


REPORT

 OPEN ACCESS  Check for updates

## Durable blockade of PD-1 signaling links preclinical efficacy of sintilimab to its clinical benefit

Jie Wang<sup>a\*</sup>, Keke Fei<sup>a\*</sup>, Hua Jing<sup>a\*</sup>, Zhihai Wu<sup>b\*</sup>, Weiwei Wu<sup>b\*</sup>, Shuaixiang Zhou<sup>a</sup>, Haiqing Ni<sup>a</sup>, Bingliang Chen<sup>a</sup>, Yan Xiong<sup>b</sup>, Yanpeng Liu<sup>b</sup>, Bo Peng<sup>b</sup>, Dechao Yu<sup>b\*</sup>, Haiping Jiang<sup>c</sup>, and Junjian Liu<sup>a</sup>

<sup>a</sup>Department of Biologics Discovery, Innovent Biologics (Suzhou) Co, Jiangsu, China; <sup>b</sup>Department of Translational Science, Innovent Biologics (Suzhou) Co, Jiangsu, China; <sup>c</sup>Department of Medical Oncology, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China

### ABSTRACT

Blockade of immune checkpoint pathways by programmed cell death protein 1 (PD-1) antibodies has demonstrated broad clinical efficacy against a variety of malignancies. Sintilimab, a highly selective, fully human monoclonal antibody (mAb), blocks the interaction of PD-1 and its ligands and has demonstrated clinical benefit in various clinical studies. Here, we evaluated the affinity of sintilimab to human PD-1 by surface plasmon resonance and mesoscale discovery and evaluated PD-1 receptor occupancy and anti-tumor efficacy of sintilimab in a humanized NOD/Shi-scid-IL2 $\gamma$  (null) (NOG) mouse model. We also assessed the receptor occupancy and immunogenicity of sintilimab from clinical studies in humans (9 patients with advanced solid tumor and 381 patients from 4 clinical studies, respectively). Sintilimab bound to human PD-1 with greater affinity than nivolumab (Opdivo<sup>®</sup>, MDX-1106) and pembrolizumab (Keytruda<sup>®</sup>, MK-3475). The high affinity of sintilimab is explained by its distinct structural binding mode to PD-1. The pharmacokinetic behavior of sintilimab did not show any significant differences compared to the other two anti-PD-1 mAbs. In the humanized NOG mouse model, sintilimab showed superior PD-1 occupancy on circulating T cells and a stronger anti-tumor effect against NCI-H292 tumors. The strong anti-tumor response correlated with increased interferon- $\gamma$ -secreting, tumor-specific CD8<sup>+</sup> T cells, but not with CD4<sup>+</sup> Tregs in tumor tissue. Pharmacodynamics testing indicated a sustained mean occupancy of  $\geq 95\%$  of PD-1 molecules on circulating T cells in patients following sintilimab infusion, regardless of infusion dose. Sintilimab infusion was associated with 0.52% (2/381 patients) of anti-drug antibodies and 0.26% (1/381 patients) neutralizing antibodies. These data validate sintilimab as a novel, safe, and efficacious anti-PD-1 mAb for cancer immunotherapy.

### ARTICLE HISTORY

Received 15 April 2019  
Revised 2 August 2019  
Accepted 6 August 2019

### KEYWORDS

PD-1; sintilimab; antibody; cancer immunotherapy; receptor occupancy; immunogenicity





## Introduction

Tumors exploit multiple mechanisms to inhibit anti-tumor immune responses and promote immune evasion. The interaction of programmed cell death protein 1 (PD-1) transmembrane protein receptor, which is found in lymphocytes and monocytes, with its natural ligands PD-L1 and PD-L2<sup>1,2</sup> is one of the major pathways exploited by cancer cells for immune evasion.<sup>3</sup> The PD-1/ligand interactions strongly counteract T cell receptor (TCR) signal transduction and subsequently attenuate cytokine production, T-cell survival, and proliferation.<sup>4</sup> Therapeutic antibodies blocking immune checkpoints restore anti-tumor immunity and lead to durable tumor regression and prolonged survival in some patients.<sup>5–8</sup> Several antibodies targeting PD-1, such as MDX-1106 (Opdivo<sup>®</sup>, nivolumab) and MK-3475 (Keytruda<sup>®</sup>, pembrolizumab), are approved by the United States Food and Drug Administration for the treatment of malignant tumors, and many more are also being tested in clinical trials.

Sintilimab, which is a fully human IgG4 monoclonal antibody (mAb) generated using yeast display technology, blocks the


binding of PD-1 to PD-L1 or PD-L2. Sintilimab has lower potency in mediating antibody-dependent cell-mediated toxicity (ADCC) and complement-mediated cytotoxicity (CDC) and displays a mechanism of action similar to MDX-1106 and MK-3475.<sup>9–11</sup> However, the high binding affinity and unique PD-1 epitopes bound by sintilimab might be responsible for its superior clinical effectiveness. In patients with relapsed or refractory classical Hodgkin's lymphoma, the objective response rate with sintilimab is 80.4%,<sup>5</sup> which is superior to MDX-1106 (69%)<sup>12</sup> and MK-3475 (69%).<sup>13</sup> Sintilimab was approved by China's National Medical Products Administration for the treatment of patients with relapsed or refractory Hodgkin's lymphoma in 2018. To date, more than 20,000 patients have been treated with sintilimab.

Like many non-chemotherapy drugs, maximum efficacy occurs when the targeted immune checkpoint receptor is saturated and blocked. This event is driven by both antibody concentration and the affinity of antibody for receptor. The sustained mean PD-1 receptor occupancy on circulating T cells of patients achieved by MDX-1106 (dose-independent) and

**CONTACT** Junjian Liu  [junjian.liu@innoventbio.com](mailto:junjian.liu@innoventbio.com)  Innovent Biologics (Suzhou) Co, 168 Dongping Street, Suzhou Industrial Park, Suzhou, Jiangsu Province 215123, China; Haiping Jiang  [jianghaiping75@163.com](mailto:jianghaiping75@163.com)  Department of Medical Oncology, First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang Province 310003, China

\*These authors contributed equally to this work.

This article has been republished with minor changes. These changes do not impact the academic content of the article.

 Supplemental data for this article can be accessed on the [publisher's website](#).

© 2019 The Author(s). Published with license by Taylor & Francis Group, LLC.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

sintilimab is reported to be >70% and >95%, respectively.<sup>5,14</sup> Although there is no comparable receptor occupancy information for MK-3745, maximum serum engagement was reached with doses greater than or equal to 1 mg/kg every 3 weeks.

