Novel insights into the contribution of human Bcl-2 protein Noxa to the metabolism and differentiation of CD4+ T cells

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By

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Abstract: The human Noxa protein was originally identified as a pro-apoptotic member of the large Bcl-2 family of apoptosis regulators but, more recently, rediscovered as a protein that can also promote growth and survival through regulation of glucose and glutamine metabolism in T leukemia cells. Preliminary studies in primary human T cells had also shown that Noxa protein was induced following TCR engagement and was required for their activation-induced switch to glutaminolysis. The research described here focuses on CD4+ T cells and tests the hypothesis that Noxa serves as a critical sensor of glutamine availability and plays a central role in driving T cell differentiation towards glutamine dependent effector phenotypes (Th1/Th17) and away from regulatory and B cell promoting (Treg /Th2) phenotypes. These studies show that Noxa is induced in co-stimulated CD4+ T cells, that the induction is glutamine-dependent and that Noxa, in turn, is required for entry of glutamine carbons into the mitochondrial TCA cycle. Knocking out Noxa does not affect mitochondrial health but increases dependence of activated knockout cells on fatty acid oxidation. Moreover, inhibiting glutaminase activity had little effect on respiration of these cells, confirming the lack of dependence on glutamine in the absence of Noxa in keeping with a Treg or Th2 phenotype. RNA-seq analysis of Noxa KO CD4+ T cells showed decreased expression of genes related to the Th17 subset of helper T cells. Overall, the results show a unique interdependence between Noxa and glutamine and suggest that this interdependence may influence metabolic choices that drive the differentiation of human CD4+T cells subsets. These studies offer a better understanding of the role of Noxa in T cell metabolism and in the generation of CD4+T cell subsets and could eventually lead to the use of Noxa as a therapeutic tool for strategies targeted at metabolic reprogramming in T cells.

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Chapter 1: Introduction

Role of Noxa in apoptosis and metabolism

Noxa is a BH3 only member of a large family of apoptosis regulator proteins referred to as the Bcl-2 family. Bcl-2 proteins can be classified as pro-apoptotic or anti-apoptotic and are grouped into subfamilies based on the number of shared Bcl-2 Homology (BH) domains (1). BH3-only proteins are generally pro-apoptotic and harbor a single BH3 domain that allows them to interact with pro-survival Bcl-2 family members to promote apoptosis (2,3)

Several years ago, the Kelekar lab demonstrated a novel pro-growth and survival function for human Noxa in hematologic malignancies. They found Noxa was stably expressed and phosphorylated on serine (S)13 in a glucose-dependent manner in T leukemia cells (4). Phospho-Noxa increased glucose uptake and promoted growth, while glucose-deprivation or kinase inhibition triggered the restoration of Noxa's apoptotic function in T-ALL cells (4). This study also demonstrated that Noxa was induced in primary human T cells activated by TCR co-stimulation. A follow-up study in T leukemia cells showed that Noxa over-expression imparted a Warburg-like proliferative phenotype to these cells, facilitating aerobic glycolysis and diversion of glucose for biosynthesis and, concurrently, enhancing glutamine utilization for oxidative phosphorylation (OxPhos) (5).

Based on Noxa's novel pro-survival role, and enhancement of glutamine utilization and its induction in activated human T cells, our lab is interested in understanding how Noxa promotes the growth and expansion of primary, normal human T lymphocytes through metabolic regulation.

Figure 1.1 shows significant induction of Noxa expression in primary human CD3+ T cells isolated from a normal donor and stimulated with anti-CD3/CD28 antibodies for 24 hours.

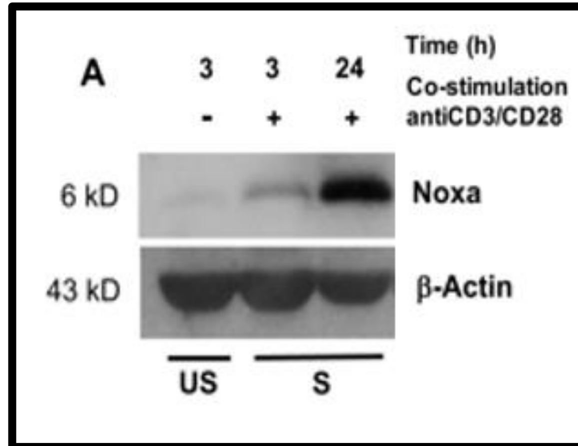


Figure 1.1. Noxa is induced in human T cells following TCR co-stimulation. Human CD3+ T cells were co-stimulated with anti-CD3/CD28 antibodies for the indicated periods of time (US = unstimulated and S = stimulated) Protein expression was analyzed via Western blot (A) Dr. Benson unpublished

The requirement for glutamine and the contribution of Noxa to this requirement during activation of the T cells was then examined. T cells failed to induce Noxa protein expression when co-stimulated in vitro for 24h in the absence of glutamine, but not in the absence of glucose. Since Noxa expression was not detected in the absence of glutamine, the mitochondrial health of the cells was evaluated using a Seahorse Mito Stress Test. When T cells are stimulated in the absence of glutamine, their oxygen consumption rate decreases significantly compared to control cells (Figure 1.2B). This suggested that glutamine was critical for Noxa expression and respiration in T cells.

Since induction of the Noxa protein required the presence of glutamine (Figure 1.2A) it was important to determine whether the switch to glutaminolysis during activation was dependent on Noxa. Noxa siRNA and control siRNA-treated activated T-cells (Figure

1.3A) were incubated with uniformly labeled [U-$^{13}C_5$]-glutamine for 12h and extracted metabolites analyzed by mass spectrometry. Glutamine uptake in activated cells was unaffected in the absence of Noxa, but glutamate-enrichment was severely impaired (Figure 1.3B). Enrichment of subsequent metabolites also decreased correspondingly (not shown). Thus, the first step in glutaminolysis, the conversion of glutamine to glutamate, is significantly impaired in the absence of Noxa protein. Despite the block in glutamate and other glutamine-derived TCA metabolites, however, mitochondrial fitness was not impaired in siNoxa cells (Figure 1.3C), suggesting they could be utilizing an alternate carbon source for OxPhos and energy generation.

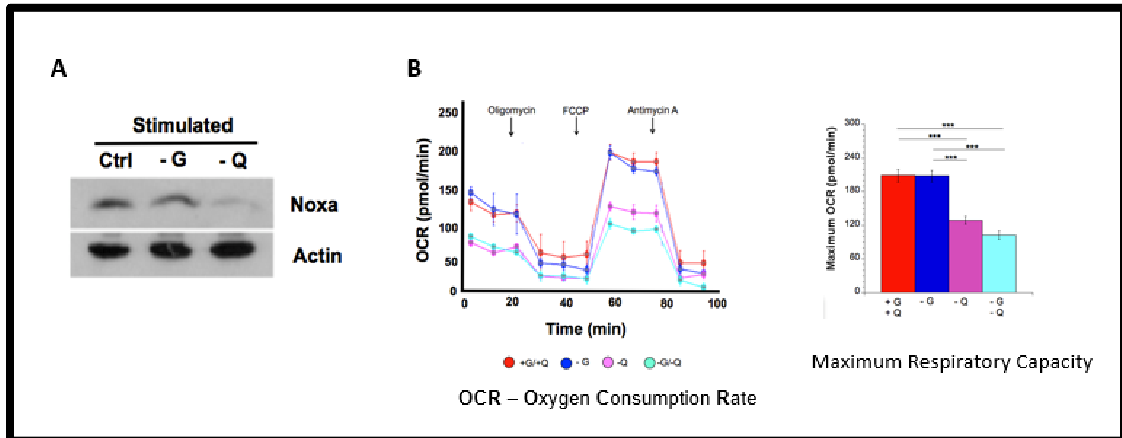


Figure 1.2. Glutamine is required for induction of Noxa protein in CD3+ T cells upon activation. Human T cells were activated with anti-CD3/ CD28 Abs for 24 h in complete medium or in the absence of glucose (--G) or glutamine (--Q). Protein expression was assessed via Western blot. **B. Mitochondrial respiratory capacity of activated T cells impaired in the absence of glutamine.** Oxygen consumption rates, OCR, (left panel) were evaluated in response to treatment with inhibitors oligomycin, FCCP, and antimycin A in a Mito Stress Test of the nutrient-deprived cells shown in A, using a Seahorse assay. The maximum OCR of uncoupled mitochondria is also shown (right panel). Similar results were observed in multiple donors (***$p<0.001$).

The previous studies were all carried out with pan (CD3+) T cells which include CD4+ and CD8+ T cells. However, CD4+ and CD8+ T cells have different immune functions and utilize different metabolic pathways. CD4+ T cells which differentiate into tumor

suppressive effector (Th) or immune-repressive regulatory (Treg) phenotypes with different fuel requirements were of particular interest and were chosen for further investigation.

