NCG/CRL as a novel (Charles River) humanized mouse model for pre-clinical oncology studies: immunophenotypic characterization and performance monitoring

P841

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1 INTRODUCTION

Human immune cell mouse models are a valuable tool in the study and development of immunologically-driven cancer therapies including checkpoint inhibitor-based interventions. For these types of rodent models NCG mice can be utilized. This strain is triple immunodeficient lacking functional murine TBNK cells in addition to reduced macrophage and DC function. In this study we characterized the hematopoietic output of humanized mice following engraftment with human umbilical cord blood derived CD34$^+$ cells to generate the HuCD34NCG model. Our results indicate that the Charles River HuCD34NCG mouse can bear a human immunophenotype with a stable xenograft. It offers an experimental model for biomedical studies that require human immune function in an *in vivo* mouse organism.

Figure 1. 36-week study plan for the characterization of human stem cell engraftment in immunodeficient NCG mice. Day 0 represents the day of injection of CD34$^+$ cells isolated from two individual donors of umbilical cord blood after X-ray radio-ablation of bone marrow. Subsequent timepoints of blood and tissue collections and *ex vivo* analyses are indicated. FCM: flow cytometry.

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 cord blood derived human CD34$^+$ cells via tail vein. 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 end point refinement for total body irradiation and humanization of NCG mice. 70th Annual American Association for Laboratory Animal Science (AALAS); 2019 Oct 13-17; Denver, CO.

Flow Cytometry

This novel humanized mouse model was phenotypically characterized by flow cytometry. Whole blood, bone marrow (BM), spleen, tumor, liver and lung tissue were collected and processed for immunophenotyping in line with the study plan (Figure 1). Tissues were homogenized into single cell suspensions using the gentleMACS™ Octo Dissociator (Miltenyi Biotech). 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 using the Cell Stimulation and Protein Transport Inhibitor Cocktails (ThermoFisher). Subsequently, cells were harvested and stained for cytokines following fixation and permeabilization. Unstained, viability and fluorescence-minus-one (FMO) controls were processed in parallel. Following instrument setup and compensation, samples were acquired using the Attune NxT Flow Cytometer 4-laser system (ThermoFisher). Post acquisition analyses were performed using FlowJo v10.4 (FlowJo) for gating and GraphPad prism (GraphPad Software) for data representation.

3a RESULTS: CHARACTERIZATION

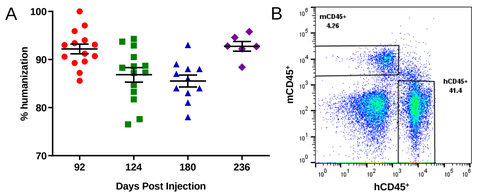

Figure 2. Human immune cell engraftment. A. Whole blood collected via submandibular bleed in lithium-heparin tubes was analyzed at Days 92, 124, 180 and 236 (Weeks 13, 18, 26 and 34) post injection using a 10-color flow cytometry panel. The presence of human lymphoid cells is calculated as percentage of human CD45$^+$ cells of total lymphocytes detected. Data are shown as mean ± SEM from n=14 (Days 92 and 124), n=12 (Day 180) and n=6 (Day 236), respectively. B. Representative dot plot depicting hCD45$^+$ and mCD45$^+$ lymphocytes as percentage of viable single cells. By sequential gating all doublets, larger cell aggregates and dead cells were initially excluded. Further TBNK cell subsets were defined within the human lymphocyte gate (complete gating strategy not depicted).

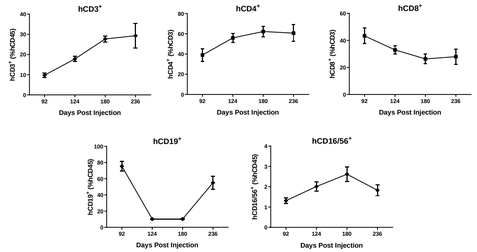

Figure 3. Longitudinal characterization of HuCD34NCG mice immunophenotypes. Whole blood collected via submandibular bleed in lithium-heparin tubes was analyzed using a 10-color flow cytometry panel at Days 92, 124, 180 and 236 post injection. Consistent levels of peripheral total T-cells (CD3$^+$), T-cell subsets (CD4$^+$ and CD8$^+$), B-cells (CD19$^+$) and NK cells (CD3$^-$CD16$^+$CD56$^+$) were detected throughout the study with initially elevated B-cell populations. Relative percentages are given as a proportion of the respective parent population. Data represent with mean ± SEM.

