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T-CELL ACTIVATION AND DYSFUNCTION IN HYPERGLYCAEMIA

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ABSTRACT

Although controversial, hyperglycaemia has been associated with immune dysregulation. We compared the expression of T-cell activation markers in Western Cape hyperglycaemic individuals with matched normoglycaemic controls. Sixty nine participants (86% being women) were included and screened for hyperglycaemia according to World Health Organisation (WHO) criteria. Standard multi-colour flow cytometry was used to measure expression of HLA-DR, CD38, CD95 and PD-1on T-cells at baseline and post incubation with 30mmol glucose. The results were compared and correlated with markers of glucose metabolism and inflammation. The 69 participants included 35 with normal glucose tolerance and 34 with hyperglycaemia. Antigen expression was similar between the two groups. However, after exposure to glucose the percentage of $CD4^+$ T-cells expressing CD95 significantly decreased (p=0.03). There was no correlation with markers of glucose metabolism, but expression did correlate with C-reactive protein. This study in a modest sample group found no relationship between T-cell activation and glucose metabolism, suggesting that other factors such as obesity may be responsible for immune dysregulation. Further studies are required to confirm these findings.

KEYWORDS

hyperglycaemia, obesity, T-cells, inflammation, Type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic inflammatory condition characterised by hyperglycaemia and insulin resistance.[1-3] Individuals with abnormal glucose metabolism and obesity have been associated with a pro-inflammatory environment which leads to immune dysfunction and complications such as cardiovascular disease.[4-6]

The effect of hyperglycaemia and obesity on innate immunity has been well described.[7] Advanced glycated end products (AGEs) interact with the receptor RAGE with consequent attraction of immune cells into adipose tissue. This amongst other mechanisms, results in secretion of pro-inflammatory cytokines, (Tumour Necrosis Factor, IL-1 and IL-6) infiltration of neutrophils, B-cells, T-cells and macrophages and production of reactive oxygen species (ROS).[8] In hyperglycaemic states, cytokine production is unbalanced and this further contributes to chronic inflammation.[9]

The effects of hyperglycaemia on peripheral T-cells still remains unclear and findings to date are controversial.[4,10] Previous studies have reported that T-cells express higher levels of activation antigens such as Human Leukocyte Antigen – antigen D Related (HLA-DR)[11] and that hyperglycaemia may in turn stimulate T-cells to release pro-inflammatory cytokines. Further reports have demonstrated that $CD4^+$ T-cells become activated and prime macrophages to stimulate T-helper (Th) pro-inflammatory subsets (Th1 and Th17).[10] In contrast however, others suggest that hyperglycaemia inhibits T-cell activation by disrupting calcium transduction major histocompatibility complex (MHC) signalling.[12]

Numerous cell surface antigens have been utilised to investigate levels of T-cell activation and include CD25, CD69, CD70, CD38 and HLA-DR.[13-16]

HLA-DR is a well-known marker of immune activation due to its increased expression on activated leukocytes.[17,18] It is a MHC class II cell surface receptor encoded by the human leukocyte antigen complex and binds to the T-cell receptor during T-cell activation.[19] CD38 on the other hand, is an immunoregulator ectoenzyme which catalyses the synthesis and hydrolysis of cyclic adenosine diphosphate-ribose (cADPR) and is crucial to T-cell activation.[20] The combination of CD38 and HLA-DR as activation markers has therefore been used in several studies of inflammatory conditions.[18,21-23]

A disruption in the balance of T-cell activation and inhibition could lead to T-cell exhaustion. This can occur when there is a deficiency of co-stimulatory molecules or the up-regulation of negative regulators such as PD-1 and Fas.[16,24-27] Programmed cell death 1 (CD279) is an immunoreceptor belonging to the CD28/CTLA-4 family that delivers a negative signal upon interacting with its ligands.[28,29] The PD-1 receptor is expressed on activated T lymphocytes and is an indication of ongoing cell

activation.[30,31] Fas (CD95 or APO-1) is a member of the tumour necrosis factor (TNF) family and can stimulate apoptotic cell death.[32,33] Thus, the upregulation of CD95 and PD-1 expression on activated T-cells could inhibit the immune response and may lead to T-cell exhaustion[18,26] and T-cell dysfunction.[3,34]

The primary aim of this study was to comparatively investigate the expression of T-cell activation and exhaustion markers in a sample of South African individuals from the Western Cape with and without hyperglycaemia. A secondary objective was to investigate the effects of high concentrations of glucose on T-cell antigen expression.

