Large-cohort humanized NPI mice reconstituted with CD34+ hematopoietic stem cells are feasible for evaluating preclinical cancer immunotherapy

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Abstract
Cancer immunotherapy has achieved impressive therapeutic effects in many cancers, while only a small subset of patients benefit from it and some patients even have experienced severe toxicity. It is urgent to develop a feasible large-cohort humanized mouse model to evaluate the pre-clinical efficacy and safety of cancer immunotherapy. Furthermore, developing potentially effective combination therapy between cancer immunotherapy and other therapies also needs humanized mouse model to adequately mimic clinical actual setting. Herein, we established a humanized mouse model engrafted with less human CD34+ HSCs than ever before and then evaluated reconstitution efficiency and the profiles of human immune cells in this humanized mouse model. Also, this humanized mouse model was used to evaluate the preclinical efficacy and safety of cancer immunotherapy. For each batch of CD34+ HSCs humanized mouse model, a relatively-large cohort with

Abbreviations: ALL, acute lymphoblastic leukemia; CAR-T, chimeric antigen receptor T cells; CDX, cell line-derived xenografts; CRC, colorectal cancer; CRS, cytokine release syndrome; CTLA4, cytotoxic T lymphocyte-associated antigen 4; FDA, Food and Drug Administration; GvHD, graft-versus-host disease; HSC, hematopoietic stem cells; Hu-NPI, humanized NPI; NOD, non-obese diabetic; NPI, NOD. Prkdc-/-; Il2rg-/-; NSCLC, non-small cell lung cancer; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; PDAC, pancreatic duct adenocarcinoma; PD-L1, programmed death ligand-1; PDX, patient-derived xenografts.

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INTRODUCTION

Checkpoint inhibitors, adoptive cell therapy, cancer vaccine, and monoclonal antibody therapy are among the most common cancer immunotherapies available so far.\(^1\^,\(^2\)

Cancer immunotherapy has emerged as a promising treatment strategy for a variety of cancers, especially recurrent or advanced metastatic cancers in recent decades.\(^3\^,\(^4\)

Due to the encouraging therapeutic effects, several cancer immunotherapy approaches have been approved by Food and Drug Administration (FDA) to treat cancers, such as checkpoint inhibitors (e.g., anti-CTLA4 and anti-PD-(L)1), chimeric antigen receptor T cells (CAR-T), and bispecific antibody (e.g., Blinatumomab).\(^5\^\text{-}^8\)

While about 20% of patients experienced remarkable tumor regressions after anti-PD-(L)1 therapy, a significant proportion of patients failed to respond to anti-PD-(L)1 immunotherapy,\(^1\)\text{-}\(^8\) and some even experienced serious side-effects.\(^9\)

Adaptive T-cell transfer therapies, including CAR-T, tumor-infiltrating lymphocytes (TILs), and genetically engineered T cell receptor (TCR) T cells, are promising and innovative therapeutic modalities for cancer patients, with precise targets and impressive efficacy.\(^2\)\text{-}\(^3\) For CAR-T cell therapy, its excellent efficiency in hematological malignancies leads to the FDA approvals of CD19-directed CAR-T cell therapy for relapsed/refractory acute B-cell lymphoblastic leukemia and diffuse large B cell lymphoma (DLBCL).\(^6\)\text{-}\(^7\)

However, low efficacy in solid tumors and its severe adverse events, ranging from tumor lysis syndrome, cytokine releasing syndrome, to fatal neurotoxicity, limit the clinical application of CAR-T cell therapy.\(^9\)\text{-}\(^10\)

Hence, it is of great importance to screen and identify the patients who will benefit most from cancer immunotherapy and at the same time have low risks of side effects. Usually, the biomarkers such as PD-L1 expression, high microsatellite instability (MSI), and high total mutation burden (TMB) are used to predict the patients who might benefit from anti-PD-(L)1 immunotherapy.\(^11\)\text{-}\(^12\)

Humanized mouse model bearing human immune cells is a good model for evaluation of the therapeutic effects and the potential side-effects of cancer immunotherapies.\(^13\)\text{-}\(^14\)

Furthermore, there is also an urgent requirement for a humanized mouse model that can adequately mimic and reflect the clinical tumor setting so as to perform preclinical evaluation and minimize failures of clinical trials, which is critical for the development of novel cancer immunotherapy or combination strategies, and a humanized mouse model with a large cohort will greatly promote preclinical evaluation of cancer immunotherapy.

Reconstitution of human immune cells in immunodeficient mice (such as NSG or NOG mice) is mainly through engraftment of human peripheral blood mononuclear cells (PBMCs) or CD34\(^+\) hematopoietic stem cells (HSC).\(^15\)

In the human PBMCs engrafted humanized mice, human T cells with activated phenotype are quickly reconstituted within 3–5 days but the mice experience lethal xeno-graft-versus-host disease (GvHD) 4–6 weeks after PBMCs injection.\(^15\)\text{-}\(^17\)

