An Improved Patient-Derived Xenograft Humanized Mouse Model for Evaluation of Lung Cancer Immune Responses

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Abstract

Human tumor xenograft models do not replicate the human immune system and tumor microenvironment. We developed an improved humanized mouse model, derived from fresh cord blood CD34+ stem cells (CD34+ HSC), and combined it with lung cancer cell line–derived human xenografts or patient-derived xenografts (Hu-PDX). Fresh CD34+ HSCs could reconstitute detectable mature human leukocytes (hCD45+) in mice at four weeks without the onset of graft-versus-host disease (GVHD). Repopulated human T cells, B cells, natural killer (NK) cells, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC) increased in peripheral blood, spleen, and bone marrow over time. Although cultured CD34+ HSCs labeled with luciferase could be detected in mice, the cultured HSCs did not develop into mature human immune cells by four weeks, unlike fresh CD34+ HSCs. Ex vivo, reconstituted T cells, obtained from the tumor-bearing humanized mice, secreted IFNγ upon treatment with phorbol myristate acetate (PMA) or exposure to human A549 lung tumor cells and mediated antigen-specific CTL responses, indicating functional activity. Growth of engrafted PDXs and tumor xenografts was not dependent on the human leukocyte antigen status of the donor. Treatment with the anti–PD-1 checkpoint inhibitors pembrolizumab or nivolumab inhibited tumor growth in humanized mice significantly, and correlated with an increased number of CTLs and decreased MDSCs, regardless of the donor HLA type. In conclusion, fresh CD34+ HSCs are more effective than their expanded counterparts in humanizing mice, and do so in a shorter time. The Hu-PDX model provides an improved platform for evaluation of immunotherapy.

Introduction

Immune checkpoint blockade has shown antitumor activity and improved survival in advanced non–small cell lung cancer (NSCLC). However, durable responses occur in only a small subset of patients. Many patients have or develop resistance to the available immunotherapies, underscoring the need to identify alternative therapeutic strategies. Immunotherapies require a functional human immune system; thus, it is difficult to test their effectiveness using conventional experimental models. Most therapeutic agents validated in current preclinical systems fail in the clinic because the mouse differs from the human in terms of immune system development, activation, and response to challenge in both the innate and adaptive arms (1, 2). For example, the syngeneic mouse model, which is currently the first-line tool for evaluation of immune-based therapy, has a significant drawback because its biology and immunity lack a human microenvironment. Genetically engineered mouse models (GEMM) and carcinogen-induced mouse models (CIMM) also have a murine tumor microenvironment (1, 3).

The patient-derived xenograft (PDX) mouse model, which is predicted to be a major advance in preclinical testing platforms, recapitulates multiple characteristics of the biological context of human cancers. However, in the context of immunotherapy, this model is not suitable because it lacks an intact immune system (4). PDX models are developed by surgically implanting tumor tissue from a patient into immunodeficient (SCID) mice, usually NOD-SCID gamma (NSG) mice, or NOD/SCIDIL2rnull (NOG) mice, that lack mature T cells, B cells, and macrophages (5–7). These models are useful for testing targeted drug efficacy and determining mechanisms of drug resistance. Several published reports seem to suggest that, in the case of some chemotherapeutic drugs, there could be good correlations between PDX models and human outcomes (4, 8, 9). We have developed and characterized more than 200 NSCLC PDXs that are clinically and molecularly annotated under the NCI PDX Development and Trial Centers...
address this need, we developed a CD34+ cell–derived human PDX (Hu-PDX) mouse model, which recapitulates the human immune system. Most humanized mice have been derived by engrafting cultured CD34+ HSCs or cultured precursor cells (HSPCs; refs. 11–13). Human peripheral blood mononuclear cells (PBMCs)-driven humanized mice are not suitable for treatment efficacy studies. They transiently harbor human immune cells and mice develop graft versus host diseases (GVHD).

Ex vivo culture of human CD34+ HSCs facilitates development of histocompatibility leukocyte antigen (HLA) partially matched PDXs (14, 15). Cultured CD34+ HSCs differentiate into myeloid, B-lymphoid, and erythroid lineages, but yield few or no T lymphocytes (12), with lower yield and purity, less proliferative potential, lower engraftment efficiency, less T-cell functionality, and more limited multilineage hematopoietic development than their fresh counterparts (11–13). Cultured CD34+ HSCs also express less CD34 and CD133, and their reconstituted T cells are reported to be functionally inactive (16). In addition, cultured cells provided delayed engraftment, which led to repopulation by differentiated T cells with low frequency (17). Thus, engraftment with cultured CD34+ HSCs does not develop fully functional humanized immune systems.

In this study, we describe the development of an improved humanized mouse model with a functional human immune system and show successful engraftment of human lung PDXs onto the humanized mice. By the use of fresh, not cultured, CD34+ HSCs, the NSG mice developed functional T and B lymphocytes, and natural killer (NK) cells (18, 19). These humanized mice had strong antitumor responses to pembrolizumab and nivolumab.