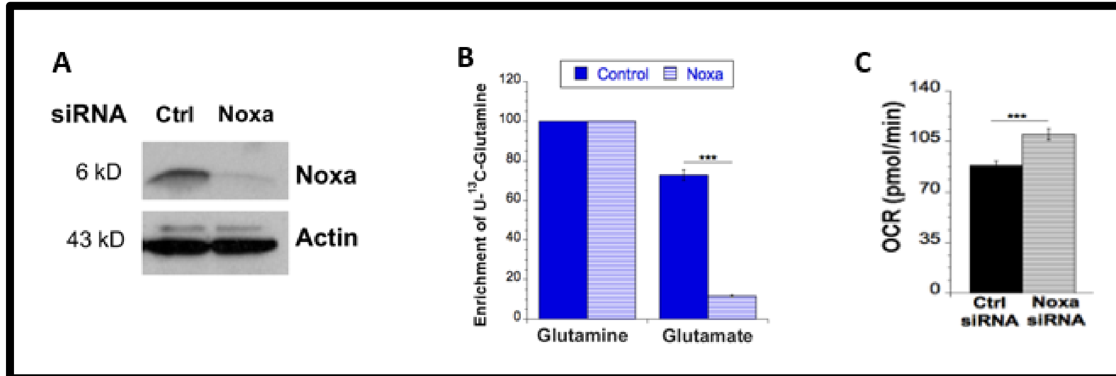


Figure 1.3. A. Noxa is required for an early step in glutaminolysis in T-cells. (A) Western blot showing Noxa protein levels in 12h activated T cells transfected with control or Noxa siRNA (B) LCMS analysis of TCA metabolites. Enrichment of M+5 labeled glutamine and glutamate in derivatized cell extracts of stimulated T cells transfected with control or Noxa siRNA and incubated with medium containing [U-$^{13}C_5$] glutamine for 12 h, n=3, ***p<0.001. **C. Mitochondrial fitness is not impaired in Noxa silenced T cells.** Maximum OCR of uncoupled mitochondria in control and Noxa siRNA cells (from A) is shown.

CD4+ T cell Differentiation and Metabolism

CD4+ T cells start as naïve cells and, after maturation, express a T-cell antigen receptor on the surface of the cell that binds to MHC class I or MHC class II antigens (6). Following binding CD4+ T cells get activated and differentiate into the subsets of helper cells, each with its own function (7). There are three main types of helper T cells: Th1, Th2 and Th17. Each cell subtype is responsible for protection against different pathogens. Th1 cells are responsible for activation of macrophages to kill intracellular pathogens through the production of IFN-γ (8). Th2 cells protect against extracellular pathogens by producing IL-4 and IL-5 and helping B cells (9,10). Th17 cells produce Il-17a and IL-17f and are involved suppression of early inflammation and helping recruit neutrophils to the sites of inflammation (10). An additional subtype of differentiated CD4+ T cell is regulatory T cells (Tregs). Tregs are responsible for suppressing autoimmune reactions within the body

and maintaining self-tolerance (11). Unlike helper T cells that require activation for their differentiation, Tregs mature in the thymus before they are released into the bloodstream and are characterized by their production of TGF-β (10) and expression of the Foxp3 transcription factor (12).

As CD4+ T cells become activated, their metabolic demands switch from energy production via mitochondrial oxidative phosphorylation to biosynthesis via glucose upregulation (13). Glycolysis and glucose uptake are increased to support the rapid growth and proliferation. At the same time, glutamine consumption is increased to support energy production (13). Upon differentiation to Th1 or Th17 cell subtypes, glycolysis is utilized for growth and division (14) while glutamine consumption is increased to generate energy through glutaminolysis and the TCA cycle (15). Unlike Th1 and Th17 cells, Th2 helper cells have been shown to increase lipid metabolism pathways (16).

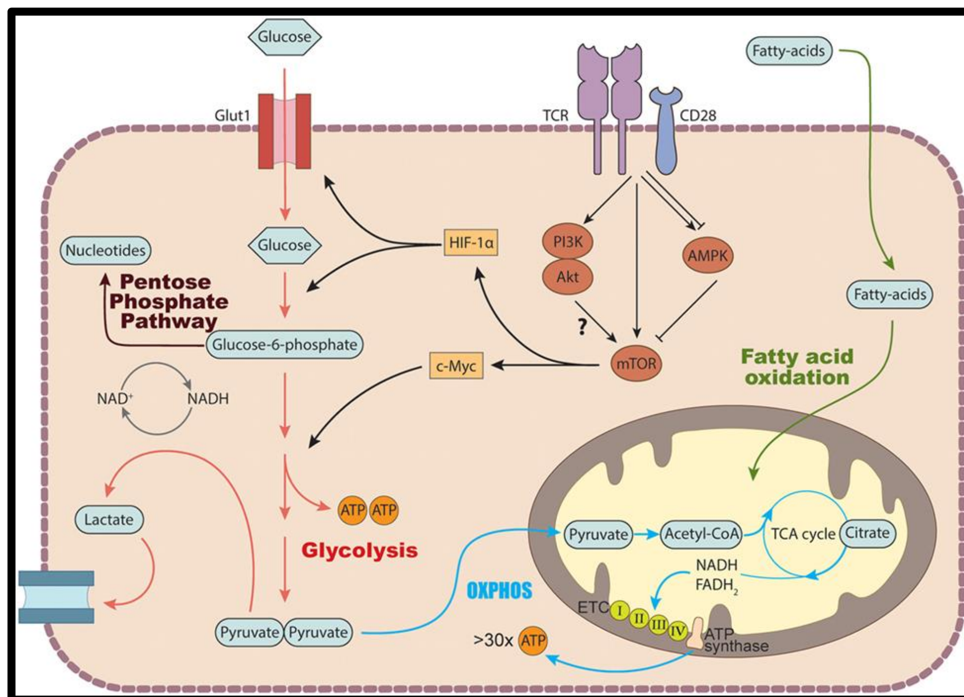


Figure 1.4. Metabolic pathways in T cells. Frontiers in Immunology. Dumitru, C., A. M. Kabat, K. J. Maloy, 2018.

It has also been shown that Th2 cells are not reliant on glutamine for the TCA cycle, but still require some glutamine to thrive although their differentiation is not hindered with decreased glutamine as in the case of Th1 cells (17). Regulatory T cells express the transcription factor Foxp3 which inhibits glycolysis. Tregs reliance on fatty acid oxidation rather than OxPhos suggests their formation is not glutamine dependent in contrast with Noxa expressing cells (18,19).

Based on the requirement of glutamine for Noxa expression (Figure 1.2A) and the impaired glutaminolysis observed when Noxa was silenced (Figure 1.3B), we hypothesize that Noxa functions to increase glutamine consumption and inhibit fatty acid oxidation in activated CD4 T cells, thus promoting their differentiation to OxPhos dominant effector cells (Figure 1.5). When glutaminolysis and glycolysis are downregulated CD4+ T cells switch to fatty acid oxidation (FAO) (20).

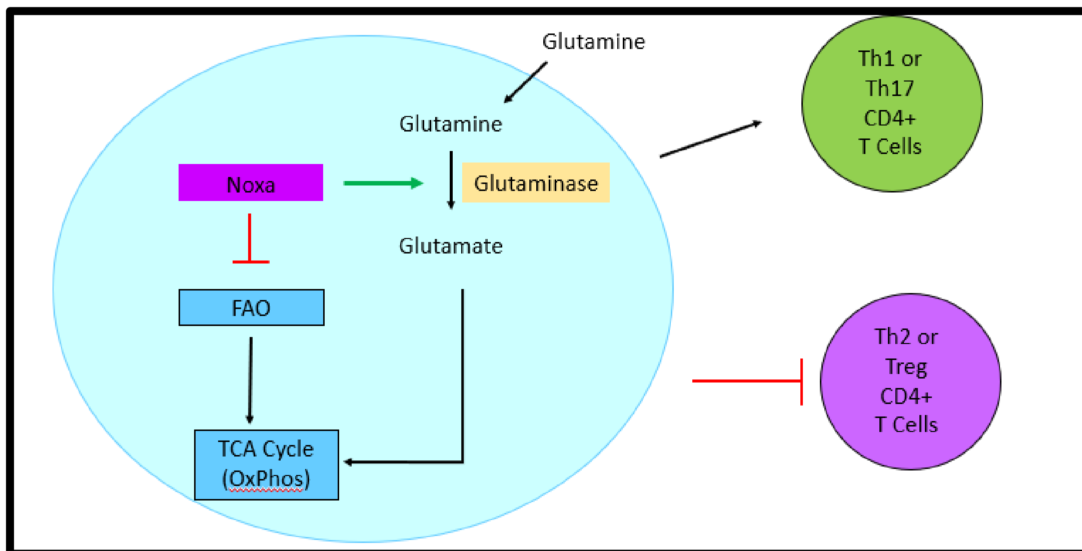


Figure 1.5. Model for Noxa's role in CD4+ T cells. Noxa expression in CD4+ T cells may increase glutamine consumption and glutaminolysis through stimulating glutaminase, while concurrently inhibiting fatty acid oxidation. CD4+ T cells that differentiate in the presence of Noxa will be subtypes that require glutamine for their metabolism, such as Th1 and Th17 cell subtypes. Treg and Th2 cell subtypes may be inhibited due to fatty acid oxidation inhibition.

Thus, my research will test the working hypothesis that Bcl-2 protein, Noxa, increases glutamine consumption and promotes differentiation of CD4+ T cells towards subtypes that require glutamine for their metabolism, such as Th1 and Th17, and away from regulatory T cell and Th2 cell subtypes.