Thus, the therapeutic observational window...
in PBMC humanized mouse model is restricted to a few weeks, which largely limits its application in the preclinical evaluation of cancer immunotherapy. In the human CD34+ HSC engrafted mice, human CD34+ HSCs from various sources such as human umbilical cord blood, adult bone marrow, or fetal liver are transplanted into newborn, young, or adult immunodeficient mice receiving myeloablative conditioning by sublethal γ-irradiation or busulfan injection. Although all human hematopoietic lineages are represented in CD34+ HSC humanized mouse model, not all lineages are functionally fully developed. CD4+ T cells show a memory phenotype, and both T and NK cells display some functional defects, maybe owing to T cells development in the mouse thymic environment. Co-transplantation of the human fetal liver with human thymus provides a human thymic microenvironment that supports the development of functional human T cells; however, it leads to a higher incidence of GvHD than that in human CD34+ HSCs-engrafted mouse models without co-transplantation of human thymus. Furthermore, in HSCs humanized mice, it is unclear how reconstituted human T cells interact with human tumors implantation from the patient and whether T cells possess anti-tumor activity in HSCs humanized mouse model. HSCs humanized mice have been successfully used in the preclinical study of checkpoint blockade, but whether in a humanized mouse model there are enough mice used for preclinical researches of various cancer immunotherapies with different mechanisms of action, such as anti-PD-1, CAR-T cells, and bispecific antibody, is rarely addressed. Hence, it is of actual importance to develop a humanized mouse model with a relatively-large cohort.

In the present study, we established a HSCs humanized mouse model with a relatively-large cohort by transplanting the low amount of human CD34+ HSCs (1 × 10^4 per mouse) into busulfan-conditioned young NOD. Prkdc-/- Il2rg-/- (NPI) mice. This humanized NPI mouse model can support the infiltration of human T cells into human tumors. Furthermore, this humanized NPI mouse model can be used for the preclinical studies of checkpoint blockade, CAR-T cells, and bispecific antibody, with in vitro and in vivo anti-tumor T cell activities.

## 2 MATERIALS AND METHODS

### 2.1 Mice and establishment of a humanized mouse model

NPI (NOD. Prkdc-/- Il2rg-/-) mice that were established from NOD mice by knocking out both Prkdc and Il2rg genes through CRISPR-Cas9 were obtained from Beijing IDMO Co., Ltd. NPI strain was similar to M-NSG strain (Shanghai Model Organisms Center, Inc.) and both lacked T cells, B cells and NK cells (Figures S1 and S2). All the animals were housed in an environment with a temperature of 22 ± 1°C, relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 h. Human umbilical cord blood cells were harvested from Changhai Hospital after written informed consent. Lin− CD34+ HSCs from fresh human umbilical cord blood were isolated according to the reference with minor modification. Briefly, mononuclear cells were depleted of Lin+ cells using biotin anti-hCD3, anti-hCD11b, anti-hCD19, and anti-hCD56 (Biolegend, San Diego, CA) and strepavidin-microbeads (Miltenyi, Germany) by negative selection. Then cells were further stained with anti-hCD34 antibody (Biolegend) and Lin− CD34+ HSCs were sorted by fluorescence-activated cell sorting (FACS) using a BD FACS ARIA II sorter (BD Bioscience, San Jose, CA). Lin− CD34+ HSCs (1 × 10^4 per mouse) were intravenously transplanted into 3~4-week-old female NPI mice, which were pre-conditioned with busulfan for depletion mouse myeloid cells. Busulfan (Sigma–Aldrich) was dissolved in DMSO (WAK-Chemi) for 25 mg/ml stock concentration and stored at −20°C and diluted with PBS when used. Busulfan solution was intraperitoneally (i.p.) injected into NPI mice (25 mg/kg body weight) 24 h prior to transplantation. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Changhai Hospital of Shanghai institutional animal care and were conducted according to the AAALAC and the IACUC guidelines.

### 2.2 Patient tumor samples

Patient tumor samples were obtained from surgical specimens of colorectal cancer (CRC), non-small cell lung cancer (NSCLC), and pancreatic duct adenocarcinoma (PDAC) patients at Changhai Hospital after written informed consent. During operation, sufficient tumor tissues were resected and immediately placed in serum-free RPMI 1640 media at 4°C for xenografting. This study was done with the approval of the Ethics Committee at the Changhai Hospital. In addition, all patients gave their permission for using their tumor specimens for the research purpose.

### 2.3 Establishment of CDX or PDX tumor model

Humanized NPI mice were used to establish cell line-derived xenografts (CDX) or patient-derived xenografts (PDX) 16~20 weeks after HSCs transplantation. Human triple-negative breast cancer cell line MDA-MB-231...
(ATCC) or human acute lymphoblastic leukemia cell line NALM6 (ATCC) was used for CDX tumor implantation. CDX tumor model was constructed as follows: 5 × 10^6 MDA-MB-231 cells, suspended in serum-free RPMI 1640 media mixed with matrigel (Corning) in 1:1, were subcutaneously injected into mammary fat pads of humanized NPI mice; or 1 × 10^6 NALM6 cells, suspended in serum-free RPMI 1640 media, were intravenously injected into humanized NPI mice. Patient fresh or passaged/cryopreserved tumor samples were used for PDX tumor xenografts. PDX tumor models were established by implantation of PDX tumor xenografts (passage 1 or 2) into humanized NPI mice as described previously.25

2.4 | In vivo tumor checkpoint inhibitor immunotherapy experiments

When CDX or PDX tumors in humanized NPI mice reached 120 mm³, checkpoint inhibitor immunotherapy was started. In the isotype or vehicle control group, isotype antibody (10 mg/kg body weight) or saline was injected intraperitoneally every 5 days until the study endpoint. Anti-CD19 (Pembrolizumab, Merck), anti-PD-L1 (Atezolizumab, Roche), or anti-CTLA4 (Ipilimumab, Bristol-Myers Squibb) was injected intraperitoneally at 10 mg/kg every 5 days until the study endpoint. The tumor was measured by caliper every 2–4 days and tumor volumes (mm³) were calculated by length*width²/2. The study endpoint was a tumor volume of 1500 mm³ in the control group.

