PRE-CLINICAL DEVELOPMENT AND CHARACTERIZATION OF A DECITABINE-INDUCED REGULATORY HLAG^{+}CD4^{+}-T CELL-ENRICHED CELL PRODUCT AGAINST GVHD

P. Christofi[1, 2*], D. Kefala[2*], M. Lysandrou[2*], P.G. Papayanni [1,2,3], C. Pieridou[4], N. Savvopoulos[2], A.-L. Chatzidaniil [1,3], M. Kyriakou[4], I. Sakellari[1], P. Costeas[4], A. Papadopoulou[1], E. Yannaki[1, 5], A. Spyridonidis[2]

[1]Gene and Cell Therapy Center, George Papanikolaou Hospital, Thessaloniki, Greece
[2] Institute of Cell Therapy, University of Patras, Rio, Greece
[3] Department of Genetics, School of Biology, Aristotle University of Thessaloniki, Thessaloniki
[4] Center for the Study of Hematological and other Malignancies, Nicosia, Cyprus
[5] University of Washington, Seattle

*equal contribution

Background Graft-vs-host-disease (GvHD) is a life-threatening complication of allogeneic hematopoietic cell transplantation(allo-HCT) for which the approved therapies are suboptimal. HLA-G is an immunosuppressive molecule playing a central role in preventing the rejection of the semi-allogeneic foetus during pregnancy, the expression of which is epigenetically regulated. We have previously reported the *ex vivo* generation of decitabine-induced HLAG^{+}CD4^{+}FOXP3^{-} regulatory T-cells exerting potent suppressive function *in vitro* (Cytotherapy 2017;19:521). Herein, we aimed to further elucidate the molecular mechanisms beyond the HLAG^{+}CD4^{+} T cell-mediated immunosuppression and produce and characterize a clinical scale product enriched for HLA-G^{+} immunsupresssive cells(iG-Tregs).

Methods Peripheral Blood Mononuclear Cells from healthy donors were enriched for T-cells through monocyte depletion, activated with CD3/CD28-beads for 3 days, followed by a 3-day decitabine treatment(n=10). The final product(iG-Treg) was assessed *in vitro* and *in vivo*(NSG mice). FACS-sorted HLAG^{+}CD4^{+} T-cells were further characterized in depth by RNA-seq (Illumina) and single cell RNA-seq(scRNA-seq) (10X Genomics).

Results The iG-Tregs contained median(Δm) 93,7% (91.6%-98.6%) CD3+cells, predominantly CD4+[Δm CD4:CD8 ratio: 2,05(0.81-3.39)]. Compared to untreated controls(PBS), iG-Tregs were enriched for HLAG^{+} cells within the total CD3+ population[Δm 15.32% (6.9-22.9%)] as well as within the CD4+[Δm 21.6% (6.5-28.9%) and CD8+[Δm 16% (8.3-26.1%)] subpopulations. iG-Tregs demonstrated a favorable safety profile over PBS-treated control cells *in vitro,* as depicted by their diminished alloreactivity against allogeneic PHA blasts (1.8±1% lysis *vs* 49±4, p<0.0001) and their reduced ability to react after PMA/ionomycin stimulation(intracellular IL-2, p=0.02, IFNγ, p=0.008, IL-17a, p=0.04]. Except IL-13 (p=0.05) no other Th1/Th2 cytokines were increased in iG-Treg supernatants. The diminished

alloreactivity of iG-Tregs over PBS-treated control cells was shown also *in vivo* after infusion in NSG mice; 6/9(67%) iG-Treg treated mice survived until sacrifice whereas all 7 PBS-control treated mice developed GvHD from day 21 and succumbed in Δm day 35. The ability of iG-Tregs in preventing GvHD was tested by their coinfusion with T-cells; while T-cell infusion resulted in lethal GvHD by day 36 in all 3 control mice, the co-administration of iG-Tregs delayed (5/6 mice died by day 66) or even prevented(1/6) the GvHD onset. NGS sequencing showed that FACS-sorted decitabine-induced $HLAG^{+}CD4^{+}$ cells had a distinct and homogenous expression profile compared to their $HLAG^{-}CD4^{+}$ counterparts. $HLAG^{+}CD4^{+}$ cells differentially expressed regulatory genes (e.g. CCL17, CCL22 and CXCL9) and interestingly also, the predominantly myeloid suppressor gene IDO-1, a finding which was further validated at mRNA (RT-PCR) and protein (FACS) level. The prominent regulatory signature of the $HLAG^{+}CD4^{+}$ cells was confirmed by single cell RNA-seq analysis.

Conclusions iG-Tregs can be easily produced by a GMP-compatible protocol and constitute a well-characterized product with a safety profile to be further evaluated in an upcoming phase I-II clinical trial for the prevention and treatment of steroid-refractory GvHD(EUDRACT 2021-006367-26). Besides HLA-G, the possible contribution of IDO and other regulatory genes in the immunosuppressive function of HLAG+CD4+ cells remain to be assessed in future functional assays. To further optimize the ability of the iG-Tregs in GvHD prevention *in vivo*, we currently explore a different treatment design, aiming to increase the persistence of the hypomethylation effect exerted by decitabine.