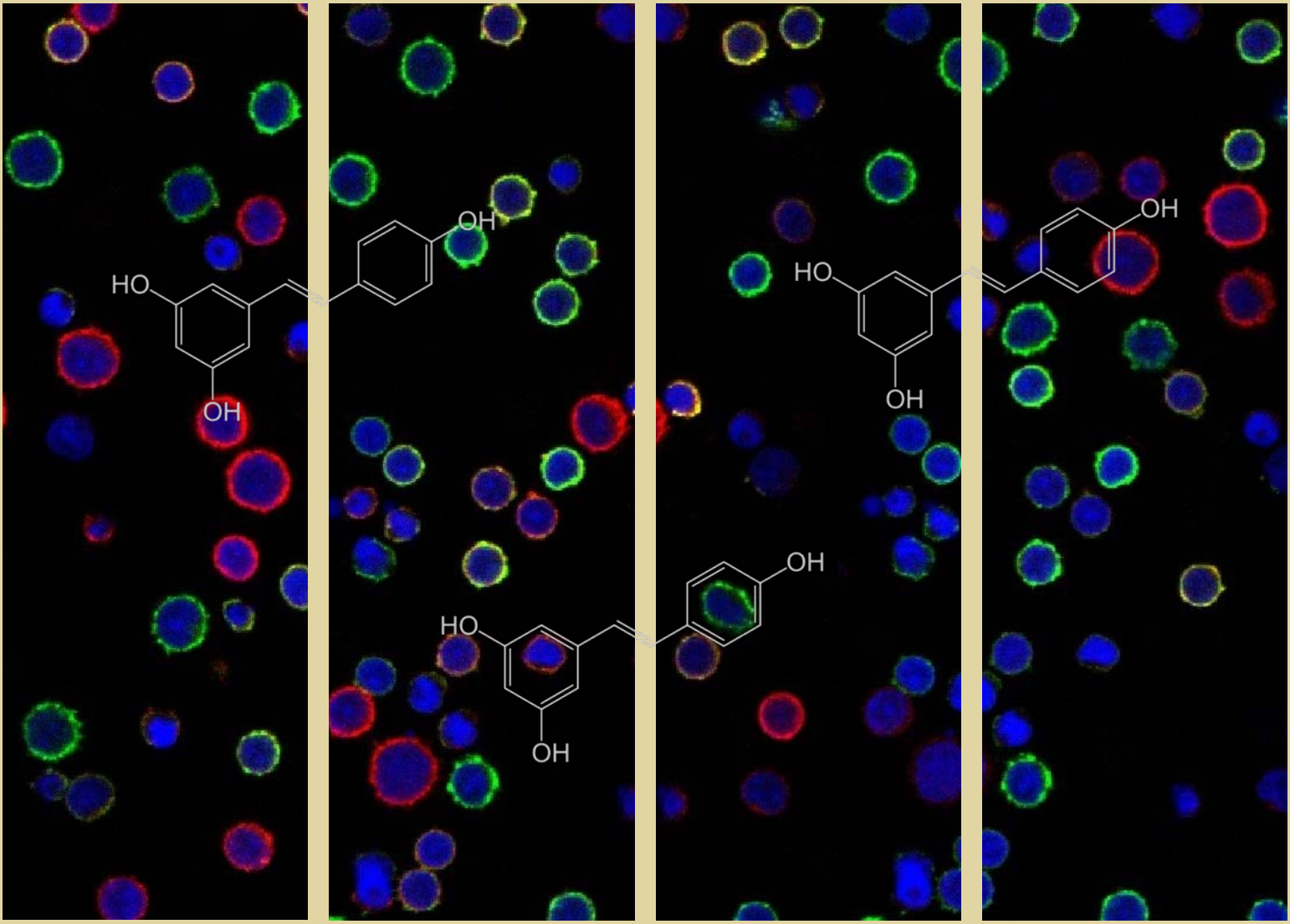

Marco Filipe Marques Craveiro

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Title (English)	**Resveratrol-induced activation of the Sirt1/AMPK axis in human CD4 T cells triggers replicative stress and an ATR-dependent cell cycle arrest**
Título (Português)	**A activação do eixo Sirt1/AMPK em linfócitos T CD4 humanos, através do uso de resveratrol, causa stress replicativo e leva a uma paragem do ciclo celular mediada pela quinase ATR.**
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Co-tutela	Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Portugal
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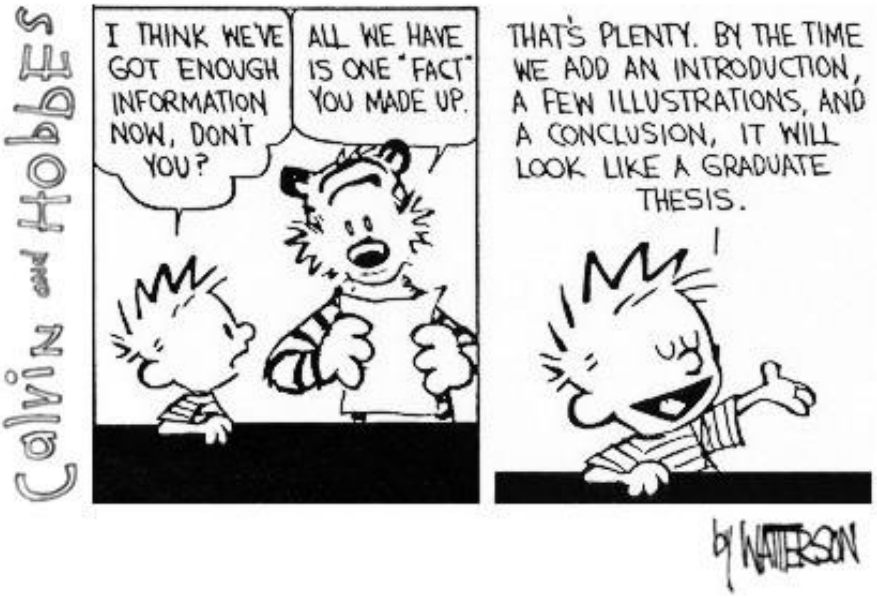
Calvin and Hobbes
I THINK WE'VE GOT ENOUGH INFORMATION NOW, DON'T YOU?
ALL WE HAVE IS ONE "FACT" YOU MADE UP.
THAT'S PLENTY. BY THE TIME WE ADD AN INTRODUCTION, A FEW ILLUSTRATIONS, AND A CONCLUSION, IT WILL LOOK LIKE A GRADUATE THESIS.
by WATTERSON

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Glossary

4E-BP1	4E-binding protein 1
acetyl-CoA	acetyl-Coenzyme A
AceCS	acetyl-CoA Synthetase
ADP	Adenosine Diphospate
Akt	Protein Kinase B
AMP	Adenosine Monophospate
AMPK	AMP-activated protein Kinase
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
ARF	Alternative Reading Frame
ASCT	Anti-Neutral amino acid transporter
ATF6	Activating Transcription Factor 6
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	ATM-Rad-3-related kinase
CaMK	Calcium-calmodulin Activated Kinase
CaMKKβ	CaMK kinase beta
cAMP	cyclic Adenosine Monophosphate
CARD11	Caspase Recruitment Domain protein 11
Cdk	Cyclin-dependent kinase
Cip	Cdk inhibitory protein
Chk	Checkpoint kinase
CHOP	C/EBP Homologous Protein
CKI	Cyclin-dependent Kinase Inhibitor
CO_2	Carbon dioxide
CR	Calorie Restriction
CREB	cAMP Response Element-Binding protein
CRTC2	CREB-regulated transcription coactivator 2
DAG	Diacylglycerol
DC	Dendritic Cell
DDR	DNA Damage Response
DL-4	Delta-like 4
DN	Double Negative (thymocyte)
DNA	Deoxyribonucleic acid
DP	Double Positive (thymocyte)
DSBs	DNA Double-Strand Breaks
E2F-DP	E2 promoter-binding factor dimerization partner
ECL	Extracellular Loops
eIF-4E	Eukaryotic translation Initiation Factor 4E
eNOSC	Energy-dependent Nucleolar Silencing Complex
ETC	Electron Transport Chain
ETP	Early Thymic Progenitor
ER	Endoplasmatic Reticulum
Erk	Extracellular signal Regulated Kinase
ERRα	Estrogen-Related Receptor
FAD	Flavin Adenine Dinucleotide
FADH	Flavin Adenine Dinucleotide Hydride
FoxO	Forkhead Box O
FoxP3	Forkhead Box P3
G_0	Gap zero phase (mitosis)
G_1	Gap one phase (mitosis)
G_2	Gap two phase (mitosis)
GADS	Grb2-related Adaptor Downstream of Shc
GAP	GTPase-Activating Protein
GATA3	GATA-binding protein 3
GDH	Glutamate Dehydrogenase
GDP	Guanosine Diphosphate
GRF	GDP releasing factor
GTP	Guanosine Triphosphate
H_2O	Dihydrogen oxide/Water
H_2O_2	Hydrogen peroxide
H2AX	Histone-2A family member X
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HIF	Hypoxia-Inducible Factor
HIV	Human Immunodeficiency Virus
HSC	Haematopoietic Stem Cell
hTERT	human Telomerase Reverse Transcriptase
IDH2	Isocitrate Dehydrogenase 2
IKK	IκB kinase complex
IL	Interleukin
IL-2Rα	Interleukin 2 Receptor alpha (CD25)
IL-2Rβ	Interleukin 2 Receptor beta (CD122)
IL-7Rα	Interleukin 7 Receptor alpha (CD127)
IMS	Intermembrane Space
INK4	Inhibitor of Kinase 4

IP3R	Inositol 1,4,5-trisphosphate Receptor
IRE1α	Inositol-Requiring Enzyme 1α
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
K (Lys)	Lysine amino acid residue
Kip	Kinase inhibitor protein
Lck	Lymphocyte-specific tyrosine kinase
LKB1	Liver Kinase B1
LKLF	Lung Krüppel-Like Factor
LXR	Liver X Receptor
MAPK	Mitogen Activated Protein Kinase
Mcm	Mini-chromosome maintenance proteins
MEK	MAPK/Erk Kinase
MEKK1	MAP/Erk Kinase 1
MMP	Mitochondrial Membrane Potential
MnSOD	Manganese Superoxide Dismutase
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTOR	Mammalian Target Of Rapamycin
M	Mitotic phase
MHC	Major Histocompatibility Complex
NAD	Nicotinamide Adenine Dinucleotide
NADH	NAD Hydride
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	NADP Hydride
NAM	Nicotinamide
Nampt	NAM phosphoribosyltransferase
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NRF	Nuclear Respiratory Factor
O_2	Dioxygen
OXPHOS	Oxidative Phosphorylation
PD-1	Programmed cell Death protein 1
PDK1	Phosphoinositide-Dependent Kinase 1
PERK	PKR-like Endoplasmic Reticulum Kinase
PHA	Phytohaemagglutin
PI3K	Phosphatidylinositide 3-kinase
PIP3	Phosphatidylinositol Trisphosphate
PGC-1α	PPAR Gamma Coactivator 1-alpha
PKC	Protein Kinase C
PKR	Protein Kinase R
PLCγ1	Phospholipase C gamma 1
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome Proliferator-Activated Receptor
PTM	Post-Translational Modification
RAG	Recombination Activating Genes
RAPTOR	Regulatory-Associated Protein of mTOR
Rb/pRb	Retinoblastoma protein
Rheb	Ras homolog enriched in brain
RICTOR	RAPTOR-Independent Companion of TOR
RNA	Ribonucleic acid
RORγt	RAR-related Orphan Receptor gamma
ROS	Radical Oxygen Species
RUNX	Runt-related transcription factor
RVT	Resveratrol
S	Synthesis phase (mitosis)
S6K	ribosomal protein S6 kinase
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SCID	Severe Combined Immunodeficiency
Sirt	Sirtuin
SGK1	Serum Glucocorticoid-regulated Kinase 1
SCF	SKP/Cullin/F-box E3 ubiquitin ligase
SLC	Solute Carrier family of transporters
SOCS	Suppressor Of Cytokine Signalling
SOD	Superoxide Dismutase
SP4	Single Positive $CD4^+$ (thymocyte)
SP8	Single Positive $CD8^+$ (thymocyte)
SREBP	Sterol-Regulatory Element Binding Protein
ssDNA	single-stranded DNA breaks
STAT	Signal Transducer and Activator of Transcription
T (Thr)	Threonine amino acid residue
Tat	Trans-Activator of Transcription
TCA cycle	Tricarboxylic acid cycle (Krebs cycle)
TCR	T Cell Receptor
TEC	Thymic Epithelial Cell
Tfh	T follicular helper
Th	T helper cell
ThPOK	Th Inducing POZ- Krüppel Factor
TNF	Tumor Necrosis Factor
T_{Reg}	regulatory T cell
TSC	Tuberous sclerosis
UPR	Unfolded Protein Response
XBP1	X-box Binding Protein 1
Y (Tyr)	Tyrosine amino acid residue
ZAP-70	Zeta-chain-associated Protein Kinase 70

Abstract

T lymphocyte activation, triggered by T cell receptor engagement and by various chemokines, can only occur when the cell has generated sufficient energy resources. The increased biosynthetic demands of an activated T cell can be met by increasing nutrient availability and adopting an anabolic metabolism, allowing an enhanced ATP generation.

In many different cell types, mitochondrial function is enhanced by the Silent information regulator (Sir) proteins, NAD^+-dependent redox-sensing histone deacetylases. However, the role of these proteins in regulating T lymphocyte metabolism is not known.

Resveratrol is a natural polyphenol that possesses anti-aging and anti-inflammatory properties, being its effects attributed to its capacity to modulate cellular metabolism via induction of the Sirtuin1 (Sirt1) histone deacetylase. Here, I show that Sirt1 is highly upregulated following TCR stimulation of primary human CD4 T lymphocytes with resveratrol further augmenting Sirt1 expression and deacetylase activity. Sirt1 upregulation is also associated with phosphorylation of serine residues in the Nuclear Localization Signal (NLS) consensus, consistent with my observations that Sirt1 is predominantly nuclear following T cell activation. Despite the increase in Sirt1 expression, I find that T cell activation leads to an increased acetylation of p53 and FoxO1, two known Sirt1 targets. Not surprisingly, the TCR-induced acetylation of p53 and FoxO1 was significantly decreased following activation of the Sirt1 deacetylase with resveratrol.

Importantly, high dose resveratrol blocked proximal TCR signaling and progression from the G_0 phase of the cell cycle. In contrast, CD4 T lymphocytes treated with low dose resveratrol were strongly activated and upregulated all cyclins and Cyclin-dependent kinases (Cdks) required for cell division. Furthermore, these cells exhibited high metabolic activity as monitored by upregulation of the Glut1 glucose transporter, increased glucose uptake and

enhanced mitochondrial function. *De facto*, when compared to control cells, activated T lymphocytes treated with low dose resveratrol show boosted mitochondrial respiration and higher intracellular ATP levels. Nevertheless, low dose resveratrol induced a G_2/M genotoxic arrest, with augmented phosphorylation of the histone H2AX double-stranded DNA break marker and the minichromosome maintenance helicase Mcm2. Phosphorylation of both H2AX and Mcm2 is associated with the onset of replicative stress, and I was able to verify that the cell cycle exit observed in lymphocytes treated with low dose resveratrol was associated with the activation of p53. Furthermore, I found that this activation of p53 was mediated both by the serine/threonine-protein kinase ATR, which is involved in sensing DNA damage and activating the DNA damage response, and the AMP-activated protein kinase (AMPK), a fuel sensor known to interact with Sirt1 and share many of its targets.

Thus, while resveratrol increased Sirt1 and AMPK activity, producing a positive energy balance in CD4 T cells, its negative regulation of DNA integrity resulted in the induction of a p53-dependent cell cycle checkpoint.

Resumo

A activação de linfócitos T, desencadeada por acoplamento do receptor de células T e por várias citocinas, pode apenas ocorrer após a célula gerar energia suficiente. A resposta às crescentes necessidades biossintéticas de uma célula T activada pode ser alcançada pelo aumento da disponibilidade de nutrientes e pela adopção de um metabolismo anabólico, permitindo optimizar a produção de ATP.

Em muitos tipos de células diferentes, a função mitocondrial é melhorada pelas proteínas reguladoras de informação silenciosa (Sir), as quais se tratam de deacetilases de histonas dependentes de NAD^+ e sensíveis a potencial redox. No entanto, desconhece-se o papel destas proteínas na regulação do metabolismo dos linfócitos T.

Resveratrol é um fenol natural com propriedades anti-envelhecimento e anti-inflamatórias, efeitos atribuídos à capacidade deste polifenol em modular o metabolismo celular por indução da actividade da deacetilase Sirtuína 1 (Sirt1). O estudo aqui apresentado mostra que a expressão da proteína Sirt1 aumenta de forma significativa em linfócitos T $CD4^+$ humanos após estimulação do receptor de células T (TCR) e que tratamento com resveratrol promove um acréscimo na actividade desta deacetilase. Esta amplificação da expressão de Sirt1 encontra-se também associada a um aumento na fosforilação dos resíduos de serina na sequência de consenso do sinal de localização nuclear (NLS), algo consistente com as minhas observações de que após activação das células T a expressão de Sirt1 é predominantemente nuclear. Curiosamente, apesar do aumento verificado na expressão de Sirt1, a activação dos linfócitos T encontra-se também associada a um incremento na acetilação dos factores de transcrição p53 e FoxO1, duas proteínas amplamente descritas na literatura como alvos da actividade de deacetilase de Sirt1. Ainda assim, pude verificar que tratamento com resveratrol resultava numa diminuição considerável dos níveis de acetilação de p53 e de FoxO1.

De destacar, que o tratamento de linfócitos T CD4 com uma dose alta de resveratrol bloqueia a sinalização proximal ao TCR, resultando numa interrupção do ciclo celular na fase G_0. Em contraste, linfócitos T tratados com uma dose baixa de resveratrol não só exibem um estado de activação normal aquando da iniciação do sinal TCR, como são capazes de amplificar as ciclinas e as quinases dependentes de ciclinas (Cdks) necessárias à normal progressão do ciclo celular. Além disso, linfócitos T activos tratados com uma baixa dose de resveratrol exibem elevada actividade metabólica, conforme demonstrado pela maior expressão do transportador de glucose Glut1, respectiva captação de glucose e da função mitocondrial. Além disso, pude ainda verificar que em comparação a linfócitos T activos controlo, os linfócitos T tratados com dose baixa de resveratrol exibem níveis de ATP intracelular mais elevados e um aumento da respiração mitocondrial. De salientar, porém, que apesar de exibirem uma capacidade metabólica notável, tratamento de linfócitos CD4 com uma dose baixa de resveratrol resulta numa paragem do ciclo celular na transição entre as fases G_2/M, consequência da ocurrência de um stress genotóxico associado ao aumento da fosforilação da histona H2AX, um marcador de quebra da cadeia dupla de ADN e da helicase de manutenção de minicromossomas Mcm2. A fosforilação tanto da histona H2AX como da helicase Mcm2 encontram-se associadas ao fenómeno de stress replicativo e no decurso do meu estudo foi-me possível verificar que o bloqueio do ciclo celular na transição G_2/M se deve à activação do guardião do genoma e regulador do ciclo celular, p53. Adicionalmente, pude também verificar que esta activação de p53 era mediada simultaneamente pela quinase ATR, um dos principais mediadores da reparação de ADN em resposta a um stress replicativo, e pela quinase AMPK, uma enzima conhecida por se associar a Sirt1 na regulação do equilíbrio energético das células.

Em conclusão, se por um lado o tratamento de linfócitos T com resveratrol resulta num incremento metabólico e energético graças a um aumento na activade das enzimas Sirt1 e AMPK, por outro, acaba por afectar a integridade genómica levando à activação de p53 e, consequentemente, a um bloqueio na progressão do ciclo celular.

CHAPTER 1:

INTRODUCTION

Introduction

1. The Immune System

The immune system consists of many different proteins, cells, organs, and tissues interacting as a dynamic network in order to protect the organism against non-self elements, such as bacteria, foreign molecules, viruses and protozoa, as well against self elements such as degenerated tumour cells.

The immune system protects organisms from infection with layered defences of increasing specificity and complexity. First, physical barriers, such as the skin, prevent pathogens from entering the body. Second, if a pathogen breaches these barriers, the innate immune system can provide an immediate response. The innate immune system encompasses the defence mechanisms that are responsible for a non-specific immune response, comprising anatomical, physiological, phagocytic and inflammatory barriers. In vertebrates, a specific response to pathogens is generated by the adaptive immune system and this response is then retained after the pathogen has been eliminated, in the form of immunological memory.

1.1. Development of the Adaptive Immune System

According to its action and function, four major properties arise from the acquired immune system: antigen-specificity, diversity, immune memory and the recognition of self vs non-self antigens. There are two major classes of acquired immune responses: humoral immunity, mediated via immunoglobulins produced by B lymphocytes, and cellular immunity, mediated by T lymphocytes. Humoral immunity consists mainly in antibody production, although it also includes accessory processes such as helper T cell (Th2) activation, cytokine production

and memory cell generation. Some antibody functions, including pathogen and toxin neutralization, classical complement activation, promotion of phagocytosis and pathogen elimination are also examples of humoral immunity. Cell-mediated immunity, as the name implies, is a type of immune response wherein immune cells are the effectors, in particular macrophages, Natural Killer (NKs) cells and T lymphocytes. These subsets of blood cells originate from haematopoietic stem cells in bone marrow niches and are responsible for the phagocytosis and killing of pathogens.

1.2. The Thymus and T cell differentiation

The haematopoietic system refers to the assembly of organs and tissues responsible for blood cell formation (haematopoiesis) (Doulatov et al., 2012). There are two types of organs in the haematopoietic system: primary or central organs, and secondary or peripheral organs. Primary haematopoietic organs are the major sites of blood cell differentiation and include the bone marrow and the thymus (Orkin and Zon, 2008). Lymph nodes and spleen constitute secondary haematopoietic organs, where blood cells continue to differentiate and are maintained (Orkin and Zon, 2008). Considering the state of differentiation, the cellular components of the haematopoietic system can be divided in four groups: stem cells, progenitor cells, precursor cells and finally, lineage differentiated cells, which include mature cells such as T lymphocytes (Blom and Spits, 2006).

The thymus is a specialized organ located in the upper chest where developing haematopoietic progenitors differentiate into functional T cells, a process known as thymopoiesis (Gordon and Manley, 2011; Ma et al., 2013). The thymus is a bilobed organ located in the thoracic cavity, anterior to the heart. Histologically, two distinct compartments can be identified in the thymus, the cortex and the medulla; each being composed of different populations of thymic epithelial cells (TEC), endothelial cells, mesenchymal cells and dendritic cells. The different cellular constitutions of these two compartments provide distinct microenvironments to developing thymic lymphocytes, also known as thymocytes. Early stages of thymocyte differentiation occur in the cortex, whereas CD4/CD8-lineage commitment occurs in the medulla (Gordon and Manley, 2011; Murphy K, 2008). Furthermore, during thymocyte differentiation, unique populations can be identified depending on the expression of specific cell surface markers, such as the T Cell Receptor (TCR), the CD3 complex (signal

transduction components of the TCR) and the CD4 and CD8 co-receptors (Singer et al., 2008).

Thymopoiesis starts with the migration of Haematopoietic Stem Cells (HSCs) from the bone marrow to the thymus where they become Early Thymic Progenitors (ETPs). In humans, the $CD34^+$ haematopoietic precursors that seed the thymus can differentiate into multiple lineages, giving rise not just to T, B and NK cells of the lymphoid compartment, but also to myeloid cells such as Dendritic Cells (DCs) and erythrocytes (Blom and Spits, 2006). Once in the thymus, Notch1 signalling instructs the progenitor cells to commit to the T-cell lineage. The Notch signalling pathway is a highly conserved cell-to-cell communication cascade present in most tissues (Radtke et al., 2013). Notch1 interaction with its ligand Delta-like 4 (DL-4), expressed by thymic stromal cells, stimulates cell survival and metabolism through activation of the PI3K/Akt pathway (Carpenter and Bosselut, 2010). How Notch1 promotes commitment to the T cell fate is not yet known; what is known is that Notch1 promotes expression of genes specific to the T lymphocyte lineage. Active Notch1 coordinates the differentiation of ETPs into Double Negative (DN) thymocytes, thus named because of the absence of CD4 and CD8 co-receptors. DN cells can also be characterized as Triple Negative because they do not express the CD3 glycoprotein either.

Considering the state of differentiation and the markers expressed, different DN populations can be identified. The DN3 stage is defined by expression of the Recombination Activating Genes (RAG) which drives the rearrangement of the *tcrb* locus and TCR β-chain expression is initiated. RAG also drives the rearrangement of the *tcrg* locus and the consequent development of a small population of γδ T cells. Commitment to the αβ-lineage requires that thymocytes are able to signal through a pre-TCR, a checkpoint step known as β-selection. The pre-TCR consists of a TCRβ chain complex with CD3 and a pre-Tα (TCRα chain not yet rearranged). Thymic αβ-lymphocytes that fail to provide a signal for T cell differentiation undergo apoptosis.

Successful TCRβ selection leads to the generation of Double Positive (DP) cells, where thymocytes begin to express CD4 and CD8 (Carpenter and Bosselut, 2010; Ma et al., 2013). CD4 and CD8 expression is followed by the rearrangement of the *tcra* locus and expression of a fully functional TCRαβ. DPs are then selected as a function of their avidity to bind Major Histocompatibility Complex peptides (pMHC) expressed by the thymic epithelium, an event known as positive selection. During this checkpoint phase, cells with low avidity for MHC

class I and MHC class II self-peptides are selected to survive, whereas thymocytes unable to bind MHC molecules are eliminated through "death by neglect" (Miller, 2011; Suzuki et al., 1995). Thymic lymphocytes that proceed through the positive selection stage move to the medullary region where they interact with self-antigens presented by TECs and DCs; this developmental step, known as negative selection, serves to eliminate self-reactive cells as DP thymocytes with high affinity for self peptides undergo apoptosis (Carpenter and Bosselut, 2010; Ma et al., 2013). The final stage of thymopoiesis is the differentiation of DP cells into CD4 single positive (SP) or CD8 SP cells, via a crucial "decision" known as CD4/CD8 lineage choice (Singer et al., 2008) [FIGURE 1].

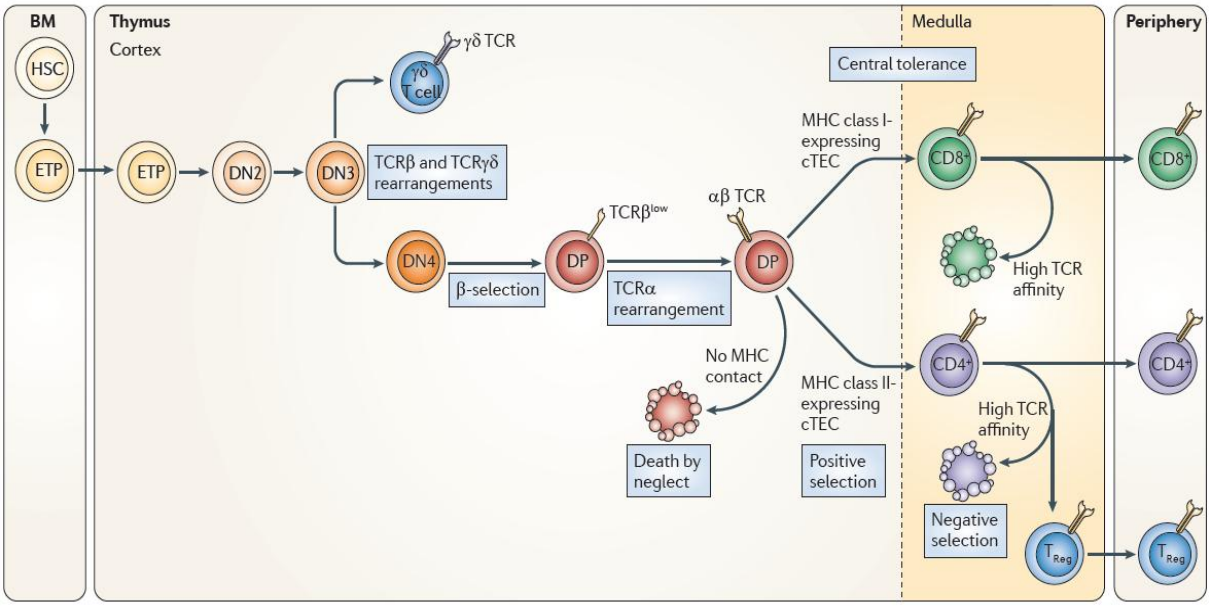

Figure 1. **Major events in thymus cell differentiation**. HSCs from the bone marrow give rise to ETPs. ETPs lose the potential to differentiate into B cells and DCs within the thymus. The DN cells, thus named because they do not express the TCR or the co-receptors CD4 and CD8, begin to undergo crucial rearrangements of TCR genes. TCRβ gene selection leads to the generation of DP cells, which express a properly rearranged TCR β-chain ($TCR\beta^{low}$) and both the CD4 and CD8 co-receptors. This is followed by the rearrangement of the TCR α-chain locus and expression of an αβ TCR. Failure of a DP thymocyte to contact MHC molecules results in death by neglect. Cells with TCRs that bind to MHC class I molecules upregulate CD8 and lose CD4, whereas cells that bind to MHC class II molecules maintain CD4 and lose CD8; this process is called positive selection. If the avidity of binding to a specific MHC– peptide ligand exceeds a certain threshold, the cells are deleted by negative selection in the medulla. Undeleted SP cells migrate out to the periphery from the medulla. A small percentage of self-reactive CD4+ thymic cells with an increased avidity for MHC class II molecules, just below the threshold for negative selection, upregulate the transcription factor forkhead box P3 (FoxP3) and exert regulatory T (T_{Reg}) cell functions. γδ T cells originate from DN cells that have not yet undergone β-selection. *Adapted From (Miller, 2011).*

1.2.1. The kinetic signalling model of CD4/CD8 lineage commitment

The kinetic signalling model [FIGURE 2] is the most widely accepted explanation for CD4/CD8 lineage choice (Singer et al., 2008). This model is based on observations made in murine thymocytes and proposes that lineage choice is determined by the duration of TCR signalling during positive selection with the Interleukin 7 (IL-7) cytokine acting as a sensor of signal duration (Singer et al., 2008). During positive selection, an initial TCR signal terminates Cd8 gene transcription, resulting in the development of an intermediate thymocyte population with a $CD4^{+}CD8^{low}$ phenotype (Park et al., 2010). Under conditions where the TCR is restricted by MHC class II presentation, signalling will continue as CD4 is still expressed, thus providing the necessary costimulation (Van Laethem et al., 2007). Moreover, persistent TCR signalling induces ThPOK expression, a specific marker of CD4 fate (Singer et al., 2008). However, if the TCR is MHC class I restricted, the signal will cease due to the lack of the required CD8 costimulation (Singer et al., 2008). The disruption of TCR signalling promotes the initiation of IL-7 signalling, inducing thymocytes to undergo co-receptor reversal and differentiate into CD8 SP thymocytes. This mechanism is mediated by expression of the transcription factor RUNX3 (Park et al., 2007b). IL-7 signalling in murine CD8 SP thymocytes results in a downregulation of *IL7r* transcripts and, as such, IL-7Rα levels are significantly lower on CD8 SP than CD4 SP thymocytes (Sudo et al., 1993; Yu et al., 2003).

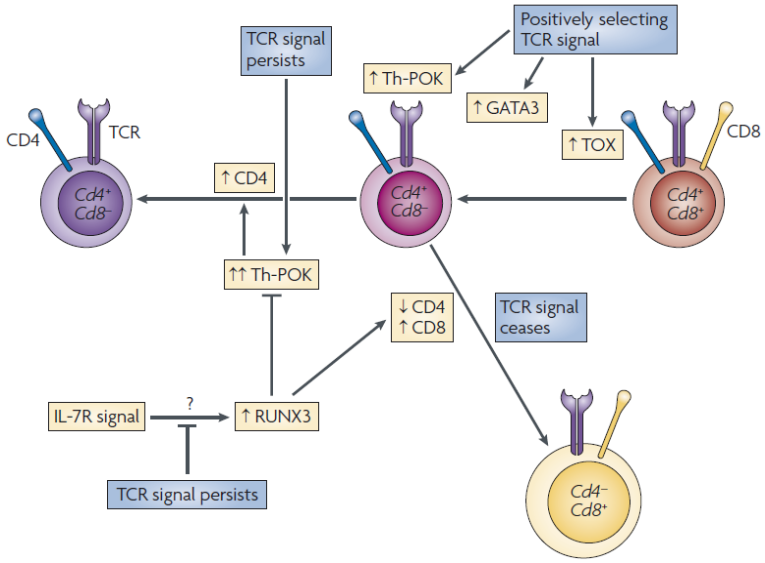

Figure 2. **The kinetic signalling model of CD4/CD8-lineage choice.** Positively selecting TCR signals upregulate TOX and induce DP thymocytes that are transcriptionally $Cd4^{+}Cd8^{+}$ to terminate *Cd8* gene expression and to convert into $Cd4^{+}Cd8^{-}$ intermediate thymocytes. Because of the absence of *Cd8* gene transcription, $Cd4^{+}Cd8^{-}$ intermediate thymocytes appear phenotypically as $CD4^{+}CD8^{low}$ cells, and these are the cells in which lineage choice is made.

Persistence of TCR signalling in *Cd4*$^{+}$*Cd8*$^{-}$ intermediate thymocytes blocks IL-7-mediated signalling and this is followed by an upregulation of GATA3 and Th-POK expression which are important for the differentiation of CD4^{+}CD8^{low} thymocytes into CD4+ T cells. Moreover, Th-POK prevents the RUNX protein-mediated silencing of the *Cd4* gene. Cessation or disruption of TCR signalling in CD4^{+}CD8^{low} allows IL-7-mediated signalling, which induces *Cd4*$^{+}$*Cd8*$^{-}$ intermediate thymocytes to undergo co-receptor reversal, gain a *Cd4*$^{-}$*Cd8*$^{+}$ phenotype and differentiate into CD8+ T cells. RUNX3, upregulated by IL-7 signalling, has three important functions that promote the differentiation of intermediate thymocytes into CD8+ T cells: first, RUNX3 binds to the *Cd4* silencer element and silences *Cd4* gene expression; second, RUNX3 binds to the E8$_1$ *Cd8* enhancer element and reinitiates *Cd8* gene expression; and third, RUNX3 silences *Zbtb7b* (zinc-finger-and-BTB-domain containing 7B, which encodes Th-POK) gene expression. *Adapted from (Singer et al., 2008).*

After the selection process, naïve mature T cells leave the thymus and migrate to the periphery where they circulate between the blood and lymphatic systems (Ma et al., 2013). Given the amazing diversity of antigens, the probability of one T cell meeting its specific antigen in circulation is very low (estimated size of clones with the same αβ TCR repertoire is 1 in 10^{15}) (Bonarius et al., 2006). This is likely why T cells have evolved to localize in secondary lymphoid organs, where the probability of interacting with an antigen presenting cell (APC) is considerably higher, since antigens also collect in secondary lymphoid organs. T cells that have not yet encountered foreign antigen are known as naïve cells, and following encounter with a specific MHC-bound antigen, they become activated. Activated T cells undergo rapid clonal expansion and gain effector functions such as cytokine production (CD4^{+} cells) and cell-mediated cytotoxicity (CD8^{+} cells). After the elimination of the antigenic agent, the majority of effector T cells disappear, and the ones that remain become memory T cells, taking up residency in secondary lymphoid tissue cells (Broere et al., 2011). M*emory* T lymphocytes accumulate over the lifetime of an organism, *c*reating a dynamic pool of antigen-experienced cells that are capable of clearing the antigenic agent much faster than naïve T cells (Sallusto et al., 2004).

1.3. T cells

T lymphocytes are characterized on the basis of their function and/or the specific markers they express. For example, cytotoxic T lymphocytes express the CD8 co-receptor and are able to directly target a given antigen-expressing cell. Helper T cells are characterized by the expression of the CD4 glycoprotein and act mainly as “assistants” to other white blood cells, including the cytotoxic CD8 cells. Memory T cells are a repository of the antigenic experience of the immune system; these are T cells that persist long-term after interaction

with their cognate antigen and are able to rapidly respond and expand if challenged again by the same antigen. Regulatory T cells are a subpopulation of T lymphocytes that express the CD4 co-receptor, the interleukin-2 receptor alpha (CD25) and the transcription factor FoxP3; they play an essential role in holding the immune system in check, thus preventing unrestricted expansion of effector T cells and the development of immune-mediated pathologies.

1.3.1. T cell receptor

The T cell Receptor (TCR) is a heterodimer of two variable disulfide-linked chains. 95% of T lymphocytes express the alpha and beta forms of these chains (αβ T cells) whereas the remaining 5% expressing gamma and delta chains (γδ T cells). Each chain of the TCR is comprised of two cysteine-linked domains: one N-terminal variable structure (V) that binds the antigen-MHC complex and one C-terminal cytoplasmic constant domain (C) that anchors the TCR to the T cell membrane via a transmembrane region composed of positively charged amino acids.

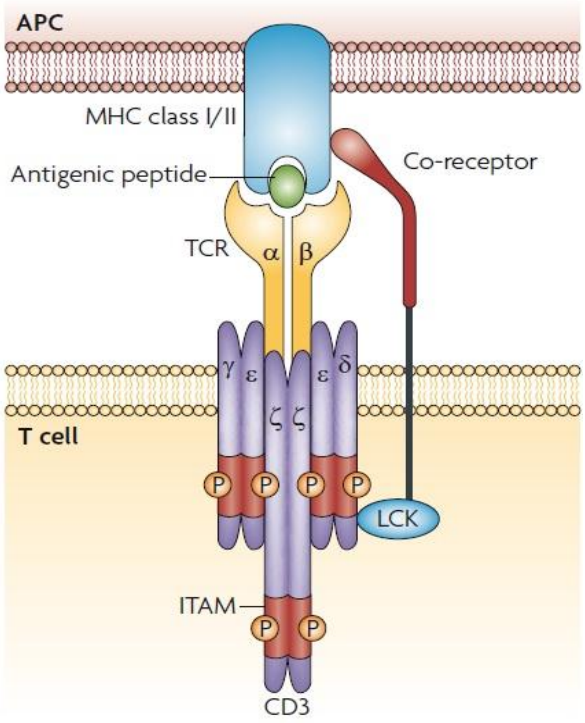

Figure 3. **Classical model of TCR-MHC interactions.** The T-cell receptor interacts with an antigenic peptide–MHC complex, and the co-receptor (CD4 or CD8) stabilizes this interaction by bringing the kinase LCK into the proximity of the TCR–CD3 complex. LCK then phosphorylates the CD3-associated immunoreceptor tyrosine-based activation motifs (ITAMs). *Adapted from (Gascoigne, 2008).*

As the cytoplasmic tails of the TCR polypeptides are very short, all proximal signalling events following a TCR-engagement are mediated through CD3, made up of five polypeptide hyper variable chains. Indeed, cell surface expression of the TCR occurs only in association with CD3. CD3 chains associate to form 3 dimers: a γε heterodimer, a δε heterodimer, and a ζζ homodimer or a ζη heterodimer. 90% of the CD3 molecules present as ζζ homodimers, with the other 10% as ζη heterodimers. The ζ and ε chains have very short external domains of 9 aa, a transmembrane domain containing a negatively charged aspartic acid involved in the interaction with the positive charges of the TCR and a cytoplasmic tail of 113 ζ aa or 155 η aa responsible for signal transduction. The cytoplasmic domains of all the CD3 chains also contain a characteristic immunoreceptor tyrosine-based activation motif (ITAM). Engagement of the TCR triggers the tyrosine phosphorylation of the ITAMs, which then orchestrate the sequential activation of the Src-related protein tyrosine kinase Lck followed by the phosphorylation of ZAP70, allowing a TCR signalling cascade to proceed [FIGURE 3].

1.3.2. The CD4 and CD8 co-receptors

In addition to the TCR and CD3, other co-receptors can bind antigen-associated MHC molecules, thus enhancing TCR signalling. These co-receptors not only ensure TCR specificity, but also prolong and stabilize the APC/T lymphocyte interaction (Gascoigne et al., 2010). They are also involved in the mobilization of protein kinases that participate in the TCR signalling, such as Lck (Holland et al., 2012).

CD4 and CD8 are examples of such co-receptors [FIGURE 3]. The extracellular domains of CD4 and CD8 connect MHC domains associated to APCs; MHC-II β2 chain for CD4 and MHC-I α2 and α3 chains for CD8. CD4 is a 55 kDa transmembrane monomeric glycoprotein. It contains 4 immunoglobulin-like extracellular domains, a hydrophobic membrane region and a long cytoplasmic tail with 3 serine residues capable of being phosphorylated. CD8 occurs either as a αβ heterodimer or a αα homodimer, with the α and β chains representing membrane glycoproteins of 30-38 kDa. Each CD8 chain has only one immunoglobulin-like extracellular domain, the hydrophobic membrane region and a small cytoplasmic tail of 25-27 aa (Alberts B, 2007a; Murphy K, 2008).

Moreover, as mentioned, CD4 and CD8 can also be used to distinguish different subsets of T lymphocytes, as CD4 is only expressed on helper T cells, whereas CD8 is specific to cytotoxic T cells (Suzuki et al., 1995).

1.4. The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a large cluster of genes found in human chromosome 6 that plays an important role in the immune system. MHC proteins are highly polymorphic and can be divided into two main subgroups, class I and class II, as well as a third and smaller group, MHC III, that encodes immune elements such as cytokines and components of the complement system (Blum et al., 2012). Classical MHC class I (MHC-I) molecules are expressed on virtually all nucleated cell types and consist of two polypeptides, the α-chain and the non-MHC β2-microglobulin subunit, which together form a stable protein (Adams and Luoma, 2012).

The role of MHC-I proteins is to present peptide antigens to killer T cells, which have receptors for class I MHC proteins (Neefjes et al., 2011). Class II MHC (MHC-II) proteins present peptide antigens derived from digested foreign particles, and this is likely why they are found only on immune cells with phagocytic capacity. MHC-II is formed of two chains, α and β, each comprised of two domains and the peptide-binding groove is formed by the heterodimer of α1 and β1 (Adams and Luoma, 2012). Helper T cells recognize antigen peptides bound to MHC class II, thus initiating antibody and cytokine production and activating killer T cells (Neefjes et al., 2011).

1.5. Cytokines

Cytokines represent a diverse family of peptides and small proteins that are secreted and act as mediators between cells (Alberts B, 2007a). Cytokines regulate cellular activity by interacting with specific cell-surface receptors. They can be autocrine or paracrine, meaning that they can affect the behaviour of the cell secreting the cytokine or modulate the activity of neighbour cells, respectively (Murphy K, 2008).

Cytokines are produced in response to activating stimuli and can have a pleiotropic behavior; individual cytokines may be produced by different cell types and exert multiple actions (Ozaki and Leonard, 2002). Cytokines can also be redundant, a consequence of overlapping functions (Ozaki and Leonard, 2002). Pleiotropism can be explained by the expression of the same receptor on different cell lineages, whereas redundancy is due to the ability of cytokines to activate multiple signalling pathways. Redundancy may also be explained by the nature of the receptors. Most cytokine receptors occur as heterodimers in which one chain is common

to several cytokines of the same family (Ozaki and Leonard, 2002). One example is CD132, also known as the Interleukin-2 receptor γ-chain. This chain is common to the heterodimer receptor of six different interleukins, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 and is therefore now referred to as the common γ-chain (γc) (Liao et al., 2013).

$CD4^+$ T cells are the main secretors of interleukin 2 (IL-2). IL-2 signals via a heterotrimer receptor formed by three IL-2 receptor subunits: IL-2Rα (CD25), IL-2Rβ (CD122) and the common γ-chain described above (Kim et al., 2006; Walsh, 2012). IL-2 production depends on calcium and Protein Kinase C signalling downstream of antigen stimulation and is mediated by multiple transcription factors including Nuclear Factor of Activated T cells (NFAT), the Jun-Fos complex and NF-κB (Liao et al., 2013). IL-2 promotes the growth and proliferation of $CD4^+$ and $CD8^+$ T cells (Morgan et al., 1976), as well of Natural Killer cells (Siegel et al., 1987) and B cells. In addition to its positive role in regulating T cell proliferation, IL-2 stimulation also promotes antibody production in B cells (Mingari et al., 1984) and T cell differentiation. In response to an antigen, IL-2 induces the activation of different transcriptional programs that drive the differentiation of naïve $CD4^+$ T cells to a T helper 1 (Th1) or Th2 fate while blocking the development of Th17 and T follicular helper (Tfh) cells (Liao et al., 2013).

IL-7 is an interleukin that plays a critical role in T cell development and homeostasis (Mackall et al., 2011). IL-7 is also a member of the common γ-chain cytokine family; the IL-7R consists of a heterodimer formed by the IL-7 receptor α-chain (CD127) and the common γ-chain (Mazzucchelli and Durum, 2007; Walsh, 2012). IL-7 is produced by stromal cells in lymphoid organs, but because the expression of this cytokine is considerably low, the spatiotemporal expression of IL-7 *in vivo* has long remained obscured and only now a full characterization of IL-7 availability is starting to be evaluated. (Kim et al., 2011b; Mazzucchelli and Durum, 2007). IL-7 mediates anti-apoptotic and proliferative signals in human T cells through the activation of the Phosphoinositide 3-kinase (PI3K) and the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways (Swainson et al., 2007; Swainson et al., 2006). Singer and colleagues have also shown that intermittent IL-7 is required for the differentiation of DP thymocytes into functionally mature $CD8^+$ T cells and for the maintenance of naïve CD8+ T cells lifelong immunocompetence (Kimura et al., 2013; Yu et al., 2003).

2. T cell activation

2.1. T cell antigen receptor signal transduction

Interaction of the TCR with antigenic peptides complexed to MHC molecules induces a series of intracellular signalling cascades that lead to cellular proliferation and differentiation, cytokine production and/or activation-induced cell death (Murphy K, 2008) [FIGURE 4]. T cell activation is also characterized by an increased metabolic activity which requires the upregulation of several metabolite transporters such as the transferrin receptor CD71 and the glucose transporter Glut1. The membrane glycoprotein CD69 is another marker transiently expressed on lymphocyte activation, believed to be an enhancer of activation and/or differentiation (Bezouska et al., 1995; Sancho et al., 2005).

TCR signalling begins with an early wave of protein tyrosine kinase activation, which is mediated by the Src-family protein kinases Lck and Fyn. Following phosphorylation of Lck and Fyn, the ZAP-70 protein kinase is recruited to the receptor complex where it is phosphorylated and then activates multiple downstream pathways, including the Extracellular signal Regulated Kinase (Erk) Protein Kinase C (PKC), and Nuclear Factor Kappa B (NF-κB) cascades and also stimulates increases in intracellular free calcium and the release of inositol phosphates. TCR engagement also triggers another T cell activation pathway that involves the recruitment of Nck following a conformational change of the CD3ε chain. Nck, the Non-catalytic region of tyrosine kinase adaptor protein, is a cytoplasmic protein that acts as a transducer of signals from receptor tyrosine kinases to downstream recipients such as members of the RAS family (Davis, 2002). Overall, these pathways lead to the activation of several transcription factors (*e.g.* Rel proteins), which ultimately regulate the expression of genes that control T cell development, homeostasis, activation, anergy, apoptosis and the acquisition of effector functions [FIGURE 4].

Complete T cell activation requires signals from various costimulatory receptors, such as those triggered by CD28 (June et al., 1990). In vivo, CD28 stimulation occurs upon interaction with the B7.1 (CD80) and B7.2 (CD86) proteins on the APC. CD86 is constitutively expressed on antigen presenting cells and CD80 is upregulated on APCs signalled by Toll-like receptor ligands (Colombetti et al., 2006; Li et al., 2001). CD28 signalling alone does not induce effector functions in resting human T cells; instead, it

provides an extra adhesion mechanism that brings the T cell and the APC membranes within a proximity sufficient to signal the TCR/CD3 complex (Acuto and Michel, 2003). In fact, it has been suggested that CD28 does not control gene transcription directly but converges with TCR signals to fully activate transcription factors, thereby preventing activation-induced anergy and augmenting T cell proliferation as a consequence of the increase in IL-2 production (Morgan et al., 1976). Thompson and colleagues have shown that CD28 signalling, via the activation of phosphatidylinositol-3-kinase (PI3K) and its downstream effector Akt, favours the metabolic mobilization of T cells by upregulating glucose transport and consumption (Frauwirth and Thompson, 2004). Lck is also activated by CD28. Phosphorylated Lck, in turn, amplifies the signals coming from the TCR/CD3 complex by increasing the phosphorylation of Fyn and ZAP-70 [FIGURE 4] (Davis, 2002). By amplifying the T cell response, CD28 signalling promotes a full cellular activation under condition of low antigen levels (Bachmann et al., 1996; Kundig et al., 1996).

ZAP-70 phosphorylation is a critical step in T cell activation and development. Indeed, patients deficient in this kinase suffer from severe combined immunodeficiency (SCID) and lack functional T cells in the periphery (Wang et al., 2010). Amongst the most important ZAP-70 targets are the raft-associated transmembrane adapter protein (LAT) and SLP-76, a cytosolic adapter protein from the Src Homology 2 family. These two adapters, together with GADS, PLCγ1 and ITK, form the backbone of the complex responsible for the spatiotemporal organization of effector molecules involved in the multiple signalling pathways downstream of the TCR/CD3 complex. Loss of any of these adapters results in a severe impairment of TCR signal transduction (Smith-Garvin et al., 2009).

PLCγ1 is directly responsible for the production of the second messengers: phosphatydilinositol trisphosphate (PIP3) and diacylglycerol (DAG). DAG is then involved in the activation of the Ras-Raf-MEK-Erk pathway that turns on the transcription machinery, whereas PIP3 binds to the IP3 receptor at the surface of the endoplasmatic reticulum (ER) promoting the release of Ca^{2+} (Lewis, 2001). Depletion of intracellular Ca^{2+} stores then triggers the influx of Ca^{2+} through store-operated Ca^{2+} channels in the plasma membrane. Amongst others, the increase in Ca^{2+} intracellular levels activates calcineurin which is responsible for the dephosphorylation of the Nuclear Factor of Activated T cells (NFAT). Once dephosphorylated, NFAT is translocated to the nucleus where it cooperates in the regulation of the transcription machinery [FIGURE 4] (Lewis, 2001).