2.5 | Construction and in vitro expansion of engineered CAR-T cells

Human CD3+ T cells were isolated from spleens of 16–20 weeks reconstituted humanized NPI mice and activated with anti-human CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a 1:1 bead: cell ratio in the presence of hIL-7/hIL-15 (500 U/ml, R&D systems) for 24 h. Then, T cells were transfected with lentivirus (LV) containing CAR genes including CD19 and 4-1BB. After 12 days, expanded engineered CAR-T cells were harvested and used for in vitro coculture with target cells or infusion into other humanized NPI mice reconstituted with CD34+ HSCs from the same donor.

2.6 | Coculture of CAR-T cells and target cells

Engineered CD19 CAR-T cells were co-cultured with CD19 expressing K562 target cells at 1:1 (Effector:Target ratio) in 96-well plates (U bottom). After 16 h, cell supernatant was harvested for IFN-γ CBA assays (BD bioscience) according to the manufacturer’s instructions.

2.7 | In vivo function of CAR-T cells depleting target CD19 B cells

Various numbers of engineered CD19 CAR-T cells (3 × 10^6 or 5 × 10^6) from humanized NPI mice were infused intravenously into humanized NPI mice from the same donor. Mock-transduced T cells (Mock-T, 3 × 10^5) group were used as control. At various time points after cell infusion, the percentage of CD19+ B cells in peripheral blood human CD45+ cells was assayed by flow cytometry.

2.8 | Establishment and validation assays of the safety switch in CAR-T cells

The safety switch, i.e., truncated EGFR was introduced into CD19 CAR by lentivirus vectors. Then, engineered tEGFR+ CD19 CAR-T cells from humanized NPI mice were infused into other humanized NPI mice from the same donor. When all the CD19+ B cells were depleted in peripheral blood (about 7 days after CAR-T infusion), cetuximab (10 mg/kg) was i.p administrated and the copy number of CAR-T cells was determined by Q-PCR in 30 ng genomic DNA on different time points.

2.9 | In vivo anti-tumor function of CAR-T cells

Various numbers of engineered CD19 CAR-T cells (3 × 10^5, 5 × 10^6 or 1 × 10^7) from humanized NPI mice were infused intravenously into ALL cell line NALM6 CDX bearing other humanized NPI mice from the same donor. PBS group and mock-transduced T cells (Mock-T, 5 × 10^6) group were used as control. At various time points after cell infusion, body weight and body temperature were detected and tumor growth was monitored by in vivo bioluminescence imaging. The survival curve of mice in different groups was drawn. And the percentage of CD19+ B cells in peripheral blood human CD45+ cells was assayed by flow cytometry and serum cytokines were assayed by CBA (BD bioscience).

2.10 | In vivo function of bispecific anti-CD19/CD3 antibody Blinatumomab

Bispecific anti-CD19/CD3 Blinatumomab (10 mg/kg) was i.v administrated to humanized NPI mice 16 weeks after
reconstituted and the percentage of CD20+ B cells in peripheral blood was assayed by flow cytometry on different time points. As a positive control, single-dose anti-CD20 Rituximab (10 mg/kg) was i.v administrated to humanized NPI mice and the percentage of CD19+ B cells in peripheral blood was assayed by flow cytometry.

### 2.11 | Multiplex immunohistochemistry staining

Multiplex immunohistochemistry staining was performed using the method described previously. Briefly, PDX tumor tissues from tumor-bearing humanized NPI mice were fixed in 10% formalin for 24 h and were then embedded in paraffin. The tissue sections with the thickness of 5 μm were deparaffinized, tissues were fixed with formaldehyde: methanol (1:10) and then antigen retrieval was performed in heated citric acid buffer (pH 6.0). The tissue sections were put through four sequential rounds of staining, each including a protein blocking with 1% BSA followed by primary antibody (listed in Table S1) and corresponding secondary horseradish peroxidase (HRP)-conjugated polymer. Each HRP-conjugated polymer mediated the covalent binding of a different fluorophore through tyramide signal amplification (TSA) using an Opal IHC kit (Perkin Elmer) according to the manufacturer's instructions. This covalent reaction was followed by additional antigen retrieval in heated citric acid buffer (pH 6.0) for 15 min to remove bound antibodies before the next step in the sequence. After all four sequential reactions, the sections were counterstained with DAPI and mounted with a fluorescence mounting medium. Multiplex stained tissue sections were imaged using the multispectral imaging system (Vectra version 3, Perkin Elmer). Then the images with spectral unmixing were obtained using inForm 2.1 image analysis software (Perkin Elmer).

