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Changes in Mitochondrial Protein Expression in Human CD4 + T cells During the Initiation and Progression of Diabetes

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During the Initiation and Progression of Diabetes

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Abstract

Introduction:

   Chronic sterile inflammation that underlies obesity can promote unhealthy cellular changes eventually leading to a decline in metabolic health and type-2 diabetes. Dysregulation of cellular processes during the initiation and progression of obesity can impair inflammatory homeostasis leading to a proinflammatory Th17 cytokine profile by $CD4^+$Tcells. Our ongoing research efforts are to understand the mechanistic link between the changes in a cellular housekeeping process known as autophagy and the regulation of Th17 cytokines in obese non diabetic and diabetic subjects.

Methods:

   We assessed cellular protein expression and colocalization using immunoblotting, immunofluorescence, flow cytometry and ELISA assays.

Results:

   We observed that diabetic obese subjects had lower autophagy ($p < 0.05$), mitochondrial fission ($p < 0.05$) and lower expression of antioxidant proteins such as nicotinamide transhydrogenase and super oxide dismutase 2(numerical reduction). The diabetic subjects had numerically higher mitochondrial fusion protein expression, significantly higher mitochondrial mass and mitochondrial colocalization with lysosomal protein LAMP1($p < 0.05$). No difference was observed in cytokines IL17A and IL-17F between the groups as assessed via intracellular staining.

Conclusion:

   The preliminary data presented in this study shows that $CD4^+$ T cells from obese diabetic subjects have significantly higher number of fused mitochondria along with lower mitochondrial antioxidant protein expression. While we observed that the cellular recycling process of autophagy is lower in the diabetic subjects the mitochondrial colocalization with lysosomal protein appears to be intact. Further experimental analysis is required to understand this discrepancy.

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Introduction

Metabolic diseases such as type 2 diabetes (T2D) and obesity are characterized by mitochondrial dysfunction, reactive oxygen generation, higher pro-inflammatory cytokine production and activation of numerous detrimental cellular signaling pathways. Perturbations in cellular metabolism and intracellular signaling can dysregulate inflammatory homeostasis. Pro-inflammatory cytokines, such as those produced by a subset of T cells known as Th17s, i.e IL-17A, IL-17F, IL-21, are produced at higher concentrations in peripheral blood mononuclear cells (PBMCs) from subjects with type 2 diabetes (T2D)(Jagannathan-Bogdan et al., 2011) (Ip et al., 2016). This Th17 signature has not been identified in mouse models of T2D or aging, suggesting that it is a distinctly human trait Our work to identify mechanisms underlying the Th17 profile showed that a partial profile was produced by cells treated *in vitro* with the dietary fatty acid palmitate, a nutrient present at higher circulating concentrations in subjects that are obese(McCambridge et al., 2019) (Boden and Shulman, 2002; Boden, 2008). The partial recapitulation of the profile by palmitate could mimic the physiology somewhere along the continuum of progression from obesity to obesity-associated T2D, a speculation that would require substantial additional investigation.

Dysfunctional mitochondria are often associated with cellular dysfunction in many cell types (Calabriso et al., 2018). Mitochondria are predominant contributors of cellular reactive oxygen species (ROS) which in turn regulates the many signaling pathways. The first report that mitochondria produce ROS came in 1966 and later Chance et al 1971 showed that isolated

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mitochondria produce hydrogen peroxide. Normal physiological levels of ROS is necessary for cellular health as ROS serves as a signaling molecule. However exaggerated ROS has been associated with metabolic and age associated pathologies. Over exuberant, damaged and dysfunctional mitochondria that produce exaggerated   Excess ROS generating organelles are cleared from the cell by the cellular clean up and recycling process of autophagy(Strzyz, 2018). Damaged, dysfunctional and excess organelles are sequestered in a double membrane structure known as autophagosome. The autophagosome then fuses with the lysosome and the contents are degraded and recycled by lysosomal enzymes. Dysregulation of autophagy would result in the accumulation of excess, damaged and dysfunctional mitochondria that generated more ROS. The cellular milieu becomes toxic if the antioxidant mechanism of the cell is unable to neutralize the ROS in concert with autophagy which removes the excess and dysfunctional ROS generating mitochondria. Exaggerated ROS is linked to aberrant effector function of T cells and generation of proinflammatory cytokines especially the Th17 cytokines that are associated with diabetes (unpublished data)(Zhi et al., 2012). Our objective was to access the expression of mitochondrial proteins, mitochondrial turn over (mitophagy) and antioxidant protein expression in α CD3/ α CD28 stimulated CD4$^+$ T cells from non-diabetic obese subjects that are generally considered "metabolically healthy obese (MHO)" and diabetic obese subjects.