Chapter 2: Results

Rationale: The goal of this study was to investigate the role of human Noxa in the activation and differentiation of human CD4+ T cells. We selected CD4+ T cells as our model for the following reason. The metabolism and fuel requirements of activated CD4+ T cells change as they expand and differentiate into functionally and metabolically distinct subsets – Th1 and Th17 cells rely primarily on glycolysis and glutaminolysis while Tregs and Th2 cells switch to fatty acid oxidation (15,17) and preliminary studies in the lab on pan (CD3+) T cells had suggested that Noxa plays a central role in the activation-induced metabolic switch from glucose to glutamine consumption for energy generation.

Noxa is induced in CD4 T cells upon activation in vitro.

CD4+ T cells were isolated from human peripheral blood mononuclear cells and activated for 48 hours in vitro using plate activation with anti-CD3/CD28 antibodies or using Immunocult™ Human CD3/CD28 T Cell Activator cocktail (StemCell Technologies). Western blot of lysates from activation experiments with two independent donors (Figure 2.1) show that Noxa expression was not detected in unstimulated controls, but significantly induced after the cells were activated. These are just two representative examples; to date, we have consistently observed Noxa induction in activated CD4+ T cells isolated from over ten independent normal human donors.

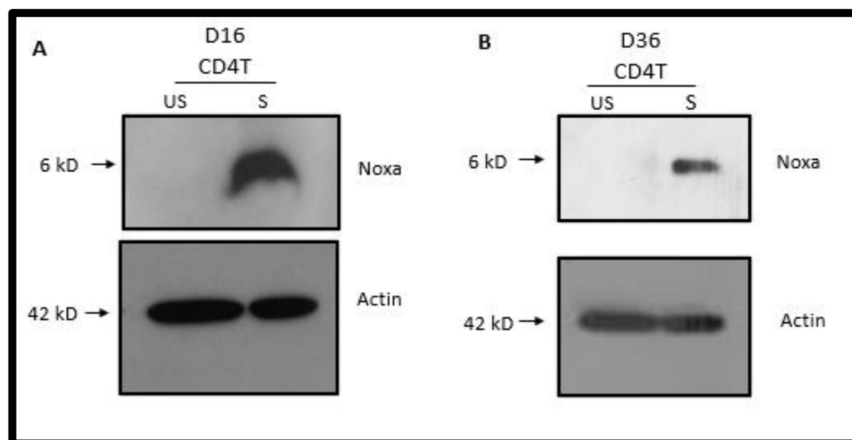


Figure 2.1. Noxa is upregulated upon activation of CD4+ T cells. Western blot of cell lysates harvested 48 hours after activation from two independent human T cell donors (D16 and D36) showing Noxa protein is induced in activated CD4 T cells. US: Unstimulated, S: Stimulated.

Noxa upregulation requires the presence of glutamine in CD4 T cells.

Noxa expression is induced as CD4+ T cells are activated in vitro. We had previously observed in pan (CD3+) T cells that this induction required glutamine (Figure 1.3A) and wanted to test this in CD4+ T cells. Western blots of lysates from CD4+ T cells activated in glutamine-free medium show that Noxa expression is significantly reduced in the absence of glutamine (Figure 2.2A & B) but restored upon re-addition of the amino acid (Figure 2.2B).

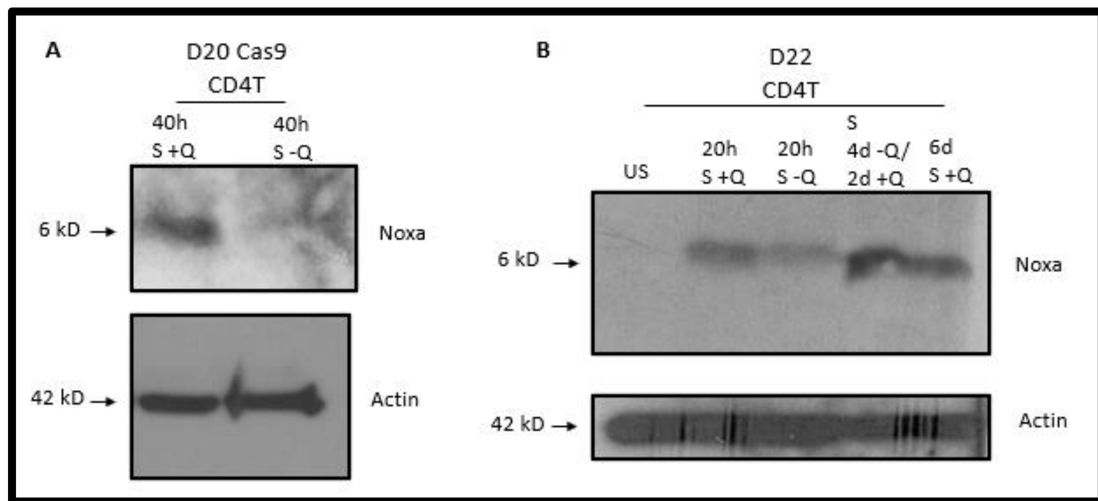


Figure 2.2. Noxa induction requires the presence of glutamine after activation in CD4+ T Cells. A and B Western blot of cell lysates harvested 20 hours or 40 hours after activation from two independent human T cell donors (D20 and D22). In **B)** glutamine (2mM on day 5 of activation) added back to the medium. US: Unstimulated; S+Q: Stimulated with glutamine; S-Q: Stimulated without glutamine.

Generation of *Noxa* KO CD4+T lines using a CRISPR/Cas9 approach

To further investigate the role of Noxa in glutamine metabolism and its contribution of CD4+ T cell differentiation we generated knockout cells using CRISPR/Cas9. Although preliminary studies had used Noxa siRNA, an RNAi approach is not optimal for long term expansion and differentiation studies. Knockouts would not only allow for long term analysis but would allow for comparison and retesting of multiple donor populations for validation experiments. Freshly isolated CD4+ T cells, activated for 48 hours were electroporated with Cas9 mRNA and chemically modified Noxa sgRNA or Cas9 mRNA

alone, allowed to expand and checked for Noxa expression two weeks later following reactivation. Figure 2.3 shows western blots of CD4+ T Noxa knockout (KO) and control populations from seven independent donors.

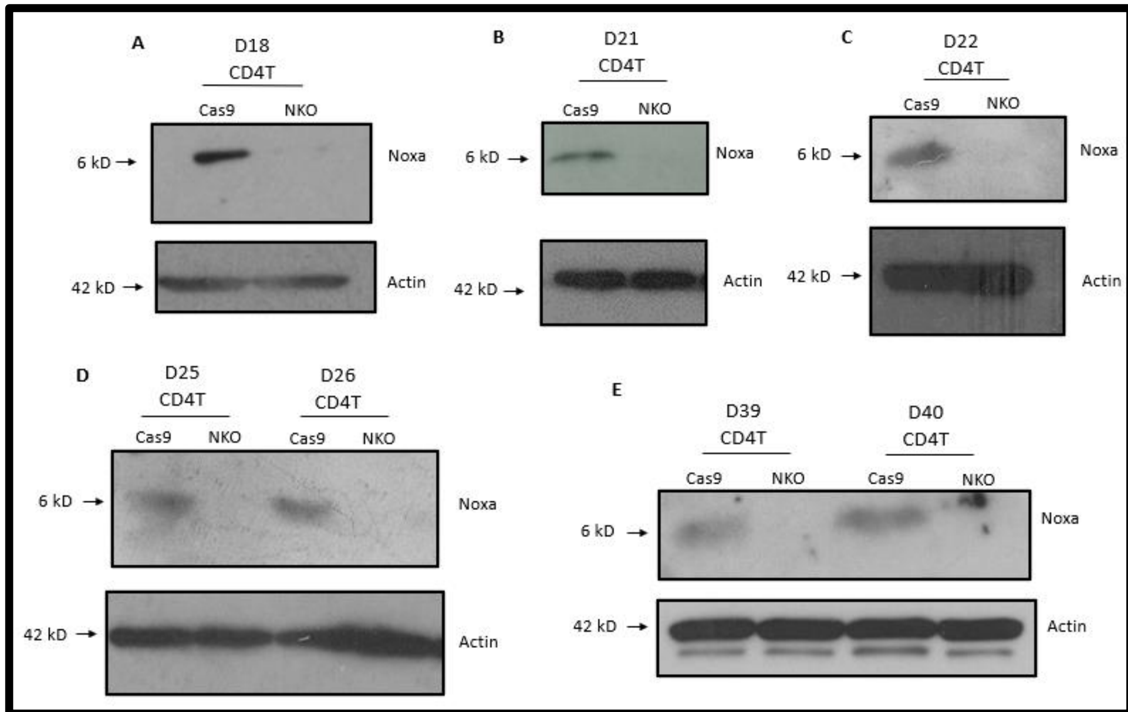


Figure 2.3. Noxa knockout cell lines. Western blot of cell lysates harvested 48 hours after reactivation of control (Cas9) and Noxa knockout (NKO) cells. The transfection occurred two weeks prior to western blotting for Noxa expression.

Foxp3 is not induced in Noxa knockout cell lines.