Humanized NSG mice

After mononuclear cells were separated from human umbilical cord blood, CD34+ HSCs were isolated using a direct CD34+ MicroBead kit (Miltenyi Biotec). Three- to 4-week-old NSG mice were irradiated with 200 cGy using a 137Cs gamma irradiator. Approximately, $1 \times 10^8$ freshly isolated CD34+ HSCs, over 90% pure, were injected intravenously into mice 24 hours after irradiation. The engraftment levels of human CD45+ cells and human immune cell populations, including CD45+, CD3+, and CD4+ CD8+ T cells, B cells, NK cells, MDSCs, and other lineage-negative cells were determined in the peripheral blood, bone marrow, and spleen tissue using a 10-color flow cytometry panel. Mice that had over 25% human CD45+ cells in the peripheral blood were considered humanized (Hu-NSG mice). Hu-NSG mice from different cord blood donors with different levels of engraftment were randomized into every treatment group in all of the experiments. All Hu-NSG mice were confirmed for humanization before tumor xenograft or PDX implantations.

Generation of humanized NSCLC xenograft tumors and PDX mice

H1299-luc and A549-luc human NSCLC cell lines were kindly provided by Dr. Frank R. Jirik (The University of Calgary, Calgary, Canada) and Dr. John Minna (The University of Texas Southwestern Medical Center, Dallas, TX). Cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS (GE Healthcare Life Sciences, HyClone Laboratories) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO₂. Both cell lines were tested negative for Mycoplasma before use in experiments. To generate subcutaneous tumors, $1 \times 10^6$ H1299-luc cells were implanted in the right flank of 6-week posthumanized NSG mice. To generate experimental lung metastases, $1 \times 10^5$ A549-luc cells were injected intravenously into NSG mice 6 weeks posthumanization. Tumor growth was measured by quantifying bioluminescence intensity with a small-animal in vivo imaging system (IVIS 200; Caliper Life Sciences).

PDXs were obtained from Dr. Bingliang Fang (Lung PDX Core Facility at MDACC, Houston, TX). All PDXs were propagated in NSG mice, harvested, and implanted into Hu-NSG mice 6 weeks after humanization. All lung PDXs used in this study were from passages F1 to F3. In brief, tumor tissues were minced to a size of 2 mm × 2 mm and were implanted subcutaneously through a tiny incision in the right flank of anesthetized Hu-NSG mice. Incisions were closed with clips, and mice underwent postsurgery care. The clips were removed 10 days after surgery, and mice were monitored daily for side effects. Two perpendicular tumor diameters were measured twice per week, and tumor surface area was calculated according to a formula $1/2 (aperture \times width^2)$. The lung PDXs used in this study were TC338, TC441, and TC241, which are completely annotated histologically and molecularly.
Humanized NSCLC xenograft tumor and PDX tumor experiments

Seven days after implantation of NSCLC cell lines, tumor growth was confirmed using IVIS imaging, and tumor-bearing Hu-NSG mice were randomized into treatment and no treatment groups. Eight to 10 humanized mice per group, from multiple donors, were used for human cell line xenograft experiments. Tumor-bearing Hu-NSG mice were treated with the anti–PD-1 agent pembrolizumab (Merck) or nivolumab (Bristol–Myers Squibb) at a concentration of 250 mg/mouse, intraperitoneally, every 3 to 4 days for three cycles. Cell line xenograft tumors were also generated in nonhumanized NSG mice, which were treated alongside the humanized mice as a control arm. Mice were monitored routinely, and tumors were imaged using the IVIS platform once a week. For immune analysis, mice from each treatment group were sacrificed 2 weeks posttreatment and organs were harvested for single-cell analysis.

For humanized PDX (Hu-PDX) tumor experiments, treatments started when implanted PDX tumors reached a volume of 100 to 200 mm³. The control group received intraperitoneal injections of the saline vehicle (Hospira). Pembrolizumab and nivolumab were injected intraperitoneally following the same dose and schedule described above. Tumors were measured with calipers every 3 to 4 days, and volumes (in mm³) were calculated using the formula (length × width²)/2. PDXs developed in nonhumanized NSG mice were used as a control arm. All measurements quantifying experimental outcomes were blinded to the intervention.

IFNγ and granzyme B assays

Two weeks following intravenous injection of A549 cells, mice were euthanized and spleens were harvested. Splenocytes were cocultured with heat-killed A549 cells (30 minutes in a 50°C water bath) for 4 hours in the presence of a protein transport inhibitor containing brefeldin A (GolgiPlug, BD Biosciences). Intracellular staining for IFNγ (BioLegend) and granzyme B (BioLegend) was performed according to the manufacturer’s protocols (BD Biosciences). Human bronchial epithelial cells (HBEC) were used as an HLA-matched control for A549 cells (HLA-A*30). When tumor volumes reached 500 to 600 mm³, in humanized and nonhumanized PDX mice, animals were euthanized and spleens and PDX tumors were harvested. Single-cell suspensions were prepared and splenocytes were cocultured with heat-killed PDX-derived cells for 4 hours in the presence of a protein transport inhibitor (GolgiPlug). HBECs were also used as a control for HLA-matched PDXs. Intracellular staining was performed according to the protocol described above. Phorbol 12-myristate 13-acetate (PMA) was used as a positive control for mitogenic stimulation of T cells.