MATERIALS AND METHODS

Ethical approval of the study

This investigation is based on the Bellville South study[5] from Cape Town that has been approved by the Research Ethics Committee (REC) of the Cape Peninsula University of Technology (CPUT) and Stellenbosch University (NHREC:REC-230 408-014 and N14/01/003, respectively). For this sub-study, ethical approval was also obtained from the CPUT Health and Wellness Sciences REC (CPUT/HW-REC 2015/H26) and was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent after all procedures were explained in the language of their choice.

Study design and procedures

This was a cross-sectional study with participants being recruited from the ongoing Cape Town Vascular and Metabolic Health (VMH) study.[5] Participants were eligible if they had undergone an overnight fast and were not on anti-inflammatory drugs for a minimum of 14 days prior to sampling. In addition, participants were excluded if they had clinical signs of recent infection, were pregnant or using immunosuppressant drugs. Data collection was based on a standardised questionnaire, which included questions regarding lifestyle and was available on a password-protected personal digital assistant. Physical examination involved body weight (to the nearest 0.1 kg) measured using an Omron body fat meter HBF-511 digital bathroom scale, height to the nearest centimetre, measured with a stadiometer and BMI which was calculated as weight per square meter (kg/m^2). Waist circumference was measured at the level of the narrowest part of the torso, as seen from the anterior view. All anthropometric measurements were performed three times and their average used for analysis.

Biochemical assays

A fasting and 2-hour blood sample was obtained after a 75g oral glucose tolerance test (OGTT) in participants with no history of doctor diagnosed diabetes mellitus. Routine biochemical parameters were analysed at an ISO 15189 accredited laboratory (PathCare, Reference Laboratory, Cape Town, South Africa). Blood glucose (mmol/L) was measured using the enzymatic hexokinase method (Beckman AU, Beckman Coulter). Insulin (mmol/L) was determined by a paramagnetic particle chemiluminescence assay (Beckman DXI, Beckman Coulter). High-density lipoprotein cholesterol (HDL-C) (mmol/L) was measured by enzymatic immune inhibition – End Point (Beckman AU, Beckman Coulter). Low-density lipoprotein cholesterol (LDL-C) (mmol/L) was measured by enzymatic selective protection – End Point (Beckman AU, Beckman Coulter). Triglycerides (TG) (mmol/L) were estimated by glycerol phosphate oxidase-peroxidase, End Point (Beckman AU, Beckman Coulter) and glycated haemoglobin (HbA1c) was measured by High Perfomance Liquid Chromotography (HPLC) (Biorad Variant Turbo, BioRad, South Africa). Ultra-sensitive C-reactive protein (U-CRP) was by Latex Particle immunoturbidimetric and serum cotinine was measured by Competitive Chemiluminescence on the Immulite 2000 (Siemens, Southern Africa). Full blood counts (FBC's) and platelets were measured on a Coulter LH 750 hematology analyser (Beckman Coulter).

History, fasting glucose, and a 2-hour glucose following OGTT were used to group participants for glucose tolerance status as normotolerant, pre-diabetes (including impaired fasting glycaemia, impaired glucose tolerance or the combination of both), screen-detected diabetes, and known diabetes, following the WHO criteria.[35] Individuals classified as diabetes mellitus (DM), impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) were included in the hyperglycaemic group.

