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THE CYTOKINE, INTERLEUKIN-7, TRANSCRIPTIONALLY REGULATES THE GENE EXPRESSION OF THE HEXOKINASE II GENE TO MEDIATE GLUCOSE UTILIZATION IN T-LYMPHOCYTES

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ABSTRACT

The cytokine, interleukin-7 (IL-7), has essential growth activities that maintain the homeostatic balance of the immune system. Little is known of the mechanism by which IL-7 signaling regulates metabolic activity in support of its vital function in lymphocytes. We observed that IL-7 deprivation caused a rapid decline in ATP levels that were attributable to loss of intracellular glucose retention. To identify the transducer of the IL-7 metabolic signal, we examined the expression of three important regulators of glucose metabolism, the glucose transporter, GLUT-1, and two glycolytic enzymes, Hexokinase II (HXKII) and phosphofructokinase-1 (PFK1), using an IL-7-dependent T-cell line and primary lymphocytes. We found that in lymphocytes deprived of IL-7 loss of glucose uptake correlated with decreased expression of HXKII. Re-addition of IL-7 to cytokine deprived lymphocytes restored the transcription of the *HXKII* gene within 2 hours, but not that of *GLUT-1* or *PFK1*. IL-7-mediated increases in HXKII, but not GLUT-1 or PFK-1, were also observed at the protein level. Inhibition of HXKII with 3-Bromopyruvate or specific siRNA decreased glucose utilization, as well as ATP levels, in the presence of IL-7, while over-expression of HXKII, but not GLUT-1, restored glucose retention and increased ATP levels in the absence of IL-7. This IL-7 mediated HXKII gene expression was abrogated with inhibition of JNK pathway. IL-7 also increased activation of AP-1 complex and DNA binding of JunD, a transcriptional complex thought to be negative regulator of proliferation. We found that over expression of HXKII caused cell cycle arrest and cell death, indicating that a potent IL-7 signal could produce negative growth signals. We conclude that IL-7 controls glucose utilization by regulating the gene expression of HXKII

through activation of JNK-JunD pathway, suggesting a mechanism by which IL-7 supports bioenergetics that control cell fate decisions in lymphocytes.

This dissertation is dedicated to the memory of the loving person who stood behind me along this long journey, and fed me with her love, courage and kindness, to the memory of my mom. This work is also dedicated the shining stars of my life, my kids Hajar and Ibrahim.

ACKNOWLEDGMENTS

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LIST OF ACRONYMS AND ABREVIATIONS

2-DOG: 2-Deoxyglucose

2-NBDG: 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose

3-OMG: 3-o-methyl-glucose

AP1: Activating Protein 1

ATP: Adenosine triphosphate

BCL-2: B-cell Lymphoma 2

BCL-XL: B-Cell Lymphoma-Extra Large

Cdc25A: Cell division cycle 25 homolog A

ChIP: Chromatin Immunoprecipitation

FBS: Fetal Bovine Serum

FITC: Fluorescein isothiocyanate

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLUT1: Glucose transporter 1

HIV: Human Immunodeficiency Virus

HXK: Hexokinase

HRP: Horse Radish Peroxydase

IL-2: Interleukin 2

IL-4: Interleukin 4

IL-7: Interleukin 7

IL-7R: Interleukin 7 receptor

IL-15: Interleukin 15

JAK1: Janus Activating Kinase 1

JAK3: Janus Activating Kinases 3

JNK: C-Jun N-terminal Kinase

MAPK: Mitogen-activated protein kinases

NADH: Nicotinamide adenine dinucleotide

PFK: Phosphofructokinase

qPCR: Quantitative polymerase chain reaction

RPMI: Roswell Park Memorial Institute medium

SAPK: Src activated protein kinase

Ser: Serine

SH2: Src Homology domain 2

siRNA: Small Interfering Ribonucleic Acid

Src: Sarcoma

STAT: Signal Transducers and Activator of Transcription

TCA: tricarboxylic acid cycle

Thr: Threonine

VDAC: Voltage dependent Anion channel

GENERAL INTRODUCTION

Numerous homeostatic mechanisms help maintain T-cells at fairly constant cell count. These mechanisms are mainly regulated by cytokines, of which Interleukin-7 (IL-7) was shown to be the most essential for T-cells survival and proliferation. IL-7 was initially described as a B-lymphocytes growth factor (1). Later, a non-redundant role of IL-7 in T-cells lymphopoiesis was described (2). Adoptive transfer of T-cells into IL-7 deficient mice or injection of monoclonal IL-7 antibodies into wild type mice hindered the survival of T-cells, while over expression of IL-7 increased the size of the naïve T cells pool establishing IL-7 as a major cytokine and growth factor for T-cells (2). Deletion of IL-7 or the IL-7 receptor decreased significantly the number of thymocytes, bone marrow B cells precursors, peripheral B-cells, CD4, and CD8 T cells (3,4).

IL-7 is a 17.5 kD cytokine produced primarily by hematopoietic stromal and epithelial cells, including fibroblastic reticular cells found in the lymph nodes, bone marrow stromal cells and thymic epithelial cells, as well as other non-lymphoid sites such as liver and intestinal epithelial cells, endothelial cells, fibroblasts, keratinocytes and smooth muscle cells (5-10). IL-7 is also produced in smaller amount by dendritic cells and macrophages (11). IL-7 can be found in multiple organs including the brain (12,13). In order to identify the tissues in which IL-7 is produced, two studies cloned the IL-7-promoter in line with fluorescent or marker gene to locate expression (14,15). Different regions of the IL-7 promoter were used in each study with disparate outcomes. In one report, only thymic production of IL-7 was found (14), while in the other study, IL-7 was produced in multiple tissues including non-lymphoid organs (15). These

results suggest that IL-7 gene expression is under tissue-specific control and that its production could be induced under conditions that are yet to be fully understood.

IL-7 delivers its signal to lymphocytes by binding to its receptor IL-7R. IL-7R is a heterodimeric receptor constituted of α and γc chains bound respectively to Janus Activating Kinases 1 and 3 (JAK1 and JAK3). Binding of IL-7 to IL-7R activates JAK1 and JAK3, which in turn phosphorylate and activate the transcription factor, STAT5. Engagement of the IL-7R by ligand can initiate other signaling pathways, in addition to the JAK/STAT pathway, which include the PI3K/AKT pathway, and, to lesser extent, the MAP kinase (MAPK) pathway (16). Engagement of the IL-7R leads to the regulation of genes expression that maintain the survival and proliferation of T-cells. Deletion of IL-7, IL-7R, γc, JAK3 or STAT5 caused a severe immunodeficiency due to defects in T cell development, both in humans and mice (17-21). IL-7 activated PI3K signaling was been implicated in cell survival (22). IL-7 activated STAT5 was essential for T-cells survival and proliferation (23). MAPK signaling pathway was identified as an important pathway for IL-7 related T-cell proliferation and activation (24). MAPK signaling was also implicated in regulation of metabolic activities (25). The MAPK family of signal transduction include different Ser/Thr kinases such as p38 MAPK, the extracellular signal related kinase (ERK), and c-Jun N terminal kinase (JNK/SAPK) (26). It has been reported that JNK kinase is activated in cancer and support tumoreginesis (27,28).

The IL-7R is expressed in T-cells at all stages of their development (29), but this expression is downregulated whenever T-cells undergo any major expansion. For instance, CD4$^-$CD8$^-$ double negative T-cells express the IL-7R and acquire IL-7 signals for their survival. Then, upon proliferation and differentiation of these cells into CD4$^+$CD8$^+$ double positive, they

abrogate expression of the IL-7R (30). This ensures that a maximum numbers of T cells are maintained by exposure to a limiting amount of IL-7 (31). Consistent with this idea, forced high expression of transgenic IL-7R on mature T cells caused a significant reduction in the overall size of the mature T cell pool (31), as well as in CD4$^+$CD8$^+$ double positive (DP) cells, and increased death of CD4$^-$CD8$^-$ double negative (DN) cells (32).

T-cells need energy to support their survival and proliferative activities. T-cells utilize glucose and glutamine as the main sources for this energy. Resting T-cells consume glucose and other nutrients at a low rate (33-35), and produce just enough energy to maintain basal housekeeping functions (36). This low rate uptake of glucose was found to be under strict regulation of cytokine signaling. When these signals are absent, glucose metabolism is abrogated and ATP levels decrease in a way that no longer sustains the survival of the cells; apoptosis is then activated, leading to cell death (37-39). It has been reported that nutrient uptake in resting T-cells depends on the ability of these cells to acquire trophic signals from IL-7; the absence of IL-7 decreased glucose uptake while incubation of deprived primary T-cells with IL-7 rescued glucose uptake (40). In a complex extracellular environment, lymphocytes are exposed to a constant flux of nutrients where the abundance of the glucose exceeds the cells needs. Normally, glucose abundance is not the limiting factor for the survival or proliferation. Rather, it is the uptake of glucose by the lymphocyte that is important. Constitutive nutrients uptake results in abnormal growth and proliferation that would lead to a malignant phenotype, while defects resulting in an abrogation of this uptake could lead to increased cell death and lymphopenia. Therefore, glucose uptake is under strict regulation, and this regulation is provided by cytokines (4,41).

Resting T-cells usually use oxidative phosphorylation to generate energy from glucose. Oxidative phosphorylation is a relatively slow but very efficient process. After the glucose is transferred to the cytosolic space by the glucose transporters, GLUT, and phosphorylated by Hexokinases (HXK), it enters the tricarboxylic acid (TCA) cycle as pyruvate; the TCA cycle produces Nicotinamide Adenine Dinucleotide (NADH) molecules, which then enter oxidative phosphorylation. At the end, one molecule of glucose is completely oxidized and yields 36 molecules of ATP, H_2O and CO_2 (41). Oxidative phosphorylation is carried on in the inner membrane of the mitochondria and involves proton transfer complexes as well as an ATP synthase.

Activation of T-cells increases their proliferative activities and their energy demands. This increase is associated with a significant rise in glucose uptake and glycolytic activities (42), but a slight increase in oxygen consumption (43,44), resulting in a significant production of lactate. Activated T-cells switch from oxidative phosphorylation to glycolysis. Glycolysis, occurring in presence of oxygen, accounts for most of the energy production of these activated T-cells. Although as inefficient as it may appear, since glycolysis only produces 2 ATP molecules for each molecule of glucose consumed, this catabolic pathway appears to be favored by the actively proliferating cells because it is the fastest pathway to produce ATP and other components needed for *denovo* synthesis of macromolecules, such as proteins and nucleic acids. This switch from the oxidative phosphorylation to glycolysis in the metabolic pathway of glucose is also observed when normal cells become tumorogenic, and is known as the Warburg effect. In 1924, Otto Warburg observed that cancer cells had a tendency to degrade glucose into lactate through anaerobic glycolysis, even in the presence of oxygen at levels sufficient to

promote oxidative phosphorylation. This led him to hypothesize that cancers result from impaired mitochondrial metabolism (45,46). Although the Warburg hypothesis was wrong, his observation was reproducible.

Glycolysis is a multistep mechanism that enables the cells to utilize glucose as source for ATP production. Glucose is first transported from the extracellular space into the cytoplasmic compartment of the cell through a twelve span transmembrane glucose transporter proteins (GLUT). There are 12 isoforms of GLUT, (1 through 12), with GLUT1 being the main glucose transporter on T-cells. GLUT1 is a 55 kD protein that has high capacity, with a relatively low Km (1-2mM) (47). Once transported, glucose is then phosphorylated by HXK. There are four isoforms of HXK, I through IV. HXK I, II, and III are about 100kD and are expressed in variety of tissues (48). HXK IV is about 50 KD and is expressed exclusively in hepatocytes and pancreatic β cells (48). While HXK I and III have only one catalytic site at the C-terminal, HXK II has two catalytic sites, and both sites are sensitive to glucose-6-phosphate (48). The N-terminal site of HXK I and II contains an amino acid sequence of 21 amino acids that is essential for the binding of these HXK to Voltage Dependent Anion Channel (VDAC) on the mitochondria (49,50). Phosphorylated glucose is then converted to fructose 6-phosphate by Phosphoglucoisomerase, then into fructose 1,6-bisphosphate by Phosphofructokinase (PFK). Subsequent steps will lead to forming glyceraldehye-3-phosphoate and then pyruvate, which will either enter the TCA cycle or be converted to lactate and released in the extracellular space.

Three key steps have been deemed important in regulation of glycolysis; transport of glucose through the cell membrane, phosphorylation of glucose, and transformation of fructose-6-phosphate to fructose 1, 6-bisphosphate. One way by which IL-7 could regulate glucose

metabolism is through the regulation of glucose transport through the glucose transporter, GLUT1. Recent studies suggested that IL-7 increased GLUT1 trafficking to the cell surface (51), while another one showed that GLUT1 gene expression in primary T-cells increased after 72 hours incubation with high dose of IL-7 (52). However, neither study could explain the immediate increase in glucose uptake after addition of IL-7 to cytokine deprived T-cells. So IL-7 could also regulate the uptake and retention of glucose – a process mediated by HXK. Abrogation of HXK binding to mitochondria or deletion of its active site was shown to trigger apoptotic cell death (53) . The third way that could be of importance in IL-7 regulation of glycolysis is the conversion of fructose 6-phosphate to fructose 1, 6-bisphosphate by PFK. This step prepares glucose for its conversion to 3 carbon molecules. Other nonrelated study have also shown that IL-3 increased activities of PFK in B-lymphocytes (4)

Because of its importance for T-cell survival and its proliferative effect, IL-7 is regarded as a potent therapeutic components for the treatment several diseases, such as cancer, HIV, and some autoimmune diseases(54). Injection of IL-7 into normal mice increased T-cells number in peripheral lymphoid organs; it also accelerated lymphoid regeneration in lymphopenic mice (55-58). IL-7 therapy enhanced the response to immunization and increased the reaction against weak antigens, which makes it a possible candidate for adjuvant therapy against cancer (59). Despite these promising data, there remain problems to be addressed. Mice over expressing IL-7 showed reduced size of the thymus and decreased thymic count, especially the CD4$^+$CD8$^+$ double positive subset (60). Sustained injection of IL-7 over long period of time caused a decline of its proliferative effect on T-cell populations and induced B cell lymphomas (61,62). This negative effect of high dose IL-7 raises concerns about the optimal dose needed to induce a

beneficial outcome for the body. It is the unknown what is the effect IL-7 dose would have on the metabolic aspect of T-cell homeostasis.

Our study sheds light on one of the most important aspects of IL-7 maintenance of the T-lymphocyte homeostasis: regulation of metabolic activity and nutrient uptake. In this study, we show for the first time that IL-7 mediates glucose uptake in T-cells through transcriptional regulation of HXKII gene expression, and that this regulation is unique in the way that it is mediated - through activation of JNK pathway and recruitment of JunD to form the AP1 complex the binds to the HXKII promoter. Our findings presents a novel understanding, from the bioenergetic aspect, as to what could be causing the negative growth outcome of high IL-7 doses, since we show that over expression of HXKII leads to cell cycle arrest and increases cell death.

INTERLEUKIN-7 MEDIATES GLUCOSE UTILIZATION IN LYMPHOCYTES THROUGH TRANSCRIPTIONAL REGULATION OF THE HEXOKINASE II GENE

Introduction

Maintenance of lymphocyte survival and proliferation is dependent upon the ability of immune cells to acquire sufficient nutrients to support cellular metabolism. Growth factors enable this process by transducing signals that promote metabolic activity. Of these, the cytokine, interleukin-7 (IL-7), first identified as a B-cell and later T-cell growth factor (18,63,64), is a potent agent for immunoreconstitution through its diverse activities supporting survival and proliferation. IL-7 is generally considered a product of stromal or accessory cells (reviewed in (30,65,66)). The receptor for IL-7 is expressed on lymphocytes and consists of the unique IL-7Rα chain (IL-7R) and the common cytokine γ chain (γc) that is also shared by the receptors for IL-2, IL-4, IL-9, IL-15, and IL-21 (67). Upon IL-7 binding, the two receptor chains heterodimerize (68), which leads to the activation of the receptor associated Janus kinases, JAK1 and JAK3 (69). The activated JAK proteins in turn phosphorylate specific residues on the IL-7R creating docking sites for signaling molecules that have Src homology 2 (SH2) domains such as the transcription factor, STAT5 (signal transducers and activators of transcription 5) (70), and to a lesser extent STAT1 and STAT3 (71). Multiple gene products are produced from engagement of the IL-7R which include anti-apoptotic proteins, signaling molecules, growth factors and receptors (72).

One of the ways by which IL-7 supports T-cell development and homeostasis is through the maintenance of survival by up-regulating the expression of anti-apoptotic members of the BCL-2 family, such as BCL-2 and BCL-XL (72-74), or down-regulating pro-apoptotic BCL-2 family members, such as BAX (75,76) or BIM (77). In addition to survival, IL-7 also has a proliferative function, promoting the replication of T-cells by preventing the degradation of the cell cycle-activating phosphatase, Cdc25A (78), or repressing the cell cycle inhibitor, p27[Kip1] (79). Yet the activities of survival and proliferation mediated by IL-7 are dependent on a T-cell's ability to uptake nutrients, such as glucose or glutamine, from the environment to support metabolic needs (16,80,81). Despite their importance, the essential metabolic targets of an IL-7 signal transduction pathway have not been characterized.

Actively dividing lymphocytes have an increased energetic demand, which is mostly met through the metabolism of glucose. Resting T-cells, in contrast, have minimal energetic requirements (82-84). T-cells can satisfy their needs for ATP from glucose metabolism through the processes of glycolysis or oxidative phosphorylation (TCA cycle). Glycolysis is a rapid process that can take place in the cytosol independent of oxygen consumption. Through glycolysis, one molecule of glucose generates two molecules of ATP. Oxidative phosphorylation is a longer process that generates more ATP (30 ATP) from glucose and pyruvate and takes place in mitochondria. It is generally accepted that quiescent T-cells utilize oxidative phosphorylation to generate ATP, while proliferating T-cells utilize glycolysis, especially when the extracellular environment is rich in glucose (44,85).

One mechanism through which cytokines, like IL-7, could control glucose metabolism in T-cells is by increased trafficking of the glucose transporter, GLUT-1, to the cell surface (51).

