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Preclinical development of the TLR9 agonist DV281 as an inhaled aerosolized immunotherapeutic for lung cancer: Pharmacological profile in mice, non-human primates, and human primary cells

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ABSTRACT

CpG-motif-containing oligodeoxynucleotides (CpG-ODN) activate innate immunity through Toll-Like Receptor (TLR) 9 signaling and generate local immune responses when delivered directly to the lung. Herein we describe pharmacological studies in mice, cynomolgus monkeys, and in human primary cells which support the development of DV281, a C-class CpG-ODN, as an inhaled aerosolized immunotherapeutic for lung cancer to be combined with an inhibitor of the anti-programmed cell death protein 1 (PD-1) immune checkpoint. In vitro, DV281 potently induced Interferon (IFN)-α from monkey and human peripheral blood mononuclear cells (PBMCs), stimulated interleukin-6 production and proliferation in human B cells, and induced TLR9-dependent cytokine responses from mouse splenocytes. Intranasal delivery of DV281 to mice led to substantial but transient cytokine and chemokine responses in the lung. Lung responses to repeated intranasal DV281 were partially to fully reversible 2 weeks after the final dose and were absent in TLR9-deficient mice. Single escalating doses of aerosolized DV281 in monkeys induced dose-dependent induction of IFN-regulated genes in bronchoalveolar lavage cells and blood. In a repeat-dose safety study in monkeys, inhaled DV281 was well-tolerated, and findings were mechanism of action-related and non-adverse. Co-culture of human PBMC with DV281 and anti-PD-1 antibody did not augment cytokine or cellular proliferation responses compared to DV281 alone, indicating that the combination did not lead to dysregulated cytokine responses. These studies support clinical development of inhaled aerosolized DV281 as a combination therapy with anti-PD-1 antibody for lung cancer immunotherapy.

1. Introduction

CpG-ODNs are agonists of the innate pattern recognition receptor TLR9, which is expressed in endosomes of plasmacytoid dendritic cells (pDCs) and B cells [1]. CpG-ODN signaling induces IFN-α from pDCs and, indirectly, IFN-γ from Natural Killer (NK) cells, promoting T helper (Th)1 responses [2]. In addition, CpG-ODNs directly promote B cell proliferation and Interleukin (IL)-6 production and up-regulation of co-stimulatory molecules on antigen presenting cells (APCs) [3,4]. These potent effects on the innate immune response serve to prime the development of adaptive T and B cell responses [5]. Accordingly, TLR9-agonists have been extensively investigated as adjuvants and immune response modifiers in nonclinical studies and their effects have successfully translated to the clinic as vaccine adjuvants [6,7] and modulators of pathological Th2 responses in allergic asthma [8,9].

There is renewed interest in combining the immunostimulatory effects of CpG-ODNs with approved immune checkpoint blockade therapies for cancer immunotherapy [10]. Specific antibodies blocking the PD-1/PD-ligand (PD-L)1 pathway have recently been approved for therapy of several solid tumors including melanoma, head and neck, and non-small cell lung cancer (NSCLC) [11]. While anti-PD-1 antibody therapy is a significant advance in treatment of cancers such as NSCLC,

Abbreviations: BAL, broncoalveolar lavage; BALF, BAL fluid; BrdU, 5-bromo-2′-deoxyuridine; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CD, Cluster of differentiation; CpG, cytidine-phospho-guanosine; Eu, Europium; GBP1, guanylate binding protein 1; IACUC, Institutional Animal Care and Use Committee; IFN, interferon; IFNA2, interferon alpha 2; IL, interleukin; IN, intranasal; IQR, interquartile range; IRF7, interferon regulatory factor 7; ISG54, interferon induced 54 kDa protein; LAL, Limulus Amebocyte Lysate; MXB, MX dynamin like GTPase 2; ODN, oligodeoxynucleotide; P28, interleukin 27 p28 subunit; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cell; RLU, relative light units; SD, standard deviation; SEB, staphylococcal enterotoxin B; SEM, standard error of the mean; TLR, toll-like receptor; TNF, tumor necrosis factor

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only about 20% of unselected NSCLC patients respond to the monotherapy [12], demonstrating the need for combination immunotherapy approaches. The combination of TLR9 agonists with PD-1/PD-L1 blockade represents an approach that has shown enhanced anti-tumor efficacy and control of metastasis in pre-clinical cancer models [13–15] and in early stage clinical studies [16].

Localized delivery of a CpG-ODN to the tumor and/or the tumor-draining lymph node appears much more effective than systemic treatment for controlling growth of the injected tumor and for generating anti-tumor immunity active at non-injected tumors [17]. In mice, direct intratumoral injection of SD-101 (a CpG-C class TLR9 agonist with strong IFN-α induction) demonstrated significant synergy with systemic anti-mouse PD-1 antibody for complete and durable elimination of subcutaneous CT26 tumors in virtually all treated animals, and generation of anti-tumor immunity that cleared the majority of non-injected distant-site tumors [15]. Mechanistically, SD-101 plus anti-PD-1 antibody greatly increased the number of multi-functional tumor antigen-specific T cells expressing both Tumor Necrosis Factor (TNF) and IFN-γ, and the proportion of memory precursor effector cells, compared to either treatment alone, suggesting a more functional and longer lasting memory T cell response to the combination treatment [15].

In lung cancers, direct intratumoral injection of TLR9 agonists is much less feasible. However, delivery by inhalation of an aerosolized CpG-ODN can achieve effective concentrations of CpG-ODN in the proximity of tumors and tumor-draining lymph nodes with minimal systemic exposure. Mouse tumor model studies have shown that inhalation delivery of CpG-ODNs in combination with Poly(I:C) or alone in microparticle form can have anti-tumor activity in murine lungs [18,19], and we have recently extended these findings to show that treatment with an inhaled aerosolized TLR9-agonist in combination with anti-PD-1 antibody leads to lasting rejection of both lung tumors and tumors outside the lungs, associated with induction of functional effector $CD8^+$ T cells in tumor-bearing lungs [20]. Previous experience with another CpG-C class TLR9 agonist, AZD1419, a therapeutic for allergic asthma, demonstrated the feasibility for translating the CpG-ODN inhalation therapy approach to the clinic. Inhaled delivery of AZD1419 was safe and well-tolerated in healthy human volunteers and demonstrated target engagement in the lungs of these individuals [8].

In the present study, we evaluated in vitro mouse, cynomolgus monkey, and human primary cell responses to a novel CpG-ODN (DV281) and conducted pharmacological and safety studies in mice and non-human primates in support of the development of DV281 as an inhaled immunotherapeutic for use in combination with systemically delivered anti-PD-1 antibody in lung cancer.

2. Materials and methods

2.1. TLR9 agonist

The CpG-motif-containing oligodeoxynucleotide DV281 was synthesized and purified by Trilink Biotechnologies (San Diego, CA) or Biosearch Technologies (Petaluma, CA) using standard techniques as previously described [21]. All lots were tested for endotoxin content and had ≤0.2 EU/mg.

2.2. Primary cell isolation

For primary cell isolation, peripheral blood was obtained from healthy human volunteers (adult males and females) who gave informed consent at Stanford Blood Center (Palo Alto, CA) in accordance with the Center's Institutional Review Board. Human PBMCs were isolated from buffy coats by Ficoll-Hypaque gradient centrifugation. Cell subsets were enriched from PBMCs by positively selecting $CD19^+$ B cells or $CD304^+$ pDCs with microbeads as per manufacturer's instructions Stemcell (Cambridge, MA) or Miltenyi Biotec (Auburn, CA). Purity of B cells, as measured by flow cytometry using anti-CD19 (H1B19) and anti-CD20 (2H7) antibodies from BD Biosciences (San Jose, CA), was typically > 85%. In the PBMC experiment with anti-PD-1 antibody, pDCs isolated from 500×10^6 total PBMCs were added back to an untouched aliquot of 50×10^6 PBMC of each donor, resulting in a 10-fold enrichment of pDCs. Peripheral blood of cynomolgus monkeys (*Macaca fascicularis*) was obtained from Valley Biosystems (West Sacramento, CA) under an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Cynomolgus monkey (males) PBMCs were isolated from 35 mL heparinized whole blood by percoll density gradient centrifugation. Murine tissues were obtained from Murigenics (Vallejo, CA) under an IACUC-approved protocol. For each experiment, single cell suspensions were generated from a pool of 4 to 8 spleens from female BALB/c, C57BL/6, or $TLR9^{-/-}$ mice by mechanically disrupting the spleens and passing the cells through a 100 μm filter. Red blood cells were lysed with lysis buffer (Sigma-Adrich, St. Louis, MO) and cells resuspended in complete mouse RPMI medium. All media and buffers are described in supplementary methods.

2.3. Primary cell stimulations

DV281 was serially diluted directly on a 96 well flat bottom plate in 2× or 4× concentrated solutions in complete RPMI in order to evaluate primary cell responses. In some experiments, a 4× solution of purified anti-PD-1 antibody (EH12.2H7; EC80 = 1.5 μg/mL) or mIgG1 isotype antibody (MOPC-21) (Biolegend, San Diego, CA) was added. Cells were added to give final concentrations of 2.5×10^6 cells/mL of human PBMCs (for cytokine assays) or 1.25×10^6 cells/mL of human PBMC (for proliferation assays); 0.75×10^6 cells/mL of enriched human B cells (for cytokine and proliferation assays); 2×10^6 cells/mL of cynomolgus PBMCs; or 3.5×10^6 cells/mL of mouse splenocytes. The cells were incubated with DV281 at 37 °C and 5% CO_2 for 24 h for the human PBMC cytokine assay, 96 h for enriched human B cell assays, and 24 h for cynomolgus PBMCs, or 48 h for mouse splenocytes (200 μL final volume for all assays). Following incubations, 180 μL of supernatant was harvested per well for cytokine assays, duplicate or triplicate wells were combined, and the supernatants were frozen at −80 °C. The stimulated human B cells were incubated a further 18 h to quantify proliferative responses. For evaluation of maturation marker expression, isolated human pDCs were incubated in vitro at 2.5×10^5 cells/mL in complete RPMI and stimulated with DV281 at 0.5 μM, or with medium alone for 24 h (37 °C and 5% CO_2).