### 2.12 | Isolation of tumor-infiltrating cells

PDX tumor tissues from tumor-bearing humanized NPI mice or NPI mice were rinsed with PBS twice and then minced into 1–3 mm³ pieces. The minced tissues were resuspended in an enzymatic cocktail containing 1 mg/ml collagenase (Sigma–Aldrich), 0.1 mg/ml hyaluronidase (Sigma–Aldrich), and 0.1 mg/ml DNase (Sigma–Aldrich) and incubated in 37°C for 30–60 min as described previously with minor modification. The cell suspension was passed through a Falcon 70 μm cell strainer (BD Biosciences) to remove any large aggregates and debris and was then subjected to centrifugation at 300 g for 5 min. The cell pellets were washed twice with PBS without performing further purification with Percoll. The isolated tissue-infiltrating cells were used for flow cytometry analysis.

### 2.13 | Flow cytometry

The phenotypes of peripheral blood cells from tumor-bearing humanized NPI mice or tumor-infiltrating cells from PDX tumor tissues were determined by flow cytometry. Cells were incubated with Fc-receptor binding inhibitor antibodies (Thermo Fisher Scientific) and then stained with various fluorescence-conjugated antibodies (listed in Table S1) in FACS buffer (PBS containing 2 mM EDTA and 1% FBS) or stained with Live/Dead fixable violet dead cell stain kit (Thermo Fisher Scientific) in PBS. After incubation at 4°C for 30 min, cells were washed once with FACS buffer and cell phenotypes were analyzed by flow cytometry. Cytometric data were collected on a NovoCyte cytometer (Agilent, Santa Clara, CA, USA) or BD LSRFortessa (BD Bioscience) and analyzed using NovoExpress 1.4.1 software (Agilent) or FlowJo X 10.0.7r2 (TreeStar).

### 2.14 | Intracellular cytokines or molecules staining

Tumor-infiltrating cells were cultured in the presence of Golgi-stop solution (10 μg/ml) for 5 h. Then cells were harvested and stained with surface markers CD8, CD4, or CD45RA and Live/Dead fixable violet dead cell stain kit (Thermo Fisher Scientific). For intracellular cytokines and transcriptional factors staining, cells were stained with fluorescence-conjugated anti-human TNF-α, IFN-γ, T-bet or Foxp3 (listed in Table S1) using Cytofix/Cytoperm kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

### 2.15 | Statistical analysis

All the data were analyzed using GraphPad Prism version 5.01 statistic software (GraphPad Software, Inc., San Diego, CA, USA). Means ± SEM were shown in all graphs. Statistical comparisons between two groups were analyzed with Student’s t test and p < .05 was considered statistically significant. For statistical comparisons of more than two groups, a two-way ANOVA test followed by a post hoc analysis (Tukey’s multiple comparison test) was used and p < .05 was considered statistically significant. Mouse survival was analyzed by log-rank test.
3 | RESULTS

3.1 | Establishment of CD34+ HSCs humanized NPI mice and the profiles of human immune cells

As depicted in Figure 1A, we purified Lin− CD34+ HSCs from fresh human umbilical cord blood and then transplanted HSCs cells into 3–4-week-old young NPI mice, which were pre-treated with busulfan for 24 h to deplete mouse myeloid cells to avoid engulfing infused HSCs. To establish a relatively-large cohort for preclinical evaluation of cancer immunotherapy, we transplanted 1 × 10^4 HSCs cells per mouse which was less than that (1 × 10^5 for each mouse) in previous other protocols and we could generate 100–200 humanized NPI mice (169 ± 44 in 7 batches for testing) from CD34+ HSCs of one donor (Table S2), depending on the yield of Lin− CD34+ HSCs (Table S3). In humanized NPI mice from the same donor, the percentage of human CD45+ cells in peripheral blood increased gradually during 4–28 weeks after the reconstitution of HSCs cells and was over 25% after 16 weeks of the reconstitution (Figure 1B). Humanized NPI mice that had over 25% human CD45+ cells in peripheral blood were considered as successful reconstitution. In seven batches of humanized NPI mice for testing, the percentage of successfully reconstituted mice in randomly sampled mice was over 80% (Figure 1C and Table S2), which could be multiplied by the average of reconstituted NPI mice numbers and predicted to generate 139 ± 54 humanized NPI mice to be used for cancer immunotherapy evaluation, implying a relatively-large cohort. Importantly, in the datasets of 3 batches of humanized NPI mice from a single donor, the percentage of successfully reconstituted mice was also over 80% during 16–32 weeks after the reconstitution of HSCs cells (Figure S3).

To evaluate the profiles of human immune cells in humanized NPI mice, the percentage of human immune cells among human CD45+ leukocytes in peripheral blood was determined by flow cytometry after transplantation of CD34+ HSCs. After 20 weeks of reconstitution, human immune cell subsets including CD19+ CD3− B cells, CD3+ CD19− T cells, CD4+ CD3+ T cells, CD4− CD3+ T cells (mainly CD8T cells), and CD14+ CD11b+ monocytes could be found in peripheral blood with the dominance of T cells (Figure 1D,E), indicating the efficient reconstitution of human adaptive and innate immune cells in CD34+ HSCs humanized NPI mice.

