ImmunoTools *special* Award 2023

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Exploring the feasibility of anti-CSPG4 vaccination for the treatment of osteosarcoma

Osteosarcoma (OSA) is one of the most common cancer in the childhood population. Conventional therapies for the management of the localized disease include surgical excision of the primary tumor in association to neo/adjuvant chemotherapy. However, OSA has a high tendency to recur and metastasize, mainly to the lungs, despite these treatments, and the advanced disease is usually incurable, with a 5-year survival rates of 20%–30%. The rarity of the disease, the young age of patients and the paucity of recognized targetable antigens, limit opportunities to robustly test new therapeutic approaches. As a step forward in this direction, we have recently demonstrated that the chondroitin sulfate proteoglycan (CSPG)4 - a cell surface proteoglycan with a key oncogenic role in several tumor histotypes - is a clinically relevant target also in OSA (*Riccardo et al., Ther. Adv. Med. Oncol., 2019; Tarone et al., Life, 2022*). Indeed, we have recently demonstrated that CSPG4 is highly expressed in OSA cell lines and in their derived osteospheres, enriched in cancer stem cells, although it is not expressed in normal tissues. Moreover, we found that CSPG4 overexpression has been related to worse prognosis in human OSA patients (*Riccardo et al., Ther. Adv. Med. Oncol., 2019*). These features and its cell surface localization make CSPG4 an ideal target for anti-cancer immunotherapy.

On these evidences, we have also recently demonstrated the safety and immunogenicity of a CSPG4-targeting DNA vaccine, exploiting highly translational preclinical models, including human OSA xenograft mouse models, and client-owned OSA-affected dogs, enrolled in a pilot veterinary trial (*Tarone et al., Mol Therapy, 2023*). Our preliminary results also suggested the potential of anti-CSPG4 vaccination in a human setting, but further evaluations are strongly needed to consider this strategy as a liable novel option for human OSA treatment.

Therefore, with the "ImmunoTools Award" we would like to have the possibility to expand our preliminary results in a human setting (*Tarone et al., Mol Therapy, 2023*) exploiting *in vitro* "surrogate" experiments. Briefly, human peripheral blood leukocytes (PBL) will be isolated by the blood of healthy subjects (provided by the local Blood Bank of Turin, Italy). Monocytes will be then isolated from PBL using CD14 MicroBeads and will be subsequently cultured with recombinant human IL-4 and GM-CSF (both from ImmunoTools), to generate immature DC. TNF-α and IL-1β (both from ImmunoTools) will be added for the final 24 h to induce DC maturation. CD14-depleted PBL will be stored in liquid nitrogen until use. mDC will be

transfected, using a DC transfection kit, with different CSPG4-coding plasmids or the empty vector and co-cultured with thawed lymphocytes for 7 days in order to simulate a DNA vaccination *in vitro*. Pre-activated lymphocytes will be then collected for further evaluation. In particular, the immunophenotyping of "vaccine" induced T cells will be evaluated. The percentage of CD4+ (ImmunoTools) and CD8+ (ImmunoTools) T cells producing IFN-γ (ImmunoTools) and granzyme (ImmunoTools), as well as the eventual induction of T regulatory expansion (ImmunoTools) will be analyzed by flow cytometry.

To evaluate the anti-tumor potential of pre-activated lymphocytes in the different conditions, PBL collected from HLA-A2 positive donors will be tested in a cytotoxicity assay against human CSPG4+ OSA, using two different HLA-A2 matched OSA cells (U2-OS and Soas-2) as targets. CFSE labeled human OSA cells will be incubated with CSPG4-transfected-DC or empty-vector-transfected-DC pre-activated lymphocytes from healthy donors at different effector:target ratios for 48 h at 37°C. After staining with 1 μg/μL of 7-aminoactinomycinD, cells will be acquired using a flow cytometer. The percentage of tumor-cell killing by mean of activated lymphocytes will be evaluated.

The ImmunoTools collection of recombinant cytokines and flow cytometry antibodies would greatly contribute to my research to set up the *in vitro* surrogate experiment and to obtain relevant insights regarding the potential of anti-CSPG4 vaccination to break the immune tolerance against the self CSPG4 molecule in a human setting and to induce a proper T cell activation with a luckily cytotoxic activity against $CSPG4^{+}$ osteosarcoma cells.

Relevant references

Riccardo F. et al., Identification of CSPG4 as a promising target for translational combinatorial approaches in osteosarcoma. Ther Adv Med Oncol 2019, https://doi.org/10.1177/1758835919855491.
Tarone L. et al., Improving Osteosarcoma Treatment: Comparative Oncology in Action. Life 2022, https://doi.org/10.3390/life12122099.
Tarone L. et al., A chimeric human/dog-DNA vaccine against CSPG4 induces immunity with therapeutic potential in comparative preclinical models of osteosarcoma. Molecular Therapy 2023, https://doi.org/10.1016/j.ymthe.2023.06.004.

ImmunoTools *special* AWARD for **Federica Riccardo** includes 10 reagents

FITC - conjugated anti-human Granzyme K

PE - conjugated anti-human CD25, IFN-γ

PerCP - conjugated anti-human CD4

APC - conjugated anti-human CD8, FoxP3

recombinant human cytokines: rh IL-4, rh GM-CSF, rh TNF-α and rh IL-1β

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