GLUT-1 is the main glucose transporter expressed in T-cells (40). However, one study showed that during T-cell activation, glucose uptake increased well before GLUT-1 expression was observed (84). Moreover, IL-7 itself was able to increase glucose uptake in resting T-cells without affecting the levels of GLUT-1 protein (51), suggesting that the activity of other factors in the glucose metabolic pathway could be targets for IL-7 regulation. Possible targets are the Hexokinase (HXK) enzymes or Phosphofructokinase 1 (PFK1). Hexokinase I (HXKI) and II (HXKII) convert glucose to glucose-6-phosphate, retaining the hexose inside the cell. In a subsequent step, PFK1 catalyzes the conversion of fructose-6-phosphate to fructose-1-6-bis-phosphate, a process that consumes ATP. Thus both HXK and PFK mediate two key steps in glycolysis – the retention of intracellular glucose after transport and the irreversible transfer of a phosphate from ATP to fructose-6-phosphate. Evidence that cytokines support glycolytic activity comes from studies with the B-cell line, FL5-12, in which IL-3 increased the activities of HXK and PFK (4). HXK may also have anti-apoptotic activities as well, since others have reported that the transfer of HXK to mitochondria may protect the cells from death induced by cytokine-withdrawal (38) . This idea is offset by the fact that increased ATP, as generated from glycolysis, can promotes the process of apoptosis (86).

Glucose metabolism is thus a multistep process that could be regulated by IL-7 at different points. To characterize the critical transducers of an IL-7 metabolic signal, we used a unique IL-7 dependent T-cell line, D1, as well as primary murine T-cells to show that IL-7 induces glucose uptake by controlling the gene expression of HXKII. We found that HXKII protein levels followed IL-7 mediated increase in HXKII mRNA and that inhibition or forced expression of HXKII in T-cells could control glucose uptake and ATP levels independently of

IL-7. Our results demonstrate that HXKII is a key regulatory target in the metabolic pathway controlled by IL-7 whose activity is decisive in determining the ultimate fate of a lymphocyte.

Materials and Methods

Cell lines, mice, and T-cell purification:

The IL-7-dependent T-cell line, D1, was established from pro-T cells isolated from a p53$^{-/-}$ mouse as previously described (72). D1 T-cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin (5000 u/ml)-streptomycin (5000 μg/ml), 50 μM β-Mercaptoethanol, and 50 ng/ml recombinant human IL-7 (Peprotech, CA). Primary lymph node (LN) T-cells from 8-12-wk-old C57BL/6 mice were isolated by mechanical teasing and cultured ($5x10^6$ cells/ml) in the presence of 150 ng/ml of IL-7 (Peprotech). To enrich for IL-7 dependent cells, as needed for experiments, we used our published method of *ex vivo* expansion and cultured LN T-cells with 150 ng/ml of IL-7 for 7 days (87). Animal use was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida.

Plasmids and Nucleofection for transient gene expression:

Plasmids used for transient expression of HXKII and GLUT1 were pcDNA-HXKII (a kind gift from Dr. Wilson, and Dr. De Xon, Michigan State University, MC) and prGT3-GLUT-1 (a kind gift from Dr. Birnbaum, Howard Hughes Medical Institute, MD). To transiently express the plasmids, D1 and primary T-cells were "nucleofected" using the Murine T-cell Nucleofection kit (Amaxa), following the manufacturer's protocol. Briefly, $1x10^6$ T-cells were

incubated with (4μg) plasmid DNA in 100μl of the mouse T-cell solution, placed in a cuvette within the Nucleofector device and electroporated with the specific program optimized for mouse T-cells. Nucleofected T-cells were incubated in the supplemented media with or without IL-7 for 4-8 hours prior to analysis.   In D1 T-cells nucleofection efficiency averaged approximately 40-50% expression of the target gene with viabilities ranging 60-80%.

Glucose uptake, ATP assay and Apoptosis assay:

D1 or primary T-cells were incubated in glucose-free, serum-free RPMI (Mediatech Inc, Manassas, VA) supplemented with or without IL-7 for 1 hour.   2-Deoxy-d-[3H] glucose (2 μCi/reaction) (Sigma) was added for 3 minutes or 3-O-Methyl-[3H]-glucose (2 μCi/reaction) (GE Healthcare) was added for 1 minute. Reactions were stopped by adding 250 μl of ice-cold 0.3 mM phloretin (Sigma). Cells were then centrifuged through a cushion of 10% Bovine Serum Albumen (BSA), and lysed using 0.1% Triton X-100. Radioactivity was measured with scintillation counter (LS6500, Beckman Coulter). To measure glucose uptake by the alternative method of 2-NBDG incorporation, IL-7 dependent T-cells were resuspended in glucose-free, serum-free RPMI 1640 for 4 hours. 2-NBDG (30 μM) was then added to the cells for 5 minutes and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Signals were acquired through the FL2 channel at an emission wave length of 550 nm.   Flow cytometry data was analyzed using the software FCS express (De Novo). Statistical analysis was done using Prism software (GraphPad).   ATP levels were quantitated using the ENLITEN rLuciferase/Luciferin Reagent (Promega) following manufacturer's guidelines. Briefly lysates were prepared from 2 x 10^6 cells, to which the reconstituted rL/Lreagent was added and immediately read in a

luminometer (Perkin Elmer). Apoptosis was measured using the FITC Annexin V Apoptosis

Detection Kit (BD Biosciences) following manufacturer's protocol. Staining was assessed by

flow cytometry as described above.

Gene expression analysis by Real-time PCR:

For RNA extraction, D1 T-cells (2.0 x10^6) were centrifuged and cell pellets were resuspended in

1 ml TRIZOL (Invitrogen). One μg of RNA was converted into cDNA using iScript cDNA

synthesis kit (BioRad). Real-time PCR reactions were performed using Fast SYBR$^®$ Green Mix

(Applied Biosystems) on a 7500 Real-time PCR system (Applied Biosystems). β-actin, a

housekeeping gene which is independent of IL-7 signaling, was used as endogenous control.

Primers sequences for β-actin were previously published (88). Primer sequences for PFK1 were

previously published (89). GLUT-1 and HXKII primer sequences were developed using Primer 3

Software (http://frodo.wi.mit.edu/). GLUT1 primers: Forward

5'GCCTGAGACCAGTTGGAAGCAC3', Reverse 3'CTGCTTAGGTAAA GTTACAGGAG5'.

HXKII primers: Forward 5'CACTGGGTACTAAGGCTCAA3', Reverse

3'CGGAGTTGTTCTGCTTTGGA5'. Relative expression or RQ values were calculated using

the following formulas: ΔCt= Target gene (GLUT-1, HXKII or PFK1) - Endogenous gene (β-

Actin); ΔΔCt= ΔCt – Calibrator gene (2 or 18 hr time points for each experiment); RQ = 2$^{-ΔΔCt}$.

Statistical analysis was done using Prism software (GraphPad).

Immunoblots for HXKII, GLUT1 and PFK1:

D1 T-cells (2×10^7) were lysed using a whole cell lysis buffer (20mM TRIS-HCl (pH7.5), 150 mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 2.5mM sodium Pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml leupeptin). Cell lysates were separated on 8% SDS-PAGE gels, then transferred onto nitrocellulose membrane and immunoblotted for HXKII, GLUT1, PFK1, and p38 MAPK (as a loading control) using monoclonal antibodies against HXKII (C-14, Santa Cruz), GLUT1 (15309, AbCam), PFK1 (L684, AbGen) and p38 MAPK (C-20, Santa Cruz) respectively. Appropriate secondary antibodies used were HRP (horseradish peroxidase) conjugated. Signal was developed by enhanced chemiluminescence (ECL) (Pierce) and visualized on BioMax ML film (Kodak).

Measurement of GLUT-1 surface and intracellular expression:

Levels of cell surface GLUT-1 was assessed using the EGFP-GLUT-1 ligand (ABCYS Biologie, Paris, France) by flow cytometry. D1 T-cells were incubated with the EGFP-GLUT-1 ligand for 30 minutes. The signal for the EGFP-GLUT-1 ligand was acquired through the FL-1 channel at an emission of 520 nm using a C6 flow cytometer (Accuri). To visualize GLUT-1 surface and intracellular protein levels, we used confocal microscopy. Briefly, D1 T-Cells (10^6 cell/ml) were incubated with 100 μl of the EGFP-GLUT-1 ligand as described above. Washed cells were fixed with 4% paraformaldehyde and attached to slides by cytospin. Images were acquired with Zeiss Confocal Microscope (LSM520) at 60X. Images were analyzed using Axiovision 4.7 software from Zeiss.

siRNA inhibition:

HXKII predesigned Accell SMART pool siRNA was purchased from Dharmacon (Thermo scientific) and was introduced into cells according to manufacturer's protocol. Briefly, SMART pool siRNA combines four different siRNAs to reduce off-target effects. The Accell siRNA is also designed for optimal delivery to hard-to-transfect cells and no transfection reagents are required to introduce the siRNAs. HXKII predesigned Accell SMART pool siRNA was introduced to murine LN T-cells or D1 T-cells in the Accell delivery media and incubated for 72 hours prior to assay for glucose uptake. Delivery efficiency and siRNA specificity was tested using Accell green (FITC) non-targeting control siRNA, GAPDH specific siRNA and an Accell non-targeting control siRNA (Dharmacon).

Results

IL-7 Regulates Glucose Utilization in T-cells.

T-cells maintain their survival and proliferative activities by acquiring sufficient nutrients to support cellular metabolism, a process dependent on cytokine signals (16). In lymphocytes, glucose is transported into the cell through the transporter, GLUT-1, and intracellular retention occurs upon the phosphorylation of glucose by HXKII, producing glucose-6-phosphate – the substrate for the next steps of glycolysis. This process is shown in Figure 1A. To measure glucose uptake and retention, we used two different glucose analogs: (1) ^{3}H-2-deoxyglucose (2-DOG), a glucose molecule which cannot be metabolized but is phosphorylated by HXKII, making it an indicator of HXKII activity and glucose use, and (2) ^{3}H-3-O-Methlyglucose (3-OMG), a non-metabolizable glucose analog that is not phosphorylated by HXKII but can be used

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to assess glucose transport (Fig. 1A). In order to determine the mechanism through which IL-7 regulates glucose uptake and metabolism in T-cells, we first assessed glucose uptake in primary LN T-cells isolated from wild type (WT) C57BL-6 mice. We and others have shown that freshly isolated T-cells from lymphoid organs are mostly naïve and require both cytokine and TCR signaling for maximal stimulation (40,87,90). In order to enrich for the small number of IL-7-responsive cells found in lymphoid organs (mainly memory CD8 T cells), we used our published method of *ex vivo* expansion and cultured LN T-cells with 150ng/ml of IL-7 for 7 days (87). This dose of IL-7 was chosen after examining the effect of low and high concentrations of IL-7 and determining that expansion of IL-7 dependent cells was best achieved using high doses of the cytokine (91). After 7 days, LN T-cells were incubated with or without 150 ng/ml of IL-7 for 4, 18 and 24 hours, and glucose use was measured through incorporation of 2-DOG, the phosphorylatable glucose analog. We observed that the levels of 2-DOG in LN T-cells incubated with IL-7 were significantly increased as compared with LN T-cells that were deprived of IL-7 (Fig. 1B), indicating that an IL-7 signal is required to support glucose uptake in T-cells.

Next, we examined glucose use in response to IL-7 using an IL-7–dependent T-cell line, D1. D1 T-cells were originally generated in the laboratory of Dr. Scott Durum (NCI-Frederick) from p53$^{-/-}$ mice and depend on IL-7 at an optimal concentration of 50 ng/ml for survival and proliferation; if the concentration of IL-7 is decreased, D1 T-cells stop dividing (78) and die after 48 hours (72). Use of this cell line enabled us to dissect the mechanism by which IL-7 controls glucose metabolism in a manner not possible with primary T-cells. D1 T-cells were cultured with or without 50 ng/ml of IL-7 for the indicated periods of time. Results shown in Figure 1C,

using D1 T-cells, validated results with LN T-cells (Fig. 1B) that glucose use, measured by 2-DOG incorporation, was consistently higher in the presence of IL-7 at all time points examined. Statistically significant differences in 2-DOG uptake in D1 T-cells cultured with or without IL-7 were observable from 4 hours through 24 hours of culture (Fig. 1C). At the 2 hr time point, increased 2-DOG import in the presence of IL-7 was observed but differences were not significant likely due to handling effects that are resolved after this time point. Earlier time points, 0.5-2 hrs were previously examined and glucose uptake was decreased in both IL-7-containing and IL-7 deprived cells due to handling stress resulting from centrifugation and media changes (80).

We examined the process of glucose import in D1 T-cells, as distinct from intracellular retention, by using the nonphosphorylatable glucose analog, 3-OMG. Results shown in Figure 1D revealed that glucose import is not dependent on IL-7 (50 ng/ml) and is in fact likely independent of IL-7. This was revealed by the apparent inconsistent results at each time point in which 3-OMG uptake was either slightly increased (6, 8 hrs) or decreased (12, 18, and 24 hrs) in the presence of IL-7. This is in contrast to the results observed with the uptake 2-DOG, which was always increased in cells cultured with IL-7 at all time points examined (Fig. 1C).

An expected, a consequence of the loss of glucose uptake would be reduced metabolism that can be indirectly assessed by measuring ATP levels. Using a luciferin-luciferase detection system, we measured total ATP levels in D1 T-cells deprived of 50 ng/ml of IL-7 for 4, 18 and 24 hours and observed significant decreases in ATP over time of IL-7 withdrawal (Fig. 1E). We concluded, therefore, as part of its growth activity, IL-7 had a necessary function in maintaining cellular energetics.

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To conclusively establish that an IL-7 signal is required to promote glucose, we deprived IL-7-dependent D1 T-cells from IL-7 for 18 hours then pulsed with 50 ng/ml of IL-7. By depriving cells of IL-7 and re-adding the cytokine, we can show that observed effects are solely due to the cytokine signal and not other environmental factors. Note that, under these conditions, D1 T-cells, though growth arrested, are still viable (data not shown) (72). After deprivation, we re-added IL-7 and measured glucose use at time points from 2 through 24 hours, using radio-labeled 2-DOG (Fig. 2A). 2-DOG uptake, indicative of glucose use, was rapidly restored after 2 hrs of IL-7 re-addition and this increase was sustained through 24 hours, restoring glucose uptake to the levels observed in the presence of the cytokine (Fig. 2A). We also noted that addition of IL-7 to deprived D1 T-cells for 30 min to 1 hour caused minimal increases in 2-DOG import (data not shown), suggesting that *de novo* synthesis could be required for IL-7 to enable the utilization of glucose. When the same experiment was repeated, measuring glucose import with the nonphosphorylatable, 3-OMG (Fig. 2B), we did not observe any significant differences upon readdition of IL-7 to cells deprived of IL-7. Collectively our findings indicate that a signal through the IL-7 receptor controls glucose utilization in a manner that is dependent, not upon import, but upon the phosphorylation and retention of the hexose.

IL-7 Regulates the Expression of Genes Involved in Glucose Metabolism.

The metabolism of glucose involves a number of enzymes, some of which are key regulators in the process of generating ATP. Of these, GLUT-1, HXKII, and PFK-1 are the essential first mediators of glucose import and metabolism (Fig. 1A). To investigate the role of each of the three proteins, we first examined the gene expression of GLUT-1, HXKII, and PFK-

1, using real time quantitative PCR (qPCR), in LN T-cells either freshly isolated and pulsed with 150 ng/ml of IL-7 for 4 hours or cultured with 150 ng/ml of IL-7 for 7 days (as described for Fig. 1D) and pulsed with or without cytokine for 18 hours. Results are shown as relative gene expression through calculations of RQ values. In Figure 3A, we observed that LN T-cells displayed elevated levels of HXKII transcripts, but not GLUT-1 or PFK1, when stimulated with 150 ng/ml of IL-7.

Next, we incubated D1 T-cells with or without 50 ng/ml of IL-7 for 0-24 hrs and measured the gene expression of GLUT-1, HXKII, and PFK-1 using qPCR as described above. We observed that, during the first 8 hours of culture without IL-7, GLUT-1 gene expression decreased as compared to D1 T-cells grown with IL-7 (Fig. 3B). However, after 12 hours of IL-7 withdrawal, GLUT1 levels increased as compared to D1 T-cells cultured with IL-7 (Fig. 3B), indicating that other factors, in addition to IL-7, may be involved in regulating GLUT-1 gene expression, as will be further discussed. Based on this result, we would expect that glucose import in IL-7 deprived cells would increase beyond 12 hours, giving time for protein synthesis to take place. This was observed in Figure 1D in which increased uptake of 3-OMG occurred at the 18 hour time point in cells cultured without IL-7 as compared to cells grown with IL-7.

We then measured gene expression of HXKII, a critical enzyme in the glycolytic pathway in D1 T-cells. HXKII generates glucose-6-phosphate from glucose, which prevents glucose from being transported out of cells. Results shown in Figure 3C demonstrated that IL-7 regulates the transcription of the HXKII gene. HXKII expression was decreased in D1 T-cells incubated without IL-7 at every time point analyzed, with results being statistically significant (p >0.05). These results correlated well with the observation that IL-7 loss caused decreased 2-DOG uptake

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(Fig. 1C). IL-7 induced HXKII gene expression increased an average of 3 to 4 fold compared to IL-7 deprived conditions. The results for HXKII (Fig. 3C) were strikingly different from those that were obtained for GLUT-1 (Fig. 3B), suggesting that HXKII gene transcription may primarily be controlled by IL-7.

A different outcome was observed with the detection of PFK1 transcripts in D1 T-cells. In contrast to the IL-7-mediated upregulation of HXKII gene expression, we observed that PFK1 gene expression was down-regulated in the presence but not absence of 50 ng/ml of IL-7 (Fig. 3D), perhaps as a result of a negative feedback mechanism through the amount of glucose 6-phosphate formed and ATP produced – more IL-7, more ATP made (Fig. 1E), less PFK1 is needed. The decrease in PFK1 gene expression in IL-7-containing D1 T-cells was statistically significant ($p < 0.05$) at every time point, other than at 24 hrs, usually by a margin of 1 to 2 fold (Fig. 3D). These results demonstrated that IL-7 directly or indirectly regulates the gene expression of all three glycolytic factors but clearly has the most direct effect upon the gene expression of HXKII.