Immune analysis by flow cytometry

Erythrocytes in the peripheral blood were lysed with ACK lysis buffer (Thermo Fisher Scientific). Single-cell suspensions were prepared from peripheral blood, bone marrow, and spleen tissue using standard procedures. A 10-color flow cytometry panel was used for immune profiling and for evaluating immune response...
Figure 2.
Characterization of humanized mice by immune profiling of human lymphocytes in mouse system. A, Gating strategy for multicolor flow cytometry for analysis of human immune cells in mouse organs. Human CD45^+ lymphocytes were gated out from mouse cells followed by stepwise gating for human T, B, and NK cells and other human immune cell subpopulations. B, Analysis of humanization status in peripheral blood at 4 weeks after CD34^+ stem cell implantation. A few drops of blood were collected without killing the mice for basic immune profiling at this stage. The inset enlarges the graph of CD56^+ NK cells. C, Analysis of humanized mice at week 6 after implantation. At this stage, deep immune analysis was performed using blood, spleen, and bone marrow. The results shown are from 1 of 4 independent experiments with similar results. D, In-depth immune analysis of blood, spleen, and bone marrow of humanized mice at week 9.
Fluorochrome-conjugated mAbs to the following human antigens were used: CD45-Alexa Fluor 700 (clone 2D1, HI30), CD45-phycoerythrin (PE; clone 2D1, HI30), CD3-PerCP/cy5.5 (clone HIT3a), CD19-PE-cyanine 7 (clone HIB19), CD8-allophycocyanin-cyanine 7 (clone RPA-T8, HIT8a), CD4-Pacific Blue (clone OKT4), CD56-PE (clone HCD56), CD69-FITC/APC (clone FN50), HLA-DR-PerCP/cy5.5 (clone LN3), CD33-PE (clone WM-53), CD11b-PE-Cy7 (clone 1CRF-44), granzyme

Figure 3.
Evaluation of human T-cell functionality in the humanized mouse system. A, Experimental timeline showing that 6 to 8 weeks after humanization, mice were injected intravenously with A549 human lung cancer cells to develop experimental lung metastasis. Two weeks after injection, mouse splenocytes were analyzed for intracellular staining of human IFNγ. B, Percentage of IFNγ⁺ cells in human CD3⁺ T-cell and human CD8⁺ T-cell gated populations in different coculture conditions. PMA was used for mitogenic stimulation, an anti-CD3 antibody was used for T-cell receptor (TCR) stimulation, and heat-killed (HK) A549 cells were used for T-cell stimulation and activation. C, Level of CD69⁺ expression on CD3⁺ and CD8⁺ T cells after the indicated stimulations. The data were derived from one (n = 3 mice) out of three independent experiments with similar results; bars, SD (**, P < 0.05; *** P < 0.005; and ****, P < 0.0005 compared with controls).
B-FITC (clone GB11), and IFNγ-APC (clone 4S.B3). A mouse CD45-FITC (clone 30-F11) antibody was used for gating out murine leukocytes. All antibodies were purchased from BioLegend. Zombie (BioLegend) viability dyes were used to stain dead cells. All samples were run for acquisition on a Gallios flow cytometer (Beckman Coulter). FlowJo and Kaluza software packages were used for data analysis.

IHC

Tissues harvested from humanized and nonhumanized NSG mice were fixed with 4% paraformaldehyde for 24 hours and paraffin-embedded for immunostaining with the indicated antibodies. Human CD45 (clone 2B11+PD2/26) and CD68 (clone PGM1) antibodies were purchased from Dako and human CD3 and mouse Gr-1 antibodies were provided by MD Anderson Histology Core Facility. Histology slides were scanned by Aperio Imaging System (Leica Biosystems) and analyzed using ImageScope software (Leica Biosystems).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 software. The tumor intensity change per time point was calculated as a relative level of tumor intensity change from baseline. Two-way ANOVA with interaction of treatment group and time point was used to determine significant differences among groups.

Figure 4.