Materials and Methods

Human Subjects Sample Collection:

Informed consent for all human samples was obtained following Institutional Review Board-approved protocols in accordance with the Declaration of Helsinki at University of

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Kentucky, The Forsyth Institute, and Boston University. Study design was cross-sectional. Obese non diabetic subjects (avg. 39.3 yrs,) and BMI-matched diabetic subjects (avg. 42 yrs) have additional characteristics shown in Table 1. Exclusion criteria were smoking, long-term or recent use of antibiotics or anti-inflammatory medications (i.e. NSAIDs; low-dose aspirin was not an exclusion), auto-immune disease and allergy medications, and pregnancy.

Cellular Analyses:

Fifty milliliters of peripheral blood were collected into acid/citrate/dextrose containing tubes by venous puncture. PBMCs were purified by histopaque 1077 followed by negative selection with $CD4^+$ cell-excluding magnetic beads (Miltenyi, Auburn, CA) for experiments on purified T cells as we published [4]. $CD4^+$ T cells were >93% pure as assessed via flow cytometry. All cells were frozen at -80°C in a Mr. Frosty apparatus (Nalgene). For multi-week storage, cells were moved to -170°C following 1-7 days at -80°C. T cells from BMI-matched ND and T2D subjects were stimulated in vitro for 40 h with αCD3/αCD28 Dynabeads (Thermo Fisher Scientific, 11132D) at 2μL/100k cells in RPMI media with 5mM glucose (euglycemic). The supernatant was stored at -80°C until analyzed in technical duplicate on a BioRad 3D instrument using a Th17 multiplex kit (Miltenyi). Cells were assayed as outlined below.

Autophagy and mitochondrial turnover:

We quantified the formation of LC3 puncta after intracellular labelling using fluorescent antibodies to LC3 II protein (Mizushima et al., 2010). Second, we measured mitochondrial turnover(Kubli and Gustafsson, 2012) we visualized mitophagy through colocalization of TOM20

with LAMP1 using immunofluorescence as detailed below. Third, we visualized mitochondrial fission protein Dynamin related protein 1(Drp1) expression using confocal microscopy. Drp1 is known to regulate autophagy and promote mitochondrial removal by enhancing mitochondrial fission.

Immunohistochemistry/Immunofluorescence:

Activated CD4$^+$ T cells from ND and T2D subjects were plated on coverslips coated with poly-D-lsine in 6-well plates. After 40 h, the cells were briefly centrifuged, washed 3 times with 1X PBS and incubated in 4% paraformaldehyde for 30 min on ice. The coverslips were washed 3X with PBS and 0.1% triton X-100 (PBST), and were blocked for 30 min in 5% BSA/PBST. Antibodies to TOM20 (Abcam, Cambridge, MA) and LAMP1 (Abcam, Cambridge, MA) were added at 1:50 dilution with incubation overnight at 4ºC. The coverslips were rinsed 3X with PBST and incubated with fluorophore-tagged secondary antibodies (TOM20 with anti-mouse Alexa 488 and LAMP1 with anti- rabbit Alexa 680) (Rockland Immunochemicals, Limerick,PA) for 2h at RT. The coverslips were washed 3X with PBST and mounted on glass slides using Fluromount G (Southern Biotech, Birmingham, AL). Cell imaging under a 63X oil immersion lens was performed in a Zeiss confocal microscope. Three cells/field and 3 fields/slide were imaged on N=4 slides and data were analyzed using Image J (Kirber et al., 2007; Vereb et al., 2000).