2.4. Primary cell proliferation assays

Proliferative responses of DV281-stimulated B cells were measured using the DELFIA assay (PerkinElmer, Waltham, MA), a fluoroimmunoassay that measures the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into newly synthesized DNA strands. The assay was performed according to the manufacturer's instructions with BrdU added after 96 h culture with DV281. Cells were then incubated for an additional 18 h incubation followed by cell fixation and detection with an anti-BrdU europium (EU) labeled monoclonal antibody. Eu-fluorescence was measured using a SpectraMax microplate reader with Softmax Pro Software version 6.0 with the following settings: TRF-EUSA LM1: 370, 616; Integration time: 1.89 milliseconds (ms); Excitation time: 0.1 ms; Number of Pulses: 45; Measurement Delay: 0.01 ms; Read from top; Read Height: 2.91 mm (Molecular Devices, San Jose, CA). All samples were tested in triplicate with mean values reported. EC_{50} values were calculated from individual donors and are presented as geometric mean ± SEM.

In the experiment with anti-PD-1 antibody, proliferative responses of DV281-stimulated PBMCs were measured by BrdU incorporation using the BrdU flow kit (BD Biosciences). The assay was performed according to the manufacturer's instructions with BrdU added after 48 h

culture with DV281, and cells incubated for an additional 18 h. Following BrdU incubation, cells were washed and stained with fluorochrome-labeled antibodies from BD Biosciences to surface markers CD3 (SK7), CD4 (SK3), CD8 (SK1), and CD19 (SJ25C1). Cells were then fixed, permeabilized and treated with DNase and stained with fluorochrome-labeled antibody to BrdU. Data was acquired on a LSR2 flow cytometer (BD Biosciences). Analysis was performed with FlowJo software (FlowJo LLC, Ashland, OR). Proliferation of $CD19^+$ B cells, $CD3^+CD4^+$ T cells, and $CD3^+CD8^+$ T cells in response to different stimulations was quantified by measuring the percentage of these cell types staining positive for BrdU.

2.5. Maturation assay

For DV281 induced maturation marker expression, cells were stained with fluorochrome labeled antibodies from BD Biosciences (San Jose, CA) for surface (CD304/BDCA4 [U21-1283], CD123 [7G3]), and maturation markers (CD80 [L307.4], CD86 [2331], and HLA-DR [U21-1283]) in flow cytometry buffer and fixed before acquiring on the LSR2 flow cytometer (BD Biosciences). Maturation marker expression was quantified as Mean Fluorescence Intensity (MFI) using FlowJo analysis software (FlowJo, LLC).

2.6. Effect of intranasal DV281 administration in mice

All mouse and non-human primate studies were compliant with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and followed respective IACUC approved protocols.

Female BALB/c mice, age 8 to 12 weeks, were purchased from Envigo (Livermore, CA). Female C57BL/6, age 12 weeks, and male and female TLR9-deficient ($TLR9^{-/-}$) mice [1], age 8 to18 weeks, were maintained at Simonsen Laboratories (Gilroy, CA). All in-life procedures were performed at Murigenics (Vallejo, CA) after mice were acclimatized for at least one week. Mice were anesthetized with isoflurane and administered DV281 doses via the intranasal route, in a volume of 50 μL saline which maximizes exposure to the lung [22]. Mice were weighed prior to dosing, 1 day after dosing, twice weekly through longer studies, and/or just prior to euthanization. DV281 doses in mg/kg were based on an average 20 g mouse weight. Cage-side clinical observations were performed approximately 2 h post-dosing and daily thereafter. At experiment end-points, blood was collected from anesthetized mice and processed to serum. After euthanization with CO_2, bronchoalveolar fluid (BALF) and BAL cells were harvested and processed as previously described [23]. Following lavage, lungs were inflated with 500 μL of 10% neutral buffered formalin and the trachea was tied off to prevent leakage. The thorax was then opened, the lungs excised and immersed in formalin. In the $TLR9^{-/-}$ mouse study, bronchial lymph nodes were also harvested and immersed in 10% neutral buffered formalin. Bronchial lymph nodes and lung tissue was subsequently processed for paraffin embedding, tissue sectioning (4 μm thickness), slide preparation and hematoxylin and eosin (H&E) staining by IDEXX BioResearch (Westbrook, ME). Lung tissue sections were scored for inflammation as previous described [24].

2.7. Single ascending dose study with inhaled aerosolized DV281 in cynomolgus monkeys

In-life procedures were performed at Lovelace Respiratory Research Institute (Albuquerque, NM). Eight healthy non-naïve female cynomolgus monkeys (*Ascaris suum*-sensitized, but were rested, i.e. not antigen-challenged, and not administered steroids or other immunomodulatory drugs for a minimum of 3 months prior to the study), ranging from 3 to 5 kg in weight, at study initiation, were pre-screened and selected based on stable baseline expression of a panel of IFN and IFN-inducible and inflammatory genes. Animals were monitored for body weight and clinical conditions throughout the study.

All animals were exposed to single escalating doses of DV281 (3 dose levels) via face mask inhalation with at least 3 weeks wash-out period between dose levels. DV281 (aqueous formulation) was aerosolized using a Pari LC Sprint compressed air jet nebulizer. Average estimated achieved inhaled lung deposited doses were 9.6, 41.3, and 91.6 μg/kg for the sequential dosing sessions, respectively. The Association of Inhalation Toxicologists standard equation for calculation of inhaled dose was used [25], incorporating the respiratory minute volume (RMV) value for monkeys sedated with ketamine [26] and application of a 20% deposition factor. BAL and whole blood draws were performed on sedated animals 24 h pre- and post-dose, respectively. BAL cells in RNeasy lysis buffer buffer/2-mercaptoethanol (Qiagen, Germantown, MD), BALF, and whole blood samples in PAXgene tubes (Qiagen) were collected and frozen for storage prior to processing.

2.8. Repeat dose safety study with inhaled aerosolized DV281 in cynomolgus monkeys

In-life procedures were conducted at Envigo CRS Limited (Huntingdon, UK). Forty naïve cynomolgus monkeys (2 to 3 kg at study initiation) were assigned to 4 groups consisting of 5 monkeys/sex/group. Animals were exposed to 5 once-weekly doses of DV281 or Vehicle (phosphate-buffered saline; control group) via face mask inhalation. DV281 (aqueous formulation) was aerosolized using a Pari LC Sprint compressed air jet nebulizer. Main study animals (3/sex/group) were sacrificed 6 days after the last administration and animals in recovery groups (2/sex/group) were sacrificed 6 weeks after the last administration. Once-weekly administration of DV281 resulted in average estimated achieved inhaled doses of 0.427, 1.39, and 3.56 mg/kg DV281. The Association of Inhalation Toxicologists standard equation for calculation of inhaled dose was used [25], incorporating the RMV value for conscious monkeys [27] and assuming 100% deposition. During the study, clinical condition, body weight, food consumption, body temperature, ophthalmic examination, electrocardiography, and respiratory inductive plethysmography, hematology, coagulation, serum chemistry, urinalysis, organ weight, macropathology and histopathology investigations were conducted. Additionally, biomarker analyses (acute phase protein measurements [C-reactive protein, and haptoglobin concentrations] and cytokines) were conducted at Envigo (data not presented). Toxicokinetic bioanalysis for content of DV281 in plasma and lung tissue were conducted at Pyxant Labs (Colorado Springs, CO; data not presented).

2.9. Cytokine quantification

BALF samples were evaporated (rotary evaporator model 4104, Thermo-Scientific, Waltham, MA) and resuspended into 300 μL LAL reagent water (endotoxin levels < 0.005 EU/mL as shown by the Limulus Amebocyte Lysate [LAL] test; Lonza, Walkersville, MD). Commercial ELISA reagents were used to measure cytokines from human PBMCs, human B cells, cynomolgus PBMCs, or mouse splenocytes, including human and cynomolgus IFN-α (Mabtech, human kit is cross-reactive for primate IFN-α [24]), human IL-6 (BD Biosciences), and mouse IL-12p40/p70 (BD Biosciences). The manufacturers' protocols were followed with the following exceptions: Nunc-immunoplate U96 ELISA plates and in-house buffers were used for the coating, blocking, dilution and wash steps; and samples were incubated overnight (see Supplementary methods for buffer descriptions). A commercial ELISA kit was used to measure mouse IFN-α per the manufacturer's instructions (eBioscience/Thermo Fisher, Waltham, MA). ELISA plates were read using a Versa Max microplate reader (Molecular Devices), measuring absorbance at 450 nm with a reference wavelength of 650 nm. The data was analyzed using SoftMax Pro Software version 5.0.1 (Molecular Devices) as previously described [23]. Samples were

tested in duplicate at 1:2 and 1:20 dilutions and the concentrations were reported as the mean ± SEM. EC_{50} values were calculated from individual donors or per experiment for mouse splenocytes and are presented as geometric mean ± SEM.

The remaining reported cytokines and chemokines were quantified using multiplex panels for mouse (CCL2, CCL3, CXCL10, IFN-γ, IL-6, IL-10, IL-12p70, and TNF; MCYTOMAG-70K) or human (CXCL10, IFN-α, IFN-γ, IL-2, IL-6, IL-10, and IL-12p70; HCYTOMAG-60K) analytes according to the manufacturer's instructions and standard ranges (MilliporeSigma, Burlington, MA). Plates with samples at 1:2, 1:20, and 1:40 dilutions were read on a MAGPIX reader (MilliporeSigma) and analyzed with eXPONENT (Luminex, Austin, TX) and Milliplex Analyst (MilliporeSigma) software. Data were graphed with Prism 7 (GraphPad, La Jolla, CA).

2.10. Gene expression analysis

RNA was isolated from BAL cell samples in RNeasy lysis buffer/2-mercaptoethanol (Qiagen) buffer and whole blood samples in PAX-gene tubes. Real-time PCR of RNA was performed as previously described [23]. Data were normalized as gene/ubiquitin ratio. See the supplemental materials and methods for the sequences of SYBR primers for indicated genes.

