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

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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-IL2rgamma (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®, MDX-1106) and pembrolizumab (Keytruda®, 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-y-secreting, tumor-specific CD8+ T cells, but not with CD4+ Tregs in tumor tissue. Pharmacodynamics testing indicated a sustained mean occupancy of ≥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.

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 is one of the major pathways exploited by cancer cells for immune evasion. The PD-1/ligand interactions strongly counteract T cell receptor (TCR) signal transduction and subsequently attenuate cytokine production, T-cell survival, and proliferation. Therapeutic antibodies blocking immune checkpoints restore anti-tumor immunity and lead to durable tumor regression and prolonged survival in some patients. Several antibodies targeting PD-1, such as MDX-1106 (Opdivo®, nivolumab) and MK-3475 (Keytruda®, 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. 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%, which is superior to MDX-1106 (69%) and MK-3475 (69%). 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

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Sintilimab is reported to be >70% and >95%, respectively.\(^5\)\(^,\)\(^14\) 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.\(^5\)\(^,\)\(^14\) Besides neutralizing therapeutic effects, immunogenicity can also cause life-threatening complications, such as anaphylaxis and immune complex-mediated disease.\(^15\)\(^,\)\(^17\) 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.\(^18\) Among 2910 MK-3475 treated patients, 1.7% had a treatment-emergent-positive ADA.\(^19\)

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-IL2\(^{\gamma}\) (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

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**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.
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

### 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-11 | 0.0266 |

### 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 culture 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 ≥ 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).
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).

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_{0-4} (h·μg·mL\(^{-1}\)) | 6597.888   | 7282.914 | 6212.197|
| AUC_{inf} (h·μg·mL\(^{-1}\)) | 7846.554   | 9349.858 | 8048.861|
| CL (mL·h\(^{-1}\))     | 0.025      | 0.021    | 0.025   |
| Cmax (μg·mL\(^{-1}\)) | 218.519    | 238.710  | 224.217 |
| t1/2 (h)                | 35.623     | 43.505   | 42.453  |
| Vss (mL)               | 1.262      | 1.299    | 1.527   |

AUC_{0-4} = area under the serum concentration–time curve from time = 0 to Study Day 5 (PK Day 4 or 96h); AUC_{inf} = area under the serum concentration–time curve extrapolated to infinity (AUC_{0-4} + Cmax/λz). CL = clearance; Cmax = maximum observed concentration; t1/2 = half-life; Vss = volume of distribution at steady state.

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
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-1106.14 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,26 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 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.

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

| Study ID          | Solid Tumors | cHL | EC | Sq NSCLC | All studies |
|-------------------|--------------|-----|----|----------|-------------|
|                   | N (%):IV     | N (%) | N (%) | N (%) | N (%)       |
| NCT02937116       | (N = 206)    | (N = 95) | (N = 49) | (N = 31) | (N = 381)   |
| Treatment emergent ADA+ | 1 (1/206, 0.49) | 0 | 0 | 2 (2/381, 0.52) |
| Treatment emergent NAb+ | 14 (14/206, 6.80) | 6 (6/67, 6.90) | 2 (2/49, 4.08) | 24 (24/381, 6.30) |
| Baseline ADA+     | 14 (14/206, 6.80) | 6 (6/67, 6.90) | 2 (2/49, 4.08) | 24 (24/381, 6.30) |

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.
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.27,28 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,29,30 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, chemother-apy, 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–Pdcd1tm1(Smo)Shoc, 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 nM 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 PBMC 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 × 10^4 DCs and 1 × 10^5 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 × 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 cytometry. 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 × 10^6) were implanted subcutaneously into the right flank of female NOG mice (n = 4) 6 days after human PBMC (2.5 × 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 μg/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 μg 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) and anti-human-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 μg/mL) of sintilimab or human IgG4. After extensive washing with BSA, samples were co-stained with anti-CD3 fluorescein isothiocyanate (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.

**Tumor model and treatment**

Six days after human PBMC (2.5 × 10^6) IV injection, NCI-H292 tumor cells (ATCC CRL-1848, 5 × 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 × width^2/2.

**Flow cytometry analysis of blood and 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, BD Pharmingen, BD Biosciences) and anti-human IFN-γ (Cat. #: 652988, BD Horizon, BD Biosciences). Flow cytometry analysis of tumor infiltrating cells using anti-PD-1 mAbs). PD-1 receptor occupancy in human patients was performed using anti-human CD3 (Cat. #:300434, Biolegend), anti-human CD8 (Cat. #: 344732, BD Pharmingen, BD Biosciences) and anti-human IFN-γ (Cat. #: 562988, BD Horizon, BD Biosciences). Intra- and extracellular IFN-γ staining, lymphocytes were incubated with IFN-γ-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 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.

**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.

**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.\textsuperscript{31,32} 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 ± 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 < 0.05, **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.

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