Characterization of human PDX and cell line xenograft tumors in the humanized mouse system. A, Comparison of the growth of subcutaneous tumors in humanized (Hu-NSG), using fresh CD34+ HSC from multiple donors of different HLA types and nonhumanized (NSG) mice. H299-luc cells were implanted subcutaneously in humanized mice (at week 6–8 after humanization). Tumor growth was determined by measuring the bioluminescence intensity at different time points. Left, representative IVIS images; middle, the difference in intensity at week 1; right, growth curves for individual mice at weeks 1, 2, and 3. This experiment was repeated three times with 8 to 10 mice/group used in each experiment. B, Comparison of the growth of experimental lung metastases in humanized and nonhumanized mice using stem cells from multiple donors of different HLA types to generate lung metastases. Left and middle, the comparison of the tumor burden in humanized and nonhumanized mice using tumor bioluminescence intensity at week 1. Right, tumor growth curves in Hu-NSG and NSG mice at weeks 1, 2, and 3. This experiment was repeated three times with 8 to 10 mice/group used in each experiment, bars, SD. ***, P < 0.0005 compared with controls. C, Growth rate of TC286 human PDX (p53 and Kras mutant) in humanized mice at the indicated time points (n = 3). D, Comparison of the growth of TC388 human PDX in humanized mice with different HLA backgrounds nonhumanized mice (n = 3).
point was performed to compare the difference of tumor intensity changes from baseline between each pair of the treatment group at each time point. Means ± SEM are shown in all graphs. The nonparametric Mann–Whitney U test was applied to compare cell numbers in different treatment groups. Differences of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

Results

Optimization of the Hu-PDX mouse model derived from fresh cord blood CD34$^+$ HSCs

The fresh cord blood–derived CD34$^+$ HSCs engrafted and repopulated into human immune subpopulations with functional T cells. Over 25% of human mature human white blood cells (hCD45$^+$) were detected within 4 weeks of stem cell implantation with no sign of GvHD. A schematic of the development of the humanized mouse model with PDX grafts is shown in Fig. 1A. Purity of CD34$^+$ HSC from cord blood was over 90% (Fig. 1B), and the recovery rate of from donor cord blood was over 1% of the total number of peripheral blood mononuclear cells (Fig. 1C).

We next compared the expression of CD34$^+$ HSCs under fresh and cultured conditions, and found that cultured cells had lower expression of CD34 (Fig. 1D). We were able to expand CD34$^+$ HSCs ex vivo (Supplementary Fig. S1; ref. 16). We infected them with a NanoLuciferase lentivirus before engraftment into irradiated mice for comparative analysis of the reconstituted human immune cell population. Although luciferase-labeled cultured CD34$^+$ HSC cells were detected in mice 3 days postinjection (Supplementary Fig. S1C), we were unable to detect any reconstituted mature human immune cells at 4 weeks, as we did with their fresh CD34$^+$ HSC counterparts.

After HLA typing of PDXs and several donor cord bloods, engraftment of PDXs was evaluated in partially matched and
Figure 6.
Antitumor immune effect of pembrolizumab on Hu-PDX and human lung metastasis model. **A**, Schematic presentation of treatment plan using pembrolizumab against A549-luc lung metastasis in humanized mice. **B**, Effect of pembrolizumab treatment on A549-luc metastases developed in Hu-NSG and NSG (nonhumanized) mice. A549-luc cells were implanted intravenously in mice 6 to 8 weeks after humanization with stem cells from multiple donors, followed by pembrolizumab treatment. (Continued on the following page.)
Immune reconstitution and distribution of human immune subpopulations was robust

Immune reconstitution and distribution of human immune subpopulations were characterized by multicolor flow cytometry of the spleen, blood, and bone marrow at different time points (Fig. 2). The flow cytometry gating strategy is shown in Fig. 2A. At week 4, 25 to 60 were hCD45 T cells (Fig. 2B). Approximately 50% of the total population of human leukocytes were CD19 T cells. In addition, human CD3 T cells were detected. On average, 10% to 15% of the human leukocytes were CD56 NK cells at every time point. Human antibodies were tested for cross-reactivity with mouse immune cells and no cross-reactivity was observed (Supplementary Fig. S2). At week 6, the percentage of hCD45 cells had increased to an average of 60% in all three analyzed sample types. The average percentage of hCD45 cells reached 80% at week 9 (Fig. 2C and D). At weeks 6 and 9, approximately 20% CD3 T cells were found in the blood and spleen, and 10% in the bone marrow. The average percentages of T cells were 25% and 50% in blood, 35% and 45% in the spleen, and 50% and 40% in the bone marrow at weeks 6 and 9, respectively. A human CD56 NK-cell population was also detected within 4 weeks post CD34 HSC engraftment. The proportion of NK cells was about 15% in all three-sample types and was consistent at weeks 6 and 9. In addition, we could detect CD56 low and CD56 high NK-cell subpopulations, which are considered cytotoxic and cytokine-producing NK cells, respectively. Approximately 30% of the total human leukocyte population were HLA-DR+ DCs. We also evaluated the status of reconstitution of humanized at week 16. The percentage of hCD45 cells was over 80%, along with substantial numbers of human CD3 T, NK, and B cells (Supplementary Fig. S3). Approximately 3% of Lin cells were identified as human HLA-DR+ CD33+ MDSCs at week 9, which increased to 5% at week 16 (Supplementary Figs. S3 and S4). Taken together, the reconstituted immune mouse model derived from CD34+ HSCs showed robust and early reconstitution with differentiated immune subpopulations.

