Gluconeogenic FBP1 plays a key metabolic role in activated T cells

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By

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Abstract: Fructose bisphosphatase-1 (FBP1) is a rate limiting enzyme in gluconeogenesis that converts fructose-1,6-bisphosphate (F1,6BP) to fructose-6-phosphate (F6P). It is active in liver, kidney and skeletal muscle cells. This study suggests that FBP1 plays a novel non-gluconeogenic role in T cells. Targeted metabolomics using [^{13}C]-6-glucose revealed a labeling pattern of F6P in stimulated CD3+ T cells that could only have resulted from FBP1 enzymatic activity. Following stimulation, T cells expressed a 27kD form of FBP1, in addition to the full-length 37kD protein. The hypothesis that T cells utilize an alternative translational start site to express a shorter, constitutively active, form is being tested. Future studies will also test the hypothesis that FBP1 activity increases carbon flux into the pentose phosphate pathway (PPP), to facilitate increased production of reducing agent and co-factor, NADPH, in preparation for proliferation. This research could contribute significantly to our understanding of T cell physiology and cancer cell metabolism.

Contents:

List of Figures:

Chapter 1: Introduction

T lymphocyte metabolism as a model for cancer and proliferative metabolism

T lymphocytes (T cells) have been used as models for studying proliferative and cancer cell metabolism (1). T cells mature in the thymus and are constantly in circulation in the blood and the lymphatic system in the naïve (unstimulated) state. They are activated via signaling pathways mediated by T cell receptors (TCRs) on these cells, upon encountering a cognate antigen. The foreign antigens are presented by antigen presenting cells (APCs) as a part of the major histocompatibility complex (MHC). Activated T cells then proliferate and differentiate into effector T cells acquiring abilities to produce substances like cytokines to combat the inflammation effectively (2).

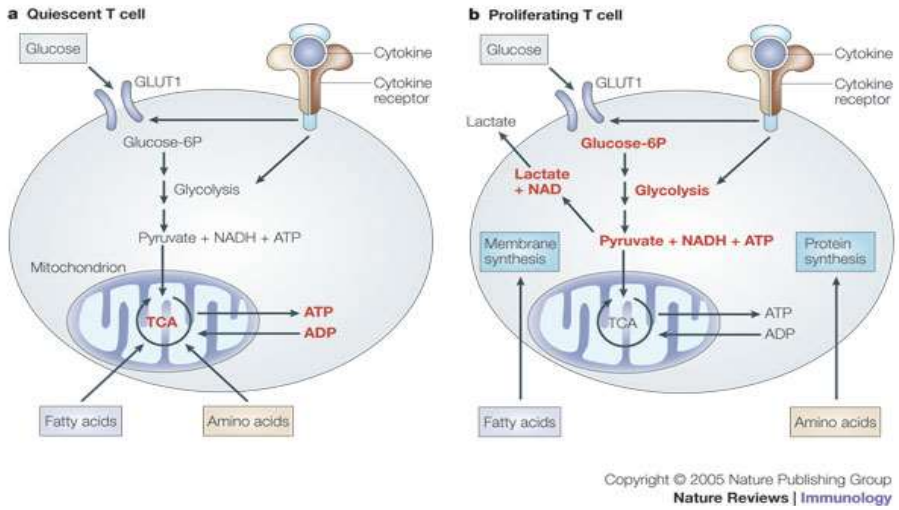

Fig. 1- Metabolic differences between quiescent and activated T cells

Quiescent and activated T cells are functionally and phenotypically quite different. The switch from a naïve cell to a proliferating cell is supported by changes in the cell's metabolic framework. Quiescent T cells have a resting metabolic phenotype, catabolizing fuels to support cell survival (Fig. 1) (1). The consumed glucose is metabolized through glycolysis, the tricarboxylic acid (TCA) cycle and subsequent oxidative phosphorylation process (OXPHOS) to generate large amounts of ATP (3). There is stagnancy in growth and cell division, and the cells maintain a status quo unless encountered by an antigen.

Activation of T cells, on the other hand results in rapid growth, proliferation and acquisition of effector functions (3). Sustaining the dramatic changes that take place in a T cell upon activation requires extensive metabolic reprogramming, and a switch to anabolic metabolism to help them grow and proliferate (4). The requirement of activated T cells for biosynthetic precursors is almost as significant as their need for ATP. Stimulated T lymphocytes become highly glycolytic and increase their rates of aerobic glycolysis to support their energetic and biosynthetic demands (4). Activated T cells thus utilize more glucose to divert it to biosynthetic intermediates, decreasing the amount of glucose entering the TCA cycle. Glycolysis as a means to generate ATP is energetically inefficient, but it branches into pathways like the pentose phosphate shunt that generate several important building blocks such as nucleotides and lipids for biomass production(1).

The proliferative signals that drive metabolic reprogramming are common to T cells and cancer cells. Aerobic glycolysis is utilized in both systems for energy and biosynthesis (1). Like activated T cells, tumors enter a stage, albeit more gradually, of rapid proliferation and growth, with an increase in cellular metabolic activity (5). Hypoxic environments and increased nutrient requirements cause these cells to adopt a similar metabolic profile during activation and tumorigenesis, respectively (1, 6). T cells thus offer a useful platform to study metabolic reprogramming in the proliferating phenotype.

Importance of the pentose phosphate pathway:

Increased glycolysis, in turn, also increases the supply of fuel to the pentose phosphate pathway (PPP), which is a branch of glycolysis, diverting G6P to form pentose phosphates and NADPH (Fig. 2). These pentose phosphate intermediates are of special importance in highly proliferative cells like activated T lymphocytes and tumor, which require them as precursors for nucleotide synthesis (4). NADPH is an important byproduct of this pathway, actively involved in neutralizing reactive oxygen species (ROS) in the cells (7). Activated T cells and tumors experience high levels of oxidative stress due to increased metabolic activity (4). The generation of NADPH via the PPP is critical in the defense against reactive oxygen species in the cells as it generates reduced

glutathione, a species that scavenges free radicals in the cell. Moreover, NADPH is also involved in the production, from Acetyl-coA, of fatty acid, used in synthesis of cellular membranes (8). Thus, mechanisms that increase the production of NADPH through the PPP are critical in sustaining growth and increasing proliferation.

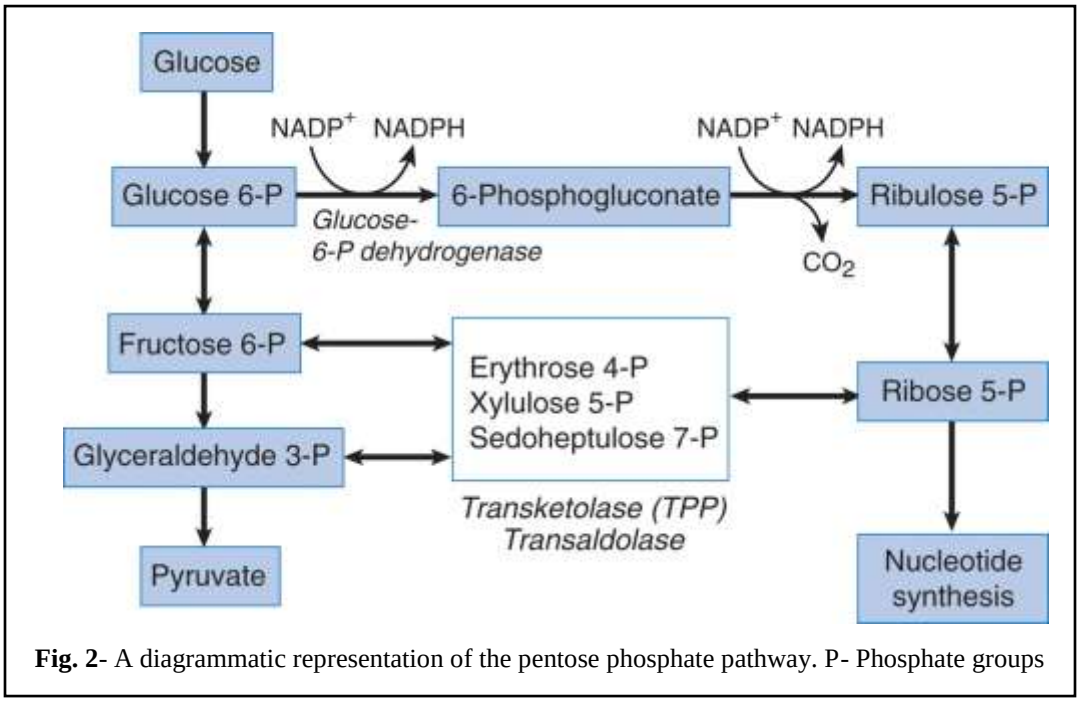

Fig. 2- A diagrammatic representation of the pentose phosphate pathway. P- Phosphate groups