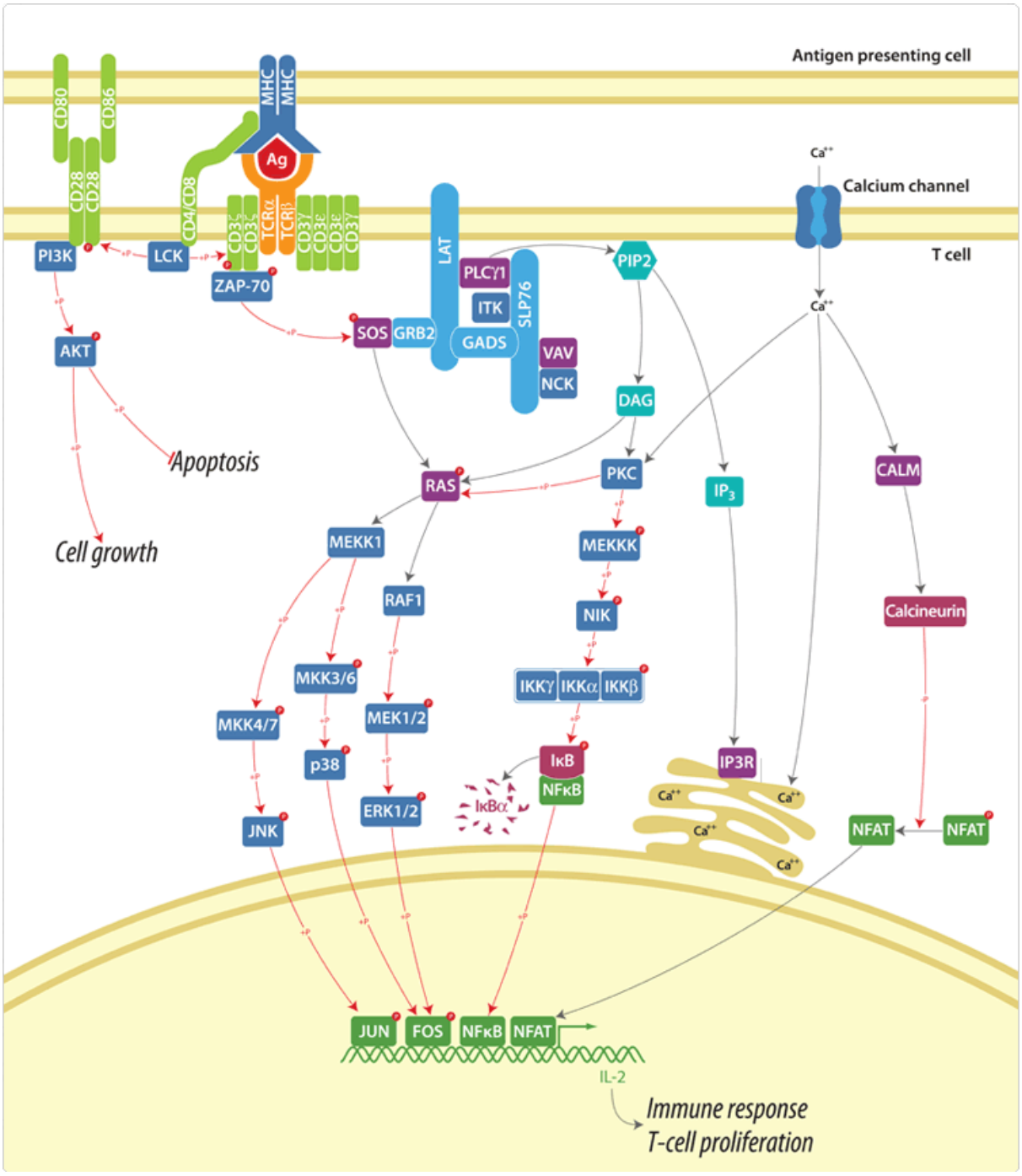

Figure 4. **Schematic representation of T cell receptor and CD28 signal transduction cascades**. Several major pathways are represented downstream of the Src-kinases, Fyn/Lck, including the PLCγ, PKC, Ras, PI3-K, Vav/Rac1, Erk, JNK, p38-MAPK and calcium pathways. *Adapted from Novex Cell Signaling Pathways by Invitrogen.*

Another signalling pathway initiated by TCR/CD28 stimulation involves the activity of the small GTPase Rac-1. Rac GTPase-transduced signals result in cytoskeletal rearrangements, cell migration and adhesion (Faroudi et al., 2010). Rac-1 in particular, modulates the catalytic activity of different Mitogen-Activated Protein Kinases (MAPKs) and controls cell

cycle progression through the G_1 phase of the cycle (Lamarche et al., 1996). Rac-1 activation requires the recruitment of the Vav GDP releasing factor (GRF) to the ZAP-70 and SLP-76 complex, where phosphorylation of Vav occurs. Once phosphorylated, Vav is assembled in a GTPase/GRF complex that induces Rac-1-mediated GDP/GTP exchanges, resulting in the activation of MEKK1 (MAP/Erk Kinase Kinase-1) (Salojin et al., 1999). MEKK1 is then responsible for the phosphorylation of p38 and the c-Jun N-terminal Kinase (JNK] and activation of the Jun-Fos transcription factors. Furthermore, in synergy with NFAT and the Ca^{2+}-dependent signalling, p38 and JNK directly phosphorylate the transcription factors Ekl1, c-Jun and ATF2, involved in the generation of IL-2 [FIGURE 4] (Li et al., 2001; Zell, 2001).

Activation of IL-2 gene transcription also requires the activation of the NF-κB transcription factor (Ghosh and Karin, 2002). Transcription factors belonging to the NF-κB family are essential for the maturation and survival of T cells as they induce the expression of genes involved in inflammatory, apoptotic and adaptive immune responses. NF-κB activation is dependent on the early wave of tyrosine phosphorylation that follows TCR activation and promotes the formation of the ZAP-70/SLP-76/PLCγ/Vav multiprotein complex. PLCγ activity generates diacylglycerol which in turn activates Protein Kinase C-θ. PKC-θ function is indispensable for TCR/CD28-dependent NF-κB recruitment (Coudronniere, 2000). Downstream of PKC-θ, the Caspase Recruitment Domain protein CARD11 and the Mitogen-Activated Protein Kinase MAP3K, participate in the activation of the IKK complex, which in turn modulates NF-κB by phosphorylating IκB. Once phosphorylated, IκB is ubiquitinated and targeted for proteosome-mediated destruction. IκB degradation frees NF-κB, which can then move into the nucleus and activate transcription [FIGURE 4] (Schmitz, 2003).

In vitro, T cell activation can be simulated through the combination of anti-CD3 and anti-CD28 monoclonal antibodies.

2.2. Role of metabolism in T cell activation

Following stimulation, T cells shift very rapidly from a quiescent (resting) to an active state. Once active, T lymphocytes grow, proliferate and undergo clonal expansion; a change in phenotype that involves the initiation of the transcriptional and translational machineries. Activation also requires lymphocytes to modulate their metabolic function, since the generation of an immune response is a very demanding bioenergetic process. Indeed, even if

past research has focused on the signal transduction pathways that emerge from the TCR, recent studies have showed that metabolism is a crucial step in lymphocyte activation and is directly involved in the regulation of T cell function and differentiation (reviewed in (Pearce, 2010; Wang and Green, 2012)) [FIGURE 13].

2.2.1. Quiescence: a controlled metabolic state

Quiescent T cells, despite residing in relatively rich nutrient environments, have slow metabolic rates and are characterized by low consumption of glucose and other essential nutrients. Quiescence is essential because it decreases the amount of resources required to maintain the vast repertoire of lymphocytes in the organism of a single individual. Small quiescent T lymphocytes use oxidative phosphorylation to break down glucose, amino acids and lipids to catabolically fuel cellular ATP demands (Krauss et al., 2001). They can also use autophagy to break down intracellular components that can then be mobilised through oxidative phosphorylation (Lum et al., 2005). Moreover, maintenance of cellular homeostasis has been shown to be controlled by extrinsic trophic signals, such as the IL-4 and IL-7 cytokines, rather than by nutrient availability (Fox et al., 2005). This suggests that quiescence is not a default pathway but rather, is the result of an enforced state that relies on the transcriptional machinery activated by cytokine and growth-factor signals (Yusuf and Fruman, 2003) [FIGURE 5].

Buckley *et al.* have demonstrated that the lung Krüppel-like factor LKLF is necessary to program quiescence in T cells (Buckley et al., 2001) [FIGURE 15]. Indeed, LKLF is up-regulated during the final stages of thymic differentiation to the single positive (SP) stage and expression is maintained upon egress from the thymus into the periphery. Interestingly, the LKLF transcription factor is rapidly downregulated following T cell activation (Kuo et al., 1997; Schober et al., 1999). The FoxO family of forkhead transcription factors is also involved in the maintenance of quiescence. Notably, FoxO3a blocks activation by promoting the expression of members of the IκB family, thus preventing the nuclear translocation of NF-κB (Liu, 2005). More recently, it was demonstrated that FoxO1 is able to regulate T cell homeostasis by sensing growth-factor availability and survival signals such as those delivered by IL-7 (Kerdiles et al., 2009). FoxO1 has also been shown to modulate important metabolic functions in insulin-sensitive tissues, a process mediated by Akst phosphorylation (Accili and Arden, 2004; Gross et al., 2008). Furthermore, IL-7 signalling in T lymphocytes

activates the PI3K/Akt pathway which coordinates the upregulation of the Glut1 transporter and subsequent increase in glucose uptake (Barata et al., 2004; Silva et al., 2011; Swainson et al., 2007). Together, these data suggests that FoxO1 may constitute a key metabolic sensor in T lymphocytes.

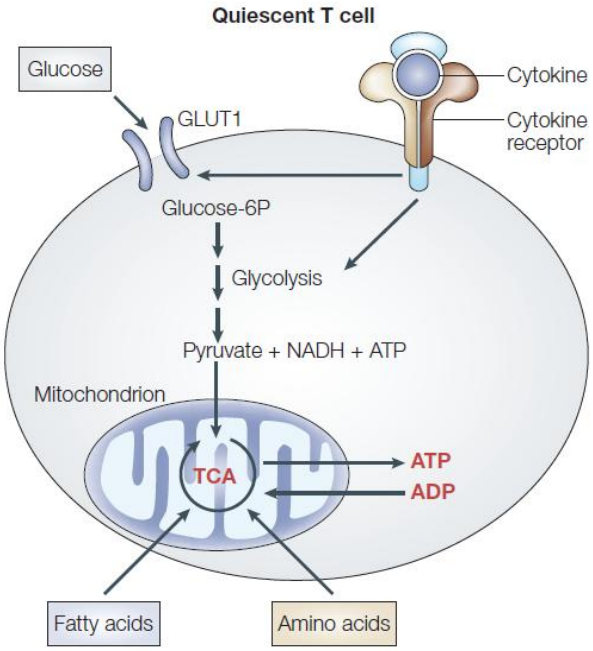

Figure 5. **ATP generation in quiescent T cells.** In quiescent T cells, cytokine signalling modulates glucose uptake and stimulates glycolytic activity, resulting in the production of ATP, pyruvate and NADH. In mitochondria, pyruvate is converted to acetyl-Coenzyme A (acetyl-CoA), a key component of the carbohydrate metabolism hub that can be used to fuel the Tricarboxylic Acid Cycle (TCA cycle) and oxidative phosphorylation processes. Fatty acids and amino-acids are other precursors of acetyl-CoA that can be mobilised by cytokine signals to generate ATP. *Adapted from (Fox et al., 2005).*

2.2.2. T cell activation: requirement for a metabolic shift

When T cells encounter a cognate antigen they shift from a quiescent to a highly proliferative state. Contrary to quiescent T cells, proliferating T lymphocytes require a high metabolic output to supply the energetic and biosynthetic requirements of an ongoing immune response (Pearce, 2010) [FIGURE 6 AND 12]. Glucose is the primary source of energy and carbon for T lymphocytes, and the most striking metabolic change observed following mitogen-induced activation is the drastic increase in glucose uptake and consumption. Without glucose, proliferating T cells fail to support their bioenergetic and biosynthetic demands and undergo apoptosis (Rathmell et al., 2000).

Stimulation induces a rapid increase in oxygen consumption but, interestingly, glucose appears to be fermented via the glycolytic pathway rather than used in a more energetic efficient reaction such as mitochondrial oxidative phosphorylation, at least *in vitro* (Michalek and Rathmell, 2010). This increase in glycolytic activity in the presence of oxygen is known as "aerobic glycolysis", as the term distinguishes it from the forced glycolysis which occurs when oxygen is a limiting factor. This metabolic condition is also known as the Warburg effect, as it was first observed by Otto Warburg in cancer cells (Warburg, 1956). The reasons as to why cancer cells, in contrast to normal cells, use high rates of glycolysis rather than relying on pyruvate oxidation in mitochondria has been a matter of debate. Several explanations ranging from the adaptation of cancer cells to low-oxygen environments to mitochondria shutdown resulting from damage or apoptosis blockade have been proposed. In T lymphocytes, the Warburg effect appears to be associated with cell proliferation. When activated, T cells increase dramatically in size (blastogenesis), a process that requires intense macromolecule synthesis and occurs prior to division.

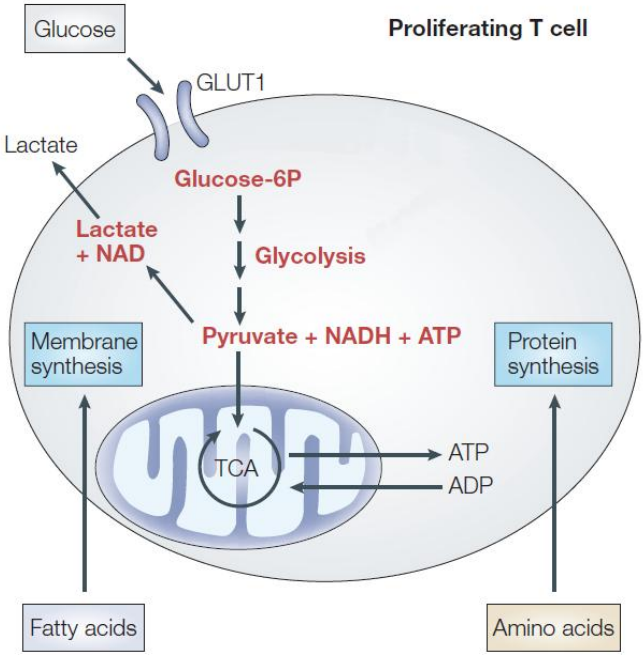

Figure 6. **Nutrient uptake in activated T cells.** T cell activation promotes glucose uptake and glycolysis, even in the presence of oxygen. This type of glycolytic catabolism is known as aerobic glycolysis and requires the presence of NAD as an electron acceptor of the reactions involved in the production of ATP. NADH can be recycled into NAD by converting pyruvate, the final product of glycolysis, to lactate or into the TCA cycle through the reduction of acetyl-CoA to ATP. Another feature of the metabolic shift in activated T lymphocytes is that amino acids and fatty acids are preferentially used in biosynthetic pathways rather than being oxidised in the mitochondria to generate ATP. *Adapted from (Fox et al., 2005).*

Glycolysis is an anabolic form of metabolism that produces NADH, pyruvate and other glucose-derived macromolecular precursors that can be incorporated in the tricarboxylic acid cycle (TCA cycle; also known as Krebs cycle) and other biosynthetic pathways [FIGURE 6 AND 12]. The preferential use of glycolysis also preserves intracellular amino acid and fatty acid levels, allowing the T cell to shunt these cellular building blocks into the biosynthesis of macromolecules (Fox et al., 2005). Finally, even if it is less energy efficient, the rate of ATP production from glycolysis is 100 times faster than the output of oxidative phosphorylation in the mitochondria (Nelson and Cox, 2004), which may help explain the biological "choice" made by proliferating T cells. Glycolysis, though, is not the only pathway of glucose metabolism recruited by T cell activation. Stimulation of T lymphocytes with mitogens also enhances the hexose monophosphate shunt, a pathway that comprises two phases: an oxidative phase in which NADPH is generated and a non-oxidative phase responsible for the production of pentose sugars necessary for nucleic acid synthesis (Frauwirth and Thompson, 2004; Sagone et al., 1974).

2.2.3. Upregulation of nutrient transporters and activation markers

Before they are able to increase in size, enter in cell cycle and proliferate, activated T cells must increase their metabolism. The supplement of nutrients required to drive the metabolism that follows ligation of both the TCR and CD28 is assured by the upregulation of several nutrient transporters at the cell-surface. The expression and function of these transporters is directly regulated by the transduction of TCR and CD28 co-receptor signalling pathways (Frauwirth et al., 2002) or alternatively, by signalling cascades induced via cytokines such as IL-7 (Barata et al., 2004; Swainson et al., 2007) or by death receptors such as the FAS receptor CD95 (Perl et al., 2002).

T lymphocytes can use glucose as a primary substrate for the generation of ATP or as a carbon source for the synthesis of macromolecules. Most mammalian cells transport glucose through a family encompassing 14 identified multimembrane-spanning facilitative nutrient transporters known as glucose transporters even though the substrates of some of these transporters are not glucose but rather fructose, dehydroascorbic acid, urate, and myoinositol amongst others (Scheepers et al., 2004). Of the glucose transporters, Glut1 appears to be the major isoform in T lymphocytes (Chakrabarti et al., 1994). Glut1 surface expression can be detected as early as 4 hours after TCR stimulation and requires de novo protein synthesis (Manel et al., 2003b). Although the precise mechanism by which T cells control Glut1

expression and trafficking have not yet been fully characterized, PI3K signalling through Akt is recognized to be essential for the process (Maciver et al., 2008). Glut1 is a uniport carrier that passively facilitates glucose transport across membranes by switching between two conformational states. It behaves as a Michaelis-Menten enzyme and contains 12 transmembrane amphipathic domains that delineate 6 extracellular loops (ECL). Six of these transmembrane regions bind together in the membrane to create a central polar channel through which glucose can cross (Carruthers et al., 2009).

Besides glucose, T lymphocytes are also capable of mobilising amino acids and fatty acids for energy supply. Glutamine, in particular, is the most abundant amino acid in the plasma and constitutes an important energy and carbon source for proliferating T lymphocytes (Aledo, 2004). Quiescent T cells express no or very low levels of neutral amino acid transporters. However, antigen recognition by the TCR leads to a strong upregulation of these transporters, notably ASCT2 (SLC1a5) (Levring et al., 2012) and SLC7a5 (Sinclair et al., 2013), amongst others. Once inside the T cell, glutamine is either hydrolysed into glutamate or partially oxidized into aspartate and CO_2, with both amino acids capable of entering into the Krebs cycle and biosynthetic pathways (Alberts B, 2007a). The precise mechanisms regulating the "choice" of a T cell as to whether to use glucose or glutamine for ATP production and whether either or both of these fuels are used for macromolecule synthesis, as sources of carbons for fatty acid synthesis and or for generation of reductive power remain to be elucidated. One key variable appears to be the kinetics with which different nutrient fuels can meet the wide diversity of metabolic requirements needed for proliferation (Aledo, 2004).

Meeting the high metabolic needs of proliferating lymphocytes also requires the transport of iron (Le and Richardson, 2004; Ponka, 2004). The transferrin receptor CD71 is a cell surface iron transport receptor that is upregulated by 24-48 hours following T cell activation and maintained during the course of the immune response (Shipkova and Wieland, 2012). CD71 is an essential factor for proliferating T cells and has been shown to participate to TCR signalling through association with the TCR ζ-chain and ZAP-70 (Salmeron et al., 1995). CD71 is considered a mid-early activation marker and defects in its upregulation are associated with T cell dysfunction (Zheng et al., 2009).

Another marker induced during T cell activation is CD69 (Leu-23), a transmembrane glycoprotein that serves as an early activation marker. Data provided by *in vitro* studies

suggest that CD69 is an activatory molecule; however the precise physiological function of this C-type lectin has not yet been elucidated, in large part due to the fact that its ligand(s) has not yet been identified (Sancho et al., 2005). CD69 is expressed at very low levels in quiescent T cells and is one of the first identified activation markers, detected as early as 1 hour following TCR stimulation. Indeed, CD69 is already expressed intracellularly in quiescent T cells and as such, its upregulation is not dependent on protein synthesis but on its translocation to the cell surface (Manel et al., 2003b). CD69 expression continues to increase during activation until it peaks between 24-48 hours, decreasing rapidly thereafter (Lim et al., 1998).

2.3. The PI3K/Akt/mTOR pathway

TCR signalling together with the costimulatory signals provided by CD28 and the IL-2 cytokine stimulate the PI3K-dependent activation of Akt, which in turn leads to the induction of the mammalian target of rapamycin (mTOR) [FIGURE 13] (Colombetti et al., 2006). mTOR is a serine/threonine protein kinase that actively participates in the integration of environmental cues such as nutrients, growth factors and stress signals into an "optimal" cellular response (Laplante and Sabatini, 2009; Sengupta et al., 2010). Furthermore, mTOR is critical in promoting protein-efficient translation and inhibiting protein degradation (Laplante and Sabatini, 2012). Recent evidence suggests that mTOR may function as a central regulator of T cell metabolism due to its sensing properties and as a direct activator of different metabolic pathways such as glycolysis, lipid synthesis and mitochondrial biogenesis (Powell and Delgoffe, 2010; Powell et al., 2012; Xu et al., 2012). mTOR also regulates T cell proliferation via its activation of cyclin D3 expression and degradation of the p27 cell cycle inhibitor (Hleb et al., 2004; Hong et al., 2008).

mTOR signalling occurs via two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [FIGURE 7]. mTORC1 and mTORC2 are characterized by the complex of mTOR with the adaptor protein RAPTOR (regulatory-associated protein of mTOR) and the scaffolder RICTOR (RAPTOR-independent companion of TOR), respectively. mTORC1 functions as a nutrient/energy/redox sensor and its upstream regulation has been extensively studied [FIGURE 13]. The role of mTORC2 in cell survival, cytoskeleton organization and metabolism has also been studied but little is known about the

upstream stimuli involved in its activation (Hay and Sonenberg, 2004; Laplante and Sabatini, 2009, 2012).

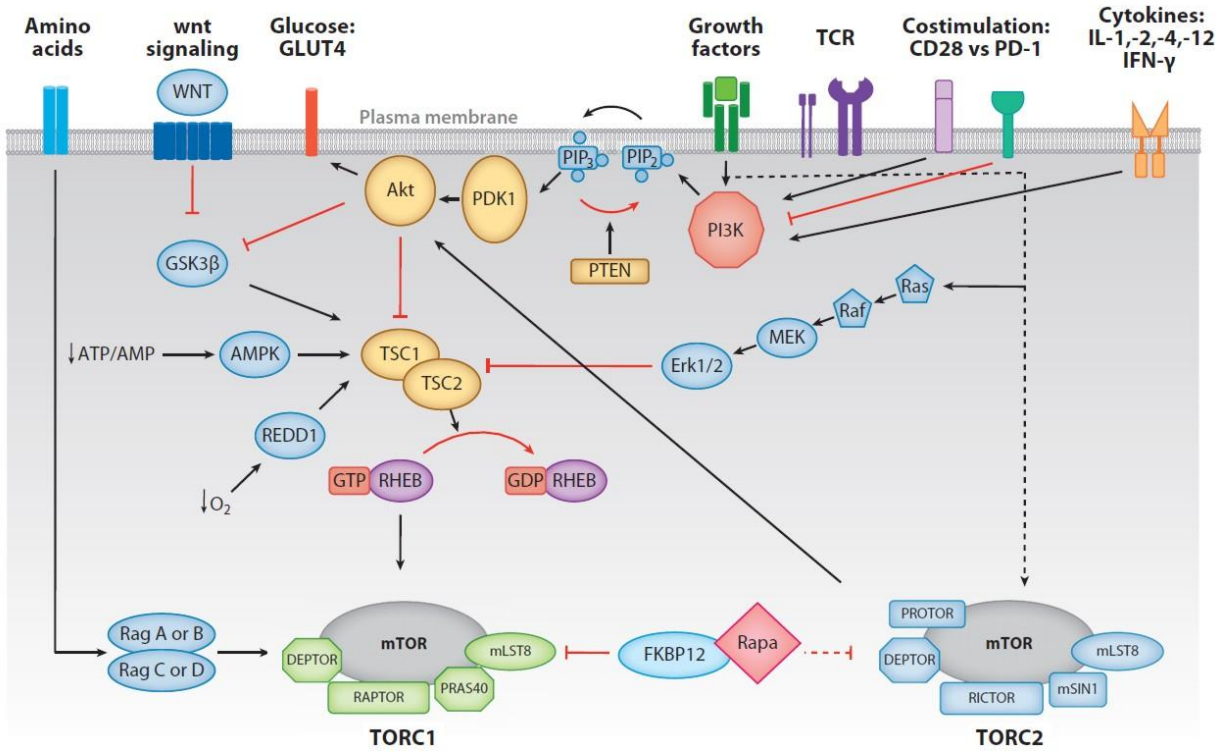

Figure 7. **Signals resulting in the induction of mTOR.** In an immune setting, growth factors, cytokines and costimulatory signals recruit PI3K to phosphorylate PIP2 into PIP3, which in turn activates PDK1 and leads to the phosphorylation of Akt (T308). Akt phosphorylates and inactivates the TSC complex. This results in the accumulation of Rheb-GTP, thus promoting mTORC1 activity. Growth factors, cytokines and costimulatory signals also trigger Erk2. Erk2 phosphorylation of TSC2 is inhibitory, thus creating an alternative pathway of mTOR induction. Following T cell activation, the availability of amino acids increases. The augmented levels of amino acids promote the binding of Rag proteins to Raptor, which in turn facilitates the relocalisation of mTORC1 with Rheb-GTP and its activation. Not all phosphorylations of TSC2 lead to the dissociation and inactivation of the TSC complex. AMPK, REDD1 and GSK3β are all factors capable of phosphorylating TSC2 in an activating manner. The AMP-activated protein kinase (AMPK) is turned on in response to a decreased ATP:AMP ratio, which is indicative of low energy levels. REDD1 (regulated in development and DNA damage responses 1), a hypoxia-inducible factor-1 target gene, plays a crucial role in inhibiting mTORC1 during hypoxic stress. The glycogen synthase kinase-3β (GSK3β) is at the crossroads of Wnt and Akt signalling. GSK3β is a serine-threonine kinase involved in energy metabolism that is capable of inhibiting mTORC1. [Black lines show activating signals, red lines show inhibitory signals, and red arrows indicate signals that indirectly lead to inhibition of mTORC1 activity.] Although growth factor signalling is known to be necessary for mTORC2 activity, the underlined mechanism has yet to be identified. [Dashed lines indicate that the exact mechanism is unknown.] *Adapted from (Powell et al., 2012).*

The small Ras-related GTPase Rheb is a crucial regulator of mTORC1. The GTP-bound form of Rheb interacts directly with mTORC1 to stimulate its activity. Rheb is controlled by the GTPase-activating protein activity (GAP) of the tuberous sclerosis complex TSC1/2 which negatively regulates mTORC1 signalling by converting Rheb into its inactive GDP-bound state. During T cell activation, the GAP activity of the TSC1/2 complex is inhibited as a consequence of phosphorylation by Akt or Erk1/2. This promotes the active form of Rheb, thus leading to the activation of mTORC1 (Powell et al., 2012; Xu et al., 2012).

Downstream, mTOR signalling essentially targets components of the translation machinery. mTORC1 activity can be assessed as a measure of the phosphorylation of S6K1 and 4E-BP1 (Hay and Sonenberg, 2004). The ribosomal protein S6 Kinase beta-1 improves mRNA translation and is activated by mTORC1-mediated phosphorylation. mTORC1 also promotes protein synthesis by inactivating the repressor of mRNA translation 4E-BP1. 4E-BP1 binds to eIF-4E, preventing its assembly into the elF4F complex and inhibiting cap-dependent translation. Phosphorylation of 4E-BP1 disrupts this binding, activating cap-dependent translation.

Akt, the serum glucocorticoid-regulated kinase 1 (SGK1) and the Protein Kinase C (PKC) are amongst the main targets of mTORC2 activity. Akt functions simultaneously as an activator and a target of mTOR. PI3K/PDK1-dependent phosphorylation of Akt at the threonine 308 residue is required for the inhibition of the TSC1/2 complex whereas phosphorylation of Akt at serine 473 is mTORC2-dependent. FoxO1 and FoxO3 regulation depends on the phosphorylation of Akt at serine 473 (Hay and Sonenberg, 2004; Laplante and Sabatini, 2009). Akt-mediated phosphorylation of these forkhead transcription factors is responsible for their sequestration in the cytoplasm, thereby inhibiting their activity (Guertin et al., 2006).

PKCα is an important mediator of actin cytoskeleton remodeling (Larsson, 2006). Thus, the ability of mTORC2 to regulate PKC activation has important immunologic consequences since actin reorganization is an important step in the T cell response to antigen-mediated activation. Furthermore, PKC activation by mTORC2 was reported recently to promote the differentiation of helper T cells to a Th2 lineage fate (Lee et al., 2010). Finally SGK1, another mTORC2 target, has been associated with the regulation of membrane potassium, sodium and chloride channels. SGK1 is also capable of phosphorylating FOXO family members, thus contributing to their cytoplasmic retention and inactivation (Garcia-Martinez and Alessi, 2008) [FIGURE 8].

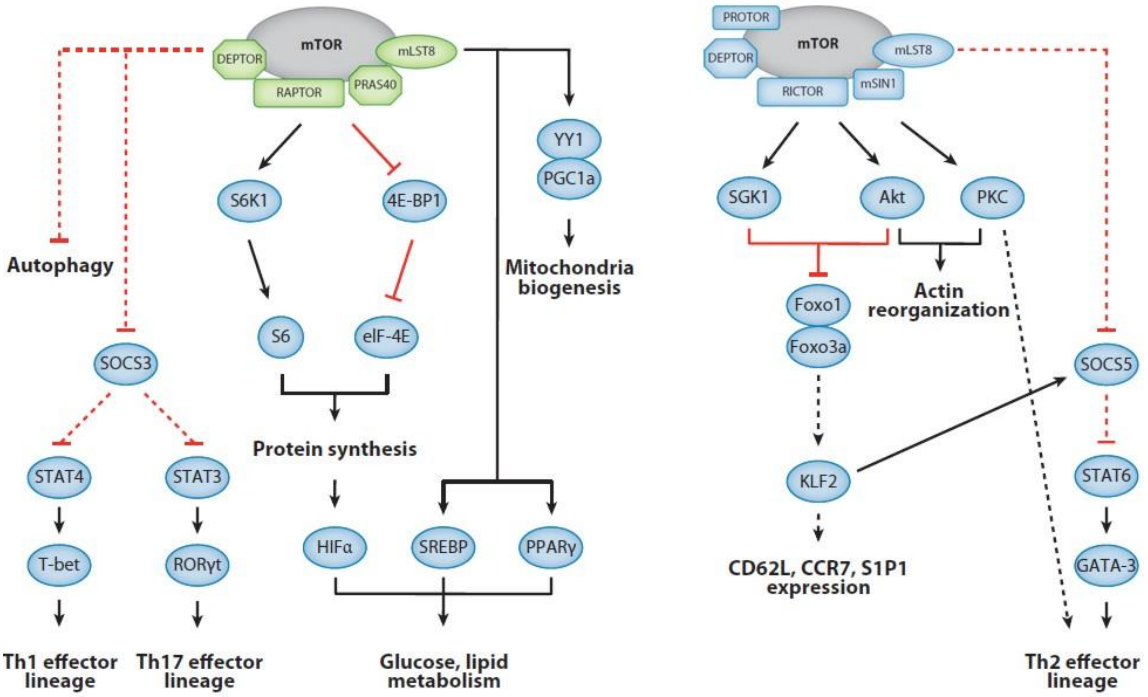

Figure 8. **Signalling cascades downstream mTOR.** S6K1 phosphorylates the ribosomal S6 protein, thus enhancing protein translation. Phosphorylation of 4E-BP1 by mTOR releases eIF-4E to participate in translation-initiation complexes. Phosphorylation of 4E-BP1 disrupts the assembly of eIF-4E into the elF4F complex that inhibits cap-dependent translation. mTORC1 promotes glucose and lipid metabolism by upregulating gene expression programs dependent on the hypoxia-inducible factor 1 alpha (HIF-1α), the sterol-regulatory element binding protein (SREBP) and the peroxisome proliferator-activated receptor (PPARγ). mTORC1 also controls mitochondrial oxidative function through the YY1–PGC1α transcriptional complex. Activation of Akt (phosphorylation at S473) and SGK1 by mTORC2 leads to the phosphorylation of FoxO family members. Phosphorylated FoxO proteins are sequestered in the cytoplasm and thus incapable of activating the transcription of target genes such as lung Krüppel-like factor LKLF2. Members of the PKC family are also targeted by mTORC2 signalling, leading to actin reorganization. Immunologically, mTORC1 activity is known to suppress the inhibitory effects of SOCS3 over STAT3 and STAT4 in an IL-12- and IL-6-dependent manner. IL-6 stimulates SOCS3 which in turn promotes RORγt activity and Th17 differentiation while IL-12 favours Th1 differentiation via activation of the STAT4/T-bet axis. mTORC2 signalling has been reported to promote Th2 differentiation via activation of PKCθ and inhibition of SOCS5. In particular, IL-4 stimulation was shown to induce Th2 differentiation via inhibition of SOCS5 in an mTORC2-dependent manner. [Dashed lines indicate that the exact mechanism is unknown, black lines for activating signals, and red lines show inhibitory signals.] *Adapted from (Powell et al., 2012).*

2.3.1. Role of mTOR in T cell metabolism

Different metabolic signatures characterize quiescent and activated T cells. As previously described, while quiescent T cells rely on catabolism to provide the necessary energy and substrates for basal levels of biosynthesis, activated T cells use anabolism to meet the increased bioenergetic demands of clonal expansion (Frauwirth and Thompson, 2004; Jones

and Thompson, 2007). mTOR, in its role as an energetic sensor, integrates the immunological stimuli and metabolic signals to modulate the dichotomy between quiescent and activated states.

As the metabolism of T cells varies as a function of environmental stimuli, so does mTOR activity. In the absence of extrinsic growth factors or activating stimuli, quiescence is promoted even when nutrients are environmentally available. Additionally, when T lymphocytes exist in a low energy status, mTOR activity is basal and macromolecule biosynthesis minimal. Therefore, in quiescent T cells ATP is generated by degradation of intracellular constituents in the autophagy pathway (Fox et al., 2005; Pua and He, 2009). Activated T cells, however, engage in high rates of aerobic glycolysis and glutaminolysis as the major suppliers of energy and intermediates for the biosynthetic surge. This increase in energy supply activates the mTORC1 pathway which in turn upregulates enzymes involved in glycolysis, glutaminolysis and lipid synthesis (Duvel et al., 2010; Wang et al., 2011b; Wieman et al., 2007). Furthermore, mTORC1 has been described as inducing the surface expression of metabolic transporters such as Glut1 and the SLC38a2 glutamine transporter, amongst others (Buller et al., 2008; Drummond et al., 2010; Nicklin et al., 2009). The metabolic reprogramming mediated by the mTOR pathway relies on the activation of downstream effectors such as the transcription factors Hypoxia-inducible Factor 1 alpha (HIF-1α), c-Myc and the sterol-regulatory element binding protein (SREBP) (Rathmell, 2011; Wang et al., 2011b; Yecies and Manning, 2011).

2.3.2. Regulation of T cell differentiation and function by mTOR

mTOR has emerged as an important regulator of T cell plasticity. Naïve $CD4^+$ T cells differentiate into distinct subsets when they are stimulated by a cognate antigen, including Th1, Th2, Th17, follicular helper T cells (Tfh) and Tregs. *In vitro* studies have also identified different cytokines capable of skewing specific fates; for example, the transcription factor T-bet which is required for Th1 differentiation is induced by the IL-12/STAT4 axis; the upregulation of GATA3 required for Th2 differentiation is induced by IL-4-mediated STAT6 phosphorylation; and IL-6-mediated activation of STAT3 enhances RORγt expression, a major regulator of Th17 polarization [FIGURE 13] (Weichhart and Saemann, 2010). *In vivo*, however, the microenvironment is more diverse resulting in a context where T cells can be simultaneously exposed to more than one cytokine, often with opposing effects, in different

nutrient and oxygen conditions (Powell et al., 2012; Xu et al., 2012). Furthermore, the transcription factors regulating T helper differentiation, such as T-bet, GATA3 and RORγt, are all induced following T cell activation, making effector lineage commitment a "grey", rather than a "black and white" mechanism. As such, $CD4^+$ T cell polarization results from the integration of diverse and opposing signals and very recent data suggest that mTOR, by sensing and integrating these environmental signals, plays an important role in this process (Ho, 2009; Powell and Delgoffe, 2010; Powell et al., 2012; Xu et al., 2012).

Indeed, mTOR-deficient T cells are unable to undergo polarization to Th1, Th2, or Th17 effector cells fates, even though they are capable of responding to an activation stimulus. This is a consequence of the inability of mTOR-deficient cells to upregulate the necessary lineage specific transcription factors, likely due to decreased phosphorylation of STAT proteins. Furthermore, Delgoffe and colleagues demonstrated that TCR engagement in the absence of mTOR promoted differentiation into $Foxp3^+$ regulatory T cells and that this differentiation was associated with hyperactive Smad3(Delgoffe et al., 2009). In addition, rapamycin, an inhibitor of mTOR, has also been shown to induce the expression of Foxp3 and promote differentiation to a T regulatory lineage, both in murine and human cells (Sauer et al., 2008). Conversely, Delgoffe *et al* showed that Th1 and Th17 polarization was specifically dependent on mTORC1 signalling, while mTORC2 activity was necessary for Th2 differentiation (Delgoffe et al., 2011). Deletion of the small GTPase Rheb, a known activator of the mTORC1 complex, prevented development of Th1 and Th17 CD4 T cells, leaving Th2 differentiation unperturbed. Furthermore, when RICTOR was deleted, CD4 T cells failed to differentiate to a Th2 cell type, but Th1 and Th17 polarization capacity was largely preserved.

Similarly to $CD4^+$ T cells, $CD8^+$ T cells switch from catabolism to anabolism upon recognition of a cognate antigen. This metabolic shift is necessary to sustain the fast proliferation induced by the engagement of the TCR and is dependent on mTOR activity. Indeed, according to data from Powell's group, mTORC1-deficient CD8+ T cells fail to become effector cells while TSC2-deleted CD8+ T cells show hyperactive mTORC1 activity and enhanced effector generation (Li et al., 2011; Powell et al., 2012). In addition, mTOR activity is involved in memory programming of cytotoxic cells. Recent work based on a model of acute lymphocytic choriomeningitis virus (LCMV) infection has shown that the mTOR inhibitor rapamycin results in an increased number and recall activity of memory $CD8^+$ T cells (O'Brien and Zhong, 2012). Furthermore, following the expansion phase of

$CD8^+$ T cells, there is a contraction phase where memory T-cell re-express markers associated with their homeostatic survival (CD62L, CD127 and Bcl-2) and this second process was also enhanced in the presence of rapamycin (Araki et al., 2009). Interestingly, rapamycin has also been shown to enhance oxidative phosphorylation in memory $CD8^+$ T cells. Memory cells, like naïve T cells, are quiescent and use essentially fatty acid oxidation and autophagy to meet their metabolic demands (Pearce et al., 2009). Together, these data suggests that rapamycin may facilitate the memory differentiation program by improving cell survival and facilitating adaptation to catabolism during the contraction phase, via an enhancement of lipid oxidation (Araki et al., 2010).

2.4. Mitochondrial biogenesis

Mitochondrial biogenesis and function is an essential feature of the metabolic reprogramming that follows T cell activation [FIGURE 12] (Waickman and Powell, 2012). Mitochondria are ubiquitous membrane-enclosed organelles that function as key regulators of metabolic homeostasis, capable of integrating signalling, cell cycle, differentiation and stress responses. The mitochondrion is the "power plant" of the cell, converting nutrients into ATP while generating important biosynthesis intermediates. It is also very important for both the production and degradation of free radicals.

Mitochondria are unique organelles; they have their own genome, they divide independently of the cell cycle by simple fission as a function of energy demand, and their internal organization comprises different compartments that carry out specialized functions [FIGURE 9]. The outer membrane entirely surrounds the inner membrane, with a small intermembrane space (IMS) between them. The inner membrane, enclosing the matrix, is loaded with proteins involved in electron transport and ATP synthesis, including ATP synthase itself. The mitochondrial matrix is where the mitochondrial DNA and ribosomes reside. The matrix also contains specialized enzymes that participate in Krebs cycle reactions and oxidation of pyruvate and fatty acids. The inner membrane also creates infoldings known as cristae that expand their surface area, thus enhancing ATP production capacity. The outer mitochondrial membrane is very similar to the eukaryotic plasma membrane and contains a large number of integral proteins. These proteins, also known as porins, create channels that allow small molecules (<5 kDa) to diffuse freely between the cytosol and the IMS (Alberts B, 2002; McBride et al., 2006). With the disruption of the outer membrane, mitochondria

release cytochrome c, leading to caspase-mediated apoptosis (Tait and Green, 2010). Furthermore, mitochondria can associate with the endoplasmic reticulum (ER) for various functions, including calcium buffering [Section 4.3.1.] and lipid metabolism (Hoth et al., 2000; McBride and Scorrano, 2013).

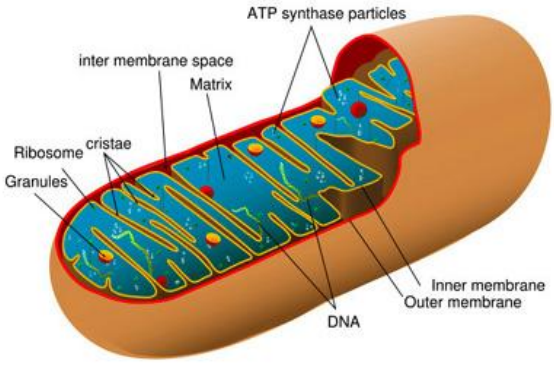

Figure 9. **Mitochondrion structure.** *Diagram by Mariana Ruiz Villarreal (source Wikimedia Commons).*

Mitochondrial biogenesis is essential not only during normal cellular homeostasis, but is critical in response to different challenges and stress conditions. For example, mitochondrial biogenesis is required to provide ATP for the energy-requiring processes induced by the activation of lymphocytes by mitogens (D'Souza et al., 2007; Van den Bogert et al., 1989). Calcium signalling, one of the pathways induced during T cell activation, is also know to modulate mitochondrial mass and function in muscle cells (Kusuhara et al., 2007; Wright, 2007).

Mitochondrial biogenesis encompasses numerous processes involved in the maintenance and growth of mitochondria, in addition to those required for their fission and segregation during the cell cycle. This process relies on the concerted and synchronized action of mitochondrial DNA with nuclear DNA because despite having their own ribosomal machinery and genome, mitochondrial DNA (mtDNA) only encodes for the 13 essential subunits of the electron transport chain (ETC). As such, the majority of mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and then imported into mitochondria. Furthermore, the regulation of mitochondrial biogenesis is governed by nuclear factors such as the nuclear respiratory factors NRF1 and NRF2, which control the expression of genes encoding for cytochrome c and cytochrome c oxidase subunits, and the family of peroxisome proliferator-activated receptor (PPAR) coactivators, including PGC-1α, PGC-1β, and the PGC-related coactivator (PRC).

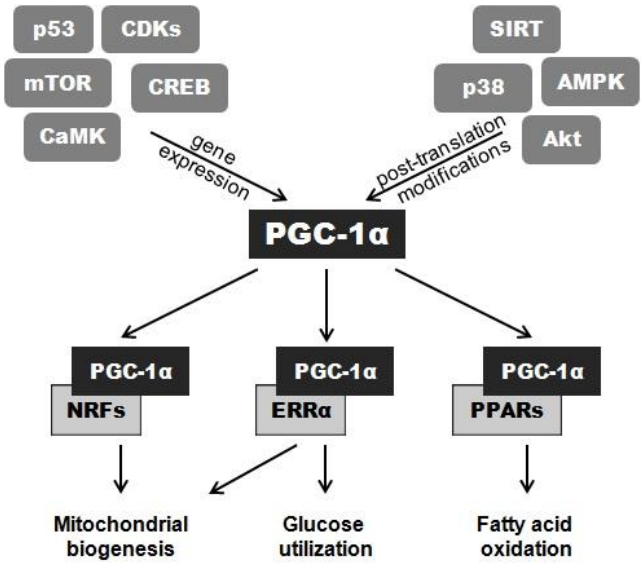

Figure 10. **Regulation of mitochondrial biogenesis by PGC-1α.** PGC-1α is regulated on both the transcriptional and post-translational levels. p53, mTOR, cAMP response element-binding protein (CREB), cyclin-dependent kinases (CDKs), calcium-calmodulin-activated kinases (CaMKs), Akt, p38 MAPK, Sirtuins (SIRT) and adenosine-monophosphate-activated kinase (AMPK) have been shown to regulate expression and/or activity of PGC-1α. PGC-1α then co-activates transcription factors such as NRFs, estrogen-related receptors ERRs and PPARs, known to regulate different aspects of energy metabolism including mitochondrial biogenesis and fatty acid oxidation. *Adapted from (Ventura-Clapier et al., 2008; Wenz, 2013).*

PGC-1α, in particular, is thought to be the master regulator of mitochondrial biogenesis [FIGURE 10]; it is a co-activator of the nuclear encoded mitochondrial factors estrogen-related receptor alpha (ERRα), NRFs and PPARγ, which regulate mitochondrial biogenesis and fatty acid oxidation, respectively (Ventura-Clapier et al., 2008; Wenz, 2013). Upstream, p53 has been demonstrated to bind and repress PGC-1α promoters (Sahin et al., 2011), while the calcium-calmodulin-activated kinases (CaMK) (Kusuhara et al., 2007) and the cAMP response element-binding protein (CREB) (Potthoff et al., 2011) promote PGC-1α gene expression. Additionally, the Cdk7/cyclin H/ménage-à-trois 1 heterotrimer (Sano et al., 2007) and mTOR (Cunningham et al., 2007) have also been shown to facilitate PGC-1α expression. Furthermore, PGC-1α activity can be modulated by several post-translational modifications. For example the NAD-dependent deacetylase Sirt1 deacetylates and activates PGC-1α (Canto and Auwerx, 2009). The adenosine-monophosphate-activated kinase (AMPK) also promotes PGC-1α activity, but in this case through phosphorylation (Canto and Auwerx, 2009). Interestingly, cytokines such as IL-1α, IL-1β, and TNFα activate and stabilize PGC-1α through phosphorylation by the p38 kinase (Puigserver et al., 2001).

2.4.1. Cellular respiration and production of reactive oxygen species

Cellular respiration is the catabolic process used by cells to biochemically convert nutrients into ATP [FIGURE 11]. Cellular respiration is an exothermic redox reaction that begins with the breakdown of glucose in glycolysis. During glycolysis, cells break down 1 molecule of glucose into 2 molecules of pyruvate in the cytoplasm with a net gain of 2 ATP. When oxygen is present, pyruvate is transported into the mitochondria, where it is oxidized to acetyl-CoA and CO_2 by the pyruvate dehydrogenase complex, a process known as oxidative decarboxylation.

Under low oxygen conditions (hypoxia), pyruvate is retained in the cytoplasm where it is reduced to lactate by the lactate dehydrogenase, recycling NADH to NAD^+ in the process. Pyruvate fermentation also occurs during aerobic glycolysis, when mitochondria are incapable of incorporating all the pyruvate being produced. Pyruvate can also be converted back to carbohydrates (such as glucose) via gluconeogenesis, and be used to produce alanine via transamination. Pyruvate can be decarboxylated into acetyl-CoA which then enters into the Krebs cycle and upon breakdown to CO_2 and H_2O, produced electrons are transferred to the shuttle molecules NAD^+ and FAD. For each molecule of glucose, the Krebs cycle produces 2 ATP, 8 NADH and 2 $FADH_2$, as well as other intermediates that can be used in biosynthetic pathways. NADH and $FADH_2$ carry the electrons to the ETC, where the majority of ATP is created (32 molecules of ATP per molecule of glucose) through oxidative phosphorylation (OXPHOS in short).

Oxidative phosphorylation occurs in the mitochondrial cristae. The electron transport chain is coupled to proton translocation, thus establishing a proton gradient across the inner mitochondrial membrane that facilitates the oxidation of NADH and $FADH_2$ and drives the phosphorylation of ADP into ATP by the ATP synthase enzyme. Electron transport phosphorylation typically produces 32 molecules of ATP and in the process, electrons are finally transferred to exogenous oxygen with the formation of H_2O after adding two protons (Lodish H, 2000; Nelson and Cox, 2004).

Mitochondrial respiration is also associated with the generation of reactive oxygen species (ROS). In the mitochondrial respiratory chain, the reduction of oxygen to water proceeds electron by electron until full reduction is achieved, with all partially reduced intermediates retained by cytochrome oxidase (complex IV). Electron leakage through complex I and III,

however, can reduce molecular oxygen to the superoxide anion O_2-. The superoxide produced by electron leakage can be reduced to hydrogen peroxide (H_2O_2) by the manganese superoxide dismutase in the mitochondrial matrix. This H_2O_2 can be then converted by catalase to harmless H_2O but can also be protonated to form the hydroperoxyl radical which can attack DNA, lipids and proteins. Thus, despite being a natural by-product of respiration, ROS accumulation can lead to the damage of DNA, proteins, and lipids, resulting in progressive cell dysfunction and even apoptosis (Nohl et al., 2005; Turrens, 2003).

