Evaluation of *in vivo* anti-tumor response of solid tumors in a novel immune cell humanized NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCrl mouse model

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1 INTRODUCTION

Checkpoint blockade inhibitors targeting PD-1 and CTLA-4 pathways are clinically approved therapies for multiple cancer types. The performance of targeted interventions has been effective, but the clinical response rates vary. *In vivo* models of human immunity in human tumor bearing mice (TBM) is an important tool for studying mechanisms of targeted therapies and developing new and effective treatments. The NCG, a recently developed triple immunodeficient model, is an effective host for both human immune cells and tumors for the study of immuno-oncology-based therapeutics. The NCG strain lacks functional murine TBNK cells in addition to reduced macrophage and DC activity. In these pilot studies we evaluated the anti-tumor effects of immune checkpoint inhibitors on colon epithelial carcinoma (RKO) and basal lung cell adenocarcinoma (A549) cell lines in a human donor immune cell-humanized mouse model (HuCD34 NCG). Our results demonstrate significant immunomodulatory anti-tumor response to standard immune checkpoint inhibitors. The Charles River HuCD34 NCG humanized mouse model sustains robust engraftment of major human immune cells and demonstrated infiltration of T-cells into tissues and tumors, thus making this mouse model ideal for immuno-oncology studies.

2 METHODS

Model Generation

The NCG (NOD-*Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/NjuCrl) model was created by simultaneous CRISPR/Cas9 editing of the *Prkdc* and *Il2rg* loci in the NOD/Nju mouse. 4-8-week old female NCG mice received a sublethal dose of X-ray irradiation and were subsequently injected with umbilical cord blood derived human CD34$^+$ cells via tail vein (HuCD34 NCG). All animals were weighed three times weekly and health status monitored according to the Charles River Humanized Mouse End Stage Illness Guidelines[1]. [1]Jen, K., Rowe, J., Festin, S. Humane Endpoint Refinement for Total Body Irradiation and Humanization of NCG mice [abstract]. In: Journal of the American Association for Laboratory Animal Science 58(5); 607-726 (Sep 2019).

Tumor Implantation

HuCD34 NCG mice were implanted subcutaneously on the flank with either RKO or A549 tumor cells. Group randomization occurred when the average tumor size reached a volume of ~100mm^3 (A549) or 30-60mm^3 (RKO). Group mean tumor volumes (mm^3) were measured tri-weekly (RKO) or bi-weekly (A549).

Checkpoint Inhibitors and Dosing Regimens

Control mice were treated with isotype control IgG antibodies. RKO TBM were treated with anti-PD-1 antibody alone (Pembrolizumab (Keytruda), 100µg/mouse, IV, biwkx4). A549 TBM were treated with anti-PD-1 (CD279, BioXCell), anti-CTLA-4 (CD152, BioXCell) antibodies independently (200µg/mouse, IP, Q3Dx8) and in combination therapy, respectively.

Flow Cytometry

This novel humanized mouse model was phenotypically characterized by flow cytometry. Whole blood, spleen and tumor tissue were collected and processed for immunophenotyping in line with the study plans (Figure 1). Tissues were homogenized into single cell suspensions using the gentleMACS^{TM} Octo Dissociator (Miltenyi Biotech) according to vendor recommendations. Cells were incubated with a cocktail of anti-human antibody conjugates for surface marker staining. For intracellular staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (ThermoFisher). Prior to intracellular cytokine staining, cells were stimulated *ex vivo* for four hours using PMA/Ionomycin in the presence of brefeldin A. Subsequently, cells were harvested and stained for cytokines following fixation and permeabilization. Unstained, viability and fluorescence-minus-one (FMO) controls were processed in parallel. A set of samples for each tissue containing no stimuli, but brefeldin A were prepared as gating controls to discriminate positive versus negative populations. Following instrument setup and compensation, samples were acquired using the Attune^{TM} NxT Flow Cytometer 4-laser system (ThermoFisher) (A549) and LSR Fortessa^{TM} 5-laser system (Beckton Dickinson) (RKO). Post-acquisition analyses were performed using FlowJo v10.4 (FlowJo) for gating of immune cell populations and GraphPad prism (GraphPad Software) for data representation.