Reconstituted T lymphocytes were functionally active

We found that reconstituted human T cells obtained from fresh CD34+ HSC-derived humanized mice that had been implanted with A549 NSCLC cells were functionally active. An intracellular IFNγ assay showed a significant number of human T cells (P < 0.005) activated by stimulation with mitogen, anti-CD3 for T-cell receptor (TCR), or heat-killed A549 cells, indicating that the mechanism of IFNγ secretion was intact (Fig. 3A and B). In addition, a significant number of human T cells and CD8+ T cells (P < 0.0005) were IFNγ-positive after stimulation, compared with unstimulated T cells. Moreover, stimulated T cells and CD8+ T cells showed significantly higher expression of CD69 than their inactivated counterparts (P < 0.05; Fig. 3C). Taken together, these results indicate that human repopulated T cells in this humanized mouse model are functionally active, as demonstrated by the T-cell response not only to mitogenic stimuli, such as PMA and IL2, but also to stimulation with allogeneic human cancer cells.

HLA-independent engraftment and growth of PDX and cell line xenograft tumors

All humanized mice were typed for most common HLA-A and HLA-B loci. Immune screening for major human immune populations, at week 4, post CD34+ HSC engraftment showed that a population of more than 25% human CD45 leukocytes was present in most mice regardless of donor HLA type. This percentage increased significantly in subsequent weeks. Reconstitution of human T, B, and NK cells did not depend on donor HLA status. All humanized mice subcutaneously implanted with H1299-luc cells developed tumors. We did not find significant tumor growth differences between humanized and nonhumanized mice at week 1, 2, and 3 post tumor cell implantation. (Fig. 4A). The representative IVIS images reflecting tumor growth in humanized and nonhumanized mice are shown in Fig. 4A (left).

For the development of the lung metastasis model, A549-luc cells were intravenously injected into humanized and nonhumanized mice. A549-luc NSCLC cells colonized the lungs within a week of implantation. At week one, engraftment of A549-luc cells was significantly lower in humanized mice than their nonhumanized counterparts (Fig. 4B, P < 0.005). IVIS imaging of humanized and nonhumanized mice showing the tumor burden are in Fig. 4B (left). The significant difference in tumor growth between humanized and nonhumanized mice reseeded with tumor progression at weeks 2 and 3, although the growth rate remained slower, but not statistically different, in humanized mice compared with nonhumanized mice (Fig. 4B, right).

PDX tissues were typed for HLA before implantation. Two PDXs of different molecular types, TC286, with mutant p53 and Kras, and TC388, with wild-type p53 and Kras, developed tumors in humanized mice derived from multiple donors (Fig. 4C and D). Approximately 60% to 80% of PDXs implanted in humanized mice developed into tumors (TC286 PDXs developed in 3 of 5 Hu-NSG mice, and TC388 PDXs developed in 8 of 10 Hu-NSG mice) regardless of donor HLA type. Simultaneously implanted TC388 PDXs grew slower in humanized than in nonhumanized mice (Fig. 4D, P = 0.08). These results indicate that HLA type does not affect xenograft and PDX growth in humanized mice.

Next, we compared infiltration of immune cells in PDX tumor tissues developed in humanized and nonhumanized NSG mice.

(Continued) A549-luc cells were simultaneously implanted in nonhumanized NSG mice, which were also treated with pembrolizumab. IVIS images show the tumor intensity in treated and untreated (control) groups among Hu-NSG and NSG mice. Table on right shows HLA status of different donors used for generating these humanized mice. C, Quantitative analysis of the effect of pembrolizumab treatment on A549 lung metastasis burden as determined by measuring bioluminescence intensity in treated and control groups. D, Effect of pembrolizumab on TC388 Hu-PDX model. TC388 human PDX tumor was implanted in humanized mice, followed by pembrolizumab treatment. Antitumor response was evaluated by comparing the tumor volume in the control and treated PDX mice. Three PDX mice were used per treatment group. The effect of pembrolizumab was also evaluated in nonhumanized mice with PDXs. E, T-cell response in humanized mice after pembrolizumab treatment. The percentage of CD3+ and CD8+ T cells was compared among splenocytes from control (black bar) and pembrolizumab (Pembro; gray bar) treatment groups. F, Immune analysis showing the percentage of activated CD8+ T cells (CD8+ CD69+ T cells), and regulatory T cells (CD3+ CD4+ CD25+ T cells), CD33+ myeloid-derived suppressor cells (MDSC), CD56+ natural killer (NK) cells (NK); and Lineage (lin)– blasts. HLA-DR+ DCs in control and treatment groups (*, P < 0.05).
Infiltration of human CD45<sup>+</sup> cells, human T cells, and human macrophages into humanized-PDX TC286 tumor tissue (Fig. 5A; Supplementary Fig. S5) was increased, compared with PDX TC286 developed in a nonhumanized counterpart, as determined by IHC. Quantitative analysis of the IHC images for hCD45<sup>+</sup> and hCD3<sup>+</sup> staining showed a significant infiltration of human hCD45<sup>+</sup> and T cells in the humanized-PDX TC286 (P < 0.0001 and P = 0.01, respectively; Fig. 5B; Supplementary Fig. S5). No CD45<sup>+</sup> human cells were detected in the same PDX engrafted in nonhumanized mice. We also detected a significant infiltration of human macrophages (CD68<sup>+</sup> cells) in Hu-PDXs, as compared with their nonhumanized counterparts (Fig. 5, P < 0.0001). However, mouse MDSC infiltration of PDX tumor tissues, as shown by Gr-1 immunostaining, was not significantly different between humanized and nonhumanized mice.