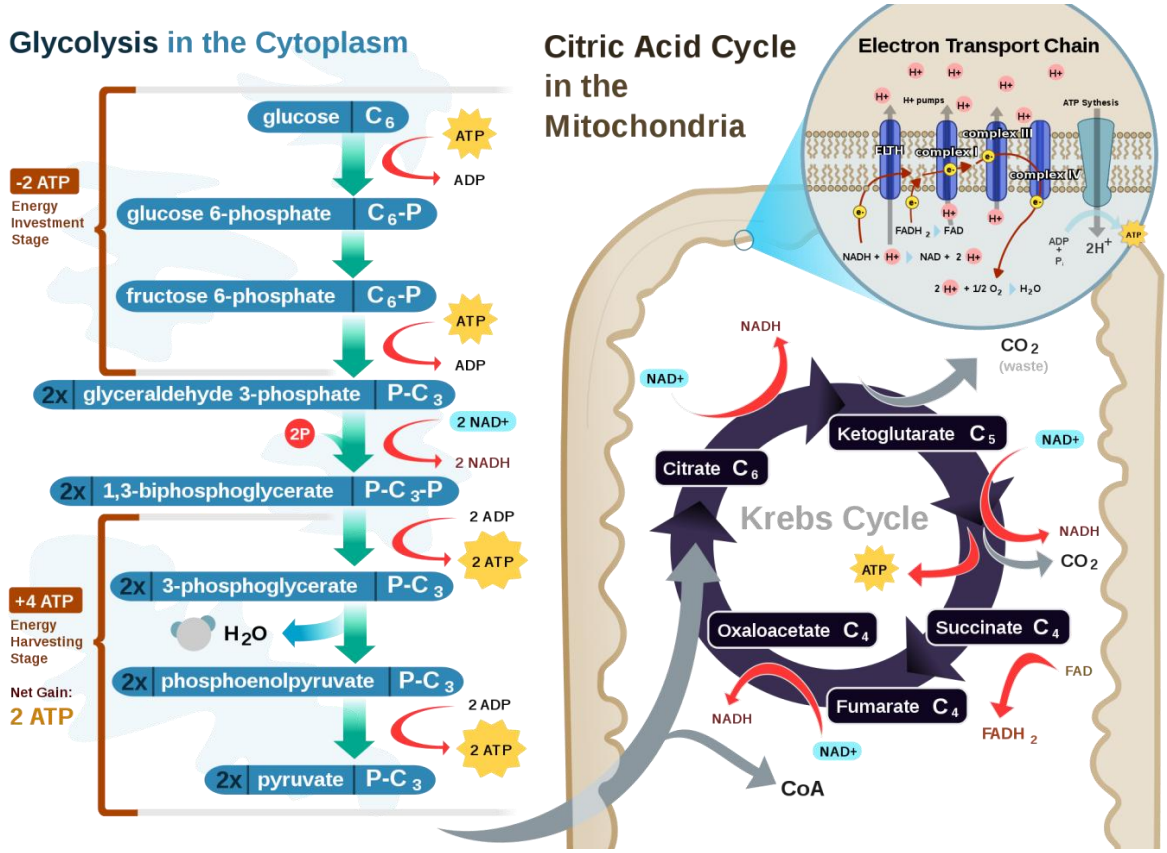

Figure 11. **Cellular respiration.** A diagram of cellular respiration presenting glycolysis, the Krebs cycle, and the electron transport chain. *Diagram by Regis Frey (source Wikimedia Commons).*

ROS and free radicals generated by the respiratory chain also modulate signalling between the mitochondria and the nucleus. In particular, ROS have been described to signal mitochondrial biogenesis via PGC-1α as an antioxidant mechanism (Wenz, 2013; Yoboue and Devin, 2012). In this feedback loop, PGC-1α upregulates the expression of catalase and superoxide dismutase (Valle et al., 2005), thereby mediating resistance to oxidative damage (St-Pierre et al., 2006). ROS production also results in the upregulation of the mitochondrial sirtuin Sirt3 via transcriptional activation by PGC-1α; Sirt3 promotes the anti-ROS system because its

deacetylation of superoxide dismutase activates this enzyme (Chen et al., 2011; Kim et al., 2010). The Akt/p38 MAPK cascade, which also signals mitochondrial biogenesis, is amongst other ROS targets that have been described in different cellular models. ROS inhibits phosphatases through redox modification of the reactive cysteine, leading to enhanced kinase signalling of this pathway(Wang et al., 2011c; Wenz, 2013; Yoboue and Devin, 2012).

2.4.2. ETC and mitochondrial membrane potential

Oxidative phosphorylation is made possible by the association of the electron carriers NADH and $FADH_2$ with the protein molecules in the ETC. There are 4 redox carriers which comprise the ETC: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome C oxidoreductase, Coenzyme Q) and complex IV (cytochrome C oxidase). These complexes guide the electrons sequentially along the respiratory chain from donors to the final acceptor, dioxide. The energetically favourable flow of electrons facilitates the pumping of protons (H^+) across the inner membrane, from the matrix to the intermembrane space, generating a pH and voltage gradient across the inner mitochondrial membrane. The pH gradient is established because small molecules equilibrate freely across the outer membrane of the mitochondrion, meaning that the pH in the intermembrane space (IMS) is close to that in the cytosol (pH=7), while the matrix has a pH of about 7.8. The voltage gradient occurs due to the net outflow of cations across the inner mitochondrial membrane, resulting in a matrix which is negatively charged and an outside region with positive charge. This voltage gradient is also known as mitochondrial membrane potential (MMP/$\Delta\Psi m$).

The MMP is particularly important because it regulates the production of high-energy phosphate by ATP synthase (Alberts B, 2002; Lodish H, 2000; Nelson and Cox, 2004). The MMP is also highly relevant in the apoptotic cascade. Condensation of the matrix as a consequence of a decrease in MMP exposes the intermembrane space to cytochrome c. The release of cytochrome c then results in the activation of caspases and subsequent apoptosis (Gottlieb et al., 2003; Scarlett et al., 2000). The transport of proteins into the matrix and across the mitochondrial inner membrane is also dependent on the MMP. As described, the majority of mitochondrial proteins are coded by the nucleus and translated in the cytoplasm by the cellular ribosomes (Wenz, 2013). The MMP facilitates the import of cleavable pre-proteins via an electrophoretic effect on the positively charged matrix-

targeting sequences and the activation of the Tim23 subunit of the mitochondrial import inner membrane translocase (Geissler et al., 2000).

2.4.3. Mitochondrial biogenesis during T cell activation

mTOR, p38MAPK, Akt, CaMK, and CREB are all stimulated during T cell receptor signalling, and all are characterized by their ability to regulate the expression and/or activity of PGC-1α. The PGC-1α transcriptional coactivator in turn controls the expression of genes involved in mitochondrial biogenesis [Section 2.4.] (Powell et al., 2012; Wenz, 2013). TCR engagement stimulates Ca^{2+}-mediated signalling pathways (Smith-Garvin et al., 2009) which regulate carbohydrate metabolism at the level of mitochondrial respiration. Mitochondria are calcium buffers that take up the Ca^{2+} released from the endoplasmic reticulum. Once in the mitochondrion, Ca^{2+} stimulates pyruvate dehydrogenase activity and other Krebs cycle enzymes resulting in increased production of biosynthesis intermediates and electron donor NADH. The decreased NAD^+/NADH ratio promotes oxidative phosphorylation in the ETC, leading to oxygen consumption and ROS accumulation (Bravo et al., 2012; Leavy, 2013; Murphy and Siegel, 2013; Sena and Chandel, 2012). Interestingly, Sena and colleagues have recently shown that mitochondrial ROS generated by complex III of the ETC are necessary for CD3/CD28-induced *Il2* expression (Sena et al., 2013). Indeed, they demonstrated that T cells lacking the iron-sulphur protein Rieske, a complex III component, failed to induce *Il2* expression and that exogenous replacement of ROS with H_2O_2 was able to restore *Il2* expression.

Induction of the OXPHOS system by calcium also leads to higher mitochondrial membrane potential while decreased MMP has been hypothesized to be a marker of apoptotic cells (Hengartner, 2000). Furthermore, hyperpolarization of the mitochondrial membrane following T cell activation is thought to promote resistance to apoptotic stimuli (Matarrese et al., 2003) and this hyperpolarization is mediated by nitric oxide (NO), a molecule known to regulate mitochondrial oxidative stress via the transcriptional coactivator PGC-1α. NO is formed enzymatically in the cytoplasm by nitric oxide synthases (NOS) via the conversion of L-arginine to L-citruline. Furthermore, as a free radical with an unpaired electron, NO competes with the electron acceptor O_2 and reversibly inhibits the cytochrome *c* oxidase (Nagy et al., 2007; Nagy et al., 2003; Wenz, 2013). At a certain level, the binding of NO to cytochrome *c* oxidase is detrimental since it inhibits respiration. However, it also

results in higher molecular oxygen availability and less ROS production (Gladwin and Shiva, 2009). Finally, the feedback loop is secured as PGC-1α, regulated or activated by mTOR, p38 MAPK, Akt, CaMK, and CREB, controls the expression of genes involved in mitochondrial biogenesis (Powell et al., 2012; Wenz, 2013).

Overall, TCR-induced metabolic reprogramming [FIGURE 12 AND 13] requires hyperpolarization of the mitochondrial membrane, stimulates the amplification of mitochondrial DNA and mitochondrial biomass and promotes oxidative phosphorylation and ROS production. These mitochondrial-specific effects of TCR stimulation are consistent with the essential role of this organelle in the growth and proliferation of activated T cells (D'Souza et al., 2007).

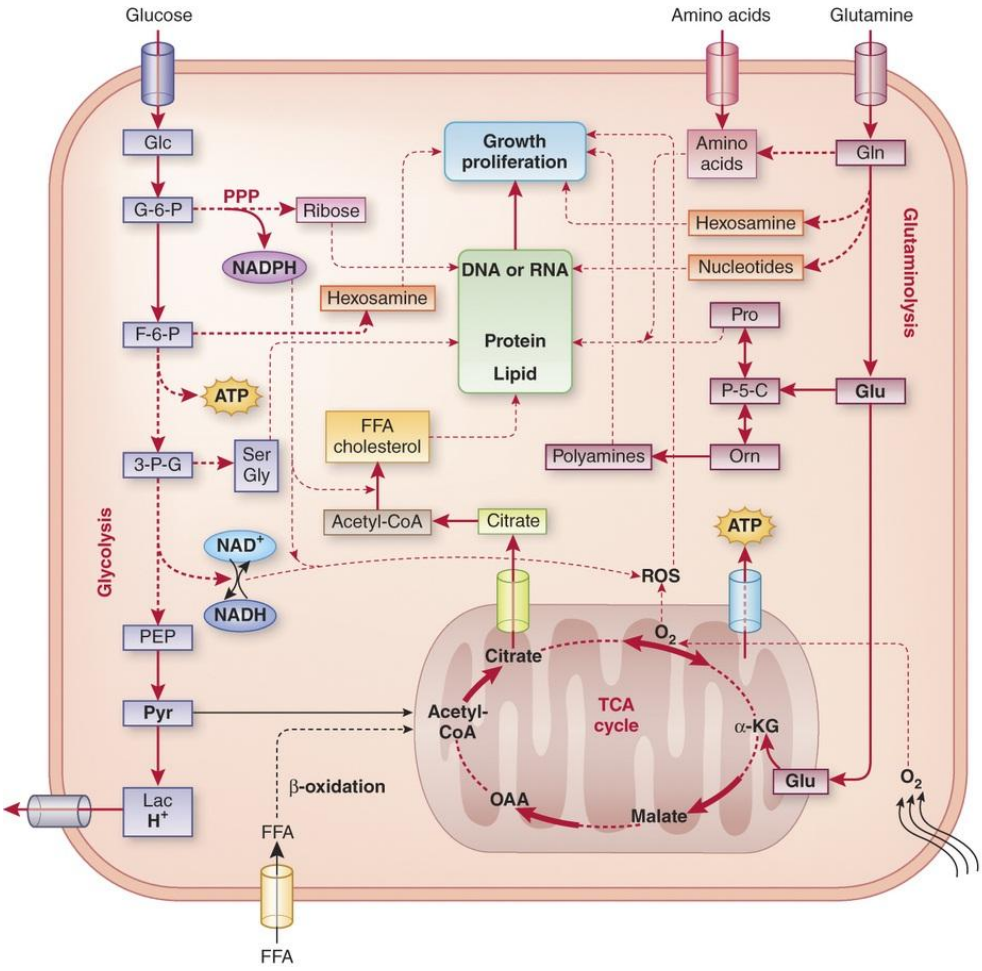

Figure 12. **T cell metabolic reprogramming.** In naive and memory T cells, mitochondria-dependent catabolic pathways, including glucose oxidation through the tricarboxylic acid (TCA) cycle and β-oxidation of fatty acids, provide most of the metabolic support for basic cellular functions. After T cell activation, β-oxidation rapidly decreases and other metabolic pathways (red), including glycolysis and glutaminolysis, increase. The glucose (Glc) catabolic pathway branches toward the production of NADPH and 5-carbon ribose (via the pentose

phosphate pathway (PPP)) at glucose-6-phosphate (G-6-P) and detours toward lactate production (aerobic glycolysis) at pyruvate. The carbons of glucose are further diverted into various synthetic pathways to generate the precursors of hexosamines, amino acids (such as serine (Ser) and glycine (Gly)) and lipids via various metabolic interconnections. Meanwhile, mitochondria are fueled by the anapleurotic substrate α-ketoglutarate (α-KG), generated via glutaminolysis. Depending on the oxygen supply and the abundance of HIF-1α, α-ketoglutarate metabolizes in either a clockwise or counter clockwise manner through the tricarboxylic acid cycle (as presented here) to provide energy and a carbon resource for lipids, respectively. In addition, glutamine (Gln) serves as an important donor of carbon and nitrogen for the biosynthesis of hexosamines, nucleotides, amino acids and polyamines. Collectively, the metabolic reprogramming after T cell activation is optimized to support cell growth and proliferation by providing carbons and ATP. [F-6-P, fructose-6-phosphate; 3-P-G, glycerate-3-phosphate; Pyr, pyruvate; Lac, lactate; FFA, free fatty acids; OAA, oxaloacetate; PEP, phosphoenolpyruvate; P-5-C, 1-pyrroline-5-carboxylate; Glu, glutamate; Orn, ornithine.]. *Adapted from (Wang and Green, 2012).*

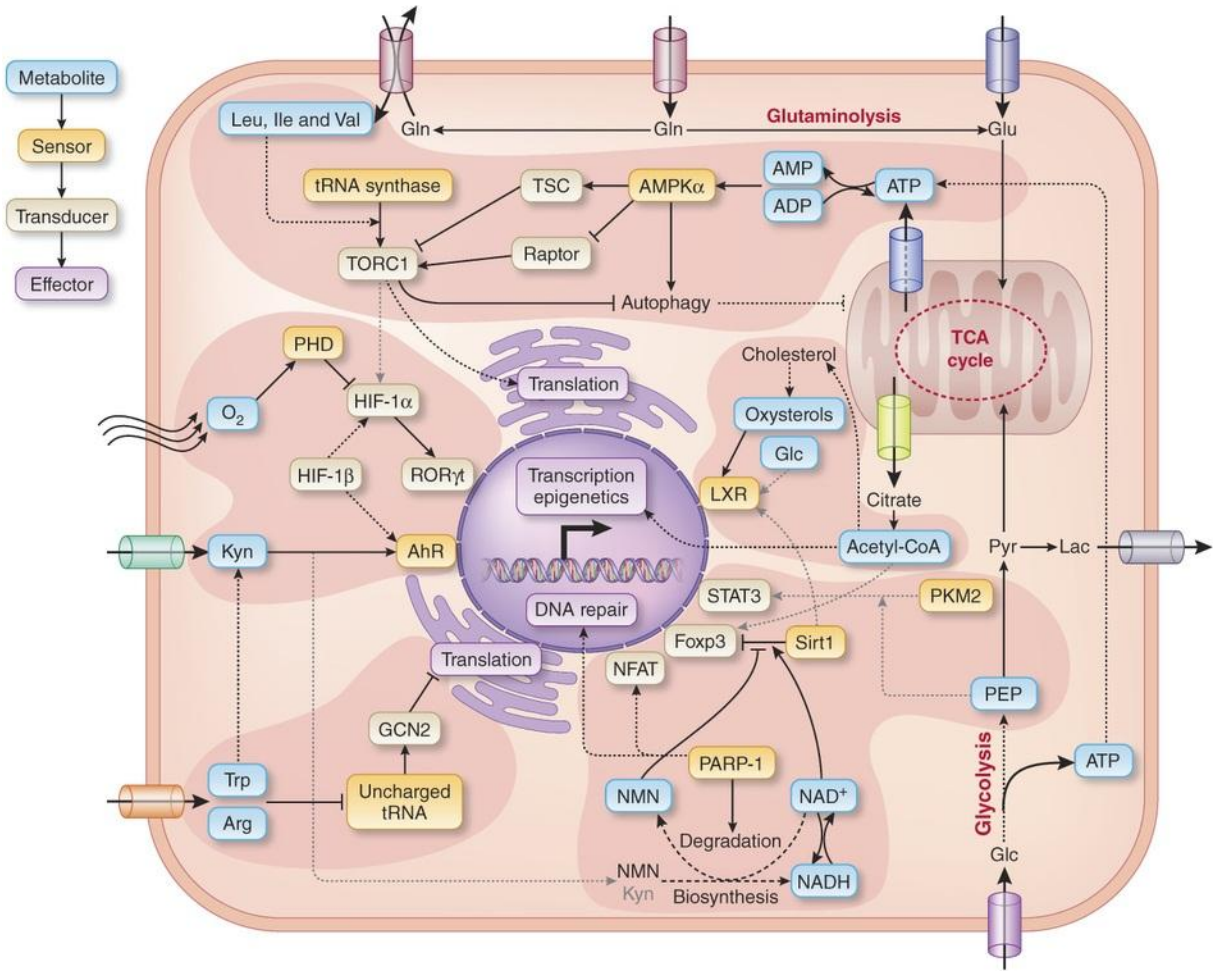

Figure 13. **Metabolic checkpoints in T cell function.** Metabolic checkpoints are cellular mechanisms that ensure the accurate 'translation' of a cell's metabolic status into a proper cellular response and are composed of metabolic signals, sensors, transducers and effectors. AMPK and mTORC1 coordinate the sensing of intracellular amino acids and ATP, and regulate autophagy, protein translation and probably HIF-1α. GCN2 (general control nonrepressed 2) is a serine/threonine-protein kinase that senses amino acid deficiency through binding to uncharged transfer RNA (tRNA) and represents another amino-acid checkpoint and

directly controls protein translation. The tryptophan-derived metabolite kynurenine (Kyn) serves as an endogenous ligand of the aryl hydrocarbon receptor (AhR), which may interact with HIF-1 α and coordinately direct T_H17 differentiation. Acetyl-CoA, the precursor of cholesterol, indirectly 'instructs' LXR (liver X receptor) [Section 4.1.3.] activity and directly regulates epigenetics via protein acetylation. As an NAD-dependent deacetylase, Sirt1 may suppress the differentiation of T_{reg} cells by modifying Foxp3 [Section 4.1.4.]. PARP-1, an NAD-consuming enzyme, may also interact with Sirt1 and serve as an NAD checkpoint. Finally, the pyruvate kinase PKM2 may use phosphoenolpyruvate (PEP) in the glycolytic pathway as a phosphate donor to modify its putative substrate STAT3, thus potentially acting as a checkpoint that responds to PEP concentrations. [NMN, nicotinamide mononucleotide]. *Adapted from (Wang and Green, 2012).*

3. Cell Cycle

The cell cycle is the biochemical and morphological process behind the production of two daughter cells as a result of the division of one cell into two during mitosis. Non-dividing quiescent T cells exist in a phase called G_0 (Gap zero). Following mitogen-stimulation, those cells enter the first stage of the cell cycle (G_1) which is characterized by cell growth, RNA production and protein synthesis (Alberts B, 2007b; Schafer, 1998). At the end of G_1 phase, an important checkpoint mechanism (restriction point; R) ensures that the cell is ready for DNA synthesis and marks the moment where the cell becomes irreversibly committed to progress into the cell cycle (Foster et al., 2010). Furthermore, based on differences in RNA content, the G1 phase of T lymphocytes can be divided into two distinct sub-compartments, G_{1A} (lower RNA content) and G_{1B} (higher RNA content) (Darzynkiewicz et al., 1980). Cells enter the S phase once R is breached and become independent of external stimulating signals; further progression through the cell cycle is controlled by the internal cell cycle signalling system. DNA replication occurs during the S phase.

The ensuing G_2 phase starts after the cell has finished the duplication of its chromosomes. During this gap, the cell will continue to grow and produce new proteins and at its end, another control checkpoint (G_2-M DNA damage checkpoint) ensures that the cell does not initiate mitosis (M) before having the chance to repair any DNA damage resulting from replication. Mitosis is the final step of the cell cycle. This M phase encompasses a series of morphological changes that begin with prophase and then pass through prometaphase, metaphase, anaphase and telophase. At the end of the cell cycle, cytokinesis drives cell division, resulting in the formation of 2 daughter cells.

The passage of a cell through the cell cycle is a tightly controlled process that involves the action of cyclin-dependent kinases (Cdks) and cyclins [FIGURE 14]. Cdk levels in a cell remain fairly stable during the cell cycle, but each must bind the appropriate cyclin, whose levels fluctuate, in order to be activated. As such, the complexes of D cyclins with Cdk4 occur essentially during the G_1 phase, whereas Cdk2 complexes with cyclins E and A are predominantly present during the S phase and Cdk1-cyclin B associations are detected during mitosis.

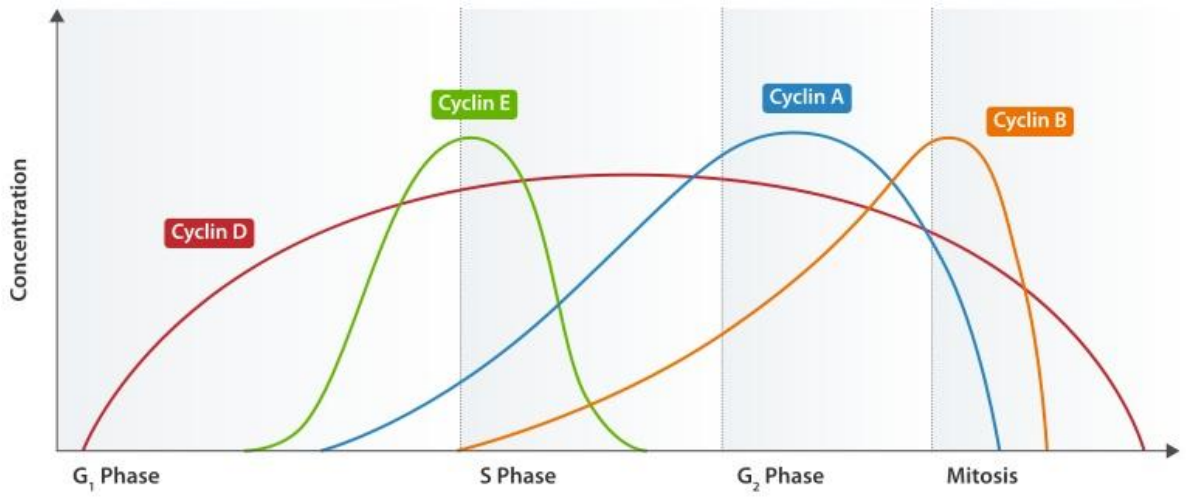

Figure 14. **Expression of cyclins during the different phases of the cell cycle.** *Image by waehrend_Zellzyklus (source Wikimedia Commons).*

The expression and functionality of Cdks and cyclins is regulated by numerous proteins, including p53, p21, p27, and retinoblastoma proteins (pRb). Association with small inhibitory proteins is the universal mechanism of Cdk regulation. Three different proteins, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, form the "CDK inhibitory Protein/Kinase Inhibitor protein" (Cip/Kip) family in mammals.

The tumour suppressor Rb is a transcriptional repressor of genes involved in S phase progression, such as cyclin E. In the unphosphorylated state, pRB bind and repress E2F transcription, stalling the cells in the G_1 phase and thereby preventing cell cycle progression. The interaction of pRb with the E2F-DP heterodimers is also involved in the recruitment of histone deacetylases (HDACs) to the chromatin, with a negative effect on the transcriptional activity of S phase promoting factors (Zhang and Dean, 2001) [FIGURE 15].

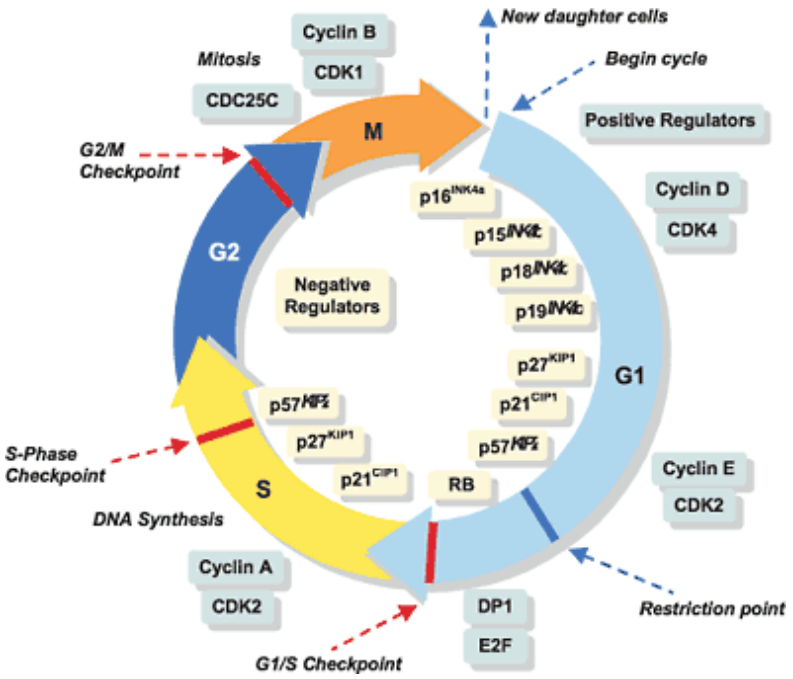

Figure 15. **Schematic illustration of the cell cycle and its regulatory mechanisms.** The cell cycle is regulated by the oscillating activities of cyclin/cyclin-dependent kinase (Cdk) complexes. Cyclin-dependent kinase inhibitors (CKIs) negatively control the activity of distinct cyclin/Cdk complexes. The CKIs of the Cip/Kip class are major regulators of the cell cycle during the initial G_1/S phase. Cip/Kip CKI include $p21^{Cip1}$and $p27^{Kip1}$, among others. *Adapted from (Kong, 2003).*

3.1. T cell activation promotes cell cycle entry

T lymphocytes spend most of their time in quiescence, a temporary and reversible state of G_0/G_1 arrest, characterized by high expression of $p27^{Kip1}$, low Cdk6 and Cdk2 kinase activity (Daignan-Fornier and Sagot, 2011), and low metabolic activity [Section 2.2.1.]. Interestingly, quiescent memory $CD8^+$ T cells have higher Cdk6 activity and express lower levels of the cell cycle inhibitor $p27^{Kip1}$ when compared to naïve cells, creating a unique "pre-activated" state that facilitates rapid division after antigen stimulation (Veiga-Fernandes and Rocha, 2004). Triggering of the TCR initiates signalling cascades that result in T cell activation, cell cycle entry and subsequent proliferation (Frauwirth and Thompson, 2004). Additional CD28-costimulatory signals accelerate entry into and progression through the cell cycle, partly due to IL-2 production and CD25 expression (Acuto and Michel, 2003). Furthermore, CD28 costimulation directly regulates cell cycle progression by inducing E2F activity and subsequent transcription of SKP2, the substrate recognition component of the SKP/Cullin/F-box (SCF) E3 ubiquitin ligase complex; in turn, the SCF complex catalyzes the ubiquitination and proteasomal degradation of $p27^{Kip1}$ (Appleman et al., 2006).

PD-1, Akt and Ras pathways are also known to regulate molecular components of the cell cycle and T cell proliferation. Engagement of the programmed cell death protein 1 (PD-1) is suggested to negatively regulate immune responses, blocking proliferation and cytokine secretion (Freeman et al., 2000). More recently, Patsoukis *et al.* demonstrated that this activation block is associated to the SCF ubiquitin ligase complex, with treatment of CD3/CD28-activated T lymphocytes with an agonistic antibody specific for PD-1 suppressing SKP2 transcription by inhibiting the PI3K/Akt and Ras-Raf-MEK-Erk pathways (Patsoukis et al., 2012). The failure to downregulate SKP2, results in $p27^{Kip1}$ accumulation and G_1 phase arrest. Moreover, PD-1 signalling led to increased levels of the G_1 phase inhibitor $p15^{INK4}$ and repressed the Cdk-activating phosphatase Cdc25A. Without the phosphatase activity of Cdc25A, Cdk2 stays inactive and is unable to phosphorylate and restrain the signal transducer Smad3, a G_1/S checkpoint inhibitor (Li et al., 2006). The Akt/PI3K and the Ras-Raf-MEK-Erk pathways are essential for pRb inactivation during G_1 phase. The Ras-Raf-MEK-Erk pathway, however, is particularly important for the exit of quiescence since it mediates the passage from G_0 to G_1 by promoting Cyclin D1 expression, which is required for pRb phosphorylation and inactivation (Coleman et al., 2004). And in addition to PD-1 signalling, cyclic adenosine monophosphate (cAMP) has also been shown to inhibit the PI3K/Akt and Ras pathways and repress T cell proliferation (Grader-Beck et al., 2003). cAMP stimulates the cAMP-dependent kinase type I (cAKI), a protein that localizes with the TCR-CD3 complex and that once activated uncouples the TCR-CD3 complex from intracellular signalling systems (Skalhegg et al., 1994). Interestingly, in the Grader-Beck paper, inefficient CD3/CD28-mediated T cell activation due to PI3K/Akt and Ras defective signalling in context of high cAMP levels was actually secondary to the impaired activation of ZAP-70 and phosphorylation of LAT. Furthermore, the authors verified that when T lymphocytes were stimulated with the phorbol ester PMA (Phorbol 12-myristate 13-acetate), which bypasses the TCR proximal signalling events, Ras signalling was functional and cells progressed into the cell cycle.

Quiescence is a tightly regulated process that it is actively enforced by a host of genes. For instance, hypophosphorylated pRb binds E2F-DP heterodimers, masking the transactivation domain of these transcription factors, and thereby maintaining cells in the G_0 phase of the cell cycle. Following TCR/CD28 stimulation, T lymphocytes upregulate the expression of D cyclins and activate the Cdk4/Cdk6 complex, which in turn phosphorylates pRb and promotes transition from G_1 into the S phase. Further phosphorylation of pRb by cyclin E and Cdk2

complexes facilitates the progression through the S phase (Hollsberg, 1999). In addition to Rb proteins, two other families of genes participate in the maintenance of quiescence by inhibiting the activity of Cdks; the Cdk-interaction protein (Cip)/cyclin-dependent kinase inhibitor (Kip) and the inhibitor of kinase 4/alternative reading frame (INK4/ARF). p21, p27 and p57 are amongst the members of the Cip/Kip family that bind and repress cyclin/Cdk complexes. For instance, induction of $p21^{Cip1}$ by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities (He et al., 2005). Deletion of $p27^{Kip1}$ in CD8 T cells enhances proliferation; furthermore, $p27^{Kip1}$ has also been implicated in regulating differentiation, transcription and migration of T cells (Jatzek et al., 2013). The INK4/ARF family encodes $p16^{INK4a}$ and $p18^{INK4c}$, which inhibit the activities of the cyclin D-dependent kinases, Cdk4 and Cdk6, and the ARF protein p14, which binds to the Mdm2-p53 complex and prevents p53 degradation (Bates et al., 1998; Sherr, 2001). In T cells, CD28 costimulation cooperates with the TCR/CD3 complex to activate the PI3K/Akt signalling pathway which in turn mediates the down-regulation of $p27^{Kip1}$ and promotes cell cycle progression (Appleman et al., 2002). $p18^{INK4c}$ has been proposed to set an inhibitory threshold in T cells and that CD28 costimulation is crucial to counteract the $p18^{INK4c}$ repressive activity on Cdk6-cyclin D complexes, thus facilitating G_1 cell cycle initiation and progression (Kovalev et al., 2001).

The mTOR pathway integrates growth factor and nutrient signalling to drive cell growth as well as mRNA and protein synthesis during the Gap 1 phase [Section 2.3.]. CD28 signalling boosts T cell activation and stimulates the Akt/PI3K which in turn promotes mTOR activity and cell growth (Acuto and Michel, 2003). Indeed, inhibition of mTOR using rapamycin blocks cell cycle progression and restoration of signalling along the mTOR-dependent S6K1 or 4E-BP1 pathways provides partial rescue (Fingar et al., 2004). Other targets of the TCR/CD28 signals are the transcription factor c-Myc and the Ras signalling molecules, which also play an important role in the control of the metabolic reprogramming and cell cycle progression upon T lymphocyte activation (Wang et al., 2011b),(Coleman et al., 2004). The Lung Krüpple-like factor which is highly expressed in naïve quiescent T cells and is down-regulated upon TCR engagement, blocks c-Myc expression and thereby inhibits cell cycle entry [FIGURE 16]. LKLF is also thought to promote quiescence via up-regulation of the cyclin inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (Di Santo, 2001). c-Myc itself regulates the expression of genes involved in G_1/S transition, replication and DNA repair, such as the E2F family members. Most E2F have a pocket protein binding domain to which proteins such as pRB and p130 may bind when hypophosphorylated, repressing transcription (Nevins, 2001). E2F also

regulates the expression of Cdc25A, the phosphatase involved in the dephosphorylation and thereby activation of Cdk2 and Cdk4, leading to the induction of S phase (Vigo et al., 1999). Interestingly, Cdc25A is specifically degraded in response to DNA damage [Section 3.2.] and stalled replication forks, a process mediated by p53 and the kinase Chk1 that results in cell cycle arrest (Demidova et al., 2009; Rother et al., 2007).

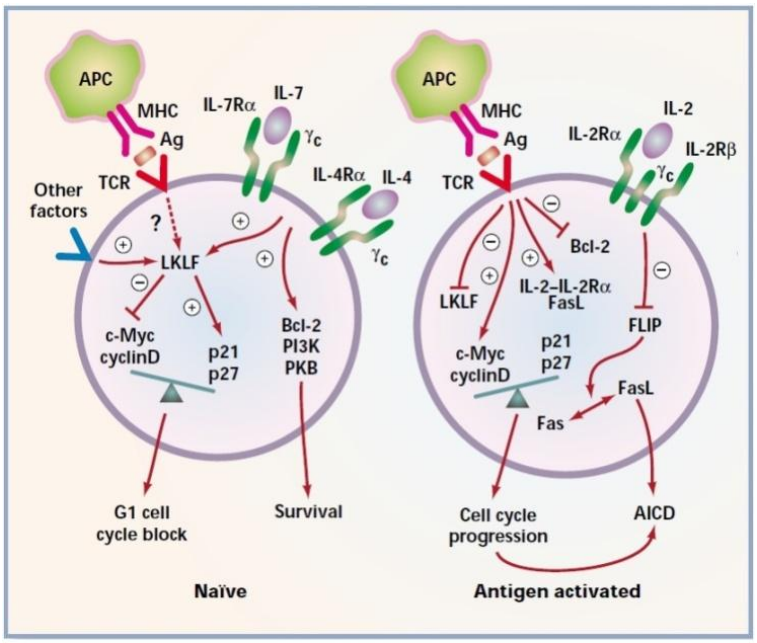

Figure 16. **T lymphocyte cell cycle regulation by LKLF and c-Myc.** LKLF promotes quiescence in T cells via a mechanism that involves the repression of c-Myc and expression of the negative cell cycle regulators $p21^{Cip1}$ and $p27^{Kip1}$. Antigen activated T cells rapidly down-regulate LKLF and up-regulate c-Myc, promoting cell cycle entry. TCR activation also results in up-regulation of IL-2/IL-2Rα and FasL, which ultimately limits cell proliferation via activation-induced cell death (AICD). *Adapted from (Di Santo, 2001).*

3.2. Replicative Stress and DNA Damage

Replication stress is defined as inefficient DNA replication that causes DNA replication forks to progress slowly or stall (Burhans and Weinberger, 2007). Chromosome replication occurs during the S phase of the cell cycle but the spatial position and replication timing of chromosomal domains are both established in early G1 phase, after the initial binding of the replication licensing factor Mcm2 to chromatin (Dimitrova and Gilbert, 1999). The replicative helicase is a hexameric complex comprised of Mini-Chromosome Maintenance proteins (Mcm2–7: Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7). The Mcm ATPases are key components of the pre-replication complex (pre-RC) and constitute a prerequisite for DNA replication licensing and the establishment of bidirectional replication forks (Evrin et al., 2009).

Amongst the Mcm proteins, Mcm2 is particularly relevant as it modulates the activity of the hexameric complex, resulting in the stabilization of replication forks. Indeed, phosphorylation and induction of Mcm2 activity has been identified to be a major aspect of the response to replicative stress (Stead et al., 2012).

Conditions challenging replication fork formation represent a major source of genomic instability. To ensure the fidelity of DNA replication, cells trigger the DNA Damage Response (DDR) pathway, a stress-response mechanism that regulates cell cycle progression, transcription, apoptosis and DNA repair, recombination and replication (Osborn et al., 2002). Cellular responses to DNA damage are coordinated by two distinct kinase signalling cascades, the Ataxia-Telangiectasia Mutated (ATM) and ATM-Rad-3-related (ATR) kinases [FIGURE 17]. The ATM-Chk2 pathway is primarily activated by DNA double-strand breaks (DSBs) caused by ionizing radiation (IR) or radiomimetic drugs whereas the ATR-Chk1 pathway is particularly sensitive to single-stranded DNA breaks (ssDNA) and instability of the replication forks (Smith et al., 2010). ATM and ATR are recruited to sites of DNA damage by related mechanisms. However, while DSBs are rare events, ATR is activated during every S phase to regulate the firing of replication origins and the repair of damaged replication forks. Furthermore ATR is of particular importance in the prevention of the premature onset of mitosis (Cimprich and Cortez, 2008).

Upon recruitment to sites of DSB DNA damage, ATM phosphorylates the Checkpoint kinase 2 (Chk2), known to inhibit the dual dual-specific phosphatase Cdc25 (cell division cycle 25) and prevent mitosis. Cdc25 activates cyclin dependent kinases by removing phosphate from residues in the Cdks active sites, thereby promoting cell cycle progression. Chk2 phosphorylation has been shown to mediate p53 stabilization as well, resulting in G_1 arrest (Sengupta and Harris, 2005). In addition, Chk2 interacts with and phosphorylates BRCA1, one of the components of the super protein complex BRCA1-associated genome Surveillance Complex (BASC), which includes ATM and is involved in the recognition and repair of aberrant DNA structures (Yang et al., 2004). Interestingly, cells and organisms are able to survive with mutations in the genes of the BASC complex, but at the cost of genomic instability and cancer predisposition (Smith et al., 2010). The ATR-interacting protein (ATRIP) recruits ATR to bind directly to replication protein A (RPA)-coated ssDNA that accumulates at stalled DNA replication forks (Jazayeri et al., 2006). ATR phosphorylates the

Checkpoint kinase 1 (Chk1); phosphorylation of Chk1 releases it from chromatin and increases its kinase activity. As Chk2, Chk1 mediates Cdc25a phosphorylation and degradation in response to DNA-damage, enabling a G_2/M checkpoint arrest (Xiao et al., 2003). Chk1 also phosphorylates the G_2/M checkpoint kinase Wee1. In turn, phosphorylated Wee1 promotes inhibition of the Cdk1 mitotic kinase that coordinates early mitotic events, leading to cell cycle arrest (Fasulo et al., 2012).

Both ATM and ATR kinases, but particularly ATM, are involved in the phosphorylation of the Histone-2A family member X (H2AX). H2AX phosphorylated on serine 139, which is then called γ-H2AX, marks the actual sites of DNA damage. MDC1, the Mediator of DNA damage checkpoint protein-1 binds to γ-H2AX and recruits additional ATM-BASC complexes that will further phosphorylate H2AX and help spread the damage signal (Cimprich and Cortez, 2008; Jazayeri et al., 2006; Pinto and Flaus, 2010).

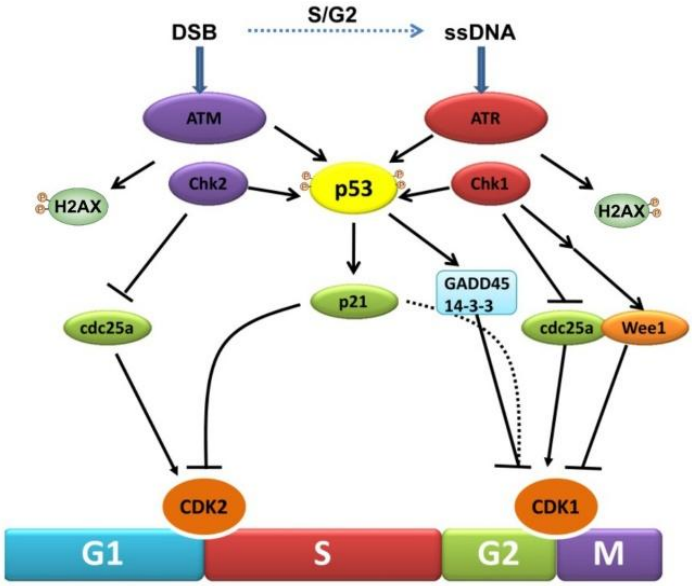

Figure 17. **ATM and ATR activation in response to DDR.** ATM and ATR orchestrate a transitory delay of the cell cycle in response to DSBs and ssDNA breaks, respectively. Whereas direct phosphorylation of Cdc25a and Wee1 allow a rapid establishment of the G_1/S and G_2/M checkpoints, p53-dependent regulation contributes to checkpoint maintenance at later time points. Excision of DSBs during S and G_2 results in the generation of ssDNA breaks and activates ATR-signalling. Furthermore, phosphorylation of the Histone-2A family member X (H2AX) in response to ATR/ATM signals, marks the actual sites of DNA damage. *Adapted from (Lopez-Contreras and Fernandez-Capetillo, 2012).*

The tumour suppressor p53, one of the most commonly mutated genes in human cancer, is another overlapping substrate of ATM and ATR. Both ATM and ATR phosphorylate p53 in response to DNA damage. Phosphorylation of p53 at N-terminal sites is hypothesized to

inhibit interactions with Mdm2, the negative modulator of p53 activity, and prevent its degradation (Lakin and Jackson, 1999). N-terminal phosphorylation of p53 is also reported to increase p53 transcriptional activity, promoting $p21^{Cip1}$ expression (Ashcroft et al., 1999). Chk1 and Chk2 also participate in the phosphorylation of serine and threonine residues in the p53 C-terminal. Phosphorylation of these residues facilitates p53 DNA binding to the promoters of its target genes and also increases p53 susceptibility to acetylation (Lakin and Jackson, 1999; Ou et al., 2005). Acetylation of p53 is an indispensable event that destabilizes the p53-Mdm2 interaction and promotes the p53-mediated stress response (Tang et al., 2008). Therefore, more than a direct player in DDR, p53 functions essentially as a node, a connection point between upstream stress signalling cascades and downstream DNA-repair and DNA-recombination pathways (Sengupta and Harris, 2005).

4. Sirtuins

Sirtuins (paralogs Sirt1 to 7) are a class III of histone deacetylases (HDACs) whose catalytic activity is NAD^+-dependent; they function as molecular sensors of the metabolic status of a cell and regulate various metabolic processes that allow cells to adapt to nutrient stress and survive (Nakagawa and Guarente, 2011). Sirtuins are the mammalian homologues of the Silent information regulator 2 (Sir2), a deacetylase that promotes longevity in budding yeast (Chang and Min, 2002). Contrary to Sir2 whose action is restricted to histone deacetylation, mammalian sirtuins are both deacetylases and ADP ribosyltransferases; furthermore, they target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (Sirt1, 2, 3, 5, 6) or ADP ribosylation (Sirt4 and 6) (Houtkooper et al., 2012; Nakagawa and Guarente, 2011). Recently, Sirt5 has also been shown to possess demalonylation and desuccinylation activity, by removing from certain proteins malonyl and succinyl moiety, respectively (Du et al., 2011; Peng et al., 2011).

Sirtuin proteins are structurally defined by two central domains that together form a highly conserved catalytic core of approximately 275 amino acids and 30 kDa, flanked by regions that are variable in length and sequence both at the N- and C-terminal. One of the domains consists in a large and structurally homologous Rossmann-fold motif, frequently found in proteins that bind nucleotides such as NAD^+. The other domain is a smaller and more structurally diverse, zinc-binding motif, thought to be structurally important for where the

Sirtuin proteins locate in the cell and for the regulation of protein-protein interactions and substrate specificity (Sanders et al., 2010). The deacetylation catalyzed by sirtuins is distinct from the class I and class II HDAC families and consists as it requires NAD^+ as a cofactor. It is a two step reaction: in the first step, sirtuins cleave NAD^+, yielding nicotinamide (NAM) and the oxocarbenium intermediate-like ADP-Ribose; the ADP-ribose moiety of NAD^+ functions as an acceptor of acetyl groups and in the second step, Sirtuins catalyze the removal of an acetyl from the ε-amino group of lysine residues within the protein targets to produce the deacetylated substrate and the unique metabolite 2'-O-acetyl-ADP-ribose [FIGURE 18].

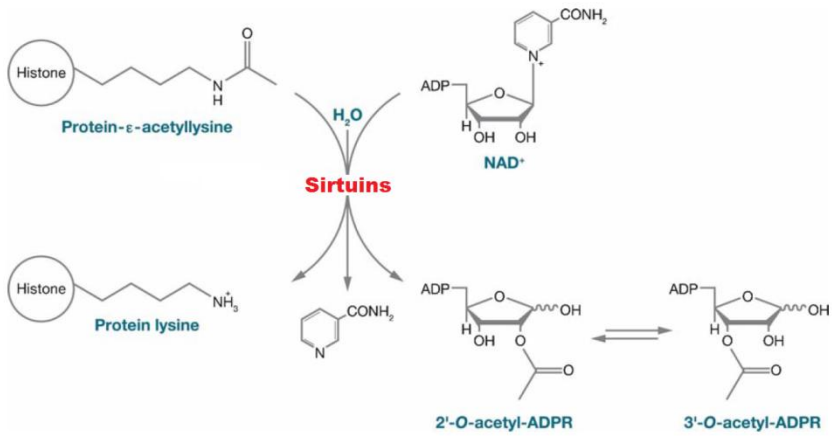

Figure 18. **Deacetylation of protein acetyl lysines catalyzed by Sirtuins.** Acetyl-group transfer to the ADP-ribose (ADPR) moiety of NAD+ occurs via Sir2p chemistry to form 2'-*O*-acetyl-ADPR. 3'-O-acetyl-ADPR is formed non enzymatically after release of 2'-O-acetyl-ADPR from the enzyme. *Adapted from (Sauve et al., 2006).*

4.1.Sirt1

Sirt1 is the most evolutionarily conserved and the best understood member of the mammalian Sirtuins. It is essentially a nuclear enzyme but in response to different environmental signals, it can also shuttle between the nucleus and the cytosol (Tanno et al., 2007). Sirt1 is the closest homologue of yeast Sir2 and has been shown to play crucial roles in health and disease. It is often associated with complex physiological processes, including transcription, DNA stability, metabolism and lipid mobilization [FIGURE 19].

Sirtuins have also been reported to play an important role in ageing; this was first discovered when overexpression of Sir2 was shown to increase replicative lifespan in *Saccharomyces cerevisiae* (Kaeberlein et al., 1999). Soon thereafter, others reported that Sir2 also mediated lifespan extension in the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). However, there

is growing evidence that Sir2 is not a longevity factor *per se* but, rather is implicated in calorie restriction (CR) pathways which extend lifespan in many organisms (Burnett et al., 2011; Garber, 2008; Kaeberlein et al., 2004). Furthermore, in a recent study, Herranz and colleagues reported that mice with systemic Sirt1 overexpression showed normal longevity but they presented with "healthier" ageing as monitored by lower levels of DNA damage and decreased expression of the ageing-associated gene $p16^{Ink4a}$ (Herranz et al., 2010). Indeed, Sirt1 is a component of the energy-dependent nucleolar silencing complex (eNoSC); eNoSC senses the energy status of a cell via the NAD^+/NADH ratio to control ribosome biogenesis, thus 'optimizing" the energy balance of the cell.

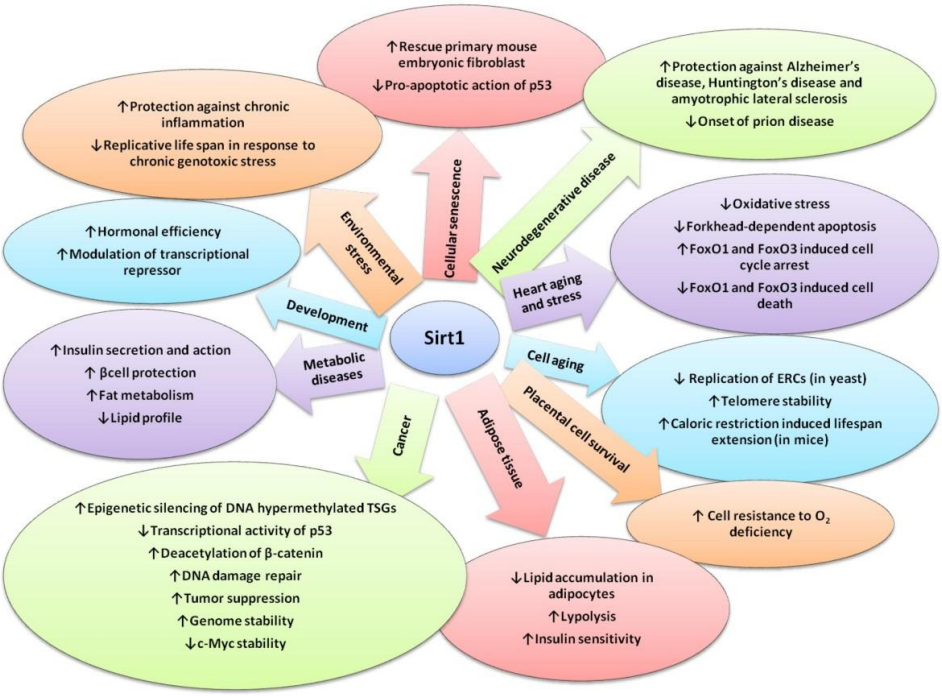

Figure 19. **Sirt1-mediated activation and inhibition of distinct cellular processes.** *Adapted from (Rahman and Islam, 2011).*

4.1.1. Sirt1 targets

Sirt1 substrates identified thus far include histones, p53, β-catenin, FoxO1, FoxO3, the Liver Kinase B1 (LKB1), PGC-1α, NF-κB, Myc, mTORC2, HIF-1α, HIF-2α and SREBP (Li and Kazgan, 2011). Recruitment of Sirt1 to its target gene promoters results in deacetylation of histone (H1, H2 and H3) polypeptides (Liu et al., 2009). Sirt1 catalyzes the deacetylation of histone H1 at lysine 26 (H1-K26Ac), promoting the formation of tightly packed chromatin structures (facultative heterochromatin). More compacted DNA makes promoter regions less accessible to binding factors, leading to inhibition of gene transcription (Vaquero et al., 2004).

