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14. ABSTRACT In Type 1 diabetes (T1D), therapies focused on decreasing T cell activation to preserve functional β-cells are a priority for the treatment of the disease. We have found that the sulfated polysaccharide dextran sulfate (DS) reduces mouse interferon-γ+CD4+ and CD8+ T cells, increases mouse FoxP3+ cells (Tregs), preserves β-cells and reverses T1D in mice (Lu et al. Diabetes, 2020, PMID: 32381645). In the DoD funded project (only Specific Aim 3 of the original application was funded), we studied the immunomodulatory effects of DS in human immune cells. Using hPBMCs from healthy individuals and flow cytometry and CyTOF techniques we have found that DS alters phenotypic markers of CD8+ and macrophage-like cells [subaim (sa) 3.1]; DS reduces expression of dendritic cells (DCs) co-stimulatory molecules, leading to enhanced Treg differentiation and reduced proliferation of activated T-cells (sa 3.2); DS does not have direct effects on T-cell activation or differentiation in whole PBMCs, isolated T-cells, or naïve CD4+-targeted differentiation with different stimulatory methods (sa 3.3); and these changes might be mediated by HGF/c-Met signaling pathway as shown in mouse myeloid cells (sa 3.4), although this requires further studies in hPBMCs. A manuscript with these observations is in preparation. In conclusion, DS induces a more tolerogenic phenotype in activated hPBMCs from healthy donors. Next, we will test whether similar DS-induced pro-tolerogenic effects occur in hPBMCs from Type 1 diabetic individuals.

15. SUBJECT TERMS
Type 1 Diabetes, Dextran sulfate, human PBMCs, CyTOF, immunomodulation

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1. Introduction

In autoimmune type 1 diabetes (T1D), self-tolerance is lost leading to the destruction of β-cells. In T1D in humans and in the NOD mouse, a spontaneous mouse model of T1D, autoreactive T cells target islet associated antigens and acquire an effector inflammatory phenotype due to co-stimulatory signals leading to islet invasion and β-cell destruction. In T1D, effector responses are prevalent over tolerogenic responses and therapies targeting both, blockade of early T cell activation and enhancement of regulatory T cells are being pursued.

Low molecular weight (6,500-10,000Da) dextran sulfate (DS) is a sulfated semi-synthetic polysaccharide with known cytoprotective actions as well as immunomodulatory properties. However, whether DS could decrease T1D development or even revert T1D was unknown. We have recently found that DS i) effectively protects human β-cells against ER stress and pro-inflammatory cytokines; ii) increases the number of mouse PD-1+CD4+ and PD-1+CD8+ T cells and Tregs; iii) diminishes the number of IFNγ+CD4+ and IFNγ+CD8+ T cells in mice; and, iv) halts progression and reverses autoimmune diabetes in non-obese diabetic (NOD) mice. These results suggest that DS has therapeutic potential for the treatment of T1D. We have published these studies in *Diabetes* in 2020 (Lu et al. *Diabetes* 69(8):1692-1707, 2020, PMID: 32381645). These studies were funded by NIH (Specific Aims 1 and 2 of the original application) and partially by the DoD award since these studies in mice guided us for performing the experiments in human immune cells funded by DoD (Specific Aim 3 of the original application).

Based on these exciting results in mice, and before initiation of clinical studies in subjects with T1D, we aimed at analyzing the DS-induced immunomodulatory effects on human immune cells in vitro, the project funded by this award. We hypothesized that DS exerts tolerogenic effects on human T cell responses by 1) skewing of APC maturation, phenotype and/or function, 2) directly modulating T cell phenotypes/function, or 3) a combination thereof. Our results suggest that DS directly induces a more tolerogenic phenotype in activated human DCs by reducing APC co-stimulatory marker presentation. DS does not have a direct effect on T-cell phenotype in differentiation conditions. Changes in T-cells are mediated indirectly; DS-treated DCs reduced CD4+ cell proliferation, and Treg phenotype induction. These studies were presented at both the Annual Boston Ithaca Islet Club Meeting in 2018 and the Network of Pancreas Organ Donors (nPOD) Meeting in 2019 and 2020. Links to the different publications are included in section 6, Products.

2. Keywords

Type 1 diabetes, dextran sulfate, immune tolerance, T cell, antigen presenting cell, flow cytometry, CyTOF, human PBMCs.

3. Accomplishments

- **What were the major goals of the project?**

The main goal of this project was to analyze **DS-induced immunomodulatory effects on human immune cells in vitro**. For that purpose and as indicated in the SOW, we developed the following subaims (SA):

Specific Aim 3: To dissect and stratify DS-induced immunomodulatory effects on human T cell responses in health and T1D disease.	Timeline	Site 1	Site 2
SA 3.1. To parse the general impact of DS on human T cell and APC populations.	Months		
SA1: Impact of DS on human T cell and APC	6	Dr. Garcia-Ocana	Dr. Homann
Milestone(s) Achieved: Analysis of general DS effects on human T cell and APC populations	6	Dr. Garcia-Ocana	Dr. Homann

For these experiments, we cultured unfractioned fresh human PBMCs from healthy donors with dextran sulfate (DS) and performed phenotypic characterization of T cell and APC populations by Cytometry by Time of Flight (CyTOF). These experiments were performed in the presence of anti-CD3/anti-CD28 stimulation to specifically evaluate the impact of DS in the context of T cell activation.

SA 3.2. To delineate the specific impact of DS on APC maturation.	Months		
SA2: Impact of DS on APC maturation	8	Dr. Garcia-Ocana	Dr. Homann
Milestone(s) Achieved: DS effects on human APC maturation	8	Dr. Garcia-Ocana	Dr. Homann

To investigate the impact of DS on APCs and in particular on human dendritic cell (DC) differentiation, purified CD14+ monocytes isolated from healthy donor buffy coats were cultured for 6 days (medium refreshed on day 3) with IL-4 and GM-CSF alone to generate immature dendritic cells (DCs). DCs were treated with LPS and/or DS for 72h for non-specific activation and analyzed for viability, maturation and co-stimulatory markers (CD80, CD83, CD86, CD40, HLA-DR) by flow cytometry. We also analyzed the functional properties of DCs by co-culturing DS±LPS-treated DCs with lymphocytes and measuring lymphocyte activation status and phenotype by flow cytometry. Endocytic capacity of DS±LPS-treated DCs was measured by DS-FITC uptake.

SA 3.3. To define the impact of DS preconditioning on islet antigen-reactive CD4+T cell responses.	Months		
SA3: Impact of DS on CD4+ T cell responses	9	Dr. Garcia-Ocana	Dr. Homann
Milestone(s) Achieved: DS effects on human CD4+ T cells	9	Dr. Garcia-Ocana	Dr. Homann

To examine the effects of DS on Th1 and Treg induction, whole PBMCs or T-cells or naïve CD4+-T-cells isolated from fresh human PBMCs from healthy donors using magnetic bead conjugated negative selection antibodies and preconditioned with differentiation media were analyzed for activation markers and cytokine production by flow cytometry.

SA 3.4. To elucidate the mechanisms of DS-induced immunomodulation.	Months		
SA4: Mechanisms of DS-induced immunomodulation: PD-1/PD-L1	8	Dr. Garcia-Ocana	Dr. Homann
Milestone(s) Achieved: DS effects on PD-1/PD-L1 axis	8	Dr. Garcia-Ocana	Dr. Homann

For these experiments we have cultured unfractioned fresh human PBMCs from healthy donors with DS for 24-48h and analyze the levels of PD1 and PDL-1 in different immune cell populations by CyTOF. These experiments were performed in the presence of anti-CD3/anti-CD28 stimulation to specifically evaluate the impact of DS in the context of T cell activation. Contrary to the results obtained in mice, results showed that PD-1 and PD-L1 were not altered or even decreased in the different immune cell types analyzed in hPBMCs. Since DS elevates circulating levels of hepatocyte growth factor (HGF) in mice and humans, we decided to focus our efforts on determining whether this growth factor through signaling via its receptor c-Met mediates DS-induced immunomodulation. These studies have been performed in circulating mouse PBMCs before to test this hypothesis in hPBMCs.