**Strong antitumor effect by anti–PD-1 on Hu-PDX and human lung metastases models**

To validate the Hu-PDX model for immunotherapy research, we used checkpoint blockade with anti–PD-1 (pembrolizumab) to treat humanized mice bearing A549-luc lung metastases and lung PDX tumors (Fig. 6A). Tumor intensity was significantly reduced at weeks 2 and 3 in pembrolizumab-treated humanized mice regardless of the donor’s HLA type (P < 0.008 and P < 0.05, respectively; Fig. 6B and C). The IVIS images show the differences in tumor burden intensity between treated and untreated humanized and nonhumanized mice (Fig. 6B). No significant antitumor effect against lung metastases was found in nonhumanized mice (Fig. 6B). Similarly, pembrolizumab showed a significant antitumor response (P < 0.05 at day 25; P < 0.005 at day 29) in TC388F2 Hu-PDX tumors independent of HLA status in humanized mice, whereas no response to pembrolizumab was observed in nonhumanized PDX-bearing mice (Fig. 6D). These results indicate that the human immune cells activated by pembrolizumab treatment are functional in tumor control.

To determine whether the antitumor effect of pembrolizumab in humanized mice was associated with the generation of an immune response, we performed immune analysis of splenocytes in humanized mice bearing A549 lung metastases. Human CD3<sup>+</sup> T cells were moderately increased but the percentages of CD8<sup>+</sup> and T cells were signifi- cantly higher following TC441 single-cell stimulation than the unstimulated control (Fig. 7C, P < 0.0005). IFN<sub>γ</sub> T cells were not detected in PDXs stimulated with HBECS (Fig. 7C), indicating that the IFN<sub>γ</sub> stimulation is specific to the TC441 PDX. Furthermore, a significant PDX-specific CTL response was also found in humanized mice challenged with PDX (TC429; P < 0.01; Supplementary Fig. S7). No significant reactivity was observed when Hu-PDX–derived T cells were cocultured with autologous human PBMCs derived from PDX (TC429) patient (Supplementary Fig. S7).

To further investigate anti–PD-1–related immune responses in Hu-PDX mice, TC241 PDXs were implanted in humanized mice, and 6 weeks later they were treated with the checkpoint inhibitor pembrolizumab or nivolumab (Fig. 7D). Nivolumab elicited a strong antitumor response. Immune analysis of human CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells showed no significant differences in the frequency of CD8<sup>+</sup> T cells comparing nivolumab-treated and untreated PDX-bearing mice (Fig. 7E). However, the percentage of granzyme B<sup>+</sup> CD8<sup>+</sup> T cells was significantly higher in nivolumab-treated mice (Fig. 7F, P < 0.0005). Intracellular staining of granzyme B showed that 73% of CD8<sup>+</sup> T cells were granzyme B<sup>+</sup> in nivolumab-treated Hu-PDX mice, compared with 19% in untreated Hu-PDX mice (Fig. 7G). These data suggest that reconstituted T cells are capable of producing antitumor immune responses in Hu-PDX mice, which is antigen-specific.

**Discussion**

Major challenges in the development of the humanized mouse model are the high cost, the lengthy time required for human immune cell reconstitution, and the integration of multidisciplinary expertise. Umbilical cord blood is a rich source of CD34<sup>+</sup> cells with high proliferative potential, which are less likely to cause severe GvHD in recipient mice (20, 21). They engraft more effectively than bone marrow–derived stem cells, as reported by prior studies (22, 23). NSG mice have several advantages over other immunodeficient mouse models. They harbor a mutation in the IL2 receptor common gamma chain (IL2γ<sup>δ</sup>), which is necessary for high-affinity binding and signaling of regulatory cytokines including IL2, IL4, IL7, IL9, IL11, and IL21 (24–29). This mutation facilitates the engraftment of human HSPCs, thus reconstituting a functional human immune system capable of T- and B-cell–dependent immune responses. In contrast to NOD-scid mice, NSG mice do not develop thymic lymphomas, have a longer lifespan, and are capable of supporting repopulated human immune cells for a much longer period (30). In addition, this mouse model at least partially expresses HLA molecules instead of mouse MHC molecules (31–34).

