity to lymphoid cells and assessed nontarget tissue binding. Reactivity of nivolumab to lymphocytes in various tissues was observed as expected; however, there was unexpected moderate-to-strong cytoplasmic staining of rare-to-occasional endocrine cells in the adenohypophysis. This was considered to be low-affinity binding, as staining occurred only at higher antibody concentrations, and is unlikely to have physiologic consequences because of limited accessibility to cytoplasmic compartments *in vivo*. Cynomolgus tissue staining showed similar lymphocyte-binding patterns, as well as cytoplasmic staining of endocrine cells in the adenohypophysis (data not shown). Potential adverse effects of this binding were not borne out in cynomolgus toxicity studies or human clinical trials (25, 26).

Vaccination studies in cynomolgus monkeys have been used as surrogates for evaluating activity of T-cell inhibitory or costimulatory molecules in the absence of tumor models (37, 43). Although anti-CTLA-4 antibody promoted significantly increased humoral responses in HBsAg-vaccinated monkeys, nivolumab did not, despite its high affinity for cynomolgus PD-1. However, slightly higher antibody titers to a cellular vaccine directed, at least in part, against the HLA-A2404 antigen expressed on SK-MEL3 cells, were detected in monkeys treated with 10 mg/kg of nivolumab. In mice, anti-PD-1 antibody can promote T-cell responses to a GVAX cellular vaccine (44) and to peptide-loaded DC vaccination (K. Bahjat et al.; unpublished data). Although not a vaccine study, it is noteworthy that simian immunodeficiency virus (SIV)–infected monkeys

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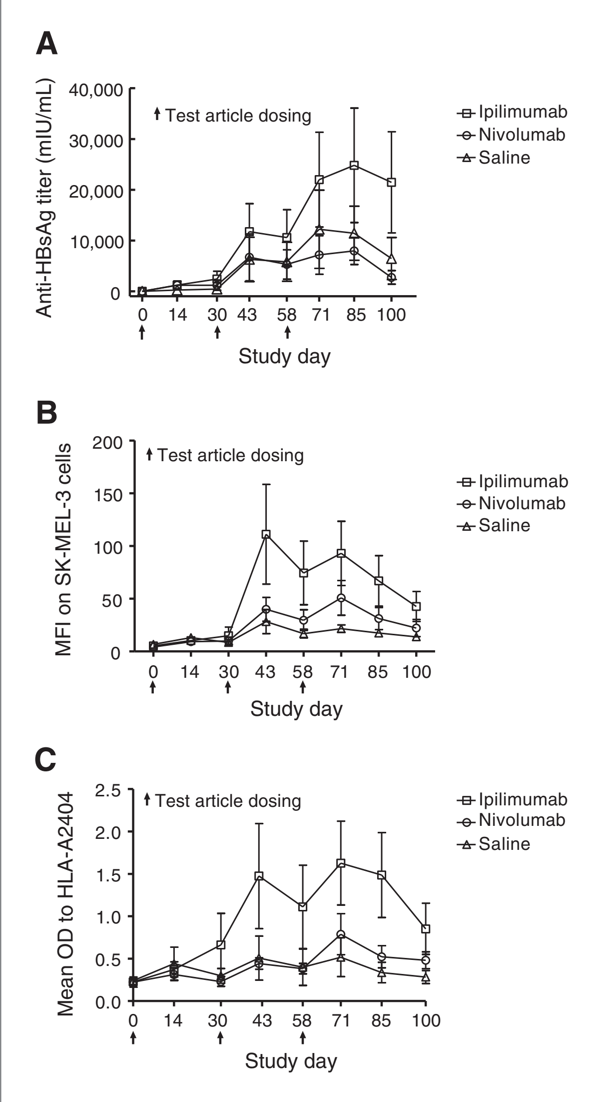

Figure 4. Effect of nivolumab or ipilimumab on immune responses to vaccination in cynomolgus monkeys. A, humoral immune responses to a particulate HBsAg vaccine by ELISA. Plasma samples obtained at the indicated times were analyzed for anti-HBsAg Abs. B, antibody responses to an SK-MEL-3 vaccine as assessed by flow cytometry. Vaccine-specific antibody responses were measured by incubation of SK-MEL-3 cells with plasma collected at 2-week intervals. Data points represent the mean $\pm$ SEM of the mean fluorescence intensity (MFI) values in each treatment group at each collection time point. C, antibody responses to HLA-A2404 were determined from plasma by ELISA. Data points represent the mean $\pm$ SEM of the mean OD values in each treatment group. All samples were analyzed at least two times with similar results.

treated with an anti-PD-1 antibody showed increased humoral responses to SIV antigens (45).

