Pre-clinical development of a decitabine-induced regulatory HLAG$^+$CD4$^+$-T cell-enriched cell product (iG-Treg) against Graft-vs-host-disease

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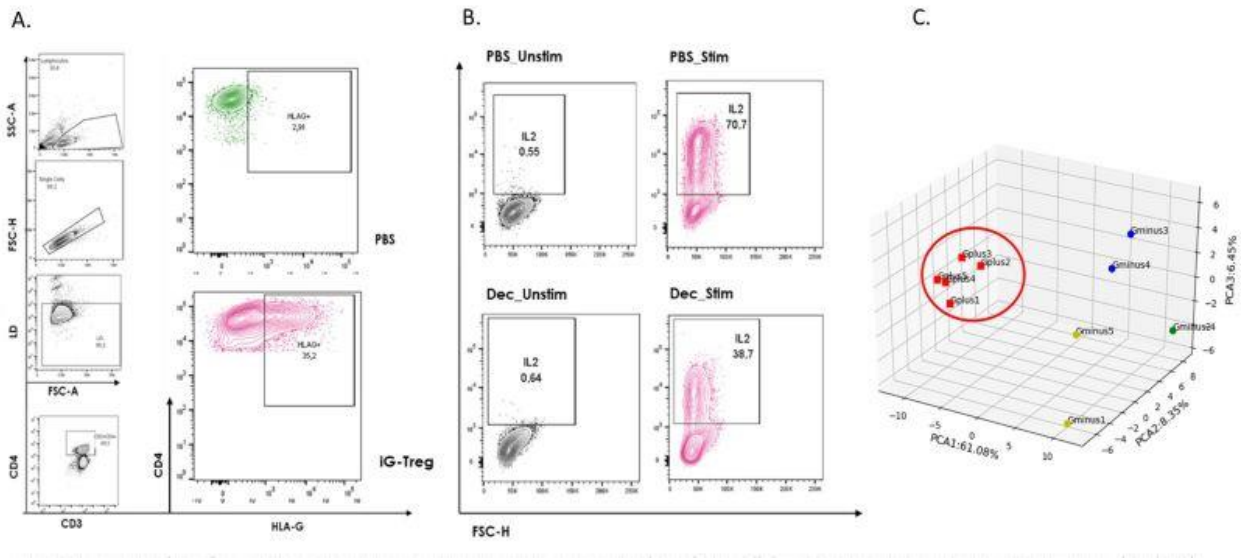
Background GvHD is a major complication of allogeneic hematopoietic cell transplantation with limited approved therapies. We have reported the simple generation through hypomethylation of HLA-G+ T-cells exerting potent *in vitro* immunosuppression *(Cytotherapy 2017;19:521)*. Herein, we aimed to generate a clinical and GMP scale product with increased number of HLAG$^+$ cells (iG-Treg) and assess its safety profile for use in a clinical GvHD study.

Methods Peripheral blood cells were enriched for T-cells by adherence-based monocyte depletion, were activated with CD3/CD28-beads and treated for 3 days with decitabine. The safety profile of the final product (iG-Treg) was assessed *in vitro* and *in vivo*. Sorted HLAG$^+$CD4$^+$ T-cells and HLAG$^-$CD4$^+$ were analysed by RNA-seq (Illumina) and the expression of the key differentially genes was validated.

Results iG-Tregs (n=20) were enriched for HLAG$^+$CD4$^+$ cells (median 24.4% vs 0.7% in PBS-treated controls, p<0.0001) (Fig. 1A). The median CD4/CD8 ratio was 1.6 (range 0.8-3.7). iG-Tregs demonstrate a favorable safety profile as depicted by their exhausted phenotype (PD-1$^+$ 61.3% vs 20.5%, p=0.04), their impaired ability to produce effector cytokines upon PMA/ionomycin stimulation [IL-2 38.1%, vs 63.3% p=0.0176, IFNγ 45.8% vs 59%, p=0.008, IL-17a 2.94% vs 5.87%, p=0.037] (Fig. 1B), and their diminished alloreactivity against allogeneic PHA blasts (% lysis, 1.8±1 vs 49±4, p<0.0001). Infusion of iG-Tregs in NSG mice reduced and prolonged the occurrence of GvHD (3/6) as compared to control-treated mice (5/5 lethal GvHD by day 35). Except IL-13 (p=0.05) no other Th1/Th2 cytokines were increased in iG-Treg supernatants. FACS-sorted HLAG$^+$CD4$^+$ cells exhibited a distinct and uniform gene expression profile compared to their HLAG$^-$CD4$^+$ counterparts, with highly differentially expressed IDO-1, CCL17, CCL22 and CXCL9 genes, among others (validated via RT-PCR and FACS for IDO-1) (Fig. 1C).

Conclusions iG-Tregs enriched for immunosupppressive HLAG$^+$CD4$^+$ cells can be effectively and reproducibly generated through a short and GMP-compatible protocol. iG-Tregs constitute a well-characterized product with the safety profile needed to be administered in an upcoming phase I-II clinical trial for GvHD (EUDRACT 2021-006367-26). The possible contribution of IDO, a predominantly myeloid suppressor gene, in the immunosuppressive function of HLAG$^+$CD4$^+$ cells remains to be assessed in functional assays.



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