Another factor influencing clinical effect is drug immunogenicity. Protein-based drugs administered to patients may induce humoral immune responses, causing the development of anti-drug antibodies (ADAs) and neutralizing antibodies (NABs) during repeated infusions.<sup>15</sup> Besides neutralizing therapeutic effects, immunogenicity can also cause life-threatening complications, such as anaphylaxis and immune complex-mediated disease.<sup>16,17</sup> For these reasons, it is important to study the incidence of immunogenicity in patients treated with anti-PD-1 mAbs in clinical studies. Among 1086 MDX-1106 treated patients, 12.7% were ADA positive and 0.8% were NAB positive at 1 time point.<sup>18</sup> Among 2910 MK-3475 treated patients, 1.7% had a treatment-emergent-positive ADA.<sup>19</sup>

To date, no head-to-head comparison of physicochemical and biological properties and efficacy of anti-PD-1 antibodies has been reported. Herein, we characterized the affinity, receptor occupancy, and anti-tumor activity of sintilimab in humanized NOD/Shi-scid-IL2rgamma (null) (NOG) mice versus MDX-1106 and MK-3475. We also assessed the pharmacodynamics and immunogenicity of sintilimab in patients from four clinical studies.

## Results

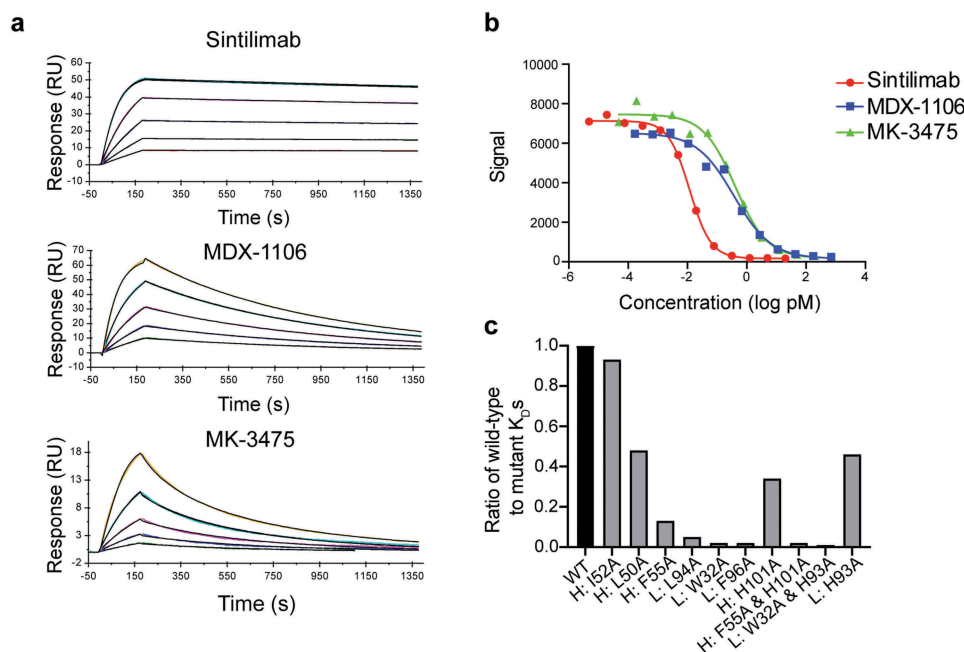
### Binding properties of sintilimab

We first evaluated the binding of sintilimab, MDX-1106, and MK-3475 to human PD-1 by surface plasmon resonance (SPR). As

shown in Figure 1a, sintilimab had the highest affinity compared with the other two anti-PD-1 mAbs. The  $K_D$  of sintilimab, MDX-1106, and MK-3475 was 74 pM, 3186 pM and 1785 pM, respectively (Table 1). Of note, sintilimab showed an extremely slow dissociation rate ( $k_d = 8.0 \times 10^{-5}$ /seconds), indicating sintilimab had very low tendency to dissociate with human PD-1 and possibly was a persistent block to the PD-1 pathway. Mesoscale discovery (MSD) analysis further confirmed that sintilimab had a stronger binding affinity to hPD-1 (Figure 1b). Sintilimab binds to a distinctive epitope compared with MDX-1106 and MK-3475 (data not shown). By mutagenesis studies and subsequent affinity measurement, we found the hydrophobic and aromatic amino acid residues (H: L50, I52, F55, H101 and L: W32, H93, L94, F96) in the complementarity-determining region (CDR) of sintilimab are critical for its interaction with PD-1 (Figure 1c).

### Sintilimab showed a high level of PD-1 occupancy *in vitro* and *in vivo*

Sintilimab showed the lowest  $k_d$  value in SPR measurements, indicating that it has a low tendency to dissociate once it binds to PD-1 molecules. To confirm this point, we incubated human peripheral blood mononuclear cells (PBMCs) with saturated anti-PD-1 mAbs *in vitro*. After thorough washing steps, we measured the percentage of CD3+ T cells that bound with different anti-PD-1 mAbs by flow cytometry. As shown in Figure 2a, sintilimab was able to bind with 41.9% of CD3+ T cells, compared to 35.6% for MDX-1106 and 32.8% for MK-3475. Correspondingly, the fluorescence intensity of PD-1 was higher in cells incubated with sintilimab, indicating that sintilimab was able to bind with more PD-1 molecules on the



**Figure 1.** Higher binding affinity of sintilimab to human PD-1 compared with MDX-1106 and MK-3475. (a) Binding affinity and kinetics of anti-PD-1 mAbs to human PD-1 determined by surface plasma resonance. Sintilimab, MDX-1106 and MK-3475 were performed with human PD-1 in multi-cycle kinetics and raw data were fitted using 1:1 binding model. (b) Meso Scale Discovery analyses of the binding between human PD-1 and anti-PD-1 mAbs. (c) Individual residue of sintilimab forming the hydrophobic core was mutated to alanine. SPR analysis reveals residue mutations impair sintilimab binding to the hPD-1 protein.

**Table 1.** Affinity of Sintilimab to human PD-1 measured by SPR.

Antibody	Antigen	$K_a$ (1/Ms)	$K_d$ (1/s)	$K_D$ (M)	Chi2
Sintilimab	hPD-1	1.090E+6	8.028E-5	7.366E-11	0.0271
MDX-1106	hPD-1	4.599E+5	1.465E-3	3.186E-9	0.0845
MK-3475	hPD-1	4.090E+6	7.301E-3	1.785E-9	0.0266

surface of CD3+ T cells compared to MDX-1106 and MK-3475 (Figure 2b).

We further measured the ability of sintilimab to promote T cell responses *in vitro* using human CD4+ T cells stimulated with DCs. As shown in Figure 2c, the PD-1 blockade with sintilimab enhanced interleukin (IL)-2 secretion over those treated with MDX-1106 or MK-3475, indicating sintilimab has superior T cell activating characteristics.

