

Hyperglycemia Activates the CD27-CD70 axis on Human PBMCs

Abstract

Obesity is now strongly associated with chronic low-grade inflammation that, without intervention, contributes to the development of pre-diabetes and eventually Type 2 diabetes. While the exact mechanism that inflammation plays in the pathogenesis from obesity to Type 2 diabetes is unclear, activated immune cells and pro-inflammatory cytokines have been found in the adipose tissue of Type 2 diabetics, implicating their role in the disease process. The CD27-CD70 pathway provides a crucial inflammatory co-stimulatory signal, with CD70 being expressed on activated antigen presenting cells, and CD27 expressed on lymphocytes. While the CD27-CD70 axis is being explored in other models of chronic inflammation, such as rheumatoid arthritis and colitis, the role played in Type 2 diabetes remains unknown. Here we report the downregulation of CD27 on CD4 T cells when co-cultured with dendritic cells primed in increasing concentrations of glucose, indicating an effector phenotype of these T cells. Importantly, we also highlight that CD70 is concurrently upregulated on dendritic cells primed in high concentrations of glucose, resulting in increased production of IFN- γ and TNF- α by the CD70 expressing dendritic cells, when compared to dendritic cells primed in a lower concentration of glucose. These results reveal a novel role for CD27-CD70 interactions in the pathogenesis of Type 2 diabetes and provide support for future investigations into this pathway. Additionally, CD27 could be analyzed as a way to further stratify pre-diabetic patients and guide diagnosticians towards the most efficient therapy.

MeSH index terms: Diabetes Mellitus, Type 2; CD4-Positive T-lymphocytes; Inflammation; Hyperglycemia; Prediabetic state; Dendritic cell;

Introduction

The most recent report from the Center of Disease Control unveils a staggering 100 million Americans are now living with diabetes or prediabetes.¹ Prediabetes is a condition that when addressed promptly and efficiently can be reversed. However, only 11.6% of those with prediabetes are aware of their prediabetic status and condition.¹ The American Diabetes Association (ADA) defines laboratory values for prediabetes as HgbA1c values between 5.7-6.4%, fasting plasma glucose values between 100-125 mg/dL, and/or an oral glucose tolerance test of 140-199 mg/dL.² Furthermore, a study by the ADA found that approximately 10% of patients with prediabetes convert to full diabetes annually.³ Laboratory testing plays an important role in distinguishing between prediabetes and diabetes, which in turn helps in disease management and even possible reversal. With the above mentioned studies bringing more attention and awareness to prediabetes and diabetes, efficient screening and patient stratifying protocols need to be standardized in order to prevent diabetic-associated complications and comorbidities.

Although the pathogenesis of Type 2 diabetes is not fully understood, strong evidence now links chronic inflammation and activated components of the immune system, such as macrophages, dendritic cells, T cells and cytokines to the pathogenesis of pre diabetes and Type 2 Diabetes. Towards this, our lab has found that human CD4 T cells co-cultured with dendritic cells primed in increasing concentrations of glucose show a dose-dependent effector phenotype, with the highest concentrations of glucose inducing the highest level of CD4 T cell activation.⁴ For activation, naïve T cells require interaction with antigen-presenting cells (APCs) along with T cell receptor: MHC engagement and co-stimulatory signaling. CD27-CD70 is a well-studied costimulatory receptor-ligand pair in the tumor necrosis factor receptor (TNFR) superfamily.⁵