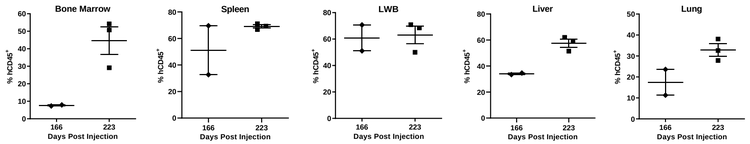

Figure 4. Human immune cell distribution in mouse tissues. Tissues collected during necropsy were analyzed using a 12-color flow cytometry panel. Viable hCD45$^+$ cells were detected in HuCD34NCG mice over time across all tissues analyzed (bone marrow, spleen, lysed whole blood (LWB), liver and lung). Relative percentages are given as a proportion of live cells. Data are shown from n=2 (Day 166) and n=3 (Day 223).

Table 1. Human lymphocyte infiltration in mouse tissues at Day 166 and Day 223 post injection. Human lymphoid and myeloid cell populations are detectable in the BM, spleen, liver and lung of HuCD34NCG mice over time. Data are shown as mean from n=2 (Day 166) and n=3 (Day 223). Frequencies of gated subsets within the tissues presented as proportion of the respective parent population: hCD3$^+$ cells: percentage of live hCD45$^+$ cells; hCD4$^+$, hCD8$^+$ and proliferating T-cells: percentage of live hCD45$^+$hCD3$^+$ cells; Treg: percentage of live hCD45$^+$hCD3$^+$CD4$^+$ cells; hCD11b$^+$ and hCD11c$^+$ cells: percentage of live hCD45$^+$hCD3$^-$ cells (data from M1 and M2 populations not shown).

	BM		Spleen		Liver		Lung	
	D166	D223	D166	D223	D166	D223	D166	D223
hCD45$^+$	7.64	44.67	51.15	69.07	34.00	57.53	17.45	32.87
hCD3$^+$	20.80	13.07	41.50	35.70	42.75	49.00	66.50	65.10
hCD4$^+$	30.25	67.03	38.95	67.07	39.85	73.73	40.70	71.37
hCD8$^+$	41.10	26.50	44.90	27.70	0.62	1.36	45.95	22.47
Treg	12.33	11.00	11.80	7.39	12.35	9.06	18.40	10.31
Prolif. T-cells	0.37	0.62	4.83	4.74	6.83	25.10	6.84	8.86
hCD11b$^+$	17.44	4.09	2.81	6.05	5.19	8.60	18.50	28.73
hCD11c$^+$	1.62	0.78	1.86	1.07	2.34	1.31	4.31	1.88

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3b RESULTS: TUMOR STUDY

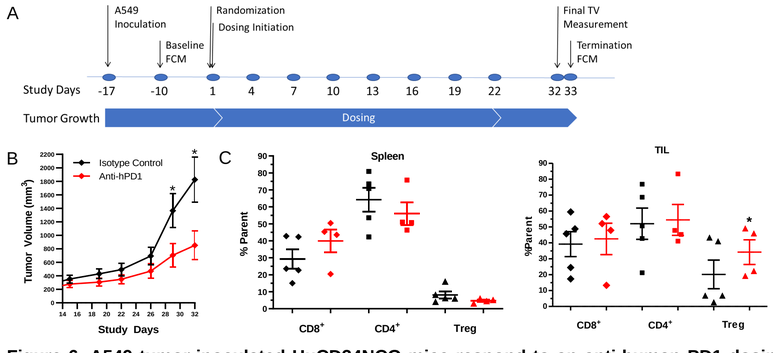

Figure 6. A549 tumor-inoculated HuCD34NCG mice respond to an anti-human PD1 dosing regimen. A. A549 (human lung carcinoma cell line) tumor study timeline. B. Tumor growth curves in HuCD34NCG mice. Tumors were implanted subcutaneously on the left flank of HuCD34NCG mice. Group mean tumor volumes (mm^3) were measured twice weekly. Data are shown as mean ± SEM for n=5 per group. C. T-cell infiltration in A549 tumor-bearing HuCD34NCG mice at termination. Spleen and tumor 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. *P<0.05 were considered statistically significant based on a one-tailed Student's t-test. TIL: tumor-infiltrating lymphocytes.

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
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

Table 2. Identification of polyfunctional T-cells within tumor-infiltrating lymphocytes. Spleen and tumor from A549 tumor-bearing HuCD34NCG 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. PMA: Phorbol 12-myristate 13-acetate.

4 CONCLUSION

The HuCD34NCG mouse has been developed as a new humanized model providing stable graft function and is a relevant model in immuno-oncology studies.

- HuCD34NCG mice can be humanized and sustain humanization through Day 236 post engraftment.
  - Phenotypic data acquired by flow cytometry demonstrate reconstitution of human immune cells.
- Human immune cells repopulate and maintain lymphoid and myeloid differentiation over time.
- T-lymphocytes are activatable and not exhausted (polyfunctional responses).
- A549 tumor-inoculated HuCD34NCG mice respond to immune check point treatment.