Production of glucose from endogenous substrates to maintain blood glucose levels- Gluconeogenesis

While most cell systems utilize circulating glucose for production of energy, liver, kidney and skeletal muscle cells generate glucose endogenously from non-carbohydrate sources such as pyruvate, lipids and amino acids, in a process termed as gluconeogenesis. Low blood glucose levels are an indicator for hepatic cells to increase their rate of gluconeogenesis (9). This is a highly energy consuming process, and cell systems in need of exogenous glucose are less likely to employ gluconeogenesis to produce glucose to be secreted out of the cell. Gluconeogenesis is a metabolic pathway that works in the reverse direction to glycolysis, and includes all the reversible reactions of glycolysis, utilizing the same enzymes (Fig. 2). It also involves 3 irreversible steps, utilizing specialized

gluconeogenic enzymes- glucose-6-phosphatase (G6Pase), fructose-1,6 bisphosphatase-1 (FBP1) and phosphoenol pyruvate carboxykinase (PEPCK).

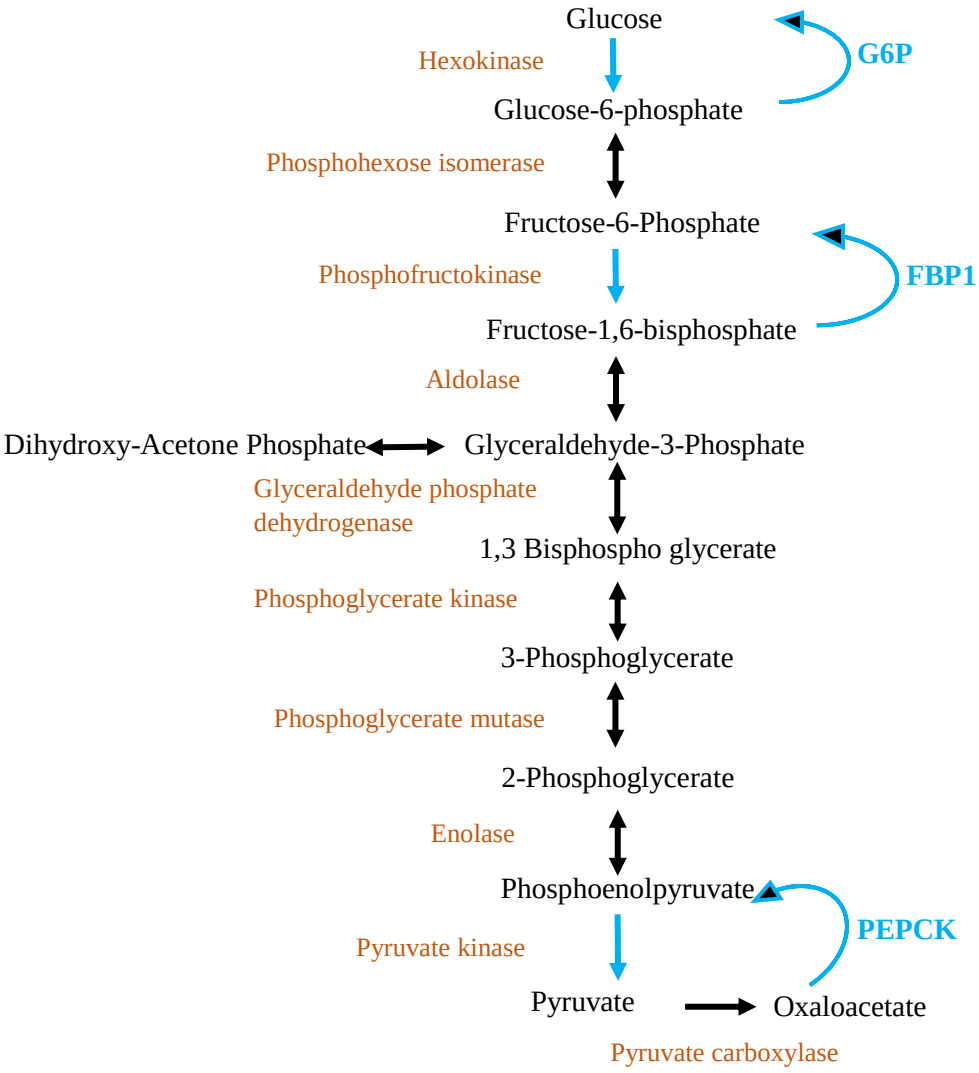

Fig. 3- Glycolysis and gluconeogenesis, with the enzymes involved in brown and blue respectively.

Fructose-1,6-bisphosphatase is the rate limiting enzyme for gluconeogenesis, which catalyzes the irreversible conversion of fructose-1,6-bisphosphate (F1,6BP) to fructose-6-phosphate (F6P). It localizes to the cytoplasm, where it actively participates in gluconeogenesis. FBP1 is a 37 kD protein which has a regulatory domain at its amino (N) terminus and a catalytic domain at its carboxy (C) terminus. AMP and cAMP bind to the

N terminus to allosterically regulate the activity of the enzyme (10). FBP1 is found to be downregulated in renal carcinomas, breast cancers and hepatocellular carcinomas and is being increasingly recognized as a tumor suppressor. Loss of FBP1 increases glucose consumption and enhances metastasis (10-12).

According to The Human Protein Atlas, FBP1 is expressed as a protein in tissues like liver, small intestine, thyroid gland, stomach and kidney. It has not been reported to be expressed in tissues originating from the bone marrow, specially lymphocytes. Presence of FBP1 mRNA was detected in an RNA sequencing study in lymphocytes (13), but its protein expression in the cell has not been studied before.

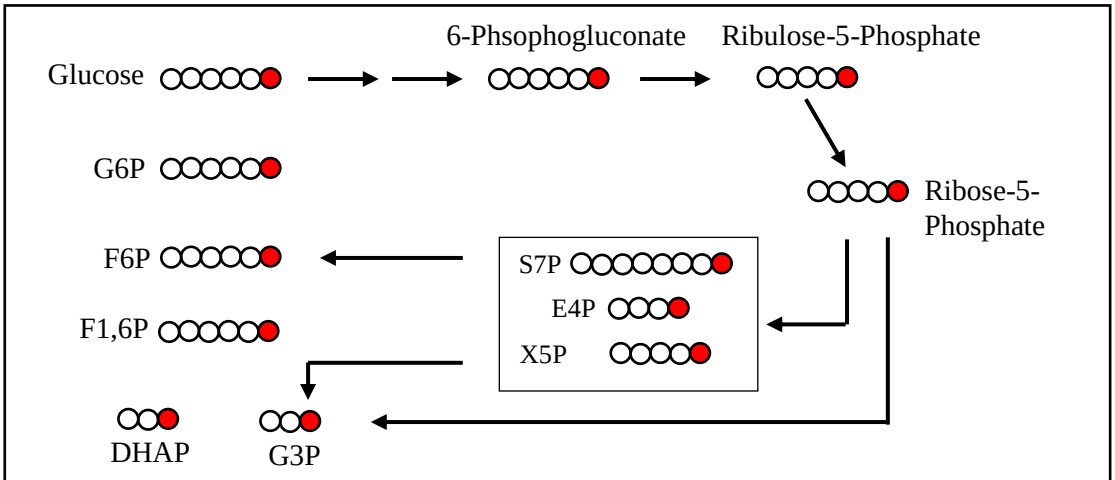

Fig. 4- Fate of [^{13}C]-6-glucose through the PPP. The labeled carbon is indicated with the red circle. S7P- Sedoheptulose-7-Phosphate, E4P- Erythrose-4-Phosphate, X5P- Xylose-5-Phosphate

Our lab is interested in metabolic alterations and reprogramming associated with the proliferating phenotype, and uses primary human T cells for modeling proliferative systems. Targeted metabolomics with non-radioactive heavy isotopomers of metabolites have been instrumental in tracing the fate of these metabolites after they are taken up by the cells. Labeling of glucose with [^{13}C] carbons at different positions enables us to study its trajectory through glycolysis, TCA cycle, or the pentose phosphate pathway. We have utilized glucose labeled at positions 1 and 2 ([^{13}C]-1,2-glucose) to study glycolysis and the TCA cycle. We also utilized glucose with a labeled carbon at position 6, to study the PPP, since the carbon at position 6 is retained during the reactions of the PPP when it

forms G3P to continue glycolysis (Fig. 4). An unexpected labeling pattern of glycolytic intermediates emerged from this approach and led us to formulate the hypothesis that is the basis of this dissertation project. We hypothesize that FBP1, a gluconeogenic enzyme, is expressed and functional in T cells, but may play a novel 'non-gluconeogenic' role. We also observed a unique shorter isoform of FBP1 in activated T cells. The experiments, described in the following chapter, investigate and begin to characterize this unique FBP1 isoform.

