Lucie Rospape

Abstract

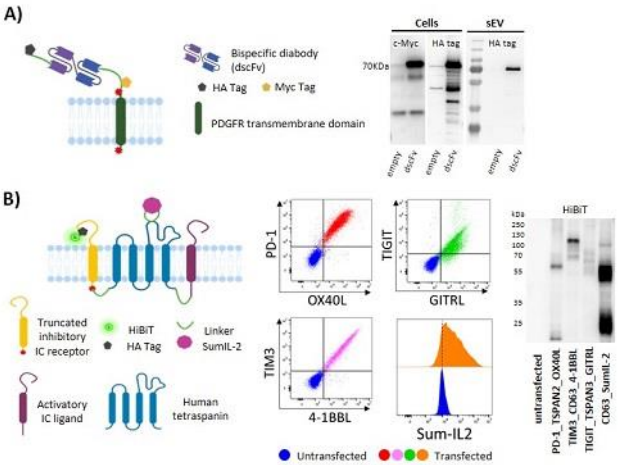
Background: Chronic Lymphocytic Leukemia (CLL), the most common leukemia in adults, is characterized by the accumulation of abnormal B-lymphocytes in blood and lymphoid organs, and by an extremely immunosuppressive microenvironment (ME). Despite significant advances in treatments, CLL remains an incurable disease with unmet clinical needs. The development of innovative immunotherapies could overcome some of these challenges. Small extracellular vesicles (sEVs) or exosomes are nano-sized vesicles secreted by all cells and involved in intercellular communications. We have previously demonstrated that sEVs are key players in CLL by promoting the formation of cancer-associated fibroblasts (Paggetti *et al*, Blood, 2015) and PD-L1+ monocytes. Importantly we showed that sEVs are indispensable for leukemogenesis *in vivo* by impairing T-cell mediated anti-tumor immunity (Gargiulo *et al*, Blood Cancer Discovery, 2023).

Aims: This project aims to design and produce bioengineered sEV (BesEV) as novel immunotherapies to counteract the immunosuppressive ME in CLL.

Methods: We designed and created, using molecular biology and vector cloning, a set of plasmids to express immune-related molecules on the surface of sEVs. Cellular models were then established using these constructs and sEVs were produced and purified using our established methods. These bioengineered sEV (BesEV) were analyzed with different methods and their functionality tested *in vitro*.

Results: First, we successfully created diabody-expressing vector (bispecific scFv against CD3 and CD19, **Fig.1A**) to express bispecific antibodies at the surface of sEVs. This construct is designed to bring CD3+ T cells in the vicinity of CD19+ CLL cells to enhance leukemic cell killing. We also generated constructs expressing truncated inhibitory immune checkpoint (ICP) receptors (e.g PD-1, TIM3) together with activating ICP ligands fused (e.g. 4-1BBL, OX40L) to tetraspanin proteins (e.g. CD63) **(Fig.1B)**. This should act as decoy for inhibitory ICP ligands (i.e. PD-L1, CD155) expressed by CLL and ME-derived cells and sEV, and at the same time stimulate activating ICP receptors (i.e. OX40, 4-1BB) on immune cells. We also created a vector containing super mutated IL-2 (SumIL-2), a modified version preferentially binding to $CD8^+$ T cells rather than Treg cells, introduced in the extracellular loop of the CD63 (**Fig.1B**). We engineered selected sEV-producing cells with these different vectors (alone or in combination). BesEV were produced, purified and characterized by WB and flow cytometry. Their activity was further tested *in vitro* with murine CD8+ T cells and TCL1-derived cells.

Conclusion: We successfully generated BesEV with immune-promoting capabilities in order to reactivate the anti-tumor immune response against CLL cells. The next step will be to test the efficacy of these different BesEV in preclinical mouse models of CLL, but we foresee BesEV as promising immunotherapies to be integrated into combinatory therapeutic strategies.



A) Representation of bispecific diabody combining anti-CD3 and anti-CD19 scFv. Western blots of c-Myc and HA tags in HEK-293F cells (left) and sEV (right) showing diabody production. **B)** Representation of an immunostimulatory molecule combining a truncated form of an inhibitory IC receptor (PD-1, TIM3 or TIGIT) and an activator IC ligand (OX-40L, GITRL, 4-1BBL) anchored in sEV membrane with a tetraspanin molecule. A super mutated form of IL-2, binding to CD8+ T cells only, was added in an extracellular loop of the tetraspanin to foster CD8+ T cells anti-tumor response. Flow cytometry overlay plots showing the expression of IC or Sum-IL2 in untransfected (blue) and transfected (colored) HEK-293F cells. The HiBiT nanoluciferase part was detected by WB in lysates of HEK-293F cell transfected with the different constructs.