The *in vivo* work was more complex, including aspects such as antibody-drug concentration and the dynamics of antigen-antibody association and dissociation that were not covered by *in vitro* analyses. To address the PD-1 receptor occupancy of different anti-PD-1 mAbs *in vivo*, we injected 1 mg/kg, 3 mg/kg and 10 mg/kg of sintilimab, MDX-1106, and MK-3475 in NOG mice reconstituted with human PBMCs. PD-1 occupancy was dose-dependent, with an occupancy of 86.8% observed 24 h after 10 mg/kg sintilimab injection. At all doses tested, sintilimab showed a higher percentage of PD-1 molecule occupancy compared with MDX-1106 and MK-3475. The occupancy rate in mice treated with 1 mg/kg sintilimab was 70%, which was comparable to that of mice treated with 10 mg/kg MDX-1106 and MK-3475 (Figure 2d). PD-1 occupancy decreased in all groups 72 h after injection of the mAbs; however, mice administered sintilimab still had the highest PD-1 occupancy 72 h after mAbs' injection (Figure 2e). We also observed a higher PD-1 receptor occupancy of sintilimab compared with MDX-1106 and MK-3475 in human PD-1 knock-in mice (Supplementary Figure 1). These data are in

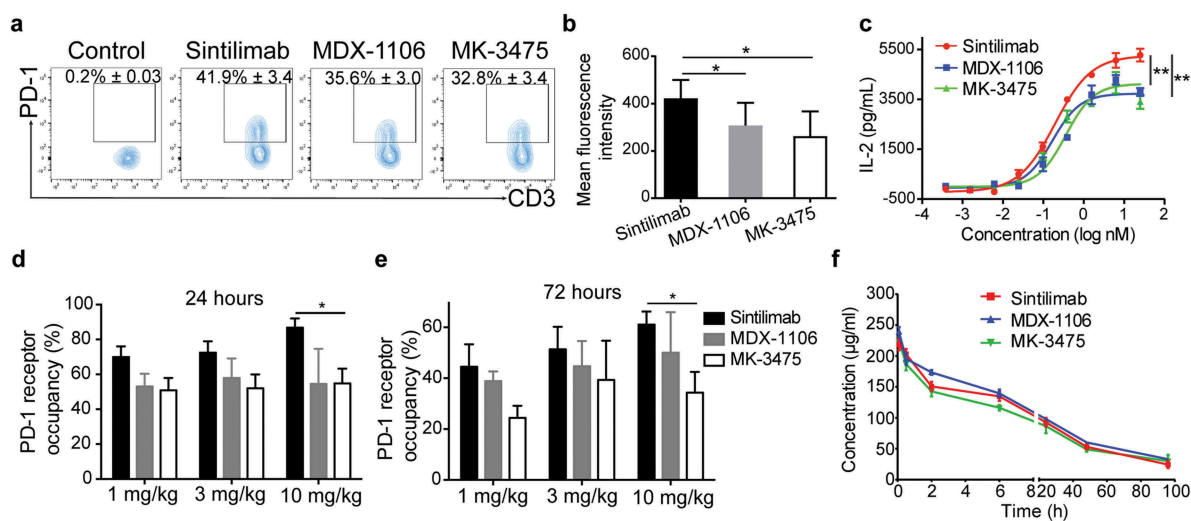
agreement with *in vitro* results that demonstrated a high affinity of sintilimab for PD-1 molecules. Interestingly, in subcutaneous NCI-H292 tumor-bearing mice, PD-1 receptor occupancy in both peripheral and CD3+ tumor-infiltrating T cells (TILs) were more than 90% 24 h after 10 mg/kg sintilimab injection. Note that CD3+ TILs expressed a higher level of PD-1 compared with peripheral CD3+ T cells (Supplementary Figure 2).

Following a single intravenous (IV) administration of anti-PD-1 mAbs at 10 mg/kg to hPD-1 knock-in mice, standard pharmacokinetic (PK) measurements of sintilimab, MDX-1106 and MK-3475 serum concentrations indicated a serum half-life ( $t_{1/2}$ ) of 35.6, 43.5 and 42.5 h, respectively. PK parameters are provided in Figure 2f and Table 2. The  $C_{max}$  and CL were comparable between these three anti-PD-1 mAbs.

### Antitumor effects of sintilimab against NCI-H292 tumors in a humanized mouse model

Next, we explored whether higher PD-1 receptor occupancy of sintilimab correlated with a better antitumor effect and stronger immune response. We first evaluated the tumor control activity of sintilimab, MDX-1106, and MK-3475 using a human tumor xenograft model in NOG mice reconstituted with human immune cells. Mice were treated with control human IgG, sintilimab (0.1, 1 and 10 mg/kg), MDX-1106 and MK-3475. While MDX-1106 and MK-3475 (10 mg/kg) inhibited tumor growth moderately, sintilimab treatment was much more effective. Moreover, tumor growth suppression in the 1 mg/kg sintilimab treatment group was significantly better compared to 10 mg/kg of MDX-1106 (Figure 3a,b).

The superior antitumor response of sintilimab correlated with a stronger increase in the number of CD3+ T cells and CD8+ T cells in the tumor (Figure 3c,d). Accordingly, the



**Figure 2.** Sintilimab showed *in vitro* and *in vivo* higher levels of PD-1 receptor occupancy. Human PBMC were stimulated to express PD-1 before incubation with sintilimab, MDX-1106 or MK-3475. Flow cytometry results showing proportions of CD3+ T cells that bind with different anti-PD-1 mAbs (a) and the mean fluorescence intensity of PD-1 (b). Data are expressed as the means ± SE of three independent experiments. (c) The effects of anti-PD-1 mAbs on mixed lymphocyte reaction (MLR) response. CD4+ T cells isolated from human PBMC were co-cultured with mature monocyte-derived dendritic cells at a ratio of 10:1 in the presence of different concentrations of anti-PD-1 mAbs. Twelve hours later, unbound mAbs was removed. Cells were co-cultured for 4 more days and the concentration of IL-2 in cultural supernatant was detected by Cisbio kit. In NOG mice reconstituted with human immune cells, PD-1 receptor occupancy on circulating CD3+ T cells 24 h (d) and 72 h (e) after anti-PD-1 mAbs intraperitoneal injection at doses of 1, 3 and 10 mg/kg ( $n \geq 3$  mice/group). (f) Mean (± SE) serum concentration-time profiles following a single IV administration of 10 mg/kg sintilimab, MDX-1106 or MK-3475 to hPD-1 knock-in mice ( $n = 3$  animals per group).

**Table 2.** Group mean non-compartmental pharmacokinetic parameters of sintilimab, MDX-1106 and MK-3475 following a single IV administration to hPD-1 knock-in mouse.