The next step was to determine whether the knockout cells expressed characteristics of CD4+ T cell subsets. Based on preliminary observations we had hypothesized that Noxa was likely to be poorly expressed or absent in Tregs (Model Fig 1.5). For a positive control experiment, CD4+ T cells isolated from a normal human donor were subject to a Treg polarization protocol. Cells were activated in the presence of TGF-B (5ng) and IL-2 (264IU). Flow cytometric analysis of the cells eight days after polarization, showed that the treated cells were almost 80% Foxp3 positive, which was a significant increase for the iTregs as compared to normal CD4+ T cells (Figure 2.4A). However, after seven days

of activation neither the Noxa KO cells nor the Cas9 controls showed increase levels of Foxp3 (Figure 2.4C). The mean fluorescence intensity (MFI) quantified and expressed as ‘fold change’ in Figure 2.4D, also showed that there was no significant difference in the intensity of Foxp3 expression in either population.

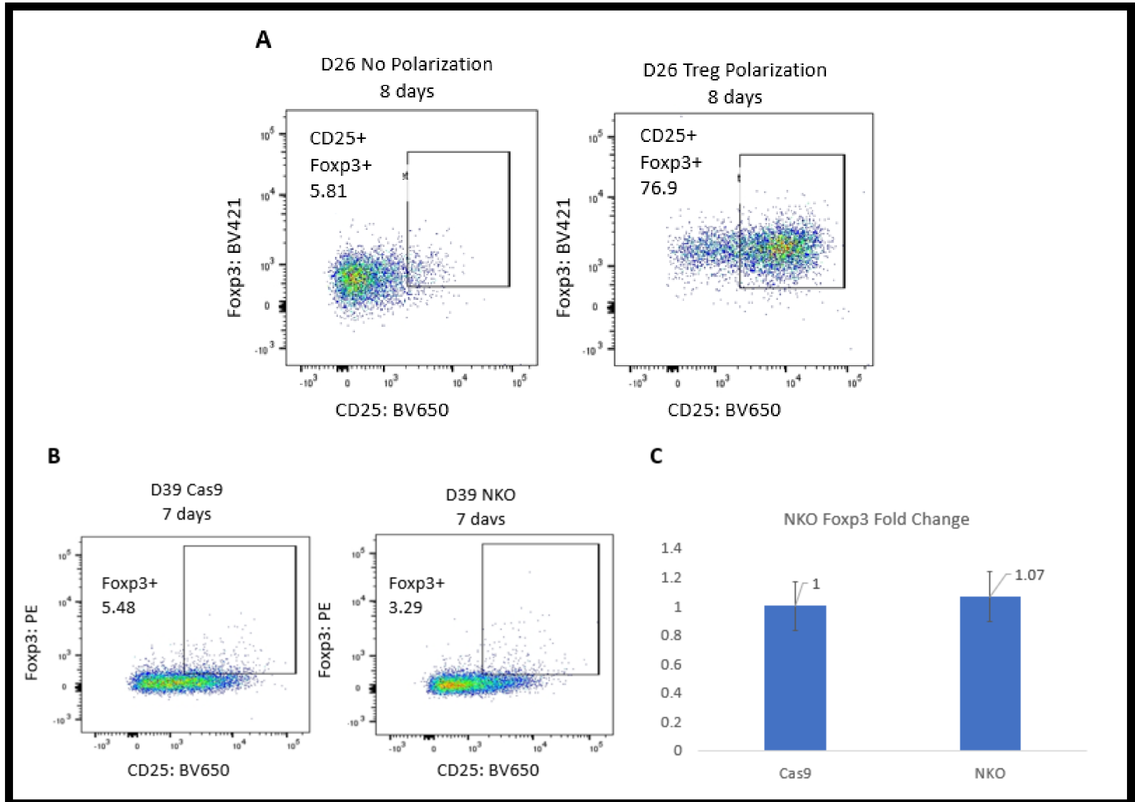


Figure 2.4. Foxp3 is not induced in Noxa KO cells. A) Raw data from FlowJo of CD4+ T cells polarized with TGF-β. B) Raw data from FlowJo of CD4+ Cas9 and Noxa knockout cells. C) Mean fluorescence intensity (MFI), quantified, and expressed as ‘fold change’ from Cas9 and Noxa knockout cells from two independent human CD4+ T cell donors.

Noxa knockout cells show greater reliance on fatty acid oxidation more than their Cas9 counterparts.

In previous studies, silencing Noxa with siRNA impeded glutaminolysis in activated cells and significantly reduced IL-2 production (not shown) without compromising mitochondrial fitness (Figure 1.3C) suggesting mitochondria utilize an alternative fuel source for OxPhos. We hypothesized that Noxa expression inhibits fatty acid oxidation,

and therefore, cells that primarily use fatty acid oxidation (FAO), such as Tregs, did not require Noxa for their metabolism. To determine if Noxa KO cells were using FAO for respiration, activated Cas9 and Noxa KO cells were subjected to a Mito Stress test in the presence and absence of Etomoxir, a FAO inhibitor. Etomoxir (Eto) is a drug that inhibits the carnitine palmitoyltransferase I (CPT-I) enzyme, the rate limiting step for fatty acid oxidation. The cells were co-stimulated in the presence or absence of glutamine for two days prior to the experiment. Eto was added to the cells four hours prior to starting the Mito Stress test. Figure 2.5A shows the oxygen consumption rate (OCR) in the presence of glutamine and Figure 2.5B shows the OCR in the absence of glutamine. The oxygen consumption is used to calculate spare respiratory capacity. The Spare Respiratory Capacity (SRC) is the difference between the maximal respiration and basal respiration.

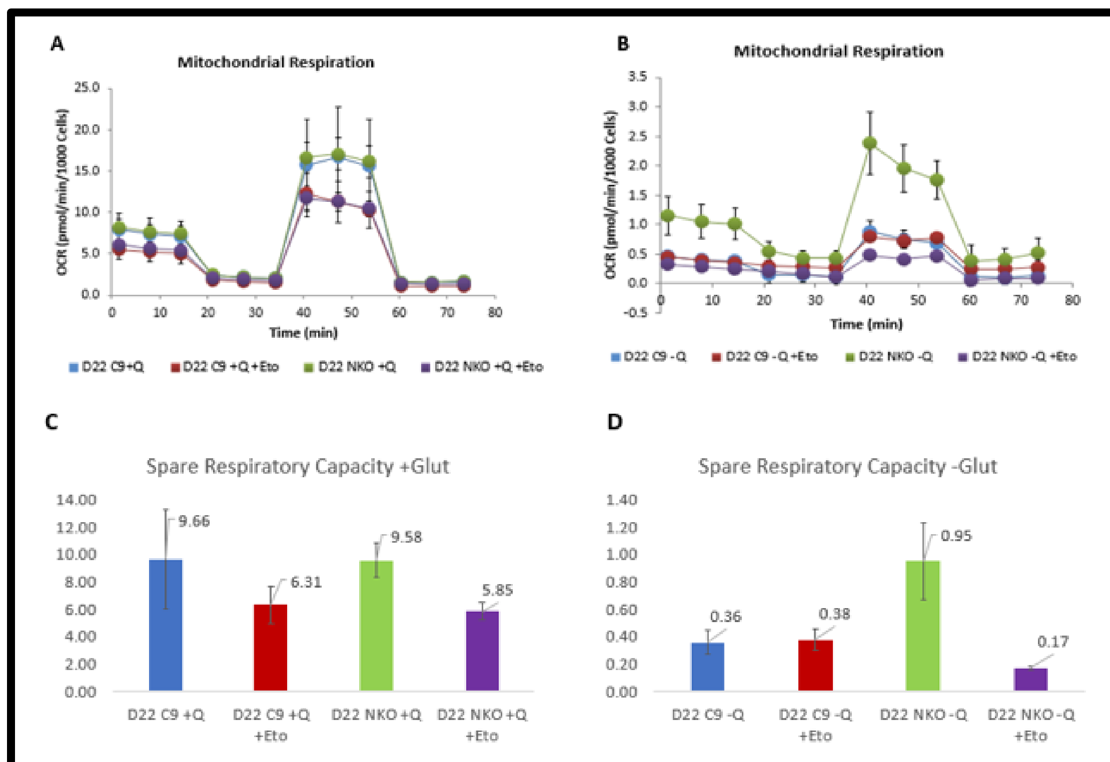


Figure 2.5. The spare respiratory capacity of Noxa knockout cells is diminished in the absence of glutamine and presence of Etomoxir. Oxygen Consumption Rate for Cas9 and Noxa KO cells in the presence of glutamine (A) or absence of glutamine (B). The spare respiratory capacity was calculated in both the presence of glutamine (C) and absence of glutamine (D).

The SRC for the Cas9 cells in the presence of Eto was not significantly affected (Figure 2.5C/D), whereas the SRC for the Noxa KO cells decreased regardless of glutamine availability (Figure 2.5C/D). These results show that Noxa KO cells are more dependent on FAO, supporting our hypothesis that cells without Noxa will primarily use FAO for their metabolism.

