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Targeting the metabolic environment to modulate T cell effector function

Modulation des fonctions effectrices des cellules T en exploitant l'environnement métabolique

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Targeting the metabolic environment to modulate T cell effector function

Abstract

T cells are stimulated upon interaction with their cognate antigen. While much research has focused on the role of antigen presenting cells (APC) and cytokines as important components of the T cell microenvironment, recent data highlight the importance of the metabolic environment in sustaining the energetic and biosynthetic demands that are induced upon antigen stimulation. The subsequent metabolic reprogramming of the T cell is conditioned by the nutrient composition and oxygen levels. Notably, this environment can be altered by pathological conditions such as tumors and data from our group, as well as others, have shown that the competition of T cells and tumor cells for limiting amounts of nutrients has a negative impact on T cells, inhibiting their anti-tumor effector functions. This effect is due, at least in part, to the distinct metabolic profiles of T lymphocyte subsets; T effector cells (including Th1 cells) are highly glycolytic while suppressive FoxP3^{+} regulatory T cells (Tregs) display a mixed metabolism with increased levels of lipid oxidation. It is therefore important to determine how changes in the metabolic programming of anti-tumor T cells impacts on their persistence and function. Indeed, in the context of my PhD research, I found that high levels of the glucose transporter GLUT1 was associated with a significantly increased level of IFNγ secretion by both CD4 and CD8 T cells. Furthermore, there was a bias of CD8 over CD4 lymphocytes in the GLUT1^{High} T cell subset. These data point to the importance of metabolic alterations in the fate and effector function of T lymphocytes and during my PhD, I focused on elucidating the metabolic parameters that regulate effector and regulatory T cells, with the goal of improving the efficacy of anti-tumor T cells. In this context, I contributed to initial studies from our group, revealing a critical role for extracellular nutrient availability in terminal CD4^{+} T cell differentiation. Activation of naïve CD4^{+} T cells under conditions of glutamine deprivation caused them to differentiate into induced Treg (iTreg). Moreover, the skewing of glutamine-deprived naïve CD4^{+} T cells to a FoxP3^{+} fate occurred even under Th1-polarizing conditions, blocking terminal Th1 differentiation. Under glutamine-deprived conditions, we found that alpha-ketoglutarate (αKG), a glutamine-derived metabolite, rescued Th1 differentiation. I then evaluated the impact of αKG under glutamine-replete

conditions in the Th1/iTreg differentiation processes. My studies showed that, under Th1-polarizing conditions, αKG markedly enhanced naïve $CD4^+$ T cell differentiation into Th1 cells and increased IFNγ secretion. Moreover, under Treg-polarizing conditions, αKG decreased FoxP3 expression and increased the secretion of inflammatory cytokines such as IFNγ, GM-CSF and IL-17. Notably, the αKG-mediated alteration in T cell differentiation was associated with an augmented oxidative phosphorylation (OXPHOS), and inhibiting the citric acid cycle and the mitochondrial complex II with malonate, an inhibitor of succinate dehydrogenase (SDH), alleviated the αKG-mediated block in Treg differentiation. Impressively, these αKG-mediated changes in the Th1/Treg balance were maintained *in vivo*, promoting a Th1-like profile in T cells expressing an anti-tumor chimeric antigen receptor (CAR) in tumor-bearing mice. Thus, our data show that low intracellular αKG content, caused by limited external glutamine availability, imposes a Treg phenotype while high αKG levels shift the balance towards a Th1 phenotype.

Altogether, the data generated during my PhD will promote the development of metabolic strategies aimed at modulating T cell function and foster the design of nutrient transporter-based approaches that can be used to select T lymphocytes with enhanced anti-tumor effector properties.

Modulation des fonctions effectrices des cellules T en exploitant l'environnement métabolique

Résumé

L'activation des cellules T est initiée suite à la rencontre avec un antigène spécifique. Les études réalisées pour mieux comprendre ce processus d'activation se sont principalement focalisées sur le rôle des cellules présentatrices d'antigènes et des cytokines. Toutefois, des données récentes soulignent également l'importance du microenvironnement métabolique pour soutenir l'augmentation des besoins énergétiques et biosynthétiques liés à la stimulation antigénique. Cette reprogrammation métabolique est conditionnée par la disponibilité en nutriments et la teneur en oxygène qui peuvent être altérés en conditions pathologiques, comme dans des tumeurs. En effet, plusieurs groupes dont le nôtre ont montré qu'en cas de faible disponibilité en nutriments, une compétition peut se créer entre les cellules tumorales et les cellules T, impactant de ce fait négativement leurs fonctions anti-tumorales. Cet effet est dû, du moins en partie, aux profils métaboliques distincts des sous-populations de cellules T ; alors que les cellules T effectrices (dont les cellules Th1) sont fortement glycolytiques, les cellules T régulatrices suppressives (Treg) présentent un métabolisme plus mixte avec des niveaux accrus d'oxydation lipidique. Il est donc important de déterminer comment les changements métaboliques des cellules T anti-tumorales affectent leur persistance et leur fonctionnalité. Ainsi, j'ai entrepris des travaux afin d'évaluer si le niveau d'expression du transporteur de glucose GLUT1 permettait d'identifier et de sélectionner des cellules T ayant des fonctions effectrices distinctes. Nous avons confirmé cette hypothèse et notamment montré que les cellules T exprimant un niveau élevé de GLUT1 possèdent un potentiel de sécrétion d'IFNγ accru.

De plus, nos travaux montrent que la disponibilité en nutriments extracellulaires est un élément clé pour la différenciation terminale des cellules Th1. En effet, l'activation des cellules T CD4 naïves en conditions limitantes en glutamine induit leur différenciation en cellules Treg FoxP3^{+}. Plus surprenant encore, cette carence induit un blocage de la différenciation Th1 même lors d'une polarisation vers ce lignage. De plus, en conditions de carence en glutamine, nous avons découvert que l'alpha-cétoglutarate (αKG), un métabolite dérivé de la glutamine, rétablit cette différenciation terminale Th1. J'ai ensuite évalué l'impact

de l'αKG dans les processus de différenciation Th1/Treg en condition non limitante en glutamine. Mes données montrent que, dans des conditions de polarisation Th1, l'ajout d'αKG améliore la différenciation des cellules T CD4 naïfs en cellules Th1 et augmente la production d'IFNγ. A l'inverse, l'ajout d'αKG s'accompagne d'une diminution des cellules $FoxP3^+$ et d'une augmentation de la sécrétion de cytokines inflammatoires dans des conditions de polarisation Treg. L'altération de la différenciation des cellules T médiée par l'αKG est notamment associée à une phosphorylation oxydative (OXPHOS) accrue ; ainsi, l'ajout d'un inhibiteur du cycle de Krebs et du complexe mitochondrial II /succinate déshydrogénase, atténue le blocage de la différenciation Treg induit par l'αKG. De façon remarquable, ces modifications de l'équilibre Th1/Treg médiées par l'αKG sont maintenues *in vivo* et impactent le devenir de cellules T exprimant un récepteur chimérique anti-tumoral (CAR) injectées chez des souris porteuses de tumeurs. En résumé, nos données montrent qu'une faible teneur en αKG intracellulaire liée à une disponibilité limitée en glutamine, favorise un phénotype Treg, alors que des niveaux élevés d'αKG modifient l'équilibre vers un phénotype Th1.

En conclusion, les données générées au cours de ma thèse devraient permettre le développement de stratégies permettant de sélectionner des cellules T ayant des propriétés effectrices anti-tumorales améliorées.

Abstract general public

T cell based-immunotherapies represent a promising approach for cancer treatment. Despite impressive successes for the treatment of hematologic cancers, it has been less fruitful on solid tumors. This is in part due to the altered metabolism generated in the tumor microenvironment that impairs T cell anti-tumor function. To overcome this problem, I have focused during my PhD on understanding the impact of metabolic parameters on T cell effector function. Our results demonstrate that glutamine availability plays a crucial role in the acquisition of effector T functions with, alpha-ketoglutarate, a glutamine-derived metabolite, acting as a metabolic regulator of T cell differentiation. In parallel, we show that T cells with distinct effector functions can be identified based on the surface expression of the glucose transporter, GLUT1. Altogether, targeting T cell metabolism could represent an original angle to modulate T cell anti-tumor function and improve cancer immunotherapies.

Résumé grand public

Malgré d'impressionnants succès pour des cancers hématologiques, les immunothérapies par transfert de cellules T s'avèrent peu efficaces pour le traitement de tumeurs solides. Ceci est en partie dû à l'altération du métabolisme par l'environnement tumoral qui limite l'efficacité anti-tumorale des cellules T. Durant ma thèse, j'ai ainsi étudié l'importance du métabolisme dans les fonctions effectrices des cellules T. Nos résultats montrent que la disponibilité en glutamine est essentielle pour l'acquisition des fonctions effectrices des cellules T et que, l'alpha-cétoglutarate, un métabolite dérivé de la glutamine, agit comme régulateur dans leur processus de différenciation. En parallèle, nous montrons que les cellules T, identifiées sur la base de l'expression du transporteur de glucose GLUT1, possèdent des fonctions effectrices distinctes. Le ciblage du métabolisme pourrait donc représenter un angle thérapeutique original pour améliorer la réponse anti-tumorale des cellules T.

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Surprisingly, even if I am someone known for my capacity to express my feelings, writing this part was not easy. It was not easy because I could write another thesis manuscript (not right now, or maybe) only to express my gratitude to all those who helped during this journey. Thus, I will try to be brief and efficient.

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Abbreviations list

3PG	3-phosphoglycerate
2HG	2-hydroxyglutarate
4E-BP1	eukaryotic translation inhibitor factor 4E-binding protein 1
5caC	5- hydroxymethylcytosine
5fC	5- formylcytosine
5hmC	5-hydroxymethylcytosine
αKG	α-ketoglutarate
αKGDH	α-Ketoglutarate dehydrogenase

A

AA	amino acids
ABCA1	ATP binding cassette A1
ABCG1	ATP binding cassette G1
ACAT	Acyl-CoA cholesterol acyltransferase
ACC	acetyl CoA carboxylase
Acetyl-coA	acetyl coenzyme A
ACT	adoptive T-cell therapies
ADC	arginine decarboxylase
AGAT	arginine: glycine amidinotransferase
AIRE	autoimmune regulator
AITL	angioimmunoblastic T-cell lymphoma
ALL	acute lymphocytic leukemia
AML	myeloid leukemias
AMP	adenosine mono phosphate
AMPK	5’ adenosine monophosphate-activated protein kinase
AP-1	activator protein-1
APC	antigen presenting antigen cells
ARG	arginase
ASCT2	ASC amino acid transporter-2
ASL	arginosuccinate lyase
ATP	Adenosine triphosphate

B

BAD	BCL-2 antagonist of cell death

BCL-6	B-cell lymphoma 6 protein
BID	BH3-interacting domain death agonist
BIM	BCL-2 interacting mediator of cell death
BLC-2	B cell lymphoma 2

C

Ca^{2+}	calcium
CAFs	cancer associated fibroblasts
cAMP	cyclic Adenosine mono phosphate
CAR	chimeric antigen receptors
CAT	cationic transporter
CCL	CC-chemokine ligand
CCR7	CC-chemokine receptor 7
CD28RE	CD28 responsive element
CD62L	CD62 ligand
CDKs	cyclin-dependent kinases
CI	complex I
CIC	citrate carrier
CII	complex II
CIII	complex III
CIV	complex IV
CLL	chronic lymphocytic leukemia
CNS	conserved non-coding DNA sequence
Con A	concanavalin A
CPT	carnitine palmitoyl transferase
CS	citrate synthase
CTCF	CCCTC-binding factor is a DNA-binding zinc-finger transcription factor
cTEC	cortex thymic epithelial cells
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTLs	cytotoxic T lymphocytes
CV	complex V
CXCL	C-X-C motif chemokine ligand
CXCR3	C-X-C motif chemokine receptor 3
CYP51	lanosterol 14α-demethylase

D

D-2HG	D-(R)-2-hydroxyglutarate

DCs	dendritic cells
DEPTOR	mTOR-interacting protein
DHAP	dihydroxyacetone phosphate
DLBCL	diffuse large B-cell lymphoma
DMK	dimethyl α-ketoglutarate
DN	double negative
DP	double positive
E	
EAE	experimental autoimmune encephalomyelitis
Eomes	eomesodermin
ER	endoplasmic reticulum
ETC	electron transporter chain
F	
F16P	fructose-1-6-P
F6P	fructose-6-phosphate
FADH2	flavin adenine dinucleotide
FAO	fatty acid oxidation
FAs	fatty acids
FAS	fatty acid synthesis
FASN	fatty acid synthase
FATP	fatty acid transport protein
Fezf2	Fez family zinc finger protein 2
FH	fumarate hydratase
FL	follicular lymphoma
FoxO1	forkhead box O1
FoxP3	Forkhead box protein P3
G	
G3P	glyceraldedehyde-3-phospate
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GDH	glutamate dehydrogenase
GEM	germline-encoded mycolyl-reactive
GLS	glutaminase
GLUT	glucose transporters

GM-CSF	granulocyte-macrophage colony-stimulating factor
GOT	glutamate oxaloacetate transaminase
GS	glutamine synthase
H	
H^+	proton
HDL	high-density lipoprotein
HDM	histone demethylases
HEV	high endothelial venules
HGMCR	hydroxymethylglutaryl-coenzyme A reductase
HGMCS	hydroxymethylglutaryl-coenzyme A synthase
HIF-1α	Inducible impact factor 1 α
HK	hexokinase
HLA	human leucocyte antigen
HMC-CoA	hydroxymethylglutaryl-coenzyme A
I	
Id2	DNA binding 2
IDH	isocitrate hydrogenase
IDO	indoleamine 2,3-dyoxyegenase
IFNα	Interferon alpha
IFNγ	Interferon gamma
IFNγR	Interferon gamma receptor
Ig	Immunoglobulin
IKK	IkB kinase
IL	interleukin
IL-12Rβ	IL-12 receptor beta
IL-23R	IL-23 receptor
IL-2R	IL-2 receptor
IL-7R	IL-7 receptor
iNK T	invariant natural killer T
INSIG	insulin-induced gene protein
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked
IRF4	interferon regulatory factor 4
ITAMs	immunoreceptor tyrosine-based activation motifs
iTreg	induced T regulatory cells
J	
JAK	janus kinase

JmjC	Jumonji C
K	
KLF	Kruppel-like transcription factor
L	
L-2HG	L-(S)-2-hydroxyglutarate
LAT	linker for activation of T cells
LCK	lymphocyte cell-specific protein-tyrosine kinase
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LFA-1	integrin CD11a
LKB1	liver kinase B1
LPS	lipopolysaccharide
LXR	liver X receptor
M	
MAA	melanoma-associated antigens
MAIT	mucosal-associated invariant T
MCT	monocarboxylate transporter
MDH	malate dehydrogenase
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complexes
mLST8	mammalian lethal with sec-13 protein 8
MS	multiple sclerosis
mTEC	medullar thymic epithelial cells
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MVK	mevalonate kinase
N	
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF- κB	nuclear factor- κB
NFAT	nuclear factor of activated T cells
NK	natural killer
NKT	natural killer T cells
NO	nitric oxide
NOS	nitric-oxide-synthase

NSCLC	non-small cell lung cancer
nTreg	natural T regulatory
O	
O2	oxygen
OAA	oxaloacetate
OAT	ornithine aminotransferase transaminase
ODC	ornithine decarboxylase
Opa1	optic atrophy 1
OXPHOS	oxidative phosphorylation
P	
P5C	pyrroline-5-carboxylate
PAMPs	pathogen-associated molecular patterns
PD-1	programmed cell death-1
PDH	pyruvate dehydrogenase
PEP	phosphoenolpyruvate
PFK1	phosphofructokinase-1
PHDs	prolyl hydroxylases
PI3K	phosphatidylinositol 3-kinase
PKCθ	protein kinase Cθ
PKM2	pyruvate kinase isozyme M2
PMBCL	primary mediastinal large B-cell lymphoma
pMHC	peptide-MHC
PNAd	peripheral lymph node addressin
PPAR	peroxisome proliferator-activated receptors
PPP	pentose phosphate pathway
PRAs40	proline-rich AKT substrate 40 kDa
PRRs	pattern recognition receptors
pTreg	peripheral-derived T regulatory cells
PUFAs	polyunsaturated fatty acids
R	
R5P	ribose-5-phosphate
RA	rheumatoid arthritis
RAG	recombination-activating gene
Raptor	regulatory-associated protein of mammalian target of rapamycin
RASGRP	RAS guanyl nucleotide-releasing protein
Rictor	rapamycin-insensitive companion mTOR

RTE	recent thymic emigrants
RUNX	Runt-related transcription factor
RXR	retinoid X receptor
S	
S1P	site-1 proteases
S2P	site-1 proteases
S6K1	S6 kinase beta-1
SCAP	SREBP cleavage-activating protein
scFv	single chain Fc
SCS	succinyl-CoA synthetase
SDF-1	stromal cell-derived factor 1
SDH	succinate dehydrogenase
SGK1	glucocorticoid-regulated kinase 1
SGLTs	sodium-glucose-linked transporters
SLC	solute carrier family
SLE	systemic lupus erythematosus
SLEC	short-lived effector cells
SLL	small lymphocytic lymphoma
SNAT	sodium-dependent neutral amino acid transporter
SP	single positive
SRC	spare respiratory capacity
SREBP	sterol regulatory element binding protein
STAT	signal transducers and activators of transcription
T	
T1D	type 1 diabetes
TAL	tyrosine ammonia lyase
TAM	tumor-associated macrophages
T-bet	T box expressed in T cells
Tc17	T cytotoxic 17
Tc2	T cytotoxic 2
Tc9	T cytotoxic 9
TCA	tricarboxylic acid
TCF-1	T cell factor 1
T_{CM}	T central memory
Tconv	T conventional cells
TCR	T cell receptor

TEC	thymic epithelial cells
T_{EFF}	Effector T cells
T_{EM}	T effector memory
TEMRA	terminal effector cells
TET	Ten-eleven translocation
T_{FH}	follicular helper T cells
TGF-β	transforming grow factor β
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
Th22	T helper 22
Th3	T helper 3
Th9	T helper 9
TILs	tumor infiltrating T cells
TKT	transketolase
TLR	toll-like receptors
TME	tumor microenvironment
TNF	tumor-necrotic factor
TNF-β	lymphotoxin
Tr1	type 1 regulatory T cells
TRA	tissue-resident antigens
TRAF-6	TNF receptor-associated factor-6
TRECs	TCR excision circles
Treg	T regulatory
T_{RM}	Tissue-resident memory T cells
TRUCK	T cell redirected universal cytokine universal cytokine killing
TSC1	tumor suppressor tuberous sclerosis complex 1
T_{SCM}	T stem cell memory
TSDR	Treg-specific demethylated region
tTreg	thymus-derived T regulatory cells
V	
VEGF	vascular endothelial growth factor
VSV	vesiculovirus
X	
XCL1	XC chemokine ligand 1
XCR1	X-C motif chemokine receptor 1

Y

y+ LAT	y+ L-type amino acid transporter 1

Z

ZAP-70	Zeta-chain-associated protein kinase 70

Introduction

I. Adaptive Immune system

General concepts

The immune response can be divided into two main groups: innate and adaptive immunity. Innate immunity, which is the first line of response against pathogens, is invariant and rapid. Pathogens recognition occurs in a non-specific manner, mediated by pattern recognition receptors (PRRs) that target pathogen-associated molecular patterns (PAMPs) expressed by microbes. PPRs are present on epithelial and immune cells notably comprising dendritic cells, macrophages, natural killer cells, mucosal-associated invariant T cells and innate lymphoid cells. Adaptive immunity represents a second line of defense and is orchestrated by B (humoral immunity) and T (cell-mediated immunity) lymphocytes. Adaptive immunity requires several days as the B and T cells must specifically recognize an antigen, undergo clonal expansion and differentiate into effector cells. While B cells recognize native antigen thanks to their surface B cell receptor, T cells, via their specific T Cell Receptors (TCR), recognize processed antigens presented by the major histocompatibility complexes (MHC) on the surface of specialized antigen presenting cells (B cells, dendritic cells, etc).

As my PhD project was centered on T lymphocytes and therefore, I will specifically focus on this cell type in the following chapter.

1) T cells

T cells recognize their cognate antigen and trigger distinct and directional responses through their specific receptor, the TCR. TCR diversity is generated from DNA rearrangements where a randomly somatic recombination of V, D and J segments, mediated by the recombination-activating genes (RAG) 1 and 2, results in the production of several and distinct associations of TCR chains (α, β, γ, δ), giving rise to roughly ~10^{18} unique TCRs in humans (Murphy et al., 2008). Based on the TCR rearrangements, T cells are classified in two main group, αβ or γδ T cells **(section I 1 A)**. Although extremely diverse, these TCRs, generated through VDJ recombination, need to be capable to recognize an enormous variety of antigens being at the same time capable to tolerate self-antigens. For that purpose, positive and negative selection takes place in the thymus in order to avoid auto-immunity and maintain self-tolerance **(section I 1 B)**.

A. T cell diversity

αβ T cells are the most represented T cell subpopulation in the periphery and "conventional" αβ T cells are divided into 2 distinct lineages: $CD4^+$ and $CD8^+$ phenotypically and functionally distinct which I will detail below. Within this αβ T cell population, there are also few subtypes classified as "unconventional" like mucosal-associated invariant T (MAIT) cells (Le Bourhis et al., 2010; Treiner et al., 2003), invariant natural killer T (iNK T) cells and germline-encoded mycolyl-reactive (GEM) T cells which recognize specifically glycolipids presented by MR1, CD1d and CD1b respectively (Beckman et al., 1994) (Van Rhijn et al., 2013).

γδ T cells are also considered as unconventional T cells and they represent between 1%-5% of all T lymphocytes. Even if this population was described for the first time in 1987 (Born et al., 1987; Tonegawa et al., 1989), the structures to which γδ T cells respond to and how are not well-characterized. Using murine models, it was established that γδ T cells are important for pathogen clearance and tumor surveillance (Carding and Egan, 2002). But the mechanisms underlying these functions are not fully characterized and, in particular, it remains unclear whether or not antigen presentation occurs similarly as for αβ T cells. It has been described so far that γδ T cells are able to bind and get activated by CD1 family members, MHC and CD1 related molecules (such as T10/T22, Qa-1, MICA and EPCR), butyrophilin-like molecules and other surface-bound proteins, soluble proteins, "phosphoantigens", haptens and free peptides (Vermijlen et al., 2018). Nevertheless, classical MHC class I and class II molecules have been as well identified as structures involved in antigen presentation (Vermijlen et al., 2018). To conclude, further studies are necessary to fully characterize unconventional T cells.

Since all my research work was centered on peripheral conventional αβ T cells, I will therefore develop the rest of the literature review on this cell type.

B. Thymopoiesis

T cell development, also called thymopoiesis, takes place in the thymus, a bilobed primary lymphoid organ. The structure of the thymus is divided in 2 regions, histologically distinct: a peripheral cortex area and a central medullar zone. These two zones of the thymus are composed of different types of thymic epithelial cells (TEC) which play a crucial role in T cell development. T cell development is initiated by the migration into the thymus of bone marrow progenitors. This development could then be characterized by the expression of the

CD4 and CD8 markers. Early thymic progenitors enter the thymus as “double negative” (DN) thymocytes as they do not express CD4 or CD8 co-receptors. In the cortex, DN thymocytes will commit into T cell lineage and differentiate into $CD4^+CD8^+$ double positive (DP) thymocytes. This process is tightly regulated by the interaction with the cortex thymic epithelial cells (cTEC) which allow a positive selection of the $CD4^+$ $CD8^+$ αβ T cells and their further differentiation into CD4 SP (single-positive) or CD8 SP T cells migrating then to the medulla (von Boehmer et al., 1989). As explained previously, these thymocytes need to be tolerant to self-peptides and, to that end, negative selection takes place in order to eliminate auto-reactive thymocytes. Therefore, in the medulla, the T cell repertoire is shaped by the interaction of the TCR expressed on thymocytes with the tissue-resident antigens (TRA) which are presented by the MHC located on the surface of thymic antigen presenting antigen cells (APC) such as medullar thymic epithelial cells (mTEC) and dendritic cells (DC). TRA expression is controlled by transcriptional regulators such as AIRE (autoimmune regulator) and Fezf2 (Fez family zinc finger protein 2) (Anderson et al., 2002; Takaba et al., 2015). Thymocytes will then be selected upon TRA presentation based on their TCR affinity. Cells that presented high affinity to self-antigens are negatively selected and eliminated (negative selection) or differentiate into a particular αβ T cell population called $FoxP3^+$ T regulatory (Treg) cells (Hogquist et al., 2005; Takaba and Takayanagi, 2017) **(Figure 1)**. $FoxP3^+$ Treg cells play an important role in the maintenance of peripheral tolerance. The differentiation, characterization and effector function of this population is discussed in **section (I 1 E a 3).**

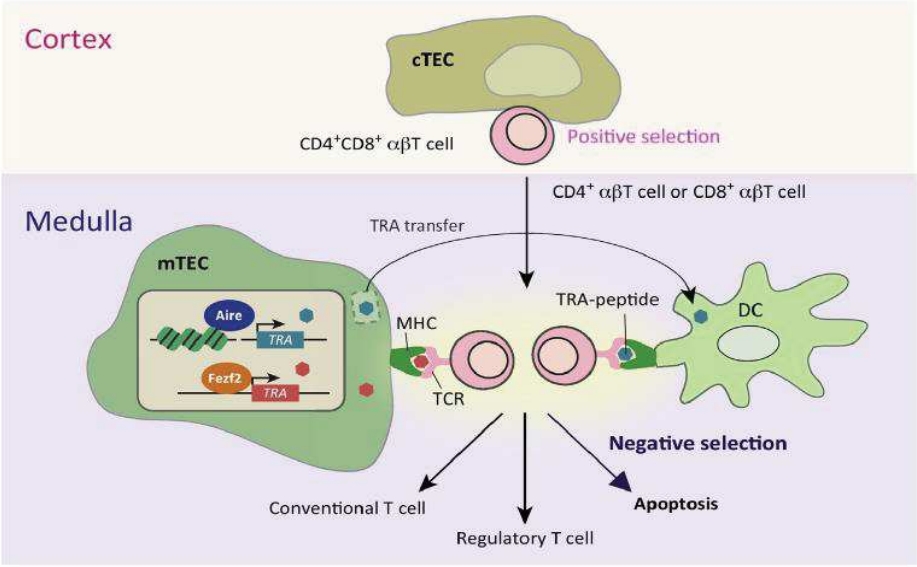

Figure 1 - αβT cell selection mechanisms in the thymus.

DN thymocytes arrive to the cortex and give rise to DP cells. DP cells are positively selected by cTEC cells and differentiate ultimately in SP CD4 or CD8 that migrate to the medulla. In the medulla, mTEC cells and DC present TRA antigens through AIRE and Fezf2 regulation. TRA presentation allows the negative selection of autoreactive T cell cells through apoptosis. AIRE (Autoimmune regulator), DN (double negative), DP (double positive), Fezf2 (Fez family zinc finger protein 2), SP (single positive) TRA (tissue-resident antigens). From (Takaba and Takayanagi, 2017)

Upon thymic selection, mature SP T cells (also known as recent thymic emigrants (RTE)) exit the thymus and are incorporated into the peripheral pool as naïve T cells. The survival of these cells is notably dependent on signaling induced by MHC-self-peptide complexes/TCR interactions and interleukin (IL) 7 (Sprent and Surh, 2012; Swainson et al., 2007). The homeostatic mechanisms involved in the regulation of the peripheral T cell pool will be discussed in detail in **section (I 1 C b).** Upon recognition of a foreign antigen specific T cell undergo a rapid activation, proliferation and effector differentiation in order to respond to this antigen. The mechanisms involved in this activation are presented in the next section.

C. Naïve T cells

a) Naïve T cell characterization and homing function

Naïve T cells have not encountered yet their cognate antigen and they are characterized by the expression of different surface markers. Both murine and human naïve T cells are characterized by the expression of lymphoid homing receptor CD62 ligand (CD62L) and CC-chemokine receptor 7 homing receptor (CCR7), by a low expression level of the CD44 adhesion molecule and by the absence of CD25 and CD69 activation markers (Boyman et al., 2009). In humans, the differential expression of the CD45 tyrosine phosphatase isoforms is also required to identify naïve T cells. Therefore, human naïve T cells also express CD45RA and do not express CD45RO which is a memory-associated marker (van den Broek et al., 2018). The different cell surface markers that characterize both murine and human naïve T cells are summarized on **Table 1.**

Naïve T cell surface markers	**Murine**	$CD62L^+$	$CCR7^+$	$CD44^{low}$	$CD25^-$	$CD69^-$	___	___
	Human	$CD62L^+$	$CCR7^+$	$CD44^{low}$	$CD25^-$	$CD69^-$	$CD45RA^+$	$CD45RO^-$

Table 1 - Naïve T cell surface markers in both mouse and human species

Naïve T cells are in constant recirculation between the lymphoid organs and the blood via the lymphatic system. This circulation is possible due to the expression of CD62L and CCR7 (Boyman et al., 2009). Interference with the expression of these 2 molecules either by using mouse CD62L mutant models (Arbones et al., 1994) or genetic deletion of CCR7 ligands (Link et al., 2007), induces a decrease in total CD4 and CD8 naïve T cell counts. These homing receptors are as well important for the recruitment of lymphocytes to the inflammatory site (Tedder et al., 1995). Expression of CCR7 and CD62L in naïve T cells are both FoxO1 (forkhead box O1)- and KLF2 (Kruppel-like transcription factor) 2- dependent factor (Carlson et al., 2006; Kerdiles et al., 2009).

b) Naïve T cell pull maintenance

The importance of thymic input for the maintenance of the naïve T cell pool seems distinct between mouse and human **(Figure 2)**. Despite the age-associated reduction of the thymus

size on both species, the human naïve T cell pool only suffers a small reduction throughout adulthood (Douek et al., 1998; Kilpatrick et al., 2008). Indeed, the absolute number of naïve $CD4^+$ T-cell compartment drops only 2-3 fold with aging (Stulnig et al., 1995; Utsuyama et al., 1992). For the maintenance of human naïve T cell pool, two mechanisms with distinct relative importance are involved **(Figure 2)**. First of all, it's important to note that despite thymus involution; T cells are still generated in the thymus during adulthood even if the number is reduced as compared to prepubescent children (Bertho et al., 1997; Jamieson et al., 1999; Poulin et al., 1999). However, the main mechanism regulating the maintenance of the human naïve T cell pool is extrathymic, and is based on the peripheral homeostatic proliferation of generated naïve T cells (Harris et al., 2005; Jamieson et al., 1999; Kilpatrick et al., 2008). Indeed, and despite a 95% decrease of TRECs (TCR excision circles produced during rearrangement; which are a marker of thymus output) between 25 and 65 years of age, the frequency of cycling $CD4^+$ T cells remain steady and their repertoire maintained highlighting the importance of homeostatic proliferation during aging (Kilpatrick et al., 2008; Naylor et al., 2005). On the other hand, the naïve T-cell pool in mice is almost fully maintained by thymic output due to a limited role of peripheral homeostatic proliferation (den Braber et al., 2012) **(Figure 2)**. In contrast to humans, naïve T cell TREC content does not significantly decline in the peripheral T compartment of aging mice (den Braber et al., 2012).

Regardless of the relative importance of the thymus output, IL-7 signaling is crucial for the survival and homeostatic proliferation of naïve T cells both in mice and humans **(Figure 2)**. Indeed, when this pathway is deficient, using either murine genetic models or blocking antibodies, the survival of naïve T cells is reduced (Vivien et al., 2001). Mechanistically, IL-7R signaling involves both the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathways in order to target its different downstream partners (Jiang et al., 2005; Rochman et al., 2009). IL-7 promotes T cell survival by increasing the expression of BCL-2 (B cell lymphoma 2) and the downregulation of pro-apoptotic factors such as BID (BH3-interacting domain death agonist), BIM (BCL-2 interacting mediator of cell death) and BAD (BCL-2 antagonist of cell death) (Jiang et al., 2005; Wojciechowski et al., 2007). In addition,

the contact between TCR and peptide-MHC complexes is also important for the survival of naïve T cells (Surh and Sprent, 2008).

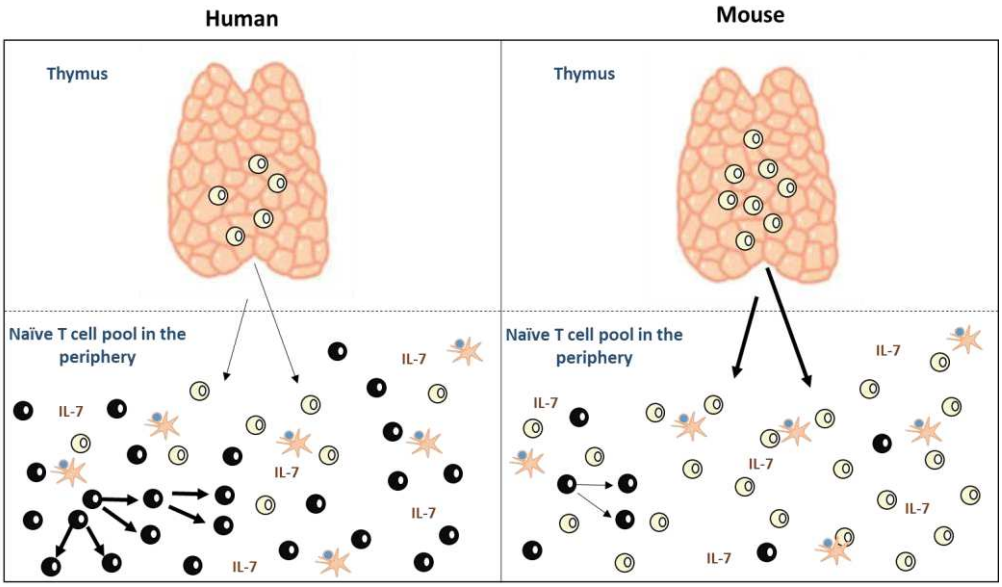

Figure 2 – Comparison of the maintenance of the naïve T cell pool in aging mice and humans.

Peripheral naïve T cell pool in mice is mainly maintained by thymic input (white cells) while in humans it is essentially maintained by the homeostatic proliferation of naïve T cells present in the periphery (black cells). For both species, both IL-7 and pMHC play a crucial role in naïve T cell survival. IL-7 (interleukin 7), pMHC (peptide- MHC complex)

D. T cell activation

Upon recognition/interaction with their cognate antigen in an adequate environment, T cells get activated, expand (clonal expansion) and differentiate into effector T cells. 3 different signals are necessary for an efficient T cell activation: TCR signaling cascade, co-stimulation and cytokine signaling. All these mechanisms are described in detail in the following paragraphs.

a) Antigen presentation by MHC molecules

As mentioned above, APCs such as B cells, macrophages and DCs are important for antigen presentation and the latter population is considered the most important for T cell activation (Sprent, 1995). DCs were first described in 1973 (Steinman and Cohn, 1973) and their increased antigen presentation capacity compare to other cell types was shown later on (Steinman and Witmer, 1978). Independently of the type of APC, as pointed out above, MHC complexes are required for antigen presentation. The MHC was first characterized in mice

after the identification of the H-2 locus as encoding for major or strong histocompatibility antigens which turned out to be the *major* histocompatibility locus in mice. However, over years, several different H-2 loci were identified (H2b, H2d, H2f among others) and the H-2 locus became finally the H-2 complex or the MHC in the mouse (reviewed in (Thorsby, 2009)). In humans, MHC classes are encoded by the human leucocyte antigen gene complex (HLA), and are located on the chromosome 6 which is the most polymorphic gene of the human genome. The classical HLA class I genes described are the HLA-A, HLA-B and HLA-C while the classical genes described for HLA class II are HLA-DR, HLA-DQ and HLA-DP (Mosaad, 2015; Shiina et al., 2017).

MHC proteins are divided in two classes: MHC class I and MHC class II. MHC I is expressed on almost all nucleated cells and MHC I complexes are recognized by $CD8^+$ T cells (Wieczorek et al., 2017). MHC I can present notably peptides from defective proteins from cell's own proteome or from invasive virus in order to induce the destruction of the damaged/infected cell. It is important to note that peptides derived from extracellular pathogens can be presented by MHC I-expressing mature DCs by a process named as cross-presentation (Joffre et al., 2012). On the other hand, MHC II expression is restricted to specific cells such as B cells, monocytes, macrophages, DCs, endothelial cells and, in humans, also in activated T cells and allows the presentation of peptides to $CD4^+$ T cells (Wieczorek et al., 2017). MHC II molecules, unlike MHC I, generally present peptides from extracellular sources upon phagocytosis of the antigen.

b) TCR signaling cascade - Signal 1

Upon TCR recognition of a cognate peptide (presented by a MHC molecule), a signaling cascade is induced, leading to T cell activation, proliferation, differentiation and effector function. CD4 and CD8 co-receptors help assisting antigen recognition through a close association with a kinase named lymphocyte cell-specific protein-tyrosine kinase (LCK). Upon activation, an immunologic synapse is formed at the site of interaction between the peptide-MHC complex (pMHC) and the TCR $\alpha\beta$ chains associated with CD3 γε, CD3 δε and CD3 ζζ dimers that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their individual cytoplasmic segments. One of the three ITAM domains present on the CD3 ζζ pairs is phosphorylated upon TCR activation by LCK proteins, inducing the recruitment and

activation of ZAP-70 (Zeta-chain-associated protein kinase 70) that phosphorylates then another molecule named linker for activation of T cells (LAT) allowing signal diversification and amplification (Malissen and Bongrand, 2015). This intracellular cascade is the first signal required to induce T cell activation and is known as "signal 1" and is represented in **Figure 3.**

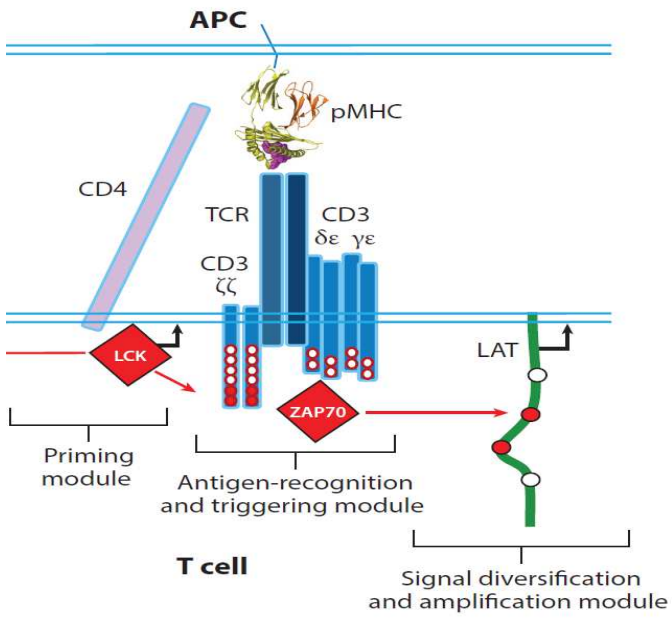

Figure 3 - TCR signaling cascade upon antigen presentation

Upon TCR-pMHC pair, single CD3γε, CD3 δε and CD3 ζζ pairs associate with TCRαβ heterodimer. The ITAM (white dots) are then phosphorylated (red dots) by LCK which induces the recruitment and activation of ZAP-70. ZAP-70 in its turn phosphorylates LAT adaptor, which allows signal diversification and amplification. ITAM (immunoreceptor tyrosine-based activation motifs), LAT (linker for activation of T cells), pMHC (peptide-MHC), ZAP-70 (Zeta-chain-associated protein kinase 70). Adapted from (Malissen and Bongrand, 2015).

c) Co-stimulation signaling – Signal 2

Other signals are however required to obtain an optimal activation. One of them is generated from the engagement of the co-stimulatory molecules, that induce a cascade of reactions known as "signal 2". Indeed, it was shown that lack of co-stimulation upon antigen recognition induces cell anergy (Jenkins and Schwartz, 1987), demonstrating that antigen presentation itself is not sufficient to induce T cell activation. A well-known and important co-stimulatory molecule is CD28 that it is constitutively expressed in naïve CD4 and CD8 T cells. CD28 is recruited at the immunological synapse upon priming by LCK and is able to recognize CD80-86 molecules present on the surface of APC. The cytoplasmic tail of CD28 is composed by two motifs: YMNM and PYAP. Once CD28 is activated, activation signals are transduced by these motifs resulting in the recruitment of the phosphatidylinositol 3-kinase (PI3K), protein kinase Cθ (PKCθ) and RAS guanyl nucleotide-releasing protein (RASGRP) (Beyersdorf et al., 2015). In particular, PI3K is important for AKT (protein kinase B) activation. The CD28-PI3K-AKT pathway contributes to T cell activation, proliferation and survival through the activation

of several molecular players such as the IkB kinase (IKK), nuclear factor- κB (NF- κB), nuclear factor of activated T cells (NFAT), BCL-XL and mammalian target of rapamycin (mTOR) among others (Chen and Flies, 2013; Wells, 2009). Current evidence suggests that CD28 co-stimulation-mediated activation of PI3K inducing mTOR and cyclin-dependent kinases (CDKs) allows cell cycle progression and is essential for optimal T cell activation (Powell et al., 1999). Moreover, CD28 co-stimulation is also important to sustain IL-2-mediated proliferation which is another mechanism crucial for optimal T cell activation. Indeed, the IL-2 gene contains a CD28 responsive element (CD28RE) as well as NFAT, NF-κB and AP-1 (activator protein-1) binding sites, highlighting the importance of the CD28 pathway and its downstream partners on IL-2 secretion (Jain et al., 1995; Serfling et al., 1995).

Upon CD28 activation, other co-stimulatory molecules are recruited such as 4-1BB and OX40, as well as co-inhibitory molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4). CTLA-4 competes for the same ligands as CD28 and upon T cell activation, its upregulation induces the internalization of CD28 and suppression of T cell responses (Rudd et al., 2009). CTLA-4 signaling is then a "stop signal" necessary to end contact between T cells and APCs consequently avoiding T cell over-activation and protection against the development of auto-proliferation and autoimmune diseases (Rudd et al., 2009). Indeed, CTLA-4- deficient mice develop a hyperproliferative syndrome and die following massive T cell tissue infiltration and organ destruction (Tivol et al., 1995; Waterhouse et al., 1995).

d) Cytokine signaling – Signal 3

An efficient T cell activation does not only require TCR engagement (signal 1), co-stimulation molecules (signal 2), but also cytokine signaling known as signal 3. IL-2 is one of the most important cytokines involved in T cell activation, induced upon TCR/CD28 engagement and acting in an autocrine/paracrine fashion (Powell et al., 1998). IL-2 binds to the IL-2 receptor (IL-2R) which can be composed by three different chains: IL-2Rα, IL-2Rβ and γ_c chain. IL-2 is capable to bind with low affinity to all of them and once these three subunits are together, the receptor affinity increases by a 1000 fold (Waldmann, 1989) **(Figure 4)**. IL-2βR is constitutively expressed in NK (natural killer), NKT (natural killer T) and memory $CD8^+$ T cells and in naïve T cells upon TCR activation. The γ_c chain is expressed by all lymphoid cells (Malek and Castro, 2010). The combination of the IL-2β and γ_c chains forms an IL-2R with

intermediate affinity that is mainly expressed on NK and memory T cells (Liao et al., 2011). Finally, high affinity IL-2R (with high CD25 (IL-2Rα) surface expression level) is expressed on Tregs and effector T cells (Liao et al., 2011) **(Figure 4)**.

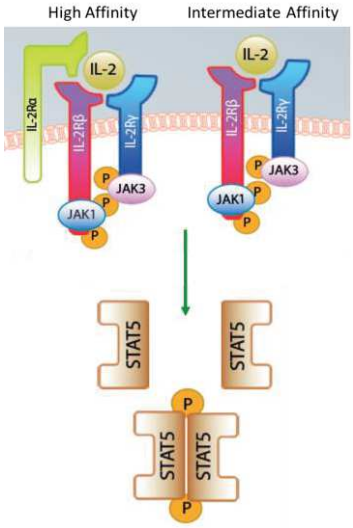

Figure 4 – IL-2 receptor affinity and STAT5 activation.

IL-2Rβ together with the IL-2γ_c (IL-2Rγ) forms a receptor with intermediate affinity. The combination of these two chains with the IL-2Rα generates an IL-2R with high affinity that is highly expressed notably on Tregs cell surface. IL-2Rα (CD25) cannot signal but it is able to induce conformational changes to IL-2 and increase its affinity to IL-2Rβ and IL-2 γ_c chains. IL (interleukin), IL-2R (IL-2 Receptor), γ_c (gamma common chain). Adapted from: InvivoGen review « IL-2: The Activator and the Controller »

IL-2 signaling is differently required *in vitro* and *in vivo* (Malek and Bayer, 2004). While *in vitro* IL-2 is essential for T cell activation, expansion and effector cell differentiation, *in vivo* IL-2 contributes mainly to T cell tolerance (Malek and Bayer, 2004).Indeed, it was demonstrated that CD25-deficient mice (in which T cells express an IL-2R with intermediate affinity to IL-2) develop massive enlargement of peripheral lymphoid organs translating an imbalance between clonal expansion and cell death following T cell activation (Willerford et al., 1995). A similar phenotype was observed in IL-2-deficient mice that developed generalized autoimmune diseases due to uncontrolled activation and proliferation of $CD4^+$ T cells (Sadlack et al., 1995).

Other cytokines can also play a role as signal 3. For instance, Keppler and colleagues demonstrated that, upon TCR stimulation, variable interplays of both IL-12 and IFNγ (interferon- gamma play an important role on CD8 T cell fate decision as well as in cytokine production and cytolytic activity in the context of Indiana vesiculovirus (VSV) and listeria infection (Keppler et al., 2012). Furthermore, cytokine signaling is also important for naïve CD4 T cell differentiation in different effector T cells subsets. The role of cytokines in CD4 and CD8 T cell differentiation is going to be discussed in the next **section (I 1 E).**

E. Effector T cells

Effector T cells (T_{EFF}) are a short-lived and large population containing several $CD4^+$ and $CD8^+$ T cell subsets. Depending on the antigen type and the surrounding environment, specific T_{EFF} cell subsets are recruited in order to respond and clear the pathogens through the production of specific effector cytokines and mediators. Murine T_{EFF} cells are characterized as $CD44^+$ $CD62L^-$. Even if human T_{EFF} cells are not found in significant proportion at steady-state, a population of terminal effector cells (T_{EMRA}) can be identified as $CD45RA^+CCR7^-$ (Kumar et al., 2018).

a) $CD4^+$ effector T cells

Within the $CD4^+$ T_{EFF} cell population, several subsets can be identified including T helper 1 (Th1), T helper 2 (Th2), T helper 9 (Th9), T helper 17 (Th17), T helper 22 (Th22) cells as well as regulatory (Treg) T cells and follicular helper T cells (T_{FH}). These subsets can be distinguished based on their cytokine profile and the expression of specific transcription factors (Raphael et al., 2015). The several $CD4^+$ T_{EFF} cell subsets and the cytokines required for their polarization are represented on **Figure 5**.

Each subset previously mentioned has a different immune cell function: Th1 cells are important for immunity against intracellular pathogens while Th2 cells are in the front line against extracellular parasites. As Th2 cells, Th9 are also implicated in host defense against extracellular parasites (primarily nematodes). In their turn, Th17 cells promote protective immunity against extracellular fungi and bacteria, with special emphasis at mucosal surfaces while T_{FH} are important for B cell class switching. Th22 cells were identified in an inflammatory skin disease context, however their role remains unclear. Finally, Treg cells are implicated in the mediation of immunosuppression and tolerogenic responses.

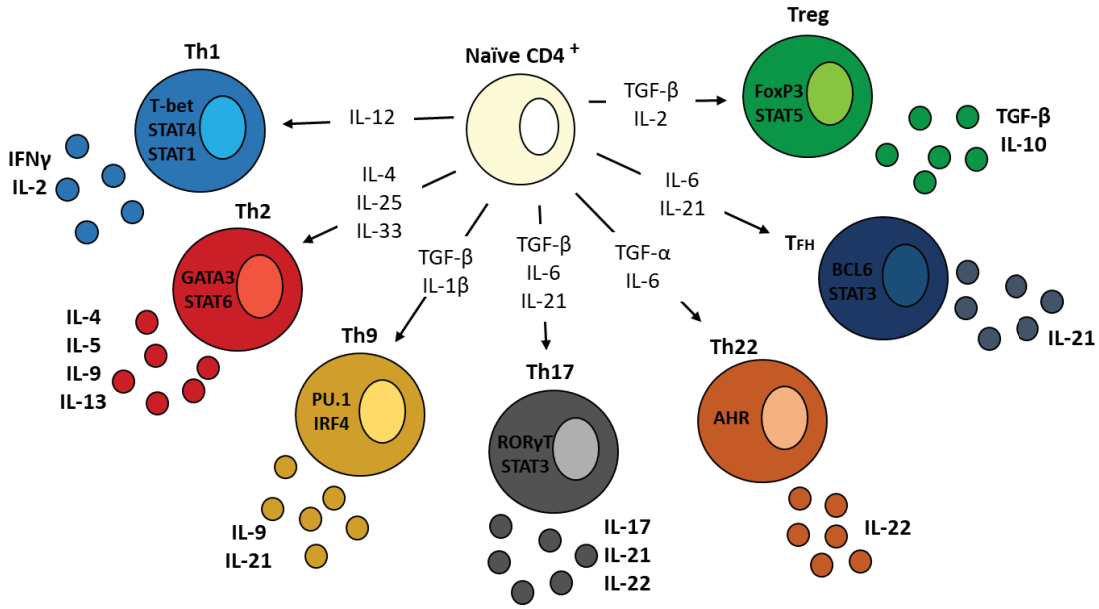

Figure 5 - Polarization of CD4⁺ naïve T cells towards distinct subsets

Upon encounter with their cognate antigen, naïve T cells differentiate into distinct T helper subsets depending on the cytokine environment and the nature of the antigen. The different subsets are characterized based on their cytokine profile and the master transcription factors expressed. AHR (aryl hydrocarbon receptor), BCL6 (B-cell lymphoma 6), FoxP3 (Forkhead box P3), GATA3 (GATA Binding Protein 3), IL (interleukin), IRF4 (Interferon regulatory factor 4), IFN (Interferon), RORγT (retinoic acid receptor-related orphan nuclear receptor gamma), TGF-β (transforming growth factor-b), T-bet (T-box expressed in T cells).

Since all my research work was centered on Th1, Th2 and Treg subsets, I will therefore develop the rest of the literature review on these $CD4^+$ T_{EFF} cell type.

1. T helper 1 cells

Th1 cells are involved in host defense against intracellular pathogens notably through the activation of macrophages by IFNγ production (Szabo et al., 2003; Zhu, 2018). Th1 cells can produce several types of cytokines including tumor-necrotic factor (TNF), lymphotoxin (TNF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF). However, this subset is mainly characterized by high levels of IFNγ and IL-2 secretion (Raphael et al., 2015; Szabo et al., 2003).

1.1. Th1 cell lineage differentiation

Different stimulus where identified to be important for Th1 differentiation, notably TCR strength signal and DCs-mediated IL-12 cytokine signaling, the lasted being the most determinant for effective Th1 cell fate.

On one hand, the strength of the TCR signal influences lineage commitment, notably by controlling the magnitude of the Ca^{2+} fluxes. In this context, Th1 cells require a stronger and specific Ca^{2+} flux as compared to Th2 cells (Sloan-Lancaster et al., 1997). Furthermore, it was demonstrated that Th1 cells have a distinct requirement of lipid-rafts (cholesterol rich pockets), comparing to Th2 cells, that are important for the aggregation of molecules involved in TCR signal initiation, regulation of Ca^{2+} signaling. Th1 polarized cells, upon re-activation, recruit rapidly TCR components to lipid rafts domains, resulting in a defined TCR/MHC clusters that have an increased sensitivity to low-affinity stimulation (Balamuth et al., 2001).

On the other hand, IL-12 cytokine signaling determinates Th1 lineage commitment by initiating a signaling cascade of events crucial for Th1 transcriptional program activation. In mice, $CD8\alpha^{+}$DCs are a IL-12 producing DC subset which preferentially induce Th1 differentiation (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). Indeed, IL-12-deficient $CD8\alpha^{+}$ DCs are not able to induce Th1 differentiation, underlying the importance of IL-12 secretion for $CD8\alpha^{+}$ DC function (Maldonado-Lopez et al., 1999). In humans, a similar subset of DCs was identified. This DC subset, named DC1, does not express CD8 but secretes as well high levels of IL-12 preferentially inducing Th1 differentiation (Collin and Bigley, 2018; Rissoan et al., 1999). Indeed, in patients presenting a weak Th1 reactivity against melanoma-associated antigens (MAA), a strong Th1 response was restored through the stimulation of patient CD4 T cells with peptide-pulsed DC1 (Wesa et al., 2007).

As mentioned above IL-12 signaling induces Th1 differentiation: IL-12 receptor is coupled to the JAK-STAT signaling pathway and activates STAT4, between others, (Kaplan et al., 1996b; Thierfelder et al., 1996) that it is important for the induction of IFNγ secretion **(Figure 6)**. Indeed, IL-12 receptor deficient mice have a defective IL-12- induced IFNγ production (Wu et al., 1997). In mice, STAT4 appeared to be skillfully specific to IL-12 (Jacobson et al., 1995). However, more recently, IL-23 was identified as well as STAT4 activator, even though the activation induced is substantially weaker as compared to the one induced by IL-12 (Parham

et al., 2002). In humans, IL-12 and IFNα (interferon alpha) (specificity in human cells) can induce STAT4 activation (Bacon et al., 1995) (Cho et al., 1996) whereas IL-23 leads to a weaker activation. (Oppmann et al., 2000; Parham et al., 2002). Mechanistically, IL-23 is a heterodimeric cytokine which binds to IL-12Rβ1 (IL-12 receptor β1) chain and to the IL-23R, whose downstream signaling activates preferentially STAT3 with weak activation of STAT4. Furthermore, IL-23 does not utilize the IL-12Rβ2 chain (expressed in IL-12R which is the crucial docking site for efficient STAT4 activation in both murine and human T cells (Watford et al., 2004). STAT4- mediated IFNγ secretion induces the activation of STAT1 (Meraz et al., 1996). Both STAT4 and STAT1 phosphorylation by IL-12 and IFNγ signaling respectively, induce the activation of the master regulator of the Th1 transcriptional program, the transcription factor T-bet (T box expressed in T cells) (Afkarian et al., 2002; Lighvani et al., 2001; Zhu et al., 2012) **(Figure 6)**. T-bet was identified for the first time in 1999 as a key transcription factor of the Th1 lineage commitment, where Szabo and colleagues showed that T-bet was a potent transactivator of the IFNγ gene (Szabo et al., 2000). T-bet is not only important for the differentiation of naïve T cell towards a Th1 fate but also plays a crucial role in suppressing the Th2 cell transcription factor, GATA3. Indeed, it was shown that T-bet is able to suppress IL-4 and GATA3 expression during T. *gondii* infection by promoting H3K27me3 alteration (suppressive histone marker) at the GATA3 (GATA Binding Protein 3 locus and by direct interaction with GATA3 (Zhu et al., 2012). Before this study, other group had already reported that T-bet was able to repress Th2 differentiation by protein-protein interaction with GATA3 interfering with its contact with its target DNA (Hwang et al., 2005).