In order to address the issue of whether GLUT-1 gene expression can be regulated by other factors in addition to IL-7, we incubated D1 T-cells with or without IL-7 (50 ng/ml) in serum free media for 18 hrs. We found that in the absence of FBS, the gene expression of GLUT1 and PFK1 were slightly higher and that this increase was independent of IL-7 (Fig. 3E), suggesting that PFK1 and GLUT1 expression may be controlled by additional factors present in the serum. In contrast HXKII gene expression was similar in IL-7-containing D1 T-cells incubated in serum free media or media with 10% FBS (Fig. 3E) which led us to infer that gene

expression of HXKII is likely under the control of IL-7 signaling, while GLUT-1 and PFK1 may be under the indirect control of IL-7 in combination with other factors.

To conclusively demonstrate that IL-7 directly regulates the transcription of HXKII, we examined the gene expression of GLUT-1, HXKII, and PFK1 during a re-addition experiment. D1 T-cells were deprived of the cytokine for 18 hrs, and then incubated with IL-7 (50 ng/ml) for specified periods of time through 24 hours. Expression of HXKII (Fig. 4A), GLUT-1 (Fig. 4B), and PFK1 (Fig. 4C) was measured by qPCR. We found that only the gene expression of HXKII was restored *immediately* after addition of IL-7 (Fig. 4A). Transcription of HXKII, after 2 hours of IL-7 re-addition, exceeded that of cells grown continuously in the cytokine (IL-7). Gene expression of GLUT-1 did eventually recover but only after 60 hours of IL-7 re-addition (Fig. 4B), indicating that HXKII is an early gene response to an IL-7 signal, while GLUT-1 is a late gene response. The gene expression of PFK1 showed no statistically significant differences after IL-7 re-addition (Fig. 4C). Our results thus establish the gene for HXKII as an important regulatory target for IL-7 signal transduction in dependent T-cells due to its rapid induction upon IL-7 re-addition.

Our studies raised the question whether the IL-7-mediated regulation of HXKII gene expression was specific to IL-7 or was it a general function of other gamma-c (γc) cytokines. To answer this, we deprived D1 T-cells of IL-7 for 18 hours then incubated the cells with different murine γc cytokines: IL-2, IL-4, IL-7, and IL-15 at the optimal dose of 50 ng/ml. After 2 hours of re-addition, we measured the gene expression of GLUT1, HXKII, and PFK1 by qPCR. We found that only IL-2 and IL-7 were able to immediately increase the transcription of the HXKII gene (Fig. 4D) but not GLUT-1 or PFK-1 genes. Re-addition with IL-4 for 2 hours had no

effect. Surprisingly, re-addition of IL-15, although slightly increasing the gene expression of HXKII, did so to a much lesser extent than IL-2 or IL-7 (Fig. 4D). Note that D1 T-cells bear receptors capable of responding to the tested murine cytokines (72). Similar experiments have been performed by others but focusing on much later time points (92). These results suggested that cytokines capable of inducing T-cell proliferation (IL-2 or IL-7) but not differentiation (IL-4) can also drive glucose utilization.

Using D1 T-cells, we next examined the effect of IL-7 upon protein expression of the two cytosolic proteins, HXKII and PFK1, in order to identify any post-translational regulatory mechanisms. Consistent with the changes in gene expression shown by qPCR (Figs. 3 and 4), protein levels of PFK1 increased and protein levels of HXKII decreased in the absence of IL-7 (Fig. 5A). These results were validated by performing IL-7 re-addition experiments in which the protein levels of HXKII were restored after 4 hours of incubation with the cytokine (Fig. 5B).

GLUT-1 is a large, glycosylated 12-membrane spanning protein that we found in primary lymphocytes to be transcribed at levels that are much lower than HXKII (Fig. 3A), potentially making protein detection more difficult. Immunoblotting GLUT-1 from lysates isolated from D1 T-cells resulted in detection of a faint band (Fig. 5B) that suggested that the protein could be detected more so in the presence than in the absence of IL-7. We detected 3-fold more total GLUT-1 in the IL-7-containing D1 T-cells and yet observed that, unlike HXKII, rapid protein induction of GLUT-1 was not detected after 4 hours of IL-7 re-addition (Fig. 5B), supporting the conclusion from the gene expression analysis (Fig. 4) that GLUT-1 was a late gene product of IL-7.

To improve the detection of GLUT-1 protein over that achieved by immunoblotting, we used a commercially available product, the EGFP-GLUT-1 ligand, which binds specifically to GLUT-1. The ligand is based in the sequence of human T cell leukemia virus (HTLV) envelope glycoprotein (HRBD) shown to bind to GLUT-1 (93). The EGFP-GLUT1 ligand binds the carboxy-terminal extracellular loop of GLUT-1, enabling detection of membrane-associated GLUT-1 by flow cytometry or fluorescence staining. Because we observed that total protein levels for GLUT-1 were elevated in the presence of IL-7 (Fig. 5B), but we did not observe any IL-7-dependent increases in glucose import using 3-OMG (Fig. 1D), it was possible that GLUT-1 could be expressed on the cell surface independently of IL-7. To address this, we examined GLUT-1 surface expression on D1 T-cells, grown with or without 50ng/ml of IL-7 for 18 hours, by confocal microscopy. As shown in Figure 5C, we were able to detect significant membrane-associated GLUT-1 in D1 T-cells regardless of the addition of IL-7, indicating that the transporter was present and that glucose transport was possible in IL-7-deprived cells.

We confirmed our microscopic observations by measuring glucose uptake with a fluorescent glucose analog, 2-NBDG, in the same D1 T-cells and under the same conditions that we assessed GLUT-1 surface expression with EGFP-GLUT-1 ligand by flow cytometry. The data in Figure 5D shows that D1 T-cells incubated with IL-7 increased glucose uptake in the presence of the cytokine and decreased glucose uptake in the absence of the cytokine, but at the same time evidenced little difference in the levels of GLUT-1 surface expression (Fig. 5E). Re-addition of IL-7 (50 ng/ml) to cytokine-deprived D1 T-cells after 4 hours cells also resulted in increased glucose uptake (Fig. 5D) that was not accompanied by an increase in the surface expression of GLUT-1 (Fig. 5E). We concluded, therefore, that IL-7 promotes the synthesis of

GLUT-1 as a late gene product (Figs. 4B, 5B), but that trafficking of GLUT-1 to surface can occur in the presence as well as the absence of IL-7 (Fig. 5C, 5E). As a control for GLUT-1 surface expression we transiently expressed GLUT-1 in D1 T-cells by the process of nucleofection (Amaxa) and detected elevated levels of the transporter in approximately 50% of the cells, which correlated well with transfection efficiency (Fig. 5E).

HXKII Regulates Glucose Metabolism in IL-7 Dependent T-Cells

Having demonstrated that IL-7 controls the expression of HXKII, we next determined whether loss of HXKII would alter glucose use in IL-7 dependent T-cells. We inhibited the enzymatic activity of HXKII using 3-Bromopyruvate (3BrPA), a synthetic brominated derivative of pyruvic acid (94). Previously, we determined that 10 μM was the least toxic dose of 3BrPA that could inhibit glucose uptake and found that, in the absence of IL-7, BrPA had minimal toxic effects (data not shown). To specifically inhibit HXKII, we also knocked down gene expression using a commercially tested product composed of four pooled small interfering RNAs (siRNA) specifically predesigned to target HXKII (Smart Pool, Dharmacon) and optimized for delivery to T-cells using supplied delivery media (Accel, Dharmacon). Using a non-targeting FITC-labeled control siRNA and GAPDH siRNA we previously determined siRNA delivery efficiency to T-cells to be about 50%. We assayed glucose use by 2-DOG incorporation and ATP production in the presence or absence of IL-7 and 3BrPA or HXKII siRNAs.

In Figure 6A, we examined the effect of HXKII inhibition upon primary LN T-cells that were isolated and cultured with 150 ng/ml of IL-7 for 7 days. Results observed showed that 2-DOG uptake was decreased in the absence of IL-7 as well as upon treatment with 3BrPA. LN T-

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cells incubated in the presence of HXK Smart Pool siRNAs also significantly decreased the uptake of 2-DOG when compared to a non-targeting siRNA control. These results suggested that HXKII is an important mediator of glucose metabolism in primary LN T-cells.

Findings obtained with LN T-cells were further investigated using D1 T-cells. We observed that in the presence of IL-7 (50 ng/ml), 3BrPA reduced glucose use in D1 T-cells by almost 2 fold, decreasing 2-DOG uptake to levels detected in the absence of the cytokine (Fig. 6B). As a consequence of reduced glycolytic activity, treatment of D1 T-cells with 3BrPA also resulted in decreased levels of ATP in the presence of IL-7 (50 ng/ml) (Fig. 6C). To confirm these results, we treated D1 T-cells with HXKII Smart Pool siRNAs. HXKII knockdown, by approximately 67%, was confirmed by immunoblot (inset, Fig. 6D). As shown in Figures 6D and 6E, specific inhibition of HXKII was able to reduce 2-DOG uptake in the presence of IL-7 (50 ng/ml) to levels comparable to those observed in IL-7-deprived D1 T-cells and also to significantly lower total intracellular ATP. These results convincingly show the essential role that HXKII plays in the metabolism of glucose in response to an IL-7 signal.

We then addressed whether expression of HXKII could replace the IL-7 signal and promote glucose use in the absence of the cytokine. To express HXKII, we used the method of nucleofection to transiently express the HXKII cDNA in the nucleus of T-cells. In T-cells, gene expression efficiencies ranged as high as 50%, eliminating the need for a selectable marker. As comparison, we also introduced by nucleofection the empty vector (pcDNA) and a plasmid encoding GLUT-1. In Figure 5E, we showed that over 50% of the nucleofected D1 T-cells expressed high levels of GLUT-1, confirming the efficiency of the gene expression system.

Using freshly isolated LN T-cells (optimized for nucleofection efficiency), we expressed either the empty vector (pcDNA) or the cDNAs for HXKII or GLUT-1 in IL-7 deprived LN T-cells and assayed for glucose uptake using 2-DOG. The results shown in Figure 7A demonstrated that the expression of HXKII was able to rescue 2-DOG uptake in LN T-cells cultured without IL-7. This is in contrast to the minimal effect of GLUT1 over expression (Fig. 7A). These results were followed by a similar experiment using D1 T-cells. HXKII or GLUT-1 was expressed in D1 T-cells grown with or without IL-7, and glucose use and HXKII activity was measured by incorporation of 2-DOG (Fig. 7B) or glucose import measured by incorporation of 3-OMG (Fig. 7C) and results compared to cells receiving the empty vector, pcDNA. We observed that nucleofection of HXKII cDNA was able to significantly increase 2-DOG uptake over that observed with pcDNA (Fig. 7B). Nucleofection of GLUT-1 also slightly increased 2-DOG import but results were not statistically different from pcDNA (Fig. 7B). Nucleofection of both GLUT-1 and HXKII together resulted in the largest increase in 2-DOG uptake (Fig. 7B), as would be anticipated by increasing the levels of the transporter and the kinase in the absence of the cytokine. In contrast, little effect of GLUT-1 or HXKII expression was observed upon 3-OMG import, confirming the need for phosphorylation of the hexose in order to promote glucose uptake and retention (Fig. 7C). Note that the same outcome was obtained with primary T-cells (p53$^{+/+}$) in Figure 7A and D1 T-cells (p53$^{-/-}$) in Figure 7B, suggesting that p53 did not contribute to our results. Because we detected only minimal effects of GLUT-1 expression upon glucose import, we confirmed that GLUT-1 was being made by measuring GLUT-1 mRNA in nucleofected D1 T-cells and found that GLUT-1 mRNA levels increased more than 1800 fold in D1 T-cells nucleofected with the GLUT-1 plasmid (data not

shown), demonstrating that GLUT-1 was being made in these cells but having modest effects upon the utilization of glucose. Thus it is the phosphorylation of glucose, mediated by HXKII, which promotes glucose retention after import by the transporter that is the critical regulatory event in the metabolic response controlled by IL-7.

To further investigate our findings that HXKII promotes glucose uptake in the absence of IL-7, we examined the levels of ATP as an indirect assessment of glucose metabolic activity. We had observed that inhibition of HXKII activity and expression resulted in decreased intracellular ATP (Figs. 6C, 6E). In Figure 7D, we found that D1 T-cells, deprived of IL-7 and nucleofected with HXKII for 4 hours, had increased total ATP approaching those levels found in IL-7 cultured cells. Increased ATP was not detected as a consequence of GLUT-1 expression (Fig. 7D). The question remained whether increased ATP as a consequence of HXKII expression would be protective or induce cell death in cells deprived of IL-7. We discovered that increased expression of HXKII for more than 8 hours in cytokine-withdrawn D1 T-cells accelerated the death process (data not shown). Moreover, in D1 T-cells maintained in IL-7 the elevated levels of ATP mediated by over expression of HXKII were ultimately toxic as seen by the increase in Annexin-V staining. (Fig. 7E). Therefore, we concluded that as a result of HXKII activity, ATP is produced that provides for the energetic needs of a cell but, in excess, can promote apoptosis.

In summary, our data shows that IL-7 regulates glucose utilization by transcriptionally controlling the expression of the *HXKII* gene, and that this process results in increased glucose metabolism that supports the growth activity of IL-7 in lymphocytes.
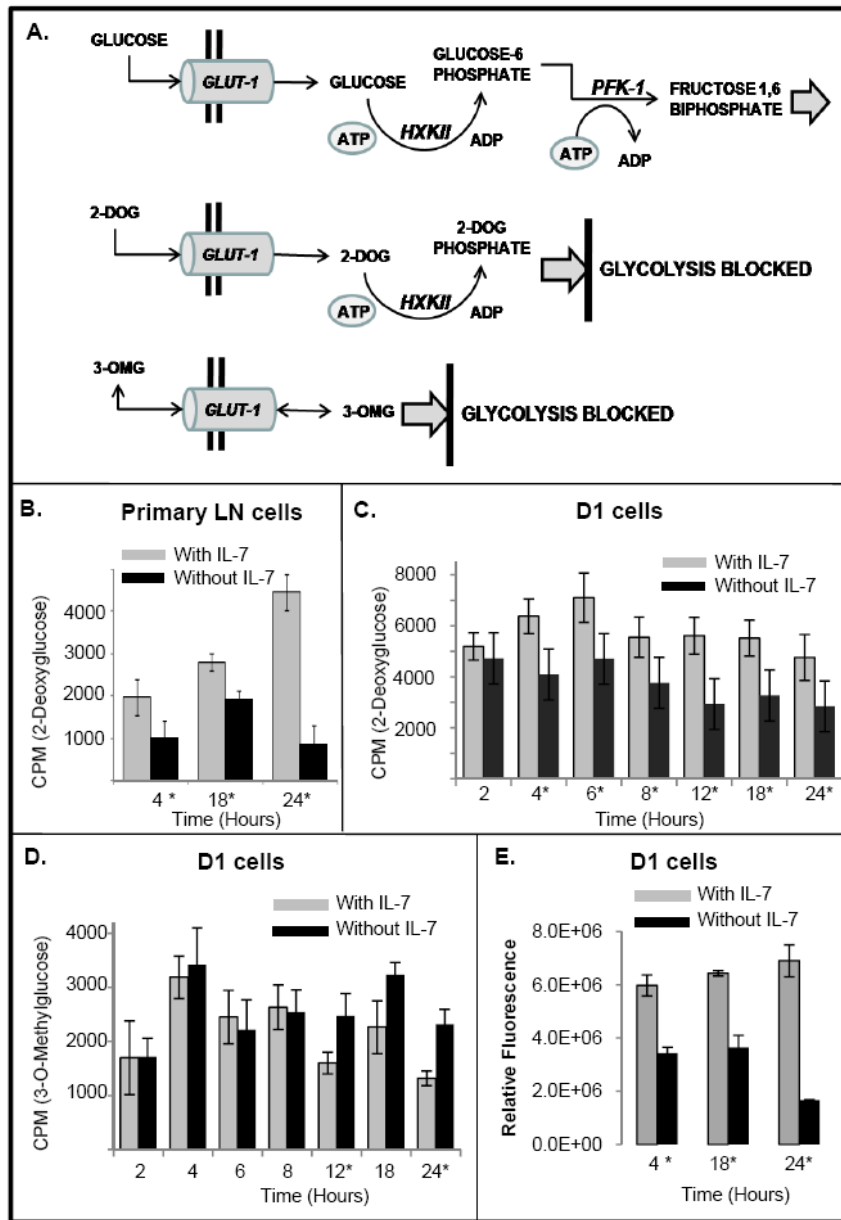
Figure 1: IL-7 signaling controls glucose retention