Vaquero and colleagues were also able to demonstrate that decreasing Sirt1 expression in human cell lines caused hyperacetylation of histone H3 lysine 9 (H3-K9Ac) and histone H4 lysine 16 (H4-K16Ac); the deacetylation of these residues is necessary for the formation of compacted chromatin, making it refractory to transcription (Johnson et al., 1998). Interestingly, deacetylation of histone H3 has also been shown to establish silent chromatin in the ribosomal DNA locus, thus repressing rRNA transcription (Murayama et al., 2008).

p53 posttranslational modifications play an important role in regulating its stability and activity. For instance, during the DNA damage response, phosphorylation of p53 at Ser15, Thr18 or Ser20 will disrupt its binding with Mdm2. Mdm2 is an E3 ubiquitin ligase that constantly monoubiquitinates p53, thus antagonizing the p53 growth-suppressive function in unstressed cells (Moll and Petrenko, 2003).

In addition, the functional activation of p53 depends also on its acetylation status. Indeed, acetylation of p53 is shown to enhance its site-specific DNA binding both *in vitro* and *in vivo* (Luo et al., 2004). Furthermore, acetylation of p53 is also required for the destabilization of the p53-Mdm2 interaction, therefore promoting the p53-mediated stress response (Tang et al., 2008) [Sections 3.2.]. Remarkably, Sirt1 is a p53-deacetylase, involved in the deacetylation of the residue Lysine 382 in its C-terminal end (K382ac). As such, Sirt1 deacetylase activity attenuates p53-mediated functions by rendering p53 unstable and decreasing its transcriptional activity. Indeed, Sirt1-mediated p53 deacetylation has been shown to repress both p53-mediated upregulation of the Cdk inhibitor p21 and p53-dependent apoptosis (Luo et al., 2001; Vaziri et al., 2001). This suggests that Sirt1 may be a double-edged sword, functioning as a survival factor and as an oncoprotein (Chen et al., 2005). However, there are also reports that describe Sirt1 as a tumour suppressor based on its role in negatively regulating survivin, an inhibitor of apoptosis that is overexpressed in various cancers, and β-catenin, a multifunctional oncogenic protein whose deacetylated form is retained in the cytoplasm and is therefore inactive. As such, these contradictory effects are possibly linked to cell-type specificity and/or a consequence of distinct regulation mechanisms of Sirt1 (Yi and Luo, 2010).

In addition to its effects on p53, mammalian Sirt1 mediates stress resistance by deacetylating the forkhead-box O transcription factors FoxO1 and FoxO3. Brunet *et al.* reported that FoxO3 acetylation in HEK-293T cells was increased in response to oxidative stress and that Sirt1 catalytic activity potentiated the ability of FoxO3 to induce cell cycle arrest and resistance to

oxidative stress while attenuating its capacity to induce cell death (Brunet et al., 2004). The FoxO factors regulate important cellular processes such as survival, proliferation and metabolism, and are also important for the homeostasis of T cells. The activation of the PI3K/Akt kinases in response to growth factors and cytokine stimulation leads to the phosphorylation of the FoxO factors and inhibition of their transcription activity due to nuclear export (Peng, 2008). Acetylation of FoxO1 at lysine residues K242, K245, and K262 alters its DNA-binding ability and attenuates its transcriptional activity. Sirt1 binds and deacetylates FoxO1 on the residues acetylated by the CREB-binding protein, increasing its affinity with the target DNA and rendering it less sensitive to phosphorylation and nuclear export (Daitoku et al., 2004; Matsuzaki et al., 2005). Furthermore, in a recent publication, Xiong *et al.* proposed the existence of an auto-feedback loop between FoxO1 and Sirt1, based on the fact that FoxO1 binds to the IRS-1 and FKHD-like responsive elements within the *sirt1* promoter and that Sirt1 deacetylates FoxO1, therefore potentiating FoxO1-dependent SIRT1 transcription (Xiong et al., 2011).

As discussed in the section above [Section 2.4.], PGC-1α constitutes a master regulator of mitochondrial biogenesis. PGC-1α transcriptional activity is tightly linked to its lysine acetylation pattern (Canto and Auwerx, 2009) and Rodgers and colleagues mapped 13 lysine sites within PGC-1α whose acetylation leads to decreased transcriptional activity. Sirt1 promotes the NAD^+-dependent deacetylation of PGC-1α, therefore enhancing PGC-1α transcriptional activity. Interestingly, the positive effect of PGC-1α deacetylation can be mimicked by mutating the lysine sites targeted by Sirt1 into arginine residues (Rodgers et al., 2005).

AMPK is also an important metabolic sensor that maintains energy homeostasis and its activity is generally closely linked with that of Sirt1. AMPK is activated by metabolic stresses that result in an increased AMP/ATP ratio, due to an inhibition of ATP production and/or an acceleration of ATP consumption. Activated AMPK restores cellular energy homeostasis by stimulating catabolic processes that generate ATP while switching off anabolic, ATP-consuming pathways (Hardie et al., 2012). Recent work from Cantó *et al.* hypothesized the existence of interdependence between AMPK and Sirt1 for metabolic adaptation; during fasting and after exercise, AMPK functions as an initial metabolic sensor capable of transforming information into transcriptional and metabolic adaptations, including Sirt1-dependent deacetylation of the transcriptional regulators PGC-1α and FoxO1 (Canto et al., 2010). In response to a decrease in the energy state, AMPK is activated at its catalytic α-

subunit by threonine 172 phosphorylation mediated by LKB1, its primary upstream kinase. Calcium/calmodulin kinase kinase-β (CaMKKβ) is also capable of phosphorylating the Thr172 residue of AMPK, in response to increases in intracellular Ca^{2+} caused by growth factor and stress signals (Ruderman et al., 2010). Initial studies demonstrated that LKB1 is acetylated on lysine residue 48 (K48ac) and more recently Lan *et al.* showed that Sirt1 overexpression in HEK-293T cells diminishes lysine acetylation of LKB1, thus enhancing the cytoplasmic/nuclear ratio of LKB1 and its binding to the STE20-related adaptor protein (STRAD), which promotes the kinase activity of LKB1 and leads to the phosphorylation of AMPK (Lan et al., 2008). Together, these data strongly suggest that AMPK and Sirt1 regulate each other and share common target molecules. Additionally, Sirt1 and AMPK both inhibit mTOR signalling. AMPK represses the mTORC pathway by activating TSC2 and binding the mTORC1 component Raptor (Gwinn et al., 2008) while Sirt1 has been shown to activate TSC2 via a direct association (Ghosh et al., 2010). In accord with this effect of Sirt1, mTOR signalling is augmented under conditions of Sirt1 deficiency (Ghosh et al., 2010).

The c-Myc transcription factor is a nodal point in many mitogenic and stress signalling cascades and its function is intricately associated with that of Sirt1. c-Myc binds to the Sirt1 promoter and induces Sirt1 expression. Furthermore, c-Myc promotes Sirt1 activity via upregulation of NAM phosphoribosyltransferase (Nampt), the rate-limiting enzyme of the NAD^+ salvage pathway that catalyzes NAD^+ biosynthesis (Menssen and Hermeking, 2012). There is also a feedback loop as Sirt1 has been shown to interact with c-Myc, and the subsequent deacetylation results in a decreased c-Myc stability (Yuan et al., 2009). Thus, while c-Myc activity promotes Sirt1 activity, Sirt1 also maintains c-Myc activity under check, controlling cellular transformation (Menssen et al., 2012). This is critical for cell division as the c-Myc regulation of gene expression, via binding to consensus sequences and recruitment of histone acetyltransferases (HATs), results in the downregulation of inhibitors of cell cycle progression (p27) and the upregulation of positive acting Cdks.

4.1.2. Regulation of Sirt1 function by phosphorylation

Regulation of Sirt1 has been attributed to changes in the NAD^+/NADH ratio, the availability of the natural Sirt1 inhibitor NAM, an end product of the deacetylase reaction, and the activity of the Nampt, which catalyzes the reconversion of NAM to NAD^+ (Ruderman et al., 2010). Furthermore, the N- and C-terminal regions of Sirt1 can also be targeted by posttranslational

modifications (PTM) that may affect its function and localization (Flick and Luscher, 2012). The N-terminal tail contains two nuclear localization sequences (NLS) and two nuclear export sequences (NES) that are sensitive to PTM modifications, in particular phosphorylation, and are important for the nucleo-cytoplasmic shuttling of Sirt1 (Tanno et al., 2007) [FIGURE 20]. Tanno *et al.* found that the nuclear localization of Sirt1 is dependent on the PI3K-Akt signalling and more recently, Nasrin *et al.* described a functional interaction between Sirt1 and JNK, a downstream effector of the PI3K/Akt pathway. Using HEK-293T and C2C12 cells, they characterized three Sirt1 residues (S27 (serine) and S47 in the N-terminus and T530 (threonine) in the C-terminus) whose phosphorylation by JNK1 increases Sirt1 nuclear localization and enzymatic activity (Nasrin et al., 2009). There is though some discrepancy, which may be related to cell type specificity, as Back *et al.* reported that phosphorylation of the S47 residue results in an inhibition of SIRT1 deacetylase activity in squamous cell carcinoma (SCC) cells, fostering survival of prematurely senescent SCC cells with DNA damage (Back et al., 2011).

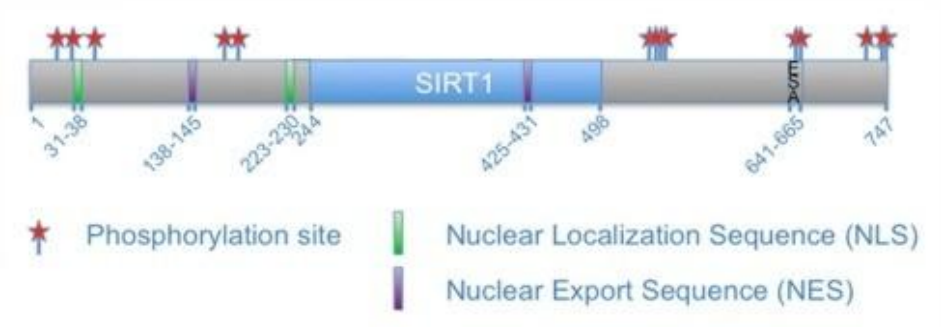

Figure 20. **Schematic overview of human Sirt1 and its phosphorylation sites.** Sirt1 is schematically shown with the blue box depicting the sirtuin-typical catalytic core domain. The catalytic domain is flanked by distinct N- and C-terminal extensions (gray boxes) and the numbers below indicate amino acid positions for orientation. PTMs (phosphorylation), nuclear localization sequences, nuclear export sequences, and proteolytic cleavage sites are indicated. *Adapted from (Flick and Luscher, 2012).*

Sasaki *et al.* identified one of these residues, T530, as well as an additional C-terminal residue, S540, as potential substrates of cyclin B/Cdk1 complexes, suggesting that these phosphorylation sites are required for normal cell cycle progression (Sasaki et al., 2008). In addition, Casein Kinase 2 (CK2), implicated in cell cycle control and DNA repair, has also been found to phosphorylate the S154, S649, S651, and S683 residues of murine Sirt1, and the human equivalents S659 and S661. Phosphorylation of these sites appears to increase

substrate affinity and therefore activity of Sirt1 towards acetylated p53 as well as p65, a major component of the NF-κB complex (Revollo and Li, 2013).

Sirt1 has also been shown to be phosphorylated at the threonine residue T522 by the pro-survival DYRK kinases, DYRK1A and DYRK3, in response to environmental stress and DNA damage in HEK-293T cells, where it promoted p53 deacetylation and cell survival (Guo et al., 2010). In a more recent publication, the same group assessed changes in Sirt1 oligomerization; they proposed that non-phosphorylated Sirt1 is aggregation-prone while Sirt1 phosphorylated on T522 is largely in a monomeric active state (Guo et al., 2012).

4.1.3. Sirt1 and metabolism

The NAD^+-dependent deacetylase Sirt1 has been implicated in a variety of metabolic processes, including glucose and lipid metabolism in the liver, fat mobilization in the adipose tissue, insulin secretion in the pancreas, nutrient sensing in the central nervous system and circadian regulation of metabolism. Furthermore, there is accumulating evidence that Sirt1 is in fact a direct link between intracellular energy resources and transcriptional activity, via regulation of chromatin structure and the function of transcription factors (Li, 2013).

Sirt1 uses the NAD^+/NADH ratio to sense the cell's metabolic status and modulate energy homeostasis [FIGURE 13]. NAD^+ is an essential coenzyme involved in redox reactions, an electron carrier that functions as an oxidizing agent. NADH, the reduced form of NAD^+, is an electron donor. Both NAD^+ and NADH are extensively used in an array of metabolic reactions, such as glycolysis, the citric acid cycle and oxidative phosphorylation, and as such, NAD^+ availability constitutes an important indicator of the cellular energy status. For instance, NAD^+ levels increase in mammalian tissues in response to nutrient stress and low-energy status such as fasting, caloric restriction (CR) and exercise. Accordingly, Sirt1 activity is enhanced by all these conditions. On the other hand, Sirt1 activity is reduced in mice submitted to high-fat diet, a high-energy status that decreases cellular NAD^+ levels (Canto and Auwerx, 2012).

In many lower organisms, CR decelerates the biological mechanism of ageing, a process thought to be mediated by the increased expression and activity of the Sir2 protein (Guarente and Picard, 2005). In higher organisms, CR is followed by important changes in metabolic pathways, hormone levels and regulatory proteins. For example, in fasting conditions, Sirt1

modulates gluconeogenesis and fatty acid β-oxidation in the liver through deacetylation of PGC-1α, FoxO1 and the CREB-regulated transcription coactivator 2 (CRTC2). During short-term fasting, circulating pancreatic glucagon promotes CRTC2 acetylation by the co-activator CBP/p300, which then supports the expression of the early gluconeogenic program (Wang et al., 2009). Prolonged fasting results in Sirt1 upregulation, which in turn deacetylates both CRTC2 and FoxO1; deacetylation renders CRTC2 inactive but FoxO1 functional, therefore leading to a switch from early to late gluconeogenic genes (Liu et al., 2008). Deacetylation of PGC-1α by Sirt1 is also an important feature of late and robust gluconeogenesis, since insulin-regulated gluconeogenesis requires an interaction between FoxO1 and PGC-1α (Puigserver et al., 2003). Together with PPARα and PPARδ, PGC-1α is also important in the coordination of fatty acid oxidation in the liver during the adaptation to CR (Liang and Ward, 2006).

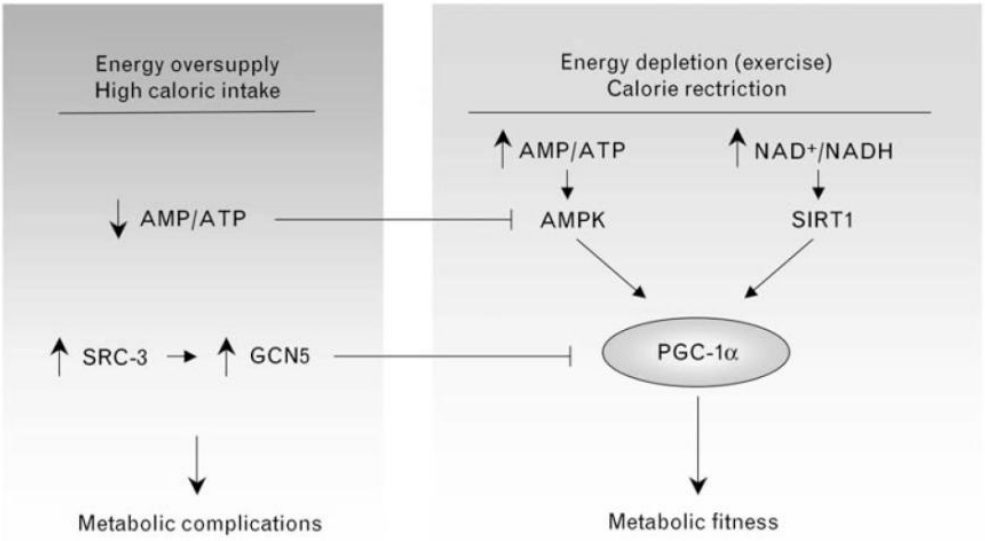

Figure 21. **PGC-1α, SIRT1 and AMPK, an energy sensing network that controls energy expenditure.** Situations of energy depletion or decreased catabolic rates or both can be sensed by different enzymes, such as AMPK and Sirt1, whose activation enhances PGC-1α dependent transcription. Upon intake of calorie rich diets or under situations when energy is not limited, AMPK activity is shut down by the high intracellular ATP levels. Similarly, high-fat diets increase SRC-3, which positively regulates the protein levels of the acetyltransferase GCN5, which, in turn, inhibits Sirt1 deacetylation of PGC-1α. When PGC-1α acetylation is highly, PGC-1α transcriptional activity is decreased. *Adapted from (Canto and Auwerx, 2009).*

As described, PGC-1α regulates mitochondrial biogenesis and oxidative phosphorylation [Sections 2.4. and 4.1.1], and it has been proposed that these effects are mediated by the

capacity of AMPK to modulate NAD^+ availability and Sirt1 activity [FIGURE 21]. Defects in energy production or increased energy consumption disturb the AMP/ATP ratio leading to AMPK activation [FIGURE 13 AND 21], through LKB1-mediated phosphorylation. AMPK activation leads to increased PGC-1α expression and phosphorylation, which results in higher transcriptional activity. Furthermore, AMPK enhances Sirt1 activity by increasing cellular NAD^+ levels, resulting in the deacetylation and increased activity of both LKB1 and PGC-1α (Canto and Auwerx, 2009; Canto et al., 2009). In mouse myocytes, Ca^{2+} influx is also know to activate both AMPK and Sirt1, a process mediated by CaMKKβ. This process similarly promotes mitochondrial biogenesis due to increased expression and decreased acetylation of PGC-1α. (Iwabu et al., 2010).

In addition, Sirt1 also targets cell specific proteins; the liver X receptor (LXR), which regulates hepatic cholesterol levels [FIGURE 13] (Li and Kazgan, 2011); PPARγ, which participates in fat mobilization in the white adipose tissue (Picard et al., 2004); and glucose-stimulated insulin secretion in pancreatic β cells through the synthesis of uncoupling protein 2 (UCP2), suggesting that Sirt1 activity may regulate whole-body glucose metabolism (Bordone et al., 2006). Mice lacking Sirt1 in the brain show normal neuronal development but reduced body size, as a consequence of specific anterior pituitary cell defects and reduced growth hormone production (Canto and Auwerx, 2012; Nakagawa and Guarente, 2011). Taken together, these findings provide strong evidence that Sirt1 plays a significant role in many metabolic processes, and that impairment of Sirt1 activity will undoubtedly result in metabolic disorders.

4.1.4. The role of Sirt1 in T cell biology

Sirt1 is ubiquitously expressed in many tissue types, and is particularly abundant in lymphoid lineage cells [FIGURE 22]. Furthermore, many Sirt1 targets, such as NF-κB, FoxO1, FoxO3 and p53 are known for their roles in T cell homeostasis, differentiation, quiescence and activation [FIGURE 23]. As such, it becomes relevant to assess whether and how Sirt1 is involved in the regulation of signalling pathways in the immune system (Kong et al., 2012).

Mice that lack Sirt1 present defective innate and adaptive immune responses as a consequence of impaired NF-κB and AP-1 signalling (Sequeira et al., 2008; Zhang et al., 2009). The NF-κB pathway executes critical functions within lymphocytes as well as within supportive cells

(such as APCs) and is essential in mediating the response to inflammatory cytokine stimulation and lymphocyte activation (Gerondakis and Siebenlist, 2010). Sirt1 represses the activity of NF-κB by enhancing deacetylation of the p65 (RelA) subunit at lysine 310 (K310), a site critical for the binding of NF-κB to specific κB consensus sequences in the chromatin and for its transcriptional activity (Yeung et al., 2004). In addition, TNF-α signalling in vascular smooth muscle cells promotes p65-mediated upregulation of Sirt1, which in turn deacetylates p65 in a repressing-manner, suggesting that Sirt1 may be essential for balancing NF-κB signals (Kong et al., 2011; Zhang et al., 2010).

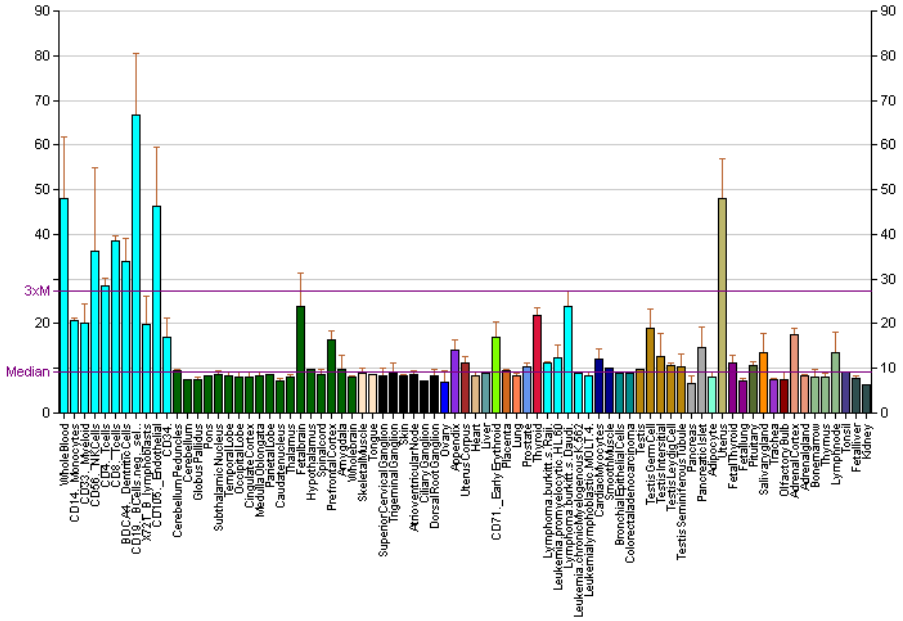

Figure 22. **Gene expression pattern of Sirt1 gene.** Tissue-specific pattern of Sirt1 mRNA expression is indicated. Light blue columns at the far left of the histogram represent the expression pattern in lymphoid and myeloid lineage cells. *Diagram created by AndrewGNF based on data from (Su et al., 2004) (source Wikimedia Commons).*

The activator protein 1 (AP-1), a heterodimeric transcription factor composed of proteins belonging to the c-Fos and c-Jun families, is another Sirt1 target. c-Jun is required for cell cycle progression through G_1, via transcriptional regulation of cyclin D1, and during T lymphocyte activation, c-Jun and c-Fos cooperate to induce proliferation, IL-2 production and T cell differentiation (Foletta et al., 1998). Recently, Zhang *et al.* showed that in activated and anergic T cells, Sirt1 physically interacts with the C-terminus of c-Jun and inhibits AP-1 transcriptional activity by specifically deacetylating c-Jun (Zhang et al., 2009). Notably though, there is a feedback loop between these factors as c-Jun also modulates Sirt1 activity by phosphorylating specific residues in the latter [Section 4.1.2].

In Sirt1-null lymphocytes, the enhanced NF-κB and AP-1 signalling results in $CD4^+$ T cells that are hyper proliferative. In addition, the proliferative response of $Sirt1^{-/-}$ T cells to TCR activation alone is as high as that detected in wild-type T cells undergoing TCR/CD28 costimulation. In response to TCR activation, $Sirt1^{-/-}$ $CD4^+$ lymphocytes produce more IL-2, IL-5 and IFN-γ, suggesting that they are more susceptible to becoming cytokine producing effector T cells. Sirt1 may also balance T cell activation via the PI3K/Akt pathway; this pathway, targeting FoxO1 and FoxO3, is also stimulated by T cell activation and IL-2 signalling (Zhang et al., 2009). FoxO1 and FoxO3 positively regulate quiescence and when they are phosphorylated by Akt, in response to cytokines or TCR signalling, they are excluded from the nucleus and their transcriptional activity is inhibited. In contrast, Sirt1 deacetylates and promotes the activity of the forkhead box O transcription factors. Thus, while the dynamics and regulation of these factors by Sirt1 in the context of T cell activation have not yet been elucidated, this might be another mechanism by which Sirt1 balances lymphocyte activation. Furthermore, and in support of this concept, Zhang *et al*. observed that NF-κB and AP-1 signalling results in the upregulation of Sirt1 in activated and anergic T cells, potentially limiting a TCR response so that a detrimental lympho-proliferative response does not occur.

Sirt1 has also been implicated in the differentiation of regulatory T cells, or rather in inhibiting Treg differentiation, based on its suppression of Foxp3 expression [FIGURE 13]. Foxp3 is widely accepted as the master transcription factor in the regulatory T cell lineage and stable Foxp3 expression is required for development of a functional T_{reg} suppressor phenotype. Importantly, Foxp3 is stabilized when it is in an acetylated state as hyperacetylation prevents its polyubiquitination and proteasomal degradation. Moreover, the acetylation status of Foxp3 is regulated by Sirt1 and the histone acetyl transferase p300 (van Loosdregt et al., 2010). Specifically, ectopic expression of Sirt1 has been shown to decrease Foxp3 acetylation while treatment with the Sirt1 inhibitor NAM increases Foxp3 acetylation and stabilization (van Loosdregt et al., 2011). Furthermore, mice with a conditional deletion of Sirt1 in either T cells (CD4-Cre) or in Treg cells (Foxp3-Cre) show enhanced Foxp3 expression and an improved suppressive function of Foxp3-expressing T cells, facilitating long-term preservation of myocardial allografts, even if conventional T-effector cell activation, proliferation, and cytokine production is unaltered (Beier et al., 2011). Using a complementary approach, Tao *et al.* found that Foxp3 gene expression, as well as the production and suppressive function of regulatory Treg cells, increases in the presence of HDAC inhibitors (Tao et al., 2007).

$CD4^+$ T lymphocyte hyperactivation and depletion is a hallmark of untreated chronic HIV-1 infection. Aberrant activation of $CD4^+$ T cells is associated with increased expression of surface activation markers, enhanced cell cycling and apoptosis (Haas et al., 2011). Recently, the group of Melanie Ott showed that the viral Trans-Activator of Transcription (Tat) promotes hyperactivation of Jurkat cells by blocking Sirt1. Tat, which is essential for efficient transcription of the viral genome, directly interacts with the catalytic domain of Sirt1 and blocks its capacity to repress and deacetylate the p65 subunit of NF-κB (Kwon et al., 2008). Along with Ott's observations, Thakur *et al.* reported that Tat-mediated inhibition of Sirt1 activity results in the functional activation of p53, secondary to p53 acetylation. While these experiments were performed in HEK-293T and HeLa cells (Thakur et al., 2012), the hypothesis is that a similar mechanism may be functional in primary T lymphocytes, resulting in T cell depletion during HIV-1 infection. Finally, it is important to note that this regulation is part of a more complex feedback loop; the Tat protein itself is acetylated by the transcriptional coactivator p300, a necessary step in Tat-mediated transactivation, while the deacetylation of Tat by Sirt1 inhibits Tat activity (Pagans et al., 2005).

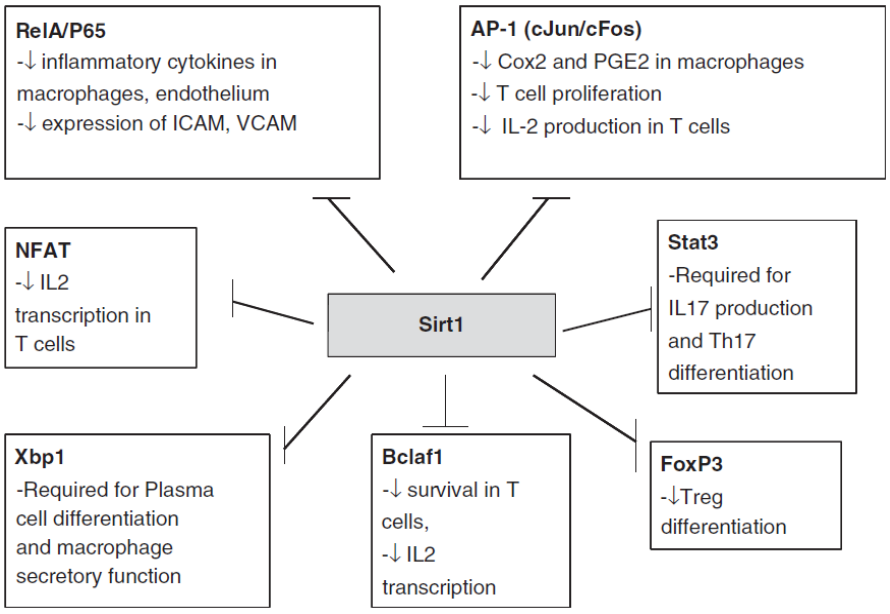

Figure 23. **Multiple Sirt1 targets are known modulators of the immune response.** Sirt1 inhibits the activity of multiple substrates via deacetylation. AP-1 and NF-κB are key transcription factors involved in the production of inflammatory cytokines and the activation of leukocytes. Xbp1 regulates endoplasmic reticulum stress, and is required for the differentiation of plasma cells and macrophages, which have complex secretory systems. Foxp3 and STAT3 are key regulators of T cell differentiation into regulatory T cells (Tregs) and Th17 cells, respectively. Bclaf1 transcription was recently found to be regulated by the NF-κB/p300/Sirt1 complex and is required for T cell proliferation after activation. *Adapted from (Kong et al., 2012).*

4.2.Sirt3

Three sirtuins have been identified as persisting in mitochondria (Sirt3, -4 and -5) where they participate in a number of processes associated with mitochondrial activity and biogenesis, such as metabolism and survival (Verdin et al., 2010). Of these sirtuins, Sirt3, a soluble protein with a mitochondrion-targeting sequence in the N-terminus, is the best characterized. Consistent with the idea that sirtuins are important cellular metabolic sensors, Sirt3 expression is higher in tissues that are metabolically active, such as brown adipose, muscle, liver, kidney, heart and brain (Li and Kazgan, 2011; Onyango et al., 2002).

Sirt3 is a major regulator of global mitochondrial lysine acetylation; Lombard and colleagues showed that Sirt3-deficient mice exhibit striking mitochondrial protein hyperacetylation while that no hyperacetylation was detected in animals lacking the two other mitochondrial sirtuins, Sirt4 and Sirt5 (Lombard et al., 2007). Sirt3 has been identified in association with the mitochondrial complexes I and II, thus regulating mitochondrial respiration and cellular energy levels. In this context, it would bind and thereby activate acetyl-CoA synthetase 2 (AceCS2) and glutamate dehydrogenase (GDH), promoting the production of the TCA cycle intermediates acetyl-CoA and α-ketoglutarate, respectively (Verdin et al., 2010). Interestingly though, Sirt1 has been identified as the only Sir2-ortholog capable of deacetylating cytoplasmic acetyl-CoA synthetase 1 (AceCS1), thus promoting AceCS1-dependent fatty-acid synthesis (Hallows et al., 2006).

Sirt3 is also involved in the deacetylation of the long-chain acyl-CoA dehydrogenase (LCAD), which is essential in fatty acid β-oxidation (Nakagawa and Guarente, 2011). The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase 2, an enzyme essential in the mevalonate and ketogenesis pathways, as well as the oxidative stress reducing enzymes, isocitrate dehydrogenase 2 (IDH2), superoxide dismutase 2 (SOD2) and Manganese Superoxide Dismutase (MnSOD), are also regulated by the Sirt3 (Li and Kazgan, 2011).

FoxO3 is a member of the forkhead box O family of transcription factors that has been reported to be a mitochondrial protein targeted by Sirt3 (Jacobs et al., 2008). FoxO3 mediates diverse cellular responses to stress and is involved in many immune mechanisms (Hedrick et al., 2012). Sirt3 deacetylates FoxO3, increasing its DNA-binding activity, leading to the upregulation of genes that are essential for mitochondrial biogenesis, ATP production and protection against oxidative stress (Peserico et al., 2013; Tseng et al., 2013).

4.3.Resveratrol

More than 300 years ago, John Ray recorded the old English proverb "there are more ways to kill a dog than hanging". A central tenet of studying biological systems is that redundancy rules. Just because one mechanism is in play does not mean that another does not occur. In fact, the most recent biochemical data show that Sirt1 can be directly activated by a large number of compounds that have been developed for therapeutic applications. We should also keep in mind that resveratrol is a natural product, and evolution may have selected for multiple ways in which this compound can activate Sirt1 orthologs, thereby maximizing the range of control over this key hub of physiology.

Leonard Guarente *(Guarente et al., 2012)*

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a polyphenolic flavonoid enriched in the skins of red grapes, and widely used as a food supplement in the US, has been shown to mimic calorie restriction and to harbour major health benefits (Aggarwal et al., 2004), including increased life span in lower organisms, antiproliferative activity (Agarwal and Baur, 2011) and cardiovascular protection in human patients (Carrizzo et al., 2013). In fact, resveratrol is currently undergoing several Phase II and III clinical trials to assess its effects in patients with Alzheimer's disease, diabetes and cancer (ClinicalTrials.gov; search word: resveratrol; link).

The mechanism of resveratrol action, however, has been a matter of intense debate. Howitz and colleagues were amongst the first to show that resveratrol directly activates Sirt1 *in vitro* (Howitz et al., 2003) [FIGURE 24] and in a later publication, Borra *et al.* proposed that binding of resveratrol to Sirt1 promotes a conformational change in the enzyme allowing the catalytic domain to bind more tightly to the acetylated substrate, lowering the Km value for the substrate and to a lesser extent that of the co-factor NAD^+ (Borra et al., 2005). It has also been demonstrated that resveratrol improves Sirt1 activity *ex vivo*, enhancing the deacetylation of FoxO1 and p53 as well as that of PGC-1α (Frescas et al., 2005; Kim et al., 2011a). Resveratrol has also been shown to promote Sirt1 activity *in vivo* (Baur et al., 2006), but the validity of this evidence has been challenged by several groups who claim that resveratrol is not a direct activator of sirtuins (Kaeberlein et al., 2005).

Still, many of the effects reported in resveratrol-treated animals require the deacetylation of Sirt1 targets, in particular PGC-1α (Lagouge et al., 2006). Indeed, in a recent publication, Price *et al.* proposed a model where moderate doses of resveratrol first activate Sirt1, which leads to deacetylation of LKB1, phosphorylation of AMPK and PGC-1α-mediated increases in mitochondrial biogenesis, while a high dose of resveratrol activates AMPK in a Sirt1-independent manner. Furthermore, they suggested that Sirt1 and AMPK do not function

independently or linearly, working instead as part of a feedback cycle where NAD^+ availability is the key factor (Price et al., 2012). In accord with this observation, Park *et al.* reported that resveratrol competitively inhibits cAMP-specific phosphodiesterases (PDE), resulting in increased intracellular Ca^{2+} levels which promote the activation of the CaMKKβ-AMPK pathway; this in turn results in increased functional AMPK, raising NAD^+ levels, and leading to enhanced Sirt1 activity (Park et al., 2012).

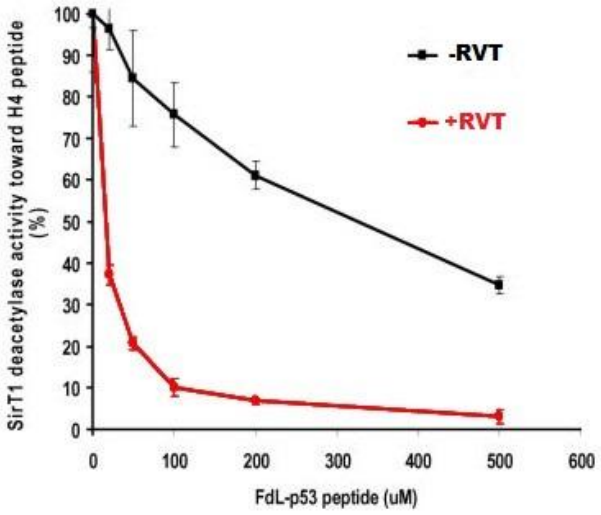

Figure 24. **Substrate-specific activation of Sirt1 by resveratrol.** Histone H4 deacetylation, measured by [3H] acetate release, in the presence of a range of concentrations of the FdL-p53 peptide substrate with and without 100μM resveratrol (resv). *Adapted from (Kaeberlein et al., 2005).*

4.3.1. Effects of resveratrol on endoplasmatic reticulum and mitochondria

The potential metabolic benefits of resveratrol and its ability to mimic calorie restriction have brought it to the forefront, but resveratrol has also been show to trigger endoplasmic reticulum (ER) stress and promote apoptosis. Resveratrol has been reported to exhibit anticancer activity *ex vivo* and in experimental animal models, via mechanisms inducing pro-apoptotic ER stress and damage to mitochondria (Sun et al., 2008). ER stress disrupts protein folding in the endoplasmic reticulum and causes the activation of the unfolded protein response (UPR) signalling network; PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) are the three ER stress sensors that implement the UPR (Bravo et al., 2013). Treatment of human colon cancer cells and multiple myeloma cells with resveratrol induces a number of signature ER stress markers, including phosphorylation of eukaryotic initiation factor-2α (eIF-2α) by the PERK kinase, activation of the stress response component IRE1α as evidenced by mRNA splicing of the X-box binding protein 1 (XBP1) and increased expression of the pro-apoptotic protein CHOP

(C/EBP homologous protein), a downstream target of both PERK and ATF6 (Park et al., 2007c; Wang et al., 2011a).

The UPR functions to restore the equilibrium between protein load and folding capacity of the ER, a process dependent on calcium by virtue of the fact that many of the chaperone enzymes require calcium. Calcium is one of the most important second messengers in the cell and the ER is the principal organelle involved in calcium homeostasis. Calcium accumulation in the ER lumen requires an appropriate supply of ATP as it relies on the activity of the Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Bravo et al., 2013). Loss of luminal Ca^{2+} causes ER stress and activates the UPR pathway (Mekahli et al., 2011) and, not surprisingly, resveratrol is reported to mediate increases in cytoplasmic calcium levels (Campos-Toimil et al., 2005; Elies et al., 2011). Furthermore, according to Park *et al.*, resveratrol promotes the phosphorylation of the ryanodine receptor 2 (Ryr2), which facilitates the release of Ca^{2+} from the ER; the subsequent increase in cytosolic Ca^{2+} promotes CaMKKβ-mediated activation of AMPK (Park et al., 2012).

Calcium transfer to mitochondria by the calcium channel inositol 1,4,5-trisphosphate receptor (IP3R) is an essential cellular process that is required for efficient mitochondrial respiration and maintenance of normal cell bioenergetics (Cardenas et al., 2010). Interestingly, IP3R-induced calcium release is increased during ER stress, including the calcium flow from the ER to mitochondria; this translates to an augmented mitochondrial respiration and ROS production (Bravo et al., 2011; Li et al., 2009). This becomes particularly relevant since resveratrol has been identified to promote mitochondrial biogenesis via Sirt1-mediated induction of PGC-1α (Lagouge et al., 2006) and promoting apoptosis through a ROS-dependent mitochondria pathway (in HT-29 human colorectal carcinoma cells) (Juan et al., 2008). Thus, the impact of resveratrol on metabolism is also likely to result from its function in the ER and mitochondria, and specifically, due to its capacity to regulate calcium homeostasis.

4.3.2. Effects of resveratrol on cell cycle progression

Resveratrol has also been shown to exert anti-proliferative actions by inducing cell cycle arrest and inhibiting cell growth. In human epidermoid carcinoma A431 cells, resveratrol treatment causes cell growth inhibition and a dose- and time-dependent induction of p21; p21 activity blocks the cyclin D1/D2-Cdk6, cyclin D1/D2-Cdk4, and cyclin E-Cdk2 complexes,

therefore imposing an artificial checkpoint at the G_1/S transition of the cell cycle (Ahmad et al., 2001) [FIGURE 25]. Using different human cancer cell lines (MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60), Joe *et al.* also observed a dose-dependent inhibition of cell growth with cells arrested in the S phase of the cell cycle, as monitored by decreased expression of cyclin D1, cyclin A and cyclin B1 (Joe et al., 2002). Interestingly, they also verified that resveratrol decreased β-catenin expression in SW480 cells, and more recently Cho *et al.* demonstrated that Sirt1 inhibits proliferation of pancreatic cancer cells via degradation of β-catenin (Cho et al., 2012). However, resveratrol-induced cell cycle arrest in malignant NK cells appears to be a consequence of inhibiting the Janus kinase 2 (JAK2)/STAT3 pathway (Quoc Trung et al., 2013). In all four NK cell lines tested (KHYG-1, NKL, NK-92 and NK-YS), resveratrol inhibited JAK2 phosphorylation but had no effect on other upstream mediators of STAT3 activation, such as PTEN, TYK2 and JAK1. This resulted in the suppression of constitutively active STAT3; loss of STAT3 signalling, led to downregulation of the anti-apoptotic proteins MCL1, Bcl-10, and survivin, while contributing to cell cycle arrest at G_0/G_1. Of interest, STAT3 acetylation decreased with resveratrol treatment and Sirt1 is known to mediate deacetylation of STAT3 (Nie et al., 2009; Wieczorek et al., 2012).

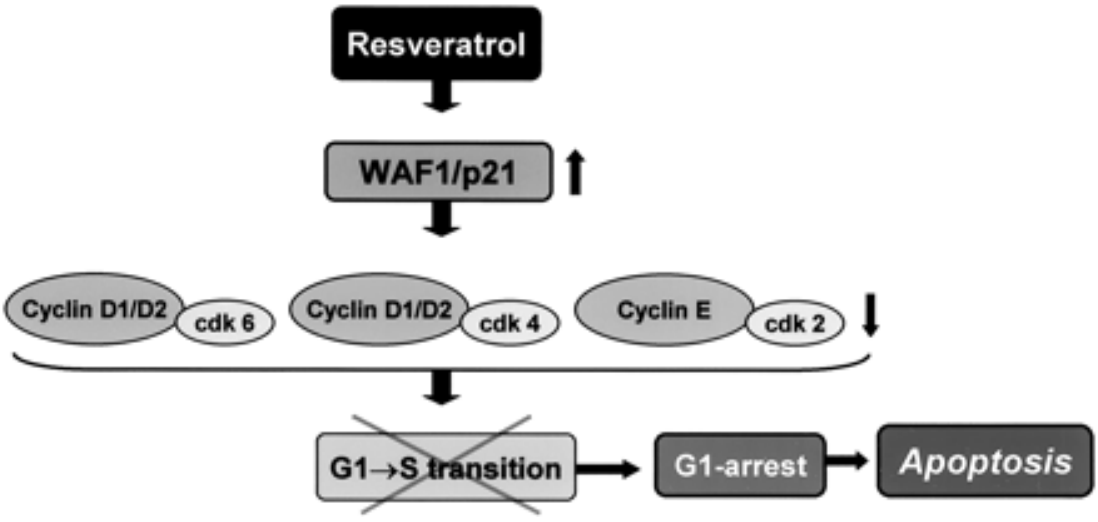

Figure 25. **Proposed model for resveratrol-mediated cell cycle arrest and apoptosis in epidermoid carcinoma A431 cells.** *Adapted from (Ahmad et al., 2001).*

Resveratrol has also been reported to affect G_0/G_1 transition and cell cycle progression of human lymphocytes. Using phytohemagglutin (PHA) to mitogenically stimulate total lymphocytes, Hsieh found that resveratrol prevented cell entry in a dose- and time-dependent manner, with 99% suppression at 100μM of resveratrol (Hsieh et al., 2002). Indeed,

lymphocytes treated with 100μM of resveratrol during 72 hours appeared to be blocked in early G_1, based on an absence of Rb phosphorylation, reduced Cdk1, cyclin E and B, and minimal RNA content.

4.3.3. The role of resveratrol and Sirt1 in cellular senescence

Senescence is the biological process of ageing that leads to the accumulation of molecular and cellular changes resulting in metabolic deterioration, disease and eventually death. Replicative cellular senescence is defined as an irreversible cell cycle arrest at the G_1 state, which *in vitro* normally happens after 50 cell divisions (Hayflick limit) (Shay and Wright, 2000). Considering its inhibitory effects on cell cycle [Section 4.3.2.], it is not surprising to find resveratrol associated with the onset of replicative senescence. For instance, chronic treatment with resveratrol induces redox stress and senescence in p53-positive cancer cells, via ATM-dependent cell cycle arrest (Heiss et al., 2007). In lung cancer cells, resveratrol induces ROS production and DNA double strand breaks (DSBs), leading to p53/p21-mediated cell cycle arrest and premature senescence (Luo et al., 2013). Mono-ubiquitination of histone H2B is associated with actively transcribed genes; in turn, reduced expression of the H2B ubiquitin ligase RNF20 is characteristic of senescent phenotype. Interestingly, resveratrol treatment is described to attenuate mono-ubiquitination of histone H2B and promotes cellular senescence in glioma cells, although the mechanism involved is not yet known (Gao et al., 2011).

Although the majority of publications state resveratrol's inhibitory effect on cell cycle, there are also contradictory studies reporting that resveratrol and Sirt1 are capable of promoting cell proliferation and antagonize cellular senescence. In an early publication, Demidenko *et al.* demonstrated that activation of the mTOR complex caused senescence when the cell cycle is blocked (Demidenko and Blagosklonny, 2008) and latter, using human fibroblasts and cell lines, they showed that at near-toxic concentrations, resveratrol inhibited the mTOR pathway (as measured by the decrease in S6 phosphorylation) and prevented cells to become senescent (Demidenko and Blagosklonny, 2009). Excessive reactive oxygen species have been shown to cause cellular senescence. Tang *et al.* recently showed that adult male Wistar rats submitted to high-fat/sucrose diet (HFS) have higher levels of ROS in the aorta and are more susceptible to develop vascular cell senescence (Tang et al., 2012). In turn, resveratrol treatment decreased the production of ROS and protected against the onset of vascular cell senescence. Furthermore, they were able to demonstrate that the decrease in ROS production was associated with the inhibition of p47phox, the cytosolic subunit of the NADPH oxidase, and

that this downregulation is dependent on Sirt1 deacetylase activity. Loss of telomerase activity is also a triggering factor for cellular senescence. Using endothelial progenitor cells (EPC) Wang et al. verified that resveratrol promoted telomerase activity by increasing the expression of the human telomerase reverse transcriptase (hTERT), therefore enhancing proliferation and functionality of EPCs and delaying senescence (Wang et al., 2011d). Enthralling, in a more recent report, Yamashita and colleagues verified that Sirt1 increased hTERT transcription in a c-Myc-dependent manner and potentiated the telomerase activity, thus preventing replicative senescence of human umbilical cord fibroblasts (Yamashita et al., 2012).

Interestingly, in a recent publication Peltz *et al.* revealed that resveratrol exerts a dosage- and duration-dependent effect on human mesenchymal stem cell self-renewal (Peltz et al., 2012); low dose resveratrol (0.1µM) inhibited cellular senescence and promoted proliferation, whereas at concentrations of 5µM or above, resveratrol repressed cell self-renewal by inducing S-phase cell cycle arrest and increasing the cell doubling time. This data provides useful insights for better understanding resveratrol's dual effects, with dose-dependency, that varies between different cell types, being one critical aspect of it.

CHAPTER 2:

PROJECT AIM

Project Aim

One of the research axes in Naomi Taylor's lab focuses on metabolic plasticity during T cell homeostasis and differentiation. In the group, I am particularly interested in the signalling pathways controlling cellular growth, proliferation and metabolic reprogramming following TCR-mediated T lymphocyte activation.

To engage in proliferation, a cell must increase its biomass and replicate its genome. To do so, activated cells require both sufficient energy and biosynthetic precursors to fuel cellular growth. Lymphocytes overcome this bioenergetic challenge by reprogramming their metabolism, switching from catabolism to anabolism.

The mammalian target of rapamycin (mTOR) is widely recognised as a central regulator of cell growth and proliferation, capable of interpreting metabolic signals from the microenvironment and programming transcription and translation, accordingly. As such, it is not surprising that mTOR provides a direct link between T cell metabolism and function. Upstream of mTOR, the AMP-activated protein kinase (AMPK) also functions as a biologic sensor of energy status and a signal transducer. During a low energy state, the decrease in the ATP/AMP ratio promotes AMPK activity which in turn inhibits mTOR by phosphorylation of TSC2 and RAPTOR, stimulating a catabolic generation of ATP and inhibiting anabolic biosynthesis. Furthermore, the interplay between mTOR and AMPK has been shown to regulate autophagy and mediate T cell differentiation.

The histone deacetylase Sirtuin 1 (Sirt1) has also emerged as a direct link between cellular metabolism and the regulation of gene expression. Due to its functional dependency upon NAD^+, Sirt1 uses the NAD^+/NADH ratio to sense the metabolic state of a cell and trigger an appropriate response through epigenetic reprogramming. Interestingly, AMPK and Sirt1 have been shown to regulate each other and they share many common target molecules. AMPK

drives NAD^+ metabolism to promote Sirt1 activity and regulate energy balance. In turn, the AMPK kinase LKB1 is a direct target of Sirt1; deacetylation promotes cytoplasmic shuttling of LKB1, facilitating its association with the STE20-related adaptor protein (STRAD), and thus enhancing its activity and subsequent phosphorylation of AMPK.

Interestingly, several Sirt1 targets such as NF-κB, FoxO1, FoxO3 and p53 play critical roles in T cell homeostasis, differentiation and metabolism. It is therefore not surprising to find that Sirt1 itself is also a key player in T cell biology. Deletion of Sirt1 in CD4 T lymphocytes results in enhanced NF-κB and AP-1 signalling, with an associated hyper proliferation. Furthermore, Sirt1 mediates the deacetylation of the Foxp3 regulatory T cell transcriptional regulator. As this modification results in Foxp3 degradation, Sirt1 deletion results in an enhanced differentiation of regulatory T cells. Nevertheless, relatively little is known about how Sirt1 activity modulates T lymphocyte function and metabolism. During my PhD, I was interested in elucidating whether Sirt1 signaling integrates with the mTOR/AMPK network to modulate antigen-driven T cell activation and proliferation.

CHAPTER 3:

RESVERATROL-INDUCED ACTIVATION OF THE SIRT1/AMPK AXIS IN HUMAN CD4 T CELLS TRIGGERS AN ATR-DEPENDENT CELL CYCLE ARREST

Results

DISCLAIMER: The following results are a summary of the experiments done in the context of my PhD project, which will soon be submitted under the running title **Resveratrol-induced activation of the Sirt1/AMPK axis in human CD4 T cells triggers an ATR-dependent cell cycle arrest.** Furthermore, I would like to acknowledge my supervisors Dr. Naomi Taylor and Professor Conceição Pedroso de Lima, as well as Dr. Gaspard Cretenet, Mr. Cédric Mongellaz and Dr. Vjekoslav Dulic for their contribution to the development and success of this project.