2.11. Statistical analysis

In different experiments, data are presented as individual values, mean ± SEM, or as median values with interquartile range, and minimum and maximum values. All statistical analyses were performed using Prism 7 (GraphPad). Comparisons of 3 or more test groups were performed using Kruskal-Wallis test with Dunn's post hoc test. P values < 0.05 were considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.)

3. Results

3.1. Pharmacological responses to DV281 in human primary cells in vitro

To determine the potency of DV281 for human cells, signaling in human pDCs or B cells was evaluated in vitro by induction of IFN-α from pDCs or induction of B cell IL-6 or B cell proliferation [28,29]. DV281 induced concentration-dependent production of IFN-α protein from pDC-enriched human PBMCs with an EC_{50} value of 34 nM (Fig. 1A). The IFN-α response demonstrated a bell-shaped curve, which is characteristic of dose-response curves observed in PBMC cultures with other CpG-ODNs, likely a reflection of negative feedback response at higher CpG-ODN concentrations [21]. Responses of B cells, the other major cell population expressing TLR9 in humans, were evaluated by induction of IL-6 production and cellular proliferation. DV281 induced concentration-dependent IL-6 production from (Fig. 1B) and cellular proliferation of (Fig. 1C) human B cells with comparable EC_{50} values: 80 and 61 nM, respectively. The ability of DV281 to induce maturation marker expression in human pDCs, indicative of increased antigen-presenting cell function, was confirmed by measuring CD80, CD86 and HLA-DR surface expression after stimulation. Compared to pDCs incubated in medium only, DV281 significantly increased expression of the maturation markers CD80 (Fig. 1D), and CD86 (Fig. 1E), as well as HLA-DR (Fig. 1F). Taken together, these data indicate that DV281 is a potent CpG-ODN stimulating key functional responses in human TLR9-expressing primary cells.

3.2. Pharmacological responses to DV281 in non-human primate and mouse primary cells in vitro

DV281 was designed to include distinct CpG motifs optimal for TLR9 engagement in rodents and in humans and non-human primates [29,30] in order to facilitate nonclinical pharmacological and toxicity evaluations in mice. Macaques have a similar TLR9 distribution pattern to humans [31], and produce similar biological responses in vitro and in vivo to CpG-ODN, and were therefore considered the primary species for evaluating toxicity and modeling human doses for DV281. However, inbred and genetically-modified mice have proved important for addressing mechanistic questions. Thus, before undertaking in vivo studies, the potency of DV281 for induction of responses from mouse and monkey primary cells in vitro was evaluated.

In monkey PBMC cultures, DV281 induced concentration-dependent production of IFN-α protein with an EC_{50} value of 108 nM (Fig. 2A), which was comparable to the potency in human PBMCs. For mice, primary cell responses were measured using DV281-stimulated splenocytes in vitro. B cell and dendritic cell cytokine responses to CpG-ODN can be reproducibly quantified by measuring IL-6 and IL-12 protein in mouse splenocyte cultures [32]. In BALB/c splenocytes, DV281 induced dose-dependent IL-6 (Fig. 2B) and IL-12 (Fig. 2C) with potency again in the nanomolar range (EC_{50} values of 76 and 3 nM, respectively). Cytokine induction in mouse splenocytes was strictly TLR9-dependent as shown by a complete absence of IL-6 and IL-12 responses in splenocytes from $TLR9^{-/-}$ mice on a C57BL/6 background (Fig. 2B and C). Splenocytes from $TLR9^{-/-}$ mice were responsive to the TLR7-selective agonist R848 for both IL-6 and IL-12 induction (data not shown), confirming that the deficiency in signaling was specific for TLR9. Taken together, these data demonstrate that DV281 has comparable potency for induction of TLR9 signaling in human, non-human primate and mouse primary cells. Thus, mice and non-human primates are suitable species in which to assess the in vivo pharmacology of the TLR9-agonist DV281.

3.3. Dose-dependent induction of bronchoalveolar lavage fluid cytokines in response to DV281 in mice

To evaluate whether DV281 demonstrates target receptor engagement in mouse lungs, we quantified induction of cytokines in the bronchoalveolar lavage fluid (BALF) of mice administered aqueous DV281 by the intranasal route. In addition to IL-6 and IL-12, we also measured other cytokines and chemokines known to be induced by CpG-ODN in mice, including IFN-α, IFN-γ, TNF, CC chemokine ligand 2 (CCL2), CCL3, and CXC chemokine ligand 10 (CXCL10). Mice were sacrificed and lungs were lavaged 24 h after intranasal administration of DV281 at a range of doses (0.002 to 1.0 mg/kg). All cytokines and chemokines were significantly increased in response to a single administration of DV281, at 1 or more dose levels, as compared to the vehicle control (Fig. 3). Peak cytokine responses varied between analytes but were observed within the range of 0.05 to 0.5 mg/kg DV281. Appreciable responses were generally not observed in the BALF of mice administered DV281 at doses lower than 0.05 mg/kg. Taken together, these data confirmed TLR9 engagement by DV281 in the lungs and identified an optimal dose range for induction of biologically relevant pharmacological responses in mouse lungs.

3.4. Kinetics of DV281-induced cytokine and chemokine responses in mouse lungs

The duration of DV281-induced BALF responses was evaluated in mice using the 0.05 mg/kg dose, which induced quantifiable levels of all cytokines and chemokines at 24 h post-intranasal administration of DV281 (Fig. 3). Response kinetics were measured over 4 h to 14 days post-intranasal DV281 administration in BALB/c mice (Fig. 4). As compared to the vehicle control, DV281 induced significant levels of BALF TNF at 4 h post-administration. All remaining BALF cytokines and chemokines were significantly induced and reached peak levels at Day 1 post-administration, with the exception of IL-12p40 which peaked at Day 4 following DV281 administration. Responses declined to background levels by Day 7, again with the exception of IL-12p40 which

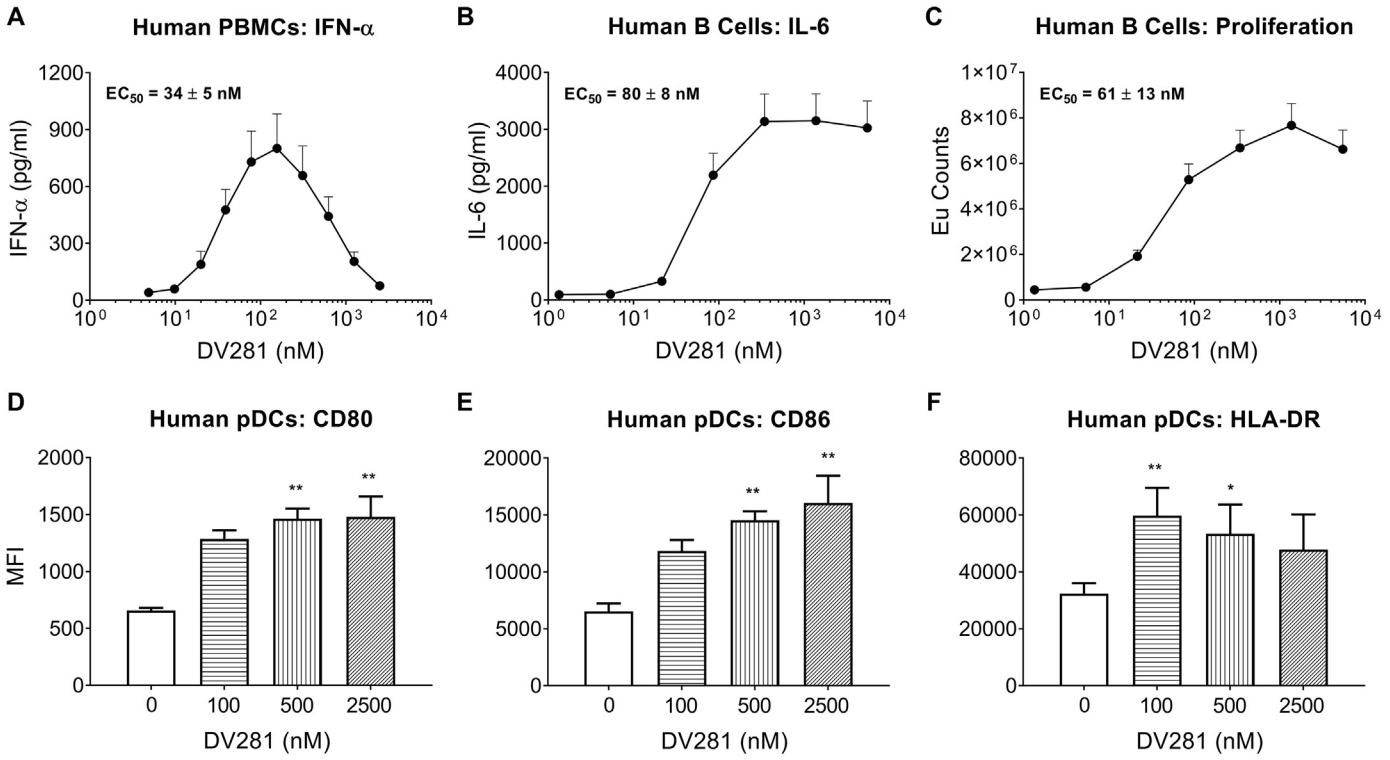

Fig. 1. Human PBMC, B cell, and pDC responses to DV281. (A) IFN-α from human PBMCs stimulated with 4.9 to 2500 nM DV281 for 24 h (arithmetic mean values ± SEM, $n = 31$). (B) IL-6 levels from and (C) proliferation of enriched human B cells stimulated with 1.3 to 5500 nM DV281 for 24 and 96 h, respectively (arithmetic mean values ± SEM, IL-6: $n = 31$; Proliferation: $n = 21$). EC_{50} values as geometric mean ± SEM indicated for (A), (B), and (C). DV281-mediated induction of maturation markers CD80 (D), CD86 (E), and HLA-DR (F) at 24 h on human pDCs is quantified as mean fluorescence intensity (MFI) ($n = 5$ donors/representative results from 1 of 3 independent experiments). Significance was assessed with Kruskal-Wallis test, followed by Dunn's multiple comparisons test for specific comparisons between the non-stimulated group and each DV281 concentration (*$P < 0.05$, **$P < 0.01$). [*double column image*].

declined to background levels at Day 14. These data indicate that cytokine and chemokine responses to intranasally-administered DV281 resolved by 7 to 14 days.