(A). Model depicts the pathway by which glucose is imported. (B-D). IL-7-cultured lymph node (LN) T-cells (B) were incubated with or without 150 ng/ml IL-7 and IL-7 dependent D1 T-cell line (C, D) were incubated with or without IL-7 (50 ng/ml) and cells assayed for glucose use using radiolabeled 2-DOG (B, C) or glucose import with radiolabeled 3-OMG (D).   (E) D1 T-cells were cultured with or without IL-7 (50 ng/ml) for 4, 18 and 24 hours and total cellular ATP measured Shown are the values for relative fluorescence that correlate with ATP concentrations. Results are representative of three independent experiments performed in triplicate (values are Average ± SD). *p<0.05 in comparison to w/IL-7 in each time point pair.
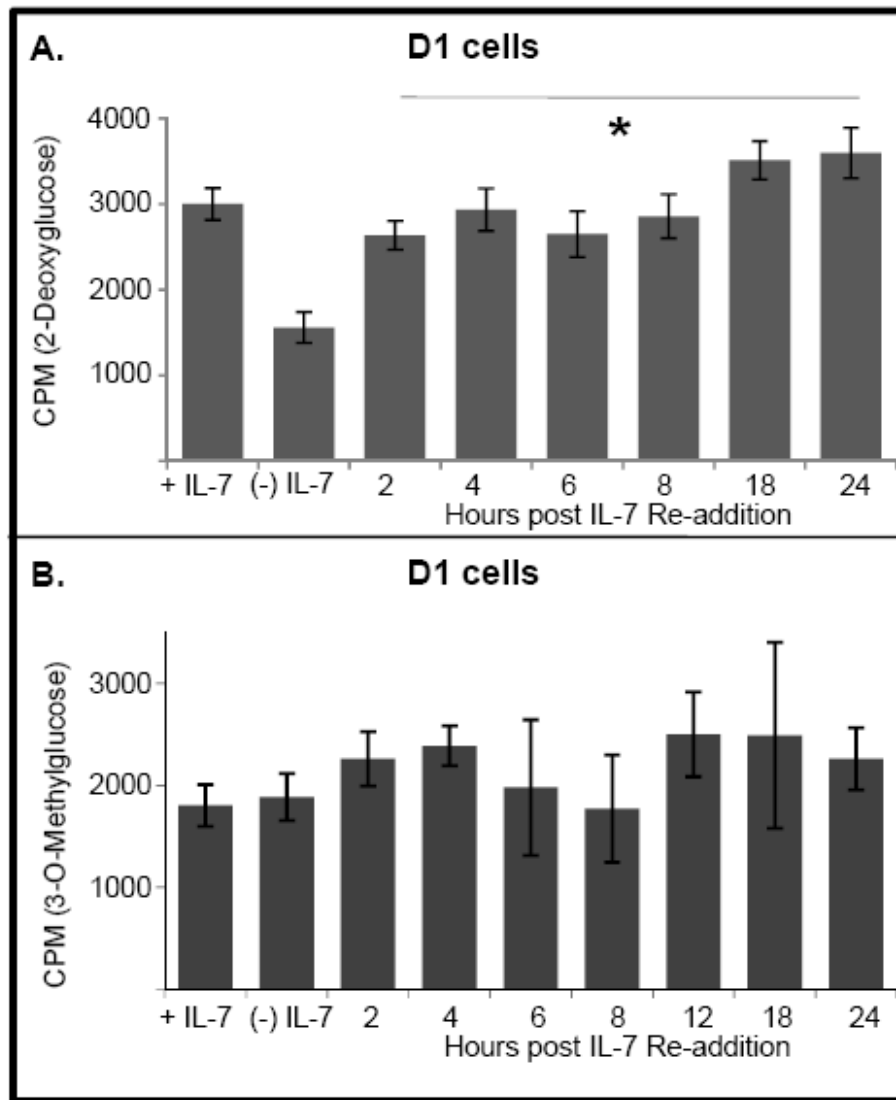
Figure 2: Restoration of glucose uptake upon IL-7 re-addition is dependent upon phosphorylation of the hexose

IL-7 dependent D1 T-cells were incubated without cytokine for 18 hours and IL-7 (50 ng/ml) re-added at the time points specified in the figures. Controls included were D1 T-cells grown continuously in IL-7 (IL-7) and D1 T-cells deprived of IL-7 for 18 hrs ((-) IL-7). D1 T-cells were assayed for glucose use with 2-DOG (A) or glucose import with 3-OMG (B). Results are representative of three independent experiments performed in triplicate (values are Average ± SD). *p<0.05 in comparison to cells cultured without IL-7 (-IL-7).
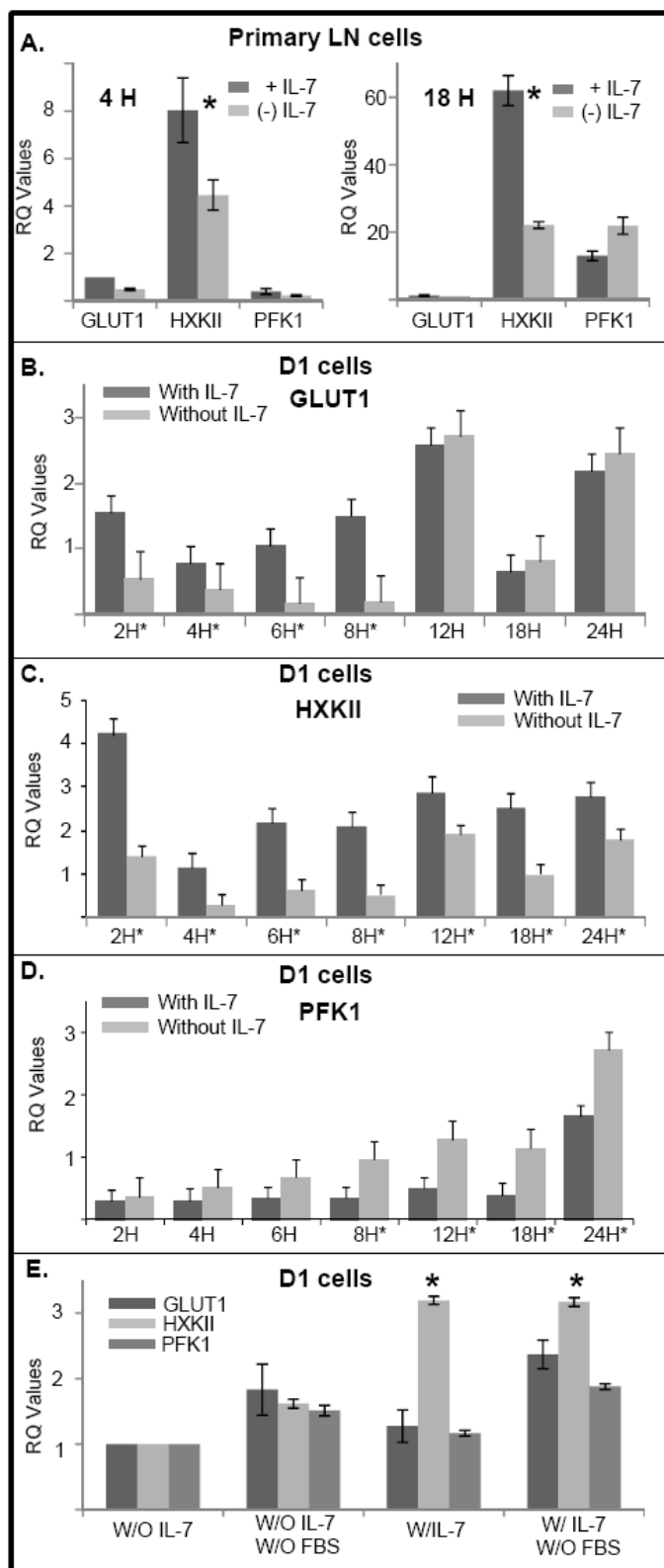
LN T-cells or D1 T-cells were incubated with or without IL-7 for various time points. qPCR was performed to measure the gene expression of GLUT-1, HXKII, and PFK1. RQ values were calculated from qPCR data to show relative gene expression. (A). Murine T-cells, isolated from lymph nodes of C57BL/6 mice, were pulsed with (+IL-7) or without ((-)IL-7) 150 ng/ml of IL-7 for 4 hours (left panel) or cultured with 150 ng/ml of IL-7 for 7 days (+IL-7) and the cytokine withdrawn for 18 hrs ((-)IL-7) (right panel) and RNA extracted for qPCR to measure transcription of GLUT-1, HXKII and PFK1 as described above. (B-D). D1 T-cells were cultured with or without 50 ng/ml of IL-7 and RNA extracted for qPCR to measure transcription of GLUT-1 (B), HXKII (C) and PFK1 (D) as described above. (E) D1 T- cells were incubated with or without 50 ng/ml of IL-7 in serum-containing or serum-free media for 18 hours and gene expression changes for GLUT-1, HXKII and PFK1 measured as described for B-D. Results are representative of at least three or more independent experiments. The calibrator sample chosen to determine RQ values was the 18 hour time point (B, C, D and E) without IL-7. The exception to this was (A) in which the GLUT-1 gene, 2 hours without IL-7 was used as calibrator. *p<0.05 in comparison to without IL-7.

Figure 3: IL-7 regulates gene expression of glycolytic enzymes involved in glucose metabolism
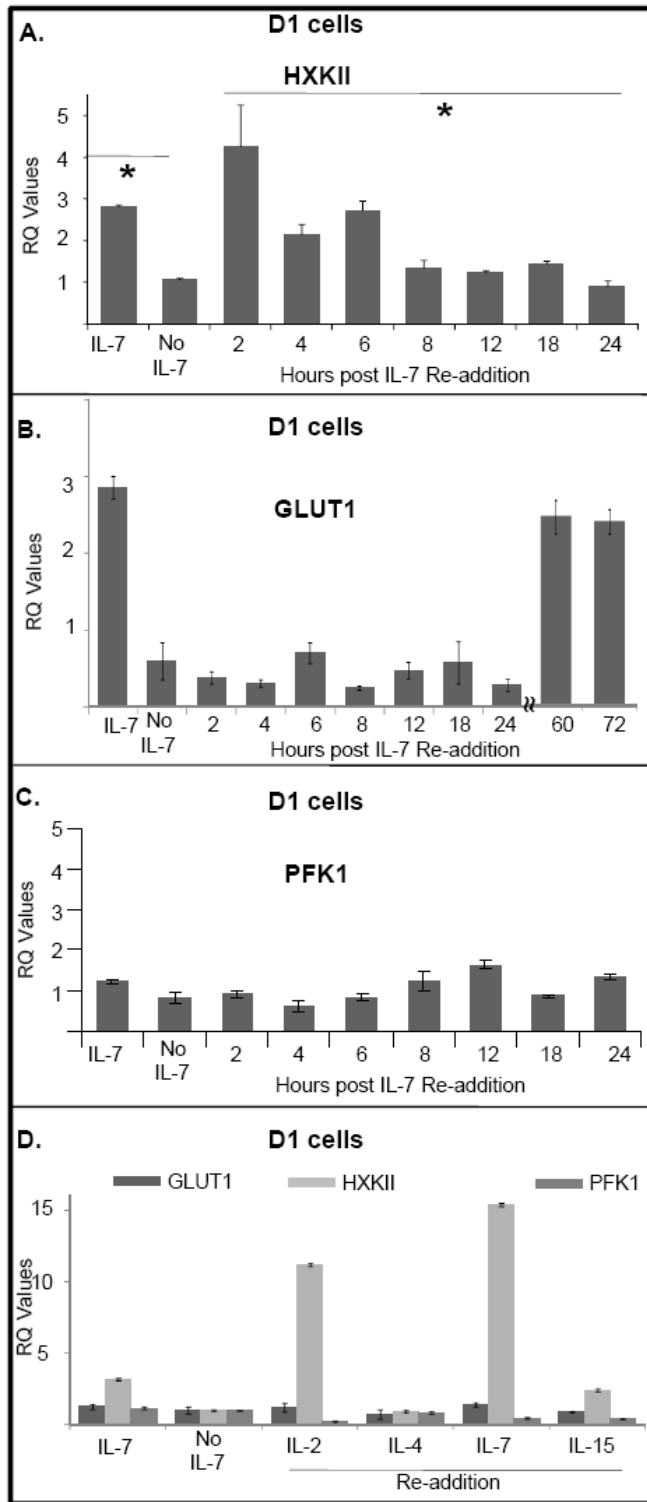
(A-C). D1 T-cells were deprived of IL-7 for 18 hours, washed, and then IL-7 (50 ng/ml) re-added for the specified periods of time in the figures. qPCR was performed to analyze the gene expression of (A) HXK II, (B) GLUT-1, and (C) PFK-1. RQ values were calculated from qPCR data to show relative gene expression. (D) D1 T-cells were deprived of IL-7 for 18 hours then incubated with IL-2, IL-4, IL-7, and IL-15 at an optimal concentration (50 ng/ml) for 2 hours. Total RNA was isolated and transcribed to cDNA and gene expression was analyzed using qPCR as described above. The calibrator sample chosen to determine RQ values was the 18 hour time point without IL-7. Results are representative of at least three independent experiments and values represent average ± SD. *$p < 0.05$ in comparison to without IL-7.

Figure 4: Re-addition of IL-7 restores gene expression of HXKII
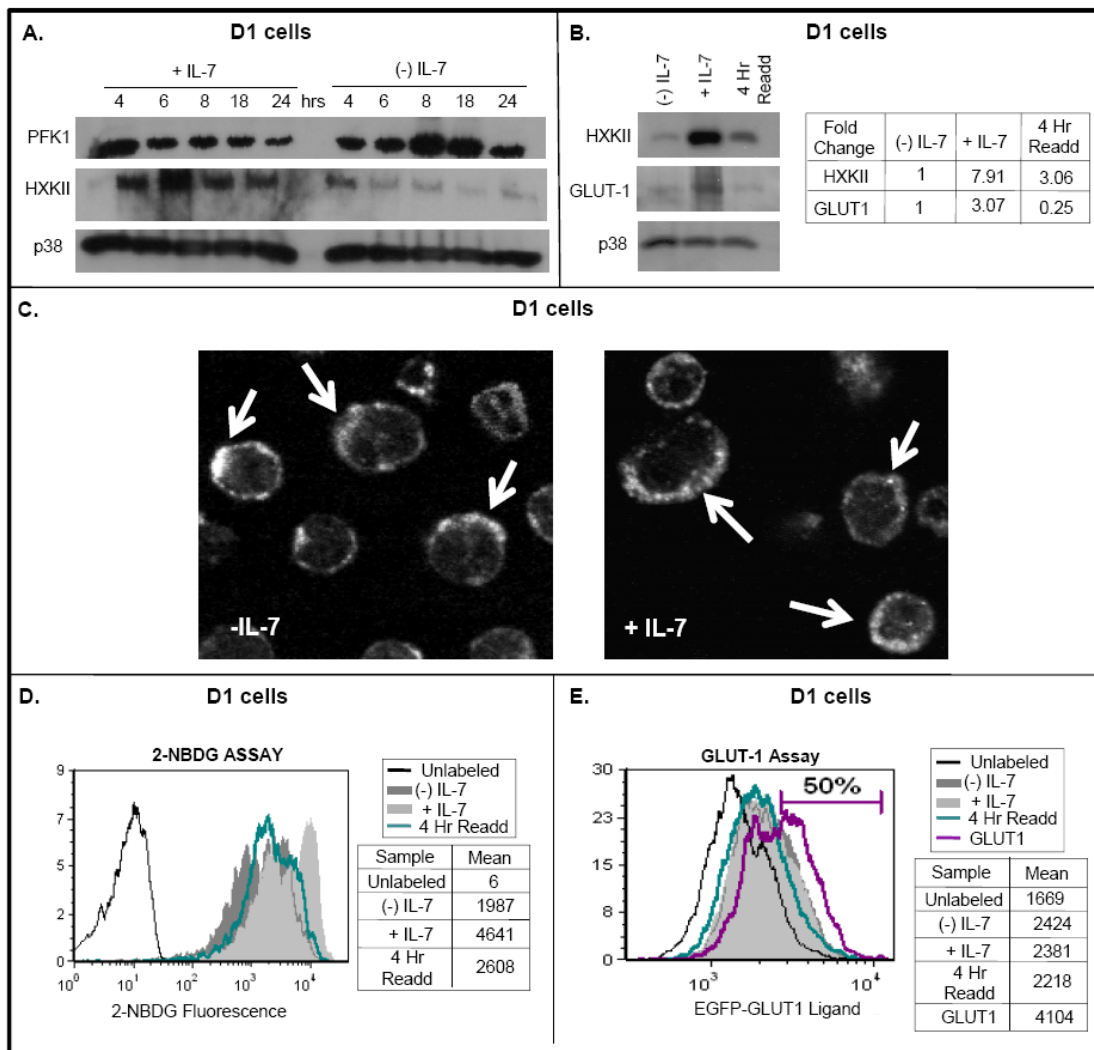
Figure 5: Protein levels of HXKII increase in response to an IL-7 signal

(A). D1 T-cells were incubated with or without IL-7 (50 ng/mL), and lysates immunoblotted for HXKII and PFK1. (B). IL-7-deprived D1 T-cells were incubated with IL-7 (50 ng/ml) and lysates immunoblotted for HXKII and GLUT-1. p38 MAPK was measured to show equal loading. (C). D1 T-cells were incubated with or without IL-7 (50 ng/ml) and EGFP-GLUT-1 ligand, Results were visualized by confocal microscopy. (D-E). IL-7-deprived D1 T-cells were cultured with IL-7 (50 ng/ml) and tested for glucose uptake using fluorescent 2-NBDG by flow cytometry, (D), or incubated with the EGFP-GLUT-1 ligand and tested for GLUT-1 surface expression by flow cytometry (green lines in figures) (E). Cells cultured with (silver) or without IL-7 (gray) for 18 hours are shown as comparison. For the 2-NBDG assay and the EGFP-GLUT-1 ligand data, controls shown are unlabeled, unstimulated cells (black lines in figures). As a positive control for measurement of GLUT-1 surface expression, D1-T cells, nucleofected with the cDNA for GLUT-1, are also shown (purple line in figure). Marker indicates the percent of cells transiently expressing high levels of GLUT-1. Data in the tables are the mean peak positions for each sample in the histograms. Controls shown are unlabeled, unstimulated cells (black lines in figures). Data in the tables are the mean peak positions for each sample in the histograms. Shown are representative results of 3 independent experiments performed.