- **What was accomplished under these goals?**

- **SA3.1: To parse the general impact of DS on human T cell and APC populations.**

Results: To address the goal in SA3.1, we performed experiments with fresh hPBMC from healthy subjects which we stimulated with anti-CD3/anti-CD28 beads in the absence/presence of 1mg/ml DS for 24h and 48h and employed our exploratory Mass Cytometry staining panel for delineation of immune cell populations. hPBMCs from healthy subjects (n=6, 66% females, 38±6 years old; Caucasian) were tested. We made multiple observations:

a) We did not observe any change in total number of CD45+ cells, T cells (CD4+ and CD8+ cells), B cells, NK cells and APCs after treatment with this dose of DS indicating that if there is any potential phenotypic change in immune cells induced by DS, this is not derived from a decrease in that specific population of cells (**Fig. 1A-B**). Similar results were observed at 24h and 48h. Only 48h results are presented here.

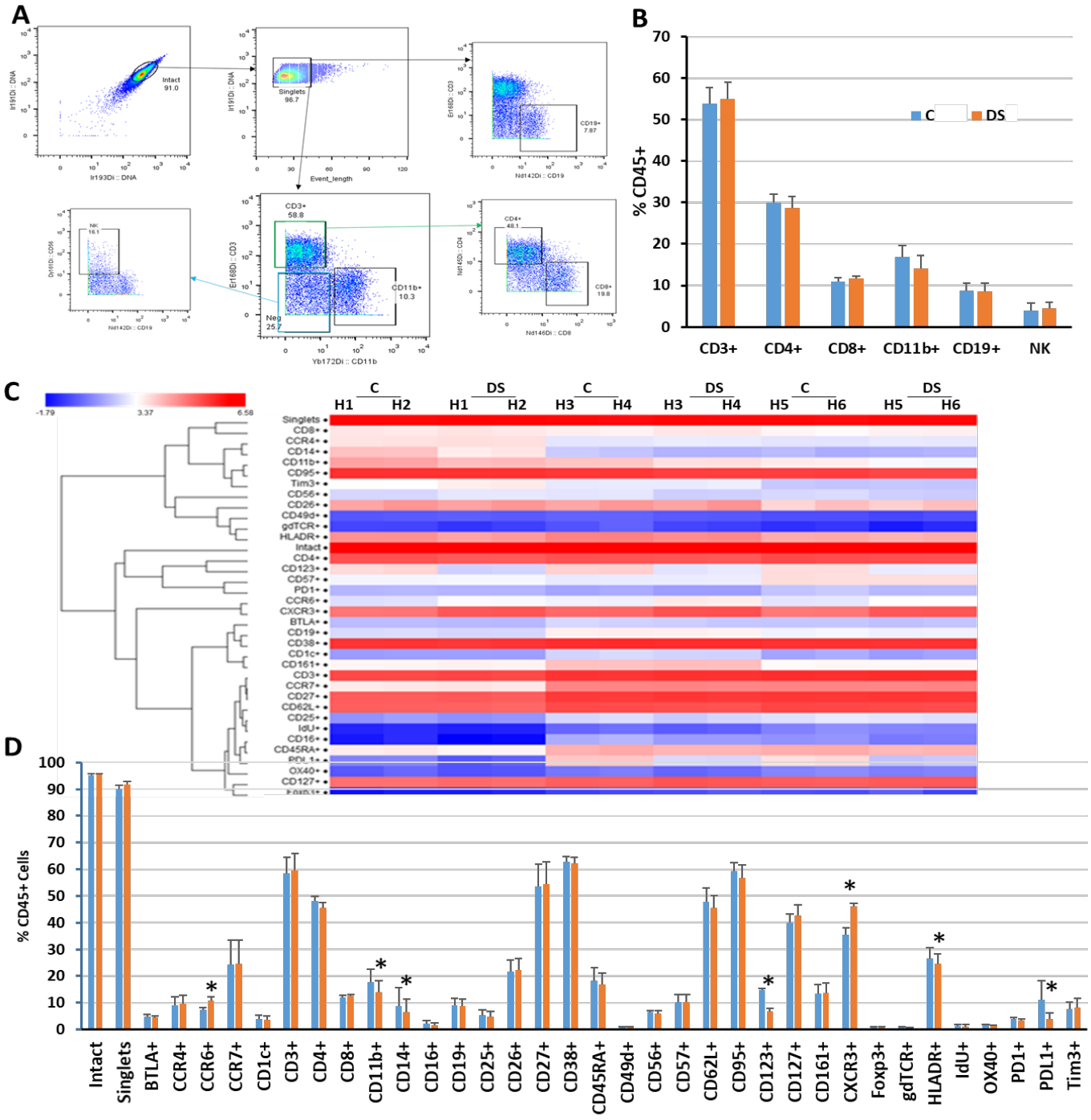


Figure. 1: CyTOF analysis of hPBMCs treated with DS. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) in the presence of anti-CD3 and anti-CD28. **(A)** Gating strategy used to determine the **(B)** percentage of different immune cell populations identified by CyTOF. Most of cells were alive and singlets. T lymphocytes were identified by CD3, CD4 and CD8 expression; myeloid cells by CD11b staining and B lymphocytes by CD19 expression**. (C)** Heatmap showing the level of expression of different immune cell markers in hPBMCs after treatment with DS for 48h. **(D)** Quantitation of the expression level of these markers in the human samples identified as H1 to H6. *p<0.05 vs C.

b) A heatmap depicting the expression levels of the different markers interrogated in the CyTOF experiments with unfractionated hPBMCs shows that CD14, CD11b, CD123, PD-L1 and HLDR are downregulated while CXCR3 and CCR6 are upregulated consistently in these studies (**Fig. 1C-D**).

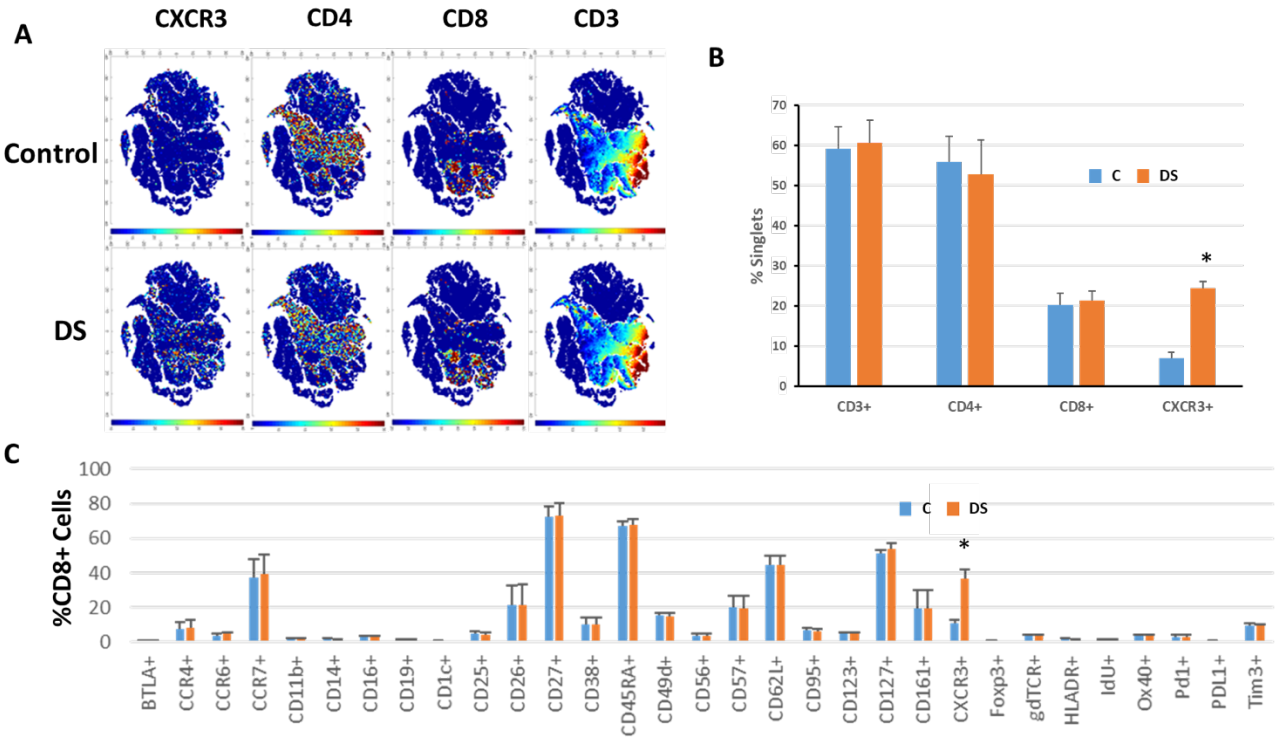


Figure. 2: Stratification of CXCR3+ cells in CD8+ activated lymphocytes. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) (see Fig. 1). **(A)** tSNE plots showing hPBMCs expressing CXCR3, CD8, CD4 and CD3 from a representative human sample (see increase in number of cells expressing CXCR3 in the CD3/CD4/CD8 cluster). **(B)** Percentage of singlets identified by CD3, CD4, CD8 and CXCR3 expression**. (C)** Quantitiation of the percentage of CD8+ cells expressing different immune cell markers in hPBMCs after treatment with DS for 48h. Notice the increase in CXCR3 expressing CD8+ cells *p<0.05 vs C.