Parameter	Sintilimab	MDX-1106	MK-3475
AUC <sub>0-4</sub> (h·µg·mL <sup>-1</sup> )	6597.888	7282.914	6212.197
AUC <sub>inf</sub> (h·µg·mL <sup>-1</sup> )	7846.554	9349.858	8048.861
CL (mL·h <sup>-1</sup> )	0.025	0.021	0.025
C <sub>max</sub> (µg·mL <sup>-1</sup> )	218.519	238.710	224.217
t <sub>1/2</sub> (h)	35.623	43.505	42.453
V <sub>ss</sub> (mL)	1.262	1.299	1.527

AUC<sub>0-96</sub> = area under the serum concentration–time curve from time = 0 to Study Day 5 (PK Day 4 or 96h) AUC<sub>inf</sub> = area under the serum concentration–time curve extrapolated to infinity (AUC<sub>0-t</sub> + C<sub>last</sub>/λ<sub>z</sub>); CL = clearance; C<sub>max</sub> = maximum observed concentration; t<sub>1/2,t</sub> = half-life; V<sub>ss</sub> = volume of distribution at steady state.

ratio of CD8+ to Treg tumor-infiltrating lymphocytes in the sintilimab treatment group was significantly higher than in mice treated with MDX-1106 (Figure 3e). Importantly, we observed a statistically insignificant increase in the total number of interferon (IFN)-γ producing tumor-specific CD8+ T cells in mice treated with sintilimab compared to mice treated with MDX-1106 and MK-3475 (Figure 3f).

### High PD-1 receptor occupancy in patients with advanced solid tumors after sintilimab treatment

Next, we asked if the preclinical receptor occupancy assessment reflected the clinical situation. Nine patients with advanced solid tumors were treated with 1, 3, or 10 mg/kg sintilimab on study day 0. Patient characteristics are summarized in Supplementary Table 1. Circulating CD3+ T cells in patients were evaluated for percent occupancy of PD-1 receptors by sintilimab. As shown in Figure 4, in all dose cohorts, we observed a sustained PD-1 receptor occupancy of more than 95% up to 4 weeks after a single sintilimab IV infusion. The high receptor

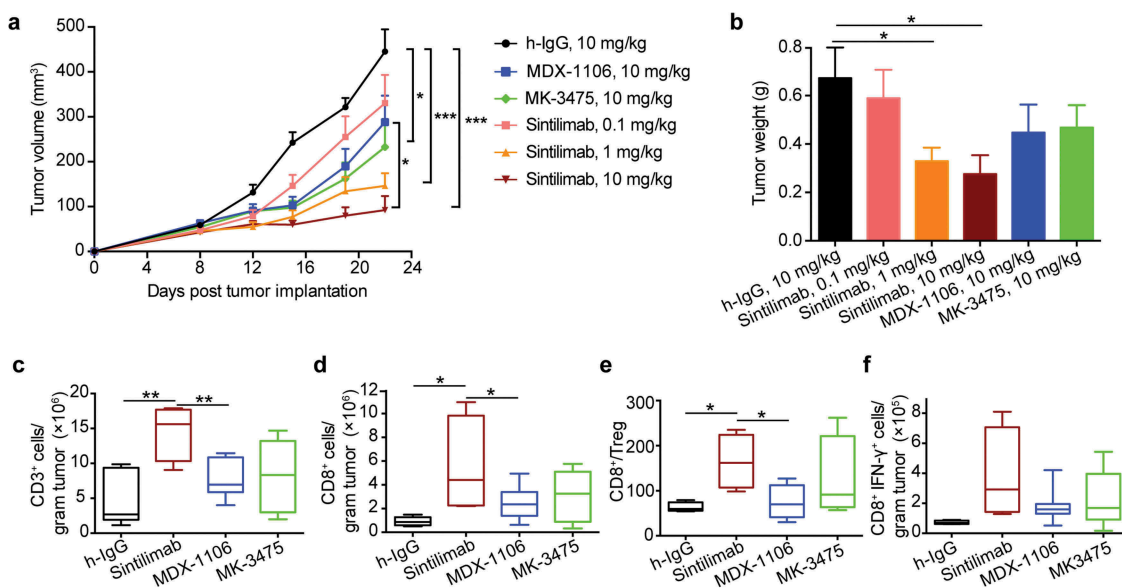
occupancy of sintilimab in patients was consistent with our preclinical data, suggesting even low doses of sintilimab treatment had a sustained, saturated receptor occupancy.

### Immunogenicity of sintilimab in cancer patients

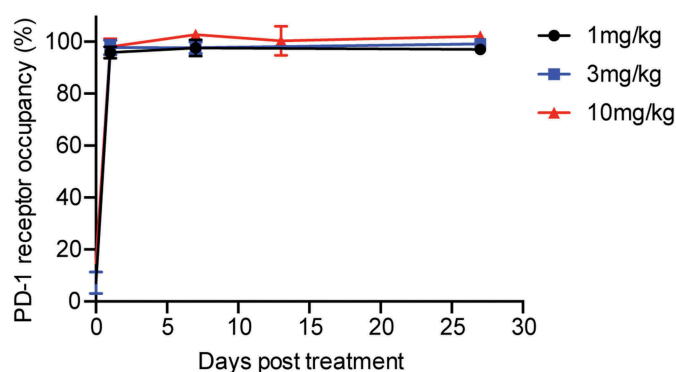
Antibody-based drugs can invoke immunogenicity, leading to the production of ADA and NAb, and influencing their clinical effects. We assessed the immunogenicity of sintilimab in patients from four clinical trials. The incidence of sintilimab-specific antibodies in patients who received sintilimab is presented in Table 3. Of 381 patients treated with sintilimab, 2 patients (0.52%) were detected as ADA-positive after sintilimab infusion. One subject with cholangiocarcinoma from the NCT02937116 study was detected ADA-positive (titer 132.8, NAb-negative) before the second cycle of sintilimab administration, which turned negative before the fourth cycle of administration. There was no causal evidence of an effect on PK (data not shown). One ADA-positive (titer 76.4) patient from the NCT03114683 study was also NAb-positive. An infusion reaction occurred 20 days after the second cycle of administration and the patient developed grade 4 thrombocytopenia and immune-related pneumonia at the same time, which led to withdrawal from study. This patient remained ADA-positive (titer 132.3) and NAb-positive during the safety follow-up period.

### Discussion

Herein, we reported on the preclinical characterization of sintilimab, a fully human IgG4 anti-PD-1 mAb. Sintilimab-bound human PD-1 has a higher affinity and slower off-rate compared with MDX-1106 and MK-3475. Drugs with slow



**Figure 3.** Better tumor control and T cell response of sintilimab treatment in NCI-H292 tumor mouse model reconstituted with human immune cells. Six days after human PBMC intravenous implantation, NCI-H292 tumor cells were implanted subcutaneously into the right flanks of NOG mice. (a) Tumor growth of NCI-H292 tumor-bearing mice treated with human IgG (10 mg/kg), sintilimab (0.1, 1 and 10 mg/kg), MDX-1106 (10 mg/kg) or MK-3475 (10 mg/kg). (b) Tumor weights at the end of the study. Three weeks after NCI-H292 cells implantation, tumors were collected and total counts of CD3+ T cells per gram tumor (c), CD8+ T cells per gram tumor (d), CD8/Treg ratios (e), IFN-γ producing tumor-specific CD8+ cells per gram tumor (f) were quantified ( $n \geq 5$  mice/group).