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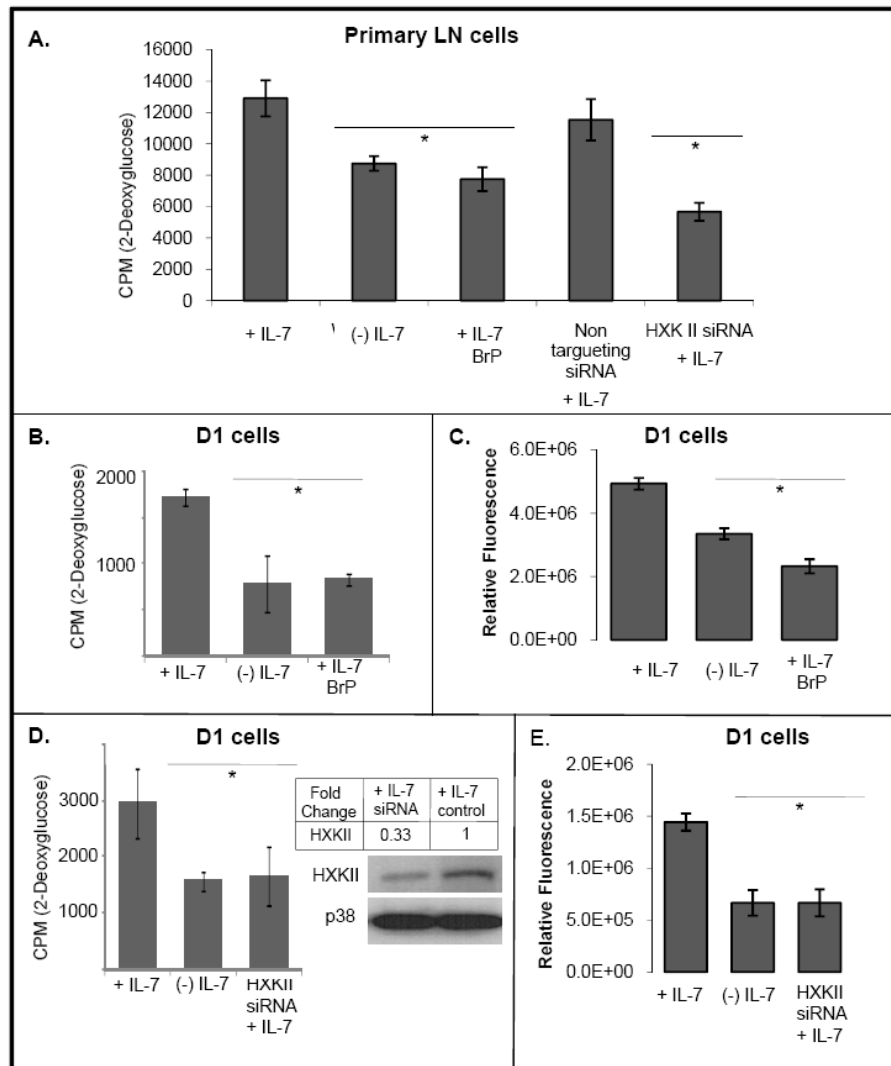
Figure 6: Inhibition of HXKII decreases glucose uptake and ATP levels independently of IL-7

(A) LN-T cells were cultured with 150 ng/ml of IL-7 for 7 days and then incubated with or without IL-7 (150 ng/ml) for 18hrs and treated with either 3-Bromopyruvate (BrP) or the Smart Pool HXKII siRNA. A non-targeting siRNA was used as control. Glucose use was measured by assaying the uptake of 2-DOG. (B) D1 T-cells were incubated with IL-7 (50 ng/ml) or without IL-7 and 3-Bromopyruvate (BrP) for 18 hours, then assayed for glucose use with 2-DOG . (C). D1 T-cells were incubated with IL-7 (50 ng/ml) or without IL-7 and 3-Bromopyruvate (BrP) as in (B), and total cellular ATP measured. Shown are the values for relative fluorescence that correlate with ATP concentrations. (D-E). HXKII gene expression was inhibited in D1 T-cells using Smart Pool HXKII siRNA. Knockout of HXKII gene expression with specific HXKII siRNA is shown in the immunoblot for HXKII (D). p38 is included as a loading control.. To assess glucose use upon HXKII inhibition, 2-DOG uptake as measured (E). Total intracellular ATP was measured as described in (C). Results are representative of three independent experiments performed in triplicate (values are Average ± SD). *$p<0.05$ in comparison to cells cultured + IL-7.
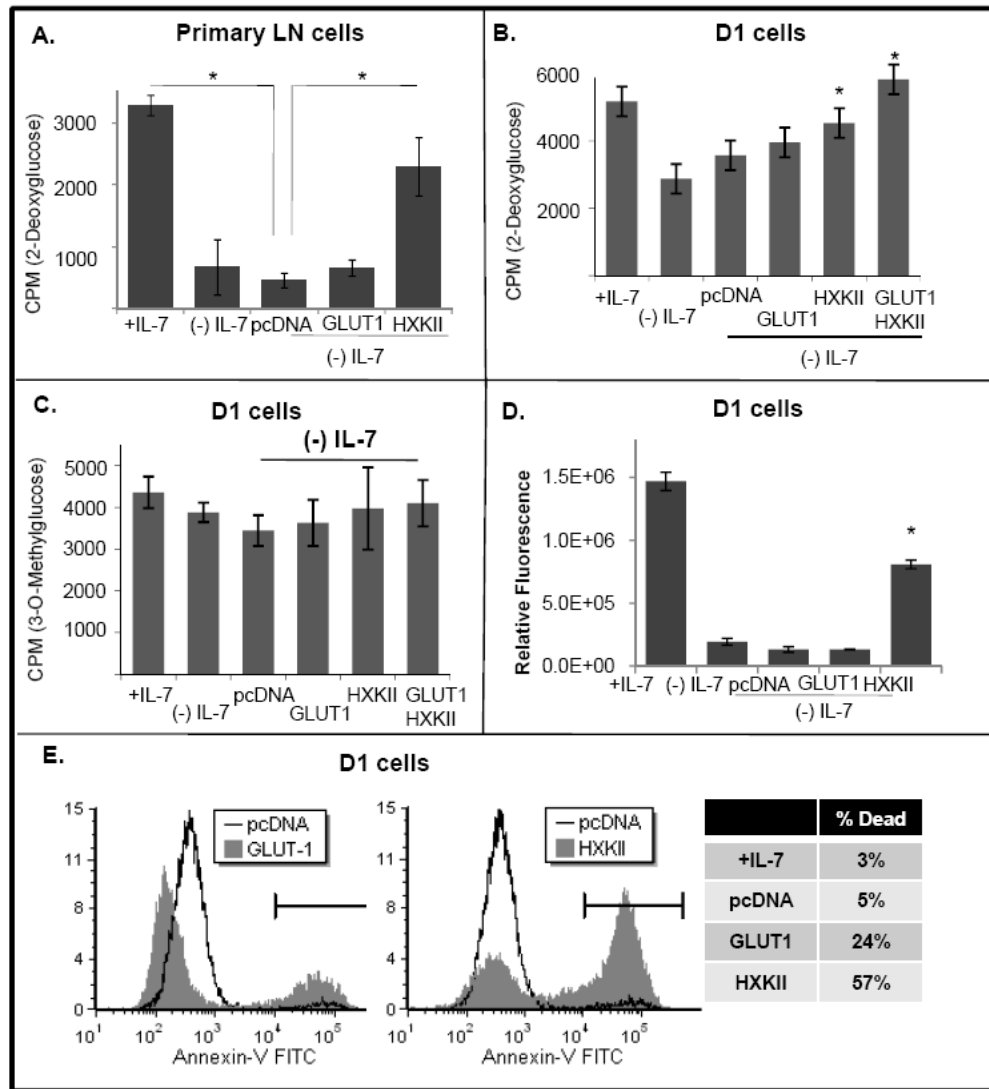
Figure 7: Over-expression of HXKII increases glucose uptake independently of IL-7

(A) Freshly isolated murine T-cells from lymph nodes were nucleofected with either empty vector (pcDNA) or the cDNAs for GLUT-1 or HXKII and glucose use by 2-DOG uptake in the absence of IL-7 measured. Cells cultured with IL-7 (+ Il-7) are shown as controls. (B-C). D1 T-cells were deprived from IL-7 for 18 hours, nucleofected with cDNAs for GLUT-1, HXK II, or empty vector (pcDNA), then incubated with or without IL-7 (50 ng/ml) for 4-8 hours, and assayed for glucose use with 2-DOG (A) or glucose import with 3-OMG (B). (D) D1 T-cells were incubated with IL-7 or without IL-7 and deprived cells were nucleofected with plasmids as described in (B). Total cellular ATP was measured. Shown are the values for relative fluorescence that correlate with ATP concentrations. (E). D1 T-cells were incubated with or without IL-7 (50 ng/ml) and IL-7-deprived cells were nucleofected with plasmids as described in (B). Cell death was assessed by staining with Annexin-V-FITC and read by flow cytometry. Results are representative of three or more independent experiments performed in triplicate (values are Average ± SD). *p<0.05 in comparison to cells cultured without (-) IL-7.

Discussion

In support of its growth activities, we report that IL-7 controls glucose use in T-cells by regulating the initial phosphorylation and subsequent intracellular retention of the hexose. Addition of IL-7 to cytokine-deprived T-cells restored the uptake of a phosphorylatable glucose analog but not a non-phosphorylatable glucose analog within 2-4 hrs. This restoration correlated with an increase in the gene expression of HXKII, but not GLUT-1 or PFK1, in response to an IL-7 signal. Moreover, over-expression of HXKII restored glucose uptake and ATP levels in cytokine deprived IL-7 dependent T-cells, while inhibition of HXKII decreased glucose uptake and ATP in the presence of IL-7. Our results therefore suggest that IL-7 promotes glucose use by directly regulating the transcription and therefore activity of HXKII.

Others have shown that incubation with IL-7 increased glucose uptake in naïve T-cells as well as activated T-cells (51). These findings are consistent with our own in which we show, with both a T-cell line and primary T-cells, that an IL-7 signal modulates glucose uptake. To address the mechanism underlying glucose transport in T-cells, we found that only *HXKII* gene expression was restored in cytokine-deprived cells upon readdition of IL-7, and that only expression of HXKII could rescue glucose uptake during IL-7 withdrawal. We did not observe that IL-7 deprived cells expressing GLUT-1 could retain significant amounts of glucose, nor did we find that GLUT-1 gene expression was induced rapidly within a few hours of IL-7 re-addition. We did note, however, that after 60 hours of IL-7 readdition, the levels of GLUT-1 mRNA did increase. Others have reported a similar trend with detection of increased GLUT-1 surface expression after 72 hours of incubation with IL-7 (53). This suggests that IL-7 may induce the production of GLUT-1 as a late gene product but that the trafficking of GLUT-1 to

the cell surface may also occur by an IL-7-independent mechanism. In support, others have shown that IL-7 induced glucose uptake without an accompanying increase in GLUT-1 protein levels (51) and that high glucose in the extracellular milieu inhibited glucose phosphorylation and glucose uptake without affecting glucose transport (95). Given that the activity of HXKII retains glucose within the cells, once the hexose is transported through GLUT-1, and that this is a critical step in the glycolytic pathway, it is likely that transcriptional control of HXKII is the most effective mechanism by which IL-7 can rapidly modulate glucose use in T-cells to supply energy as needed.

Further support of our findings comes from studies in which the regulation of HXKII activity was found to be subject to growth factor regulation (96). However, reports that growth factors like IL-3 increased GLUT-1 protein trafficking to the cell surface (97), and that IL-7 induced GLUT-1 expression and glucose uptake in proliferating thymocytes, T-ALL cells, and RTEs seem to contradict our results (52,98,99). In similar studies, the phosphoinositide 3-kinase (PI3K) signaling pathway was implicated in the synthesis and trafficking of GLUT-1 (3,100). Our results do not rule out that IL-7 could controls GLUT-1 expression, perhaps through PI3K, signaling. Rather, we put forward the concept that regulation of GLUT-1 activity by IL-7 is not the principal mechanism by which IL-7 promotes glucose utilization, but rather it is through the rapid induction of HXKII synthesis that IL-7 enables glucose metabolism in response to the energetic needs of T-cells.

We also observed that IL-7 negatively regulated PFK1. The most probable explanation for this observation is that a negative feedback mechanism, perhaps through accumulation of ATP, which is dependent upon IL-7, exists. Hence, the expression of PFK is down-regulated

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when glucose is being transported and metabolized in response to IL-7 to control the production of ATP. We observed that over expression of HXKII was not protective but did result in elevated levels of ATP that proved detrimental to cells, inducing apoptosis even in the presence of IL-7. There is support for the concept that ATP levels control the induction of apoptosis. Others have reported that increasing glucose concentrations, thereby increasing ATP, resulting in a switch from necrosis to apoptosis, with higher amounts of ATP driving the apoptotic process (101,102). Such studies also showed that lowering ATP prevented apoptosis, and that lowering ATP followed by recovery of ATP caused cell death (103).

Our results that IL-7 controls glucose uptake through the transcription of the *HXKII* gene is significant given the cytokine's critical role in T-cell development and homeostasis, but also has implications for the therapeutic use of the cytokine. IL-7 has been listed among the top five immunotherapy compounds by the National Cancer Institute (104). IL-7 is typically given to patients in super physiological concentrations. Our findings suggest that excessive glycolytic activity could result from receiving a large dose of IL-7, mimicking the tumorogenic activation of glycolysis. Tumor cells tend to generate their energy through glycolysis instead of oxidative phosphorylation – a phenomenon known as the Warburg effect (105). Tumor cells are also known to over express HXKI and HXKII (106) By showing that the increased synthesis of HXKII by T-cells is a direct result of engagement of the IL-7R, we have revealed a previously unknown activity of the cytokine that has implications for the development of IL-7 in the treatment of cancer, immunodeficiencies and other disorders of the hematopoietic system.

IL-7 REGULATES HEXOKINASE II GENE EXPRESSION THROUGH ACTIVATION OF JNK/JUND PATHWAY

Introduction

IL-7 plays a major role in T-cell survival, development and proliferation by activating a cascade of signaling pathways downstream the IL-7 receptor. IL-7 also regulates glucose metabolism as a mean to regulate T-cell homeostasis (51,80). We previously showed that this function is partially fulfilled trough transcriptional control of the gene expression of HXKII (107). The regulation of glucose retention by IL-7 is crucial for T-cells since increases in aberrant HXKII gene expression have been described as an important phenotype in cancer.

How IL-7 regulates HXKII gene expression is unknown. The mouse HXKII cDNA is approximately 5.5 k in length with an open reading frame of 2751 base pairs that encodes 917 amino acids to form the protein (108). A study of the mouse HXKII promoter region conducted by Heikkinen et al showed that the promoter region of HXKII is about 4.8 kb - of which the 550 base pairs of the proximal promoter region contain most of the transcription factors binding motifs (108). Some of the recognized factors include, SP-1 whose activity is regulated by insulin, NF-Y, CREB, Glucose binding site, and the AP-1 complex.

AP-1 complexes are implicated in activating a myriad of genes involved in proliferation, differentiation, and activation of T-cells (109). The activation of AP-1 is an important component for cell signaling in response to growth factors, cytokines, T-cell activators, and UV radiation (110-112). AP-1 complexes bind to palindromic DNA sequences defined as TPA response elements (TRE) or cyclic AMP response elements in the promoters and enhancers of

target genes (113). The AP-1 complex is mainly composed of transcription factors belonging to the Fos and Jun families of transcription factors. These proteins, when activated, form homo or heterodimers. Induction of AP-1 is regulated by MAPK signaling. MAPK kinases are family of serine/threonine kinases. These enzymes are activated in response to variety of extracellular signals through sets of threonine/ tyrosine dual specificity kinase kinases (MAPKK), namely MEK1/2, MAPKK3, 4, 6, AND 7 (24) and can induce cell proliferation, activation, and differentiation. Three MAPK kinase pathways have been identified in mammalian cells: the ERK, JNK, and p38 MAPK kinase pathway. Each of these kinases phosphorylates different substrates.

The JNK pathway is one of the three sub-groups of MAPKs. It is activated by MAPKK 4/7 mainly in response to cytokine stimulation and exposure to environmental stress (114). Three different members have been described: JNK1, JNK2, and JNK3. JNK1 and 2 are ubiquitously expressed, while JNK3 is selectively expressed in neuronal and cardiac tissues (115). JNK 1 and 2 regulated differently the cell proliferation in fibroblasts; JNK1 positively regulated the increase in cell numbers, while JNK 2 negatively regulated that increase (116). JNK1 and 2 behaved also differently in CD4$^+$ and CD8$^+$ T-cells. While JNK1 stimulated CD8$^+$ expansion and survival, JNK2 negatively regulated CD8+ T-cell proliferation. Also JNK2 stimulated TH1 differentiation, when JNK1 negatively regulated this aspect (115).

The Jun family of transcription factors comprises c-Jun, Jun B, and Jun-D, and is phosphorylated by JNKs. While c-Jun and Jun-B are considered activating factors and expressed as early genes (117-119), Jun-D was first described as a negative regulator of T-cell proliferation (120-122). Over expression of Jun-D in immortalized fibroblasts decreased proliferation (122),

while Jun-D$^{-/-}$ fibroblasts exhibited an increased proliferation (123,124). In contrast to earlier reports, Weitzman et al. showed that Jun-D promoted proliferation. In their study, primary mouse fibroblasts lacking Jun-D proliferated less and displayed premature senescence. It was suggested that Jun-D possessed a protective effect against apoptosis as Jun-D-deficient fibroblasts displayed increased sensitivity to UV irradiation or TNF-a treatment (123,124). In this regard, JNK-activated Jun-D was shown to cause an increase in gene expression of anti-apoptotic proteins (125). IL-7, IL-15, and also IL-2 stimulated the DNA-binding of JunD; moreover, IL-7 increased the Jun-D–DNA binding after 30 min of its re-addition, which implied that IL-7 activated an existing JunD protein without requiring *denovo* synthesis (126).

The activities of JNK and the AP-1 complex in the context of IL-7 signaling are poorly understood. The role of MAPKs in the regulation of metabolic activity in T-cells also remains ill-defined. In this study we investigated the mechanism controlling the induction of HXKII gene expression in response to IL-7 stimulation. We found that the IL-7-driven synthesis of HXKI, and resulting glucose uptake, involved the activation of the AP-1 complex via the JNK pathway and discovered that the AP-1 complex induced by JNK in response to IL-7 was composed of Jun-D homodimers.

Material and Methods

Cell lines, mice, and T-cell purification

The IL-7-dependent T-cell line, D1, was established from pro-T cells isolated from a p53-/- mouse as previously described (22). D1 T-cells were maintained in RPMI 1640

supplemented with 10% fetal bovine serum (FBS), 1% penicillin (5000 u/ml)-streptomycin (5000 µg/ml), 50 µM β-Mercaptoethanol, and 50 ng/ml recombinant human IL-7 (Peprotech, CA). Primary lymph node (LN) T-cells from 8-12-wk-old C57BL/6 mice were isolated by mechanical teasing and cultured (5x106 cells/ml) in the presence of 150 ng/ml of IL-7 (Peprotech). To enrich for IL-7 dependent cells, as needed for experiments, we used our published method of *ex vivo* expansion and cultured LN T-cells with 150 ng/ml of IL-7 for 7 days (24). Animal use was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida.