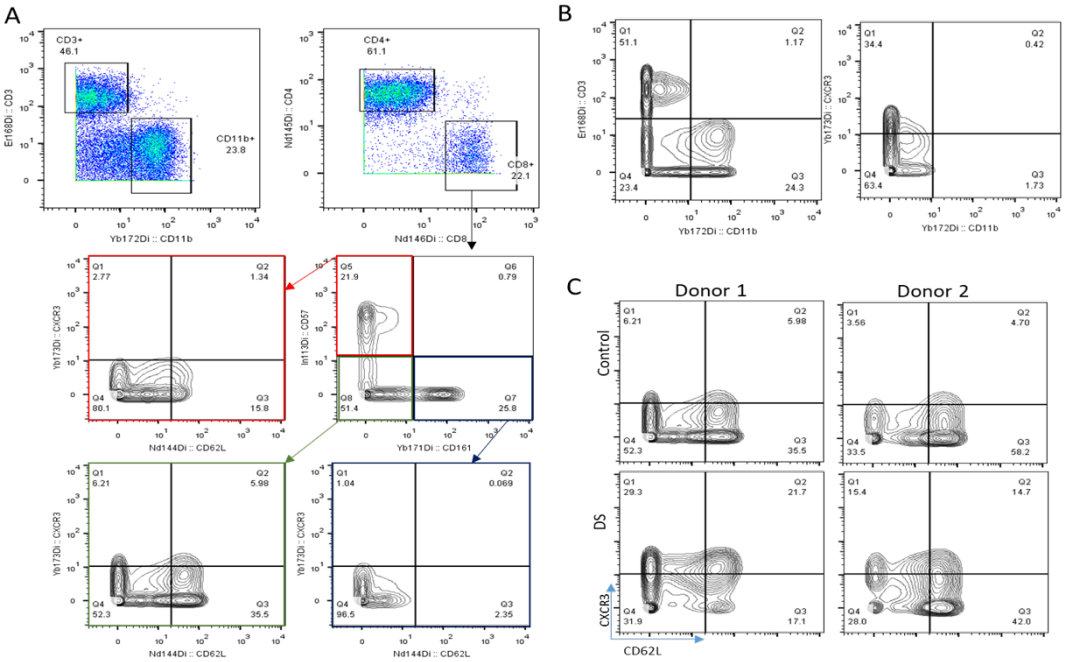


Figure. 3: Activation of CXCR3+CD8+ cells based on C57, CD161 and CD62L gating. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) (see Fig. 1). **(A)** Gating strategy used to separate CD3, CD8, CD57, CD161, CD62L and CXCR3 expressing cells. **(B)** Demonstration of gating strategy provides only CD3+ but not CD11b+ cells expressing CXCR3. **(C)** Representative results in two human PBMCs preparations showing an increase in the percentage of CXCR3+CD62L+ activated T cells by DS treatment.

c) Among the changes observed, we made the unexpected observation that DS promoted a selective increase of CXCR3 expression by activated CD8+T cells (**Fig. 2**). Recruitment of CXCR3-expressing T cells into the pancreas *via* binding to the CXCR3 ligands CXCL9, CXCL10 and/or CXCL11 has long been implicated in T1D pathogenesis and targeting this molecular pathway has been proposed as a preventive approach in murine models of T1D, though the complexities and practical utility of harnessing these interactions for therapeutic purposes remain a matter of debate (Homann D. *Diabetes*, 4(12):3990-2, 2015, PMID: 26604174). Nevertheless, expression of CXCL10 in the context of human T1D appears to be a robust pathogenetic feature that warrants further elucidation of this pathway (Sarkar et al. *Diabetes* 2012 Feb;61(2):436-46, PMID: 22210319).

d) Further stratification of activated CD8+T cells at the level of CXCR3 expression was performed and it is depicted in (**Fig. 3**). We found that regardless of broad CD8+T cell subset (Tnaive, Teff, Tem, Tcm, Tscm), greater proportions of these cells expressed CXCR3 in the presence of DS. *In vivo,* CXCR3 expression is dynamically regulated in response to T cell-specific stimuli promoting an initial upregulation, subsequent downregulation and over time, a gradual re-expression by long-term memory T cells (Eberlein J et al. *J Clin Invest.,*126(10):3942-3960, 2016, PMID: 27617858). How these kinetics relate to the in vitro stimulation system employed here is currently unclear, i.e. CD8+T cell stimulated in the presence of DS may exhibit greater induction or delayed downmodulation of CXCR3; further time course analyses to resolve this question are pending.

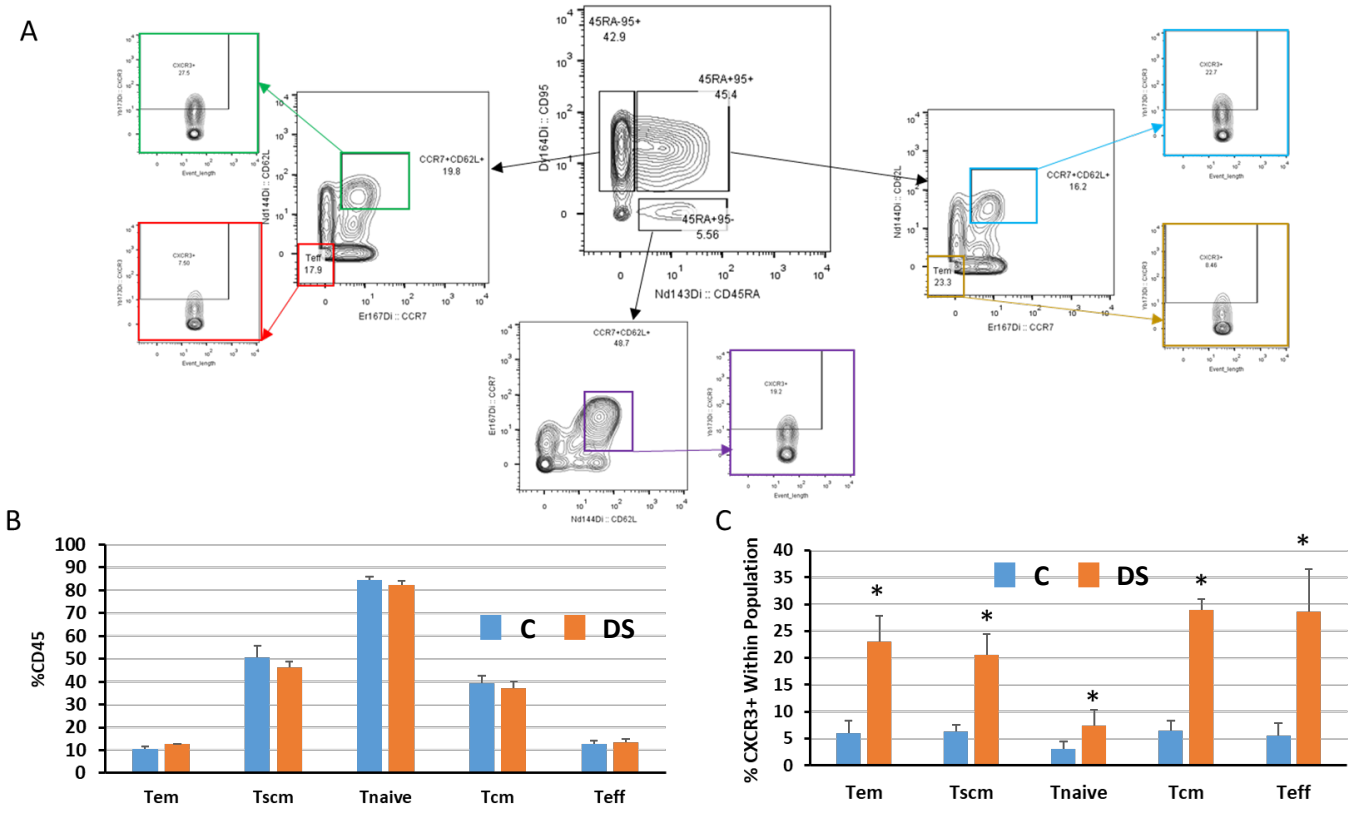


Figure 4: Analysis of the effect of DS on the presence of CXCR3 in different subsets of differentiated T cells. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) (see Fig. 1). **(A)** Gating strategy to define T naïve, Tscm (stem central memory), Tcm (central memory), Tem (effector memory) and Teff (effector) cells and CXCR3 expression. **(B)** Percentage of cells at the different stages of differentiation after DS treatment. **(C)** Percentage of CXCR3+ cells within the different T cell populations during differentiation. *p<0.05.