Immunoblotting:

Immunoblotting was used to quantify protein expression as we published [14,15]. The procedure was modified according to the cell type from which the proteins were extracted. Thirty μl of 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA, US) was added to 1x10^6 cells and incubated on ice for 20 min. Cells were then centrifuged at 13,000 rpm for 20 min and

supernatant was collected. A Bicinchoninic Assay (Thermo Fisher Scientific, Waltham, MA, US) assessed protein concentration. Twenty μg protein was loaded in polyacrylamide gels and electrophoresis was performed at 100 V for 1 h. Transfer of protein to polyvinylidene difluoride (PVDF) membrane was performed at 45 V for 5 h. The membrane was blocked for 30 min at room temperature (RT) in blocking buffer containing 2% bovine serum albumin in TBST followed by overnight incubation at 4°C in the respective primary antibodies. The membrane was washed 3X with 1X TBST and incubated with secondary antibodies for 2h at RT, then imaged. Table 2 lists the antibodies used in this study. All antibodies were used at a dilution of 1:500 except α-actin which was used at 1:10,000. We quantified NNT, MnSOD and OPA1 expression on western blots using Image studio lite (Licor, Lincoln, NE, US) [16].

Statistical analyses:

Data are presented as mean ± standard error of the mean (SEM). Unpaired 2-tailed *t*-tests compared means of two values. Significance was accepted when *p*< 0.05.

Results and Discussion

T cells from obese diabetic subjects had lower autophagy protein expression:

CD4$^+$ T cells were activated for 40 h with α CD3/α CD28 beads. The supernatant was collected after the completion of incubation for cytokine profiling. The cells were prepared for observing protein expression via confocal microscopy. Cells were probed with LC3 antibody that would bind the autophagosomes that are formed upon induction of autophagy and LC3 puncta would increase. Upon activation of CD4$^+$ T cells autophagy induction is expected to occur in order to meet the increased biosynthetic needs of the cell as well as to neutralize the activation induced

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ROS generation. We observed that CD4$^+$ T cells from obese diabetic subjects had lower LC3 puncta formation compared to CD4$^+$ T cells from obese non diabetic subjects (Fig 1 panels A (images) and B(quantification)).

T cells from obese subjects had lower Drp1 and higher OPA1 expression:

The mitochondrial fission protein Drp1 and fusion protein OPA1 regulate the mitochondrial dynamics and function. It is know that mitochondria that are excessively fused are not effectively removed by mitophagy(Twig et al., 2008b). When the cell prepares for removal of damaged, dysfunctional or excess mitochondria, it upregulates the expression of Drp1 resulting in mitochondrial fission (Twig et al., 2008a). The cell also downregulates Opa1 thereby enhancing mitochondrial fission which will enable the mitochondria to be removed by autophagic machinery(Twig et al., 2008b). It is also reported that mitochondrial fission is necessary for effector function of T cells (Schumann et al., 2014). When mitochondrial fission occurs the mitochondrial move to regions of immunological synapses(IS) and position themselves in close proximity to the IS. This results in calcium influx into the cells and the influx is tightly regulated by mitochondria that are positioned close to the IS. Calcium influx into the cell promotes the effector cytokine production. Schumann et al, 2014 showed that in a mouse "knock in" model of TCAIM protein, TCAIM over expression resulted in reduction of Drp1 expression which prevented the movement of mitochondria to the IS causing a decrease in calcium influx and decreased effector cytokine production. We observed that the cells from T2D subjects had significantly lower levels of Drp1 expression (Fig 2 A (image) and B (quantification) and higher levels of OPA1 expression (Fig 2C). Our results show that mitochondrial fission is prevented

whereas the fusion of mitochondria is enhanced. Lower Drp1 expression along with lower autophagy indicates accumulation of fused mitochondria in T2D subjects.

T2D increases T cell mitochondrial accumulation:

Mitochondrial outer membrane protein translocase of outer membrane 20 (TOM20) expression was assessed via confocal microscopy. We observed that T cells from diabetic subjects had higher expression of TOM20 compared to the non-diabetic subjects indicating an increase in mitochondrial mass Fig 3 A and B. Next, we sought to determine if there is a change in the colocalization of TOM20 with the lysosome associated protein 1(LAMP1). An increased colocalization would indicate that mitochondria are in close proximity to the lysosome thereby implicating mitochondrial turnover. Surprisingly, our data showed that increased colocalization of mitochondrial protein TOM20 with LAMP1 occurred in T2D subjects when compared to the ND subjects Fig 4 A and B. Autophagy (LC3 puncta Fig 1A, B) being low but mitochondrial turn over being high is a discrepancy. Although not unusual this needs further investigation. No difference in LAMP1 expression was observed Fig 3 C and D.