Chapter 2: Results

1. Targeted metabolomics suggest catalytically active fructose bisphosphatase-1 is expressed in activated T cells -

We described in the previous chapter how isotope tracer experiments are useful for tracking the metabolism of nutrients like glucose and glutamine through the various metabolic pathways in a cell. Replacing glucose carbons at various positions with a heavier isotope, [^{13}C], labels the carbon atom and can be detected by mass spectrometry as the glucose is taken up and metabolized. Each added [^{13}C] increases the mass number of the molecule by 1 unit (m+1, m+2 and more). The position of the labeled carbon on the isotopomer is selected based on the focus of the experiment. Glucose with labeled carbon at position 1 and 2 ([^{13}C]-1,2-glucose) or at all six positions (uniformly labeled) is used to study glycolysis and TCA cycle. We used singly labeled [^{13}C]-6-glucose for our experiments, to study the trajectory of the glucose molecule through the PPP (Fig. 4). Human CD3+ T cells were isolated from peripheral blood mononuclear cells (PBMCs), and co-stimulated *in vitro* with anti-CD3 and anti-CD28 antibodies. These cells were cultured in [^{13}C]-6-glucose following co-stimulation, harvested 24 hours later and cell extracts analyzed by mass spectrometry. An unexpected labeled metabolic intermediate was detected in activated T cells (Fig. 5A and 5B). Along with the predicted m+1 labeled F6P and F1,6BP, close to 20% of m+2 labeled F6P and F1,6BP were also detected in activated cells (Fig. 5C and 5D). Dihydroxy acetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) can each acquire the labeled carbon atom from glucose in the interconversion reaction. They can combine to form fructose-1,6-biphosphate (F1,6BP) (m+1 or m+2) via the bidirectional reaction catalyzed by aldolase. Thus, the presence of m+2 F1,6BP was not surprising. The presence of doubly labeled F6P, however, was unexpected as the enzyme phosphofructokinase (PFK) that converts F6P to F1,6BP is unidirectional unlike most other glycolytic enzymes, with the reverse reaction F1,6BP back to F6P requiring a different unidirectional enzyme fructose bisphosphatase 1.

The dual labeling of F6P can only be explained by FBP1 actively converting F1,6BP back to F6P. This unexpected labeling was the initial observation that led us to

hypothesize that FBP1 is expressed and enzymatically active in activated T cells. The presence of an active FBP1 suggested that gluconeogenesis, leading to production and secretion of glucose, may be occurring in T cells. We, therefore, looked for the expression of other two gluconeogenic enzymes, PEPCK and G6P in the T lymphocytes.

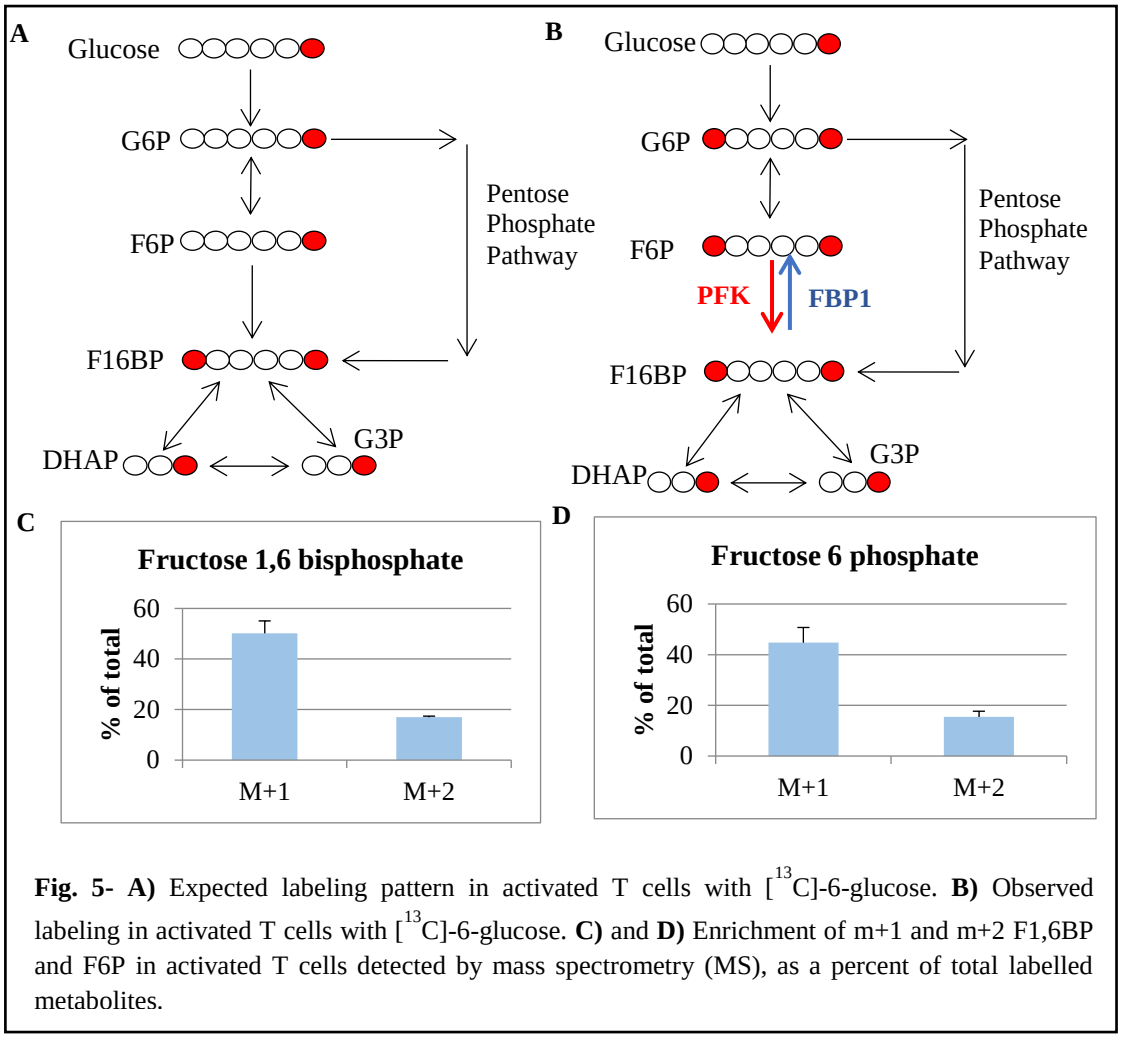

Fig. 5- A) Expected labeling pattern in activated T cells with [^{13}C]-6-glucose. **B)** Observed labeling in activated T cells with [^{13}C]-6-glucose. **C)** and **D)** Enrichment of m+1 and m+2 F1,6BP and F6P in activated T cells detected by mass spectrometry (MS), as a percent of total labelled metabolites.