Flow cytometry

A volume of 2-3ml of venous blood was collected into 4.5ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose, CA). Measurements of T-cell activation were performed pre- and post-glucose incubation using fresh blood samples stained within 1-2 hours of collection and analysed immediately. A multi-colour panel of antibodies was set up to measure the levels of activation and exhaustion markers on T-cells and included: CD3-PC7 (clone IM2469), CD4-PE (clone 13B8.2), CD8-APC (clone B9.11), CD95-FITC (clone UB2), (Beckman Coulter, Miami, USA), HLA-DR-Alexa Fluor 700 (clone L243), PD-1-APC (clone EH 12.2H7) (BioLegends, San Diego, USA) and CD38-PerCP-Cy5.5 (clone HIT2) (Becton Dickinson,USA). The cells were stained as previously described[14] and were processed immediately using the Navios cytometer and analysed with the Kaluza V 1.3 software (Beckman Coulter, USA) using the gating strategy demonstrated in Figure 1. Daily cleaning of the flow cell fluidics was performed using Isoflo and Clenz® (Beckman Coulter, USA). For daily monitoring of instrument performance, flow set beads and flow check pro beads (Beckman Coulter, USA) were used. Antibody titrations were performed to determine optimal antibody concentrations and colour compensation was performed using the versa comp antibody capture beads (Beckman Coulter, USA).

Incubation with high concentrations of glucose

A sub-group of 31 metabolically similar participants (16 hyperglycaemic and 15 normoglycaemic) were randomly selected and 50µl of whole blood was incubated with 30mmol/l glucose (AlphaPharm, South Africa) mimicking hyperglycaemia respectively.[36] The whole blood/glucose mixture was incubated at 37°C for 45 minutes and thereafter stained and analysed using flow cytometry as previously described. The results were then compared to the sample which had not been incubated.

Statistics Analysis

Statistical analysis was performed using Statistica statistical software version 13. Diego CA, USA). The Mann-Whitney U-test and Student's t-test were used to compare quantitative variables