T2D lowers antioxidant protein expression:

Since T2D is associated with mitochondrial ROS we assessed the expression of important mitochondrial antioxidants protein such as nicotinamide nucleotide transhydrogenase (NNT) and manganese super oxide dismutase (MnSOD). NNT maintains the levels of NADPH and reduced glutathione (GSH) where as MnSOD detoxifies superoxide formed in the mitochondria. An impaired expression or function of these proteins would result in uncontrolled production of ROS

which will promote cellular dysfunction (McCambridge et al., 2019). We observed that the levels of both NNT (Fig 5A) and MnSOD (Fig 5B) were numerically lower in T2D cells compared to the cells from ND. This experiment was limited by the low N.

Cytokine expression:

We assessed cytokine expression either via intracellular staining and flow cytometry (Fig 6 A and B) or by enzyme linked immunosorbent assay (ELISA- Fig 6 C). We did not observe a significant difference in IL17A or IL-17F production via flow analysis. However ELISA revealed that the non-diabetic obese subjects had higher levels of IL17A/F cytokine compared to the T2D subjects. The result was unexpected as we hypothesized that the T2D subjects would have higher production of the proinflammatory Th17 cytokine. Since the data set is derived from a limited number of subject's further investigations with more subjects is required. According to Schumann et al, lower Drp1 expression and autophagy may blunt effector function of T cells resulting in impaired cytokine production. This might be one conclusion. However, substantial investigation is required to further analyze our data and understand its physiological relevance in the context of obesity and diabetes.
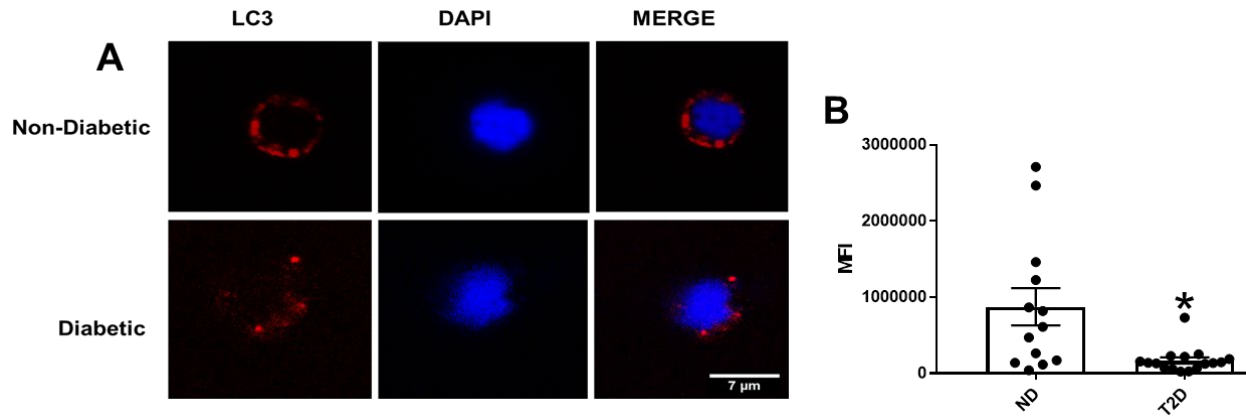
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Figure 1: T cells from diabetic subjects had less LC3 puncta formation. Lower punctuate structures(A) quantification (B) was observed in CD4+T cells from obese diabetic subjects compared to obese normoglycemic subjects A&B n=4, 3 cells/field and 3 fields/slide were imaged using 63X oil immersion in Zeiss microscope. each n represents T cells isolated from one subject. * p<0.05 vs. ND,
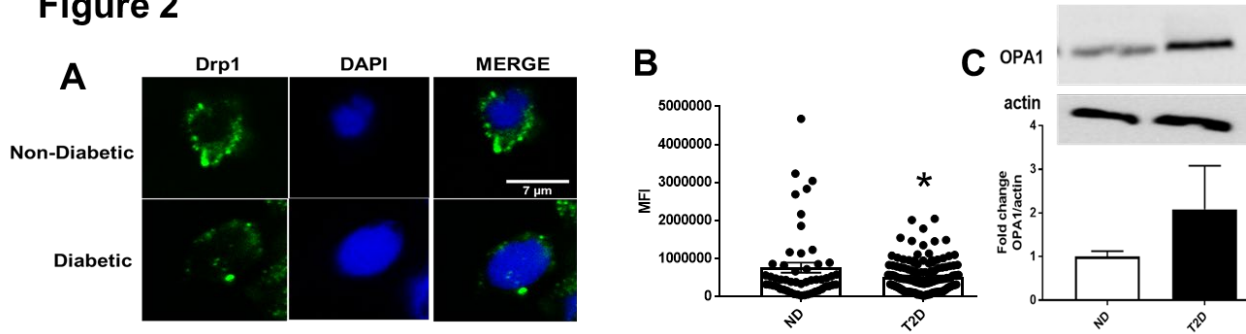