2. FBP1 is the only gluconeogenic enzyme expressed in T cells

Gluconeogenesis is a major metabolic pathway used by humans and other primates to maintain and restore blood glucose levels. It occurs primarily in the liver, and occasionally in the kidneys and small intestine. The results from the labeling experiments

(Fig. 5) were surprising as T cells are not known to carry out gluconeogenesis. Moreover, the glucose-rich cell culture medium in which the T cells were activated *in vitro* made it unlikely that they would need to produce glucose. The targeted metabolomics suggested that at least one gluconeogenic enzyme, FBP1, was expressed in T cells, so we next checked for expression of the other two, PEPCK and G6Pase. We activated T cells for 24 hours and analyzed RNA from unstimulated and stimulated cells for expression of FBP1, PEPCK and G6Pase mRNA by RT-PCR using the hepatocellular carcinoma cell line HepG2 as a positive control. We detected FBP1 mRNA, but no PEPCK or G6Pase expression, in the T cells using this approach (Fig. 6A and B). We conclude, based on these data, that T cells are not gluconeogenic and hypothesize that active FBP1 may have a unique, non-gluconeogenic role in activated T cells.

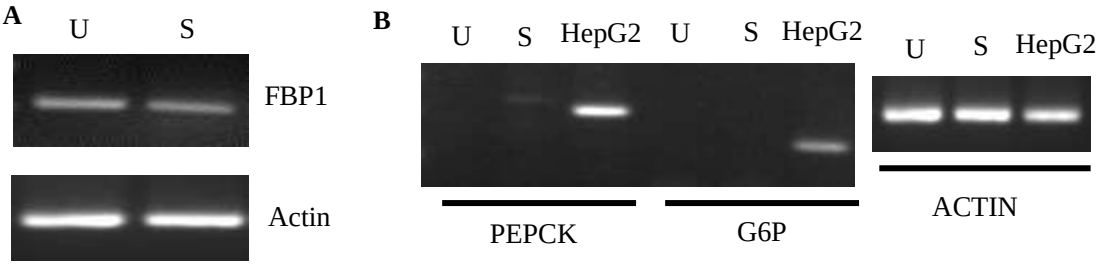

Fig. 6- PCR amplification of gluconeogenic mRNA in T cells. **A)** amplification of FBP1 transcript in unstimulated (U) and stimulated (S) T cells. **B)** amplification of PEPCK, G6P and actin mRNA in T cells, using HepG2 cells as positive control.

3. Western blotting reveals two isoforms of FBP1 expressed in stimulated T cells.

Analysis of T cell mRNA revealed the presence of FBP1 transcripts in both unstimulated and stimulated T cells. However, the labeling pattern of F6P indicative of an active FBP1, was observed only in stimulated T cells. Next, lysates from CD3+ T cells activated for 24 hours *in vitro* with anti-CD3 and anti-CD28 antibodies, were analyzed for FBP1 protein expression by western blotting (Fig 7). Acute myeloid leukemia cell line, HL60, was used as a positive control for FBP1 expression (16). FBP1 was detected in unstimulated as well as CD3+ stimulated T cell lysates at 37 kD, the reported size of

the full-length protein. The FBP1 antibody also detected a prominent 27 kD band only in stimulated T cells. A 27 kD band had previously been observed in western blots of mouse liver cell lysates and in HEK293 cells transfected with an FBP1 expression plasmid, but had not been described as an FBP1 isoform (18). To eliminate the possibility that the 27 kD band was a result of non-specific binding of the FBP1 antibody, we tested four independent monoclonal and polyclonal antibodies from different sources. All four detected the shorter 27 kD band. Representative data with two of these antibodies are shown in Fig. 7A and 7B. The data strongly suggest that the 27 kD protein is an isoform of FBP1 induced in activated T cells.

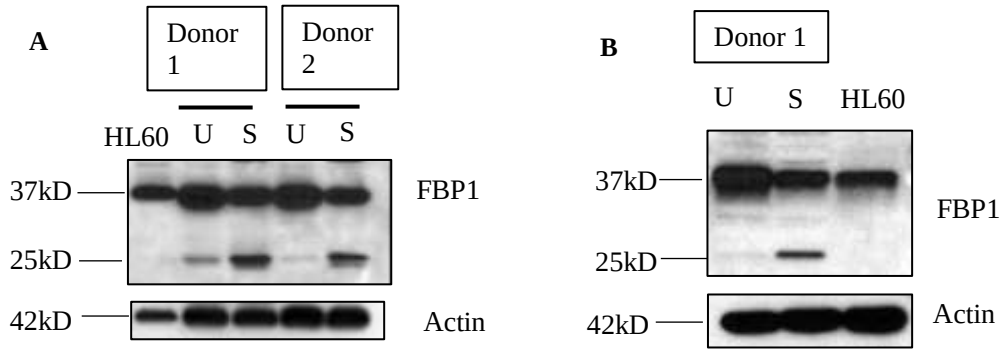

Fig. 7- Western blot of cell lysates from two independent human T cell donors showing the shorter isoform at 27kD detected by **A)** ThermoFisher anti-FBP1 antibody (PA5- 30028) and **B)** Santa Cruz anti-FBP1 antibody (sc-166145). U: Unstimulated S: Stimulated. HL60 cells were used as a positive control for FBP1 expression

4. Both isoforms of FBP1 are highly stable proteins in T cells

To further confirm the identity of the putative shorter FBP1 isoform we attempted to knock down protein expression through RNA interference using siRNA. Human CD3+ T cells were activated 24 hours after transfection with FBP1 specific siRNA and analyzed for FBP1 protein and mRNA expression following 24 hours after activation. Despite strong silencing at the RNA level (Fig. 8A and 8B) western blots showed no decrease in the levels of either isoform of FBP1, in unstimulated or in stimulated T cells (Fig. 8C). However, FBP1 specific siRNA decreased mRNA as well as protein expression in control