Plasmids and Nucleofection for transient gene expression

Plasmids used for transient expression of HXKII and GLUT1 were pcDNA-HXKII (a kind gift from Dr. Wilson, and Dr. De Xon, Michigan State University, MC) and pcDNASTAT-5a-CA (a kind gift from Dr. Qiong (NCI-Frederick). To transiently express the plasmids, D1 and primary T-cells were "nucleofected" using the Murine T-cell Nucleofection kit (Amaxa), following the manufacturer's protocol. Briefly, 1x106 T-cells were incubated with (4µg) plasmid DNA in 100µl of the mouse T-cell solution, and electroporated with the specific program optimized for mouse T-cells. Nucleofected T-cells were incubated in the supplemented media with or without IL-7 for 4-8 hours prior to analysis. In D1 T-cells nucleofection efficiency averaged approximately 40-50% expression of the target gene with viabilities ranging 60-80%.

Cell proliferation assay

To measure cell proliferation, 2×10^6 of nucleofected D1cells were pulsed with BrdU (10 μM) overnight, and BrdU incorporation was measured with a commercially available kit (BD Biosciences) according to manufacturer's protocol. Briefly, washed, fixed, and permeabilized prior to incubation with a FITC-conjugated anti-BrdU antibody. The cells were analyzed by flow cytometry using Accurai flow cytometer (Accurai, Ca). Data was analyzed using FCS software.

Glucose uptake assay

D1 or primary T-cells were incubated in glucose-free, serum-free RPMI (Mediatech Inc, Manassas, VA) supplemented with or without IL-7 for 1 hour. 2-Deoxy-d-[3H] glucose (2 μCi/reaction) (Sigma) was added for 3 minutes or 3-O-methyl-[3H]-glucose (2 μCi/reaction (GE Healthcare) was added for 1 minute. Reactions were stopped by adding 250 μl of ice-cold 0.3 mM phloretin (Sigma). Cells were then centrifuged through a cushion of 10% Bovine Serum Albumen (BSA), and lysed using 0.1% Triton X-100. Radioactivity was measured with scintillation counter (LS6500, Beckman Coulter or Microbeta 2450, Perkin Elmer). Statistical analysis was done using Prism software (GraphPad).

Gene expression analysis by Real-time PCR

For RNA extraction, D1 cells (2.0×10^7) were centrifuged and cell pellets were resuspended in 1 ml TRIZOL (Invitrogen). One μg of RNA was converted into cDNA using iScript cDNA synthesis kit (BioRad). Real-time PCR reactions were performed using Fast

SYBR® Green Mix (Applied Biosystems) on a 7500 Real-time PCR system (Applied Biosystems). β-actin was used as endogenous control. Primers sequences for β-actin were previously published(88), JunD primers were as follow: Forward: 5' GCCGCCTCCAAGTGCCGCAAG3' Reverse: 5'CGGACTCAGTACGCGGGCACCTG3'and were designed using Primer 3 Software (http://frodo.wi.mit.edu/). Data analysis was done using Graphpad (prism, ca)

Immunoblots for JunD

D1 cells (5×10^6) were lysed using a whole cell lysis buffer (20mM TRIS-HCl (pH7.5), 150 mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium Pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO4, 1μg/ml leupeptin). Cell lysates were separated on 12% SDS-PAGE gels, then transferred onto nitrocellulose membrane and immunoblotted, the primary JunD antibody was an anti-rabbit antibody ab5799 (Calbiochem, LaJolla, Ca) Rabbit anti Lamin B antibody (119D5-F1) (Calbiochem, Ca) was used to detect the levels of Lamin B as loading control.

siRNA inhibition

Predesigned Accell SMART pool siRNA against JunD was purchased from Dharmacon (Thermo scientific) and was introduced into cells according to manufacturer's protocol The Accel siRNA was introduced to D1 cells and incubated for 72 hours.

JNK kinase activity

JNK kinase activity was measured using KinaseSTAR kit (BioVision, Ca) and following the manufacturer protocol. Briefly, D1 (10^7 cell) were incubated in glucose-free, RPMI (Mediatech Inc, Manassas, VA) supplemented with or without IL-7 for the indicated period of time, cells were then lysed using the JNK extraction buffer provided in the kit, cell lysates were then centrifuged and supernatants were used for JNK immunoprecipitation. Protein concentration was measured using Nanodrop 8000 (ThermoScientific, Ca). Equal amount of proteins were used throughout all samples. JNK immunoprecipitation was then carried out using JNK specific antibody provided in the Kit Samples were then immunoprecipitated using JNK specific antibody provided in the Kit and Protein A sepharose beads. Kinase assay was then carried out. Samples were incubated with C-Jun protein / ATP mixture, and immunoblotted for phospho-C-Jun. westernblot was carried as described in chapter one, Phospho-C-Jun (Ser 73) antibody was provided with the Kit. Samples were separated on 8% SDS-PAGE gels, and then transferred onto nitrocellulose membrane and immunoblotted for Phospho-C-Jun using monoclonal antibodies against Phosph-C-Jun (provided with the kit. Anti-rabbit secondary polyclonal antibodies used were HRP (horseradish peroxidase) conjugated. Signal was developed by enhanced chemiluminescence (ECL) (Pierce) and visualized on BioMax ML film (Kodak).

Nuclear extracts and Electrophoretic mobility shift assay

Nuclear extraction and Electrophoretic mobility shift assay were performed according to the method described by Jaganathan et al. (127,128). Briefly, Nuclear extract were prepared in Hypertonic buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20%

glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 0.5 mM PMSF, 0.1 mM aprotinin, 1 mM leupeptin, 1 mM antipain). Normalized extracts, containing 3 μg of total protein, were incubated with a double-stranded ^{32}P-radiolabeled AP1 oligonucleotide probe prepared by radiolaeling double strand AP1 probe with [α32P]dCTP (3000 Ci/mmol) and [α32P]dATP (3000 Ci/mmol). Protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography.

For supershift, anti-c-Jun (39309, Active Motif), anti-C-Fos (101311, Active Motif), and anti-JunD (39328, Active Motif) antibodies were incubated with nuclear extract for 30min before adding radiolabeled probe. Electrophoresis was carried as described above.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP-IT kit (Active Motif, Ca) according to the protocol provided by the manufacturer. Briefly, D1 cells were incubated overnight with or without IL-7, and treated with 37% formaldehyde to crosslink protein to DNA. Cells were then lysed using ice cold lysis buffer supplemented with the kit and dounce homogenizer. Nuclei pellets were then sonicated and precleard using protein G beads provided with the kit. Precleard Chromatin was then incubated with anti JunD antibody (ab28837) (AbCam, Ca) overnight, and immunoprecipitated using protein G beads according to the manufacturer protocol, DNA was then eluted from the beads and purified following the manufacturer protocol. We then performed PCR analysis using AP1 primers designed be Primer3 software, *Forward Primer:*

5'Gggctctaggcgctgatt3' *Reverse Primer*: 5'ggagttggtgcaacaatgtg3', PCR products were then run on 1% agarose gel. Real Time PCR was also carried out using Fast SYBR® Green Mix (Applied Biosystems) on a 7500 Real-time PCR system (Applied Biosystems).

Results

STAT-5 is not involved in IL-7 regulation of Hexokinase gene expression

IL-7 supports T-cell survival, activation and proliferation by maintaining glucose utilization. We found that this process is controlled through transcriptional regulation of HXKII gene expression (107). While regulation of GLUT1 activity has been extensively studied, signaling pathways involved in the regulation of HXKII gene expression have yet to be determined. To determine whether IL-7 controlled the synthesis of HXKII by activating the transcription factor, STAT5, we isolated lymph node T-cells from mice expressing a constitutively active (CA) form of STAT5, STAT5b-CA, and measured HXKII gene expression by qPCR. As a positive control, we measured the basal gene expression of HXKII in T-cells from WT mice. As a negative control we also treated T-cells from WT mice with an anti-IL-7 antibody to block IL-7 signal transduction. Shown in Figure 8A are the results indicating that the gene expression of HXKII in T-cells from STAT5b-CA mice was markedly higher than in WT T-cells, suggesting that mice with high levels of STAT5 had increased synthesis of HXKII.

However, because the STAT5b-CA T-cells came from a transgenic mouse that produces IL-7, it was possible that other IL-7-mediated signals could synergize with STAT5 to drive

HXKII synthesis in T-cells. To address this and determine whether in the absence of IL-7, STAT5 could induce HXKII gene expression, we nucleofected a constitutively active (CA) STAT5a plasmid into D1 cells deprived of IL-7 and measured HXKII synthesis by qPCR. D1 cells, incubated with IL-7 and nucleofected with STAT5a-CA, exhibited a level of gene expression similar to that of non-nucleofected cells incubated with IL-7, while nucleofection of D1 cells expressing STAT5a-CA, lacking IL-7, failed to increase the gene expression of HXKII (Figure 8B). A similar outcome was seen when D1cells, nucleofected with STAT5a were tested for glucose uptake using 3H-deoxyglucose (2-DOG) (Figure 8C). Moreover, STAT5a over-expression did not increase the transport of 3-0-methyl-glucose (3-OMG), which we had found was not affected by IL-7 withdrawal (Figure 8D). These results indicated that expression of an active form of STAT5 could not promote the gene expression of HXKII in the absence of IL-7.

To demonstrate that a STAT5-independent, but still an IL-7 dependent signal, controlled HXKII gene expression, we nucleofected D1 cells with a chimeric IL-4/IL-7 wild-type receptor or with a chimeric IL-4/IL-7 receptor bearing a mutation in the STAT5 binding site, Y449. Normally, when Y449 is phosphorylated it binds STAT-5, therefore mutation of this site would prevent STAT5 activation. D1 cells, nucleofected with the chimeric receptors, were cultured with human IL-4. Gene expression of HXKII was measured by qPCR (Figure 8E), and glucose uptake was measured using 2-DOG (Figure 8F). We found that both gene expression and glucose uptake was not affected by the Y449 mutation and they were comparable to that of D1 cells incubated with IL-7, used as positive control. The use of STAT-5 inhibitor also did not decrease the HXKII gene expression (Figure 8G). Taken together, these data suggest that STAT-5 is not involved in regulating HXKII gene expression by IL-7.

JNK activity is regulated by IL-7 and controls glucose uptake in response to the cytokine

In order to investigate the signaling pathway involved in IL-7 activation of HXKII gene expression, we deprived D1 cells from IL-7 for 18 hours, then re- added IL-7 to culture for four hours in the presence of the MAPK inhibitor, PD169316, a MEK1/2 inhibitor, or the PI3K inhibitor, wortmannin. We then measured the glucose uptake using 2-DOG. Only D1 cells incubated with the MAPK inhibitor had decreased glucose uptake (Figure 9A). We, next, incubated IL-7-deprived D1 cells with specific inhibitors for JNK or p38 MAPK, while re-adding IL-7 to the media, and measured glucose uptake using 2-DOG. D1 cells incubated with the JNK inhibitor, but not the p38 MAPK inhibitor, showed decreased glucose uptake upon IL-7 addition (Figure 9B). To validate our finding, we sought to investigate the effect of JNK inhibitor on primary T-cells. We cultured enriched T-cells, extracted from lymph nodes of C57BL6 mice, without IL-7 for 18 hours, and then restored IL-7 for 2 hours while adding the JNK inhibitor. Similar to its effect on D1 cells, JNK inhibitor reduced the uptake of 2-DOG in T –cells from murine lymph nodes (Figure 9C), establishing that JNK plays an essential role in the IL-7 regulation of glucose uptake.

To examine the pattern of JNK activity in response to IL-7, a JNK kinase activity assay, based on immunoprecipitation of the kinase and detection of the phosphorylated c-Jun substrate, was used. A shown in Figure 10A, we incubated D1 cells with or without IL-7 for the indicated periods of time. We extracted JNK from each sample by immunoprecipitation then measured its activity. Western blot for phospho-c-Jun showed that D1 cells incubated with IL-7 had higher

levels of JNK kinase activity than those deprived from IL-7 (Figure 10A). The increase in JNK

kinase activity was also concentration dependent. D1 cells incubated with 50ng/ml of IL-7 had

more JNK kinase activity than D1 cells incubated with 10ng/ml, while D1 cells deprived of IL-7

had the lowest activity (Figure 10B).

Jun-D containing AP-1 complexes are activated upon IL-7 stimulation through the JNK

Pathway

An analysis of HXKII promoter region revealed that this promoter included a binding

region for AP1 complex (108). Since JNK is known to regulate the components that lead to AP-

1 activation(116), we performed an EMSA in which an oligonucleotide probe, containing the

AP-1 DNA-binding consensus site, was incubated with nuclear extracts from D1 cells incubated

with or without IL-7 for 18 hours, as well as from D1 cells incubated without IL-7 for 18 hours

to which IL-7 was added to the medium for four hours.   Shown in Figure 11A are the results

revealing an increase in the amount of active AP-1 complex that bound DNA when D1 cells

were incubated with IL-7, or when IL-7 was added to the media after 18 hours of deprivation,

compared to D1 cells grown without IL-7. This suggested that IL-7 induced the activation of the

DNA binding AP-1 complexes (Figure 11A). To confirm that the protein binding the AP-1

probe was specific, we performed a competition assay with excess cold AP_1 probe (unlabeled)

as well as with a mutant AP-1 probe (Figure 11B). We found that cold AP-1 probe effectively

reduced the binding of protein to the AP-1 DNA probe, while the mutant AP-1 probe had no

effect (Figure 11B). We also co-incubated the nuclear extract made from D1 cells grown with

IL-7 (Figure 11B) or without IL-7 (data not shown) with antibodies against the different components of the AP-1 complex, namely c-fos, c-Jun and Jun-D, and then combined the mixture with the AP-1 radiolabeled DNA probe, performing a supershift assay to identify the transcription factor components involved in the formation of IL-7-induced AP1 complex. We found that c-Fos and c-Jun antibodies did not cause a supershift, while the Jun-D antibody prevented protein binding to the AP-1 DNA probe by sterically interfering with the interaction of the protein to the DNA. We concluded that Jun-D homodimers formed the AP-1 complex in response to IL-7 stimulation (Figure 11B).

Since we answered the question about the nature of the transcription factor involved in forming the AP1 complex, we wanted next to confirm that in fact the Jun-D-containing AP-1 complexes were binding to AP-1 site on HXKII promoter to induce the transcription of HXKII. We performed a chromatin immunoprecipitation assay (ChIP). We incubated D1 cells with and without IL-7 for 18 hours and used the JunD antibody to immunoprecipitate the sheared chromatin to pull-down AP-1-bound DNA. Then we performed qPCR using primers specific to AP-1 region on the HXKII promoter to amplify a sequence of 150 base pairs. Data in Figure 11C shows a slight increase in AP-1 DNA detected in samples from D1 cells grown with IL-7. Although additional optimization is required, this experiment confirmed that JunD-containing AP-1 complexes bind to the AP1 site on HXKII promoter region in an IL-7 dependent manner.

In our initial report, we found that HXKII gene expression increased 2 hours after IL-7 re-addition to deprived cells (107). This rapid increase suggested that the transcription factor involved in this activation would need to be readily activated. To determine whether the JNK

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pathway, which we found dependent on IL-7 (Fig. 9B, C), was involved in synthesis and activation of Jun-D as part of inducing HXKII transcription, we examined, by qPCR, the gene expression levels of Jun-D in the presence of p38MAPK and JNK inhibitors. D1 cells were grown with or without IL-7 for 18 hrs and IL-7 re-added for 2 hours to a separate group of IL-7-deprived cells. Loss of IL-7 caused a decrease in the gene expression of Jun-D that was restored upon a two hour re-addition of IL-7 (Figure 12A). We also confirmed at the protein level, by immunoblot, that Jun-D was decreased in the absence of IL-7 (Figure 12B). We next found, upon inhibition of JNK but not p38 MAPK, that the gene expression of Jun-D was reduced to a level below that of D1 cells deprived of IL-7 (Figure 12A). To confirm our findings with D1 cells, we measured Jun-D gene expression by qPCR using primary T-cells isolated from the lymph nodes of C57BL6 mice. Primary T-cells were cultured with IL-7 for 7 days to expand the IL-7 dependent T-cells, and then IL-7 was withdrawn for 18 hours and then re-added for two hours in the presence or absence of the JNK inhibitor. As we observed with D1 cells, re-addition of IL-7 induced the expression of Jun-D in a manner that was inhibited by the JNK inhibitor (Figure 12C).

IL-7-dependent JNK activity drives glucose uptake through the synthesis of HXKII

To investigate whether Jun-D activity mediated the IL-7 driven increase in glucose uptake that we previously reported was mediated by HXKII (107), we incubated D1 cells with Jun-D siRNA and measured the uptake of 2-DOG. Inhibition of Jun-D with siRNA reduced the uptake of glucose to levels observed in the cells cultured without IL-7 (Figure 13A). This result

indicated that AP-1 complexes containing Jun-D were in large part responsible for the uptake of glucose detected upon IL-7 stimulation.

Next, we examined whether the increased HXKII gene expression that followed IL-7 readdition was also associated with the activity of JNK. We deprived D1 cells from IL-7 for 18 hours, then added IL-7 and the JNK inhibitor for two hours. The gene expression of HXKII was measured by qPCR. We observed that the synthesis of HXKII was reduced by about 70% upon treatment with the JNK inhibitor (Figure 13B). We also incubated D1 cells with the p38 MAPK inhibitor, but did not see any effect on gene expression of HXKII (Figure 13B). This data confirmed that the IL-7 driven increase in HXKII gene expression is mediated by JNK activity and not by p38 MAPK. We also examined primary T-cells from lymph nodes of C57BL6 mice and also observed decreased IL-7 driven HXKII gene expression after incubation with JNK kinase inhibitor, validating the data obtained by using D1 cells (Figure 13C)

Over expression of HXKII causes cell cycle arrest.