TITLE

Resveratrol-induced activation of the Sirt1/AMPK axis in human CD4 T cells triggers replicative stress and an ATR-dependent cell cycle arrest

ABSTRACT

Resveratrol possesses anti-aging and anti-inflammatory properties, effects attributed to its capacity to modulate cellular metabolism via induction of the Sirtuin1 (Sirt1) histone deacetylase. Here, we show that Sirt1 is highly upregulated following TCR stimulation of primary human CD4 T lymphocytes with resveratrol further augmenting Sirt1 expression and deacetylase activity. While high dose resveratrol blocked proximal TCR signaling and progression from the G0 phase of the cell cycle, lymphocytes treated with low dose

resveratrol were strongly activated and upregulated all cyclins and Cdks required for cell division. Furthermore, these cells exhibited high metabolic activity as monitored by glycolysis and mitochondrial function. Nevertheless, low dose resveratrol induced a G2/M genotoxic arrest, associated with a high level of replicative stress. Cell cycle exit was mediated by an ATR/Chk1-dependent and AMPK-dependent activation of p53. Thus, resveratrol induces a coordinated regulation of cell-cycle control in primary T lymphocytes *via* a Sirt1/AMPK/p53 network activated in response to replication stress.

BACKGROUND

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenolic flavonoid produced by plants in response to environmental stress (Aggarwal et al., 2004). Resveratrol appears to mimic the effects of caloric restriction, and amongst other effects, it has been found to increase life span in lower organisms (Agarwal and Baur, 2011). On the basis of a significant amount of very promising data emerging from *ex vivo* and preclinical animal models, resveratrol has been tested in more than 30 clinical trials involving over 1000 individuals (Smoliga et al., 2013; Tome-Carneiro et al., 2013; Vang, 2013). Indeed, resveratrol has been found to be efficacious in several populations including the elderly, patients with type 2 diabetes (Timmers et al., 2013) and individuals with cardiometabolic dysfunction (Carrizzo et al., 2013). Nevertheless, the clinical effects of resveratrol remain limited and safety concerns remain unresolved (Smoliga et al., 2013; Tome-Carneiro et al., 2013; Vang, 2013), despite the clear positive health effects of caloric restriction.

Caloric restriction was first found to extend life span in *Saccharomyces cerevisiae* via the activity of Sir2, a NAD^+-dependent deacetylase (Lin et al., 2000). Sirt1 is the closest mammalian homologue of yeast Sir2, functioning as a molecular sensor of the cell's metabolic status and thereby facilitating adaptation to conditions of nutrient stress (Nakagawa and Guarente, 2011). The activity of Sirt1 has been intimately linked to resveratrol as shown by a direct activation of this deacetylase both *in vitro* (Gertz et al., 2012; Howitz et al., 2003) and *in vivo* (Baur et al., 2006). The Sirtuin (Sirt) class III histone deacetylases (HDAC) cleave

NAD^+, yielding nicotinamide (NAM) and the oxocarbenium intermediate-like ADP-Ribose and then catalyze the removal of an acetyl from the ε-amino group of lysine residues within the substrate target. This results in the production of a deacetylated protein and a unique 2'-O-acetyl-ADP-ribose metabolite.

Irrespective of the precise clinical effects of resveratrol, Sirt1 has been found to regulate complex physiological processes in cell and animal models, including transcription, DNA stability, lipid mobilization and metabolism. Indeed, overexpression of Sirt1 in mice has been found to confer protection to a diverse array of diseases including diabetes, cancer, and neurodegenerative disease, amongst others (Banks et al., 2008; Donmez and Guarente, 2010; Donmez et al., 2010; Herranz et al., 2010; Herranz and Serrano, 2010; Pfluger et al., 2008). Moreover, Sirt1 activity has been associated with a protection from inflammation; global deletion of Sirt1 resulted in autoimmunity in several murine models (Cheng et al., 2003; Purushotham et al., 2009; Sequeira et al., 2008; Zhang et al., 2009). While this effect may be due to a hyperproliferative T lymphocyte response (Zhang et al., 2009), due to enhanced NF-κB and AP-1 signalling (Sequeira et al., 2008; Zhang et al., 2009), this point is still being debated as a recent study reported that T effector function is not altered by the absence of Sirt1 (Beier et al., 2011). In this regard, it is interesting to note that Sirt1 is highly expressed in lymphoid lineage cells (Kong et al., 2012) and has been found to be upregulated in murine T cells following TCR stimulation (Gao et al., 2012; Zhang et al., 2009).

Upon activation of T lymphocytes by an encounter with their cognate foreign antigen, they undergo a rapid clonal expansion. This proliferation is fairly unique to the adaptive immune system and requires a high expenditure of energy and cellular resources. There is an increase in oxygen consumption but interestingly, glucose appears to be fermented via the glycolytic pathway rather than used in the more energetic efficient mitochondrial oxidative phosphorylation, at least *in vitro* (Michalek and Rathmell, 2010). This increase in glycolytic activity in the presence of oxygen is known as aerobic glycolysis, or Warburg metabolism, as it was first observed by Otto Warburg in cancer cells (Warburg, 1956). In this process, phosphate transfer from glycolytic intermediates to ADP results in the generation of ATP and

the production of nicotinamide adenine dinucleotide (NADH) reducing equivalents. The regeneration of NAD^+ from NADH is critical for maintaining flux through the glycolysis pathway and occurs as a result of energy transfer to NAD^+ during beta oxidation, the citric acid cycle and glycolysis (reviewed in (Pearce and Pearce, 2013)).

Given the critical role of NAD^+ in T cell energy homeostasis and Sirt1 activity, it is surprising that we have only limited knowledge of whether Sirt1 deacetylase-enhancing agents modulate T cell activity. Additionally, based on the plethora of health benefits attributed to resveratrol action, it is important to assess whether resveratrol impacts on human T cell activation, either as a desired target or as an off-target effect in the diverse array of ongoing and future clinical trials. Here, we show that in primary human CD4 T cells, Sirt1 is highly upregulated upon TCR activation and resveratrol induced the deacetylation of two Sirt1 targets that play critical roles in T cell homeostasis, p53 and FoxO1. While high dose resveratrol severely attenuated proximal TCR signalling resulting in a block at the G_1 phase of the cell cycle, TCR-engaged lymphocytes treated with low dose resveratrol revealed a stimulated phenotype with upregulation of all cyclins and Cdks required for cell division. Moreover, these cells exhibited a significantly increased energy homeostasis with upregulation of the Glut1 glucose transporter and an augmented TCR-stimulated oxidative phosphorylation resulting in a more than 50% increase in intracellular ATP levels as compared to control TCR-stimulated T cells. Nevertheless, low dose RVT-treated cells failed to divide and we find that they underwent a G_2/M cell cycle arrest, secondary to replicative stress with high levels of H2AX-phosphorylated DNA damage foci. This then triggered activation of the ATR/Chk1 signaling cascade and together with AMPK activity, induced the phosphorylation of p53 on serine 15 and exit from the cell cycle. Thus, while resveratrol increased Sirt1 and AMPK activity, producing a positive energy balance in CD4 T cells, its negative regulation of DNA integrity resulted in the induction of a p53-dependent cell cycle checkpoint.

RESULTS

Increased expression and nuclear localization of the NAD$^+$-dependent deacetylase Sirt1 in primary human CD4 T cells following TCR stimulation.

The Sirt1 deacetylase has been shown to be upregulated in murine T cells following TCR stimulation (Gao et al., 2012; Zhang et al., 2009) but its expression in human T lymphocytes has not been established. Primary CD4 T cells were activated with anti-CD3/CD28 antibodies and Sirt1 levels as well as localization were evaluated. Nuclear localization and nuclear export sequences within the protein are highly sensitive to post-translational modifications and it has been shown that the nuclear localization of Sirt1, at least in transformed cell lines, is dependent on PI3K-AKT signalling (Tanno et al., 2007). Sirt1 expression in freshly isolated quiescent T cells was detected as punctate nuclear staining and interestingly, within 3 days of culture in cytokine-starved conditions (non-stimulated), the vast majority of Sirt1 exited from the nucleus [FIGURE R.1A]. Notably, TCR stimulation dramatically changed the expression pattern of Sirt1; there was a significant increase in nuclear and aggregated Sirt1. The density of nuclear Sirt1 staining was even higher in T cells activated in the presence of low doses of resveratrol, a direct activator of Sirt1 enzymatic activity (Borra et al., 2005; Gertz et al., 2012; Howitz et al., 2003). This was also detected by flow cytometry where the mean fluorescence intensity of Sirt1 staining was significantly increased by TCR stimulation and further augmented upon low dose resveratrol treatment [FIGURE R.1A]. Interestingly though, TCR-stimulated cells treated with high dose RVT showed a pattern of staining similar to that detected in quiescent cells and there was no significant increase in protein levels [FIGURE R.1A].

The generation of TCR-induced T cell blasts was increased in the presence of low dose RVT [FIGURE R.2] and as such, it was of interest to determine whether blast formation was associated with augmented Sirt1 expression. Indeed, distinguishing subsets of TCR-stimulated CD4 T cells on the basis of their FSC/SSC profiles demonstrated that Sirt1 expression was proportional to cell size and granularity [FIGURE R.1A]. Moreover, Sirt1 immunofluorescence, assessed as a function of T cell size, showed a punctate staining in small cells while larger cells revealed a significantly more dense and aggregate staining pattern [FIGURE R.1B]. Thus, Sirt1 expression and its nuclear pattern are highly affected by the activation state of human CD4 T cells and these parameters are further modulated by resveratrol treatment.

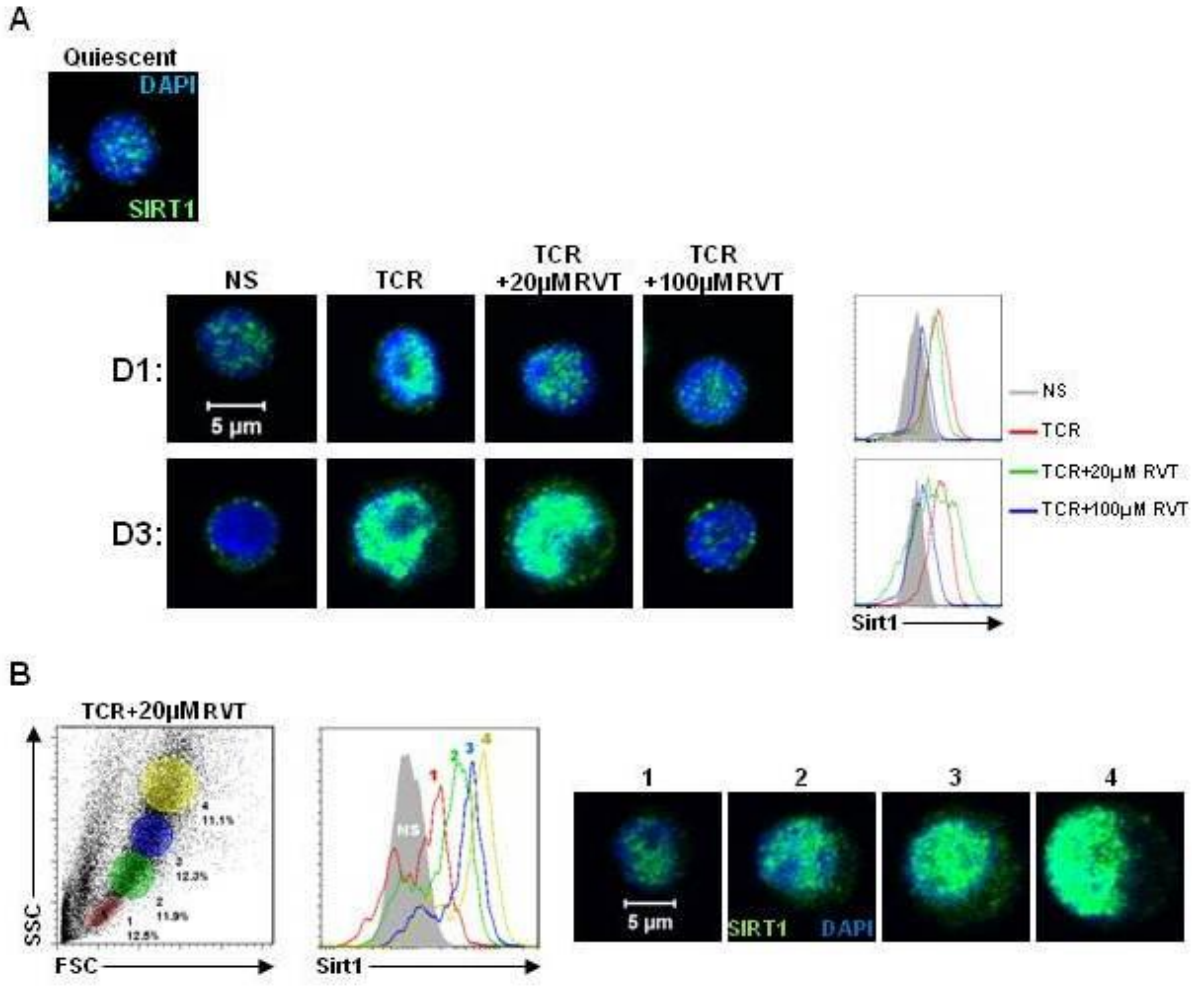

Figure R.1. **TCR-mediated upregulation of the NAD$^+$-dependent deacetylase Sirt1 in human CD4 T cells.** (**A**) Sirt1 expression in freshly isolated quiescent human CD4 T cells was assessed by staining with a Sirt1 polyclonal antibody (green) and a DAPI nuclear stain (blue). Expression and localization were also assessed after one (D1) or three (D3) days in non-stimulating conditions (NS) or following TCR activation (TCR) in the absence or presence of resveratrol (RVT: 20 and 100μM). Representative images are shown. Sirt1 expression was also monitored by flow cytometry and representative histograms showing expression levels in quiescent and TCR-activated cells in the absence or presence of resveratrol (RVT: 20 and 100μM) at day 3 are shown. (**B**) The expression of Sirt1 in resveratrol-treated activated T cells (20μM) was monitored as a function of FSC/SSC profile. Four populations were distinguished on the basis of FSC/SSC characteristics (left plot) and representative histograms and immunofluorescence showing expression of Sirt1 in each population are shown.

Sirt1 activity has been found to be increased by phosphorylation of the S27, S47 and T530 residues of the protein (Tanno et al., 2007) but it is not known whether TCR stimulation changes the phosphorylation state of Sirt1. Activation with anti-CD3/CD28 antibodies resulted in an augmented phosphorylation of Sirt1, as assessed using antibodies directed

against both the phosphorylated S27 and S47 residues, and total Sirt1 levels were also increased [FIGURE R.3A]. Low dose resveratrol treatment did not significantly alter the TCR-induced phosphorylation of Sirt1 but phosphorylation was diminished in the presence of high doses of resveratrol. Interestingly though, Sirt1 activity was significantly induced by resveratrol in TCR-stimulated T cells, as monitored by the generation of O-acetyl-ADP-ribose (OAADPr), a reaction product of Sirt-catalyzed NAD+-dependent protein deacetylation. This augmented deacetylation was even more pronounced when evaluated as a function of Sirt1 levels [FIGURE R.3B].

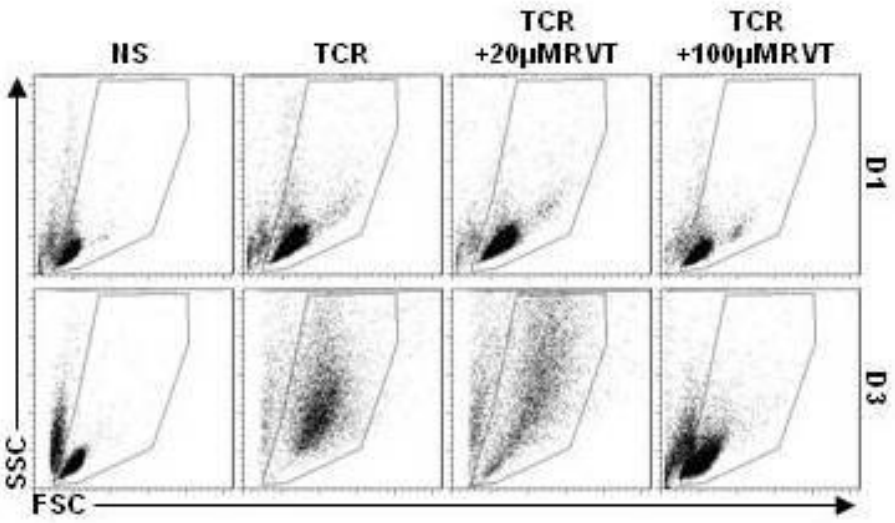

Figure R.2. **The generation of TCR-induced blasts is modulated by resveratrol treatment.**
Freshly isolated quiescent CD4 T cells were either left non-stimulated (NS) or activated with anti-CD3 and anti-CD28 antibodies (TCR) in the absence or presence of resveratrol (RVT: 20 and 100μM). Forward (FSC) and side (SSC) scatter profiles at days 1(D1) and 3 (D3) of stimulation are shown.

Multiple Sirt1 targets have been identified, at least two of which are critical for T cell homeostasis and survival; p53 and FoxO1. Deacetylation of p53 at lysine 382 attenuates p53-mediated functions (Luo et al., 2001; Vaziri et al., 2001) while deacetylation of FoxO1 results in augmented function, increasing its affinity to target DNA and rendering it less sensitive to phosphorylation and nuclear export (Daitoku et al., 2004; Matsuzaki et al., 2005). p53 has previously been shown to be upregulated in TCR-stimulated murine T cells (Wang et al., 2011a) but its acetylation status, as a function of either TCR stimulation or Sirt1 activation, has not been described. p53 was indeed highly upregulated following TCR engagement of human CD4 T cells and this induction was also detected in the presence of resveratrol

[FIGURE R.3C]. Moreover, p53 was highly acetylated by 3 days post TCR engagement, as assessed using a pan anti-acetylated lysine antibody or an antibody directed against the acetylated lysine 382 residue of p53 [FIGURE R.3B]. As expected from the deacetylase activity of Sirt1, treatment of cells with 20 and 100 µM of resveratrol resulted in a decrease in p53 acetylation, by approximately 2-fold and 50-fold, respectively. Furthermore, RVT treatment similarly reduced TCR-induced acetylation of FoxO1 [FIGURE R.3B]. Thus, while Sirt1 is highly upregulated upon TCR engagement, TCR-stimulation itself actually induces the acetylation of several Sirt1 targets. Notably though, treatment with the resveratrol polyphenol decreases this acetylation.

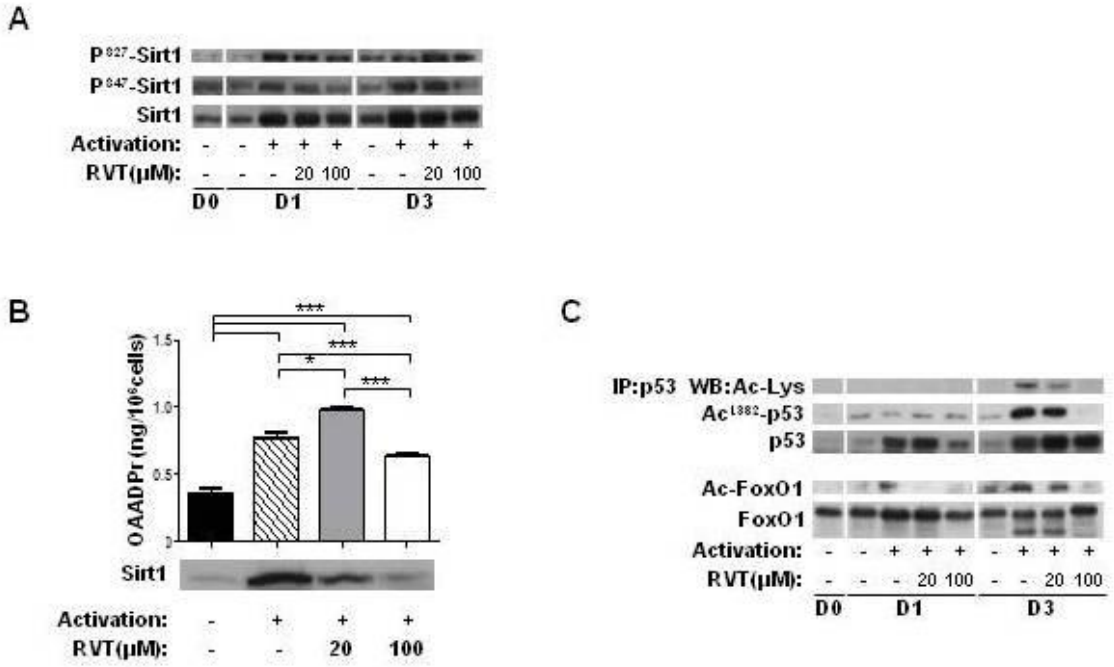

Figure R.3. **Resveratrol enhances Sirt1 deacetylase activity in TCR-activated cells.** (**A**) Phosphorylation of Sirt1 was assessed in CD4 T cells stimulated in the absence or presence of RVT (20 µM and 100µM). Phosphorylation was monitored at days 1 and 3 of activation by immunoblot analyses with phospho-specific serine 27 and 47 Sirt1 antibodies. Sirt1 levels were assessed with a pan-Sirt1 antibody. (**B**) Sirt1 deacetylase activity was monitored as a function of O-acetyl-ADP-ribose (OAADPr) generation, a reaction product of Sirt-catalyzed NAD^+-dependent protein deacetylation. OAADPr levels ± SD in each condition are shown. Corresponding levels of Sirt1 in extracts of cells from each condition were assessed by immunoblotting and representative blots are presented. (**C**) TCR-mediated acetylation of two Sirt1 targets, p53 and FoxO1, was evaluated by immunoblot analysis on days 1 and 3 of stimulation. p53 was immunoprecipitated from total lysates of CD4 T cells and acetylation was analysed using an acetylated-lysine antibody. Acetylation was also monitored using antibodies directed against acetylated L382 of p53 and L259/262/271 of FoxO1. Total p53 and FoxO1 levels in each condition are shown.

Low and high dose resveratrol induce distinct G_1 and G_2 cell cycle arrests in TCR-stimulated CD4 T cells.

In light of the RVT-mediated changes in the size and granularity of TCR-stimulated lymphocytes, we assessed whether TCR-induced cell cycle progression was altered by resveratrol treatment. TCR stimulation resulted in cell cycle entry with approximately 15-20% of quiescent CD4 T cells entering G_1 by day 1 and by day 3, greater than 60% of lymphocytes were actively cycling in a representative experiment [FIGURE R.4A AND R.4B]. This progression was associated with a rapid proliferation and the majority of CD4 lymphocytes underwent 1-3 rounds of division by day 3 [FIGURE R.4B]. Interestingly, virtually none of the high dose RVT-treated cells progressed into late G_1, as monitored by an absence in TCR-induced RNA levels [FIGURE R.4A], consistent with the lack of blast formation [FIGURE R.2]. As expected from these data, the cells did not undergo division [FIGURE R.4B]. Remarkably though, while T cells treated with low dose resveratrol exhibited a cell cycle entry progression equivalent to that observed in control TCR-stimulated cells, with approximately 30% of cells having entered into the S phase by day 3 of stimulation, their proliferation was dramatically attenuated [FIGURE R.4A AND R.4B].

The passage of a cell through the proliferation cycle is a tightly controlled process involving the action of cyclin-dependent kinases (CDKs) and cyclins. To understand the molecular bases underlying the cell cycle arrests induced by different doses of resveratrol, we investigated the regulation of components of the cell cycle machinery. Cyclins D2, E1 and A2, and B1, as well as their cognate kinases, Cdk4/Cdk6, Cdk2, and Cdk1 respectively, were strongly upregulated upon T cell activation, coinciding with pRb and p130 hyper-phosphorylation [FIGURE R.4C and R.4D]. While this induction was not affected by low dose RVT, it was severely attenuated in the presence of high dose RVT [FIGURE R.4C AND R.4D].

Interestingly though, by day 3 of activation, high dose RVT-treated cells showed increased cyclin D2 and cyclin E1 levels but these cells did not progress into S phase; cyclin A was not upregulated and enhanced phosphorylation of pRb and p130, hallmarks of S phase, was not induced. Furthermore, the assembly of functional holoenzyme complexes between cyclins and specific cyclin-dependent kinases (Cdk) regulating G_1 progression could not proceed under these conditions as neither Cdk4, Cdk6, Cdk2 nor Cdk1 were upregulated [FIGURE R.4C].

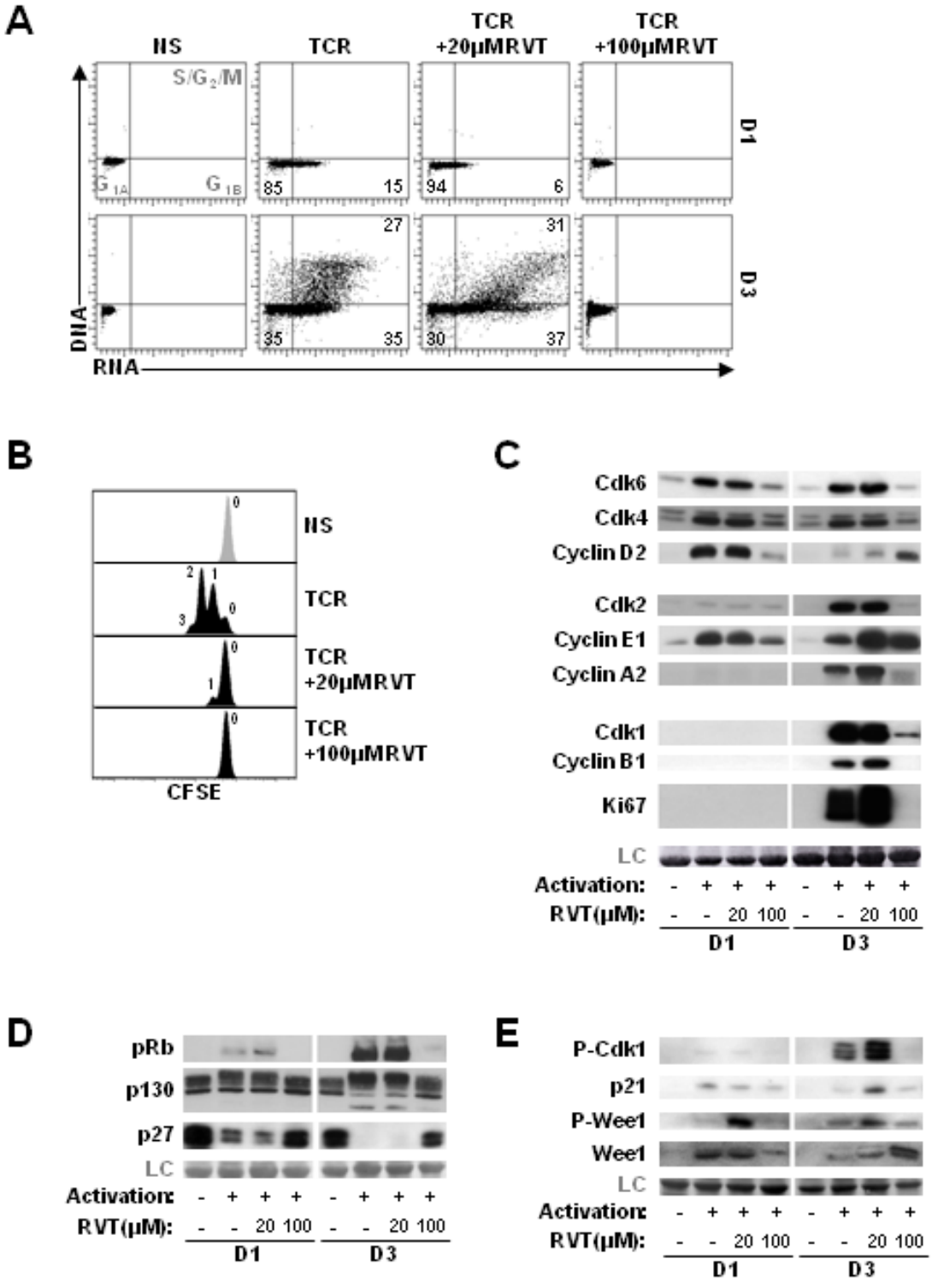

Figure R.4. **Low and high dose resveratrol block TCR-induced cell cycle progression at distinct stages of the cell cycle.**

(**A**) Cell cycle entry following TCR stimulation was monitored by simultaneous staining of DNA and RNA with 7-aminoactinomycin D and pyronin Y, respectively. Representative dot plots of non-stimulated (NS) and TCR-activated CD4 T cells, in the absence or presence of resveratrol, are shown. The percentages of cells in G_0/G_{1A}

(LL quadrant), G_{1B} (LR quadrant) and $S/G_2/M$ (UR quadrant) are indicated. (**B**) T cell proliferation in the indicated conditions was monitored by CFSE labeling and dilution of the fluorescent dye was assessed at 72h. The number of division peaks are indicated in each histogram. (**C**) Expression of the cyclin-cdks that regulate cell cycle entry, including cyclins D2, E1, A2 and B1 and Cdk4, Cdk6, Cdk2, and Cdk1 were monitored and representative immunoblots at days 1 and 3 of activation are shown. Phosphorylation of Cdk1 as well as induction of the Ki67, mid-G_1 marker, was assessed. (**D**) Expression of the cyclin kinase inhibitors including the retinoblastoma protein (pRb) and the pRb-related p130 protein as well as p27 was monitored in the indicated conditions. (**E**) Cdk1 activity was monitored as a function of its phosphorylation and by the activity of the Wee1 kinase and the Cdk1 inhibitor p21. Representative immunoblots at days 1 and 3 of activation are shown. Loading controls (LC) for all blots are presented.

As cell cycle entry of lymphocytes treated with high dose RVT was associated with a G_1 phase arrest, we assessed expression of the p27 Cdk inhibitor. This Kip family member physically interacts with cyclin-Cdk complexes, inhibiting their activation, and TCR stimulation has been shown to significantly decrease p27 levels by inducing its ubiquitin-dependent degradation (Appleman et al., 2002) (Appleman et al., 2006). Furthermore, it has been shown that FoxO1 mediates cell cycle arrest via enhanced transcription and protein expression of $p27^{kip1}$ (Stahl et al., 2002). Indeed, while the abundance of p27 was dramatically reduced by 24h post TCR stimulation of control and low dose RVT-treated cells, it remained elevated in CD4 lymphocytes treated with high dose RVT [FIGURE R.4C], consistent with the sustained unmodified expression of FoxO1 [FIGURE R.3C].

It is important to note that investigation of these multiple molecular components did not reveal the cause of the proliferation defect in lymphocytes treated with low dose resveratrol. These cells were not blocked until late G_2 as documented by upregulation of Cdk1, G_2/M cyclins and proliferation marker Ki67 [FIGURE R.4C]. Furthermore, pRb and p130 were hyperphosphorylated at levels equivalent to that detected in control TCR-stimulated lymphocytes [FIGURE R.4D]. Association of Cdk1 with cyclin B is essential for activation of the kinase and entry into M phase and Cdk1 was highly upregulated in low dose RVT-treated cells. Notably though, the Cdk1-cylin B complex is inactive under conditions where the T14 and Y15 residues are phosphorylated, likely via a direct effect on phosphotransfer activity during G2 of the cell cycle. The Wee1 kinase drives this phosphorylation and it exhibited higher phosphorylation in low dose RVT-treated cells than in control TCR-stimulated cells. Under these conditions, the dephosphorylation of Cdk1, required for mitotic entry, may be impaired and indeed, Cdk1 was phosphorylated at significantly higher levels in RVT-treated

cells, concordant with a G_2 arrest [FIGURE R.4D]. Furthermore, the p21 inhibitor directly inhibits Cdk1 activity and this protein was expressed at higher levels in low dose RVT-treated cells [FIGURE R.4E]. Thus, while high dose resveratrol blocks cell cycle entry of TCR-stimulated T cells in early G_1, these data show that T cells treated with low dose resveratrol are blocked at the G_2/M transition.

Resveratrol-mediated Sirt1 activation modulates TCR signalling cascades.

To determine whether the effect of resveratrol in blocking cell cycle progression was mediated at the level of TCR signalling, we first assessed proximal signalling intermediates. As shown in Figure R.5A, ZAP-70 was phosphorylated by 1 minute post TCR engagement, remained high at at 10' and decreased by 30', irrespectively of the presence of resveratrol. Further downstream signalling was monitored as a function of Erk1/2 and AKT phosphorylation. Phosphorylation of Erk1/2 as well as AKT proceeded with similar kinetics in TCR-stimulated cells in the absence or presence of 20 μM RVT but phosphorylation in the presence of 100 μM RVT was significantly attenuated [FIGURE R.5B].

To determine whether the changes in proximal TCR signalling resulted in a modulated induction of cell surface activation markers, expression of CD69, the IL-2Rα subunit (CD25) and the transferrin receptor (CD71) were followed. CD69, a marker whose rapid upregulation is due to the translocation of intracellular stores to the cell membrane without a requirement for protein synthesis (Manel et al., 2003b), was highly upregulated in all conditions, irrespective of the presence of resveratrol [FIGURE R.5C]. In marked contrast, CD25 and CD71 expression, both of which are dependent on *de novo* protein synthesis for their induction, were not upregulated in the presence of 100 μM RVT but were not affected by 20 μM RVT [FIGURE R.5C]. Together these results indicate that TCR signalling itself is impeded in the presence of high, but not low, dose resveratrol.

TCR signalling stimulates the PI3K-dependent activation of Akt, which in turn leads to the induction of mTOR (Colombetti et al., 2006; Delgoffe et al., 2009; Sauer et al., 2008). mTOR is a serine/threonine protein kinase that integrates environmental cues such as nutrients, growth factors and stress signals into an "optimal" cellular response (Laplante and Sabatini, 2009; Sengupta et al., 2010). Due to its direct effect on different metabolic pathways such as

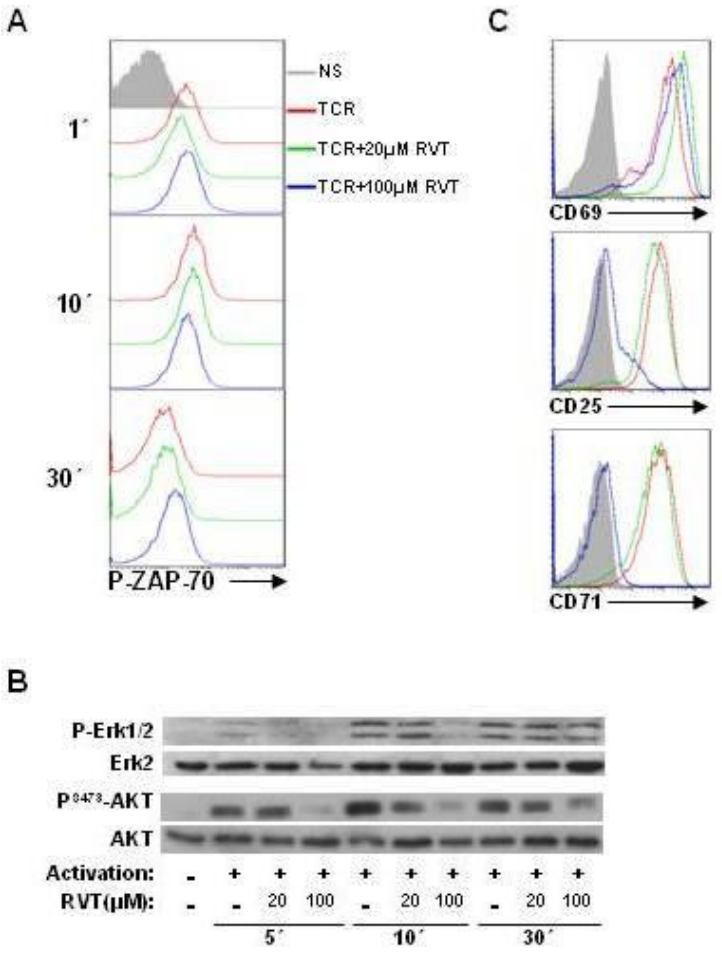

Figure R.5. **Proximal TCR signalling is severely attenuated by high, but not low, dose resveratrol treatment.** (**A**) Phosphorylation of ZAP-70 at residue Y319 was monitored by flow cytometry using a phospho-specific following TCR engagement of CD4 T cells with anti-CD3/CD28 antibodies in the absence or presence of RVT (20μM or 100μM). Representative histograms showing phosphorylation at 1, 10 and 30 minutes post activation are presented relative to non-stimulated cells (filled histogram). (**B**) Phosphorylation of Erk1/2 and Akt (S473) was monitored by immunoblotting and representative blots showing phosphorylation status following 5, 10 and 30 min of activation are shown. Immunoblots of total Erk1/2 and Akt levels are presented. (**C**) Induction of the CD69, IL-2Rα (CD25) and transferrin receptor (CD71) activation markers was assessed by flow cytometry following TCR activation in the presence or absence of resveratrol. Representative histograms at day 3 of activation are shown.

glycolysis, lipid synthesis and mitochondrial biogenesis (Powell and Delgoffe, 2010; Powell et al., 2012; Xu et al., 2012), mTOR plays a critical role in regulating T cell metabolism. While Sirt1 has been found to inhibit mTOR signalling in cell lines and transformed cells (Ghosh et al., 2010; Gwinn et al., 2008), its role in primary T cells is not known. Phosphorylation of mTOR itself, monitored as a function of phosphorylation on residues

S2448 and S2481, was markedly augmented by TCR stimulation and was not significantly affected by low dose resveratrol. This also translated to an increased phosphorylation of mTOR substrates; S6 ribosomal protein and the 4E-BP1 repressor of the eIF4F eukaryotic translation initiation complex. In marked contrast, the induction of these mTOR substrates was significantly decreased in the presence of 100 μM RVT [FIGURE R.6A]. Phosphorylation of TSC2 on threonine 1462, by Akt, inhibits TSC1/TSC2 activity, resulting in a continued activation of mTORC1 while phosphorylation of TSC2 on Ser 1387, by AMPK, inactivated mTORC1. Importantly, TCR stimulation resulted in an early induction of Thr1462 phosphorylation, consistent with an activation of mTORC1 [FIGURE R.6B]. As expected from the experiments assessing mTOR induction, TSC2 was highly phosphorylated in the presence of low, but not high, dose resveratrol [FIGURE R.6B]. Thus, mTOR signalling in TCR-stimulated T cells is severely attenuated by high dose RVT whereas at low doses, the activity resulting from TCR engagement, rather than a direct contribution from RVT, appears to be dominant.

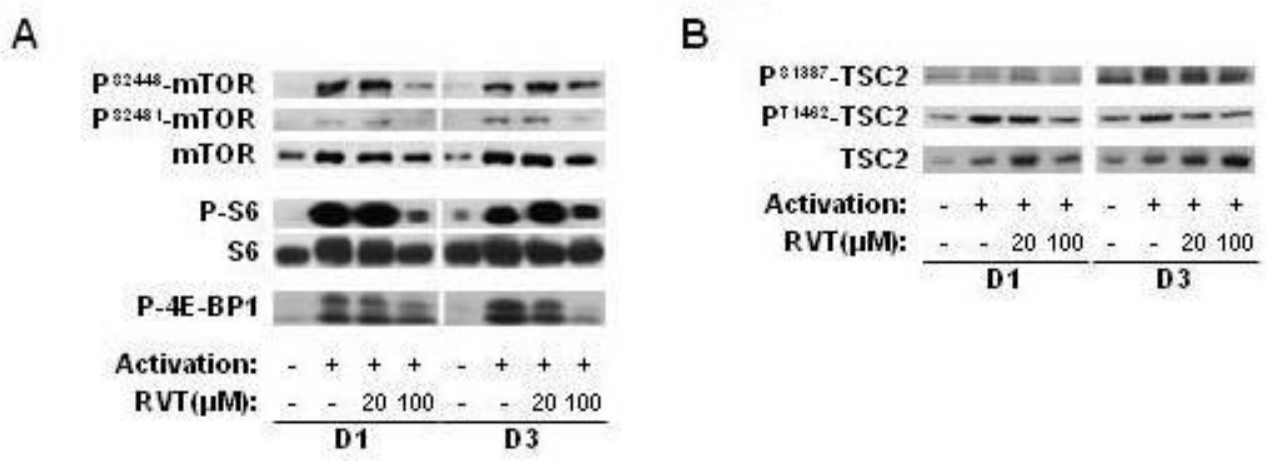

Figure R.6. **Decreased mTOR signalling in TCR-activated T cells exposed to high dose resveratrol.**
(**A**) TCR-induced mTOR activation was monitored in the presence of RVT using antibodies directed against the phosphorylated S2448 and S2481 residues of mTOR. Representative immunoblots at days 1 and 3 of stimulation are shown and total mTOR levels are presented. Phosphorylation of mTOR substrates, S6 ribosomal protein and 4E-BP1, were monitored using phospho-specific antibodies. (**B**) Phosphorylation of TSC2 at Ser 1387 and Thr 1462, activating and inhibiting TSC1–TSC2, respectively, with subsequent disparate roles on mTORC1 was monitored by immunoblotting. Representative blots at days 1 and 3 of activation are shown and total TSC2 levels are presented.

Sirt1 induction alters the metabolic balance in TCR-activated CD4 T cells.

To understand why lymphocytes treated with low dose resveratrol did not proceed through mitosis, even though proximal TCR signalling was intact and the mTOR pathway was activated, we explored the impact of Sirtuin activation on the metabolism of these TCR-engaged T cells. Indeed, proliferation can only occur when a lymphocyte has generated sufficient energy to meet its increased energy demands. We hypothesized that Sirt1 activation may modulate energy homeostasis in TCR-stimulated lymphocytes as this protein functions as a sensor, using the cellular NAD^+/NADH ratio to control energy expenditure (Canto et al., 2009).

Glucose is a key nutrient in mediating T cell activation and its entry is regulated by expression of the Glut1 glucose transporter (Chakrabarti et al., 1994; Frauwirth et al., 2002; Kinet et al., 2007; Michalek and Rathmell, 2010; Wang et al., 2011a). Glut1 was upregulated following TCR activation and was highly expressed at the cell surface by day 3 of activation [FIGURE R.7A]. There was a similar high level induction of Glut1 in TCR-stimulated T cells treated with low dose RVT but this upregulation was abrogated in the presence of high dose RVT [FIGURE R.7A]. Glut1 functions to facilitate glucose transport into the cell and indeed TCR engagement stimulated a greater than 50 and 85-fold increase in glucose uptake at day 1 and 3, respectively [FIGURE R.7A]. Interestingly, in the presence of low dose RVT, glucose uptake, while increased, was lower than that detected in control TCR-activated cells [FIGURE R.7A]. This correlated with a significantly lower production of lactate and extracellular acidification [FIGURE R.7B]. Interestingly, while lactate production was also highly upregulated in the presence of low dose RVT (10-fold), extracellular acidification was less striking in these conditions, with a mean pH of 7.25 [FIGURE R.7B]. As TCR-mediated glucose catabolism, and specifically glycolysis, has been shown to be dependent on c-Myc expression (Wang et al., 2011a), we assessed its upregulation under these conditions. While c-Myc was upregulated by day 3 of stimulation in the presence of low-dose resveratrol, it was not highly induced at day 1, in marked contrast with the expression detected in control TCR-stimulated T cells. Furthermore, c-Myc was significantly reduced in high dose RVT cells, in accord with minimal Glut1 induction, glucose uptake, lactate production, and extracellular acidification [FIGURE R.7C]. Thus, c-Myc upregulation was closely correlated with glucose metabolism in these TCR-stimulated lymphocytes.

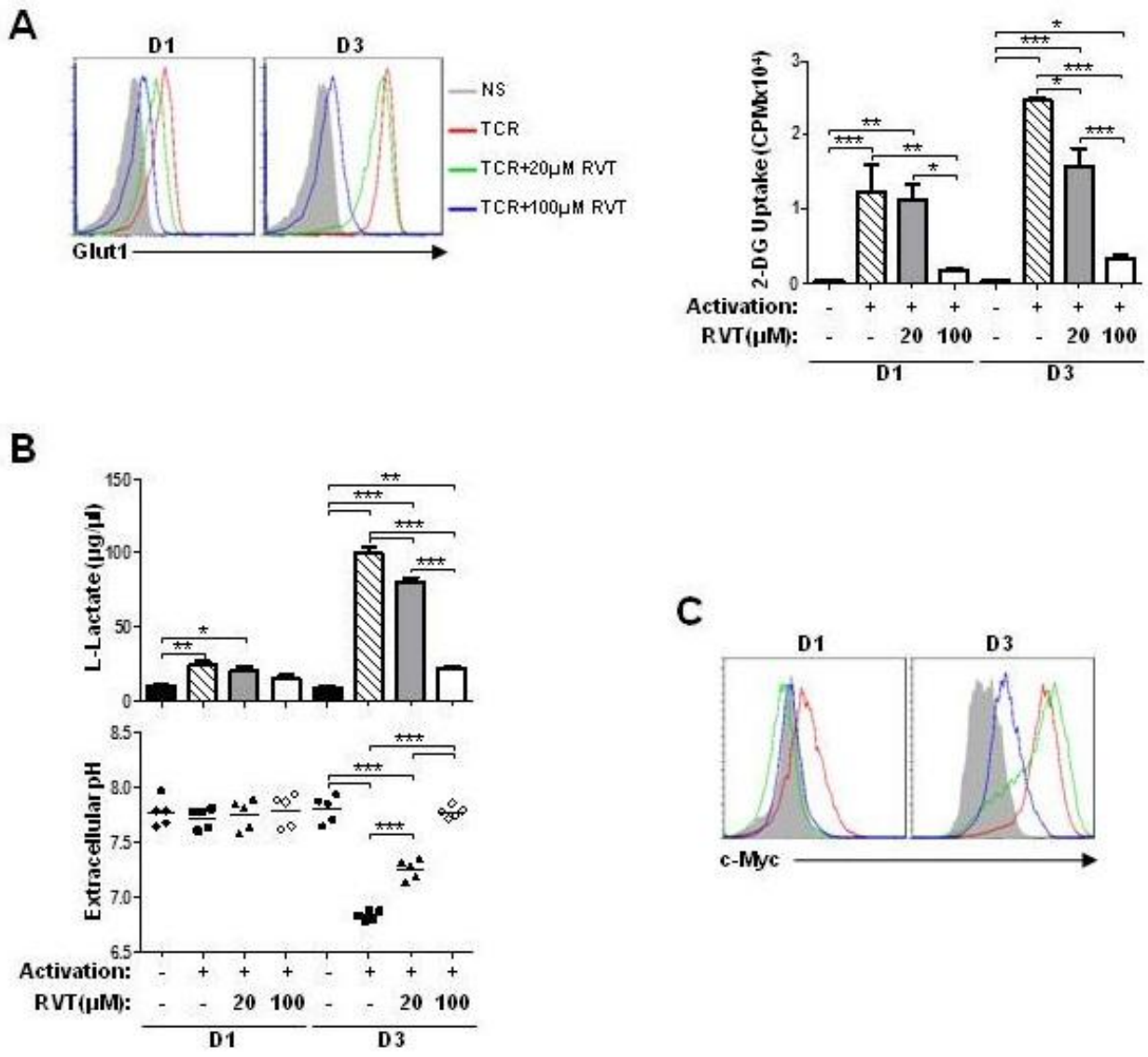

Figure R.7. **Resveratrol treatment results in decreased glycolysis in TCR-stimulated CD4 T cells.**

(**A**) Cell surface expression of the Glut1 glucose transporter was monitored by flow cytometry. Representative histograms showing expression levels in non-stimulated CD4 T cells and in CD4 T cells following TCR engagement in the absence and presence of resveratrol (RVT: 20 or 100µM) are presented (left). Glucose uptake was monitored by incubation of lymphocytes ($1x10^6$) with the non-hydrolyzable 2-deoxy-D[1-3H]glucose (2-DG, 2 µCi) analogue for 10 min at room temperature. Results are expressed as mean CPM ± SD for triplicate samples. (**B**) Lactate production (top) and extracellular pH (bottom) were monitored at days 1 and 3 of activation as a function of resveratrol concentration. Mean lactate levels ± SD are presented and each pH point represents an independent activation. Horizontal lines depict the mean extracellular pH in each condition. (**C**) c-Myc levels were monitored by intracellular staining with a c-Myc-specific antibody and representative histogram profiles at day 1 and 3 of TCR stimulation in the presence or absence of RVT are shown.