e) Upon contact with APCs, memory T cells differentiate from naïve T cells in a stepwise manner as they progress from Tscm (stem central memory) to Tcm (central memory), Tem (effector memory) to then Teff (effector) cells. We next addressed in what step of T cell differentiation CXCR3 was appearing after DS treatment (**Fig. 4**). We found that DS promoted greater CXCR3 expression by all major T cell subsets regardless of differentiation status. This would indicate facilitated access to pancreatic islets, presumably via CXCL10 expressed at elevated levels by islet cells in the context of T1D.

f) Importantly, however, further stratification of the CXCR3+CD8+T cell subset in DS cultures revealed that these cells significantly downregulated canonical T cell activation markers such as CD25, CD38, Ox40 and HLA-DR (**Fig. 5**). Thus, **reduced T cell activation, despite elevated CXCR3 expression, may limit the local elaboration of effector functions and associated islet cell damage.**

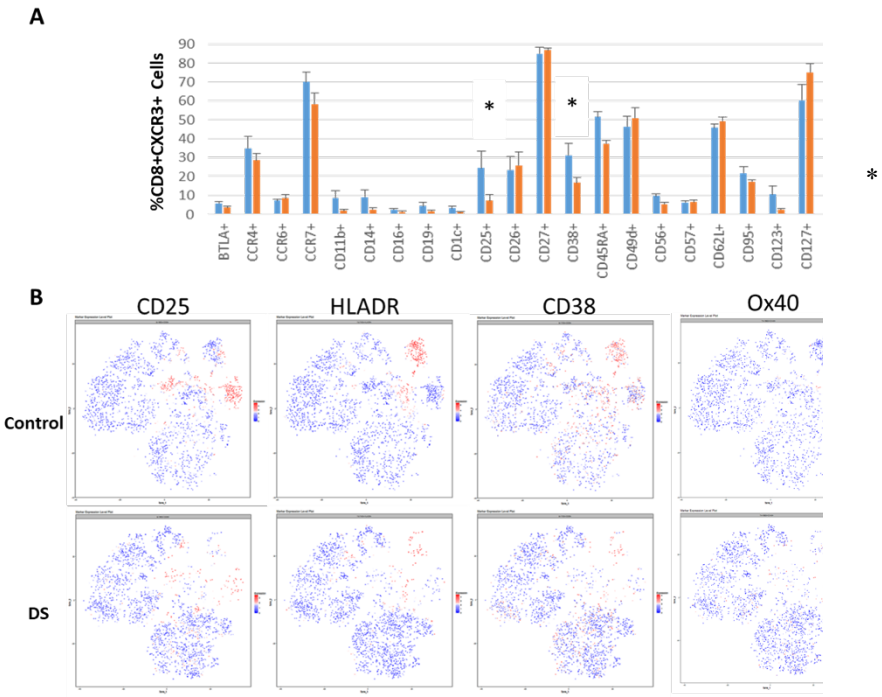
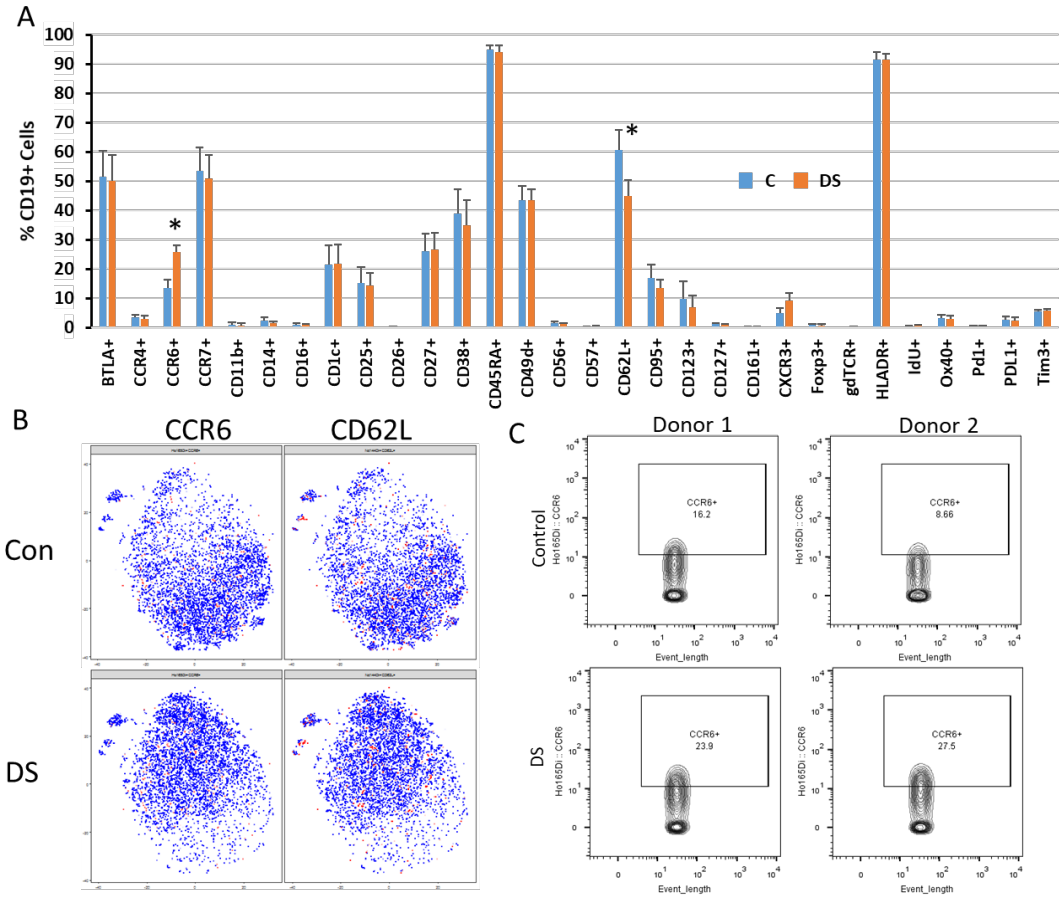


Figure. 5: Expression of other activation markers in CD8+CXCR3+ cells. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) (see Fig. 1). **(A)** Percentage of cells expressing those markers within the different CD8+CXCR3+ cell population. **(B)** Representative tSNE plots from one of the human PBMCs preparations treated with DS and anti-CD3 and anti-CD8 for 48h showing a decrease in the number of cells expressing CD25, HLADR, CD38 and Ox40.

Figure 6: Changes in B lymphocytes (CD19+) induced by DS in hPBMCs. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (C) (see Fig. 1). **(A)** Percentage of CD19+ cells expressing the different markers depicted in the graph. *p<0.05 **(B)** tSNE plot of a representative sample showing expression of CCR6+ and CD62L+ cells induced by DS treatment. **(C)** Representative results in two human PBMCs preparations showing an increase in the percentage of CD19+CD62L+CCR6+ cells by DS treatment.



g) Analysis of expression markers in B cells (CD19+) in hPBMCs treated with DS reveals a significant change in the number of cells expressing reduced levels of CD62L while increased levels of CCR6 (**Fig. 6**). The relevance of these observation is difficult to assess since the role of B cells as relevant participants in T1D pathogenesis, i.e. beyond the production of autoantibodies as a signature for disease progression, remains incompletely defined (Smith MJ et al. *Nat Rev Nephrol*. 13(11): 712-720, 2017, PMID: 29038537).

h) Furthermore, DS treatment for 48 hours leads to loss of macrophage-like populations in hPBMCs (**Fig. 7**). Macrophages and related "innate" immune cell populations have been proposed early on to contribute to T1D pathogenesis (PMID: 2491626) but it is only of late that *in situ* analyses of macrophages in the type 1 diabetic pancreas have reinvigorated this hypothesis (Willcox A, et al. *Clin Exp Immunol.*155(2):173-81, 2009, PMID:19128359; Damond N et al. *Cell Metab.* 29(3):755-768, 2019, PMID: 30713109; Wang YJ et al. *Cell Metab*. 29(3):769-783, 2019, PMID:30713110; Zirpel H et al. *Front Endocrinol (Lausanne), 1*2:666795, 2021, PMID:33912139). Of note, macrophages may exert both pathogenetic and protective effects in T1D, and their targeted manipulation is emerging as an area of intense interest.