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3a RESULTS: TUMOR STUDY

Figure 1

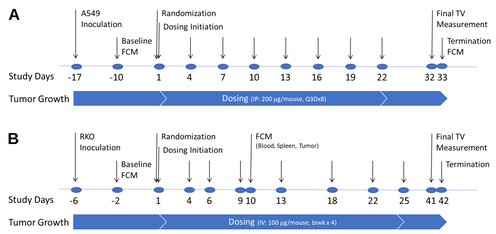

Figure 1 (Panel A and B). Tumor Study Timelines. A. A549 (human basal lung cell adenocarcinoma cell line) tumor study timeline. **B**. RKO (human colon epithelial carcinoma cell line) tumor study timeline.

Figure 2

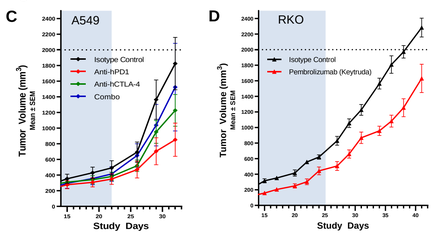

Figure 2 (Panel C and D). A549 and RKO tumor-inoculated HuCD34 NCG mice respond to immune checkpoint inhibitor dosing regimens. Tumor growth curves in HuCD34 NCG mice. Data are shown as mean ± SEM for n=5 per group (A549) and n=5-7 per group (RKO). Blue band corresponds to dosing window.

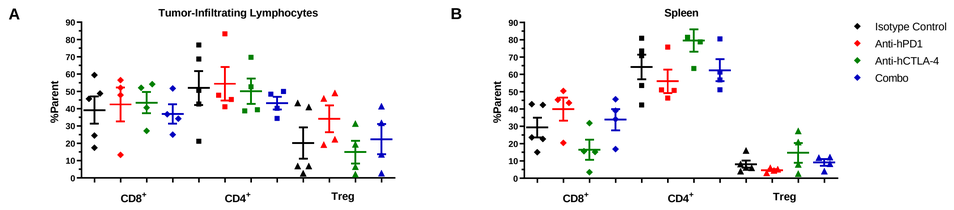

Figure 3. Human T-cell infiltration in A549 tumor-bearing HuCD34 NCG mice at termination. Tumor **(A)** and spleen **(B)** samples were collected at study termination for immunophenotyping by flow cytometry. Frequencies of analyzed subsets presented as proportion of their respective parent population in the gating hierarchy: CD4$^+$ and CD8$^+$ T-cells as percentage of CD3$^+$ T-cells and Treg as percentage of CD4$^+$ T-cells.

Fold Change		hCD3$^+$			hCD4$^+$			hCD8$^+$		
		IFN-γ$^+$	IL-2$^+$	TNF-α$^+$	IFN-γ$^+$	IL-2$^+$	TNF-α$^+$	IFN-γ$^+$	IL-2$^+$	TNF-α$^+$
Spleen	Isotype Control	28.88	29.13	0.97	15.64	35.85	24.42	120.63	20.12	20.79
	Anti-hPD1	22.11	21.16	0.65	17.82	30.85	28.12	57.21	32.40	14.12
	Anti-hCTLA-4	332.63	19.08	55.65	287.34	123.74	65.64	1004.89	14.29	37.28
	Combo	91.70	29.72	46.31	37.73	86.51	40.82	606.78	6.05	45.23
TIL	Isotype Control	14.14	2.75	14.19	12.66	15.25	17.80	22.11	7.96	16.01
	Anti-hPD1	8.54	2.20	12.36	5.74	2.40	9.55	61.00	0.35	14.03
	Anti-hCTLA-4	174.74	8.48	51.13	182.47	13.99	59.79	180.66	2.40	46.94
	Combo	6.88	4.26	8.71	5.41	2.69	12.74	11.44	52.24	7.90

Table 1. Identification of polyfunctional T-cells within tumor-infiltrating lymphocytes. Spleen and tumor from A549 tumor-bearing HuCD34 NCG mice were processed at study termination for intracellular cytokine staining (n=10). The frequency of IFN-γ, IL-2 and TNF-α producing cells are reported as proportion of human total T-cells (CD3$^+$) and subsets (CD4$^+$ and CD8$^+$), respectively. For each study group sample fold changes were calculated as mean percentages of lymphocytes stimulated with PMA/Ionomycin divided by the mean percentages of unstimulated lymphocytes. Double and triple positive cytokine excreting T-cells were detectable (data not shown). PMA: Phorbol 12-myristate 13-acetate. TIL: tumor-infiltrating lymphocytes.