CD27 is constitutively expressed on naïve CD4 T cells but is down-regulated when T cells differentiate into effector cells.⁶ With expression largely limited to activated antigen presenting cells (APCs), CD70 is tightly regulated and is the sole ligand for CD27.⁷ Infection and immunization models have identified a crucial role played by the CD27-CD70 pair in T cell priming.⁸⁻¹¹ However, this has not been investigated in diabetes and chronic hyperglycemia. We therefore sought to investigate CD27 and CD70 on human CD4 T cells and dendritic cells derived from peripheral blood mononuclear cells (PBMC) in our hyperglycemia model. Here we demonstrate that when co-cultured in hyperglycemic conditions, CD4 T cells down regulate their expression of CD27 while dendritic cells simultaneously up regulate their CD70 expression. In addition, the inflammatory status of the dendritic cells was confirmed through their increased cytokine production, with both interferon gamma (IFN- γ) and tumor necrosis factor –alpha (TNF- α) found to be increased. These results indicate that in conditions with elevated blood glucose such as diabetes, the CD27-CD70 axis is activated and both CD4 T cells and dendritic cells exhibit a pro-inflammatory phenotype and function. This study provides support for future investigations into analyzing the expression of CD70 and CD27 on the surface of dendritic cells and CD4 T cells from the peripheral blood of patients with pre diabetes or diabetes, and provides a mechanism to stratify the patients most at risk for inflammatory-related comorbidities or the rapid progression from prediabetes to full diabetes.

Materials and Methods

Cell separation and isolation of peripheral blood mononuclear cells

Isolation of human peripheral blood mononuclear cells (PBMCs) was carried out using Histopaque® (MP Bio) density-gradient centrifugation. Human whole blood used for PBMC

isolation was provided as a unit of de-identified whole blood from LifeSource (Chicago, IL). The blood was diluted 1:2 with Phosphate Buffered Saline, (PBS) (MP Bio) and layered over Histopaque® (MP Bio). This was then centrifuged at room temperature (20-25°C) for 30 minutes at 2000 rpm. The interface (buffy coat) was recovered and washed three times with PBS (MP Bio). The resulting PBMCs were subject to positive selection of CD14+ and CD3+ cells via magnetic-activated cell sorting (MACs) technology. This study was exempt from IRB approval as all blood used was completely de-identified and donors did give their informed consent to donate peripheral blood.

Immature dendritic cell and T cell isolation from PBMCs

CD14 is a pan-monocyte marker that identifies peripheral blood mononuclear cells that can differentiate into antigen presenting cells such as macrophages and dendritic cells. Therefore, monocytes were purified from the isolated PBMCs by positive selection using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec). The PBMCs were incubated with the anti-CD14-conjugated microbeads for 20 minutes at 4 °C, washed with MACs buffer (Miltenyi Biotec) before being applied to a MACS magnetic column (Miltenyi Biotec). The column was then washed three times with MACS buffer to wash away any non-CD14+ cells before the column was eluted for CD14+ monocytes (purity >85%). Concurrently, T cells were purified from the isolated PBMCs by positive selection using anti-CD3-conjugated magnetic microbeads (Miltenyi Biotec). A separate fraction of PBMCs were incubated with the anti-CD3-conjugated microbeads for 20 minutes at 4 °C, washed with MACs buffer (Miltenyi Biotec) and then applied to a MACS magnetic column (Miltenyi Biotec). The column was then washed three times with

MACS buffer to wash away any non-CD3⁺ cells before the column was eluted for CD3⁺ T cells (purity 87.6%). Both purity measurements were determined through flow cytometry.

Generation of monocyte-derived DCs

Isolated immature CD14⁺ monocytes (1×10^5 /mL) were cultured for six days in 6-well tissue culture plates containing glucose-free HyCloneTM RPMI media supplemented with 10% fetal bovine serum (Gibco), 1mM sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (Gibco) and 1% penicillin/streptomycin (Gibco). Conventionally, peripheral blood monocytes are differentiated into myeloid dendritic cells by adding granulocyte-macrophage-colony stimulating factor (GM-CSF) and IL-4 to the culture media.¹² Accordingly, in our culture system, GM-CSF (100ng/mL), and IL-4 (50ng/mL) were added to the culture media of CD14⁺ monocytes to differentiate them into immature dendritic cells. Cells were incubated at 37°C with 5% CO₂ with media changed every two days. On day 6 of culture, the resulting immature DCs were subject to varying concentrations of glucose (Fisher Scientific) and incubated for an additional 24 hours to allow sufficient time for glucose stimulation. The conditions included complete absence of glucose, the addition of 5.5 mmol/L (physiologic), 15 mmol/L (prediabetic) or 30 mmol/L (hyperglycemic) glucose concentrations.

