DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Targeting T cell metabolism: A new tool for modulating T cell function

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação da Doutora Naomi Taylor (Universidade de Montpellier) e do Professor Doutor Carlos Duarte (Universidade de Coimbra)

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2015

Todas as actividades experimentais foram realizadas no Institut de Génétique Moléculaire de Montpellier (França).O trabalho apresentado nesta tese foi realizado sob a orientação científica da Dra. Naomi Taylor e co-orientação científica da Dra.Valérie Dardalhon.

Acknowledgement

To my lab and my friends in Montpellier:

During these 10 months in Montpellier I had the opportunity to growth as a scientist and to work in a fantastic laboratory. I could write a lot of pages about all the adventures and the scientific achievements. Expressing all of it by writing is not always easy, however I will try to do my best.

To Naomi, I would start to say a big thank you. Thank you for the opportunity that you gave me to do my master thesis in your lab and for all your support during these 10 months. You have not only a remarkable career but also an amazing personality. Despite all the time passed in the U.S.A, it was like if you were always there with us in the lab: you were always aware of my work, always ready to help me in case of necessity, always available to discuss and teach me how to do the things in a better way and always interested in helping me with my future steps. And all this help and availability were always marked with your special and kind way to talk and to be. Thank you for everything!

To Valérie D, firstly, I would like to say: "Santa Valérie". Since the beginning (and even before I came to Montpellier) you were always ready to help me. You were always available to teach me everything that I could need in the lab and you were there in all the moments where was necessary to solve problems outside the lab. Also, you thought me how to be less stressed (this process is still in progress) and to look at the issues that arise in a positive way. It is important to add that during these 10 months you had constantly a lot of things to do and you never, even for a little moment, stopped helping me. You are an amazing supervisor and an amazing person! Thank you!

To all the other NTL members, a big thank you! Dorota, with you I learned a lot and what you thought me will always be the bases for my future work. Gaspard, a really special thank you for you! I learned a lot with you and was a pleasure and really funny to be part of "Crenet/Iberica" Team. Valérie Z. and Sandrina, thank you both because you were always there to advise me and help when was necessary. Together with Valérie D. you also thought me that I should breath when I am speaking (process still in progress too). Cédric pour toi j'ai dit primairement que j'adore le bruit. Merci pour d'être le meilleur dictionnaire Français-Portugais,

pour être toujours disponible pour m'aider ("may day may day") et de me dire où je dois mettre ("colloquer") les choses. Isabelle, thank you for making me feel like I was in Portugal with our conversations and thank you Marco for all the directions that you gave me before coming to Montpellier. Marie, Leal, Anne Sophie, Sarah and Carmen, working with you was really funny! Thank you for being there for me and making me laugh when I was stressed. Peggy, your way to be is so funny (and fashionable). Thank you for all the lunch moments and I will never forget the M&M times! Also to Daouda: your kind personality is remarkable. Myriam, your support in the FACS room was really important. Thank you for teaching me a lot of things and also for all the times that you helped me entering the system when I forgot my passwords. Finally, thank you Patrice for all the moments where you made me laugh, for all the desserts that you, so gentle, offered me in order to help me with my sugar addiction, for the polar bear in my desk and for taking care of my computer.

To my friends in Montpellier: Lupita, Uriel, Ambra, Klara, Leonor, Marco E. and Nico, a big thank you for all the moments that we spent together.

Aos meus Professores, família e amigos em Portugal:

Estes cinco anos da minha vida académica passaram a correr. Muitos desafios surgiram e hoje posso dizer que um após outro os superei. Contudo, e apesar da muita dedicação e do meu empenho, este sucesso não seria possível sem as inúmeras pessoas que estiveram ao meu lado e que me apoiaram desde o primeiro momento.

Em primeiro lugar, começo por agradecer a todos os Professores que fizeram parte do meu percurso académico. Aos Professores de Mestrado, o meu muito obrigada por todas as fantásticas aulas, pela excelente preparação académica para os desafios futuros e por toda a disponibilidade em ajudar sempre que preciso. Um especial obrigada ao Professor Carlos Duarte por todo o apoio, ajuda e por me ter dado a conhecer o fantástico laboratório , onde tive oportunidade de trabalhar neste presente ano letivo. À Professora Emília, Coordenadora do Mestrado, obrigada por me ter dado a possibilidade de entrar neste Mestrado que é, sem dúvida, pautado de uma de grande excelência e que prepara os alunos, com enorme qualidade, para a exigente vida científica.

A todos os meus Professores de Licenciatura, muito obrigada por todo o conhecimento que me transmitiram durante os meus primeiros três anos de Universidade e que serão sempre as bases do meu conhecimento. Gostaria, igualmente, de agradecer à Doutora Teresa Martins por me ter dado a oportunidade de estagiar no seu laboratório no IPO, no meu último ano de licenciatura.

Obrigada à minha Mãe, ao meu Irmão e ao meu Pai por me terem apoiado sempre em todos os momentos deste meu percurso. É graças a vocês que tive a oportunidade de chegar até aqui. Sempre que alcançava mais um objetivo, foi em vocês que eu pensava em primeiro lugar. Ao meu tio Zé e à minha tia Zita um grande e especial obrigada por terem estado sempre presentes em todas as etapas da minha vida. À minha avó e a todos os meus tios e primos, obrigada por terem acompanhado o meu crescimento e por me terem apoiado nesta fase final.

Seguidamente, um grande obrigada aos amigos que estiveram sempre prontos para me apoiarem. Adriana, Soraia, Tati e Joana Diniz, vocês foram as melhores colegas de casa que poderia ter desejado e são como irmãs para mim. Assim como tu, Mariana, que seguimos sempre caminhos tão parecidos e, ao mesmo tempo, tão distantes. Obrigada por, mesmo estando longe, a nossa amizade ter-se mantido exactamente na mesma e por teres estado sempre presente em todos os momentos. Por fim, obrigada, Rafael, por todo o apoio que me deste antes e durante a minha aventura por Montpellier.

Aos meus colegas de licenciatura, são muitos os agradecimentos que quero prestar. À minha família de praxe um enorme obrigada por me terem apoiado desde o meu primeiro dia na Universidade. Um especial obrigada à minha Madrinha Inês, aos meus afilhados (Lu, Bibi e Diogo) e ao meu Tio Rojões que, tendo sindo meu colega de Licenciatura e Mestrado, esteve sempre presente no meu percurso. À Susana, à Filipa Brito e à Maria Inês Verrísimo obrigada pelo lugar especial que ocupam no meu coração. Aos meus colegas de Mestrado do dia-a-dia, obrigada pela constante boa disposição. Vilaça, obrigada pela amiga fantástica que foste e que continuas a ser e por teres estado sempre presente desde o primeiro mês de aulas. És, sem dúvida, a grande amiga que levo deste Mestrado e que levo para a vida. Ao Alberto e à Daniela, também um especial obrigada.

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Abbreviations

AML1 – acute myeloid leukemia -1

AMPK - 5' adenosine monophosphate-activated protein kinase

APC – antigen presenting cells

ASCT2 - ASC amino-acid transporter 2

ATP – adenosine tri-phosphate

BCR – B cell receptor

Blimp-1 – B lymphocyte-induced maturation protein 1

CCL – CC-chemokine ligand

CCR7 – C-C chemokine receptor type 7

CD – cluster of differentiation

CPT1 – carnitine palmitoyl transferase

CTL – cytotoxic T lymphocyte

CTLA-4 - cytotoxic T-lymphocyte-associated protein 4

DC – dendritic cells

Eomes – transcription factor eomesodermin

FAO – fatty acid oxidation

FoxP3 - forkhead box P3

GATA3- trans-acting T-cell-specific transcription factor GATA-3

GITR - glucocorticoid-induced TNFR family related gene

GLS – glutaminase

Glut1 – glucose transporter 1

HEV - high endothelial venules

HIF-1α – hypoxia-inducible factor

IFN-γ – interferon γ

IL-12Rβ2 – IL-12 receptor beta 2

IL-4Rα – IL-4 receptor alpha

IPEX- immune dysregulation polyendocrinopathy X linked

IRF4 – interferon-regulatory factor 4

iTreg - inducible T regulatory

KLF2 – kruppel-like transcription factor 2

KLRG1 – killer cell lectin-like receptor subfamily G, member 1

LFA-1 - leukocyte function-associated antigen-1

LTα – lymphotixin α

MHC – major histocompatibility complex

mTOR – mammalian target of rapamycin

mTORC – mammalian targer of rapamycin complex

NFAT – nuclear factor of activated T cells

nTreg – natural T regulatory

OXPHOS – oxidative phosphorylation

PDK1 – 3-phosphoinositide-dependent protein kinase-1

PPR – pattern recognition receptors

ROR – retinoid-related orphan receptor

ROS – reactive oxygen species

RTE – recent thymic emigrants

Runx –runt related transcription factor

STAT – signaling transducer and activator of transcription

S6K1– S6 kinase beta-1

Tbet – T box transcription factor

TCA – tricarboxylic acid

TCR – T cell receptor

TGF-β – transforming growth factor beta

T_{FH} – T follicular B helper cells

Th1 – T helper 1

Th17 –T helper 17

Th2 – T helper 2

Th9 – T helper 9

Tr1 – Type 1 regulatory T cells

Treg – T regulatory

VHL – von Hippel-Lindau

α-KG – α-ketoglutarate

Abstract

T cells play a central role in the development of efficient immune responses. When a naïve T cell interacts with its cognate antigen, multiple pathways are activated, leading to T cell differentiation and specialization into specific functions. During the past 20-30 years, significant research efforts have been mobilized to decipher the molecular mechanisms orchestrating T cell differentiation and the establishment of T cell effector responses. The majority of these studies focused on the impact of signaling via the T cell receptor (TCR), cytokines, chemokines and hormones as well as cell-cell interactions, demonstrating important roles for each of these components in T cell activation. However, studies of less "classical" parameters of the T cell microenvironment, such as the availability of nutrients, amino acids, mineral, vitamins and oxygen, were not at the forefront. It is only in the past few years that we have begun to appreciate the impact of metabolism in T cell activation. Nutrient availability is particularly important as T cell activation requires that the lymphocyte meet increased energetic and biosynthetic demands. Furthermore, in pathological situations such as cancer, the direct environment of intra-tumoral T lymphocytes can be altered by nutrient availability. As such, it becomes important to determine how nutrient availability affects T cell activation and the potential of these T cells to mediate different effector functions. My project in Naomi Taylor's lab focused on the impact of glutamine and glucose metabolism as well as oxygen tension on T cell fate.

During my rotation, I found that activation of naïve $CD4^+$ or $CD8^+$ T cells under conditions of glutamine deprivation decreased their proliferation potential and resulted in a skewing of both subsets to a $Foxp3^+$ T cell fate (Tregs). Moreover, glutamine-deprived $CD4^+$ T cells activated under Th1-polarizing conditions lost their potential to differentiate into Th1 cells but instead, a significant percentage adopted a $Foxp3^+$ Treg fate. Notably, we found that α-ketoglutarate (αKG), the glutamine metabolite that enters into the mitochondrial citric acid cycle, functions as a metabolic regulator between these alternative CD4+ T cell effector fates. Supplementing glutamine-deprived cells with a cell-permeable αKG induced expression of Tbet, a master

transcriptional regulator of Th1 differentiation, and restored Th1 differentiation, as monitored by IFN-γ secretion.

I was also interested in determining whether the profile of expression of the Glut1 glucose transporter can be used to distinguish activated T lymphocyte cells with different phenotypes, both under conditions of normoxia (20%) and hypoxia (1%). We found that Glut1 was expressed at higher levels under conditions of hypoxia and normoxia and moreover, sorting of T lymphocytes on the basis of their Glut1 profiles revealed Glut1^{high} cells to have a higher proliferation potential, more rapidly acquiring a memory cell phenotype. There was also a higher percentage of CD8 T cells within the Glut1^{high} than Glut1^{low} lymphocyte subsets. Finally, our preliminary data suggest that both oxygen tension and Glut1 expression levels correlate with differential cytokine secretion profiles. Specifically, Glut1^{high} CD4 T cells secrete significantly higher levels of IL-17 than the Glut1^{low} subset and cytokine expression is more than 3-fold higher under normoxic oxygen conditions. The ensemble of these results reveal T cell metabolism as an important modulator of T cell fate and strongly suggest that the specific targeting of metabolic pathways may allow the development of innovative strategies to improve T cell immunotherapies.

Keywords: T cell metabolism ● Differentiation ● Glutamine● Glut1● Oxygen Tension

Resumo

As células T detêm um papel central no desenvolvimento de respostas imunitárias eficientes. Quando uma célula T naive interege com o seu respectivo antigénio, múltiplas vias são activadas resultando na diferenciação e especialização das células T em funções específicas. Nos últimos 20-30 anos, esforços ciêntificos significativos têm sido feitos com o objectivo de decifrar os mecanismos moleculares envolvidos da diferenciação das células T assim como no estabelecimento de resposta efectoras. A maioria dos estudos tiveram como principal foco o impacto da sinalização mediada pelo receptor das células T, citoquinas, quimiocinas e hormonas assim como interacções célula-célula, demonstrando o papel importante de cada um destes componentes na activação das células T. No entanto, estudo de parâmetros menos "clássicos", como por exemplo, a disponibilidade de nutrientes, aminoácidos, minerais, vitaminas e oxigénio, não foram igualmente considerados. Apenas nos últimos anos, é que estes parâmetros começaram a ser considerados como tendo um possível impacto na activação das células T. A disponibilidade dos nutrientes é, particularmente, importante para as células T visto que a sua activação induz um aumento dos requerimentos energéticos e biosintéticos. Além disso, em situações patológicas, como por exemplo cancro, o ambiente que envolve as células T anti-tumorais pode ser alterado pela disponibilidade de nutrientes. Assim sendo, é importante determinar como é que a disponibilidade dos nutrientes pode afectar a activação das células T bem como afectar o seu potencial em mediar funções efectoras diferentes. O meu projecto no laboratório da Dr. Naomi Taylor focou-se no impacto do metabolismo da glutamina e da glucose assim como no papel da tensão de oxigénio no destino das células T.

Durante a minha rotação, eu observei que a activação das células T $CD4^+$ ou $CD8^+$, na ausência de glutamina, induz uma diminuição no potencial de proliferação e redirecciona ambas as populações para o mesmo fenótipo: $FoxP3^+$ (Treg). Ainda, células activadas na ausência de glutamina e sob polarização Th1 perdem a sua capacidade de diferenciação em células Th1 e, por outro lado, grande parte adoptam um fenótipo de células $FoxP3^+$ Treg. De maneira notável, nós observámos que adicionando α-cetoglutarato (α-KG), um metabolito da glutamina que entra mitocôndria por via do ciclo de krebs, funciona como um regulador metabolico de diferentes subpopulações efectoras de células T $CD4^+$. Adicionando α-KG às células mantidas na

ausência de glutamina, é verificado uma indução da expressão de Tbet, um importante regulador transcripcional na diferenciação das células Th1, e uma restauração da diferenciação das células Th1, como observado pela secreção de IFN-γ.

Para além do papel da glutamina no metabolismo das células T, também procurei determinar se diferentes perfis de expressão do transportador de glucose Glut1 poderiam ser utilizados para distinguir células T activadas com diferentes fenótipos, ambas sob condições de normoxia (20%) e hipoxia (1%). Nós observámos que Glut1 é expresso a níveis elevados sob condições de normoxia e hipoxia e, ainda, fazendo um sorting das células T baseado nos perfis de expressão de Glut1, foi observado que células $Glut1^{high}$ têm um maior potencial de proliferação, adquirindo mais rapidamente um fénotipo de memória. Foi também verificado uma maior percentagem de células CD8 nas células $Glut1^{high}$ comparativamente com as células $Glut1^{low}$. Finalmente, os nossos resultados perliminares sugerem que em ambas as tensões de oxigénio e em ambos os perfis de expressão de Glut1 estão correlacionados com diferentes padrões de expressão de citoquinas. Especialmente, células T CD4 $Glut1^{high}$ secretam significativamente níveis mais elevados de IL-17 que a subpopulação $Glut1^{low}$ e a expressão de citoquinas é 3 vezes maior sob condições de normoxia. O conjunto destes resultados revela que o metabolismo de células T é um importante modulador da diferenciação das células T e sugere fortemente que, actuando directamente nas vias metabólicas, pode permitir o desenvolvimento de novas estratégias com o objectivo de melhorar imunoterapias que têm por base o uso de células T.

Palavras-chave: Metabolismo de células T ● Diferenciação ● Glutamina ● Glut1 ● Tensão de oxigénio

Chapter 1 Introduction

1 Immune system

1.1 General concepts

The immune system can be divided into two main categories: innate and adaptive immunity. Innate immunity, which is the first line of response against pathogens, is genetically fixed and invariant. This recognition occurs in a non-specific manner, mediated by pattern recognition receptors (PRRs) that target pathogen-associated molecular patterns expressed by microbes. These receptors are present on epithelial and immune cells including dendritic cells, macrophages, natural killer cells, mucosal-associated invariant T, and innate lymphoid cells (Bremers & Parmiani 2000). Adaptive immunity represents a second line of defence and is mediated by B (humoral immunity) and T (cell-mediated immunity) lymphocytes. Adaptive immunity requires several days before the organism can respond as the B and T cells must specifically recognize an antigen, undergo clonal expansion and differentiate into effector cells.

1.1.1 B cells

B lymphocytes develop and mature in the bone marrow. During their development, cells go through different stages and the main phases are pro-B, pre-B and immature B cells. Differentiation occurs only if there are successful rearrangements of the immunoglobulin heavy and light chains and pre-B cell receptor (BCR). Elimination of potentially auto-reactive B cells (BCRs), recognizing self-antigens, occurs during late stage differentiation. After this selection, immature B cells migrate from the bone marrow to the spleen, where they become transitional B cells (Eibel et al. 2014). B cells produce antibodies which are key elements in adaptive immunity and play a major role in protection against a vast variety of pathogens (Pieper et al. 2013). Antibodies can promote the neutralization of toxins, activation of the complement system and phagocytosis of extracellular microbes. When a B cell interacts with its antigen, it differentiates into a plasma cell which produces large amounts of antibodies and/or memory B cells which are able to more rapidly and efficiently respond in the event of a second contact

with the same antigen. These memory B cells do not produce immunoglobulins but can rapidly give rise to new plasma B cells if the same antigen is presented. They are also renewed such that they can continually respond to a new challenge (Mcheyzer-williams et al. 2014).

1.1.2 T cells

My project is centred on classical T lymphocytes and therefore, I will specifically focus on this cell type in the following chapter/paragraphs.

TCR-$\alpha\beta$ T cells can be divided into 2 distinct lineages: $CD4^+$ and $CD8^+$ T cells. Both CD4 and CD8 T cell progenitors are generated in the bone marrow but the differentiation process occurs in the thymus and is called thymopoiesis. They leave the thymus as a mature but not activated, naïve T cells that are referred to as recent thymic emigrants (RTE). The T cell repertoire is shaped by the interaction of the T cell receptor on the surface of thymocytes with self-peptide Major Histocompatibility Complexes (MHC) that are displayed by thymic antigen-presenting cells (APCs). Only those thymocytes that interact with antigen-MHC at a defined level of low reactivity are able to continue their maturation process (positive selection) while thymocytes with high affinity for self-antigens are eliminated (negative selection) (Hogquist et al. 2005).