Studies using ex vivo–expanded CD34<sup>+</sup> HSPCs for mouse humanization have repeatedly shown delayed engraftment. Engraftment of 25% mature human white blood cells (hCD45<sup>+</sup>), the threshold for successful humanization, takes almost 12 weeks, and inactive human CD3<sup>+</sup> T cells are not detected until 15 to 16 weeks post stem cell implantation. We have developed an improved humanized NSG mouse model system, derived from fresh, not expanded, cord blood CD34<sup>+</sup> HSPCs. As low as 100,000 stem cells, over 90% pure, were able to reconstitute

**Antigen-specific responses by reconstituted T cells in Hu-PDX tumors**

To determine whether T cells in humanized mice bearing PDX tumors could elicit an antigen-specific immune response, we challenged humanized mice with PDX TC441 (HLA-A’02) and assayed intracellular IFN<sub>γ</sub> assay in harvested splenocytes (Fig. 7A). HBECs with the same HLA-A’02 type were cocultured with T cells derived from challenged humanized mice (Fig. 7B). The percentages of IFNγ<sup>+</sup> CD3<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup> T cells from TC441 bearing humanized mice were significantly higher following TC441 single-cell stimulation than the unstimulated control (Fig. 7C, P < 0.0005). IFNγ<sup>+</sup> T cells were not detected in PDXs stimulated with HBECS (Fig. 7C), indicating that the IFNγ stimulation is specific to the TC441 PDX. Furthermore, a significant PDX-specific CTL response was also found in humanized mice challenged with PDX (TC429; P < 0.01; Supplementary Fig. S7). No significant reactivity was observed when Hu-PDX–derived T cells were cocultured with autologous human PBMCs derived from PDX (TC429) patient (Supplementary Fig. S7).

To further investigate anti–PD-1–related immune responses in Hu-PDX mice, TC241 PDXs were implanted in humanized mice, and 6 weeks later they were treated with the checkpoint inhibitor pembrolizumab or nivolumab (Fig. 7D). Nivolumab elicited a strong antitumor response. Immune analysis of human CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells showed no significant differences in the frequency of CD8<sup>+</sup> T cells comparing nivolumab-treated and untreated PDX-bearing mice (Fig. 7E). However, the percentage of granzyme B<sup>+</sup> CD8<sup>+</sup> T cells was significantly higher in nivolumab-treated mice (Fig. 7F, P < 0.0005). Intracellular staining of granzyme B showed that 73% of CD8<sup>+</sup> T cells were granzyme B<sup>+</sup> in nivolumab-treated Hu-PDX mice, compared with 19% in untreated Hu-PDX mice (Fig. 7G). These data suggest that reconstituted T cells are capable of producing antitumor immune responses in Hu-PDX mice, which is antigen-specific.

**Discussion**

Major challenges in the development of the humanized mouse model are the high cost, the lengthy time required for human immune cell reconstitution, and the integration of multidisciplinary expertise. Umbilical cord blood is a rich source of CD34<sup>+</sup> cells with high proliferative potential, which are less likely to cause severe GvHD in recipient mice (20, 21). They engraft more effectively than bone marrow–derived stem cells, as reported by prior studies (22, 23). NSG mice have several advantages over other immunodeficient mouse models. They harbor a mutation in the IL2 receptor common gamma chain (IL2γ<sup>δ</sup>), which is necessary for high-affinity binding and signaling of regulatory cytokines including IL2, IL4, IL7, IL9, IL11, and IL21 (24–29). This mutation facilitates the engraftment of human HSPCs, thus reconstituting a functional human immune system capable of T- and B-cell–dependent immune responses. In contrast to NOD-scid mice, NSG mice do not develop thymic lymphomas, have a longer lifespan, and are capable of supporting repopulated human immune cells for a much longer period (30). In addition, this mouse model at least partially expresses HLA molecules instead of mouse MHC molecules (31–34).

Studies using ex vivo–expanded CD34<sup>+</sup> HSPCs for mouse humanization have repeatedly shown delayed engraftment. Engraftment of 25% mature human white blood cells (hCD45<sup>+</sup>), the threshold for successful humanization, takes almost 12 weeks, and inactive human CD3<sup>+</sup> T cells are not detected until 15 to 16 weeks post stem cell implantation. We have developed an improved humanized NSG mouse model system, derived from fresh, not expanded, cord blood CD34<sup>+</sup> HSPCs. As low as 100,000 stem cells, over 90% pure, were able to reconstitute
Figure 7. Cytotoxic T-cell response in Hu-PDX tumors. A, Schematic of the assessment of antigen-specific T-cell response in humanized PDX mice. Humanized mice were implanted with PDXs, and the spleen and PDX were harvested when tumor volume reached 500 to 600 mm³ for in vitro coculture assays to detect intracellular IFNγ. B, Determination of the HLA status of TC441 PDXs. HBECs with the same HLA-A*02 type as the PDX were used as a control in coculture assays for alloreactivity. C, The percentage of IFNγ⁺ T cells under different stimulation conditions. Single-cell suspensions of PDX were mixed with splenocytes for stimulation of T cells. This T-cell stimulation cocktail was used as a positive control. ***, P < 0.0005. D, A single Hu-PDX mouse trial (SMT) was performed to evaluate the response to nivolumab treatment as determined by tumor volume measurements. TC241 human PDXs, a representative of n = 3 different PDXs with similar results, were developed in humanized mice and treated with nivolumab (Nivo) or untreated. E, Splenocytes of PDX-bearing mice were analyzed to determine T-cell responses by evaluating the status of CD3⁺, CD4⁺, and CD8⁺ T-cell subsets among the gated human lymphocyte population. F, The percentage of granzyme B⁺ T cells was also determined by intracellular staining of splenocytes derived from PDX-bearing humanized mice. G, Scatterplots showing the percentage of granzyme B⁺ CD8⁺ T cells in the treated and untreated groups.
Human immune populations, in peripheral blood, in just 4 weeks post stem cell engraftment, compared with 12 weeks or longer for ex vivo CD34+ HSPCs (14, 15). We found more than 25% hCD45+ cells in peripheral blood and detected human CD3+ T cells, CD19+ B cells, and NK cells. The rate of repopulation increased significantly at 6 weeks and 9 weeks, as shown in peripheral blood, spleen, and bone marrow of mice. At 16 weeks postengraftment, reconstituted human immune cell populations were still very high. The flow cytometry data (shows that most of the non-CD34+ HSCs are CD34-CD3+ cells that do not cause GvHD).