Dendritic Cell: T cell Co-Culture

Naïve autologous CD3⁺ T cells (1×10^6 /mL) were added to the cultured DCs after the DCs had been incubated with or without glucose for 24 hours. The culture medium was supplemented with IL-2 (80U/mL; Fisher Scientific). It is possible that the dendritic cells utilized all of the glucose before the T cells were exposed; therefore T cells co-cultured with dendritic cells in the

absence of glucose provided a valuable internal control. For the positive control, T cells were stimulated with CD3/CD28 beads (Tonbo Biosciences) to mimic *in vivo* antigen presentation through T cell-co-receptor and co-stimulatory molecule engagement. The DCs and T cells were co-cultured for 7 days before harvesting for flow cytometry assays.

Flow Cytometry assays

Lymphocytes were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) directed against human CD3 (SK7), CD4 (SK3), CD27 (O323), all from Tonbo Biosciences, and IFN- γ (B27) and TNF-alpha (Mab11) and CD70 (Ki-24) from BD Biosciences. Monocyte-derived dendritic cells were stained with CD11b (ICRF44), CD14 (61D3) and CD11c (3.9) from Tonbo Biosciences and CD70 (Ki-24), IFN- γ (B27) and TNF-alpha (Mab11) from BD Biosciences. For intracellular staining, lymphocytes and dendritic cells were cultured for 5 hours with 1 mg/ml GolgiPlug (BD Biosciences) then stained for surface markers (CD3 and CD4 for T cells, CD14, CD11b and CD11c for dendritic cells). Cells were then fixed and permeabilized using Cytotfix/Cytoperm solution (BD Bioscience) for 20 min at 4°C, and were subsequently stained with anti-IFN γ , anti-TNF α , or the corresponding isotype controls (BD Biosciences). Finally, cells were washed twice and analyzed by flow cytometry. The combined use of cell surface markers with intracellular staining allows for the identification of cytokines being produced on a single-cell basis. Flow cytometry was performed on a BD LSRII instrument and data was analyzed with FlowJo software.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean (SEM) of independent experiments. Statistical significance was determined by Student's two-tailed paired *t*-test assuming equal variances or ANOVA. P values less than 0.05 were considered statistically significant.

Results

CD4 T cells have an activated effector phenotype after co-culture with glucose stimulated dendritic cells.

The functional state of CD4 T cells can be divided into distinct subsets: naïve, effector, or memory T cells based on their phenotype, i.e. the expression (or lack of expression) of diverse cell surface receptors.¹³ One surface receptor that is frequently utilized as a marker of effector state, or activated CD4 T cells is the tumor-necrosis family member (TNFR) CD27.¹⁴ Previous studies have shown a step-wise downregulation of CD27 on the surface of naïve CD4 T cells differentiating into effector and further into memory CD4 T cells.^{15, 16} Therefore, we sought to evaluate the CD27 expression on CD4 T cells from co-culture with glucose-primed dendritic cells. Because CD27 is highly expressed on naïve CD4 T cells, we included the analysis of CD4 T cells prior to being added to culture with dendritic cells as a comparison. In accordance with this finding we observed that our naïve CD4 T cells expressed high levels of CD27 (86%) prior to being added to the co-culture (Figure 1A-1B). Interestingly, flow cytometric analysis revealed a dose-dependent gradual decrease in CD27 expression on CD4 T cells in co-culture with dendritic cells primed in increasing concentrations of glucose with the 30 mmol/L (hyperglycemic) priming condition showing the smallest percentage of CD27+ CD4 T cells

(16%, Figure 1B-1C). While the dendritic cells that were primed in the absence of glucose also induced the downregulation of CD27 on subsequent CD4 T cells encountered, our data shows a statistically significant decrease in CD27 expression between CD4 T cells co-cultured with dendritic cells primed with 0 mmol/L or 5.5 mmol/L (physiological) of glucose and CD4 T cells co-cultured with dendritic cells primed with 30 mmol/L of glucose (p -value = 0.033 and 0.027 respectively).