**Figure 4.** PD-1 receptor occupancy on circulating CD3+ T cells in patients with advanced solid tumors following infusion with sintilimab. Sintilimab was administered at doses of 1, 3 or 10 mg/kg on study day 0. Blood was collected on day 0, 1, 7, 13 and 27.

off-rates occupy the binding site for a long time after the plasma concentration has decreased, potentially giving a longer duration of drug effect.<sup>20,21</sup> Therefore, it is reasonable to speculate that anti-PD-1 mAbs with the longest dissociative half-life can approach complete physiological inhibition. The higher affinity of sintilimab is possibly a result of hydrophobic and aromatic amino acid residues (H: L50, I52, F55, H101 and L: W32, H93, L94, F96) in the CDR of sintilimab.

Anti-PD-1 mAbs bind to PD-1 receptors, blocking immune-suppressing ligands from interacting with PD-1, thus restoring T-cell and immune responses. Therefore, high PD-1 receptor occupancy is likely to result in a better anti-tumor effect and a stronger immune response. Indeed, we observed initially higher PD-1 receptor occupancy in mice injected with sintilimab, and consequently a better tumor control effect. In fact, 1 mg/kg sintilimab infusion in mice was capable of occupying more than 60% of PD-1 molecules on the surface of circulating T cells, which is comparable to that of 10 mg/kg MDX-1106 or MK-3475 infusion. We did not observe any difference in serum concentration between these three mAbs in a preclinical study. Compared with clinical pharmacodynamics data, the receptor occupancy in mouse models is relatively lower. There are at least 2 possible reasons: 1) we used intraperitoneal (IP) injection in mice, but patients received IV injections, and the IP injection may have caused lower absorption efficiency; 2) in two mouse models we used in this study, PBMC are at the stage of rapid proliferation, causing rapid rate of target turnover. This might be another reason for the relatively low RO in mouse models.

To date, preclinical development of antibodies that target immune checkpoints has been based on the use of surrogate, murine-specific antagonist antibodies and syngeneic immune

competent mouse models. These models have demonstrated that blocking the murine PD-1/PD-L1 axis can result in immune-mediated anti-tumor activity.<sup>22,23</sup> However, the use of syngeneic murine-derived cancer models in immune-competent mice has limitations because of the intrinsic differences between the murine and human immune systems.<sup>24,25</sup> We have shown previously that sintilimab significantly inhibits tumor growth in a human PD-1 knock-in mouse model of colon adenocarcinoma.<sup>9</sup> Here, we evaluated the anti-tumor efficacy of anti-PD-1 mAbs in NOG mice reconstructed with human immune cells, which can capture the distinct features of the corresponding human immune system and tumor microenvironment. In this humanized mouse model, we found that both 1 mg/kg and 10 mg/kg sintilimab treatment lead to a strong anti-tumor effect against NCI-H292 tumors. The superior anti-tumor effect of sintilimab treatment compared to MDX-1106 or MK-3475 correlated with an increased number of CD8+ T cells and tumor-specific effector T cells. In accordance with receptor occupancy results, the anti-tumor activity of 1 mg/kg sintilimab treatment is comparable, or even better, than that achieved by 10 mg/kg MDX-1106 or MK-3475 treatment.

In patients with advanced solid tumors, we observed consistently high levels of PD-1 occupancy on circulating T cells for up to 4 weeks after a single sintilimab administration across all concentrations. Of note, even patients treated with 1 mg/kg sintilimab showed more than 95% of receptor occupancy, which is higher than what has been reported for MDX-1106.<sup>14</sup> Until now, we have not addressed the issue of whether the findings of circulating lymphocytes reflect PD-1 occupancy on lymphocytes in tumors and secondary lymphoid organs. Given the fact that T cells continuously redistribute between blood, lymph, and tissues,<sup>26</sup> it is reasonable to speculate that PD-1 occupancy after sintilimab infusion is also high in other tissues besides blood. Indeed, in a simplified subcutaneous NIC-H292 xenograft model, we observed a more than 90% of PD-1 receptor occupancy in tumors 24 h after 10 mg/kg sintilimab injection, which is comparable to that in blood. However, taking into consideration the inefficient penetration and heterogeneous distribution of antibodies in tumor tissue of patients, a relatively higher dose should be selected, although PD-1 occupancy saturation in peripheral blood was reached after 1 mg/kg sintilimab infusion. Sintilimab monotherapy has demonstrated considerable activity in a Phase 2 trial in patients with relapsed and refractory classical Hodgkin's lymphoma at a dose level and schedule of 200 mg every 3 weeks.<sup>5</sup> Our preclinical data suggests sintilimab may have a favorable long-term

**Table 3.** Summary of immunogenicity test results from 4 clinical studies.

	NCT02937116	NCT03114683	NCT03116152	NCT03150875	
	la+lb	II	II	III	
	Solid Tumors	cHL	EC	Sq NSCLC	All studies
N (%)	(N = 206)	(N = 95)	(N = 49)	(N = 31)	(N = 381)
Treatment emergent ADA+	1 (1/206, 0.49)	1 (1/95, 1.05)	0	0	2 (2/381, 0.52)
Treatment emergent NAb+	0	1 (1/95, 1.05)	0	0	1 (1/381, 0.26)
Baseline ADA+	14 (14/206, 6.80)	6 (6/87, 6.90)	2 (2/49, 4.08)	2 (2/31, 6.45)	24 (24/381, 6.30)

effect without the need for repeated high-dose infusion. Additional studies are necessary to determine the proper dose and schedule for different types of tumors.

Evaluating immunogenicity is a critical component in the clinical development of protein-based drugs. The formation of ADAs and NAbs may have profound consequences on PK, as well as safety and efficacy.<sup>27,28</sup> We analyzed samples from 381 patients who participated in 4 clinical trials and found only 0.52% and 0.26% of patients developed ADA and NAbs, respectively. In one of the ADA-positive patients, we did not observe any causal evidence of an effect on PK. Given the low percentage of ADA and NAbs positive patients and low ADA titers in positive patients, sintilimab carried a low risk of immunogenicity.

On the basis of clinical trials for other PD-1/PD-L1 therapies showing that anti-PD-1 is highly synergistic in combination with other treatment methods,<sup>29,30</sup> we anticipate that more effective uses of sintilimab for cancer therapy will involve combination therapies with other agents that boost endogenous anti-tumor immunity. Treatment regimens combining sintilimab with other immunomodulators, chemotherapy, and molecularly targeted therapies are already under evaluation in the laboratory or in clinical trials (Trial identifiers: NCT03794440, NCT03798743, and NCT03765775).