Reports have shown that over expression and forced phosphorylation and activation of JunD increased cell death and forced cells into cell cycle arrest (85). Previously, we found that D1 cells nucleofected with the HXKII plasmid increased ATP production and underwent cell death (Figure 7D, E). To determine whether over expression of HXKII caused cell cycle arrest prior to inducing apoptosis, we nucleofected D1 cells with the cDNA for HXKII and assessed proliferation by measuring BrdU incorporation. We found that 70% of the cells expressing HXKII were growth arrested (did not incorporated BrdU), compared to 25% of the D1 cells expressing the empty vector. These results are suggestive that increased expression of HXKII,

likely mediated through the JNK-JunD pathway, can confer a negative growth signal, causing cell cycle arrest and eventually apoptosis.

In summary, we have shown that IL-7 promotes the uptake of glucose by inducing the gene expression of HXKII through a signaling pathway involving the activation of JNK. In response to IL-7, JNK, in turn, initiates the synthesis and activation of the AP-1 transcriptional complex composed of Jun-D homodimers. Given these findings, deregulation of HXKII expression, and likely its upstream activators, has the potential to cause inhibition of T-cell growth.
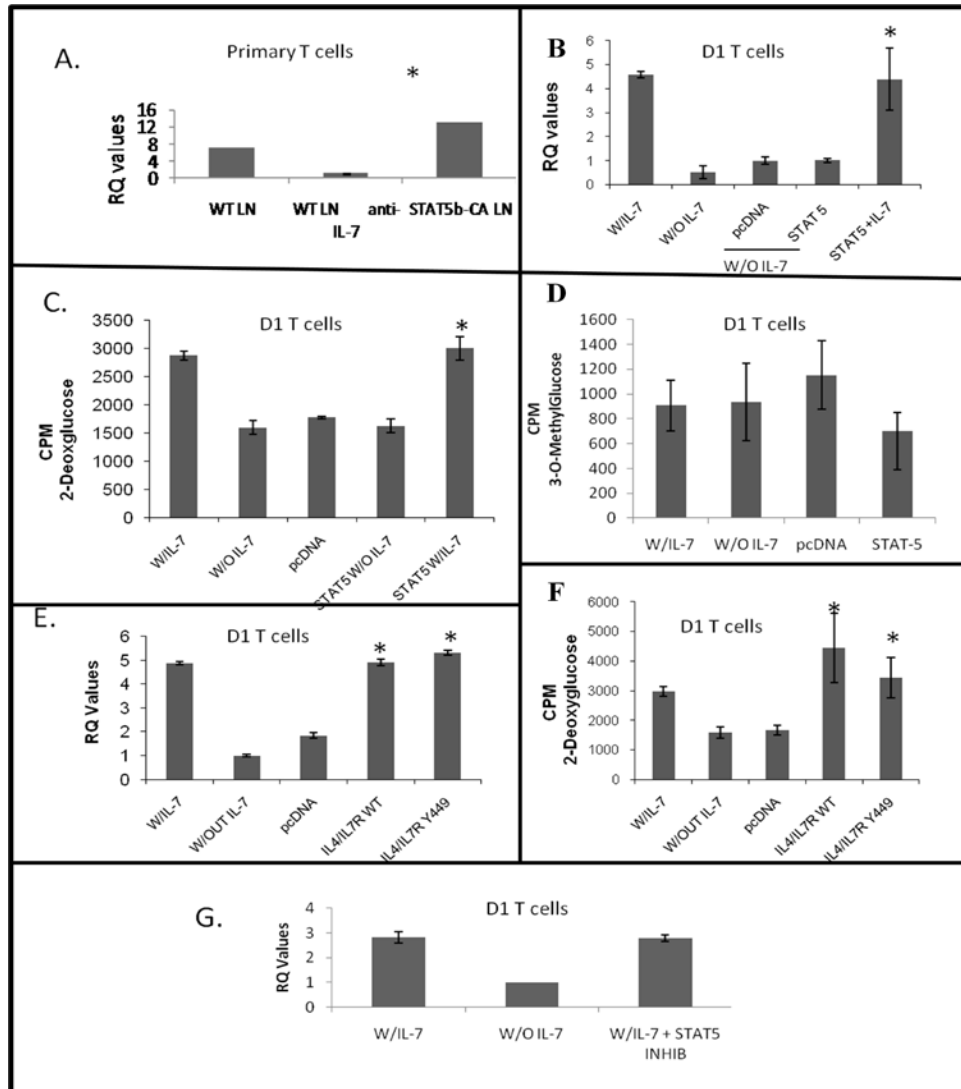
Figure 8: STAT5 Enhances but is not sufficient for expression of HXKII.

(A, B) Primary T-cells isolated from the lymph nodes of mice expressing STAT5b-CA (A) or D1 cells nucleofected with pcDNA (empty vector) or STAT5a-CA (B) were assayed for HXKII gene expression. Total RNA was extracted and transcribed to cDNA and qPCR was performed to analyze the gene expression of HXK II. Results are representative of two independent experiments performed in triplicates (values are average ± SD). (C, D) D1 cells were deprived from IL-7 for 18 hours, nucleofected with constitutively active STAT5a-CA or pcDNA, incubated with or without IL-7 for 8 hours, and then assayed for glucose uptake using ^{3}H-deoxyglucose (C) or 3-O-MethylGlucose (D). (E,F) D1 cells were nucleofected with pcDNA, IL4R/IL-7R-WT or IL-4R/IL-7R-Y449A cDNAs, stimulated with human IL-4, and assayed for HXKII gene expression (E) or glucose uptake with 2-deoxyglucose (F) as described above. Results are representative of three independent experiments performed in triplicate (values are average ± SD). *p<0.05 in comparison to w/o IL-7

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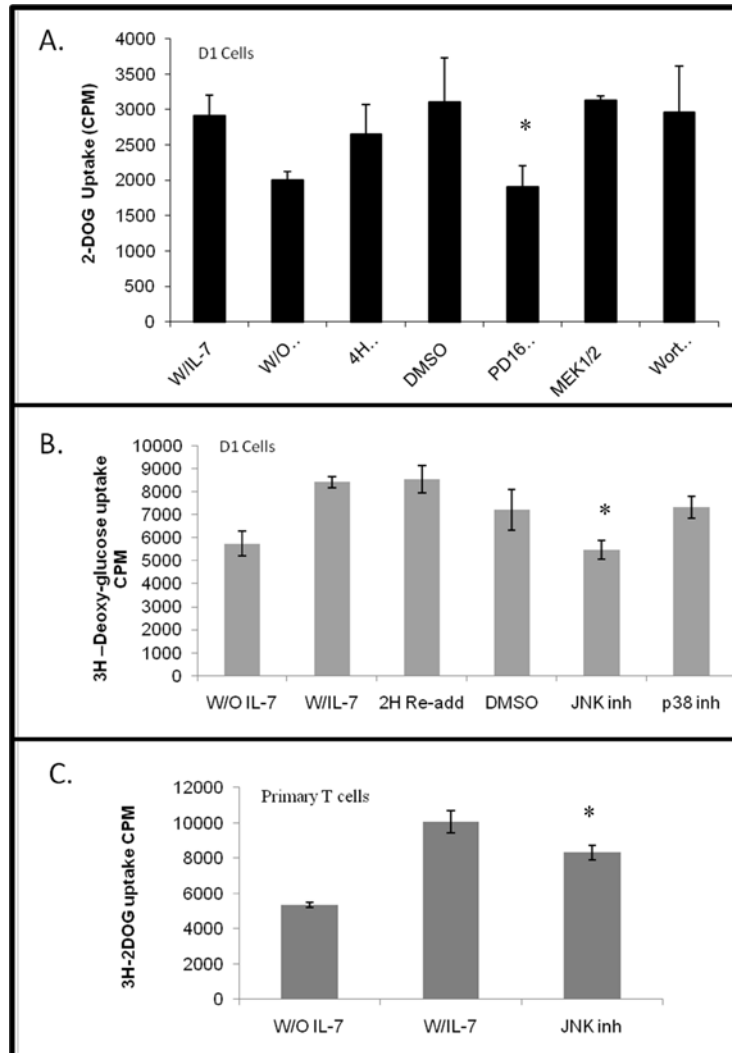
Figure 9: Inhibition of JNK prevented glucose uptake in cells cultured with IL-7.

 (A) D1 cells were grown with IL-7 or without IL-7 for 18 hours. To IL-7-deprived cells, IL-7 was re-added for four hours in presence of the vehicle control (DMSO), the MAPK inhibitor, PD169316, the MEK1/2 inhibitor and the PI3K inhibitor, wortmannin. Glucose uptake was measured by radiolabeled 2-DOG uptake. (B) Same experiment as in (A) except that IL-7 was re-added for 2 hours and specific inhibitors for JNK and p38 MAPK were used. (C) Primary T cells isolated from lymph nodes were cultured for 7 days with IL-7, then deprived of the cytokine for 18 hours. JNK inhibitor was used to treat the cells in the presence of IL-7. Results are representative of three experiments performed in triplicate (values are average ± SD). (values are average ± SD). *p<0.05 in comparison to 4hr IL-7 re-addtion.

A.

+ IL-7	0H	1 H	2 H
Intensity	79	82	82
- IL-7	0 H	1 H	2 H
Intensity	69	35	48

B.

IL-7 ng/ml	0	10	50
Intensity	11	25	32
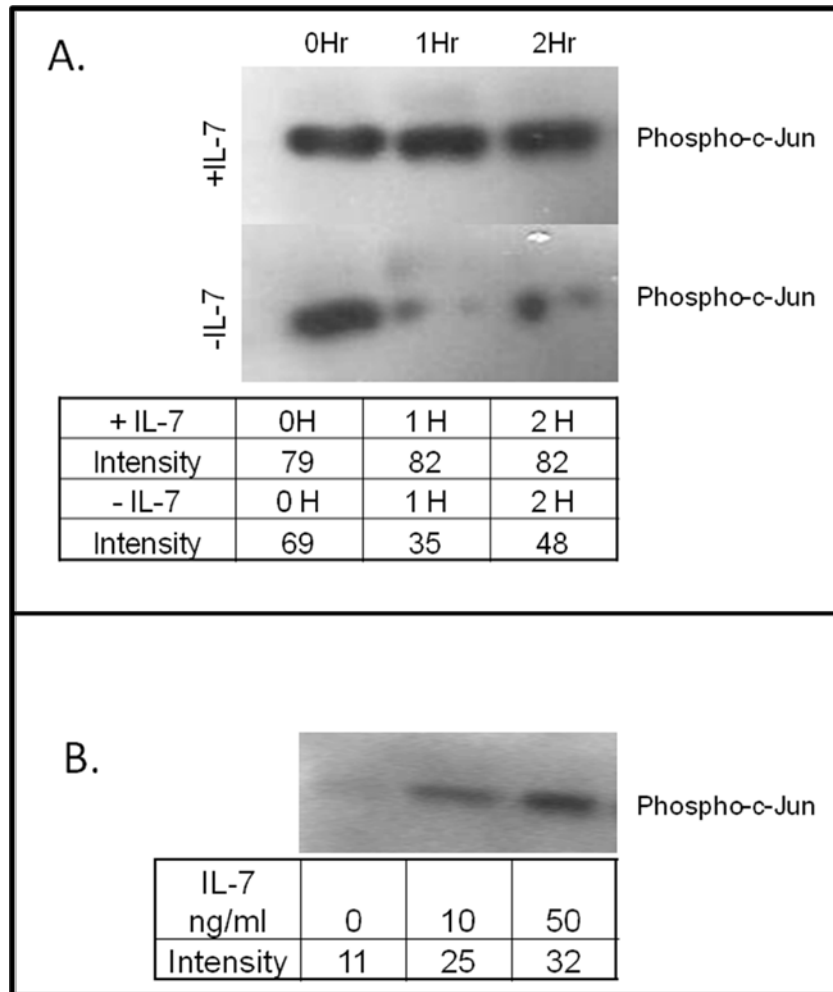
Figure 10: JNK activity increases with IL-7 in concentration-dependent manner.

 (A). D1 cells were grown with or without IL-7 for 0-2 hours.   JNK activity was measured by immunoprecipitating JNK and assaying the phosphorylation of a c-Jun substrate by immunoblot. (B) D1 cells were cultured for 18 hours with 0, 10 or 50 ng/ml of IL-7 and JNK activity assayed as described in (A).   For both blots, relative band intensities were determined and listed in the tables below.

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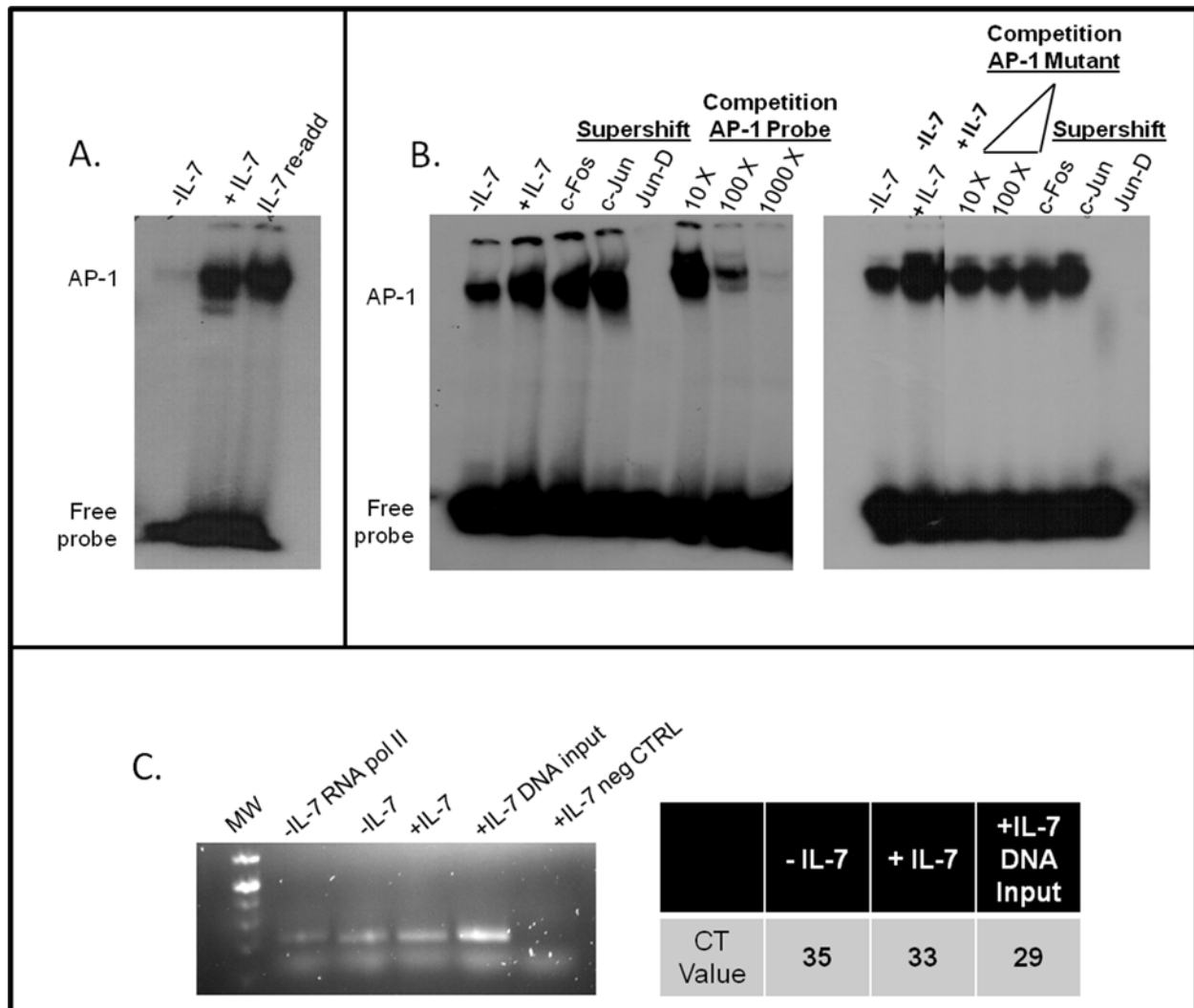
Figure 11: IL-7 induces AP-1 complexes containing Jun-D.