CD70 is upregulated on dendritic cells primed in hyperglycemic conditions.

CD70 is also a member of the TNFR family, and is currently the only known ligand for CD27, with CD27-CD70 acting as a costimulatory receptor-ligand pair.¹⁷ While CD27 is expressed on naïve and memory T cells as well as some subsets of B cells and NK cells, CD70 is primarily expressed on APCs.^{10, 18} Therefore, we analyzed the expression of CD70 on the CD11b+ dendritic cells in our culture system, primed in increasing concentrations of glucose. As shown in Figure 2A-2B, there is an increase in CD70 expression as the concentration of glucose used to prime dendritic cells increases (p -value = 0.003). While CD70 is primarily expressed on activated APCs, it can be expressed on activated T cells as well. Therefore, we analyzed the T cells in co-culture with the dendritic cells for their expression of CD70. This analysis revealed similar expression irrespective of the concentration of glucose used in cultures (5.5 mmol/L-30 mmol/L, Figure 2C). However, T cells co-cultured with dendritic cells in the absence of glucose reported a significant decrease in their CD70 expression (p -value =0.002, 0.001 and 0.002 when compared to 5.5 mmol/L, 15 mmol/L and 30 mmol/L of glucose respectively). Collectively, this data implicate the CD27-CD70 costimulatory pair to play a role in the activation of naïve CD4 T cells in hyperglycemic conditions.

Dendritic cells produce IFN- γ and TNF- α when primed in hyperglycemic conditions.

While surface markers are one indication of the activation status of an immune cell, cytokines produced also give insight into the function of that cell. Therefore, we sought to examine whether the cells in our co-cultures produced the inflammatory cytokines IFN- γ or TNF- α . In our co-culture system, T cells produced very little of either cytokine (data not shown). However, the dendritic cells displayed a dose-specific increase in their cytokine production as the respective priming glucose concentration increased (Figure 3A-B). This was true for both IFN- γ (Figure 3A, $p=0.001$) and TNF- α (Figure 3B, $p=0.002$) when dendritic cells primed in physiological (5.5 mmol/L) glucose and hyperglycemic (30 mmol/L) conditions were compared. Together, these results confirm that dendritic cells primed in a hyperglycemic environment display an activated phenotype and function, which results in subsequent activation of CD4 T cells, skewing them towards an effector/inflammatory phenotype.

Discussion

Dendritic cells play a critical role in the activation of CD4 T cells due to their crucial role in antigen-presentation to T cells. In order to carry out antigen presentation, dendritic cells need to express co-stimulatory molecules to induce efficient T cell responses. Once such co-stimulatory molecule is CD70, which is the only known ligand for the receptor CD27.¹⁸ Here we demonstrated that the expression of CD70 on human PBMC-derived dendritic cells is up regulated as the concentration of glucose used to prime these cells was increased. While the expression and function of CD70 on human immune cells is not fully understood yet, our results are in accordance with others highlighting that CD27-CD70 plays a crucial role in the adaptive immune response in humans.^{19, 20} However, this study is the first to our knowledge looking at

the CD27 and CD70 profiles on immune cells in a model of hyperglycemia. In one study utilizing an adenovirus infection model, it was found that regardless of the mechanism by which CD70 was induced on activated dendritic cells, a blockade of CD70 on the activated dendritic cells significantly reduced their immunogenicity.²⁰ Additionally, in a mouse model of colitis anti-CD70 antibody was found to be effective in the prevention and reversal of disease due to the significant reduction in IFN- γ produced by dendritic cells. Results from our model show dendritic cells as the primary producers of IFN- γ , highlighting an important pathway in Type 2 diabetes or pre-diabetes that could be explored for future therapies. Furthermore, CD27 function and eventual down-regulation on T cells only happens through interaction with CD70. This means that the inflammatory response as a result of CD27-CD70 signaling, would fail to occur in the absence of CD70. Recent evidence points to chronic and low-grade inflammation as the major driving force for the pathogenesis of pre-diabetes into Type 2 diabetes with immune cells such as antigen presenting cells, T cells and B cells being the key players in inflammation.^{21,22} We suggest that CD27-CD70 signaling is one pathway by which immune cells become activated in hyperglycemic conditions and propose future studies to investigate the utilization of targeted CD70 therapy to control and selectively reduce the chronic inflammation driving the pathogenesis of diabetes. Towards this, our data also shows that the dendritic cells are the predominant cells expressing CD70 in our model of hyperglycemia. Therefore, this selective targeting of dendritic cells would potentially avoid generalized immunosuppression. Although the expression of CD27 on B cells has not been investigated in our model, it is an area that warrants future research. Additionally, CD27 could be analyzed to predict progression of prediabetes to Type 2 diabetes, or to detect the subclinical inflammatory processes that drive its progression and risks for comorbidities. Perhaps when analyzed together with CD11a, per our

