Pitfalls in preclinical development of immunotherapies for ER+ breast cancer: estrogen as an immunomodulator potentially influencing pembrolizumab efficacy in a breast cancer model in humanized mice

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Introduction

Immunotherapies have the potential to improve outcomes in triple-negative breast cancer patients but evidence is less consistent in estrogen receptor-positive (ER+) patients.

To advance preclinical development and to understand the effects of immunotherapies against ER+ breast cancer, we aimed to establish a novel orthotopic ER+ breast cancer model in humanized mice and to study efficacy of pembrolizumab in the model.

Materials and Methods

Female CIEA NOG® (NOG) mice and NOG mice engrafted with human CD34+ hematopoietic stem cells (huNOG, Taconic Biosciences) were implanted with 5 µg/day estradiol (E2) -releasing implants (PreclinApps), and one week later inoculated with ER+ MCF-7 (ATCC) human breast cancer cells into the mammary fat pad (n=12 per group). One group of huNOG mice did not receive E2 implants. Orthotopic tumor growth was followed by caliper measurements. At study week 2, the E2 supplemented huNOG mice were stratified to receive either human IgG4 isotype control (CrownBio) or anti-PD-1, pembrolizumab (5 mg/kg, i.p., Q5D, MSD Finland) until the end of the study. The study was terminated at study week 7 and tumors were processed for histology and immunohistochemical (IHC) stainings. Changes in blood cell counts were assessed by flow cytometry (BD LSRFortessa™) performed at Turku Bioscience Centre, Cell Imaging and Cytometry Core, Finland and hematology (VetScanHM5) performed at Central Animal Laboratory, University of Turku, Finland.

Table 1. Information of the antibodies used in IHC.

Marker	Antibody information
Human leukocyte common antigen (CD45)	(2B11+PD7/26) 1:60 cat: AZC-050, Nordic Biosite
Human T-cell (CD3)	(BSR10) 1:100 cat: BSH-3000-1, Nordic Biosite
Human helper T-cell (CD4)	(BSR4) 1:200 cat: BSH-3008-1, Nordic Biosite
Human cytotoxic T-cell (CD8)	(BSR5) 1:100 cat: BSH5001-1, Spring Bioscience
Human Granzyme B	(BSR150) 1:200 cat: BSH-3014-1, Nordic Biosite
Human programmed cell death 1 (PD-1)	(BSR1) 1:200 cat: BSH-3001-1, Nordic Biosite
Human programmed death ligand 1 (PD-L1)	(BSR90) 1:200 cat: BSH-4003-1, Nordic Biosite

Table 2. Information of the antibodies used in flow cytometry.

Target	Antibody
Human CD3	CD3-FITC, REA613 130 113 138, Miltenyi
Human CD4	CD4-APC, REA623 130 113 222, Miltenyi
Human CD8	CD8-APC-Vio770, REA734 130 110 681, Miltenyi

Tumor growth and survival

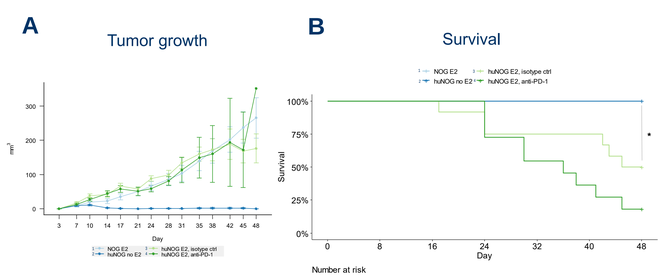

FIGURE 1. A) Tumor growth *in vivo*. Tumor growth regressed in huNOG mice that were not supplemented with E2. B) Kaplan-Meier analysis of survival. During the study, mice were sacrificed when they met pre-defined sacrifice criteria. The median survival time was 48, 48, 40 and 35 days in the study groups 1 – 4, respectively. 100%, 100%, 50% and 22% of the mice were alive at study end day in study groups 1 – 4, respectively.