3b RESULTS: TUMOR STUDY

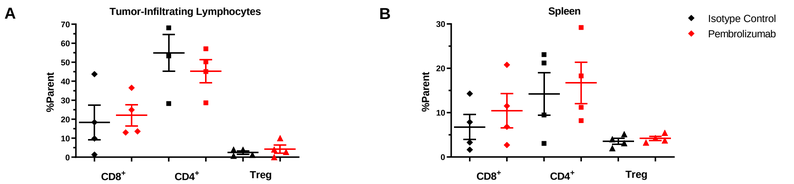

Figure 4. Human T-cell infiltration in RKO tumor-bearing HuCD34 NCG mice at termination. Tumor **(A)** and spleen **(B)** samples were collected at study termination for immunophenotyping by flow cytometry. Frequencies of analyzed subsets presented as proportion of their respective parent population in the gating hierarchy: CD4$^+$ and CD8$^+$ T-cells as percentage of CD45$^+$ cells and Treg as percentage of CD4$^+$ T-cells (hCD25$^+$ hFoxP3$^+$). Data are shown as mean ± SEM for n=4 per group.

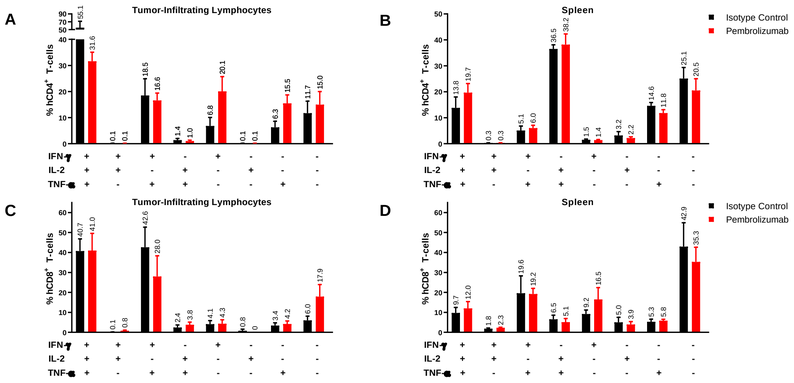

Figure 5. Identification of polyfunctional T-cells. Tumor **(A and C)** and spleen **(B and D)** from RKO tumor-bearing HuCD34 NCG mice were processed at study termination for intracellular cytokine staining after stimulation with PMA/Ionomycin. The frequency of IFN-γ, IL-2 and TNF-α producing cells are reported as percentage of human CD4$^+$ (A and B) and human CD8$^+$ (C and D) T-cells, respectively. Data are shown as mean ± SEM for n=4 per group. PMA: Phorbol 12-myristate 13-acetate.

4 CONCLUSION

The HuCD34 NCG mouse has been developed as a new humanized model providing stable graft function suitable for long-term tumor studies.

- A549 and RKO tumor-inoculated HuCD34 NCG mice respond to standard immune check point treatment.
- Anti-PD1 monotherapy significantly inhibits RKO and A549 tumor growth.
- Anti-CTLA-4 dosing, but not combined blockade of CTLA-4 and PD-1 increases intratumoral CD8$^+$ T-lymphocytes in A549 tumor-bearing HuCD34 NCG mice.
- Human immune cell engraftment levels confirmed by flow cytometry in peripheral blood (data not shown), spleen and tumor specimen collected from HuCD34 NCG mice.
- Human T-cell infiltration is observed in both A549 and RKO tumors with the majority of live T-cells responsive post infiltration.
- Human cytokines (IFN-γ, IL-2 and TNF-α) are released by tumor-infiltrating total T-cells.
- Triple positive IFN-γ, IL-2 and TNF-α secreting T-cells is highest in the tumor of RKO tumor-inoculated HuCD34 NCG mice.
- T-lymphocytes are activatable and not exhausted as indicated by polyfunctional responses.