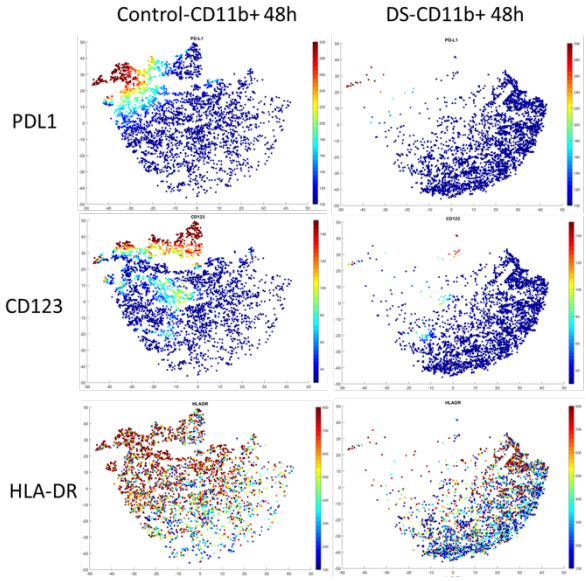


Figure 7: Changes in the expression of markers of macrophage-like cells (CD11b+) induced by DS in hPBMCs. Fresh human PBMCs were treated for 48h with medium containing 1mg/ml dextran sulfate (DS) or medium alone (Con) (see Fig. 1). tSNE plot of a representative sample showing expression of PDL1, CD123 and HLADR in CD11b+ cells induced by DS treatment.

i) In conclusion, in vitro DS treatment is shown here to promote robust changes in immune cell phenotypes that pertain to the major cell populations implicated in disease initiation and progression. How these changes precisely affect the balance between beta cell destruction and the prevention thereof is in part addressed by our complementary murine investigations that demonstrate amelioration of T1D disease following in vivo DS conditioning.

j) Milestone(s) Achieved: Analysis of general DS effects on human T cell and APC populations.

k) Future studies: we will performed phenotypic characterization of T cell and APC populations by CyTOF in fresh human PBMCs from type 1 diabetic donors treated in culture with DS)..

- **SA3.2: To delineate the specific impact of DS on APC maturation.**

Results: To address the goal in SA3.2, we interrogated DS modulation of antigen presenting capacity in LPS-stimulated APCs, in particular dendritic cells (DCs) as deduced from expression of HLA-DR, CD80, CD83, CD40 and CD86. We analyzed these parameters by using flow cytometry and following the protocol depicted in **Fig. 8**. We found that:

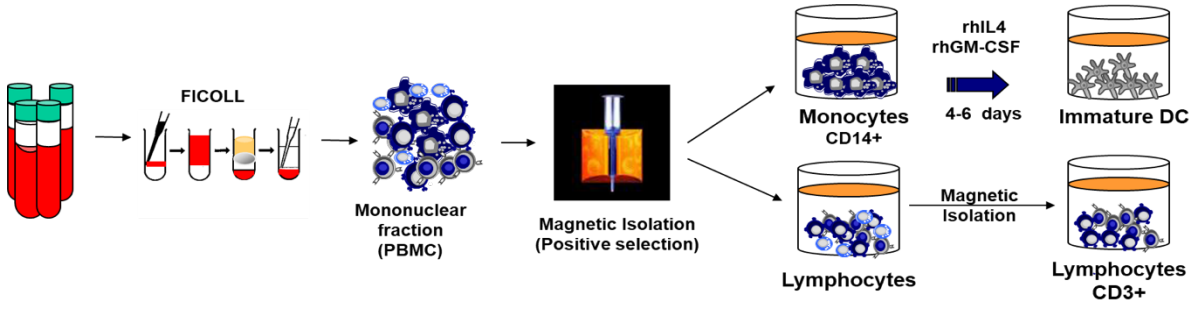


Figure 8: Human DCs maturation and functional characterization after treatment with DS. Blood samples were obtained and PBMCs isolated by Ficoll gradient centrifugation. Specific cell types (CD14+, CD3+) were then isolated using magnetic bead sorting. CD14+ cells were treated with GM-CSF and IL-4 for six days to generate immature dendritic cells (DCs). DCs were treated with LPS and/or DS for 72h for non-specific activation and analyzed for viability, maturation and co-stimulatory markers (CD80, CD83, CD86, CD40, HLA-DR) by flow cytometry.

a) DS decreases the mean fluorescence intensity (MFI) and the number of DCs expressing the co-stimulatory molecules CD80 and CD86, and MHCII expression, but did not alter CD83 or CD40 (not shown) (**Fig. 9**). This indicates that DS can affect the maturation of DCs and potentially their capacity to present antigens.

b) In conclusion, a phenotype consistent with reduced antigen-presenting function adds to the potentially unique function of DS as an agent for the concurrent tolerogenic modulation of APCs, T cells and beta cells.

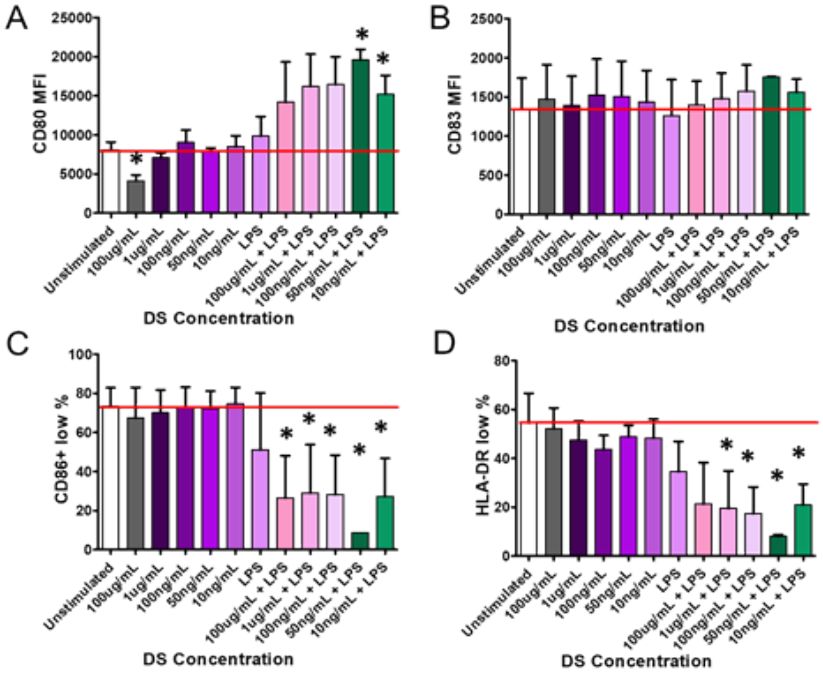


Figure 9: Human DCs maturation and functional characterization and treatment with DS. DCs from healthy controls were treated with different concentrations of DS and activated with LPS. DS treatment altered CD80 (**A**), CD86 (**C**) and MHCII (HLA-DR) (**D**) expression on DCs, but did not affect CD83 (**B**) expression. N=3 different human PBMC preparations. *p<0.05 vs. unstimulated or LPS.

c) Milestone(s) Achieved: DS effects on human APC maturation

d) Future studies, we will address whether DS effects on DCs occur in hPBMCs from type 1 diabetes donors.