After leaving the thymus, RTE are incorporated into the peripheral pool of mature T cells. Their survival is dependent on signalling by the IL-7 cytokine and self peptide-TCR (T cell receptor) interactions (Tan et al. 2001) (Spent & Surh 2012). IL-7 acts as a pro-survival factor, at least in part by upregulating the Bcl-2 anti-apoptotic factor (Rathmell et al. 2001). Indeed, in IL-7Rα deficient mice, T cell survival can be rescued by overexpression of Bcl-2 (Maraskovsky et al. 1997) (Akashi et al. 1997). IL-7 also plays a crucial role in Glut1 upregulation and glucose uptake (Barata et al. 2004) .

When naïve T cells encounter foreign antigens presented by APC's they are rapidly activated and undergo proliferation and effector differentiation, allowing them to efficiently respond to antigen (Boyman et al. 2009). The mechanisms resulting in this activation are presented in the next section.

1.1.2.1 T cell activation

As mentioned above, APCs, such as B cells, macrophages and dendritic cells, play a crucial role in the activation of naïve T cells, with the latter considered to be the most important (Banchereau & Steinman 1998). Indeed, the TCR recognizes a specific antigen in the context of its presentation by a MHC molecule expressed on an APC. There are 2 main types of MHC molecules: MHC class I and MHC class II. These molecules have the ability to present oligopeptides fragments to CD8 and CD4 T cells, respectively (Banchereau & Steinman 1998). Whereas MHC I generally presents intracellular peptides, MHC II presents extracellular peptides and endocytic processes are required for peptide fragmentation. MHC II is notably but not exclusively expressed on APCs including dendritic cells, B cells and monocytes/macrophages (Rocha & Neefjes 2008) (Vyas et al. 2008). Concisely, the responses of T cells are initiated when naïve T cells encounter DCs that present foreign antigens (Lanzavecchia 2000).

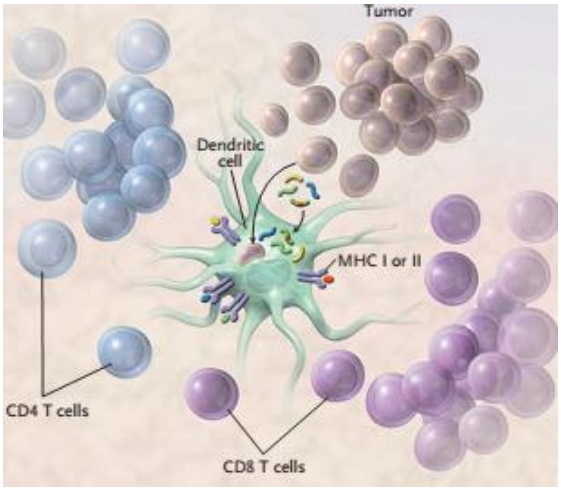

Figure 1 - Antigen presenting cells and their function in Finn, O. J. Cancer immunology. N. Engl. J. Med. **358,** 2704–15 (2008)

APCs play a crucial role in TCR triggering and the induced signalling ceases if contact is abrogated. Thus, the time/strength of TCR activation conditions the force of T cell activation, differentiation and, even an activated-induced cell death (Lanzavecchia et al. 1999). Co-stimulatory signals are essential for efficient T cell activation and CD28 is one of the major co-stimulatory molecules. Following TCR recruitment, a cascade of signalling molecules is activated, amplifying the signal transduction cascade and reaching an activation threshold (Viola et al. 1999).

1.1.2.1.1 Naïve T cells

Naïve T cells are present in lymphoid organs and are distinguished by their high expression of CD62L (lymph-node homing receptor) and CCR7 (C-C chemokine receptor type 7) (Picker et al. 1993) (Boyman et al. 2009). The expression of these receptors is dependent on the activity of kruppel-like transcription factor KLF2 and the FoxO1 transcription factor (Kerdiles et al. 2009) (Carlson et al. 2006) and their absence inhibits the homeostasis of naïve T cells (Link et al. 2007) (De Rosa et al. 2001). Furthermore, both human and murine naïve T cells are $CD44^{low}$ and do not express the CD25 (IL-2R-alpha chain) and CD69 activation markers (Woodland & Dutton 2003) (R. W. Dutton, L. M. Bradley 1998) (Boyman & Sprent 2012). In the human system, expression of two isoforms of the CD45 tyrosine phosphatase, a regulator of multiple antigen and cytokine signalling cascades, distinguishes naïve and memory T cells (Trowbridge & Thomas 1994). Naïve and memory T cells express the CD45RA and CD45RO isoforms, respectively (De Rosa et al. 2001). This distinction does not occur in the murine system, but naïve T cells are $CD45RB^{high}$ (R. W. Dutton, L. M. Bradley 1998).

Activation of the TCR on a naïve T cell, by contact with its MHC-presented cognate antigen, induces its proliferation and differentiation, with the acquisition of a memory or effector phenotype.

1.1.2.1.2 Memory T cells

Memory cells can be characterized by two main phenotypes: central memory and effector memory. Central memory cells are found in lymph nodes, spleen, blood and bone marrow while effector memory T cells are found in spleen and blood but also in non-lymphoid organs including lung, liver, intestinal tract, reproductive tract, kidney, adipose tissue and heart (Mueller et al. 2013). Changes in localization are due, at least in part, to distinct chemokine receptor profiles. Central memory T cells are $CCR7^{+}$. They produce higher levels of IL-2 and lower levels of effector cytokines and based on their presence in secondary lymphoid organs, they are able to rapidly respond to secondary contact with antigen. They have little or no

effector function but can proliferate quickly and differentiate into effector cells following antigen stimulation (Sallusto et al. 2004) (Mahnke et al. 2013). The presence of CCR7, also known as CD197, promotes responsiveness to the CCL19 (CC-chemokine ligand 19) and CCL21 (CC-chemokine ligand 21) ligands. These ligands are present on the luminal surface of high endothelial venules (HEV), recruiting naïve T cells, allowing their extravasation and inducing migration into the T cell zone. CCR7 ligands also recruit APC, promoting a robust antigen presentation (Okada & Cyster 2007) (Worbs et al. 2007) (Cyster 2000). In contrast, effector memory T cells are $CCR7^-$ and produce high levels of effector cytokines depending on the cell type (IFN-γ, IL-4, IL-5) (Mahnke et al. 2013).

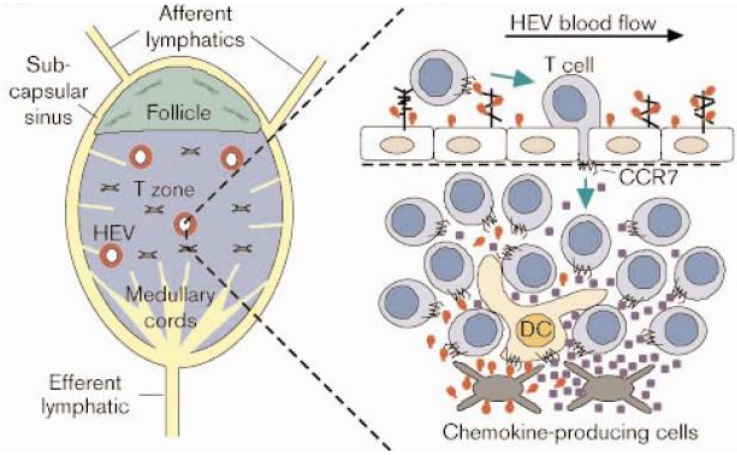

Figure 2 – Migration of T cells to the T cell zone in Leukocyte Migration: Scent of the T Zone. *Current Biology* 10 (1): 30–33.

CD62L expression also characterizes central memory T cells (Sallusto et al. 2004). Like CCR7, CD62L serves as a homing receptor, promoting the migration of central memory T cells to secondary lymphoid organs. Another important memory T cell marker is CD44, involved in the regulation of T cell adhesion and migration to inflammation sites (Baaten et al. 2012). CD44 signalling promotes cytoskeletal changes needed for T cell extravasation from blood into tissues and its presence serves as an activation marker.

As discussed above, memory T cells are more potent than naïve T cells due to their ability to rapidly produce cytokines upon antigen restimulation (Mackay et al. 1990). However, antigen-mediated signalling can also culminate in the generation of effector T cells.

1.1.2.1.3 $CD4^+$ Effector T cells

Within the CD4 T cell population, there are different effector subsets including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and T helper 9 (Th9) cells as well as a suppressor

regulatory (Treg) subset. Moreover, CD4 T cells also can differentiate into T Follicular B helper cells (T_{FH}) that are crucial for B cell activation mediating its selection and survival (Crotty 2014).

1.1.2.1.3.1 T helper 1 cells

The Th1 $CD4^+$ T cell subset is involved in cell-mediated immunity and produces mainly interferon-γ (IFN-γ). However, these cells can also produce lymphotoxin α (LTα) and IL-2 (Zhu & Paul 2015). They are induced following infection by intracellular pathogens such as bacteria or viruses and can induce macrophage activation, resulting in the destruction of intracellular bacteria (Geginat et al. 2014). Their activation induces expression of the IL-12Rβ2 which is then maintained by IL-12 and IFN-γ signals (Szabo et al. 1997). Differentiation of Th1 cells requires the function of the Tbet, (T box transcription factor) and this factor also represses a Th2 program (Szabo et al. 2015). The STAT4 (signalling transducer and activator of transcription 4) transcription factor is also important, inducing IL-12 and IFN- γ that consequently increase T-bet expression leading to a positive feedback loop (M H Kaplan et al. 1996) (Thierfelder et al. 1996). Indeed, in the absence of STAT4, activated CD4 T cells acquire a Th2 phenotype (M H Kaplan et al. 1996). Runx3 (runt related transcription factor 3) is also upregulated in Th1 cells, functioning together with Tbet to silence interleukin 4 expression (Djuretic et al. 2007) and induce IFN-γ (Naoe et al. 2007). The IL-12 production required for Th1 differentiation is notably produced by activated dendritic cells (Zhu & Paul 2015).

1.1.2.1.3.2 T helper 2 cells

The Th2 $CD4^+$ T cell subset is generally involved in humoral immunity, enabling the organism to optimally mediate responses against parasites (Coffman & Osmann 1989). Based on this phenotype, they are also implicated in the persistence of asthma and other allergic diseases. Th2 cells produce mainly IL-4, IL-5, and IL-13 but they also can produce IL-9, IL-10 and IL-25 (Zhu & Paul 2015). IL-4 is the most important cytokine and is required for Th2 cell differentiation (Szabo et al. 2015) (Swain et al. 1990). IL-5 is involved in eosinophil recruitment (Coffman et al. 1989) and IL-9, among other functions, leads to mucin production in epithelial

cells (Longphre et al. 1999). IL-13 is also produced by Th2 cells and its production can result in an alternative activation of macrophages (Wynn 2003). Finally, it was shown that IL-25 can promote Th2 differentiation and its enhancement of Th2 cytokine production makes it a critical factor in regulating adaptive allergic responses (Angkasekwinai et al. 2007). The principal receptor expressed on Th2 cells is IL-4Rα. CD25 is also highly expressed in Th2 cells as compared to Th1 cells, likely due to its upregulation by a Th2 transcription factor (c-maf) (Hwang et al. 2002). In contrast to STAT4 in Th1 cells, STAT6 is required for Th2 signalling downstream of the IL-4 and IL-13 receptors (Kaplan et al. 1996) (Shimoda et al. 1996). The main transcription factor in Th2 cells is GATA3 and its absence results in a loss of Th2 differentiation (Pai et al. 2004). Moreover, it has been shown that the absence of GATA3 not only impairs Th2 differentiation but it induces Th1 differentiation in the absence of IL-12 and IFN-γ (Zhu et al. 2004). GATA3 also serves a role downstream of the IL-4/Stat6 pathway since ectopic expression of GATA3 in STAT6-deficient cells results in the production of Th2 cytokines production (Ouyang et al. 2000).

1.1.2.1.3.3 T helper 17 cells

The Th17 $CD4^+$ T subset plays a major role in mucosal immunity and inflammation (Annunziato & Romagnani 2009) and is associated with multiple auto-immune diseases. These cells are characterized by the production of IL-17a, IL-17f, IL-21 and IL-22. Both IL-17a and IL-17f can bind to IL-17RA chain, however IL-17a binds with a higher affinity (Hymowitz et al. 2001). Th17 differentiation requires IL-6 as well as TGF-β (transforming growth factor β) signalling (Veldhoen et al. 2006). It was shown that IL-6-deficient mice do not develop Th17 cells, but rather generate large number of regulatory T cells. In parallel, it was also demonstrated that IL-21 could induce an alternative Th17 differentiation pathway (Korn et al. 2007). Thus, IL-6 plays a crucial role in the differentiation of naïve T cells to a Th17 vs Treg fate. IL-23 is also important for Th17 differentiation, although there is not an absolute requirement, at least during the first steps of the differentiation, since the absence of its receptor does not impair Th17 differentiation. It is though required for the pathogenic inflammation induced by Th17 cells (Torchinsky & Blander 2010).

Regarding the transcriptional regulation of Th17 differentiation, signalling through a retinoid orphan nuclear receptor (RORγT) has been found to be critical (Ivanov et al. 2006). A related nuclear receptor, RORαT is also involved with its absence resulting in a lower level of IL-17 expression (Yang et al. 2008). Furthermore, signalling through IL-6, IL-21 and IL-23 is mediated via STAT3 and this transcription factor is required for optimal induction of RORγT as well as IL-23R (Harris et al. 2007). Finally, it is also important to mention the importance of Interferon-regulatory factor 4 (IRF4). IRF4 deficiency inhibits Th17 differentiation and loss of IRF4 within T helper cells results in the induction of regulatory T cells expressing the forkhead box P3 (Foxp3) transcription factor (Brüstle et al. 2007).

1.1.2.1.3.4 T helper 9 cells

The Th9 $CD4^+$ T cell subset was one of the most recently described, and is notably playing a role in allergic inflammation. This subset, characterized by the production of IL-9, is induced by concomitant TGF-β and IL-4 signaling (Veldhoen et al. 2008) (Dardalhon et al. 2008) (Zhao et al. 2013). IL-4 is a STAT6 and IRF4 activator, while TGF-β activates PU.1 and represses GATA3 and Tbet (Perumal & Kaplan 2011). PU.1 appears to be the main transcription factor involved in Th9 cell differentiation, with the capacity to bind to the IL-9 promoter (Chang et al. 2010). This T helper population is closely related to the Th2 subset as Th2 cell express both IL-4 and IL-9. During the initial phase of differentiation, the IL-4 produced by Th2 cells is crucial for Th9 differentiation with exogenous TGF-β sufficient to convert the cells to a Th9 fate (Veldhoen et al. 2008).

1.1.2.1.4 $CD8^+$ effector T cells

$CD8^+$ T cells acquire cytotoxic properties following antigen stimulation, promoting the direct killing of cells expressing the targeted antigen.

1.1.2.1.4.1 Cytotoxic T lymphocytes

$CD8^+$ T lymphocytes differentiate into cytotoxic T cells (CTL) that are able to kill cells expressing a specific antigen such as virus-infected cells. After activation, expression of CD62L and CCR7 is reduced while there is an upregulation of CD44, LFA-1 and the α4β1 integrin, promoting migration out of lymphoid organs to inflammatory sites (Weninger et al. 2002). Cytotoxicity can be induced via two major pathways; perforin/granzyme secretion and stimulation of the Fas pathway, with $CD8^+$ CTL using mainly the former. Briefly, after activation, there is an increase in perforin/granzyme (granzyme A/granzyme B) which then mediate granule-dependent killing of the target cell (Shresta et al. 1998).

Several cytokines has been shown to be important for CTL differentiation. IL-2 is one of the most important and plays a crucial role during the CTL differentiation process. IL-2 is produced by $CD4^+$ T cells but $CD8^+$ T cells also produce lower levels of this cytokine (Malek & Castro 2010). Obar *et al.* showed that expression of the CD25 subunit of the IL-2 receptor is dependent on $CD4^+$ T helper cells as well as activation of the co-stimulatory CD28 receptor and interaction with its receptors (CD80/CD86) expressed on APCs (Obar et al. 2010). Pipkin *et al.* demonstrated the crucial role of IL-2 stimulation in upregulating CTL transcription of the eomesodermin (Eomes) regulator and perforin while simultaneously repressing memory CTL markers as Bcl6 and IL-7Rα (Pipkin et al. 2010). IL-2 also induces the transcriptional repressor B lymphocyte-induced maturation protein 1 (Blimp-1) which enhances $CD8^+$ effector cell differentiation (Boulet et al. 2014). In the absence of Blimp-1, granzyme B, perforin and KLRG1 (Killer cell lectin-like receptor subfamily G, member 1) expression are decreased while expression of memory CD8 T cell markers such as CD127, CCR7, CD62L, CD27 and IL-2 are increased (Zhang & Bevan 2011).

1.1.2.1.5 Regulatory T cells (Treg)

1.1.2.1.5.1 $CD4^+$ Regulatory T cells

As indicated above, in addition to giving rise to effector helper T cell subsets, $CD4^+$ T cells can also give rise to suppressive regulatory T cells (Treg) (Lanzavecchia 2000), controlling potential excessive immune responsiveness and autoimmune disease. Treg cells can be divided into two main types: natural Treg cells (nTreg) that differentiate in the thymus and inducible Treg cells (iTreg) that are generated in the peripheral circulation from naïve T cells. The initial phenotype of Treg was defined as $CD4^+CD25^+$ with a critical role of IL-2 signalling in this subset (Zhu & Paul 2015). It was then found that expression of the FoxP3 transcription factor was critical for Treg development and function (Hori et al. 2003). Indeed, mutations in the *Foxp3* gene results in an autoimmune disease in humans called IPEX syndrome (immune dysregulation, polyendocrinophaty, X-linked). IPEX patients develop autoimmune enteropathy, dermatitis, thyroiditis and type I diabetes with a poor prognosis and death in the two first years of life unless they undergo autologous hematopoietic stem cell transplantation (Bennett et al. 2001) (Van Der Vliet & Nieuwenhuis 2007). In mice, a spontaneous loss-of-function mutation of the Foxp3 gene is responsible for the scurfy phenotype, with a complete loss of Treg and death at 3-4 weeks of age due to a CD4 T cell-mediated lymphoproliferation (Brunkow et al., 2001). Notably though, there are also regulatory T cells that do not express FoxP3 or at very low level such as type 1 regulatory T cells (Tr1) and Th3 cells. Tr1 cells produce high levels of IL-10 and TGF-β, moderate levels of IL-5, minimal amounts of IL-2 and IFN-γ and do not secrete IL-4 (Groux et al. 1997) (Battaglia et al. 2006). Th3 regulatory cells were identified in mice that underwent oral tolerance induction and are characterized by their production of TGF-β, low amounts of IL-4 and IL-10 and no IFN-γ or IL-2 (Chen et al. 1994). As part my research focused on $FoxP3^+$ Treg, these cells will be described in more detail below.

