**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 CD3+ hematopoietic stem cells (huNOG, Taconic Biosciences) were implanted with 5 μg/day estradiol (E2) -releasing implants (PrecinAppSs) 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 palpation measurements. At study week 2, the E2 supplemented huNOG mice were stratified to receive either human IgG4 isotype control (CrowBio) or anti-PD-1, pembrolizumab (5 mg/kg, i.e., QSD, 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.

**Flow cytometry**

**Hematology**

**Tumor growth and survival**

**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.

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**Table 1** Information of the antibodies used in IHC

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