Results from the metabolic tracer study using Noxa siRNA showing that the conversion of glutamine to glutamate was severely reduced but mitochondrial fitness was not impaired (Figure 1.3) had suggested that T cells were utilizing an alternate source for energy generation in the absence of Noxa. To test the possibility that T cells lacking Noxa were not dependent on glutamine for the TCA cycle, Cas9 and Noxa KO cells were treated with CB839, a glutaminase inhibitor. CB839 blocks the entry of glutamine carbons into the TCA cycle by blocking the conversion of glutamine to glutamate in the first step of glutaminolysis. Results from a Mito Stress test on Cas9 and Noxa KO cells activated in the presence or absence of glutamine or treated with CB839 in the presence of glutamine for three days are shown in Figure 2.6. The SRC of Cas9 cells diminished significantly in the presence of CB839 suggesting severe impairment of mitochondrial respiratory function in the absence of glutamate and downstream metabolites (Figure 2.6C). Noxa KO cells, on the other hand, showed only a small reduction in maximum OCR and SRC in response to the glutaminase inhibitor, suggesting that mitochondrial fitness was not significantly affected (Figure 2.6D). The severe reduction of OCR in KO cells in the absence of glutamine may be due to an overall reduction in viability and suggests that the glutamine taken up by the cell may be essential to support cell division by contributing directly to *de novo* pyrimidine biosynthesis or phospholipid and nucleic acid biosynthesis (21,22). Taken together the results from Figures 2.5 and 2.6 support

the possibility that Noxa KO cells rely on fatty acid oxidation for mitochondrial respiration and energy generation.

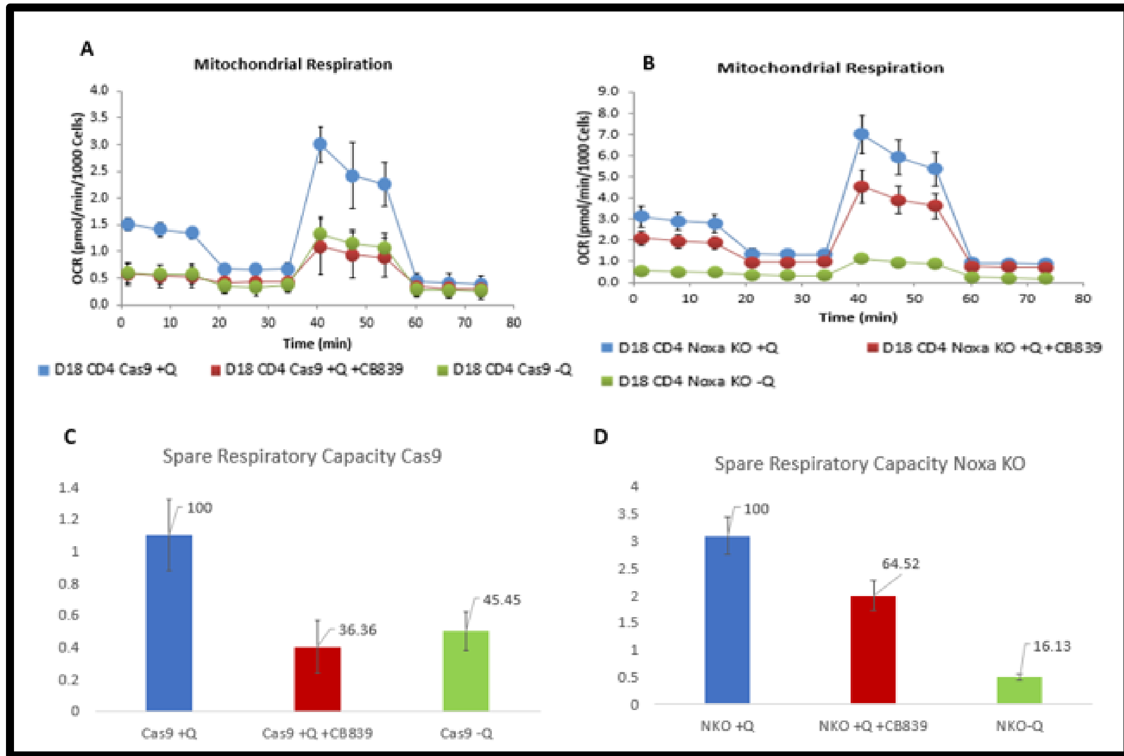


Figure 2.6. Glutaminase inhibition with CB839 did not affect the spare respiratory capacity of Noxa knockout cells as much as Cas9 cells. The oxygen consumption rate (OCR) is shown for Cas9 (A) and Noxa KO (B) cells. When the Cas9 cells were treated with CB839, the OCR drops to a similar rate as when no glutamine is present, but when the Noxa KO cells are treated with CB839 the drop in OCR is not as significant. The spare respiratory capacity is shown for Cas9 (C) and Noxa KO (D) cells. The percentages are calculated as a percent of the control for each group (Cas9 +Q and NKO +Q). The spare respiratory capacity is affected more in the Cas9 cells than the Noxa KO cells when treated with CB839.

Noxa KO cells required glutamine but not for mitochondrial function, the response of Noxa induction was tested with the glutaminase inhibitor, CB839 and a glutamine antagonist, 6-Diazo-5-oxo-L-norleucine (DON). DON competes with glutamine, rendering the amino acid unavailable for all downstream pathways, thus effectively creating a glutamine deprived state (22,23). CD4+ T cells were isolated from a normal donor and activated for thirty-six hours in the presence of DON or CB839 and checked for Noxa expression by western blot (Figure 2.7A/B). Results show that Noxa is induced in CB839 treated

activated cells, but not detected in cells activated in the presence of DON. A Mito Stress test revealed a small decrease in OCR and SRC of CD4+ T cells after treatment with CB839 as compared to untreated cells (Figure 2.7D/E). However, treatment with DON, reduced the SRC to levels comparable to those observed in glutamine deprived cells (Figure 2.7D/E). These results strongly suggest that glutamine is required for Noxa protein induction and that the amino acid is, in turn, dependent on Noxa for its entry into the mitochondrial TCA cycle for ATP synthesis in activated T cells.

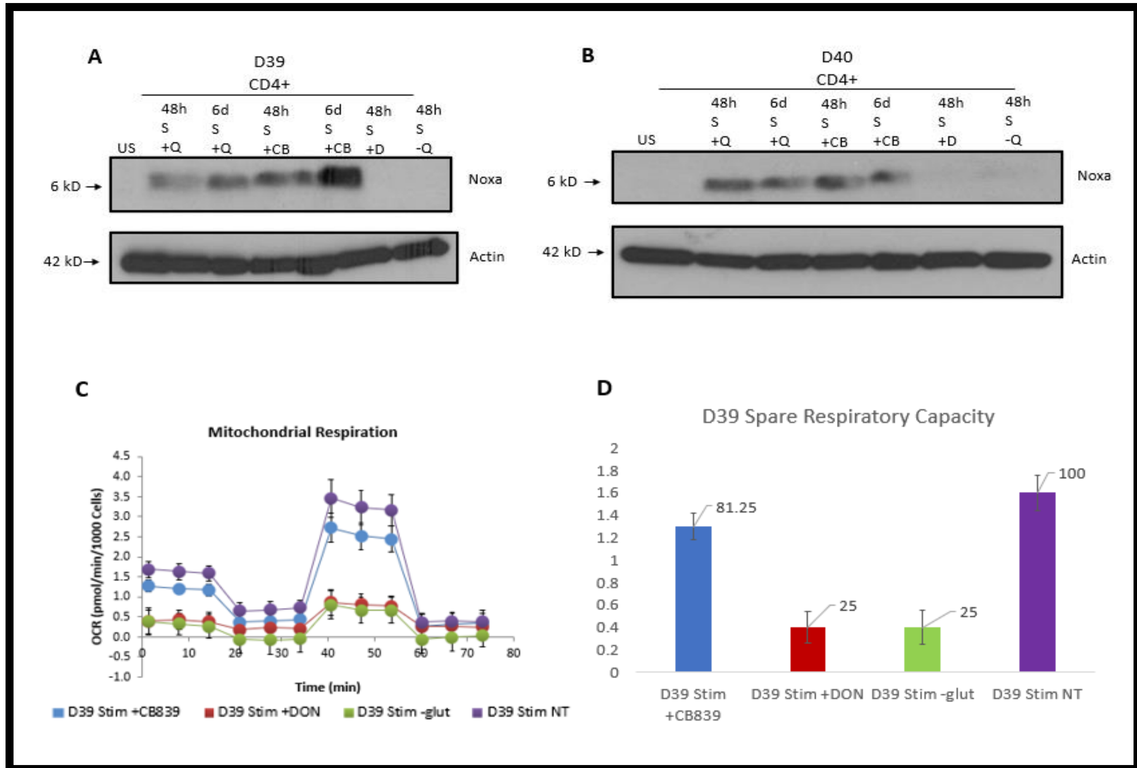


Figure 2.7. Noxa is expressed when treated with CB839 but is not expressed when treated with DON. A and B) Western blot lysates harvested 48 hours or 6 days after treatment with the inhibitors, in two independent donors. US: Unstimulated; S+Q: Stimulated +glutamine; S+CB: Stimulated +CB839; S+D: Stimulated +DON; S-Q: Stimulated -glutamine. C) Oxygen consumption rate of normal CD4+ T cells from a human donor after treatment with CB839 or DON. D) Calculated spare respiratory capacity of CD4+ T cells after treatment with DON or CB839.