Figure 2: T cells from diabetic subjects had lower Drp1 expression. Lower expression of DRP1 (A) quantification (B) was observed in CD4+T cells from obese diabetic subjects compared to obese normoglycemic subjects. Higher expression of mitochondrial fusion protein OPA1 was observed in T cells from diabetic subjects(C) A&B n=4, 3 cells/field and 3 fields/slide were imaged using 63X oil immersion in Zeiss microscope. C n= 2-3. each n represents T cells isolated from one subject. * p<0.05 vs. ND,
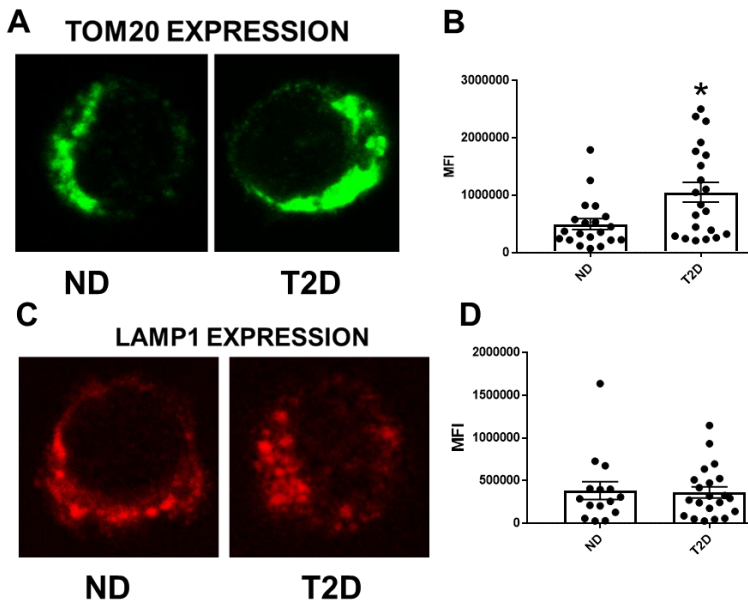
Figure 3

Figure 3: T cells from diabetic subjects had higher mitochondrial mass. Higher expression of TOM 20 (A) quantification (B) was observed in CD4^{+}T cells from obese diabetic subjects compared to obese normoglycemic subjects indicating an increase in mitochondrial mass. No change in lysosomal protein LAMP1 was observed between the two groups (C and D). A-D n=3-4, 3 cells/field and 3 fields/slide were imaged using 63X oil immersion in Zeiss microscope. each n represents T cells isolated from one subject. * $p<0.05$ vs. ND**,**