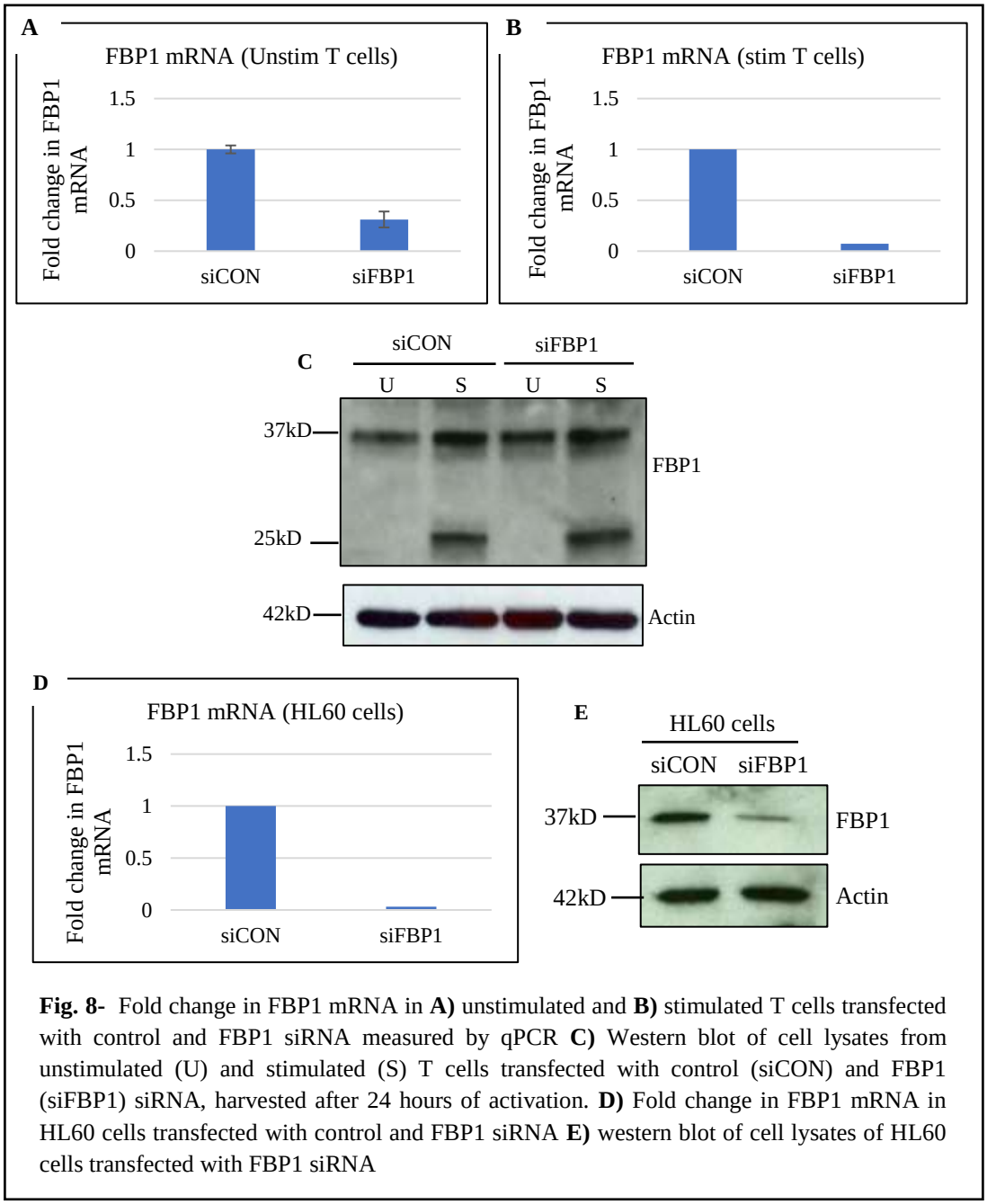

Fig. 8- Fold change in FBP1 mRNA in **A)** unstimulated and **B)** stimulated T cells transfected with control and FBP1 siRNA measured by qPCR **C)** Western blot of cell lysates from unstimulated (U) and stimulated (S) T cells transfected with control (siCON) and FBP1 (siFBP1) siRNA, harvested after 24 hours of activation. **D)** Fold change in FBP1 mRNA in HL60 cells transfected with control and FBP1 siRNA **E)** western blot of cell lysates of HL60 cells transfected with FBP1 siRNA

HL60 cells (Fig. 8D and 8E). This suggested that both isoforms of FBP1 are highly stable in T cells. To test this possibility, we looked at expression of both isoforms of FBP1 in T cells exposed to the protein synthesis inhibitor cycloheximide. CD3+ unstimulated and stimulated T cells were incubated with cycloheximide and protein expression was

determined at the indicated times by western blot (Fig. 9A and 9B). Levels of both isoforms of FBP1 were unaffected for as long as 30 hours in both unstimulated and stimulated T cells. The loss of expression of Noxa, a Bcl-2 protein consistently induced in activated T cells (14) shown in Fig 9C confirmed that the cycloheximide treatment had been effective. Based on these data we conclude that both isoforms of FBP1 are highly stable proteins in T cells.

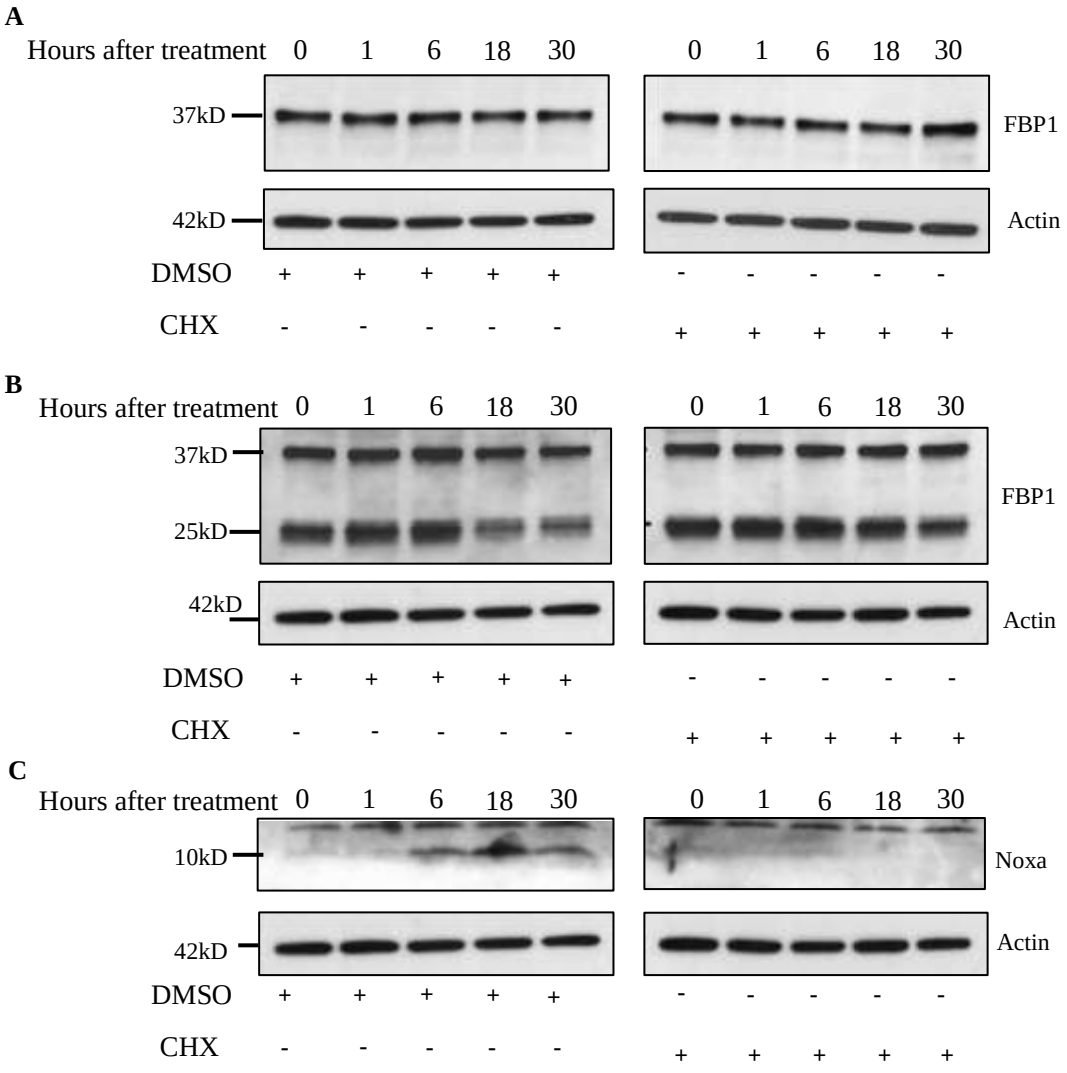

Fig. 9- Western blot showing protein expression of FBP1 in A) unstimulated and B) stimulated T cells indicated hours after treatment with DMSO or 10µg/mL Cycloheximide (CHX) C) Protein expression of Bcl-2 protein Noxa in stimulated T cells after treatment with DMSO or 10µg/mL CHX indicating effectiveness of CHX treatment.

5. Mechanism of regulation of FBP1 expression in T cells.

We have determined that T cells express a shorter isoform of FBP1, upon activation *in vitro*. A similar short isoform was observed previously in lysates of HEK293 cells transfected with a full-length FBP1 expression plasmid, and in western blots of mouse liver lysates (18). There are three possible regulatory mechanisms that could lead to the generation of the short isoform:

1) Alternative splicing of FBP1 mRNA could result in a shorter transcript, however, RT–PCR with different sets of primers showed full length transcripts of FBP1 to be present in both stimulated and unstimulated cells (data not shown), eliminating alternative splicing as a possible mechanism.

A

MADQAPFDTDVNTLTRFVMEEGRKARGTGELTQLLNSLCTAVKAISSAVRKAGIAHLYGIAGST
NVTGDQVKKLDVLSNDLVMNMLKSSFATCVLVSEEDKHAIIVEPEKRGKYVVCFDPLDGSSNID
CLVSVGTIFGIYRKKSTDEPSEKDALQPGRNLVAAGYALYGSATMLVLAMDCGVNCFMLDPAIG
EFILVDKDVKIKKKGKIYSLNEGYARDFDPAVTEYIQRKKFPPDNSAPYGARYVGSMVADVHRTL
VYGGIFLYPANKKSPNGKLRLLYECNPMAYVMEKAGGMATTGKEAVLDVIPTDIHQRAPVILGS
PDDVLEFLKVYEKHSAQ

B

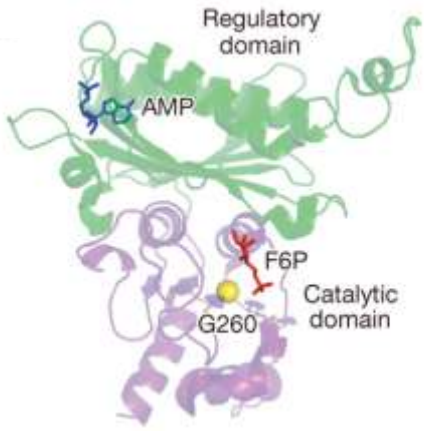

Fig. 10 A) Sequence of FBP1 protein with the methionine at position 1, 83 and 85 indicated in bold. **B)** Crytsal structure of FBP1 showing the regulatory domain (green) with bound AMP molecule and the catalytic domain (purple) bound F6P molecule.