3.2 | CD34+ HSCs humanized NPI mice support the infiltration of T cells into patient-derived xenograft

Humanized NPI mice bearing PDX tumors as illustrated in Figure 2A were used to assess whether humanized NPI mice can support tumor growth and the infiltration of T lymphocytes into PDX tumors. The growth of PDX tumors in humanized NPI mice was slightly slower than that in NPI mice (p > .05, Figure S4A). As shown in Figures 2B and S4B, the percentage of human CD45+ leukocytes in tumor-infiltrating cells from PDX-bearing-humanized NPI mice was significantly higher than that in NPI mice bearing PDX tumors. Also, multiplex immunohistochemistry staining data showed that PD-1 was expressed on tumor-infiltrating human CD4 T cells and CD8 T cells whereas Foxp3 was mainly expressed on CD4 T cells (Figure 2C). Furthermore, tumor-infiltrating CD8+ T cells, in humanized NPI mice bearing PDAC or NSCLC PDX tumors, expressed high-level of exhaustion molecules PD-1 and 2B4, low to medium-level TIM3, but expressed low-level of costimulatory molecules CD28 and OX40 (Figures 2D and 3E). These results demonstrate that our CD34+ HSCs humanized NPI mice can recapitulate tumor infiltration of T cells with the exhausted phenotype and potential immune checkpoint inhibition of anti-tumor immune response.

3.3 | Checkpoint blockade suppresses tumor growth in CD34+ HSCs humanized NPI mice possibly through activating tumor-infiltrating CD8 T cells

Previous studies suggest that human T cell functions in humanized NSG or NOG mice reconstituted with CD34+ HSCs are defective due to the lack of human thymus. To validate whether human T cells that reconstituted in CD34+ HSCs humanized NPI mice have anti-tumor activity and whether our humanized NPI mice can be used for preclinical evaluation of cancer immunotherapy, we first assessed the efficacy of checkpoint blockade such as anti-PD-1, anti-PD-L1, and anti-CTLA4 in rejecting tumor growth and enhancing T cell function as illustrated in Figure 3A. In this study, the HLA genotypes in HSC donors used for humanized NPI mice were partially matched with those in both CDX tumor and PDX tumors (Table 1), trying to reduce the rejection of tumors by allogeneic responses of human T cells. In the MDA-MB-231 tumor model of humanized NPI mice, both anti-PD-1 antibody and anti-PD-L1 antibody significantly inhibited tumor growth compared with isotype control antibody (Figure 3B). Anti-PD-1 antibody also markedly suppressed tumor growth in both CRC and NSCLC PDX tumor models of humanized NPI mice (Figure 3C,D), whereas it had no effects on tumor growth in PDAC PDX tumor model of humanized NPI mice (Data not shown), showing the variation of the efficacy of anti-PD-1 antibody in different cancer types may be due to PD-L1 expression or MSI/TMB status of the original tumor.
To demonstrate whether anti-PD-1 antibody can enhance T cell functions, we analyzed the number, phenotype, and functional status of tumor-infiltrating CD8 T cells in NSCLC PDX tumor model of humanized NPI mice, which showed a good response to anti-PD-1 therapy. As shown in Figure 3E, anti-PD-1 treatment did not increase the tumor-infiltration...
of CD8 T cells and had no effects on TIM3⁺ or 2B4⁺ CD8 T cells, however, it increased the proportion of CD28⁺ CD8 T cells, implying the partial reverse of CD8 T cell exhaustion by anti-PD-1 treatment. Importantly, anti-PD-1 treatment greatly enhanced the expression of IFN-γ, TNF-α or T-bet in tumor-infiltrating CD8 T cells, which were significantly higher compared to the vehicle control group (IFN-γ⁺ TNF-α⁺ cells: 21.6% vs. 0%, IFN-γ⁺ T-bet⁺ cells: 52.3% vs. 1.72%, TNF-α⁺ T-bet⁺ cells: 24.8% vs. 4.31%; Figure 3F), indicating the enhancement of CD8 T cell activation in tumor by anti-PD-1 treatment.

Furthermore, we found that anti-CTLA4 treatment efficiently depleted CD4⁺ Foxp3⁺ Treg cells in NSCLC PDX tumor of humanized NPI mice (Figure 3G), though without the suppression of tumor growth (Figure 3D). Notably, significant loss of body weight was found after anti-CTLA4 treatment in humanized NPI mice bearing NSCLC PDX tumors, while the mice's body weight remained normal after anti-PD-1 therapy (Figure S5), which was consistent with greater toxicity of anti-CTLA4 in a clinical setting.