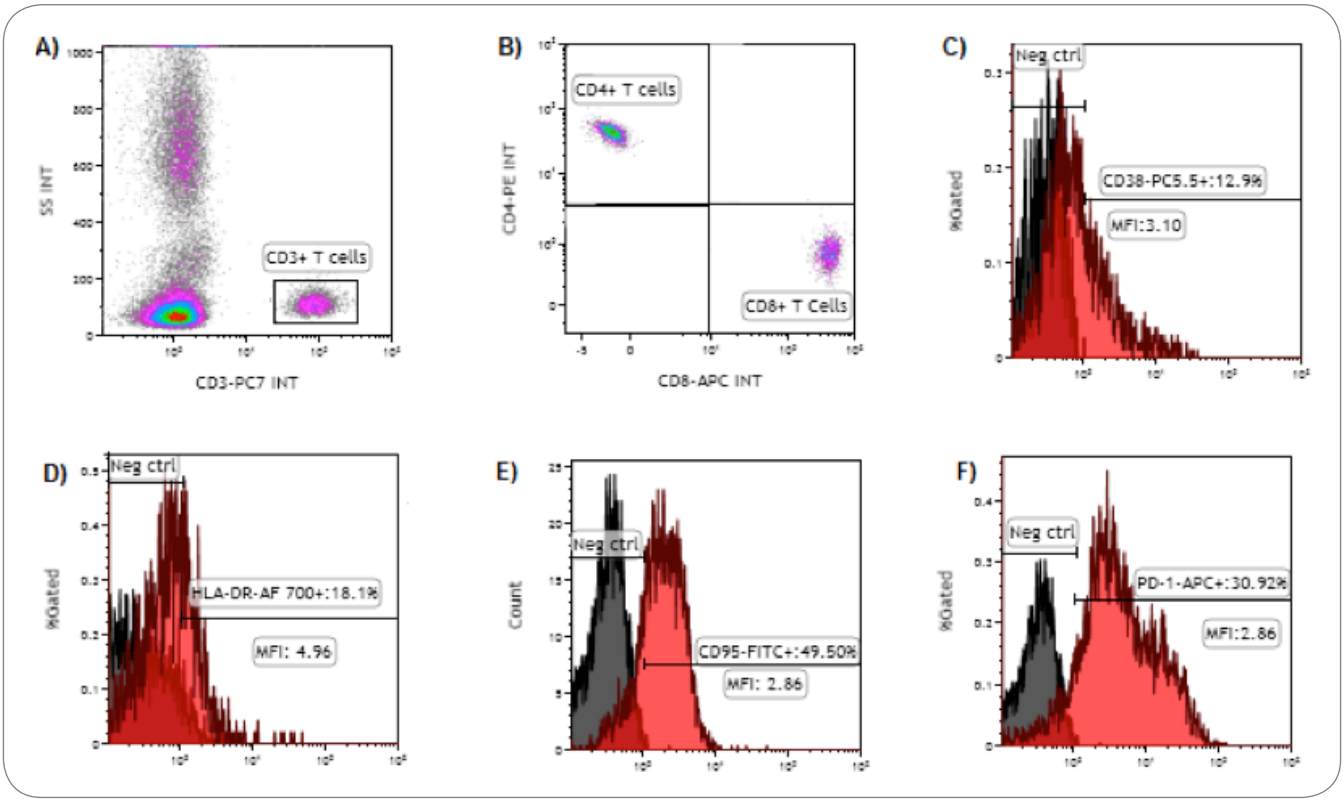

Figure 1: The figure illustrates the gating strategy used to discriminate T lymphocytes based on size and internal complexity. The colour dot plot **(A)** illustrates the discrimination of T-cells from other haemopoietic cells using CD3-PC7 and side scatter (SS). **(B)** Using the primary gate the $CD3^+$ T-cells were subdivided into $CD4^+$ and $CD8^+$ subsets. Plots **(C)-(F)** illustrates the use of histograms to measure the percentage of T-cells expressing CD38-PerCP-Cy5.5, HLA-DR-Alexa Fluor 700, CD95-FITC and PD-1-APC respectively, on the T-cells. A fluorescent minus one (FMO) negative control was used to differentiate between positive and negative events.

and values were reported as median and 25^{th} - 75^{th} percentiles or mean and standard deviation. Spearman rank correlations were used to assess the continuous association between quantitative variables. A p-value of <0.05 represents statistical significance.

RESULTS

General characteristics of the participants

The study comprised of 69 participants (86% being women) which included 35 (51%) with normal glucose tolerance and 34 (49%) with hyperglycaemia (pre-diabetes and screen-detected diabetes). The overall median (25^{th}-75^{th} percentile) age was 56 (47-62) years and 30.5 (26.2-34.8)kg/m^2 for BMI, and 97.5 (88.5-106.8)cm for waist circumference. These measurements were similar between the two groups. However, as expected the measures of glucose homeostasis were significantly different between the groups, all p≤0.009 (Table1).

Activation antigen expression on circulating T-lymphocytes

The median percentage and Mean Fluorescent Intensity (MFI) of T-cells expressing the apoptotic antigen CD95, and activation antigens CD38 and HLA-DR was not significantly different between the hyperglycaemic and normoglycaemic participants at baseline (pre-incubation with glucose) (all p≥0.05). A similar pattern was observed when the exhaustion antigen PD-1 was analysed (p=0.884).

T-cell antigen expression post glucose incubation

Thirty one, metabolically similar individuals, of the sixty nine samples were incubated with 30mmol/L glucose. Although there was no difference in the expression of CD38, CD95 and PD-1 before incubation, between the two groups, the percentage of $CD4^+$ T-cells expressing HLA-DR was lower in the hyperglycaemic group 4.2% (range: 2.4-5.3) compared to 7.4% (range: 7.3-8), in the normoglycaemic group, p=0.025. After incubation, there was a significant decrease in the percentage of $CD4^+$ T-cells expressing CD95 in the hyperglycaemic group from baseline 49.3% (range: 44.1-55.8) to post-incubation 41.73% (range: 29.3-53.8), p=0.035. Asimilar pattern was observed in the normoglycaemic group (Figure 2). There was no difference in the expression of CD95 (p.0.05), CD38 (p>0.05) and PD-1 (p>0.05) on all other T-cell subsets.