(A) Nuclear lysates were prepared from D1 cells grown with IL-7, without IL-7 for 18 hours or without IL-7 to which IL-7 was readded after deprivation. AP-1 complexes were measured by EMSA using a radiolabeled DNA probe containing the AP-1 consensus binding site. (B) Nuclear lysates were prepared from D1 cells as described in (A) and supershifts were performed with antibodies against c-Fos, c-Jun or Jun-D. Competition was also performed using 10X, 100X or 1000X excess unlabeled AP-1 probe or AP-1 mutant probe. (C) ChIP assay was performed using DNA from D1 cells cultured with or without IL-7 for 18 hours. PCR-amplified AP-1 promoter DNA was visualized by ethidium bromide staining on an agarose gel and quantitative results from the qPCR are shown in the table.
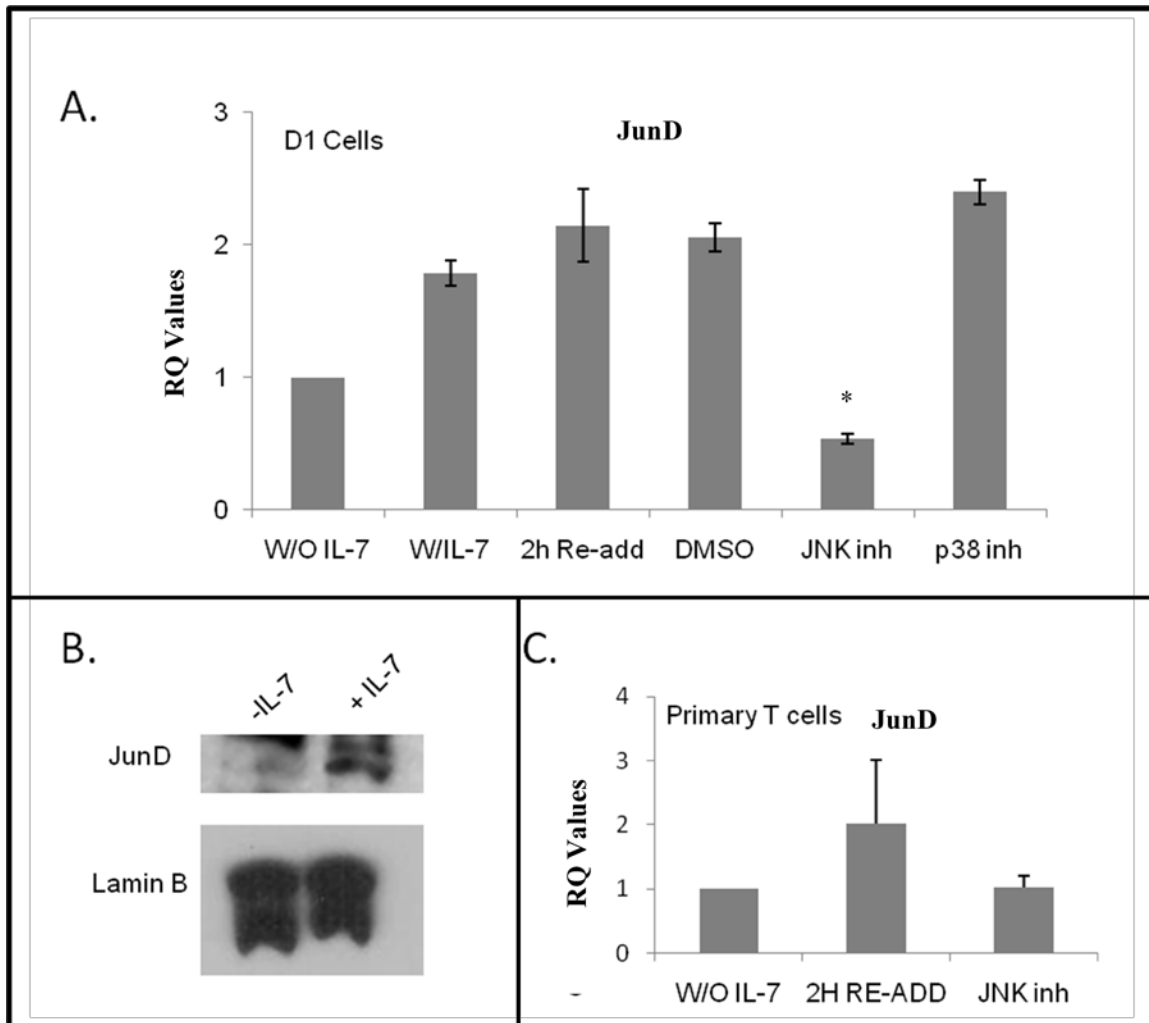
Figure 12: IL-7 promotes the gene expression of JunD through the JNK pathway.

(A) D1 cells were grown with IL-7, without IL-7 for 18 hours or without IL-7 to which IL-7 was readded after deprivation. The gene expression of JunD was measured by qPCR in the presence or absence of p38 MAPK or JNK inhibitors. (B) Protein lysates were prepared from D1 cells and immunoblotted with JunD antibodies to detect JunD protein levels in the cells cultured with or without IL-7. (C) Primary T cells were isolated from the lymph nodes of C57BL6 mice, cultured with IL-7, and then deprived of IL-7 for 18 hours. IL-7 was re-added in the presence or absence of the JNK inhibitor. qPCR was used to measured the gene expression of JunD. Results are representative of three experiments performed in triplicate (values are average ± SD). (values are average ± SD). *$p<0.05$ in comparison to 2hr IL-7 re-addtion.
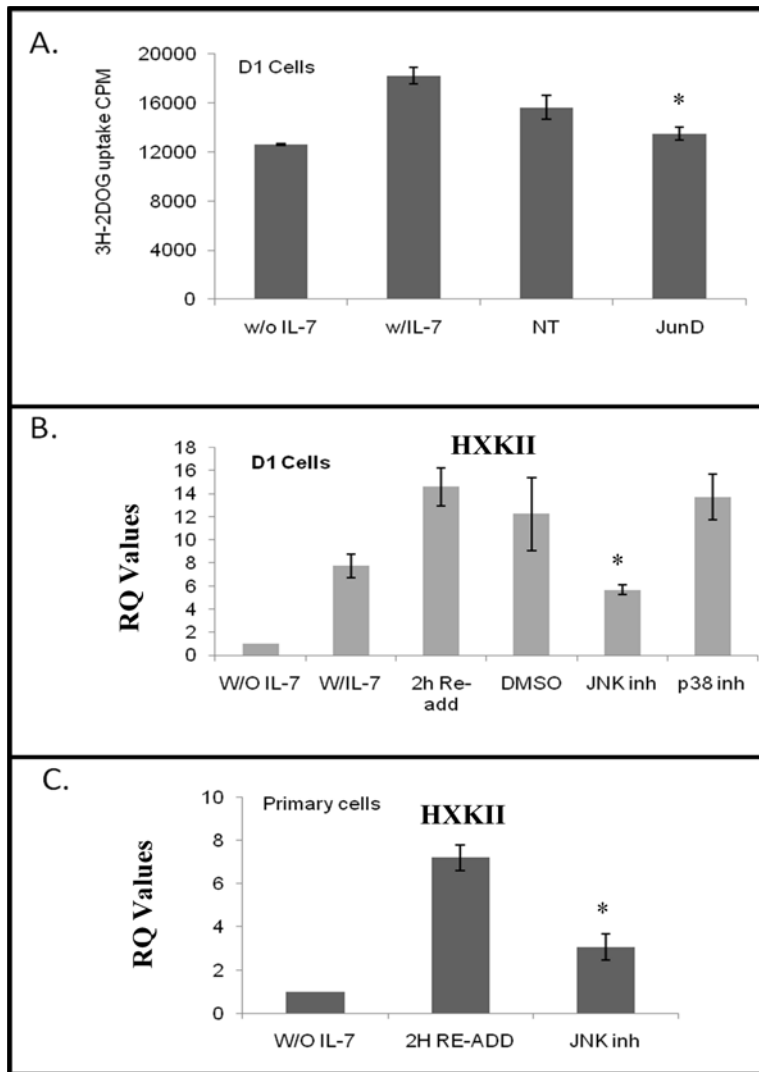
Figure 13: Inhibition of JunD or JNK Decreases Glucose Uptake and the Synthesis of HXKII.

(A) D1 cells were grown with IL-7, without IL-7 after introduction a non-specific siRNA (NT) or a JunD specific siRNA. Glucose uptake was measured by 2-DOG incorporation. (B). D1 cells were cultured with or without IL-7 and also re-added to a group of deprived cells in the presence or absence of JNK or p38 MAPK inhibitors. Gene expression of HXKII was measured by qPCR. (C) Primary T cells were isolated from the lymph nodes of C57BL6 mice, cultured with IL-7, and then deprived of IL-7 for 18 hours. IL-7 was re-added in the presence or absence of the JNK inhibitor. qPCR was used to measured the gene expression of HXKII. **Results** are representative of three experiments performed in triplicate (values are average ± SD). (values are average ± SD). *$p<0.05$ in comparison to 2hr IL-7 re-addtion.

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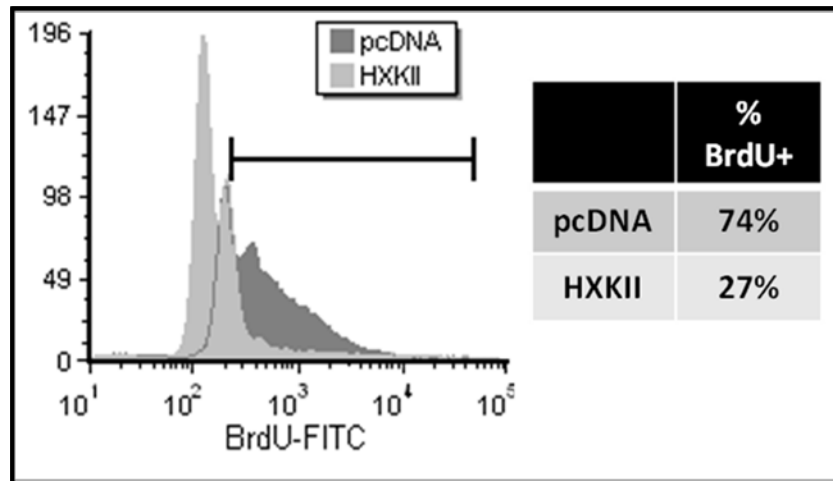**Figure 14: Over expression of HXKII induces cell cycle arrest.**

D1 cells were grown with IL-7 were nucleofected with empty vector (pcDNA) or the cDNA encoding HXKII. Proliferation was measured by assaying BrdU incorporation by flow cytometry.

Discussion

Herein we report that IL-7 contributes to the metabolic regulation of T-cells by mediating HXKII gene expression through the JNK-JunD pathway. We found that constitutively active STAT5 is not sufficient to induce the gene expression of HXKII, but that a STAT5-independent IL-7-mediated signal was involved. We observed that IL-7 signaling increased the activity of JNK in concentration-dependent manner, and that inhibition of the JNK pathway reduced glucose uptake. Moreover, engagement of the IL-7R by ligand increased the activation of nuclear AP-1 complexes containing Jun-D and promoted the binding of Jun-D homodimers to the AP-1 DNA binding site on HXKII promoter. Inhibition of JNK reduced the gene expression of Jun-D and decreased the synthesis of HXKII – specific inhibition of Jun-D also decreased glucose uptake. Others reported that Jun-D over expression repressed T-cells proliferation and differentiation (124). In agreement with these reports, our data showed that over expression of HXKII induced cell cycle arrest. These findings strongly suggest that IL-7 regulates HXKII gene expression through increased activity of JNK, which results in the activation of JunD and binding to the AP-1 site on HXKII promoter.

STAT5 had been implicated in the IL-7-dependent maintenance of T-cell proliferation and survival by activating gene expression of anti-apoptotic proteins of BCL2 family (51). STAT5 was also suggested to maintain glucose metabolism in the B cell derived cell line FL5.12 by supporting GLUT1 trafficking to cell surface (51). Using our IL-7 dependent T-cell line D1 and primary T-cells from C57BL6 mice, we found that the IL-7-mediated increase of HXKII

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gene expression was independent from STAT5 activation, suggesting that IL-7 uses a STAT5 independent pathway to regulate glucose uptake in T-cells.

Many studies had reported that PI3K regulated glucose metabolism in T-cells (99). We investigated whether PI3K mediated the IL-7 regulated gene expression of HXKII in T-cells. Using the PI3K inhibitor, wortmanin, we found that inhibition of PI3K signaling did not reduce glucose uptake, indicating that this pathway was not responsible for regulating glucose metabolic activity in response to IL-7. Rather, we discovered a role for the MAPKs in IL-7-driven gene expression of HXKII. This finding is supported by reports that the MAPK pathway can be induced by IL-7 signaling (129).

Addition of IL-7 to cytokine-deprived T-cells induced rapid increase in the gene expression of HXKII that was detectable after two hours. This rapid response required that the transcription factor mediating this increase be readily available. Our finding that c-Jun was not involved in the AP-1 complex forming at the HXKII promoter correlates well with this rapid increase, since c-Jun is an inducible gene and needs to be newly synthesized upon addition of IL-7. This absence of c-Jun also suggests that JNK-mediated HXKII gene expression likely causes the down regulation of T-cell proliferation, since others have showed that the proliferative activity of JNK pathway is mostly conducted by c-Jun (130).

Interestingly, we found that the AP-1 complex induced by IL-7 signaling contained Jun-D homodimers. Initial reports of Jun-D activity suggested that it was a negative regulator of proliferation (122). Over expression of Jun-D in immortalized fibroblast reduced their proliferation (122). Fibroblasts from JunD$^{-/-}$ mice expressed higher levels of Cyclin D1 and

displayed increased proliferation (123), while over expression of JunD induced cell death in T-cells (124). However, JunD was also reported to positively regulate cell survival and proliferation (131). Our data are supported by the findings of Lamb et al. that Jun-D-induced survival activities and activation of anti-apoptotic genes was mediated by JNK kinase signaling (125), as well as that of Meixner et al. showing that JunD $^{-/-}$ mice exhibit reduced T- cell count (124).

The proliferative activity of IL-7 and its ability to sustain T-cell survival and homeostasis, have raised the prospective of its immunotherapeutic use in cancer and HIV therapy. Use of IL-7 to enhance the immune competence in the elderly is also being considered. In cancer therapy, the immunoreconstitutive character of IL-7 can help mitigate the lymphopenic condition, which ensues as a consequence of chemotherapy, leading to reduced T-cell count, especially in the CD4+ T cell population (132,133). In HIV patients, in vitro studies reported that IL-7 increased the CD4+ T-cells population as well as HIV virus specific CD8+ (134).

This optimistic prediction of IL-7 therapeutic success is nevertheless challenged by certain reports where a high dose of IL-7 could have a reciprocal effect on the homeostasis of T-cells, our data indicates that IL-7 increases the activation of Jun-D, promoting HXKII gene expression, glucose uptake and elevated ATP levels. Meixner et al. reported that mice over expressing Jun-D have strongly reduced numbers of peripheral lymphocytes, and that JunD over expression in lymphocytes does not protect from numerous apoptotic insults (124). In this study, it was also reported that transgenic T cells proliferated poorly and exhibit impaired activation due to reduced levels of IL-4, CD25 and CD69 (124). Our data reports that, in addition to these effects, that overexpression of HXKII induced cell cycle arrest in T-cells and increased cell

death. We also found that JNK activity rose when we increased the dose of IL-7. This suggests

the existence of a direct link between the IL-7 dose, JunD activation and HXKII gene expression

and indicate that further research is required to determine the optimal dose of IL-7 needed for an

effective T-cell immune response.

GENERAL CONCLUSIONS AND DISCUSSION

IL-7 is an important cytokine with potential activating role in T-cell survival, proliferation, and differentiation. Upon binding to the IL-7R, IL-7 transmits stimuli involving the activation a number of signaling pathways such as JAK/STAT, PI3K, and MAPK pathways to activate an array of genes, such as those encoding anti-apoptotic proteins. Regulation of the T-cell response to IL-7 is reported to be mediated through modulation of IL-7 availability. IL-7 is normally produced at low levels, but higher levels of IL-7 can be induced under conditions of disease or stress. For example, IL-7 production was induced in hepatocytes in response to Toll-like Receptor (TLR) signaling (135). In addition to cytokine production, the response to IL-7 can also be controlled through modulation of the of its receptor IL-7Rα levels(31). We found that IL-7 is involved in the regulation of T-cells homeostasis at different stages, and the control of glucose metabolism can play a pivotal role in the regulation of the survival and proliferation of T-cells.

IL-7 induced a rapid increase in glucose uptake. Our data demonstrated that IL-7 mediated glucose uptake through a rapid increase of HXKII gene expression. While others have proposed that IL-7 regulates glucose metabolic activity by controlling glucose trafficking through GLUT1 and found that GLUT1 was expressed by IL-7 as a late gene 72 hours after cytokine stimulation(53), we discovered that, instead, IL-7 induced the expression of HXKII as an early gene product made in response to the cytokine. In fact, we also observed an increase in GLUT1 gene expression after 60 hours of IL-7 addition to cytokines deprived D1 T-cells. We also reported in this study that over expression of HXKII rescued glucose uptake and increased intracellular ATP levels, while overexpression of GLUT1 had a minimal effect on glucose

uptake. This increase in ATP levels was, however, associated with an increase in apoptosis, implicating a tie between high level of intracellular ATP and cell death that was suggested by others (86,103). Our data offered an explanation to the rapid increase induced in glucose uptake by IL-7 that was observed in our study as well as in other studies (51,53)..

Our data showed for the first time that IL-7 induces the gene expression of HXKII through JNK activation and the increase of Jun-D binding to AP1 consensus on HXKII promoter region. IL-7 activation of JNK increased with higher doses of IL-7. This would lead to an increase in Jun-D activation and it's binding to AP1 consensus DNA on virtually all the genes that would have an AP1 consensus on their promoter. Some of these genes play critical roles in cell cycling such as Cyclin D1, and CDK4. These two genes were reported to be down regulated when JunD is activated (123,136). Interesting enough, Cyclin D1 was reported to down regulate HXKII in mammary epithelia of transgenic mice expressing ErbB2 (137). Other genes induced by Jun-D belong to the anti-apoptotic family of proteins BCL2; these genes were reported to be activated through the transcriptional activity of Jun-D (123), associating it with cell survival. It is possible that these Jun-D-dependent genes that encode anti-apoptotic molecules would increase their activities in parallel with the strength of the IL-7, and could contribute to maintenance of the size of T-cell pool.

However, over expression of Jun-D increased apoptotic cell death and reduced the number of T-cells in transgenic mice (124). In a parallel manner, the over expression of HXKII increased intracellular ATP, induced cell cycle arrest, and increased apoptotic cell death. Given that IL-7 induced an increase in Jun-D activity through activation of JNK, and that the IL-7-induced JNK activation increased when we increased the IL-7 dose, this suggest that there is a

dose dependent effect that could possibly trigger similar effects upon Jun-D or HXKII over expression. The question remains as to would this also occur upon therapeutic administration of high, non-physiological doses of IL-7.

The initial optimistic prediction of IL-7 therapeutic success in the treatment of cancer is challenged by reports where a high dose of IL-7 has a reciprocal effect on the homeostasis of T-cells. Moreover, an initial transient depletion of lymphocytes has accompanied most reports of the therapeutic use of IL-7(138). Finally, limited clinical benefit, in terms of cancer regression, has resulted from the first group of patients undergoing phase clinical trials to test the use of IL-7 (13). Our data reported that over expression of HXKII induced cell cycle arrest in T-cells and increased cell death and proposed a direct link between IL-7 dose, Jun-D activation and HXKII gene expression.

Given the importance of IL-7 in T-cell proliferation and activation, and its prominent therapeutic use, it is vital to take in consideration the potential negative effects that administration of high doses of IL-7 could have. Based on reports from others and based on the data reported in our studies, treatment with large, non-physiological amounts of IL-7 could lead to an increase in T-cell death, rather than cell proliferation, and could reduce the size of the certain T-cell subsets, skewing the balance needed for an effective immune response. This phenotype had been reported and considered a challenge for the development of IL-7 immunotherapy.

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