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

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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 CD4+ T-cells (small scale production, n=17) through immunodensity gradient selection (StemCell Technologies) or for CD3^{+} T cells through monocyte depletion (clinical scale production, n=10), activated with CD3/CD28-beads for 3 days, followed by a 3-day decitabine treatment. The final product (iG-Treg) was characterized *in vitro* at a cellular and molecular level using flow cytometry and in depth RNA-seq (Illumina) and single cell RNA-seq (scRNA-seq) (10X Genomics) of FACS-sorted HLAG^{+}CD4^{+} T-cells. IG-Tregs were also assessed for their safety profile *in vitro* and *in vivo* (NSG mice). For GMP validation experiments, iG-Treg products (n=2) were manufactured under controlled environment using cGMP reagents and were assessed by immunophenotyping and *in vitro* assays.

Results

The generation of HLA-G$^+$ T cells from 8 different donors was consistent and robust both in small and clinical scale production. Specifically, through small scale production HLA-G was induced on CD4$^+$ T cells with a median [Δm] expression of 16.4% (5.3% - 32.5%). The clinical scale iG-Tregs contained 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 HLA-G$^+$ 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 with the HLA-G$^+$CD4$^+$ compartment being comparable between small and clinical scale production (p=0.34).

Bulk RNA-seq showed that FACS-sorted decitabine-induced HLAG$^+$CD4$^+$ cells are a distinct and transcriptionally homogenous population compared to their HLAG$^-$CD4$^+$ counterparts. HLAG$^+$CD4$^+$ cells differentially expressed regulatory genes (e.g. CCL17, CCL22 and CXCL9) and interestingly the highest differentially expressed gene was the predominantly myeloid suppressor gene IDO-1, a finding which was further validated at mRNA (RT-PCR, n=4, median fold change=10.82) and protein (FACS, n=8, p=0.001) level. Following scRNA-seq profiling HLAG$^+$CD4$^+$ T cells displayed 6 discreet clusters, 4 of which were strongly associated with regulatory T cell signatures enriched in genes indicative of Treg function and co-stimulation.

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 hyperresponsiveness upon restimulation with PMA/ionomycin in the presence of monensin with reduced intracellular cytokine production (IL-2, p=0.02, IFNγ, p=0.008, IL-17a, p=0.04]. Assessment of iG-Treg cell culture supernatants for cytokine secretion profiling revealed that no Th1/Th2 cytokines were increased, except IL-13 (p=0.05). 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 (p=0.0019). 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.

Finally, iG-Treg production was validated under GMP conditions where the percentage of HLA-G+ T cells, the viability as well as immunophenotypic characteristics were comparable to the clinical scale products (p=ns). Moreover, the favourable safety profile was also confirmed *in vitro* utilizing cytotoxicity assays resulting in reduced alloreactivity against allogeneic PHA blasts (p<0.001).

Conclusions iG-Tregs can be easily produced by a GMP-compatible protocol and constitute a well-characterized product with a favourable safety profile. 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 different treatment designs. In conclusion, iG-Tregs are currently undergoing clinical evaluation in the active phase I-II clinical trial for the prevention of GvHD in patients receiving HLA-matched sibling allo-HCT (EUDRACT 2021-006367-26).