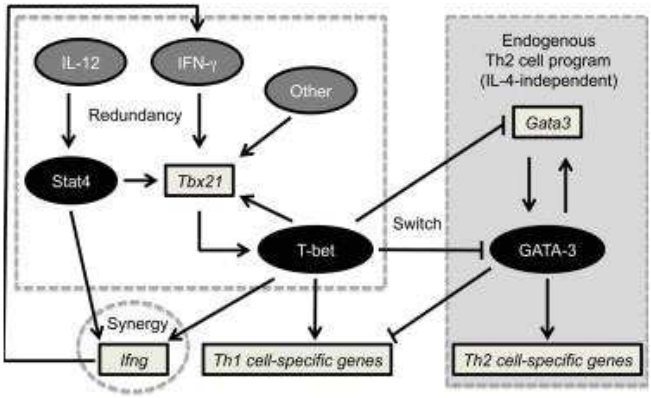

Figure 6- IL-12 and IFNγ signaling pathways during Th1

IL-12 induces STAT4 activation which will then induce IFNγ secretion and T-bet transcription. T-bet once expressed induces Th1 cell-specific genes and contributes to the repression of the Th2 program. IFNγ and T-bet work synergistically. T-bet induces IFNγ secretion while IFNγ activates STAT1 which together with STAT4, induces and maintains T-bet expression (Zhu et al., 2012)

1.2. Th1 cell effector function

IFNγ is a potent pro-inflammatory cytokine involved in many processes. Notably, it increases the expression of toll-like receptors (TLR) by innate immune cells and as a result, their responsiveness (Bosisio et al., 2002). It also promotes MHC I and MHC II transactivation resulting in increased antigen presentation (Butticè et al., 2006; Giroux et al., 2003; Martini et al., 2010; Vegh et al., 1993). Moreover, it induces chemokine signaling such as C-X-C motif chemokine ligand (CXCL) 9, CXCL10 and CXL11 from epithelial cells and it up-regulates the surface expression of CXCR3 (C-X-C motif chemokine receptor 3) on Th1 cells, facilitating their migration to inflammatory sites (Rotondi et al., 2003). IFNγ also primes macrophage activation and increases phagocytosis (Schroder et al., 2004). Studies done in interferon gamma receptor (IFNγR) $^{-/-}$ mice or using tumor cells expressing dominant-negative IFNγR mutations revealed the importance of IFNγ in tumor rejection (Dighe et al., 1994; Kaplan et al., 1998). Indeed, Th1 cell infiltration in tumors tissue is generally associated with a good prognosis in cancer patients (Ling et al., 2016; Tosolini et al., 2011; Xu et al., 2016). Altogether IFNγ appears as a potent weapon in anti-tumor responses (Ikeda et al., 2002; Schroder et al., 2004; Tannenbaum and Hamilton, 2000).

Dysregulations of Th1 function can be associated with several autoimmune pathologies including autoimmune type 1 diabetes (T1D), rheumatoid arthritis (RA) and multiple sclerosis (MS) (Skurkovich and Skurkovich, 2005). In experimental autoimmune encephalomyelitis (EAE) studies (animal model for multiple sclerosis), it was notably demonstrated that myelin-autoreactive T cells were producing high levels of IFNγ, therefore suggesting that EAE was induced because of Th1-aberrant responses (Ando et al., 1989; Olsson, 1992; Renno et al., 1994). This model was however challenged when studies demonstrated that IFNγ-deficient (Ferber et al., 1996), IFNγR deficient (Willenborg et al., 1996), or in IL-12 (Becher et al., 2002; Gran et al., 2002) and IL12R deficient (Zhang et al., 2003) mice developed normal or even stronger progression of EAE compared to WT mice. In the early 2000's it was then described by several groups that IL-23 and not IL-12 was the critical cytokine for the development of EAE (Becher et al., 2003; Cua et al., 2003) through the action of CD4 $^{+}$ IL-17-producing T cells (Langrish et al., 2005). These CD4 $^{+}$ IL-17 producing T cells were then named Th17 cells (Bettelli et al., 2006; Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005) and consensually accepted as one of the main mediators of EAE.

2. T helper 2 cells

Th2 cells are involved in allergic and humoral immunity, with an important role in the mediation of immune responses against extracellular parasites, including helminths (Zhu, 2018). This subset can produce several cytokines such as IL-4, IL-5, IL-9 and IL-13 (Walker and McKenzie, 2018) **(Figure 5)**.

2.1 Th2 cell lineage differentiation

Th2 differentiation requires the expression of its master transcription factor: GATA 3 (Pai et al., 2004; Zhang et al., 1997; Zheng and Flavell, 1997) and its regulation is done by a combination of several and distinct molecular pathways: TCR signaling strength, CD28-co stimulation, priming by other immune cells and cytokine signaling (mainly IL-4 and IL-2).

First of all, strength of the TCR signaling was shown, in vitro, to be important for Th2 differentiation as it was demonstrated that low-dose of peptides (and not the contrary) were able to induce GATA3 (Yamane et al., 2005). Moreover, co-stimulatory molecules are also crucial for Th2 differentiation. Indeed, co-stimulation through CD86 is essential for initial IL-4 secretion (a Th2 cell-promoting cytokine) and Th2 development, while another co-stimulation molecule, CD80, seems to be less required (De Becker et al., 1998; Freeman et al., 1995; Ranger et al., 1996).

Several immune subsets were demonstrated to induce Th2 differentiation through IL-4 secretion. For instance, it was demonstrated that a population of NK cells was able to secrete IL-4 and promotes the development of Th2 cells (Yoshimoto and Paul, 1994). Moreover, basophils were also shown to be able to secrete IL-4 and induce Th2 differentiation (Sokol et al., 2008; Sokol et al., 2009). Finally, other elegant studies demonstrated that $CD4^+$ T cells themselves are able to induce Th2 differentiation through IL-4 secretion (Noben-Trauth et al., 2002; Yamane et al., 2005)

In contrast to Th1 cells, the role of DCs in Th2 differentiation is controversial since DCs are not commonly described to produce IL-4 (Walker and McKenzie, 2018) and therefore it was not consensual that DCs could mediate Th2 differentiation. However, several studies demonstrated that DCs can mediate Th2 differentiation, through IL-4-independent pathways. It was shown that the administration of peritoneal macrophages pulsed with antigen (Keyhole

limpet hemocyanin) were capable to induce Th2 differentiation (De Becker et al., 1998). Moreover, in humans, it was shown that Th2 differentiation was induced by DC2, using an IL-4-independent mechanism (Rissoan et al., 1999). More recent studies showed that CD301b$^+$ dermal dendritic cells were important to drive Th2 immunity (Kumamoto et al., 2013) and to prime T cells to IL-4 production upon helminth infection. Finally, other elegant studies demonstrated that DCs that express the transcription factor interferon regulatory factor 4 (IRF4) and KLF4 are crucial for Th2 induction (Gao et al., 2013; Tussiwand et al., 2015; Williams et al., 2013). IRF4 targets IL-10 and IL-33 genes which are important for DC-driven Th2 differentiation (Williams et al., 2013). Unlike IRF4, the role of KLF4 is not well understood yet. However, it remains to clarify whether IRF4-dependent and KLF4-dependent DCs polarize directly or indirectly Th2 cells (Walker and McKenzie, 2018).

Concerning cytokine signaling, there are two cytokines with an important role for Th2 differentiation: IL-4 and IL-2. Once IL-4 binds to its receptor, JAK-STAT signaling pathway is activated, and STAT6 is phosphorylated resulting in the induction of a set of genes including *Gata3* (Kaplan et al., 1996a; Kurata et al., 1999; Zheng and Flavell, 1997). IL-2 was also demonstrated to be involved in Th2 differentiation (Ben-Sasson et al., 1990; Le Gros et al., 1990). Mechanistically, IL-2 is involved in STAT5 activation which consequently contributes to the stabilization of the IL-4 gene transcription (Cote-Sierra et al., 2004). C-Maf is another key player during Th2 differentiation, as it transactivates the IL-4 promoter and attenuates Th1 differentiation program (Ho et al., 1998; Kim et al., 1999). GATA3 was also described to have functions in the repression of Th1 cell differentiation program through repression of the IFNγ locus and independently of IL-4 mechanisms (Hwang et al., 2010; Ouyang et al., 1998). IL-25 and IL-33 were also reported as cytokines inducing Th2 responses (Fort et al., 2001; Saenz et al., 2008; Schmitz et al., 2005).

2.2. Th2 cell effector function

Th2 cells secrete several types of cytokines which are involved in distinct functions:
1) IL-4 is important for the induction of other Th2-cytokines such as IL-5, IL-9 and IL-10 and is required for the induction of B-cell immunoglobulin (Ig) switching to IgG1 and IgE (Kopf et al., 1993).

2) IL-5 is involved in the recruitment of eosinophils, an immune subset important to eliminate parasites and certain infections (Coffman et al., 1989).
3) IL-13 allows the induction of smooth muscle's movement and epithelial cell's mucus production (Kuperman et al., 2002; Urban et al., 1998) and it is also important for IgG4 and IgE class switching and upregulation of the MHC II expression by monocytes (McKenzie et al., 1993; Punnonen et al., 1993). IL-13 in combination with IL-4 can also induce alternatively activated macrophages (Gordon, 2003), which means that both cytokines are a sufficient stimuli and no priming is required.

Initially, Th2 cells were characterized as anti-inflammatory due their capacity to inhibit cell-mediated or Th1 models of disease (Berger, 2000). Indeed, IL-4 is known to powerfully suppress the development of Th1 cells, antagonizing Th1 cells functions (Swain et al., 1990). IL-4 is capable to inhibit Th1-activated macrophages and suppress the secretion of several potent pro-inflammatory cytokines (Rocken et al., 1996).

However, later on, deregulation of Th2 cells was also discovered as mediators in tissue inflammation and some immunopathologies. The pathogenic role of Th2 cells is well documented in antibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE) (Dean et al., 2000; Luzina et al., 1999; Nakajima et al., 1997). Th2 cells are also associated with allergic/hypersensitivity immune responses. Allergen-specific Th2 cells are activated by APCs resulting in pathophysiological responses including eosinophilic inflammation, oedema, smooth muscle contraction and increased mucus production (Garlisi et al., 1995; Larche et al., 2003; Leigh et al., 2004; Robinson et al., 1992). Th2 cytokines such as IL-4, IL-5 and IL-13 have been described as main players in murine models of allergic airway inflammation as well was in human asthma (Umetsu and DeKruyff, 2006). It was shown that several Th2 transcription factors impact on the regulation of asthma pathology. For instance, STAT6, a downstream signal transducer of IL-4 and IL-13, is implicated in airway inflammation as STAT6-deficient mice displayed reduced eosinophil infiltration and airway hyper responsiveness and decreased mucus production (Akimoto et al., 1998).

The role of Th2 cells in anti-tumor responses is still unclear due to discrepancies in the results demonstrating either a pro- or anti- tumor activity. It was shown that IL-4 has a negative impact on tumor growth as it induces infiltration of granulocytes that are anti-angiogenic (Modesti et al., 1993; Pericle et al., 1994; Tepper et al., 1992). Similarly, IL-13 was

also described to induce granulocyte-mediated tumor clearance (Lebel-Binay et al., 1995; Ma et al., 2004). However, IL-13-derived NKT cells were able to inhibit cytotoxic T lymphocytes (CTL) through macrophage secretion of TGF-β (Terabe et al., 2000). Furthermore, tumor infiltrates composed of high ratios of Th2 cells were associated with a more aggressive cancer and poorer prognosis in pancreatic cancer patients (De Monte et al., 2011; Tassi et al., 2008).

3. $CD4^+$ regulatory T cells

Treg cells participate to the regulation of immunotolerance and control several types of immune responses, including allergy, autoimmunity, inflammation and tumor immunity (Zhao et al., 2017). In 1970, Gherson and Kondo were the first to report the existence of a T cell subset that was able to inhibit immune responses (Gershon and Kondo, 1970) and in 1995, Treg cells were described for the first time as a subset which expresses high levels of CD25 (Sakaguchi et al., 1995). Few years later, FoxP3 was identified as the transcription factor controlling Treg cell development (Fontenot et al., 2003; Hori et al., 2003). In the early 90's, a mouse strain, named Scurfy, developing a X-linked recessive autoimmune and inflammatory diseases due to inability to control the expansion and the reactivity of T cells was described (Blair et al., 1994; Clark et al., 1999; Godfrey et al., 1991). The origin of the pathology was however not characterized at that time, but few years later, Brunkow and colleagues identified *Foxp3* as the defective gene (Brunkow et al., 2001). Further studies showed that FoxP3 is expressed in naturally arising (thymic) $CD4^+$ Tregs (Hori et al., 2003) correlating with the Treg deficiency in Scurfy and as well as $FoxP3^{null}$ mice and explaining the fatal systemic autoimmunity developed in these mouse lines (Fontenot et al., 2003). These two publications established the link between uncontrolled T cell reactivity, FoxP3 expression and Treg cells. In humans, mutations in the *Foxp3* gene also generate an impaired development or dysfunction of Treg cells, and result in the development of the immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) which is associated with severe autoimmune diseases, inflammatory bowel disease, allergy or even insulin-dependent diabetes mellitus (Bennett et al., 2001; Chatila et al., 2000; Gambineri et al., 2003; Wildin et al., 2001). Interestingly, IPEX identification (Powell et al., 1982) as well as FoxP3 mutation were firstly identified/characterized in humans (Chatila et al., 2000) with Bennett et al. and Wildin et al. confirming later that it was the same gene (FoxP3) which was mutated in both IPEX patients and scurfy mice (Bennett et al., 2001; Wildin et al., 2001). Therefore, the

characterization of the scurfy mice brought important clues and allowed to understand this human pathology as well as Treg cell differentiation and function. Since all these discovers, Tregs have been extensively studied and over the years new findings have emerged.

3.1. Classification and nomenclature

Until now, three main types of $CD4^+$ Treg cells have been described based on their suppressive functions: Two of them do not express FoxP3 and are IL-10- producing Tr1 (type 1 regulatory T cells) (Bacchetta et al., 1994; Cottrez et al., 2000; Groux et al., 1997) and TGF-β-producing $CD4^+$ Th3 (T helper 3) (Chen et al., 1994; Weiner, 2001). A third population characterized by $CD4^+$ $CD25^+$ and $FoxP3^+$ expression is the most abundant Treg subset. Depending on their origin, FoxP3-expressing cells can be classified as: thymus-derived Treg cells (tTreg), previously named as natural Treg cells (nTreg), and peripheral-derived Treg (pTreg) formerly called induced Treg (iTreg). The term iTreg is still used but exclusively for *in-vitro* generated Treg cells in order to distinguish them from the ones physiologically generated *in vivo* in the periphery (Abbas et al., 2013). I will center my literature review on FoxP3-expressing Treg cells.

3.2. Cytokine-induced signaling in Treg differentiation

In order to induce the polarization of naïve T cells to a Treg program, cytokines such as TGF-β (Chen et al., 2003; Marie et al., 2005) and IL-2 are required **(Figure 5)**. TGF-β in combination with TCR signaling results, in vitro, in the induction of Smad3 and NFAT and then FoxP3 expression (Tone et al., 2008). As discussed above, Treg cells express high levels of cell surface expression of CD25, which means that they express an high affinity IL-2 receptor (Sakaguchi et al., 1995) indicating the important role of IL-2 in Treg cells (Zhu and Paul, 2008). Indeed, IL-2 is important for Treg cells cycling *in vivo* (Smigiel et al., 2014). Moreover, another study demonstrated that IL-2R-dependent activation of STAT5 is important for their suppressive function (Chinen et al., 2016)

3.3. FoxP3 gene and its regulation

As mentioned above, FoxP3 is a critical transcription factor for the development and function of $CD4^+$ $CD25^+$ Treg cells (Fontenot et al., 2003; Hori et al., 2003); not only

contributing to the induction of Treg transcriptional program but it is also important in the lineage stability (Gavin et al., 2007). The *foxp3* gene has several conserved noncoding sequences, designated as conserved non-coding DNA sequence (CNS) 0-3 with each of them implicated in different signaling pathways **(Figure 7)**.

CNS0, also known as super-enhancer, is upstream of the transcriptional starting site and it is crucial for the expression of FoxP3 in Treg cells. This region is one of the binding sites for Stab1 that allows the modulation of the chromatin and the subsequent activities of the other CNS elements (Kitagawa et al., 2017).

CNS1 is the binding site for NFAT, AP-1, Smads and retinoic acid (Tone et al., 2008) and is required for TGF-β signaling mechanisms, a crucial cytokine involved in the differentiation of naïve T cells to a pTreg/iTreg fate. Indeed, CNS1 KO (knockout) mice showed a reduced differentiation of pTreg/iTreg cells through TGF-β signaling but a normal development of tTreg in the thymus (Zheng et al., 2010).

The CNS2 enhancer is activated by TCR expression and IL-2 and contains the binding sites of other transcription factors such as STAT5, runt-related transcription factor (RUNX), CREB and FoxP3 itself (Burchill et al., 2007; Kim and Leonard, 2007; Kitoh et al., 2009; Zhao et al., 2017; Zheng et al., 2010). Nevertheless, it was demonstrated that the activation status of this region is considerably different between tTregs and iTregs. Indeed, it was shown that tTreg cells are more stable than iTreg cells. This is in part due to the CpG islands hypomethylation pattern in the CNS2 region of the FoxP3 locus (Feng et al., 2014; Li et al., 2014) that is maintained irrespectively of extracellular conditions, cell proliferation and FoxP3 inducing signals (Ohkura et al., 2013). This demethylation is crucial for the recruitment of several transcription factors mentioned previously. IL-2 signals are essential for the demethylation induction of CNS2 which allows a stable FoxP3 expression and the acquisition of a Treg phenotype for iTreg cells (Chen et al., 2011; Freudenberg et al., 2018). Conversely, in the absence of IL-2 signaling, this region is rarely demethylated in iTreg cells (Kanamori et al., 2016). Several elegant studies demonstrated that FoxP3 stability could be increased through augmented CNS2 demethylation by Ten-eleven translocation (TET) dioxygenases (Nair et al., 2016; Someya et al., 2017).

Finally, CNS3 is essential for FoxP3 induction during thymic and induced Treg cell differentiation by enrolling c-Rel and other transcription factors (Zheng et al., 2010). The non-coding regions and its regulation is represented on **Figure 7.**

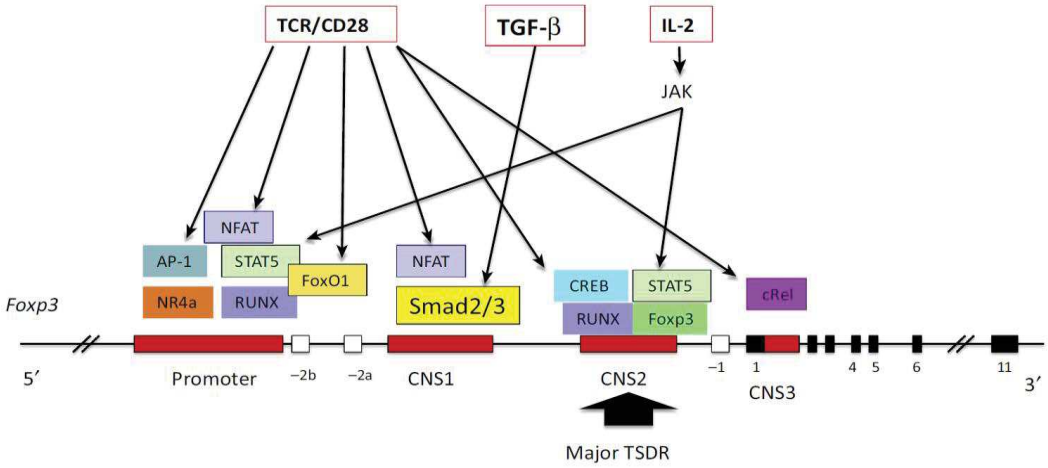

Figure 7 - Scheme of non-coding regions, signals and transcription factors involved in FoxP3 induction and stabilization.

TGF-β induces Smad2/3 activation and its recruitment to CNS1 region which is crucial to induce Treg differentiation. TCR activation and IL-2 signaling activate CREB and STAT5, respectively, activating the CNS2 enhancer which is a binding site for RUNX and FoxP3 itself. This region is hypomethylated in tTregs (major TSDR) and heavily methylated in recently generated iTregs causing FoxP3 instability. CNS3 region is activated through TCR/CD28 signaling which induces cREL activity which contributes to FoxP3 induction in nTregs, pTregs and iTregs. AP-1 (activator protein), CREB (C-AMP Response Element-binding protein) JAK (Janus kinase), NFAT (nuclear factor of activated T cells), RUNX (Runt-related transcription factor), STAT (signal transducer and activator of transcription), TCR (T cell receptor), TGF (transforming growth factor), TSDR (Treg-specific demethylated region) (Kanamori et

Despite the importance of FoxP3, other studies demonstrated that ectopic retroviral transduction of FoxP3 in T conventional (Tconv) cells failed to recapitulate certain Treg cell gene signatures (Hill et al., 2008; Sugimoto et al., 2006), highlighting that FoxP3^{+} cells are not always functional Tregs and also, that certain signature molecules can be expressed in absence of FoxP3 expression. Indeed, and even if FoxP3 is a master regulator of Treg development, other findings suggest that FoxP3 expression *per se* might not be sufficient for lineage maintenance, Treg suppressive functions or even for Treg cell phenotyping. For example, human Tconv cells, unlike murine cells, express FoxP3 transiently and at low levels upon TCR activation, without showing suppressive functions (Allan et al., 2007). Furthermore, in human blood, three phenotypically and functionally distinct subsets that express FoxP3

were described and not all of them showed suppressive functions: CD45RA^{+} FoxP3^{low} (resting Treg cells) and CD45RA^{-}FoxP3^{high} (activated Treg cells) were proved to be suppressive *in vitro* while CD45RA^{-}FoxP3^{low} where non-suppressive T cells (Miyara et al., 2009). Furthermore, CD45RA^{-}FoxP3^{high} cells show a fully demethylated CNS2 while FoxP3^{low} non-Treg cells have been described with only a partially demethylated CNS2 region.

3.4. Treg suppressive mechanisms

Treg cells can mediate suppressive functions through different mechanisms including the direct targeting of DCs, through cytolysis and metabolic disruption and also through secretion of suppressive cytokines **(Figure 8)**. The most predominant suppressive mechanism is the inhibition of DCs through CTLA-4. CTLA-4 shares with CD28 two ligands (CD80 and CD86) and it was shown that CTLA-4 binding was able to induce trans-endocytosis of both CD80 and CD86 (Qureshi et al., 2011). Treg cells express high CTLA-4 surface levels and several studies demonstrated that both *in vivo* and *in vitro* Treg suppressive functions could be reversed by blocking CTLA-4 (Read et al., 2000; Takahashi et al., 2000). Selective deletion of CTLA-4 results in autoimmune diseases and potent anti-tumor immunity (Wing et al., 2008). CLTA-4 can also stimulate the expression of the enzyme indoleamine 2,3-dyoxyegenase (IDO) by DCs resulting in tryptophan depletion in T cell environment, a known immunoregulator mechanism (Grohmann et al., 2002; Munn et al., 1999). Furthermore, Treg cells can also deliver negative signals to responder T cells by inducing an up-regulation of intracellular cyclic adenosine mono phosphate (cAMP) (Bopp et al., 2007) and through the generation of pericellular adenosine catalyzed by CD39 (ectonucleoside triphosphate diphosphohydrolase 1) and CD73 (ecto-5'-nucleotidase) found on Tregs cell surface. (Bopp et al., 2007; Grant et al., 2015). All these mechanisms can induce a metabolic disruption in Tconv cells. How metabolic changes impact T cell activation, differentiation and effector function will be discussed in detail in **section III**. Treg cells can as well secrete several types of inhibitory cytokines like TGF-β, IL-10 and IL-35 which are able to inhibit the differentiation, proliferation and activation of T_{EFF} cells in addition to their cytokine secretion (Asseman et al., 1999; Collison et al., 2007; Fahlen et al., 2005; Marie et al., 2005). Furthermore, TGF-β is likewise able to induce the conversion of naïve cells towards suppressor T cells (Chen et al., 2003). In addition, Treg cells suppress polyclonal T cell activation by inhibiting IL-2 production (Thornton and Shevach, 1998). Moreover, another study proposed a different mechanism where Treg cells, due to their

expression of high affinity IL-2R, 'steal' IL-2 produced by other cells and consequently limits the survival and proliferation of other T cells (Pandiyan et al., 2007). Therefore, the role of IL-2 in Treg suppression activity is still under debate. Finally, Treg cells can induce T cell cytotoxicity by perforin-dependent or granzyme B-dependent mechanisms (Cao et al., 2007; Gondek et al., 2005).

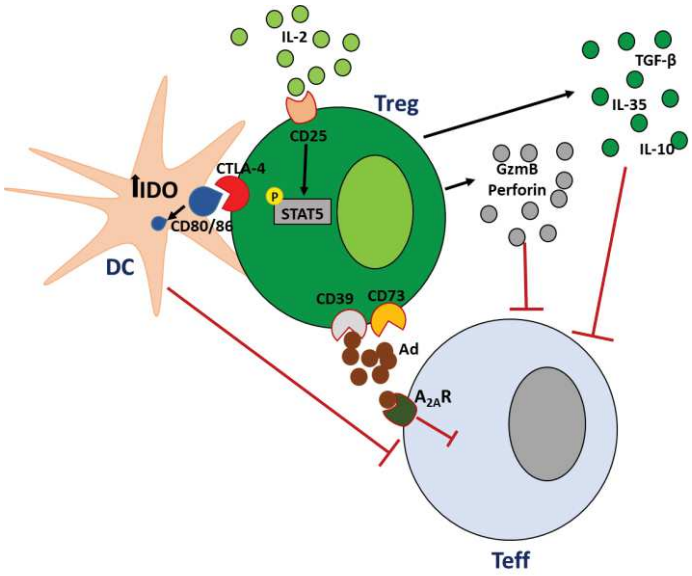

Figure 8 – Treg-mediated suppression mechanisms/pathways.

Treg cells can suppress T_{EFF} cells through different mechanisms: 1) inhibition of DCs through cytotoxic T-lymphocyte-associated protein 4 (CTLA-4); 2) metabolic disruption due to adenosine production; 3) through inhibitory cytokine secretion such as IL-10, IL-35 and TGF-β; 4) competing with T_{EFF} cells for IL-2; 5) cytolysis due to GzmB and perforin secretion. Ad (adenosine), $A_{2A}R$ (adenosine A2A receptor), CTLA-4 (cytotoxic T-lymphocyte-associated protein), DC (dendritic cell), GzmB (granzyme B) IL (interleukin), IDO (indoleamine 2,3-dioxygenase), STAT5 (signal transducers and activators of transcription 5), Treg (T regulatory), Teff (T effector).

On one hand, it is widely accepted that defects in Treg function are associated with the pathogenesis of autoimmune diseases in humans (Long and Buckner, 2011). Autoimmune diseases develop due to reduced frequencies of Treg cells and/or Treg dysfunctions resulting in the lack of control of auto-reactive effector T cells (Grant et al., 2015). For instance, defective $FoxP3^+$ Treg function can result in T1D development, an autoimmune disease characterized by insulin producing beta cells destruction (Hull et al., 2017). On the other hand, given their suppressive properties, Tregs can have a pro-tumor function as demonstrated notably by the poor prognosis associated with the tumor infiltration of this T cell subset (Bates et al., 2006; Curiel et al., 2004; Hiraoka et al., 2006; Hou et al., 2017; Ormandy et al., 2005; Wolf et al., 2003). In this context, the depletion of Treg cells represents therefore an

interesting strategy which appeared to be efficient in several studies (Kurose et al., 2015; Mahne et al., 2017; Soares et al., 2015). Due to the importance of CTLA-4 in the mediation of Treg suppressive functions, it is also important to highlight the contribution of the anti-CTLA-4 immunotherapies (such as ipilimumab) in cancer treatment (Seidel et al., 2018) .

b) $CD8^+$ effector T cells

As $CD4^+$ effector T cells, $CD8^+$ effector T cell are divided into several types depending on the profile of the cytokines secreted and the expression of transcription factors: T cytotoxic 1 (Tc1- also known as cytotoxic T cells (CTLs), T cytotoxic 2 (Tc2), T cytotoxic 9 (Tc9), T cytotoxic 17 (Tc17) and $CD8^+$ Treg cells. Each subset contributes specifically to immune responses. Despite this heterogeneity already described, CTLs are the best-characterized subpopulation and I will focus on this $CD8^+$ effector T cell subset.

CTLs play an important role in the clearance of intracellular pathogens and in the killing of malignant cells. Due to their cytotoxic capacity, CTLs are able to kill cells by releasing cytotoxic molecules like granzymes and perforin into the immunological synapse and secrete cytokines such as IFNγ and TNF-α (Mittrucker et al., 2014). CTLs are well known for their anti-tumor function due to their secretion of IFNγ, TNFα, perforin and granzymes (Reiser and Banerjee, 2016). Indeed, $CD8^+$ T cells tumor infiltration correlates with good prognosis in many types of cancer such as glioblastoma, lung, breast and colorectal tumors (Djenidi et al., 2015; Galon et al., 2006; Kmiecik et al., 2013). CTL differentiation requires the combination of several and distinct cellular and molecular pathways such as DC antigen presentation, cytokine and chemokine signaling and transcription factor activation.

Antigen presentation by DCs to $CD8^+$ T cells is important for the acquisition of effector functions. In particular, $CD8\alpha^+$ DCs (as mentioned in **(section I 1 E a 1.1)**, the IL-12 secreting DC subset implicated in Th1 differentiation), play a crucial role in the antigen presentation to $CD8^+$ T cells mainly through the receptor pair XCL1-XCR1. XCR1 (X-C motif chemokine receptor 1) is highly expressed on $CD8\alpha^+$ DCs while its ligand XCL1 (X-C motif chemokine ligand 1) is produced by activated $CD8^+$ T cells **(Figure 9)**. Upon TCR activation, this interaction plays a role to reach maximal priming and expansion of $CD8^+$ T cells (Dorner et al., 2009). Moreover, certain chemokines such as CCL (C-C motif chemokine ligand) 3, CCL4 and CCL17 secreted by

DCs upon interaction either with $CD4^+$ or NK cells can attract cognate $CCR5^+$ and $CCR4^+$ naïve $CD8^+$ T cells in a process called cross-priming (Castellino et al., 2006; Semmling et al., 2010).

Several transcription factors are required for effector differentiation **(Figure 9)**. T-bet is one of the transcription factors required for the effector functions and it's highly expressed in SLEC (short lived effector cells). (Intlekofer et al., 2005; Joshi et al., 2007). Blimp-1 (also known as PR domain zinc finger protein 1) is another transcription factor required for terminal differentiation, crucial for the repression of central memory properties acquisition (Kallies et al., 2009; Rutishauser et al., 2009). The IRF4 is another transcription factor required for CTL differentiation. IRF4 is not only indispensable for early activation but also crucial for sustained expansion and effector differentiation as it is involved in the expression and function of T-bet and Blimp-1 (Raczkowski et al., 2013; Yao et al., 2013). DNA binding 2 (Id2) is as well upregulated in $CD8^+$ T cells upon infection. Despite the fact that Id2-deficient naïve T cells are able to differentiate into effector cells, these cells are more susceptible to apoptosis which impacts on the accumulation of $CD8^+$ effector T cells (Cannarile et al., 2006). Therefore, Id2 is not necessary for CTL differentiation but instead for the magnitude of the effector responses.

In addition to transcription factors, cytokine signaling is also required for CTL differentiation and function **(Figure 9)**. IL-12 is essential for the terminal effector differentiation of CTLs. Mechanistically, IL-12 induces T-bet expression (Curtsinger et al., 2003; Joshi et al., 2007) and can also repress Eomes (a memory T cell transcription factor) (Takemoto et al., 2006). Signaling mediated by IL-2 is also required for CTL effector function. IL-2 is mainly produced by $CD4^+$ T cells while activated $CD8^+$ T cells secrete lower amounts (Malek and Castro, 2010). Activated $CD4^+$ T cells instruct DCs to produce IL-12 which induces CD25 expression in CD8 T cells, allowing the expression of high affinity IL-2R which increase the responsiveness to IL-2 (Obar et al., 2010; Wiesel et al., 2010). Several elegant studies demonstrated that persistent IL-2 stimulation is needed for promoting terminal-effector differentiation (Kalia et al., 2010; Pipkin et al., 2010).Finally, IL-2 through STAT5 activation regulates as well T-bet and Eomes expression (Grange et al., 2013).

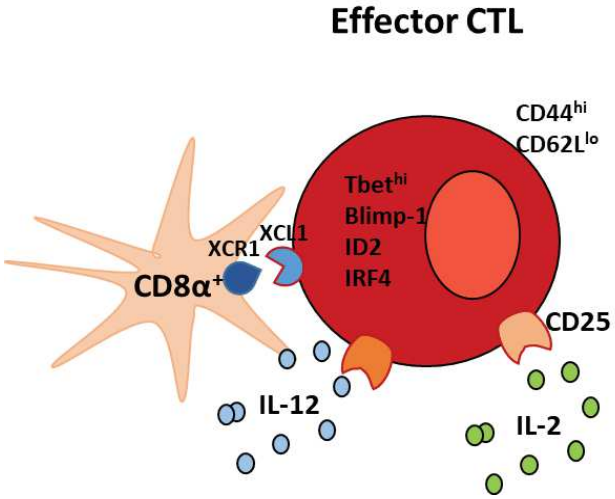

Figure 9 – Cytotoxic T cell differentiation and phenotype.

The phenotype of CTL (TF and surface cell markers) as well as the mechanisms involved in CTL differentiation are represented. IRF4 (interferon regulatory factor 4), Id2 (DNA binding 2), T-bet (T box transcription factor), XCR1 (X-C motif chemokine receptor 1), XCL1 (X-C motif chemokine ligand 1).

F. Memory T cells

Memory T cells are able to rapidly produce cytokines upon antigen restimulation and respond rapidly to antigens stimulation, which is what make this T cell subset crucial in immediate protection in peripheral tissues and in rapid responses in secondary lymphoid organs. The origin of memory T cells is still a subject of debate with several studies done in $CD8^+$ T cells proposing two different models represented in the figure below **(Figure 10)** (Arsenio et al., 2014; Flossdorf et al., 2015; Joshi and Kaech, 2008; Restifo and Gattinoni, 2013). In this literature review I will not detail the discovers done on that matter and I will focus on memory T cell phenotype and functions.

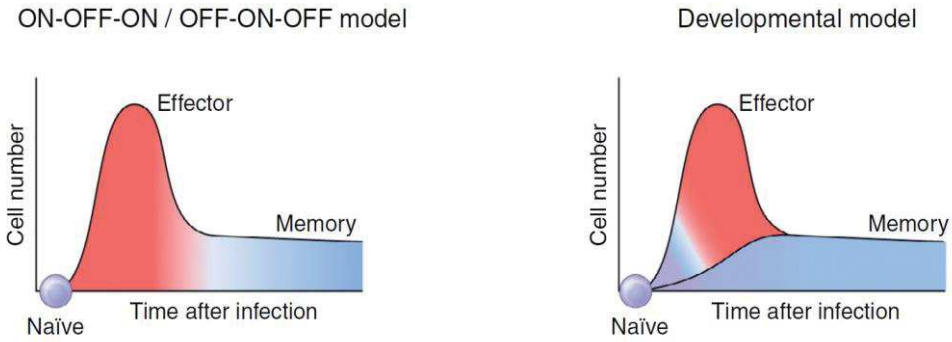

Figure 10 - Two models proposed for T cell differentiation.

The ON-OFF-ON/ OFF-ON-OFF model proposes that memory T cells have always effector T cells as precursors. The developmental model on the other hand, defends that memory cells can be directly generated from naïve T cells (Restifo and Gattinoni, 2013)

Several transcription factors are required for memory T cell differentiation. For instance, B-cell lymphoma 6 protein (BCL-6) in one of the transcription factor involved in the generation and maintenance of memory $CD8^+$ T cells (Ichii et al., 2002) as well T cell factor 1 (TCF-1) (Zhou et al., 2010), FoxO1 (Hess Michelini et al., 2013; Kim et al., 2013; Tejera et al., 2013) and Id3 (Ji et al., 2011). Finally, T-box brain protein 2 or Eomesodermin (Eomes) is also involved for memory differentiation (Intlekofer et al., 2005; Pipkin et al., 2010).

Memory T cells are highly heterogeneous in regards to their surface-receptor expression, effector function, location and trafficking properties. In 1999, the classification of circulating memory T cells was revisited and gave rise to a division of this vast T cell subset into two distinct groups: T central memory (T_{CM}) and T effector memory (T_{EM}) (Sallusto et al., 1999). The T_{CM} and T_{EM} subsets, even if initially described in humans, were rapidly demonstrated to have parallels in mice (Huster et al., 2004; Masopust et al., 2001; Reinhardt et al., 2001; Wherry et al., 2003). More recently, a population of non-circulating memory T cells known as tissue resident memory T cells (T_{RM}) was also described and will be discussed later on in this section.

Despite their differences, few markers are expressed on both T_{CM} and T_{EM}. On humans, T_{CM} and T_{EM} cells are characterized as $CD45RO^+$ $CD45RA^-$ (Merkenschlager and Beverley, 1989; Sanders et al., 1988; Smith et al., 1986) **(Table 2)**. Both murine and human T_{CM} and T_{EM} express CD44 that is a carbohydrate-binding type 1 transmembrane protein involved in cell adhesion and migration to inflammatory sites (Baaten et al., 2012; Camp et al., 1993; Nandi et al., 2004). Its signaling promotes cytoskeletal changes required for T cell extravasation from blood into tissues and its expression is also associated with T cell activation.

To distinguish between T_{CM} and T_{EM} in humans, a combination of surface markers are used: T_{CM} are characterized as $CD62L^+$, $CCR7^+$ $CD127^+$, $CD69^-$ and $CD103^-$ (α_E $integrin^-$) (Mueller et al., 2013) **(Table 2)** and they have the ability to rapidly respond to secondary contacts with a cognate antigen. This subset can be found in lymph nodes, spleen, blood and bone marrow mainly because of the expression of lymphoid homing receptor ligands that allows the passage of T_{CM} into the LN through specialized high endothelial venules (HEV) (Gowans and Knight, 1964). Endothelial cells express CCL19 and CCL21 which are recognized by CCR7-expressing T cells, allowing their extravasation and migration into the T cell zone (Okada and Cyster, 2007; Worbs et al., 2007). The binding of CD62L to the peripheral lymph node

addressin (PNAd) is another mechanism leading to T cell trafficking into LN (Butcher and Picker, 1996). Despite the fact that they have little/no effector function, T_{CM} cells can proliferate quickly and give origin to an effector response upon antigenic stimulation (Sallusto et al., 2004). T_{CM} cells can produce high levels of IL-2, but they also can secrete IFNγ and TNFα at moderate levels (Farber et al., 2014; Sallusto et al., 1999).

Unlike T_{CM}, T_{EM} are essentially located in peripheral tissues and are dedicated to immediate effector response (Sallusto et al., 2004). T_{EM} are characterized as $CD62L^-$, $CCR7^-$ $CD127^+$, $CD69^-$ and $CD103^-$ **(Table 2)** and they can be found in spleen and blood but also in non-lymphoid organs including liver, lung, intestinal tract, reproductive tract, kidney, adipose tissue and heart (Mueller et al., 2013). T_{EM} cells can produce high levels of IFNγ and TNFα but they can also secrete IL-2 at moderate levels (Farber et al., 2014; Sallusto et al., 1999).

Apart of T_{CM} and T_{EM}, other memory T cell subsets were described. As previously mentioned, T_{RM} define a population of non-circulating memory cells that can permanently reside in peripheral tissues after infection clearance (Masopust et al., 2010; Masopust et al., 2001). Most T_{RM} are $CD103^+$ $CD69^+$ (Schenkel and Masopust, 2014), although in certain tissues such as the lamina propria, brain parenchyma, salivary glands and female reproductive tract they can be $CD103^-$ or/and $CD69^-$ (Takamura, 2018; Topham and Reilly, 2018). T_{RM} cells can also be characterized by the expression of CD49a, an integrin which is an adhesion molecule. Furthermore, as the other memory T cell subsets, T_{RM} express CD44 (Amsen et al., 2018). The study of human T_{RM} has been more challenging as it requires the study of human tissues and there are no in vitro systems optimized so far for this purpose. Despite the difficulties, several studies contributed already to the description of this memory T cell population in humans, as review in (Steinbach et al., 2018). T_{RM} are important in response to infections in non-lymphoid tissues and its presence have been associated to a good prognosis in cancer patients. Dysfunction on T_{RM} responses have been associated with certain chronic inflammatory diseases such as MS (Steinbach et al., 2018). T_{RM} cell surface markers are summarized in **table 2**.

T central memory (T_{CM})		T effector memory (T_{EM})		Tissue Resident memory (T_{RM})	
Mouse	**Human**	**Mouse**	**Human**	**Mouse**	**Human**
$CD62L^+$	$CD62L^+$	$CD62L^-$	$CD62L^-$	$CD62L^-$	$CD62L^-$
$CCR7^+$	$CCR7^+$	$CCR7^-$	$CCR7^-$	$CCR7^-$	$CCR7^-$
$CD44^+$	$CD44^+$	$CD44^+$	$CD44^+$	$CD44^+$	?
$CD69^-$	$CD69^-$	$CD69^-$	$CD69^-$	$CD69^+$	$CD69^+$
-	$CD45RO^+$	-	$CD45RO^+$	-	?
-	$CD45RA^-$	-	$CD45RA^-$	-	?
$CD103^-$	$CD103^-$	$CD103^-$	$CD103^-$	$CD103^+$	$CD103^+$
$CD49a^-$	$CD49a^-$	$CD49a^-$	$CD49a^-$	$CD49a^+$	$CD49a^+$

Table 2 –T_{CM}, T_{EM} and T_{RM} surface markers in both mouse and human species

Stem cell memory T cells (T_{SCM}). is another memory T cell subset described which, interestingly, shares common markers with naïve T cells (CD45RA, CD62L and CCR7). However, it can be distinguished from naïve T cells based on the expression of CD95, CD122, CXCR3 and the integrin CD11a (LFA-1) (Fuertes Marraco et al., 2015; Gattinoni et al., 2011; Gattinoni et al., 2017) They have high proliferative capacity and they can further differentiate into other T cell subsets such as T_{CM} and T_{EM} (Gattinoni et al., 2011).

During this section, I reviewed and highlighted the role of antigen presentation, TCR, co-stimulation and cytokine signaling and also gene regulation on T cell activation, differentiation and effector function. Though, there are other factors that can impact and shape T cell function, notably the metabolic environment which was the main subject of research during my PhD. Therefore, the next sections are going to be centered on the impact of the metabolic environment on T cell activation, differentiation and effector function.

II. Metabolism

General concepts

Proliferating and differentiating cells have increased requirements of energy and "building blocks". These requirements are essentially fulfilled by the metabolism of essential nutrients like glucose, glutamine, other amino acids and lipids. Energy-sensing mechanisms that allow the detection of nutrient availability as well as energy levels regulate several metabolic pathways. They engage anabolic reactions in nutrient-replete condition whereas catabolic reactions are triggered when nutrients and energy are scarce, in a process called metabolic reprogramming. Amongst several energy sensing pathways, two are particularly implicated in T cell differentiation and function: mTOR and the 5' adenosine monophosphate-activated protein kinase (AMPK).

During my thesis, I studied the role of different metabolic/ energy sensing pathways and their impact on T cell differentiation and effector function. Therefore, in this **part II** will provide a general overview about the metabolic pathways. In **part III**, I will discuss in more detail the literature regarding the metabolic pathways implicated in T cell biology.

1) Glycolysis and the pentose phosphate pathway

The glycolytic pathway, commonly referred as glycolysis, starts with the uptake of extracellular glucose. It is essentially mediated by two major families of transporters, GLUT (glucose transporters) and SGLTs (sodium-glucose-linked transporters). Once in the cell, glucose is metabolized by hexokinase (HK) to glucose-6-phosphate (G6P), a metabolite that can be further used either in the pentose phosphate pathway (PPP) or in the glycolytic pathway **(Figure 11)**.

In the first situation, G6P can enter through the oxidative branch of PPP resulting in the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH), crucial for glutathione reduction and fatty acid synthesis. Moreover, the PPP is the only source of Ribose-5-phosphate indispensable for nucleotide formation and amino acid precursors. Glycolysis is also linked with the PPP through its non-oxidative branch. In this case, the PPP is fulfilled through fructose-6-phosphate (F6P) and glyceraldedehyde-3-phospate (G3P) (another

intermediates of the glycolysis pathway) resulting also in the generation of Ribose-5-phosphate, without production of NADPH **(Figure 11)**.

In the second situation, glucose-6-phosphate, after multistep-reactions, is converted into two molecules of pyruvate along with the production of two molecules of reduced nicotinamide adenine dinucleotide (NADH) and four molecules of adenosine triphosphate (ATP), however two of them are used to restart another round of glycolysis **(Figure 11)**. Pyruvate can then either be reduced into lactate through the lactate dehydrogenase (LDH) (to maintain the glycolytic flux by upholding the oxidized nicotinamide adenine dinucleotide (NAD^+) levels) or enter directly into the tricarboxylic acid (TCA) cycle as acetyl coenzyme A (acetyl-coA) **(discussed in the next section)**. Unexpectedly, lactate can be produced as well in aerobic conditions in response to mitogenic or inflammatory signals and this process is called aerobic glycolysis. This phenomenon, initially described in cancer cells, was described by Otto Warburg and is known as “Warburg effect” (Warburg et al., 1958, 1960).

Glycolysis is not the most efficient metabolic pathway in terms of ATP production. However, it can be rapidly activated and quickly produce several intermediates important for a rapid cell growth. These intermediates are not only important to fulfill the PPP and the TCA cycle but they also represent biomass elements for the synthesis of serine, glycine and alanine. Glycolysis can also provide dihydroxyacetone phosphate (DHAP) important for glycerol generation that is important for the cell membrane structure. (Gaber et al., 2019; O'Neill et al., 2016) **(Figure 11)**.

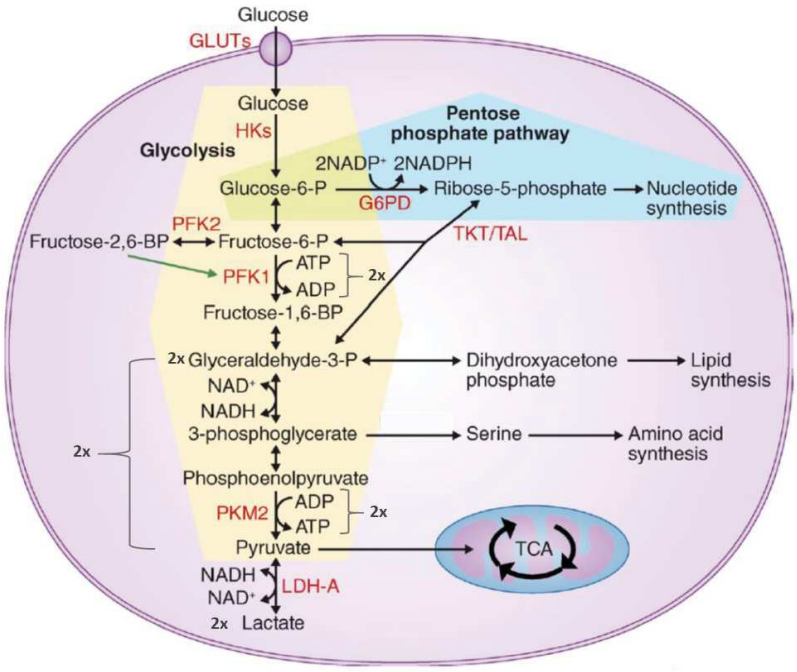

Figure 11 - Glycolysis and the pentose phosphate pathway (PPP)

Glucose enters the cell by glucose transporters (GLUTs) and, is metabolized by the hexokinases (HKs) into Glucose-6-P (G6P). G6P is converted into Fructose-6-P (F6P) and then into fructose-1-6-P (F16P) by the phosphofructokinase-1 (PFK1). This process requires 2 ATP molecules. After F16P is metabolized and gives rise to 2 Glyceraldehyde-3-P (G3P) molecules. Each one will be metabolized into 3-phosphoglycerate (3PG). 3GP is then converted into phosphoenolpyruvate and then into pyruvate through the pyruvate kinase isozyme M2 (PKM2). Once pyruvate is produced it can either enter in the mitochondria to fulfill the TCA cycle or be metabolized into lactate by the lactate dehydrogenase (LDH-A). G6P produced during glycolysis can also enter in the oxidative branch of the PPP and be metabolized into ribose-5-phosphate (R5P) by the glucose-6-phosphate dehydrogenase (G6PD). This process results in the generation of 2 NADPH molecules which are important for lipid synthesis. F6P and G3P can also fulfill the PPP through its non-oxidative branch and be metabolized into R5P due the action of transketolase (TKT) and TAL (tyrosine ammonia lyase) respectively. Glycolysis can also provide dihydroxyacetone phosphate (DHAP) from G3P and serine through 3PG. Adapted from (Hamanaka and Chandel, 2012)

2) TCA cycle and oxidative phosphorylation

The TCA cycle (also known as citric acid cycle or Krebs cycle) takes place in the matrix of mitochondria and is closely associated with the electron transporter chain (ETC). The TCA cycle can be fed from different metabolites generated in distinct metabolic pathways through: (I) acetyl-CoA produced either from: (I) pyruvate catabolism by the pyruvate dehydrogenase (PDH) or from fatty acid oxidation (FAO); (II) α-ketoglutarate (αKG) that is an end product of the glutaminolysis pathway (discussed on **section II 4**). From the TCA cycle

results, the production of 3 molecules of NADH and one molecule of reduced flavin adenine dinucleotide ($FADH_2$) that are crucial for the optimal activity of complexes I and II of the ETC. The complex I (CI) together with CIII and CIV, export electrons out of the mitochondrial membrane in order to create a proton gradient between the matrix and the intermembrane space of the mitochondria. These electrons located in the intermembrane space, are then used to drive the function of the complex V, also known as ATP synthase. Together, the TCA cycle and ETC, from one single molecule of glucose, produce 36 ATP molecules by a process named oxidative phosphorylation (OXPHOS) **(Figure 12)**. Moreover, TCA cycle also give rise to several metabolites important for fatty acid synthesis and crucial for other cellular and molecular reactions (Gaber et al., 2019; O'Neill et al., 2016).

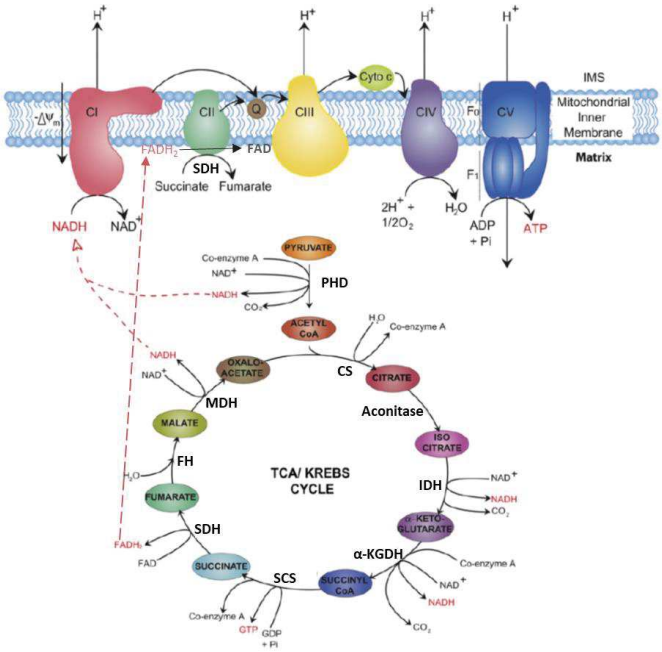

Figure 12 - The tricarboxylic acid (TCA) cycle and its connection with the electron transport chain and OXPHOS

Pyruvate is converted into Acetyl-CoA by PDH and enters in the TCA cycle. Once in the TCA cycle, a cascade of metabolic reactions occurs with the generation of several metabolites. From this cycle results the formation of NADH and $FADH_2$ which are proton (H^+) donors crucial for CI and CII activity of the electron transport chain and initiation of the OXPHOS pathway. While H^+ are pumped out to the IMS space and create a transmembrane proton electrochemical gradient, electrons are transported from the CI and CII to the CIII and CIV until they reach the terminal electron acceptor (O_2). This process ends up with the entry of the H^+ pumped out from the first 4 complexes through the CV which generates ATP. αKGDH (α-Ketoglutarate dehydrogenase), CS (citrate synthase), CI (complex I), CII (complex II), CIII (complex III), CIV (complex IV), CV (complex V), FH (fumarate hydratase), $FADH_2$ (reduced flavin adenine dinucleotide), IDH (isocitrate hydrogenase), NADH (reduced nicotinamide adenine dinucleotide), MDH (malate dehydrogenase), PDH (pyruvate dydrogenase), SDH (succinate dehydrogenase), SCS (succinyl-CoA synthetase), TCA (tricarboxylic acid). Adapted from: (Osellame et al., 2012)

3) Lipid synthesis and Fatty acid oxidation

Lipid metabolism is a dynamic and a complex process that includes lipid synthesis (anabolic reactions) and oxidation (catabolic reactions). During lipogenesis, lipids, such as fatty acids (*de novo* fatty acid synthesis pathway) and cholesterol (mevalonate pathway or cholesterol biosynthesis pathway), are synthetized and used as building blocks for cell structures (such as cell membrane). During lipid oxidation, lipids and specific fatty acids (FAs), can be broken down and used as a cell energy source in a process known as β-oxidation or fatty acid oxidation (FAO).