Humanized NSG mice derived from cultured CD34+ HSCs harbor human T cells that lack the ability to recognize antigens in an HLA-restricted manner (35) and are functionally inactive (11–13). In contrast, in our model, T cells were functionally active with the intact machinery of IFNγ signaling. They showed an antigen-specific response, and were reactive to both mitogenic stimulation, such as PMA and IL2, and allogeneic human cancer cells. The reconstituted T cells derived from humanized mice challenged with tumor cells showed no alloreactivity when cocultured with identical HLA-type human bronchial epithelial cells. KRAS or other mutation-specific CTL response was not determined due to the weak antigenicity of those proteins (36). We found that reconstituted T cells were granzyme B+, indicating that they are cytotoxic.

Next, we examined tumor growth and any corresponding immune responses and responses to checkpoint blockade in humanized mice implanted with molecularly tagged NSCLC xenografts or PDXs. We analyzed growth of two NSCLC xenografts, subcutaneous and metastatic, and three PDXs in humanized and nonhumanized NSG mice. The slightly slower tumor growth we observed in humanized mice was most likely due to the presence of an active immune system. Both tumor xenografts were successfully engrafted with no rejections, and PDX engraftment success rate was approximately 70%. Although our data indicated that PDX growth did not depend on donor HLA type, we are currently investigating the exact role of HLA in PDX engraftment and antitumor immune responses.

To provide additional validation that this improved humanized mouse model can recapitulate the responses to immunotherapy observed in patients, we treated animals implanted with either NSCLC metastatic tumor xenograft or PDX with pembrolizumab, a checkpoint inhibitor. Pembrolizumab has shown durable antitumor activity in NSCLCs that were previously not considered immune-responsive (37–41). We observed a strong antitumor response to pembrolizumab in humanized mice but not in their nonhumanized counterparts, thus highlighting the role of reconstituted human immune cells that are activated by checkpoint blockade therapy. After treatment, the populations of T cells (both CD3+ and CD8+ T cells) and active T cells (CD69+CD8+ T cells) were increased, whereas the population of myeloid-derived suppressor cells decreased. This is consistent with the results of our previous study, in a syngeneic mouse model, showing no significant effect of pembrolizumab effect on NK cells (42). In addition, the antitumor immune response to another anti–PD-1 checkpoint inhibitor, nivolumab, was evaluated in Hu-PDXs. A strong antitumor response to nivolumab was observed. Whereas the frequency of granzyme B+ cytotoxic T cells increased significantly, the total number of T cells did not change after nivolumab treatment. Further studies are needed to understand how antitumor immune responses are generated in HLA-mismatched humanized mice by analyzing the clonal variations of T cells (14, 15). A clinical study on allogeneic adaptive T-cell therapy reported antigen-specific CTL response in HLA-mismatch settings without initiating GvHD (43).

In conclusion, these findings provide evidence that humanization with fresh CD34+ HSCs, compared with cultured stem cells, can influence engraftment success, is less time consuming, and reconstitutes mice with enduring, substantial populations of multiple lineages of human immune cells. There is a significant difference in prolonged reconstitution between mice engrafted with fresh CD34+ HSCs have significantly prolonged reconstitutions compared with grafts of cultured stem cells. We found that our model replicates the human tumor response to checkpoint blockade, mounts an immune response to tumor-associated antigens and not alloantigens, and is not stem cell donor-dependent for its immune response. Coengrafting mice with human tumors that retain heterogeneity and human stem cells that are pure with high proliferative and stem cell activity will allow us to investigate the complex relationship between the human immune system and human tumors and to more accurately assess the efficacy of novel immunotherapeutic agents.

Disclosure of Potential Conflicts of Interest

J. A. Roth reports receiving a commercial research grant from The University of Texas MD Anderson Cancer Center, sponsored research agreement from Genprex, Inc, has ownership interest (including stock, patents, etc.) in Genprex, Inc, and is a consultant/advisory board member for Genprex, Inc. No potential conflicts of interest were disclosed by the other authors.

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References
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