Correlation between T-cell markers, glucose metabolism and inflammation

Although no significant correlation could be detected between the expression of T-cell activation antigens and fasting or 2-hour glucose, a significant association was found between the percentage of $CD3^+$ and $CD4^+$ T-cells expressing CD95 and CRP and fasting insulin.

DISCUSSION

The results of this study demonstrated that the percentage of T-cells expressing activation antigens was increased and that there was no difference between normoglycaemic and hyperglycaemic participants. However, after exposure to high glucose concentrations the expression of CD95 on $CD4^+$ T-cells decreased in both groups. Activation antigen expression did not correlate with markers of glucose metabolism, but was associated with the inflammation marker CRP.

Table 1: Biochemical and general characteristics of participants

	Total, N69	Normal, N35	Hyper-Glycaemia, N34	
	Median (P25-75)			P-value
Age (years)	56.0 (47.0-62.0)	53.0 (40.0-61.5)	56.0 (52.0-62.0)	0.057
Body mass index (kg/m^2)	30.5 (26.2-34.8)	29.2 (24.4-34.5)	31.3 (29.3-34.9)	0.104
Waist circumference (cm)	97.5 (88.5-106.8)	94.0 (80.3-102.8)	101.0 (92.8-105.0)	0.087
Fasting capillary Glucose (mmol/L)	5.40 (5.00-5.90)	5.10 (4.65-5.40)	5.90 (5.40-6.20)	<0.0001
2-hour glucose 2 (mmol/L)	7.55 (5.80-9.20)	5.85 (4.85-7.00)	9.25 (8.40-11.00)	<0.0001
Glycated haemoglobin (HbA1c) (%)	6.00 (5.70-6.40)	5.75 (5.40-6.05)	6.30 (6.00-6.60)	<0.0001
Fasting insulin (mIU/L)	8.30 (5.70-12.00)	6.55 (4.10-9.35)	9.95 (7.20-16.30)	0.009
Triglycerides (mmol/L)	1.34 (1.06-2.11)	1.14 (0.95-1.56)	1.63 (1.28-2.36)	0.137
Measured LDL cholesterol (mmol/L)	3.80 (3.20-4.50)	3.65 (3.20-4.55)	3.95 (3.20-4.50)	0.959
Cholesterol HDL (mmol/L)	1.25 (1.10-1.50)	1.20 (1.10-1.50)	1.40 (1.10-1.50)	0.640
Total cholesterol (mmol/L)	5.75 (4.90-6.60)	5.45 (5.00-6.50)	5.80 (4.90-6.60)	0.940
Ultrasensitive C-reactive protein (mg/L)	5.21 (2.89-11.38)	4.84 (2.49-6.83)	8.72 (3.37-18.36)	0.176
Cotinine (ng/mL)	10.0 (10.0-241.0)	10.0 (10.0-224.0)	10.0 (10.0-248.0)	0.643
Red Cell Count (x10E12/L)	4.69 (4.37-5.00)	4.61 (4.19-4.90)	4.76 (4.54-5.01)	0.262
Haematocrit (L/L)	0.41 (0.38-0.44)	0.41 (0.38-0.43)	0.41 (0.41-0.44)	0.221
Haemoglobin (g/dL)	13.2 (12.5-14.1)	12.9 (12.4-14.0)	13.6 (12.9-14.3)	0.206
Mean cell volume (fl)	88.0 (83.0-92.0)	88.0 (83.0-92.0)	87.5 (84.0-92.0)	0.830
Mean cell haemoglobin (pg)	28.5 (27.0-30.0)	29.0 (26.0-30.0)	28.0 (27.0-30.0)	0.798
Mean corpuscular haemoglobin concentration (g/dL)	33.0 (32.0-33.0)	32.0 (32.0-33.0)	33.0 (32.0-33.0)	0.705
Red cell distribution width (%)	14.3 (13.5-15.0)	14.4 (13.5-15.0)	14.2 (13.5-15.0)	0.376
White Cell Count (x10E9/L)	7.20 (6.00-8.80)	6.60 (5.60-7.90)	7.50 (6.50-9.40)	0.144
Lymphocytes %	31.4 (25.4-39.0)	30.7 (25.5-40.0)	31.5 (25.4-39.0)	0.902
Lymphocytes ABS (x10E9/L)	2.30 (1.80-2.80)	2.20 (1.80-2.55)	2.35 (1.90-3.00)	0.111
Monocytes %	5.90 (4.70-7.20)	6.05 (5.05-7.60)	5.30 (4.50-7.10)	0.189
Monocytes ABS (x10E9/L)	0.40 (0.37-0.50)	0.40 (0.36-0.50)	0.40 (0.37-0.50)	0.697
Neutrophils %	59.1 (51.5-66.6)	58.9 (50.2-65.6)	59.3 (51.8-68.0)	0.730
Neutrophils ABS (x10E9/L)	4.35 (3.00-5.70)	3.95 (2.85-4.90)	4.75 (3.30-6.00)	0.234
Basophils %	0.40 (-0.30-0.50)	0.40 (0.30-0.50)	0.40 (0.30-0.50)	0.558
Basophils ABS (x10E9/L)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.01 (0.01-0.01)	0.136
Eosinophils %	1.95 (1.20-3.30)	2.15 (1.55-3.80)	1.65 (1.20-2.80)	0.264
Eosinophils ABS (x10E9/L)	0.18 (0.10-0.20)	0.20 (0.10-0.20)	0.10 (0.10-0.20)	0.603
Platelet Count (x10E9/L)	262 (229-319)	264 (229-308)	259 (226-327)	0.981