Fatty acid synthesis (FAS) is highly dependent on metabolites produced by other metabolic pathways like glycolysis, PPP and the TCA cycle. As such, the acetyl-coA produced either through TCA cycle-derived citrate, glycolysis-derived pyruvate or from acetate catabolism, is carboxylated into malonyl-CoA by the acetyl CoA carboxylase (ACC) and after into malonyl-ACP **(Figure 13)**. The elongation of the nascent fatty acid starts then with a Malonyl-ACP molecule acting as a two carbons donor to other acetyl-CoA molecule. After several metabolic reactions, a butyryl-CoA molecule is formed through the enzymatic activity of fatty acid synthase (FASN) in a NADPH-dependent manner (first elongation cycle). This elongation cycle is repeated six more times with another 6 malonyl-CoA molecules (one at each cycle) giving their carbons in a NADPH-dependent manner. This process ends up with a 16-carbon palmitic acid saturated FA straight chain **(Figure 13)**. For the synthesis of alternative-length chain FAs (with more than 16 carbons), palmitic acid is used as a substrate for elongation and as a substrate for desaturation reactions for the generation of unsaturated fatty acids. The synthesis of other classes of FAs, known as branched chain fatty acids, require specific amino acids like valine, leucine and isoleucine to generate the methyl branches on the carbon chain. (Gaber et al., 2019; O'Neill et al., 2016).

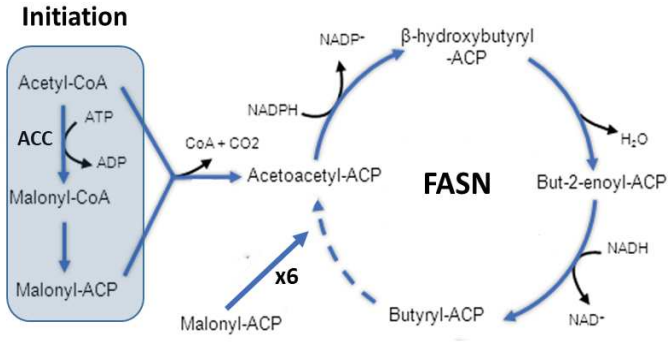

Figure 13 – Fatty acid synthesis.

Acetyl-CoA is metabolized into malonyl-CoA by the ACC. Manolyl-CoA is metabolized into Malonyl-ACP which together with another Acetyl-CoA molecule generate Acetoacetyl-ACP (initiation). After, several reactions take place in the FASN protein complex, to produce butyryl-ACP. Once this metabolite is generated, this cycle restarts 6 times up to palmitate synthesis (16:0). Each cycle extends the Acyl Chain by 2 carbons. ACC (acetyl CoA carboxylase), ACP (acyl carrier peptide), FASN (fatty acid synthase). Adapted from (Jawed et al., 2016).

Cholesterol synthesis (also known as mevalonate pathway) requires a distinct metabolic pathway. Acetyl-CoA is one of the essential precursors that is metabolized into acetoacetyl-CoA by the thiolases. After, this acetoacetyl-CoA molecule in combination with another Acetyl-CoA molecule is converted in hydroxymethylglutaryl-coenzyme A (HMC-CoA) by the hydroxymethylglutaryl-coenzyme A synthase (HGMCS). Then, HMC-CoA is reduced by the hydroxymethylglutaryl-coenzyme A reductase (HGMCR) and mevalonate is generated. After, through a multistep enzymatic reaction, several intermediates are produced such as squalene and lanosterol until the end product: cholesterol **(Figure 14)**. Cholesterol is transported as a lipoprotein either in the form of HDL (high-density lipoprotein) or LDL (low-density lipoprotein) (Bietz et al., 2017) and its influx and efflux are regulated by several transporters: cholesterol enters in the cells through CD36 and/or through the LDL receptor and exit the cell through ATP binding cassette (ABC) A1 and ABCG1 **(Figure 15)**.

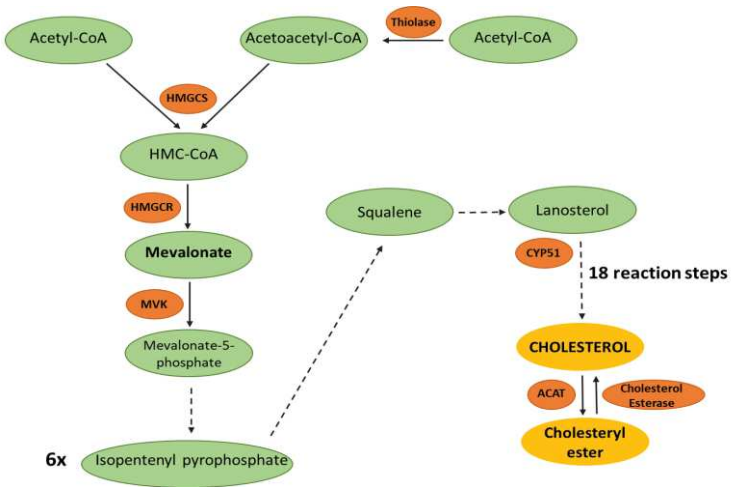

Figure 14 – The Mevalonate pathway.

Acetyl-CoA and Acetoacetyl-CoA through the HMGS enzymatic activity give rise to HMC-CoA, that is then, metabolized into mevalonate through the action of HMGCR. Mevalonate is converted into Mevalonate-5-phosphate by MVK, which through multiple step enzymatic processes, ends up in the generation of isopentenyl pyrophosphate. 6 isopentenyl pyrophosphate molecules are required for the production of one squalene molecule. Squalene is then metabolized into lanosterol and after a 18-steps reaction, cholesterol is generated. Cholesterol can be stored into the cell through the enzymatic activity of ACAT that transforms it into cholesteryl ester. ACAT (Acyl-CoA cholesterol acyltransferase), CYP51 (Lanosterol 14α-demethylase), HMGCR (hydroxymethylglutaryl-coenzyme A reductase), HMGCS (hydroxymethylglutaryl-coenzyme A synthase), MVK (mevalonate kinase).

The expression of *de novo* lipid synthesis-related enzymes are regulated by sterol regulatory element binding protein (SREBP). SREBP are a family of transcription factors that regulate both fatty acid and cholesterol synthesis. There are 3 different SREBPs isoforms with distinct physiological roles: SREBP1a (involved in global lipid synthesis), SREBP1c (the most active form in driving expression of genes related to fatty acid synthesis regulation) and SREBP2 (the most active form in the regulation of genes related to cholesterol synthesis) (Shimano and Sato, 2017; Ye and DeBose-Boyd, 2011). SREBP activation is highly regulated and achieved by a feedback mechanism that senses cholesterol and fatty acids intracellular levels **(Figure 15)**. Mechanistically, SREBP proteins are associated with SCAP (SREBP cleavage-activating protein) in the endoplasmic reticulum (ER) membrane where it is retained due to its binding to the protein INSIG (insulin-induced gene protein). When cholesterol levels are low, INSIG and SCAP are no longer associated; the SREBP-SCAP complex is not retained in the ER and moves to the Golgi. There, SREBP is cleaved by site-1 and 2 proteases (S1P and S2P) giving rise to the SREBP activated form which, after migration to the nucleus, activates genes involved in cholesterol biosynthesis and other lipids. When extracellular levels of cholesterol

are high, INSIG and SCAP bind to each other, the SREBP-SCAP complex remains in the ER not allowing the generation of the SREBP mature form and the activation of genes involved in cholesterol and other lipids biosynthesis (Bietz et al., 2017; Horton et al., 2002; Ye and DeBose-Boyd, 2011) **(Figure 15)**. However, this regulatory mechanism also applies to other lipids; for example, fatty acid synthesis is controlled by the levels of palmitolyl-CoA; high levels inactivating ACC activity. Furthermore, fatty acids are also able to inhibit proteolytic activation of SREBP1 by increasing the amount of INSIG protein in the ER. However, this inhibition is not independent of sterols, that are crucial for the binding of INSIG and SCAP (Ye and DeBose-Boyd, 2011).

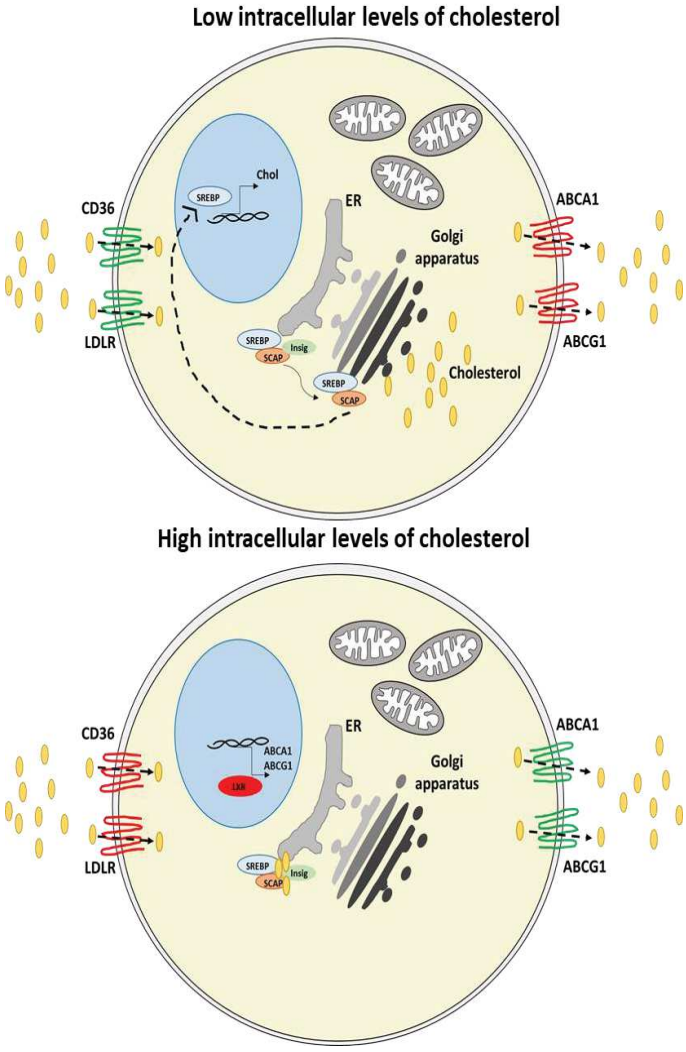

Figure 15 – Intracellular Cholesterol levels homeostasis

When intracellular levels of cholesterol are low, INSIG no longer binds to the SREBP-SCAP complex in the ER and allows its migration to the Golgi apparatus. Once in the Golgi, proteases SP1 and SP2 allow the generation of the active form of SREBP that migrates to the nucleus and induces the regulation of cholesterol-synthesis-related genes. When intracellular levels are high, cholesterol binds to SCAP, which results in the increased affinity with INSIG. Once INSIG binds to the SREBP-SCAP complex, it is retained in the ER and cholesterol synthesis is then downregulated. In the same conditions, LXR is activated, increasing the surface expression of ABCA1 and ABCG1 cholesterol efflux transporters. ABCA1/ ABCG1 (ATP binding cassette A1/G1), INSIG (insulin-induced gene protein), ER (endoplasmic reticulum), LDLR (low density lipoprotein receptor), SCAP (SREBP cleavage-activating protein), SREBP (sterol regulatory element binding protein).

Liver X receptor (LXR) plays an opposite role to SREBP as it gets activated under high intracellular cholesterol conditions. Its activation and assembly with the retinoid X receptor (RXR) induces the upregulation of ABCA1 and ABCG1, crucial transporters for cholesterol efflux (Tall and Yvan-Charvet, 2015) **(Figure 15)**. Furthermore, LXR is also involved in the regulation of the SREBP-1c gene. LXR-mediated regulation of the SREBP-1c induces the expression of genes involved in the elongation and unsaturation of fatty acids resulting in an increased synthesis of long-chain polyunsaturated fatty acids (PUFAs) (Tall and Yvan-Charvet, 2015; Ye and DeBose-Boyd, 2011).

The FAs produced or imported into the cells (which is mediated by CD36 and by FATP (fatty acid transport protein) (Howie et al., 2017b)) can also be catabolized in the cell in order to produce energy (ATP). FAs present in the cytosol are first converted to give rise to fatty acid acyl-CoA. Short-fatty acids are able to pass through the mitochondrial membranes without any other mediator. However, medium-chain and long-chain fatty acids need to be conjugated with carnitine by the carnitine palmitoyl transferase (CPT) 1 to be shuttled into the mitochondria. Once in the mitochondria, the carnitine is removed by CPT2 and FAO, also known as β-oxidation, is initiated. Aside of ATP production, from FAO also results the production of NADH and $FADH_2$ that are important for the ETC function. (Bietz et al., 2017; Gaber et al., 2019; O'Neill et al., 2016).

4) Amino acid metabolism

Amino acids (AA) are important precursors for protein synthesis, *de novo* synthesis of branched-chain fatty acids and *de novo* purine and pyrimidine synthesis. They can be classified into three different classes: essential, conditionally essential and non-essential. Essential AA cannot be synthetized by the cell itself or are inadequately synthetized; therefore they need to be provided from the diet. Conditionally essential AA can be synthesized in necessary amounts by the organism. However, when the utilization rate is higher than the synthesis, they need to be provided from the diet. Finally, non-essential AA can be synthetized physiologically in adequate amounts to meet optimal requirements (Wu, 2009). There are several amino acids required for normal cell physiology and function but considering the topics of my PhD work, I will focus on glutamine and arginine.

Glutamine is the most abundant amino acid in the human plasma corresponding to 20% of the total pool and is a non-essential AA (Newsholme and Parry-Billings, 1990; Roth et al., 1990). Glutamine is important for cell growth, represents an alternative source for ATP production and can be metabolized into αKG, through a process called glutaminolysis. Glutamine is first converted into glutamate and ammonia by the glutaminases (GLS) and then glutamate is converted into αKG either through dehydrogenation in the mitochondria by the enzyme glutamate dehydrogenase (GDH) or through a transamination reaction mediated by the glutamate oxaloacetate transaminase (GOT) and the ornithine aminotransferase transaminase (OAT) in the mitochondria or in the cytosol (DeBerardinis et al., 2007). αKG can fulfill the TCA cycle through a process called anaplerosis resulting in ATP production through OXPHOS. Furthermore, glutamine-derived αKG can be a precursor for citrate production which can be used for fatty acid synthesis. Moreover, glutamine along with aspartate are important for *de novo* purine and pyrimidine synthesis (Gaber et al., 2019; O'Neill et al., 2016) **(Figure 16)**.

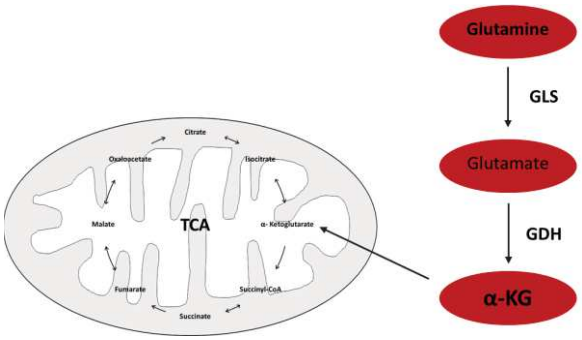

Figure 16 – Glutaminolysis

Glutamine is converted into glutamate via actions of glutaminases. Glutamate is then converted to αKG, which enters and fuels OXPHOS through the TCA cycle. αKG (α-ketoglutarate), GLS (glutaminase), GDH (glutamate dehydrogenase), TCA (tricarboxylic acid).

Arginine is classified as a conditionally essential AA (Luiking et al., 2012) and its imported into the cells by the cationic transporters (CAT) **(discussed in section III 2 E)**. Arginine metabolism results notably in the production of urea and ornithine by the arginase enzymes (ARG) **(Figure 17)**. The urea produced enters in the urea cycle while the ornithine is used for the production of polyamines (putrescine, spermidine and spermine) that are crucial for DNA and RNA stabilization, transcription and translation, cell growth, cell proliferation among others. Arginine can also be metabolized into citrulline by the nitric-oxide-synthase (NOS). From this reaction results the production of nitric oxide (NO), a well-known signaling molecule involved in many physiological processes **(Figure 17)**. *De novo* arginine production is dependent on citrulline availability which can therefore give rise to argino-succinate and be

metabolized through arginosuccinate lyase (ASL) into arginine which represents 10-15% of whole body arginine production under normal conditions (Castillo et al., 1996; Dejong et al., 1998) **(Figure 17)**. These metabolic processes take place mainly in the intestinal-renal axis, where citrulline is highly produced in the intestines and arginine in the kidney (Ligthart-Melis et al., 2008; van de Poll et al., 2007; van de Poll et al., 2004; Windmueller and Spaeth, 1981; Yu et al., 1996). Despite the capacity of the body to produce arginine, about 80% of it results from recycled amino acids released from protein breakdown (Szefel et al., 2018)

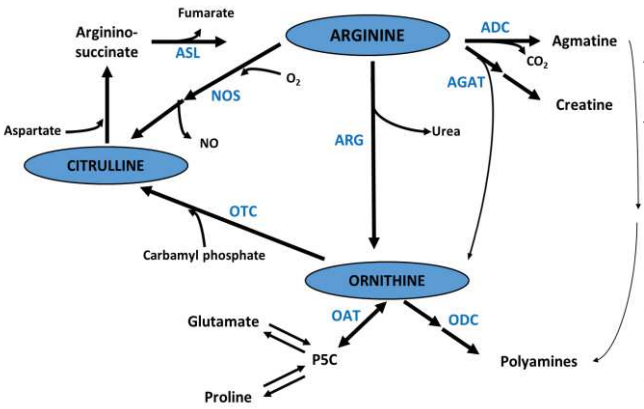

Figure 17 – Arginine metabolism

Arginine can be converted into two major metabolites: Ornithine through the action of ARG and Citrulline by NOS. From ARG enzymatic activity, it results the production of Urea and from NOS action, NO generation. Ornithine can be metabolized into polyamines by ODC while Citrulline can give rise to fumarate creating a link with the TCA cycle. ADC (arginine decarboxylase), AGAT (arginine: glycine amidinotransferase), ARG (arginase), ASL (argininosuccinate lyase), OAT (ornithine aminotransferase), ODC (ornithine decarboxylase), OTC (Ornithine transcarbamylase), NO (nitric oxide), NOS (nitric oxide synthase), P5C (pyrroline-5-carboxylate), TCA (tricarboxylic acid).

5) mTOR and AMPK signaling pathways

mTOR functions as an energy-sensor pathway that is responsive to several immunological (TCR stimulation, co-stimulation, cytokines) and environmental cues (Pollizzi and Powell, 2015). In environments where nutrient availability is not sufficient to sustain cell growth and expansion, mTOR is inhibited. Signaling from mTOR is mediated through two distinct complexes: mTOR complex (mTORC) 1 and mTORC2 that are associated with several scaffold proteins. Both complexes share the mTOR catalytic subunit, the mammalian lethal with sec-13 protein 8 (mLST8) and the DEP domain containing mTOR-interacting protein (DEPTOR). In contrast, only mTORC1 is associated with the regulatory-associated protein of mammalian target of rapamycin (Raptor) and with proline-rich AKT substrate 40 kDa (PRAs40) while mTORC2 is associated with rapamycin-insensitive companion mTOR (Rictor), with mammalian

stress-activated map kinase-interacting protein 1 (mSin1) and protein observed with rictor 1 and 2 (protor 1/2) (Laplante and Sabatini, 2012) **(Figure 18)**.

Despite the fact that both complexes share the same catalytic subunit, they have distinct functions. Thus, signaling mediated by mTORC1 promotes protein synthesis, lipogenesis and energy metabolism inhibiting at the same time autophagy and lysosome biogenesis. mTORC1 activation results in the phosphorylation of S6 kinase beta-1 (S6K1) and the eukaryotic translation inhibitor factor 4E-binding protein 1 (4E-BP1). In contrast, mTORC2 signaling regulates cytoskeletal organization and cell survival/metabolism. It also induces a metabolic switch from OXPHOS to glycolysis (Laplante and Sabatini, 2012; Pollizzi and Powell, 2015). mTORC2 activation results in the phosphorylation of the AKT kinase at serine 473 and the glucocorticoid-regulated kinase 1 (SGK1) **(Figure 18)**.

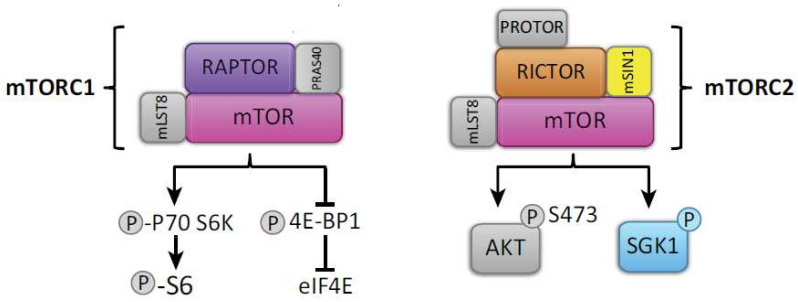

Figure 18 – mTOR complexes and its targets.

mTOR pathway via two different complexes: mTORC1 and mTORC2. mTORC1 that contains the adaptor protein RAPTOR while mTORC2 contains RICTOR. Activation of mTORC1 and mTORC2 leads to the phosphorylation of different targets as illustrated in this figure. AKT (mechanistic target of rapamycin), mTOR (), mLST8 (mammalian lethal with sec-13 protein 8), mSIN1 (map kinase-interacting protein 1), PRAs40 (proline-rich AKT substrate 40 kDa), RAPTOR (regulatory-associated protein of mammalian target of rapamycin), RICTOR (rapamycin-insensitive companion mTOR), SGK1 (), S6K (S6 kinase), 4E-BP1 (4E-binding protein 1). Adapted from (Pollizzi and Powell, 2015)

AMPK complex is another metabolic regulator, highly conserved, that senses intracellular adenosine nucleotide levels. AMPK is constituted by 3 subunits: the catalytic subunit α and two other regulatory subunits, β and γ **(Figure 19)**. When the AMP (adenosine mono phosphate)/ATP ratio decreases, AMPK activation is induced through the phosphorylation of the Thr172 present in the α subunit by liver kinase B1 (LKB1) (Shaw et al., 2004; Woods et al., 2003). Furthermore, AMP is able to directly bind to the γ regulatory subunits, inducing a conformational change protecting AMPK against dephosphorylation, and thus, its inactivation (Xiao et al., 2011). Activation of AMPK due to AMP increased levels can also happen through

allosteric activation of the phosphorylated enzyme (Suter et al., 2006). Once activated, AMPK triggers several metabolic pathways to generate ATP while inhibiting cell growth, biosynthesis and other ATP-dependent/consuming processes. Importantly, AMPK activity inhibits mTOR-dependent pathways, promoting catabolism and inhibiting biosynthesis and cell growth (Hardie et al., 2006).

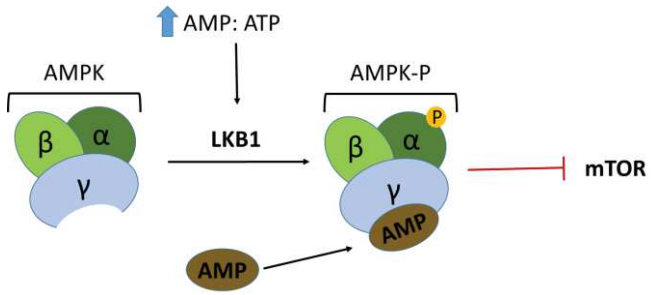

Figure 19 – The AMPK pathway

AMPKα can be activated by phosphorylation on Thr172 by LKB1 kinase. LKB1 promotes AMPK phosphorylation under high AMP: ATP ratio. High AMP levels also induce allosteric activation of the phosphorylated enzyme. One biological outcome of AMPK activity is the inhibition of mRNA translation under low-energy conditions through inhibition of mTORC1 activity. AMPK (AMP-activated protein kinase), AMPK-P (AMPK phosphorylated) AMP (Adenosine monophosphate), ATP (Adenosine triphosphate), mTOR (mechanistic target of rapamycin).

Several metabolic pathways are engaged upon TCR stimulation. However, the energy and the metabolic requirements of each T cell subset are distinct. In **section III**, the metabolic changes that occur throughout T cell lifecycle are dissected in order to specifically highlight the importance of metabolism on T cell activation, differentiation, effector function and survival.

III. T cell metabolism

1) Naïve T cells

In the periphery, naïve T cells are continuously migrating through the secondary lymphoid organs, surveying for antigens. For example, cytoskeletal rearrangements, sustained by ATP production, occur constantly and energetic resources are needed in order to fulfill their energetic demands. It has been shown that naïve T cell metabolism relies mainly on OXPHOS (Verbist et al., 2012). Therefore, naïve T cells have a metabolic balance that favors energy production over biosynthesis of new cellular compounds (MacIver et al., 2013). Thus, and despite the classification of naïve T cells as quiescent T cells, they are metabolically active.

It will be tempting to believe that the maintenance of a quiescent metabolic state that characterizes naïve T cells is a “by-default” program in the absence of activation signals. On the contrary, several studies suggest that naïve T cells metabolic state is a process actively maintained. For naïve T cell survival and homeostatic proliferation, cytokine signaling (IL-7) and weak TCR interactions with self-peptides presented by MHC molecules are required (Geltink et al., 2018). Indeed, IL-7 and TCR stimulation are important for the maintenance of naïve T cell state and are associated with GLUT1 transporter upregulation at the cell surface and increased glucose uptake. Furthermore, this process recruits the PI3K /Akt /mTOR pathway with constitutively active Akt promoting the trafficking of GLUT1 to cell surface (Barata et al., 2004; Jacobs et al., 2010; Rathmell et al., 2001; Swainson et al., 2007; Swainson et al., 2006; Tan et al., 2001; Verhoeyen et al., 2003; Wofford et al., 2008). Contradictory, full mTOR signaling can impact naïve T cells homeostasis since the loss of TSC1 (tumor suppressor tuberous sclerosis complex 1) function (which inhibits mTOR activation) results in loss of quiescence state, increased glycolytic metabolism and cell growth disrupting naïve T cell homeostasis (Yang et al., 2011). All together, these data suggest that naïve T cell metabolic profile requires mTOR activation but at low level comparing to activated T cells (Geltink et al., 2018).

2) Metabolic requirements and reprograming upon TCR activation

Upon TCR stimulation, naïve T cells get activated. In order to sustain their rapid proliferation, a “metabolic switch” from OXPHOS to aerobic glycolysis occurs, even if the oxygen supply is not limiting. This phenomenon is not specific to T cells and, as mentioned earlier, was initially described in cancer cells as Warburg effect (Warburg et al., 1958, 1960) **(previously discussed in the section II 1)**. As previously discussed, the Warburg effect can appear at first glance as counterproductive since the ATP production is lower compared to OXPHOS (2 ATP molecules instead of 36). However, this metabolic pathway is faster to engage – notably because of the fast upregulation of glycolytic enzymes expression as compared to the ones involved in OXPHOS (mitochondrial biogenesis is consequently relatively slow). Moreover, it allows the generation of several "building blocks" important, for instance, for the PPP or the FAS pathways (Donnelly and Finlay, 2015). It is important to keep in mind that upon activation, even if the glycolytic rate is increased (Chang et al., 2013; Michalek et al., 2011), the TCA cycle and OXPHOS remain essential to fulfill T cell energetic requirements (Carr

et al., 2010; Dimeloe et al., 2017; Sena et al., 2013; Wang et al., 2011). Altogether, it would be more accurate to describe this “metabolic switch” as a change in the ratio between glycolysis/OXPHOS: biosynthetic intermediates for new cellular components (which result mainly from glycolysis) are a priority when compared to ATP production (more efficiently produced by OXPHOS) (Gaber et al., 2019). How nutrients and the metabolic pathways are used upon T cell activation will be further discussed in the next parts of this section.

A. mTOR and AMPK signaling

As mentioned previously in the **section II 5**, mTOR is a central regulator of cell growth and metabolism (Pollizzi et al., 2015). Mechanistically, TCR engagement induces the activation of the PI3K-Akt axis which in turn, activates mTOR (Bantug et al., 2018). Furthermore, recruitment of the CD28 co-stimulation signaling, is also crucial to reach an optimal mTORC1 activation (Zheng et al., 2007). mTOR activation will then induces the up-regulation of two important transcription factor: inducible impact factor 1α (HIF-1α) and c-Myc. HIF-1α alters the range of glycolytic-associated genes resulting in increased glycolysis rate and enhances IFNγ secretion (Roman et al., 2010; Semenza et al., 1994). c-Myc is tightly involved in the increase of the glycolysis and glutaminolysis rates, and as consequence of the PPP pathway and TCA cycle, respectively (Wang et al., 2011). mTOR activity is also crucial for the activation of SREBP and PPAR (peroxisome proliferator-activated receptors) which are two crucial transcription factors involved in lipid production (Angela et al., 2016; Kidani et al., 2013). PPARs are transcription factors that are part of the nuclear hormone receptors family. They are activated by lipophilic molecules and mostly control genes involved in lipid metabolism (Sonoda et al., 2008). The role of mTOR in T cell activation and metabolic reprogramming is represented on **Figure 20.**

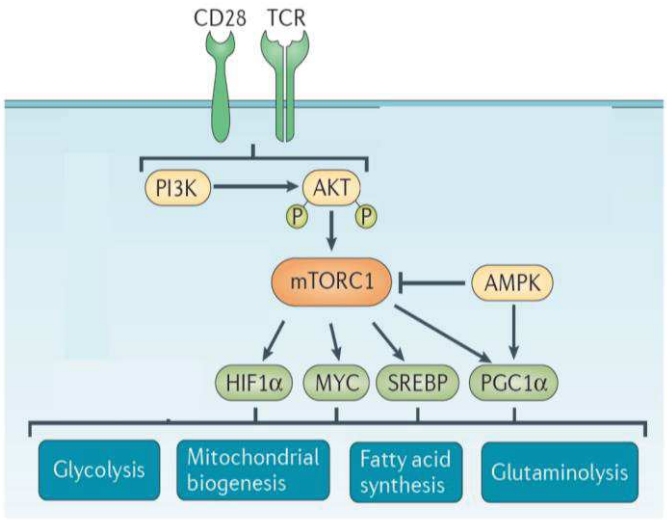

Figure 20 – Role of mTOR activation in metabolic reprogramming.

Upon TCR/CD28 activation, mTORC1 is activated and several transcription factors recruited. These transcription factors allow the engagement of several metabolic pathways necessary for the reprograming required to sustain T cell activation. Akt (protein kinase B), AMPK (AMP-activated protein kinase), HIF1α (Hypoxia-inducible factor 1-alpha), mTOR (mechanistic target of rapamycin), PI3K (Phosphoinositide 3-kinases), PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha). Adapted from: (Bantug et al., 2018)

AMPK is also activated by TCR signaling and it is important for T cell activation (Tamás et al., 2006). AMPKα deficient T cell demonstrated defects in T cell activation, metabolism and inflammatory cytokine production. In the same line, LKB1-deficient T cells exhibited defects in cell proliferation, viability and altered glycolytic and lipid metabolism (MacIver et al., 2011). Activated AMPK induced FAO and inhibited fatty acid synthesis (Davies et al., 1990; Merrill et al., 1997) and its activation also downregulated mTORC1 activity under nutrient deprived conditions (Blagih et al., 2015). These AMPK functions seem to be count productive considering the metabolic requirements of activated T cells. Thought, AMPK activation by TCR is transient, decreasing over the time to low levels in proliferating T effector cells (Kishton et al., 2016; Tamás et al., 2006).

B. Glycolysis

Upon activation, increased glycolysis is crucial to sustain T cell activation and proliferation (Gerriets and Rathmell, 2012). Glucose can enter in the cells either through the SGLTs or through the facilitative glucose transporters, a family of transporters named GLUTs (Navale and Paranjape, 2016). SGLTs are glucose transporters which transport glucose together with

sodium ions (in the same direction) and they are mainly expressed in intestinal cells. The GLUTs family is composed of 13 isoforms in mice and 14 in humans (Uldry and Thorens, 2004; Zhao and Keating, 2007). All Gluts are highly glycosylated proteins harboring a N-linked glycosylation site and, based on their localization, they can be divided into three classes. GLUT1 is the main glucose transporter in T lymphocytes. However, it also important to mention that upon TCR activation, GLUT3, GLUT6 and GLUT8 were also detected at mRNA level even if it was at lower levels as compared to GLUT1 (Macintyre et al., 2014; Vaeth et al., 2017). T cell activation is associated with an increased glucose uptake and an upregulation of GLUT1 cell surface expression (Gerriets and Rathmell, 2012; Kinet et al., 2007; MacIver et al., 2013; Manel et al., 2003b). GLUT1 can be detected at the cell surface as early as 4 hours following TCR stimulation (Manel et al., 2003b) and it is expressed in both $CD4^+$ and $CD8^+$ T cells (Kinet et al., 2007). Despite the fact that the precise mechanism by which T cells control GLUT1 expression and trafficking has not been yet fully characterized, it is known that PI3K signaling through Akt is essential for this process (Wieman et al., 2007) and CD28 signaling is crucial for Akt regulation of GLUT1 trafficking (Frauwirth et al., 2002; Jacobs et al., 2008). Also, another recent study demonstrated that store-operated Ca^{2+} was involved in the regulation of glucose transporters, glycolytic enzymes through the activation of NFAT (nuclear factor of activated T cells) (Vaeth et al., 2017). Interestingly, over-expression of GLUT1 did not impact T cell development or homeostasis but was associated with increased cell size, glucose uptake, proliferation rate and IFNγ and IL-2 secretion and lead to inflammatory diseases (Cretenet et al., 2016; Jacobs et al., 2008; Michalek et al., 2011). Additionally, under glucose-deprived conditions, proliferating T cells undergo apoptosis (Jacobs et al., 2008; Rathmell et al., 2000).

C. Glutamine metabolism

Glutamine is also crucial for T cells as low extracellular concentrations decrease human leukocyte proliferation (Parry-Billings et al., 1990) whereas a glutamine-rich diet is associated with an increased lymphocyte proliferation and increased cytokine secretion (Kew et al., 1999). As observed for glucose, activation of the TCR signaling resulted in increased glutamine import, notably through upregulation of the cell surface expression of neutral amino acid transporters (Sena et al., 2013). Several transporters can uptake glutamine including the

sodium-dependent neutral amino acid transporter 1/2 (SNAT1/SNAT2) and the system ASC amino acid transporter-2 (ASCT2 or SLC (solute carrier family) 1A5) (Carr et al., 2010; Nakaya et al., 2014; Ren et al., 2017). Glutamine is a rate limiting step for mTOR activation and its entry in Hela cells through the transporter ASCT2 regulates mTOR activation (Nicklin et al., 2009). Mechanistically, glutamine entry through ASCT2 allows the functioning of the SLC7A5/SLC3A2 heterodimer, a bidirectional transporter which controls the import of extracellular leucine in exchange with intracellular glutamine export. This process activates mTORC1 and highlights the role of glutamine flux in mTOR activation. (Nicklin et al., 2009). In addition, another elegant study demonstrated that activated ASCT2$^{-/-}$ T cells displayed defective phosphorylation of mTOR targets S6K1 and 4EBP1, highlighting again the importance of ASCT2 in the activation/recruitment of mTOR signaling pathway (Nakaya et al., 2014).

D. Lipid metabolism

The induction of the lipid biosynthetic pathway is also engaged upon TCR activation program (Lochner et al., 2015). Thus cholesterol as well as FA synthesis has been shown to be highly upregulated upon T cell activation (Bietz et al., 2017). Indeed, Wang and colleagues demonstrated that 24-30 hours following T cell activation, an increase of metabolites from the FAS pathway was detected (Wang et al., 2011). The FAS pathway is induced by SREBP processing and LXR signaling inhibition (Bensinger et al., 2008). Mechanistically, TCR-stimulated *de novo* lipid synthesis, is dependent on mTORC1 which activates SREBP and mediates the process (Kidani et al., 2013; Yang et al., 2013). C-myc also contributes to lipid biosynthesis in T cells through its implication in glucose catabolism (from which result important intermediates to sustain lipid synthesis) (Wang et al., 2011).

Cholesterol can also modulate T cell activation due to its contribution on lipid rafts formation, which are structures where lipid-lipid and lipid-protein interactions occur and contribute to the optimal clustering of signaling proteins at the membrane to facilitate signal transduction (Hou et al., 2016). In T cells, it was demonstrated that cholesterol present in the lipid rafts was important for the formation of TCR dimers which determined the sensitivity of the TCR to external stimuli (Molnar et al., 2012). Lipids, and in particular cholesterol, can modulate TCR signaling through the promotion of different conformational state of the CD3

chain (Guo et al., 2017). Furthermore, cholesterol can directly bind to the TCR and modify it via allosteric regulation of TCR phosphorylation (Swamy et al., 2016). Moreover, membrane fluidity and spatial control of the receptors is also important to maintain the immunological synapse (Dustin, 2014). Finally, cholesterol efflux also plays an important role on T cells being LXR-dependent ABCG1 expression regulation **(section II 3)** important for T cell proliferation regulation (Bensinger et al., 2008).

E. Arginine metabolism

Arginine availability is essential for optimal immune responses and more specifically for T cell proliferation and function (Bronte and Zanovello, 2005). However, another study revealed that arginine was not equally important for both $CD4^+$ and $CD8^+$ T cell showing that $CD4^+$ T cell proliferation was not dependent on arginine, whereas $CD8^+$ T cell proliferation appeared to be arginine concentration-dependent (Ochoa et al., 2001). However, this study goes against other works mentioned in this section, where total T cell proliferation was impacted either by arginine deprivation either by reduced arginine uptake (Werner et al., 2016; Werner et al., 2017). In human cells, arginine is mostly transported by members of the SLC7 (Closs et al., 2006; Fotiadis et al., 2013) comprising three CATs known as SLC7A1-3 or CAT1-3 and three other subfamilies transporting not only cationic amino acids but also neutral amino acids which are y^+ L-type amino acid transporter 1 (y^+ LAT1 or SLC7A7), y^+ LAT2 or SLC7A6 and $b^{0,+}$ AT or SLC7A9 (Fotiadis et al., 2013). On one hand, CAT transporters can mediate arginine import (Closs et al., 2006; Fotiadis et al., 2013) in most eukaryotic cells fueling arginine-dependent metabolic pathways and protein synthesis, while, on the other hand, y^+ LAT proteins mediate arginine export in exchange for neutral amino acids import (Closs et al., 2006; Fotiadis et al., 2013). From all these arginine transporters, CAT-1 appears as the most expressed on T cells. Recently it was shown that human CAT-1 mRNA is the most upregulated upon T cell activation and also that CAT-1 is necessary for activation-induced arginine uptake and human T cell proliferation (Werner et al., 2016).

It was shown that, in low-arginine environments, CD3ζ expression is down-regulated, most likely because of a decreased synthesis of the chain (Taheri et al., 2001; Zea et al., 2004). Moreover, these cells also demonstrated lower IFNγ, IL-5, IL-10 but not IL-2 production (Zea et al., 2004). Furthermore, T cells stimulated in the absence of arginine failed to progress

through cell cycle and remained in G0-G1 phase (Rodriguez et al., 2007). Besides, in absence of arginine, T cells undergo autophagy to get arginine intra-cellularly to preserve cell viability (Garcia-Navas et al., 2012). An alternative strategy in arginine-deprived environments relies on citrulline catabolism through ASS and ASL into arginine (Husson et al., 2003). Macrophages can take up citrulline from the extracellular environment and therefore produce arginine (Baydoun et al., 1994), demonstrating the importance of citrulline-arginine pathway in this immune cell type. Similar mechanisms were recently demonstrated in human T cells, where it was showed that citrulline could be transported by LAT1 transporter as siRNA-mediated LAT1 knockdown in activated T cells resulted in reduced citrulline import (Werner et al., 2017). Furthermore, citrulline supplementation was able to restore $CD4^+$ and $CD8^+$ proliferation under arginine-deprived concentrations being this process correlated with ASS induction. However, in the complete absence of arginine, citrulline was not able to restore T cell proliferation to the levels observed in conditions where arginine was available (Werner et al., 2017). Another elegant study highlighted the importance of ASS as they demonstrated that ASS1 deficiency resulted in reduced spleen size, reduced T cell numbers and abnormal T cell differentiation and function (Tarasenko et al., 2015).

The global T cells metabolic feautures before and after TCR stimulation are described in **Figure 21.**

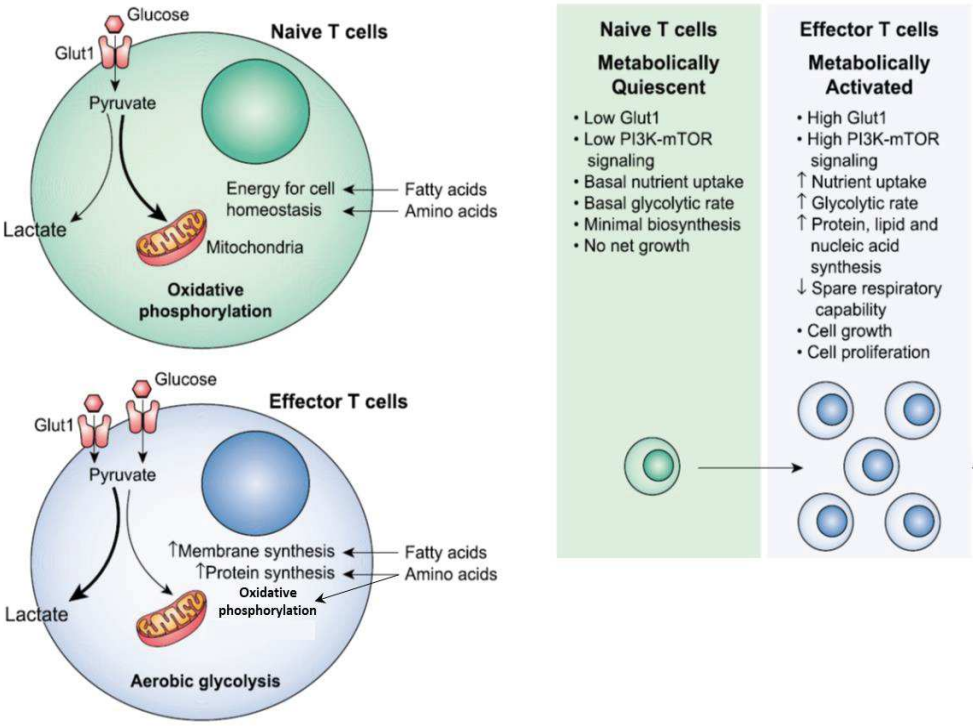

Figure 21- Metabolic reprogramming of T cell upon TCR activation

Naïve T cells use glucose, amino acids and fatty acids to produce energy through OXPHOS. Nutrient uptake occurs at low levels (comparing to T_{EFF} cells), enough to maintain naïve T cell homeostasis and cell quiescence. Upon TCR activation, nutrient uptake is increased followed by augmented protein, lipid and nucleotide synthesis to sustain T cell growth and proliferation. Glucose is used as a main energy source through aerobic glycolysis, a faster pathway to engage and from which results several metabolic intermediates important to sustain the increased biosynthetic demands. mTOR (mechanistic target of rapamycin), OXPHOS (oxidative phosphorylation), PI3K (Phosphoinositide 3-kinases). Adapted from: (Palmer et al., 2016b)

3) Metabolic requirement and control during T cell differentiation – Effector and regulatory functions.

A. Glycolysis

Glucose not only plays a crucial role in T cell activation but also in T cell differentiation and acquisition of effector functions. Besides the general importance of glucose in T cell biology several elegant studies demonstrated that glycolysis is not equally required for all T cell subsets and can differentially impact their fate. Indeed, and despite the fact that both human $CD4^+$ and $CD8^+$ T cells increased their glycolytic flux upon stimulation, $CD4^+$ T cells had increased HKII expression and were more dependent on aerobic glycolysis comparing to $CD8^+$ T cells (Jones et al., 2017). It has also been shown that upon TCR activation, murine $CD4^+$ and

$CD8^+$ T cells rely highly on glycolysis. However, in contrast to the previous study, the authors describe that $CD4^+$ murine T cells had a higher ratio OXPHOS/glycolysis than $CD8^+$ T cells. These discrepancies can probably be explained by the differences on the time points chosen to measure these parameters and by the fact that the studies were done in the distinct systems (human vs murine). Moreover, glucose requirements among the different $CD4^+$ T cell subsets appear to be different as well. Indeed, naïve $CD4^+$ T cell differentiation into Th1 and Th17 cells was highly dependent on glycolysis, though these two different subsets exhibited clear difference in the activation of HIF-1α for their glycolytic engagement (Dang et al., 2011; Michalek et al., 2011; Shehade et al., 2015; Shi et al., 2011). Differentiated Th2 cells were also highly dependent on glycolysis (Michalek et al., 2011), although the role of glucose in the polarization of this lineage is less understood (Bantug et al., 2018). Due to the importance of glycolysis in Th1, Th2 and Th17 cells, it was not surprising that GLUT1 deletion, as well as glucose deprivation, altered Th1, Th2 and Th17 differentiation (Macintyre et al., 2014; Michalek et al., 2011). In the context of my thesis, we also demonstrated that GLUT1 cell surface levels could be used to select human $CD4^+$ and $CD8^+$ T cells subsets with distinct effector functions. Cells that express high levels of GLUT1 were more proliferative, secreted more IL-2 and IFNγ and had more effector-like phenotype (Cretenet et al., 2016). Other elegant studies showed as well that increased glycolysis enhanced effector functions. For instance, in GLUT1 transgenic mice, it was observed a selective increase in T_{EFF} cells and in IFNγ, IL-4 and IL-17 secretion (Michalek et al., 2011). Moreover, other two studies demonstrated that reduced T effector function in tumor bearing mice could be overcome by increased T cell glycolytic activity (Chang et al., 2015; Ho et al., 2015). Controversially, in a study were T cells were segregated based on glucose uptake level (based on the uptake of a fluorescent 2-NBDG glucose analogue), high glucose uptake was associated with a terminal differentiated effector state and decreased *in vivo* effector function (Sukumar et al., 2013a).

Glycolytic enzymes activity can also impact on T cell polarization. For instance, PDH activity seems to be important for Treg cell but not for Th17 cell differentiation (Gerriets et al., 2015). Furthermore, LDH-A (another enzyme from the glycolytic pathway) was demonstrated to be important in promoting an efficient Th1 polarization through a direct role on the epigenetic regulation of IFNγ expression (Peng et al., 2016). Finally, Chang and colleagues showed that the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was

important to control effector cytokine production, notably IFNγ due the regulation of its locus (Chang et al., 2013).

B. Glutaminolysis

Despite its role in T cell activation, glutamine is also involved during T cell differentiation and acquisition of effector functions. ASTC2 knockout revealed an important role of glutamine and its transporter in the induction of signaling pathways required for T cell polarization and effector function. Indeed, our lab among others demonstrated that ASCT2 knockout as well as glutamine deprivation in vitro (on both murine and human cells) impaired the induction of both Th1 and Th17 cells (Klysz et al., 2015; Metzler et al., 2016; Nakaya et al., 2014) via a sub-optimal activation of mTORC1. However, glutamine deprivation or glutaminolysis inhibition did not alter Th2 and Treg differentiation on both murine and human T cells (Klysz et al., 2015; Metzler et al., 2016). Furthermore, work from our lab showed that, in glutamine-deprived conditions, naïve T cells failed to differentiate to a Th1 fate and resulted instead with a Treg phenotype, highlighting the role of glutamine availability in the differentiation of $CD4^+$ T cells into different Th subsets (Klysz et al., 2015).

C. TCA cycle metabolites

As already described in **section II 2**, TCA-cycle metabolic intermediates can derive from the catabolism of glucose, glutamine and fatty acids**.** These metabolites are important for T cell activation and acquisition of effector functions by activating different metabolic pathways. For instance, citrate and acetyl-coA are not only fulfilling the TCA cycle, but also have an interconnection with lipid synthesis and gene expression. TCA cycle-produced citrate can be transported into cytosol by the citrate carrier (CIC) and then used for lipid and cholesterol synthesis (Palmieri, 2004). In the same line, acetyl-coA is important for FA and cholesterol synthesis. How these metabolites are used in the fatty acid and cholesterol metabolism is explained in the **section II 3.** Importantly, citrate and acetyl-coA are also main players on epigenetic regulation **(Figure 22) (section III 3 C b)**.

a) Alpha-ketoglutarate

αKG is involved in many processes besides its role in fueling the TCA cycle. Thus, it was demonstrated that αKG contributes to lipid metabolism. For instance, it was demonstrated that αKG supplementation, was able to decrease through unknown mechanisms the serum content in total cholesterol, LDL and triglycerides, and increase HDL concentration (Radzki et al., 2009). Moreover, αKG was demonstrated to have also a role as regulator of gene expression and cell signaling pathways. For instance, αKG was identified as a ligand of a G protein-coupled receptor (GPR99 also known as 2-oxoglutarate receptor 1) which, once activated, is able to modulate cell metabolism and signaling resulting in increased cell proliferation (He et al., 2004). Another better-described role of αKG consists in its capacity to repress HIF-1α stabilization by fostering the activity of the metabolic sensor prolyl hydroxylases (PHDs) (Pan et al., 2007). Indeed, when amino acids are depleted, reduced αKG levels lead to PHDs inactivation and HIF-1α stabilization (Durán et al., 2012). Furthermore, HIF-1α has many functions in the regulation of T cell cellular metabolism and function. An elegant study revealed that HIF-1α was important to sustain glycolysis during T cell response, but not required during early T cell activation (Wang et al., 2011). The role of HIF-1α in T cell differentiation and notably on the Th17/iTreg balance is however not fully characterized (Dang et al., 2011; Shi et al., 2011). While Shi and colleagues demonstrated that HIF-1α deficiency inhibited Th17 differentiation and promoted iTreg differentiation due a reduction of the glycolytic metabolism (Shi et al., 2011), Dang and colleagues revealed that Th17 differentiation was altered due to the inhibition of RORγT expression and, on the contrary, iTreg differentiation was promoted because of the absence of HIF-1α-dependent inhibition of FoxP3 expression (Dang et al., 2011). Contradictory, another study demonstrated that HIF-1α was required for FoxP3 expression and optimal Treg function (Clambey et al., 2012). However, this study was performed in a hypoxia context and in iTreg polarizing conditions while the previous ones were done in normoxia conditions (Shi et al., 2011) and in a Th17 polarizing context (Dang et al., 2011; Shi et al., 2011), which can potentially explain the differences observed. In CD8 T cells, it was shown that HIF-1α increased the expression of effector molecules (such as IFNγ and TNF) and the cytolytic activity (Doedens et al., 2013; Finlay et al., 2012). αKG also promotes mTOR signaling through PHDs activation**,** blocks autophagy and induces cell growth and proliferation (Duran et al., 2012; Durán et al., 2012). αKG has also an

important role as a nitrogen-acting scavenger to protect cells from ammonia exposure (Nilsang et al., 2008).

αKG plays also an important role in gene regulation through its implication in epigenetic regulation. Indeed, αKG is a co-factor for 2 families of dioxygenases that mediate demethylation and hydroxylation reactions of DNA and histones: the TET proteins family of 5-methylcytosine (5mc) hydroxylases and the Jumonji C (JmjC) domain containing histone demethylases (HDM)(JHDM) (Kaelin and McKnight, 2013; Loenarz and Schofield, 2011). These dioxygenases not only required αKG as a co-factor, but also oxygen and iron (Kaelin and McKnight, 2013) **(Figure 22)**. TET proteins are able to oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5- formylcytosine (5fC) and 5- hydroxymethylcytosine (5caC) (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009) leading to demethylation.

Several studies have recently demonstrated that TET proteins are important for T cell differentiation. Indeed, mice deficient in TET2 were unable to produce Th1 and Th17 cytokines, although Th2 differentiation was not impacted. Therefore, different Th subsets exhibit a differential dependence on TET2-mediated 5hmc and H3K4me3 epigenetic modifications (Ichiyama et al., 2015). Furthermore, several studies have shown a distinct epigenetic regulation of Treg differentiation as compared to other Th subsets (Morikawa et al., 2014). FoxP3 expression has been demonstrated to be regulated by epigenetic mechanisms, particularly through the methylation of an element located in the 5'untranslated region of the FoxP3 locus known as TSDR (Treg-specific demethylated region) (Polansky et al., 2008). TET1, 2 and 3 have also been shown to be important for FoxP3 stability in Treg cells through epigenetic regulation mechanism (Someya et al., 2017; Yang et al., 2015; Yue et al., 2016). Mechanistically, Yang and colleagues demonstrated that IL-2 and TGF-β signaling, through SMAD3 and STAT5a phosphorylation, activate TET1 and 2 facilitating the demethylation of CNS1 and CNS2 regions and consequently allowing FoxP3 stability (Yang et al., 2015). In the absence of IL-2, TSDR remained methylated and FoxP3 expression was transient and unstable (Nair et al., 2016). Furthermore, it was recently discovered that αKG was able to regulate certain CTCF (CCCTC-binding factor is a DNA-binding zinc-finger transcription factor) binding sites inducing CTCF association and impacting the Th1 program in a TET-dependent manner (Chisolm et al., 2017).

Alteration of αKG availability and dysregulation of associated enzymes can interfere with T cell differentiation and it is often associated with oncogenicity. Mutations in a single allele of the isocitrate dehydrogenase (IDH) 1 and 2 can create a neomorphic enzyme with increased affinity to αKG. Mutant IDH1/2 enzymes result in diminished αKG/ isocitrate inter-conversion and the generation of the 2-hydroxyglutarate (2HG) oncometabolite: D-(R)-2-hydroxyglutarate ((R)-2HG or D-2HG) (Dang et al., 2009; Rendina et al., 2013; Ward et al., 2012). As such, elevated levels of D-2HG have been detected in patients with myeloid leukemias (AML), gliomas and angio-immunoblastic T-cell lymphoma (AITL) (Dang et al., 2009; Lemonnier et al., 2016; Ward et al., 2010). αKG can also be converted into a different 2HG enantiomer known as L-(S)-2-hydroxyglutarate (S-2HG or L-2HG). Interestingly, this enantiomer does not result from a mutation in IDH enzymes and can be generated either from LDH-A and malate dehydrogenase (MDH)1 activity in cytosol , either from MDH2 activity in the mitochondria under conditions such as hypoxia, mitochondrial impairment or specific pH conditions (Intlekofer et al., 2015; Intlekofer et al., 2017; Oldham et al., 2015).