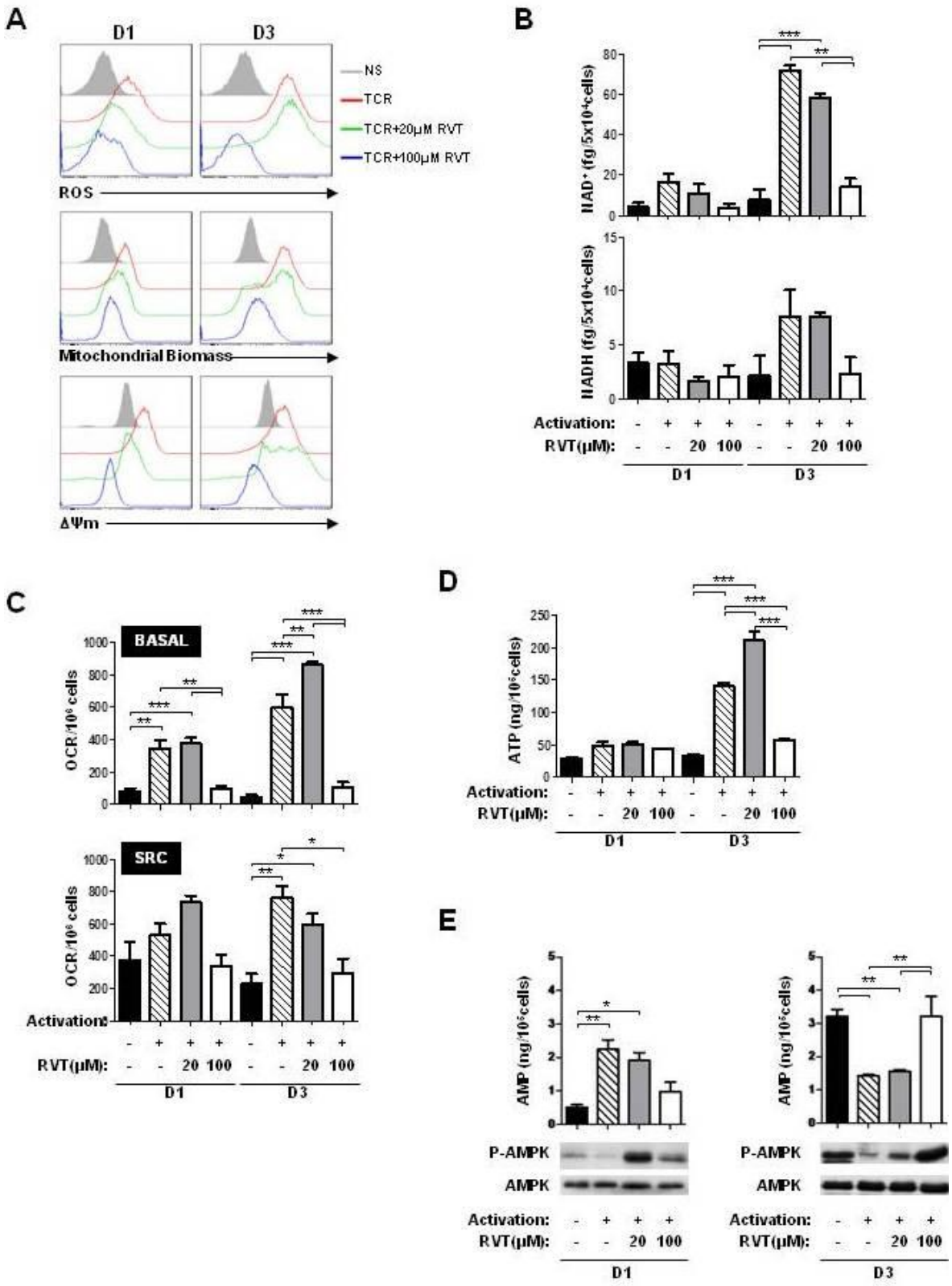

Figure R.8. **Low dose resveratrol augments oxidative phosphorylation and TCR-stimulated intracellular stores in CD4 T cells.**

(**A**) The level of reactive oxygen species (ROS), mitochondrial biomass and mitochondrial membrane potential were monitored by staining with Rhodamine 123, Mitotracker green, and Mitotracker red, respectively. Representative histograms showing relative staining at days 1 and 3 of activation are presented. (**B**) Total NAD+

and NADH levels were measured by colorimetric assays and means ± SD for triplicate samples are shown. (**C**) Oxygen consumption rates (OCR) were measured under basal conditions (2.5 mM glucose) and spare respiratory capacity (Maximal respiration) was monitored in the presence of the uncoupling agent FCCP (5 μM) and mean levels are presented. (**E**) ATP levels were measured by luminescence and mean intracellular levels in 5 independent experiments are presented for each condition. (**F**) AMP levels were monitored at days 1 and 3 of activation in the indicated conditions and mean levels are presented. Phosphorylation of AMPK in each condition was assessed by immunoblotting with a phospho-specific antibody. Total AMPK levels are shown as a control. ***p≤0.0001, **p≤0.01, *p≤0.05.

TCR-engaged T cells exhibit an upregulation of aerobic glycolysis as well as oxidative phosphorylation and the relative contribution of these two processes have been shown to modulate T cell activation and function. Mitochondrial respiration results in the production of ROS and levels were similar in TCR-stimulated and low dose RVT-treated cells [FIGURE R.8A]. While some reports have found that low dose resveratrol upregulates mitochondrial biogenesis (Baur et al., 2006; Dasgupta and Milbrandt, 2007; Lagouge et al., 2006), ROS levels themselves can also signal mitochondrial biogenesis resulting in an antioxidant feedback loop (Wenz, 2013; Yoboue and Devin, 2012). Interestingly, TCR-induced mitochondrial biogenesis, already detected at day 1 of stimulation, was detected in control conditions and in the presence of low dose RVT but was almost completely abrogated in the presence of high dose RVT [FIGURE R.8A].

In order to assess mitochondrial activity, a process shown to be affected by Sirt1, we assessed NAD^+/NADH levels, an essential redox pair, whose generation is tightly associated with the metabolic state of the cell. Furthermore, the concentration of NAD^+ is the rate limiting step for Sirtuin-mediated enzymatic reactions and as such, Sirtuins serve as metabolic sensors of intracellular NAD^+, reflecting the redox state of the cell (Imai et al., 2000) (Wang et al., 2012). TCR engagement increased levels of the oxidized redox equivalent NAD^+ by 9-fold, but interestingly this augmentation was detected only following 3 days of stimulation [FIGURE R.8B]. Notably, levels in RVT-treated cells, wherein Sirt1 deacetylase activity was induced, were also highly augmented, but by 7-fold, potentially due to NAD^+-dependent Sirt1 activity. As levels of the NADH reducing equivalent were similar in the two conditions, this yielded a decreased redox ratio under conditions where Sirt1 was activated. In marked contrast with TCR stimulated cells activated in the absence or presence of low dose RVT, high dose RVT-

treated lymphocytes demonstrated a severely attenuated NAD^+/ NADH generation [FIGURE R.8B].

Both glycolysis and oxidative phosphorylation regulate the energetic homeostasis of a cell. In order to assess the level of oxidative phosphorylation, we directly monitored the oxygen consumption rate (OCR) and found that both the basal and spare respiratory capacity (SRC; maximal respiration) of CD4 T cells were augmented within 24h following TCR stimulation. Interestingly, the SRC was further significantly augmented by low dose resveratrol [FIGURE R.8C]. Furthermore, by day 3 of activation, the increase in basal respiration mediated by low dose RVT treatment was even more striking ($p<0.001$ compared to TCR-stimulated cells). This extremely high basal level may account for the inability of these cells to consume additional oxygen in response to uncoupling of ATP synthesis from electron transport (a measure of SRC). Nevertheless, it is important to note that the increased early respiration in low dose resveratrol-treated cells is an important parameter as it potentially allows the cell to proliferate vigorously and survive under conditions of stress. High dose RVT-treated cells did not show an augmented OCR as compared to quiescent cells, again consistent with their low metabolic state [FIGURE R.8C]. An augmentation of ATP in TCR-stimulated cells required a longer time period than that needed to augment oxygen consumption but importantly, the data were completely consistent with the measured oxygen consumption. These data are in accord with a significantly higher ATP generation from oxidative phosphorylation than from glycolysis and reveal a higher energetic state in low dose RVT-treated cells [FIGURE R.8D]. As expected from the minimal levels of glycolysis and mitochondrial activity in high dose RVT-treated cells, ATP levels were not induced above that detected in quiescent lymphocytes [FIGURE R.8D]. Indeed, by day 3, AMP levels were significantly higher in quiescent and high dose RVT-treated cells than in TCR-stimulated conditions and this was associated with a significant increase in the phosphorylation of AMPK [FIGURE R.8E]. The ensemble of these experiments show that low, but not high, dose resveratrol induces mitochondrial function resulting in the production of high levels of intracellular ATP. On the basis of these data, it was therefore still not clear as to why a low dose RVT-treated T cells, with augmented respiratory capacity and intracellular ATP levels was not capable of undergoing TCR-stimulated division.

Resveratrol-mediated Sirt1 stimulation results in the induction of a replicative stress in TCR-engaged CD4 T cells.

The metabolic effects of low dose resveratrol on ATP production could not, in itself, account for the cell cycle arrest of these lymphocytes. In this context, it is important to note that cell cycle progression can be blocked, even when the cell cycle machinery is fully engaged, under conditions of DNA damage. This was not the first hypothesis that we evoked to explain thecell cycle arrest in RVT-treated CD4 T cells as it has previously been shown that Sirt1 can actually promote DNA integrity; In Sirt1-deficient mice, there is a reduced ability to repair DNA damage while conversely, RVT-mediated activation of Sirt1 reduces tumorigenesis (Wang et al., 2008). Furthermore, in several cellular models, resveratrol, directly and indirectly through its actions on Sirt1, contributes to the maintenance of genomic stability, promoting recovery from DNA damage and decreasing reactive oxygen species that lead to oxidative damage (Denissova et al., 2012; Gatz and Wiesmuller, 2008). Nevertheless, the effects of Sirt1 activation, and more specifically of RVT, may be cell-specific as RVT has also been reported to induce DNA breaks (Gatz and Wiesmuller, 2008; Heiss et al., 2007; Hussain et al., 2011).

To specifically assess DNA damage following RVT treatment, we monitored the phosphorylation of H2AX (γH2AX) as this histone protein identifies DNA damage foci. Twenty four hours post TCR stimulation, at a time point where the CD4 lymphocytes had not yet entered into S phase, γH2AX staining in control cells was low, not surpassing 5% [FIGURE R.9A]. However, in RVT-treated cells, the percentage of γH2AX+ cells was significantly higher (20-40%). It is important to note that this augmentation specifically reflects DNA damage as none of the cells were replicating. Furthermore, γH2AX induction was detected following treatment with aphidicolin, an inhibitor of replication polymerases resulting in stalled replication forks and a subsequent late G_1 phase arrest (Ward and Chen, 2001) [FIGURE R.9A]. The RVT-mediated induction of γH2AX foci was not immediate as only low levels were detected by 12 hours post TCR stimulation. Moreover, it is important to note that this induction required activation and entry into the cell cycle; non-stimulated T cells treated with equivalent doses of resveratrol exhibited only minimal induction of γH2AX foci [FIGURE R.9B].

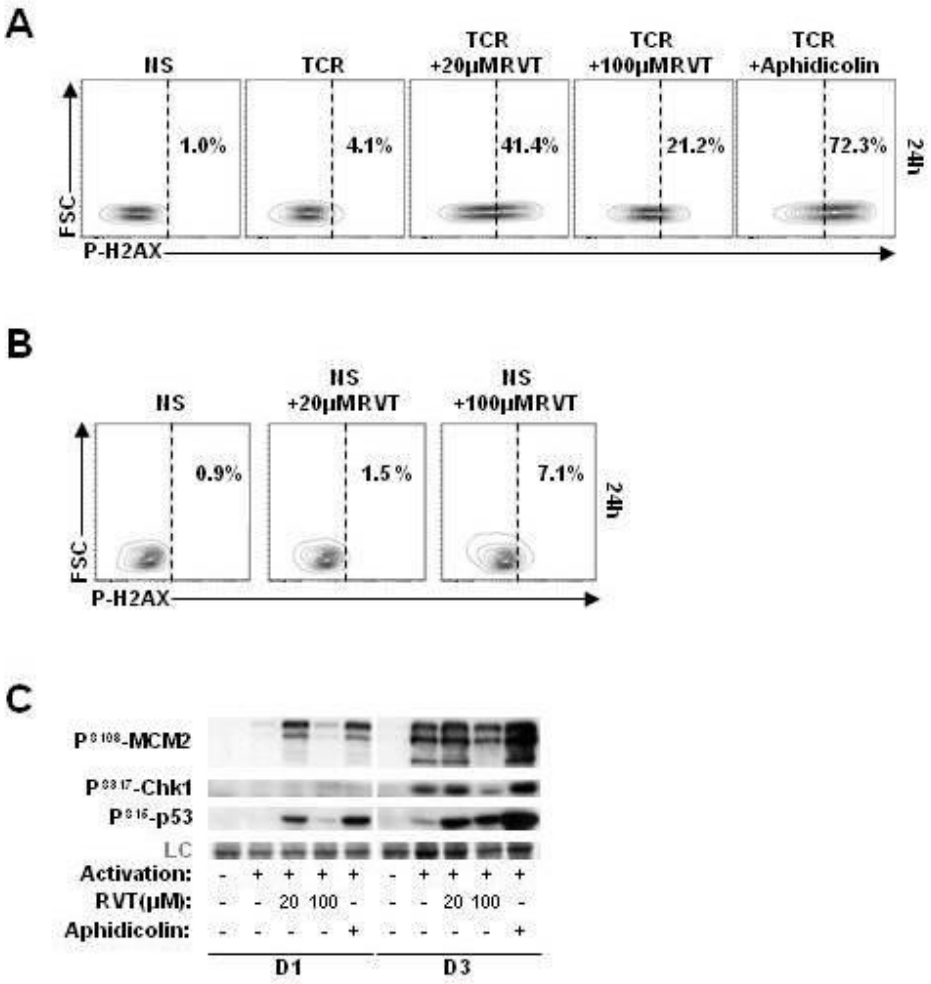

Figure R.9. **Replication stress in resveratrol treated CD4 lymphocytes results in an ATR/Chk1/AMPK-mediated induction of p53 and cell cycle exit.**

(**A**) DNA damage response in response to double-stranded DNA breaks was monitored as a function of H2AX phosphorylation at serine 139. CD4 T cells were activated with anti-CD3/anti-CD28 mAbs for 24h in the absence or presence of RVT or aphidicolin and phosphorylation was assessed using a phospho-specific antibody. Representative dot plots are presented and the percentages of positively-stained cells are indicated for each condition. (**B**) The induction of H2AX foci in non-stimulated (NS) T cells are shown at 24h post treatment with low and high dose resveratrol. The percentages of positively-stained cells are indicated for each condition. (**C**) Phosphorylation of the mini-chromosome maintenance MCM2 helicase protein, the checkpoint kinase Chk1 and p53 were monitored with the appropriate phospho-specific antibodies. Representative plots and a loading control (LC) are presented.

The response to DNA damage is coordinated by two distinct kinase signalling cascades, the ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-related) pathways, which are activated by DNA double-strand breaks (DSBs) and a wide variety of single-stranded DNA

lesions, respectively. The overall effect of activating the ATR pathway is to stabilize DNA replication forks via protein complexes on the replisome. In this context, the replicative helicase, a hexameric complex comprised of mini-chromosome maintenance proteins (MCM), plays a critical role (reviewed in (Cimprich and Cortez, 2008)). MCM proteins are components of the pre-replication complex (Evrin et al., 2009) and are directly phosphorylated and activated by ATM/ATR (Cortez et al., 2004). MCM2 has been identified as playing a critical role in the response to replicative stress (Stead et al., 2012) and is directly phosphorylated by ATR (Cortez et al., 2004). Notably, at a time point of TCR stimulation prior to entry of the cells into S phase (24h), MCM2 was only phosphorylated following RVT or aphidicolin treatment [FIGURE R.9C]. As expected, this was not the case by 72h post stimulation, a time point at which the vast majority of control TCR-activated cells had already progressed through S phase, and as such MCM2 was also phosphorylated in these conditions. However, it is significant that MCM2 was also highly phosphorylated in high dose RVT-treated cells that had not progressed past the G_1 phase of the cell cycle.

Based on these data, we assessed the phosphorylation of checkpoint kinase 1 (Chk1); the ATR-dependent phosphorylation of this kinase regulates the signal transduction axis activated by genotoxic stress (Toledo et al., 2008), downregulating Cdk activity even during unperturbed S phase (Shechter et al., 2004; Sorensen et al., 2004). Notably, Chk1 was highly phosphorylated in non-proliferating resveratrol-treated cells, at levels similar to that detected following aphidicolin treatment [FIGURE R.9C]. Thus, the importance of the ATR cascade in non-replicating RVT-treated CD4 T cells is underscored by the phosphorylation of the MCM2 and Chk1 downstream targets.

Another ATR target is the Ser15 residue of p53, a site whose phosphorylation increases p53 activity. Intriguingly, this p53 residue is also phosphorylated in response to glucose deprivation and AMPK activation (Feng et al., 2005; Jones et al., 2005). AMP-activated protein kinase (AMPK) is induced under conditions of energetic stress, allowing the cell to respond to declining fuel supplies and promote a catabolic metabolism (Hardie et al., 2012). Resveratrol itself also activates AMPK, and at low doses this activation appears to occur in a Sirt1-dependent manner without decreasing energy (Dasgupta and Milbrandt, 2007; Park et al., 2007; Price et al., 2012; Suchankova et al., 2009). Indeed, AMPK was phosphorylated in RVT-treated cells, even under conditions where AMP levels were not augmented. Furthermore, this induction was significantly higher than that detected under conditions of TCR stimulation alone and was already prominent by 24h post stimulation [FIGURE R.8E].

As expected from our findings that both the ATR-Chk1 and AMPK signalling cascades are highly activated in RVT-treated CD4 T cells, p53 was highly phosphorylated on Ser15. Moreover, this phosphorylation was much higher than that detected in control TCR-activated lymphocytes, where p53 phosphorylation was not even detected at day 1 of stimulation [FIGURE R.9C]. Thus, the response to the genotoxic stress induced by low dose RVT is mediated by the ATR-Chk1 signalling cascade and AMPK induction, with activation of a p53-dependent checkpoint triggering a G_2/M cell cycle arrest.

CHAPTER 4:

DISCUSSION

Discussion

Sirtuins have emerged as central players in many cellular processes, including stress response, cell survival, apoptosis, chromosome stability, gene transcription and DNA repair. They also constitute important energy sensors that directly link environmental signals to metabolic homeostasis. Sirt1, the most important and well-studied protein of the sirtuin family, has also been shown to be a critical regulator of T cell immune responses as its deletion results in autoimmunity (Cheng et al., 2003; Purushotham et al., 2009; Sequeira et al., 2008; Zhang et al., 2009). Furthermore, Sirt1 may balance T cell activation by targeting FoxO1, a positive regulator of T cell quiescence, (Zhang et al., 2009) as well as by repressing NF-κB activity (Yeung et al., 2004). Nevertheless, its role is clearly complex as the specific deletion of Sirt1 in CD4 T cells inhibits immune responsiveness to foreign antigens by augmenting the numbers of $Foxp3^{+}$ suppressor T cells (Beier et al., 2011), likely as a result of stabilized Foxp3 expression under conditions of increased acetylation (Tao et al., 2007; van Loosdregt et al., 2011; van Loosdregt et al., 2010). Furthermore, pharmacological induction of Sirt1 activity by resveratrol has been associated with improvements in several autoimmune disease settings including arthritis and diabetes (Elmali et al., 2007; Kong et al., 2012; Lee et al., 2011) but the mechanisms regulating the responsiveness of T lymphocytes in these conditions have not been elucidated.

Here, I report that under conditions of antigen-driven T cell stimulation, the resveratrol-mediated induction of Sirt1 results in the cell cycle arrest of human CD4+ lymphocytes. High dose resveratrol severely attenuates proximal TCR signaling and mTOR activation but low dose resveratrol inhibits division via a distinct mechanism. In the latter condition, cells actually respond extensively to TCR stimulation; low dose resveratrol-treated T cells demonstrate induction of mTOR, cyclins, and mitochondrial function following TCR engagement. Moreover, these lymphocytes show a metabolic profile which is enhanced as

compared to control TCR-stimulated lymphocytes, at least as regards several parameter. They present with an augmented basal respiration and higher intracellular ATP stores. Nevertheless, they demonstrate a p53-mediated cell cycle exit secondary to induction of the ATR-Chk1 DNA damage pathway and AMPK function.

The complex interplay and feedback between Sirt1 and AMPK functions often results in similar physiological consequences for the cell. Activation of these two proteins facilitates the capacity of the cell to respond to stress and/or to a low energy homeostasis (Hardie et al., 2012). AMPK enhances Sirt1 activity by increasing cellular NAD^+ levels (Canto and Auwerx, 2009) and conversely, Sirt1 deacetylation of the upstream LKB1 kinase augments AMPK activity (Lan et al., 2008). Resveratrol itself stimulates AMPK activity in multiple cell types as well as *in vivo* (Baur et al., 2006; Dasgupta and Milbrandt, 2007; Lan et al., 2008; Park et al., 2007; Price et al., 2012; Suchankova et al., 2009). At low doses (25 μM), this activation has been shown to be completely dependent on the function of Sirt1, while at high doses (50 μM), it can occur in a Sirt1-independent manner (50 μM) (Baur et al., 2006; Price et al., 2012). In the experiments described here, both low (20 μM) and high doses (100 μM) of resveratrol induced AMPK phosphorylation in primary T cells. While resveratrol has been reported to activate AMPK by acting as an ATPase or complex III inhibitor (Gledhill et al., 2007; Hawley et al., 2010; Zini et al., 1999), this was not the case in low dose-treated T cells where ATP levels were extremely high. Moreover, in the presence of low dose resveratrol, the induction of AMPK phosphorylation occurred even without an increase in AMP levels. Thus, AMPK activity appears to be directly related to Sirt1 activity and independent of a AMP/ATP-mediated feedback loop.

In the majority of studies performed in muscle, liver, and neural cells, resveratrol has been reported to increase ATP levels by enhancing mitochondrial activity (Dasgupta and Milbrandt, 2007) (Price et al., 2012; Suchankova et al., 2009). It is important to note that quiescent T lymphocytes differ from the majority of cell types wherein resveratrol activity has been tested thus far in that they are in the G_0 phase of the cell cycle with extremely low basal energy resources, corresponding to minimal cellular ATP demands (Maciver et al., 2013; Pearce and Pearce, 2013; van der Windt and Pearce, 2012). This low energetic condition is also attested to by the low mitochondrial biomass of T cells and indeed, in my experiments, resveratrol did not alter the homeostasis of non-activated T cells (unpublished observations). Notably though, the metabolic state of a T cell is rapidly changed by TCR engagement; within 15 minutes of TCR stimulation, mitochondrial ROS are generated, creating a positive

feedback loop for TCR signalling (Devadas et al., 2002; Kwon et al., 2010). Importantly, it has recently been shown that fueling mitochondrial ROS production is a prerequisite for antigen-specific T cell expansion in mice (Sena et al., 2013). While we show here that *ex vivo* TCR stimulation triggers a high induction of ROS and mitochondrial biosynthesis in human T cells, we found that resveratrol treatment did not markedly augment either of these parameters. This was somewhat surprising as one of the characteristics attributed to resveratrol is its ability to stimulate mitochondrial biogenesis (Baur et al., 2006; Dasgupta and Milbrandt, 2007; Lagouge et al., 2006; Verdin et al., 2010), even if this effect has recently been challenged (Higashida et al., 2013). I therefore propose that under conditions where TCR engagements results in a maximal induction of mitochondrial biogenesis, there is no further inducing effect of resveratrol treatment. Nevertheless, it is interesting to note that resveratrol positively affected mitochondrial function, as monitored by oxidative phosphorylation. At an early time point post TCR stimulation, prior to cell division (24h), the spare mitochondrial respiratory capacity was significantly increased by low dose resveratrol. This was monitored as a function of the oxygen consumption rate, an indicator of oxidative phosphorylation. Interestingly, while the maximal OCR was substantially higher, lactic acid production and associated extracellular acidification, markers of glycolysis, were lower. Thus, while high dose resveratrol blocks TCR-induced metabolic changes, low dose resveratrol alters the balance between glycolysis and oxidative phosphorylation, favouring the latter. As OXPHOS produces significantly more ATP molecules than glycolysis, these data are concordant with the significantly higher levels of ATP that we detected in low dose resveratrol-treated cells.

The role of resveratrol in protecting against carcinogenesis has been the subject of intense study and multiple reports have shown that it functions by means of its action in preventing DNA damage formation as well as by improving DNA damage removal (Gatz and Wiesmuller, 2008; Jang et al., 1997; Wang et al., 2008). Resveratrol affects multiple aspects of DNA metabolism including DNA replication, recombination, repair, and telomere maintenance as well as the redox state, thereby promoting the integrity of genomic DNA. However, *in vitro*, resveratrol has also been shown to mediate DNA cleavage, although it appears that this requires DNA-bound copper (Cu(II)) ions (Ahmad et al., 2001; Fukuhara et al 2006; Burkitt and Duncan, 2000; Fukuhara and Miyata, 1998; Gatz et al., 2008; Subramanian et al., 2004). On the basis of these diverse studies, it is nearly impossible to draw clear-cut conclusions on the effects of either Sirtuins or resveratrol on genome stability.

Both reduction of DNA breaks and an inhibition of replicative senescence (Gao et al., 2011; Luo et al., 2013; Peltz et al., 2012; Tang et al., 2012; Wang et al., 2011b; Yamashita et al., 2012) as well as induction of DNA breaks with associated senescence (Gao et al., 2011; Luo et al., 2013; Peltz et al., 2012) have been reported (reviewed in (Bosch-Presegue and Vaquero, 2011)). Here, I find a high level of DNA breaks in RVT-treated cells that were not yet in S phase, as shown by the presence of phosphorylated H2AX DNA damage foci in 20-40% of cells. This effect was dependent on T cell activation as low dose of resveratrol did not induce DNA damage in the absence of TCR stimulation.

Following DNA damage, the ATM/ATR kinase signaling cascades trigger a DNA damage response. Under conditions of single-stranded DNA breaks or instability of replication forks prior to S phase, it is the ATR pathway that is activated (Smith et al., 2010). ATR phosphorylates checkpoint kinase 1 (Chk1), releasing it from chromatin and increasing its kinase activity towards targets regulating cell cycle transition/checkpoints. This is indeed what I detected in T lymphocytes. One of the ATR/Chk1 targets is the p53 tumor suppressor. Phosphorylation of p53 on serine 15 mediates cell cycle arrest, serving as a node between upstream stress signalling cascades and downstream DNA repair pathways (Sengupta and Harris, 2005). This pathway was clearly activated in low dose resveratrol-treated CD4 T lymphocytes, coupling DNA damage to cell cycle arrest. It is though important to note that in resveratrol-treated T lymphocytes, this coupling was also likely stimulated via a second mechanisms. While the role of AMPK in mediating cell cycle progression in primary T cells has not been evaluated, activation of this kinase has been shown to induce cell cycle arrest in primary fibroblasts via the phosphorylation of p53 on serine 15 (Jones et al., 2005). As indicated above, in TCR-stimulated T cells AMPK was activated at both low and high doses of resveratrol. Thus, in the presence of low dose resveratrol, wherein T cells progressed towards G2 of the cell cycle, the joint induction of ATR/Chk1 and AMPK promoted p53 phosphorylation and a virtual abrogation of T cell proliferation. Furthermore, AMPK was activated in high dose resveratrol-treated cells, and while these lymphocytes did not highly upregulate cyclins/Cdks associate with cell cycle progression, p53 was also phosphorylated on Ser15 in these conditions.

Considering that resveratrol is currently being evaluated in clinical trials and is envisaged for the therapeutic treatment of pathological cardiovascular, hepatic, or metabolic conditions, it will be critical to evaluate the potential impact of inhibiting the clonal expansion of T lymphocytes in response to foreign antigen. Conversely, my data supports a role for

resveratrol as a therapeutic strategy under conditions of T-mediated autoimmunity, potentially in settings where the rapamycin immunomodulator is presently being used. Both agents inhibit mTOR signal transduction pathways with resveratrol also stimulating the Sirt1/AMPK axis, triggering an ATR/Chk1/p53-mediated cell cycle arrest.

CHAPTER 5:

PERSPECTIVES

Perspectives

Sirt1 is highly upregulated following TCR-mediated T lymphocyte activation and this increased expression is associated with phosphorylation of Sirt1 in the nuclear localization sequence (NLS) region. These data are consistent with my finding that Sirt1 exhibits a predominantly nuclear localization after T cell activation [FIGURE R.1A]. Treatment of CD4 T cells with the Sirt1 activator, resveratrol, interferes with T lymphocyte activation in a dose-dependent manner, with high dose-treated cells (100μM) failing to undergo blastogenesis or upregulation of activation markers such as CD25 and CD71 [FIGURES R.2 AND R.5C]. These data are consistent with a previous publication showing that deletion of Sirt1 in mice results in a CD4 hyperproliferation while Sirt1 overexpression inhibits T cell activation, resulting in anergy (Zhang et al., 2009). According to Zhang and colleagues, CD4 hyperproliferation following Sirt1 deletion is the consequence of enhanced NF-κB and AP-1 signaling. Additionally, Sirt1 has been suggested to regulate NF-κB signalling: the subunit of NF-κB, RelA/p65, mediates the upregulation of Sirt1, which in turn deacetylates p65, repressing NF-κB transcriptional activity (Kong et al., 2011; Yeung et al., 2004; Zhang et al., 2010). Hence, it becomes important to investigate whether the upregulation in Sirt1 expression observed upon TCR-engagement is mediated by NF-κB, and whether this increase functions to balance TCR signalling.

Preliminary data shows that, in mature human T cells cultured *ex vivo*, neither shRNA-mediated downregulation of Sirt1 nor treatment with Sirtinol, a Sirt1 inhibitor, significantly altered cell cycle progression or proliferative capacity [FIGURES P.1A AND P.1B]. During the course of my experiments I had the opportunity of test another Sirt1 inhibitor, CHIC-35, which proved to be more efficient in inhibiting Sirt1 deacetylase activity than Sirtinol (data not shown), but, it also did not significantly change the proliferation of activated CD4 T cells [FIGURE P.1C]. This is not entirely surprising since, in contrast to the murine experiments, the

deletion of Sirt1 is not complete and is carried out following thymocyte differentiation. As regards the latter point, it is interesting to note that deletion of Sirt1 in mature murine T cells did not modulate, T effector function (Beier et al., 2011).-Nevertheless, it is possible that Sirt1 inhibition modulates T cell proliferation in response to cytokines or suboptimal TCR stimulation or with a different kinetics. Thus, a closer evaluation of these parameters is justified.

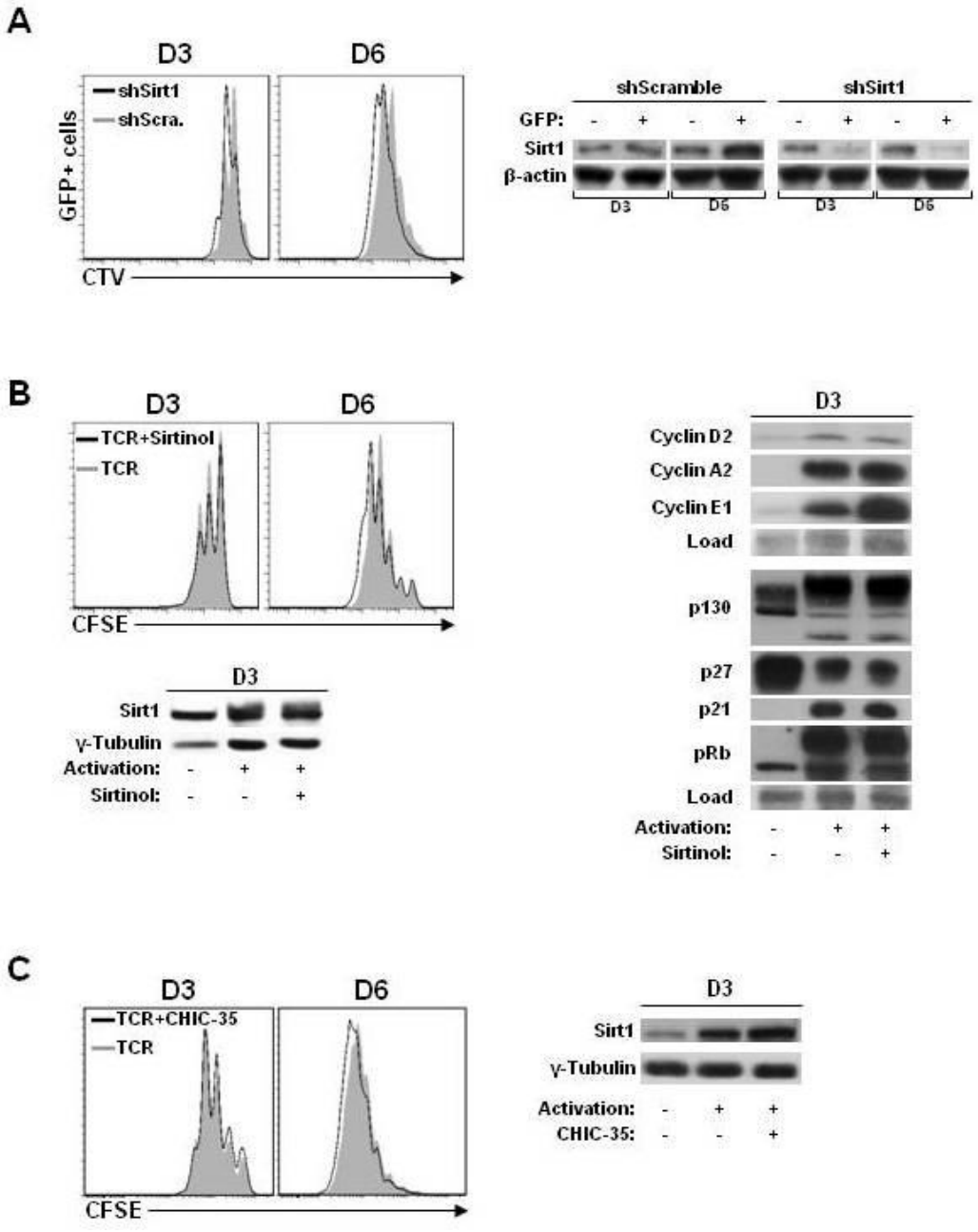

Figure P.1. **Effects of Sirt1 downregulation on T cell proliferation.** (**A**) Sirt1 levels in primary T cells were downregulated by transduction with a lentiviral vector harboring a Sirt1-specific shRNA together with a GFP marker (shSirt1). Sirt1 levels were monitored at days 3 and 6 post transduction by immunoblotting and control

levels in lymphocytes transduced with a scrambled shRNA (shScramble) are presented. Loading was monitored by assessing total actin levels. Proliferation was monitored as a function of CTV staining and representative profiles comparing cells transduced with shScrambled and shSirt1 vectors are shown at days 3 and 6 following re-stimulation with anti-CD3/anti-CD28 mAbs. (**B**) Sirt1 levels were monitored in non-stimulated T cells and in T cells stimulated in the presence or absence of the Sirt1 inhibitor Sirtinol. A representative immnoblot at day 3 of stimulation is shown. Proliferation of T cells stimulated in the absence or presence of Sirtinol was monitored as a function of CFSE dilution and representative profiles at days 3 and 6 of stimulation are shown.Expression of cyclins D2, A2, E1 and cell cycle regulatory proteins p130, p27, p21 and pRb were monitored as a function of TCR stimulation in the presence of Sirtinol. Representative immunoblots and loading controls are presented. (**C**) Sirt1 levels were monitored in non-stimulated T cells and in T cells stimulated in the presence or absence of the Sirt1 inhibitor CHIC-35. A representative immnoblot at day 3 of stimulation is shown. Proliferation of T cells stimulated in the absence or presence of CHIC-35 was monitored as a function of CFSE dilution and representative profiles at days 3 and 6 of stimulation are shown.

Interestingly, although Sirtinol treatment did not alter the early proliferation of TCR-stimulated CD4 T lymphocytes, it modulated the expression profiles of both FoxO1 and p53. Sirtinol treatment accentuated the mobility shift in FoxO1 observed following TCR stimulation, a shift that is inhibited by resveratrol [FIGURE P.2]. Furthermore, while p53 levels were significantly augmented in RVT conditions, expression in Sirtinol-treated cells was not augmented as compared to control TCR-activated cells. As FoxO1 and p53 regulate quiescence and cell cycle, respectively, these data suggest that sirtinol treatment may inhibit the return of CD4 T lymphocytes to a quiescent state, altering their long-term responsiveness.

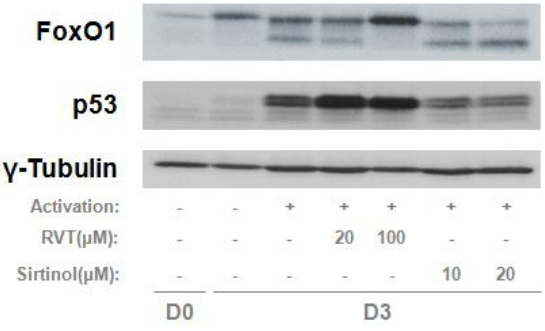

Figure P.3. **Expression of FoxO1 and p53 altered by resveratrol and Sirtinol treatment.** Expression of FoxO1 and p53 was monitored in freshly isolated human CD4 T lymphocytes (Day 0; D0) and 3 days post TCR stimulation in the presence or absence of resveratrol (RVT; 20 uM and 100 uM) or Sirtinol (10 uM and 20 uM). Immunoblots of whole cell extracts were revealted with specific antibodies. Expression of γ-Tubulin is shown as a control.

The lower expression of p53 in sirtinol-treated cells may inhibit their capacity to respond to stress conditions. This hypothesis is supported by recent work showing that autophagy, a mechanism which can confer protection under conditions of metabolic stress, is induced by the deacetylation of p53 (Contreras et al., 2013), a HDAC1-mediated process. Moreover, the downregulation of p53 expression in sirtinol-treated cells is surprising as acetylation has been shown to be indispensable for p53 activation (Tang et al., 2008) and Sirt1 inhibition is associated with increased expression and transcriptional activity of p53, at least in certain cell types (Vaziri et al., 2001). However, in other cell types including mammary epithelial cells, inhibiting Sirt1 catalytic activity has revealed no effect on cell growth, viability, or p53-controlled gene expression (Solomon et al., 2006). In T lymphocytes, it will be important to determine the precise mechanisms controlling TCR-mediated p53 upregulation and the functional consequences of p53 acetylation/deacetylation are yet to be determined.

In addition to p53 and FoxO1, Sirt1 also targets factors such as PGC-1α and Myc (Li and Kazgan, 2011). Myc, in particular, participates to T lymphocyte homeostasis, being essential during the metabolic adaptation occurring during clonal expansion (Wang et al., 2011b). Similarly to NF-κB, c-Myc promotes Sirt1 expression, which in turn regulates c-Myc activity, as deacetylation of c-Myc results in decreased stability (Menssen et al., 2012; Yuan et al., 2009). Interestingly, by day 3 of TCR activation I verified that the profile of c-Myc and Sirt1 share similar profiles of expression, and are similarly regulated by resveratrol [FIGURES R.1A AND R.7C]. In future work, it will therefore be interesting to assess the correlation between c-Myc and Sirt1 activities.

Nuclear Sirt1 has been associated with enhanced mitochondrial biogenesis, likely via the deacetylation and activation of PGC-1α (Gurd et al., 2011). In accord with these findings, resveratrol has been shown to enhance mitochondrial biogenesis in a dose-dependent manner, both *in vitro* and *in vivo* (Csiszar et al., 2009; Lagouge et al., 2006; Price et al., 2012). Nevertheless, in a recent publication, Higashida and colleagues found that resveratrol did not improve mitochondrial biogenesis and that deacetylation of PGC-1α was not associated with enhanced mitochondrial function (Higashida et al., 2013a). Still, considering that the mitochondrion is a key regulator of the metabolic activity of the cell and that in my studies low dose resveratrol enhanced oxidative phosphorylation, it will be relevant to investigate whether the modifications verified in mitochondrial function by resveratrol treatment were the consequence of altered PGC-1α activity or transformed TCR signalling.

Additionally, it will be of importance to determine the role of Sirt3, the major mitochondrial sirtuin (Onyango et al., 2002), during the metabolic reprogramming associated with T cell activation. Sirt3 deacetylates and activates the manganese superoxide dismutase to scavenge ROS (Chen et al., 2011) and is also known to protect mitochondria against oxidative damage by deacetylating FoxO3 (Tseng et al., 2013). FoxO3 has been described to regulate the adaptation to hypoxia by reducing mitochondrial mass and oxygen consumption, in addition to being involved in the regulation of cell survival and apoptosis via modulation of ROS levels (Hagenbuchner and Ausserlechner, 2013). Interestingly, FoxO3 is the second major Forkhead box O paralogue expressed in T cells and has been associated with T cell homeostasis, playing a role in differentiation, cell growth and proliferation (Hedrick et al., 2012).

As Sirt1 is a histone deacetylase and low dose resveratrol treatment of activated CD4 T cells promoted dose-dependent cell cycle arrest in association with the onset of replicative stress, it will be pertinent to explore how histone acetylation is regulated in conditions of Sirt1 activation or inhibition. The organization of chromatin has a major impact on all DNA-directed processes, including transcription, replication, recombination, and repair (Fernandez-Capetillo and Nussenzweig, 2004). Acetylation of histones influences chromatin organization at the nucleosomal level, with consequences for the switch between permissive and repressive chromatin structure (Eberharter and Becker, 2002). De facto, Sirt1 is known to associate with mitotic chromatin and contribute to chromosomal condensation (Fatoba and Okorokov, 2011), block acetylation of H2AX and repress excessive activation of the DNA damage response (Yamagata and Kitabayashi, 2009), and act synergistically with Mcm10 in DNA replication fork initiation (Fatoba et al., 2013). Therefore, it will be interesting to determine whether resveratrol-mediated activation of Sirt1 in CD4 T cells is promoting deacetylation of lysine residues that are essential for the fluctuation between closed and open chromatin, with consequences for gene transcription and cell cycle progression.

While studies of Sirt1 activity have been assessed in murine T cells, it is not known whether Sirt1 activity in human T cells can be augmented or decreased by its overexpression or by expression of a dominant-negative (DN) Sirt1 (H363Y) or shSirt1, respectively. In this regard, it of interest to determine whether expression levels are sufficient to change Sirt1 activity or alternatively, whether the rate-limiting step of Sirt1 activity in T lymphocytes is the availability of NAD^+ that is required for the enzymatic activity of Sirt1. Furthermore, the catalytic domains of sirtuins have been described to be monomeric (Finnin et al., 2001;

Sanders et al., 2010), with oligomerization of the Sirt1 protein resulting in a less-active form (Guo et al., 2012; Lakshminarasimhan et al., 2013). Therefore the increase in NAD^+ availability and Sirt1expression alone, observed following T cell activation, may not be enough to enhance the deacetylase activity of Sirt1. Still, to respond to this important question, I have already begun both shRNA knockdown experiments as well as lentiviral-mediated introduction of WT and DN Sirt1 proteins in T lymphocytes. One of the difficulties in carrying out these experiments is that neither the shRNAs nor the genes themselves can be efficiently introduced into primary T cells in the absence of activation (required for lentiviral transduction). Thus, the cells are already in an activated state at the time point wherein Sirt1 is downregulated or overexpressed. To assess how this deacetylase modulates T cell activation itself, I have allowed the transduced cells to return to a resting state and then monitored their capacity to respond to a re-stimulation. In these preliminary experiments, neither overexpression [FIGURE P.3] nor inhibition [FIGURE P.1] appears to significantly change the proliferative capacity of the transduced cells but it will be important to study their cytokine secretion profile, especially as Sirt1 may modulate the Th1/Th17 balance (Alvarez et al., 2012) and enhance Treg differentiation by stabilizing Foxp3 expression (Beier et al., 2011). Thus, Sirt levels and/or activity may also regulate the relative percentage of effector to regulatory T cells.

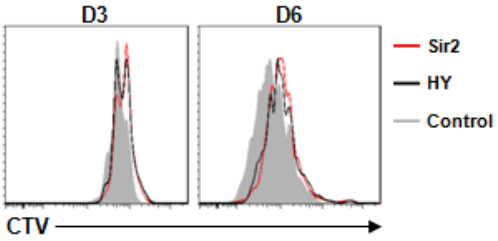

Figure P.3. **Effects of Sirt1 overexpression on T cell proliferation.** Primary T cells were transduced with a lentiviral vector encoding Sirt1 (Sir2) or the dominant negative form of Sirt1 (HY). Proliferation was monitored as a function of CTV staining and representative profiles comparing transduced GFP+ cells are shown at days 3 and 6 following re-stimulation with anti-CD3/anti-CD28 mAbs.

Finally, in the studies presented here, I have only considered lysine acetylation in the context of Sirt1. Acetylation is a posttranslational modification that plays a key role in regulating protein activity and gene expression and, besides Sirtuins, there are three other classes of protein deacetylases involved in the regulation of lysine acetylation (Minucci and Pelicci, 2006). Additionally, the dynamics of lysine acetylation is also dependent on the activity of

acetyltransferases, enzymes that catalyze the transfer of acetyl groups (Grunstein, 1997). And although much is known about gene-specific control by acetylation, little is understood about the importance of acetylation in T lymphocytes, in particular its relevance during the process of metabolic reprogramming and clonal expansion. Therefore, and considering that the transcription factors p53 and FoxO1 are highly acetylated following TCR engagement [FIGURE R.3C], it will be of interest to study the expression of other deacetylases as well as acetyltransferases, such as CBP/p300, and their respective targets during the process of T cell activation.

Given the capacity of Sirtuins to modulate T cell metabolism and potentially alter the effector profiles of activated T cells, it is clearly an interesting pharmacological target under clinical conditions where changing T cell responsiveness would be an advantage. Particularly, conditions where decreasing T cell responsiveness would be an advantage such as autoimmune diseases like arthritis and type I diabetes and following transplantation of allogeneic stem cells or grafts. Indeed, treatment with resveratrol has been found to decrease the severity of diabetes and prevent chronic obstructive pulmonary disease in different murine models (reviewed in (Kong et al., 2012)). However, at the present time, it is not clear whether the effects of Sirt1 activation in general, and modulators such as resveratrol in particular, are equivalent in murine and human lymphocytes. In the context of potential clinical trials in patients, it will first be important to resolve this issue in order to determine the relevance of preclinical murine models. Our findings that Sirt1 activation changes the metabolic environment of human T cells and results in a reversible cell cycle exit (data not shown) reveal this protein to be a promising target for the development of therapies aimed at inhibiting T cell responses in patients with autoimmune disorders or following allogeneic transplantation.

CHAPTER 6:

METHODS

Methods

T cell isolation and culture. CD4+ T cells were isolated from adult peripheral blood (APB), obtained from healthy donors after informed consent. Cells were purified using negative-selection Rosette tetramers (StemCell Technologies) and the purity of the cell population was monitored on a FACS-CantoII (BD Biosciences). Lymphocytes were cultured in RPMI medium 1640 + GlutaMAX (Gibco-Life technologies) supplemented with 10% FCS and 2% penicillin/streptomycin (Gibco-Life technologies). For TCR stimulation, 24 well plates were coated with anti-CD3 (clone OKT3, Biolegend) and anti-CD28 (clone 9.3, kindly provided by Carl June) mAbs at a concentration of 1µg/ml. As indicated, resveratrol (Sigma) was added to T cell cultures at concentrations of 20µM or 100µM 1h prior to TCR stimulation. Sirtinol (Sigma) was used at a concentration of 20µM.

Immunofluorescence. Cells were collected and coated on poly-L-lysine-treated slides. Cells were fixed in a paraformaldehyde solution 4% (PBS 4% PFA) at 37ºC for 15 minutes, permeabilized in PBS 3% BSA/0.1% saponin for 10 minutes and blocked for non-specific protein binding using 10% FCS. Staining with primary anti-Sirt1 ab (Santa Cruz) and a secondary AlexaFluor 488-coupled anti-rabbit IgG (Invitrogen) was performed in PBS 3% BSA for 1h at RT. Nuclei were then labelled by DAPI staining for 10min at RT.

Flow cytometry analyses. To detect expression of surface markers, cells were incubated with the appropriate fluorochrome-conjugated mAbs and expression was monitored in comparison with isotype controls. Antibodies against CD4, CD25, CD69, and CD71 were from Beckman Coulter. Expression of Y^{319}-phosphorylated ZAP-70 (BD Biosciences), c-Myc (Santa Cruz), and phosphorylated H2AX (BioLegend) was monitored following cell fixation and permeabilization. Surface Glut1 expression was monitored by binding to a recombinant human T lymphotrophic virus (HTLV)-2-envelope-receptor-binding domain (HRBD) fused to

the eGFP coding sequence (HRBDeGFP) as described (Kim et al., 2004; Kinet et al., 2007; Manel et al., 2003a; Swainson et al., 2005). Proliferation was monitored as a function of CFSE (Invitrogen) or CTV (Invitrogen) dilution.

Cell cycle analysis was performed by simultaneous staining for DNA and RNA, using 7-amino-actinomycin-D (7AAD; 20μM; Sigma) and pyronin Y (PY; 5μM; Sigma), respectively. ER biomass, mitochondrial biomass, mitochondrial membrane potential and ROS levels were monitored by staining with ER-tracker blue-white DPX (Invitrogen), mitotracker green (Invitrogen), TMRE (Sigma) and 123 Rhodamine (Sigma), respectively. Calcium influx was monitored by preloading cells with Fluo-4 AM (Invitrogen) and fluorescence was analysed using the 530/30 filter. Data was acquired using FACS-CantoII or BD LSRII-Fortessa (BD Biosciences) and analysed using Diva (BD Biosciences) or FlowJo (Tree Star) software.

Biochemical Measurements. Oxygen consumption rates were measured on an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience) in XF media under basal conditions (10 mM glucose) and in response to fluoro-carbonyl cyanide phenylhydrazone (FCCP; 1.5 μM). ATP, NAD/NADH and L-lactate were measured according to the standard procedures of the ATPLite kit (Perkin Elmer), NAD/NADH kit (Sigma) and L-Lactate kit (Eton Bioscience), respectively. The Mammalian Cell Lysis solution from the ATPLite kit (Perkin Elmer) was used to lyse the cells and AMP was measured according to the standard procedures of the AMP-Glo Assay (Promega). Extracellular pH was measured immediately after harvesting of medium using a standard pH meter.

Sirt1 deacetylase activity was assessed using the SIRT1 Assay Kit (Sigma). Cell lysis and total protein purification for the Sirt1 deacetylase assay was done using the M-PER extraction reagent (Thermo Scientific).

Glucose uptake assays. Cells (2×10^6) were starved by incubation at 37°C in serum/glucose-free RPMI for 30 min. Radiolabeled 2-deoxy-D-[1-3H] glucose (Amersham Biosciences) was added to a final concentration of 0.1 mM (2μCi/mL). Cells were incubated for 10 min at room temperature, washed in cold serum/glucose-free RPMI, and solubilised in 500μL 0.1% SDS. Radioactivity was measured by liquid scintillation.

shRNA-mediated downregulation of Sirt1. Lentiviral pLKO.1 plasmids harbouring a specific shRNA against Sirt1 and a scrambled control (Sigma) were obtained and eGFP was inserted in place of the puromycin gene in the unique BamHI and KpnI restriction sites. Virions were

generated by transient transfection of 293T cells with these vectors together with the Gag-Pol packaging construct 8.91 and a plasmid encoding the VSV-G envelope, pCMV-VSV-G. Viral supernatant was harvested 48h post-transfection and used to transduce CD4+ T cells following 24h post TCR receptor activation. Transduced cells were detected as a function of eGFP expression.

Total Protein Extraction and Analyses. Cells were lysed in lysis buffer containing 20 mM HEPES (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% Nonidet P-40, 0.15 mM spermin, 0.5 mM spermidin, 1 mM DTT, and a protease inhibitor cocktail. After a 30min incubation on ice, extracts were centrifuged and supernatants were harvested. Extracts (20 µg) were resolved on SDS-PAGE gels (8.5%-12%) and transferred electrophoretically to PVDF membranes. Membranes were immunoblotted with the indicated antibodies (Table 1) for 1h at RT or overnight at 4ºC, incubated with horseradish peroxidase-conjugated α-goat, α-rabbit or α-mouse secondary Abs and immunoreactive proteins were visualized using enhanced chemiluminescence.