Figure 4

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Figure 4: T cells from diabetic subjects had more colocalization of mitochondria with the lysosome. Diabetic CD4^{+} T cells had more mitochondrial colocalization with lysosomal protein which may indicate a higher mitochondrial turnover. A (images), B(quantification). A and B n=2-3, 3 cells/field and 3 fields/slide were imaged using 63X oil immersion in Zeiss microscope. each n represents T cells isolated from one subject. * p<0.05 vs. ND,
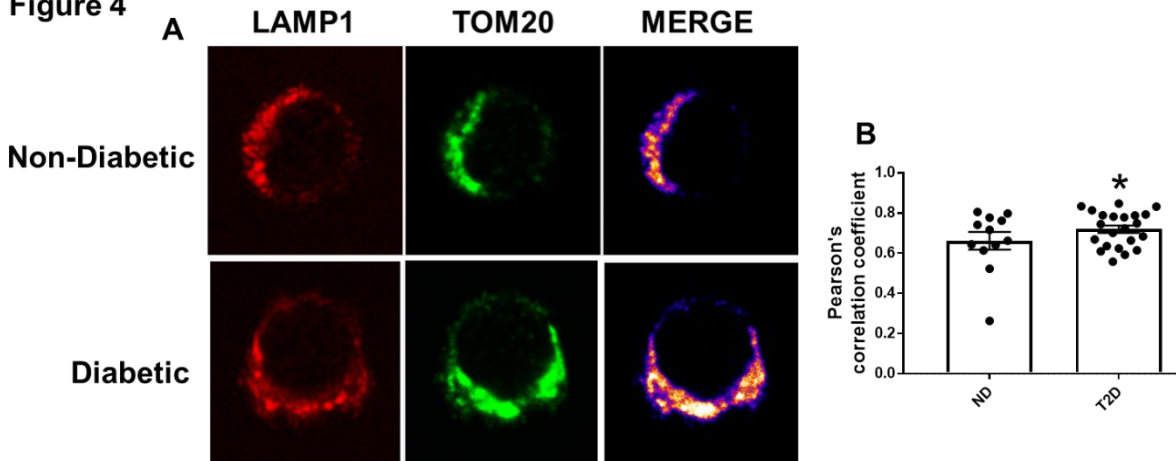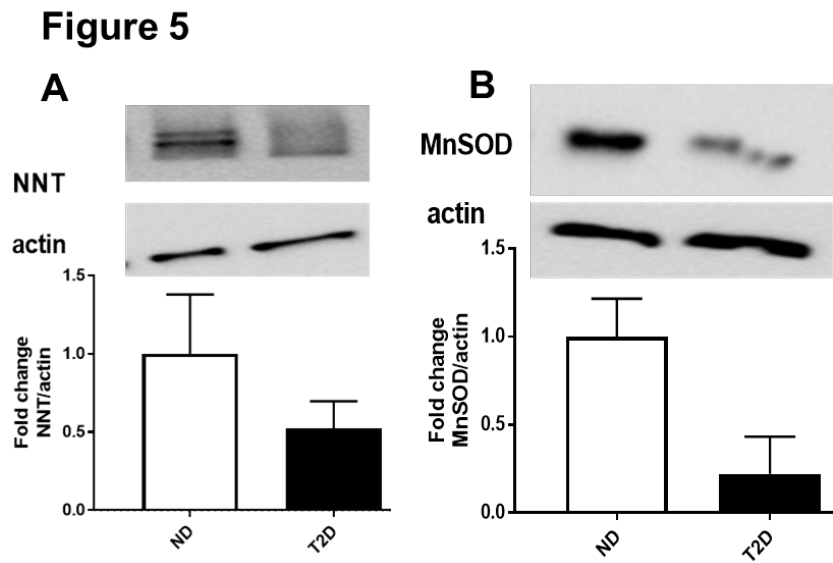

Figure 5: T cells from diabetic subjects had lower expression of mitochondrial antioxidant proteins. Diabetic CD4^{+} T cells had numerically lower expression of mitochondrial antioxidant proteins NNT (A) and MnSOD (B). A and B n=2-3, each n represents T cells isolated from one subject.
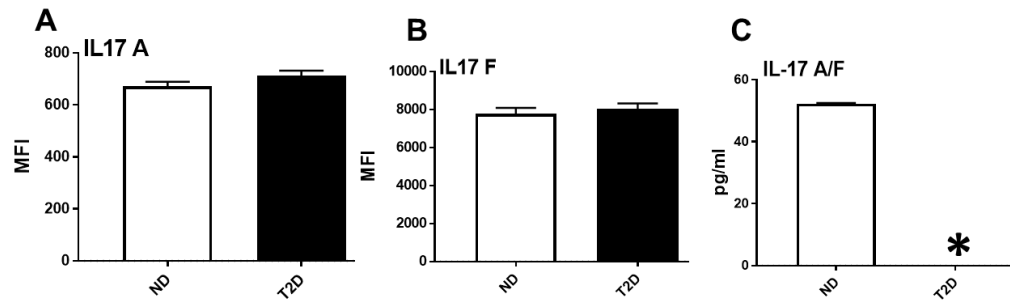
Figure 6

