IN VITRO CHARACTERIZATION OF DECITABINE-INDUCED CD4$^+$HLAG$^+$ SUPPRESSOR CELLS

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Objectives

Graft versus Host disease (GvHD) is unique in allogeneic haematopoietic stem cell transplantation, as the transplanted tissue-donor T-cells recognize the genetically disparate and immunosuppressed recipient as non-self, leading to organ damage. Focusing on the HLA-G immunosuppressive molecule which is vital in pregnancy for prevention of semi-allogeneic fetus rejection, we have previously shown that HLA-G$^+$ and not their HLA-G$^-$ T-cell counterparts from Decitabine-treated T-cell cultures display potent suppression function. These cells do not express FOXP3 and their suppression functions are not exclusively HLA-G dependent. As DNA hypomethylation is associated with increased gene expression, we hypothesize that other regulatory function genes may also be induced. RNA sequencing was performed on Dec-induced sorted CD4$^+$HLA-G$^+$ and CD4$^+$HLA-G$^-$ cells. Comparison of gene expression profiles enabled the identification of gene transcripts upregulated in CD4$^+$HLA-G$^+$ cells. Findings were validated by Real-Time PCR, flow cytometry and multiplex immunoassays.

Methods

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized whole blood by density gradient sedimentation over Ficoll-Paque PLUS (GE Healthcare). PBT-cells from PBMCs were isolated by negative magnetic cell selection using MACS Pan-T-Cell Isolation Kit II Human (Miltenyi Biotec). Enriched T-cells were activated for 3 days in the presence of anti-CD3/CD28 beads (Invitrogen, UK). Activated cells were incubated in the presence of 50U/mL IL-2 (Peprotech) and 7.5μM Decitabine (Sigma-Aldrich) or PBS as control, for an additional 3 days. Sorting of CD4$^+$HLA-G$^+$ and CD4$^+$HLA-G$^-$ cells was performed using the BD FACSAriaIII platform. Following RNA extraction and cDNA synthesis, Real-Time PCR was performed for expression analysis of genes identified to be upregulated in Dec-induced sorted CD4$^+$HLA-G$^+$ through RNA sequencing. In another instance, sorted Dec-induced CD4$^+$HLA-G$^+$ and CD4$^+$HLA-G$^-$ cells were cultured for another 2 days with or without TCR stimulation and the supernatants from these dedicated cultures were used in multiplex immunoassays for the investigation of cytokine/chemokine secretion profiles. Flow cytometry was also performed for intracellular IDO-1 detection.

Results

Real-Time PCR was performed using TaqMan® Gene Expression Assays for the investigation of HLA-G, IDO1, CXCL9, CCL22, CCL17 and IL-6 gene expression. Whilst HLA-G expression was confirmed, IDO-1 ($p=0.0476$), CCL22 ($p=0.0328$), CCL17 ($p=0.0490$) and CXCL9 ($p=0.0389$) were also upregulated $CD4^+HLA\text{-}G^+$ sorted cells.

Supernatants were harvested and used in multiplex immunoassays on the Bio-Plex200 system. The Human Cytokine Th1/Th2 Assay (Bio-Rad Laboratories) and a custom panel (ProtATonce LTD) enabled the interrogation of cytokines/chemokine cell signalling molecules in complex extracellular events and signalling pathways. Interestingly, at a resting state IL-4 ($p=0.002$), IL-6 ($p=0.011$), IFN-γ ($p=0.045$) and CCL17 ($p=0.027$) and at a stimulated state IL-4 ($p=0.019$), IL-13 ($p=0.011$), IL-6 ($p=0.003$), CCL17 ($p=0.0019$) and CCL22 ($p=0.0021$) were significantly increased in $CD4^+HLA\text{-}G^+$ sorted cells, compared to PBS-treated controls.

IDO-1 protein expression was also investigated since, to our knowledge, it has not been previously observed on T-cells. Interestingly, IDO-1 levels were increased in Dec-treated $CD4^+HLA\text{-}G^+$ sorted cells compared to both $CD4^+HLAG^-$ ($p=0.001$) and PBS-treated controls ($p=0.014$).

Conclusions

Following investigation of certain gene transcripts, a correlation is observed at RNA and protein levels for IDO-1 and CCL17. The possible contribution of other regulatory genes will be assessed through functional studies.