- **SA3.3: Analyze the impact on DS preconditioning on CD4+T cell responses.**

Results: To address the goal in SA3.3, whole fresh PBMCs from healthy individuals or CD4+ or CD8+ T-cells isolated with magnetic bead conjugated negative selection antibodies were treated with activators (PMA/ionomycin, PHA, 5h-24h) or anti-CD3 and anti-CD28 (48h) plus DS or vehicle. Cells were analyzed for activation markers IFNg+ (Th1), IL-4+ (Th2), IL-17+ (Th17), and CD25+FoxP3+CD127low (T-reg) by flow cytometry. We found that:

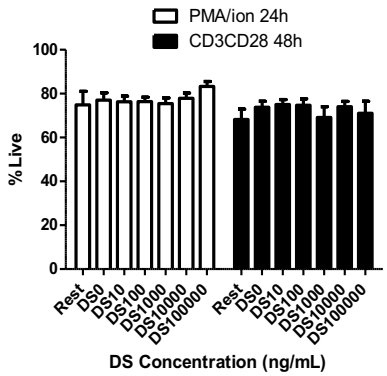


Figure 10: Effect of DS on the viability of hPBMCs activated with PMA/ionomycin or anti-CD3 and anti-CD8. No significant differences were observed.

a) DS did not decrease the viability of activated hPBMCs (or non-activated, resting) when added for 24h or 48h at different concentration from 10ng to 100 µg/ml (**Fig. 10**). Therefore, any potential phenotypic change induced by DS on these cells will not be related to changes in cell viability.

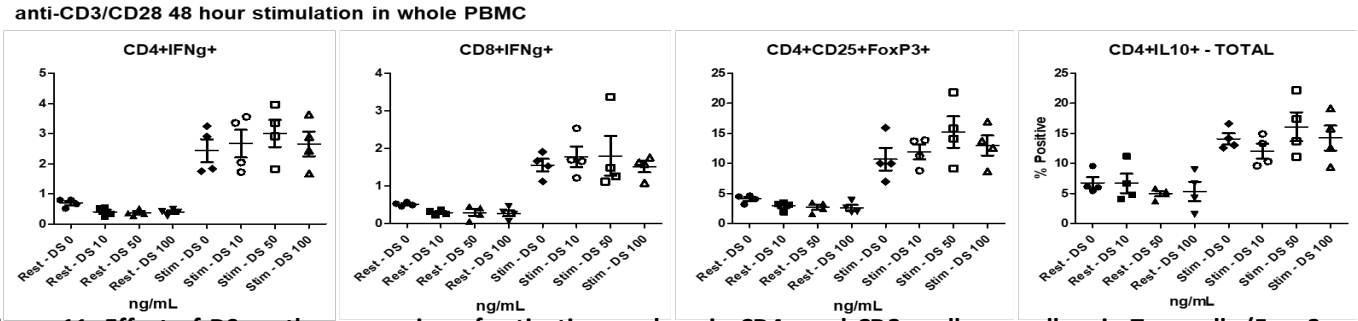


Figure 11: Effect of DS on the expression of activating markers in CD4+ and CD8+ cells as well as in Treg cells (Foxp3+ and IL10+) after treatment of whole PBMCs with (stim) or without (rest) anti-CD3 and anti-CD8 for 48h. No significant differences were observed. Four different human PBMC preparations were used.

b) Treatment of whole PBMCs with different doses of DS did not alter the number of CD4+IFNg+ (Th1) cells, CD4+IL4+ (Th2) cells (not shown), CD4+IL17A (Th17) cells (not shown), CD8+IFNg+ cells or Tregs (CD4+CD25+FoxP3+ or CD4+IL10+) (**Fig. 11**). Identical results were obtained with PMA/ionomycin activation for 24h (not shown). Therefore, DS does not have a direct effect on T-cell differentiation or activation in whole PBMC stimulated with anti-CD3/anti-CD28 or PMA/ ionomycin.

c) Treatment of isolated CD3+ lymphocytes with different doses of DS did not significantly alter the number of Th1, Th2, Th17 or Tregs after PMA/ionomycin activation for 5h (**Fig. 12**). Similar results were observed when cells were activated with anti-CD3 and anti-CD28 (not shown). Therefore, DS does not have a direct effect on T-cell differentiation or activation in isolated T-cells stimulated with anti-CD3/CD28 or PMA/ionomycin.

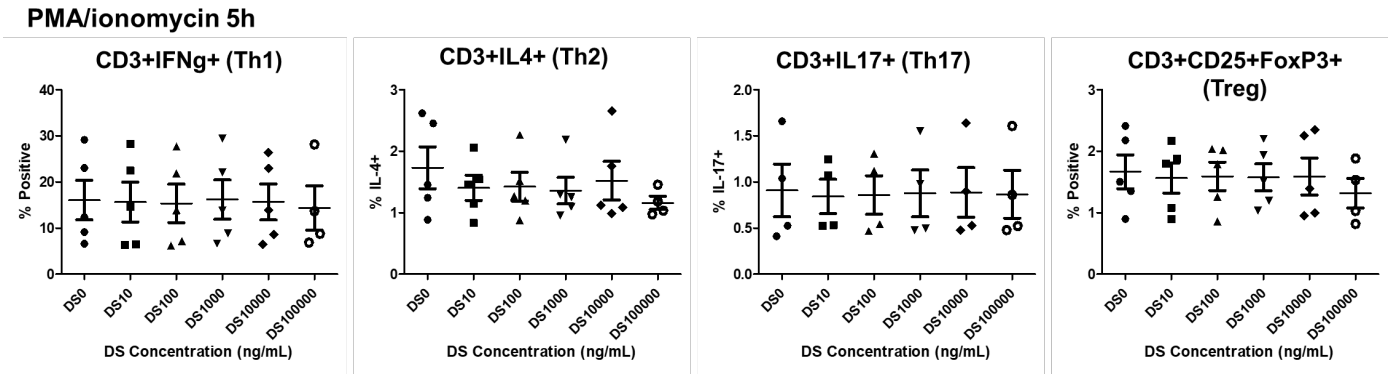


Figure 12: Effect of DS on the expression of activating markers (Th1, Th2, Th17 and Tregs) in isolated CD+3 cells after treatment with (stim) or without (rest) PMA/ionomycin for 5h. No significant differences were observed. Four different human PBMC preparations were used.

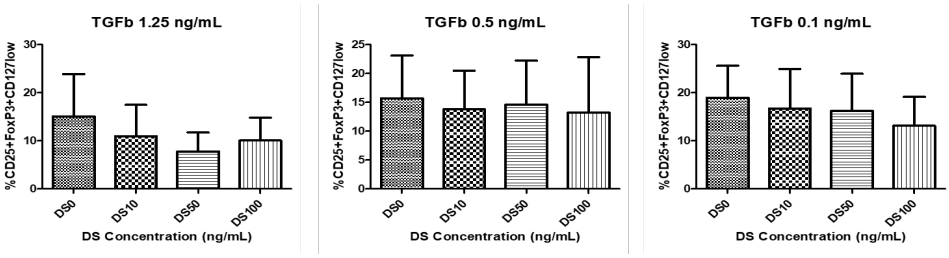


Figure 13: Effect of DS on T-cell differentiation in isolated naïve CD4+ T-cells when preferentially polarized. No significant differences were observed. Four different human PBMC preparations were used.

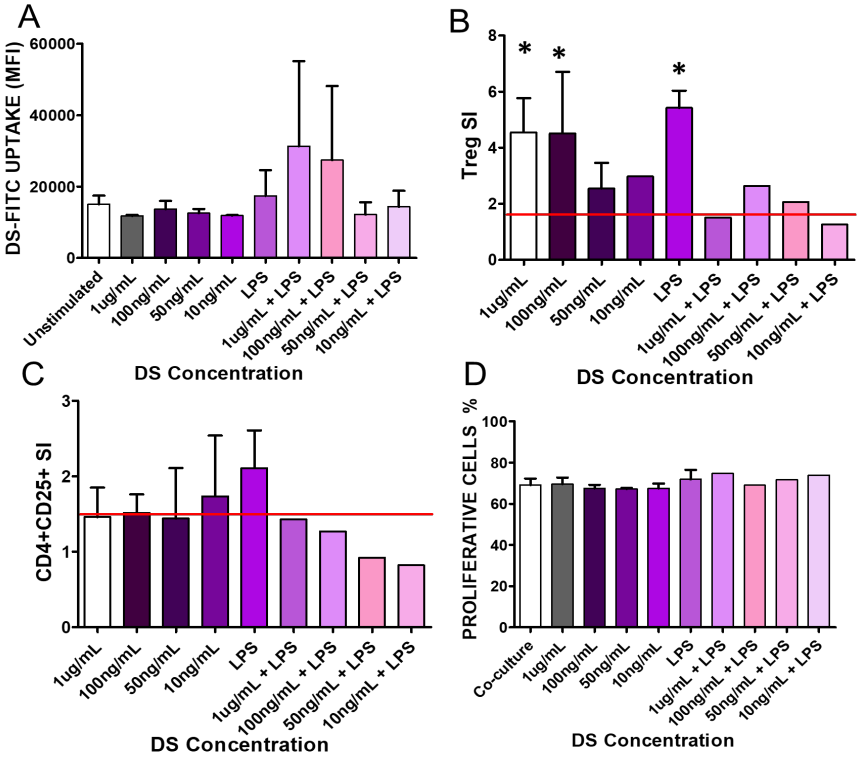


Figure 14: Effect of DS on the interaction between DCs and autologous lymphocytes. (**A**) DCs pre-treated with DS uptake similar amounts of DS-FITC regardless of DS concentration. (**B**) DCs treated with DS increased differentiation of T-reg with co-culture of naïve CD4+ cells. (**C**) T-cell proliferation was reduced with DS- and LPS-treated DCs, (**D**) but not with allogeneic lymphocytes, indicating a MHC-required response. *p<0.05 vs. unstimulated.