## Materials and methods

### Reagents

Sintilimab was produced by Innovent Biologics Co., LTD (Suzhou, China). Human IgG, MDX-1106, and MK-3475 were purchased from Equitech-Bio (Kerrville, Texas, USA), Bristol-Myers Squibb (New York City, New York, USA), and Merck & Co. (Kenilworth, New Jersey, USA), respectively.

### Mice

All animal experiments were performed in accordance with regulations for care and use of laboratory animals at Innovent Biologics, and were approved by Innovent's Institutional Animal Care and Use Committee. NOG mice were purchased from Vital River Laboratory Animal Technology Co., Ltd (strain: 408; Beijing, China). Human PD-1 knock-in mice were purchased from Shanghai Model Organisms Center, Inc. (strain: C57BL/6J-Pdcd1<sup>em1 (hPDCCD1)/Smoc</sup>; Shanghai, China). All mice were kept in specific pathogen-free conditions.

### Surface plasma resonance analysis of monoclonal antibody binding kinetics

SPR analysis was performed in HBS-EP+ (BR-1006-69, GE Healthcare, Chicago, Illinois, USA) running buffer using the GE Biacore T200. In each running cycle, antibody was captured to a Protein A sensor chip (29127555, GE Healthcare) at 1 µg/ml for 30 s, followed by injection of serial 2-fold dilutions of human PD-1 (with a starting concentration of 40 nM or 10 nM, PD1-H5221, Acro Biosystems Inc., Newark, Delaware, USA) for binding detection as well as running buffer for subtraction. Each concentration of human PD-1

flowed over the antibody with an association time of 180 s and a dissociation time of 600 s. At the end of each cycle, a pulse injection of 10 mM glycine-HCl (pH 1.5, BR-1003-54, GE Healthcare) was used for sensor regeneration. Raw data were processed by Biacore T200 evaluation software version 3.1 using a 1:1 binding model.

### Mesoscale discovery (MSD) assay

A mixture containing 100 µl of Biotin-rhPD-1-His (100 pM, PD1-H82E4, Acro Biosystems) and 100 µl of serial 4-fold mAbs dilutions was incubated in 96-well plates overnight at room temperature. Multi-Array 96-well plates (L15XA-3, Meso Scale Diagnostics, Rockville, Maryland, USA) plates were coated with 100 µl of PBSF (1 × phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA)) containing 20 nM of sintilimab, MDX-1106, or MK-3475 and incubated overnight at 4°C; MSD plates were washed three times with PBST (1 × PBS + 0.5% Tween-20) and blocked with 250 µl of 3% BSA at room temperature for 2 h. Thereafter, plates were washed three times with PBST. Twenty-five µl of the prepared mAb-antigen mixture was pipetted into MSD plates and incubated for 150 s. The plates were then washed three times with PBST and 25 µl of MSD sulfo-tag labeled streptavidin (250 ng/ml, R32AD-5, MSD) was transferred into each well. After thorough washing, 150 µl of 1 × MSD read buffer with surfactant (R92TC-1, MSD) was added into each well. The electrochemiluminescent signal was then measured on the MSD SQ120 instrument.

### In vitro PD-1 receptor occupancy

PBMCs (AllCells, Alameda, California, USA) were activated by human dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) for 48 h to induce PD-1 expression and were then incubated with sintilimab, MDX-1106, or MK-3475 at a concentration of 150 ng/µl. Cells were stained with anti-human CD3 (Cat. #: 300434, Biolegend). The binding of different PD-1 mAbs to PMBC was detected by anti-human-IgG Fc antibody (Cat. #: 409304, Biolegend).

### Mixed lymphocyte reactions

CD4+ T cells were isolated from PBMCs (AllCells, Alameda, California, USA) using EasySep human CD4+ T cells enrichment kit (StemCell Technologies, Vancouver, Canada). Dendritic cells (DCs) were generated by incubating PBMCs (Saily Bio, Shanghai, China) first with IL-4 (1000 U/ml) and GM-CSF (1000 U/ml), followed by maturation in media containing tumor necrosis factor (1000 U/ml), IL-1β (5 ng/ml), IL-6 (10 ng/ml), and prostaglandin E2 (1 µM) for 2 days. 1 × 10<sup>4</sup> DCs and 1 × 10<sup>5</sup> CD4+ T cells were seeded in RPMI medium containing 1 ng/ml Staphylococcal enterotoxin E in a 96-well plate and incubated with serial 4-fold dilutions of anti-PD-1 mAbs (with a starting concentration of 100 nM overnight. Dissociated antibodies were then washed away. The concentration of IL-2 in culture supernatants was measured by a Cisbio kit (Bedford, Massachusetts, USA) 4 days later.

### **PD-1 receptor occupancy in NOG mice**

Human PBMCs were activated by dynabeads Human T-Activator CD3/CD28 for 48 h to induce PD-1 expression.  $2.5 \times 10^6$  of activated human PBMCs were injected (IV) into female NOG mice. Nine days later, PBMCs were collected and co-stained with anti-human CD3 (Cat. #: 300434, Biolegend) and a commercial anti-human PD-1 antibody (Cat. #: 367404, Biolegend) and detected with flow cytometric. One day later, mice were injected IP with 1 mg/kg, 3 mg/kg and 10 mg/kg of anti-PD-1 mAbs. Twenty-four hours after injection of the mAbs, PBMCs were collected and stained with anti-human CD3 (Cat. #: 409304, Biolegend) and anti-human IgG Fc antibody (Cat. #: 100341, Biolegend). PD-1 receptor occupancy was estimated as the ratio of CD3+ T cells stained positive with anti-human IgG Fc antibody to CD3+ T cells positive with the commercial anti-PD-1 (before PD-1 mAbs injection, indicating total available binding sites).

To compare the PD-1 receptor occupancy in blood and tumor, NCI-H292 tumor cells (ATCC CRL-1848,  $5 \times 10^6$ ) were implanted subcutaneously into the right flank of female NOG mice ( $n = 4$ ) 6 days after human PBMC ( $2.5 \times 10^6$ ) IV injection. Three weeks after tumor cells implantation, mice were injected IP with 10 mg/kg sintilimab. Twenty-four hours later, aliquots of single-cell suspensions from blood and tumor were preincubated (30 min at 4°C) with a saturating concentration (20  $\mu$ g/mL) of sintilimab or left untreated. After extensive washing, samples were co-stained with anti-human CD3 (Cat. #: 300434, Biolegend) and anti-human IgG Fc antibody (Cat. #: 409306, Biolegend). PD-1 occupancy in CD3+ T cells was calculated as the ratio of cells positive for anti-human IgG Fc to that saturated with sintilimab.

