TITLE: **Simultaneous over-expression of graft protective hHO-1, hCD73 and hCD39: effects against cell cytotoxicity and cell death induced by the inflammatory cytokine TNF-α in an in vitro model**

AUTHORS: Alessandro Cinti[1], Marco De Giorgi[2], Claudia Arena[1], Elisa Chisci[1], Gloria Galimberti[1], Cristina Bugarin[3], Maria Grazia Cerrito[1], Giuseppe Gaipa[3], Ryszard Tom Smolenski[1,2], Roberto Giovannoni[1], and Marialuisa Lavitrano[1]

AFFILIATIONS: [1]Department of Surgery and Interdisciplinary Medicine, University of Milano-Bicocca, Monza, Italy; [2]Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland. [3]Pediatric Department, Laboratory of Cell Therapy Stefano Verri, University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy

AIM: In order to obtain a donor animal that is compatible for xenotransplantation several modifications of the genome are required. We sought to test the effects of simultaneous expression of a combination of three selected human genes, namely Heme Oxygenase 1 (hHO1), Ecto 5' Nucleotidase (hCD73) and Ecto Nucleoside Triphosphate Diphosphohydrolase 1 (hCD39), involved in the down-regulation of the inflammatory response, in an in vitro model of TNF-α-induced injury.

METHODS AND RESULTS: A triple cistronic vector, named pCX TRI-2A, harbouring hHO-1, hCD73 and hCD39 coding sequences, has been produced by cloning in frame the sequences of the three genes by using the F2A-based technology. This vector has been stably transfected in mouse NIH3T3 cell line. The simultaneous expression of the three genes has been demonstrated at RNA level by RT-PCR and at protein level by immunofluorescence, fluorescence activated cell sorting (FACS) and western blotting.
Transgenic (TG) and wild type (WT) NIH3T3 were tested in a TNF-α mediated cytotoxicity assay and caspase activation assay. Both test showed a significant reduction in cytotoxicity (-26% at 24h, -44% at 48h) and caspase activation (-27% at 16h) in TG cells in comparison with WT cells. Moreover, there is a significant reduction in number of dead cells in TG compared to WT cells, as assayed by trypan blue exclusion counting (-10% at 24h, -46% at 48h) and by FACS detection of Propidium Iodide (PI) staining (-35% PI positive at 24h, -37% PI positive at 48h).
Incubation of TG and WT NIH3T3 with enzymatic substrates of HO-1 and CD39/CD73, hemin and ATP respectively, promotes resistance of murine cells to inflammatory stimuli (TNF-α). This effect is more evident and further increased in TG cells over-expressing the three genes. In fact, 24 hours of TNF-α treatment induced a cytotoxicity of about 27% and 20% in WT and TG cells respectively, while the addition of hemin and ATP decreased the cytotoxicity up to 16% in WT cells and markedly more in TG cell reaching only 5% of cytotoxicity.
The differences in the activation of TNF-a signaling pathway were analysed at molecular level by using an RT^2 Profiler TM PCR array system in which the expression of 84 TNF-related pathway genes were evaluated by real-time PCR. The activity of the hCD39/hCD73 genes in TNF-α-treated TG cells maintained up-regulated the anti-inflammatory *Tnfaip3* gene and the activity of hHO1 in TNF-α-treated TG cells up-regulated the anti-apoptotic *Traf3* gene as compared to WT cells.

CONCLUSIONS: The simultaneous over-expression of hHO-1, hCD73 and hCD39 in murine cells is protective against human TNF-alpha mediated cytotoxicity and apoptosis. The protective effect given by the simultaneous expression of hHO1, hCD73 and hCD39 genes against TNF-alpha toxicity is characterized by the up-regulation of specific genes inhibiting the NfkB pathway.