Nivolumab was well tolerated when administered to cynomolgus monkeys as twice-weekly i.v. injections for 3 months at doses up to 50 mg/kg, with no adverse effects on any parameters. Although there was a low incidence of anti-nivolumab antibodies, they were not associated with any adverse effects (i.e., hypersensitivity reactions), and had no substantial impact on PK parameters. Furthermore, anti-drug antibody responses in animals are not considered predictive of responses in humans (46). Thus, the results of the nonclinical studies in monkeys suggested a favorable risk:benefit ratio to support initial clinical trials with nivolumab. Although nivolumab seems to lack toxicity in monkeys, toxicities have been observed in human clinical trials. In a phase I trial, nivolumab had a favorable safety profile (26). Adverse events were generally similar to those observed with ipilimumab, although with lower incidence and of less severity, and comprised gastrointestinal, endocrine, and skin toxicities, and pulmonary inflammation. Interestingly, pneumonitis has been observed in PD-1-deficient mice bred onto the MRL genetic background (8), but not in PD-1-deficient mice with other genetic backgrounds (6, 7). In cynomolgus toxicity studies with anti-CTLA-4 (ipilimumab), no or only rare toxicities were observed, although they were evident in human studies. These observations highlight the difficulty of predicting toxicities in humans with antibodies mediating checkpoint blockade, such as anti-PD-1 or anti-CTLA-4 antibodies, from results in mice and nonhuman primates.

Increased numbers of $CD8^+$ T-effector memory cells were detected in cynomolgus monkey peripheral blood after repeated treatment at the highest dose of nivolumab for 3 months. Although recent data (24) suggest that activated T cells are not potential pharmacodynamic markers of nivolumab treatment, it remains to be determined whether nivolumab treatment increases $CD8^+$ effector memory cells in humans with cancer. A marked accumulation of $CD8^+$ effector memory cells in lymphoid organs and tissues of PD-1-deficient mice has been described previously (47). Increases in $CD11c^+$ DCs were also observed in the cynomolgus monkey safety study, although the underlying mechanism is unclear.

In early clinical trials, nivolumab produced durable responses and stable disease, and an encouraging survival profile, in patients with advanced melanoma, lung, and renal cancers. In some patients, tumor regression persisted after discontinuation of nivolumab (26, 27, 48). Nivolumab was generally well tolerated, even with prolonged dosing (26, 27, 48). Another PD-1-blocking antibody, pembrolizumab, has shown similar activity and safety in metastatic melanoma (49). These studies further validate the concept of modulating immune responses with checkpoint blockade for cancer immunotherapy, as first demonstrated in clinical trials with ipilimumab (1, 2).

Exploration of nivolumab combinations with other immuno-oncology approaches, as well as standard of care therapies, is warranted. PD-1 pathway blockade combined with anti-CTLA-4 or anti-LAG-3 antibody showed synergistic antitumor activity superior to the single agents in murine tumor models (15–17). Preliminary clinical data in patients with melanoma receiving nivolumab plus ipilimumab showed rapid and durable responses: 31% of responders had tumor regression of 80% or more by week 12, a superior profile to monotherapies (50). The combination is currently being clinically evaluated in multiple tumor types.

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Disclosure of Potential Conflicts of Interest

C. Wang, M.J. Selby, X.-T. Wang, and A.J. Korman have ownership interest (including patents) in Bristol-Myers Squibb stock. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

All pivotal toxicology studies were conducted in compliance with the Good Laboratory Practice Regulations for nonclinical Laboratory Studies of the U.S. Food and Drug Administration (21 CFR Part 58) and were approved by the laboratory's Institutional Animal Care and Use Committee.

Authors' Contributions

Conception and design: C. Wang, X.-T. Wang, H. Huang, D. Blanset, M.J. Selby, A.J. Korman

Development of methodology: C. Wang, K.B. Thudium, X.-T. Wang, H. Huang, D. Feingersh, M. Srinivasan, S. Wong, N. Garner, D. Blanset, M.J. Selby

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Wang, K.B. Thudium, X.-T. Wang, H. Huang, D. Feingersh, C. Garcia, Y. Wu, M. Kuhne, M. Srinivasan, S. Singh, S. Wong, N. Garner, M.J. Selby

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Wang, K.B. Thudium, M. Han, X.-T. Wang, H. Huang, D. Feingersh, C. Garcia, Y. Wu, M. Kuhne, M. Srinivasan, S. Singh, S. Wong, N. Garner, T. Bunch, D. Blanset, M.J. Selby, A.J. Korman

Writing, review, and/or revision of the manuscript: C. Wang, K.B. Thudium, M. Han, X.-T. Wang, H. Huang, D. Feingersh, M. Kuhne, M. Srinivasan, S. Wong, N. Garner, H. Leblanc, T. Bunch, D. Blanset, M.J. Selby, A.J. Korman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Wang, K.B. Thudium, H. Huang, M. Kuhne, S. Wong, M.J. Selby

Study supervision: C. Wang, H. Huang, H. Leblanc, D. Blanset, M.J. Selby, A.J. Korman

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Cancer Immunology Research

In Vitro Characterization of the Anti-PD-1 Antibody Nivolumab, BMS-936558, and *In Vivo* Toxicology in Non-Human Primates

Changyu Wang, Kent B. Thudium, Minhua Han, et al.

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