While D-2HG has been commonly detected in different cancers, both D-2HG and L-2HG enantiomers are competitive inhibitors of αKG due their capability to bind to αKG-dependent dioxygenases. As a result, increased levels of 2HG can inhibit the TET2 and JmjC mediated-demethylation (Chowdhury et al., 2011; Lu et al., 2012; Xu et al., 2011). Moreover, mutations in TET2 have also been reported in myeloid cancer patients, strongly suggesting that epigenetic deregulation and histone modifications can promote an oncogenic phenotype (Delhommeau et al., 2009; Langemeijer et al., 2009).

b) Other TCA cycle metabolites

As discussed on the **section III 3 C a**, αKG is a TCA intermediate with a preponderant role in epigenetic regulation. However, other TCA cycle metabolites are also crucial. Succinate and fumarate are two metabolites that can directly compete with αKG, inhibiting αKG-dependent dioxygenases and PHDs activation (Vatrinet et al., 2017) **(Figure 22)**. Indeed, in succinate dehydrogenase- (SDH) and fumarate hydratase (FH) (two enzymes classified as tumor suppressors) deficient tumors, an increased stability of HIF1-α was observed even in normoxic conditions (Pollard et al., 2005). Furthermore, it was also shown that ectopic expression of tumor-derived SDH and FH mutants inhibited histone demethylation and hydroxylation of

5mC (Xiao et al., 2012). However, the precise impact of SDH and FDH mutations in T cell differentiation and function remain unclear.

Epigenetic control not only requires methylation/demethylation events, but also involves acetylation consisting in post-transcriptional modifications mediated by histone acetyl-transferases. These enzymes use Acetyl-CoA as substrate which can be originated from the citrate, another TCA cycle metabolite (Gaber et al., 2019). Actually, acetyl-CoA-mediated histone acetylation **(Figure 22)** was already demonstrated to be important to regulate the transcription of *Ifng* , thus promoting Th1 cell differentiation (Peng et al., 2016).

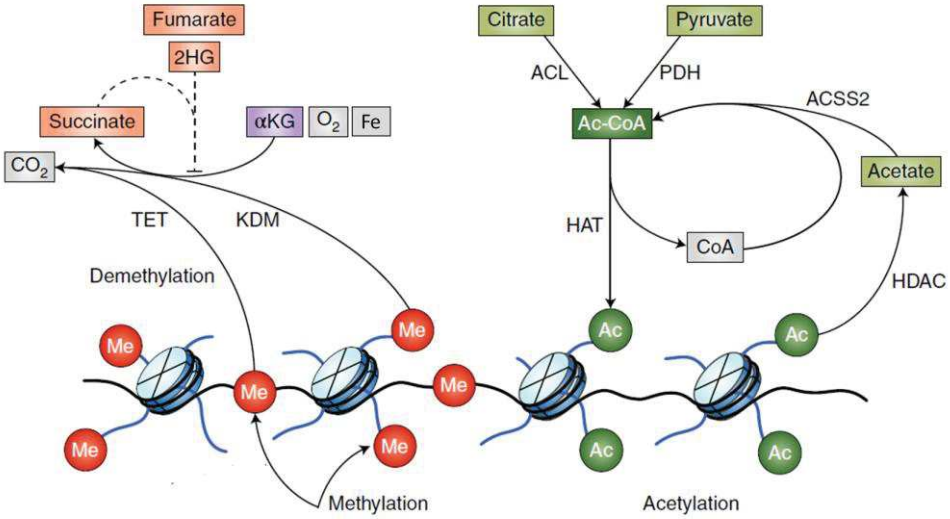

Figure 22 – Epigenetic regulation mediated by TCA cycle metabolites

Demethylation can be mediated by TET enzymes and KDM which require αKG, O_2 and Fe as co-factors. High levels of succinate, fumarate and 2HG inhibit TET and KDM demethylation activity. Acetylation is mediated by HAT which requires the utilization of the acetyl group from the acetyl-CoA. Acetyl-CoA can derive from nuclear/cytosolic citrate and pyruvate, which are the substrates for ACL and PDH, respectively. HDAC can generate acetate as a product that can be used by ACSS2 to regenerate acetyl-CoA. 2HG (2-hydroxyglutarate), αKG (Alpha-Ketoglutarate), ACL (acetyl-CoA-generating enzymes), ACSS2 (Acyl-CoA Synthetase Short Chain Family Member 2), HAT (Histone acetyltransferases), HDAC (histone deacetylase), KDM (Lysine demethylases), PDH (Pyruvate dehydrogenase), TET (ten-eleven translocation hydroxylases). Adapted from: (Intlekofer and Finley, 2019)

D. Lipid metabolism

Depending on the T cell subset, FAS and FAO are more or less engaged. The role of fatty acids in $CD8^+$ T cells is mainly associated with the generation of murine memory cells (Pearce et al., 2009) since $CD8^+$ effector cells rely mainly on glycolysis (**discussed in section III 4**). Within murine $CD4^+$ T cell compartment, Treg cells as compared to Th cells, were less

glycolytic and rely mainly on FAO (Michalek et al., 2011). However, another study done in human T cells showed that freshly isolated Treg cells were highly glycolytic comparing to Tconv cells and that FAO was upregulated only upon activation and during proliferation (Procaccini et al., 2016). Furthermore, Cluxton and colleagues demonstrated that human Treg cells not only exhibited increased FAO but also increased glycolysis upon TCR stimulation (Cluxton et al., 2019).The recruitment of FAO metabolic program in Treg cells could explain the capacity of these cells to adapt to environments rich in FAs as it was demonstrated FoxP3 expression is sufficient to upregulate FAO and OXPHOS related proteins and therefore favors the survival of Treg cells (Howie et al., 2017a). Moreover, it was demonstrated that FoxP3 induces OXPHOS and increases the NAD/NADH ratio to overcome lactate-mediated inhibition of proliferation (Angelin et al., 2017). These mechanisms could justify the capacity of Treg cells to adapt to environments rich in FAs, lactate and poor in glucose. Another study demonstrated that FAO is required for Treg differentiation by using etomoxir (FAO inhibitor through CPT1) (Michalek et al., 2011). However, two studies demonstrated recently that, when used at high concentrations (as in the study by Michalek and colleagues), etomoxir induces CPT1a-independent off-targets resulting in severe oxidative stress and decreased OXPHOS instead of long-chain fatty acid oxidation inhibition per se (O'Connor et al., 2018; Raud et al., 2018). Furthermore, using $CPT1^{-/-}$ mice or etomoxir at non-toxic concentrations, it was shown that FAO inhibition did not impact Treg differentiation (Raud et al., 2018). Finally, another study showed that pharmalogical/genetic inhibition of FAS did not impact Treg development since Treg cells used mainly exogenous FAs from the surrounding environment (Berod et al., 2014). Though, other study indicated that intratumoral Treg cells displayed an intracellular lipid accumulation due and increased rate on FAS that complement glycolysis to sustain Treg cell expansion (Pacella et al., 2018). Therefore, the overall picture of FAS/FAO role in Treg differentiation/function remains a controversy subject of ongoing investigation.

Th17 cells were described as a T cell subset that favors a lipogenic mode of metabolism for their development where ACC1 is highly required (Berod et al., 2014). In this study, they showed that Th17 cells depend on fatty acid synthesis to produce cellular membrane phospholipids which were important for their metabolic program (Berod et al., 2014). Another very recent study done in human T cells went in the same direction highlighting the

importance of FAS for Th17 cell function (Cluxton et al., 2019). Furthermore, an elegant study also established that the balance between saturated and polyunsaturated FAs, which is controlled by CD5L expression, can determine the pathogenic vs non-pathogenic profile of Th17 cells (Wang et al., 2015). Down-regulation of CD5L promoted pathogenic Th17 cells by changing the fatty acid composition and increasing cholesterol synthesis (Wang et al., 2015) which was previously demonstrated to be important for Th17 function since is a precursor of RORγT ligands (Santori et al., 2015; Soroosh et al., 2014).

The role of cholesterol in T cell activation is well described, however, its role on T cell differentiation and its importance among the different T cell subsets have been so far less addressed. It is known that levels of cholesterol in the microenvironment can impact T cell differentiation. For instance, it was demonstrated by different studies that hypercholesterolemia promoted inflammation and enhanced TCR stimulation, T cell proliferation and Treg cell development (Maganto-Garcia and Lichtman, 2008; Mailer et al., 2017a; Mailer et al., 2017b).

T cell differentiation and effector function can also be impacted by the cholesterol biosynthesis pathway (discussed on **section II 3**). Several studies pointed out that statins-mediated inhibition of cholesterol synthesis was associated with enhanced Treg differentiation (Forero-Pena and Gutierrez, 2013). However, another study showed that the inhibition of the cholesterol pathway blocked Treg suppressive capacity (Zeng et al., 2013). Therefore, the role of cholesterol biosynthesis on Treg differentiation is not fully understood. Nevertheless, it was shown that cholesterol esterification in CD8 T cells through the inhibition of the enzyme ACAT1 potentiated antiviral functions (Yang et al., 2016b).

Finally, LXR also have a role in T cell differentiation. Indeed, in EAE model, the treatment with a LXR decreased Th17 differentiation resulting in decrease disease severity (Xu et al., 2009). Furthermore, it was shown that the use of LXR agonists enhances Treg differentiation (Herold et al., 2017).

More indirectly, ABCA1/G1 deficient-dendritic cells resulted in enhanced T cell activation, increased T cell numbers on lymph nodes and enhanced Th1 and Th17 polarizations due to inflammasome activation, GMCSF and inflammatory cytokines secretion, highlighting the

importance of cholesterol efflux transporters in maintaining immune tolerance. (Westerterp et al., 2017).

The metabolic signatures of Teff and Treg cells is summarized on **Figure 23**.

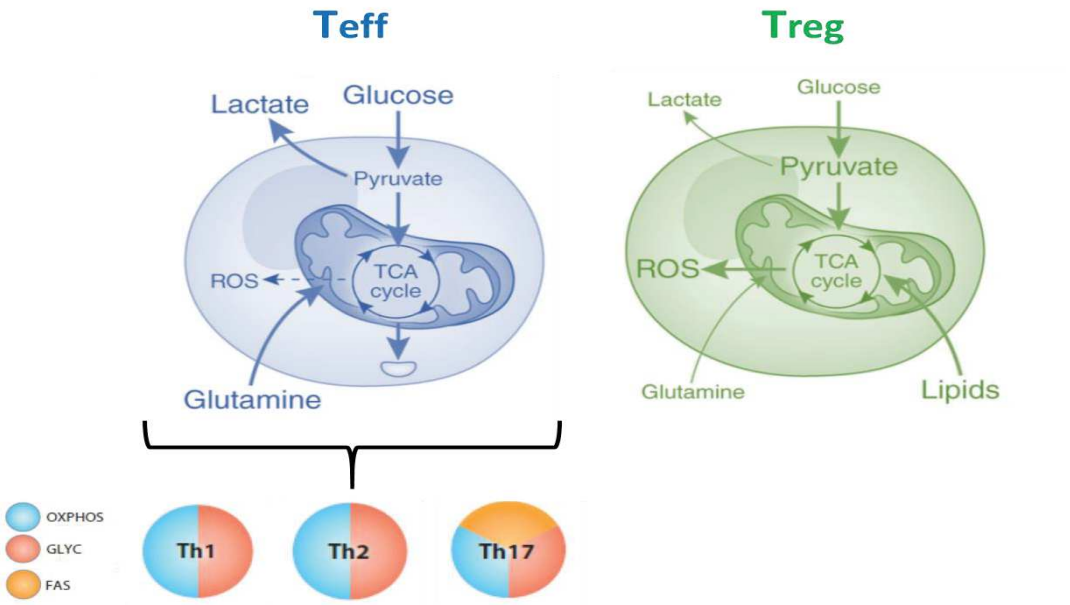

Figure 23- Metabolic signatures of T_{EFF} (Th1/Th2/Th17) and Treg cells

Th1, Th2, Th17 cells depend mainly on carbon substrates such as glucose and glutamine in order to fulfill their biosynthetic demands and to produce energy through aerobic glycolysis and OXPHOS. Additionally, Th17 cells rely also on *de novo* fatty acid synthesis for their differentiation. Despite the fact that Treg cells engage glycolysis, this subset depends on FAO for suppressive function. FAS (fatty acid synthesis), GLYC (glycolysis), OXPHOS (oxidative phosphorylation), Th (T helper), T_{EFF} (T effector), Treg (T regulatory), Adapted from: (Geltink et al., 2018; Newton et al., 2016)

E. Arginine metabolism

A recent study demonstrated extracellular concentrations of arginine could impact on the T cell phenotype and effector function. Geiger and colleagues showed, in mice, that high extracellular arginine levels modulated the regulation of several metabolic programs, downregulating glycolysis and upregulating OXPHOS (Geiger et al., 2016). Furthermore, high extracellular arginine levels limited T cell differentiation and maintain cells in a central-memory-like state with great anti-tumor properties (Geiger et al., 2016). Finally, another recent study performed in infantile rats reveals that arginine supplementation enhances Th1 response following activation by concanavalin A (Con A) or lipopolysaccharide (LPS) (Lee et

al., 2018). Moreover, both arginine and citrulline based-therapies in infantile rats could modulate Treg immune effects by increasing IL-10 levels (Lee et al., 2018).

F. mTOR and AMPK signaling pathway

Despite its role in metabolic regulation and T cell activation, mTOR has also a crucial implication in T cell differentiation. The conditional deletion of mTOR altered Th1, Th2 and Th17 differentiation while promoted Treg differentiation (Delgoffe et al., 2009). Furthermore, as mTOR can be part of 2 different complexes (mTORC1 and mTORC2), Delgoffe and colleagues further demonstrated that mTORC1 was required for Th1 and Th17 differentiations, while Rictor and therefore mTORC2 was required specifically for the Th2 development (Delgoffe et al., 2011). Controversially, further studies demonstrated that deletion of Raptor (as discussed in **section II**, a mTORC1-associated protein) was important for Th17 differentiation but not for Th1 development (Kurebayashi et al., 2012). Moreover, Raptor deletion showed as well an important mTORC1 role in Th2 development (Yang et al., 2013).

Interestingly, and despite the studies from Delgoffe and colleagues showing that the absence of mTOR resulted in T cells with a Treg phenotype (Delgoffe et al., 2011), the role of mTOR in Treg cells is still debated. Indeed, few other studies demonstrated that deletion of mTOR signaling pathway components induced Treg generation (Battaglia et al., 2012; Chang et al., 2012; Haxhinasto et al., 2008). However, other groups showed that mTOR activity was important to support Treg proliferation and it was increased in human Treg cells (Battaglia et al., 2006; Procaccini et al., 2010; Valmori et al., 2006). Another elegant study demonstrated that mTORC1 was a pivotal determinant of Treg suppressive activity while it did not directly affect Foxp3 expression (Zeng et al., 2013). Finally, another recent study done in mice revealed that mTOR inhibition promoted generation of long-lived central Tregs with a memory-like phenotype while mTOR function was required for the generation of effector Treg cells (Sun et al., 2018). Thus, a balanced mTOR activity is required to maintain Treg cells stability and function (Zeng and Chi, 2017). The roles of mTOR complexes during T cells differentiation are depicted in **Figure 24**.

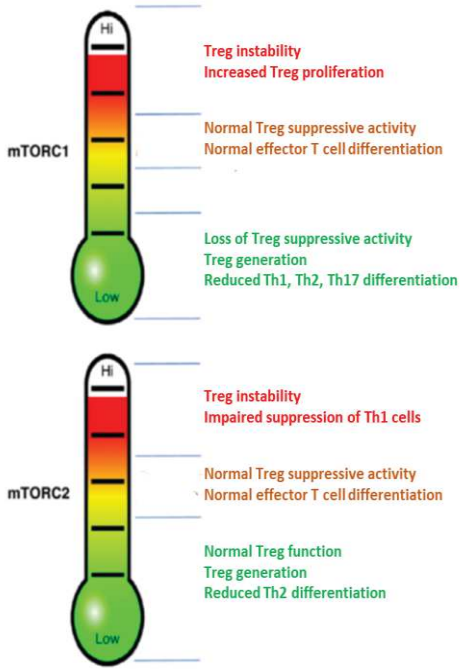

Figure 24- mTOR signaling function in T cell differentiation

mTORC1 and mTORC2 play a role in T cell differentiation. In this figure it is detailed the role of mTOR complexes in Th/Treg cell differentiation. mTOR (mechanistic target of rapamycin), mTORC (mTOR complex). Adapted from: (Zeng and Chi, 2017)

AMPK, as mentioned in the **section II 5** is another energy sensor involved in the regulation of cell metabolism. Activation of AMPK inhibits mTOR signaling, favoring catabolism. Therefore, it was not surprising that AMPK activation could impact Th/Treg cell differentiation. Indeed, studies performed in $CD4^+$ T cells showed that AMPK activation by metformin prevented the efficient polarization of Th1 and Th17 cells (Kang et al., 2013; Zhao et al., 2015), suggesting a role of AMPK as negative regulator in Th1 and Th17 differentiation. However, the role of AMPK on Treg cells appears to be different. TFG-β signals that were important for iTreg differentiation (Marie et al., 2005) are also required for AMPK activation (Xie et al., 2006). Indeed, AMPK activation was required to promote FAO which promotes Treg differentiation (Gualdoni et al., 2016; Michalek et al., 2011). Furthermore, under Th17 polarizing conditions, AMPK activation dampened Th17 induction and induced FoxP3 expression (Gualdoni et al., 2016). Moreover, Treg-specific LKB1 deletion (one of the activators of AMPK, discussed on **section II 5**) impacted Treg function and survival due to defective mitochondria, compromised OXPHOS, altered cellular metabolism and decreased ATP levels (He et al., 2017).

4) Memory T cells

At steady state, memory T cells share some common features with naïve T cells, illustrated by 95% similarity in gene expression (Weng et al., 2012). Thus, it is not surprising that memory T cells present a metabolism similar to that of naïve T cells relying mainly on OXPHOS and FAO (Almeida et al., 2016; Verbist et al., 2012). However, upon TCR activation, memory T metabolic response is faster and more intense as compared to naïve T cells (Gubser et al., 2013; van der Windt et al., 2013). Furthermore, because of increased mitochondrial biogenesis, $CD8^+$ memory T cell also have a greater mitochondrial mass than naïve T cells displaying an increased spare respiratory capacity (SRC) (Dimeloe et al., 2016; van der Windt et al., 2012; van der Windt et al., 2013). In line with this result, another study revealed that $CD8^+$ T cells with low glucose uptake (based on the uptake of a fluorescent 2-NBDG glucose analogue) correlated with memory T cell phenotype (Sukumar et al., 2013), highlighting the low glycolytic profile of memory T cells.

Several factors are involved in the regulation of memory T cell metabolism, among which is IL-15. In $CD8^+$ T cells, IL-15-mediated signaling increases mitochondrial biogenesis and expression of CPT1 resulting in an increased SRC and FAO, respectively (van der Windt et al., 2012). However, the concentrations of etomoxir used in this study were further demonstrated to have CPT1a- independent off-targets, as discussed in the **section III 3 D,** which makes complicated the interpretation of the results (O'Connor et al., 2018; Raud et al., 2018). Mitochondrial dynamics is also important for memory T cell generation as it was demonstrated, in mice, that the differentiation of memory cells required the expression of Opa1 (optic atrophy 1), a molecule involved in mitochondrial fusion and promoting efficient ETC activity (Cogliati et al., 2013). Indeed when mitochondrial fusion is enforced in effector cells, they acquire memory T cell characteristics (Buck et al., 2016).

Energy-sensing pathways were also shown to have a preponderant role in memory T cell differentiation. Contrary to its role in $CD8^+$ effector T cells, mTOR was shown to be a negative regulator in memory T cell generation. In a mice, mTOR inhibition through rapamycin treatment induced $CD8^+$ T cell differentiation to a memory phenotype (Araki et al., 2009). Furthermore, siRNA-mediated reduction of mTORC1 signaling as well as inhibition of mTORC2 in $CD8^+$ T cell was also resulting in memory T cell generation (Berezhnoy et al., 2014).

Moreover, it was demonstrated that AMPK promoted memory T cell formation (Rolf et al., 2013) and TRAF-6 (TNF receptor associated factor 6) was important for effective AMPK activation and consequent increase on FAO (Pearce et al., 2009). Also, the activation of AMPK by metformin in activated T cell resulted in increased FAO and $CD8^+$ memory T cell differentiation (Pearce et al., 2009). Further, AMPK activity on memory $CD8^+$ T cells (at the expense of mTOR activation) increased CPT1 activity and supported the mitochondrial uptake of fatty acids. These mechanisms resulted in a decreased dependence on glucose metabolism (Buzzai et al., 2005).

IV. The tumor metabolic environment and Chimeric antigen receptor (CAR) T cell immunotherapies

1) Immunity shapes the tumor – cancer immunoediting

The role of the immune system in controlling cancer was not always a subject of debate. In early 1900s, Paul Ehrlich had the theory that without the immune system, cancer would be probably a pathology with a higher incident rate (Ehrlich, 1909). However, since little was known about the immune system at that time it was difficult to prove this theory. For a long time, the contribution of the immune system to tumor surveillance was under debate and it was only in late 1950s that Burnet and Thomas proposed, for the first time, a major implication in cancer immunosurveillance and that immune cells play an important role in preventing cancer development in immunocompetent hosts (Burnet, 1957; Thomas, 1959). Since then, many scientific discoveries supported the role of immune cells in anti-tumor responses. However, in 2001, another discovery led to the revision of the cancer immunosurveillance hypothesis proposed by Burnet and Thomas (Shankaran et al., 2001). Indeed, when intracellular tumor suppressor mechanisms fail, upon cellular transformation, several immune mechanisms are involved in the recognition and elimination of these transformed cells. Despite the pressure of the immune system, an equilibrium phase establishes and tumor cells with reduced immunogenicity escape immunosurveillance and, as a result, pursue transformation by genetic and epigenetic changes. In the final phase, these alterations will allow escape from immune detection and elimination, resulting in the

expansion of the cell clones transformed at the equilibrium phase and generation of a tumor. All these different phases (elimination, equilibrium, escape) demonstrate that the immune system not only protects the host from tumor generation but also shapes tumor immunogenicity in a process called cancer immunoediting (Schreiber et al., 2011). This gap in the host's immune system can lead to alterations in tumor cells that can notably affect tumor recognition (downregulation of MHC molecules) or induce T cell apoptosis and condition the TME (tumor microenvironment) (Dunn et al., 2004). The TME is a really complex environment, composed of different actors in which tumor cells take part. The explanation of how the TME is composed and how it can impact T cell anti-tumor function is described in the next section.

2) Tumor microenvironment

Solid tumors are not only composed of clones of cancer cells but they also include several other cell types such as endothelial cells, stromal fibroblasts, adipocytes, extracellular matrix molecules and immune cells in a highly disorganized version of what we would find in normal organs (Egeblad et al., 2010). This structure favors the development of hypoxic environments that represents an adverse prognostic factor for patient outcome (Vaupel and Mayer, 2007). Furthermore, these structures vary between tumors, which add to the complexity of solid tumors.

In 2000, an iconic review came out describing the six hallmarks of cancer. In this review, the authors discussed about the remarkable diversity of neoplastic diseases due the capacity of tumor cells to sustain proliferative signaling, to evade growth suppressor genes, to activate invasion and metastasis, to enable replicative immortality, to induce angiogenesis and finally to resist cell death (Hanahan and Weinberg, 2000). Eleven years later, the same authors pursued their initial discussion to integrate new raised questions and to highlight new mechanistic concepts and notably the following two hallmarks: Dysregulation of cellular energetics and mechanisms to avoid immune destruction (Hanahan and Weinberg, 2011). Therefore, not only the capacity of tumor cells to evade immunological destruction by B and T cells was discussed but also the capacity of cancer cells to modify and reprogram cellular metabolism was pointed out as a crucial factor to support neoplastic proliferation (Hanahan and Weinberg, 2011). As a result, in the past years, the concept that tumors are more than an assembly of tumorigenic cells but instead a complex system where the TME should be

taken into account was consolidated (Hanahan and Weinberg, 2011). How the TME impacts on T cell anti-tumor functions, in particular through nutrient availability and hypoxia, will be discussed in the next two sections **(section IV 2 A and IV 2 B)**.

A. Nutrient competition

As explained in the **section III**, several metabolic pathways are crucial for T cells and their engagement plays then an important role to sustain T cell activation, differentiation and effector functions. Tumors are poorly vascularized and highly glycolytic resulting in the generation of a surrounding environment with reduced glucose and amino acid levels (Johnson et al., 2016) and high levels of waste products such as lactate (Romero-Garcia et al., 2016). Once tumor-infiltrating T cells (TILs) infiltrate the tumor, they face an adverse TME that not only can impact their activation and differentiation, but also their anti-tumor function.

a) Glucose deprivation

Tumor cells are highly glycolytic (Warburg, 1925) and several recent studies have demonstrated the importance of metabolic pathways coordinating glucose and lactate metabolisms in several cancer types (Doherty and Cleveland, 2013; Draoui and Feron, 2011; Hensley et al., 2016; Kennedy et al., 2013; Ruan and Kazlauskas, 2013). Cancer cells and endothelial cells in the TME are able to regulate lactate transport via the monocarboxylate transporter (MCT) 1 which is released by glycolytic cancer cells mainly through MCT4 (Sonveaux et al., 2008; Vegran et al., 2011). Inhibition of MCT1 can impact the metabolic preferences of cancer cells forcing them to use glucose, reducing the availability of lactate for tumor cells in hypoxic environments and thereby impacting their survival (Sonveaux et al., 2008). Moreover, the inhibition of MCT4 directly targets tumor cells in hypoxic environments and promotes cell death through the intracellular accumulation of lactate (Draoui and Feron, 2011). These works highlight the importance of lactate as a cell metabolic fuel. However, cancer cells are not the only ones requiring high amount of glucose in the TME. A study performed in prostate cancer showed that cancer associated fibroblasts (CAFs) present high GLUT1 expression, glucose uptake, MCT4 expression and lactate release (Fiaschi et al., 2012). In presence of CAFs, the surrounding cancer cells increase their lactate intake highlighting the importance of the tumor-stromal interplay (Fiaschi et al., 2012; Rattigan et al., 2012).

As discussed in the **section III 3** we, among others, demonstrated that glucose is important for IFNγ secretion and for pro-inflammatory functions (Cham et al., 2008; Cretenet et al., 2016; Macintyre et al., 2014). Thus, it is expected that the low glucose availability generated in the TME could negatively impact on T cell anti-tumor function. For instance, in a mouse sarcoma model, Chang and colleagues demonstrated that glucose restriction on the TME reduced the glycolytic capacity, mTOR activity and IFNγ secretion of tumor-infiltrating T cells, therefore favoring tumor progression (Chang et al., 2015). Another elegant study demonstrated that reduction of glycolysis resulted in a decrease in phosphoenolpyruvate (PEP), a glucose-derived metabolite, impacting calcium signaling and therefore anti-tumor responses (Ho et al., 2015). Also, using an human ovarian cancer chimeric model in a tumor-imposed glucose restriction context, microRNAs miR-101 and miR-26a were induced limiting the expression of the methyltransferase EZH2 which therefore is important for Notch pathway activation and consequently T cell cytokine production induction (Zhao et al., 2016). The lactate that results from cancer glycolytic activity also impairs T cell effector capacity (Romero-Garcia et al., 2016), with several studies demonstrating that lactate contributes to T cell suppressive functions (Calcinotto et al., 2012; Fischer et al., 2007). High lactate concentration exposure impairs NFAT activation as well as IFNγ secretion (Brand et al., 2016) and in addition, T cell mobility and cytolytic functions were impacted as well. However, even if low-glucose/high-lactate environments impact negatively T cell function, FoxP3-expressing Treg cells seem to have the metabolic capacity to overcome this negative TME environment (Angelin et al., 2017). Indeed, Treg cells have been associated with pro-tumor activity in several cancer types (Curiel et al., 2004; Jiang et al., 2014; Sayour et al., 2015; Tang et al., 2014; Tao et al., 2012; Yang et al., 2006) and bad prognosis.

b) Glutamine deprivation

Glutaminolysis is also a crucial metabolic pathway for some cancer cells types (Medina et al., 1992; Souba, 1993). Several studies revealed that cancer cells such as non-small cell lung cancer (NSCLC), breast, brain and liver tumor cells are highly dependent on glutamine for their growth and survival (Choi and Park, 2018). It was demonstrated that glutamine synthase (GS) is an important enzyme for fueling nucleotide biosynthesis and to support the growth of glutamine-restricted glioblastoma (Tardito et al., 2015). Furthermore, another study showed that both GS activity in CAFs and glutaminases in cancer cells are two important enzymes for

tumor growth and metastasis (Yang et al., 2016a). Thus, it is not surprising that in some cases, TILs will find a glutamine-depleted TME. As detailed in the section **section III 3 B**, glutamine is also essential during naïve T cells activation/differentiation to a Th1 fate. Furthermore, glutamine deprivation pushes naïve T cells to differentiate into Tregs which, as mentioned before, are important pro-tumor players (Klysz et al., 2015; Metzler et al., 2016).

c) Arginine deprivation and byproducts

In the TME, myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM) and DC are immune cells that also play a crucial role in the maintenance of an hostile environment for anti-tumor effector T cells (Marigo et al., 2008). It is well known that one of the metabolic features of both MDSC and TAMs is their capacity to rapidly degrade arginine (Lyssiotis and Kimmelman, 2017; Marigo et al., 2008) which is either catabolized by Arg1 or NOS2 and results in urea and ornithine production or in citrulline and NO production respectively (Bronte et al., 2003). As a consequence, arginine catabolization results in reduced arginine availability (which is crucial for T cells as discussed in the **section III 2 E)** and also in the generation of byproducts (such as NO) that can impact T cell function. In a murine lung carcinoma model, the production of Arg1 in the TME by myeloid cells was already shown to inhibit T cell receptor CD3ζ chain surface expression and antigen-specific T cell proliferation (Rodriguez et al., 2004). Another study in renal cell carcinoma patients demonstrated that these patients had low levels of arginine and high levels of ornithine in the plasma due myeloid cells activity, highlighting the role of Arg1 in tumor progression (Zea et al., 2005). Moreover, NO released from these heterogeneous groups of MDSC was able to inhibit T cell responses due to an impairment of the IL-2R signaling pathway (defects in the phosphorylation of janus kinase JAKs, STAT5, ERK and AKT) (Bingisser et al., 1998; Mazzoni et al., 2002).

d) Tryptophan degradation and IDO activity

Another important player in the TME are IDO (indoleamine 2,3 deoxygenase)-expressing cells that can be found in both murine and human tumor contexts (Munn and Mellor, 2007). IDO is an enzyme which catabolizes tryptophan and in response to TGF-β, acts as signal transducer important for plasmocytoid DCs tolerogenic phenotype (Chen, 2011). IDO

enzymatic activity is important for the creation of a suppressive TME which, through the reduction of tryptophan availability, suppresses T cell responses and enhances Treg-mediated suppression (Munn and Mellor, 2007). An elegant study demonstrated that most human tumors constitutively express IDO and the tumors that express IDO are resistant to immune rejection (Uyttenhove et al., 2003). IDO activity can also affect T cell priming: the presence of a small population of IDO-expressing plasmacytoid DCs in tumor-draining lymph nodes was described to create a suppressive microenvironment impacting anti-tumor T cell responses (Munn et al., 2004). Moreover, this same IDO-expressing DC population could directly activate mature Treg cell in tumor-draining lymph nodes (Sharma et al., 2007). Blocking IDO activity is considered as a potential strategy to enhance anti-tumor immunity (Munn, 2012).

As discussed above, the metabolic activity of myeloid cells can have a negative impact on anti-tumor functions. Nevertheless, it is essential to take in consideration that the TME can impact myeloid cell functions. Immune responses are characterized as transient going through “on” (cytotoxic activity and cytokine release) and “off” (immunosuppression) phases (Bercovici and Trautmann, 2012). Both T cells and myeloid cells go through these phases that are not necessarily synchronized between them (Bercovici and Trautmann, 2012). One possible model is that when T cells infiltrate the TME, myeloid cells (which are recruited first) are already in this long “off” phase conditioned by the surrounding environment. The cooperation between T –myeloid cells occurs at the tumor site (Bercovici et al., 2008; Thoreau et al., 2015) where the TME can interfere with it, pushing myeloid cells to have a pro-tumor role. Therefore, the role of the TME on myeloid and its cooperation with T cells should be also taken in consideration to improve anti-tumor responses.

e) Lipid metabolism

Another feature associated with cancer cells is the increased lipid biosynthesis (Zaidi et al., 2013). *De novo* FAS contributes to generate lipids and phospholipids required for the plasma membrane as well as lipid droplets in proliferating cancer cells (Zaidi et al., 2013). This high lipid synthesis is associated with a membrane composed mainly by high saturated/ mono-unsaturated fatty acids that allow cancer cells to be more resistant to oxidative stress (Rysman et al., 2010). Fatty acids can also represent a source of energy for tumors that are less glycolytic such as prostate tumors (Effert et al., 1996; Liu et al., 2010) and human

leukemia cells (Samudio et al., 2010). Increased level of FASN expression and activity are detected in cancer cells and correlate with cancer progression (Menendez and Lupu, 2007). However, cancer cells also uptake exogenous fatty acids to fulfill their energetic demands (Kuemmerle et al., 2011). Several studies demonstrated that targeting several lipid synthesis pathway–related enzymes could result in tumor regression (Chajes et al., 2006; Hanai et al., 2012; Kridel et al., 2004; Mashima et al., 2009; Menendez and Lupu, 2007). Lipid availability is important for T cell activation, differentiation and function, as summarized in the **sections III 2 D and III 3 D**, and this should be taken in consideration considering the high demand on lipids by tumor cells. Indeed, lipid and cholesterol intra-tumoral metabolism can impact anti-tumor function. It was shown that TILs have an abnormal lipid metabolism when compared to non- tumor infiltrated T cells that can be reversed by inhibiting programmed cell death-1 (PD-1) which was initially induced by TME (Yin et al., 2016). It was also demonstrated that lipid metabolism is also impacted in tumor-associated DCs from the tumor-bearing host, resulting in the accumulation of lipid bodies that impair proper MHCI trafficking and antigen presentation to $CD8^+$ T cells (Ramakrishnan et al., 2014; Veglia et al., 2017). As a result, targeting immune cells lipid metabolism could also represent a strategy in cancer treatment. Indeed, it was recently revealed that $CD8^+$ anti-tumor responses could be potentiated by modulating cholesterol metabolism (Yang et al., 2016b), highlighting the importance of lipids on anti-tumor function. A summary of the principal points concerning nutrient competition in the TME is described in **Figure 25.**

B. Hypoxia

Hypoxia is a well-known characteristic of solid tumors associated with a wide range of malignancies (Vaupel and Mayer, 2007) **(Figure 25)**. It is also known that hypoxia levels among different solid tumors is distinct (Brown and Wilson, 2004; Byrne et al., 2014) and that within a tumor tissue, hypoxia levels vary as well notably between the periphery and center of the tumor. In the periphery, tumor cells are better irrigated when compared to the central parts of the tumor tissue, which therefore modulates differently the TME and tumorigenesis (Campillo et al., 2019; Herman et al., 2011; Lewis et al., 2016; Quail and Joyce, 2013; Rofstad et al., 2014). It is commonly accepted that the levels of oxygen (O_2) within the tumor tissues are low (between 1% and 2%) when compared to the respective healthy organs (Muz et al., 2015). In human organs, oxygen concentration will vary between 9.5% in the renal cortex

(Muller et al., 1998) and 4.6% in the brain (Dings et al., 1998; Hoffman et al., 1996). In murine lymphoid organs, the oxygen tension range between 0.5% and 4.5% (Caldwell et al., 2001). Thus, when TILs arrive into the TME, they will most likely encounter an environment lower in O_2 availability comparing to their initial location. Hypoxia is an environmental factor impacting cell signaling through activation of the HIF pathway, of which HIF (a transcription factor) is important for cellular adaptation to low oxygen environments (Muz et al., 2015; Semenza and Wang, 1992). Hypoxia is linked to several signaling pathways such as PI3K/AKT/mTOR (Agani and Jiang, 2013; Courtnay et al., 2015), MAPK/ERK (Minet et al., 2000; Sanchez et al., 2012) and NFκB pathways (Koong et al., 1994) that, once activated, are able to induce the HIF pathway. HIFs mediate through gene regulation several physiological processes that are crucial for tumor development (Semenza, 2012). For instance, it is involved in the promotion of angiogenesis through induction of vascular endothelial growth factor (VEGF) expression (Ahluwalia and Tarnawski, 2012; Lin et al., 2004) and stromal cell-derived factor 1 (SDF-1) (Zagzag et al., 2005). Furthermore, the HIF pathway is important for cell apoptosis regulation and cell survival via the induction of BNIP-3 (Guo et al., 2001), the stabilization of p53 (An et al., 1998), and activation of TGF-β and bFGF (Muz et al., 2015). Hypoxia also inhibits the expression of genes related to cell-surface integrins, thus promoting tumor cell detachment and potential metastasis (Hasan et al., 1998). Moreover, HIF pathway is important for the regulation of GLUT1, GLUT3 and other glycolytic enzymes (Iliopoulos et al., 1996; Iyer et al., 1998; Semenza, 2013). Indeed, the induction of HIF-1α increased tumor cell utilization of glucose and lactate release (Eales et al., 2016). Using a glucose tracing in healthy lung tissue and in tumor tissue of non-small lung cell carcinoma patients, an elegant study demonstrated that, even within the same type of tumor, heterogeneity of glucose utilization was observed when compared to benign tumor (Hensley et al., 2016). Combining a glucose tracing approach and dynamic contrast-enhanced magnetic resonance imaging, Hensley and colleagues demonstrated that in less-perfused regions, glucose was the main source of energy. However, in more-perfused regions, lactate was used as an alternative fuel (Hensley et al., 2016) that, as described in the beginning of this section, can be transported into cancer cells by MCT1 and used as carbon source. This highlights how oxygen availability in the TME impacts metabolites utilization by tumor cells.

Hypoxia, indeed, impacts T cell metabolism, proliferation and effector function. During my PhD, we showed that, in hypoxic conditions, GLUT1 surface expression was higher as compared to normoxic conditions, and in contrast proliferation and cytokine secretion was diminished (Cretenet et al., 2016). Furthermore, and as explained in the **section III 2 A,** HIF-1α plays many roles in T cell metabolism and function, notably in the regulation of cellular metabolism (Kim et al., 2006) and T cell differentiation. However, the role of HIF-1α in Treg cells is still under debate (Clambey et al., 2012; Dang et al., 2011; Shi et al., 2011) and in CD8 T cells it was demonstrated that HIF-1α increased effector molecules expression and the cytolytic activity (Doedens et al., 2013; Finlay et al., 2012). Therefore, it was expected that the hypoxic environment in the TME could modulate HIF1-α activation and, as a result, potentiate T cell anti-tumor responses. This is actually not the case (Buck et al., 2017) and several reasons could explain this result. One of those is related to the induction of programmed cell death ligand 1 (PD-L1) on tumor cells, TAMs and MDSCs by HIF-1α (Noman et al., 2014). PD-L1 is the ligand of PD-1 expressed on T cell surface following activation which is maintained upon chronic activation mediated for instance by tumor cells. (Simon and Labarriere, 2017). When PD1 binds to its ligand, the TCR signaling and CD28-costimulation are impaired (Hui et al., 2017; Yokosuka et al., 2012) resulting in reduced proliferation, cytokine secretion and effector function. $PD1^+$ TILs upon binding to PD-L1 expressed by tumor cells or other immune cells have an impaired anti-tumor function, known as checkpoint inhibition (Simon and Labarriere, 2017). Another parameter to take in consideration is the difficulty to perform experiments in hypoxic conditions, making it hard to mimic the actual physiological conditions found in the TME, which could explain the discrepancies of the previous studies mentioned before.

All these findings highlight hypoxia as an important determinant in the TME microenvironment which should be taken in consideration for the design of new therapies. Further studies are necessary to understand the impact of hypoxia in T cell differentiation and effector function, notably on Treg cells.

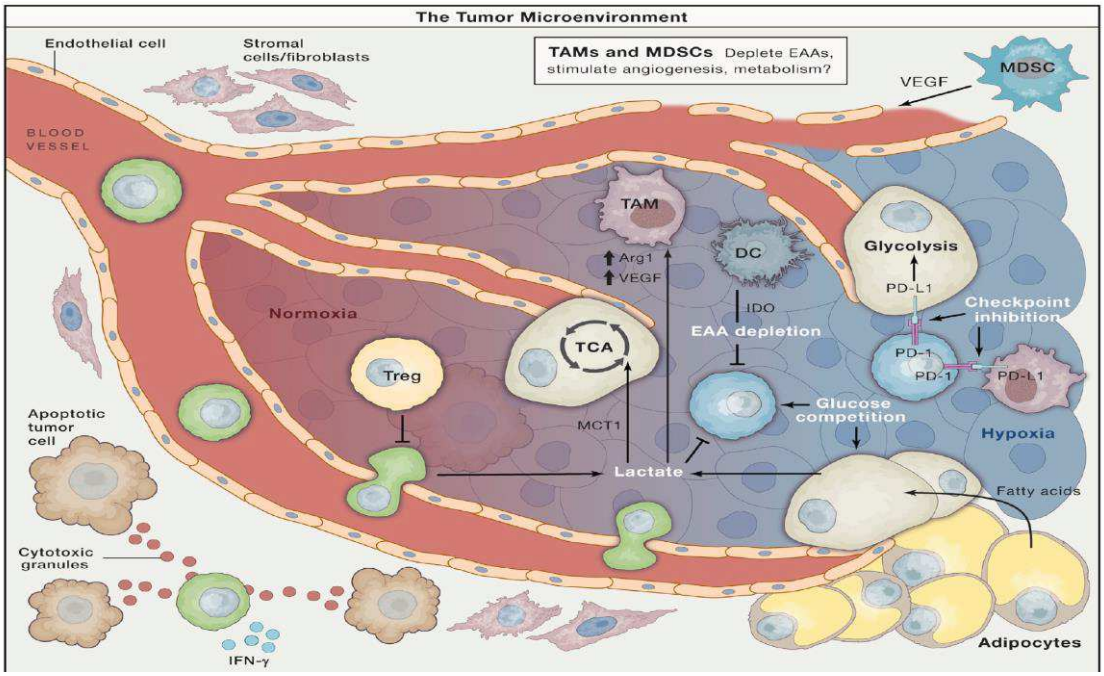

Figure 25 – The tumor microenvironment

Tumor cells consume high levels of glucose, amino acids and fatty acids resulting in a deprived environment in nutrients crucial for T cell anti-tumor function. As a consequence of high glycolytic tumor activity, tumor cells secrete high levels of lactate, which is a toxic for T cells. Despite that, tumor cells are able to use lactate as an energy source exporting it by the MCT1. In the TME, other immune cells are present, favored by the surrounding environment and able to impact negatively T cell anti-tumor function. TAM and MDSC metabolic activity deprive the TME from arginine (through the action of Agr1) while DCs are well known for their capacity to deplete the TME in tryptophan through the activity of IDO. Furthermore, Tregs present in the TME downregulate T cell effector function. Finally, PD-1 expression by T cells, as a result of cell exhaustion, results in the downregulation of T cell anti-tumor function through the binding with PD-L1 present on the tumor cells surface. Hypoxia levels in the tumor site are also a negative factor in the TME which impact T cell function.

Arg1 (arginase 1), DC (dendritic cell), EAA (essential amino acids), IDO (indoleamine 2,3-dioxygenase), MDSC (myeloid derived suppressor cells), MCT1 (monocarboxylate transporter 1), PD1 (programmed cell death-1), PD-L1 (PD ligand 1), TAM (tumor-associated macrophages), TME (tumor microenvironment), Treg (T regulatory T cells). From: (Buck et al., 2017)

3) Chimeric Antigen Receptor (CAR) T cell Immunotherapies

The tight relation between cancer cells and the immune system described in **section IV 1** opened the doors to the development of immunotherapies. The idea that immune cells could be directly targeted and manipulated *ex vivo* to overcome immune tolerance resulted in the development of several therapies. Since T cells are the main players in immunosurveillance, the first attempts at immunotherapy were centered on T cell manipulation (Kantoff et al., 2010; Rosenberg et al., 2011). Ever since, adoptive T-cell therapies (ACT) have been in continuous development.

ACT consists on the re-infusion of autologous tumor-antigen-specific T cells that were previously collected from the patient, expanded ex vivo and thus re-injected in order to generate an anti-tumor response. The current T-cell therapies are centered in two general approaches: the use of T cells isolated from tumor zones called TILs and redirected T cells (Cheadle et al., 2014). This latter category results from advances in genetic engineering leading to recombinant T cell receptors (TCR) or to chimeric antigen receptors (CAR). Both approaches result in the generation of T cells with increased specificity to specific tumor antigens (Miliotou and Papadopoulou, 2018). In this section, I will particularly focus on CAR T cells.

TILs are isolated from tumor biopsies and can be activated *ex vivo* (free from a potentially suppressive tumor environment) and demonstrate *in vitro* tumor killing (Rosenberg et al., 1986). These cells are then cultured in presence of IL-2 in order to get the largest number possible. After testing their reactivity, TILs are re-infused into the patient. Despite the interesting therapeutic results from TILs treatment, this approach presents some disadvantages. This method is laborious, patient-specific, time-consuming and highly dependent on the antigen levels expressed on the tumor. Furthermore, not all the TILs isolated are tumor-specific which obliges the use of enrichment strategies that increases time spent in culture which may alter their phenotype or induce anergy (Geukes Foppen et al., 2015). However, the studies and the clinical trials that have been performed with TILs not only highlighted the therapeutic potential of T cell-based immunotherapies but also have been extremely important and crucial to understand, evaluate and improve the conditioning regimens used prior to TILs injection (chemotherapy and radiotherapy) as well as the optimal

IL-2 doses to administrate to the patients (Andersen et al., 2016; Dudley et al., 2005; Dudley et al., 2008; Goff et al., 2016; Rosenberg et al., 2011; Tseng et al., 2014).

The approach using a modified TCR results from the ectopic expression of α and β chains to T cells to redirect their antigen specificity. These α and β chains are associated with constant regions by disulphide bonds and also with the γ, δ, ε and ζ chains of the CD3 complex (Uttenthal et al., 2012). The advantage of this gene-modified T cells is that the antigen recognition mechanisms are equal to non-modified TCRs. However, with this approach the responses against tumor antigens depend on MHC presentation, which is therefore not compatible with the treatment of patients expressing a different MHC haplotype (Cheadle et al., 2014). Furthermore, it also represents an important downside for the treatment of tumors with low or no MHC expression (Garrido and Algarra, 2001; Seliger et al., 2000). These problems promoted scientific investigations on approaches to provide antigen specificity to T cells and to overcome disadvantages related to modified TCRs.

CARs, or T-bodies, are generated by joining as a single chain variable fragment (scFv) molecule, the light and heavy chains of variable regions of a monoclonal antibody. The construct contains a hinge domain, transmembrane domain, and cytoplasmic signaling domains derived from the CD3ζ chain or Fc receptor γ chains (Sadelain, 2009). A specificity of the CAR approach is that MHC antigen presentation is not required, since the antigens can bind directly to the CARs and trigger T cell responses. This approach therefore overcomes the problems discussed earlier associated with the lack of MHC recognition in some tumor environments. However, contrary to TCRs, CARs cannot recognize antigens that are not localized on tumor cells surface which, therefore, need to be processed, presented by APCs and recognized through the MHC molecules. (Uttenthal et al., 2012).

The development of the CAR strategy led to the generation of several generations of CAR constructs. Until now, 4 different CAR constructions were developed and they can be distinguished based on their intracellular tail composition which results in distinct features: first generation, second generation, third generation and fourth generation; also called TRUCK (T cell redirected universal cytokine killing) (Cheadle et al., 2014; Wilkins et al., 2017). The first generation, initially designed by Zelig Eshhar, includes only the CD3ζ as intracellular domain (Eshhar et al., 1993; Gross et al., 1989). The second generation also expresses one co-stimulatory domain (provided from endodomains of co-stimulatory molecules) and therefore

the intracellular tail contains two signaling domains. The idea of adding a co-stimulatory molecule in this second generation CAR construct was to have a co-stimulatory signal which could promote IL-2 synthesis in order to complete T cell activation and to improve the CAR T cell proliferation, cytotoxicity and to prolong the lifespan of CAR T cells by avoiding apoptosis (Zhang et al., 2017). The intracellular domain of the third generation is composed of the CD3ζ and two co-stimulatory domains. These domains could be, for instance, CD28, CD137 (also named 4-1BB) or CD134 (also known as OX40) (Finney et al., 2004). The presence of two co-stimulatory domains was combined with the intention of increasing cytokine production, killing ability and to respond better to recurrent exposition to tumor antigen (Miliotou and Papadopoulou, 2018; Zhang et al., 2017). The fourth generation is distinct as the vector encoding the CAR construct also includes a cytokine cassette that is expressed upon engagement of the CAR. The expression of these cytokines leads to the activation of a CAR-responsive promoter that could be a nuclear factor of activated T cells that potentiate anti-tumor immune responses (Chmielewski et al., 2011; Pegram et al., 2012). The structure of the different CAR constructs is represented in **Figure 26**.

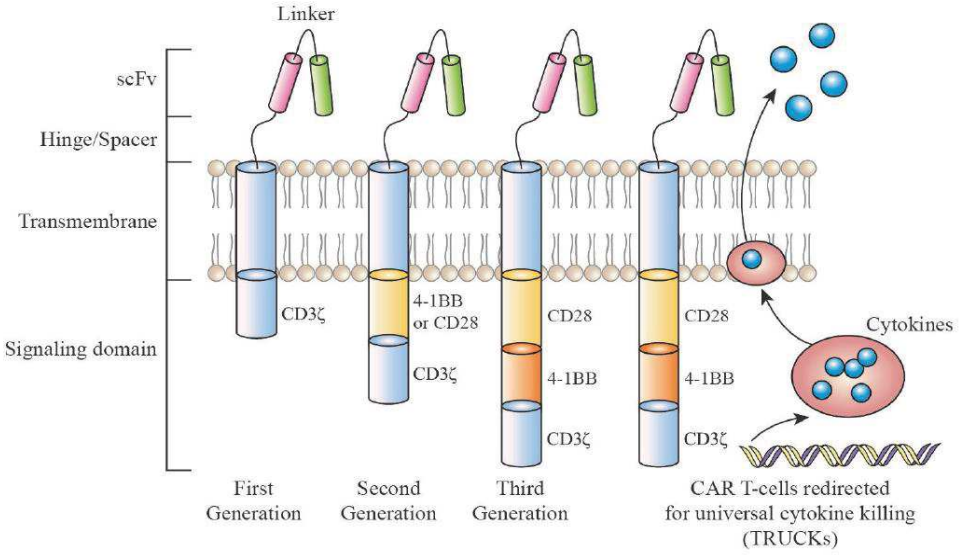

Figure 26 – The four generations of CAR T cell constructs.

All CAR generations contains a scFv domain derived from a monoclonal antibody. This extracellular part is specific to a desired tumor-antigen and does not require MHC presentation. The different CAR T cell generations are characterized by different cytoplasmic tails. The first generation only contains a CD3ζ domain in its cytoplasmic tail, while the second generation contains two domains: a CD3ζ and co-stimulatory domain (here represented by CD28 or 4-1BB). The third generation, as the other generations, contains three cytoplasmic domains being two of them two different co-stimulatory molecules. Structurally, the fourth generation (TRUCK) is similar to the third generation, with the capacity to induce the secretion of pro-inflammatory cytokines. From: (Miliotou and Papadopoulou, 2018)

CAR T cell therapies represent a very promising approach for the treatment of hematologic malignancies. These malignancies express high levels of surface antigens and T cells have a tendency to migrate to hematologic organs like blood, bone marrow and lymph nodes, which facilitates contacts between CAR T cell and malignant cells (Maus et al., 2014). The most successful CAR therapies so far are directed against CD19. The expression of lineage specific antigens like CD19 (which is expressed at all B cell stages and also in B-cell malignancies) ensure that CAR T cells can target cancer cells in the absence of antigen heterogeneity (Maus and June, 2016). Moreover, CD19 is not expressed in hematopoietic stem cells which reduces the risk of autoimmunity and bone marrow toxicity (Brentjens et al., 2011). However, it is important to point out that as normal B cells also express CD19, this approach depletes normal B cells (Grupp et al., 2013; Kochenderfer et al., 2010; Wilkins et al., 2017). Therefore, to overcome this problem, patients are provided with gamma globulins which compensates for the lack of B cells (Savoldo and Dotti, 2013). However, the CAR T cell approach in hematologic malignancies is not always efficient and successful. For example, studies demonstrate that engineered T cells are more efficient in acute lymphocytic leukemia (ALL) than in chronic lymphocytic leukemia (CLL) or indolent lymphomas (Maude et al., 2014; Porter et al., 2015). Several factors could modulate the efficiency of the response: host T cell defects, inhibitory impact of the tumor microenvironment, pre-conditioning treatments, age of the patients, number and/or effector capacity of transduced T cells and loss or downregulation of the target antigen in tumor cells (Majzner and Mackall, 2018; Maus et al., 2014). For instance, it was recently demonstrated that CAR T cell can provoke reversible antigen loss through trogocytosis, resulting in decreased density of antigen on tumor cells and impacting T cell anti-tumor function (Hamieh et al., 2019). Moreover, intravital imaging in B-cell lymphoma bearing mice revealed that CAR T cell activity was higher in bone marrow than in lymph nodes, which suggests anatomical heterogeneity in CAR T cell function (Cazaux et al., 2019). Also, in this study they demonstrated that exists a functional heterogeneity as not all CAR T cell contacts resulted in tumor cell killing (Cazaux et al., 2019). All these issues can vary from one type of tumor to another and as a result, impact the overall efficiency of the therapy. Moreover, despite the impressive success of CD19 CAR T therapies, it is necessary to reduce the variables and understand the differences between clinical approaches. Moreover, the research in B malignancies is very complex and there is not a single standardized optimized CD19-specific CAR construct. Nevertheless, most clinical trials

are based on second generation of CARs like CD19-CD28-ζ or CD19-4-1BB-ζ (Cheadle et al., 2014).