Immunoblotting Antibodies. The following antibodies were used throughout this study: anti-Sirt1 (sc-15404, Santa Cruz), anti-Phos-Sirt1.S27 (#2327, CST), anti-Phos-Sirt1.S47 (#2314, CST), anti-Phos-Sirt1.T530 (ab-156585, Abcam), anti-p53 (#2524, CST), anti-Ac-p53.K382 (#2525, CST), anti-Phos-p53.S15 (#9286, CST), anti-AKT (#4991, CST), anti-Phos-AKT.S473 (#4060, CST), anti-FoxO1 (#2880, CST), anti-Ac-FoxO1.K259.K262.K271 (sc-49437, Santa Cruz), anti-Erk2 (#9108, CST), anti-Phos-Erk1/Erk2.T202.Y204 (#9101, CST), anti-mTOR (#2983, CST), anti-Phos-mTOR.S2448 (#2971, CST), anti-Phos-mTOR.S2481 (#2974, CST), anti-RPS6 (#2217, CST), anti-Phos-RPS6.S235.S236 (#2211, CST), anti-Phos-4EBP1.T37.T46 (#2855, CST), anti-AMPK (#2603, CST), anti-Phos-AMPK.T172 (#2535, CST), anti-Ac-Lysine (#9441, CST), anti-Phos-Chk1.S317 (#8191, CST), anti-Cyclin A2 (#4656, CST), anti-Cyclin B1 (sc-752, Santa Cruz), anti-Cyclin D2 (sc593, CST), anti-Cyclin E1 (sc-247, Santa Cruz), anti-Cdk2 (sc-163, Santa Cruz), anti-Cdk4 (sc260, Santa Cruz), anti-Cdk6 (sc-177, Santa Cruz), anti-p21 (sc-397, Santa Cruz), anti-p130 (sc-317, Santa Cruz), anti-p27 (sc-528, Santa Cruz), anti-Ki67 (ab16667, Abcam), anti-γTubulin (T6557, Sigma), anti-Phos-Rb.S780 (M0453, MBL), anti-Phos-MCM2.S139 (#12958,CST), anti-Phos-Chk1.S317 (#12302, CST), anti-Phos-Cdk1.Y15 (ab-133463, Abcam)

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CHAPTER 7:

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ANNEXES

Project 2

IL-7 signalling and lineage choice in the human thymus

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IL-7 signalling and lineage choice in the human thymus

BACKGROUND: Throughout thymocyte development, immature double positive (DP) cells that express both CD4 and CD8 co-receptors undergo a differentiation process in the thymus cortex based on their ability to recognize peptide antigens presented on self major histocompatibility complex (MHC) molecules (Miller, 2011). MHC class I restricted thymocytes commit to the cytotoxic CD8 lineage, whereas thymocytes that recognize MHC class II antigens become single positive (SP) CD4 helper cells. However, the mechanism underlying lineage commitment is more than simple MHC interaction and recognition.

According to the kinetic model proposed by the group of Alfred Singer (Singer et al., 2008), during murine thymopoiesis, TCR signalling forces DP thymocytes to terminate *Cd8* gene expression but not *Cd4*, therefore becoming phenotypically $CD4^{+}CD8^{low}$ cells. And if positively selecting TCR signals persist, these $CD4^{+}CD8^{low}$ intermediate thymocytes upregulate the transcription factor ThPOK which directs differentiation into CD4SP cells. Cessation or disruption of TCR signalling, however, allows intermediate thymocytes to be signalled by the cytokine IL-7 and upregulate Runx3, undergoing co-receptor reversal and specifying into CD8SP cells. Still, only TCR-signalled murine DP thymocytes are capable of responding to IL-7 as DP thymocytes themselves do not express the IL-7Rα and furthermore, DP cells express high levels of the cytokine signaling suppressor SOCS1 (Yu et al., 2006). Additionally, in accord with this differentiation and the action of IL-7 in negatively regulating expression of its own receptor, murine SP8 thymocytes express lower IL-7Rα levels than SP4 thymocytes (Yu et al., 2003).

In contrast to the murine system, some reports have shown that human DP thymocytes express the IL-7Rα and are capable of responding to IL-7 as measured by phosphorylation of STAT-5 and increased survival rate (Marino et al., 2010; Napolitano et al., 2003; Young and Angel, 2006). Therefore, and given the specificity of IL-7 signalling during lineage commitment in the murine thymus, we wondered if T lineage differentiation proceeded differently in humans.

METHODS: Human thymus tissue was obtained, with informed consent, from patients aged 1 week to 6 years submitted to pediatric cardiac surgery at the Hôpital de la Timone (Marseille, France). Thymus tissue was homogenized by gently mincing, and single cell

thymocyte suspensions were generated by forceful disruption of thymuses with 50-ml syringe plungers on 40µm cell strainers (BD Falcon). Human thymocyte subpopulations were discriminated using fluorochrome-coupled anti-TCRαβ, -CD4, -CD8, -CD11b antibodies (Beckman Coulter). Sorting and analysis of thymocyte subsets were performed on LSRII Fortessa and FACS CantoII (BD Biosciences) flow cytometers, respectively.

Sorted thymic populations were cultured in RPMI 1640 medium supplemented with 10% FCS and 2% penicillin and streptomycin. To study IL-7 signalling, thymocytes were cultured in the presence of 10 ng/mL of recombinant IL-7 (Cytheris). For lineage commitment studies, sorted DP thymocytes were either continuously or transiently stimulated with 0.1 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 0.1 g/ml ionomycin for 3h (Sigma-Aldrich), then washed and cultured in medium alone or medium with 10 ng/mL rIL-7.

To detect surface expression of CD4, CD5, CD8 and CD127 (IL-7Rα), cells were incubated for 20 minutes on ice with the appropriate fluorochrome-conjugated mAbs (Beckman Coulter). Background fluorescence was measured using isotype-matched irrelevant antibodies. Thymocytes were prepared for intracellular staining by fixation and permeabilization (Cytofix and PhosFlow Perm III; BD Pharmingen). STAT5 phosphorylation state and control fluorescence were assessed using an anti-phospho-STAT5 (Y694) antibody coupled to Alexa Fluor 647 and an anti-IgG Alexa Fluor 647–coupled antibody (BD Pharmingen), respectively.

Total RNA was purified using the RNeasy Micro Kit (Qiagen) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Expression of RUNX3 (distal isoform), ThPOK, IL-7Rα, SOCS1, CD4 and CD8 mRNA was assessed by real time quantitative PCR using the LightCycler 480 Real-Time PCR (Roche).

RESULTS: To address the question of whether human and mice thymocytes share the same dynamics of lineage commitment, we started by assessing the level of IL-7Rα transcripts in the different human thymocyte populations. In marked contrast to the situation in mice, we found that IL-7Rα was expressed throughout human thymic ontogeny, including the DP stage. Furthermore, we found that IL-7Rα transcript levels were 3-fold higher in SP8 thymocytes than SP4 cells [FIGURE 1]. This correlated with higher surface expression of IL-7Rα on the

SP8 population and, in contrast to mice, SP4 cells were more responsive to IL-7 as monitored by STAT-5 phosphorylation.

IL-7Rα expression on human DP thymocytes had a functional relevance as IL-7 stimulation resulted in the phosphorylation of STAT-5 [FIGURE 1]. Furthermore, IL-7 stimulation was an important survival signal for DP thymocytes, as sorted DP cells cultured in absence of IL-7 would not survive past 48h (not shown). Additionally, DP thymocytes upregulated SOCS1 and downregulated the IL-7Rα in response to IL-7, further evidence that DP cells exhibit proximal IL-7 signaling responses [FIGURE 2B]. Interestingly, IL-7 promoted the upregulation of the distal form of Runx3 (dRunx3) [FIGURE 2B], which in the murine model is required for CD8-lineage commitment (Park et al., 2010); however the expression of the lineage–specific co-receptors CD4 and CD8 was unaltered, both at transcriptional level and at surface expression of the respective proteins [FIGURE 2A].

Based on the important differences in IL-7Rα expression and IL-7 responsiveness observed between human and murine DP thymocytes, we then addressed the question of lineage differentiation. As expected, TCR-signalled human DP thymocytes showed a differentiation to a CD4 SP fate, but this fate was significantly promoted in the presence of rIL-7 [FIGURE 3]. This was surprising as Singer and colleagues have elegantly shown that TCR-primed thymocytes treated with IL-7 are strongly skewed towards a CD8 lineage fate, whereas we only observe persistent CD8 expression in DP cells that have never encountered IL-7. Remarkably, in the early days following TCR-priming we observe a surface downregulation of the CD4 co-receptor, with cells gaining a characteristic intermediate phenotype ($CD4^{low}CD8^{+}$), and only at late time points we observe CD4 resurfacing [FIGURE 3]. Again, this is different from the evidence supporting the kinetic model (Singer et al., 2008), where murine DP thymocytes that are signalled by the TCR undergo positive selection and become intermediate $CD4^{+}CD8^{low}$ cells.

Pending the specific kinetics observed in TCR-primed DP thymocytes cultured in the presence or absence of IL-7, we considered analysing the mRNA levels CD4, CD8, ThPOK and dRunx3, all markers proposed to be lineage-specific by the kinetic model. CD4 transcripts were only detected in cells that expressed the CD4 co-receptor at the surface phenotypically (sorted populations A and C), whereas CD8 transcripts were exclusive to cells that expressed CD8 at the surface after 6 days of culture [FIGURE 4A]. In addition, the observed CD8 expression only occurred in TCR-primed DP thymocytes culture in the absence of rIL-7,

while CD4 expression was observed both in cells cultured in the absence and presence of rIL-7. Interestingly, dRunx3 was upregulated in all cells that were IL-7 stimulated. Furthermore, dRunx3 was also highly upregulated in TCR-primed cells that expressed the CD8 co-receptor but were never treated with the IL-7 cytokine [FIGURE 4B]. Therefore, we propose that Runx3 is simultaneously an IL-7 responsive gene and a transcription factor required for the specification of the cytotoxic lineage. ThPOK transcripts were observed in all sorted populations, with higher expression in cells expressing the CD4 co-receptor. Yet, ThPOK transcripts were lower when TCR-primed DP thymocytes were cultured in presence of IL-7, which may be secondary to Runx3 expression in these cells, as cross-repression between Runx3 and ThPOK is a necessary event during lineage choice (Wang and Bosselut, 2009).

It has also been proposed that lineage choice is dictated by the strength of the signals received through the TCR and the major co-receptors during positive selection. For instance, co-engagement of the TCR and CD4 generates strong signals, while TCR and CD8 co-stimulation is generates weaker signals (Bosselut, 2004). To address the importance of the signal strength and if somehow IL-7 stimulation resulted in modulation of that signal, we decided to analyse the expression of CD5, which directly correlates with the intensity of TCR signaling (Azzam et al., 1998). Notably, IL-7 signals potentiate TCR signaling on differentiating human DP thymocytes as CD5 levels were markedly upregulated [FIGURE 5; sorted populations C and D]. Still, this upregulation was clearly dependent on TCR signals, since non-primed DP thymocytes treated with IL-7 (DP+IL-7) did not increase CD5 levels. Additionally, we verified that immature DP thymocytes ($TCR\alpha\beta^{low}$) show significantly increased sensitivity to TCR- and IL-7-mediated differentiation, as the relative numbers of thymocytes originating from this population are highly increased during differentiation [FIGURE 6]. Interestingly, we found that the immature DP population is characterized by higher surface expression of the IL-7Rα [FIGURE 7A], which may be the reason why immature DP thymocytes show increased sensitivity to TCR- and IL-7-mediated differentiation.

Remarkably, a closer analysis of the immature human DP population revealed that these thymocytes are particularly active, expressing higher levels of metabolic markers such as the Glut1 glucose transporter and the transferrin receptor, CD71. Glut1 has been shown to be an IL-7 responsive gene (Swainson et al., 2007), therefore its expression may be associated with the fact that immature DP cells are more sensitivity to IL-7 signals. Still, T cell development is marked by striking changes in metabolism and there is growing evidence that the metabolic pathways control important T cell functions and drive T cell differentiation (Pearce, 2010; van

der Windt and Pearce, 2012a; Waickman and Powell, 2012). Therefore, it is possible that manipulation of thymocyte metabolism in the context of TCR and cytokine signalling represents an important aspect of lineage commitment.

CONCLUSION: Human DP thymocytes express the IL-7Rα and exhibit proximal IL-7 signaling responses, as measured by phosphorylation of STAT-5, upregulation of SOCS1 and Runx3, and transcriptional downregulation of *IL7r*. Moreover, the present study reveals that IL-7 promotes the differentiation of human TCR-signaled DP thymocytes *to a CD4 lineage fate*. In these cells, IL-7 stimulation increases TCR signaling intensity as measured by upregulation of CD5. It is however interesting to note that IL-7-mediated upregulation of Runx3 in these phenotypic SP4 thymocytes correlates with significantly lower levels of the CD4 lineage regulator ThPOK. Together, these data reveal that murine and human DP thymocytes react differently to IL-7 stimulation, with implications on lineage commitment.

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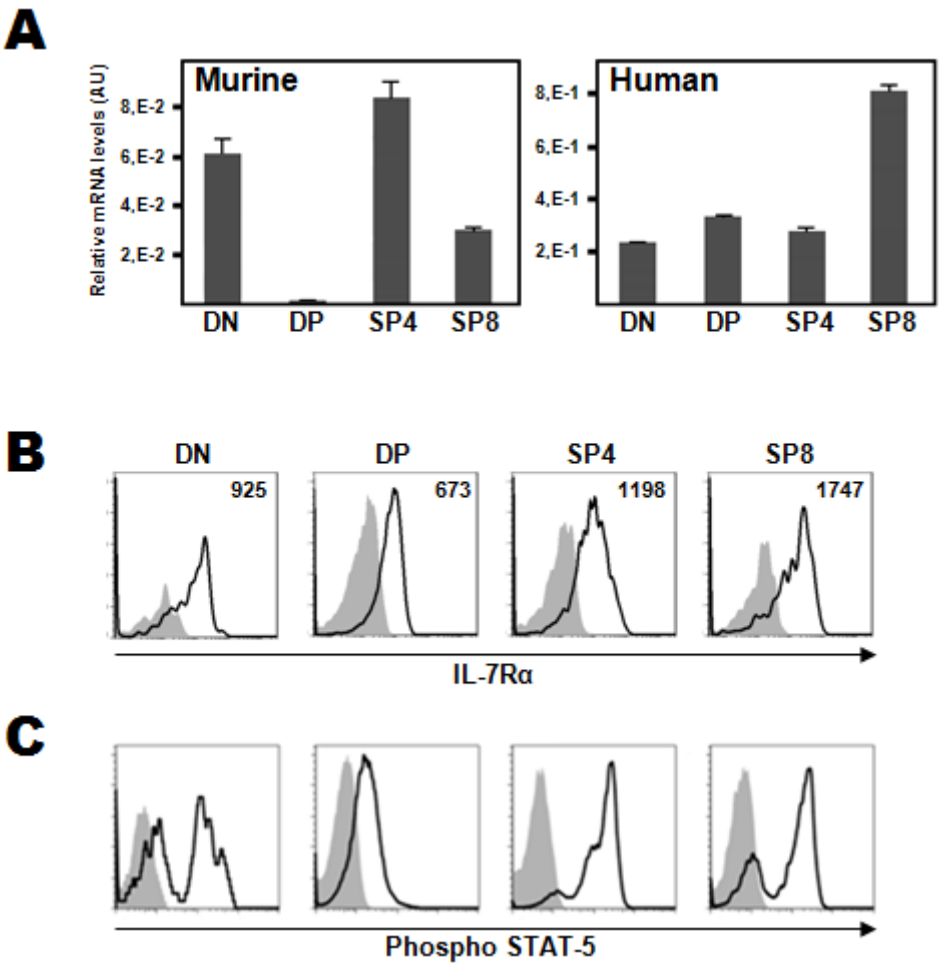
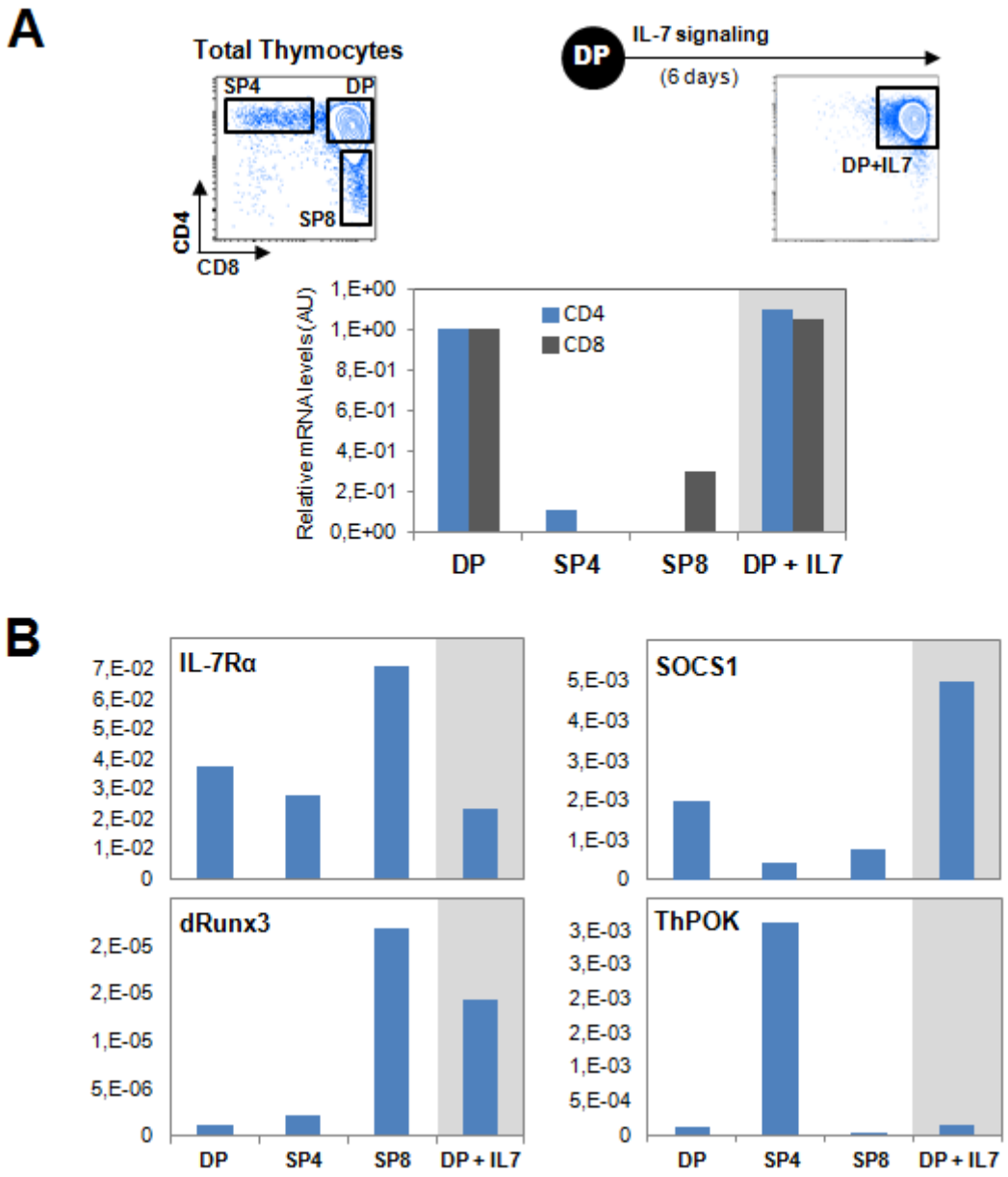
Figure 1. **Human DP thymocytes express the IL-7Rα and respond to IL-7 signals**



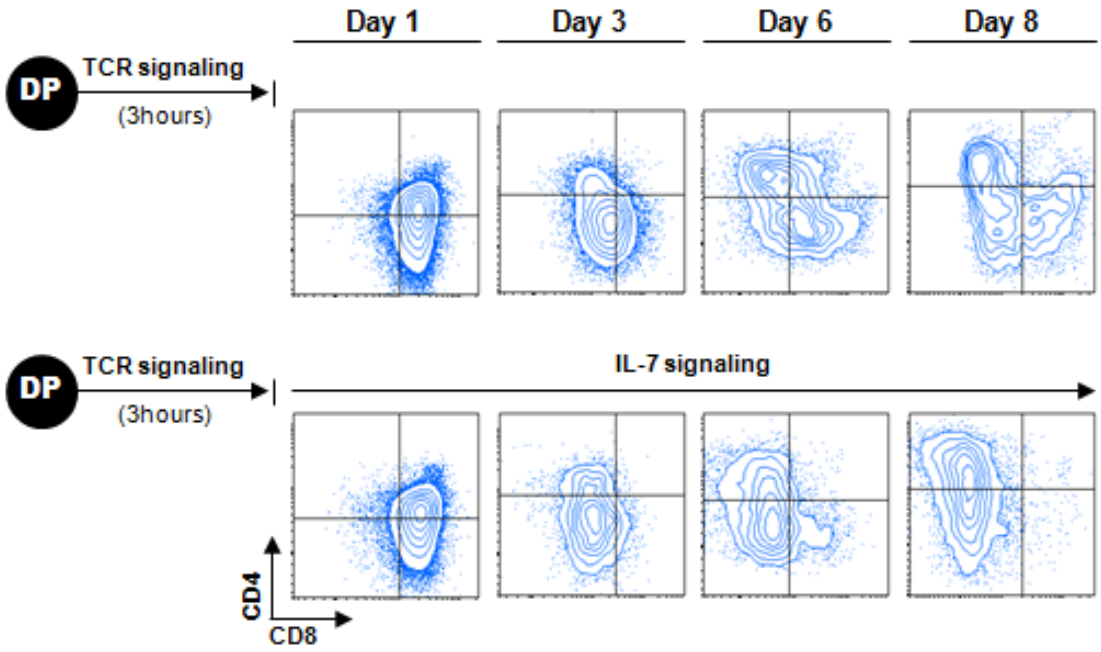
A Double Negative (DN), Double Positive (DP), Single Positive CD4 (SP4) and Single Positive CD8 (SP8) murine and human thymocytes were isolated by flow cytometry cell sorting. Levels of IL-7Rα were assessed by qRT-PCR and the relative quantities (arbitrary units) of IL-7Rα relative to actin were determined using the Second Derivative calculation method. **B** Surface expression of IL-7Rα on human thymocytes was detected by flow cytometry using a PC7-conjugated anti-IL-7Rα antibody (black line) and control fluorescence, corresponding to the IgG control, is shown in grey. Mean fluorescence intensity (MFI) of IL-7Rα levels are indicated in each histogram. C Total human thymocytes were stimulated with IL-7 (10ng/ml) and phosphorylation of STAT5 was monitored in the DN, DP, SP4 and SP8 subsets as a function of CD4/ CD8 staining. Expression of the Tyr694-phosphorylated form of STAT-5 is shown as a black line and fluorescence of the corresponding IgG control is shown in grey.

Figure 2. **Human DP thymocytes upregulate SOCS1 and Runx3 in response to IL-7 stimulation**



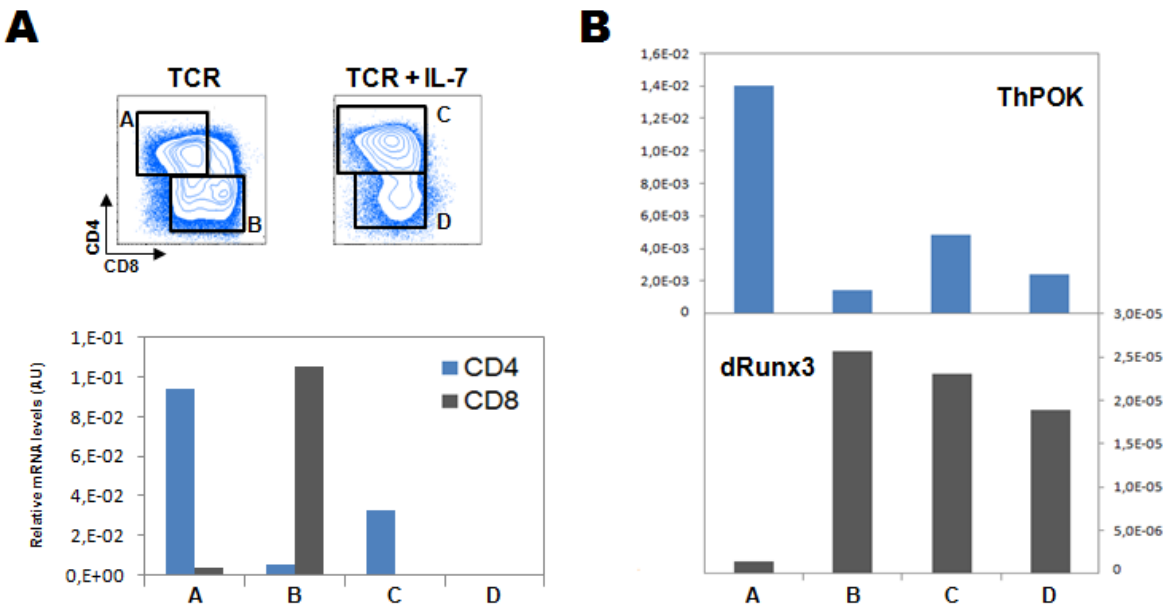
A The levels of CD4 and CD8 lineage markers were monitored in freshly sorted DP, SP4 and SP8 human thymocyte subsets as well as in sorted DP thymocytes stimulated with IL-7 (10ng/ml) for 6 days. CD4/CD8 staining on total purified thymocytes, used to sort the SP4, SP8 and DP populations are shown. CD4/CD8 staining on sorted DP thymocytes stimulated with IL-7 for 6 days is shown as well. Levels of RNA transcripts on purified subsets were assessed by qRT-PCR. The relative quantities (arbitrary units) relative to actin were determined using the Second Derivative calculation method and normalized to the freshly isolated DP population. **B** Levels of IL-7 responsive genes including IL-7Rα itself, SOCS1 and Runx3 (distal promoter transcript) were monitored in freshly isolated DP, SP4 and SP8 subsets and compared with levels in IL-7-stimulated DP cells. Levels of the CD4 regulator ThPOK were also monitored. RT-qPCR was performed and mean units relative to actin are shown.

Figure 3. **IL-7 modulates the phenotype of TCR-signaled Human DP thymocytes**



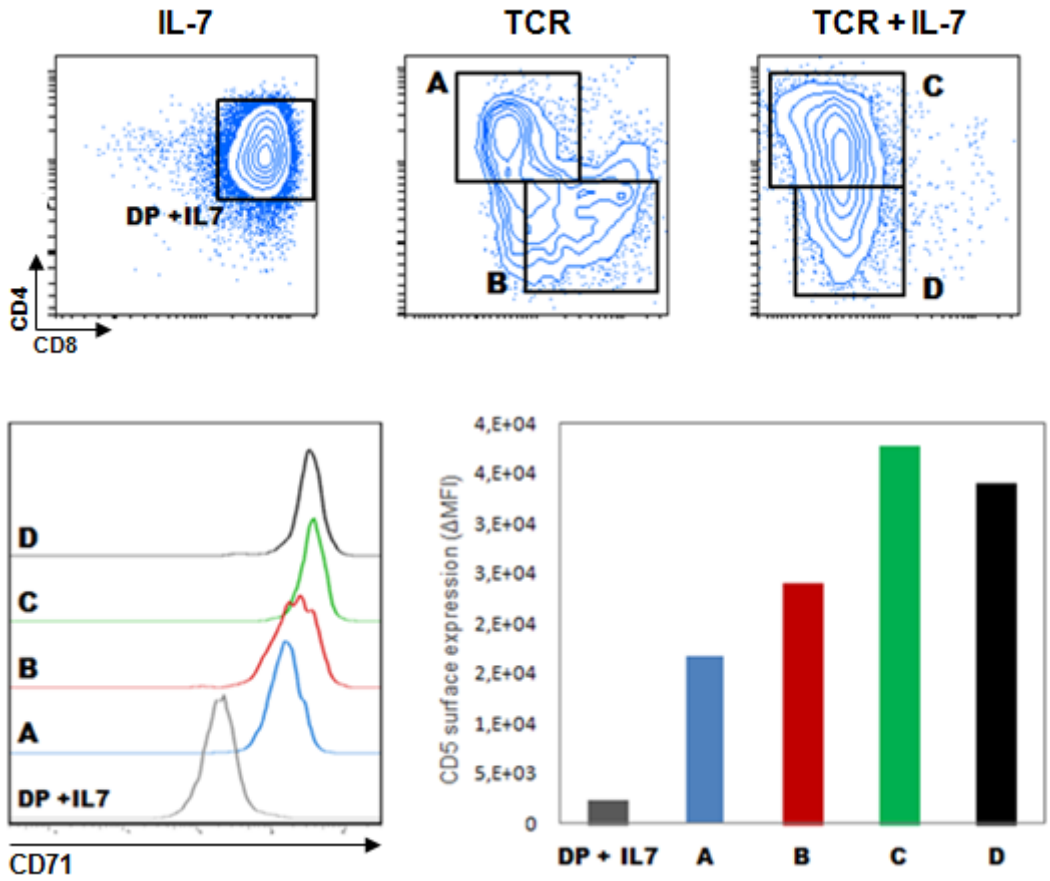
Human DP thymocytes were purified by flow cytometry and TCR-signaled with PMA (0.1ng/mL) plus ionomycin (0.1mg/mL) for 3 hours. Thymocytes were then cultured in the absence (upper panels) or presence of IL-7 (lower panels). Expression of the CD4 and CD8 co-receptors was assessed 1, 3, 6 and 8 days post stimulation by flow cytometry and representative density plots are shown.

Figure 4. **IL-7 inhibits ThPOK upregulation in TCR-signaled DP thymocytes**



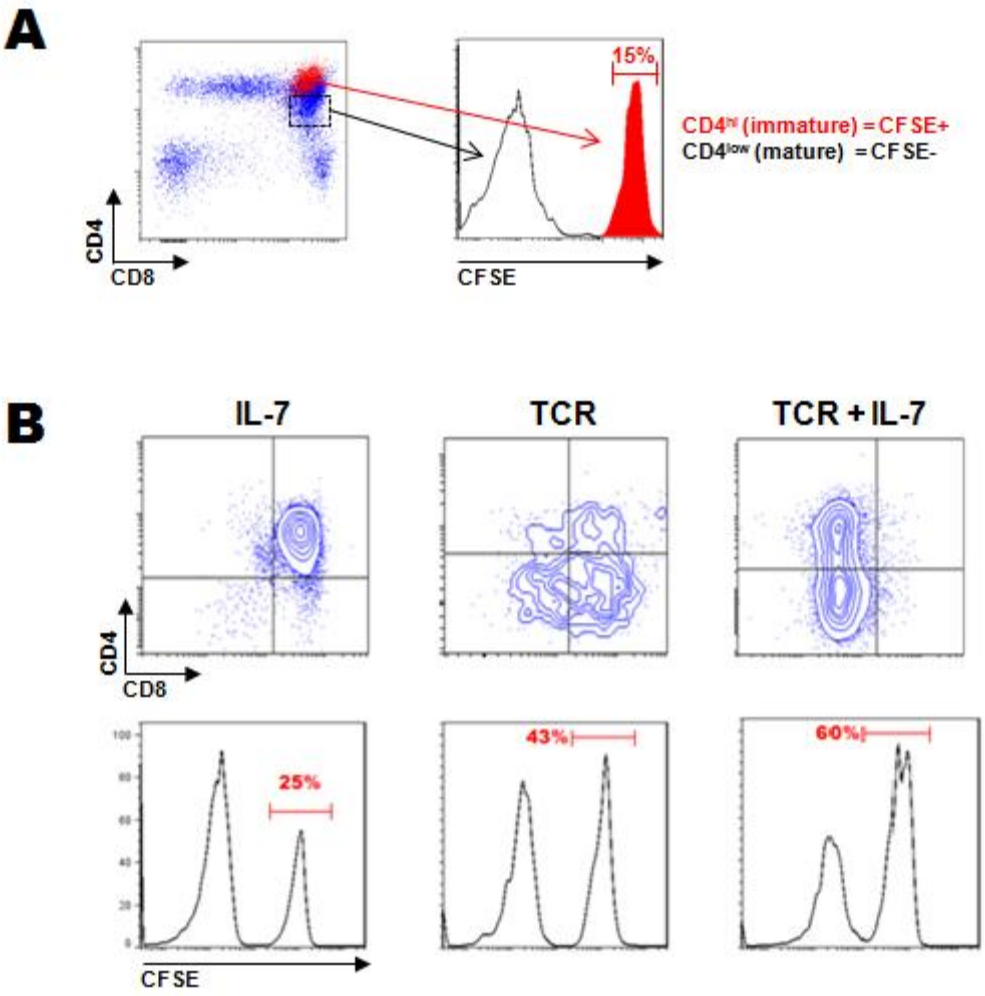
A Purified human DP thymocytes were TCR-signaled by a PMA plus ionomycin (TCR) stimulation during 3 hours and then cultured in the absence or presence of rIL-7 as indicated in figure 3. Six days post stimulation, four different populations were sorted by flow cytometry as a function of CD4 and CD8 co-receptor expression. TCR-stimulated cells were separated into $CD4^{+}CD8^{-}$ (A) and $CD4^{-}CD8^{+}$ (B) phenotypic subsets while TCR+IL7-stimulated cells were sorted as $CD4^{+}CD8^{-}$ (C) and $CD4^{-}CD8^{-}$ (D) populations. Levels of CD4 and CD8 RNA transcripts were assessed by RT-qPCR and mean quantifications relative to actin are shown. **B** Levels of the ThPok and Runx3 regulators of CD4/CD8 lineage commitment were assessed in the 4 subsets by RT-qPCR. Relative quantities (arbitrary units) relative to actin were determined using the Second Derivative calculation method and mean levels are presented

Figure 5. **IL-7 induces CD5 expression on TCR-signaled DP thymocytes**



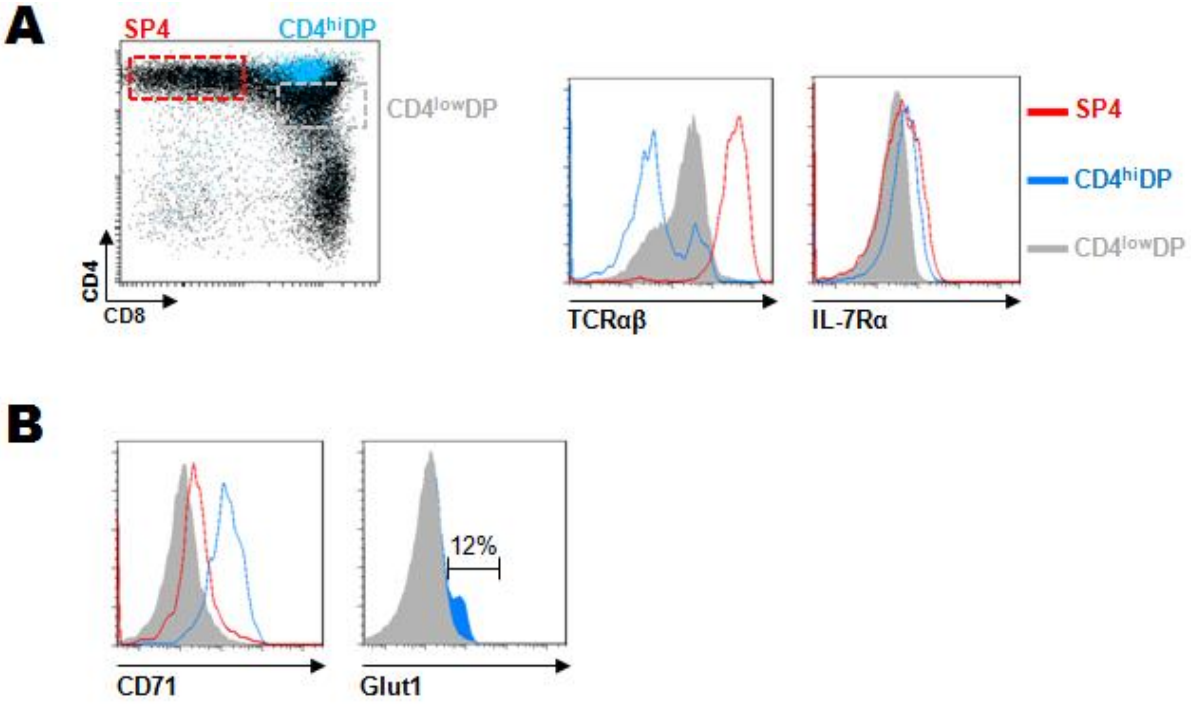
Purified human DP thymocytes were either cytokine-stimulated in the continuous presence of rIL-7 (10ng/mL) or TCR-signaled with PMA plus ionomycin as described above and then cultured in the absence or presence of rIL-7. Surface CD5 expression, a measure of TCR signaling intensity, was assessed 8 days post stimulation by flow cytometry and data are presented as a histogram. MFI- mean fluorescence intensity.

Figure 6. **Immature DP thymocytes show significantly increased sensitivity to TCR- and IL-7-mediated differentiation**



A Mature ($CD4^{low}$; black) and immature ($CD4^{hi}$; red) human DP thymocytes were purified and the immature population was loaded with CFSE, a dye widely used for cell tracking and proliferation studies. After CFSE loading, the DP population was reconstituted and the relative percentages of each population were assessed by flow cytometry, with immature DP cells constituting 15% of the total. **B** The reconstituted DP population was either IL-7 stimulated or TCR-signaled with PMA plus ionomycin (TCR) during 3 hours and then cultured in the absence or presence of rIL-7. Six days post stimulation, the relative contribution of the mature and immature populations for the differentiating thymocytes were assessed by flow cytometry and the respective percentages are depicted.

Figure 7. **Immature DP thymocytes express higher levels of IL-7Rα and are metabolically more active.**



A CD4/CD8 staining on total purified thymocytes was used to sort the SP4, CD4^{hi}DP and CD4^{low}DP populations. Surface expression of TCRαβ and IL-7Rα was detected by flow cytometry using a PE-conjugated anti-TCRαβ antibody and a PC7-conjugated anti-IL-7Rα antibody. Mean fluorescence intensity (MFI) of TCRαβ and IL-7Rα levels for the SP4 (red line), CD4^{hi}DP (blue line) and CD4^{low}DP (full grey) populations are indicated in each histogram. **B** Cell surface expression of the transferrin receptor (CD71) and of the Glut1 glucose transporter was monitored by flow cytometry using an APC-conjugated anti-CD71 antibody and the recombinant Human T Lymphotrophic Virus 2-envelope-receptor-binding domain (HRBD) fused to the eGFP coding sequence (HRBDeGFP), respectively. MFI of CD71 levels for the SP4 (red line), CD4^{hi}DP (blue line) and CD4^{low}DP (full grey) populations are indicated in each histogram. MFI of Glut1 levels is indicated for the CD4^{hi} (full blue) and CD4^{low} (full grey) DqP populations.

Original Article

Glut1-mediated glucose transport regulates HIV infection

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The authors declare no conflict of interest.

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Cell cycle entry is commonly considered to positively regulate HIV-1 infection of CD4 T cells, raising the question as to how quiescent lymphocytes, representing a large portion of the viral reservoir, are infected in vivo. Factors such as the homeostatic cytokine IL-7 have been shown to render quiescent T cells permissive to HIV-1 infection, presumably by transiently stimulating their entry into the cell cycle. However, we show here that at physiological oxygen (O_2) levels (2–5% O_2 tension in lymphoid organs), IL-7 stimulation generates an environment permissive to HIV-1 infection, despite a significantly attenuated level of cell cycle entry. We identify the IL-7–induced increase in Glut1 expression, resulting in augmented glucose uptake, as a key factor in rendering these T lymphocytes susceptible to HIV-1 infection. HIV-1 infection of human T cells is abrogated either by impairment of Glut1 signal transduction or by siRNA-mediated Glut1 down-regulation. Consistent with this, we show that the susceptibility of human thymocyte subsets to HIV-1 infection correlates with Glut1 expression; single-round infection is markedly higher in the Glut1-expressing double-positive thymocyte population than in any of the Glut1-negative subsets. Thus, our studies reveal the Glut1-mediated metabolic pathway as a critical regulator of HIV-1 infection in human CD4 T cells and thymocytes.

thymus | metabolism | hypoxia

The productive infection of both naive and memory CD4 T cells by HIV-1, at least outside the context of lymphoid tissues (1, 2), requires a transition into the G1b stage of the cell cycle (3). As most circulating peripheral T lymphocytes are in the G0 resting state, this would theoretically preclude their infection by HIV-1. However, within lymphoid aggregates, HIV-1 infection can occur in quiescent naive T cells (1, 2), although this phenomenon has recently been shown to be associated with the expression of activation markers (4, 5). Under what conditions can HIV-1 infection be achieved in quiescent T lymphocytes that remain phenotypically naive? One possibility is that the in vivo infection of naive $CD4^+$ T cells occurs following exposure of these lymphocytes to cytokines such as IL-7. Indeed, IL-7 stimulation is currently thought to be critical for activating the latent HIV reservoir (6–8). Thus, the infection status of quiescent T cells under physiological conditions, either in the absence or presence of IL-7, remains to be addressed.

IL-7 is a 25-kDa glycoprotein produced in thymus, intestine, skin, lymph nodes, and other sites (reviewed in ref. 9). It plays a major role in the in vivo maintenance of polyclonal naive and memory T cells, positively regulating the survival, differentiation, and proliferation of thymocyte and peripheral T-lymphocyte populations. The homeostatic maintenance of this polyclonal T-cell pool is required for the persistence of immunologic memory as well as for the maintenance of naive T cells with the potential to respond to novel antigens. Under conditions of lymphopenia, IL-7 supports a transient homeostatic T-cell proliferation, promoting the expansion of T cells with a diverse T-cell receptor (TCR) repertoire. However, under physiological conditions, the vast majority of peripheral lymphocytes are quiescent, perhaps because homeostatic levels of IL-7 are low relative to peripheral T-cell numbers (reviewed in ref. 10). Indeed, serum IL-7 levels are increased in patients with lymphopenia, secondary to either HIV-1 infection or chemotherapy (reviewed in ref. 10). Consistent with these findings, administration of exogenous IL-7 in preclinical murine and primate studies has been shown to promote T-cell survival and proliferation with short-term expansion of de novo generated recent thymic emigrants (reviewed in ref. 10). Moreover, in recent clinical trials, the administration of recombinant IL-7 led to a transient entry of T lymphocytes into the cycle, albeit with a rapid return to quiescence (11–15).

To date, studies of IL-7 stimulation and IL-7 signaling intermediates ex vivo have been performed almost exclusively in incubators maintained at atmospheric O_2 levels (20% O_2). However, tissue O_2 levels in vivo are substantially lower: direct in vivo measurements of O_2 levels in murine lymphoid organs have revealed partial pressures of 0.5–4.5% (16), somewhat higher O_2 levels in peripheral blood, and 14% in alveoli (17, 18). Thus, although lymphocytes encounter fluctuations in O_2 levels in vivo, the physiological range of O_2 levels to which they are exposed is at least two to six times lower than the 20% O_2 levels maintained in standard incubators. Culturing at atmospheric O_2 levels has been shown to result in altered cell proliferation rates and other skewed T-cell responses (16, 19–23). Thus, because the goal of in vitro studies is generally to reveal information of in vivo significance, our studies here focus on findings with peripheral blood T cells cultured at physiological O_2 levels. Here we assessed how naive and memory T cells respond to IL-7 at the O_2 levels to which they are exposed in vivo and determined their susceptibility to HIV-1 infection.

Results

Physiological O_2 Levels Decrease IL-7–Induced Cell Cycle Entry Without Modulating Proximal IL-7 Signaling. IL-7 has long been known to be a survival factor for T lymphocytes both ex vivo and in vivo. Nevertheless, the effects of IL-7 on T lymphocytes cultured under physiological O_2 concentrations have not been evaluated. IL-7 stimulation of freshly isolated CD4 T lymphocytes promoted a high level of viability, irrespective of whether the cells were cultured at 20% (atmospheric) or 2.5% (physiological) O_2 concentrations (Fig. S1). Both forward scatter (FSC), a measure of cell size, and

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side scatter (SSC), a function of cell granularity, profiles of the cells were increased by IL-7 stimulation, but the increase was significantly higher under atmospheric compared with physiological O_2 conditions. Specifically, in both naive and memory CD4 T cells, FSC and SSC were increased by ~20% and 50–60%, respectively, following IL-7 stimulation at atmospheric O_2, whereas these parameters were only increased by 5% and 20% at physiological O_2 (Fig. S1; $P < 0.05$ for both naive and memory T cells; $n = 3$). Differences in these physical parameters were the first indication that O_2 concentration impacts on IL-7–mediated effects.

Indeed, IL-7–induced cell cycle entry, monitored as a function of DNA/RNA levels, was significantly lower under conditions of 2.5% O_2 for both naive and memory CD4 T cells ($P < 0.0001$ and $P < 0.01$, respectively, at day 9; $n = 4$; Fig. 1*A* and Fig. S2). Indeed, the percentages of IL-7–stimulated naive or memory CD4 T cells entering into S/G2/M at day 9 were 10-fold lower under physiological compared with atmospheric O_2, and this phenomenon was observed throughout the 12 d of IL-7 stimulation (Fig. S2). Furthermore, this difference was not restricted to the DNA replication phase of the cell cycle, as levels of Ki67, expression of which is acquired as early as mid-G1, were also significantly lower following IL-7 stimulation of naive and memory CD4 T cells at physiological compared with atmospheric O_2 concentrations ($P < 0.05$ for both subsets at day 9; $n = 3$; Fig. S2).

Given the significant differences in IL-7–mediated cell cycle entry under 20% and 2.5% concentrations, it was important to determine whether IL-7 was able to efficiently induce proximal signaling pathways under the latter conditions. Interaction of IL-7 with its receptor is known to result in receptor internalization and decreased receptor transcription, resulting in a decrease in surface IL-7Rα levels (24–26). We indeed observed IL-7Rα down-regulation on naive as well as memory CD4 T cells, irrespective of the O_2 concentration at which the lymphocytes were cultured (Fig. 1*B*). Moreover, IL-7 stimulation resulted in a similar phosphorylation of STAT5 at 20% and 2.5% O_2 concentrations [nonsignificant (NS), $P > 0.05$; $n = 5$]. STAT5 phosphorylation was detected under both conditions between days 1 and 6 following IL-7 stimulation (Fig. 1*C*), strongly suggesting that proximal IL-7 signaling was not inhibited by the O_2 concentration at which the T cells were cultured. However, upon assessment of a late activation marker, the CD71 transferrin receptor, significant induction was detected only upon IL-7 stimulation of naive and memory CD4 T cells at atmospheric O_2 and only at late time points (Fig. 1*D* and Fig. S3). Thus, IL-7 signaling is induced under both atmospheric and physiological O_2 conditions, but transmission of this signal, as assessed by CD71 up-regulation and cell cycle entry, is dependent on the O_2 concentration to which the T cells are exposed.

Susceptibility of IL-7–Stimulated CD4 T Cells to Single-Round HIV-1 Infection Is Maintained Under Physiological O_2 Concentrations. HIV-1 infection of CD4 T cells is known to be highly dependent on T cell activation and cell cycle entry and, indeed, the permissivity of quiescent lymphocytes to HIV-1 infection is extremely inefficient (reviewed in ref. 5). As we found that expression of activation markers and cell cycle entry were markedly inhibited at 2.5% O_2 (Fig. 1), we postulated that HIV-1 infection would be reduced at 2.5% O_2 relative to 20% atmospheric O_2 levels. To address this issue, we performed single-round infections of IL-7–stimulated T lymphocytes with X4-HIV-1 virions harboring the *eGFP* transgene. Interestingly, equivalent infection levels were observed at physiological and atmospheric O_2 (NS, $P > 0.05$ for both naive and memory T cells; $n = 3$). However, memory CD4 T cells were always infected at significantly higher levels than their naive counterpart (~30% and 10% at day 6, respectively, in the representative donor shown; Fig. 2*A*).

Given this result, it was critical to determine whether the unexpectedly high levels of infection at 2.5% O_2 were due to changes in coreceptor expression. IL-7 has previously been shown to augment CXCR4 levels (27), and we found this to be the case in both naive and memory T cells. At physiological O_2 concentrations, CXCR4 levels were modestly increased by a further 16% in the memory subset ($P = 0.03$; $n = 8$). In naive CD4 T cells, CXCR4 levels were comparable, irrespective of O_2 concentration (NS, $P > 0.05$; $n = 8$; Fig. S4), making it unlikely that the infection detected at physiological O_2 levels resulted from changes in coreceptor levels.

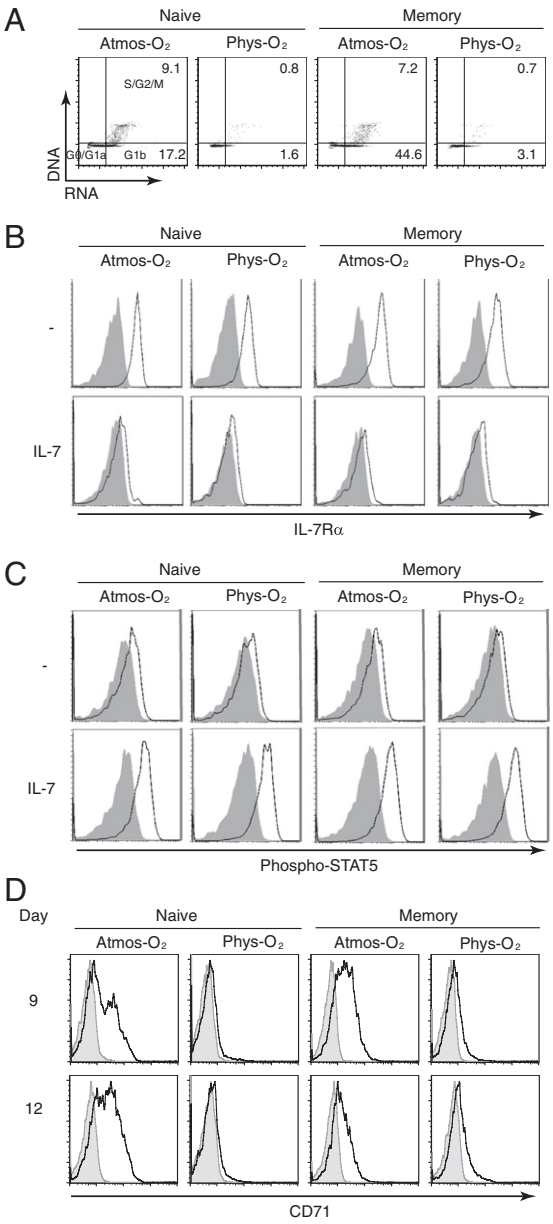

Fig. 1. Physiological O_2 levels diminish IL-7–induced cell cycle entry while maintaining proximal IL-7Rα signaling. Naive and memory CD4 T cell populations isolated from adult peripheral blood (APB) were stimulated with IL-7 (10 ng/mL) under 20% (Atmos-O_2) or 2.5% (Phys-O_2) O_2 conditions. (*A*) Cell cycle entry was monitored as a function of DNA and RNA levels, using 7-amino-actinomycin-D (7AAD) and pyronin Y (PY), respectively. PY/7AAD cell cycle staining is shown at day 9, and the percentages of cells in the G1b (lower right quadrant) and S/G2/M stages (upper right quadrant) are indicated. Cells in the lower left quadrant are in G0/G1a. (*B*) Expression of CD127, the IL-7Rα chain, was monitored at day 3, and specific staining (open histograms) compared with control isotype (closed histograms) is shown. (*C*) STAT5 phosphorylation was measured using an anti–phospho-STAT5 polyclonal antibody. Specific (open histograms) relative to control isotype staining (closed histograms) is shown in the presence or absence of IL-7. (*D*) CD71 transferrin receptor expression (open histograms) and isotype controls (closed histograms) were monitored at days 9 and 12 of IL-7 stimulation. Data are representative of three independent experiments comprising three to six different donors.