d) To address whether DS could require polarization with TGF beta to enhance the number of Tregs from hPBMCS, we treated isolated CD4+ T-cells with different doses of TGF beta and DS and determine the number of CD25+FoxP3+CD127low (Tregs) generated by flow cytometry. As shown in **Fig. 13**, DS does not have a direct effect on T-cell

differentiation and Treg generation in isolated naïve CD4+ T-cells when preferentially polarized.

d) Analysis of the endocytic capacity of DCs was not significantly altered with DS treatment. (**Fig. 14A**). Interestingly, however, the interaction between DS-treated DCs and autologous lymphocytes leads to a significant increase in the number of T-regs (CD4+CD25+FoxP3+) (**Fig. 14B**) and a potential decrease in proliferation of CD4+ T cells (**Fig. 14C**) when stimulated with LPS (needs to be confirmed). On the other hand, the incubation of DS-treated DCs with allogenic lymphocytes did not result in proliferation changes (**Fig. 14D**).

e) In conclusion, these results suggest that direct effects of DS in T cells do not lead to changes in their activation or regulatory profile. However, DS induces a more tolerogenic phenotype in activated human DCs leading to an increase in the number of Tregs and decreased CD4+ T cell proliferation.

f) Milestone(s) Achieved: DS effects on human APC maturation.

g) Future studies: we will decipher the impact of DS on DCs as the underlying mechanism decreasing T-cell activation.

- **SA 3.4. To elucidate the mechanisms of DS-induced immunomodulation.**

Results: Mechanisms of DS-induced immunomodulation: PD-1/PD-L1. To address the goal in SA3.4, we performed experiments with fresh hPBMC from healthy subjects which we stimulated with αCD3/αCD28 beads and CyTOF analysis was performed as indicated in Fig. 1.

a) Contrary to the results we found in mice (Lu et al. *Diabetes*, 2020, Aug;69(8):1692-1707, Fig. 6, PMID: 32381645) where DS *in vivo* treatment of NOD mice led to increased numbers of PD-1+CD4+ and PD-1+CD8+ in spleen and pancreatic lymph nodes, PD-1, expression levels did not change in hPBMCs after DS treatment (**Figs. 1, 2, 5 and 6**). These differences in the results could be due to the different immune compartment tested, to the difference between *in vivo* and *in vitro* effects of DS, to the different methods used for the analysis, to differences in doses and timing, and also to the species difference.

b) As shown in **Figs. 1** and **7**, PD-L1 expression is decreased when looking at whole PBMCs and more specifically in myeloid–derived cells. Combining both observations, these results indicate that at least in hPBMCs *in vitro*, DS does not increase the expression PD-1/PDL1 pathway suggesting potential alternative mechanisms for DS effects.

c) We next turned our attention on the HGF/c-Met pathway since DS has been shown to increase HGF circulating levels in mice (unpublished observation) and humans (Schmidt P et al. *Transplantation*. 86(11):1523-30, 2008, PMID: 19077884) and this pathway is known to regulate multiple immune functions (Molnarfi N, et al. *Autoimmun Rev.* 14(4):293-303, 2015, PMID: 25476732). Interestingly, we made the observation that inhibition of c-Met signaling with the specific c-Met inhibitor Ph665752 blocks the upregulation of PD-1 expression in CD4+ and CD8+ cells induced by DS in pancreatic lymph nodes and splenocytes from NOD treated mice (**Fig. 15A**). We have also made the important observation that the number of myeloid-derived suppressor cells (MDSCs) (CD11b+Ly6CG+) increases with DS treatment in mouse PBMCs and this increase is suppressed by the c-Met inhibitor (**Fig. 15B**). These studies were partially funded by NIH R01DK113079 and are the base for future studies in human immune cells. Experiments with combination of DS plus HGF should be performed in hPBMCs to determine whether the tolerogenic effects of DS depend on PD1/PD-L1 via HGF/c-Met signaling. These studies are planned and will be performed in the near future.

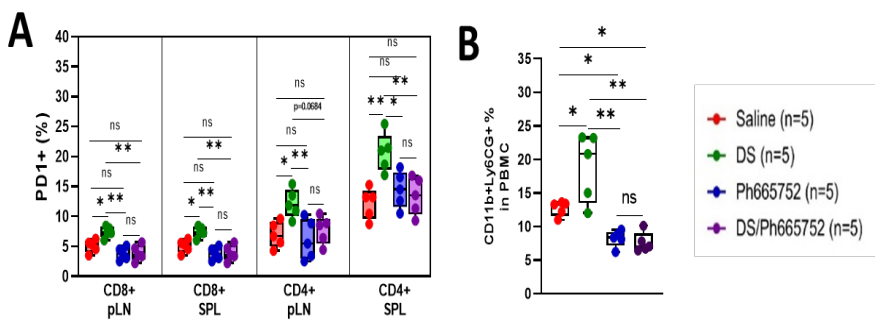


Figure 15: Effect of the c-Met inhibitor Ph665752 on the DS-induced (A) upregulation of PD-1 in CD4+ and CD8+ cells in spleen (SPL) and pancreatic lymph nodes (pLN) and (B) the increased number of MDSCs (Cd11b+Ly6CG+) in PBMCs of the same mice. Five mice per condition were treated for four weeks in these studies with 10mg/Kg DS. *p<0.05; **p<0.01 vs. saline or DS.

d) In conclusion, our results suggest that PD-1/PD-L1 is not altered in hPBMCs by DS and might not play a role in DS-induced tolerogenic effects in human cells. However, HGF/c-Met system can play a role in these

effects since DS increases the levels on HGF in the circulation in mice and humans and inhibition of c-Met signaling impairs the upregulation of PD-1 in T cells and the increase in numbers of MDSCs.

e) Milestone(s) Achieved: DS effects on PD-1/PD-L1 axis. We have further explored the potential role of HGF/c-Met in DS effects at least in mouse PBMCs.

f) Future studies: We will address whether HGF/c-Met signaling plays a role in the tolerogenic effects of DS in human immune cells.

- **What opportunities for training and professional development has the project provided?**

- Training on Flow Cytometry was provided by:

- Dr. Jordi Ochando, PhD; Assistant Professor, Oncological Sciences, Medicine, Nephrology and Pathology at Icahn School of Medicine at Mount Sinai.

Training on CyTOF data analysis was provided by:

- Dr. Adeeb Rahman, PhD, Assistant Professor, Genetics and Genomic Sciences, Director of Technology, Human Immune Monitoring Center at Icahn School of Medicine at Mount Sinai.

- Dr. Amir Horowitz, PhD, Assistant Professor, Oncological Sciences, Precision Immunology Institute at Icahn School of Medicine at Mount Sinai.

- For the period 2019-2020, John G Graham, MD fellow from the Division of Endocrinology, Diabetes and Bone Disease performed one year of research training on this project funded by Icahn School of Medicine at Mount Sinai.