In general, suppression by $Foxp3^+$ Tregs is due to their production of TGF-β, IL-10 and IL-35. Other suppressive mechanisms have also been described: 1) tryptophan catabolism induction through CTLA-4; 2) cytotoxicity mediated by perforin/ granzyme; and 3) binding of the TGF-β receptor *via* the membrane-bound TGF β present in Treg cells (Miyara & Sakaguchi 2007).

TGF-β is also required for the differentiation of Treg from naïve T cells (Zhu & Paul 2015) and as indicated above, Foxp3 is the major transcription factor involved in Treg differentiation and function. Foxp3 is induced by the Signal Transducer and Activator of Transcription 5 (STAT5) which binds directly to its promoter (Burchill et al. 2007). FoxP3 then induces CD25, T cell-associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family related gene/protein (GITR) while reducing the production of IL-2, IFN-γ and IL-4 (Sakaguchi et al. 2008). The control of Treg function by FoxP3 is well described, although, complex. Briefly, FoxP3 interacts with other transcription factors such as AML1 (acute myeloid leukemia-1)/Runx1 (runt-related transcription factor 1) and NFAT (nuclear factor of activated T cells) that are crucial for T effector cell differentiation. Upon interaction of FoxP3 with these transcription factors, inhibits transcription of IL-2 and IFN-γ-encoding genes are inhibited (Wu et al. 2006) (Ono et al. 2007).

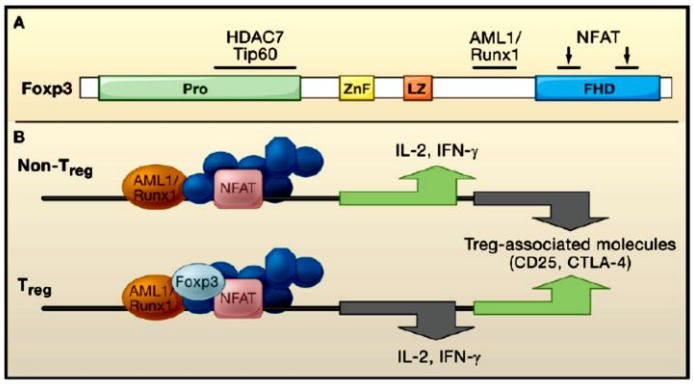

Figure 3- Treg function controlled by FoxP3 expression in Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* 133, 775–787 (2008).

It is important to note that in human cells, FoxP3 is upregulated upon T cell activation and is therefore not a dependable marker of Tregs (Gavin et al. 2006) (Wang et al. 2007). There has been a significant amount of research looking for new human Treg markers with studies focusing on changes in CD25, IL-7Ra (CD127), GITR, CTLA4, ICOS, Helios, neuropilin and most recently, CD15s (sialyl Lewis x) (Liu et al. 2006) (Miyara et al. 2015), but it does not appear that any of these changes are exclusively associated with Treg. At present, downregulation of CD127 and upregulation of CD25 are used to identify human Treg cells

1.1.2.1.5.2 $CD8^+$ Regulatory T cells

There are also $CD8^+$ regulatory T cells but these cells only represents 1% in the peripheral circulation (Wang & Alexander 2009). As such, they are a difficult population to

isolate and characterize and thus, for many years, the scientific community focused on CD4 Treg. Recent work though indicates an important role for CD8 Treg in the control of intestinal immunity, immunopathology, autoimmunity, oral tolerance and prevention of graft-versus-host disease as well as graft-rejection (Pomié et al. 2008).

Several CD8 Treg populations have been described (Pomié et al. 2008). Regulatory function of these CD8 cells have been identified in the thymus and peripheral lymphoid organs of mice (Bienvenu et al. 2005), albeit potentially less than that of the CD4 Treg subset (Mayer et al. 2011). Differentiation of this Treg population, like that of CD4 Tregs, requires TCR as well as TGF-β stimulation (Mayer et al. 2011), and they express common markers such as GITR, CTLA-4 and TGF-β1 (Transforming growth factor β1) (Mahic et al. 2008) (Cosmi et al. 2003). Moreover, these cells also produce IL-10 and TGF-β but suppressive function appears to require cell-cell contact. This is controversial though as Mahic *et al.* reported that blocking CTLA-4, CD80 or CD86 does not impair suppression (Mahic et al. 2008) while Cosmi *et al.* found that anti-CTLA-4 and anti-TGF-β1 antibodies abrogate suppressive activity (Cosmi et al. 2003).

Thus, in summary, CD4 T helper cells, CD8 cytotoxic lymphocytes and regulatory T lymphocytes together orchestrate the adaptive T lymphocyte response. Under optimal conditions, this system is able to efficiently eliminating foreign antigens while minimizing adverse autoimmune events. Nevertheless, as responsiveness is not always optimal, with ineffective responses to antigen-bearing tumors or chronic infections and over-responsiveness resulting in allergy/asthma and autoimmune disease, T cell differentiation and function is an exciting target for the development of new therapeutic approaches.

1.2 T cell metabolism

To sustain their activation and effector functions, T cells undergo metabolic reprogramming to meet the requirements of these energetically demanding processes.

The capacity of naïve cells to give rise to effector or memory T cells is dependent on increases in their metabolic activity (Mockler et al. 2014). The capacity of T cells to undergo through the different activation steps requires a transport and/or generation of the necessary metabolites, with their utilisation processed in the context of distinct transcriptional programs (Verbist et al. 2012). Briefly, the phenotype alterations that occur in T cells are conditioned by the metabolites to which they have access to and the manner in which they are metabolized.

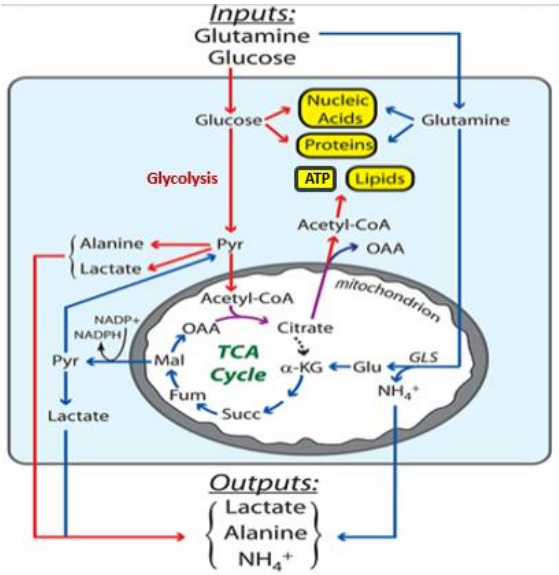

Figure 4- Glycolysis and OXPHOS in DeBerardinis, R.J., and Cheng, T. (2009). Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 29, 313-324

It was long thought that cells preferentially use oxidative phosphorylation (OXPHOS) as their main source of energy in the presence of oxygen, resulting in the production of 36 molecules of ATP from glucose. However, Warburg showed that many cancer cells preferentially use glycolysis (aerobic glycolysis) over oxidative phosphorylation, resulting in the production of lactic acid (Warburg 1956). As this process is less efficient in ATP production (2 net ATPs per molecule of glucose), this metabolic "choice" clearly indicates that cancer cells have metabolic requirements that extend beyond the synthesis of ATP. Indeed, the carbons and electrons (NADPH) required for production of macromolecular precursors such as acetyl-CoA for fatty acids, glycolytic intermediates for amino acids, and ribose for nucleotides are more efficiently provided by aerobic glycolysis, promoting a rapid growth and proliferation of a cancer cell (Vander Heiden et al. 2009). Notably, these metabolic features, favouring glycolysis over oxidative phosphorylation, have also been identified in activated T cells (Gerriets & Rathmell 2012). This suggests that the anabolic pathways leading to the production of important cellular building blocks, such as amino acids and fatty acids, which can be used to produce proteins and cell membrane condition the capacity of a T cell to respond to antigen stimulation (Fox et al. 2005)(Pearce 2010).

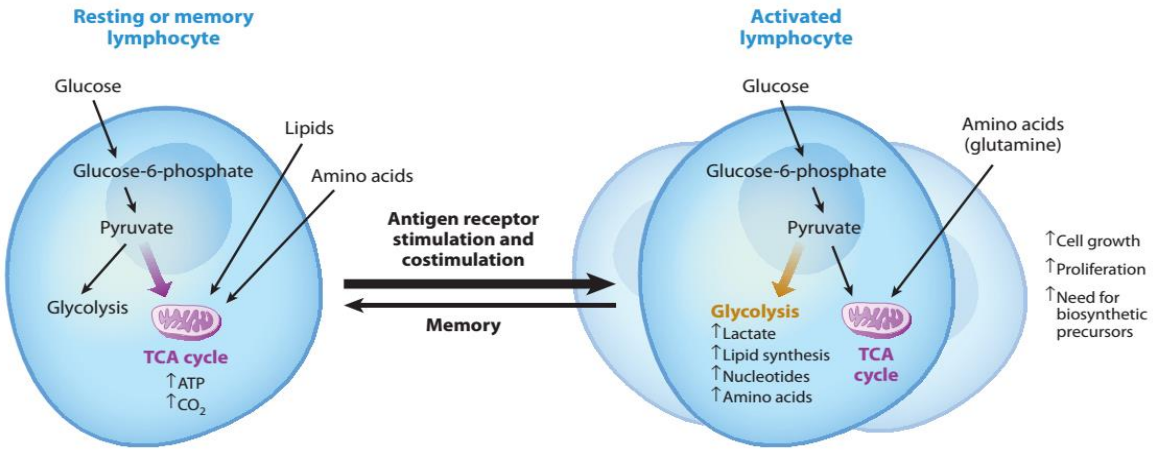

Figure 5 - T cell metabolism in MacIver, N. J., Michalek, R. D. & Rathmell, J. C. Metabolic regulation of T lymphocytes. Annu. Rev. Immunol. 31, 259–83 (2013).

1.2.1 Metabolism of Naïve, Memory and Effector T cells

Resting lymphocytes require energetic resources for their maintenance, supporting their survival and continuous migration from and to secondary lymphoid organs. Cytoskeletal rearrangements, supported by ATP consumption, occur persistently and this metabolism has been shown to rely on OXPHOS, suggesting a low level of glycolytic-dependent biosynthesis (MacIver et al. 2013) (Guppy et al. 1993). These metabolic processes involve crucial metabolites, namely pyruvate, amino acids and fatty acids that are catabolised to generate Acetyl-CoA. This Acetyl-CoA enters the TCA cycle, resulting in ATP production, but only minimal generation of carbon dioxide and free electrons in the form of NADH and FADH2 (Verbist et al. 2012).

Low level cytokine signalling and stimulation through the TCR are required for nutrient uptake (Frauwirth & Thompson 2004). Indeed, extracellular cytokine (IL-7) and TCR stimulation is required for glucose uptake in T lymphocytes because the induced signalling pathways result in the upregulation of the Glut1 glucose transporter at the cell surface (Barata et al. 2004) (MacIver et al. 2013). While the mechanisms *via* which these signals induce Glut1 surface expression is not well understood, likely involving changes in transcription, translation and transport, it has been found that this process involves activation of the PI3K/Akt/mTOR pathway with constitutively active Akt promoting the trafficking of *Glut1* to the cell surface (Swainson et al. 2007) (Wofford et al. 2008) (Silva et al. 2011). Notably, these studies show that naïve T cells

are not “inactive” and their quiescent state requires the maintenance of several metabolic pathways.

Memory T cells share common features with naïve T cells, with 95% similarity in expressed genes (Weng et al. 2012). Furthermore, memory T cells present a metabolism that is similar to that of naïve T cells. The mechanisms regulating the change in metabolism from activated state to a memory state are not yet fully understood (Pearce 2010) (Verbist et al. 2012) but the latter rely mainly on OXPHOS and fatty acid oxidation (Verbist et al. 2012).

Interleukin-15 (IL-15) appears to play an important role in the metabolic switch occurring in effector T cells relative to memory T cells. In $CD8^+$ T cells, IL-15 signalling improves mitochondrial biogenesis and expression of carnitine palmitoyl transferase (CPT1), an enzyme involved in fatty acid oxidation (FAO). This results in an increase in oxidative metabolism and mitochondrial spare respiratory capacity (van der Windt et al. 2012). The $CD8^+$ TNF receptor associated factor 6 (TRAF6) has also been identified as an important factor in inducing fatty acid oxidation in memory CD8 T cells (Pearce et al. 2009). Moreover, AMPK activity in memory $CD8^+$ T cells (at the expense of mTOR activation) promotes the mitochondrial uptake of fatty acids and, similarly, increases CPT1 activity. These mechanisms result in a decreased dependence on glucose metabolism, contrasting with the glycolytic metabolism of effector cells (Buzzai et al. 2005). Moreover, Restifo and Gattinoni *et al.* showed that decreasing glycolysis results in an increased maintenance of memory $CD8^+$ T cells and a decreased differentiation of CD8 T cells to a terminal effector state. Indeed, they found that CD8 T cells with low and high glucose uptake (based on uptake of a fluorescent 2-NBDG glucose analogue) correlated with memory and shorted lived Teff phenotypes, respectively (Sukumar et al. 2013).

T cell activation is followed by an increase of glucose uptake due to increased surface Glu1 expression (Kinet et al. 2007) (Gerriets & Rathmell 2012) (MacIver et al. 2013). This activation leads to an increase in glycolysis and lipid synthesis, supporting growth and proliferation (Gerriets & Rathmell 2012). As described above, the aerobic glycolysis used by activated T cells is less efficient in ATP production than OXPHOS but it promotes the production of important metabolic intermediates required for cell growth and proliferation. Furthermore,

OXPHOS, but not glycolysis, is associated with the production of reactive oxygen species (ROS) and under some conditions, this can severely compromise T cell survival (Fleury et al. 2002).

1.2.2 Glutamine metabolism in activated T cells

In addition to glucose and fatty acids, glutamine is extremely important for T cell activation and function. This amino acid is the most abundant in the body and can be produced in sufficient quantities in physiological conditions. However, it is already known for many years that small reductions in glutamine bio-availability decreases human leukocyte proliferation (Billings et al. 1988). In fact, Kew *et al.* demonstrated that a diet rich in glutamine may increase lymphocyte reactivity (Kew et al. 1999). Moreover, Nakaya *et al.* showed that impeding glutamine uptake through the ASCT2 amino acid transporter in murine T cells following antigen receptor activation inhibits Th1 and Th17 differentiation, resulting in attenuated inflammatory responses (Nakaya et al. 2014).

Glutamine is utilized by the cells *via* glutaminolysis (DeBerardinis, Sayed, et al. 2008). Glutamine is metabolized into glutamate which can then give rise to α-ketoglutarate (α-KG), fuelling the TCA cycle through a process called anaplerosis. Furthermore, α-KG can be broken down to malate, which can then leave the mitochondria and be metabolized into pyruvate, which can then either re-fuel the TCA cycle or be used in glycolysis with the formation of lactate (Heikamp & Powell 2012). Thus, glutamine can be used for energy production through OXPHOS as well as glycolysis (van der Windt & Pearce 2012). The utilisation of glutamine is regulated at many levels, and first at the level of transporter expression. As indicated above, ASCT2 is upregulated by TCR stimulation (Nakaya et al. 2014) and the glutamine anti-porter CD98 (Wang et al. 2011). The latter is regulated by c-Myc whose activity is closely related to that of mTOR. Following glutamine entry, its utilisation is dependent on expression of the glutaminase enzyme (GLS), catalysing the hydrolysis of glutamine to glutamate. GLS1 activity is promoted by Myc

whereas GLS2 activity is promoted by p53 (Suzuki et al. 2010). Thus, there are multiple complex levels regulating the utilization of glutamine in the TCA cycle (Wise et al. 2008).

Glutamine can also be involved in lipid synthesis. Fuelling the TCA cycle, the glutamine-derived citrate can be exported to the cytosol and be metabolized into acetyl-CoA for lipid synthesis (DeBerardinis, Sayed, et al. 2008). Endogenous lipid synthesis is crucial for Th17 differentiation and its impairment was shown to result in the conversion of T cells to a Treg fate. Indeed, Tregs, but not Th17 cells, are able to utilize extracellular fatty acids (Berod et al. 2014). Glutamine also plays a crucial role in amino acid synthesis. The conversion of glutamine to glutamate (*via* GLS) results in the release of ammonia. Due the activity of transaminases, the released amino groups can be used for the generation of nonessential amino acids such as alanine (alanine aminotransferase) and aspartate (aspartate aminotransferase) (DeBerardinis & Cheng 2010). Finally, glutamine also contributes nucleotide biosynthesis, promoting an abundant nucleotide pool and DNA replication (Tong et al. 2009). Glutamine can donate its amide nitrogen group to purine or pyrimidines, giving rising to glutamate (DeBerardinis, Lum, et al. 2008). These reactions take place in the cytoplasm and are catalyzed by glutamine-dependent aminotransferases (Massière F 1998).

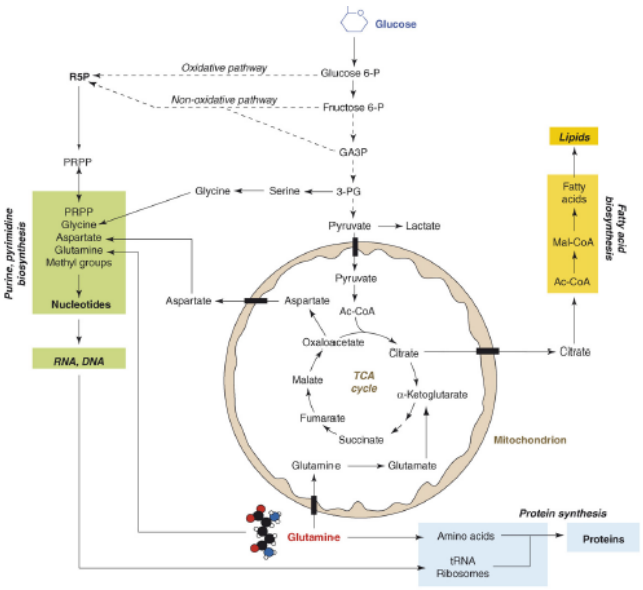

Figure 6 – Glucose and glutamine pathways in DeBerardinis, R. J., Sayed, N., Ditsworth, D. & Thompson, C. B. Brick by brick: metabolism and tumor cell growth. *Curr. Opin. Genet. Dev.* 18, 54–61 (2008).

1.2.3 Pathways regulating T cell metabolism

As mentioned previously, several transduction pathways regulate the metabolic profiles of activated T cells and, in this review, I will be focusing on some of them.