A study performed by Savoldo *et al.* demonstrated that the presence of co-stimulatory molecule CD28 improves *in vivo* expansion and persistence of CAR-modified T cells when compared with T cells with only one signaling domain (first generation CAR) (Savoldo et al., 2011). Additionally, the levels of CAR CD19-CD28-ζ detected in tumor biopsies suggested a better efficient tumor infiltration (Savoldo et al., 2011). In another study, Brentjens *et al.* tried to evaluate the putative role of the cytoplasmic signaling co-stimulatory domain in the increased efficiency of second generation CARs. They observed that the presence of CD28 in the CAR structure was associated with an enhanced T-cell proliferation and cytokine secretion. However, this was not the case with other signaling domains, such as 4-1BB, DAP-10 or OX40 (Brentjens et al., 2007). On the other hand, other studies demonstrated that 4-1BB and OX40 co-signaling domains contribute to an enhanced co-stimulation in second-generation CARs by increasing cytokine secretion upon antigen stimulation and specific cytotoxicity (Finney et al., 2004; Imai et al., 2004). These discrepancies could be notably related to the type of antigen targeted and/or the CAR construct.

Furthermore, it is described that the co-stimulatory domain CD28 is important to promote IL-2 secretion (Hombach et al., 2006; Hombach et al., 2001a; Hombach et al., 2001b) by CAR T cells, a feature not observed in second generation CAR constructs containing 4-1BB and OX40 co-stimulatory domains (Koehler et al., 2012). 4-1BB and OX40 are not associated with an increase of IL-2 production, but both domains can lead to enhanced IFNγ production. Nevertheless, OX40 and 4-1BB are better for preventing induced cell death mechanisms and promoting T cell survival (Koehler et al., 2012). With these results, we can conclude that CD28, 4-1BB and OX40 can bring distinct advantages to potentiate the function of CAR T cells. As a result, the third CAR generation was initiated by combining two different co-stimulatory domains. In this regard, an elegant study compared CD28-ζ, OX40-ζ and CD28-OX40-ζ CAR constructions and showed that the third generation CAR demonstrated higher NFκB activity, IL-2 secretion, continuous proliferation and enhanced anti-tumor activity (Pule et al., 2005). Furthermore, other studies showed that the combination of 4-1BB and CD28 improved persistence, survival, anti-tumor efficiency and induced multifunctional cytokine secretion as

compared to second generation CARs (Carpenito et al., 2009; Tammana et al., 2010; Wang et al., 2007; Zhong et al., 2010).

Despite the huge success of CAR T cell approaches in hematological malignancies, up to 90% clinical response rates in the case of ALL (Maude et al., 2014), the overall clinical efficiency to treat solid cancer has been much less rewarding (Yong et al., 2017). Several cases describing a lack of therapeutic response or toxic side effects have been reported when using CAR T cells for solid tumors treatment (Kershaw et al., 2006; Lamers et al., 2006; Maus et al., 2013; Morgan et al., 2010). One potential issue in this case is the nature of the surrounding TME microenvironment which is more complex in solid tumors. This TME not only restricts the CAR T cells infiltration into the solid tumor mass due to the presence of stromal and epithelial cells, but also alters nutrient availability, which transforms the TME into a metabolic barrier (discussed in **the section IV 2 A**). Finally, the low levels of O_2 availability and immune checkpoint ligands such as PD-L1 can also dampen effector T cell responses (discussed in the **section IV 2 B**).

The importance of the TME and the metabolic reprograming on CAR T cell treatment and other T cell based immunotherapies has been recently addressed and highlighted (Le Bourgeois et al., 2018). Kawalekar and colleagues demonstrated that depending on the co-stimulatory domain expressed in the CAR construct, the redirected T cells recruit distinct metabolic programs and are engaged into different differentiating programs (Kawalekar et al., 2016). In this study, they demonstrated that 4-1BBζ CAR T cells have better persistence, high SRC, higher mitochondrial biogenesis, higher oxidative metabolism and increased frequency of T_{CM} as compared to 28ζ CAR T cells. These latter ones, on the other hand, showed reduced persistence, higher glycolytic metabolism and an increased frequency of T_{EM} cells (Kawalekar et al., 2016). These findings demonstrate that the CAR construct itself could impact the metabolism of the redirected-T cells and suggesting that this parameter should be considered for the design of CAR therapies. Recently, another group developed a CAR T cell construct based on another feature of the TME: hypoxia. This CAR express an oxygen sensitive domain of HIF1-α which is fused to a CAR scaffold, allowing the CAR-expressing T cells to better respond to hypoxic environments. Furthermore, another team demonstrated that metabolic modulation of CAR T cell could already be performed before CAR T cell injection with a positive impact for anti-tumor functions. Through the combination of IL-7 and IL-15

with CD3 and CD28 stimulation, T_{SCM} and T_{CM} phenotypes were promoted which were described to have better persistence and anti-tumor immunity (Alizadeh et al., 2019; Gargett and Brown, 2015; Gomez-Eerland et al., 2014). A study performed by Sukumar and colleagues highlighted the importance of metabolic reprograming and the TME in T-cell based immunotherapies. They show that T cell activation in the presence of a glycolysis inhibitor resulted in an increased generation of memory like cells with increased anti-tumor function comparing to the non-treated T cells with a higher glycolytic flux (Sukumar et al., 2013). These results clearly demonstrate that T cell metabolic reprogramming can be used to select T cells for ACT therapies. Moreover, Chang *et al* demonstrated that within the tumor, there is a competition for glucose between tumor cells and T cells which impacts negatively anti-tumor function. They showed that this could be overcome by PD-L1 blockade, which dampened the glycolytic rate of tumor cells and improved consequently anti-tumor functions (Chang et al., 2015). In an evident manipulation of the TME, this study highpoint the impact of the metabolic competition between T cells and tumor cells and why it is important to take it into consideration.

As described in this literature review, modulation of T cell metabolism can impact their activation, differentiation and effector function. Moreover, nutrients availability required to fulfill cell function is decreased in the TME, which as a consequence, impacts on anti-tumor responses. Therefore, it becomes crucial to further understand how CAR T cell metabolism is regulated and to develop new strategies to overcome the metabolic barriers imposed by the TME and to optimize CAR T cell efficiency.

Results

Chapter 1
Selection of different T cell subsets based on nutrient transporters expression

Introduction

Irrespective of the T cell subset utilized in an adoptive therapy, the metabolic needs of T cells have begun to be appreciated as a key factor in their anti-tumor activity. The activation, proliferation and effector functions of T cells are energy-"hungry" processes and recent studies have shown the importance of metabolic pathways in optimizing immune functions. When activated, lymphocytes upregulate their energetic state, increasing both oxidative phosphorylation and aerobic glycolysis but in general the latter increases to a greater level than the former (Buck et al., 2017; Clerc et al., 2019; Geltink et al., 2018) . This metabolic programming results in an increase in the input and use of nutrients. Indeed, the optimal activation of T cells and their effector activity requires, in particular, an increase in the surface expression of glucose (GLUT1) and arginine (CAT-1)transporters, resulting in an augmented use of these nutrients by lymphocytes (Jacobs et al., 2008; Loisel-Meyer et al., 2012; Macintyre et al., 2014; Wang et al., 2011; Werner et al., 2016). In addition, recent studies have shown that distinct subpopulations of T lymphocytes have disparate metabolic profiles; effector T cells are highly glycolytic but also lipogenic, while regulatory T cells present a heterogeneous metabolism with an increase in the level of lipid oxidation (Michalek et al., 2011). From this point of view, it is interesting to note that Treg, but not effector T cells, can be generated in the absence of glucose, glutamine or leucine transporters (Macintyre et al., 2014). Similarly, the restriction of glucose or glutamine in the cellular environment limits the development of effector cells but not Tregs (Cham et al., 2008; Chang et al., 2013; Klysz et al., 2015; Macintyre et al., 2014; Metzler et al., 2016; Nakaya et al., 2014). This is of particular interest as the metabolism generated in the tumor microenvironment, wherein nutrient availability may be limiting, would then contribute to prevent effective anti-tumor responses against a solid tumor. Indeed, the dysregulated growth of cancer cells can directly influence the extracellular environment and multiple studies now strongly suggest that the metabolic phenotype of the tumor governs the ensuing immune response (reviewed in (Ackerman and Simon, 2014; Mayers and Vander Heiden, 2017)). Thus, the metabolic fitness of an effector T cell in a tumor environment is likely to differ from its fitness in healthy lymphoid tissue.

It is interesting to note that within an activated T cell population, cells that are capable of transporting higher levels of glucose or exhibiting higher mitochondrial activity have an enhanced effector phenotype (as monitored by higher IFNγ secretion levels), while

populations with low glucose incorporation or low mitochondrial activity have a memory phenotype. Furthermore, arginine metabolism also activates mitochondrial respiration(Geiger et al., 2016). Thus, metabolic parameters can be used to distinguish T cells with different characteristics (Sukumar et al., 2013; Sukumar et al., 2015).

Based on the importance of glucose and arginine metabolism in T cell function, we initiated studies to assess whether the levels of these nutrient transporters (focusing on the GLUT1 glucose transporter and CAT-1 arginine transporter) on activated T lymphocytes can be used to select for T cells with distinct anti-tumor effector functions. Using GLUT1 and CAT-1 surface expression, we were able to select T cells expressing distinct GLUT1 and CAT-1 surface levels. These sorted cells were then studied and their phenotype as well as their effector function was evaluated. The data resulting from these studies are discussed in this chapter.

Article I

SCIENTIFIC REPORTS

OPEN

Cell surface Glut1 levels distinguish human CD4 and CD8 T lymphocyte subsets with distinct effector functions

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CD4 and CD8 T lymphocyte activation requires the generation of sufficient energy to support new biosynthetic demands. Following T cell receptor (TCR) engagement, these requirements are met by an increased glycolysis, due, at least in part, to induction of the Glut1 glucose transporter. As Glut1 is upregulated on tumor cells in response to hypoxia, we assessed whether surface Glut1 levels regulate the antigen responsiveness of human T lymphocytes in both hypoxic and atmospheric oxygen conditions. Notably, Glut1 upregulation in response to TCR stimulation was significantly higher in T lymphocytes activated under hypoxic as compared to atmospheric oxygen conditions. Furthermore, TCR-stimulated human T lymphocytes sorted on the basis of Glut1-Lo and Glut1-Hi profiles maintained distinct characteristics, irrespective of the oxygen tension. While T cells activated in hypoxia divided less than those activated in atmospheric oxygen, Glut1-Hi lymphocytes exhibited increased effector phenotype acquisition, augmented proliferation, and an inverted CD4/CD8 ratio in both oxygen conditions. Moreover, Glut1-Hi T lymphocytes exhibited a significantly enhanced ability to produce IFN-γ and this secretion potential was completely dependent on continued glycolysis. Thus, Glut1 surface levels identify human T lymphocytes with distinct effector functions in both hypoxic and atmospheric oxygen tensions.

The response of a T lymphocyte to antigen stimulation is conditioned by the cell's energetic and biosynthetic resources. T cell proliferation and effector function require the generation of ATP, phospholipids, nucleotides and NADPH. Their production is regulated by an augmented nutrient transport and utilization. Interestingly though, different T lymphocyte subsets display distinct metabolic profiles resulting from the differential utilization of glucose, fatty acids, and amino acids such as leucine and glutamine. T effector cells exhibit much higher glycolysis than suppressive regulatory T cells (Tregs) and differentiation of the latter subset is dependent on fatty acid oxidation[1–4]. Furthermore, the generation of T effector cells, but not regulatory T cells, requires a high level of amino acid metabolism[5–8].

In the context of glucose utilization, its transport into cells is often a rate-limiting step in its metabolism. Indeed, Glut1, the major glucose transporter in T lymphocytes (reviewed in[9]), is not expressed at significant levels on the surface of quiescent T cells[10–12] but is highly upregulated following TCR or cytokine stimulation[12–19]. Many studies have found that increasing glycolysis results in enhanced effector function, monitored as a capacity to produce IFN-γ[1,20–23]. Conversely, decreased glycolysis has been shown to inhibit both IFN-γ and IL-17 production[24–28]. However, a contradictory phenomenon has also been reported with T lymphocytes segregated on the basis of high glucose uptake exhibiting a terminally differentiated state with decreased effector function[21]. Notably though, the ensemble of these studies were all performed in a murine system and it is not known whether the level of glucose transport *per se*, irrespective of downstream glycolytic enzymes, modulates the fate of human T lymphocytes.

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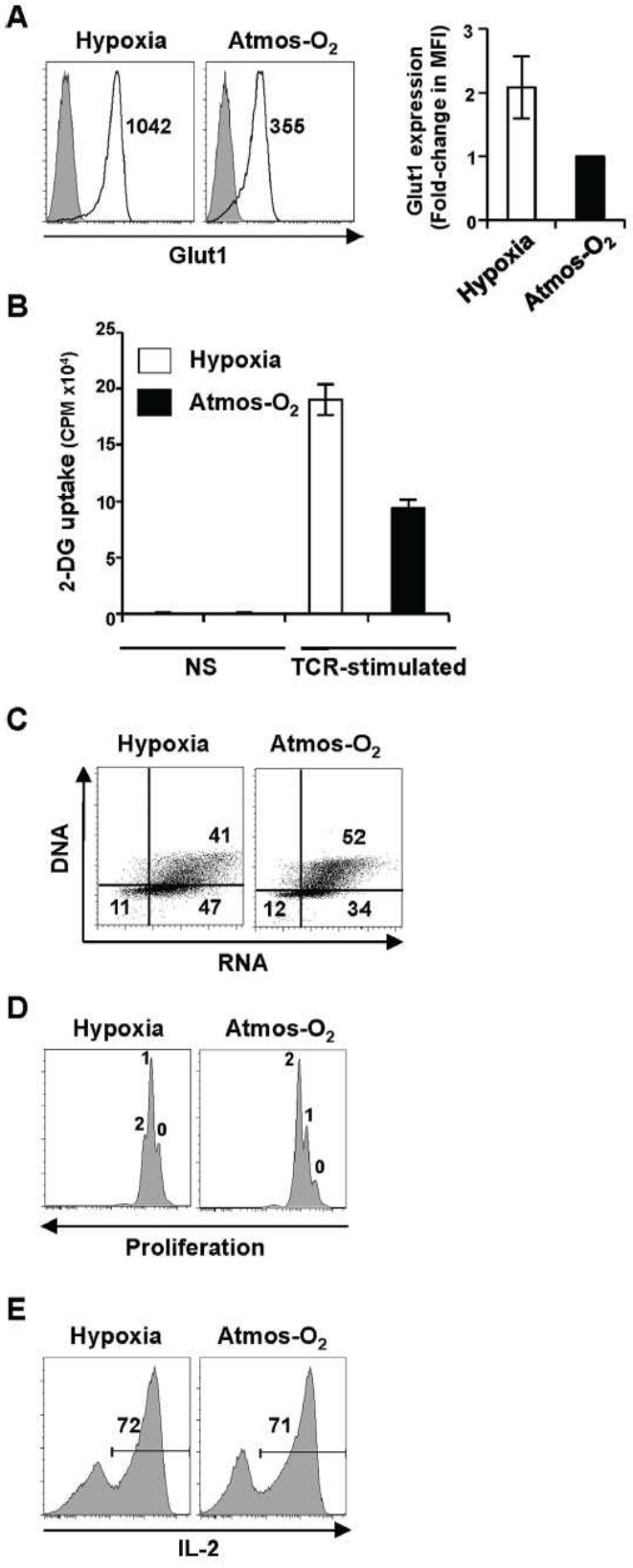

Figure 1. Glut1 expression and glucose uptake are significantly increased following activation of T cells under hypoxic conditions. (**A**) Human $CD3^+$ T cells, activated by anti-CD3/anti-CD28 monoclonal antibodies, were cultured under hypoxic (2%) or atmospheric (Atmos-O_2, 20%) oxygen conditions. Levels of the Glut1 glucose transporter were assessed at day 2–3 following activation and representative histograms are shown with Glut1 expression and control staining in black and filled grey histograms, respectively. Mean fluorescence intensities are indicated. The fold change in the MFI of Glut1 staining between atmospheric and hypoxic oxygen conditions was quantified in 6 independent experiments with the MFI in the former arbitrarily set at 1. Means ± SD are presented. (**B**) Glucose uptake was assessed in non-stimulated (NS) and TCR-stimulated T cell populations (1×10^6) by incubation with 2-deoxy-D[1-^{3}H]glucose (2-DG, 0.1 mM) for 10 min at RT. Uptake is expressed as mean counts per minute (CPM) for triplicate samples in 1 of 3 representative experiments; $p < 0.05$ by 2-tailed Mann-Whitney and Student's t-test. (**C**) Cell cycle status was monitored by simultaneous staining of

DNA and RNA with 7-aminoactinomycin D (7-AAD) and pyronin Y (PY), respectively. The percentages of cells in G1b (LR quadrant) and S/G2/M (UR quadrant) are indicated and are representative of 1 of 3 independent experiments. (**D**) T cell proliferation was monitored by CFSE labeling and dilution of the fluorescent dye was assessed at day 3. Each division peak is noted and represents 1 of 3 experiments. (**E**) IL-2 production was monitored at day 7 of stimulation following a 4 h PMA/ionomycin stimulation in the presence of brefeldin (**A**). Cells were fixed, permeabilized and intracellular IL-2 levels were analysed by staining with a fluorochrome-coupled antibody. Data are representative of 2 independent experiments.

It is also important to note that T lymphocyte responsiveness can be conditioned by oxygen tension. In hypoxic conditions, there is a stabilization of the hypoxia-inducible transcription factors, HIF-1α and HIF-2α[29]. Moreover, HIF-1α enhances glycolysis and Th17 effector function but its effects on Foxp3 transcription and the associated differentiation of murine Treg differentiation are controversial[30–34]. Hypoxic conditions can also potentially augment Glut1 levels on T lymphocytes[35] by interaction of HIF-1 with a consensus site in the *glut1* promoter[36,37]. This may have important *in vivo* significance as oxygen tensions in lymphoid tissues range from 0.5–4.5% and tumor microenvironments are often hypoxic[38–40]. However, whether and how Glut1 expression can predict the capacity of a human T lymphocyte to respond to antigen stimulation is unclear. Here, we report that cell surface induction of Glut1 conditions the fate of human CD4 and CD8 T lymphocytes in hypoxic as well as atmospheric oxygen conditions, with proliferation and effector function increasing with Glut1 expression.

Results

The glucose transporter Glut1 is highly upregulated in hypoxic conditions but TCR-induced T cell proliferation is significantly lower than at atmospheric oxygen. As indicated above, the oxygen tensions to which lymphocytes are exposed *in vivo* are dramatically lower than the 20–21% oxygen levels present in standard incubators. Furthermore, as the tumor microenvironment is often hypoxic, lymphocytes that infiltrate into tumors can be exposed to oxygen conditions that are often <1–2%. The capacity of T cells to respond to TCR stimulation requires increased metabolism, and it is interesting to note that in tumor cells, hypoxia results in the upregulation of the Glut1 glucose transporter, potentially allowing an increased level of glycolysis[29]. We therefore assessed whether the TCR-mediated upregulation of Glut1 on primary T cells is influenced by oxygen tension. Notably, Glut1 transporter expression was significantly higher in human T cells activated under hypoxic conditions with a mean increase in Glut1 fluorescence intensity of 2-fold in 6 different donors (Fig. 1A). Moreover, this increase in Glut1 levels was associated with a significantly augmented glucose transport, monitored as a function of ^{3}H-2-deoxyglucose uptake ($p < 0.05$; Fig. 1B). However, in hypoxic conditions, there was a lower percentage of cells in S/G_2/M (Fig. 1C), resulting in a decreased division. While the vast majority of cells in hypoxia had undergone a single division by day 3 post stimulation, the corresponding lymphocytes at 20% O_2 had undergone two divisions (Fig. 1D). Despite the lower proliferation (Fig. 1D), T lymphocytes in both conditions maintained the capacity to produce IL-2 (Fig. 1E).

Ectopic Glut1 expression results in increased TCR-induced proliferation of human T lymphocytes. Given the role of Glut1 in T cell metabolism, it was intriguing that T lymphocytes activated under hypoxic conditions expressed higher levels of this transporter but underwent decreased proliferation as compared to T lymphocytes activated in atmospheric O_2 conditions. While we hypothesized that this was due to the significantly higher amount of energy that is generated under aerobic versus anaerobic conditions, it was important to determine whether Glut1 expression *per se* was associated with a higher level of TCR-induced proliferation in human T cells. To test this hypothesis, we introduced ectopic Glut1 into mature human T cells by lentiviral transduction of a Glut1-dsRed fusion construct downstream of the SFFV promoter. This resulted in an approximate 5-fold increase in Glut1 levels as monitored by mean fluorescence intensity (Fig. 2A). We then assessed whether Glut1 levels were directly associated with changes in the proliferation profiles of T lymphocytes activated at 20% oxygen, and this was indeed the case. The majority of cells with low Glut1 (defined as population 1) had not divided whereas more than 70% of T cells expressing high levels of Glut1 (defined as population 4) had undergone 2 or more divisions (Fig. 2B). Thus, for T cells activated in atmospheric oxygen conditions, Glut1 levels directly correlate with the proliferation potential of the lymphocyte.

TCR-induced Glut1 levels distinguish T lymphocyte subsets with distinct proliferation and CD4/CD8 profiles. Based on the results described above, it was of interest to determine whether endogenous Glut1 expression influences T cell fate under hypoxic and/or atmospheric oxygen conditions. To this end, T lymphocytes were activated in both conditions and 48 h post TCR stimulation, lymphocytes were isolated as a function of surface Glut1 expression. Specifically, the 10% of lymphocytes expressing the lowest and highest levels of Glut1 were isolated on a FACSAria cell sorter and are hereafter referred to as Glut1-Lo and Glut1-Hi, respectively (Fig. 3A). Interestingly, at both 1% and 20% oxygen conditions, the percentages of CD8 T cells in the Glut1-Hi subsets were significantly higher than in the Glut1-Lo subsets, with a mean CD4/CD8 ratio of 6 in the Glut1-Lo subset and 3 in the Glut1-Hi subset ($p < 0.01$, Fig. 3A,B). It is important to note that this was not due to differences in the percentages of dividing versus non-dividing cells as proliferation at 48 h was minimal (Supplementary Figure 1). Furthermore, Glut1-Lo and Glut1-Hi subsets did not appear to be differentially polarized as the relative mRNA levels of transcription factors such as Tbet and GATA3 and effector cytokines such as IFNγ and IL-17 were not altered in the two subsets (data not shown). Consistent with a higher metabolic activity in Glut1-Hi cells, their forward/side scatters were significantly higher than those of Glut1-Lo cells, both at 1% and 20% oxygen tensions (Fig. 3C). Notably, the sorted Glut1-Lo and Glut1-Hi T cells remained distinct with

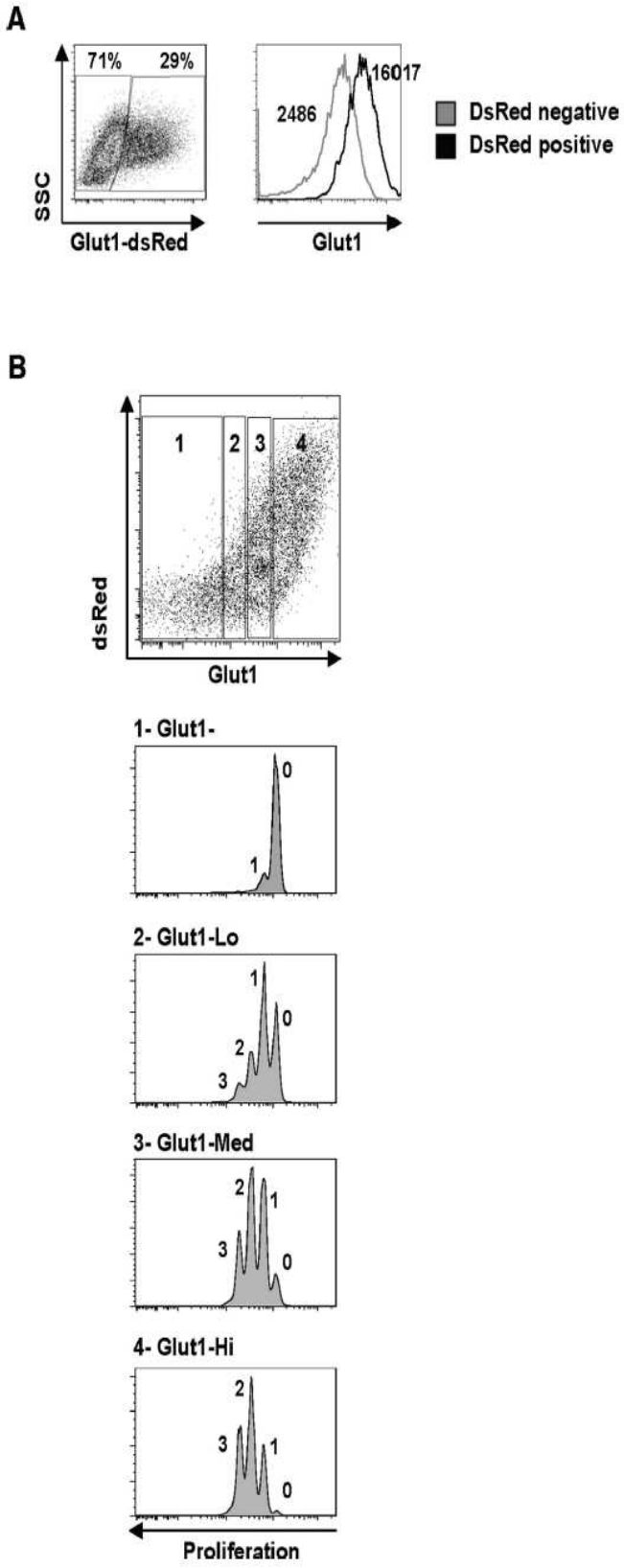

Figure 2. Ectopic Glut1 expression resulted in a significantly increased level of TCR-induced T cell proliferation . (**A**) $CD4^+$ T cells were transduced with a dsRed-expressing Glut1 lentiviral vector. Expresssion of Glut1 in dsRed + (black lines) and dsRed- cells (grey line) are shown in a representative histogram and the respective MFIs are indicated. (**B**) To determine the relative proliferation of Glut1- versus Glut1+ and Glut1-overexpressing cells, lymphocytes were labeled with the VPD450 dye and cell division was monitored 2 days post transduction with the dsRed vector. Division profiles of cells expressing different levels of Glut1 were assessed on gated subsets, denoted as Glut1- (1), Glut1-Lo (2), Glut1-Med (3) and Glut1-Hi (4), and the number of division peaks are indicated. Data are representative of 3 independent experiments.

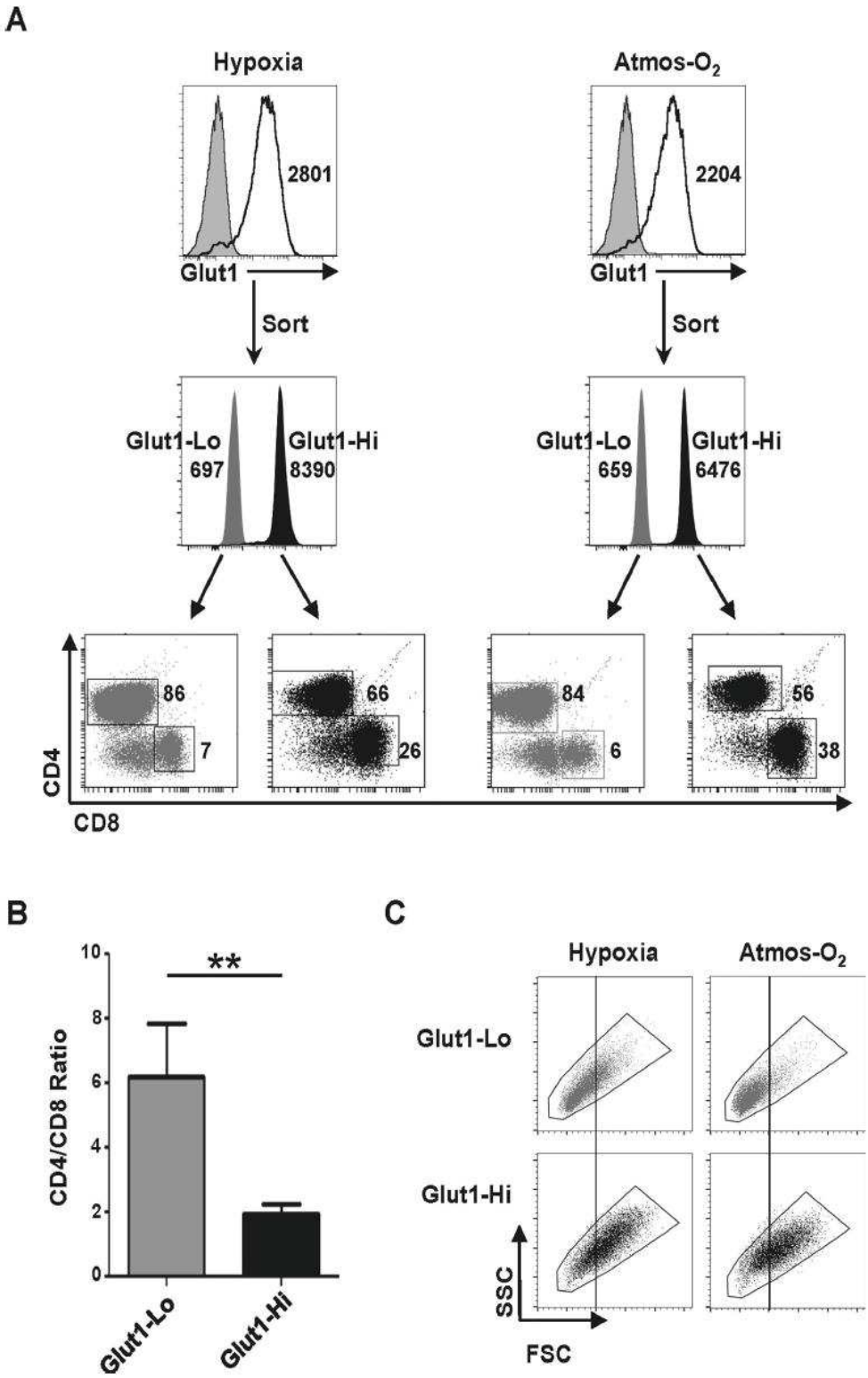

Figure 3. Differential Glut1 expression on TCR-activated T cells is associated with distinct repartitions of CD4 to CD8 T cells. (A) Purified human $CD3^+$ T cells were VPD450-labeled and TCR-activated in hypoxic and atmospheric oxygen tensions. Two days post stimulation, T cells were stained for Glut1 expression and cells with the 5–10% lowest and highest levels of surface Glut1 were sorted on a FACSAria cell sorter as shown and are hereby designated as Glut1-Lo and Glut1-Hi, respectively. The repartition of CD4 and CD8 T cells within each subset was assessed immediately after sorting and representative dot plots are shown. (B) Quantification of the ratio of CD4/CD8 T cells in the sorted Glut1-Lo and Glut1-Hi subsets. Mean ratios ± SEM of 5 independent experiments are presented and statistical differences were determined by a 2-tailed Mann-Whitney test; $^{**}p < 0.01$. (C) The forward (FSC) and side (SSC) scatter profiles of the Glut1-Lo and Glut1-Hi FACS-sorted subsets activated at hypoxia and atmospheric oxygen are presented. Data are representative of 3 independent experiments.

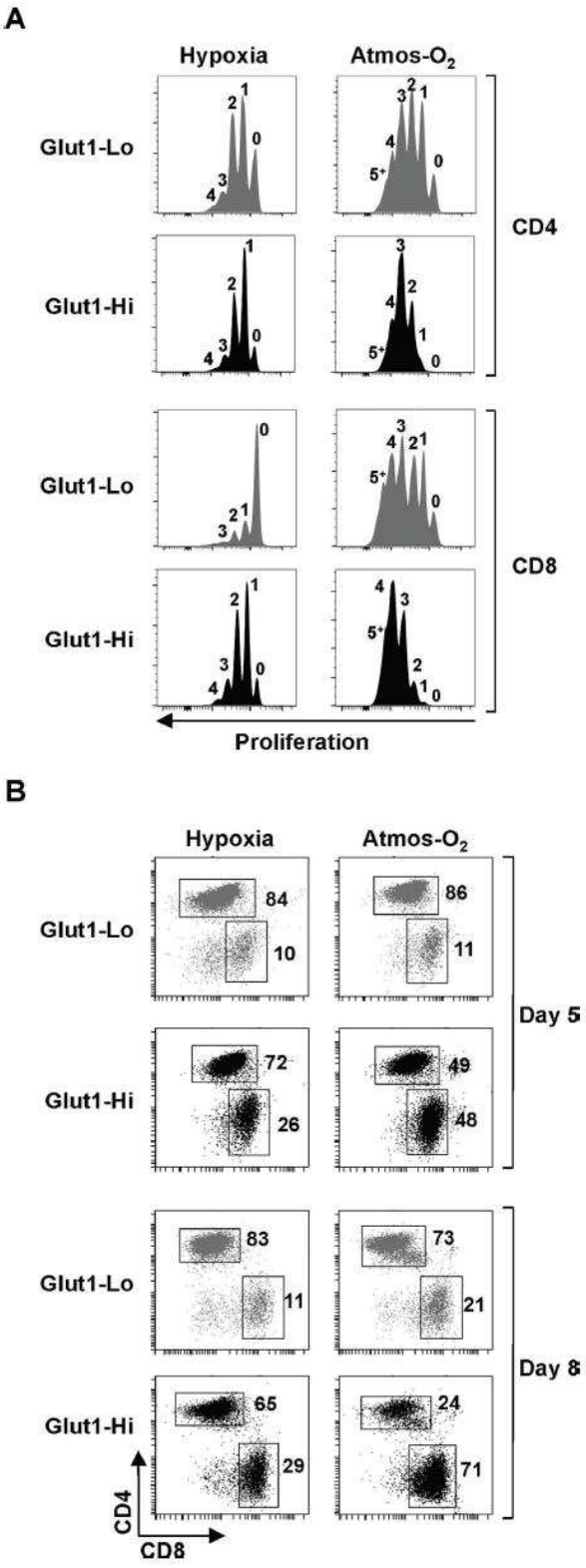

Figure 4. Glut1-Hi T cells maintain a distinct phenotype with enhanced proliferation and an increased proportion of CD8+ lymphocytes. (**A**) T lymphocytes activated in hypoxia and atmospheric oxygen were sorted at day 2 on the basis of Glut1 expression. The proliferation of Glut1-Lo and Glut1-Hi subsets were monitored at day 5 post stimulation as a function of VPD450 fluorescence and representative histograms showing the number of divisions are presented. (**B**) The repartition between CD4 and CD8 T cells within the Glut1-Lo and Glut1-Hi subsets are presented at days 5 and 8 post stimulation. The percentages of cells within each quadrant are indicated. Data are representative of 3 independent experiments.

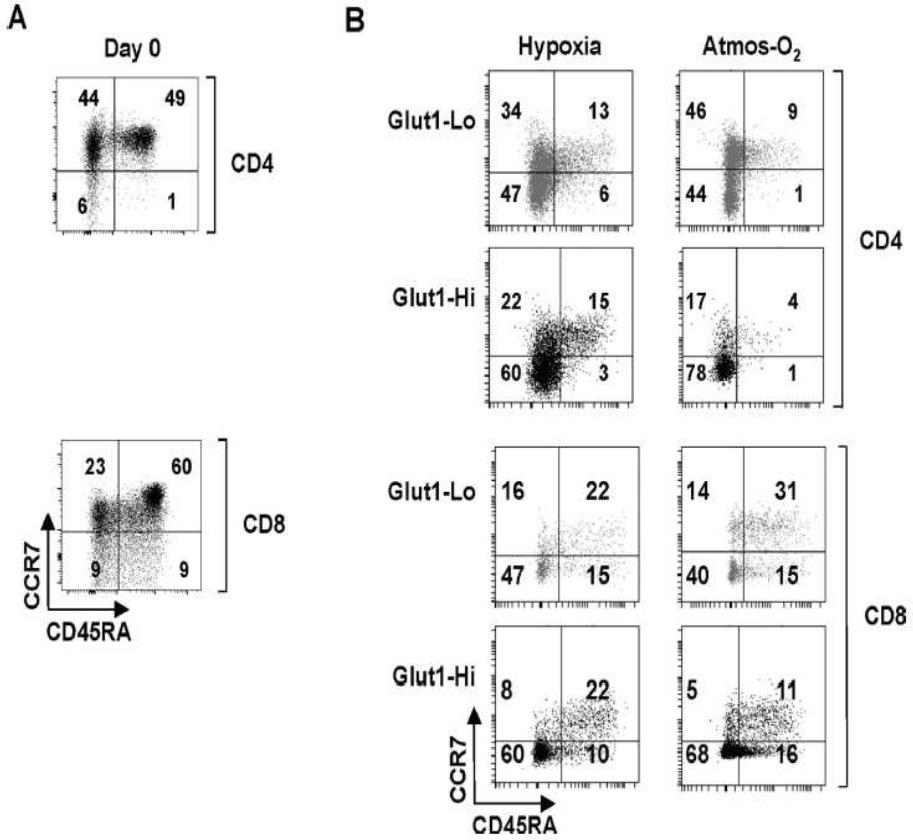

Figure 5. T lymphocytes expressing high surface Glut1 levels exhibit increased acquisition of an effector phenotype. (**A**) The naïve, memory and effector phenotypes of freshly isolated CD4 and CD8 T lymphocytes were monitored as a function of CCR7 and CD45RA expression (CCR7+CD45RA+, naïve; CCR7+CD45RA-, central memory; CCR7-CD45RA-, effector memory). Representative plots are shown. (**B**) T lymphocytes were TCR-activated for 2 days and sorted on the basis of Glut1-Lo and Glut1-Hi expression. Purified subsets were then maintained in culture in the presence of rIL-2 in hypoxia and atmospheric oxygen as indicated. The naïve, memory and effector phenotypes of the CD4 and CD8 populations were distinguished by CCR7/CD45RA staining. Representative plots of 3 independent experiments are presented and the percentages of T cells in each quadrant are indicated.

significantly higher levels of Glut1 detected on the latter 24 h post FACS isolation, in both hypoxic and atmospheric oxygen conditions (data not shown).

The fate of Glut1-Lo and Glut1-Hi cells was maintained during continued culture of the cells in the presence of IL-2. While all Glut1 subsets proliferated to a greater extent at 20% than 1% oxygen, in agreement with the data presented in Fig. 2, a greater percentage of Glut1-Hi cells divided as compared to Glut1-Lo cells (Fig. 4A). These data corresponded to a striking bias towards CD8 T cells within the Glut1-Hi subset at 20% oxygen; more than 70% of lymphocytes in the Glut1-Hi subset were CD8+ by day 8 as compared to 21% in the Glut1-Lo subset (Fig. 4B). At 1% oxygen, the percentage of CD8 T cells in the Glut1-Hi subset was also augmented relative to the Glut1-Lo subset but the differences were less marked; 29% in Glut1-Hi compared to 11% in the Glut1-Lo subset (Fig. 4B). This correlated with a decreased proliferation of all subsets at 1% oxygen relative to 20% oxygen. The ensemble of these data demonstrates the importance of the initial TCR-induced cell surface upregulation of Glut1 in modulating the subsequent fate of these lymphocytes. T cells selected on the basis of high Glut1 expression exhibit a significantly higher proliferation rate than their Glut1-Lo counterpart and this distinction results in a selective advantage for the CD8 T cell population.

Distinct phenotypes and proliferation profiles of Glut1-Lo and Glut1-Hi T cells. To follow the phenotype of FACS-sorted Glut1-Lo and Glut1-Hi T cells, we assessed CCR7 and CD45RA expression. Naïve T cells are identified as CCR7+/CD45RA+ while central memory cells are CCR7+/CD45RA- and effector memory cells are CCR7-/CD45RA-. In freshly isolated quiescent T lymphocytes from healthy adult donors, approximately 50% of cells are naïve (Fig. 5A). Upon TCR engagement, naïve cells rapidly lose CD45RA expression, with the vast majority of cells acquiring memory or effector phenotypes. Interestingly, acquisition of central memory and effector memory phenotypes was similar in 1% and 20% oxygen conditions, but Glut1 levels significantly influenced T cell fate. The percentage of effector memory T cells, characterized as CCR7-CD45RA-, was significantly higher in the Glut1-Hi than the Glut1-Lo subsets, with percentages ranging from 60–78% versus 40–47%, respectively (Fig. 5B). These data show that acquisition of an effector memory phenotype correlates with surface Glut1 levels.

Nevertheless, based on the experiments presented above, we could not exclude the possibility that the differences detected in T cells expressing distinct levels of Glut1 levels were determined by the cell's initial phenotype. Indeed, CD4 and CD8 T cells are heterogeneous with naïve (N), central memory (CM) and effector memory

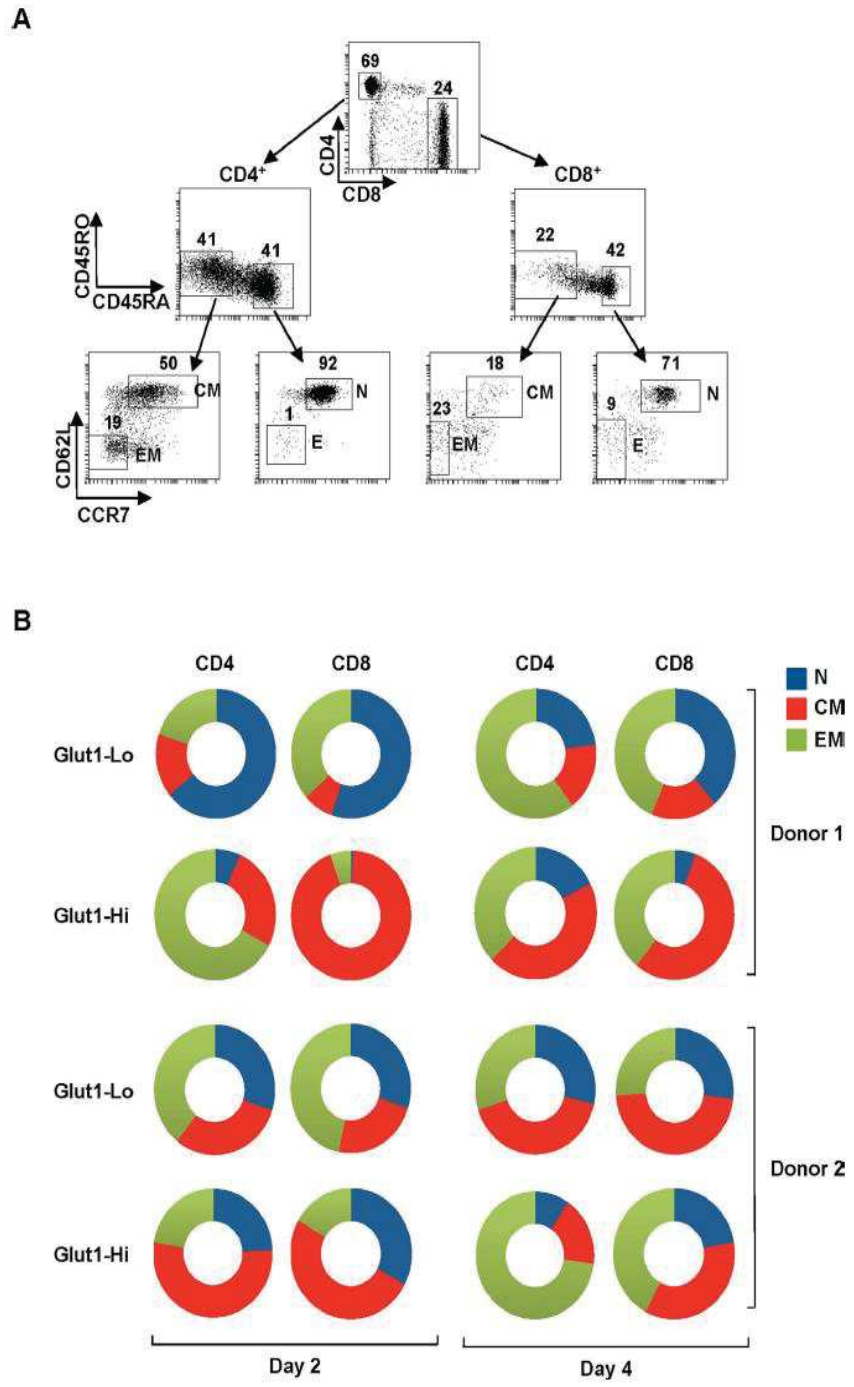

Figure 6. TCR-induced Glut1 expression varies as a function of T cell phenotype and the kinetics of T cell activation. (**A**) Sorting strategy for isolation of freshly isolated CD4 and CD8 T cell subsets. Representative plots show CD4/CD8, CD45RO/CD45RA, and CD62L/CCR7 repartitions. Naïve (N), central memory (CM) and effector memory (EM) CD4 and CD8 subsets were sorted as CD45RA+CD45RO-CD62L+CCR7+, CD45RO+CD45RA-CD62L+CCR7+, and CD45RO+CD45RA-CD62L-CCR7- phenotypes, respectively. Percentages of cells in each gate are indicated and subsets were sorted on a FACSAria to >90% purity. (**B**) Sorted subsets were activated and Glut1 expression was assessed at days 2 and 4 post TCR stimulation. Glut1-Lo and Glut1-Hi cells were defined as a function of the 10% lowest and highest levels of surface Glut1. Pie charts present the relative repartition of naïve, central memory and effector memory CD4 and CD8 cells within the Glut1-Lo and Glut1-Hi gates in 2 healthy donors at days 2 and 4 post TCR stimulation. Raw data for one donor are presented in Supplementary Figure 1.

(EM) subsets presenting lymphocytes with diverse response histories. We therefore FACS-sorted these six subsets (CD4-N, CD8-N, CD4-CM, CD8-CM, CD4-EM, CD8-EM) from fresh peripheral blood samples (Fig. 6A) and assessed whether they exhibited differences in the kinetics of TCR-induced Glut1 upregulation and proliferation. TCR stimulation resulted in a rapid Glut1 upregulation in all T cell subsets. However, as shown in Fig. 6B, there is clearly diversity between donors; while there was a higher percentage of naïve CD4 and CD8 T cells within the Glut1-Lo population at day 2 post stimulation, a significant percentage of naïve CD4 and CD8 T cells from donor 2 were also Glut1-Hi. A higher percentage of CM cells were present in the Glut1-Hi than the Glut1-Lo subsets

for both CD4 and CD8 T cells. Importantly, the percentages of N, CM and EM T cells within the Glut1-Lo and Glut1-Hi subsets changed with time post stimulation. At day 4 post stimulation, varying percentages of N, CM, and EM populations were found in both Glut1-Lo and Glut1-Hi subsets (Fig. 6B and Supplementary Figure 1). While N, CM and EM T cells all underwent extensive TCR-induced proliferation by day 4, cell surface Glut1 levels on all subsets were higher on T cells that had undergone at least 1 round of division. Moreover, the surface expression of Glut1 on T cells following TCR stimulation is not static but rather evolves with time (Supplementary Figure 1). These data are in agreement with previous studies showing that surface Glut1 decreases to undetectable levels as T lymphocytes return to a resting state[12].

Glut1-Hi cells exhibit enhanced effector function. The data presented above suggested that the effector function of human T cells might be more affected by surface Glut1 expression than by oxygen concentration. Notably, IFN-γ secretion was significantly higher in the Glut1-Hi subsets of both CD4 and CD8 T cells than in the Glut1-Lo counterparts; in CD4 T cells, the mean percentage of IFN-γ-expressing cells increased from 20% in the Glut1-Lo subset to 39% in the Glut1-Hi subset while in CD8 T cells, it increased from 32% to 53% ($p < 0.05$; Fig. 7A,B). The same trend was also detected for IL-17 secretion by CD4 T cells, increasing from a mean of 3% to 8% but this difference was not significant due to variability between donors (Fig. 7A,B). Based on these data, we assessed whether the relative contribution of glycolysis to cytokine secretion differed in Glut1-Lo versus Glut1-Hi T cells. In this regard, previous studies have elegantly shown that inhibiting glycolysis in murine T cells results in a significant attenuation of IFN-γ production while conversely, increasing glycolysis promotes IFN-γ secretion from murine T cells[20,22,23,25,41,42]. Indeed, we found that blocking glycolysis in human T cells with a non-metabolizable 2-deoxyglucose (2-DG) analogue dramatically inhibited IFN-γ secretion. Importantly, this inhibition occurred in both Glut1-Lo and Glut1-Hi subsets ($p < 0.05$; Fig. 7B). Thus, maintaining glucose metabolism is critical for the IFN-γ secretion potential of T lymphocytes, irrespective of their level of Glut1 expression. However, under conditions where glycolysis is maintained, the potential of T cells to secrete IFN-γ is significantly enhanced in lymphocytes expressing high Glut1 levels. These data attest to the importance of nutrient resources and their intracellular transport in modulating the function of human T lymphocytes.

Discussion

Recent findings reveal the critical role of metabolic programs in conditioning T cell activation and differentiation, suggesting new strategic avenues for modulating T cell function. As many studies have found that genetic manipulations resulting in enhanced glycolysis lead to augmented T cell effector function[1,20–23,26,43], it was of interest to determine whether differences in Glut1 surface expression are associated with distinct cell fates. Glut1 is rapidly upregulated following T cell stimulation, peaking at approximately 2–3 days post TCR engagement and then slowly returning to baseline levels[12,15,16,18,19]. Here we show that Glut1 levels distinguish the behavior of human T lymphocytes following TCR stimulation. Notably, segregating T lymphocytes on the basis of Glut1 levels at an early time following TCR stimulation (2 days), identified subsets with unique phenotypic and functional traits. Glut1-Hi CD4 as well as CD8 T lymphocytes preferentially acquired an effector phenotype with enhanced effector function.

Glut1 regulation is complex, controlled at the level of transcription, translation and transport to the cell surface. This regulation is critical as glucose uptake across the plasma membrane is often the rate-limiting step in the production of ATP. In accord with the importance of Glut1 regulation, augmented Glut1 levels at the cell surface optimize the "fitness" of tumor cells to their hypoxic environment (reviewed in[44]). Our finding that hypoxia also increases TCR-induced Glut1 levels on T lymphocytes suggests a potential competition between tumor cells and infiltrating T lymphocytes for glucose. Indeed, two recent studies have elegantly shown that increased tumor glycolysis severely impedes the capacity of infiltrating T cells to control tumor growth. However, manipulations that increase T cell glycolysis within the tumor microenvironment resulted in bolstered effector functions, promoting IFN-γ secretion[22,23]. Furthermore, it appears that even the beneficial effects of checkpoint inhibitors (αCTLA-4, αPD-1 and αPD-L1) are regulated by T cell metabolism; glucose concentrations within the tumor increase, resulting in a higher glycolysis in infiltrating T cells[22]. While we found that human T lymphocytes divided less robustly in hypoxia, augmented cell surface levels of Glut1 may have evolved in order to promote an efficient and rapid adaptive immune response against infectious agents and tumor antigens, even in oxygen-deprived regions of the body. Indeed, the potential of CD4 as well as CD8 T lymphocytes to secrete IFN-γ is significantly enhanced in the Glut1-Hi populations.

Conversely, manipulations that decrease the glycolysis of T lymphocytes may improve the outcome of patients with T cell-mediated autoimmune disease. In this regard, it is significant that blocking glucose metabolism in murine T cells results in a significant attenuation of IFN-γ secretion[20,25,41]. In accord with these data, we found that glucose metabolism is a *sine qua non* for IFN-γ generation by activated human T cells, at least *ex vivo*. Moreover, a combined block of glycolysis and hexokinase activity reverses the development of an autoimmune disease, systemic lupus erythematosus, in a murine model[27,28]. As Glut1 is the major glucose transporter on T lymphocytes, these data suggest that Glut1 levels may remain elevated in autoimmune conditions. Furthermore, it is likely that the subset of T lymphocytes with high Glut1 levels early following TCR stimulation is distinct from the subset with high Glut1 levels at late time points, during the contraction phase of an immune response. This phenomenon may explain the apparent contradictions between studies reporting enhanced versus decreased effector functions for highly glycolytic murine T cells[1,20–23]. Indeed, we show here that while there is an initial enrichment of naïve T lymphocytes in the Glut1-Lo population and central memory CD8 lymphocytes in the Glut1-Hi population, surface Glut1 expression on the different subsets evolves with time post TCR stimulation. Nonetheless, it is important to note that under physiological conditions, surface Glut1 levels return to low baseline on all T cell subsets as they return to a resting state, at late time points following TCR stimulation.