Hematology

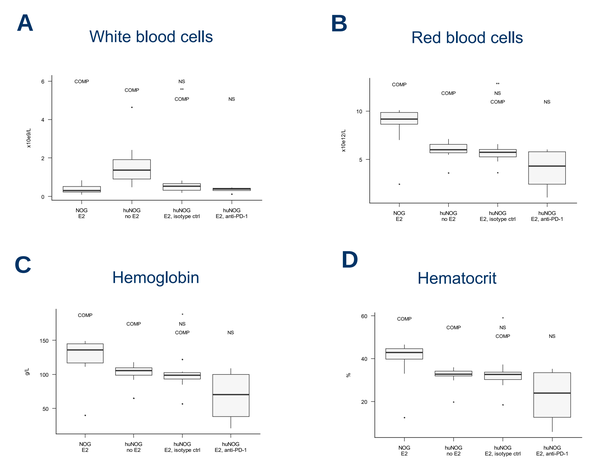

FIGURE 2. A) White blood cell count (x10e9/l), B) red blood cell count (x10e12/l), C) hemoglobin (g/L) and D) hematocrit (%) analyzed from blood samples collected before sacrifice at study end. The number of mice in analysis is 10, 12, 8 and 4 for the groups shown in the figures. All figures are presented as median ± IQR25% ± min/max.

Flow cytometry

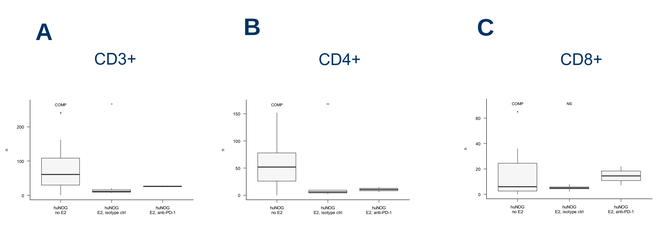

FIGURE 3. A) Number of live CD45+CD3+ T cells B) number of CD4+ helper T cells and C) number of CD8+ cytotoxic T cells analyzed from blood samples collected before sacrifice at study end. All figures are presented as median ± 25%IQR ± min/max.

Tumor IHC stainings for TILs, PD-L1 and PD-1

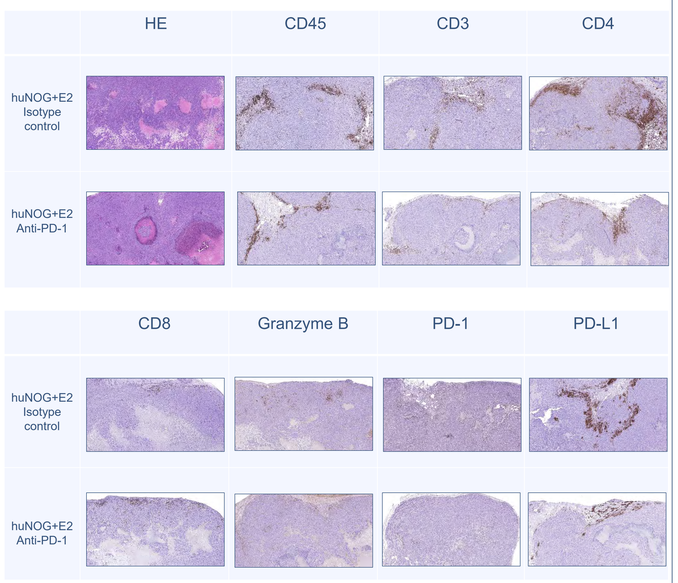

FIGURE 4. Histology and IHC staining of orthotopic MCF-7 tumors grown in huNOG mice supplemented with E2 and treated with isotype control or anti-PD-1. HE= hematoxylin-eosin staining (magnification x 5).

Summary

- ER+ orthotopic MCF-7 breast tumors grew only in the presence of E2 supplement. No clear anti-tumor effects were observed with pembrolizumab treatment.
- General condition of huNOG mice started to decrease after 3 weeks of E2 supplementation and their survival was decreased compared to huNOG mice without E2 supplement.
- Hematological analysis indicated that E2 decreased the levels of white and red blood cells, hemoglobin and hematocrit.
- Flow cytometry analysis confirmed lower numbers of CD3+, CD4+ and CD8+ T cells in the blood of E2 supplemented huNOG mice.
- IHC staining showed low number of TILs and low expression of PD-1 and PD-L1 in tumors grown in huNOG mice supplemented with E2.

Conclusions

Estrogen had immunomodulatory effects and induced adverse effects including anemia in humanized mice. No clear anti-tumor effects of pembrolizumab were observed in this ER+ model.

Caution should be taken when evaluating efficacy of immunotherapies in hormone-dependent preclinical cancer models. Preferably, the use of ER+ breast cancer models where tumor growth is supported by local microenvironment instead of E2 supplementation, such as bone metastasis models, should be considered.

Acknowledgements

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