LDL – Low density lipoprotein cholesterol

The strengths of this study were that efforts were made to avoid artefactual T-cell activation by minimising the time between sample collection and analysis. The major limitation was that the number of participants studied was low and therefore we were unable to group participants across all glucose tolerance groups.

Previous researchers have shown significant differences in the expression of T-cell activation antigens between normoglycaemic and hyperglycaemic individuals. One study was conducted on newly diagnosed and known diabetics with myocardial infarction using random glucose levels to classify the participants. The authors demonstrated significantly increased percentages of T-cells expressing HLA-DR together with elevated levels of U-CRP in hyperglycaemic patients.[11] Similarly, other researchers investigating the expression of PD-1 (an inhibitor of T-cell activation) demonstrated increased positivity in association with diabetic complications.[27,37] In contrast however, others have reported no differences in the expression of CD95 between diabetics and normal controls.[38]

We found no correlation/s between T-cell antigen expression and glucose metabolism. This difference could be attributed to the method used to classify hyperglycaemia and the severity of complications. In our study, participants were all assessed using the OGTT and classified according to WHO guidelines.[35]

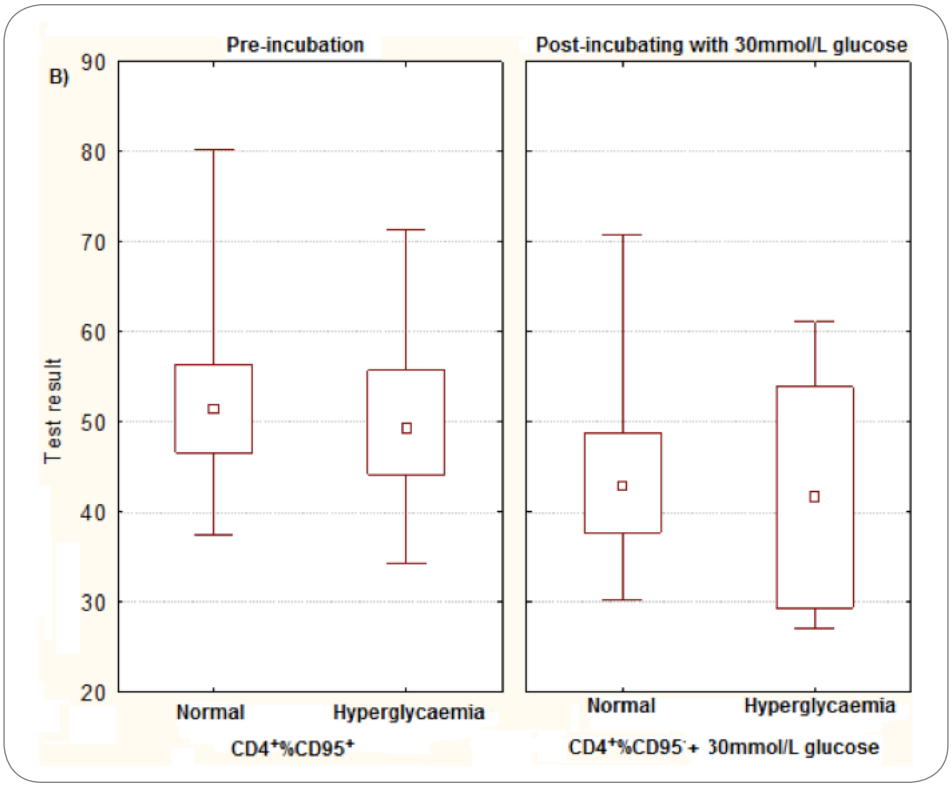

Figure 2: The percentage of CD4+ T-cells expressing CD95 in normoglycaemia and hyperglycaemia. The median % of CD4+ T-cells expressing CD95 was significantly decreased in both normoglycaemic and hyperglycaemic participants after incubation with 30 mmol/L glucose, p=values 0.0213 and 0.0349 respectively.

None had complications such as cardiovascular disease and therefore, differing selection criteria may have significantly contributed to the differences in the findings.

Obesity is a major cause of chronic inflammation.[7] In this study both groups of participants had increased BMI, with the majority satisfying all the criteria of obesity. This could further explain the similar expression of T-cell activation antigens between the two groups and is supported by the high levels of CRP in both the normoglycaemic and hyperglycaemic participants. However, no significant correlation between the BMI and T-cell activation could be detected. Chronic inflammation in obesity is initiated by adipocytes becoming hypertrophic in an effort to accommodate excess triglycerides.