previously published results.⁴ As most clinical laboratories contain a flow cytometer, even a basic 3-4 monoclonal antibody panel could detect the CD27 and CD11a expression on a patient's T cells in order to determine the proportion of naïve vs. effector T cells present. Taken in context with the patient's pre diabetic or diabetic diagnosis, this could help guide subsequent therapeutic options.

Patients with diabetes often contract diseases and infections at a higher frequency than non-diabetics.^{23,24} However, the underlying mechanism behind this process and why this occurs in a state of chronic inflammation is currently unknown. One recent study found that patients with Type 2 diabetes had a higher frequency of B cells and effector T cells, as well as a normal cytokine profile from myeloid and NK cells.²⁵ While our study focuses exclusively on CD4 T cells and CD11+ PBMC-derived dendritic cells, it is true that the phenotypic and functional capacity of both dendritic cells and CD4 T cells is not only preserved, but also increased with elevated glucose concentrations. Interestingly, the CD4 T cells in our model had an increased effector phenotype in terms of mobilization and co-stimulation, but did not produce the inflammatory cytokines IFN- γ and TNF- α after 7 days in co-culture with dendritic cells. An important aspect to note is that this study only looked at one time-point (7 days) post co-culture and priming of the CD4 T cells. In a study investigating the role of CD70 in human-monocyte-derived dendritic cells to be used in tumor immunotherapy, investigators showed an increase in cytokine production from CD4 T cells after being in co-culture with dendritic cells for 8 days. This was dependent on IFN-alpha being present to in the priming conditions for the dendritic cells.²⁶ Therefore, it is possible that if we looked at other time points in our co-culture system or used a different method of stimulation, we would observe CD4 T cell cytokine production. Nonetheless our study recognizes that it may seem counterintuitive for a Type 2 diabetic to have

an increased effector T cell population and activated dendritic cells, yet still be more prone to infections than a non-diabetic individual. However, our work corroborates previously published studies on the immune profile of diabetic individuals. It should be noted that there are many other aspects of the immune system that could be defective in Type 2 diabetics. For example, the innate arm and the cells/defenses it comprises could have significant defects in Type 2 diabetics that are yet to be discovered.

In conclusion, we have shown that dendritic cells matured in hyperglycemic conditions display an activated phenotype, confirming a recent study.²⁷ Additionally, we demonstrated that activated dendritic cells alter the phenotype and function of the CD4 T cells they subsequently prime. This supports the notion that immune cells play a crucial role in chronic inflammation observed in patients with Type 2 Diabetes or pre-diabetes. The CD27-CD70 co-stimulatory pair has been linked to inflammation in a variety of different autoimmune and chronic inflammatory conditions.²⁸⁻³¹ The results presented in this study provide novel evidence that the CD27-CD70 axis is activated in diabetes or hyperglycemic conditions and support future examination of cell surface markers, particularly CD27 and other CD4 T cell markers of effector function, to further stratify pre-diabetic and diabetic patients by level of chronic inflammation and identify patients most at risk of comorbidities. Collectively, results reported from this study lend novel insight into the role that immune cells play in hyperglycemia and diabetes.