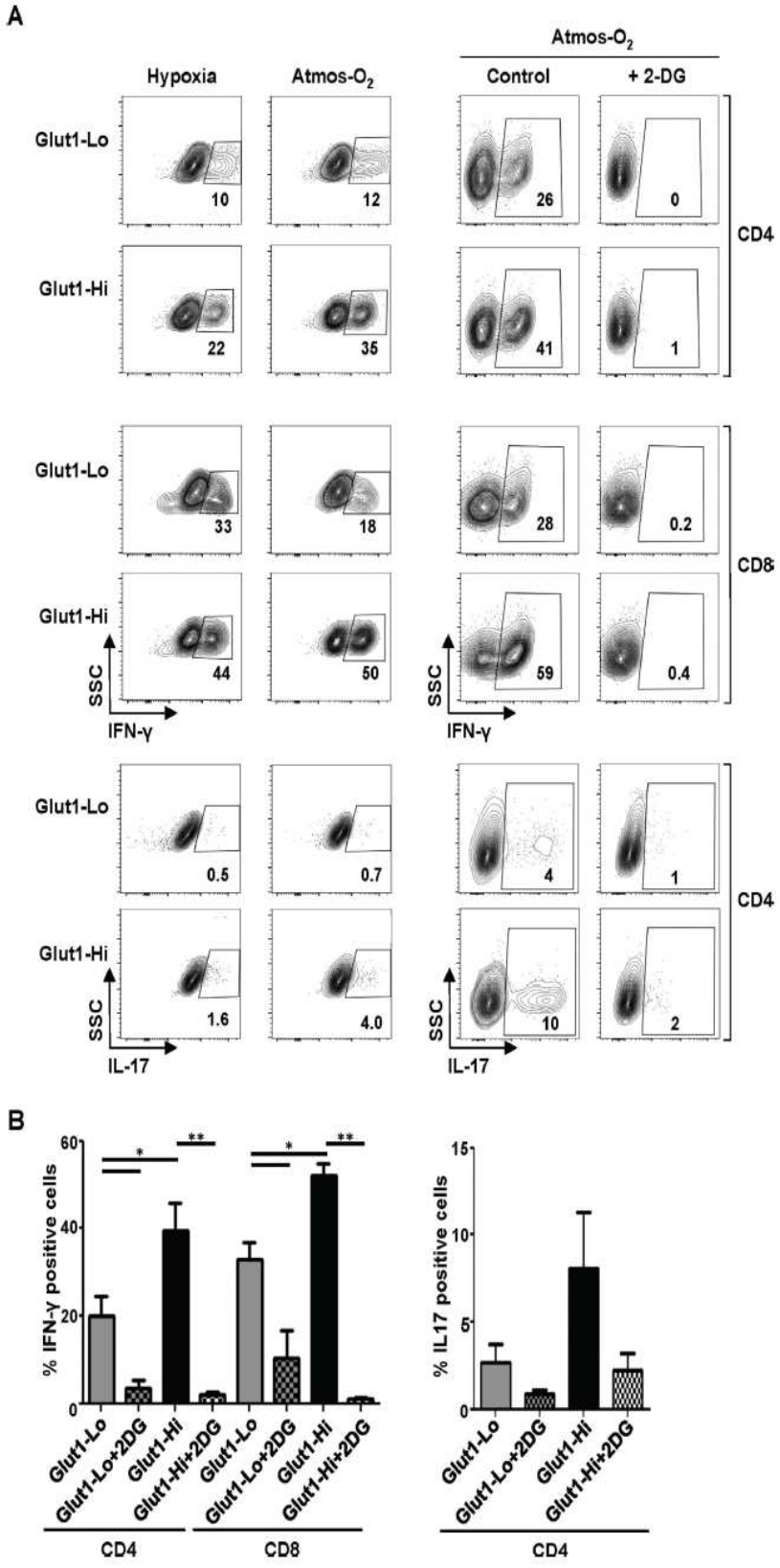

Figure 7. Surface Glut1 levels modulate the cytokine secretion potential of CD4 and CD8 T lymphocytes at both hypoxic and atmospheric oxygen conditions. (**A**) TCR-stimulated $CD3^+$ human T cells were FACS-sorted at day 2 on the basis of Glut1 levels and cultured for an additional 3 days at either hypoxia or atmospheric oxygen as indicated. Production of IFN-γ by CD4 and CD8 T cells as well as IL-17 by CD4 T cells was monitored in the Glut1-Lo and Glut1-Hi subsets. Intracellular staining was performed 4 h post PMA/ionomycin treatment in the presence of brefeldin A (left plots). IFN-γ and IL-17 production was also monitored simultaneously in CD4 and CD8 subsets that were restimulated with PMA/ionomycin in the absence or presence of 2-deoxyglucose (2-DG) and data from 1 of 5 independent experiments are shown (right plots). (**B**) Quantification of IFN-γ and IL-17 production in Glut1-Hi and Glut1-Lo subsets as a function of restimulation in the absence or presence of 2-DG. Means ± SEM of 5 independent experiments are presented and statistical difference was monitored by a 2-tailed Mann-Whitney test; $^*p < 0.05$; $^{**}p < 0.01$.

In conclusion, our data reveal a robust proliferation and cytokine secretion in human T lymphocytes exhibiting an early TCR-induced upregulation of Glut1. Furthermore, cell surface expression of Glut1 on naïve, central memory and effector memory T cells can be used as a biomarker of recent T cell activation. Future studies will allow us to determine whether there is a differential proportion of Glut1-Lo and Glut1-Hi T lymphocyte subsets in immunopathological conditions such as autoimmunity, chronic inflammation and cancer, potentially opening new avenues for targeting immune responses.

Methods

T cell isolation and culture. $CD3^+$ T cells were isolated from healthy adult donors. All experiments using primary human cells were conducted in accordance with the Declaration of Helsinki and IRB approval to the French Blood Bank (Etablissement Français du Sang). T lymphocytes were purified by negative-selection using Rosette tetramers (StemCell Technologies) and the purity was monitored by flow cytometry. Lymphocytes were cultured in RPMI medium 1640 + GlutaMAX (Gibco-Life technologies) supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco-Life technologies).

T cell activations were performed using plate-bound anti-CD3 (clone OKT3, Biolegend) and anti-CD28 (clone 9.3, kindly provided by Carl June) mAbs at a concentration of 1 µg/ml. rIL-2 (100 U/ml) was added every other day starting at day 2 post-activation. Cells were maintained in a standard tissue culture incubator containing atmospheric (20%) O_2 or an air-controlled incubator where O_2 conditions were maintained at 1–2% by nitrogen injection (Heraeus incubator; Sanyo) and 5% CO_2.

Flow cytometry analyses and cell sorting. Immunophenotyping of cells was performed using fluorochrome-conjugated mAbs (BD Bioscience or eBioscience) against CD4, CD8, CD45RA, CD45RO, CCR7, CD62L and isotype controls (Beckman Coulter). Cell cycle analysis was performed by staining cells for DNA and RNA content with 7-amino-actinomycin-D (7AAD; 20 µM, Sigma) and pyronin Y (PY; 5 µM, Sigma), respectively. Proliferation was monitored by labeling cells with either CFSE (Life Technologies; 2.5 µM) or VPD450 (BD Biosciences; 1 µM) for 3 min at RT. Surface Glut1 expression was monitored by binding to the Glut1 ligand fused to eGFP as previously described[19,45,46] (Metafora biosystems). For sorting of cells based on Glut1 expression, $60 \times 10e^6$ cells were stained at day 2 post stimulation and the 10% of lymphocytes expressing the highest and lowest levels of Glut1 were sorted on a FACSAria (BD Biosciences). For sorting of CD4 and CD8 naïve, central memory and effector memory subsets, freshly isolated T cells were stained with fluorochrome-conjugated mAbs against CD4, CD8, CD45 RA, CD45RO, CCR7 and CD62L. Cells were FACS-sorted based on the gating strategy shown in Fig. 6. For intracellular cytokine staining, lymphocytes were re-activated with PMA (Sigma-Aldrich; 100 ng/ml) and ionomycin (Sigma-Aldrich; 1 µg/ml) in the presence of brefeldin A (Sigma-Aldrich; 10 ug/ml) for 3.5–4h at 37 °C. Re-activations were performed either in the absence or presence of 2-deoxyglucose (50 mM, Sigma) to inhibit glycolysis. Intracellular staining for IL-2, IFN-γ and IL-17 was performed following fixation/permeabilization using the eBioscience kit or using 4% PFA (Thermo-scientific)/ 0.1% saponin (Sigma). Cells were analysed on a CantoII or LSRII-Fortessa (BD Biosciences) and data were analysed using Diva (BD Biosciences) or FlowJo (Tree Star) software.

Virus production and T cell transduction. For expression of ectopic Glut1, a Glut1-dsRed fusion construct[45] was inserted into the HIV1-derived pCSGW lentiviral vector, harboring the SFFV promoter. Self-inactivating single-round HIV-1 virions were generated by $CaCl_2$ transfection of 293T cells with pCSGW-Glut1-dsRed together with the 8.91 Gag-Pol packaging construct and the CMV-VSV-G plasmid. Viral supernatant was harvested 48 h post transfection and concentrated by ultracentrifugation (25,000 rpm) for 2 h at 4 °C. For T cell transductions, 1×10^6 lymphocytes were activated for 24 h and transduced with VSV-G–pseudotyped HIV-1 vector at MOI of 1 in IL-2-containing RPMI media for 48 h. Transduction was determined as the percentage of DsRed+ cells on an LSRII Fortessa (BD Biosciences).

Glucose uptake assays. Prior to transport analyses, T cells (0.5×10^6) were starved in glucose-free RPMI for 30 min at 37 °C. Glucose uptake was initiated by addition of 2-deoxy-D[1-^{3}H]glucose (2 µCi; Perkin Elmer) for 10 min at room temperature. Cells were then washed and lysed in 0.1% SDS (500 µl) and radioactivity was measured by liquid scintillation. Uptake for each cell subset is expressed as mean counts per minute (CPM) for triplicate samples and error bars indicate standard deviation (SD).

Statistical analyses. p values were determined using a Mann-Whitney test with a 2-tailed distribution (Graph Pad Software, La Jolla, CA).

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Addendum I

T cell selection based on CAT-1 surface expression

Results and Discussion

Selection of TCR-stimulated human T cells on the basis of surface CAT-1 expression

Based on our previous work, demonstrating that the cytokine secretion potential of human TCR-stimulated T cells can be distinguished on the basis of GLUT1 levels, we assessed whether human T cells selected on the basis of the surface expression of an independent nutrient transporter would exhibit distinct characteristics. We focused on the arginine transporter, CAT-1/SLC7A1. Briefly, arginine uptake in T cells is dependent on CAT-1 and its expression is important for T cell proliferation (Werner et al., 2016). Indeed, upon TCR stimulation, CAT-1 surface expression is highly upregulated, to a higher level even than GLUT1 expression (CAT-1 deltaMFI 20467 compared to GLUT1 deltaMFI 4703 at day 2 and CAT-1 deltaMFI 24974 compared to GLUT1 deltaMFI 7748 at day 4 (**Figure 1**).

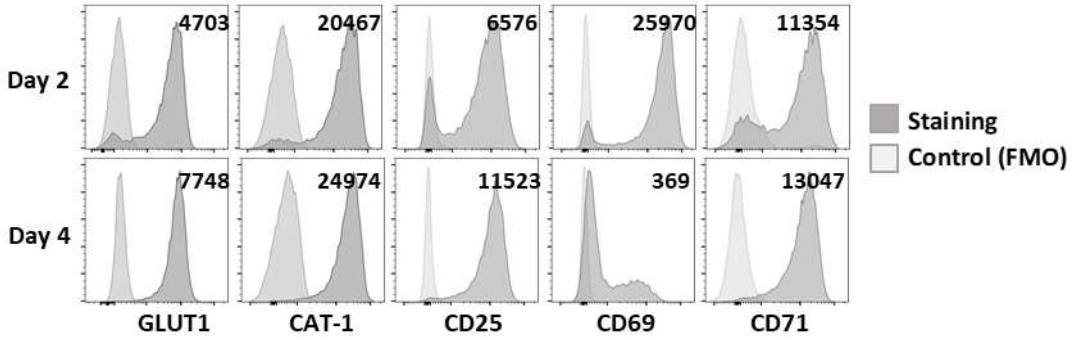

Figure 1 - Upregulation of CAT-1 surface expression upon TCR stimulation

Purified human $CD3^+$ T cells were TCR-activated with anti-CD3/CD28 mAbs. At day 2 and 4 post stimulation, T cells were stained for GLUT1, CAT-1 as well as the activation markers CD25, CD69 and CD71. Data are representative of 2 independent experiments.

To address this question, $CD3^+$ T cells were isolated from the blood of healthy donors and TCR-stimulated using anti-CD3/anti-CD28 mAbs. Following 48h of TCR activation, T cells were stained with a CAT-1 ligand (CAT-1-RBD) in order to detect CAT-1 surface expression. $CD3^+$ T cells expressing the lowest and highest levels of CAT-1 (~15%) were FACS-sorted and distinguished as CAT-1^{Low} and CAT-1^{High}, respectively. Since CAT-1 was not detected on quiescent T cells and was induced following TCR stimulation, we hypothesized that the CAT-1^{High} subset would have a higher metabolic activity. In agreement with this hypothesis, the forward/side scatters of CAT-1^{High} cells were higher than those of CAT-1^{Low} cells **(Figure 2).**

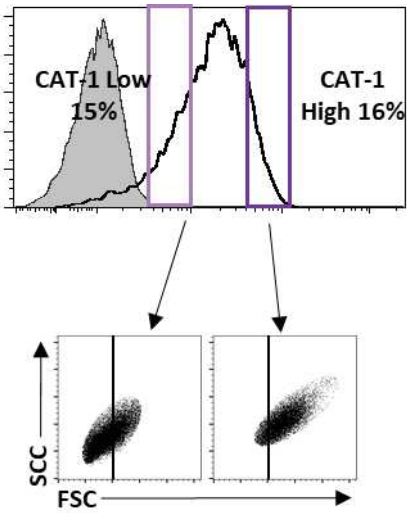

Figure 2 - Selection of TCR-stimulated human T cells on the basis of CAT-1 surface expression

Purified human $CD3^+$ T cells were TCR-activated with anti-CD3/CD28 mAbs. Two days post-stimulation, T cells were stained for CAT-1 expression and cells with the lowest and highest levels of surface CAT-1 (appr. 15%) were sorted on a FACSAria cell sorter as shown and are hereby designated as CAT-1^{Low} and CAT-1^{High}, respectively. The forward (FSC) and side (SSC) scatter profiles of the sorted CAT-1^{Low} and CAT-1^{High} subsets are presented. Data are representative of 4 independent experiments.

Increased percentages of CD8 T cells in the CAT-1^{High} as compared to the CAT-1^{Low} subset following TCR stimulation

We next assessed whether there was a difference in the CD4/CD8 ratios within the CAT-1^{High} vs. CAT-1^{Low} T cell subset. Notably, the relative percentages of $CD4^+$ T cells were significantly higher in the CAT-1^{Low} than the CAT-1^{High} T cell subset (81% versus 50%) at day 2 post stimulation and this correlated with a lower percentage of $CD8^+$ T cells (17% versus 47% in a representative experiment, **Figure 3A)**. Thus, while the total T cell population (CAT-1^{Total}, all cells were sorted) exhibited a CD4:CD8 ratio of approximately 3:1 following 2 days of TCR stimulation, the ratio in the CAT-1^{Low} subset was >4:1 and the ratio in the CAT-1^{High} subset was approximately 1:1 **(Figure 3A)**. Thus, we detected a striking bias towards $CD8^+$ T cells in lymphocytes with a high induction of CAT-1. Interestingly, the ex vivo culture of sorted CAT-1^{Low} and CAT-1^{High} T cells in the presence of IL-2 resulted in an increased proportion of CD8 T cells in both subsets (day 8) but the relative percentages of CD8 T cells remained higher in the latter, resulting in a CD4:CD8 ratio of approximately 0.7 in the former and 0.3 in the latter **(Figure 3A)**. The bias towards $CD8^+$ T cells was likely due to their faster proliferation as compared to $CD4^+$ T cells **(Figure 3B)**. Furthermore, as monitored by VPD dilution in Figure 2B, the percentages of $CD4^+$ T cells that underwent extensive proliferation in the CAT-1^{Low} subset was substantially lower than in the CAT-1^{High} subset (80% as compared to 54%). Within the CD8 subset, 64% of CAT-1^{Low} cells had undergone >4 divisions whereas 72% of CAT-1^{High} cells had already reached this level **(Figure 3B)**. Notably, this alteration in cell division was not due to a defect in activation as both CAT-1^{Low} and CAT-1^{High} cells exhibited an equivalent

upregulation of activation markers such as CD69 and CD25 (data not shown).

Altogether, these data show that CAT-1^{High} cells exhibit a higher level of proliferation than CAT-1^{Low} cells in response to TCR stimulation and moreover, that the high induction of CAT-1 is biased to the highly proliferating CD8 T cell subset.

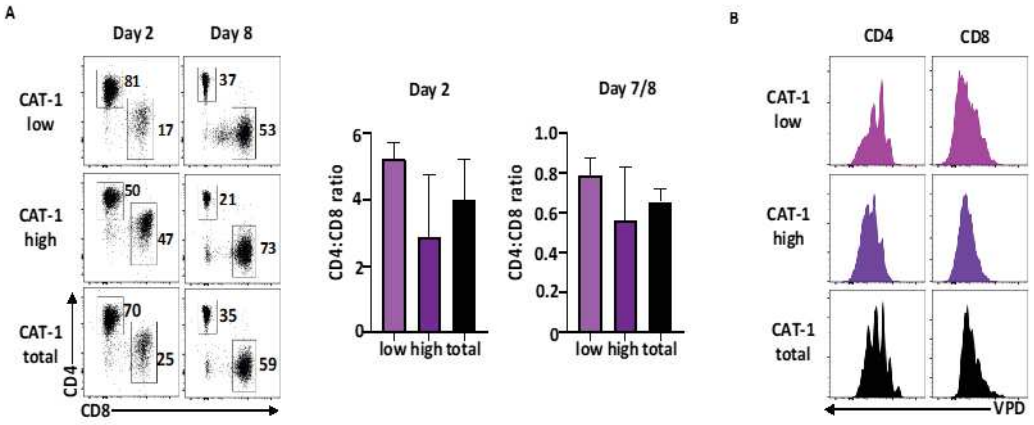

Figure 3- CAT-1 is highly upregulated on CD8 T cells and is associated with an increased proliferation of CD8 as compared to CD4 T cells.

(A) Purified human CD3$^+$ T cells were VPD450-labeled and TCR-activated using anti CD3/CD28 mAbs and FACS-sorted on the basis of CAT-1 expression as described in Figure 1. The repartition of CD4 and CD8 T cells within each subset was assessed immediately after sorting (day 2) and at day 8 post-TCR activation and representative dot plots of 2 independent experiments are shown. **(B)** The proliferation of sorted CAT-1^{Low} and CAT-1^{High} CD4 and CD8 subsets, as well as control total sorted T cells (CAT-1^{Total}) were monitored at day 7 post-stimulation as a function of VPD450 fluorescence and representative histograms

CAT-1^{High} CD4$^+$ T cells exhibit higher effector function than CAT-1^{Low} CD4$^+$ T cells

To assess whether the selection of human T cells on the basis of CAT-1 expression Is associated with the enrichment of naïve versus memory T cells, we monitored expression of the CD45RA and CD45RO markers which are generally expressed on naïve and memory cells, respectively. As expected, the percentages of CD45RA$^+$ T cells decreased between days 2 and 8 of stimulation, consistent with the loss of naïve cells following TCR stimulation, with a more pronounced loss in the CD4$^+$ subset. Notably though, there were no striking alterations in the percentages of CD45RA$^+$ and CD45RO$^+$ cells within the CAT-1^{Low} as compared to CAT-1^{High} sorted subsets, at either time point **(Figure 4)**. These data suggest that CAT-1 surface expression does not distinguish CD4 or CD8 T cell subsets on the basis of their naïve/ memory phenotype. Interestingly, these data differ from our previous results based on Glut-1

expression wherein high Glut-1 levels were associated with the acquisition of an effector memory phenotype.

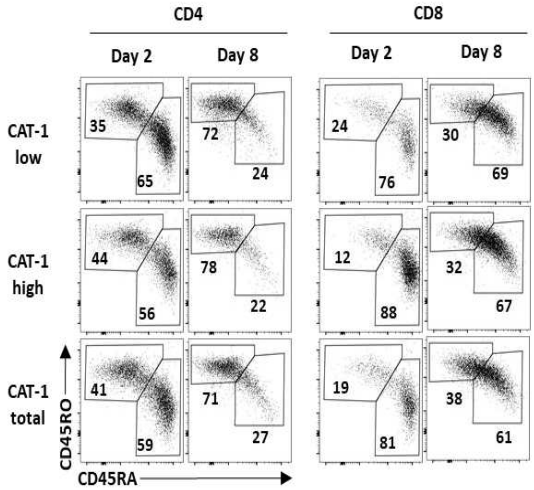

Figure 4 - CD45RA and CD45RO expression on CAT-1^{Low} and CAT-1^{High} sorted cells

Purified human CD3$^+$ T cells were TCR-activated, sorted on the basis of CAT-1 expression at day 2 as indicated and then cultured ex vivo in the presence of IL-2. The CD45RA/CD45RO phenotype of CD4$^+$ and CD8$^+$ T cells is shown at days 2 and 8 and the percentages of cells in each gate are presented. Representative plots from 1 of 2 independent experiments are shown.

We next assessed whether there were differences in the effector potential of CAT-1^{Low} and CAT-1^{High} T cells by evaluating their cytokine secretion capacity. While the percentages of CD4 and CD8 T cells secreting IL-2 was high in both the CAT-1^{Low} and CAT-1^{High} subsets (mean of >50%), it is notable that IL-17 secretion was substantially higher in the CD4$^+$CAT-1^{High} subset (6% as compare to <2% in the CAT-1^{Low} subset). Furthermore, IFN-γ secretion was slightly increased in CAT-1^{High} as compared to CAT-1^{Low}CD4$^+$ T cells and total CD4 T cells (29 vs 23%). Interestingly, these percentages were not altered in the CD8 T cell compartment, potentially due to the high level proliferation of CD8 T cells in both the CAT-1^{Low} and CAT-1^{High} subsets **(Figure 5)**.

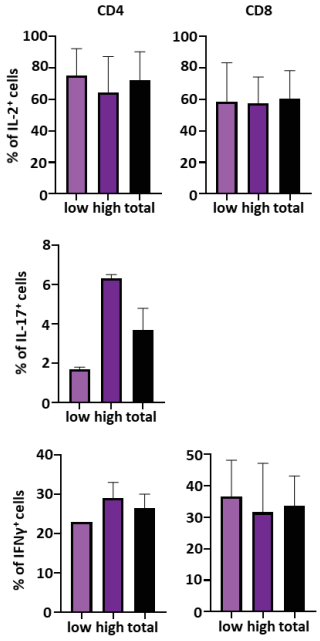

Figure 5 - High CAT-1 expression is associated with high IL-17 and IFN-γ expression in the CD4 T cell subset.

Purified human CD3$^+$ T cells were TCR-activated and sorted on the basis of CAT-1 expression at day 2 as indicated. Production of IL-2, IL-17, and IFN-γ was assessed in the CAT-1^{Low}, CAT-1^{High} and total CD4 and CD8 T cell subsets by intracellular staining at day 5/6, following restimulation with PMA/ionomycin in the presence of Brefeldin A for 4h. Means ± SEM of 2 independent experiments are presented.

Considering our previous work on the characterization of T cells selected based on Glut1 surface expression and the proliferation level of $CD4^+CAT\text{-}1^{High}$ cells, it was quite surprising not to detect a higher percentage of $IFN\gamma^+$ cells or memory phenotype associated to this subset. As a result, the cytokine/phenotype pattern associated to each subset appears as distinct and the major characteristic at this point seems that IL-17-producing cells are enriched in $CD4^+CAT\text{-}1^{High}$ cells as compared to $CD4^+CAT\text{-}1^{low}$. It is important to keep in mind that these results are representative of two independent experiments and further experiments are therefore required. However, and even if these results are preliminary, selection based on GLUT1 and CAT-1 surface expression does not seem to select/enrich similar T cell subsets. Therefore, these data open new perspectives regarding the use of distinct nutrient transporters to select T cells with different phenotype and effector functions.

Material and Methods

Human T cell isolation and culture. $CD3^+$ T cells were isolated from healthy adult donors. All experiments using primary human cells were conducted in accordance with the Declaration of Helsinki and IRB approval to the French Blood Bank (Etablissement Francais du Sang). T lymphocytes were purified by negative-selection using Rosette tetramers (StemCell Technologies) and the purity was monitored by flow cytometry. Lymphocytes were cultured in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco-Life technologies). T cell activation was performed using plate-bound anti-CD3 (clone OKT3, Biolegend) and anti-CD28 (clone 9.3, kindly provided by Carl June) mAbs at a concentration of 1 µg/ml. rIL-2 (100 U/ml) was added every other day starting either at day 2 or day 3 post-activation. Cells were maintained in a standard tissue culture incubator containing atmospheric (20%) O_2 and 5% CO_2.

Flow cytometry analyses. Immunophenotyping of cells was performed using fluorochrome-conjugated mAbs (BD Bioscience, Beckman Coulter or eBioscience) against CD4, CD8, CD45RA, CD45RO, CCR7 and CD62L and cell activation monitored using fluorochrome-conjugated mAbs BD Bioscience, Beckman Coulter or eBioscience) against CD25, CD69 and CD71. Proliferation was monitored by labeling cells with VPD450 (BD Biosciences; 1 µM) for 3 min at RT. For intracellular cytokine staining, lymphocytes were re-activated with PMA

(Sigma-Aldrich; 100 ng/ml) and ionomycin (Sigma-Aldrich; 1 µg/ml) in the presence of brefeldin A (Sigma-Aldrich; 10 ug/ml) for 3.5–4h at 37 °C. Intracellular staining for IL-2, IFN-γ and IL-17 was performed following fixation/ permeabilization using the eBioscience kit (Thermo-scientific) or using the BD kit (BD Biosciences). Cells were analyzed on LSRII-Fortessa (BD Biosciences) and data was analyzed using FlowJo (Tree Star) software.

Nutrient transporters surface expression detection. GLUT1 and CAT-1 expression at cell surface was monitored using RBD (receptor binding domain) ligands fused to rFc tail or eGFP (Kim et al., 2004; Kinet et al., 2007; Manel et al., 2003a). RBDs were incubated during 30 min at 37 °C. If required, after RBD incubation, an anti-rFc secondary antibody coupled to AF647 (life technology) was incubated during 30 min at 4 °C. The surface expression level was monitored by flow cytometry and was calculated as a function of the difference in average fluorescence intensity (ΔMFI) between the sample and its control condition (non-stained cells or cells incubated only with anti-rFc secondary antibody coupled to AF647).

Cell sorting based on surface CAT-1 expression. $CD3^+$ T cells maintained in Nutr+ medium and VPD450-labeled were stained at day 2 post TCR stimulation and the 15-16% of lymphocytes expressing the highest ($CAT\text{-}1^{High}$) and lowest levels of CAT-1 ($CAT\text{-}1^{Low)}$ were sorted on a FACSAria (BD Biosciences). Lymphocytes were cultured in RPMI medium 1640 supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco-Life technologies) in the presence of rIL-2.

Chapter 2
Impact of amino acid availability on T cell differentiation and effector function

Introduction

In the previous chapter we demonstrated that GLUT1 and CAT-1 surface expression could be used to select T cells with distinct phenotypes and effector functions. Altogether, our data suggest that nutrient transporters can be used to select T cells with increased anti-tumor function and a potentially better metabolic fitness. However, and despite of the advantages of injecting specific T cell subsets in cancer patients, it remains important to evaluate the impact of nutrient availability on T cell differentiation and effector function. T cell-based therapies have had much more success in the treatment of hematological malignancies than in the treatment of solid malignancies. One challenge in the latter context is that the ability of immune cells to maintain an effective response is often negatively modulated by the metabolic environment of the tumor. This environment is conditioned by nutrient composition, "waste" products, oxygen concentration, pH and physical forces, amongst others. The dysregulated growth of cancer cells can directly influence the extracellular environment and multiple studies now strongly suggest that the metabolic phenotype of the tumor governs the ensuing immune response (reviewed in (Ackerman and Simon, 2014; Mayers and Vander Heiden, 2017). Furthermore, recent data suggest that several metabolites, generated by distinct cells within the tumoral microenvironment (TME), may represent a form of intercellular communication modulating both tumor cell growth and response to therapy (Xu et al., 2019). One feature associated with the tumoral metabolic environment and directly linked to the proliferation of the tumor cells, is the depletion of nutrients in the TME. Indeed, in the context of several solid tumors, including hepatocellular carcinomas, stomach/colon tumors, and pancreatic tumors, quantitative metabolite profiling has revealed decreased intra-tumoral concentrations of glucose as well as glutamine (Bode and Souba, 1999; Hirayama et al., 2009; Kamphorst et al., 2015). In this context, our group as well as others have shown that the competition of T cells and tumor cells for limiting amounts of nutrients has a negative impact on the former, inhibiting T cell cytolytic and effector functions (Cham et al., 2008; Chang et al., 2013; Klysz et al., 2015; Macintyre et al., 2014; Metzler et al., 2016; Nakaya et al., 2014). Glucose and glutamine represent essential carbon/nitrogen sources, sustaining the proliferation and activity of T effector cells (Carr et al., 2010; Gerriets and Rathmell, 2012). This is illustrated by the upregulation of glucose and glutamine transporters following TCR stimulation as well as the associated increase in glucose

and glutamine metabolism (Carr et al., 2010; Gerriets and Rathmell, 2012; Kinet et al., 2007; MacIver et al., 2013; Manel et al., 2003b; Nakaya et al., 2014; Ren et al., 2017). However, T cells also strongly rely on others amino acids. Indeed, the presence of arginine-degrading enzymes, arginases, in the TME can result in arginine depletion and T cell anergy (Rodriguez et al., 2007). Furthermore, arginine supplementation was described to contribute to the production of pro-inflammatory cytokines and to the survival of central memory T cells in an *in vitro* setting (Geiger et al., 2016; Rodriguez et al., 2007). Thus, the metabolic fitness of an effector T cell in a nutrient-deprived tumor environment is likely to differ from its fitness in healthy lymphoid tissue. Finally, considering the divergent metabolic requirements of different T cell subsets (reviewed in (Buck et al., 2017), the TME may differentially promote the survival and/or proliferation of one subtype as compared to another.

It is therefore of critical importance to determine how metabolic modulation of anti-tumor T cells impact on their persistence and function, especially in the context of “hostile” tumor tissue with high nutrient consumption. As a result, part of my PhD research focused on advancing our understanding of how T cell responses are modulated by their metabolic environment and more specifically to elucidate the impact of glutamine and arginine deprivation, which represent some hallmarks of the TME on T cell function.

Article II

IMMUNOLOGY

Glutamine-dependent α-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation

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T cell activation requires that the cell meet increased energetic and biosynthetic demands. We showed that exogenous nutrient availability regulated the differentiation of naïve CD4$^+$ T cells into distinct subsets. Activation of naïve CD4$^+$ T cells under conditions of glutamine deprivation resulted in their differentiation into Foxp3$^+$ (forkhead box P3–positive) regulatory T (T_{reg}) cells, which had suppressor function in vivo. Moreover, glutamine-deprived CD4$^+$ T cells that were activated in the presence of cytokines that normally induce the generation of T helper 1 (T_H1) cells instead differentiated into Foxp3$^+$ T_{reg} cells. We found that α-ketoglutarate (αKG), the glutamine-derived metabolite that enters into the mitochondrial citric acid cycle, acted as a metabolic regulator of CD4$^+$ T cell differentiation. Activation of glutamine-deprived naïve CD4$^+$ T cells in the presence of a cell-permeable αKG analog increased the expression of the gene encoding the T_H1 cell–associated transcription factor Tbet and resulted in their differentiation into T_H1 cells, concomitant with stimulation of mammalian target of rapamycin complex 1 (mTORC1) signaling. Together, these data suggest that a decrease in the intracellular amount of αKG, caused by the limited availability of extracellular glutamine, shifts the balance between the generation of T_H1 and T_{reg} cells toward that of a T_{reg} phenotype.

INTRODUCTION

When T cells interact with their cognate foreign antigens, they undergo rapid activation, which requires considerable energy and cellular resources. These metabolic needs are secured by the augmented uptake and use of nutrients. Indeed, a sine qua non for optimal T cell proliferation and effector function is the T cell receptor (TCR)–stimulated increase in the cell surface amounts of glucose and glutamine transporters. The resulting increase in glucose and glutamine metabolism (*1*–*9*) results in a metabolic shift from the fatty acid oxidation that characterizes quiescent T cells (*10*–*12*).

Studies have shown that distinct T lymphocyte subsets exhibit disparate metabolic profiles; effector T (T_{eff}) cells are highly glycolytic and even lipogenic, whereas suppressive regulatory T (T_{reg}) cells display a mixed metabolism with increased lipid oxidation (*13*–*16*). Cellular metabolism is regulated, at least in part, by the mammalian target of rapamycin (mTOR) pathway. The mTOR complex 1 (mTORC1) is critical for the differentiation of naïve T cells into T helper 1 (T_H1) and T_H17 cells, as well as for the cytolytic activity of CD8$^+$ memory T cells, whereas mTORC2 signaling promotes the differentiation of naïve CD4$^+$ T cells into T_H2 cells (*17*–*21*). Conversely, inhibition of mTOR activity blocks the generation of T_{eff} cells, instead promoting the generation and function of Foxp3$^+$ (forkhead box P3–positive) T_{reg} cells (*22*–*24*). Consistent with these observations, the activation of mTOR blocks the differentiation of naïve CD4$^+$ T cells into T_{reg} cells and blocks T_{reg} function (*25*–*27*). Furthermore, decreasing mTOR activity by genetic deletion of nutrient transporters that are responsible for the uptake of glucose, leucine, or glutamine (*8*, *9*, *28*) inhibits the generation of T_{eff} cells without affecting T_{reg} cell generation.

Because pathological microenvironments can alter the nutrients available to a T cell, it is important to determine whether the external nutrient environment regulates the intrinsic differential potential of that cell. Indeed, nutrient concentrations within tumor microenvironments are generally reduced compared to those in normal tissues. Specifically, quantitative metabolomics profiling has revealed that the intratumoral concentrations of glucose and glutamine are reduced in patients with hepatocellular carcinomas and stomach and colon tumors (*29*, *30*). Furthermore, alkylating chemotherapies decrease the intracellular generation of the antioxidant glutathione because of limited glutamine availability (*31*–*34*).

Here, we demonstrated that nutrient availability plays a major role in regulating the differentiation of naïve CD4$^+$ T cells into different subsets. Stimulation of the TCR under conditions in which the amount of glutamine was limited resulted in the conversion of naïve CD4$^+$ T cells into Foxp3$^+$ T cells. This phenomenon was specifically a result of glutamine catabolism because it was recapitulated by a glutaminase inhibitor. The converted Foxp3$^+$ T cells exhibited increased proliferation in vivo as compared to conventional Foxp3$^-$ T cells, and they protected recombinase activating gene (Rag)–deficient mice from the development of T_{eff} cell–mediated autoimmune colitis. This de novo generation of T_{reg} cells occurred even under T_H1-polarizing conditions, abrogating the differentiation of naïve cells into T_H1 cells. This

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block in differentiation was associated with attenuated mTOR signaling and decreased expression of the gene encoding glutaminase 2 (GLS2), the enzyme that catalyzes the first step in the generation of α-ketoglutarate (αKG) from glutamine. Because αKG is incorporated into the tricarboxylic acid (TCA) cycle, the major anaplerotic step in proliferating cells (*35*, *36*), we assessed whether the glutamine-derived production of αKG was required for the generation of T_H1 cells. We found that supplementing glutamine-deprived $CD4^+$ T cells with a cell-permeable αKG ester under T_H1-polarizing conditions resulted in a marked increase in the abundance of Tbet, a transcription factor required for T_H1 cell differentiation, as well as interferon-γ (IFN-γ), a signature cytokine of T_H1 cells. This was associated with the activation of mTORC1, as demonstrated by increased phosphorylation of the ribosomal protein S6. Together, these data suggest that extracellular glutamine availability governs the concentration of intracellular αKG, which in turn functions as a metabolic regulator that determines whether naïve T cells differentiate into T_H1-type T_{eff} or T_{reg} cells.

RESULTS

T_{eff} cell function is differentially modulated by the deprivation of glucose or glutamine

To assess whether the relative availability of nutrients in the external environment regulated the differentiation potential of naïve $CD4^+$ T cells, we stimulated lymphocytes with activating antibodies (anti-CD3/CD28) specific for the TCR complex component CD3 and the co-receptor CD28 in either complete medium or medium deficient in glucose or glutamine. Whereas the early TCR-stimulated increases in the production of the cytokines interleukin-2 (IL-2) and IL-17 were not inhibited, glutamine withdrawal resulted in an increase in the percentage of $Foxp3^+$ cells (Fig. 1A). Furthermore, we also measured the increased abundance of *foxp3* mRNA in cells cultured under glutamine-deprived, but not glucose-deprived, conditions (Fig. 1B). Thus, this increased gene expression did not represent a response to nutrient deprivation per se but rather was a specific consequence of glutamine starvation. To determine whether the increased Foxp3 expression in cells cultured under glutamine-deprived conditions was specifically a result of changes in glutamine catabolism, we blocked glutaminolysis with 6-diazo-5-oxo-L-norleucine (DON), a glutaminase inhibitor that functions only when the enzyme is active (*37*). There was an approximately 45-fold increase in *foxp3* mRNA abundance in DON-treated cells compared to that in cells cultured under normal nutrient conditions (Fig. 1B). Thus, either limiting the available amount of extracellular glutamine or inhibiting intracellular glutaminolysis resulted in increased *foxp3* expression in stimulated naïve $CD4^+$ T cells.

The increased percentage of $Foxp3^+$ T cells under glutamine-deprived conditions was detected 96 hours after TCR stimulation (Fig. 1C) and was not a result of their preferential proliferation as compared to that of $CD4^+Foxp3^-$ T cells. Whereas $CD4^+$ T cell proliferation was reduced in the absence of glutamine or in the presence of DON, the relative proliferation profiles of the $Foxp3^-$ and $Foxp3^+$ cells were similar under all conditions (Fig. 1C, right panels). Moreover, although glucose deprivation also reduced the TCR-stimulated proliferation of $CD4^+$ T cells, there was no accompanying increase in the percentage of $Foxp3^+$ cells (Fig. 1C). Thus, the biased TCR-stimulated generation of $Foxp3^+$ T cells was specific to conditions of glutamine deprivation. Furthermore, this biased generation of $Foxp3^+$ T cells was reversed by the addition of low amounts of glutamine to the cell culture medium (fig. S1).

These data gave rise to two nonexclusive hypotheses. First, the survival of $Foxp3^+$ cells relative to that of $Foxp3^-$ cells is modulated when glutamine abundance is limiting. Second, $Foxp3^-CD4^+$ T cells are converted to $Foxp3^+$ cells under conditions of glutamine deprivation. To test the second hypothesis, we purified $CD4^+CD62L^+CD44^-CD25^-GFP^-$ conventional naïve T cells from reporter mice expressing green fluorescent protein (GFP) under the control of the *foxp3* promoter (Foxp3-GFP) (*38*, *39*) and found that deprivation of glutamine, but not glucose, led to an increase in the percentage of $Foxp3^+$ cells that were generated in response to T cell stimulation (Fig. 1D, $P < 0.0001$). These data suggest that glutamine deprivation in the context of T cell stimulation favors the conversion of $Foxp3^-$ cells to $Foxp3^+$ cells.

The conversion of naïve $CD4^+$ T cells into $Foxp3^+$ $CD4^+$ T cells under glutamine-deprived conditions results from TGF-β–dependent signaling

Given the central role of transforming growth factor–β (TGF-β) in the conversion of naïve T cells to $Foxp3^+$ T_{reg} cells, we evaluated its potential involvement in the enhanced generation of these cells under glutamine-deprived conditions. Although we had not added exogenous TGF-β to the cell culture medium, we evaluated the possibility that either the low amount of TGF-β present in the fetal calf serum (FCS) or any TGF-β produced by the cells themselves was sufficient to drive the differentiation of naïve $CD4^+$ T cells into $Foxp3^+$ T cells under glutamine-deprived conditions. Indeed, a neutralizing anti–TGF-β antibody reduced the percentage of $Foxp3^+$ T cells generated under glutamine-deprived conditions in a proliferation-independent manner (Fig. 2A). Furthermore, SB431542, an inhibitor of the TGF-β receptor 1 (TGFβR1) signaling pathway (*40*), statistically significantly decreased the generation of $Foxp3^+$ cells under conditions in which glutamine abundance was limiting (Fig. 2B, $P < 0.0001$). Finally, we evaluated the T_{reg} cell–specific demethylated region (TSDR), an enhancer element of the *Foxp3* locus that is selectively demethylated in stable $Foxp3^+$ T_{reg} cells (*41*–*43*), and found that the methylation status of the TSDR in TGF-β–induced $Foxp3^+$ T_{reg} (iT_{reg}) cells was similar to that in $Foxp3^+$ cells generated in the absence of glutamine (fig. S2).

$Foxp3^+$ T cells generated in the absence of glutamine show enhanced proliferation in vivo

Our earlier experiments demonstrated that glutamine depletion promotes the in vitro differentiation of naïve $CD4^+$ T cells into $Foxp3^+$ T cells. It was nonetheless not clear whether these $Foxp3^+$ T cells would be maintained after their transfer into lymphopenic hosts and how their proliferation in vivo would compare with that of the $Foxp3^-$ T cells derived from the same cultures. To evaluate this question, we activated naïve $CD4^+$ T cells from C57BL/6-Thy1.1 mice for 5 days in nutrient-replete or glutamine-deprived conditions or in the presence of DON (Fig. 3A). Under the glutamine-altered conditions, the percentages of $Foxp3^+$ T cells increased between 15 to 35% (Fig. 3A). The cells were then labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and adoptively transferred into sublethally irradiated C57BL/6-Thy1.2 recipient mice. The ratio of $Foxp3^+$ to $Foxp3^-$ donor T cells from both the glutamine-depleted cultures and the DON-treated cultures remained substantially greater than that of cells derived from the nutrient-replete control culture on day 7 after adoptive transfer (means of 20 to 40% as compared to 10%; $P < 0.005$) and was associated with an increased in vivo proliferation of the $Foxp3^+$ cells (Fig. 3B). Thus, the $Foxp3^+$ T cells generated ex vivo by culturing under glutamine-deprived conditions were maintained after their adoptive transfer to recipient mice.

$Foxp3^+$ T cells generated under glutamine-deficient conditions show suppressive activity in vivo

To evaluate the in vivo suppressive potential of $Foxp3^+$ T cells generated under glutamine-deprived conditions in vitro, we assessed their ability to prevent inflammatory bowel disease (IBD) in $Rag2^{-/-}$ mice injected with naïve $CD4^+$ T_{eff} cells. Briefly, naïve $CD4^+$ T cells isolated from Foxp3-GFP reporter mice were stimulated under glutamine-deprived conditions

for 5 days before being sorted on the basis of GFP abundance (fig. S3). Consistent with previous reports (*44–48*), injection of Rag2$^{-/-}$ mice with T_{reg} cells generated in the thymus of wild-type mice [which are referred to as thymic T_{reg} or natural T_{reg} (nT_{reg}) cells] protected the Rag2$^{-/-}$ mice from IBD induced by CD4$^+$CD45RB^{hi} T_{eff} cells during the entire 9-week follow-up period (Fig. 4, A and B). Injection with Foxp3$^+$ cells generated in vitro under glutamine-deprived conditions also fully protected Rag2$^{-/-}$ mice co-injected with T_{eff} cells from IBD during the entire follow-up period, an effect that was associated with reduced cell surface abundance of the effector molecule CD44 on the injected T_{eff} cells (Fig. 4C). Moreover, the Foxp3$^+$

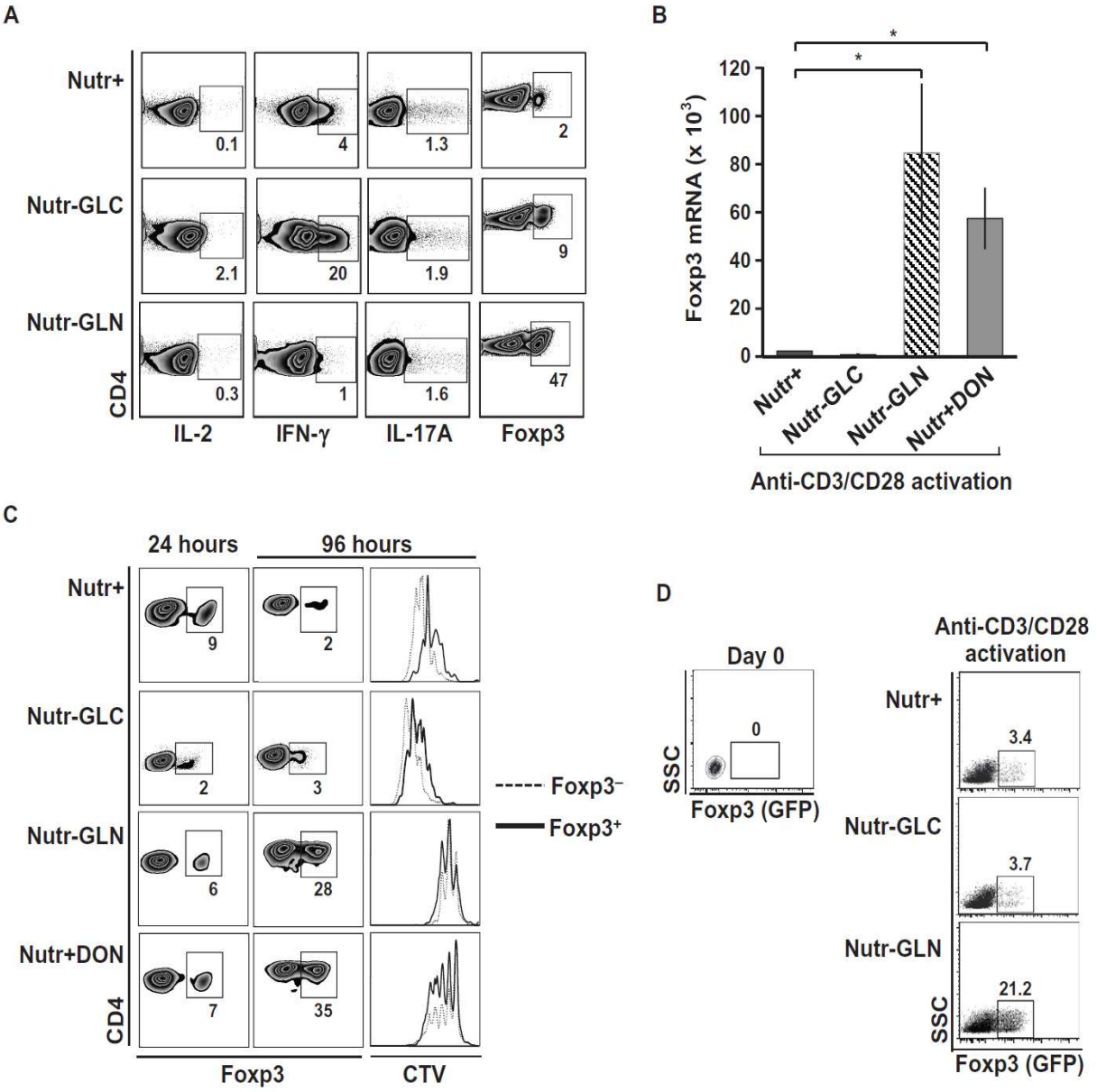

Fig. 1. Glutamine deprivation promotes the conversion of naïve CD4$^+$ T cells into Foxp3$^+$ T cells. (A) Naïve mouse CD4$^+$ T cells were activated by immobilized anti-CD3 and anti-CD28 monoclonal antibodies (anti-CD3/CD28 activation) in nutrient-replete medium (Nutr+), glucose-free medium (Nutr–GLC), or glutamine-free medium (Nutr–GLN). After 96 hours, the percentages of CD4$^+$ cells that were positive for IL-2, IFN-γ, IL-17A, or Foxp3 were determined by intracellular staining and flow cytometric analysis. Percentages are indicated in the dot plots, which are representative of four experiments. (B) Naïve CD4$^+$ T cells cultured under the indicated conditions and activated for 96 hours with anti-CD3/CD28 were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine the relative amounts of *Foxp3* mRNA normalized to that of *Hprt* mRNA. Data are means ± SD of triplicate samples from one experiment, which is representative of four independent experiments. *$P < 0.05$ by analysis of variance (ANOVA). (C) Left: Freshly isolated CD4$^+$ lymph node T cells loaded with Cell Trace Violet (CTV) and cultured under the indicated conditions were activated for 24 or 96 hours and then were analyzed by flow cytometry to determine the percentages of Foxp3$^+$ cells. Right: At 96 hours, the proliferation profiles of Foxp3$^-$CD4$^+$ T cells (dashed line) and Foxp3$^+$CD4$^+$ T cells (solid line) were monitored by flow cytometric analysis of the dilution of CTV. Dot plots and histograms are representative of three experiments. (D) Naïve conventional CD4$^+$CD62L$^+$CD44$^-$CD25$^-$GFP$^-$ T cells were sorted by FACS (fluorescence-activated cell sorting) from Foxp3-GFP reporter mice, and their purity was monitored by flow cytometry (left panel; day 0). The cells were then activated under the indicated conditions, and the appearance of Foxp3$^+$ cells was determined by flow cytometric analysis after 96 hours. The percentages of Foxp3$^+$ cells are indicated in the dot plots. Data are representative of four independent experiments. SSC, side scatter.

cells generated in vitro under glutamine-deprived conditions exhibited enhanced persistence compared to that of nT_{reg} cells (Fig. 4D). These data suggest that glutamine withdrawal promotes the suppressive activity and in vivo persistence of the generated T_{reg} cells.

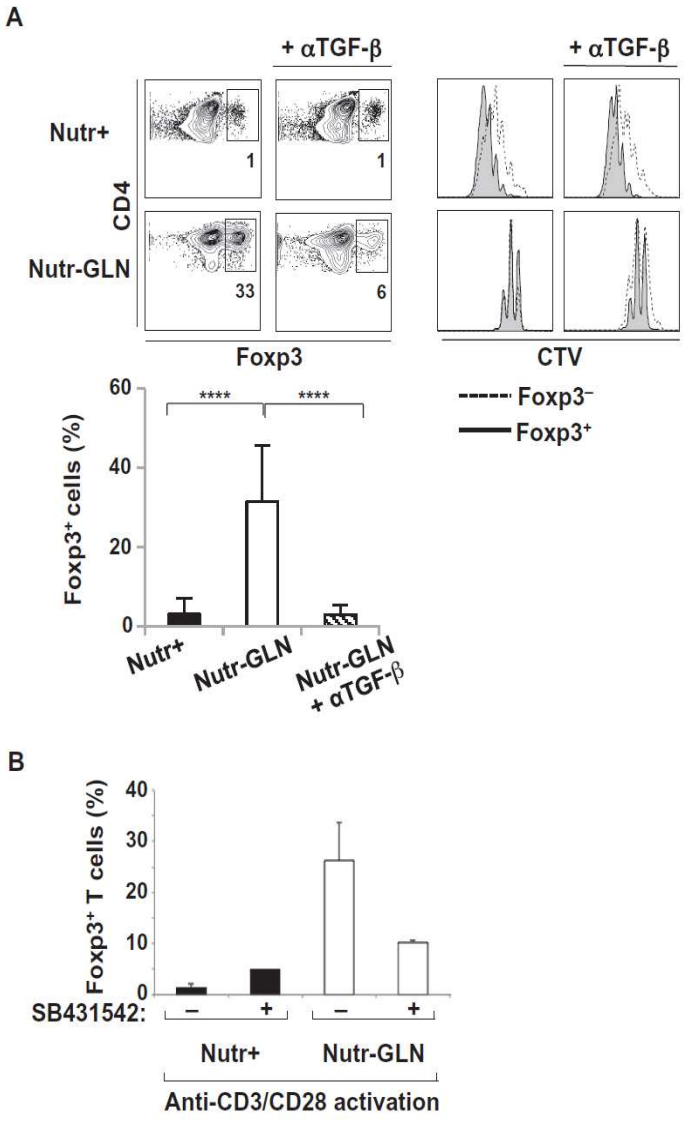

Fig. 2. Glutamine deprivation promotes the TGF-β–dependent conversion of naïve $CD4^+$ T cells into $Foxp3^+$ T_{reg} cells. (A) Naïve $CD4^+$ T cells loaded with CTV were activated for 4 days under the indicated nutrient conditions in the absence or presence of a neutralizing anti–TGF-β antibody (αTGF-β). Left: The percentages of $Foxp3^+$ cells on day 4 of activation are indicated in the dot plots. Right: The relative proliferation profiles of $Foxp3^-CD4^+$ and $Foxp3^+CD4^+$ T cells under each condition were monitored on day 4 of activation as a function of CTV fluorescence and are presented in histograms. Bottom: Quantification of the percentages of $Foxp3^+$ T cells generated under glutamine-deficient conditions, in the presence or absence of TGF-β. Data are means ± SD of three independent experiments. $^{****}P < 0.0001$ by χ^2 test. (B) Naïve $CD4^+$ T cells were activated for 5 days under the indicated nutrient conditions in the presence or absence of the TGF-β receptor signaling inhibitor SB431542. The percentages of $Foxp3^+$ T cells on day 5 of activation were assessed by flow cytometric analysis. Data are means ± SD of two experiments.

Glutamine deprivation inhibits the differentiation of naïve $CD4^+$ T cells into T_H1 cells and increases the generation of $Foxp3^+$ cells

Next, we investigated whether glutamine deprivation affected the generation of Foxp3-expressing cells after the activation of naïve T cells under cell-polarizing conditions. As expected, most naïve $CD4^+$ T cells cultured under iT_{reg}-polarizing conditions became $Foxp3^+$ cells, and this percentage was not modulated by glutamine availability (Fig. 5A). These data suggest that the potential of a naïve $CD4^+$ T cell to differentiate into an iT_{reg} cell occurs in a glutamine-independent manner.

To determine whether glutamine availability conditions the potential of a cell to differentiate into a specific T_{eff} cell type, we stimulated naïve $CD4^+$ T cells under T_H1- or T_H2-polarizing conditions and monitored their differentiation as a function of the abundances of the mRNAs for the transcription factors Tbet and GATA3, respectively. Glutamine deprivation blocked the expression of *Tbet* under T_H1-polarizing conditions but had no effect on the expression of *GATA3* under T_H2-polarizing conditions (Fig. 5B). Furthermore, this change in *Tbet* expression in glutamine-deprived cells was associated with an almost complete absence of IFN-γ secretion by $CD4^+$ T cells exposed to T_H1-polarizing cytokines (Fig. 5C, $P < 0.0001$). In marked contrast, glutamine deprivation resulted in enhanced IL-4 production by cells cultured under T_H2-polarizing conditions (Fig. 5C). Note that the differentiation of naïve cells into T_H1 cells was not inhibited by glucose starvation (fig. S4), suggesting that there are substantial differences in the capacity of energetic fuel sources to support T_{eff} cell differentiation.