3.5. Pharmacological responses to multiple weekly DV281 administrations in wild type and TLR9$^{-/-}$ mice

As part of a combination treatment regimen for lung malignancies, it is envisioned that inhaled aerosolized DV281 will be administered as a multi-dose regimen. To evaluate induction and resolution of DV281-induced responses after repeated treatments, naïve BALB/c mice were administered intranasal DV281 at doses of 0.05, 0.25 and 1 mg/kg 4 times at 2 week intervals. This repeat dose DV281 regimen was well tolerated with no adverse clinical signs observed over the course of the study. BALF cytokines and histopathological changes in lung tissue were evaluated 24 h and 2 weeks following the fourth administration of DV281. BALF cytokines were elevated at 24 h at all DV281 dose levels (Fig. 5A), but were not detectable 2 weeks after the dosing regimen completed (data not shown), indicating resolution of the cytokine response. To provide a semi-quantitative measure of leukocyte infiltration around airways and blood vessels as well as other possible reactive changes, such as epithelial and goblet cell hyperplasia or vasculitis, lung tissue sections were scored on a graded scale of 1 to 5 [24]. Supplemental Fig. 1 shows images of H&E stained tissue sections representing each grade of the scoring scale used to evaluate DV281-induced lung inflammation. Histological changes in the lung tissue were dose-dependent at 24 h following the fourth administration of DV281, but differences relative to saline control were reduced for all DV281 dose levels tested by 2 weeks post-fourth DV281 administration (Fig. 5B). Together, these data suggest that inflammatory responses to a series of DV281 administrations are partially to fully resolved by 2 weeks following completion of the multi-dose regimen.

To evaluate the potential for off-target effects in response to a multi-dose DV281 regimen, TLR9$^{-/-}$ mice and their C57BL/6 wild-type counterparts were administered DV281 intranasally once weekly for 5 weeks. In this experiment, we tested a more aggressive once-weekly regimen of 1.5 mg/kg DV281 doses and, in TLR9$^{-/-}$ mice, a 4.5 mg/kg dose regimen that would cause severe toxicity if given to wild-type mice. While significant BALF cytokine and chemokine responses were evident in wild-type mice 24 h after the fifth administration of 1.5 mg/kg DV281, repeated DV281 administration at either 1.5 or 4.5 mg/kg did not induce appreciable cytokine or chemokine levels in the BALF of TLR9$^{-/-}$ mice (Fig. 6A). As administration of a high dose of a CpG-ODN by the intransasal route in mice can result in transient weight loss [24], we monitored weight changes in both C57BL/6 and TLR9$^{-/-}$ mice. DV281 administration to wild-type mice resulted in approximately 10% body weight loss from 1 to 3 days post-first dose and transient weight loss following each successive administration of DV281, while saline-administered control mice steadily gained weight (Fig. 6B). In contrast, intranasal administration of DV281 to TLR9$^{-/-}$ mice at either 1.5 or 4.5 mg/kg dose levels did not induce any weight loss or impairment of weight gain as compared to saline-administered TLR9$^{-/-}$ mice. Consistent with the lack of BALF cytokine induction and body weight loss in response to intranasal DV281 administration in TLR9$^{-/-}$ mice, there were no lung histopathological changes (data not shown) or adverse clinical signs in the TLR9-deficient mice. Taken together, these data suggest that DV281 does not induce off-target toxicity in mice at any dose tested.

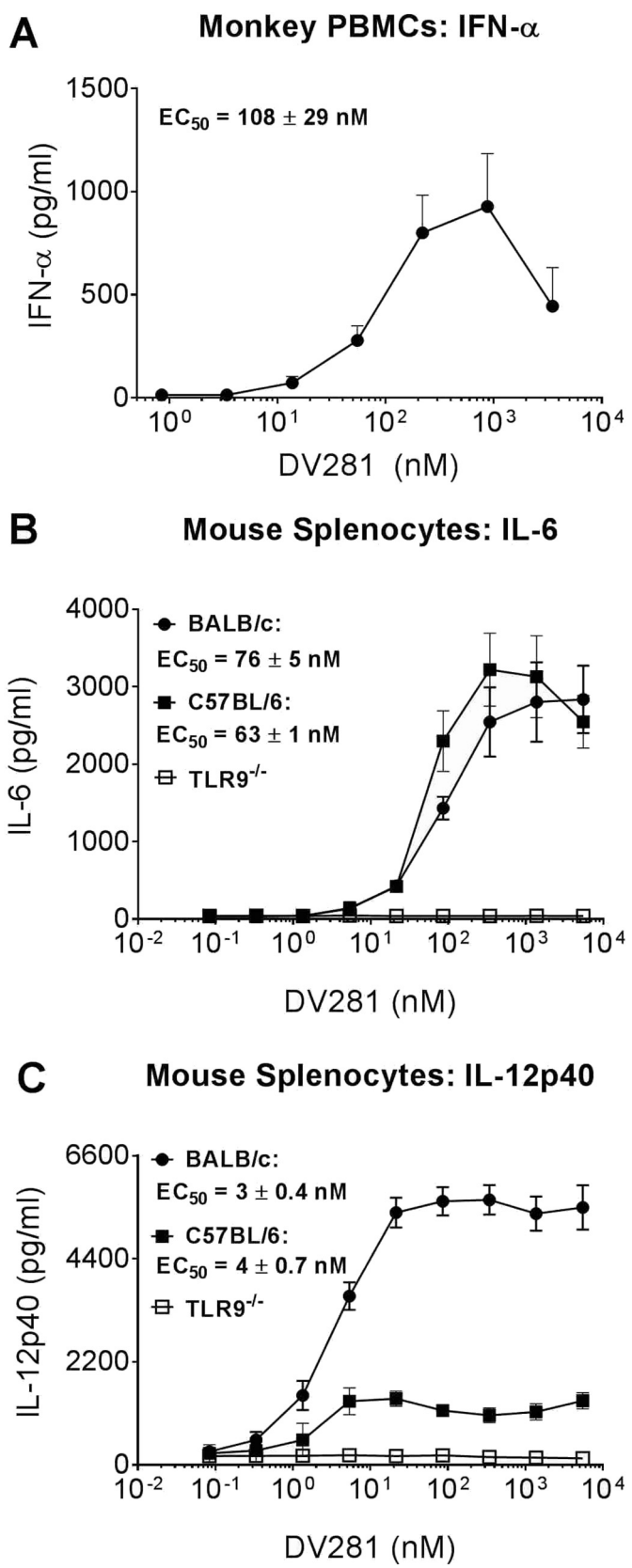

Fig. 2. Monkey PBMC and murine splenocyte responses to DV281. (A) IFN-α from cynomolgus monkey PBMCs stimulated with 0.85 to 3500 nM DV281 for 24 h (arithmetic mean values ± SEM, $n = 12$). (B) IL-6 and (C) IL-12p40, from BALB/c, C57BL/6, and TLR9$^{-/-}$ murine splenocytes stimulated with 0.084 to 22,000 nM DV281 for 48 h (arithmetic mean values ± SEM, $n = 2$ to 4 pooled splenocyte experiments). EC_{50} values as geometric mean ± SEM indicated for (A), (B), and (C). [*single column image*].

3.6. Responses to single ascending doses of inhaled aerosolized DV281 in cynomolgus monkeys

Demonstration of DV281 receptor-target engagement in mouse lungs, resulting in induction of dose-dependent cytokines with defined kinetics supported testing of DV281 as an inhaled therapeutic in mouse models of lung cancer and guided in vivo pharmacology study design in non-human primates. While TLR9 responses in pDC and B cells are comparable between mice and humans, TLR9 expression is broader in rodents than in primates, with monocytes and myeloid dendritic cells also expressing TLR9 in mice but not in humans and non-human primates [31,33]. To bridge our mouse pharmacology data to humans, we tested for induction of IFN-α protein and IFN-inducible genes in cynomolgus monkeys administered inhaled aerosolized DV281. We have previously demonstrated that other CpG-ODN molecules induce IFN responses in the lungs of cynomolgus monkeys exposed by the inhalation route and that data from this species is highly relevant for the establishment of doses to evaluate in human studies [8]. To test responses to aerosolized DV281, a single escalating dose study was designed with target lung deposited doses of 10, 30, and 90 μg/kg for the 3 dosing sessions, each separated by a 3-week washout period (see schematic, Fig. 7A). Eight healthy female monkeys were placed on study after demonstrating stable baseline expression of a panel of IFN and IFN-inducible and inflammatory genes. Pharmacologic responses to DV281 inhalation were evaluated by measuring the induction of genes compared to pre-dose samples in BAL cells and in whole blood, as well as measuring IFN-α protein in the BALF.

Achieved DV281 lung deposited doses were 9.6, 41.3 and 91.6 μg/kg, respectively (Fig. 7A), and inhaled DV281 was well tolerated in all animals with no adverse clinical signs. Induction of IFN and IFN-inducible genes *GBP1*, *IFNA2*, *IRF7*, *ISG54*, *MXB*, and *P28* in BAL cells was observed in a proportion of animals at the 9.6 μg/kg DV281 dose and responses, both in terms of numbers of animals and magnitude of response, increased in a dose-dependent fashion at the 41.3 and 91.6 μg/kg DV281 dose levels (Fig. 7B). While *IFNA2* gene expression was not detected in the blood, IFN-regulated genes were increased in blood post-dosing in a dose-dependent manner (Fig. 7D), likely reflecting a response of blood cells to IFN-α protein induced in the lung (Fig. 7C) and subsequently released into the blood stream. In addition to the core IFN and IFN-inducible genes, we also quantified induction of genes for IFN-γ, TNF and the chemokines CXCL9 and CXCL10 in the BAL cells and blood. *IFNG*, *CXCL9*, and *CXCL10* gene induction was evident in BAL cells. In the blood, *CXCL9* and *CXCL10* expression were induced in only some of the animals at the 41.3 and the 91.6 μg/kg DV281 doses. *TNF* expression was not induced in BAL cells or whole blood cells in response to any dose of DV281 tested (Supplemental Fig. 2), consistent with previous human dosing data with other CpG-ODNs [24]. While this study was not formally designed to define a Minimum Anticipated Biological Effect Level (MABEL), the response observed at the 9.6 μg/kg DV281 dose is suggestive of an approximate MABEL dose. Taken together, these data demonstrate that DV281 induces dose-dependent pharmacological responses when administered in aerosolized form by inhalation in non-human primates, an exposure route intended to be employed in the clinic for patients with lung malignancies.