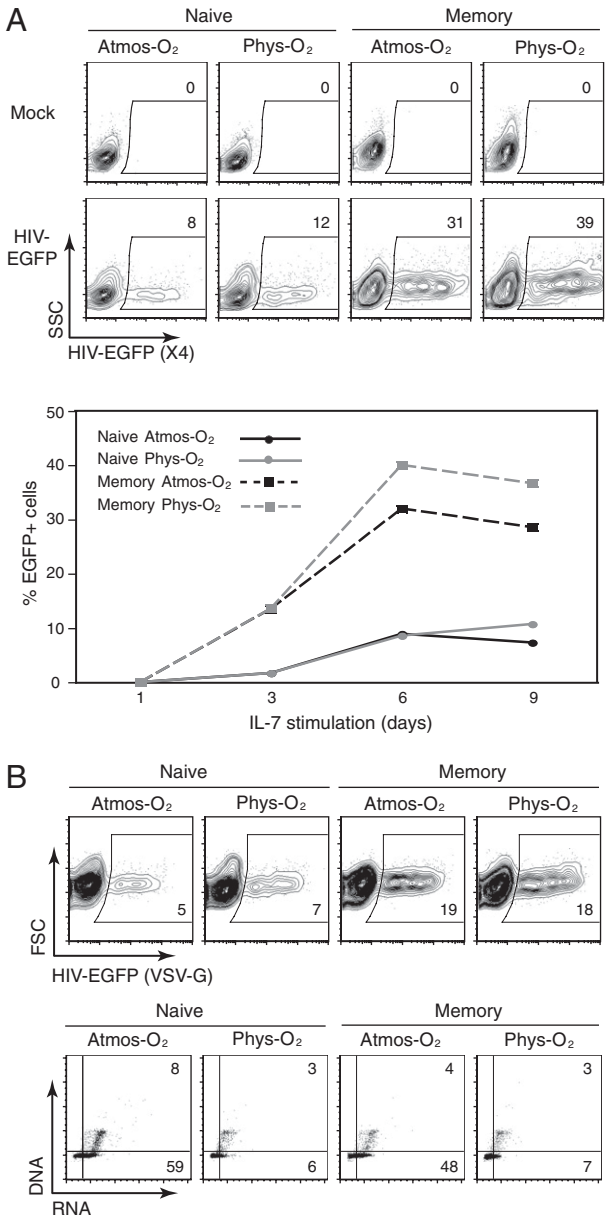

Fig. 2. Permissivity of IL-7–stimulated CD4 T lymphocytes to infection by X4- and VSV-G–pseudotyped HIV-1 virions does not correlate with cell cycle kinetics under physiological O_2 concentrations. (*A*) Naive and memory CD4 T cell populations isolated from APB were stimulated for 1, 3, 6, or 9 d with IL-7 (10 ng/mL) under 20% (Atmos-O_2) or 2.5% (Phys-O_2) conditions and then infected with an X4-tropic HIV-1 vector harboring the EGFP reporter gene. Single-round infected cells were detected by monitoring EGFP expression 48 h postinfection. Representative dot plots are shown for infections performed at day 9 of IL-7 stimulation, and the percentages of infected EGFP$^+$ cells are indicated (*Upper*). The *Lower* panel shows a quantification of the percentages of EGFP$^+$ cells from infections performed at the indicated time points. (*B*) Nine days post–IL-7 stimulation, naive and memory CD4 T cells were infected with a VSV-G envelope-pseudotyped HIV-1 vector expressing EGFP. The percentages of EGFP$^+$ cells are indicated (*Upper*). The corresponding level of cell cycle entry, assessed at the time of infection by PY/7AAD staining, is shown (*Lower*). The percentages of cells in the G1b (lower right quadrant) and S/G2/M (upper right quadrant) phases of the cell cycle are indicated (*Lower*). Data are representative of three independent experiments comprising three to eight different donors.

To exclude the possibility that the surprisingly high infection at physiological O_2 was due to enhanced gp120–CXCR4 interactions, we assessed single-round infection using HIV-1–based virions pseudotyped with the VSV-G envelope glycoprotein. The receptor for this envelope glycoprotein appears to be ubiquitously expressed on all mammalian cells. As shown in Fig. 2*B*, infection by VSV-G–pseudotyped HIV-1 virions was not decreased at 2.5% O_2 (NS, $P > 0.05$ for both naive and memory T cells; $n = 8$). Notably, though, memory CD4 T cells remained significantly more susceptible to infection than naive T cells, irrespective of the O_2 concentration (18–19% compared with 5–7% infection, respectively; Fig. 2*B*). Thus, neither X4- nor VSV-G–mediated HIV-1 infection is decreased at physiological O_2 concentrations despite a highly reduced level of activation and cell cycle entry (Fig. 2*B*).

Recent thymic emigrants (RTE) show increased proliferation to IL-7 compared with long-term resident peripheral T cells (28–30). It was therefore of interest to determine whether IL-7 stimulation of RTE under physiological O_2 concentrations would also result in a diminished cell cycle entry and how this would impact on the permissivity of these cells to HIV-1 infection. RTE, characterized by a naive immature phenotype, represents the vast majority of T cells present in neonates and umbilical cord blood (UCB). IL-7 stimulation of UCB CD4 T cells at 2.5% O_2 resulted in a significantly attenuated level of cell cycle entry ($P < 0.05$; $n = 3$), with an effect similar to that which we observed in mature peripheral blood CD4 T cells (Fig. S5). Moreover, like mature CD4 T cells, the infection of IL-7–stimulated UCB CD4 T cells was not attenuated at 2.5% O_2 (NS, $P > 0.05$; $n = 5$; Fig. S5), providing further evidence that decreased cell cycle entry did not modulate infection of IL-7–stimulated CD4 T cells at physiological O_2 concentrations.

Stimulation of CD4 T Cells at Physiological O_2 Concentrations Results in Enhanced Surface Glut1 Expression and Glucose Uptake. It has long been postulated that the inability of quiescent T cells to be efficiently infected by HIV-1 is likely due, at least in part, to their very low metabolic state. Whereas this characteristic was once thought to be the default mode of T lymphocytes, it is now known that many factors actively control this quiescence (reviewed in ref. 5). One intriguing possibility to explain the permissivity of IL-7–stimulated T cells to HIV infection at low O_2 concentrations, even in the absence of cell cycle entry, is an enhanced metabolic activity and, specifically, an augmented uptake and utilization of glucose. This hypothesis is based, at least in part, on previous data showing that expression of the ubiquitous glucose transporter Glut1 is up-regulated in response to low O_2 in several cell types (31). Although Glut1 has not been detected at significant levels at the surface of quiescent T cells (32–34), it is induced by TCR stimulation (35, 36), as well as by the IL-7 cytokine (34, 37, 38).

Surface Glut1 expression was significantly augmented following IL-7 stimulation at 2.5% O_2 conditions compared with 20% O_2 (Fig. 3*A*; $P < 0.05$; $n = 8$). Furthermore, this increase in surface Glut1 expression was accompanied by a 300% increase in glucose uptake at physiological O_2 concentrations, as measured by the ability to uptake nonhydrolyzable 2-deoxy-D-[1-^{3}H]glucose (Fig. 3*B*).

HIV-1 Infection Is Abrogated in the Absence of Surface Glut1. Glut1 is a downstream substrate of the AKT/PI3K pathway, as assessed in both lymphocyte and nonlymphocyte lineage cells (reviewed in refs. 39 and 40). Moreover, expression of an activated transgenic Akt in murine T cells was found to result in an augmented glycolysis (32). To determine the role of this pathway in IL-7–induced surface Glut1 induction and subsequent HIV-1 infection, we used a PI3K inhibitor, LY294002. Incubation of IL-7–stimulated T cells with the LY294002 inhibitor resulted in an almost complete suppression of surface Glut1 as well as glucose uptake, both under 20% and 2.5% O_2 conditions (Fig. 3 *A* and *B*). This LY294002-mediated Glut1 inhibition was sufficient to completely abrogate single-round HIV-1 infection under either O_2 condition (Fig. 3*C*).

To verify that this effect was mediated by the Glut1 transporter itself, we used siRNAs directed against the Glut1 3′ UTR to specifically down-modulate endogenous Glut1 expression (41). Transfection of a mix of three different Glut1 siRNAs led to an approximate 70% decrease in Glut1 mRNA, to levels similar to those detected in nonstimulated CD4 T cells (Fig. S6).

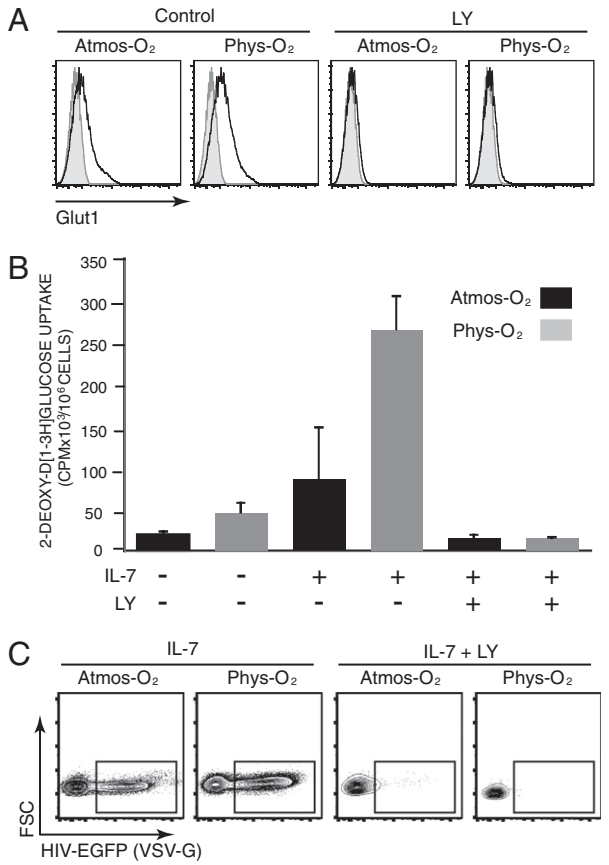

Fig. 3. IL-7–induced expression and function of the Glut1 glucose transporter are enhanced under physiological O_2 conditions, and its PI3K-mediated abrogation results in attenuated HIV-1 infection. (*A*) IL-7–stimulated APB CD4 T cells were cultured in the absence or presence of the PI3K inhibitor LY294002 (LY; 15 μM) and, at day 3, Glut1 expression was monitored. Representative histograms show specific Glut1 staining (open) relative to control staining (filled). (*B*) Glucose uptake was monitored in 1×10^6 CD4 T cells cultured for 3 d in the absence (–) or presence (+) of IL-7 and the LY inhibitor, as above. Lymphocytes were incubated with 2-deoxy-D-[1-^{3}H]glucose (2 μCi) for 10 min at room temperature. Results are expressed as mean cpm ± SD for triplicate sample analysis from cells cultured under 20% (black) or 2.5% (gray) O_2 conditions. (*C*) IL-7–stimulated CD4 T cells, cultured in the absence or presence of LY, were infected with VSV-G–pseudotyped HIV-EGFP virions. Infection was monitored 48 h later and the percentages of EGFP$^+$ cells are indicated. Data are representative of results obtained in six independent experiments comprising seven different donors.

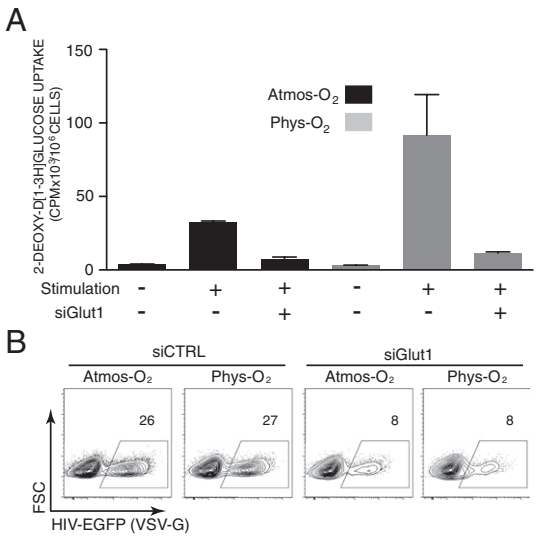

Fig. 4. Inhibition of Glut1 up-regulation attenuates HIV-1 infection under atmospheric and physiological O_2 conditions. (*A*) CD4 T cells from APB were transfected with Glut1-specific siRNAs (siGlut1) and then stimulated under 20% (Atmos-O_2) or 2.5% (Phys-O_2) conditions for 24 h. Glucose uptake was monitored by incubating cells with 2-deoxy-D-[1-^{3}H]glucose (2 μCi) for 10 min at room temperature. Results are expressed as mean cpm ± SD for triplicate samples. (*B*) CD4 T cells stimulated following transfection with control (siCTRL) or Glut1-specific siRNAs were infected with VSV-G–pseudotyped HIV-EGFP virions. Infection was monitored at 48 h and the percentages of EGFP$^+$ cells are indicated. Data are representative of results obtained in four independent experiments comprising four different donors.

Moreover, this decrease in Glut1 mRNA resulted in a 70–80% attenuation in glucose uptake under both physiological and atmospheric O_2 conditions (Fig. 4*A*). The decrease in Glut1 and glucose uptake resulted in an average 70% inhibition of HIV-1 infection under both atmospheric and physiological O_2 conditions (Fig. 4*B*). Thus, the susceptibility of CD4 T cells to HIV-1 infection, at both physiological and atmospheric O_2 conditions, is regulated via the activity of the Glut1 glucose transporter.

Glut1-Expressing Human Double Positive (DP) Thymocytes Present a Significantly Enhanced Susceptibility to HIV-1 Infection. Thymocyte destruction by X4-tropic HIV-1 initially occurs in the DP population (42), and preferentially in those DP cells expressing higher levels of CD4 (CD4hiDP) and CXCR4 (43). Intriguingly, we previously found that Glut1 is expressed on ∼10% of all human thymocytes, and this marker characterizes a subset of DP cells with high CD4 expression (44). As shown in Fig. 5*A*, CD4hiDP thymocytes express significantly higher levels of Glut1 and CXCR4 than CD4loDP thymocytes or single-positive (SP) SP8 or SP4 thymocytes. On the basis of the data obtained in CD4 T cells, it was possible that the high metabolic activity of CD4hiDP thymocytes conditioned their susceptibility to HIV-1 infection. We hypothesized that high surface Glut1 expression may result in an augmented susceptibility of these cells to HIV-1 infection, notwithstanding their high CD4 and CXCR4 receptor levels.

To test this hypothesis, we needed to infect thymocytes in a manner that bypassed the CD4/CXCR4 entry requirement. As such, we infected thymocytes with HIV-1 virions pseudotyped with the pantropic VSV-G envelope and, to avoid bias resulting from thymocyte mortality, freshly isolated total thymocytes were infected for only 12 h. At that time, thymocyte populations were sorted and immediately lysed for DNA extraction. Nested PCR was performed to analyze viral reverse-transcription products (GFP) and integrated provirus (Alu-LTR products). Notably, the HIV-1 core virions infected Glut1+DP thymocytes with significantly higher efficacy than other thymocyte populations. Enhanced infection was largely generated at a stage in the viral life cycle that occurred before or during reverse transcription, with a 6.5-fold higher level of GFP DNA in CD4hiDP compared with CD4loDP thymocytes. Levels of nuclear entry and integration were correspondingly higher, with seven- to ninefold increases in integrated Alu-LTR products in this subset compared with either Glut1-CD4loDP or SP subsets (Fig. 5*B*). The preferential infection of the CD4hiDP population could potentially promote the transmission of HIV-1 to more mature thymocyte progeny, resulting in a reservoir of infected T lymphocytes in vivo (43). This finding has important implications for the subsequent maintenance of the virus in a quiescent T-cell pool.

Discussion

Here we show that IL-7–mediated activation and cell cycle entry are strikingly altered by the O_2 concentration to which CD4 T cells are exposed. Notably, though, IL-7 stimulation renders CD4 T lymphocytes susceptible to HIV-1 infection, irrespective of the O_2 concentration. We find that it is Glut1-mediated glucose uptake, rather than cell cycle entry per se, that regulates HIV-1 infection in

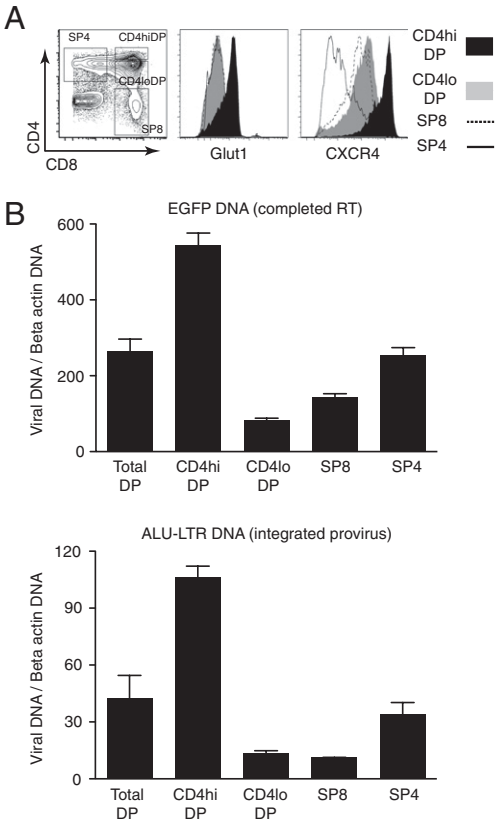

Fig. 5. Glut1 expression is associated with increased permissivity of the CD4hiCXCR4hi DP human thymocyte subset to VSV-G–pseudotyped HIV-1 infection. (*A*) Glut1 and CXCR4 staining in the CD4hiDP, CD4loDP, SP4, and SP8 human thymocyte subsets are shown as histogram overlays. The gates used to define and FACS sort these subsets are shown, and the percentages of cells in each quadrant are indicated. (*B*) Freshly isolated human thymocytes were infected for 12 h with a single-round VSV-G–pseudotyped HIV-1. Thymocyte populations were subsequently sorted by FACS and quantitative PCR analysis was performed to detect EGFP DNA (*Upper*), indicative of completed reverse transcription, as well as a two-step nested PCR for Alu-LTR DNA to detect integrated provirus (*Lower*). Signals were normalized to β-actin DNA. Results for each subset are expressed as means ± SD of triplicate samples.

these cells, establishing a unique paradigm for HIV-1 infection. Permissivity to HIV-1 infection was directly dependent on Glut1 activity, as Glut1-specific siRNAs inhibited infection of both X4- and VSV-G–pseudotyped viruses. Furthermore, within the human thymus, where Glut1 is differentially expressed, HIV-1 infection was markedly higher in the Glut1-expressing DP subset compared with Glut1-negative thymocytes. Therefore, we identify the Glut1-mediated metabolic pathway as a critical regulator of HIV-1 infection in human CD4 T cells and thymocytes.

Regulation of Glut1, the major glucose transporter in T lymphocytes, is quite complex, as it is controlled at the level of transcription, translation, and transport to the cell surface. Indeed, in most cell types, it is glucose uptake across the plasma membrane that is the rate-limiting step in the production of ATP. In response to low O_2 levels, the enhanced expression and function of Glut1 is mediated, at least in part, by the hypoxia-inducible factor 1 transcription factor (31). Augmented Glut1 levels have been shown to play an important role in the survival and "fitness" of tumor cells adapted to hypoxic conditions (reviewed in ref. 45) but, to our knowledge, this is a unique report showing that Glut1 levels on T cells can be modulated by the O_2 conditions to which they are exposed.

Surface Glut1 levels on CD4 T cells were augmented at 2.5% O_2 levels following IL-7 stimulations (Fig. 3). The high repercussion on glucose uptake, with a dramatic 300% increase in transport, is likely due not only to increased Glut expression but also to conformational changes in Glut1 itself. It has been shown that cytoplasmic ATP inhibits Glut1-mediated glucose uptake by favoring an interaction between the Glut1 C terminus and Glut1 cytoplasmic loop 6–7 (46). Therefore, under conditions where ATP levels are lower at physiological O_2 concentrations compared with atmospheric O_2, the Glut1 conformation would be, at least, partially released from this inhibitory effect.

It is well-known that efficient HIV-1 infection is highly dependent on T-cell activation and cell cycle entry, with quiescent lymphocytes showing very inefficient HIV-1 infection (reviewed in ref. 5). This paradoxical observation, in the context of a lentivirus otherwise shown to infect nondividing cells, has been puzzling. The factor(s) controlling infection in resting T cells has not yet been completely elucidated, but recent studies indicate important roles for cellular c-Jun N-terminal kinase (47) and chemokine-induced changes in the actin cytoskeleton (48). The role of the PI3K pathway in HIV infection of primary T cells is somewhat controversial, as early studies, performed in transformed cell lines and activated T cells, reported that PI3K negatively impacts HIV-1 transcription (48), whereas later work found that PI3K signaling is required for HIV integration in chemokine-treated quiescent $CD4^+$ T cells (49). The results reported here are in agreement with the more recent study, as blocking PI3K signaling in IL-7–stimulated resting T cells abrogated HIV-1 infection. Furthermore, our data suggest that inhibition of HIV-1 infection by PI3K inhibitors is mediated, at least in part, via the suppression of Glut1 expression and function.

Our finding that IL-7–mediated glucose uptake is associated with a sustained permissivity to HIV-1 infection at physiological O_2 levels brings unique perspectives to previous reports showing that G0-phase T cells can be infected by HIV-1 when present in lymphoid aggregate cultures (1, 2, 50). Specifically, in these latter conditions, aimed at more faithfully reproducing the in vivo system, T cells within the aggregates would be exposed to significantly lower levels of O_2 than those delivered to suspension T cells cultured under atmospheric conditions. Although we reveal the Glut1-mediated pathway as critically impacting on HIV-1 infection under both atmospheric and physiological O_2 concentrations, it is likely that O_2 modulates IL-7–mediated HIV-1 infection via other pathways as well. Intriguingly, the transactivational properties of the HIV-1 Tat protein promote HIV-1 infection at physiological but not atmospheric O_2 conditions (51). Thus, it will be of interest to reassess some of the earlier ex vivo HIV studies to determine whether the data were modulated by the O_2 tensions at which the experiments were performed.

The ensemble of the data presented here strongly suggests a role for IL-7 in reversing HIV-1 latency in the low O_2 environments found in spleen, lymph nodes, and thymus, thereby eliminating latent reservoirs (reviewed in ref. 8). It will be important to assess whether IL-7 administration modulates glucose uptake and metabolism in T lymphocytes of HIV-1–infected patients and to determine whether the outcome of IL-7 cytokine therapy differs in the high O_2 environment of the peripheral circulation compared with the relatively low O_2 state of lymphoid organs.

Materials and Methods

Cell Isolation and Culture. T cells were isolated from adult peripheral blood, umbilical cord blood, and thymi as described in *SI Materials and Methods*. Cells were cultured in RPMI media supplemented with human recombinant IL-7 (10 ng/mL; kindly provided by Cytheris) as described in *SI Materials and Methods* and, when indicated, LY294002 was added.

Flow Cytometry. To detect expression of surface markers, cells were incubated with the appropriate fluorochrome-conjugated mAbs (BD Biosciences), or with 7-amino-actinomycin-D and pyronin Y as described in *SI Materials and Methods*. Surface Glut1 expression was monitored by binding to a recombinant human T-lymphotrophic virus-2 envelope receptor-binding domain (HRBD) fused to the EGFP coding sequence (HRBDEGFP) as described (41, 52).

Glucose Uptake Assays. Uptake was performed for 10 min at room temperature upon addition of 2-deoxy-D-[1-^{3}H]glucose (Amersham Biosciences) as described in *SI Materials and Methods*.

siRNA Transfections for Glut1 Inhibition and Analyses of Glut1 Transcripts. Purified T cells were transfected with the indicated synthetic siRNAs, complementary to the Glut1 3′ UTR (41), as described in *SI Materials and Methods*. Quantitative PCR for Glut1 and 18S was performed as described in *SI Materials and Methods*.

Virus Production and Infections. Self-inactivating single-round HIV-1 virions were generated by transient transfection of 293T cells as published (53) and described in *SI Materials and Methods*. For single-round infections, lymphocytes were infected with HIVenv712-pseudotyped vector or VSV-G–pseudotyped HIV-1 vector as indicated in *SI Materials and Methods*.

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Review Article

Metabolic pathways as regulators of HIV infection

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Metabolic pathways as regulators of HIV infection

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Purpose of review
Activation of the immune system only occurs when stimulated cells generate sufficient energy to support their growth and proliferation. Moreover, efficient HIV-1 infection requires that $CD4^+$ T cells meet the energy demands involved in completing the different steps of the virus life cycle. In this review, we highlight recent studies revealing the importance of nutrient fuels, nucleotide metabolism and the oxygen microenvironment in regulating HIV-1 infection, T-cell differentiation and the generation of HIV-1-specific immune responses.

Recent findings
Glucose uptake via the Glut1 glucose transporter is required for efficient HIV-1 infection of $CD4^+$ lymphocytes. Other nutrients can also be used as sources of energy and their utilization conditions the differentiation of $CD4^+$ T cells to distinct effector fates. The conversion of ATP to adenosine inhibits HIV-specific effector cells and the hydrolysis of dNTPs by SAMHD1 restricts infection. Furthermore, oxygen concentration modulates metabolic status, thereby altering T-cell differentiation and potential to mediate a specific immune response.

Summary
The availability and use of energy resources in fluctuating environments regulate T-cell function and susceptibility to HIV-1 infection. Identification of the targets coordinating the selected metabolic pathways will advance new strategic avenues for controlling HIV-1 disease progression.

Keywords
glucose, glut1, HIV-1, hypoxia, metabolism

INTRODUCTION

The activation of quiescent $CD4^+$ T cells renders them highly susceptible to HIV-1 infection. In this regard, it is important to note that T-cell activation cannot proceed without an increased nutrient availability, to meet the new energetic and biosynthetic demands of the cell. The generation of ATP, the synthesis of nucleic acids and phospholipids, de-novo lipogenesis and the production of reducing equivalents in the form of NADPH are fueled by an augmented nutrient transport and utilization. We have recently found that glucose uptake via the Glut1 glucose transporter is required for efficient HIV-1 infection of $CD4^+$ lymphocytes. However, it is important to note that the metabolic state of a $CD4^+$ T-cell not only regulates its susceptibility to HIV-1 infection but also conditions its differentiation to distinct effector fates. This review provides an overview of recent research revealing the importance of immune cell metabolism in HIV-1 disease progression. We focus on the routes via which nutrient fuels, nucleotide metabolism and the oxygen microenvironment govern HIV-1 infection, T-cell activation and differentiation and HIV-1-specific immune responses.

TEXT OF REVIEW

Glu1-mediated glucose transport is required for efficient HIV-1 infection of $CD4^+$ lymphocytes

Efficient HIV-1 infection of $CD4^+$ T cells requires cytokine stimulation or T-cell receptor (TCR) engagement as quiescent lymphocytes are relatively refractory to infection. The diverse stimulations that promote cell cycle entry of lymphocytes that are in

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KEY POINTS

- The susceptibility of $CD4^+$ T cells to HIV-1 infection is regulated by cell metabolism, requiring Glut1-mediated glucose uptake.
- The utilization of nutrient fuels conditions the differentiation of $CD4^+$ and $CD8^+$ T cells to distinct effector fates.
- gp120-triggered ATP release is required for HIV-1 infection and the CD39/CD73-mediated conversion of ATP to adenosine induces Treg suppressive activity, inhibiting HIV-specific effector T-cell function.
- SAMHD1 inhibits HIV-1 infection in dendritic cells, monocytes and quiescent $CD4^+$ T cells by hydrolyzing dNTPs to levels below that required for efficient reverse transcription but this effect is antagonized by the HIV-2 and SIVsm/SIVmac Vpx proteins which target SAMHD1 for proteasome-dependent degradation.
- Oxygen tensions of 2–3%, such as those found in lymphoid organs, modulate T-effector differentiation and metabolism.

the G0 phase of the cell cycle enhance susceptibility to HIV-1 infection [1,2]. Thus, the environment of a $CD4^+$ lymphocyte regulates its capacity to promote HIV-1 reverse transcription, nuclear import, integration and the expression of viral proteins necessary to complete the viral life cycle.

Elucidating the precise changes that foster HIV-1 infection following T-cell activation, either via cytokine stimulation or TCR engagement has been the focus of intense study for many years. T-cell stimulation results in the induction of a myriad of signaling pathways and research from many groups has added to our understanding of the mechanisms regulating kinase phosphorylation and activation, recruitment of adapter/scaffold proteins, induction of transcription factors and transcription of cytokines. However, studies assessing the role of nutrient resources in T-cell activation have been much more recent. This latter field of investigation is critical as we now recognize that the activity of nutrient transporters as well as fuel availability and utilization modulate the function and fate of a T lymphocyte [3,4].

Glucose provides cells with a key source of energy and the transport of this nutrient is a universally conserved property of living organisms. In vertebrates, Glut1 appears to be the main glucose transporter (reviewed in [5]). Glut1 expression is induced by various treatments (reviewed in [6]), including glucose starvation [7,8], hypoxia [9], inhibition of oxidative phosphorylation [10] and osmotic stress [11]. Whereas the Glut1 protein does not appear to be expressed at significant levels at the surface of quiescent T cells [12–14], we and others have shown that it is induced by TCR stimulation [15,16], as well as by the IL-7 cytokine [14,17–21].

The interest of our groups in this domain was motivated, at least in part, by our identification of the Glut1 glucose transporter as the human T-cell leukemia virus (HTLV) receptor ([14,22–24]. Notably, cell surface up-regulation of metabolite transporters is a rate-limiting step for nutrient uptake following cell stimulation. The induction of these metabolite transporters is often controlled by their translocation from intracellular compartments to the cell surface and not necessarily by an increased transcription and/or translation [25,26]. Whereas it had been difficult to assess this parameter on live cells because of a lack of reliable antibodies directed against extracellular determinants, the development of tagged HTLV receptor binding domain fusion proteins [27,28] has allowed surface Glut1 expression to be directly monitored.

The up-regulation of Glut1 on T lymphocytes as well as thymocytes increases their ex-vivo susceptibility to HIV-1 infection [29▪▪,30▪▪]. Moreover, Glut1 levels have recently been shown to be increased on $CD4^+$ T cells during chronic HIV-1 infection *in vivo* [30▪▪]. The Glut1-mediated function of glucose transport is proposed to be responsible for its positive regulation of HIV-1 infection. Nevertheless, it is important to note that Glut1 can also transport the 2-electron oxidized form of ascorbic acid, L-dehydroascorbic acid (DHA) [31–33] and in some conditions, glucose does not competitively inhibit this transport [34–36]. As ascorbic acid is one of the most potent-reducing agents, Glut1 function may also modulate the redox potential of the cell. Thus, whereas Glut1-mediated function is now known to condition the susceptibility of a $CD4^+$ T cell to HIV-1 infection, it will be of interest to determine whether this effect is regulated entirely at the level of glucose uptake or whether DHA transport also plays a role in this process.

Nutrient transport and utilization conditions T-cell effector differentiation

Glucose is a key nutrient in mediating T-cell activation but the increased metabolic requirements of proliferating cells can be provided by energy sources other than glucose, including lactate, fatty acids, and glutamine. $CD4^+$ T cells polarized to different effector fates appear to have distinct glycolytic requirements; Th1, Th2, and Th17 cells show much higher glycolysis than suppressive regulatory T cells (Tregs) [37▪,38▪]. In contrast, Tregs exhibit high rates of fatty acid oxidation

and blocking this pathway appears to impair Treg differentiation but not differentiation of other effector subsets [37▪].

Within the $CD8^+$ T-cell population, the generation of a long-lived memory $CD8^+$ T-cell subset (from $CD8^+$ T-effector cells) depends on a switch in energy metabolism from glucose to fatty acid metabolism [3,39,40▪]. This switch requires an increase in mitochondrial biogenesis and up-regulation of carnitine palmitoyl transferase (CPT1α), an enzyme controlling the rate-limiting step to mitochondrial fatty acid oxidation (FAO) [3,39,40▪]. Amino acid availability may also be required for the development of memory $CD8^+$ T cells [39] and importantly, Sundrud *et al.* [41] found that amino acid availability conditions $CD4^+$ T-cell polarization. Specifically, they showed that low levels of cysteine and methionine amino acids are not detrimental to Th1, Th2, or Treg polarization, but abrogate Th17 function [41].

There is, therefore, a need for research addressing the question of whether the forced utilization of one 'fuel' as compared with another, potentially by changing the relative levels of ATP generation, lipogenesis and nucleotide, as well as amino acid biosynthesis, modulates HIV-1 infection (Fig. 1) and/or HIV-specific immune responsiveness. Although it is well known that memory $CD4^+$ T cells are significantly more permissive to HIV-1 infection than naïve $CD4^+$ T cells, the vast majority of research has focused on differences in HIV co-receptor levels as well as to integrins such as α4β7, which can bind HIV-gp120 [42]. Expression of CCR6 (on Th1 as well as Th17 subsets) has also been shown to be associated with increased susceptibility to HIV-1 infection [43]. These cell surface proteins have been proposed to enhance HIV-1 infection by altering virus attachment and T-cell migration (i.e. CCR6-mediated trafficking to the intestine as

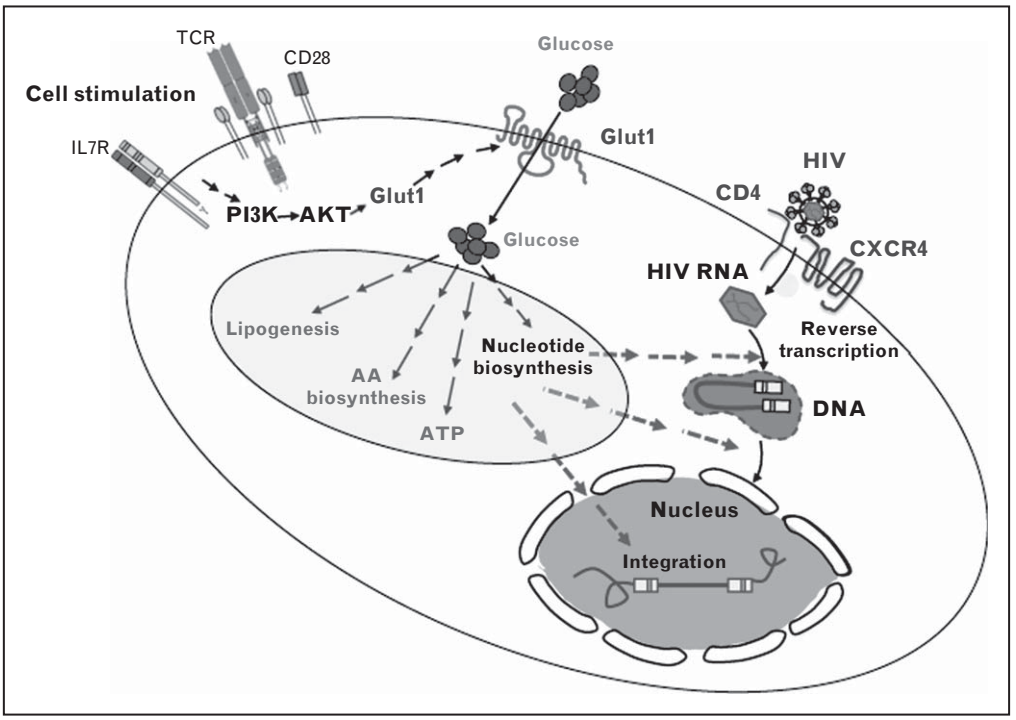

FIGURE 1. HIV infection is promoted by TCR/cytokine-mediated up-regulation of nutrient transporters. HIV infects $CD4^+$ T lymphocytes via CD4 and the CXCR4 or CCR5 co-receptor (CD4/CXCR4 is shown in the figure). However, once this occurs, uncoating of the entering virion, reverse transcription, nuclear entry and integration of the viral DNA are critical for a productive infection. These steps are inefficient in quiescent T lymphocytes but TCR and/or cytokine stimulation results in a significantly higher level of infection. Differences between quiescent and activated T cells are numerous but one major change is the metabolic state of the cells. Upon activation (TCR and IL-7 stimulations are represented here), there is a cell surface up-regulation of multiple nutrient transporters including the Glut1 glucose transporter via a PI3K/AKT-mediated pathway. The enhanced transport of glucose results not only in increased ATP production but also in nucleotide biosynthesis, amino acid biosynthesis, and lipogenesis (indicated in the pale green circle). We and others have found that blocking glucose transport in T cells inhibits HIV-1 infection [29▪▪,30▪▪], but the precise metabolic processes regulating HIV-1 infection remain to be determined. The possible steps wherein glucose metabolism can influence HIV-1 infection are presented as dashed arrows. TCR, T-cell receptor.

compared with lymphoid organs). However, in the future, it will be important to assess whether infection is modulated by the metabolic state of these effector subsets.

Nucleotide metabolism regulates HIV-1 infection and immune responsiveness

Purinergic receptors (P2X and P2Y subclasses) are activated by extracellular adenosine triphosphate (ATP) and it has recently been shown that the interaction of the HIV gp120 envelope with its receptor stimulates a local release of ATP, thereby directly triggering this receptor family [44▪▪,45▪▪]. Importantly, inhibiting any of the constituents of this pathway blocks HIV infection in both primary T cells and macrophages, showing a direct effect of extracellular nucleotides on the viral life cycle [44▪▪,45▪▪]. This impact of extracellular nucleotides on HIV replication is also likely to be associated with the activation state of the cell; TCR stimulation induces the release of cellular ATP and positive feedback through purinergic receptors is required for sustaining this activation [46–48].

The second messenger cAMP also impacts on HIV-1 infection by several distinct processes. The CD39/CD73-mediated conversion of ATP to adenosine, via hydrolysis of ATP to ADP/5-AMP followed by cleavage to adenosine, respectively, induces the expansion as well as suppressive activity of Tregs [49]. In accord with these results, adenosine stimulation via the adenosine A(2A) receptor promotes the induction of Treg while inhibiting the development of cytotoxicity and effector function in conventional T cells [50–52]. Importantly, in HIV-infected patients, blocking CD39 activity on Treg results in improved HIV-specific effector T cell function [53▪▪]. Furthermore, it is intriguing to note that a CD39 polymorphism associated with lower CD39 expression on Treg results in a slower progression to AIDS [53▪▪]. Nevertheless, the effects of adenosine production are likely to be multiparametric as inhibition of CD39 activity also blocks the Treg-mediated suppression of HIV replication [54,55].

The availability of nucleotide pools also plays direct as well as indirect roles in HIV-1 progression. The failure of HIV-1 to infect dendritic cells and the refractoriness of monocytes to HIV-1 infection was recently shown to be due to the effects of SAMHD1 [56▪▪,57▪▪]. Interestingly, prior to the discovery of its role in HIV-1 infection in 2011, there were less than 20 references to SAMHD1 in PubMed even though previous elegant work had shown that it is one of the genes responsible for Aicardi-Goutières syndrome (AGS) [58▪▪]. AGS is a rare multisystemic genetic neurodegenerative disorder with early-onset encephalopathy resembling congenital viral infection. These latter symptoms, with up-regulation of interferon-stimulated genes, provided the first evidence that SAMHD1 may act as a negative regulator of innate antiviral responses [58▪▪]. Importantly, SAMHD1 was found to hydrolyzes deoxynucleoside triphosphates (dNTPs), thereby lowering their concentration to levels below that which are required for the efficient reverse transcription of HIV-1 [59▪▪]. Thus, SAMHD1 plays a critical role in cells with low dNTP pools such as dendritic cells, monocytes, and quiescent $CD4^+$ T cells [56▪▪,57▪▪,60▪,61▪]. Notably, the effects of SAMHD1 are antagonized by the Vpx protein of HIV-2 and SIVsm/SIVmac; this viral protein promotes the proteasome-dependent degradation of SAMHD1 [56▪▪,57▪▪]. The recent studies described above have focused on the direct effects of SAMHD1 in impairing HIV-1 reverse transcription and productive infection but it will also be of interest to determine whether the Vpx-mediated degradation of SAMHD1 induces an interferon-mediated innate immune response due to the accumulation of nucleic acid debris.

Oxygen-mediated regulation of T-cell metabolism, T-cell fate and HIV-1 infection

The progression of HIV-1 infection is often evaluated as a function of peripheral $CD4^+$ T-cell counts as well as the viral load within those peripheral T cells. However, peripheral blood T cells account for only approximately 2% of the body's T cells, as estimated in humans and precisely evaluated in rhesus macaques [62,63]. Moreover, it is important to note that while SIV infection results in decreased $CD4^+$ cell counts in the blood (in accord with some of the earliest research in HIV-1-infected humans), $CD4^+$ cell counts in spleen and lymph nodes actually increase during early infection and remain stable in nonlymphoid organs [63]. These data are critical for our understanding of the progression of HIV-1 disease and raise important questions as to the differences in the microenvironments in which peripheral blood T cells and lymphoid organ T cells are found. There are clearly a multitude of differences, including the cytokine microenvironment, cell–cell contacts, cell density, local nutrient availability and local oxygen concentrations. Given the context of this review, we will focus on how oxygen concentration changes the metabolic state of a T cell, thereby altering its function and polarization. The potential for these effects to modulate HIV-1 infection will then be discussed.

It is known that the in-vivo oxygen tensions in lymphoid organs range from approximately

0.5–4.5%, whereas partial oxygen pressures in the arterial peripheral blood increase up to 13% [64]. Thus, the oxygen concentrations to which $CD4^+$ T cells are exposed in the lymph node/spleen are significantly lower than those to which they would be exposed in the peripheral circulation. Furthermore, the oxygen pressure used in the vast majority of ex-vivo T-cell activation experiments and HIV-1 infection assays is even higher as it reflects the atmospheric concentration of 21%. This is important as the responses of T cells to TCR stimulation are modulated by oxygen concentrations: Studies comparing TCR responses at 2–5% oxygen and 21% oxygen found lower levels of proliferation [65], calcium flux [66] and activation-induced cell death [67,68] as well as changes in secreted cytokines [64,69].

At least some of the changes in T-cell responsiveness at lower oxygen concentrations are regulated by the oxygen-dependent stabilization of the hypoxia-inducible transcription factors, HIF-1α and HIF-2α [70]. Indeed, several of the consequences of HIF-1α deletion or activation correspond precisely to the effects observed in response to changes in oxygen concentration; deletion of HIF-1α results in a markedly-enhanced cytokine secretion [71], whereas constitutive activation of HIF-1α results in decreased calcium flux [72].

In the context of HIV-1 infection, HIF-1α activation can alter T effector function and polarization. Two recent studies reported that deletion of HIF-1α severely impairs Th17 differentiation while enhancing the generation of Tregs [73,74▪]. However, two other studies found that hypoxia induces expression of the Treg transcription factor Foxp3, via a direct transcriptional activation by HIF-1α, and enhances Treg abundance both *in vitro* and *in vivo* [73,74▪]. These differences may be due to distinct experimental approaches assessing the effect(s) of HIF-1α in atmospheric as compared with hypoxic conditions but the ensemble of these studies points to an important role for oxygen in modulating T-cell fate.

The effects of oxygen also interrelate significantly with the metabolic/nutritional state of the T cell. This occurs, at least in part, because Glut1 levels in T lymphocytes are induced under conditions of hypoxia [9]. Glut1 transcription is directly regulated by HIF-1 binding to *cis* acting sites located within the 5′ flanking region of the glut1 gene [75,76]. The glucose transport function of Glut1 may also be augmented in hypoxia as ATP itself reduces net glucose transport by inducing conformational changes in the Glut1 molecule [77]. Finally, HIF-1 has recently been shown to be required for sustaining glucose metabolism and glycolysis in effector $CD8^+$ cytotoxic T lymphocytes, regulating their differentiation [78].

Upon T-cell activation by cytokines, such as IL-7 or following TCR engagement, Glut1 is up-regulated as described above [14–18,21], and in our most recent studies, we found that this up-regulation is significantly enhanced at 2% oxygen conditions [29▪▪]. Moreover, under low oxygen conditions, glucose uptake is augmented by more than three-fold, likely due to a lower level of ATP in the cells [29▪▪]. Interestingly, despite this increase glucose metabolism, both IL-7 and TCR-stimulated T cells show lower levels of cell cycle progression and proliferation as compared with equivalent stimulations at 20% oxygen [29▪▪].

As the susceptibility of quiescent T cells to HIV-1 infection is extremely low [79], we assessed whether infection would continue to be inefficient under low oxygen conditions. This finding was of particular interest as cytokine stimulation under low oxygen results in an environment wherein glucose metabolism is significantly increased as compared with freshly isolated quiescent lymphocytes but cell cycle entry is only minimal. Importantly, we found that the susceptibility of these lymphocytes to HIV-1 infection, at least at the levels of single round infection, is directly dependent on Glut1 activity and function [29▪▪].

These data may help explain reports of HIV-1 infection in quiescent T cells that are located within lymphoid aggregates cultures [80–82]. In the latter context, the microenvironment likely results in oxygen concentrations that are significantly lower than the atmospheric oxygen levels to which suspension lymphoid cells are exposed and as such, lymphocytes may exhibit higher Glut1 levels and associated glucose transport activity. We also found that in the human thymus, Glut1-expressing thymocytes exhibit a seven-fold higher susceptibility to single round infection as compared with Glut1-negative thymocytes [29▪▪,83]. Notably though, HIV-1 infection at lower oxygen concentrations is clearly regulated by multiple parameters and not only by changes in glucose metabolism. At low oxygen (3%), Tat-induced HIV-1 transcription is reduced, due to decreased CDK9/cyclin T1 activity [29▪▪,83], but it also appears that recombinant Tat primes cells for HIV-1 infection at 5% but not 20% oxygen conditions [29▪▪,83]. Altogether, these data establish a new paradigm for HIV-1, showing the impact of the metabolic state of the lymphocyte in mediating susceptibility to infection.

Effects of antiretroviral therapy on cellular energy status

Life expectancy of individuals infected with HIV-1 has increased to more than 25 years, in large part

due to the introduction of combination antiretroviral therapy. These drugs can be divided into different classes and consist of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors, as well as newer fusion inhibitors, integrase inhibitors and chemokine receptor antagonists. These therapies are undoubtedly beneficial but they have also been associated with significant adverse side effects. Many of these fall into the category of metabolic anormalies including hyperglycemia/insulin resistance, hepatobiliary complications, dyslipidemia, lipodystrophy, subcutaneous fat wasting, abdominal obesity, pancreatitis, and lactic acidosis amongst others. These adverse drug reactions result from a number of cause including direct mitochondrial toxicity (reviewed in [84]).

Protease inhibitors are associated with hyperbilirubinemia, hyperlipidemia, hyperglycemia, and dyslipidemia, all of which will affect the metabolic microenvironment in which immune cells are found. Indeed, metabonomic analyses (by NMR and mass spectroscopy) have elegantly demonstrated significant changes in the metabolite composition of biofluids in HIV-1-treated patients (reviewed in [85▪▪]). It is also important to note that the positive effects of NRTIs are often limited by their direct mitochondrial toxicity; they reduce mitochondrial DNA replication in a manner similar to that by which they block HIV DNA replication. The ensuing mitochondrial dysfunction results in an impaired oxidative phosphorylation with subsequent decreases in oxidative phosphorylation. This result in turn leads to an inability to oxidize fatty acids, with accumulation of metabolites such as adipic and suberic acids, and the triggering of glycolysis. For the patient, the disrupted glycose/carbohydrate metabolism can translate to serious symptoms of hepatic steatosis and lactic acidosis [84,86–88].

The metabolic consequences of NRTIs and protease inhibitors are likely to be more pronounced in the context of HIV-1 infection as it is known that HIV-infected cells have a high energy demand. Recent metabonomics analyses have shown increased glucose and triglyceride levels in HIV-1-infected individuals associated with high IL-6, a cytokine contributing to the progression of inflammatory diseases [85▪▪]. In light of the data described above, it will be critical to directly evaluate metabolic alterations of $CD4^+$ and $CD8^+$ lymphocyte subsets in the context of HIV-1 infection and determine the impact of treatments with NRTIs and other HIV-1 inhibitors on T-cell metabolism. These studies will enable us to determine how metabolic changes associated with infection and treatment modulate the susceptibility of $CD4^+$ T cells to infection as well as their capacity to undergo specific effector fates needed for the development of an optimal HIV-1-specific immune response.

CONCLUSION

The finding that changes in metabolic programs condition T-cell activation and differentiation as well as susceptibility to HIV-1 infection opens new strategic avenues for controlling disease progression. Furthermore, elucidating the metabolic/energetic parameters that modulate the capacity of immune cells to mediate an efficient HIV-specific immune response will be critical for future immune-based anti-HIV therapies. However, harnessing the benefits of such therapies may be complex as molecules such as cAMP can inhibit viral replication while also suppressing antiviral immune responses. Future studies should be aimed at identifying new targets regulating the metabolic demands of T lymphocytes in order to develop novel therapeutic strategies restraining viral replication and enhancing antiviral immune responses.

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Conflicts of interest

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