The co-stimulatory molecule CD28 plays a crucial role during T cell activation. Activation of CD28 induces the PI3K/AKT pathway, positively regulating glucose metabolism by inducing Glut1 surface translocation and increasing glucose uptake (Maciolek et al. 2014). Specifically, interaction of CD28 with its ligands leads to the activation of PI3K resulting in the production of phosphatidylinositol 3-phosphate that recruits AKT isoforms and 3-phosphoinositide-dependent protein kinase-1 (PDK1) to the cell membrane (Rathmell et al. 2003). PDK1 phosphorylates Akt at the cell membrane resulting in the activation of mTORC1. Both Akt and mTORC1 are involved in the promotion of aerobic glycolysis in T cells, increasing Glut1 expression and decreasing its internalization (Barthel et al. 1999)(Frauwirth et al. 2002) (Wieman et al. 2007) (MacIver et al. 2013).

mTOR functions as an energy sensor that is responsive to environmental inputs. mTOR signals come from to distinct complexes: mTORC1 and mTORC2. These complexes are associated with different scaffolding proteins: Raptor is the scaffolding protein of mTORC1 while Rictor is the scaffolding protein of mTORC2. Activation of mTORC1 results in the phosphorylation of S6 kinase beta-1 (S6K1) and the translation initiation factor 4E-BP1 while activation of mTORC2 results in the phosphorylation of the AKT kinase at serine 473 (Pollizzi & Powell 2015). As indicated, mTOR is involved in stimulating aerobic glycolysis but it also regulates T cell differentiation. Delgoffe *et al.* showed that mTOR-deficient $CD4^+$ T cells are not capable of differentiating into Th1, Th2 or Th17 effector cells whereas Treg differentiation is

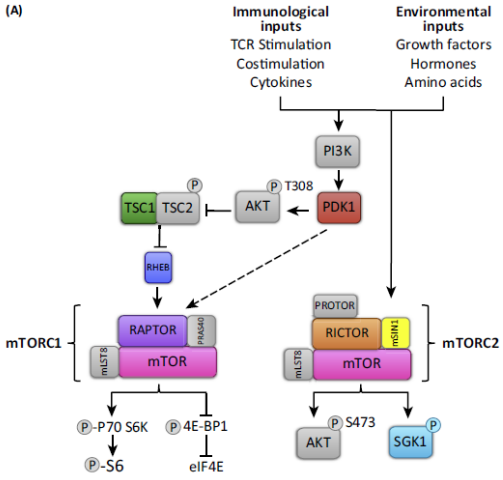

Figure 7 – mTOR signalling in Pollizzi, K. N. & Powell, J. D. Regulation of T cells by mTOR: the known knowns and the known unknowns. *Trends Immunol.* 36, 13–20 (2015).

intact (Delgoffe et al. 2009). Indeed, pharmacological inhibition of mTORC by rapamycin also favors Treg differentiation (Battaglia et al. 2005) (Kang et al. 2008) (Kopf et al. 2007). Notably though, mTORC1 and mTORC2 activities are differently required in T helper subsets; mTORC1 is critical for Th1 and Th17 differentiation whereas mTORC2 is important for Th2 differentiation (Delgoffe et al. 2011).

In addition to the mTOR pathway, Myc and Hypoxia-inducible factor 1α (HIF-1α) also regulate T cell metabolism. Although both are involved in the induction of genes involved in glycolysis, only Myc appears to be necessary to the metabolic switch in activated T cells, at least in a murine system (Wang et al. 2011). Myc regulates glycolysis, glutamine oxydation and polyamine synthesis (Rathmell 2011). mTOR promotes the translation of Myc, due to the phosphorylation and inhibition of the translational repressor 4E-BP1 and subsequent activation of eIF-4e (Ruggero et al. 2004). In addition to inducing the expression of genes involved in glycolytic flux (Wang et al. 2011), Myc also directly targets several mitochondrial genes, increasing mitochondrial biogenesis and function (Li et al. 2005).

HIF-1α, induced under conditions of hypoxia, decreases mitochondrial oxygen consumption by limiting the entrance of pyruvate in the TCA cycle and enhancing glycolysis (Papandreou et al. 2006). Importantly, HIF-1α is critical for Th17 differentiation and in its absence, Th effectors adopt a Treg fate. Thus, while the metabolic mechanisms are not clear HIF-1α-induced pathways are required for Th17 but not Treg differentiation (Shi et al. 2011).

AMP- activated protein kinase (AMPK) complex is another metabolic regulator of T cell survival and activation(MacIver et al. 2013). AMPK leads to the activation of macro-autophagy, a process that ensures the generation of metabolites that are required for proliferation (Hubbard et al. 2010). Importantly, AMPK activity opposes mTOR-dependent pathways, promoting mitochondrial oxidative metabolism and limiting glycolysis (Hardie et al. 2006). AMPK is crucial for Treg differentiation (Hardie et al. 2006), promoting the exogenous fatty acid oxidation that is required by these cells (O'Neill & Hardie 2013). Finally, Blagih *et al.* recently showed that AMPK is important in the metabolic reprogramming of Teff under under limiting glucose conditions.

Following glucose starvation, AMPK induces the expression of genes involved in glutamine uptake and glutaminolysis, promoting a metabolic adaptation (Blagih et al. 2015).

In summary, T cell metabolism is a complex topic that has been the focus of much work in the past few years. Understanding T cell metabolism may allow the development of novel therapeutic strategies modulating T cell differentiation and function.

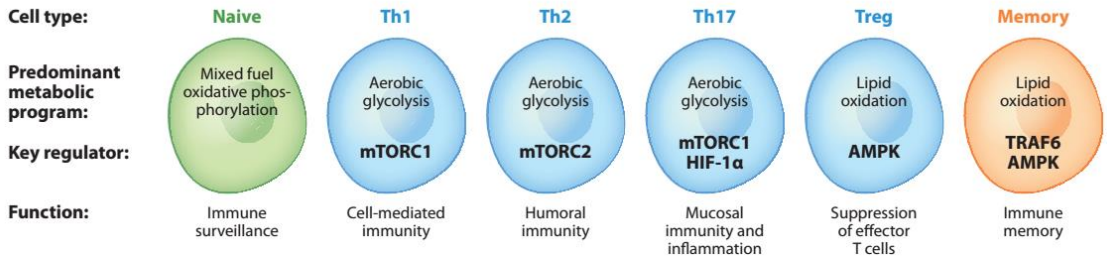

Figure 8 - Metabolic profiles and their key regulators in MacIver, N. J., Michalek, R. D. & Rathmell, J. C. Metabolic regulation of T lymphocytes. Annu. Rev. Immunol. 31, 259–83 (2013)

1.2.4 Hypoxia

As indicated above, in the presence of oxygen, cells can direct pyruvate trough the TCA cycle in order to produce maximal amounts of energy in a process termed the "Pasteur effect"(E. 1974). However, under hypoxic conditions, pyruvate does not enter in the TCA cycle and, rather, lactate is produced through anaerobic glycolysis. Notably though, this latter process can also occur in aerobic conditions, as in T lymphocytes, and is therefore termed aerobic glycolysis (Vander Heiden et al. 2009). As explained above, this process produces less energy, although it leads to the production of metabolic intermediates crucial for cell growth and proliferation.

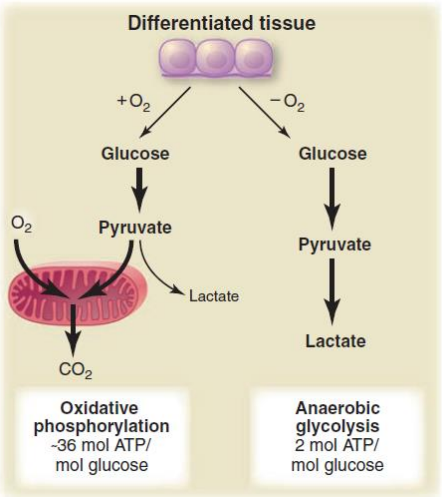

Figure 9 – Metabolic shift under different oxygen tensions in Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–33 (2009).

In the context of T lymphocytes, it is interesting to note that the oxygen tension in vivo ranges between 0.5 and 4.5% (Caldwell et al. 2001). These values are very different from the atmospheric oxygen concentration (approximately 20%) in which the vast majority of *ex vivo* T cell experiments are performed. Furthermore, hypoxia, at levels lower than 2% oxygen, is a major characteristics of many tumors, associated with tumor progression and resistance to therapy (Hockel & Vaupel 2001).

The hypoxic environment can lead to different alterations in T cells responses (Mockler et al. 2014). Hypoxia leads to alterations in cell metabolism and gene expression, with the majority of these alterations associated with increased expression of the hypoxia-inducible factor (HIF-1α), a transcription factor. HIFs are constitutively synthesized but are also rapidly targeted for proteasomal degradation following hydroxylation of at least one of two critical proline residues, mediating their interaction with the von Hippel-Lindau (VHL) E3 ubiquitin-ligase complex. This hydroxylation is catalyzed in humans by HIF prolyl 4-hydroxylases whose activity is inhibited by even small decreases in oxygen concentration. Under these conditions, HIFs escape and modulate expression of numerous hypoxia-regulated genes. HIFs can also be regulated by stimuli non-related to oxygen tension such as cytokines and growth factors (Déry et al. 2005) (Roman et al. 2010) as well as by glycolytic intermediates (Fischer et al. 2007). The transcriptional programs regulated by HIF are clearly important for T cell differentiation as it has been shown that the absence of HIF-1α inhibits the capacity of a naïve T cell to undergo Th17 differentiation and redirect T cells to an Treg fate (Shi et al. 2011) (Dang et al. 2011). Furthermore, hypoxia can alter cytokine secretion profiles in T lymphocytes, increasing IFN-γ (Roman et al. 2010) and decreasing IL-2 transcription (Zuckerberg AL, Goldberg LI 1994), and inhibiting Kv1.3 channels, involved in membrane potential and cell function (Conforti et al. 2003).

Following hypoxia-induced HIF-1a expression, there is a transcriptional activation of genes involved in glucose uptake (GLUT1), glycolysis, angiogenic molecules, survival and growth factors, proteins as well as other factors playing an important role in tumor invasiveness, chaperones and therapy resistance (Hockel & Vaupel 2001). Hypoxia also inhibits the expression of genes related to cell-surface integrins, thereby promoting tumor cell detachment and

potential metastasis (Hasan, NM and Adams, GE and Joiner, MC and Marshall, JF and Hart 1998). As integrins promote migration of lymphocytes to inflammatory zones, this phenomenon can also negatively affect the migration and persistence of T lymphocytes to a tumor microenvironment. While the effects of hypoxic conditions on T cells has not been fully elucidated, it is known that changes in the environment of a T lymphocyte, between lymph node organs and other tissues, will markedly alter the oxygen availability to which it is exposed.

Elucidating the role of changing oxygen concentrations on T cell differentiation and proliferation is important in optimizing the potential impact of adoptively transferred anti-tumor T cells.

Objectives

Previous work from Naomi Taylor's lab, as well as others, showed that TCR activation of CD4 T cells results in increased surface Glut1 expression and an accompanying augmentation in glucose uptake, with more recent studies showing that expression of the ASCT2/SLC1A5 glutamine transporter as well as glutamine uptake is also highly upregulated. Moreover, the group found that inhibiting glucose or glutamine uptake results in decreased T cell activation and proliferation. It was therefore of interest to determine whether deprivation in these nutrients affected T cell fate. Importantly, the group's unpublished data strongly suggested that activation of murine $CD4^+$ T cells under conditions of glutamine deprivation causes them to terminally differentiate into $Foxp3^+$ regulatory T cells (Tregs) with potent *in vivo* suppressor function. Thus, during my lab rotation, I was interested in determining the degree of glutamine deprivation required for this effect, assess whether it was also detected in the context of CD8 T cells and determine whether the same phenomenon exists in human CD4 T cells. Furthermore, I undertook experiments to elucidate the mechanisms via which glutamine derivation inhibits Th effector differentiation and results in a skewing of naïve T cells to a $Foxp3^+$ T cells fate. To this end, I focused on the importance of glutamine-derived TCA cycle intermediates.

In addition to determining the fundamental importance of glutamine in T cell function, these studies can have potential impact in the anti-tumor efficacy of adoptively transferred T cells because tumors themselves may alter the nutrients available to a T lymphocyte. Indeed, nutrient concentrations within tumor microenvironments are generally lower than in normal tissues. Specifically, quantitative metabolomics profiling has revealed lower intratumoral glucose as well as glutamine concentrations in patients with hepatocellular carcinomas, stomach and colon tumors (Bode & Souba 1999) Furthermore, alkylating chemotherapies have been found to decrease the intracellular generation of the glutathione antioxidant due to limiting glutamine levels (Welbourne 1979) (DeLeve & Wang 2000).

In this context, I was also very interested in determining whether changes in the nutrient transporter profile of a CD4 T cell would change its effector phenotype, potentially altering its ability to respond to tumor. Therefore, during my rotation, I also focused on expression of the

Glut1 glucose transporter and its regulation by hypoxia. Specifically, I assessed whether differential expression of the Glut1 glucose transporter in hypoxic or normoxic conditions can distinguish CD4 T cells; at the level of phenotype, proliferation and/or effector phenotype.

Thus, the ensemble of my experiments focused on studying the importance of glutamine and glucose metabolism in T cell activity and function. The overall goal of the research to which I contributed is to determine how specific metabolic pathways, may enhance T lymphocyte anti-tumor immunity.

Chapter 2 Material and Methods

2 Material and Methods

2.1 Material

2.1.1 Products and materials

Company	Product
Life Technologies	Dynabeads Untouched Mouse $CD8^+$ cells kit
	Dynabeads untouched Mouse T cells kit
	RPMI 1640 media
	RPMI 1640 + GlutaMAX
	CTV (Cell Tracer Violet)
	Glutamine
	Penicillin/Streptomycin
Myltenyi Biotec	MACS $CD4^+$ T cell isolation kit
Sigma- Aldrich	Dymethyl alpha ketoglutarate (DMK)
	PMA (phorbol 12-myristate 13-acetate)
	Ionomycin
	Brefeldin A
	Histopaque 10707
	PBS
	B-mercaptoethanol
	Glucose

Qiagen	RNeasy Micro Kit
	QuantiTect Reverse transcription Kit
Roche	LightCycler 480 SYBR Green I Master Kit
Thermo Fisher	24 well non-treated plates
	Tissue-treated 24 well plates
	Tissue-treated 6 well plates
Eurobio	FCS (Fetal calf serum)
Dominique Dutscher	FCS (Fetal calf serum)
Stem Cell	RosetteSep Human T cell enrichment cocktail
	RosetteSep Human $CD8^+$ T cell enrichment cocktail
eBioscience	Fixation/permeabilization $FoxP3^+$ Kit
	VDP
Eurogentec	Actin primer
IDT	FoxP3 primer
Proleukin	Human recombinant IL-2
BioXcell	Murine anti-IL-4 (clone 11b11)
Peprotech	Murine recombinant IL-12

2.1.2 Panels of Antibodies use for immunophenotyping (Flow Cytometry Technique)

Type of cells	Experiment	Fluorochrome-conjugated antibodies	Clone	Company
Murine cells	Naïve T cell sorting	CD4-PE	RM4-4	BD Pharmingen
		CD8 -PcP	53-6.7	BD Pharmingen
		CD25 –APC-eFluor 780	PC61	BD Pharmingen
		CD44- Pc7	IM7	BD Pharmingen
		CD62L- APC	MEL-14	BD Pharmingen
	Activation/Phenotype	CD4- APC-eFluor 780	RM4-5	eBioscience
		CD8-PcP	53-6.7	BD Pharmingen
		CD25-PE	PC61	BD Pharmingen
		CD44-APC	IM7	BD Pharmingen
		CD62L-FITC	MEL-14	BD Pharmingen
	Transcription factors	Tbet- PE	eBio4B10	eBioscience
		FoxP3-Pc7	FJK-16S	eBioscience
	Cytokine	IFN-γ- APC	XMG1.2	eBioscience
	General phenotype	CD4- APC-eFluor 780	RTA-T4	eBioscience
		CD8-PcP Cy5.5	SFC121Thy2D3	Beckman

Human cells		CD45RA-PE	ALB11	Beckman
		CCR7-Pc7	3A9	BD Pharmigen
	Treg phenotype	CD4- APC-eFluor 780	RTA-T4	eBioscience
		CD8-PcP Cy5.5	SFC121Thy2D3	Beckman
		CD25- FITC	BC96	Biolegend
		CD127- Pc7	R34.34	Beckman
		FoxP3- PE	236A/E7	eBioscience
	Sorting based on Glut1 expression	H2-GFP	-	Marc Sitbon Laboratory – IGMM, France
		CD71- PE	YDJ1.2.2.	Beckman
	Cytokine	IL-17- PE	64CAP17	eBioscience
		IFN-γ- Pc7	B27	BD Pharmigen

2.2 Methods

2.2.1 Murine T cell isolation and activation

Lymph nodes were harvested from C57BL/6 mice and mashed on cell strainers to obtain cell suspension. CD4 and CD8 T cells were enriched using MACS $CD4^+$ T cell isolation kit and dynabeads Untouched Mouse $CD8^+$ cells kit respectively. Total $CD3^+$ T cells were purified using dynabeads untouched Mouse T cell kit. After enrichment, T cells were labeled with the proliferation dye CTV for 3 min at RT and the reaction was stopped with PBS 2% FCS.

T cell activation was performed on anti-CD3 (clone 2C11; 1 µg/ml) and anti-CD28 (clone PV-1; 1 µg/ml) mAbs coated non-treated 24 plates. T cells were activated and maintained in RPMI 1640 media supplemented with 10% FCS, 1% PS, β-mercaptoethanol , glucose (11mM) in

presence of different concentrations of glutamine (2, 0,4, 0,3 and 0 mM). Exogenous recombinant IL-2 (100 U/ml) was added every other day starting at day 2 post-activation.

For differentiation experiments, after CD4 T cell enrichment, naïve $CD4^{+}CD62L^{hi}CD44^{-}CD25^{-}$ T cells were sorted on a FACSaria flow cytometer (BD Biosciences). To induce Th1 polarization, recombinant murine IL-12 (10 ng/ml) and anti-IL-4 (5 µg/ml) were added during T cell activation. When indicated, dimethyl alpha ketoglutarate (3.5 mM) was also added to the cell culture.

2.2.2 Human T cell isolation and activation

Total $CD3^{+}$ T cells were isolated from healthy donor blood samples using RosetteSep Human T cell enrichment cocktail. After incubation with the RosetteSep cocktail for 20 minutes at RT, blood was centrifugated on a ficoll gradient and enriched CD3 T cells were collected at the interface of the ficoll. As described for murine T cells, human CD3 T cells were VDP labelled for 3 min at RT and then washed with PBS 2% FCS.

T cell activation was performed using with coated anti-CD3 (clone OKT3; 1 µg/ml) and anti-CD28 (clone 9.3; 1 µg/ml) mAbs on non-treated 24 well plates (plate-bound). Total $CD3^{+}$ T cells were activated and maintained in RPMI 1640 media supplemented with 10% FCS, 1% PS, glucose (11 mM) and in the presence or absence of glutamine (2 mM). Exogenous recombinant IL-2 (100 U/ml) was added every other day starting at day 2 post-activation. For some experiments, T cells were incubated in parallel at 20% and 1% of oxygen tension.