3.7. Responses to repeat doses of inhaled aerosolized DV281 in the cynomolgus monkey safety study

To evaluate tolerability to higher doses of DV281 and establish a reasonably safe starting dose for humans before initiation of clinical studies in lung cancer patients, we performed a repeat-dose inhalation toxicity study in cynomolgus monkeys with 5 once-weekly administrations. DV281 was administered at achieved delivered doses of 0.427, 1.39, and 3.56 mg/kg/week, and a control group (Vehicle, phosphate buffered saline) was included. The high dose in this study was expected to provide a level of exposure above that of the highest anticipated

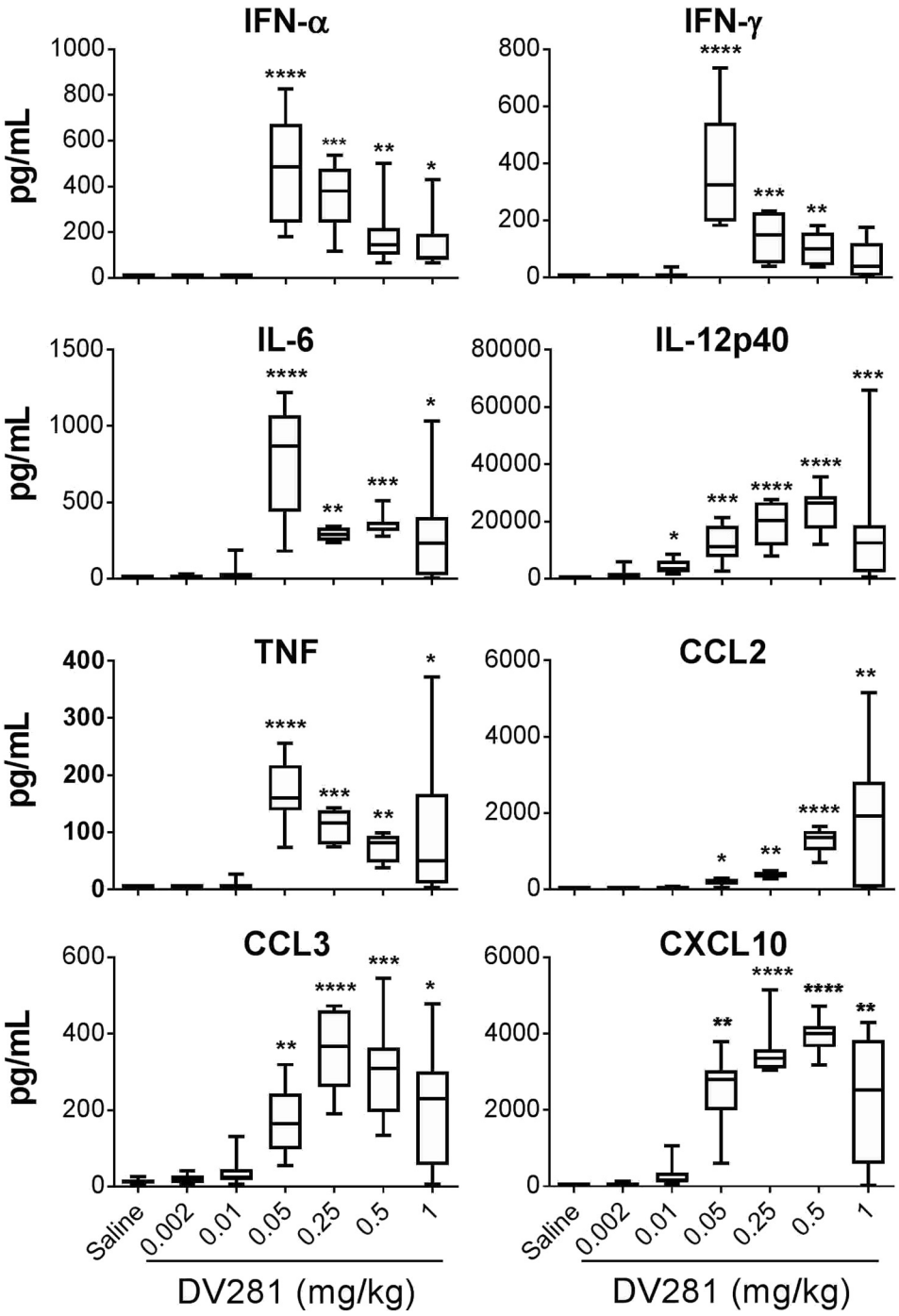

Fig. 3. Dose response for BALF cytokines and chemokines in BALB/c mice administered intranasal DV281. Data at 24 h after single administrations ($n = 8$/group) are presented as median, interquartile range (IQR; boxes), and minimum and maximum values. Significance was assessed with Kruskal-Wallis test, followed by Dunn's multiple comparisons test for specific comparisons between the saline group and each DV281 dose (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). [*1.5 column image*].

clinical exposure level, while the low dose was an approximation of the highest clinical dose on a body weight basis (mg/kg). Animals were sacrificed 7 days (main study: 3/sex/group) after the fifth administration and following a 6 weeks non-dosing period (recovery group: 2/sex/group) after the fifth administration. Once-weekly inhalation of DV281 was well tolerated and there was no mortality or significant toxicities. At 7 days post-dosing, histopathological non-adverse changes in the lungs related to immune stimulation included increased incidence and severity (minimal to slight) in bronchus-associated lymphoid tissue (BALT) hyperplasia as well as perivascular/peribronchiolar lymphoid infiltrate, but with no apparent dose relationship which may reflect the small number of animals per group. There was partial (in males) to complete (in females) recovery in the lungs after a 6 weeks non-dosing period (Table 1). In the tracheobronchial lymph nodes, slightly increased germinal center development was evident, was usually correlated with macroscopic enlargement of these draining lymph nodes and was fully resolved by 6 weeks post-dosing. All these findings were consistent with changes related to the local pharmacological action of a TLR9 agonist, that is the expected immunostimulatory properties of DV281. As there were no treatment-associated inflammatory or

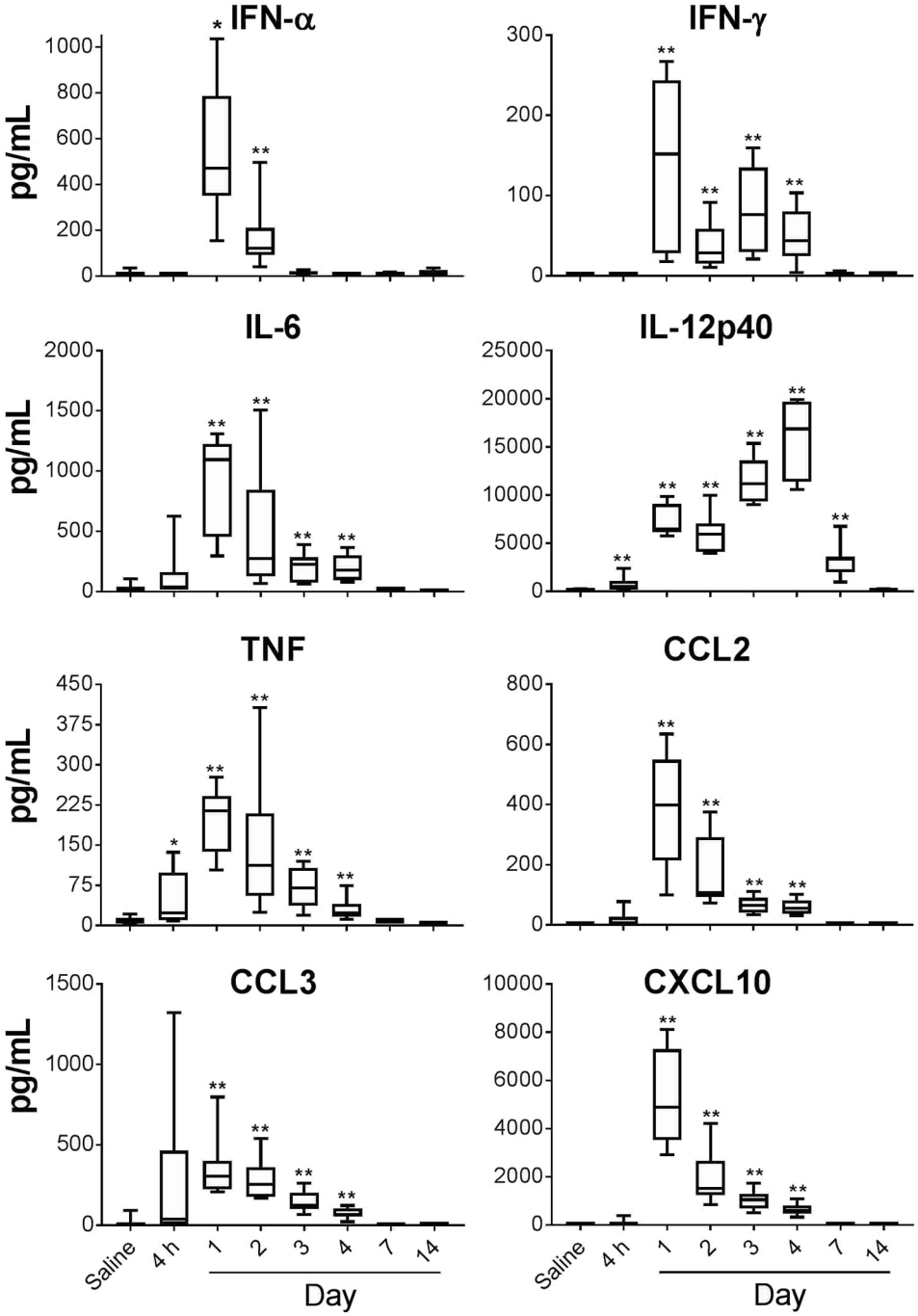

Fig. 4. Duration of BALF cytokine and chemokine responses in BALB/c mice administered intranasal DV281. Responses to 0.05 mg/kg DV281 (n = 8/group) are presented as median, interquartile range (IQR; boxes), and minimum and maximum values. Data for each group of time-point matched control animals administered saline is combined. (n = 35, 7 timepoints, n = 5/group). Significance was assessed with Kruskal-Wallis test, followed by Dunn's multiple comparisons test for specific comparisons between the saline group and each DV281 time point (*$P < 0.05$, **$P < 0.01$). [*1.5 column image*].