Given that glutamine-deprived naïve $CD4^+$ T cells were unable to undergo differentiation into T_H1 cells under T_H1-polarizing conditions, we next monitored *Foxp3* expression in these cells. We found that the percentage of $Foxp3^+$ cells was markedly increased under T_H1-polarizing, but not T_H2-polarizing, conditions in the context of glutamine deprivation (Fig. 5D, $P < 0.0001$). Furthermore, *Foxp3* mRNA abundance was increased fivefold in glutamine-deprived cells compared to that in cells cultured with complete nutrients (Fig. 5D). These data suggest that the nutrient milieu affects the expression of *Foxp3*, even in cells cultured in the presence of cytokines that would be expected to bias a naïve $CD4^+$ T cell to differentiate into a specific T_{eff} cell type.

On the basis of these data, it was of interest to determine whether endogenous TGF-β was also responsible for the emergence of $Foxp3^+$ T cells under T_H1-polarizing conditions. Indeed, anti–TGF-β antibodies inhibited the conversion of naïve $CD4^+$ T cells to $Foxp3^+$ cells under T_H1-polarizing conditions (Fig. 6A). However, even though the generation of $Foxp3^+$ cells was dependent on TGF-β, neutralizing this cytokine was not sufficient to promote T_H1 cell polarization under glutamine-deprived conditions. Specifically, Tbet abundance was not restored in cells treated with the anti–TGF-β antibody and cultured under T_H1-polarizing conditions (Fig. 6B), and cells cultured under these conditions did not produce sufficient amounts of IFN-γ (Fig. 6C). In contrast, the differentiation of naïve $CD4^+$ T cells into T_H2 cells was not sensitive to the inhibition of TGF-β (Fig. 6B). Together, these results indicate that, under limiting glutamine conditions, the conversion of $Foxp3^-$ cells into $Foxp3^+$ cells under T_H0- or T_H1-polarizing conditions was dependent on TGF-β. Nevertheless, inhibiting TGF-β signaling was not sufficient to enable T_H1 cell generation under these conditions of glutamine deprivation.

Generation of the glutamine-derived metabolite αKG is the rate-limiting step in the differentiation of naïve cells into T_H1 cells

To determine how glutamine deprivation affected the intracellular metabolism of the cell, we first assessed glutamine uptake. Whereas T cell stimulation increased glutamine uptake by >25-fold compared to that of unstimulated cells, T cell stimulation of glutamine-deprived or DON-treated cells statistically

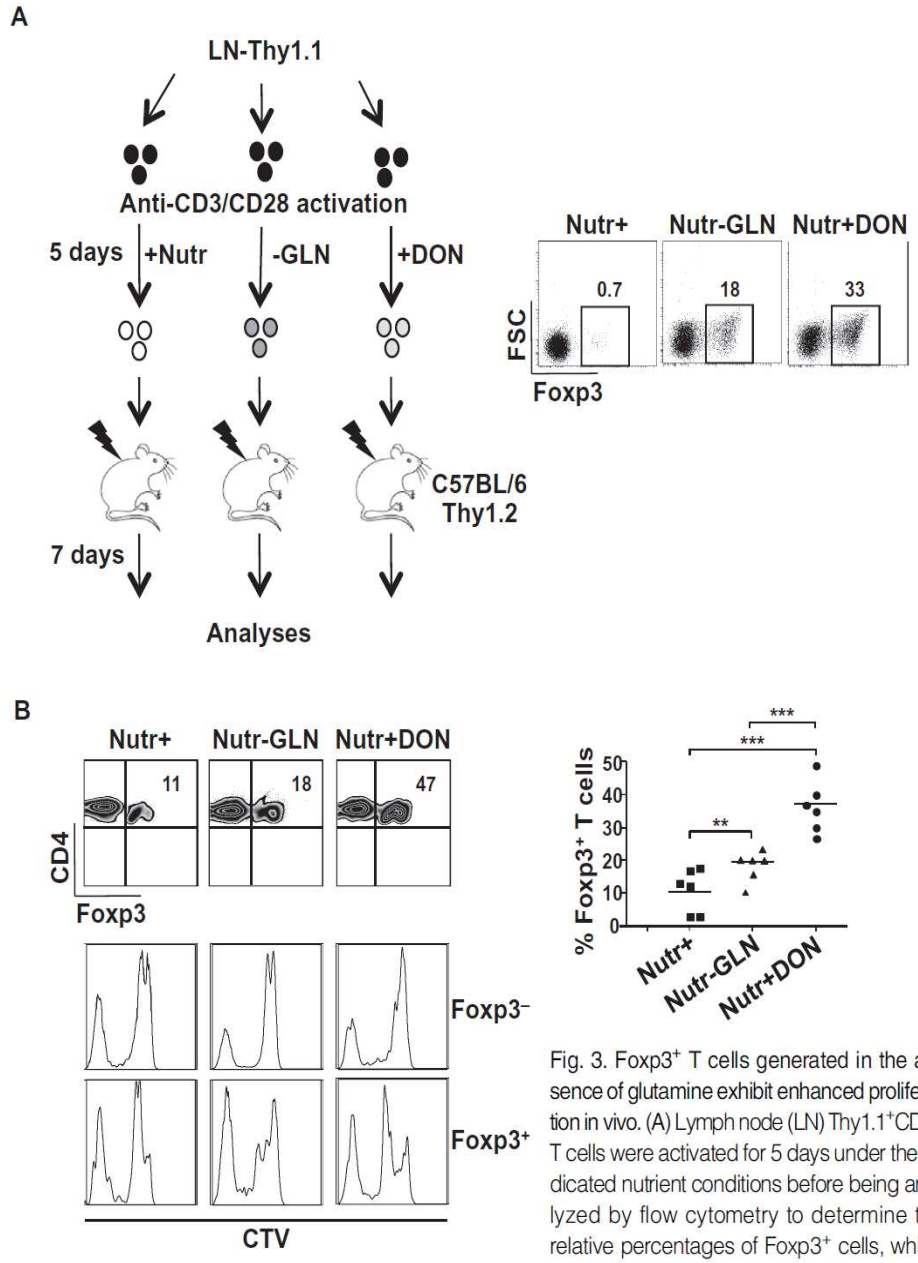

Fig. 3. Foxp3$^+$ T cells generated in the absence of glutamine exhibit enhanced proliferation in vivo. (A) Lymph node (LN) Thy1.1$^+$CD4$^+$ T cells were activated for 5 days under the indicated nutrient conditions before being analyzed by flow cytometry to determine the relative percentages of Foxp3$^+$ cells, which are shown in the representative dot plots (right). The cells were then labeled with CTV and transferred into congenic Thy1.2$^+$ C57BL/6 mice that were previously rendered lymphopenic by irradiation (5.5 Gy). The mice were sacrificed 7 days later, and the status of the adoptively transferred T cells was assessed by flow cytometry. FSC, forward scatter. (B) The engraftment of the transferred T cells was monitored as a function of Thy1.1 expression (dot blots, top left), and the presence of Foxp3 within the indicated transferred CD4$^+$ T cell populations was also assessed (right panel). The percentages of Foxp3$^+$ cells are indicated in each dot plot. Right: Quantification of the percentages of Foxp3$^+$ donor T cells. Each point represents data from a single mouse. Means are indicated by horizontal lines. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Student's t test and ***$P < 0.001$ by ANOVA. The proliferation of Foxp3$^-$CD4$^+$ T cells relative to that of Foxp3$^+$CD4$^+$ T cells was monitored in individual mice as a function of CTV fluorescence. Representative histograms for the indicated conditions are shown (bottom panels). Data are representative of two independent experiments and each experiment was performed with three or four mice per group.

significantly decreased their glutamine uptake (Fig. 7A, $P < 0.05$). Furthermore, glutamine deprivation attenuated the T cell–stimulated increase in the abundance of the mRNA encoding GLS2 (Fig. 7B), a key enzyme in the conversion of glutamine to glutamate. Because the catabolism of glutamine contributes to the metabolism of T cells, these data suggest that the metabolic phenotypes of CD4$^+$ T cells activated under nutrient-replete or glutamine-deprived conditions are distinct.

In cells cultured under nutrient-replete conditions, TCR stimulation resulted in a >10-fold increase in the basal oxygen consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS); however, the OCR was statistically significantly decreased in glutamine-deprived cells (Fig. 7C, $P < 0.001$). These data are concordant with a study that showed that T_{reg} cells have a lower level of mitochondrial OCR than T_{eff} cells (*49*). Furthermore, T_{reg} cells exhibit reduced glycolysis compared to T_{eff} cells, as determined by measurement of the extracellular acidification rate (ECAR) (*49*). Indeed, we found that stimulated glutamine-deprived T cells showed a 10-fold decrease in basal ECAR compared to stimulated control T cells (Fig. 7C, $P < 0.001$). On the other hand, T_{reg} cells have a high spare respiratory capacity (SRC) (*49*), which potentially enables them to respond to starvation conditions in which glucose is the only fuel, and this was indeed the case for glutamine-deprived CD4$^+$ T cells (Fig. 7C). As expected from the decreased OXPHOS and ECAR in glutamine-deprived CD4$^+$ T cells, they had a statistically significantly decreased adenosine triphosphate (ATP) concentration compared to that of cells cultured with complete nutrients (Fig. 7C, $P < 0.01$), revealing an important role for glutaminolysis in the energy homeostasis of an activated CD4$^+$ T cell.

Glutamine is catabolized to generate αKG, which supports energy production through TCA cycle anaplerosis (Fig. 7D). We therefore assessed whether glutamine-derived αKG was critical for the commitment of activated CD4$^+$ T lymphocytes to become T_H1 cells. To this end, glutamine-deprived naïve CD4$^+$ T cells activated under T_H1-polarizing conditions were supplemented with a cell-permeable αKG derivative, dimethyl αKG (DMK). When supplemented with DMK, glutamine-deprived cells showed an increased abundance of Tbet, the transcription factor required for the generation of T_H1 cells, which was comparable to that in control T_H1-polarized cells (Fig. 7E). Furthermore, DMK-supplemented cultures exhibited a substantial increase in the percentage of IFN-γ$^+$ cells (Fig. 7E). Supplementing glutamine-deprived CD4$^+$ T cells with DMK also decreased the generation of Foxp3$^+$ cells (Fig. 7E). Thus, the reduced abundance of αKG correlates with the increased production of Foxp3.

The differentiation of naïve CD4$^+$ T cells into T_H1 cells depends on the activation of the mTORC1 pathway (*17*, *22*, *50*), and glutamine transport and the subsequent catabolism of glutamine are required to stimulate mTOR signaling pathways (*9*, *51*–*54*). Furthermore, the extent of mTOR signaling inversely correlates with the generation of T_{reg} cells (*22*–*27*). Indeed, we

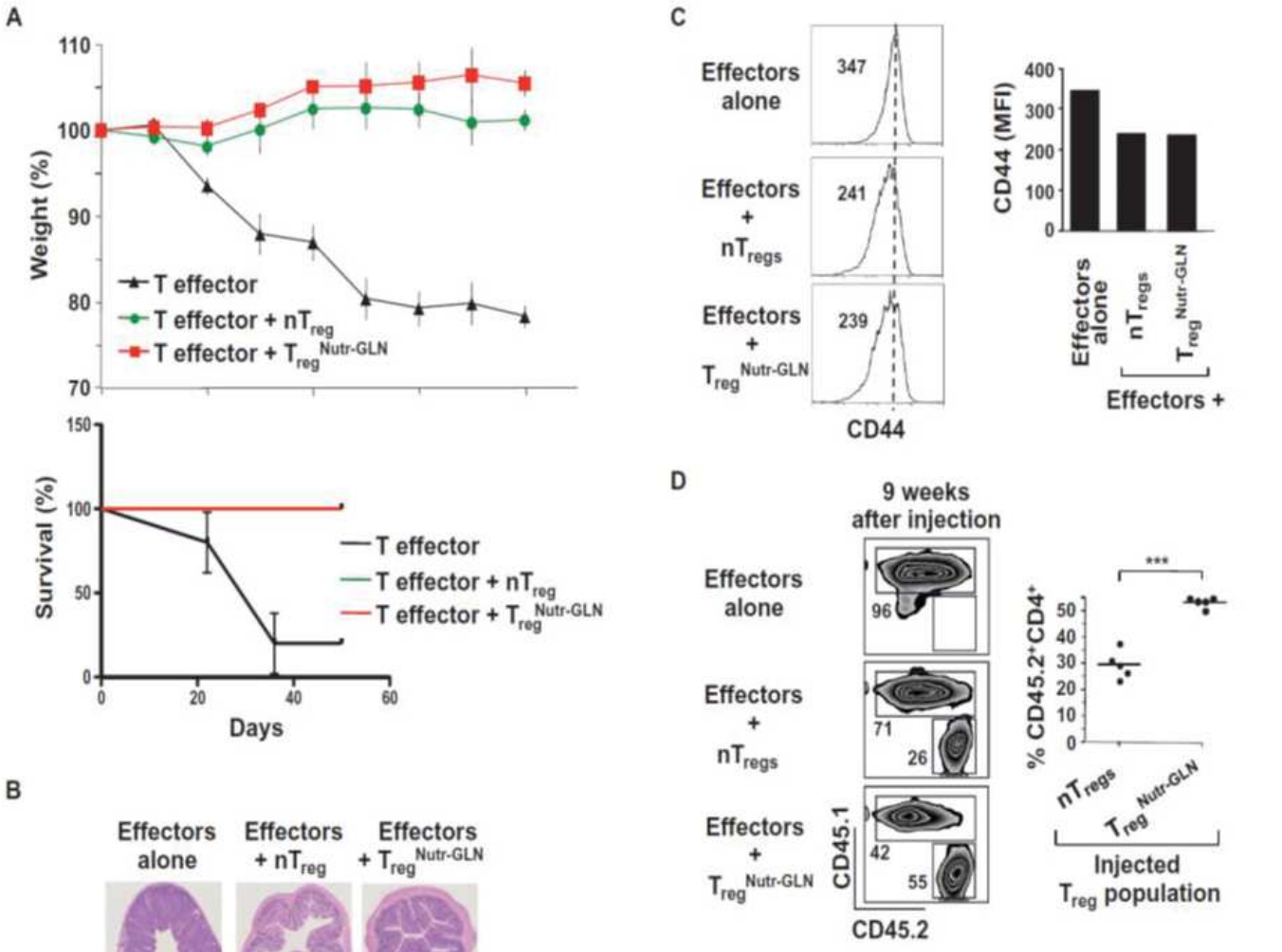

Fig. 4. T_{reg} cells generated under glutamine-deficient conditions inhibit effector T cell–mediated autoimmune colitis. (A to D) Rag2-deficient mice were injected with sorted $CD4^+CD45RB^{hi}$ ($CD45.1^+$) T_{eff} cells alone or together with purified nT_{reg} cells or glutamine-deprived in vitro–generated $Foxp3^+CD4^+$ T cells ($T_{reg}^{Nutr\text{-}GLN}$). (A) Top: The injected Rag2-deficient mice were examined for weight loss on a weekly basis, and the mean weights in each group are presented as a function of the initial weight. Data are means ± SD of five mice per group. Bottom: The survival rate in each group is presented at the indicated times as means ± SD. The log-rank (Mantel-Cox) test and test for trend indicate *P* values of 0.0089 and 0.0087, respectively. (B) Colons from mice injected with the indicated T cells were collected, fixed, and stained with hematoxylin and eosin (H&E). Representative sections from each group of mice are shown. The magnifications in the upper and lower panels are ×5 and ×10, respectively. (C) $CD45.1^+CD4^+$ T_{eff} cells isolated from Rag2-deficient mice injected with the indicated T cells were analyzed by flow cytometry to determine the cell surface abundance of the activation marker CD44. Representative histograms are shown. The vertical dotted line delineates the mean fluorescence intensity (MFI) of CD44 staining on $CD45.1^+CD4^+$ T cells isolated from Rag2-deficient mice injected with T_{eff} cells alone. Right: Mean MFIs of CD44 staining in each group. Data are means ± SD of five mice in each group. (D) Left: The relative recovery of injected T_{eff} cells ($CD45.1^+$) and T_{reg} cells ($CD45.2^+$) in the different groups of mice was assessed 9 weeks after adoptive transfer. Representative dot plots discriminating between $CD45.1^+$ T_{eff} cells and $CD45.2^+$ T_{reg} cells in the mesenteric lymph node (mLN) of the mice are presented for each condition. Right: The proportion of $CD45.2^+$ T_{reg} cells recovered from the mLN of individual injected Rag2-deficient mice from each group is quantified, and each point represents data from a single mouse. Data are means from five mice per group. Means are indicated by horizontal lines. ***$P < 0.001$ by Student's *t* test.

found that the phosphorylation of ribosomal protein S6, a readout of mTORC1 activity, was reduced by more than fourfold in $CD4^+$ T cells undergoing T_H1 polarization in glutamine-deprived as compared to nutrient-replete conditions (Fig. 7E). Because αKG directly activates mTORC1 signaling in multiple primary and transformed cell lines (*51*), we next assessed whether DMK affected mTORC1 signaling in glutamine-deprived cells. We found that phosphorylated S6 (pS6) was increased two- to threefold in abundance in DMK-treated cells compared to that in glutamine-deprived cells (Fig. 7E, $P < 0.05$). Together, these data suggest that increasing the intracellular concentration of αKG enhances mTORC1 signaling, which supports the differentiation of naïve cells into T_H1 cells.

DISCUSSION

Our data suggest that glutamine availability is a key determinant of the differentiation of naïve $CD4^+$ T cells. Low glutamine availability promoted the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ T_{reg} cells, which displayed highly effective regulatory function. The marked increase in the generation

of Foxp3$^+$ T cells under these conditions was dependent on signaling by endogenous TGF-β. The Foxp3$^+$ T cells that were generated under glutamine-deprived conditions exhibited robust in vivo proliferative potential as well as suppressor activity, controlling autoimmune colitis in a mouse model of T cell adoptive transfer. Moreover, the skewing of glutamine-deprived naïve CD4$^+$ T cells toward Foxp3$^+$ cells occurred even under T_H1-polarizing conditions, blocking their differentiation into T_H1 cells. Our data identify a metabolic program that conditions T_H1 lineage specification. We found that the

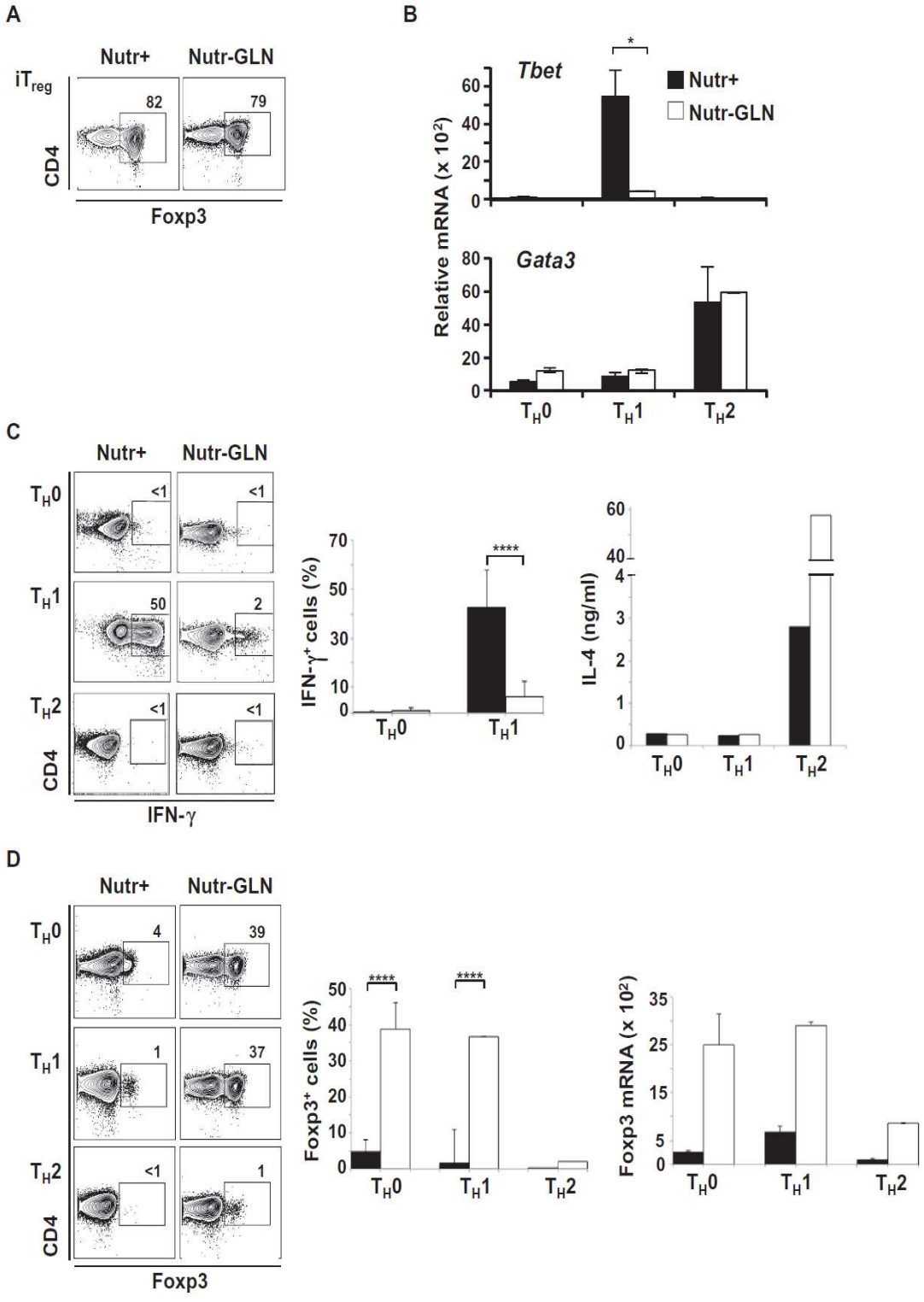

Fig. 5. Glutamine deprivation inhibits the generation of T_H1 cells but promotes the generation of T_{reg} cells. (A) Naïve CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ T cells were activated under iT$_{reg}$-inducing conditions in the presence or absence of glutamine. After 6 days of activation, the percentages of Foxp3$^+$ cells were determined by intracellular staining and flow cytometric analysis. Dot plots are representative of three experiments. (B) Naïve CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ T cells cultured under the indicated polarizing and nutrient conditions were analyzed after 30 hours by qRT-PCR to determine the relative amounts of *Tbet* (top) and *Gata3* (bottom) mRNAs normalized to that of *Hprt* mRNA. Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments. *$P < 0.05$ by Student's *t* test. (C) Naïve CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ T cells were activated for 6 days under T_H0-, T_H1-, or T_H2-polarizing conditions in complete or glutamine-deficient medium, as indicated. Left: The percentages of CD4$^+$ T cells that were positive for IFN-γ were determined by intracellular staining and flow cytometric analysis. Dot plots are representative of eight experiments. Middle: Mean percentages ± SD of IFN-γ–secreting cells ($n = 8$ experiments). Statistical difference was determined by χ^2 (****$P < 0.0001$) and Mann-Whitney tests ($P < 0.001$). Right: IL-4 production by the indicated cells was assessed by cytometric bead array. Data are means from one experiment and are representative of two independent experiments. (D) Naïve CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ T cells were activated under T_H0-, T_H1-, or T_H2-polarizing conditions in nutrient-replete or glutamine-depleted medium. Left: After 6 days of activation, the percentages of Foxp3$^+$ cells were determined by flow cytometric analysis. Dot plots show the percentages of Foxp3$^+$ cells. Middle: Quantification of the percentages of Foxp3$^+$ T cells. Data are presented as means ± SD of eight experiments. *P* values were determined by χ^2 (****$P < 0.0001$) and Mann-Whitney tests ($P < 0.05$). Right: The indicated cells were analyzed by qRT-PCR to determine the relative amounts of *Foxp3* mRNA normalized to that of *Hprt* mRNA. Data are means ± SD of triplicate samples from a single experiment and are representative of two independent experiments.

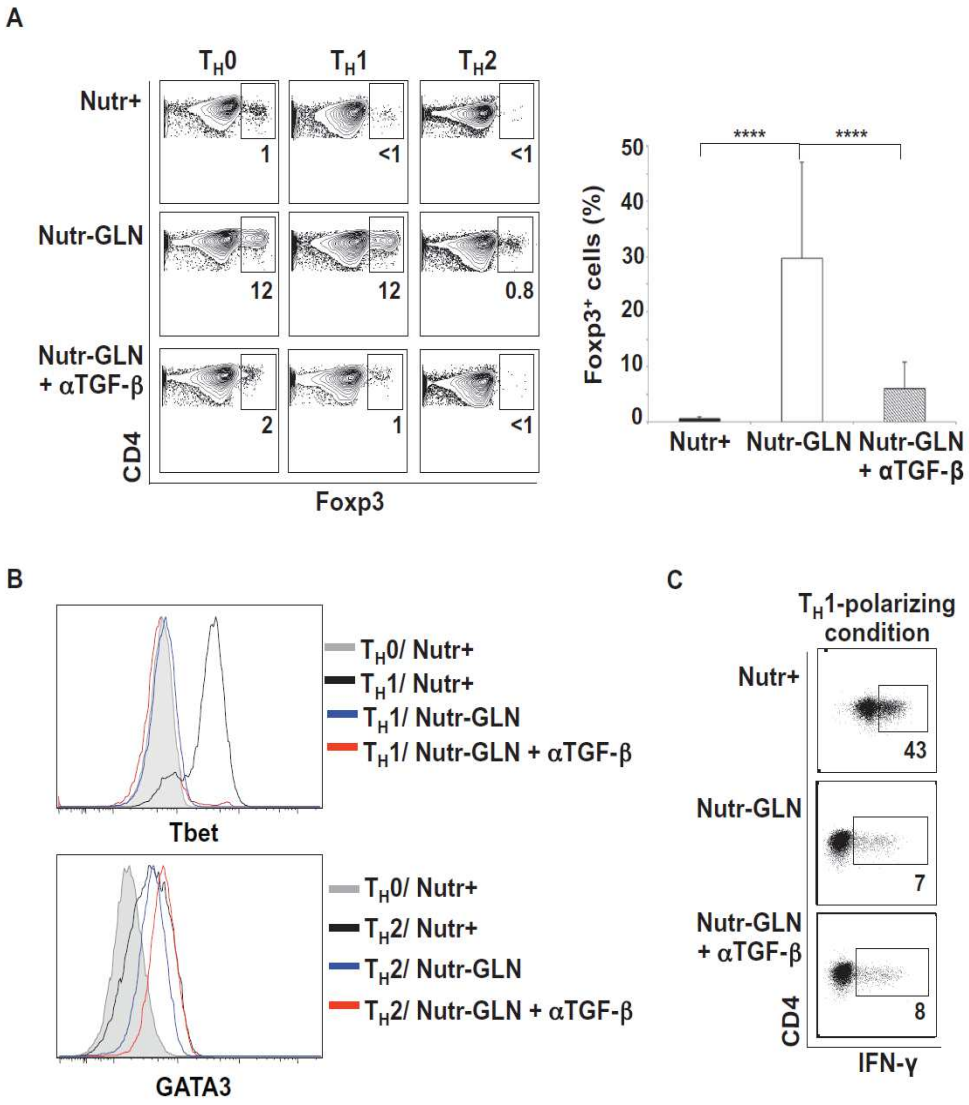

Fig. 6. Inhibition of TGF-β signaling abrogates the conversion of glutamine-deprived CD4$^+$ T cells to T_{reg} cells but cannot support T_H1 cell generation. (A to C) Naïve CD4$^+$ T cells were stimulated under T_H0-, T_H1-, or T_H2-polarizing conditions in nutrient-replete or glutamine-deprived medium in the presence or absence of an anti–TGF-β neutralizing antibody. (A) After 96 hours of activation, the CD4$^+$ T cells were analyzed by flow cytometry to determine the percentages of Foxp3$^+$ cells. Left: Dot plots show the percentages of Foxp3$^+$ cells. Right: Quantification of the percentages of Foxp3$^+$ T cells. Data are means ± SD of three independent experiments. ****$P < 0.0001$ by χ^2 test. (B) After 96 hours of stimulation, the CD4$^+$ T cells were analyzed by flow cytometry to determine the relative abundances of the transcription factors Tbet (top) and GATA3 (bottom). Histograms are representative of four independent experiments. (C) On day 6 of activation, the CD4$^+$ T cells were analyzed by flow cytometry to determine the percentages of IFN-γ–producing cells. Dot plots show the percentages of IFN-γ$^+$ cells and are representative of three independent experiments.

differentiation of naïve cells into T_H1 cells required that glutamine be catabolized to αKG, replenishing the pools of metabolic intermediates in the TCA cycle and substantially increasing mTORC1 signaling. Under conditions in which glutamine use was limiting, the addition of an αKG ester restored the capacity of a cell to generate Tbet and adopt a T_H1 cell phenotype.

These data raise the question of why glutamine-derived αKG is required for the differentiation of naïve cells into T_H1-type T_{eff} cells, but not into anti-inflammatory T_{reg} cells. The entry of metabolic intermediates into the TCA cycle is central to energy metabolism, whereas their exit fosters the synthesis of biological molecules that are 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 (*36*, *55*, *56*). By analogy, this would suggest that T_H1 cells, but not T_{reg} cells, present a metabolic state that requires the support of a high level of anabolism. Indeed, T_{eff} cells show high rates of glycolysis, whereas suppressive T_{reg} cells exhibit a higher dependence on fatty acid oxidation (*13*, *14*). Furthermore, although T_{reg} cells can take up exogenous fatty acids, T_H17 cells depend on the de novo fatty acid synthesis, which is costly in terms of ATP (*16*). Together, these data suggest that T_{eff} cells have higher metabolic requirements than T_{reg} cells. In this regard, it is interesting to note that the addition of αKG to cultures of glutamine-deprived naïve cells not only stimulated their differentiation into T_H1 cells but also inhibited their differentiation into T_{reg} cells. Thus, our findings suggest that altering the concentrations of intracellular metabolic intermediates conditions the balance between T_H1 cell effector functions and T_{reg} cell suppressor functions.

However, it is also important to note that TCA cycle intermediates can regulate the epigenetic state of an activated T cell. Specifically, Tet2 (ten-eleven translocation 2) is an αKG-dependent enzyme that alters DNA methylation by conversion of 5-methylcytosine to 5-hydroxymethylcytosine. Deficiency in Tet2 inhibits the differentiation of naïve CD4$^+$ T cells into T_H1 cells, but not T_H2 cells, whereas their conversion into T_{reg} cells is enhanced (*57*). Because these changes in differentiation directly parallel those 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 the generation of T_H1 cells, but not T_H2 cells.

iT_{reg} cells can be generated de novo by the stimulation of naïve CD4$^+$ T cells through the TCR in the presence of exogenous TGF-β. We found that this conversion process was independent of glutamine abundance; large numbers of T_{reg} cells were generated irrespective of the glutamine concentration. However, it was only under glutamine-deficient conditions that endogenous TGF-β, or the low abundance of TGF-β in FCS, was sufficient to promote the differentiation of naïve cells into T_{reg} cells. Furthermore, the TSDR methylation status detected in TGF-β–induced T_{reg} cells was similar to that of Foxp3$^+$ T cells generated under glutamine-deficient conditions. These glutamine-deprived Foxp3$^+$ T cells were also highly proliferative in vivo and were recovered at higher percentages than nT_{reg} cells when cotransferred with T_{eff} cells into Rag2-deficient mice. This conversion, which we detected under either T_H0- or T_H1-type stimulations, was abrogated under conditions in which

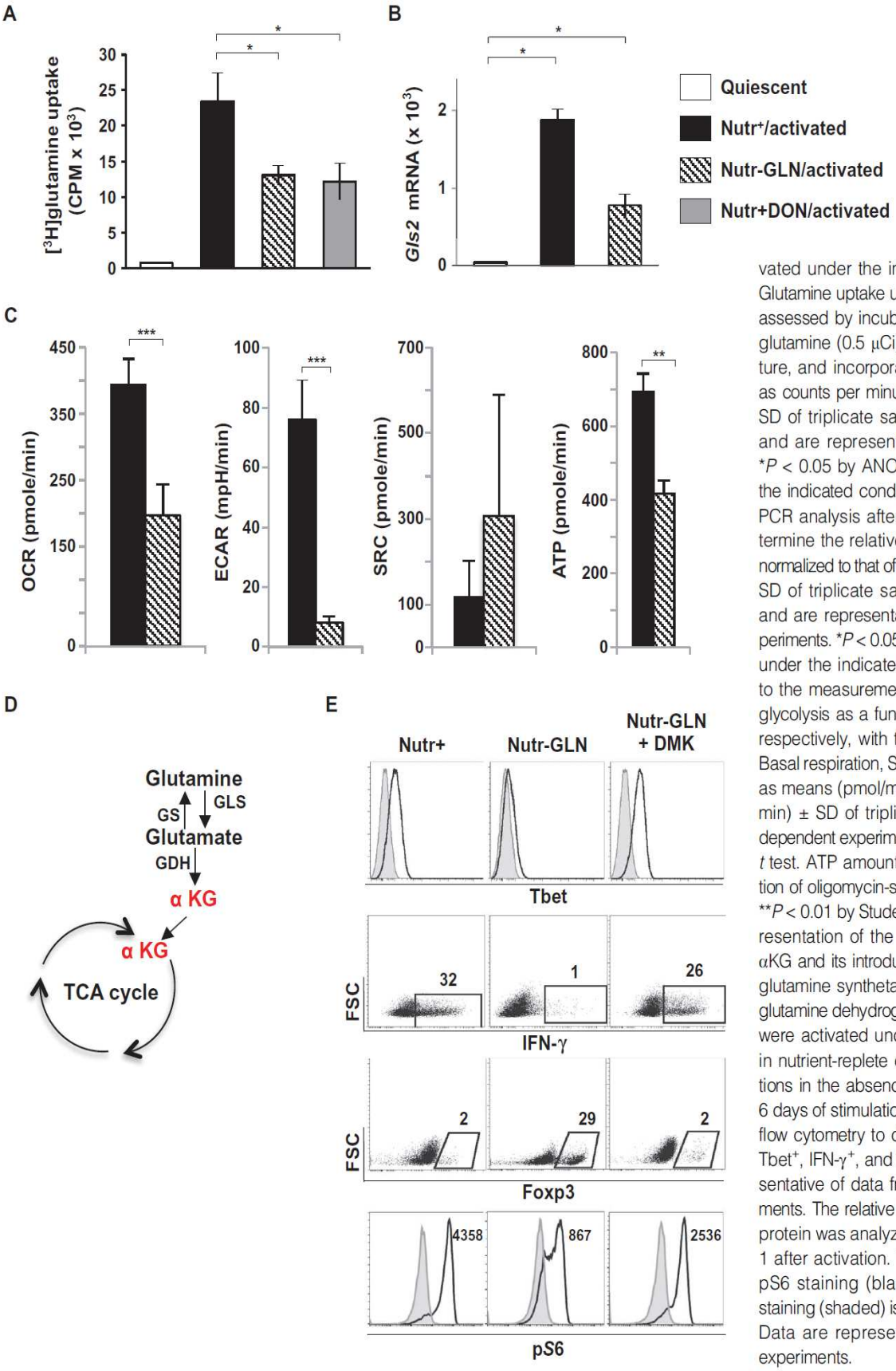

Fig. 7. The cell-permeable αKG analog DMK rescues the generation of T_H1 cells from naïve $CD4^+$ T cells under glutamine-deprived conditions. (A) Naïve $CD4^+$ T cells (5×10^5) were cultured for 3 days in the absence of any activating agents (quiescent) or were activated under the indicated nutrient conditions. Glutamine uptake under each condition was then assessed by incubating cells with L-[3,4-^{3}H (N)] glutamine (0.5 μCi) for 5 min at room temperature, and incorporation per cell was measured as counts per minute (CPM). Data are means ± SD of triplicate samples from one experiment and are representative of three experiments. *$P < 0.05$ by ANOVA. (B) Cells cultured under the indicated conditions were subjected to qRT-PCR analysis after 2 days of activation to determine the relative abundance of *Gls2* mRNA normalized to that of *Hprt* mRNA. Data are means ± SD of triplicate samples from one experiment and are representative of two independent experiments. *$P < 0.05$ by ANOVA. (C) Cells cultured under the indicated conditions were subjected to the measurement of cellular respiration and glycolysis as a function of the OCR and ECAR, respectively, with the Seahorse XF24 analyzer. Basal respiration, SRC, and ECAR are presented as means (pmol/min per 1×10^6 cells or mpH/min) ± SD of triplicate samples from three independent experiments. ***$P < 0.001$ by Student's *t* test. ATP amounts were assessed as a function of oligomycin-sensitive oxygen consumption. **$P < 0.01$ by Student's *t* test. (D) Schematic representation of the metabolism of glutamine to αKG and its introduction into the TCA cycle. GS, glutamine synthetase; GLS, glutaminase; GDH, glutamine dehydrogenase. (E) Naïve $CD4^+$ T cells were activated under T_H1-polarizing conditions in nutrient-replete or glutamine-depleted conditions in the absence or presence of DMK. After 6 days of stimulation, the cells were analyzed by flow cytometry to determine the percentages of $Tbet^+$, IFN-γ^+, and $Foxp3^+$ cells. Plots are representative of data from five independent experiments. The relative abundance of pS6 ribosomal protein was analyzed by flow cytometry on day 1 after activation. The difference in the MFI of pS6 staining (black lines) relative to control staining (shaded) is indicated in each histogram. Data are representative of five independent experiments.

TGF-β signaling was inhibited. These data are consistent with previous elegant work that demonstrated that the low abundance of endogenous TGF-β is sufficient to generate in vitro iT_{reg} cells in an mTOR-deficient background (*22*). Indeed, decreased glutaminolysis resulted in decreased mTOR signaling in $CD4^+$ T cells stimulated ex vivo, whereas supplementation of DMK activated mTORC1 and inhibited Foxp3 mRNA and protein expression.

Although it is not yet known how the intricate coordination of nutrient processing regulates the formation of specific metabolic intermediates in

T cell subsets, our data point to the extracellular nutrient environment as a key factor in this equation. This may have substantial physiological consequences in microenvironments, such as those that occur because of tumors or infections, wherein the nutrient milieu into which a T cell enters can be altered (*29*, *30*, *58*). Indeed, glutamine abundance is reduced in patients with hepatocellular, colon, and stomach tumors (*29*, *30*). This finding may explain the data that show that T_{reg} cells preferentially accumulate in and around murine tumors, especially as the tumors progress (*59*). Furthermore, $Foxp3^+$ T cells are often recruited to a tumor before T_{eff} cells are recruited, which prevents the T cell–mediated eradication of the tumor cells (*60*). Thus, the glutamine-deficient microenvironment of a tumor, potentially caused by the "addiction" of tumor cells to glutamine (*61*, *62*) or as an undesirable result of chemotherapy (*32*–*34*), appears to be a critical factor in enforcing a T_{reg} cell phenotype even under conditions in which the $CD4^+$ T cells recruited into the tumor are exposed to conditions that promote their differentiation into effectors. Manipulating the metabolic state of T cells may modulate the balance between effector and suppressor functions, potentially opening new avenues for the development of immunotherapies. From an evolutionary perspective, it is tempting to speculate that it is in the interest of the organism to evade an energetically costly immune response under conditions of amino acid starvation.

MATERIALS AND METHODS

Mice

C57BL/6 mice and C57BL/6-Thy1.1 mice were purchased from Charles River laboratories, whereas Foxp3-GFP reporter mice have been previously described (*38*, *39*). The mice were housed in conventional, pathogen-free facilities at the Institut de Génétique Moléculaire de Montpellier and at the National Cancer Institute at Frederick. Animal care and experiments were performed in accordance with the National Institutes of Health (NIH) and French national guidelines.

Cell isolation and activation

$CD4^+$ T cells were purified with the MACS $CD4^+$ T cell isolation kit (Miltenyi Biotec). For experiments with $CD4^+$ T cells depleted of $Foxp3^+$ cells, enriched $CD4^+$ T cells from Foxp3-GFP reporter mice were sorted on the basis of a $CD4^+CD62L^{hi}CD44^-GFP^-$ expression profile on a FACSAria flow cytometer (BD Biosciences). For T_H1-, T_H2-, and iT_{reg}-polarizing conditions, IL-12 (10 ng/ml) and anti–IL-4 antibody (5 μg/ml), IL-4 (10 ng/ml) and anti–IFN-γ antibody (10 μg/ml), or human TGF-β (3 ng/ml), respectively, were added to the cultures. TGF-β or the TGF-β signaling pathway were neutralized by the addition of an anti–TGF-β monoclonal antibody (clone 1D11, 10 μg/ml) or the inhibitor SB431542 (5 μM, Sigma), respectively. For rescue experiments, cultures were supplemented with DMK (3.5 mM, Sigma-Aldrich). Cell activations were performed with plate-bound anti-CD3 (clone 17A2 or 2C11, 1 μg/ml) and anti-CD28 (clone PV-1 or 37.5, 1 μg/ml) monoclonal antibodies in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS and IL-2 (100 U/ml) in the presence or absence of 2 mM glutamine or 11 mM glucose. In some experiments, glutamine was added at the concentrations indicated in the figure legends. Exogenous IL-2 (100 U/ml) was added every other day starting on day 2 after stimulation. To block glutaminolysis, $CD4^+$ T cells were activated in the presence of 3 μM L-DON (Sigma-Aldrich). Cell proliferation was monitored by labeling the cells with 2.5 μM CFSE (Life Technologies) or 5 μM CTV (Life Technologies) at 37°C for 3 or 8 to 10 min, respectively, for in vitro and in vivo manipulations.

Gene expression analysis

RNA was isolated from purified $CD4^+$ T cells with the RNeasy Micro Kit (Qiagen) and then was reverse-transcribed into cDNA by oligonucleotide priming with the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR analysis was performed with the LightCycler 480 SYBR Green I Master kit (Roche) and the following specific primers: *Tbet* sense, 5′-TCCCCCAAGCAGTTGACAGT-3′; *Tbet* antisense, 5′-CAACAACCCCTTTGCCAAAG-3′; *Gata3* sense, 5′-AGTTCGCGCAGGATGTCC-3′; *Gata3* antisense, 5′-AGAACCGGCCCCTTATCAA-3′; *Foxp3* sense, 5′-CCCAGGAAAGACAGCAACCTT-3′, *Foxp3* antisense, 5′-TTCTCACAACCAGGCCACTTG-3′; *Gls2* sense, 5′-AGCGTATCCCTATCCACAAGTTCA-3′; *Gls2* antisense, 5′- GCAGTCCAGTGGCCTTCAGAG-3′; *Hprt* sense, 5′-CTGGTGAAAAGGACCTCTCG-3′; *Hprt* antisense, 5′-TGAAGTACTCATTATAGTCAAGGGCA-3′.

Immunophenotyping and flow cytometric analysis

Immunophenotyping of cells was performed with fluorochrome-conjugated antibodies, and intracellular staining was performed after the fixation and permeabilization of the cells (intracellular staining kit, eBioscience). Phosphorylation of S6 was assessed after fixation in 4% paraformaldehyde and permeabilization by staining with an anti-pS6 antibody (clone 91B2, Cell Signaling) and revealed with a secondary anti-rabbit immunoglobulin G antibody conjugated to Alexa Fluor 647 (Life Technologies). IL-4 production was assessed on day 6 of polarization with a Cytometric Bead Array (CBA) Kit (BD Biosciences). Before being subjected to staining for intracellular cytokines, cells were activated with phorbol myristate acetate (100 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml Sigma-Aldrich) in nutrient-replete medium in the presence of brefeldin A (10 μg/ml, Sigma-Aldrich) for 3.5 to 4 hours at 37°C. Cells were analyzed with a FACSCanto flow cytometer (BD Biosciences) or were sorted on a BD FACSAria flow cytometer. Data analysis was performed with FlowJo Mac version 8.8.7 software (Tree Star) and FCAP Array Software (CBA analysis).

Bisulfite pyrosequencing

For each analyzed cell type, cells (1×10^6) were subjected to FACS to >95% purity. Genomic DNA was isolated from the sorted cells with the DNeasy kit (Qiagen) and bisulfite-converted with the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. The murine TSDR was amplified by PCR in a reaction containing 20 ng of bisulfite-converted genomic DNA, 25 μl of ZymoTaq PreMix (Zymo Research), and forward and reverse primers (0.4 μM each) in a final volume of 50 μl. PCR conditions were as follows: 95°C for 10 min; 45 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; 72°C for 7 min; and 4°C for > 4 min. The PCR products were analyzed by gel electrophoresis. The PCR product (40 μl), PyroMark Gold Q96 reagent (Qiagen), PyroMark buffer (Qiagen), streptavidin Sepharose (GE Healthcare), and the sequencing primer were used for pyrosequencing on a PSQ96MA instrument (Qiagen) according to the manufacturer's protocol. The primers mTSDR-amp forward (TAAGGGGGTTTTAATATTTATGAGGTTT), which was biotinylated at the 5′ end, and mTSDR-amp reverse (CCTAAACTTAACCAAATTTTTCTACCA) were used for TSDR amplification, whereas the primers mTSDR-seq1 (CCATACAAAACCCAAATTC), mTSDR-seq2 (ACCCAAATAAAATAATATAAATACT), mTSDR-seq3 (ATCTACCCCACAAATTT), and mTSDR-seq4 (AACCAAATTTTTCTACCATT) were used for pyrosequencing. Male mice were used to analyze DNA methylation status to avoid artificial recalculation because of X chromosome inactivation in female mice.

Glutamine uptake assay

Before being analyzed for glutamine uptake, $CD4^+$ T cells (0.5×10^6) were starved in glutamine-free RPMI for 30 min at 37°C. Glutamine uptake assays were initiated by the addition of L-2,3,4-[^{3}H]glutamine (0.5 μCi, PerkinElmer) for 5 min at room temperature. Cells were solubilized in 500 μl of 0.1% SDS, and radioactivity was measured by liquid scintillation.

Metabolic flux analysis

OCR and ECAR were measured with the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells (1×10^6) were placed in XF medium (nonbuffered Dulbecco's modified Eagle's medium containing 2.5 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate) and monitored under basal conditions and in response to 1 μM oligomycin, 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), 100 nM rotenone, and 1 μM antimycin A (Sigma). The basal respiration rate was calculated as the difference between the OCR under basal conditions and the OCR after the inhibition of mitochondrial complexes 1 and 3 with rotenone and antimycin A, respectively. SRC was determined as the difference between the readings obtained under basal conditions and after injection of the ionophore FCCP. Mitochondrial ATP synthesis was estimated from the decrease in oligomycin-sensitive oxygen consumption using a phosphate/oxygen ratio of 2.3 as described previously (*63*).

Adoptive T cell transfer and induction of colitis

To induce colitis, $Rag2^{-/-}$ C57BL/6 mice were injected with sorted $CD45.1^+CD4^+CD45RB^{hi}$ T cells (2.5×10^5) alone or in combination with sorted $CD4^+Foxp3/GFP^+$ T cells (2.5×10^5) from $CD45.2^+$Foxp3-GFP mice. The mice were injected with either freshly isolated T_{reg} cells or $Foxp3^+GFP^+$ T cells obtained after 5 days of stimulation with anti-CD3 and anti-CD28 antibodies under glutamine-depleted conditions (fig. S3). After they were injected with the T cells, the mice were weighed weekly for 9 weeks or were sacrificed if their weight loss exceeded 20% of their total body weight. Lymphoid tissues were harvested, and colons were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E.

Statistical analyses

P values were determined with an unpaired Student's *t* test, ANOVA, Mann-Whitney with a two-tailed distribution, or a χ^2 test when indicated. Survival differences during the development of colitis were analyzed with a Mantel-Cox test (GraphPad Software).

SUPPLEMENTARY MATERIALS

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Fig. S1. Reduced amounts of glutamine in the culture medium inhibit the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ T cells.

Fig. S2. The methylation status of the TSDR is similar in iT_{reg} and T_{reg} cells generated in the absence of glutamine.

Fig. S3. Strategy for the purification of $Foxp3^+CD4^+$ T cells by flow cytometry.

Fig. S4. Naïve $CD4^+$ T cells secrete IFN-γ after stimulation under T_H1-polarizing conditions in glucose-depleted medium.

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Addendum II
Impact of arginine availability on human T cell function

Results and Discussion

Arginine deprivation impairs T cell activation and proliferation. Arginine concentrations can be significantly modulated within the tumor environment (Kim et al., 2018; Lyssiotis and Kimmelman, 2017; Marigo et al., 2008; Szefel et al., 2018) with important consequences on T cell function. Moreover, the arginine transporter CAT-1 is significantly upregulated upon TCR stimulation (presented in **Chapter I, Addendum I, Figure1**), suggesting a dependence of activated T cells on arginine uptake to sustain their new metabolic requirements.

To evaluate the impact of arginine deprivation on human T cell activation, $CD3^+$ T cells were isolated from the blood of healthy donors and TCR-stimulated using anti-CD3/anti-CD28 mAbs in the absence (-Arg) or presence (Nutr+) of arginine at 200 µg/ml which represents the « classical » concentration used in RPMI media). At day 4 following TCR-stimulation, T cells activated in arginine-deprived conditions exhibited high viability but were significantly smaller as compared to T cells activated in nutrient replete media, as assessed by microscopy and forward/side scatters parameters **(**data not shown and **Figure 1A).** Indeed, expression of the CD25 and CD71 activation markers, encoding the IL-2Rα chain and the transferrin receptor, respectively, were strongly reduced in conditions of arginine depletion (**Figure 1B**). Interestingly, CD69, an early activation marker whose cell surface upregulation is independent of transcription(Manel et al., 2003b), was upregulated even in arginine-depleted conditions and its downregulation was delayed as compared to control Nutr+ conditions (>45-fold higher MFI at day 4 for both CD4 and CD8 T cells, **Figure 1B**). Consistent with the suboptimal upregulation of T cell activation markers, there was a massive reduction of T cell proliferation in arginine-depleted conditions. T cells stimulated in -Arg conditions only initiated a first round of division at day 7 post stimulation whereas the vast majority of both CD4 and CD8 T cells activated in Nutr+ conditions had divided by day 3 (**Figure 1C**). Lower concentrations of arginine (decreasing from 200µg/ml to 50 µg/ml) did not appear to significantly impact CD4 T cell proliferation but CD8 T cell proliferation was delayed (14% of undivided cells at day 3 as compared to 8% of undivided cells in 50 µg/ml vs 200ug/ml arginine, respectively, **Figure 1C)**. Notably though, the concentration of 50 µg/ml is higher than plasma arginine levels (14 µg/ml; 82µM) suggesting that arginine levels in the T cell microenvironment may limit optimal T cell responsiveness *in vivo*.

Arginine deprivation does not inhibit the acquisition of a memory phenotype or the cytokine secretion potential of TCR-stimulated T lymphocytes. To determine whether arginine deprivation alters the proportion of CD4/CD8 T cells or the acquisition of a memory phenotype, we monitored T cell phenotype at day 4 post-stimulation in the presence or absence of arginine. Interestingly, neither the CD4/CD8 ratio (**Figure 2A**) nor the CD45RA/RO ratio (**Figure 2B**) was altered by activation in the absence of arginine. The percentages of CD4 and CD8 T cells producing IL-2, IL-17 and IFNγ were also not significantly modulated in the absence of arginine (**Figure 2C**). While these data differ from reported decreases in T cell-mediated IFNγ secretion in the absence of arginine (Feldmeyer et al., 2012), it is notable that the previous study assessed the levels of secreted cytokines whereas our experiments evaluated the potential of individual cells to produce cytokines following PMA/ ionomycin restimulation. Indeed, Geiger and colleagues found that even though high extracellular arginine levels resulted in a lower secretion of IFNγ from TCR-stimulated human CD8 T cells, PMA/ionomycin stimulation of the same cells resulted in the opposite effect, with an observed increase in IFNγ stimulation (Feldmeyer et al., 2012; Geiger et al., 2016; Munder et al., 2006; Rodriguez et al., 2007). Collectively, these data indicate that while both arginine deprivation and excess can decrease the level of cytokine secretion by TCR-stimulated T cells, they do not diminish the PMA/ionomycin-induced potential of these cells to secrete effector cytokines.

Downregulation of the CAT-1 arginine transporter decreases TCR-mediated CD4 and CD8 T cell proliferation. The CAT-1/SLC7A1 arginine transporter has been shown to be important for T cell proliferation (Werner et al., 2016) and indeed, we found that it is highly upregulated following TCR stimulation as shown in (**Chapter I, Addendum I, Figure1**). In an attempt to modulate arginine availability in CD4/CD8 T cells, we employed a CAT-1-mediated shRNA strategy. T cells were simultaneously activated with anti-CD3/anti-CD28 mAbs and transduced with a lentiviral vector harboring an shCAT-1 or control scramble (shCTRL) sequence. The transduction efficiency for both constructs, evaluated at day 3 by GFP expression, reached approximately 50% (**Figure 3A, left panel**). As expected, CAT-1 surface expression was not decreased in either CD4 or CD8 T cells following transduction with the

shCTRL vector but notably, the mean florescence intensity of CAT-1 expression in shCAT1-transduced lymphocytes was decreased by approximately 50% (**Figure 3A, right panels**). shRNA-mediated knockdown of CAT-1 did not alter either the CD4/CD8 ratio (**Figure 3B**) nor the upregulation of CD25 or CD71 following stimulation (**Figure 3C**) and cytokine secretion was not impacted (**Figure 3D**). Furthermore, the proliferation of CD4 as well as CD8 T cells transduced with the shCAT-1 vector did not differ from cells transduced with the shCTRL vector. Interestingly though, those T cells that were susceptible to lentiviral transduction, irrespective of the shRNA construct, proliferated to a greater extent than non-transduced cells, suggesting that lymphocytes that had progressed further into the cell cycle were more susceptible to lentiviral transduction. It is likely that the absence of a detectable impact of CAT-1 downregulation on CD4/CD8 proliferation is due to the experimental setup wherein lymphocytes were already in an activated state when transporter levels were decreased. Importantly, CD4 T cells with downregulated CAT-1 expression, but not cells with a control shRNA, were negatively selected during the expansion period, as monitored by a loss of GFP (**Figure 3E**). Moreover, when T cells transduced with a CAT-1-specific shRNA were restimulated (12-14 days post initial TCR stimulation), cells with downregulated CAT-1 exhibited a significantly lower level of proliferation, as monitored by >50% of undivided cells as compared to <20% in control conditions. Conversely, <5% of CAT-1-downregulated T cells underwent 2 divisions whereas >20% of control T cells underwent division (**Figure 3E**). Thus, CAT-1 upregulation is required for optimal cell cycle entry of human T cells and its continued expression is required for the expansion of these lymphocytes.