For experiments in which T cells were sorted based on Glut1 expression, at day 2 post-TCR activation, cells were collected and incubated during 30 min at 37 ^{o}C with H_{RBD}EGFP (Glut1 ligand coupled with GFP). Briefly, H_{RBD}EGFP consists in recombinant HTLV-2 envelope receptor-binding domain (H_{RBD}) fused to the enhanced green fluorescence protein–coding sequence (H_{RBD}EGFP). Cell sorting was then performed based on Glut1 level of expression: the 10% highest and lowest Glut1 expressing T cells (glut1hi and glutlo T cells respectively) were sorted and put back to culture.

2.2.3 Flow cytometry analyses

Immunophenotyping of the cells was performed at the indicated time points using fluorochrome-conjugated antibodies (Abs detailed in the above table).Cells were incubated during 20 minutes on ice for extracellular stainings and over-night in intracellular stainings. Intracellular staining was performed following fixation/permeabilization using the ebioscience fixation kit. For cytokine detection, T cells were re-activated with (100 ng/ml)/ Ionomycin (1 µg/ml) in the presence of brefeldin A (10ug/ml) for 3.5-4h at 37^{o}C prior to cytokine staining. Cells were analyzed on a FACScanto II or on FACSfortessa (BD Biosciences). Data analyses were performed using FlowJo Pc version 10 software (Tree Star).

2.2.4 Gene expression analyses

RNA was isolated from purified CD4^{+} or CD8^{+} T cells using the RNeasy Micro Kit and reverse transcribed into cDNA by oligonucleotide priming with the QuantiTect Reverse Transcription Kit. Quantitative RT-PCR was performed using the LightCycler 480 SYBR Green I Master kit using specific primers . Foxp3 mRNA expression was normalized as compared to actin.

Foxp3: S: 5'-CCCAGGAAAGACAGCAACCTT, AS: 5’-TTCTCACAACCAGGCCACTTG

Actin: S: 5’-TGAGACCTTCAACACCCCAGCC, AS: 5’- GGAGAGCATAGCCCTCGTAG

Chapter 3 Results

Results

Part 1: Impact of glutamine concentration on T cell activation and differentiation.

To mimic in vivo situations in which glutamine can be a limiting factor such as cancers and chemotherapies several experiments were designed to evaluate the potential impact of metabolites on T cells. Glucose and glutamine represent 2 crucial sources of energy required to fulfill the needs of the cell during T cell activation and differentiation. To evaluate the specific contribution of glutamine and how it impacts on T cell activation, we undertook experiments comparing different doses of extracellular glutamine. To perform these experiments, lymph nodes were collected from C57BL/6 mice and T cells were enriched using a negative selection approach. 94-98% efficiency of enrichment was obtained in average in all performed experiments (**Figure 1**).

The phenotype of the freshly isolated T cell populations was assessed prior to activation, monitoring the CD4/CD8 ratio as well as the expression of CD62L and CD44 to determine the percentages of naïve ($CD62L^+CD44^-$), effector ($CD62L^-CD44^-$) and memory ($CD62L^+CD44^+$) T cells. We also determined the percentage of regulatory T cells by measuring the expression of their master regulator, the transcription factor FoxP3. The CD4/CD8 ratio was in physiological levels (approximately 1,5:1) and as expected, most $CD4^+$ and $CD8^+$ T cells expressed a naïve phenotype (74% and 78% respectively). The percentage of Foxp3-expressing T cells was also in a physiological range, 14,5% and 0,5% for $CD4^+$ and $CD8^+$ T cells, respectively (**Figure 1**).

To monitor the effect of glutamine on the TCR responsiveness of $CD3^+$ T lymphocytes, the enriched cells were activated with plate-bound α-CD3 and α-CD28 antibodies in the presence of different concentrations of extracellular glutamine (0-2 mM, with physiological serum concentrations at approximately 0.8 mM and levels in culture media generally set at 2 mM). Of note, even at 0 mM glutamine in the extracellular media, the cells were exposed to glutamine as in the fetal calf serum (FCS) resulting in an approximate final concentration of 0,08%.

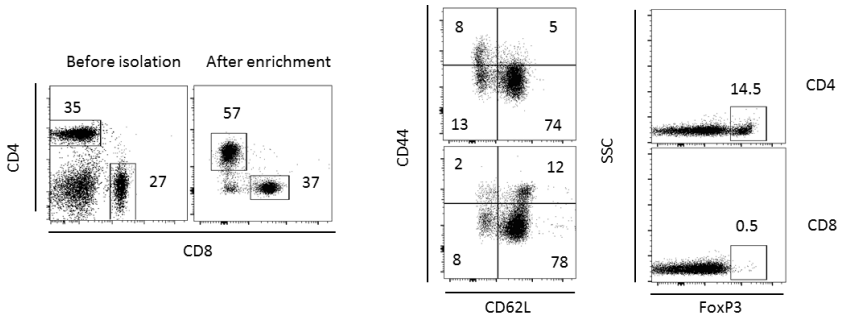

Figure 1: Phenotype of purified lymph node T cells

$CD3^+$ T cells were purified from freshly isolated C57BL/6 lymph node suspensions by MACS selection. A representative dot plot showing the relative percentage of $CD4^+$ to $CD8^+$ T lymphocytes is presented (left). The relative percentages of naïve ($CD62L^+CD44^-$), central memory ($CD62L^+CD44^+$), effector (CD62L-$CD44^+$) and suppressor ($Foxp3^+$) cells within the $CD4^+$ (top) and $CD8^+$ (bottom) subsets is presented.

Impact of glutamine concentration on forward and side scatter parameters and proliferation of $CD3^+$ murine T cell populations.

The impact of limiting glutamine concentration was already detectable by eye, looking at the cell cultures by microscopy, where the cells were less “blast”-like and less clumped in the absence of glutamine. Flow cytometry, allowing forward (FSC) and side (SSC) scatters, a measure of approximate size and granularity, to be monitored revealed that these parameters were decreased in the absence of extracellular glutamine (**Figure 2A**). Furthermore, by day 6 there was a small decrease in viability, but only in the absence of any extracellular glutamine (**Figure 2A**). Furthermore, there was a decrease in the relative percentage in $CD4^+$ T cells at lower glutamine concentrations, potentially explained by an increased cell death/sensitivity or decreased proliferation (**Figure 2B**).

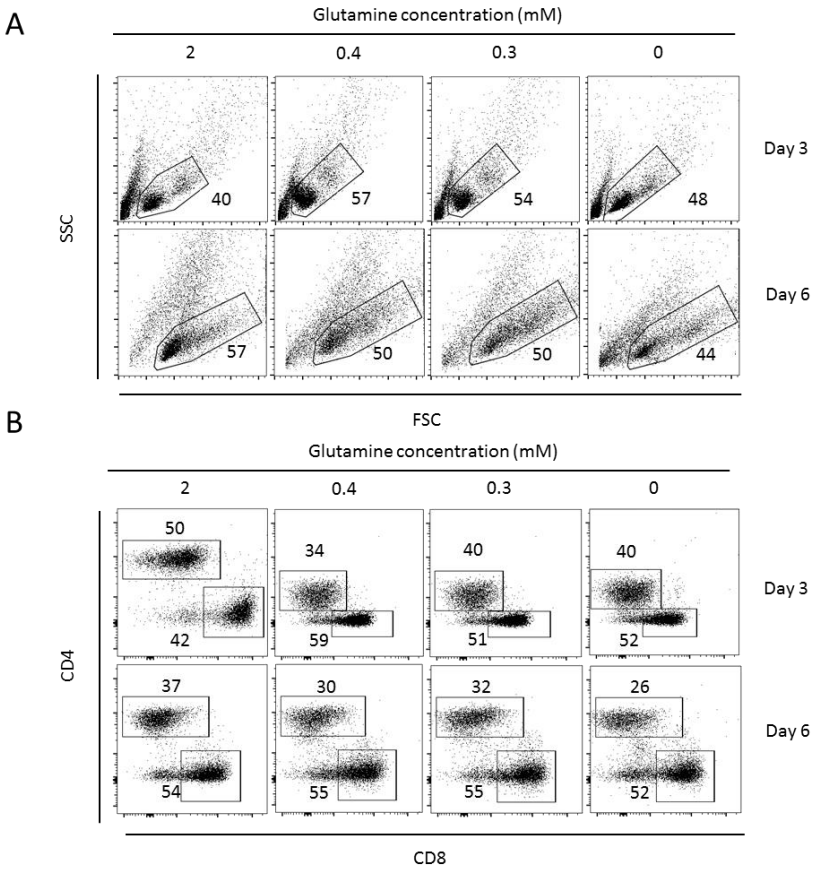

Figure 2: Impact of glutamine concentration on forward/side scatter and CD4/CD8 ratios following TCR stimulation. (A) Purified $CD3^+$ T cells were activated with immobilized CD3/CD28 mAbs in the presence of 0 to 2 mM glutamine. Representative plots showing forward/side scatter at days 3 and 6 are presented. The percentages of viable cells in each plot are noted. (B) CD4/CD8 profiles at days 3 and 6 are presented and relative percentages are indicated.

We then assessed the proliferation of $CD4^+$ and $CD8^+$ T cells activated under the different extracellular glutamine concentrations. To monitor cell division, freshly purified T cells were labeled with a fluorescent dye (CTV) which binds to cytoplasmic protein and is diluted by 50% at each division, allowing division peaks to be evaluated by flow cytometry. At day 3 following activation, T cells activated under optimal conditions had undergone up to 3-4 rounds of division while this level was progressively decreased with lower concentrations of glutamine, with only 1 division peak in its absence (**Figure 3A**). Interestingly, even at 0.4 mM glutamine, division was decreased in both $CD4^+$ and $CD8^+$ T cells (2 division peaks). While proliferation of both $CD4^+$ and $CD8^+$ T cells was affected, there was less overall proliferation in the former subset. However, at day 6, the differences in proliferation were not as marked, and cells underwent proliferation in all conditions, with extensive division in 0.4 and 0.3 mM

glutamine conditions. These data suggest that TCR-induced proliferation of both $CD4^+$ and $CD8^+$ T cells is delayed under conditions of glutamine deprivation (**Figure 3B**).

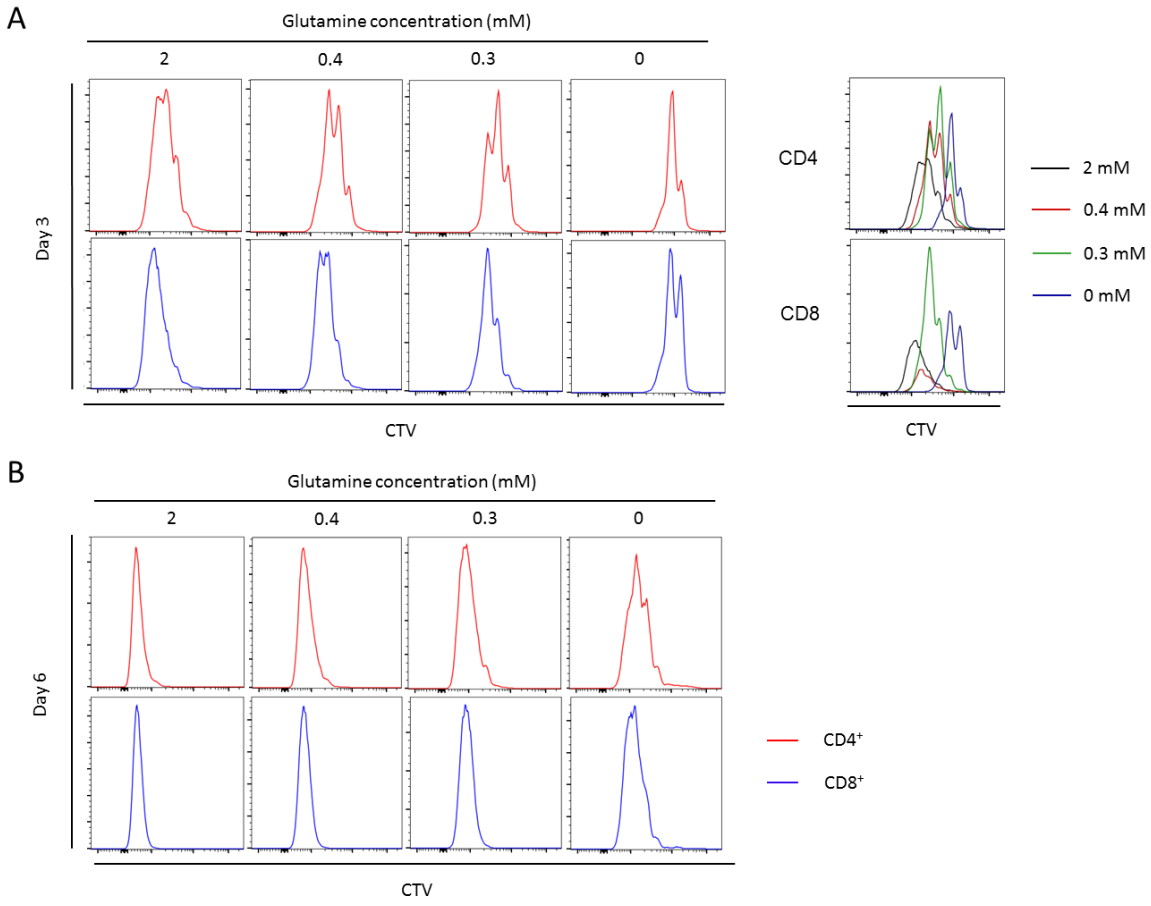

Figure 3: Decreasing extracellular glutamine results in a delay in TCR-stimulated proliferation of $CD3^+$ T cells.

The effect of glutamine concentration (0-2 mM) on TCR-induced proliferation of $CD3^+$ T cells was assessed. T cells were labeled with the fluorescent CTV dye and division of daughter cells was monitored as a function of CTV dilution at days 3 (A) and 6 (B) post stimulation. Representative histograms are shown.

Decreased extracellular glutamine results in an increase in Foxp3-expressing $CD4^+$ T cells following TCR stimulation.

To assess whether the phenotype of activated $CD4^+$ and $CD8^+$ T cells was affected by the concentration of extracellular glutamine, we analyzed the phenotype based on the CD62L/ CD44 profiles at days 3 and 6 after TCR stimulation. These analyses also revealed a delayed activation and upregulation of CD44 expression in conditions of glutamine starvation. The percentages of

naïve $CD4^+$ T cells ($CD62L^+CD44^-$) were higher in the absence of glutamine but it is important to note that both for $CD4^+$ and $CD8^+$ T cells, there was a conversion to a memory phenotype ($CD62L^+CD44^+$) irrespective of the concentration of extracellular glutamine. Thus, even in sub-optimal glutamine concentrations, TCR activation results in a differentiation of naïve T lymphocytes to a memory phenotype (**Figures 4A and 4B**).

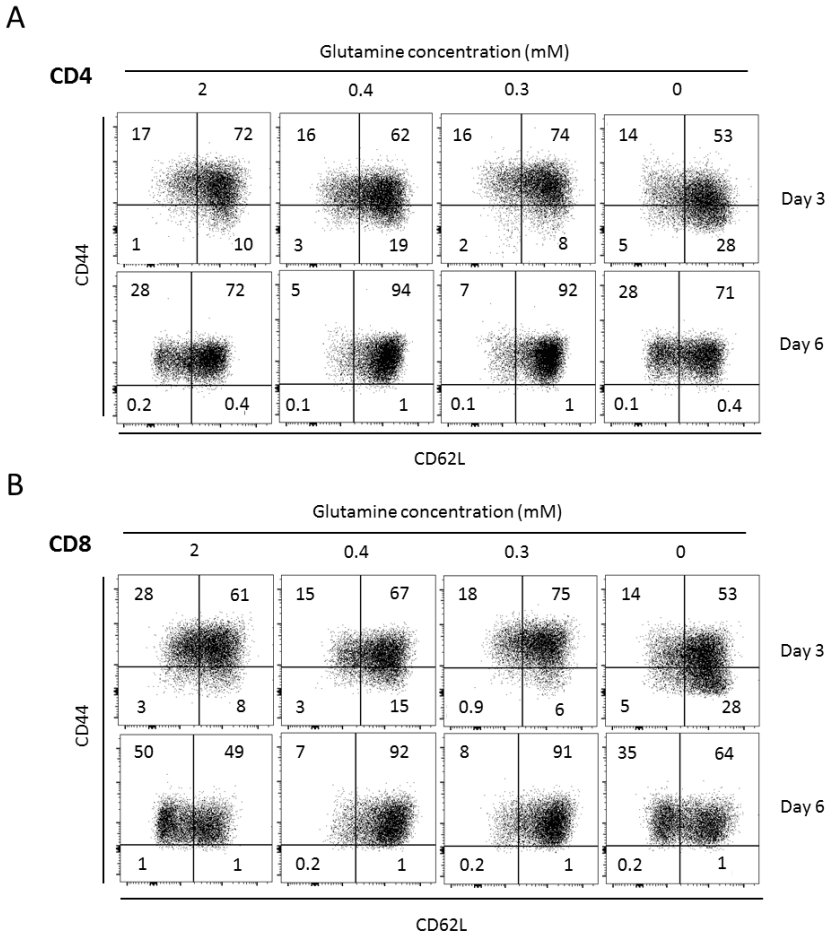

Figure 4: Low extracellular glutamine results in decreased effector differentiation of TCR-stimulated $CD4^+$ and $CD8^+$ T cells.

Purified $CD3^+$ T cells were TCR-activated in the presence of different glutamine concentrations. The naïve, memory and effector phenotype of $CD4^+$ (A) and $CD8^+$ T cells (B) was assessed at days 3 and 6 post stimulation on the basis of CD62L and CD44 expression as in Figure 1 (CD62L+CD44-: Naïve; CD62L+CD44+: memory; CD62L-CD44+: effector). The percentages of cells in each quadrant are indicated.

As my lab previously found that the absence of glutamine biased $CD4^+$ T cells to a regulatory T cell fate, I assessed whether there was a change in the percentage of FoxP3-expressing $CD4^+$ as well as $CD8^+$ T cells under different extracellular glutamine concentrations.

FoxP3 is the master regulator of regulatory T cell development and expression of this transcription factor is also used as a reliable marker of most murine regulatory T cell populations, at least in the murine system. Notably, the percentage of $FoxP3^+$ cells within the $CD4^+$ T cell population increased significantly in the absence of extracellular glutamine, approximately 8-fold as compared to physiological conditions (4% to 31%). Moreover, the percentages of $CD4^+FoxP3^+$ T cells were already elevated at intermediate glutamine concentrations of 0.4 mM to 14% (**Figure 5A**). Furthermore, a very similar trend was detected in the $CD8^+$ T cell population, although the change didn't reveal a linear inverse proportion with glutamine concentrations. Increases from 2% to 8-14% between 2 mM and low glutamine levels (0-0.4 mM) were detected (**Figure 5A**). We also confirmed these results at the transcriptional level as qRT-PCR analyses demonstrated an almost 16-fold increase expression in FoxP3 mRNA levels (**Figure 5B**).