degenerative changes in the parenchymal tissues of the lung, or clinical or pathological systemic effects, findings were considered to be non-adverse. After 6 weeks recovery there was evidence of reversibility following cessation of treatment, and no evidence of progression or delayed appearance of treatment effects. Thus, the no observed adverse effect level (NOAEL) in the 5-week monkey study was determined to be the highest dose tested, specifically 3.56 mg/kg/week DV281. These findings are consistent with safety data generated using other inhaled CpG-ODNs in non-human primates and in humans [8,34], and these findings supported clinical studies in lung cancer patients with inhaled DV281.

3.8. Effect of immune checkpoint blockade with anti-PD-1 antibody on DV281-induced responses from human primary cells

DV281 is being developed for clinical application as an inhaled therapy to be combined with systemic administration of PD-1/PD-L1 pathway blocking antibody. The combination of an immunostimulant with immune checkpoint blockade is an attractive approach for potentially improving patient anti-tumor responses, but raises the potential of toxicity due to a dysregulated cytokine response [35,36]. To address this concern, we conducted studies of DV281 combined with anti-PD-1 antibody in human PBMC and in vivo in mice. For the human

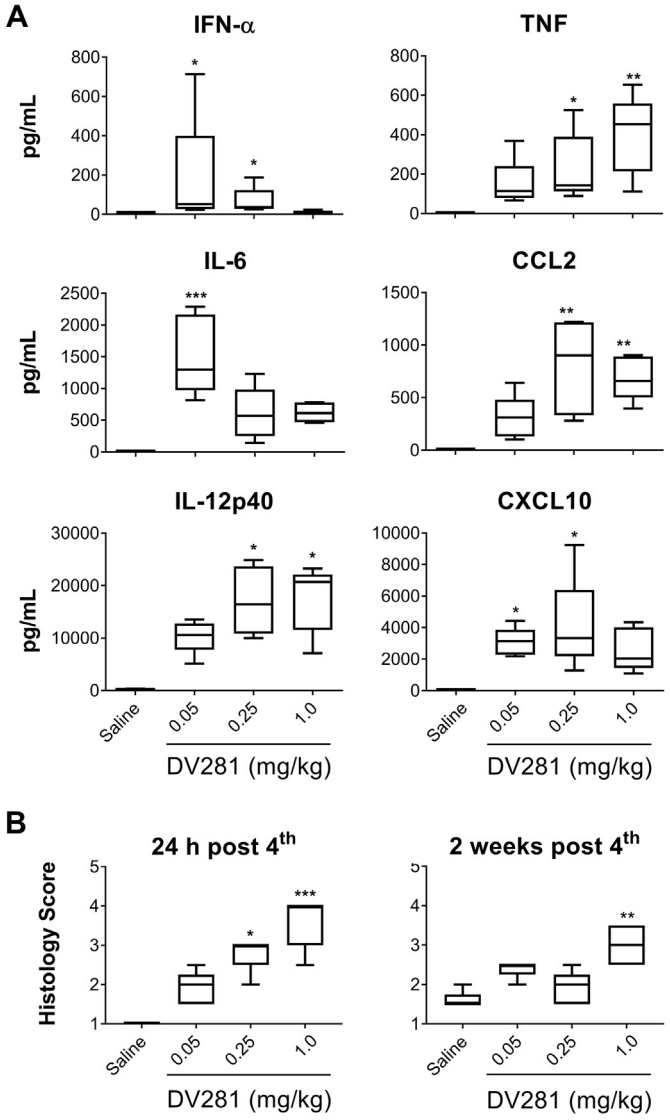

Fig. 5. Lung responses in BALB/c mice to intranasal DV281 administered every two weeks for a total of 4 doses. Mice were euthanized either 24 h or 2 weeks after the fourth DV281 dose at 0.05, 0.25, or 1 mg/kg. (A) BALF cytokines and chemokines at 24 h post-fourth DV281 doses. (B) H&E-stained lung tissue sections were scored on a scale of 1 to 5 (1 = no change, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) to evaluate the extent of inflammation around airways and blood vessels at both timepoints. All data are graphed as median, interquartile range (IQR; boxes), and minimum and maximum values. Significance was assessed with Kruskal-Wallis test, followed by Dunn's multiple comparisons test for specific comparisons between the saline group and each DV281 dose group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). [*1.5 column image*].

PBMC study, the anti- human PD-1 antibody was used at 2 μg/mL, which was slightly above the calculated IC_{80} value for inhibitory activity (data not shown). Human PBMCs (enriched 10-fold for pDCs) were stimulated with DV281 alone at a range of concentrations, or in combination with anti-PD-1 antibody, or isotype control antibody. Cytokine responses to DV281, including IL-2, IL-6, and TNF levels, were not affected by presence of anti-PD-1 antibody (Fig. 8A). Furthermore, DV281 stimulation with or without anti-PD-1 antibody induced similar B cell proliferation responses (Fig. 8B) and, as expected, did not result in appreciable $CD4^+$ or $CD8^+$ T cell proliferation (Fig. 8B). In contrast, the superantigen staphylococcal enterotoxin B induced strong proliferation of $CD4^+$ and $CD8^+$ T cells (data not shown). To test the effects of anti-PD-1 antibody on DV281 effects in vivo, aqueous DV281 was administered via the intranasal route to naïve mice with or without prior administration of an anti-mouse PD-1 antibody (see Supplemental Methods). The data showed that a single injection of anti-PD-1 antibody 24 h prior to intranasal DV281 did not have a significant effect on serum or lung (BALF) cytokine and chemokine responses (Supplemental Fig. 3). Together, these findings suggest that DV281 administration to humans in which the PD-1/PD-L1 feedback inhibition pathway is blocked by antibody therapy will not lead to dysregulation of DV281-induced cytokine or cell proliferation responses.

4. Discussion

Inhaled delivery of aerosolized CpG-ODN has the potential to distribute therapeutic concentrations of the drug in proximity to tumor lesions in the lung tissue and to tracheobronchial lymph nodes, while simultaneously restricting systemic exposure. In mouse models, direct intratumoral injection of the CpG-ODN SD-101 has significant anti-tumor activity and can complement PD-1 blockade leading to rejection of both injected and non-injected tumor and long-term survival [15]. For primary lung cancers and lung metastases of other tumor types, inhaled delivery of a CpG-ODN represents an alternate approach to achieve localized immune stimulation. In mouse models of lung metastases, inhaled delivery of a CpG-ODN can initiate $CD8^+$ T cell infiltration and, when combined with anti-PD-1, leads to rejection of tumor lesions in the lung and other sites, conferring durable survival [20]. The combination of an inhaled CpG-ODN and a PD-1 inhibitor has the potential to address an unmet need for NSCLC patients that do not respond to PD-1 inhibition as a monotherapy. To support this approach, we conducted pharmacological studies to define the in vitro and in vivo activity of DV281, a novel CpG-ODN, in development for inhaled delivery to NSCLC patients receiving antibody to block the PD-1/PD-L1 pathway.

In the present study we demonstrated that DV281 has very similar potency (low nanomolar range) on primary cells across species (mice, monkey, human), while the lack of responses from $TLR9^{-/-}$ mouse splenocytes demonstrated TLR9-dependence for cytokine induction in vitro. Comparable inter-species potency for DV281-induced responses supported pharmacodynamics evaluation in the selected animal species. In additional to comparable in vitro potency, DV281 induced similar maximum levels of IFN-α protein (~900–950 pg/mL) in humans and monkeys, further supporting safety evaluation in the non-human primates. High potency in combination with moderate peak IFN-α production would, in theory, facilitate evaluation of DV281 efficacy over a potentially wide treatment window in patients.

Given similarities in both cellular distribution of TLR9 and biological responses to CpG-ODNs in humans and cynomolgus monkeys, data from the latter species have proved informative for selection of doses to go into human clinical studies [8]. Through both a single ascending dose study and a separate repeat dose safety study in cynomolgus monkeys, we were able to evaluate responses to inhaled aerosolized DV281 over a broad dose range in non-human primates. The low level *IFNA2* and IFN-inducible gene responses in BAL as well as IFN-α protein levels in BALF of monkeys administered the 9.6 μg/kg dose identified an approximate MABEL comparable to the MABEL previously defined for a related CpG-ODN (AZD1419; 6.6 μg/kg), also administered by the inhalation route [8]. For DV281, further induction of genes and IFN-α protein was evident in lungs at the 41.3 and 91.6 μg/kg dose levels. We did not measure levels of DV281 in the lungs or plasma in the single ascending dose study. However, DV281 concentrations were quantified in the repeat dose safety study with mean values of 69, 269, and 463 ng DV281/g of lung tissue for the 0.427, 1.39, and 3.56 mg/kg achieved lung doses, respectively. In the plasma, DV281 levels were below the limit of quantitation even at the highest dose tested (3.56 mg/kg), suggesting DV281 is largely retained in the lung tissue, which is consistent with previous studies on oligonucleotide inhalation [8,37]. Thus, the lack of *IFNA2* expression in the blood in the single ascending dose study is most likely reflective of low DV281 in circulation, with the