2) Full length translated FBP1 protein could be subject to cleavage by proteolytic enzymes such as caspases, following activation. A scan of the amino acid sequence of FBP1 located a few caspase cleavage sites none of which, if utilized, was likely to yield a protein fragment of size 25-27kD.

3) Utilization of an internal translation start site on the full-length transcript could result in translation of a shorter protein. This appears to be the most likely mechanism. We hypothesize that the T cells are utilizing an alternative internal start site to translate the shorter isoform. The amino acid sequence of FBP1 (Fig. 10A) contains two methionine residues at position 83 (M83) and 85 (M85), either of which can potentially serve as initiator methionines for translation. The size of a protein translated starting at either of these residues is predicted to be 25-27 kD. The methionine residue at position 85 is conserved in mouse FBP1, where the shorter isoform was previously detected (18).

An alternative internal start site for FBP1 could have the following regulatory effect. As described in the introductory chapter, FBP1 harbors a regulatory domain at the N terminus and a catalytic domain at the C-terminus (Fig. 10B). The N-terminal regulatory domain can interact with AMP which inhibits its activity. We hypothesize that utilization of either M83 or M85 for initiation of translation by activated T cells would generate a constitutively active enzyme harboring only the catalytic domain.

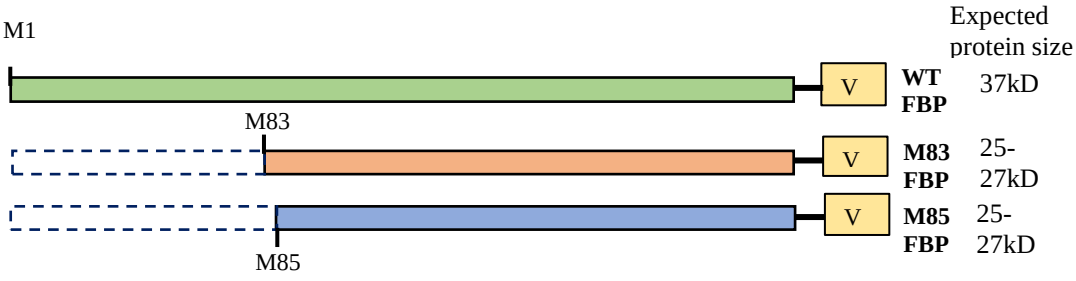

Fig. 11- Diagrammatic representation of truncated FBP1 isoforms translated from the FBP1 expression plasmids, with M1, M83 and M85 as the starting methionine, and their expected protein sizes

To begin to test the hypothesis that the shorter FBP1 is constitutively active, we subcloned full length FBP1, M83 and M85 cDNAs carrying a 3' V5 tag into EcoRI and HindIII restriction sites of the pCDNA 3.1(-) mammalian expression plasmid (Fig. 11).

Selected constructs were sequenced for accuracy, purified and used as templates for *in vitro* translation in the TnT Quick Coupled Transcription/Translation System (ProMega Biotech). *In vitro* translated full length wildtype FBP1 as well as the M83 and M85 forms were detected at the expected size after immunoprecipitation with anti-V5 tag antibody and subsequent western blotting with anti-FBP1 antibody (Fig. 12A and 12B). *In vitro* translated isoforms of FBP1 were also recognized by an antibody that could detect the shorter isoform in activated T cells. We also checked for intracellular expression by transfecting the plasmids into human embryonic kidney cell line HEK293 and subjecting cell extracts to immunoprecipitation with anti-V5 antibody. Along with the full length FBP1 band at 37kD, we observed an additional band at 27kD in HEK293s transfected with WT FBP1. The full-length construct expressed both forms in HEK293, confirming previous reports and validating in part that the shorter peptide is an FBP1 isoform. However, M83 FBP1 and M85 FBP1 were not being translated in HEK293 cells. Possible implications of this observation will be discussed in the next section.

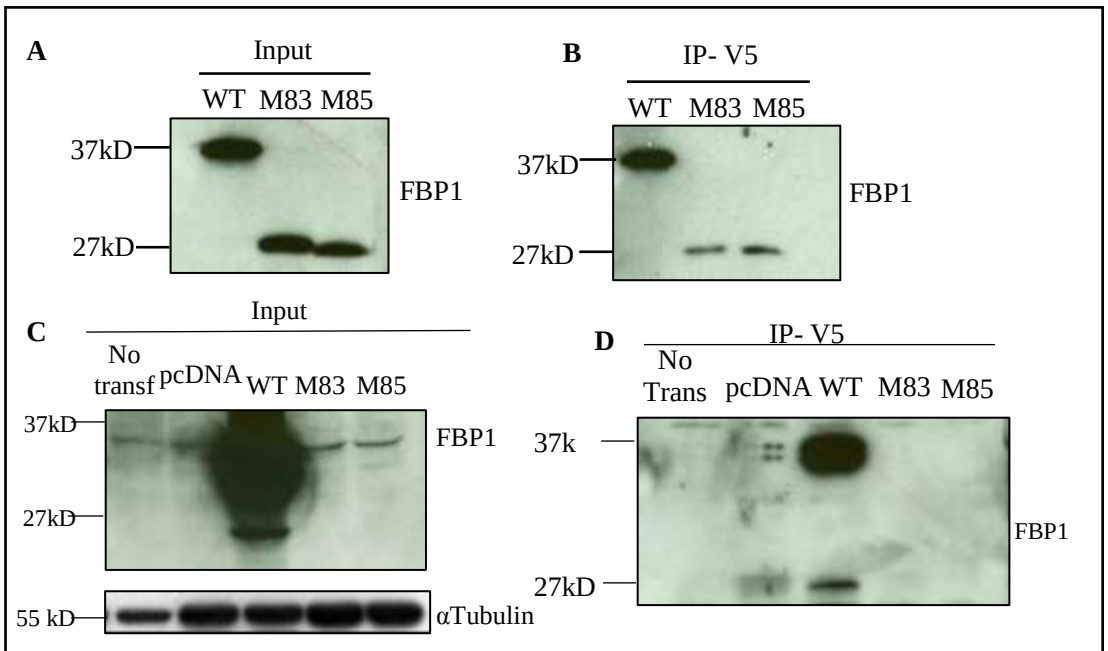

Fig. 12- A) and **B)** Immunoprecipitation of FBP1 constructs translated in vitro, with anti-V5 tag antibody, and subsequent western blotting with ThermoFisher anti FBP1 antibody **C)** and **D)** Immunoprecipitation of FBP1 constructs with anti-V5 tag antibody from lysates of HEK293 cells transfected with FBP1 constructs. No Transf- No transfection, pcDNA- HEK298 cells transfected with empty pcDNA 3.1(-) vector.

These results strengthen the hypothesis that the additional shorter band detected in T cells is indeed FBP1. The shorter isoform that appeared in HEK293s was after immunoprecipitation by an anti-V5 tag antibody, which suggests an intact C-terminus. This further supports the hypothesis that it is the N-terminus of the protein that is lost in the shorter isoform. Targeted metabolism had suggested the presence of a catalytically active FBP1 in stimulated T cells, which we hypothesize is likely to be a constitutively active shorter isoform.