### **PD-1 receptor occupancy in human PD-1 knock-in mice**

Mice (4 mice per group) were injected IP with 10  $\mu$ g IL-2 to induce PD-1 expression. Five days later, mice were dosed with PBS or 1 mg/kg, 3 mg/kg and 10 mg/kg of anti-PD-1 mAbs by IP injection. Twenty-four hours later, PBMCs were collected and co-stained with anti-mouse CD3 (Cat. #: 100206, Biolegend) and a commercial anti-human PD-1 (Cat. #: 8004666, BD Bioscience, for mice injected with PBS) or anti-mouse CD3 and anti-human-IgG Fc (Cat. #: 409306, BD Bioscience, for mice injected with anti-PD-1 mAbs). PD-1 receptor occupancy was estimated as the ratio of percent cells stained positive with anti-human-IgG Fc to percent cells stained positive with the commercial anti-human PD-1 in the PBS group (indicating total available binding sites).

### **Pharmacokinetic study in human PD-1 knock-in mice**

Female human PD-1 knock-in mice were injected IV with 10 mg/kg anti-PD-1 mAbs via tail vein. Blood samples were collected from each mouse at the following time points ( $n = 3$ / time point): 5 min, 30 min, 2 h, 6 h, 24 h, 48 h and 96 h after mAbs injection) and processed for serum and ELISA detection. PK analysis was conducted using a non-compartmental model of PKSolver program.

### **Tumor model and treatment**

Six days after human PBMC ( $2.5 \times 10^6$ ) IV injection, NCI-H292 tumor cells (ATCC CRL-1848,  $5 \times 10^6$ ) were implanted subcutaneously into the right flank of female NOG mice. Human IgG (10 mg/kg), sintilimab (0.1 mg/kg, 1 mg/kg and 10 mg/kg), MDX-1106 (10 mg/kg) and MK-3475 (10 mg/kg) were injected IP 1 day, 8 days, 12 days, and 15 days after tumor cell implantation. Tumor growth was monitored by caliper measurements in units. Tumor volume was calculated using the formula: length  $\times$  width<sup>2</sup>/2.

### **Flow cytometry analysis of tumor infiltrating cells**

Flow cytometry analysis of single-cell suspensions from tumors was performed using anti-human CD3 (Cat. #:300434, Biolegend), anti-human CD8 (Cat. #: 344732, Biolegend), anti-human FoxP3 (Cat. #: 320014, Biolegend), anti-human CD4 (Cat. #: 552838, BD Pharmingen, BD Biosciences) and anti-human IFN- $\gamma$  (Cat. #: 562988, BD Horizon, BD Biosciences). Staining of intracellular FoxP3 and IFN- $\gamma$  were performed following the manufacturer's instructions (eBioscience, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Before intracellular IFN- $\gamma$  staining, lymphocytes were incubated with IFN- $\gamma$ -stimulated (50 IU/ml; 48 h) NCI-H292 cells for 6 h at 37°C in the presence of brefeldin A (Biolegend). Cells were analyzed using a BD FACSCelesta (BD Biosciences) flow cytometer with FlowJo software (FlowJo, LLC, Ashland, Oregon, USA).

### **PD-1 receptor occupancy in human patients**

PD-1 receptor occupancy evaluation was performed with blood samples obtained from 9 patients enrolled in clinical study CIBI308A101 (NCT02937116). Patients diagnosed with advanced solid tumor were treated with IV sintilimab (1, 3, or 10 mg/kg) on day 0, day 28, and once every 2 weeks thereafter. Receptor occupancy of sintilimab on circulating CD3 T cells was investigated by flow cytometry of blood samples collected on day 0, day 1 (post-sintilimab injection), day 7, 13 and 27 (before sintilimab injection). Briefly, peripheral blood aliquots were pre-incubated (30 min at 37°C) with a saturating concentration (30  $\mu$ g/mL) of sintilimab or human IgG4. After extensive washing with BSA, samples were co-stained with anti-CD3 fluorescein isothiocyanate (Cat. #: 555339, BD Biosciences) and biotin mouse anti-human IgG4 (Cat. #: 555879, BD Biosciences) plus APC-streptavidin. PD-1 occupancy in CD3 + T cells was calculated as the ratio of cells positive for anti-human IgG4 (of aliquot saturated with IgG4) to that saturated with sintilimab.

### **Immunogenicity characterization**

Ongoing immunogenicity evaluations have been performed with samples from 381 patients enrolled in 4 clinical studies (Trial identifiers: NCT02937116, NCT03116152, NCT03114683, and NCT03150875). Patients were treated with 200 mg of sintilimab IV every 3 weeks. Three hundred and eighty-one patients were included, with a maximum of 38 treatment cycles for a total of 1514 samples. A validated, qualitative electrochemiluminescence

(ECL) bridging immunoassay was used to detect the presence of ADA in patient serum samples. All analyses were performed using a three-tiered testing approach (screen, confirm, and titer) in accordance with applicable guidelines.<sup>31,32</sup> ADA-positive samples were further tested for the presence of NAb using a validated competitive ligand binding assay. The overall immunogenicity incidence was defined as the proportion of patients detected ADAs/NAbs positive to the total number of evaluable patients.

### Statistical analyses

Results are presented as mean  $\pm$  standard error means (SEM). Statistical analyses were performed using GraphPad Prism 6.0 statistical software (GraphPad Software Inc., San Diego, California, USA). A  $p$ -value of  $<0.05$  was considered significant ( $*p < .05$ ,  $**p < .01$  and  $***p < .001$ ).

### Disclosure of potential conflicts of interest

Haiping Jiang has no potential conflicts of interest to disclose. All other authors are employees of Innovent Biologics (Suzhou), who are commencing the commercialization of Sintilimab.