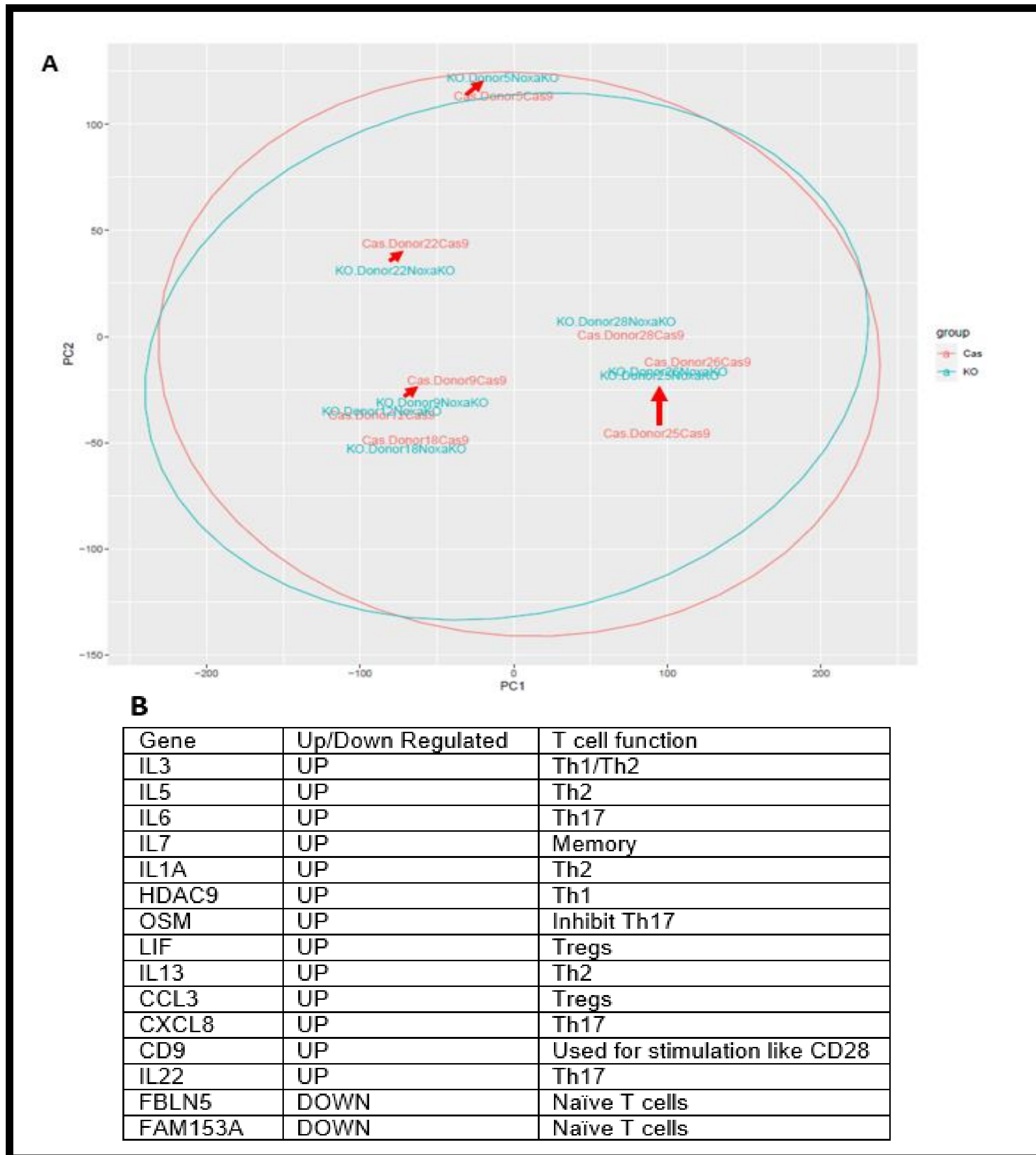
RNA-Seq analysis of eight independent Noxa knockout cell lines.

Given the central role of Noxa in T cell activation and metabolic reprogramming that could affect T cell fate and function, we hypothesize that Noxa expression triggers specific

downstream signaling events and pathways relevant to differentiation. Thus, comparative RNA-Seq analysis of Noxa KO T cells and controls generated from multiple normal donors would be expected to reveal valuable information about Noxa-regulated signaling pathways and T cell subtypes. Generation of CRISPR/Cas9 knockouts of Noxa was described in an earlier section. Metabolic studies suggested that Noxa KO cells showed dependence on FAO and may be similar to regulatory T cells (Figure 2.5) although Foxp3 detection did not yield clear results (Figure 2.4C). A small pilot proteomic study of CD4+ T Noxa KO and control cells from a single donor identified genes related to Tregs in the KO populations. Given this, we predicted that RNA-seq analysis would reveal upregulation of Treg related transcripts in the Noxa KO cells.

Eight CD4+ T cell Cas9/Noxa KO pairs were selected for RNA-seq analysis. Viable cells were recovered by ficoll density gradient centrifugation and stimulated in vitro for 48 hours. Activated cells were harvested for RNA isolation, following the manufacturer's protocol from the Qiagen RNeasy Mini kit, and stored at -80°C until required. A total of 2 µg of RNA was plated in a semi-skirted PCR plat for RNA-seq analysis before transferring to the University of Minnesota Genomics Center (UMGC). After a QC test on the RNA samples to ensure quality, UMGC staff created individual TruSeq stranded mRNA libraries from all sixteen samples, which were then sequences via a NovaSeq Charter Service. The data was then released for further analysis. UMGC also assisted with the initial data analysis. Upon initial review a principal component analysis (PCA) plot was generated, and the four 'best' donors were selected for further analysis based on distance between Cas9 and Noxa KO pairs as indicated by red arrows on the PCA plot (Figure 2.8A). Fifteen differentially expressed genes were shared among these donors, most of which were shared among three donors and only two, IL3 and CXCL8, emerging in all

four donors. As shown in Figure 2.8B, no single T cell subset emerged. Thus, the data were inconclusive.



Gene	Up/Down Regulated	T cell function
IL3	UP	Th1/Th2
IL5	UP	Th2
IL6	UP	Th17
IL7	UP	Memory
IL1A	UP	Th2
HDAC9	UP	Th1
OSM	UP	Inhibit Th17
LIF	UP	Tregs
IL13	UP	Th2
CCL3	UP	Tregs
CXCL8	UP	Th17
CD9	UP	Used for stimulation like CD28
IL22	UP	Th17
FBLN5	DOWN	Naïve T cells
FAM153A	DOWN	Naïve T cells

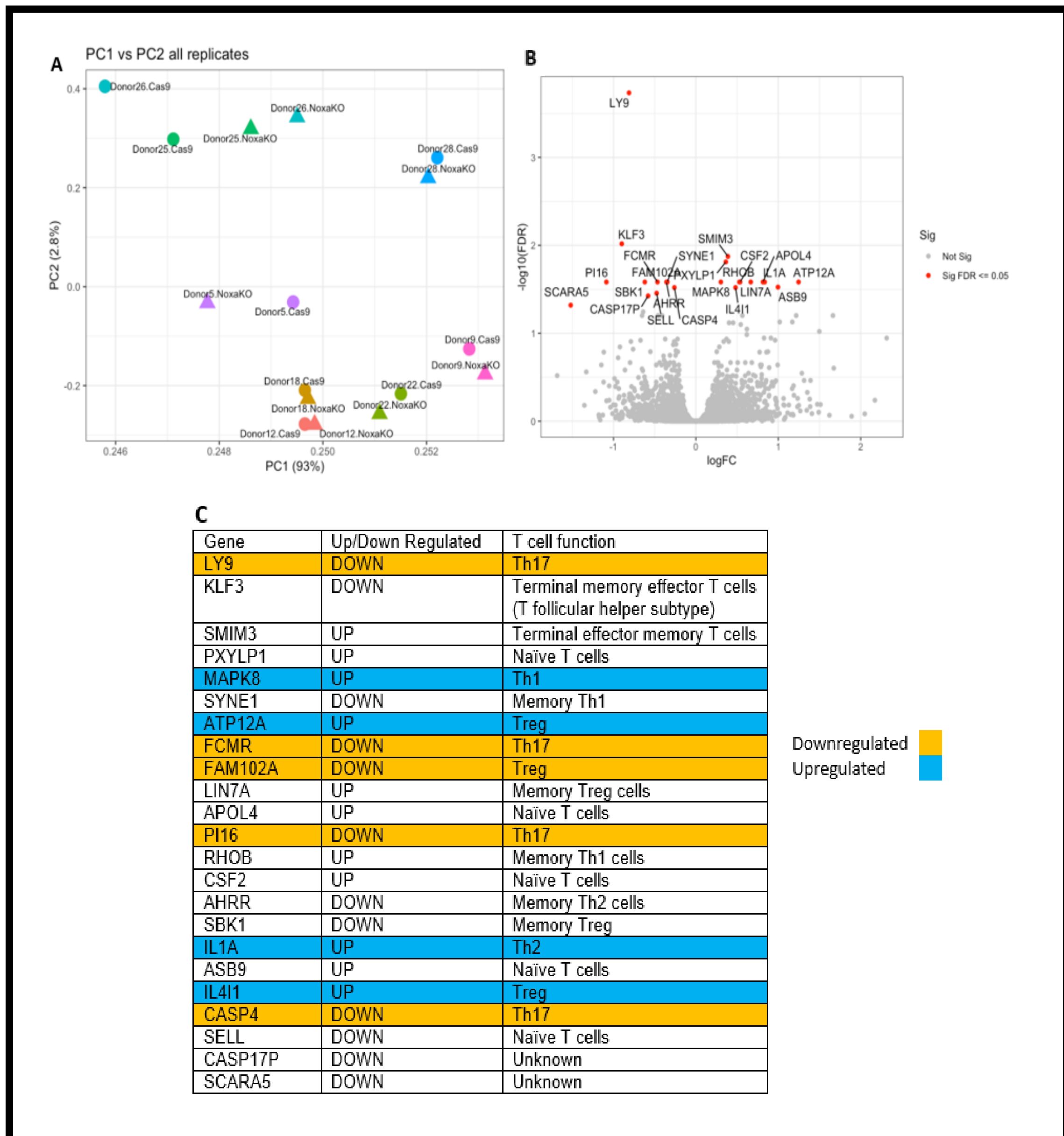
Figure 2.8. RNA-Seq Analysis from four of eight Noxa KO human CD4+ T cell donors. A) Original principal component analysis (PCA) of RNA-Seq data. Original four donors chosen for further analysis from red arrows because of separation of the Cas9 and Noxa KO samples. B) List of differentially expressed genes and T cell functions.