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Figure Legends

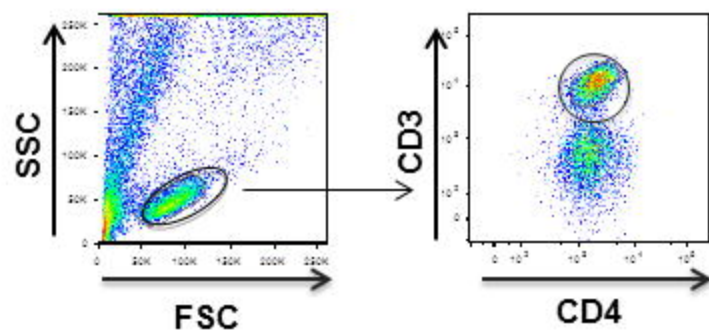
Figure 1: CD27 expression on CD3+CD4+ T cells in co-culture with dendritic cells primed in increasing concentrations of glucose. Peripheral blood derived CD3+ CD4 T cells were co-cultured with autologous dendritic cells primed in varying concentrations of glucose. After 7 days in culture, the expression of CD27 on the surface of the T cells was analyzed via flow cytometry. (A) Gating scheme of CD3+CD4+ T cells gated from total lymphocytes in cell culture. (B) Representative dot plots of this CD27 expression and (C) bar graphs are shown. Data represents 2 independent experiments with 3-6 wells per culture condition. * $p \leq 0.05$

Figure 2: PBMC derived dendritic cells increase their CD70 expression when primed in increasing concentrations of glucose. Peripheral blood derived human CD11b+ cells were primed in varying concentrations of glucose. Twenty four hours after the addition of glucose, autologous CD3+ T cells were added at a 1:10 DC: T cell ratio. After 7 days in co-culture, expression of the co-stimulatory molecule CD70 on CD11b+ dendritic cells or CD3+ CD4 T cells was determined using flow cytometry. (A) Gating scheme of CD11b+ DCs gated from total cells in cell culture. (B) Bar graphs showing the expression of CD70 on CD11b+ DCs. (C) Bar graphs showing the expression of CD70 on CD3+CD4+ T cells. Data represents 4 independent experiments with 3-6 wells per culture condition. * $p \leq 0.05$; ** $p \leq 0.01$;

Figure 3: PBMC derived dendritic cells but not T cells secrete pro-inflammatory cytokines after 7 days of co-culture in hyperglycemic conditions. Peripheral blood derived human CD11b+ cells were primed in varying concentrations of glucose. After 7 days in co-culture with autologous CD3+ T cells, the production of the inflammatory cytokines was analyzed from both cell types via intracellular staining. (A) Bar graphs showing the production of IFN- γ and (B) TNF- α from CD11b+ DCs. Data represents 2 independent experiments with 3-6 wells per culture condition. Unstained and isotype controls were used as negative gates for cytokine expression.