The above results demonstrate that human T cells in CD34⁺ HSCs humanized NPI mice possess efficient antitumor activity that can be further enhanced by checkpoint blockade and CD34⁺ HSCs humanized NPI mice are feasible for preclinical efficacy and safety evaluation of checkpoint blockade in cancer immunotherapy.
FIGURE 3 Checkpoint blockade suppresses tumor growth in humanized NPI mice possibly through activating tumor-infiltrating CD8 T cells. (A) Study design diagram of checkpoint blockade experiments in humanized NPI mice bearing CDX or PDX tumors. (B–D) The humanized NPI mice, reconstituted for 16–20 weeks, were implanted with MDA-MB-231 cells (B), CRC PDX (C), or NSCLC PDX (D) tumor tissues subcutaneously in mammary fat pads or the right flank. Dosing of anti-PD-1, anti-PD-L1, or anti-CTLA4 (i.p., 10 mg/kg, Q5d) began on day 0 in mice with established tumors (group mean 120 mm³). The study endpoint was a tumor volume of 1500 mm³ in the control group. Mean tumor growth results are shown. *, p < .05; ***, p < .001. (E–G) In humanized NPI mice bearing NSCLC PDX tumors, dosing of anti-PD-1 (i.p. 10 mg/kg, Q5d) or anti-CTLA4 (i.p. 10 mg/kg, Q5d) began on Day 0 in mice with established tumors (group mean 120 mm³). After anti-PD-1 treatment, phenotypes and cytokines production of CD8⁺ TILs were determined by flow cytometry analysis (E) and intracellular cytokine staining (F). After anti-CTLA4 treatment, depletion of Treg cells was analyzed by flow cytometry (G).
3.4 | Engineered CAR-T cells from humanized NPI mice are functional in vitro and in vivo

To further validate the functions of human T cells in humanized NPI mice, we isolated human CD3+ T cells from spleens of humanized NPI mice, prepared engineered CD19 CAR-T cells, and then evaluated the functions of these engineered CAR-T cells in vitro and in vivo as depicted in Figure 4A. Figure 4B data showed that human CD3+ T cells from humanized NPI mice could be activated and expanded with anti-hCD3/anti-hCD28 beads/cytokines and the activated CAR-T cells released high-level of INF-γ after coculture with target cells in vitro, validating the activating and proliferating properties of human T cells from humanized NPI mice. Notably, CD8 effector T cells prepared from the spleen of humanized NPI mice could be efficiently induced to express CD107a (the marker of degranulation), TNF-α, IFN-γ, and granzyme B after activation by anti-CD3 antibody (Figure S6). Furthermore, after infusion of these CD19 CAR-T cells into humanized NPI mice from the same donor, they could efficiently decrease the number of target CD19+ B cells in peripheral blood in time-dependent and dose-dependent manners (Figures 4C and S7), demonstrating that engineered CAR-T cells from humanized NPI mice are able to deplete target cells in vivo. Interestingly, 150 days after infusion of these engineered CAR-T cells into humanized NPI mice, target CD19+ B cells in peripheral blood were still very low (Figures 4C and S7), indicating the long-term persistence of these engineered CAR-T cells in vivo continuously depleting target CD19+ B cells. As CD19 CAR-T cells target both CD19+ tumor cells and CD19+ normal cells such as B cells, it is necessary to remove or reduce these infused CAR-T cells to avoid on-target off-tumor toxicity after depleting target tumor cells. Hence, we designed the safety switch, i.e., truncated EGFR on engineered CD19 CAR-T cells from humanized NPI mice. Anti-EGFR Cetuximab was administrated when CD19+ B cells were depleted in peripheral blood, and we found that the number of CAR-T cells decreased quickly in humanized NPI mice (Figure 4D). Importantly, NK cells from the spleen of humanized NPI mice could be induced to express CD107a, TNF-α, IFN-γ, and granzyme B after activation by poly I: C/IL-15 (Figure S8). This result demonstrated that our humanized NPI mice could be used to study CAR-T depleting mediated by clinically approved therapeutic antibody if the CAR-T cells were engineered to express the specific cell surface antigen targeted by this therapeutic antibody.

3.5 | Engineered CD19 CAR-T cells from humanized NPI mice suppress acute lymphoblastic leukemia progression in vivo

To mimic the clinical application of CD19 CAR-T cells in human acute lymphoblastic leukemia (ALL), we infused engineered CD19 CAR-T cells from humanized NPI mice into humanized NPI mice from the same donor bearing ALL cell line NALM6 and evaluated the efficacy and side effects of these engineered CD19 CAR-T cells in vivo as depicted in Figure 5A. We observed that the infusion of CD19 CAR-T cells at various doses significantly suppressed the growth of ALL CDX tumors (Figure 5B,C) and prolonged the survival of mice (Figure 5D). Also, we found that compared with PBS and Mock-transduced T cells groups, there were higher levels of systemic human IL-6 and IFN-γ cytokines in CAR-T groups but without loss of body weight and increase of body temperature (Figure S9A–C). Furthermore, on-target off-tumor effects of CD19 CAR-T cells were confirmed by CD19+ B cells counting by flow cytometry (Figure 5E), displaying one of the side effects of CAR-T cell infusion in humanized NPI mice-bearing CDX tumors.

3.6 | Bispecific antibody Blinatumomab depletes target cells in vivo

Considering that the results of checkpoint blockade and engineered CAR-T cells above had demonstrated that human T cells from our humanized NPI mice are functional, we wonder whether bispecific antibody anti-CD19/
CD3 will deplete target cells in vivo expectedly through activating human CD3⁺ T cells in humanized NPI mice. As depicted in Figure 6A, bispecific antibody anti-CD19/CD3 Blinatumomab or control anti-CD20 Rituximab was administrated to humanized NPI mice, and the percentage of B cells in peripheral blood was assayed by flow cytometry. As expected, a single-dose of Rituximab (positive control) could efficiently deplete B cells through antibody-dependent cell-mediated cytotoxicity (Figures 6B and S10A). Meanwhile, a single-dose of Blinatumomab could also significantly deplete B cells and the percentage of B cells partially recovered after 10 days (Figures 6C and S10B). These results indicate that bispecific antibody Blinatumomab can deplete target cells in vivo in CD34⁺ HSCs humanized NPI mice.