Bioinformatics specialist at the Minnesota Supercomputing Institute (MSI), Dr. Sarah Munro, determined that all eight donors could be used for analysis and the donor effect taken into account. Using the edgeR database, a new PCA plot was created that showed

93% of the differences among the donors occurred across the x-axis (Figure 2.9A). Although some of the Cas9 and Noxa KO pairs appear close together on the PCA plot, all donors were analyzed as many genes were missed during the first analysis and the bulk of the data are spread across the x-axis. A volcano plot shows all the differentially expressed genes in the Noxa KO cells (Figure 2.9B), with the genes showing the greatest distance from other differentially expressed genes being LY9. LY9 drives differentiation toward the Th17 helper T cells subset (24). Other genes associated with Th17 differentiation include: FCMR, PI16, and CASP4, which are all also significantly downregulated in Noxa KO cells. Conversely, the genes ATP12A and IL4I1 are both upregulated in Noxa KO cells are these both function to increase regulatory T cells (Figure 2.9C). There are multiple genes that are associated with Memory CD4+ T cell differentiation and no conclusion can be drawn from them, because these genes are both up and downregulated in the cells. The dataset was also recently tested against the Gene Set Enrichment Analysis database, MSigDb C7, which contains immunological signatures. Further analysis of the hits in the C7 database will be analyzed in the future. Overall, the RNA-seq data reveal that Noxa (a) is likely involved in the differentiation of Th17 cells, and (b) may inhibit regulatory T cell formation, supporting early predictions.

The results of these studies have provided insight into how Noxa functions in metabolism and differentiation of CD4+ T cells. Importantly our results show that -

- Noxa is induced upon activation of CD4+ T cells.
- Glutamine is required for Noxa induction.
- Noxa is required for entry of glutamine into the mitochondrial TCA cycle.
- Noxa may prevent T cells from utilizing fatty acid oxidation for metabolism.
- Noxa may be required for generation of Th17 cells.



Gene	Up/Down Regulated	T cell function
LY9	DOWN	Th17
KLF3	DOWN	Terminal memory effector T cells (T follicular helper subtype)
SMIM3	UP	Terminal effector memory T cells
PXYLP1	UP	Naïve T cells
MAPK8	UP	Th1
SYNE1	DOWN	Memory Th1
ATP12A	UP	Treg
FCMR	DOWN	Th17
FAM102A	DOWN	Treg
LIN7A	UP	Memory Treg cells
APOL4	UP	Naïve T cells
PI16	DOWN	Th17
RHOB	UP	Memory Th1 cells
CSF2	UP	Naïve T cells
AHRR	DOWN	Memory Th2 cells
SBK1	DOWN	Memory Treg
IL1A	UP	Th2
ASB9	UP	Naïve T cells
IL4I1	UP	Treg
CASP4	DOWN	Th17
SELL	DOWN	Naïve T cells
CASP17P	DOWN	Unknown
SCARA5	DOWN	Unknown

Figure 2.9. RNA-Seq analysis of eight independent Noxa KO human CD4+ T cell donors. A) New PCA with all eight donors being further analyzed. 93% of separation occurs on the x-axis, so even a slight shift in difference between Cas9 and Noxa KO point for each donor will represent a significant amount of data. B) Volcano plot showing the significant genes that are downregulated (negative) or upregulated (positive) in Noxa KO cells after analysis using all eight independent donors. C) List of differentially expressed genes and function in T cells.

Chapter 3: Discussion

The research described here was designed to test the hypothesis that human Bcl-2 protein, Noxa, regulates glutamine metabolism, and promotes differentiation of CD4+T towards subtypes that require glutamine for their metabolism, such as Th1 and Th17, and away from regulatory T cell and Th2 cell subtypes. The hypothesis was based not only on preliminary studies using pan (CD3+) T cells that revealed interdependence between Noxa and glutamine, but also on the knowledge that CD4+T cells differentiate into functionally and metabolically distinct subsets that differ in their utilization of glutamine. Our studies showed that the Noxa protein was induced in a glutamine dependent manner when CD4+ T cells were stimulated to expand and differentiate, confirming previous observations using pan T cells. Metabolic assays and RNA-Seq studies described in the dissertation have offered valuable clues into Noxa's role in metabolic reprogramming and in driving differentiation of CD4+ T cells through glutamine utilization. The early conclusion from our studies is that Noxa may be essential for the expansion and differentiation of human Th1 and Th17 cells, but dispensable for Th2 and Treg differentiation.

We have generated CRISPR/Cas9 Noxa knockout CD4+ T cell populations from multiple donors for our studies. Previous studies had relied on RNA silencing, but the transient nature of siRNA knockdowns limits the potential for long term studies. CRISPR/Cas9 knockouts were, therefore, preferred over an RNA silencing approach. However, one serious limitation to a CRISPR approach is the long waiting period prior to testing for successful knockout during which cells may undergo changes unrelated to Noxa that could confound interpretation of experimental results. Another limitation of knockout approach in primary T cell populations is the challenge of obtaining clonal lines, given that insertions or deletions could occur in multiple different sites within the gene. Single

cell cloning experiments of the Noxa knockout cells are ongoing, although early attempts have been unsuccessful. Single cell clones would offer a more rigorous platform for analysis, allowing multiple different knockouts from the same donor to be used as biological replicates. Overall, CRISPR/Cas9 is the best available method to study Noxa as there is no mouse model available. Murine Noxa differs from the human protein both structurally and in its regulation (25).

The spare respiratory capacity of Noxa KO CD4+ T cells was severely impaired in the presence of the FAO inhibitor, Eto, suggesting these cells relied on FAO, rather than glutamine for mitochondrial respiration. The use of two independent chemical toxins, one a glutaminase inhibitor and the other a glutamine antagonist, provided additional support for the hypothesis that T cells are not reliant on glutamine for mitochondrial metabolism in the absence of Noxa. When only glutaminase was inhibited in Noxa KO cells, the cells respired normally, however the respiration of the Cas9 cells was significantly inhibited. On the other hand, the glutamine antagonist, DON, which effectively renders glutamine unavailable, was toxic to CD4+ T cells. This was further supported by western blot data showing Noxa was induced in the presence of CB839, but not in the presence of DON. Thus, our studies reveal a unique interdependence between Noxa and glutamine. Noxa requires glutamine for induction, and glutamine, in turn, relies on Noxa for its use as a carbon source in the TCA cycle.

The pilot RNA-seq study showed differential expression of 23 genes in the Noxa KO cells. Four of those genes were related to Th17 and were downregulated in Noxa KO’s, notably LY9, which was the most significantly separated from the rest of the genes. There were also two genes that were related to Tregs, upregulated in the Noxa KO cells (Figure 2.9).

These finding indicate that Noxa may be involved in differentiation into Th17 cell subtypes. This was only a pilot RNA-seq study, so we chose eight of the best donor knockouts we had generated to use for our study. However, if this were completed on a large scale with many more donors and multiple replicates from each donor, the findings would be much more significant, and would help strengthen the data.

The next steps of this study include validation of the RNA-seq data. Cell subtype differentiation will continue to be tested using flow cytometry and western blots. Each time cells are harvested for studies; the supernatant will be collected for a cytokine ELISA which will help determine the cytokines present at each timepoint over the differentiation process. To better understand the metabolism of Noxa KO cells, metabolic tracer studies will be conducted with labeled glutamine as well as labeled fatty acids. Finally, a comparative proteomic analysis could support the RNA-Seq studies and offer valuable information about Noxa-regulated signaling pathways in T cell subtypes.

Our studies have shown a novel role for Noxa, a Bcl-2 protein, in CD4+ T cell activation, differentiation, and metabolic regulation. Based on our observations, Noxa is required for glutamine consumption and when Noxa is knocked out, the cells are resistant to glutaminase inhibition. It is important to have a better understanding of the metabolic needs of T cells as they undergo differentiation into helper, effector, memory, and regulatory subsets because this would allow for subtype specific target therapeutics. For example, if Noxa is shown to suppress Treg formation, knocking out Noxa *ex vivo* could have applicability in the treatment of graft versus host disease (GvHD) or other diseases. The research described here also offers valuable insight into the role of metabolism in T

cell function and, in the future, could help in characterizing metabolic signatures of human T cell subsets.