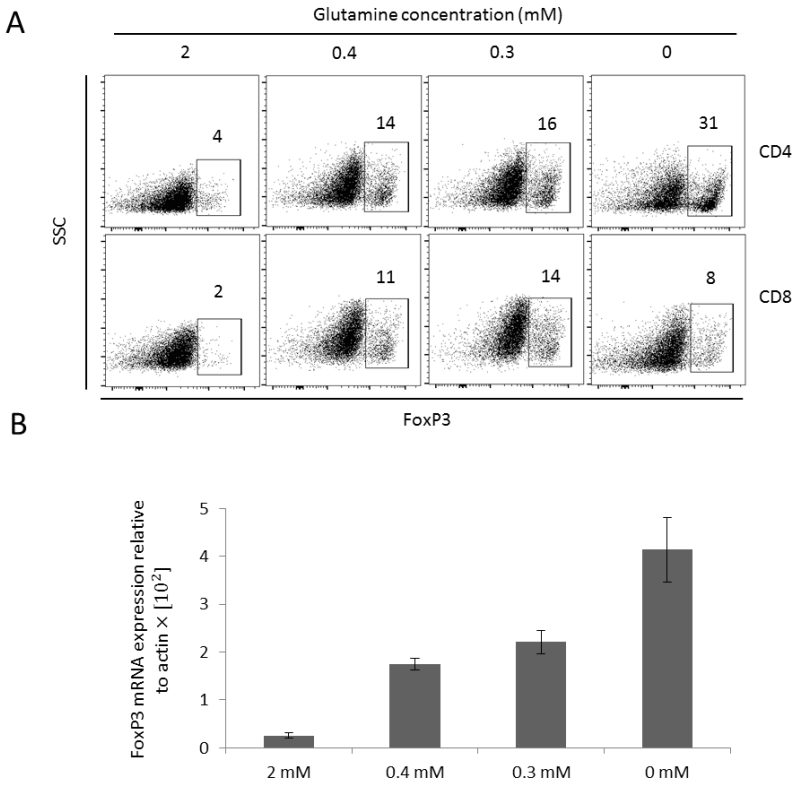

Figure 5: Extracellular glutamine modulates the induction of Foxp3 expression in activated $CD4^+$ T cells. (A) The percentages of $Foxp3^+$ cells within the CD4+ and $CD8^+$ T cell populations were monitored 6 days post TCR stimulation as a function of the exogenous glutamine concentration, as indicated (0-2 mM). Representative dot plots are presented. (B) The levels of Foxp3 transcripts in the indicated conditions were quantified by qRT-PCR and normalized relative to actin. Means +/- SD are presented.

The impact of glutamine concentration on TCR-activated $CD8^+$ T cells is independent of the presence of $CD4^+$ T cells.

As the fate of $CD8^+$ T cells was modulated by activation under limited-glutamine concentrations, we were interested in determining whether $CD4^+$ T cells played a role in this process. Indeed, in the experiments presented above, the fate of $CD8^+$ T cells was monitored in cultures where $CD4^+$ T cells were also present. $CD8^+$ T cells were therefore isolated from murine lymph nodes with an enrichment of 93% and TCR-stimulated in the presence of different glutamine concentrations (0-2 mM) as described above. 2 mM, 0.4 mM, 0.3 mM and 0 mM. Interestingly, the viability of activated CD8 T cells was actually higher in the absence of extracellular glutamine, possibly reflecting a lower level of TCR-induced cell death (data not shown). Notably though, and as detected in the presence of $CD4^+$ T cells, proliferation of $CD8^+$ T cells after 3 days of TCR stimulation was decreased at lower levels of glutamine, with lowest level proliferation at 0 mM glutamine, decreased from 3 to 1 maximal rounds of division (**Figure 6A**). Division continued over the next 3 days (day 6) but was again lower in the absence of glutamine (**Figure 6A**).

Notably, the increase in $FoxP3^+$ $CD8^+$ T cells following stimulation in glutamine-deprived conditions was also detected in the absence of $CD4^+$ T cells. Furthermore, this increase already reached maximal levels in the presence of 0.3 mM glutamine, increasing from 1% to 14% (**Figure 6B**). Interestingly though, the level of FoxP3 mRNA, increased between 0.3 mM and 0 mM glutamine, reaching levels more than 10-fold higher than that detected at 2 mM glutamine (**Figure 6C**). Thus, while the increase in the percentage of $FoxP3^+CD8^+$ T cells was already maximal at 0.3 mM glutamine, Foxp3 mRNA continued to rise in a manner that correlated inversely with glutamine levels (**Figure 6C**). These data therefore show that the conversion of $CD8^+$ T cells in Treg cells is not due to their co-culture with $CD4^+$ T cells. This redirection of T cell fate is only dependent on the glutamine availability and occurs in a $CD4^+$ T cell-independent manner.

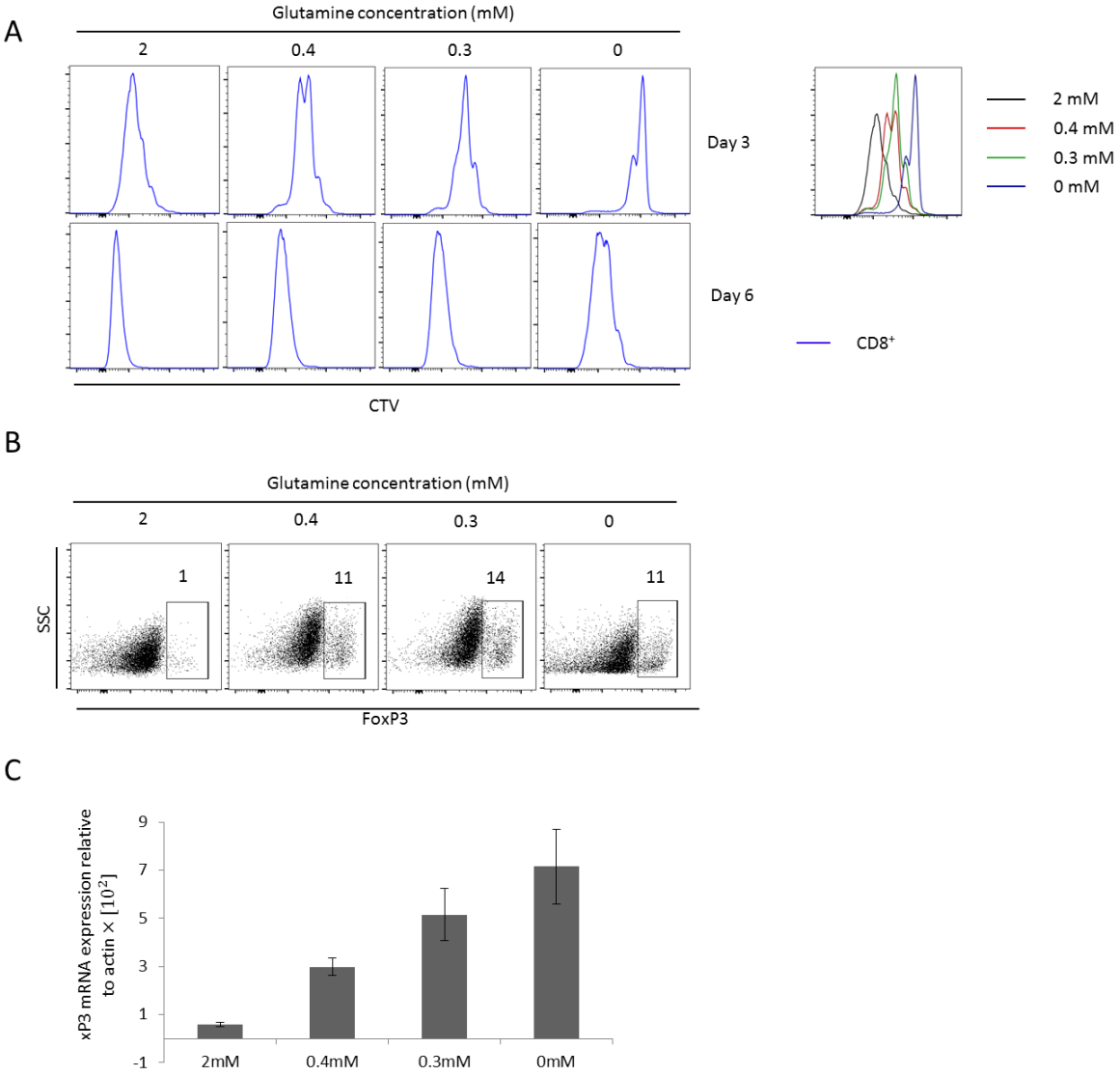

Figure 6: Decreased extracellular glutamine inhibits proliferation and increases Foxp3 expression in CD8$^+$ T cells in a CD4-independent manner. (A) The effects of glutamine concentration (0-2 mM) on the proliferation of purified CD8$^+$ T cells was assessed as a function of CTV dilution at days 3 and 6 post TCR stimulation. Representative histograms are shown. (B) The percentages of Foxp3$^+$ T cells within the CD8+ T cell population were monitored 6 days post TCR stimulation and representative dot plots are presented. (C) The levels of Foxp3 transcripts in the indicated conditions were quantified by qRT-PCR and normalized relative to actin. Means +/- SD are presented.

Th1 polarization of naïve CD4$^+$ T cells is abrogated under limiting glutamine conditions and cells are biased to a regulatory (Foxp3$^+$) T cell fate

Our results clearly show that sub-optimal glutamine concentrations impact T cell activation and surprisingly promote Treg cell differentiation both in CD4$^+$ and CD8$^+$ T cell subsets. During my rotation, I was also interested in determining whether CD4$^+$ T cells would also be biased to a Treg fate when they are activated under conditions that support effector T cell differentiation, with a specific focus on Th1 effectors. To address this question, naïve sorted T cells (CD4$^+$, CD62L$^+$, CD44$^-$, CD25^{hi}, FoxP3$^-$) were TCR stimulated using immobilized α-CD3 and α-CD28 antibodies under Th1 polarizing conditions (in presence of IL-12 and anti-IL-4) and in the presence or absence of glutamine. Th1 differentiation, monitored as a function of IFN-γ expression, was significantly impaired in the absence of glutamine (22% vs 2%; **Figure 7**) and a high percentage of these cells, activated under Th1 polarizing conditions, adopted a regulatory T cell fate (5.5 to 14%; **Figure 7**). Therefore, the nutrient milieu regulates the Foxp3 expression profile, even in cytokine environments that would be expected to bias a naïve CD4$^+$ T cell to a distinct effector fate.

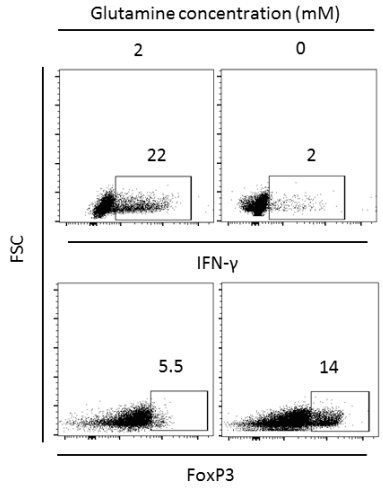

Figure 7: Polarization of naïve CD4$^+$ T cells to a Th1 fate is inhibited in the presence of low extracellular glutamine resulting in an upregulation of Foxp3$^+$ regulatory T cells. (A) Naïve CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ T cells were activated under Th1 polarizing conditions in the presence or absence of glutamine and the percentages of IFN-γ$^+$ and FoxP3$^+$ cells within the Th1 CD4$^+$ T cell population were assessed at day 5 by intracellular staining. Representative plots are presented.

As described in the introduction, glutamine contributes to several energetic cellular pathways. Glutamine is first converted to glutamate by glutaminase, which is then converted into α-ketoglutarate, and the latter can directly fuel the TCA cycle (**Figure 8A**). We were therefore interested in assessing whether the impaired Th1 differentiation observed under glutamine deprived condition could be restored by refueling the TCA cycle with α-ketoglutarate. To evaluate this hypothesis, naïve $CD4^+$ T cell were sorted, activated under Th1 polarizing conditions in the presence or absence of glutamine and in the presence or absence of a cell permeable α-ketoglutarate (DMK). As expected, in the absence of glutamine, there was negligible induction of Tbet, the master transcriptional regulator of Th1 differentiation (**Figure 8B**). Notably though, supplementing glutamine-deprived lymphocytes with DMK resulted in the induction of Tbet, to levels at least equivalent to that detected in control Th1-polarized cells (**Figure 8B**). Moreover, after 5 days of TCR stimulation under polarizing conditions, we monitored the percentage of IFN-γ-expressing T cells and supplementing cultures with DMK, markedly increased the percentage of IFN-γ producing cells (from 2% to 38%) while the percentage of Foxp3-expressing T cells decreased to control levels (**Figure 8C**). Interestingly, in glutamine-replete conditions, the presence of DMK resulted in an even higher level of Th1 differentiation (22% to 46%; **Figure 8C**). These results clearly demonstrate that the decreased αKG production occurring in glutamine-deprived $CD4^+$ T cells is directly involved in Th1 effector differentiation and the induction of an alternative $Foxp3^+$ Treg fate.

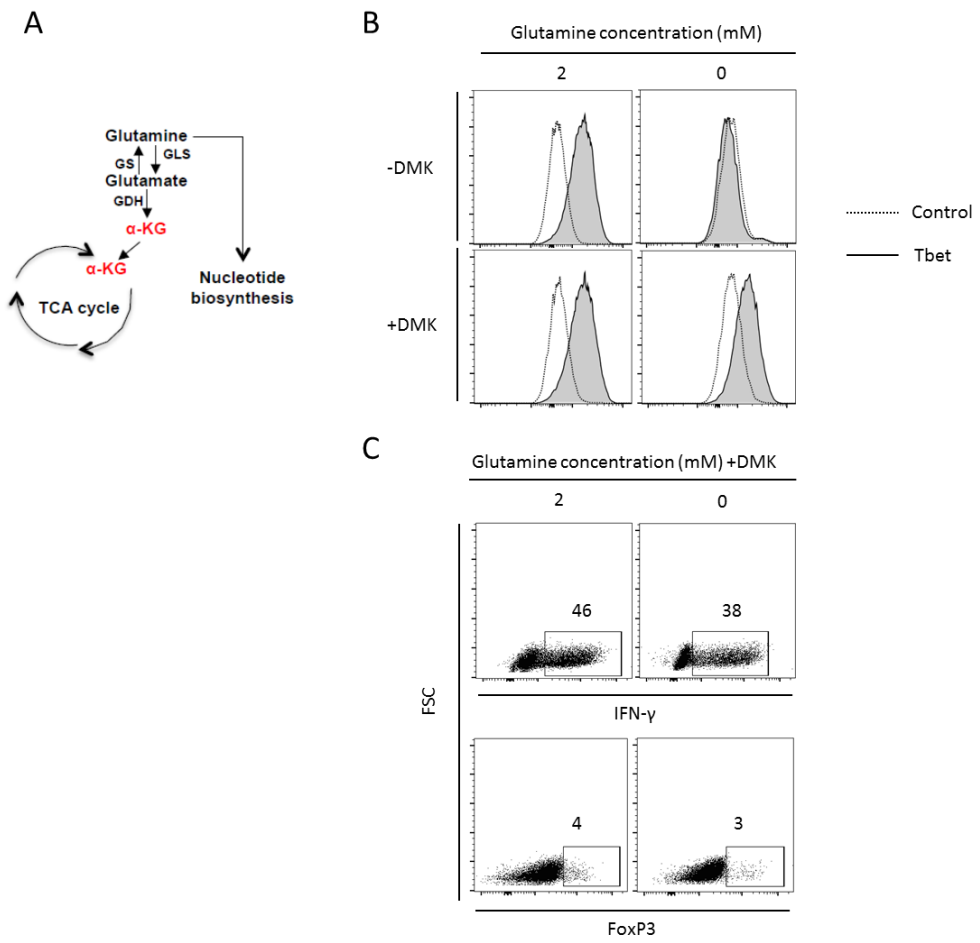

Figure 8: Cell-permeable α-ketoglutarate rescues terminal Th1 differentiation under glutamine-deprived conditions. (A) Schematic representation of glutamine metabolism to α-KG and its introduction into the tricarboxylic acid (TCA) cycle. Enzymes involved in key reactions are indicated (glutamine synthetase, GS; GLS, glutaminase; GDH, glutamine dehydrogenase). (B) Naïve $CD4^+$ T cells were stimulated under Th1-polarizing conditions in the absence or presence of glutamine. The contribution of glutamine-derived αKG was assessed by supplementing glutamine-deprived cultures with dimethyl αKG (DMK). Tbet, IFNγ, and Foxp3 were monitored by intracellular staining at day 5 post stimulation and representative plots are presented.

Glutamine deprivation promotes the regulatory $CD3^+$ $FoxP3^+$ $CD127^{low/-}$ human cells

Our results show that limiting the availability of glutamine conditions murine T cell proliferation, activation and differentiation. Considering the potential therapeutic outcome of these results, we were also interested in assessing the impact of glutamine deprivation on human T cells.

$CD3^+$ T cells were isolated from fresh blood samples from healthy donors and enrichment reached an average efficiency of 94%. The phenotype of the freshly isolated T cells

was assessed prior to activation by profiling CD45RA and CCR7 levels. As expected for healthy donors, most CD4 and CD8 T cells were CD45RA^{+} CCR7^{+} and CD45RA^{-} CCR7^{+}, representative of naïve and central-memory T cell phenotypes, respectively. The percentage of regulatory cells, defined as Foxp3^{+}CD25^{hi}CD127^{low}, were in a normal range of 2.4 % and 0.1% in the CD4 and CD8 subsets respectively (data not shown).

To assess proliferation in response to TCR stimulation, isolated cells were activated with immobilized α-CD3 and α-CD28 antibodies in the presence or absence of glutamine. Similar to the results we obtained with murine T cells, proliferation of human CD4^{+} and CD8^{+} T cells was lower under glutamine-deprived than -replete conditions (**Figure 9**). Interestingly, neither CD4^{+} nor CD8^{+} T cells had undergone significant division at day 3 in glutamine-deprived conditions (**Figure 9**).