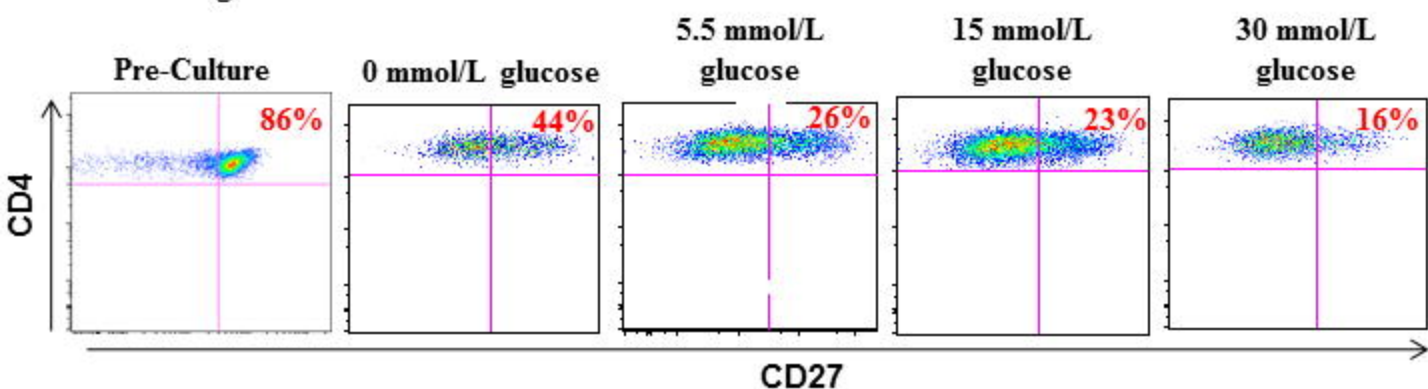
** $p \leq 0.01$; *** $p \leq 0.0001$

1A.



1B.

DC Priming Condition



1C.

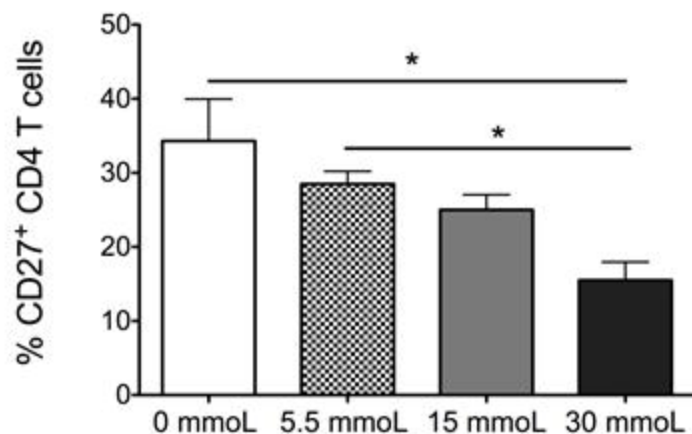


Figure 1: CD4 T cell expression of CD27 after co-culture with dendritic cells primed in increasing concentrations of glucose