4 | DISCUSSION

In this study, we successfully established a large-cohort humanized NPI mouse model using a relatively small amount of CD34⁺ HSC cells (1 × 10⁴ for each mouse), which was feasible for the preclinical evaluation of cancer immunotherapy including checkpoint blockade, CAR-T cell therapy, and bispecific antibody therapy. Meanwhile, it was demonstrated that the reconstituted human T cells...
Engineered CD19 CAR-T cells from humanized NPI mice suppress acute lymphoblastic leukemia progress in vivo. (A) Study design diagram of in vivo function of engineered CD19 CAR-T cells from humanized NPI mice. Human CD3⁺ T cells were isolated from spleens of humanized NPI mice 16–20 weeks post-reconstitution and the engineered CD19 CAR-T cells were prepared as described in Figure 4A. Then, various numbers of engineered CD19 CAR-T cells were infused into humanized NPI mice from the same donor bearing acute lymphoblastic leukemia (ALL) cell line NALM6 CDX. PBS group and Mock-transduced T cells (Mock-T) group (5 × 10⁶ cells) were used as control. The number of mice in each group was 6. (B and C) Tumor growth was monitored by in vivo imaging. At various time points after cell infusion, tumor sizes were calculated (B). Representative images on day 7 and day 14 were shown (C). (D) The survival curve of mice in different groups was drawn. (E) The percentage of CD19⁺ B cells in human CD45⁺ cells was assayed by flow cytometry at various time points after the infusion of engineered CD19 CAR-T cells. CAR-T L: 3 × 10⁶ cells infusion, CAR-T M: 5 × 10⁶ cells infusion, and CAR-T H: 1 × 10⁷ cells infusion. **, p < .01 versus Mock-T group.
possessed anti-tumor efficacy in our humanized NPI mice bearing PDX or CDX tumors in the context of cancer immunotherapy.

In the human CD34⁺ HSC-engrafted mouse model, the success of engraftment is variable, mainly depending on HSC source and cell number, age and strain of mice, and myeloablative conditioning regimen. For HSC sources, it is very popular using CD34⁺ HSC from human umbilical cord blood, probably due to the high engraftment efficiency and easy access to the sample. For HSC cells number, it has not been fully investigated previously. In this study, we demonstrated that although less number of HSCs than previously reported were transplanted, human adaptive and innate immune cells could be efficiently reconstituted in our humanized NPI mice and therefore a larger-cohort humanized mice would be generated, which provided adequate humanized mice feasible for evaluating cancer immunotherapy. When considering the age and strain of mice for generating humanized mice, newborn or young mice (up to 4 weeks of age) allows accelerated T cell development compared with adult mice, and the most commonly used strain of mice is NSG or NOG. Notably, the NPI mice used in this study lack T, B, and NK cells, similar to NSG mice. For the myeloablative conditioning regimen, both sublethal γ-irradiation and busulfan injection are feasible, and the latter will be a good choice if there is no γ-irradiator available. Herein, we transplanted human CD34⁺ HSCs cells into 3–4-week-old young NPI mice receiving myeloablative conditioning by busulfan injection. In our established humanized NPI mice, most human lymphoid and myeloid cells were efficiently reconstituted, including T cells, B cells, and monocytes. Furthermore, the percentage of successfully reconstituted mice was over 80% after 16 weeks of reconstitution with a relatively-large cohort (about 139 ± 54 mice), which will allow this model to be used in most preclinical experiments of cancer immunotherapy.

It is well known that the interaction between tumor cells and immune cells in the tumor microenvironment can facilitate cancer development and progression.
Similar to one recent study, in our humanized mouse model bearing human tumors, it was found that cancer could educate the immune system and induce the infiltration of exhausted T cells expressing checkpoint molecules and regulatory T cells, which might support tumor growth in humanized NPI mice. Certainly, it is not excluded that other immunosuppressive cells such as tumor-associated macrophage and myeloid-derived suppressor cells (MDSCs) may contribute to the immunosuppressive tumor microenvironment in our humanized NPI mice, being worth further investigation.

It has been shown that humanized mice reconstituted with human CD34+ HSCs can be used to evaluate the efficacy and even side-effects of anti-PD-1 or anti-CTLA4 in cancers. Wang M et al. found that anti-PD-1 therapy produced significant tumor growth inhibition in CDX and PDX tumors of humanized NSG mice, dependent on human CD8 T cells, as demonstrated by antibody-mediated depletion. Zhao et al. demonstrated that anti-PD-1 or anti-CTLA4 suppressed tumor growth of human hepatocellular carcinoma PDX in humanized NSG mice probably by re-activating CD8 T cells and altering the tumor immune microenvironment, and mice treated with anti-CTLA4 but not anti-PD-1 lost weight significantly and showed cachexia, indicating side effects of anti-CTLA4. In our humanized NPI mouse model, we found that anti-PD-(L)1 showed therapeutic efficacy, depending on cancer types, which is consistent with the clinical setting. More importantly, compared to the vehicle control group, anti-PD-1 treatment-induced re-activation of CD8 T cells and altering the tumor immune microenvironment, and mice treated with anti-CTLA4 but not anti-PD-1 lost weight significantly and showed cachexia, indicating side effects of anti-CTLA4.3

Also, its side-effects might be mediated by human T cells-induced autoimmune-like responses. Thus, our humanized mouse model will be valuable for the evaluation of both therapeutic and side effects of checkpoint inhibitors. It is still unknown whether alloreactive T cell responses or tumor antigen-specific T cell responses are involved in anti-tumor effects of checkpoint inhibitors, requiring further studies.