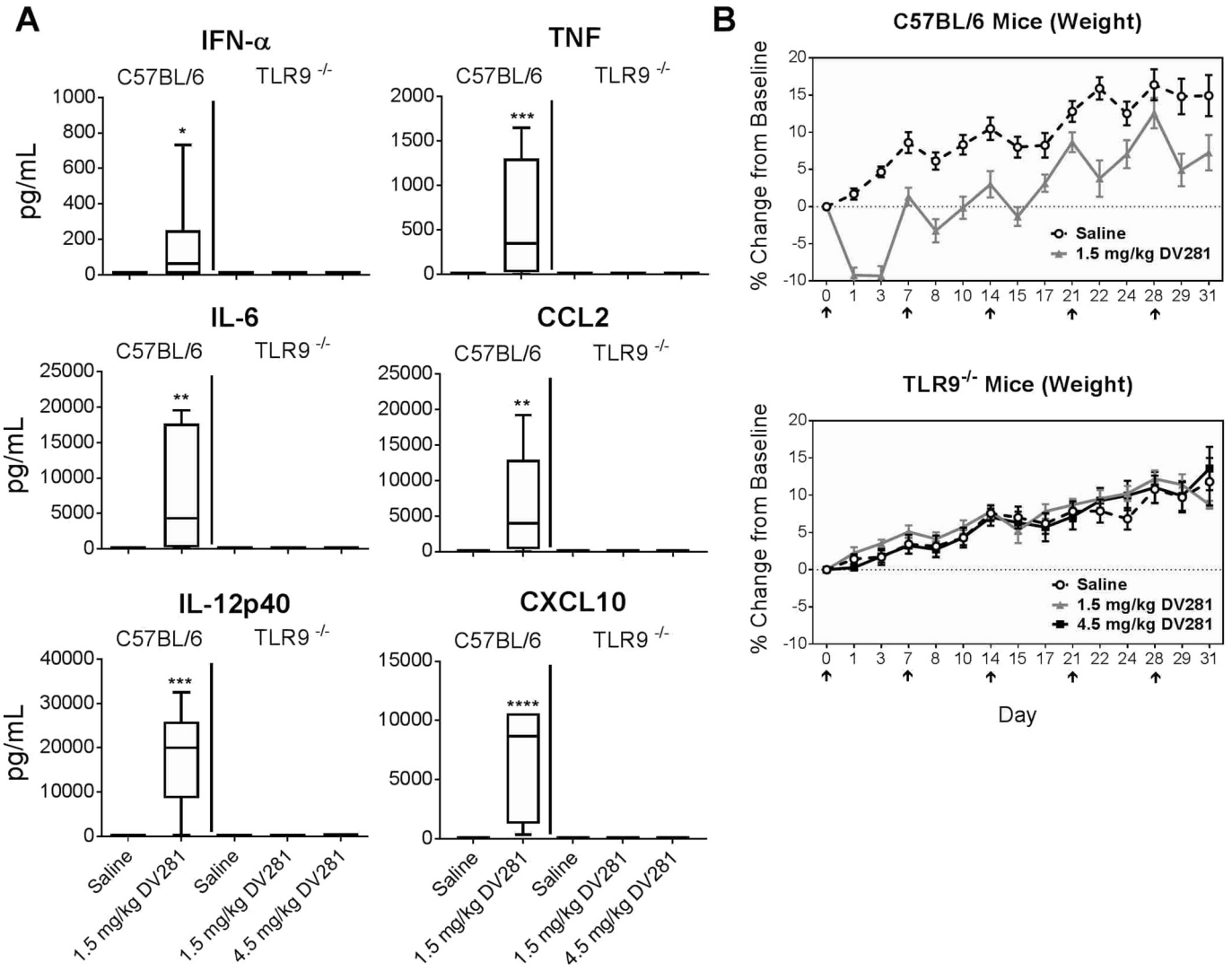

Fig. 6. Responses in C57BL/6 and TLR9$^{-/-}$ mice to intranasal DV281 administered every week for a total of 5 doses. (A) Cytokine/chemokine data from BALF of TLR9$^{-/-}$ mice and wildtype (C57BL/6) that was harvested 24 h after a fifth weekly intranasal dose of DV281. Data are graphed as median, interquartile range (IQR; boxes), and minimum and maximum values. Significance was assessed with Kruskal-Wallis test, followed by Dunn's multiple comparisons test for specific comparisons between saline groups and respective DV281 dose groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). (B) Mice were weighed twice weekly throughout study and at sacrifice on Day 29 or Day 31 ($n = 12$/group for Day 0 to Day 29; $n = 6$/group for Day 31). Arrow heads represent DV281 intranasal dose administration time points. Data are graphed as cumulative percent change from baseline weight at Day 0 (arithmetic mean ± SEM). [*2 column image*].

activation of IFN-responsive genes in blood being a secondary response to IFN-α protein released by the lung. Inhalation dosing with DV281 in both the single ascending dose study and the repeat dose toxicity study was well tolerated at all doses tested with no notable clinical signs or mortality. In the repeat dose toxicity study, the highest dose tested, 3.56 mg/kg/week, was determined to be the NOAEL. Observations in the lungs were consistent with the known immunostimulatory effects of CpG-ODN and partial to complete recovery in the lungs was evident 6 weeks after dosing cessation. These studies in non-human primates with inhaled DV281 establish a clear dose-response relationship and establish safety margins, parameters important for determining the doses to be evaluated in human studies.

Although the responses of non-human primates to TLR9 agonists more closely reflects human responses, studies of inhaled DV281 in mice are useful to address mechanistic questions requiring lung tissue harvest, such as dose course and kinetics, and to conduct preliminary safety assessments. In mice, intranasally administered DV281 induced dose-dependent cytokine and chemokine responses at 24 h but the shape of dose-response curves differed between the analytes measured, perhaps reflecting a mixture of cell sources and indirect as well as direct responses. Nevertheless, peak cytokine responses generally occurred at 24 h post dosing and were at background levels by Day 7 (with the exception of IL-12p40), supporting weekly to bi-weekly dosing regimens. To evaluate resolution of lung responses after a multi-dose regimen and to test for signs of off-target toxicity at high DV281 doses, we ran experiments in mice testing a 4 dose regimen in BALB/c mice (dosing at 0.05–1 mg/kg, once every 2 weeks) and 5 dose regimens in C57BL/6 and TLR9$^{-/-}$ mice on a C57BL/6 background (dosing at 1.5 or 4.5 mg/kg once every week). The former experiment demonstrated that BALF cytokine responses and histopathological changes evident in lung tissue 24 h after a 4th intranasal dose were absent or largely resolved, respectively, by 2 weeks after dosing cessation. In the latter experiment, BALF cytokine responses and transient body weight loss, previously shown to be TNF-dependent [24], was evident in wild-type C57BL/6 but not TLR9$^{-/-}$ mice, which also showed no clinical or pharmacological responses to DV281 dosing at 1.5 or 4.5 mg/mg, levels up to about 100 times that required for induction of cytokine responses in the lung. Of note, inhaled DV281 did not induce appreciable TNF responses in monkey lungs or blood, consistent with previous observations with other CpG-ODNs [24]. Taken together, the multi-dose