[39] They then undergo apoptosis and trigger production of pro-inflammatory cytokines leading to chronic inflammation with the consequent activation of T-cells.[2] Our findings support the fact that obesity as well as hyperglycaemia could be the important drivers of chronic inflammation. Most probably due to low participant numbers no correlation between T-cell activation and BMI was observed.

The incubation of cells with high concentrations of glucose resulted in significantly decreased expression of CD95 on CD4+ T-cells in both groups. This observation seems to suggest that increased glucose may inhibit CD4+ T-cell activation and induce a state of immune exhaustion. CD4+ T-helper cells play an important role in the adaptive immune response and evidence suggests that they play a role in the pathogenesis of T2DM and the initiation of low grade chronic inflammation.[40] The role of CD4+ T-cells in obesity and insulin resistance has also been described. Obesity increases MHC class II expression on adipocytes, thereby activating CD4+ cells and initiating tissue inflammation.[41] In addition, pro-inflammatory CD4+ subsets (Th1 and Th17) release cytokines and this further contributes to the pro inflammatory environment.[42] The expression of CD95 could be important as it plays a role in cellular glucose transportation, by inhibiting the affinity of the glucose transporter 1 (GLUT1) which also initiates activation-induced cell death.[43] The exact effects of decreased expression of CD95 is still unknown and more studies are needed to investigate this phenomenon.

In conclusion, this study suggests that in this cohort of participants, there is no difference in the expression of T-cell activation antigens between hyperglycaemic and normoglycaemic individuals and those other factors such as obesity may be important. High concentrations of glucose appear to suppress the expression of CD95 on CD4+ T-cells and further investigations are required to investigate this further. These findings may be important as increased levels of immune activation and inflammation have been associated with the development of thrombotic plaques and cardiovascular complications and further longitudinal studies that include long term diabetics with complications are recommended.

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