6. Both isoforms of FBP1 are in separate larger complexes-

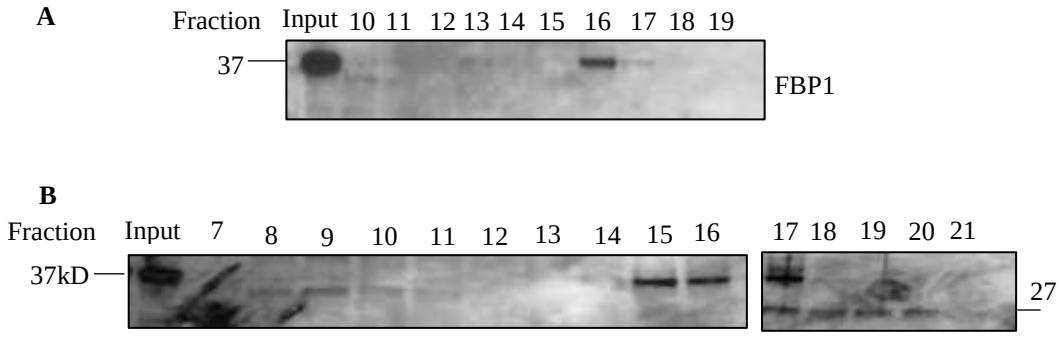

Fig. 13. Western blots of protein fractions collected from the Superose column. **A)** 37kD isoform of FBP1 fractionates in a high molecular weight complex in unstimulated T cells. **B)** Both isoforms on FBP1 in stimulated T cells elute in different high molecular weight complexes. Cells were lysed in Buffer A, and loaded on Superose 6 columns. 1mL fractions were collected every 10 mins, and purified protein was analyzed by SDS-PAGE

We have begun to characterize the two isoforms as we test our hypothesis that the shorter form is an enzymatically active protein, while the full-length lacks activity. To determine whether the two isoforms fractionate together or are components of independent protein complexes, cell extracts from unstimulated and stimulated T cells were fractionated on a Superose 6 gel filtration column, which separates cellular protein complexes by size. Western blots of fractionated extracts (Fig. 13A and 13B) show that the 37 kD isoform of FBP1 consistently eluted between fraction 15 and 17 (~200 kD), whereas the 27 kD

isoform emerged in fractions 17 to 20 (~80 kD) The ability to physically separate the two isoforms in their native forms will enable us to test them individually for catalytic activity. The multiprotein complexes are likely to be physiologically relevant and may offer valuable insights into the regulation and catalytic activity of this interesting metabolic enzyme.

Chapter 3: Discussion

The activation process in T lymphocytes is accompanied by significant reprogramming of metabolic networks including a switch to aerobic glycolysis, to fuel increased biosynthetic demands of proliferation (3). Similar metabolic alterations occur in cells as they acquire tumorigenicity. The Kelekar lab is interested in T cell metabolism as a model for metabolic reprogramming in proliferating systems. Early studies with a specific singly-labeled glucose isotopomer led to the discovery that FBP1 was expressed in T cells. In fact, FBP1was the only gluconeogenic enzyme expressed in T cells, leading us to hypothesize that it harbors a novel, non-gluconeogenic function.

We also report, for the first time, the presence of a shorter isoform of FBP1 unique to activated T cells, that is induced within an hour of activation, and remains upregulated for 96 hours, post stimulation. We hypothesize that the cells use an internal methionine residue either at position 83 or 85 as a start site for translation of the short peptide. The methionine at position 85 is conserved in mouse FBP1, where the shorter isoform was previously described (18). The codon for M85 also has a better Kozak consensus, favoring its probability as a start site. AMP and cAMP bind to the N-terminus of the protein, allosterically inhibiting its catalytic activity. Thus, a shorter protein devoid of the N-terminal regulatory domain would be predicted to be constitutively active.

To further characterize this isoform and test our hypotheses, we generated plasmid constructs that would express the predicted shorter FBP1 proteins. These isoforms could be translated in an *in vitro* system, but were unable to produce detectable protein when transfected into HEK293 cells. However, both the shorter isoform and the full-length protein were detected, in HEK293 cells transfected with the WT FBP1 plasmid construct. It is possible that the shorter isoforms, being constitutively active, are not favored by the tumor cell line, in keeping with reports that have found FBP1 to be downregulated in cancer. Alternatively, an unknown regulatory protein may need to interact with the region of FBP1 transcript coding for the N terminus (missing in the deletion constructs) to facilitate intracellular translation from an internal methionine residue.

We hypothesize that the short FBP1 isoform is constitutively active. However, its catalytic activity remains to be determined. The enzymatic activity of *in vitro* translated

products immunoprecipitated with the V5 antibody will be assayed in the following manner. Translated protein or cell lysates will be added to a reaction mixture containing the required substrates NADP+ and fructose-6-phosphate. A readout for the phosphatase activity will be obtained by coupling it with exogenous phosphoglucose isomerase and glucose-6-phosphate dehydrogenase and measured by following NADPH formation (15). Using this assay, we will also evaluate FBP1 activity in T lymphocyte extracts fractionated by size-exclusion chromatography (shown in Fig 13). This approach will allow us to test our hypothesis of a constitutively active isoform in activated T cells and the inhibitory effect of AMP on the enzymatic activity.

In the metabolic framework of a cell, FBP1 occupies a crucial junction between glycolysis, the PPP and gluconeogenesis. The observed dual labeling suggests a molecule of glucose being diverted to the PPP more than once, for which catalytic activity of FBP1 is necessary (Fig. 5B). The absence of other gluconeogenic enzymes indicates a non-gluconeogenic role for FBP1 which may be related to the PPP in activated T cells. We hypothesize that FBP1 facilitates an increased flux of glucose into the pentose phosphate pathway, to increase the production of NADPH in the cells. Soon after activation, T cells experience a phase of high metabolic activity, which can lead to increased free radical generation in the cells. There is also an elevated demand for biosynthetic precursors like lipids, for synthesis of membranes (4). Increased NADPH may serve both these purposes, being utilized to decrease the ROS in the cells, or to increase the rate of lipid synthesis. Preliminary observations indicate an inverse correlation between levels of FBP1 isoform and ROS over time in activated T cells. The enzyme may also have a role in increasing nucleotide synthesis through the PPP and enhancing proliferation, which can be monitored at the time cells enter a phase of rapid cell division. An immediate and stable induction of FBP1 upon stimulation indicates a significant physiological role for FBP1 in the metabolic reprogramming in T cells. More investigations, such as the use of an FBP1 specific inhibitor, would help to accurately describe the physiological role of FBP1 in T cells. Approaches to further investigate FBP1 expression in T cells include studying its expression under metabolic stress, analyzing the effect of glucose deprivation on FBP1

expression. It would also be important to investigate FBP1 expression in differentiated T cell populations and to study the effects of FBP1 inhibition on T cell differentiation in *vitro* as well as in *vivo* using mouse models.

Experiments are also underway to determine the sequence identity of the shorter isoform via mass spectrometric analysis. Size exclusion based chromatography suggest that both isoforms of FBP1 are components of larger multiprotein complexes. Identification of the components of these complexes could offer clues to their physiological role in T cell activation. FBP1 was also the only gluconeogenic enzyme expressed in the acute myeloid leukemia cell (AML) line HL60 (data not shown), which we have used as a positive control in our studies. FBP1 has been reported to be expressed in HL60 cells upon differentiation, but little is known about its physiological significance (16).

Recent publications have shown an association of gastric, breast and renal cancers with decreased FBP1 expression (10-12, 17). Our observations in T cells suggest that FBP1 plays a significant role in T cell activation and proliferation. It would also be interesting to study its expression in AML and other such malignancies. Expression of FBP1 in a non-gluconeogenic setting, such as a proliferative cell system, is yet to be explored. The results obtained so far lay the foundation for a significant role for FBP1 in T lymphocyte activation, differentiation, and effector functions via T cell metabolism. Further investigations into novel functions of FBP1 could offer useful insights into T cell physiology, autoimmune disorders and cancer immunotherapy, and could eventually aid in the development of effective therapeutics.

Chapter 4: Materials and Methods

Cell lines and cell culture-

HL60, cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, NEAA and 4 mM L-glutamine. HepG2 and HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Primary T cell isolation and activation-

Human PBMCs were obtained from healthy donors via Memorial Blood Centre, St. Paul, MN, USA and purified from red blood cells using Histopaque (Sigma Aldrich). CD3+ T cell were isolated from PBMC's by negative separation using Pan T cell isolation kit (Miltenyi). Briefly, PBMCs were passed through MACS separation columns (Miltenyi), and allowed to recover in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, NEAA and 4 mM L-glutamine overnight. At 18 hours, live T cells were recovered by Ficoll density gradient centrifugation and allowed to rest for 2 hours in RPMI medium. Culture dishes were coated with 5µg/mL of anti-CD3 antibody (BioLegend, San Diego, CA, USA) and incubated for 2 hours at 37°C. T-cells were then transferred to the coated culture plates and 5µg /mL of anti-CD28 antibody was added to the culture medium. The cells were collected 24 hours after activation unless specified otherwise.