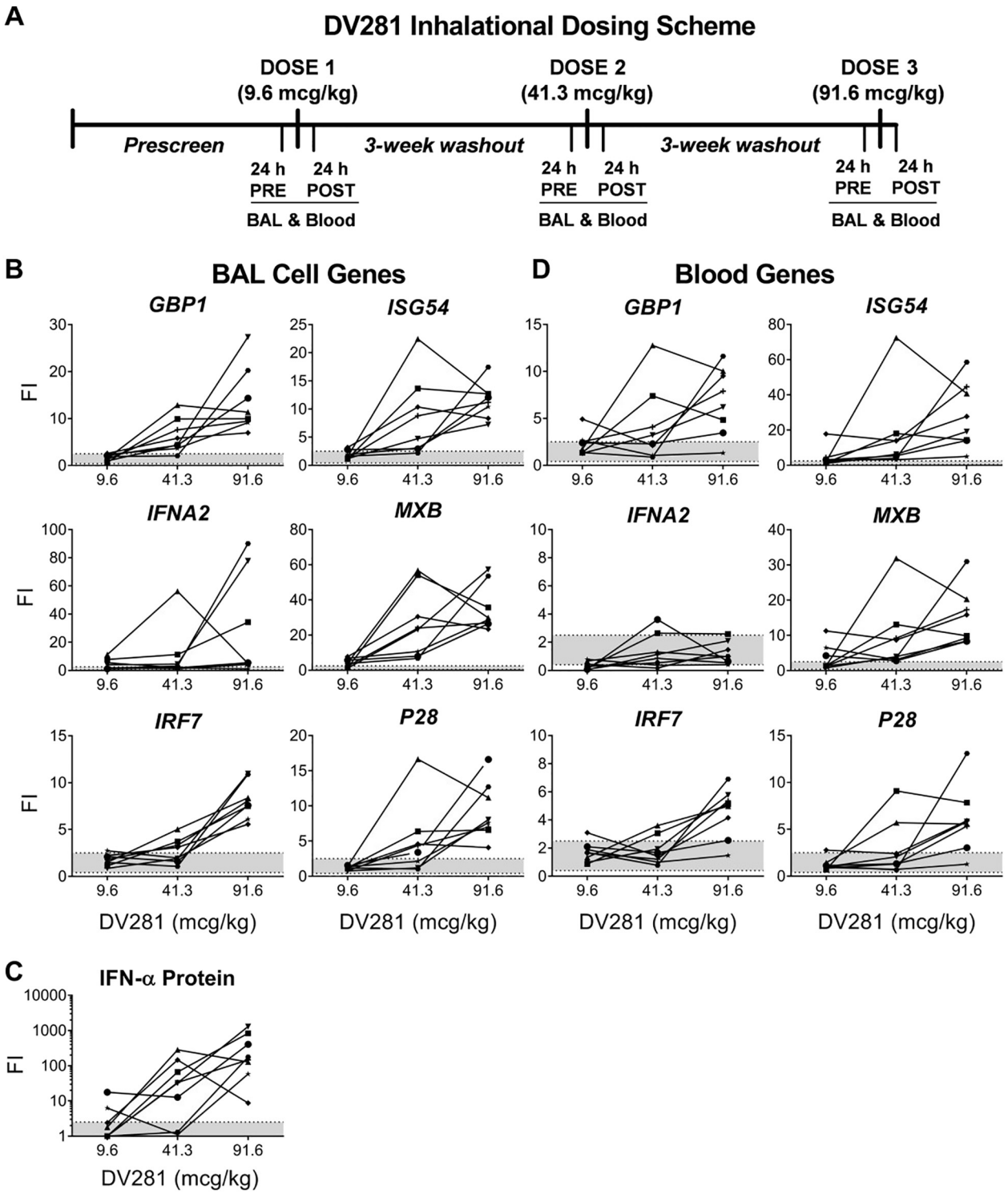

Fig. 7. Cynomolgus monkey responses to escalating doses of inhaled aerosolized DV281. A) Schematic representation of aerosolized DV281 inhalation dosing of monkeys, including achieved deposited doses and sampling time points. B) IFN-related gene expression (*GBP1, IFNA2, IRF7, ISG54, MXB,* and *P28*) in the BAL, C) IFN-α protein in the BALF. D) IFN-related gene expression in whole blood. Relative threshold cycle (Ct) of each gene to the housekeeping gene *UBC* was calculated. For each DV281 administration (9.6, 41.3, or 91.6 μg/kg), fold induction (FI) of gene and protein responses were calculated compared to the respective baselines (−24 h pre-dose). Data for individual animals are shown. Fold induction over 2.5 (shaded line) was considered above background. [*2 column image*].

regimen experiments in mice demonstrate that lung tissue responses to DV281 dosing are strictly TLR9-dependent and resolve within weeks after dosing cessation. In $TLR9^{-/-}$ mice there was no evidence of toxicity upon gross examination post mortem, suggesting a lack of off-target toxicity. Collectively, these data support a weekly DV281 dosing regimen as is envisioned for clinical application in lung cancer patients.

As DV281 will be used in combination with antibody blockade of the PD-1/PD-L1 pathway in the clinic, the effect of anti-PD-1 antibody on DV281-induced cytokine and cell proliferative responses was evaluated in vitro with human cells and in vivo in mice to determine if the combination resulted in any dysregulation of DV28-induced responses. In vitro, human PBMC cultures were 10-fold enriched for pDCs in order

Table 1
Summary of DV281 treatment-related findings in the lungs of cynomolgus monkeys sacrificed 7 days (main study) or 6 weeks (recovery) after 5 weeks of inhalation. Values represent the number of animals demonstrating incidence of the given parameter.

	Main study animals (Sacrificed 7 days post 5th weekly DV281 administration)								Recovery animals (Sacrificed 6 weeks post 5th weekly DV281 administration)							
Group/sex	1 M	2 M	3 M	4 M	1F	2F	3F	4F	1 M	2 M	3 M	4 M	1F	2F	3F	4F
Achieved dose (mg/kg/week)	0	0.427	1.39	3.56	0	0.427	1.39	3.56	0	0.427	1.39	3.56	0	0.427	1.39	3.56
BALT hyperplasia[a]																
Minimal	1	1	2	0	1	1	1	1	0	1	0	1	0	1	1	0
Slight	0	1	1	2	0	2	1	1	0	0	0	1	0	0	0	0
Total	1	2	3	2	1	3	2	2	0	1	0	2	0	1	1	0
Perivascular/peribronchiolar lymphoid infiltrate[a]																
Minimal	1	1	2	0	1	1	1	1	0	1	0	1	0	1	1	0
Slight	0	1	1	2	0	2	1	1	0	0	0	1	0	0	0	0
Total	1	2	3	2	1	3	2	2	0	1	0	2	0	1	1	0
Number of tissues examined	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2

[a] Both findings were seen in all affected animals. BALT, Bronchus-Associated Lymphoid Tissue.

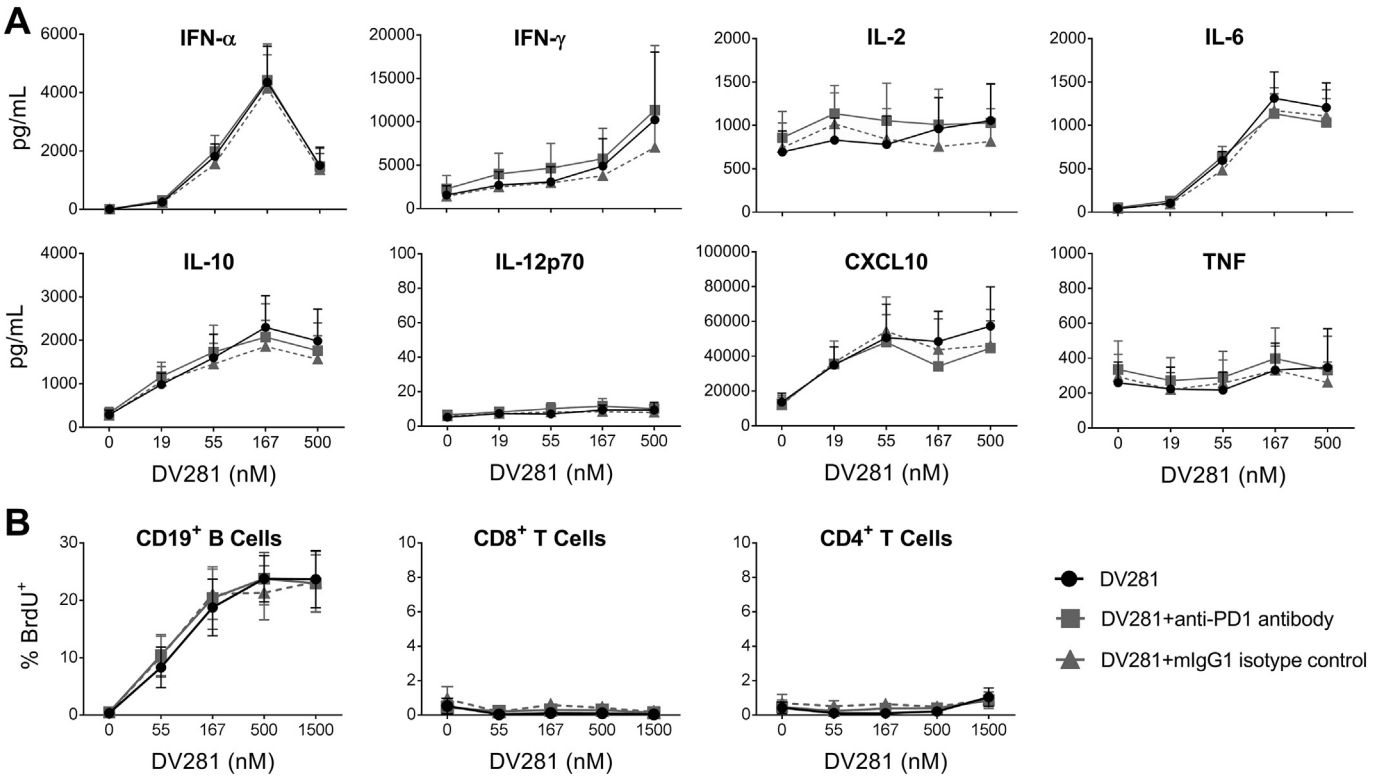

Fig. 8. Effect of anti-PD-1 antibody on human PBMC responses to DV281. (A) Human pDC-enriched PBMC cytokine responses after 24 h DV281 stimulation with or without anti-PD-1 or mIgG1 isotype control antibodies, respectively (arithmetic mean ± SEM, $n = 5$). (B) Human $CD19^+$ B cell, $CD4^+$ and $CD8^+$ T cell proliferation responses to DV281 with or without anti-PD-1 or mIgG1 isotype control antibodies, respectively (48 h stimulation, followed by BrdU-incorporation over 18 h) (arithmetic mean ± SEM, $n = 8$).

to test the effect of PD-1/PD-L1 pathway blockade on a background of augmented DV281-induced IFN-α responses. However, the immunostimulatory effects of DV281 in vitro or in vivo were not significantly altered in the presence of PD-1/PD-L1 pathway blockade. While intranasal administration of DV281 at 1.0 mg/kg (but not 0.1 mg/kg) in anti-PD-1 treated mice resulted in lower BALF cytokines, this effect was not significant. DV281-induced human PBMC cytokine and B cell proliferative response curves were similar in the presence or absence of anti-PD-1 antibody. These findings suggest anti-PD-1 antibody has no appreciable effect on DV281 induced responses in innate immune cells and is unlikely to result in dysregulated responses in patients.

In summary, this set of pharmacology and safety studies support the clinical development of DV281 as an inhaled therapeutic for application in combination with anti-PD-1 in lung cancer. DV281 is a potent activator of TLR9 pathway responses in vitro in human cells and in vitro and in vivo in mice and non-human primates. Additionally, DV281 appears safe and well tolerated over a range of dose levels in mice and primates and does not present safety concerns when used in combination with anti-PD-1 antibody, as is intended in the clinic. As a proof of concept for this lung tumor therapy approach, separately published mechanism of action studies in mouse tumor models demonstrated synergy between an inhaled CpG-ODN and anti-PD-1 antibody for induction of tumor infiltration by polyfunctional $CD8^+$ T cells, leading to durable rejection of both lung tumors and extra-pulmonary lesions [20]. For DV281, both in vitro primary cell potency data and responses of monkeys to inhalational dosing informed starting dose calculations for evaluation of DV281 in human trials. A Phase 1b trial to evaluate

the safety and preliminary efficacy of inhaled DV281, a novel CpG-C class ODN, in combination with an approved anti-PD-1 inhibitor for the treatment of NSCLC has been initiated (NCT03326752).

Conflict of interest

All authors were employees of Dynavax Tecnologies at the time that this work was conducted.

Author contributions

Participated in research design: Kell, Traquina, Coffman, Campbell

Conducted experiments and performed data analysis: Kell, Kachura, Renn, Traquina, Campbell

Wrote or contributed to the writing of the manuscript: Kell, Coffman, Campbell

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.019.

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