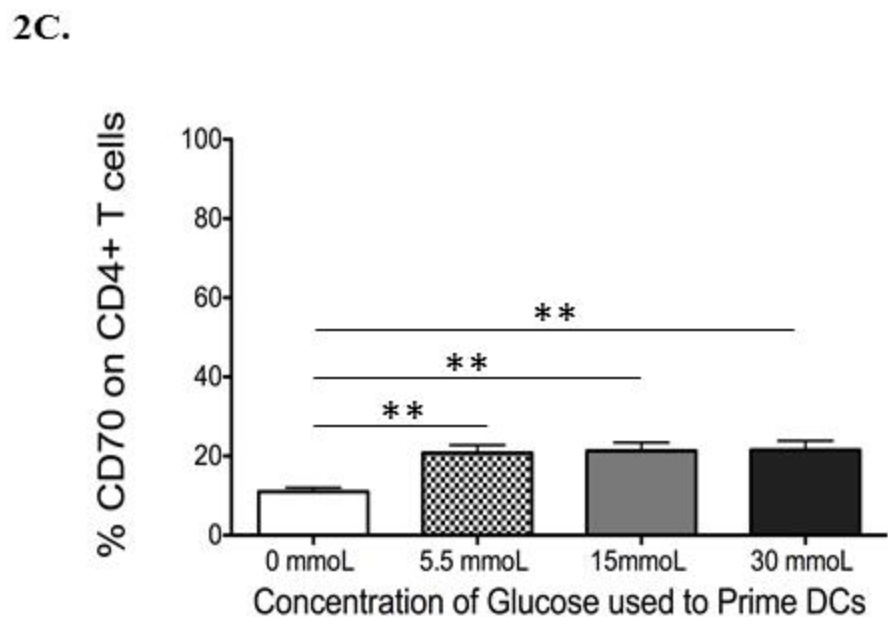
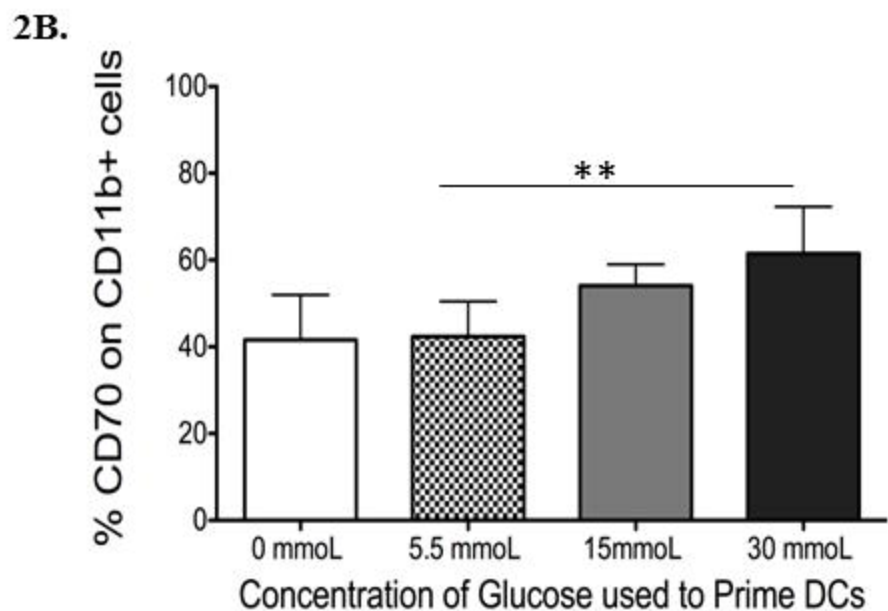
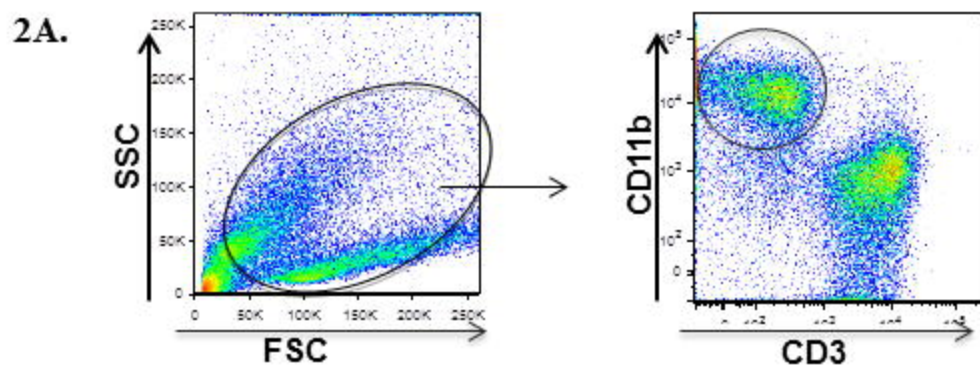
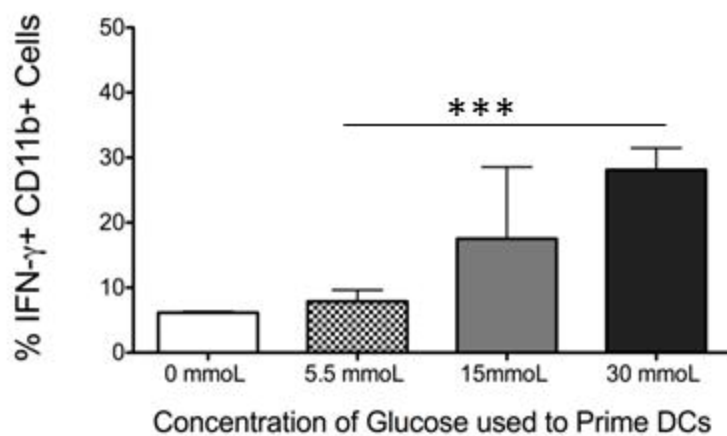


Figure 2: CD70 expression on CD11b+ dendritic cells derived from CD14+ peripheral blood monocytes and CD4+ T cells after being primed in increasing concentrations of glucose.

3A.



3B.



Figure 3: Pro-inflammatory cytokine production from CD11b+ dendritic cells after being primed in increasing concentrations of glucose.