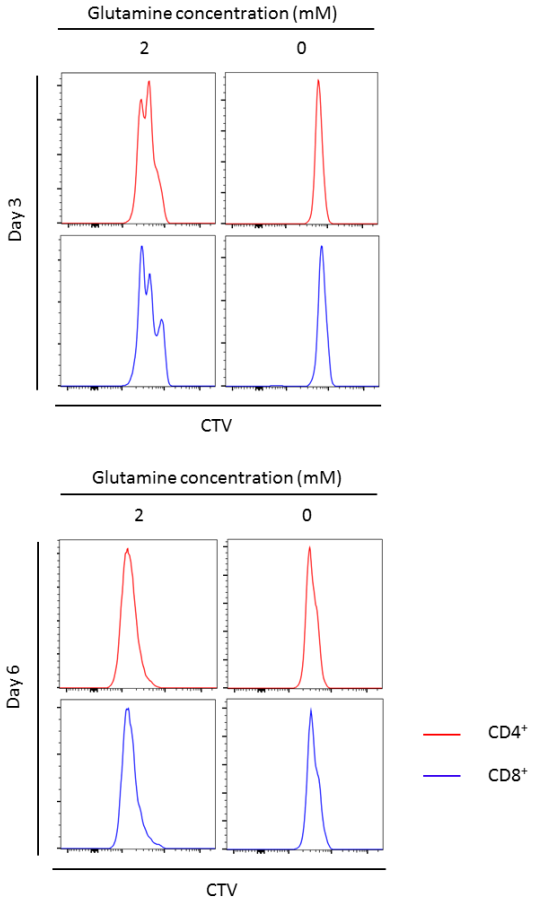

Figure 9: Deprivation in extracellular glutamine results in a delayed TCR-induced proliferation of human CD4^{+} and CD8^{+} T cells. Human T cells were activated with immobilized CD3/CD28 mAbs in the presence of 0 or 2 mM of extracellular glutamine. TCR-induced proliferation was monitored as a function of CTV dilution at days 3 and 6 post-activation.

Similarly to murine T cells, the TCR-induced loss of a naive phenotype in human $CD4^+$ T cells was slower in the absence of glutamine as compared to its presence (54 vs 43% at day 6 and 30 vs 15% at day 9; **Figure 10**). However, within the $CD8^+$ T cell subset, the acquisition of a memory phenotype and loss of naïve cells were similar in the two conditions. Notably though, the percentages of cells with a Treg phenotype, identified as $CD127^{low/-}$ $FoxP3^+$ were significantly increased within both $CD4^+$ and $CD8^+$ human subsets activated in glutamine deprived conditions. Therefore, the ensemble of our data clearly suggest that under conditions of decreased glutamine availability, activation of T lymphocytes results in an increased differentiation to a $Foxp3^+$ fate (**Figure 11**).

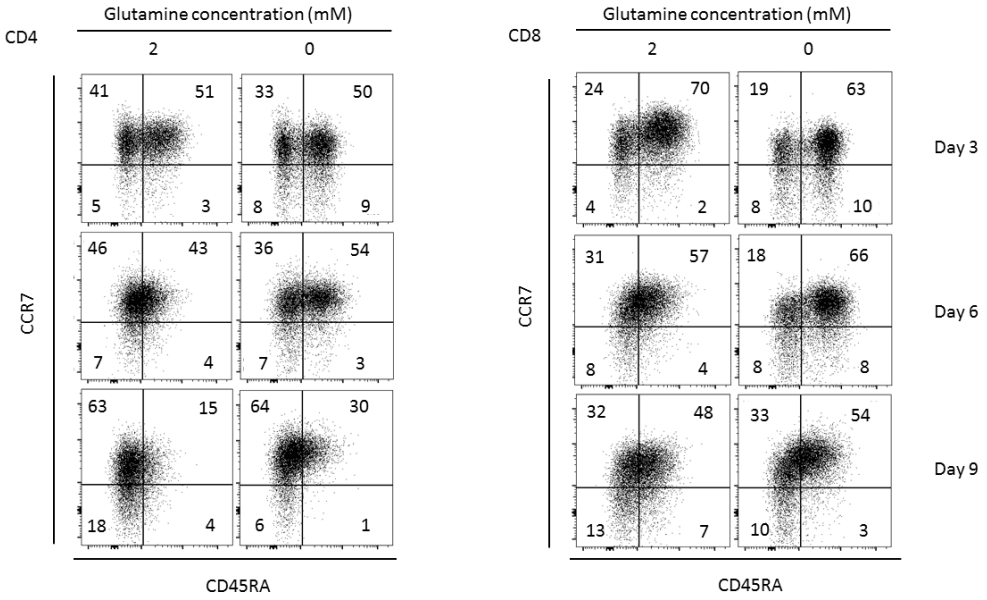

Figure 10: $CD4^+$ and $CD8^+$ T cells acquire a memory phenotype following TCR activation in the presence and absence of extracellular glutamine. The naïve and memory phenotypes of $CD4^+$ (left) and $CD8^+$ T cells (right) was assessed at days 3, 6 and 9 post stimulation on the basis of CCR7 and CD45RA expression (CCR7+CD45RA+: Naïve; CCR7-CD45RA+:memory). The percentages of cells in each quadrant are indicated.

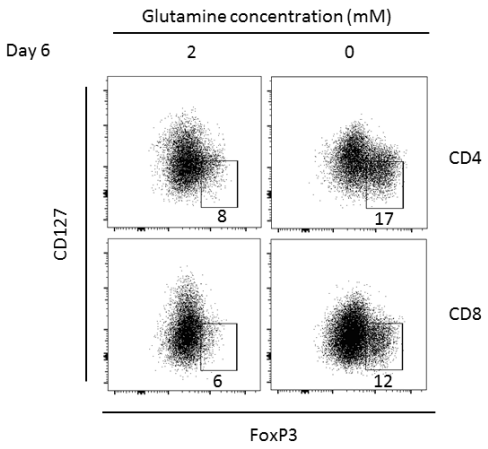

Figure 11: Increased Foxp3 expression in $CD4^+$ and $CD8^+$ T cells activated in the absence of glutamine. The percentages of $Foxp3^+CD127^{low}$ cells, corresponding to a Treg phenotype, within the $CD4^+$ and $CD8^+$ T cell populations were monitored 6 days post TCR stimulation as a function of exogenous glutamine. Representative dot plots are presented and the percentages of cells are indicated.

Part 2

Glut1 expression allows discrimination of activated $CD4^+$ and $CD8^+$ T cells with different phenotypes.

Glutamine is a crucial source of energy for the cell, but as indicated in the introduction, other nutrients such as glucose are also essential. Glucose can enter into the cell via several transporters. One of the must well studied glucose transporters is Glut1 and its expression is upregulated upon TCR activation, mediating increased glucose uptake and allowing T cells to respond to augmented metabolic demands. Moreover, following T cell activation, it appears that higher glucose uptake is associated with Teff cells while lower uptake is associated with memory T cells. As such, our lab is interesting in assessing whether different levels of Glut1 expression are associated with different T cell fates. Furthermore, Glut1 is induced by hypoxic conditions and the tumor environment is often hypoxic. Thus, in the context of anti-tumor immunotherapies, it is of interest to determine whether Glut1 expression in a hypoxic environment alters T cell function and fate.

To assess this question, human $CD3^+$ T cells were purified from the blood of healthy donors and activated with immobilized α-CD3 and α-CD28 antibodies at 1% and 20% oxygen. After 2 days of stimulation, the vast majority of the T cells were Glut1-positive and were sorted on the basis of the lowest or highest 10% of Glut1 expression as shown in **Figure 12**. Following cell sorting on a FACSAria, culture was continued at the same initial oxygen tension. Notably, at the time of cell sorting, the $Glut1^{high}$ cells had a significantly higher FSC/SSC than the $Glut1^{low}$ cells (**Figure 13**). Moreover, the FSC/SSC profiles of these two subsets were higher following activation in 1% than 20% oxygen (**Figure 13**).

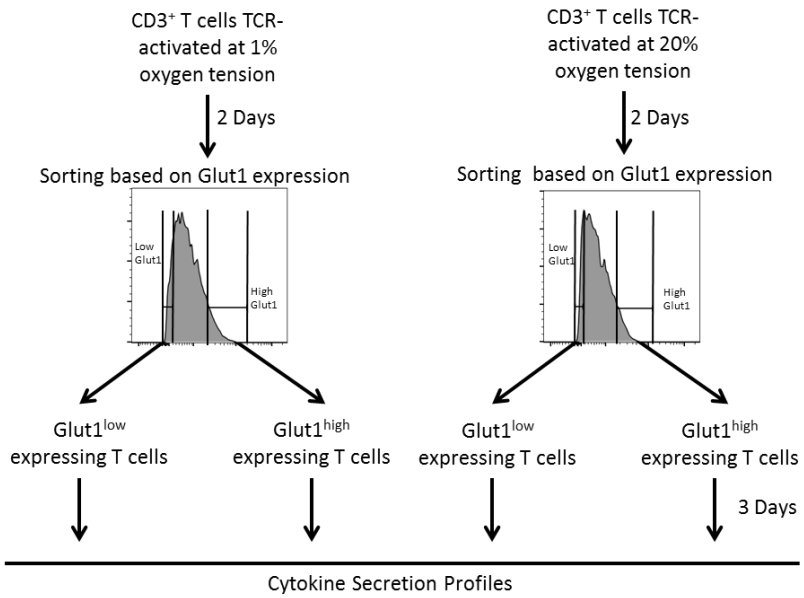

Figure 12: Strategy for the sorting of TCR-stimulated human T lymphocytes on the basis of Glut1 expression. Purified human $CD3^+$ T cells were VDP-labeled and TCR-activated at 1% and atmospheric (20%) oxygen. Two days post stimulation, T cells were stained for Glut1 expression using the HTLV-receptor binding domain ligand (HRBD). Cells with the 5-10% lowest and highest levels of surface Glut1 were sorted on a FACSAria as shown. Culture of sorted populations was continued for an additional 3 days prior to evaluation of cytokine expression.

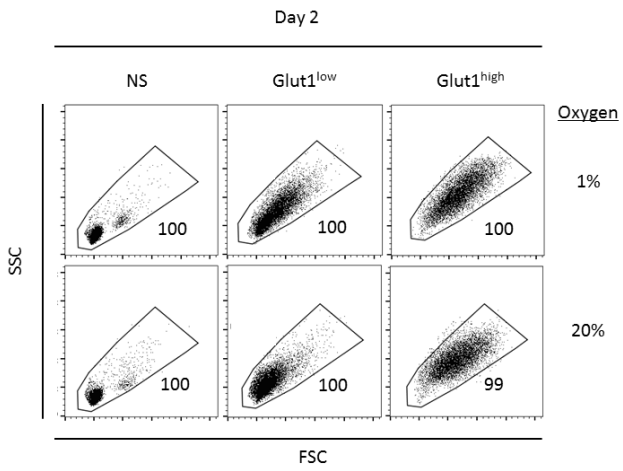

Figure 13: Surface Glut1 expression on TCR-stimulated CD3$^+$ T cells correlates with forward and side scatter profiles. Following sorting of activated CD3$^+$ T cells on the basis of low and high Glut1 expression, forward (FSC) and side (SSC) scatters of the purified lymphocytes were assessed and compared with that detected in non-stimulated (NS) T cells. Representative plots of T cells activated at 1% and 20% oxygen are presented.

While the relative ratio of CD4:CD8 T cells differed neither between the Glut^{high} and Glut^{low} subsets nor between 1% and 20% oxygen conditions at day 2 of stimulation (**Figure 14**; top panels), significant differences were detected by day 5 of stimulation. The relative percentage of CD8$^+$ T cells was significantly higher within the Glut1^{high} subset, both at 1% and 20% oxygen conditions (**Figure 14**). Increased percentages of CD8 T cells also correlated with an increased proliferation within the Glut1^{high} subset for both CD4$^+$ and CD8$^+$ T cell subsets. Moreover, within each Glut1 subset, proliferation was significantly higher in atmospheric oxygen conditions (20%) as compared to hypoxia (1%) (**Figure 15**).

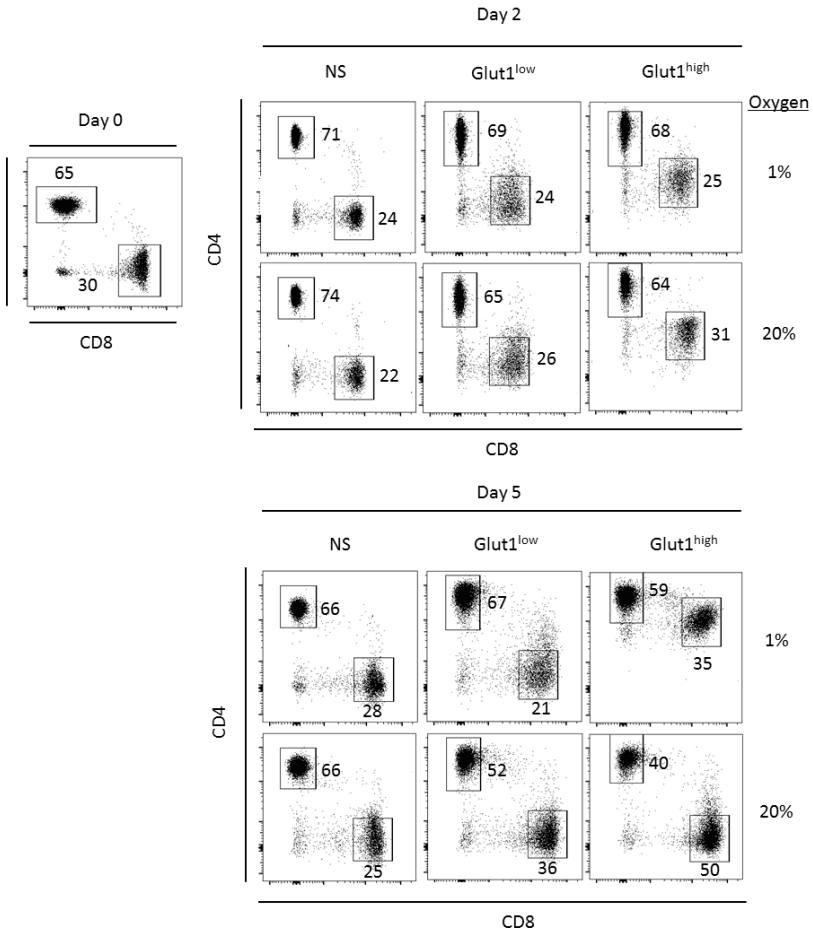

Figure 14: High surface Glut1 is associated with a decreased CD4:CD8 ratio in activated CD3$^+$ T cell populations. CD3$^+$ T cells were activated in 1% or 20% oxygen concentrations, sorted at day 2 on the basis of surface Glut1 levels and then cultured for an additional 3 days at the initial oxygen concentration. The relative percentages of CD4$^+$ and CD8$^+$ T cells was assessed at days 0, 2, and 5 and representative dot plots are presented. The percentage of cells in each quadrant is indicated.

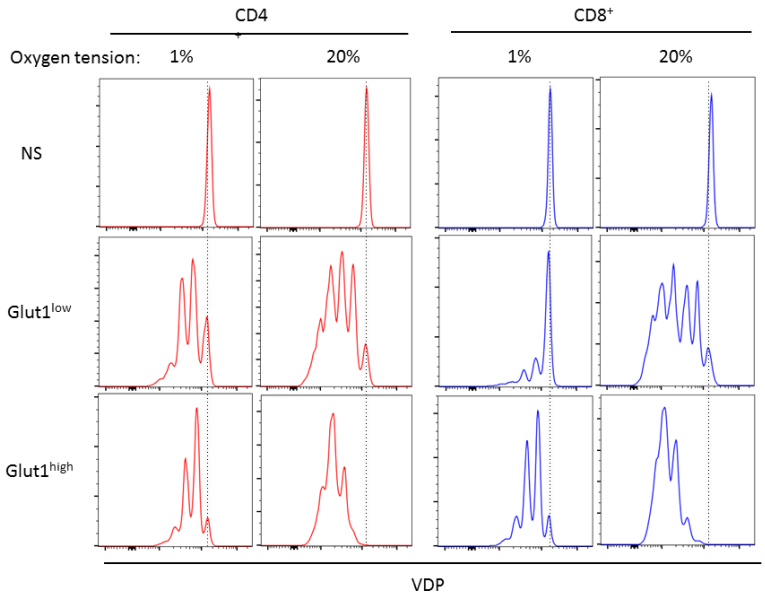

Figure 15: Proliferation of TCR-stimulated CD4$^+$ and CD8$^+$ T cells is modulated by oxygen concentration and surface Glut1 levels. Proliferation of CD3$^+$ T cells was monitored as a function of dilution of the VDP fluorescent dye. Cells were either non-stimulated (NS) or TCR-stimulated at 1% or 20% oxygen concentration, sorted at day 2 on the basis of Glut1 levels and cultured for an additional 3 days prior to analysis of VDP fluorescence. Representative proliferation profiles are presented. Dotted vertical lines indicate the level of VDP fluorescence in non-divided cells.

In parallel with the proliferation pattern, we also evaluated the expression of CD71 as an additional monitor of activation level and metabolic status. CD71 is the transferrin receptor, a measure of T cell activation, and it has previously been shown that its knockout results in impaired T cell differentiation and activation. Indeed, CD71 is upregulated within 24-48h of TCR stimulation (data not shown) and our assessments at day 5 are therefore a monitor of the loss of CD71 as the cells return to a resting state. While Glut1^{high} cells expressed higher levels of CD71 than the Glut1^{low} subset, the most striking differences were between 1% and 20% oxygen tensions (**Figure 16**). CD71 expression on Glut1^{high} cells activated in conditions of hypoxia were higher than on the equivalent subset activated at 20% oxygen. It is not clear whether this represents a slower upregulation of this marker in hypoxia or alternatively, just an altered downregulation. Expression of this marker as well as other more rapidly upregulated activation markers such as CD69 will be evaluated.

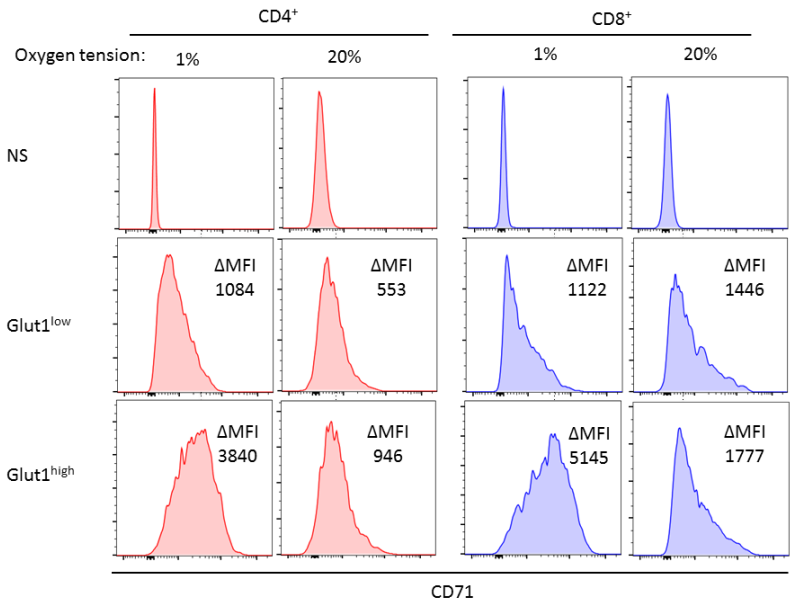

Figure 16: High Glut1 expression is associated with prolonged TCR-induced upregulation of the CD71 transferrin receptor. $CD3^+$ T cells were either non-stimulated (NS) or TCR-stimulated at 1% or 20% oxygen concentration, sorted at day 2 on the basis of Glut1 levels and cultured for an additional 3 days. Expression levels of the CD71 transferrin receptor was monitored and representative histograms are shown. Dotted vertical lines indicate the mean fluorescence intensity in the $Glut1^{hi}$ cells in each condition.

