**Pre-clinical development and characterisation of a decitabine-induced regulatory HLAG$^+$CD4$^+$-T cell-enriched cell product against GvHD**

M. Lysandrou[1], D. Kefala[1], P. Christofi[1, 2], C. Pieridou[3, 4], P.G. Papagianni[1, 2, 5], N. Savvopoulos[1], M. Kyriakou [3, 4], P. Stamou [1], A. Papadopoulou [2], P. Costeas [3, 4], E. Yannaki [2, 6], A. Spyridonidis [1]

[1]University of Patras, Patras, Greece, [2]Gene and Cell Therapy Center, George Papanikolaou Hospital, Thessaloniki, Greece, [3]Center for the Study of Hematological and other Malignancies, Nicosia, Cyprus, [4]Cyprus Cancer Research Institute (CCRI), Nicosia, Cyprus, [5]Aristotle University of Thessaloniki, Thessaloniki, Greece, [6]University of Washington, Seattle, United States

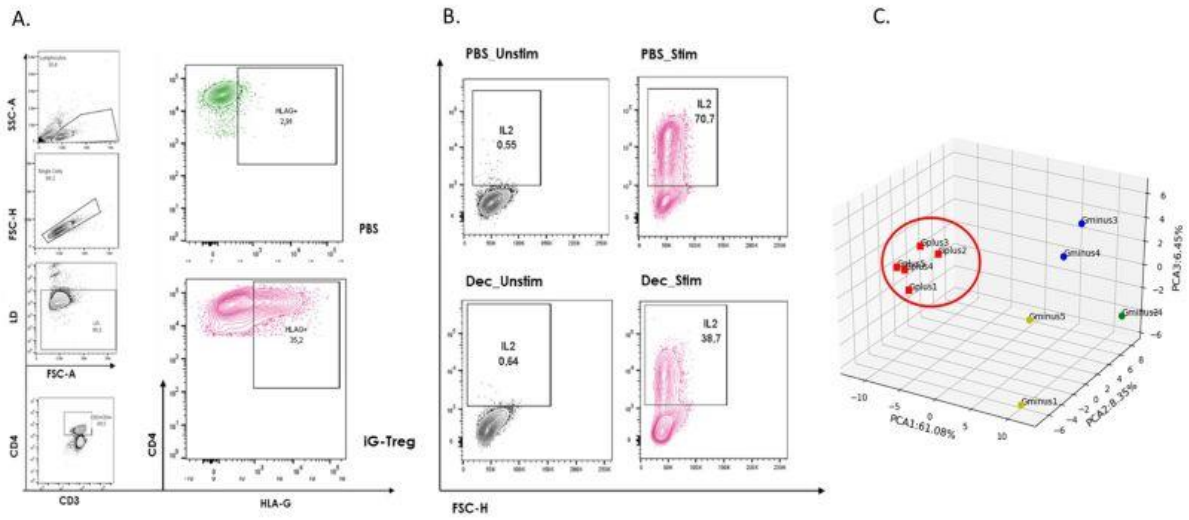
**Background** Graft-vs-host-disease (GvHD) is a life-threatening complication of allogeneic hematopoietic cell transplantation (allo-HCT) with limited approved therapies. HLA-G is an immunosuppressive molecule playing a central role in the acceptance of the semi-allogeneic foetus during pregnancy, the expression of which is epigenetically regulated. We have previously reported the small-scale generation of HLAG$^+$CD4$^+$FOXP3$^-$ regulatory T-cells through hypomethylation exerting potent suppressive function *in vitro* (Stamou et al, 2017). Herein, we aimed to produce and characterise a clinical scale HLAG$^+$ product (iG-Treg), assess its safety profile and elucidate the molecular mechanisms of HLAG$^+$CD4$^+$ T cell-mediated suppression.

**Methods** Peripheral blood mononuclear cells from healthy donors were enriched for T cells through monocyte depletion and were activated with CD3/CD28-beads for 3 days and followed a 3-day decitabine treatment (n=9). The final product was tested for immunophenotype, expression of exhaustion markers and ability to produce effector cytokines following 4-hour stimulation with PMA/ionomycin via flow cytometry (n=3) as well as for cytokine secretion in supernatants during production using a multiplex magnetic bead-based immunoassay (n=9). Sorted HLAG$^+$CD4$^+$ T-cells and HLAG$^-$CD4$^+$ were analysed by RNA-seq and the expression of the key differentially genes were validated via RT-PCR and/or flow cytometry.

**Results** The iG-Treg product (n=9) contains 95,8% CD3+ of which 58,6% are CD4+ and 37,8% are CD8+. Compared to untreated controls (PBS), iG-Tregs are enriched for HLAG$^+$CD4$^+$ T-cells (25.6% vs 0,7%, p<0,0001), are PD-1+ (61.25% vs 20.46%, p=0.04), with impaired ability to produce effector cytokines as was evident by diminished intracellular IL-2 (38.1%, vs 63.3% p=0.0176), IFNγ (45.8% vs 59%, p=0.008) and IL-17a (2.94% vs 5.87%, p=0.0368). Assessment of culture supernatants interestingly displayed increased production of IL-13 (653.3pg/ml vs 291pg/ml, p=0.0496) without concomitant increase of other Th1/Th2 cytokines (p=ns). RNA-seq revealed that HLAG$^+$CD4$^+$ T cells have a distinct and uniform gene expression profile compared to HLAG$^-$CD4$^+$ with highly differentially expressed IDO-1, CCL17, CCL22 and CXCL9 transcripts ($log_2$(fold change)>1.5 & q<0.05), findings which were validated via RT-PCR. Notably, the expression of IDO-1 on HLAG$^+$CD4$^+$ cells was further validated with flow cytometry (p=0.02).

**Conclusions** Our data indicate that iG-Tregs, which are enriched in HLAG$^+$CD4$^+$ T cells, can be effectively produced through a short and GMP-compatible protocol. iG-Tregs demonstrate a favourable safety profile as depicted in the exhausted phenotype associated with high levels of PD-1, the impaired ability to produce effector cytokines that are typically associated with GvHD exacerbation and the absence of Th1/Th2 polarized cytokine secretion in supernatants despite the increase in IL-13. Moreover, we describe, for the first time, the presence of the predominantly myeloid suppressor gene IDO-1 on regulatory HLAG$^+$CD4$^+$ T cells. The exact

effect on immunosuppression mediated through IDO-1 remains to be assessed through functional. In parallel, iG-Tregs are being evaluated for their GVHD and GVL effect in *in vivo* models. Collectively, iG-Tregs constitute a well-characterized and safe immunosuppressive product able to be administered against GvHD in the initiated phase I clinical trial (EUDRACT number: 2021-006367-26).




**A**: Representative plots of HLAG expression on iG-Tregs vs PBS, **B**: Representative plots of intracellulat IL-2 staining on iG-Tregs vs PBS on unstimulated and stimulated conditions, **C**: Principal Components Analysis (PCA) of RNA seq data showing HLAG+ unique and distinct transcriptional profile (red circle on the left)