### ORCID

Zhihai Wu  <http://orcid.org/0000-0001-7293-9250>

Weiwei Wu  <http://orcid.org/0000-0001-5605-6837>

Dechao Yu  <http://orcid.org/0000-0003-0563-3248>

### References

- Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol*. 1996;8:765–72. doi:10.1093/intimm/8.5.765.
- Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo J*. 1992;11:3887–95.
- Marin-Acevedo JA, Dholaria B, Soyano AE, Knutson KL, Chumsri S, Lou Y. Next generation of immune checkpoint therapy in cancer: new developments and challenges. *J Hematol Oncol*. 2018;11:39. doi:10.1186/s13045-018-0582-8.
- Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. 2013;13:227–42. doi:10.1038/nri3405.
- Shi Y, Su H, Song Y, Jiang W, Sun X, Qian W, Zhang W, Gao Y, Jin Z, Zhou J, et al. Safety and activity of sintilimab in patients with relapsed or refractory classical Hodgkin lymphoma (ORIENT-1): a multicentre, single-arm, phase 2 trial. *Lancet Haematol*. 2019;6:e12–e9. doi:10.1016/S2352-3026(18)30192-3.
- Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature*. 2014;515:563–67. doi:10.1038/nature14011.
- Tan DSW. Changing the natural history of non-small-cell lung cancer through upfront programmed death protein 1/programmed death-ligand 1 blockade. *J Clin Oncol*. 2017;35:2735–36. doi:10.1200/JCO.2017.73.6215.
- Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, Schuster SJ, Millenson MM, Cattray D, Freeman GJ, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2015;372:311–19. doi:10.1056/NEJMoa1411087.
- Zhang S, Zhang M, Wu W, Yuan Z, Tsun A, Wu M, Chen B, Li J, Miao X, Miao X, et al. Preclinical characterization of sintilimab, a fully human anti-PD-1 therapeutic monoclonal antibody for cancer. *AntibodyTherapeutics*. 2018;1:65–73.
- Wang C, Thudium KB, Han M, Wang XT, Huang H, Feingersh D, Garcia C, Wu Y, Kuhne M, Srinivasan M, et al. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer Immunol Res*. 2014;2:846–56. doi:10.1158/2326-6066.CIR-14-0040.
- Homet Moreno B, Ribas A. Anti-programmed cell death protein-1/ligand-1 therapy in different cancers. *Br J Cancer*. 2015;112:1421–27. doi:10.1038/bjc.2015.124.
- Armand P, Engert A, Younes A, Fanale M, Santoro A, Zinzani PL, Timmerman JM, Collins GP, Ramchandren R, Cohen JB, et al. Nivolumab for relapsed/refractory classic hodgkin lymphoma after failure of autologous hematopoietic cell transplantation: extended follow-up of the multicohort single-arm phase II checkmate 205 trial. *J Clin Oncol*. 2018;36:1428–39. doi:10.1200/JCO.2017.76.0793.
- Chen R, Zinzani PL, Fanale MA, Armand P, Johnson NA, Brice P, Radford J, Ribrag V, Molin D, Vassilakopoulos TP, et al. Phase II study of the efficacy and safety of pembrolizumab for relapsed/refractory classic Hodgkin lymphoma. *J Clin Oncol*. 2017;35:2125–32. doi:10.1200/JCO.2016.72.1316.
- Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010;28:3167–75. doi:10.1200/JCO.2009.26.7609.
- Talotta R, Rucci F, Canti G, Scaglione F. Pros and cons of the immunogenicity of monoclonal antibodies in cancer treatment: a lesson from autoimmune diseases. *Immunotherapy*. 2019;11:241–54. doi:10.2217/imt-2018-0081.
- Sathish JG, Sethu S, Bielsky MC, de Haan L, French NS, Govindappa K, Green J, Griffiths CEM, Holgate S, Jones D, et al. Challenges and approaches for the development of safer immunomodulatory biologics. *Nat Rev Drug Discov*. 2013;12:306–24. doi:10.1038/nrd3974.
- Schellekens H, Jiskoot W. Eprex-associated pure red cell aplasia and leachates. *Nat Biotechnol*. 2006;24:613–14. doi:10.1038/nbt0606-613.
- Agrawal S, Statkevich P, Bajaj G, Feng Y, Saeger S, Desai DD, Park J-S, Waxman IM, Roy A, Gupta M. Evaluation of immunogenicity of nivolumab monotherapy and its clinical relevance in patients with metastatic solid tumors. *J Clin Pharmacol*. 2017;57:394–400. doi:10.1002/jcph.818.
- van Vugt M, de Greef R, Freshwater T, Mangin E, van Aarle F, AK. Immunogenicity of pembrolizumab (pembro) in patients (pts) with advanced melanoma (MEL) and non-small cell lung cancer (NSCLC): pooled results from KEYNOTE-001, 002, 006, and 010. *J Clin Oncol*. 2016;34:3063. doi:10.1200/JCO.2016.34.15\_suppl.3063.
- Berezov A, Zhang HT, Greene MI, Murali R. Disabling erBB receptors with rationally designed exocyclic mimetics of antibodies: structure-function analysis. *J Med Chem*. 2001;44:2565–74. doi:10.1021/jm000527m.
- Copeland RA, Pompliano DL, Meek TD. Drug-target residence time and its implications for lead optimization. *Nat Rev Drug Discov*. 2006;5:730–39. doi:10.1038/nrd2082.
- Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793–800. doi:10.1038/nm730.
- Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, Rietz C, Flies DB, Lau JS, Zhu G, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res*. 2005;65:1089–96.
- Rongvaux A, Takizawa H, Strowig T, Willinger T, Eynon EE, Flavell RA, Manz MG. Human hemato-lymphoid system



- mice: current use and future potential for medicine. *Annu Rev Immunol.* 2013;31:635–74. doi:10.1146/annurev-immunol-032712-095921.
25. Shay T, Jovic V, Zuk O, Rothamel K, Puyraimond-Zemmour D, Feng T, Wakamatsu E, Benoist C, Koller D, Regev A. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proc Natl Acad Sci U S A.* 2013;110:2946–51. doi:10.1073/pnas.1222738110.
  26. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, Gherardini PF, Prestwood TR, Chabon J, Bendall SC, et al. Systemic immunity is required for effective cancer immunotherapy. *Cell.* 2017;168:487–502 e15. doi:10.1016/j.cell.2016.12.022.
  27. Hoofring SA, Lopez R, Hock MB, Kaliyaperumal A, Patel SK, Swanson SJ, Chirmule N, Starcevic M. Immunogenicity testing strategy and bioanalytical assays for antibody-drug conjugates. *Bioanalysis.* 2013;5:1041–55. doi:10.4155/bio.13.10.
  28. Sauerborn M, van Dongen W. Practical considerations for the pharmacokinetic and immunogenic assessment of antibody-drug conjugates. *BioDrugs.* 2014;28:383–91. doi:10.1007/s40259-014-0096-z.
  29. Johnson CB, Win SY. Combination therapy with PD-1/PD-L1 blockade: an overview of ongoing clinical trials. *Oncoimmunology.* 2018;7:e1408744. doi:10.1080/2162402X.2017.1408744.
  30. Gotwals P, Cameron S, Cipolletta D, Cremasco V, Crystal A, Hewes B, Mueller B, Quarantino S, Sabatos-Peyton C, Petruzzelli L, et al. Prospects for combining targeted and conventional cancer therapy with immunotherapy. *Nat Rev Cancer.* 2017;17:286–301. doi:10.1038/nrc.2017.17.
  31. Shankar G, Devanarayan V, Amaravadi L, Barrett YC, Bowsher R, Finco-Kent D, Fiscella M, Gorovits B, Kirschner S, Moxness M, et al. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal.* 2008;48:1267–81. doi:10.1016/j.jpba.2008.09.020.
  32. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research. Immunogenicity testing of therapeutic protein products -developing and validating assays for anti-drug antibody detection: guidance for industry. 2019.