In a first step to assess the differentiation potential of these cells, we evaluated their cytokine secretion profile as a function of Glut1 expression, albeit on cells those were activated under neutral TCR stimulation conditions. To monitor intracellular cytokine protein levels, the different groups of T cells underwent a 4h re-stimulation with PMA/ionomycin in the presence of brefeldin A following 5 days of TCR stimulation. As expected for IL-17 secretion, it was extremely low in $CD8^+$ T cells but increased in $CD4^+$ T cells. Notably though, within the $CD4^+$ T culture conditions, there were significant differences; IL-17 expression was markedly higher in $Glut1^{high}$ than $Glut1^{low}$ cells and in both Glut1 subsets, expression was higher at 20% oxygen (**Figure 17**, left panels). In contrast with IL-17, the production of IFN-γ was detected in all $CD4^+$ as well as $CD8^+$ T cell subsets. While it appears that there was actually a higher percentage of cells producing IFN-γ within the $Glut1^{low}$ subsets (i.e. 49% vs 15% at 1% oxygen and 41% vs 29% at 20% oxygen), it is difficult to interpret the data because of the high spontaneous level of IFN-γ that was detected in non-stimulated cells (**Figure 17**, right panels). Further studies are therefore necessary in order to determine whether Glut1 expression levels distinguish T

lymphocyte subsets with different cytokine secretion profiles and it will also be important to perform these experiments under T cell polarization conditions.

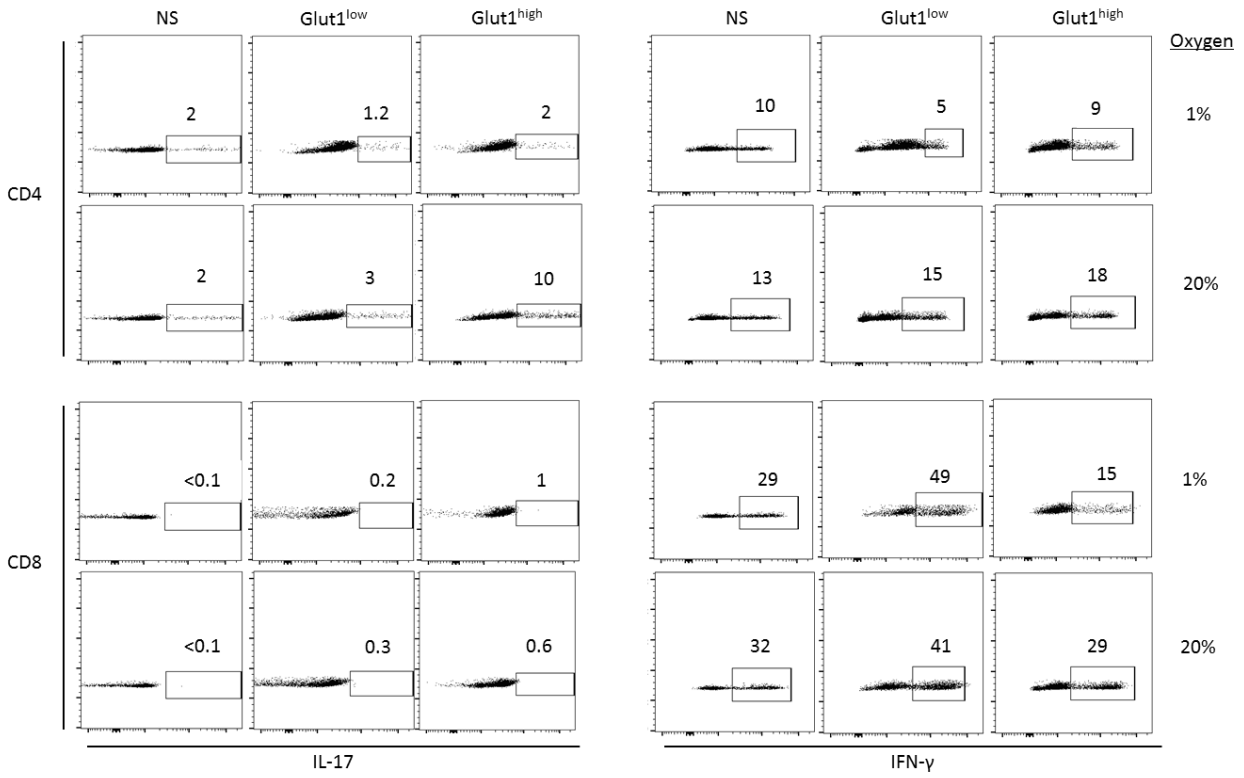

Figure 17: Production of IL-17 and IFN-γ by TCR-stimulated CD4 and CD8 T lymphocytes is modulated by oxygen conditions and Glut1 surface expression. $CD3^+$ T cells were either non-stimulated (NS) or TCR-stimulated at 1% or 20% oxygen concentration, sorted at day 2 on the basis of Glut1 levels and cultured for an additional 3 days. Production of IL-17 and IFN-γ by $CD4^+$ and $CD8^+$ T cells was monitored by intracellular cytokine staining. Representative plots are presented and the percentages of stained cells are indicated.

Chapter 4 Discussion

DISCUSSION

The first part of the work I developed during my internship demonstrates that glutamine availability is a key determinant of the functional fate of naïve $CD4^+$ T cells. T cell activation performed under limiting glutamine concentrations (0.4mM, 0.3mM and 0mM as compared to 2 mM) resulted in an altered proliferation of both CD4 and CD8 T cells and was associated with a proportional increase of Foxp3-expressing T cells. Moreover, the skewing of glutamine-deprived naive $CD4^+$ T cells to a $Foxp3^+$ fate occurred even under Th1-polarizing conditions, blocking terminal Th1 differentiation. Our data identify a metabolic switch that conditions Th1 lineage specification. We found that Th1 differentiation requires that glutamine be catabolized to αKG, replenishing the pools of metabolic intermediates in the TCA cycle. Under conditions where glutamine utilization was limiting, the addition of an αKG ester restored the cell's capacity to upregulate Tbet and adopt a Th1 lineage fate.

The altered level of proliferation of T cells activated under glutamine sub-optimal conditions is in agreement with several published studies demonstrating the importance of glutamine for T lymphocyte division (Newsholme et al. 1985). However, it is important to note that previous results from the laboratory showed no significant difference between murine T cells activated in presence of 0.6 mM or 2 mM glutamine concentration (data non shown). Notably though, decreases to 0.4mM extracellular glutamine was not sufficient for optimal proliferation. Altogether these data suggest that there is a window of glutamine concentrations that allow optimal T cell proliferation and a level of 0.6 mM may identify the minimal glutamine concentration required for this process.

Several metabolic aspects could explain the delay in T cell activation/proliferation under low glutamine conditions. As discussed in the introduction of this report, glucose is a crucial nutrient during T cell activation. During glycolysis, glucose is metabolized into glucose-6-phosphate that can be used into two main metabolic pathways: 1) the pentose phosphate pathway (PPP) which can be used to generate ribose for RNA or DNA synthesis and NADPH for fatty acid synthesis; 2) conversion into pyruvate which can be metabolized into lactate or fully oxidized in the TCA cycle as acetyl Co-A (Wasinski et al. 2014). During activation, T cells rely mainly on aerobic glycolysis

with most of the generated pyruvate converted into lactate (around 91%) and therefore not used to fulfill the TCA cycle. Therefore, to sustain cellular functions, it is probably that the TCA cycle needs to be filled by other sources of energy. In this context, it is known that glutamine, the most abundant amino acid in plasma, can be metabolized into glutamate and then α-ketoglutarate, feeding the TCA cycle. As a result, the altered T cell proliferation level observed under limiting glutamine conditions could be explained by a lower level of oxidative phosphorylation. As T cell proliferation of glutamine-deprived cells increased at later time points, it is likely that other sources of energy and/or compensatory metabolic mechanisms may be activated, allowing the T cell to produce the energy required.

Our data also show that under glutamine-deprived conditions both CD4 and CD8 T cells are skewed to Foxp3+ T cell fate. Interestingly, this skewing occurred even under Th1-polarizing conditions, blocking terminal Th1 differentiation. Conversion of naive $CD4^+$ T cells to a $Foxp3^+$ T cell fate was associated with diminished mTORC1 activity and a decreased energetic state of the cell, as shown by lower mitochondrial function and intracellular ATP levels (data not shown; research from other lab members). mTOR is a critical factor in the metabolic reprogramming of TCR-stimulated T cells and serves to integrate environmental cues such as nutrients, growth factors and stress signals into an “optimal” cellular response (Laplante & Sabatini 2009) (Sengupta et al. 2010). Indeed, inhibition of mTOR activity has been found to foster the generation and function of Foxp3-expressing Treg (Delgoffe et al. 2009) (Powell et al. 2012) (Sauer et al. 2008) while the mTORC2-mediated activation of Foxo1 and Foxo3a abrogates Foxp3 expression (Kerdiles et al. 2010) (Ouyang et al. 2010).

Similarly, activation of mTORC1, via deletion of the upstream TSC1 negative regulator, results in a loss of Foxp3 expression and a gain of effector function (Park et al. 2013). Nevertheless, the relationship between the mTOR pathway and regulatory T cells is not completely clear as disruption of mTORC1 was recently shown to reduce Treg activity (Zeng et al. 2013). In transformed cell lines, glutamine withdrawal as well as glutaminolysis inhibition can decrease mTORC1 activation in an energy-independent manner, i.e. without any alterations to intracellular ATP levels (Durán et al. 2012). However, in primary T cells, glutamine withdrawal not only resulted in significant decreases in mTOR activation but ATP levels were reduced (data

from the laboratory, not shown). A rate-limiting step in the activation of mTOR is glutamine transport (Nakaya et al. 2014) (Durán et al. 2012) (Fuchs et al. 2007). In this context, it is interesting to note that the deletion of the SLC7A5 amino acid transporter completely blocks the clonal expansion and effector differentiation of CD8 T cells (Sinclair et al. 2013) and more recent work has shown the critical importance of the ASCT2 glutamine transporter in Th1 and Th17 effector differentiation (Nakaya et al. 2014). Furthermore, and in accord with the data presented here, knockout of the CD98 heavy chain (SLC3A2), regulating integrin signaling as well as amino acid transport, has been shown to increase the generation of regulatory T cells (Bhuyan et al. 2014). Taken together, these results strongly suggest that it the TCR-stimulated induction of mTORC1 regulates the generation of $Foxp3^+$ cells. Our experiments reveal a critical sensitivity of T lymphocytes to extracellular glutamine levels, with replenishment of even 20% of extracellular levels inhibiting the biasing of conventional T cells to a $Foxp3^+$ fate.

Our data also demonstrate that under limiting glutamine conditions, the addition of an αKG ester restores the cell's capacity to upregulate Tbet and adopt a Th1 lineage fate. These results raise the question as to why glutamine-derived αKG is required for Th1 effector differentiation but not for the development of anti-inflammatory regulatory T cells. The entry of metabolic intermediates into the TCA cycle is central to energy metabolism while their exit fosters the synthesis of biological molecules required for cell growth and division. In cancer cells, the heightened need for biosynthetic intermediates results in a disproportionate dependency on glutamine which undergoes anaplerotic reactions to form αKG (DeBerardinis et al. 2007) (Lyssiotis et al. 2013) (Son et al. 2013). By analogy, this would suggest that Th1 cells, but not Tregs, present a metabolic state that requires support of a large number of anabolic processes. Indeed, effector T cells show high rates of glycolysis whereas suppressive Tregs exhibit a higher dependence on fatty acid oxidation (Michalek et al. 2011) (Shi et al. 2011). Furthermore, while Tregs can take up exogenous fatty acids, Th17 effector cells depend on the ATP-costly process of de novo fatty acid synthesis (Berod et al. 2014). Together, these data strongly suggest that Th effector cells have higher metabolic requirements than Tregs. In this regard, it is interesting to note that the addition of αKG not only pushed glutamine-deprived cells to a Th1 fate but

inhibited Treg differentiation. Thus, our findings the balance between Th1 effector and Treg suppressors is conditioned by intracellular metabolic intermediates.

It is, however, also important to note that TCA cycle intermediates can regulate the epigenetic state of an activated T lymphocyte. Specifically, Tet2 is an αKG-dependent enzyme that alters DNA methylation by conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and it has recently been shown that Tet2 deficiency inhibits Th1, but not Th2, differentiation while promoting Treg conversion (Ichiyama et al. 2015). As this phenotype directly parallels the differentiation that we observed under conditions of glutamine deficiency, the metabolism of this amino acid may be critical for Tet2-mediated demethylation and regulation of an epigenetic state that is required for a Th1 fate but not a Th2 or Treg fate.

While it is not yet known how the intricate coordination of nutrient processing regulates the formation of specific metabolic intermediates in T lymphocyte subsets, our data point to the extracellular nutrient environment as a key factor in this equation. This may have significant physiological consequences in microenvironments, such as those due to tumor or infection, wherein the nutrient milieu into which a T cell enters can be altered (Bode & Souba 1999) (Hirayama et al. 2009) (Karinch et al. 2001). Indeed, glutamine levels have been reported to be diminished in patients with hepatocellular, colon and stomach tumors (Bode & Souba 1999) (Hirayama et al. 2009). This may explain data showing that Tregs preferentially accumulate in and around murine tumors, especially as they progress (Lin et al. 2009). Furthermore, $Foxp3^+$ T cells are often recruited to a tumor prior to T effector cells, preventing a lymphocyte-mediated eradication (*62*). Thus, the glutamine-deficient microenvironment of a tumor, potentially due to the glutamine "addiction" of cancer cells (*63, 64*) or secondary to chemotherapies (DeLeve & Wang 2000) (Abraham et al. 2011), appears to be a critical factor in enforcing a Treg phenotype even under conditions where the $CD4^+$ T cells recruited into the tumor are exposed to effector polarizing conditions. In this context, it is important to note that activation of human CD4 T cells under conditions of glutamine deprivation also resulted in a significant increase in the relative percentage of $Foxp3^+/CD127^{low}$ cells. Altogether, our results highlight a previously unknown relationship between glutamine availability and the establishment of a tolerogenic environment.

In the second part of the work that I developed during my internship, I focused on the impact of oxygen tension on the survival and phenotype of T lymphocytes. In parallel, I also assessed whether the expression profile of the Glut1 glucose transporter could allow us to segregate T lymphocytes with distinct phenotypes and functions. To this end, T lymphocytes were activated at low (1%) and high (20%) oxygen conditions. Glut1 expression was significantly higher at 1% as compared to 20% oxygen, potentially due to increased HIF1a activity under hypoxic conditions (data not shown). In both conditions, cells expressing the highest and lowest levels of Glut1 were sorted as described in the results section. Importantly, Glut1 expression on sorted cells remained distinct during short term ex vivo culture, with MFIs for Glut1 staining approximately 32-fold higher in $Glut1^{high}$ versus $Glut1^{low}$ cells (data not shown). Notably, $Glut1^{high}$ and $Glut1^{low}$ cells also exhibited distinct characteristics as assessed by their proliferation and phenotypic profiles. It will therefore be important to determine whether these diferences are maintained following the adoptive transfer of these two subsets into lymphocyte-replete as well as lymphopenic mice. In the latter case, it will be of interest to determine whether the capacity of the T cell to fill the lymphocyte compartment differ.

Interestingly, there was an increased percentage of $CD8^+$ T cells in the $Glut1^{high}$ as compared to $Glut1^{low}$ population. Several non-exclusive reasons could explain this result. This may be due to a higher TCR-induced proliferation of $CD8^+$ as compared to $CD4^+$ T cells and/or to a more highly glycolytic metabolism in the former (Cao et al. 2014). Therefore the skewed CD4/CD8 ratio detected in $Glut1^{high}$ T cells may be explained by an initial bias toward CD8 T cells at the time of the cell sorting based on Glut1 expression and/or to a metabolic advantage of these cells within the Glut1-high gate. In this regard, it is interesting to note that mTOR activation has been reported to be higher in $CD8^+$ T cells as compared to CD4 T cells (Cao et al. 2014). As reviewed in the introduction section, mTOR can upregulate Glut1 expression and metabolism. $Glut1^{high}$ cells also proliferated to a higher degree than $Glut1^{low}$ cells and in general, proliferation was more pronounced at 20% than 1% oxygen tensions. These differences in $Glut1^{high}$ and $Glut1^{low}$ cells also appears to be associated with differences in their capacity to produce effector cytokines but these data are preliminary and need to be repeated.

Different T cell subsets exhibit distinct anti-tumor functions. A T cell memory phenotype on anti-tumor T cells has been associated with a higher level of tumor regression. Indeed Sukumar et al. found that the adoptive transfer of memory T cells in a tumor mouse, injection of T cells with a higher memory pattern led to tumour regression and higher survival (Sukumar et al. 2013). The memory phenotype associated with a low glycolytic potential was beneficial in the context of an anti-tumor response. As IFNγ plays a crucial role in anti-tumor responses (Lin & Young 2013) (Schroder et al. 2004), it will be of interest to assess whether Glut1^{high} and Glut1^{low} subsets can be used to distinguish T lymphocytes with different effector and anti-tumor reactivities.

In conclusion, our data together with other recent elegant studies (Lyssiotis et al. 2013) (Michalek et al. 2011) (Shi et al. 2011) (Son et al. 2013) (Walker et al. 2014) (Willems et al. 2013) suggest that the targeting of metabolic pathways may present an attractive new strategy for modulating immune responses. This is especially interesting in the context of the tumor microenvironment where fluctuations in nutrient availability may force T cells to adapt their metabolism, resulting in modifications of their effector functions.

Chapter 5 Conclusion

CONCLUSION

The majority of the studies from the past 20-30 years focused on the impact of signaling via the T cell receptor (TCR), cytokines, chemokines and hormones as well as cell-cell interactions, demonstrating important roles for each of these components in T cell activation. However, studies of less “classical” parameters of the T cell microenvironment, such as the availability of nutrients, amino acids, mineral, vitamins and oxygen, were not at the forefront. It is only in the past few years that we have begun to appreciate the impact of metabolism in T cell activation. Indeed, while it is not yet known how the intricate coordination of nutrient processing regulates the formation of specific metabolic intermediates in T lymphocyte subsets, our data point to the extracellular nutrient environment as a key factor in this equation.

Overall, the ensemble of these results reveal T cell metabolism as an important modulator of T cell fate and strongly suggest that the specific targeting of metabolic pathways may allow the development of innovative strategies to improve T cell immunotherapies. This is especially interesting in the context of the tumor microenvironment where fluctuations in nutrient availability may force T cells to adapt their metabolism, resulting in modifications of their effector functions.

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