Metabolomic labelling experiments-

Live, healthy T cells were recovered by Ficoll density gradient centrifugation and activated as described earlier. At T_0 $10E^6$-$20E^6$ cells per sample were pelleted and washed in glucose free medium. For glucose labeling experiments, cells were resuspended at 1E6 cells/ml in glucose free medium supplemented with 10% dialyzed FBS, NEAA, and 4mM L-glutamine for 1 hour. After starvation, cells were supplemented with 10mM [6-^{13}C] labeled glucose for 3 hours. Cells were then pelleted and washed 1X in ice cold PBS.

Pellets were then resuspended in 100-200µl -20°C methanol, snap frozen and stored at -80C. Liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) were used for identification and quantification of labeled metabolites of all samples. [6-^{13}C] glucose was purchased from Cambridge Isotopes (Tewksbury, MA USA).

FBP1 knockdown experiments-

T cells were isolated from PBMCs as described before, and live cells were recovered by Ficoll density gradient 18 hours after isolation. The cells were rested for 2 hours after recovery, and transfected with 100mM of FBP1 specific and non-targeting siRNA respectively (Dharmacon), using the Neon Transfection system (Invitrogen). The cells were cultured overnight in RPMI 1640 medium supplemented with 10% FBS, NEAA and 4mM L-glutamine. On the next day, plates were coated with anti CD3 antibodies for 2 hours as described before, and 24 hours after transfection, the cells were activated as described earlier. Protein and mRNA were harvested from the cells 24 hours after activation.

Plasmid Constructs-

FBP1 pcDNA 3.1 V5-His-TOPO plasmid construct was a generous gift from from Dr. M. Celest Simon. WT FBP1 (full length), FBP1 M83 and FBP1 M85 were amplified from the source vector to carry a 5' EcoRI and a 3' HindIII restriction site along with a 3' V5 tag. The forward primers used for the PCR reaction were:

FBP1 full length- 5'- CATGATGAATTCACCATGGCTGACCAGGCGCCCTTC- 3',

FBP1 M83- 5'- CATGATGAATTCACCATGAACATGTTAAAGTCA- 3',

FBP1 M85- 5'- CATGATGAATTCACCATGTTAAAGTCATCCTTT- 3'.

The reverse primer used was 5'- CATGATAAGCTTTCACGTAGAATCGAGACC- 3'. The vector, pcDNA 3.1 (-) was dephosphorylated with alkaline phsophatases. The amplified PCR band was PCR purified by Qiagen PCR purification kit, digested with EcorI and HindIII, and cloned into pcDNA 3.1(-), digested with the same restriction

enzymes. The inserts were ligated with the digested plasmid in 1:4 (plasmid: insert) ration using T4 DNA ligase. The plasmid was then transformed into DH5α competent E. coli cells. After selection of transformed bacteria by Carbenicillin, E. Coli were cultured in large quantities, and the plasmid DNA was isolated using Qiagen Maxi Prep kit. The DNA insert cloned into pcDNA 3.1 was sequenced to confirm that there were no mutations.

In-vitro translations of plasmid constructs-

In vitro translations of plasmid constructs were carried out using the TnT Quick Coupled Transcription/Translation Systems (Promega). The components for a 20µL mix were: 16µL TnT Quick Master mix, 0.5µL Methionine, 1 µL plasmid DNA (0.5µg/ µL), 2.5µL nuclease free water. The mixture was incubated at 30°C for 90 minutes and stored at -20°C until further use.

HEK293 Transfections-

Transfections of 293s was done in 6- well plates. 3µL of Fugene reagent (Promega) and 97µL of Opti-MEM medium (Promega) were incubated together for 5 minutes. To this mixture, 1µg of respective construct (WT, M83 and M85) was added and incubated for 15 minutes. The cells were fed with DMEM medium with 10% serum, and the transfection mix was added to the wells. The cells were harvested 48 hours after transfection and lysed in Dignam Buffer A.

Western Blots-

Cells were washed with 1x cold PBS and lysed with RIPA buffer (50mM Tris-HCl [pH 7.5]), 150mM NaCl, 0.5% v/v sodium deoxycholate, 1% v/v Nonidet P-40, 0.1% SDS supplemented with protease and phosphatase inhibitor cocktails. Lysates were incubated on ice for 10 minutes and spun at 10000 rpm for 10 minutes at 4°C. The supernatant was collected for further analysis. 30-40 µg of protein was boiled in 2x Laemlli buffer and resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies specific for FBP-1 (ThermoFisher-PA5-30028, Santa Cruz- 166145, Santa

Cruz- 376388) Chemiluminescence reactions were carried out using ECL plus kit (Amersham).

Immunoprecipitation-

HEK293 cells were lysed in Dignam Buffer A (10mM HEPES, 0.015mM $MgCl_2$, 10mM KCl, 0.05% IGEPAL). Cell lysate equivalent to 200µg of protein was incubated with 1.5µg of anti V5 tag antibody (ProteinTech 66007-1-Ig) at 4°C overnight. The antibody-protein complex was captured on ProteinG-Agarose beads (Invitrogen). Immunoprecipitates were resolved by SDS-PAGE and detected by western blotting as described earlier. For immunoprecipitations with in-vitro translations, 6µL of translated mix was incubated with 1.5µg of anti V5 tag antibody overnight. The complex was captured and processed as described earlier.

RNA isolation-

RNA was isolated from HepG2 and T cells with TRIZOL reagents (Invitrogen) as per the manufacturer's protocol. The isolated RNA was dissolved in RNase free water. RNA concentrations were determined using the Thermo Scientific Nano Drop 2000 Spectrophotometer. Samples were stored at -80°C until further use.

Reverse Transcription-

cDNA synthesis was performed with 500-800ng RNA using the DyNAamo Synthesis Kit for qRT-PCR (Thermo Scientific) per the manufacturer's protocol using random hexamer primers. The reaction program is: primer extension at 25°C for 10 min, cDNA synthesis at 37°C for 1 hour and reaction termination at 85°C for 5 min. cDNA products were stored at -20°C until further use.

PCR to measure gluconeogenic enzyme mRNA levels in T cell, HepG2 cell mRNA-

The oligonucleotide sequences for each primer are as follows-

Primer	*Sequence*
FBP1 full length FWD	5’-ACAGCAGTCAAAGCCATCTC-3’
FBP1 full length REV	5’-GGTTCCACTATGATGGCGTG-3’
G6P FWD	5’-GAGACTGGCTCAACCTCGTC-3’
G6P REV	5’-CCTGGTCCAGTCTCACAGGT-3’
PEPCK FWD	5’-AAGAGACACAGTGCCCATCC-3’
PEPCK REV	5’-ACGTAGGGTGAATCCGTCAG -3’

Thermocycler PCR conditions were as follows: 1) 94°C for 2 min 2) 94°C for 30 sec 3) annealing temperature for 30 sec 4) 72°C for 1 min 5) Repeat steps 2-4 for 35 cycles 6) 72 °C for 4 min. The annealing temperature for FBP1 primers was 57°C, for G6P and Actin primers was 59°C, and for PEPCK primers was 62°C. PCR products were run on a 1.5% agarose gel, and bands were visualized under UV light.

Fractionation of proteins/FPLC-

The Superose 6 column was pre-equilibrated with Dignam Buffer A. T cells were isolated and activated as described previously, and collected after 24 hours of activation and washed with 1X cold PBS. Cells were then lysed in Dignam Buffer A and subsequently fractionated on a GE/Amersham Superose 6 FPLC column. For each fractionation, 1 mL fractions were collected every 10 minutes and samples were analyzed by SDS-PAGE and western blot after TCA precipitation.

TCA precipitation-

Protein was precipitated from fractionation samples by adding 250µL of trichloroacetic acid (TCA) to 1mL of sample, incubated at 4°C for 10 mins, and spun at 140000 rpm for 5 mins at 4°C. The protein pellet was washed with cold acetone and heated at 95°C for 10-15 minutes to drive off the acetone. The samples were then prepared for western blot as described before.

FBP1 half-life-

CD3+ T cells were isolated from human PBMCs and cultured overnight as described before. After recovery of live cells by Ficoll gradient, the cells were activated with anti-CD3 and anti-CD28 antibodies. Unstimulated and stimulated T cells were then treated with DMSO (control) or 10µg/mL Cycloheximide (Sigma). The cells were harvested at each time point after treatment, lysed in RIPA buffer and analyzed for protein expression by western blotting as described before.

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