Figure 6: Cytokine expression. No change in Th17 cytokines IL-17A and IL-17F was observed between the groups after intracellular staining and flow cytometry analysis (A and B). However, ELISA assay (C) showed that non-diabetic subjects had significantly higher levels of IL17A/F compared to the diabetic subjects. A and B n=6-8, C n=3. each n represents T cells isolated from one subject. * $p<0.05$ vs. ND,

Table 1. Description of research subjects

	ND	T2D
Total N	6	6
Age, years [mean (range)]	(33-45)	(34-47)
A1c, % [mean (range)]	(4.5-5.6)	(6.2.-11)
BMI, kg m^{-2} [mean (range)]*	(29.1-36.2.)	(30.4-38.2)
Females [N (%)]	4	4
Males [N (%)]	2	2

Table 2. Antibodies used in this study

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All antibodies were used at a dilution of 1:500 except α-actin which was used at 1:10,000.

Protein	Catalog #	Company
LC3	L7543	Sigma Aldrich
TOM20	ab56783	Abcam
LAMP1	ab24170	Abcam
DRP1	146475	Cell signaling technology
OPA1	67589	Cell signaling technology
B-Actin	3700	Cell signaling technology
NNT	ab110352	Abcam
MnSOD	sc-137254	Santa Cruz Biotechnology

Signaling events upon T cell activation a brief overview:

CD4$^+$ T cells are a type of lymphocyte that plays a major role in the body's immune response by secretion of cytokines. T cell activation that results from the presenting of cognate antigen by antigen presenting cells such as dendritic cells and macrophages is shown to create profound changes in cellular process such as metabolism and effector function. Activation causes CD4$^+$ T cells to differentiate into different subtypes; Th1 cells, Th2 cells, Th17 cells, and regulatory T cells (Tregs). There are three main signaling pathways that happen with activation; PI3K-AkT, mTORC, and MAPK pathways. The activation of the kinases according to the signal received determines if the cells would differentiate into pro or anti-inflammatory effectors.

It is known that T cells upon activation prefers aerobic glycolysis for ATP generation. During glycolysis glucose is converted to lactate instead of undergoing oxidative phosphorylation. Although the yield of ATP via glycolysis is significantly less than the ATP generated via

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oxidative phosphorylation, cells prefer glycolysis upon activation for generation of redox balance

regulators such as glutathione. The redox regulators are critical to neutralize activation induced

ROS generation and for preserving cellular health. CD4^{+} T cells produce cytokines, unlike naïve

or memory cells. Naïve and memory cells are metabolically different from the effector cells and

prefer mitochondrial oxidative phosphorylation while effector T cells prefer glycolysis due to the

need for rapid biosynthesis and for antioxidant defenses.

T cell activation requires engagement of the T-cell receptor (TCR) and costimulation

through CD28 (Wahl et al., 2012). CD28 signaling activates the kinase Akt which results in

translocation of GLUT1 transporter to the T cell membrane which is followed by glucose uptake

and glycolysis. Akt-mTOR signaling can also promote glycolysis through various mechanisms

such as phosphorylating GLUT1, modifying hexokinase; and transcriptionally regulating

metabolism (Revu et al., 2018). CD28 costimulation, acting through phosphatidylinositol 3-

kinase (P13K) and Akt, is required to increase glycolytic rate in T cells (Clavijo and Frauwirth,

2012). CD28 can amplify signals and activate kinase cascades like Akt and mTOR, which leads

to activation and proliferation of the T cell.