The unprecedented efficacy of CD19 CAR-T cell therapy is accompanied by long-lasting B cell aplasia, severe cytokine release syndrome (CRS), and even neurotoxicity. It is attractive and difficult to establish a preclinical mouse model to reproduce these side effects in CD19 CAR-T cell therapy. Recently, Norelli et al. describe a humanized SGM3 mouse model recapitulating key features of CRS and neurotoxicity, and demonstrate that human monocyte-derived IL-1 and IL-6 are differentially required for CRS and neurotoxicity due to CAR T cell therapy. Notably, in these humanized SGM3 mice there are significantly more CD14+ monocytes than that in humanized NSG mice which might contribute to the reproduction of key features of CRS and neurotoxicity. CD3+ T cells from our humanized NPI mice were successfully transduced by CD19-CAR and these engineered CD19 CAR-T cells were shown to be functional in vitro and in vivo with normal proliferation, IFN-γ secreting, and target cell-killing abilities. In humanized NPI mice bearing ALL CDX, engineered CD19 CAR-T cells showed significant therapeutic effects and also induced the depletion of normal B cells, demonstrating that our humanized mouse model could reproduce the feature of B cell aplasia. We also tested the feasibility of depleting the infused CAR-T cells using truncated EGFR knock-in and Cetuximab (anti-EGFR) administration and found that it was practical, demonstrating normal antibody-dependent cellular cytotoxicity in our humanized mouse model. Our humanized mouse model could not mimic the features of CRS (high fever, body weight loss, and high-level serum IL-6), maybe owing to the relatively low burden of the tumor, or the low percentage of monocytes (<10%, less than that in humanized SGM3 mice).14

T cell engaging bispecific antibodies, such as Blinatumomab, recruit and activate T cells to lyse cancer cells, achieving impressive efficacy in hematologic malignancies. However, similar to CAR-T therapy, T cell engaging bispecific antibody therapy is commonly accompanied by CRS and neurologic toxicity, which are the primary challenge hindering the broad use of T cell engaging bispecific antibodies.5,32 Previously, humanized mice reconstituted with PBMCs have been used to evaluate the preclinical efficacy of some T cell engaging bispecific antibodies, such as anti-CD19/CD3, anti-CLDN6/CD3, and anti-GPC3/CD3. Humanized mice reconstituted with CD34+ HSC are rarely used to evaluate the efficacy or side-effects of T cell engaging bispecific antibodies. In a previous study, Sun et al. used humanized NSG mice engrafted with human CD34+ HSC to evaluate the effects of anti-CD20/CD3 bispecific antibody and found that 3 weekly doses of this antibody at 0.5 mg/kg robustly depleted B cells and there was a 10-fold increase in CD8+ T cells in peripheral blood by day 7 and they gradually decreased by day 14 and 21. In our humanized mouse model, anti-CD19/CD3 Blinatumomab could deplete B cells in vivo. This indicates that our humanized mouse model can be used for the preclinical evaluation of bispecific antibodies.

In the future, this humanized mouse model will be further used for the preclinical evaluation of novel checkpoint inhibitors, CAR-T cell therapy, or bispecific antibody.
Also, it is worth investigating whether this humanized mouse model is feasible for evaluating novel cancer immunotherapy modalities, such as cancer neoantigen vaccine and engineered TCR T cell therapy.

In summary, this large-cohort humanized NPI mouse model engrafted with human CD34+ HSCs is feasible for evaluating the therapeutic effects and partial side-effects of various cancer immunotherapy approaches including checkpoint inhibitor, CAR-T, and bispecific antibody. Furthermore, our study validates the anti-tumor efficacy of human T cells in the humanized NPI mice engrafted with human CD34+ HSCs, enhancing the feasibility of the application of this humanized NPI mouse model in cancer immunotherapy.

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DISCLOSURES
The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
Conceived and supervised the study: Gang Jin, Baohua Qian, and Shiwei Guo. Designed the experiments: Xiongfei Xu, Shiwei Guo, and Suizhi Gao. Performed the experiments: Xiongfei Xu, Haihui Gu, Hongwei Li, Siying Peng, Xiaohan Shi, Peng Cheng, Zhanshan Cha, and Jing Shen. Provided all the clinical information: Bo Li and Huan Wan. Analyzed all the data: Xiongfei Xu, Kaiilian Zheng, and Zhuo Shao. Wrote the manuscript: Xiongfei Xu. Revised the manuscript: Gang Jin, Shiwei Guo, Yongzhan Nie, and Zhaoshen Li. All authors reviewed the results and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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