- **How were the results disseminated to communities of interest?**

The results of these studies have been presented at the Boston-Ithaca Islet Club in 2018 and the network of Pancreas Organ Donors (nPOD) in 2019 and 2020.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Final report, nothing to report.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

The unexpected confluence of dextran sulfate-dependent chemokine expression modulation at the level of both T cells and islet cells, the decrease in human T cell activation, together with a phenotype consistent with reduced antigen-presenting function highlights the unique function of dextran sulfate as an agent for the concurrent tolerogenic modulation of APCs, T cells and pancreatic beta cells. In conclusion, our studies developed during the time of the award highlight the specific immunomodulation by dextran sulfate treatment that could clearly favor a “tolerogenic” phenotype in autoimmune type 1 diabetes. This highlights the therapeutic potential of dextran sulfate for treating type 1 diabetes. Next step will be to determine the tolerogenic potential of DS for diabetes performing *in vitro* studies in hPBMCs from subjects with T1D at different stages of the disease (early-onset vs already established disease). Funding for these studies is being pursued.

- **What was the impact on other disciplines?**

Importantly, our studies can have an important impact in other medical disciplines. Our results show an immunomodulatory effect of dextran sulfate in antigen presenting cells and T cells highlighting the therapeutic potential of this agent for autoimmune type 1 diabetes. Therefore, dextran sulfate can have potential applicability for other autoimmune diseases such as lupus, rheumatoid arthritis, thyroiditis, etc.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. Changes/Problems

No changes or problems in the project or its direction were encountered during the award period.

6. Products

- **Publications, conference papers, and presentations**

i. **Journal publications.**

- Lu G, Rausell-Palamos F, Zhang J, Zheng Z, Zhang T, Valle S, Rosselot C, Berrouet C, Conde P, Spindler MP, Graham JG, Homann D, Garcia-Ocaña A. Dextran Sulfate Protects Pancreatic β-Cells, Reduces Autoimmunity, and Ameliorates Type 1 Diabetes. ***Diabetes***. 2020 Aug;69(8):1692-1707. doi: 10.2337/db19-0725. Epub 2020 May 7. PMID: 32381645; PMCID: PMC7372066. *Published; federal support (yes, funded by NIH R01DK113079 and partially by W81XWH-17-1-0363 and W81XWH-17-1-0364).* https://diabetes.diabetesjournals.org/content/69/8/1692

ii. **Books or other non-periodical, one-time publications.**

Nothing to Report.

iii. **Other publications, conference papers, and presentations.**

- Zheng Z, Lu G, Alvarez-Perez JC, Homann D and Garcia-Ocana A. Immunomodulatory and beta cell cytoprotective actions of dextran sulfate in T1D. **26th Annual Boston Ithaca Islet Club Meeting. 2018.** UMass Medical School, Worcester, MA.

- Lu G, Homann D and Garcia-Ocana A. Dextran Sulfate Ameliorates Type 1 Diabetes by Enhancing Beta Cell Survival and Mitochondrial Function and Increasing Regulatory T Cells. **11th nPOD Annual Meeting. 2019.** Hollywood, FL.

- Graham JG, Lopez S, Lu G, Levy C, Homann D, Garcia-Ocana A. Tolerogenic Effects of Dextran Sulfate in Human Dendritic Cells and T Lymphocytes. **12th nPOD Annual Meeting. 2020.** Orlando, FL.

- **Website(s) or other Internet site(s)**.

Nothing to Report.

- **Technologies or techniques.**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	Dirk Homann
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0002-7622-5754
Nearest person month worked:	3.0 CM
Contribution to Project:	Directing, reviewing and interpreting the results of the project.
Funding Support:	No additional funding for this project.

Name:	Adolfo Garcia-Ocana
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0002-6883-6176
Nearest person month worked:	3.0 CM
Contribution to Project:	Directing, reviewing and interpreting the results of the project.
Funding Support:	No additional funding for this project.

Name:	Zihan Zhang
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12 CM
Contribution to Project:	hPMBCs treatments, flow cytometry and analysis of CyTOF data.
Funding Support:	No additional funding for this project.

Name:	Jessica Wilson
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.6 CM
Contribution to Project:	Cell culture and hPMBCs treatments.

Funding Support:	No additional funding for this project.

Name:	John G Graham
Project Role:	MD Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12 CM
Contribution to Project:	hPMBCs isolation, culture, treatments, Flow cytometry and analysis of data.
Funding Support:	Supported by Icahn School of Medicine. Endocrine MD research fellow.

Name:	Joe Schragenheim
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.0 CM
Contribution to Project:	Flow cytometry analysis.
Funding Support:	No additional funding for this project.

Name:	Geming Lu
Project Role:	Senior Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0002-1322-3997
Nearest person month worked:	6.0 CM
Contribution to Project:	hPMBCs treatments, Flow cytometry and analysis of data.
Funding Support:	No additional funding for this project.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Previous Grant Support that Ended:

2017-2018

- NIH/NIDDK R01 DK098437-01(Garcia-Ocana, Co-I): “FoxO1 in Beta Cell Compensation” ended on 11/30/17

2018-2019

- NIH/NIDDK DK-012893 (Garcia-Ocana: Co-I): “Osteoprotegerin and the Pancreatic Beta Cell” ended on 01/31/2019

2019-2020
- Pfizer, Inc. (Homann: Co-I) “Rat Diabetes Microgliosis Investigation”. 9/1/18 – 8/31/19
- 2-SRA-2017-514-S-B. (Garcia-Ocana PI, Stewart, co-PI); 09/01/17-8/31/20; “Combined harmalog-TGFß inhibitors for human ß cell expansion”. Juvenile Diabetes Research Foundation
- JDRF 201305402 (Homann: MPI); 11/01/2018 - 8/31/2020 “Center for mass cytometry analysis of TrialNet samples” Juvenile Diabetes Research Foundation
- NIH/NIDDK; R01 DK015015 (MPI); “Dyrk Inhibitors for Human Beta Cell Expansion”; PROJECT DATES: 4/1/16 – 3/31/20

Pending Grants that are now Active:

2017-2018
- R01 DK110156 (Garcia-Ocana, Co-I; Scott, PI); 04/01/17 – 03/31/21; “ChREBP Alpha, Keap1-Nrf2 and Glucose-stimulated Beta Cell Proliferation”. NIH/NIDDK
- R01 DK116904 (Garcia-Ocana, Co-I; DeVita, Stewart Co-PIs); 04/01/18-03/30/23; “Type II Kinase Inhibitors to Treat Diabetes”. NIH/NIDDK
- R01 DK114338 (Garcia-Ocana, Co-I; Scott, PI); 07/01/18 – 06/30/23; “Nrf2 and the adaptive expansion of beta cell mass”. NIH/NIDDK
- UC4DK116284 (Homann, Co-I) 09/15/17 – 08/30/21 “Human islet infiltrating T cell biology: reactivity, structure, and function”. NIH/NIDDK.
- R01AI134971 (Homann, MPI) 12/07/17 – 11/30/22 “Integrated functional histopathology of the diabetic human pancreas”. NIH/NIAID.

2018-2019
- NIH/NIDDK U01DK123716 (Homann, MPI) 09/22/19 – 08/31/24 “Human Pancreas Analysis Program-T2D”

2019-2020
- P30 DK020541 (Garcia-Ocana, Site PI) 4/1/20 – 3/31/25 “Einstein-Mount Sinai Diabetes Research Center (ES-DRC)-Human Islet and Adenovirus Core“ NIH/NIDDK
- P30 DK020541 (Homann, Site PI) 4/1/20 – 3/31/25 “Einstein-Mount Sinai Diabetes Research Center (ES-DRC)-Immuno-Technology Core” NIH/NIDDK.
- W81XWH2010345 (Garcia-Ocana: Co-I; Stanley, PI) 6/1/20 – 5/31/23. “Evaluating Pancreatic Neuromodulation for Prediabetes and Diabetes”. Department of Defense
- R01 DK125285 (MPI: Garcia-Ocana, DeVita and Stewart) 7/1/20 – 6/30/25. “Biological and Medicinal Chemistry Approaches to Human Beta Cell Regeneration”. NIH/NIDDK
- R01 DK126450-01 (MPI: Garcia-Ocana and Scott) 7/1/20 – 6/30/24. “Myc Physiology in the Pancreatic Beta Cell”. NIH/NIDDK
- NIH supplement (Homann, Co-I) 09/01/20 – 08/21/21 “Multiparametric mapping of Covid-19 immune responses in kidney transplant recipients”

  - **What other organizations were involved as partners?**

Nothing to Report.

8. Special Reporting Requirements

- **COLLABORATIVE AWARDS:**

None

- **QUAD CHARTS:**

None

9. Appendices

None