TCR signaling is known to promote activation of pyruvate dehydrogenase kinase 1

(PDHK1) (Revu et al., 2018). This pathway activation inhibits mitochondrial import of pyruvate,

facilitating the breakdown into lactate (Revu et al., 2018). Another major signaling pathway is

mTOR, which interrupts the catabolic metabolism and mitochondrial oxidative phosphorylation

as well as induces transcription of glycolytic enzymes to increase glycolysis. Akt activates

mTOR signaling to promote protein synthesis of ATP citrate lyase to promote lipid synthesis

(Ron-Harel et al., 2015). MTOR activation stimulates the activity of transcription factor Myc as

well as HIF-1α that enables the expression of enzymes of glycolytic pathways, thereby

regulating the balance between pro and anti-inflammatory differentiation. Myc is associated with

the upregulation of several glycolytic enzymes including GLUT1, hexokinase,

phosphofructokinase, and lactate dehydrogenase (Wahl et al., 2012). Hexokinase is further

induced by Erk after TCR activation.

STAT5, Erk, and MAPKinases (MAPK) are other signaling pathways that may regulate cell

metabolism depending on the condition. Activation of p38 MAPK requires dual phosphorylation

at Thr180 and Tyr182 and can be mediated by two different pathways in T cells. Recruitment of

linker for activation of T cells (LAT) and the activation of guanine nucleotide exchange factors

(GEFs) is essential for activation of MAPK (Dodeller and Schulze-Koops, 2006). MAPK pathway

results in regulation of cytokines expression such as TNF, IL-2, IL-4, IL-5 and IL-13. The

activation of p38 MAPK is necessary for the expression of IL-10 and of the T helper type 2 (Th2)

cytokines 1L-4, IL-6, and IL-10, but not for that of the Th1 cytokines IFN-y(Dodeller and Schulze-

Koops, 2006). CD4 T cells maintain their ability to proliferate and secrete cytokines at low levels

of glucose (Wahl et al., 2012). (Seki and Gaultier, 2017) Glycolytic enzymes (Seki & Gaultier,

2017). The glycolysis enzymes that play major roles in metabolic inflammation are Hexokinase II,

Glyceraldehyde 3-phospahte dehydrogenase, α-Enolase, Pyruvate Kinase Isoform M2, and

Lactate Dehydrogenase A. Hexokinase II (HK-II) catalyzes the phosphorylation of glucose to

glucose 6-phosphate (G6P). HK-II appears to be closely linked to activation of inflammatory

pathways in immune cells. AKT stabilizes the localization of HK-II to the outer mitochondrial

membrane where HK-II has more access to mitochondria ATP. Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) catalyzes conversion of glyceraldehyde 3-phosphate to 1, 3-

bisphosphoglycerate. GAPDH is an energy sensor that regulates translation of inflammatory cytokine mRNA in response to the availability of glucose in the cell. α-Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP) in glycolysis. Pyruvate Kinase (PK) Isoform M2 is the ATP-generating enzyme that catalyzes the conversion of PEP to pyruvate during glycolysis. Lactate dehydrogenase is a tetrameric enzyme compose of A and B subunits that, when combined, form a complex with the capability of converting pyruvate to lactate, which is the defining step of glycolysis. This enzyme is also critical for the production of the inflammation-promotingcytokineIFN-γ. (Seki and Gaultier, 2017)

Mitochondrial antioxidant proteins:

NNT-

Nicotinamide nucleotide transhydrogenase (NNT) is an integral protein of the inner mitochondrial membrane. This protein plays a role in the production of NADPH, which is involved in removing reactive oxygen species (ROS) that could damage the membrane and its function. NNT catalyzes the reduction of $NADP^+$ by NADH and the conversion of NADH to NAD^+· We saw that low levels of NNT also caused a reduction in antioxidant expression such as NADPH. We found circulatory free fatty acids resulted in decreased function and expression of NNT, a key redox regulator, which lead to a redox imbalance and higher ROS levels.

MnSOD-

Manganese superoxide dismutase (MnSOD) is the essential mitochondrial antioxidant enzyme that detoxifies superoxide, a free radical. Its dismutase function is fully activated in the

mitochondria to detoxify free radical O2 and is upregulated by oxidative stress (Candas & Li,

2014). This enzyme constitutes an important control switch in the process of activation-induced

oxidative signal generation in T cells(Kaminski et al., 2012). Kaminski et al showed the

association between T cell receptor (TCR)-triggered ROS production and higher MnSOD

abundance/activity and a shut-down phase of oxidative signal generation (Kaminski et al., 2012).

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