Chapter 4: Materials and Methods

Cell Lines and Cell Culture

Human CD4+ T cells were cultured in T cell expansion basal medium (TCEM), supplemented with Optimizer CTS (Gibco), CTS Immune Cell SR (Gibco), 100U/ml penicillin, 100µg/ml streptomycin (Gibco), and 2mM L-Glutamine (VWR Life Science). Cells were grown in a humidified environment with 5% CO_2 at 37°C.

Primary T Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples obtained from healthy donors (Memorial Blood Center St. Paul, MN, USA) using density gradient centrifugation (10 min, 25°C, 900 g without break) over Histopaque®-1077 (density 1.077 g/mL, Sigma Alrich) and were washed in phosphate-buffered saline free of calcium and magnesium (PBS). Cells were allowed to rest in Immunocult™-XF T Cell Expansion Medium (StemCell Technologies) overnight before living cells were recovered by density gradient centrifugation (25 min, 25°C, 1000 g without break) over Ficoll-Paque™ PLUS (density 1.077 g/mL, Cytiva). Two hours following live cell separation, CD4+ T cells were isolated from PBMCs by negative selection with CD4+ isolation kit (Miltenyi Biotec, catalog: 130-096-533 or StemCell Technologies, catalog: 17952RF) according to the manufacturers' instructions. For the Miltenyi Biotec separation, PBMCs in MACS buffer (PBS containing 2 mM EDTA and 0.5% FBS) were passed through magnetic separation columns (Miltenyi Biotec). The StemCell Technologies protocol requires a magnet and polystyrene tubes for isolation.

Primary T Cell Activation

Human CD4+ T cells were plate-activated with anti-CD3/CD28 antibodies (BioLegend) or with Immunocult™ Human CD3/CD28 T Cell Activator cocktail (StemCell

Technologies, catalog: 10791). For plate activation, culture plates were coated with 0.5 mg/mL of anti-CD3 antibodies (BioLegend, catalog: 300437) diluted 1:100 in PBS and incubated for two hours at 37°C. T cells in TCEM were later transferred to the treated plate and 0.5 mg/mL of anti-CD28 antibodies (BioLegend, catalog: 302933) was added and IL-2 (Peprotech, catalog: AF-200-02-10) at a final concentration of 4 ng/mL (66 IU/ng). For activation with Immunocult™ Human CD3/CD28 T Cell Activators, 25 uL/mL of cocktail was added to cells at a density of $1x10^6$/mL followed by IL-2 (4 ng/mL).

Noxa Knockout Experiments

$2x10^6$ CD4+ T cells were isolated and activated for 48 hours as described above. Cells were then harvested, washed once in 1X PBS, and resuspended in T Buffer (Invitrogen). Half of the cells were used as transfection controls only mixed with 1 µg Clean Cap 3XNLS Cas9 mRNA (TriLink Biotechnologies, catalog: L-606-100). The remaining cells were intended to be Noxa knockout cells and mixed with 1µg Cas9 and 100pmol PMAIP1 sgRNA, sequence: CACCGGCGGAGAUGCCU – Modified (Synthego, catalog: PMAIP1+59900181). The seq 10µL tips from Invitrogen were used twice to transfect 1 million cells (500,000 cells/tip). The transfections were completed using the Neon Transfection System (Invitrogen), with parameters: 1400 Volts, 10 pulse width, 3 pulses. After the transfection was completed, the cells were cultured in TCEM without antibiotics for 48 hours before re-addition of Pen/Strep.

Western Blots

Cells were harvested and washed once with cold PBS and lysed with RIPA buffer (50mM Tris-HCL [pH 7.5], 150mM 0.5% v/v sodium deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS supplemented with protease (Inhibitor Cocktail III, Calbiochem) and phosphatase

inhibitors (inhibitor cocktail III, Millipore). Lysates made with RIPA were immediately spun at 10000 rpm for 10 minutes at 4°C. The supernatant was collected, and 40 µg of protein was boiled in 2x Laemlli buffer (BioRad) for 3 minutes at 85°C. Protein lysates were resolved by SDS-PAGE, transferred onto Amersham™ Protram™ nitrocellulose (GE Healthcare Life Sciences) blocked in TBS-TM (20 mM Tris-HCl pH 7.6, 37 mM NaCl, 0.1% Tween 20®, 5% skim milk) and then incubated with Noxa (1:500 – 1:1000) (Cell Signaling Technologies, catalog: 14766S) or β-actin (1:7000) (Santa Cruz Biotechnology, catalog: sc-96879) primary antibodies diluted in TBS-T at 4°C overnight. After primary antibodies were removed the membranes were washed three times for five minutes in TBS-T. Secondary anti-mouse or anti-rabbit HRP-conjugated antibodies (Invitrogen, mouse catalog: NA931VS, rabbit catalog: NA934VS) were diluted (1:5000) in 5% w/v milk in TBS-T and the membranes were incubated for one hour at room temperature. After the secondary antibody incubation, the Noxa membrane was washed three times for ten minutes in TBS-T and the Actin membrane was washed three times for five minutes in TBS-T. Chemiluminescence reactions were carried out using ProSignal Pico or Femto ECL Reagent Kits (Prometheus) or Pierce ECL Western Blotting Substrate (Thermo Scientific).

Flow Cytometry

Human CD4+ T cells were analyzed via flow cytometry. 500,000 cells were harvested and washed (5 minutes, 4°C, 436g) once with cold 1x PBS. The cells were stained with Zombie NIR Viability dye (1:1000) (BioLegend, catalog: 423106). One tube was used as a control and the cells were killed by heating in a thermomixer at 65°C for 1.5 minutes. The tubes were incubated on ice in the dark for 20 minutes. The cells were then washed twice, once with 1x PBS and once with FACS buffer (1% FBS and 0.1% NaN_3 in PBS).

After washing, the cells were stained with 1µL of dye per 500,000 cells with CD4-BV510 (BioLegend, clone: A161A1, catalog: 357420), CD25-BV650 (BioLegend, clone: BC96, catalog: 302634), CD69-FITC (BioLegend, clone: FN50, catalog: 310903), and CXCR3-PerCP (BioLegend, clone: G025H7, catalog: 353739) and incubated at room temperature in the dark for 20 minutes. After two washes with FACS buffer, flow cytometry was performed on a BD LSR II flow cytometer and the data were analyzed using Flowjo version 10.7.1.

Intracellular Flow Cytometry

The extracellular staining process was completed as above. The cells were fixed and permeabilized according to the manufacturer protocol for 5mL tubes (True-Nuclear Transcription Factor Buffer Set – BioLegend, catalog: 424401). Cells were stained with 1µL of dye per 500,000 cells with Tbet-Pe/Cy7 (BioLegend, clone: 4B10 catalog: 644824) and FoxP3-BV421 (BioLegend, clone: 206D, catalog: 320123) or FoxP3-PE (BioLegend, clone: 206D, catalog: 320108) and incubated at room temperature in the dark for 45 minutes. After staining, the cells were washed once with 1x Perm buffer and once with FACS buffer and flow cytometry was performed on a BD LSR II flow cytometer. The data was analyzed using Flowjo version 10.7.1.

RNA Isolation

RNA was isolated from CD4+ T cells using the RNeasy Kit following the Qiagen manufacturer) protocol (catalog: 74104). The isolated RNA was resuspended in RNase-free water. The concentration of RNA was determined using Thermo Scientific Nano Drop 2000 Spectrophotometer. Samples were stored at a concentration of 0.1 µg/µL at -80°C until further use.

Agilent Seahorse XFe96 Cell Mito Stress Test

The Seahorse cartridge was hydrated first with water (HyClone) then calibrant (Agilent) according to the manufacturer protocol. Cell-Tak (Corning) was mixed with Sodium Bicarbonate 7.5% (Gibco) and added to a cell culture plate. After a 45-minute incubation, at room temperature the wells were washed out twice with 100 µL of water. The plate was stored at room temperature until use. Seahorse Medium +HEPES, pH 7.4 (Agilent) was made with 10mM Glucose (Agilent), 2mM Sodium Pyruvate (Agilent), and 2mM Glutamine (Agilent). Cells were harvested and washed once in cold 1x PBS and resuspended in prewarmed Seahorse Medium. 250,000 cells were plated per well in 180 µL of Seahorse Medium. Seahorse medium was added to any empty well, but at minimum must be added to the four corners for a background reading for the experiment. The plate was spun down to secure cells to adhesive (100 g without brake for 5 seconds) flipped and repeated to ensure evenness before being placed in the 37° incubator with no CO_2 for 1 hour. Mito Stress Kit drugs were prepared in Seahorse media (Oligomycin 1.5µM, FCCP 2.0µM, and Rotenone/ Antimycin A 0.5µM) and added to cartridge drug ports. Hoechst (5.2 µM) (AnaSpec.) was prepared in Seahorse media and added to the final port. The cartridge was placed in the Seahorse for calibration. The bottom of the cell plate was wiped with a Kim wipe saturated with 70% ethanol and placed in the Biotek Cytation 5 to count the cells using a brightfield scan (Seahorse XF Imaging and Counting Software). After completion of the metabolism experiment the cell plate was put back into the Biotek Cytation 5 for a fluorescent cell count. The cell count was imported into the Wave software for normalization of the metabolism experiment.

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