Arginine-mediated polyamine synthesis, but not NO production, is required for optimal T cell activation and proliferation. Arginine is used in different metabolic pathways, contributing to the production of iNOS, creatine, agmantine, polyamines, as well as the hypusine modification on the eIF5A translation factor (**Figure 4A**). To determine the potential contributions of these different pathways to T cell activation and proliferation, the following inhibitors were used: DL-α-(Difluoromethyl)ornithine (DFMO), an inhibitor of putrescine synthesis; asymmetric dimethylarginine (ADMA), a methyl derivate of arginine that impairs nitric oxide (NO) synthesis; and N1-monoguanyl 1,7-diaminoheptane (GC7), a spermidine analogue that inhibits the hypusination (conjugation of the aminobutyl moiety of spermidine to lysine) of the eIF5A translation factor (**Figure 4A**). Neither the absence of arginine nor any

of the tested inhibitors negatively impacted on T cell viability (**Figure 4B**) but inhibiting polyamine synthesis markedly decreased, in a dose-dependent manner, T cell proliferation (**Figure 4C**). Interestingly, inhibiting the production of NO (with ADMA) or eIF5A hypusination did not attenuate T cell proliferation (**Figure 4C**) nor the upregulation of activation markers (**Figure 4D**). The potential importance of NO production (and NOS activity) in the relative survival/proliferation of CD4 and CD8 T cells will be further evaluated but given the significant decrease in T cell proliferation following inhibition of polyamine biosynthesis, we further investigated its impact on the induction of T cell activation markers. Notably, the upregulation of CD25 was similarly decreased in the absence of arginine as compared to the presence of DFMO, at levels approximately 2-fold lower than control activated T cells (days 3 and 5 of activation, **Figure 4D, 4E**). Consistent with a decreased activation state of DFMO-treated T cells, there was a delay in CD69 downregulation, similarly to that detected in the absence of arginine; 80% vs 60% and 50% vs 10% compared to control cells, at days 3 and 5, respectively (**Figure 4D, 4E**).

T cell activation results in the upregulation of genes involved in polyamine biosynthesis, including ODC and S-adenosylmethionine decarboxylase (Fidelus et al., 1984; Hesterberg et al., 2018; Hunt and Fragonas, 1992; Kay and Lindsay, 1973; Kay and Pegg, 1973; Puleston et al., 2017; Scott et al., 1985) and polyamine levels increase upon T cell activation (Wang et al., 2011). While the specific role of polyamines as well as the genes regulating their biosynthesis (i.e. ODC) have not been evaluated in T cell development and activation, Myc, a critical transcription factor in both of these processes, regulates polyamine biosynthesis (Hesterberg et al., 2018). The ensemble of these data point to the importance of the arginine-polyamine pathway and future studies will specifically evaluate the role of this axis in regulating T cell activation and effector function.

Figure 1

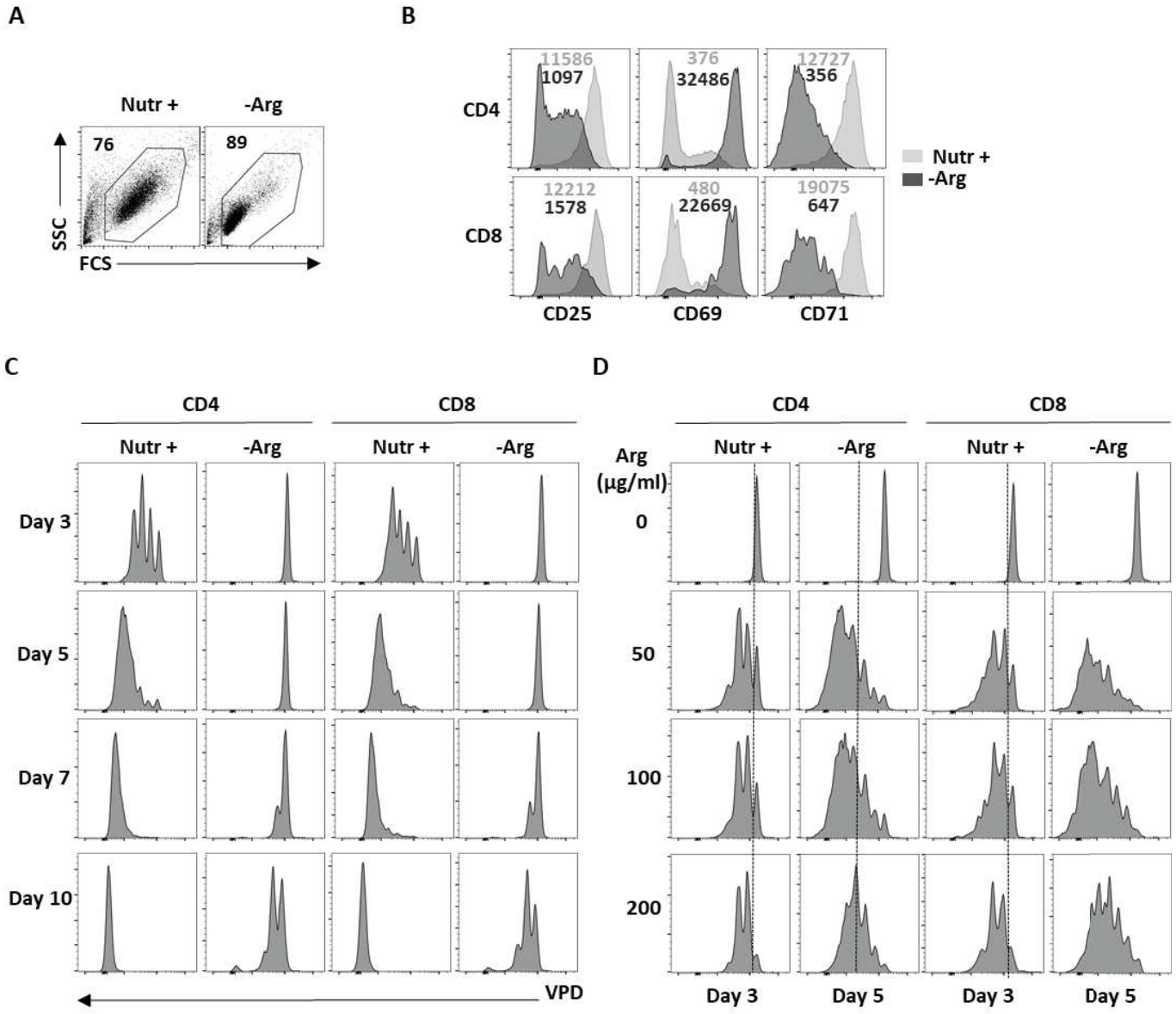


Figure 1- Arginine deprivation inhibits T cell activation and proliferation

(A) Purified human $CD3^+$ T cells were TCR-activated with anti-CD3/CD28 mAbs in nutrient-replete (Nutr+, 200 ug/ml arginine), or in the absence of arginine (-Arg). Forward (FSC) and side (SSC) scatter profiles were evaluated at day 4 of activation and representative plots are presented. **(B)** Expression of the CD25, CD69, and CD71 activation markers was monitored in CD4 and CD8 T cells at day 4 of stimulation in the indicated conditions (data are representative of 1 of 5 independent experiments). **(C)** The proliferation profiles of TCR-stimulated CD4 and CD8 T cells activated in the presence or absence of arginine were monitored at days 3, 5, 7, and 10 as a function of VPD450 fluorescence and representative histograms are shown (representative of 1 of 2 independent experiments). (**D)** Proliferation profiles of CD4 and CD8 T cells activated in the presence of 0, 50, 100 and 200 ug/ml of arginine are shown at days 3 and 5 following TCR stimulation.

Figure 2

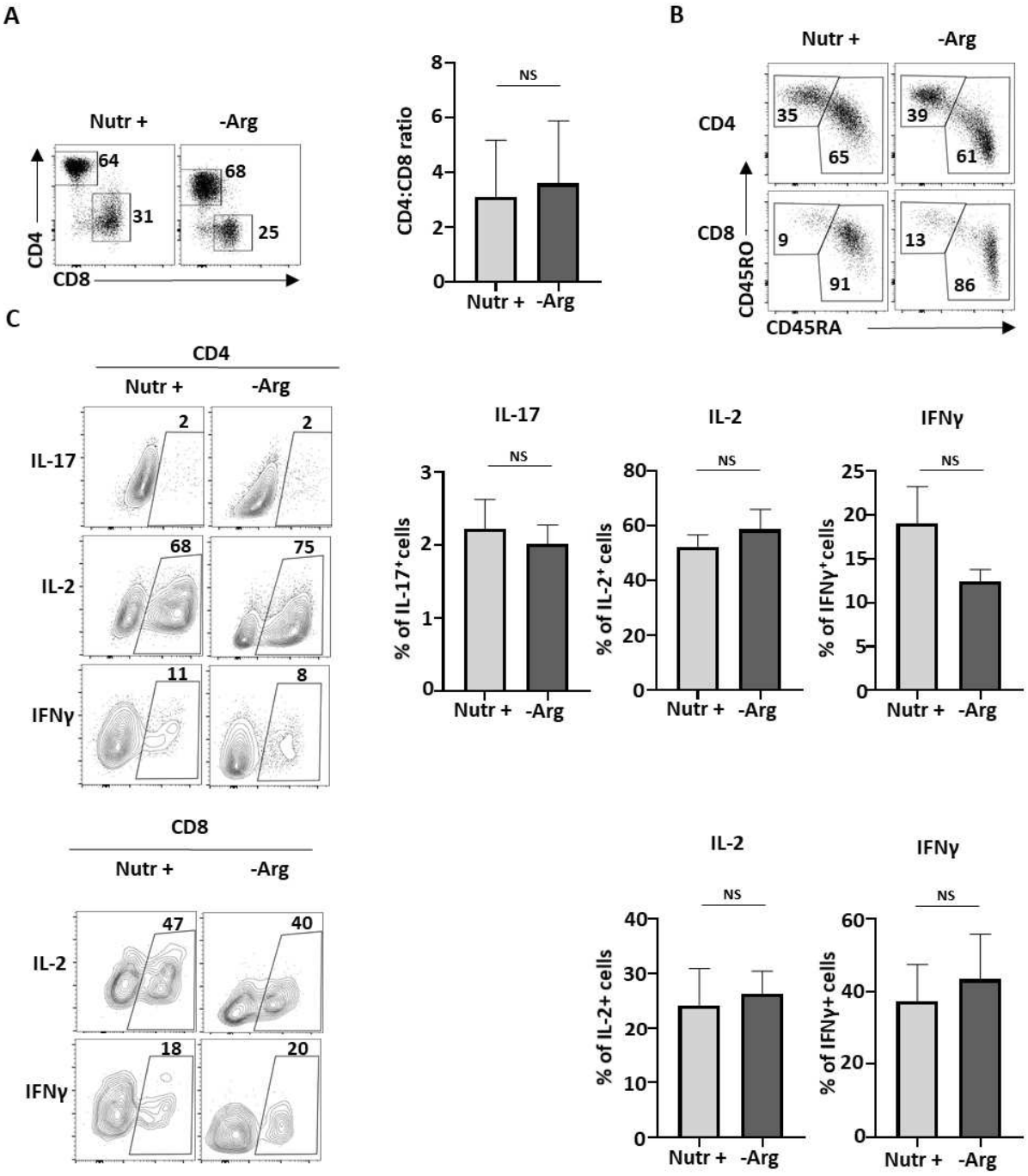
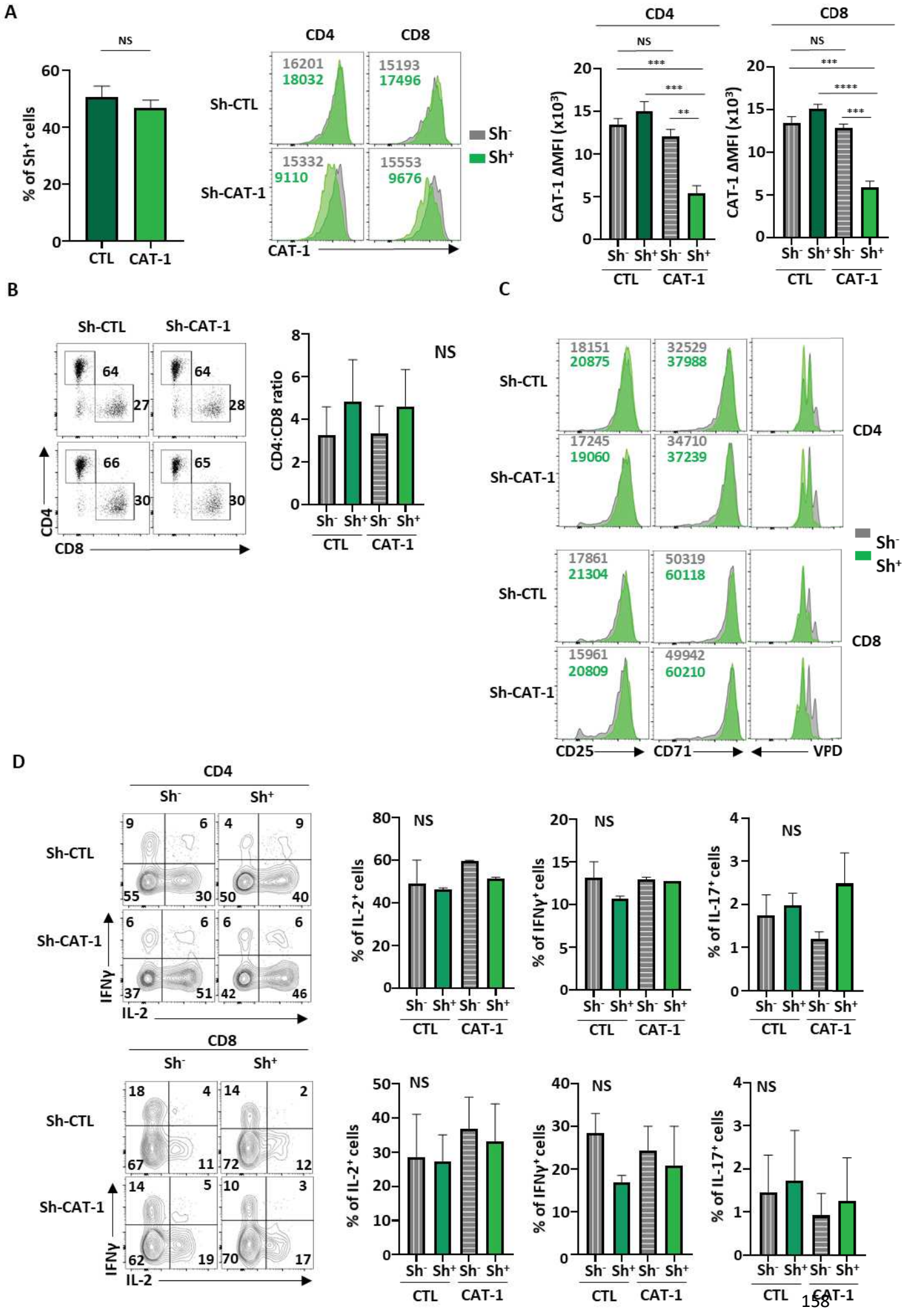


Figure 2- T cell phenotype is not significantly altered under conditions of arginine deprivation

(A) The repartition of CD4 and CD8 T cells was assessed at day 4 post-TCR activation in nutrient-replete (Nutr+) and arginine-deprived (-Arg) conditions by flow cytometry and representative dot plots are shown (1 of 5 independent experiments). **(B)** The CD45RA/CD45RO phenotype of $CD4^+$ and $CD8^+$ T cells is shown at day 4 and the percentages of cells in each gate are presented. Representative plots from 1 of 5 independent experiments are shown. **(C)** Production of IL-17, IL-2, and IFNγ was assessed in the T cells activated in the presence or absence of arginine at day 4 of TCR engagement following restimulation with PMA/ionomycin in the presence of Brefeldin A for 4h. Cytokine production was monitored by intracellular staining with the indicated fluorochrome-conjugated antibodies and representative plots are shown (left). Quantification of means ± SEM of 5 independent experiments are presented as historgrams; ns=non-significant.

Figure 3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20809
49942
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% of IL-2+ cells
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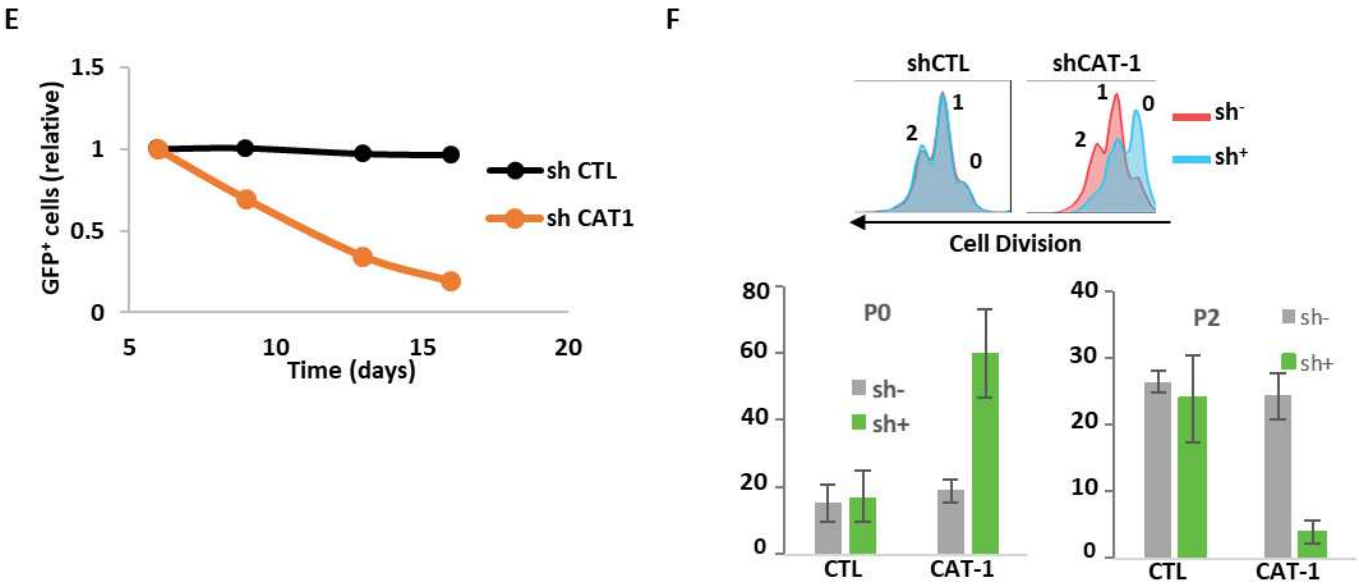
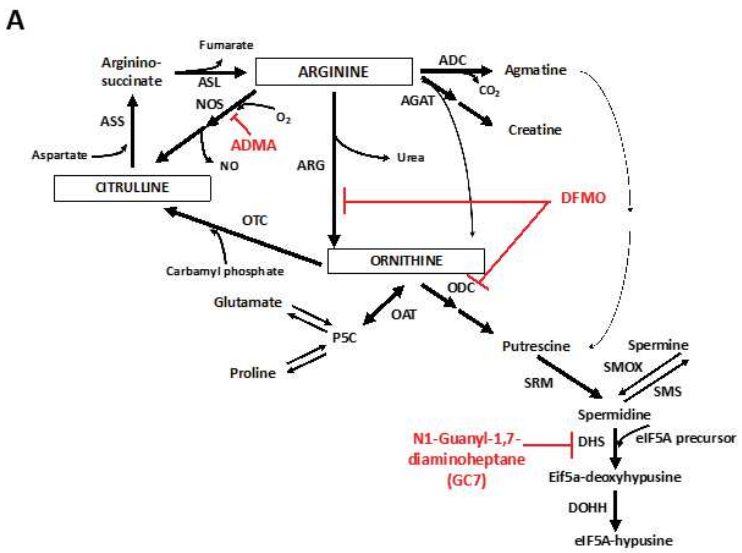


Figure 3- Downregulation of CAT-1 expression by lentiviral-mediated shRNA based approach inhibits T cell proliferation following restimulation

(A) Purified human CD3+ T cells were activated with anti-CD3/CD28 mAbs and simultaneously transduced with a lentiviral-based vector harboring a scrambled (CTL) or CAT-1-specific shRNA and encoding GFP as a marker protein. Transduction efficiency was monitored as a function of GFP expression at day 3 and means ± SEM of 3 independent experiments are presented (left panel). Surface CAT-1 expression was monitored in non-transduced (GFP-/sh-, grey histograms) as compared to transduced (GFP+/sh+, green histograms) CD4+ and CD8+ T cells and representative histograms with indicated mean fluorescence intensities (MFI) are presented (middle panels). Quantification of MFI in non-transduced and transduced CD4 and CD8 T cell subsets are presented as means ± SEM of 3 independent experiments (right panels). *NS=non-significant; *, p<0.05; **, p<0.01; ***, P<0.001. **(B)** The repartition of CD4 and CD8 T cells within non-transduced (sh-) and transduced T cell subsets was assessed at day 3 post-TCR activation and representative dot plots with the indicated percentages are shown (left panels). Quantification of CD4/CD8 subsets in 3 independent experiments are shown as means ± SEM (right). **(C)** Induction of the CD25 and CD71 activation markers was monitored in non-transduced (sh-) and transduced (sh+) CD4 and CD8 subsets at day 3 of activation and representative histograms with the corresponding MFIs are shown (left). Proliferation was monitored by VPD450 fluorescence dilution and day 3 profiles of sh- and sh+ subsets are overlaid in representative histograms in 1 of 3 independent experiments. **(D)** Production of IL-17, IL-2, and IFNγ was assessed in sh- and sh+ CD4 and CD8 T cell subsets at day 5 by intracellular staining following restimulation with PMA/ionomycin in the presence of Brefeldin A for 4h. Representative plots are shown (left) and means ± SEM of 3 independent experiments are presented (right). **(E)** The percentages of T cells transduced with shCTL and shCAT-1 vectors were followed over time (day 6 to 16) as a function of GFP fluorescence and normalized to "1" (representing 40-60% transduction at day 3). **(F)** Cell division of non-transduced (sh-) and transduced (sh+) T cells was evaluated following CD3/CD28 restimulation of T cells at day 14. Representative proliferation profiles are shown as a function of VPD450 fluorescence and the mean percentages ± SEM of undivided cells (P0) and cells that divided twice (P2) within the sh- and sh+ subsets are presented.

Figure 4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NO
ARG
Urea
CITRULLINE
DFMO
OTC
ORNITHINE
Carbamyl phosphate
ODC
Glutamate
OAT
P5C
Putrescine
Spermine
SMOX
Proline
SRM
SMS
Spermidine
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DHS
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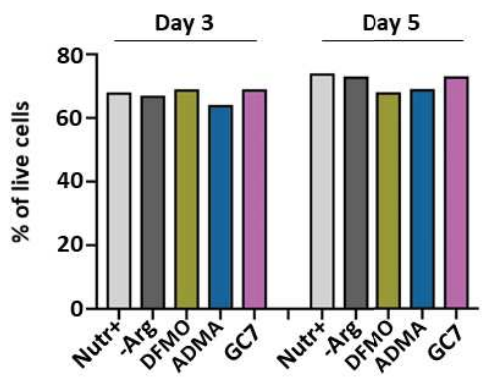
Eif5a-deoxyhypusine
DOHH
eIF5A-hypusine


B
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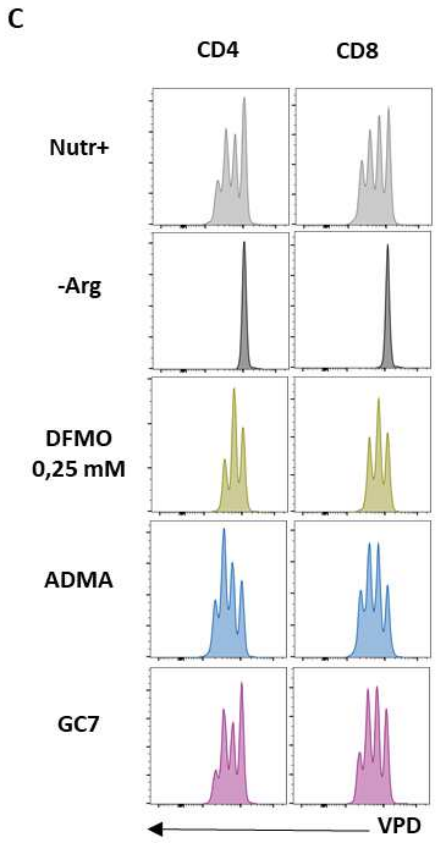
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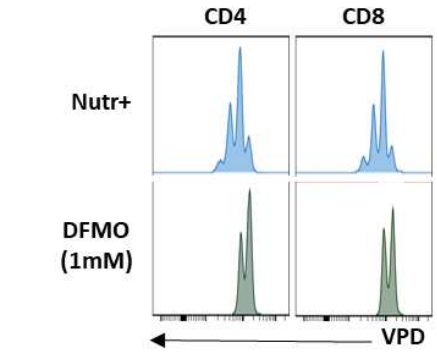
CD4
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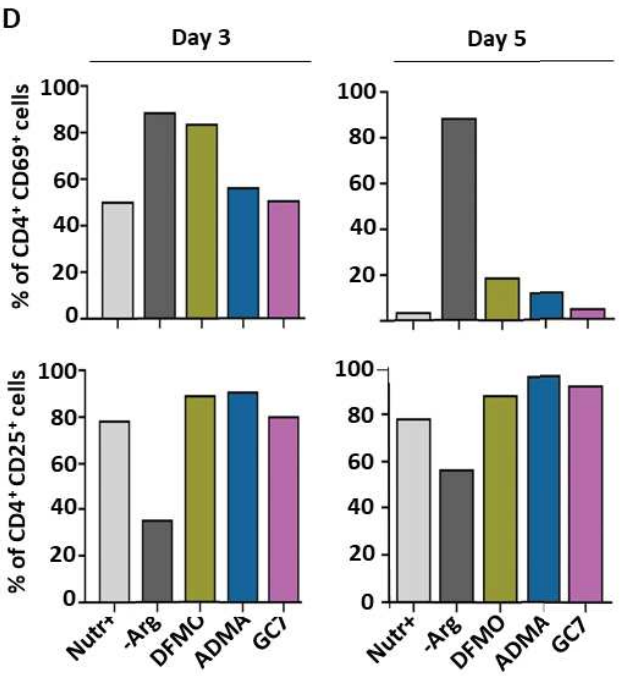
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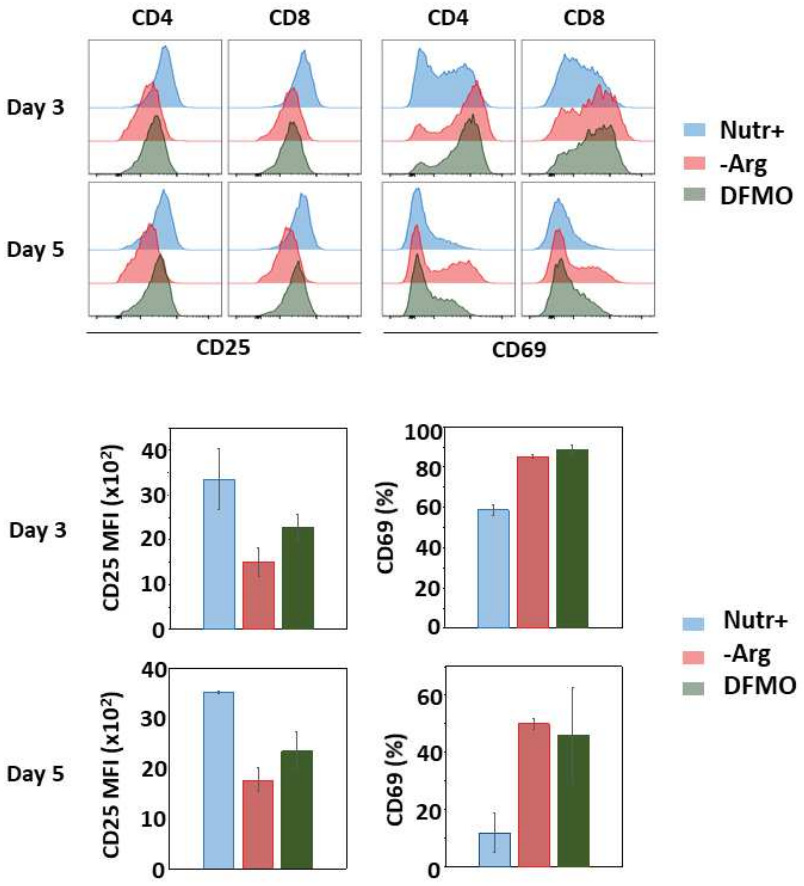

Figure 4.-Arginine-based generation of polyamines is required for optimal T cell activation and proliferation

(**A**) Scheme of arginine utilization in different metabolic pathways resulting in the generation of nitric oxide (NO), agmatine, creatine, polyamines (putrescine/ spermine) and hypusine. (**B**) The viability of T cells activated in control conditions (Nutr+), in the absence of arginine (-Arg), and in control conditions in the presence of inhibitors of polyamine (DFMO), NO (ADMA) and hypusine (GC7) generation were evaluated at day 3 and day 5 of stimulation. Data from one representative experiment are shown. (**C)** The proliferation of CD4 and CD8 T cells activated in the indicated conditions was evaluated by VPD450 fluorescence and representative histograms are shown at day 3 of activation. Data are representative of 3 independent experiments. **(D)** The percentages of $CD25^+$ and $CD69^+$ cells in the different conditions are presented at days 3 and 5 of activation. **(E)** Histogram profiles of CD25 and CD69 are presented in control conditions (Nutr+), in the absence of arginine (-Arg), and in the presence of the DFMO polyamine inhibitor (top panels). Quantifications of the mean MFI ± SEM of CD25 fluorescence and the percentages of CD69+ cells are shown (n=3, bottom panels).

Materials and Methods

Human T cell isolation and culture. $CD3^+$ and $CD4^+$ T cells were isolated from healthy adult donors. All experiments using primary human cells were conducted in accordance with the Declaration of Helsinki and IRB approval to the French Blood Bank (Etablissement Francais du Sang). T lymphocytes were purified by negative-selection using Rosette tetramers (StemCell Technologies) and purity was monitored by flow cytometry. Lymphocytes were cultured in nutrient-replete (Nutr+) RPMI SILAC medium 1640 supplemented with lysine (0.2mM) and arginine (1.15mM) or in the absence of arginine (-Arg). Both Nutr+ and -Arg medium were supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco-Life technologies). In some experiments, alpha-difluoromethylornithine (DFMO, 0,25 mM or 1mM), asymmetric dimethylarginine (ADMA, 120mM) or GC7 (0,5 µM) were added as indicated.

T cell activation was performed using plate-bound anti-CD3 (clone OKT3, Biolegend) and anti-CD28 (clone 9.3, kindly provided by Carl June) mAbs at a concentration of 1 µg/ml. rIL-2 (100 U/ml) was added every other day starting either at day 2-3 post-activation. Cells were maintained in a standard tissue culture incubator containing atmospheric (20%) O2 and 5% CO2.

Flow cytometry analyses. Immunophenotyping of cells was performed using fluorochrome-conjugated mAbs (BD Bioscience or eBioscience) against CD4, CD8, CD45RA and CD45RO and cell activation was monitored using fluorochrome-conjugated mAbs against CD25, CD69 and CD71 (BD Bioscience, Beckman Coulter or eBioscience). CAT-1 (SLC7A1) cell surface expression was monitored by binding to its Fc-coupled receptor binding domain (RBD) ligand (Ivanova et al, manuscript in preparation; patent WO2017085271A1) for 30 min at 37°C following by a secondary AF647-coupled anti-rFc secondary antibody (Life Technologies). Proliferation was monitored by labeling cells with VPD450 (BD Biosciences; 1 µM) for 3 min at RT. For intracellular cytokine staining, lymphocytes were re-activated with PMA (Sigma-Aldrich; 100 ng/ml) and ionomycin (Sigma-Aldrich; 1 µg/ml) in the presence of brefeldin A (Sigma-Aldrich; 10 ug/ml) for 3.5–4h at 37 °C. Intracellular staining for IL-2, IFNγ and IL-17 was performed following fixation/ permeabilization using the eBioscience kit (Thermo-scientific)

or using the BD kit (BD Biosciences). Cells were analyzed on an LSRII-Fortessa (BD Biosciences) and data was analyzed using FlowJo (Tree Star) software.

shRNA-mediated inhibition of CAT-1 mRNA expression in T lymphocytes.

shRNA-encoding lentiviral vectors were generated by transient transfection of 293T cells as described previously (Verhoeyen et al., 2003). Briefly, co-transfection was performed with PsPax2, encoding Gag, Pol, Rev and tat, the VSV-G pseudotyping envelope, pCMV-VSV-G and the HIV-1 derived shRNA-encoding vectors (pLKO.1, ShRNA mission, Sh-CAT-1 ref# TRCN0000042967 and Sh-scramble ref# shC002 (non mammalian sh-scr)). Viral supernatant was harvested 48 hours post-transfection, and concentrated by centrifugation (3000 RPM O/N, 4°C). Titers were assessed on Jurkat cells to determine transducing units (TU/ml) and transduction was determined as the percentage of GFP^+ cells by flow cytometry.

For T cell transductions, 1×10^6 lymphocytes were transduced with shRNA-encoding lentiviral particles (MOI 5) and activated on anti-CD3 /CD28-coated plates in RPMI media. Transduction was evaluated at day 3 as a percentage of GFP^+ cells on an LSRII Fortessa (BD Biosciences).

Chapter 3
Role of αKG as a metabolic regulator of Th1/iTreg differentiation

Introduction

In the previous chapter, I described our work showing that glutamine deprivation had a pronounced effect, abrogating the differentiation of naïve T cells to a Th1 effector fate and skewing them towards a regulatory suppressive T cell function.

As naïve $CD4^+$ T cells are unable to differentiate to an IFNγ-secreting Th1 fate in glutamine-deprived conditions and rather, generate FoxP3-expressing suppressive T cells, it was important to determine the mechanism(s) responsible for this alteration. We found that this defect is specifically due to a deficiency in intracellular α-ketoglutarate, an intermediate that feeds the tricarboxylic acid cycle (TCA). We further demonstrated that an exogenous cell permeable form rescued Th polarization in these conditions. In the past years, several studies have demonstrated an important role of αKG-dependent hydroxylases and demethylases during T cell differentiation and effector function through epigenetic regulation (Chisolm et al., 2017; Polansky et al., 2008; Someya et al., 2017; Xu et al., 2017; Yang et al., 2015). However, this is clearly not the only mechanism via which aKG (or the ratio between aKG and other TCA metabolites) can impact on T cell functions and it becomes therefore crucial to decipher them. In this context, the major aspect of my PhD project has focused on the role of αKG in controlling T cell differentiation and the data resulting from these studies are presented in this section. Briefly, these data have revealed a critical role of αKG, positively regulating IFNγ effector function in $CD4^+$ T cells activated under Th1-polarizing conditions. Interestingly, αKG markedly upregulated IFNγ secretion in naive T cells activated under Treg polarizing conditions and inhibited the induction of FoxP3. Our work unrevealed a new role of αKG on Th1 and iTreg differentiation.

Article III

The balance between Th1 and Treg differentiation is regulated by α-ketoglutarate-mediated alterations in TCA cycle flux

Abstract

The proliferation and differentiation of T lymphocytes is regulated by antigen and cytokine signals but the induction of metabolic pathways is also required, resulting in an integration of environmental cues in cell fate decisions. Indeed, T lymphocyte activation is regulated by the metabolism of glucose, fatty acids and amino acids, allowing the cell to meet increased energetic and biosynthetic demands. Notably, we recently found that glutamine availability is a key determinant of T cell differentiation; CD4 lymphocytes undergo differentiation to IFNγ-secreting Th1 effectors under conditions of optimal glutaminolysis but are converted to FoxP3^{+} regulatory T cells (Tregs) when glutamine catabolism is abrogated. Here, we show that the glutamine-derived metabolite, α-ketoglutarate (αKG), significantly augmented IFNγ secretion while dramatically attenuating Treg generation. Specifically, αKG-mediated attenuation of FoxP3 transcription resulted in the differentiation of CD4 T cells with an enhanced inflammatory profile. Under these conditions, αKG promoted the effector activity of chimeric antigen receptor (CAR)-engineered T cells against the ErbB2 tumor antigen in tumor-bearing mice, increasing IFNγ secretion by >10-fold under conditions of Treg polarization. Mechanistically, αKG altered the transcription of genes in the cholesterol biosynthesis pathway and significantly increased membrane concentrations of polyunsaturated fatty acid species. Furthermore, an αKG-mediated augmentation in TCA cycle metabolism and downstream oxidative phosphorylation was identified as a critical component of CD4 T cell differentiation. Inhibition of succinate oxidation and decreasing the αKG/succinate ratio resulted in a significantly augmented Treg polarization and decreased levels of IFNγ. Thus, manipulation of TCA cycle intermediates may promote the development of novel strategies aimed at enhancing anti-tumor CAR T cell effector functions.

Results and Discussion

αKG enhances Th1 differentiation while conversely, inhibiting Treg polarization. To assess whether high αKG levels modulate Th1 differentiation potential, naïve murine CD4 T lymphocytes were TCR-stimulated under Th1 conditions in the absence or presence of exogenous αKG. Notably, αKG significantly augmented the percentage of CD4 T cells secreting IFNγ, increasing the mean level from 24% to 52% ($p<0.0001$, **Figure 1A**). This increase was regulated at the transcriptional level with a >10-fold augmentation in Ifng mRNA levels ($p<0.01$) and a markedly altered visualization of RNASeq reads mapped to the *Ifng* gene (**Figure 1B**). Thus, high levels of αKG augment the potential of CD4 T cells to produce IFNγ following stimulation in Th1-polarizing conditions.

As the potential of a CD4 T cell to generate an effector Th1 phenotype is often inversely related to its potential to adopt a suppressive Treg phenotype (1-3), we assessed whether αKG attenuates Treg polarization. Indeed, while the majority of naïve T cells stimulated under Treg polarization conditions exhibited an upregulation of the master transcription factor FoxP3, the percentage of $FoxP3^+$ T cells differentiated in the presence of exogenous αKG was approximately 3-fold lower (decreasing from a mean of 60% to 28%, $p<0.0001$; **Figure 1C**). This decrease in Treg polarization efficiency/potential was associated with 3-fold lower levels of FoxP3 mRNA levels and an attenuation of RNAseq reads in the *Foxp3* gene (**Figure 1D**). Moreover, Treg differentiation was attenuated even when T cells were initially stimulated in control Treg polarization conditions and αKG was added only 24h post-activation (**Supplementary Figure 1**). Importantly though, the αKG-mediated inhibition of FoxP3 upregulation was specific to Treg polarizing conditions because the activation of natural Treg (nTreg), expressing FoxP3 in a quiescent state, was not altered by αKG (**Figure 1E**). Together, these data reveal the importance of αKG in regulating the balance in the polarization of naïve CD4 T cells to a Th1 versus a Treg fate.

The inflammatory phenotype of αKG-induced CD4 T cells is associated with enhanced tumor infiltration of ErbB2-CAR T cells in mice bearing $ErbB2^+$ tumors. As αKG decreased the potential of CD4 T cells to upregulate FoxP3 expression following stimulation under Treg polarization conditions, it was of interest to assess the characteristics of these cells.

Interestingly, the percentages of IFNγ-secreting cells was significantly augmented ($p<0.0001$, **Figure 2A**) and this correlated with an upregulation of the master Th1 transcription factor, T-bet, at both the protein and mRNA level (**Figure 2B, 2C**). Furthermore, these CD4 T cells exhibit an upregulation of granzyme B (**Figure 2C**), a marker of CD4 T cells with CTL activity (4). While this αKG-induced CD4 T cell phenotype was not associated with an increase in TNFα or GM-CSF at the mRNA level, the secretion of these two cytokines as well as that of IFNγ, IL-17 and IL-2 were remarkably elevated by αKG following stimulation of naïve CD4 T cells in Treg-polarizing conditions (**Figure 2D, 2E**). The increase in IL-2 levels is in agreement with previous work showing that αKG regulates the association of CCTC-binding factor (CTCF) with select loci including that of the IL-2 gene, specifically increasing IL-2 expression (5).

The data presented above suggested that αKG might alter the phenotype and persistence of anti-tumor T cells in an *in vivo* setting. This is especially critical in the "hostile" environment of a solid tumor that consumes high levels of nutrients and is often anaerobic (6). To test this hypothesis, we evaluated the effector function of murine T cells harboring a CAR directed against the ErbB2/HER2 tumor antigen in immunodeficient $RAG2^{-/-}$ mice bearing the 24JK fibrosarcoma, engineered to express ErbB2 (24JK-ERB) (**Figure 3A**) (7). We first assessed whether αKG altered the differentiation potential of ErbB2-CAR CD4 T cells similarly to that of polyclonal T cells. αKG treatment resulted in a >2-fold increase in IFNγ-secreting cells following activation in Th1 conditions and a decrease in $FoxP3^+$ cells, irrespective of whether the T cells were polyclonal or harboring the ErbB2-CAR construct (**Supplemental Figure 2**). ErbB2-CAR T cells were then activated under Th1 or Treg polarizing conditions in the absence or presence of αKG for 4 days and adoptively transferred into 24JK-ERB tumor-bearing mice. At day 20 of tumor growth, the percentages of ErbB2-CAR CD4 T cells within lymph nodes (LN) were not significantly different in an of the groups (**Figure 3B**). Notably though, within the tumor, the percentages of ErbB2-CAR CD4 T cells were significantly higher following stimulation in either Th1 or Treg+αKG polarizing conditions as compared to control Treg polarizing conditions (***, $p<0.005$; **Figure 3C**). Furthermore, the percentages of $FoxP3^+$ ErbB2-CAR T cells in both LNs and tumors were significantly decreased by the initial *ex vivo* polarization in the presence of αKG ($p<0.005$) and the IFNγ potential of ErbB2-CAR cells in LN, but not in tumors, was significantly augmented ($p<0.01$; **Figures 3B and 3C**). While these data

point to the critical role of the metabolic tumor environment in diminishing the effector function of anti-tumor T cells (8), they strongly indicate that an *ex vivo* modification of intracellular metabolite levels can alter the migration and/or persistence of anti-tumor T cells. Furthermore, αKG treatment significantly decreased the percentage of intratumoral FoxP3^{+} cells. This is particularly important in the solid tumor setting where recruitment of Tregs negatively impacts on the potential of effector T cells to eradicate the tumor (9).

The transcriptional landscape of CD4 T cells polarized to a Treg fate is significantly impacted by αKG. To gain insights into the mechanisms via which αKG negatively impacts on Treg differentiation but not on Th1 differentiation, RNASeq analyses were performed. In accord with the alteration in T cell fate under Treg but not Th1 conditions, αKG-mediated changes in gene expression were more marked in the former as assessed by volcano plot representations (**Figure 4A**). This corresponded to substantially more upregulated and downregulated genes in Treg as compared to Th1 conditions (**Figure 4B**). Upregulation of transcription factors such as T-bet ($p<1x10^{-20}$) were expected based on our initial qRT-PCR analyses (**Figure 2**) but other factors such as Irf1, Irf8 and Stat1 were also highly upregulated, suggesting increased signaling in these cells (**Figure 4C**). Interestingly though, other factors were markedly downregulated by αKG (i.e. Fos, Klf4, Sox4) whereas others were unaffected (Rpl11, Ube3c; **Figure 4C**). Most importantly, gene ontogeny analysis revealed the importance of membrane proteins, glycoproteins, which are often present at the cell membrane, and signal peptides, which result in the translocation of proteins to organelles and the cell membrane (**Figure 4D**). These data highlight the importance of cell surface proteins in the αKG-mediated inhibition of CD4 T cell polarization to a Treg fate.

αKG alters cholesterol metabolism and incorporation of poly-unsaturated fatty acids in the membrane of Treg-polarized lymphocytes. In association with the data presented above, we found that genes involved in cholesterol biosynthesis were significantly downregulated by αKG treatment (**Figure 5A**). Cholesterol is an integral part of the lipid membrane and its incorporation is coordinated with efflux via the ATP-binding cassette transporters ABCA1 and ABCG1 (10, 11). Notably, loss of these transporters in dendritic cells and macrophages resulted in increased inflammatory responses (12-14) and the loss of ABCG1 in T cells has been associated with an increased responsiveness to antigen and altered lipid raft formation

(15, 16). Conversely, the knockout of ABCG1 has also been associated with an increase in the differentiation of natural Tregs (17), but its impact on Treg polarization is not known. Importantly, we find that αKG significantly decreased mRNA levels of both ABCA1 and ABCG1 in CD4 T cells activated in Treg polarization conditions ($p<0.0001$, **Figure 5B** and **Supplementary Figure 3**). While αKG did not impact on these transporters in Th1 conditions, it is important to note that the levels of both ABCA1 and ABCG1 were significantly higher in Treg- than Th1-polarized cells (**Figure 5B**).

As the low cholesterol biosynthesis in αKG-treated CD4 T cells activated under Treg conditions was potentially compensated by decreased levels of ABCA1 and ABCG1 efflux transporters, it was not apparent as to whether cholesterol levels were altered. Using a quantitative lipidomics approach (18), we found that while total lipids were substantially increased by αKG treatment, the percentage of cholesterol as well as other lipid classes were unchanged (**Figure 5C**). Consistent with these data, cholesterol supplementation did not augment Treg differentiation, either in the presence or absence of αKG (**Figure 5D**).

While cholesterol supplementation did not impact on Treg differentiation, despite the massive alterations in the expression of cholesterol biosynthesis genes and ATP-binding cassette transporters, the physical parameters of a membrane are also regulated by changes in the packing of saturated and polyunsaturated fatty acids, altering lateral organization, membrane permeability, tension, bilayer thickness and fluidity, amongst others (19-22). The incorporation of polyunsaturated fatty acids into phospholipids can alter the order of microdomains and the proximity of signaling proteins, thereby impacting on signaling pathways. Indeed, one of the lipid species whose percentage was increased in αKG-treated Tregs was triacylglycerol (TAG) and cellular stores of TAG in lipid droplets release fatty acids that undergo β-oxidation and regulate gene transcription through different signaling pathways (23). Importantly, a comparison of the number of double bonds in phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the most abundant phospholipids in mammalian membranes (24) revealed a significant increase in unsaturation in response to αKG treatment ($p<0.0001$,$p<0.05$; **Figure 5E**). While polyunsaturated phospholipids are generally associated with a higher disorder, it has been shown that polyunsaturated PE can exert a differential structural effect, increasing molecular order

through cholesterol redistribution (25). Using the polarity sensitive C-Laurdan probe and a fluorescence correlation spectroscopy (FCS) combined with super-resolution stimulated emission depletion (STED) microscopy (21, 26), we determined that αKG treatment results in an increased generalized polarization (GP). Thus, the αKG-mediated decrease in CD4 T cell polarization to a Treg fate is accompanied by significant changes in membrane composition, with increased TAG and membrane ordering likely regulating the behavior of membrane receptor signaling pathways.

Augmented αKG-dependent TCA cycling and oxidative phosphorylation negatively regulates Treg differentiation

The metabolism of CD4 T cell subsets is distinct; T effector cells exhibit a highly glycolytic metabolism (27, 28) and suppressive regulatory T cells (Tregs) display a mixed metabolism with augmented lipid oxidation (29-32). However, while mitochondrial metabolism is critical for Treg differentiation and function (33-37), long chain fatty acid oxidation does not appear to be required (38) and the precise role of the TCA cycle in fueling oxidative phosphorylation in Tregs is not known. Interestingly, the level of basal oxygen consumption (correlating with oxidative phosphorylation, OXPHOS) as well as spare respiratory capacity was higher in CD4 T cells polarized to a Th1 as compared to a Treg fate (**Figure 6A**). These data are in accord with a recent study that reported a lower OXPHOS and glycolysis in activated nTreg as well as iTreg as compared to Th0 and Th1 polarized cells (39). The higher OXPHOS in Th1 cells (as compared to Treg) was associated with an augmented glycolysis (measured as a function of the extracellular acidification rate, ECAR); as such, the OCR/ECAR ratios in Th1 and Tregs were equivalent (data not shown). Thus, CD4 T cells polarized to a Treg fate exhibit a lower energetic profile than cells polarized to a Th1 fate.

αKG directly affected the metabolism of Treg-polarized cells because its injection into the flux analyzer immediately increased oxygen consumption (**Figure 6A, middle**) and 2 days later, both the basal and spare respiratory capacity were markedly elevated (**Figure 6A, right**). This increase in OXPHOS resulted in an elevated OCR/ECAR ratio (data not shown) and in accord with the enhanced ATP production from oxidative phosphorylation as compared to aerobic glycolysis, the ratio of ATP/ADP was significantly higher in αKG-treated cells ($p<0.05$; **Figure 6B**). These data strongly suggested an increased level of cycling of TCA intermediates

(**Supplemental Figure 4A**) and indeed, while αKG levels were markedly elevated ($p<0.0001$), as expected from the ectopic addition of mM quantities, it was surprising that MS metabolite analysis did not reveal significant increases in either succinate or malate (**Figure 6C**). Accordingly, we detected a substantial utilization of ectopic αKG in the TCA cycle, monitored by the tracing of glutamine and glucose uniformly labelled with heavy carbon ($^{13}C_5$ and $^{13}C_6$, respectively), as shown by an increased level of utilization of non-labelled (C_0) in the generation of the different intermediates (**Supplemental Figure 4B**). The enhanced level of TCA cycling was also associated with a decreased generation of intracellular lactate (**Supplemental Figure 4C**), in agreement with the increased OCR/ECAR ratio in the presence of αKG.

To assess whether increased αKG levels and the associated augmentation in the αKG/succinate ratio regulated Treg polarization, we monitored the impact of ectopic succinate. While the addition of succinate did not significantly alter Treg polarization, it significantly attenuated the negative impact of αKG (**Figure 6D**). Succinate, which often accumulates due to mutations in the succinate dehydrogenase (SDH) gene, can function by inhibiting prolyl hydroxylase (PHD), thereby leading to stabilization of the HIF-1α transcription factor and decreased TCA flux through the αKG dehydrogenase complex (40-42). To distinguish between potential cytosolic and mitochondrial effects of succinate, we inhibited succinate oxidation with malonate, using a cell-permeable dimethyl malonate (DMM) molecule, impairing the oxidation of succinate to fumarate without altering PHD activity in the cytoplasm (43). Malonate directly decreased oxygen consumption in the presence of αKG, as shown by a diminished spare respiratory capacity following its injection into the Seahorse machine (**Figure 6E**). Notably, under these conditions, malonate not only restored the induction of $FoxP3^+$ T cells in the presence of αKG ($p<0.0001$) but increased FoxP3 levels under control conditions (mean of 63% to 75% $FoxP3^+$ cells, $p<0.01$; **Figure 6F**). Furthermore, malonate massively reduced the generation of inflammatory cytokines including IFN-γ, IL-17, IL-2, TNFα and GM-CSF ($p<0.005$, **Figure 6G**). Similarly, malonate was recently found to decrease IFN-γ secretion in Th1-polarized cells (44). Together, these data show that maintaining a low level of TCA flux is central to Treg differentiation. Indeed, TCA flux is inversely proportional to Treg polarization; conditions that increase TCA flux inhibit Treg polarization while decreased flux augments Treg polarization.

The ensemble of these data provide a mechanistic explanation for the observation by several groups, including our own, that Treg, but not T_{EFF}, generation is maintained under conditions where glucose and amino acid transport are limiting (3, 45-48). Furthermore, together with the finding that Th1 differentiation is augmented under conditions of complement-mediated increases in OCR and ECAR (49), our results support a mechanism whereby IFN-γ secretion by $CD4^+$ T cells is positively regulated by an increased energetic state and an elevated OCR/ECAR ratio. These results have important implications for the modulation of anti-tumor effector function by αKG. Indeed, we found that the negative impact of αKG on Treg polarization was also observed in human CD4 T cells (**Supplementary Figure 5**). And finally, our data showing that the intratumoral migration of effector CAR-T cells is significantly augmented by pre-treatment with αKG suggests that this TCA cycle intermediate may promote the persistence and efficacy of anti-tumor CAR T cells.

Figure 1- Exogenous αKG augments Th1 polarization and inhibits the polarization potential of naïve CD4 T cells towards a Treg fate

(**A**) Naïve CD4 T cells were stimulated with plate-bound αCD3/αCD28 and polarized under Th1 conditions in the absence (control) or presence of exogenous αKG (3.5mM). The percentages of IFNγ$^+$ cells were evaluated by intracellular staining and representative dot plots showing the percentages of IFNγ$^+$ cells are presented (left). Quantification of 20 independent experiments are showed with means ± SEM presented as horizontal lines (right panels) and statistical difference determined by an unpaired 2-tailed t-test; p<0.0001. (**B**) IFNγ mRNA levels were assessed by qRT-PCR at day 4 of Th1 polarization and normalized to HPRT. Each point represents an independent experiment (left panel, n=7) and statistical difference was assessed by an unpaired 2-tailed t-test; p=0.0023. RNAseq read densities of the *Ifng* gene in Th1-polarizing cells in the absence or presence of αKG are presented (n=3, right panels). (**C**) Naïve CD4 T cells were stimulated under Treg polarizing conditions in the absence (control) or presence of exogenous αKG and the percentage of FoxP3$^+$ Tregs was evaluated at day 4. Representative histograms are shown (left) and quantifications of 42 independent experiments are presented (right). Means ± SEM are shown as horizontal lines; p<0.0001 (**D**) FoxP3 mRNA levels were evaluated by qRT-PCR and normalized to HPRT. Each point represents an independent experiment (left, n=8) and means ± SEM are shown as horizontal lines. RNAseq read densities of the *Foxp3* gene in Treg-polarized samples in the absence of presence of αKG are presented (n=3, right panels). (**E**) FoxP3$^+$ nTregs were isolated from FoxP3-GFP reporter mice and activated with αCD3/αCD28 mAbs in the absence or presence of αKG. At day 4, the percentages of FoxP3$^+$ cells were evaluated by flow cytometry (left) and quantification of means ± SEM of 7 independent experiments are presented (right). All significant differences were evaluated by an unpaired 2-tailed t-test.

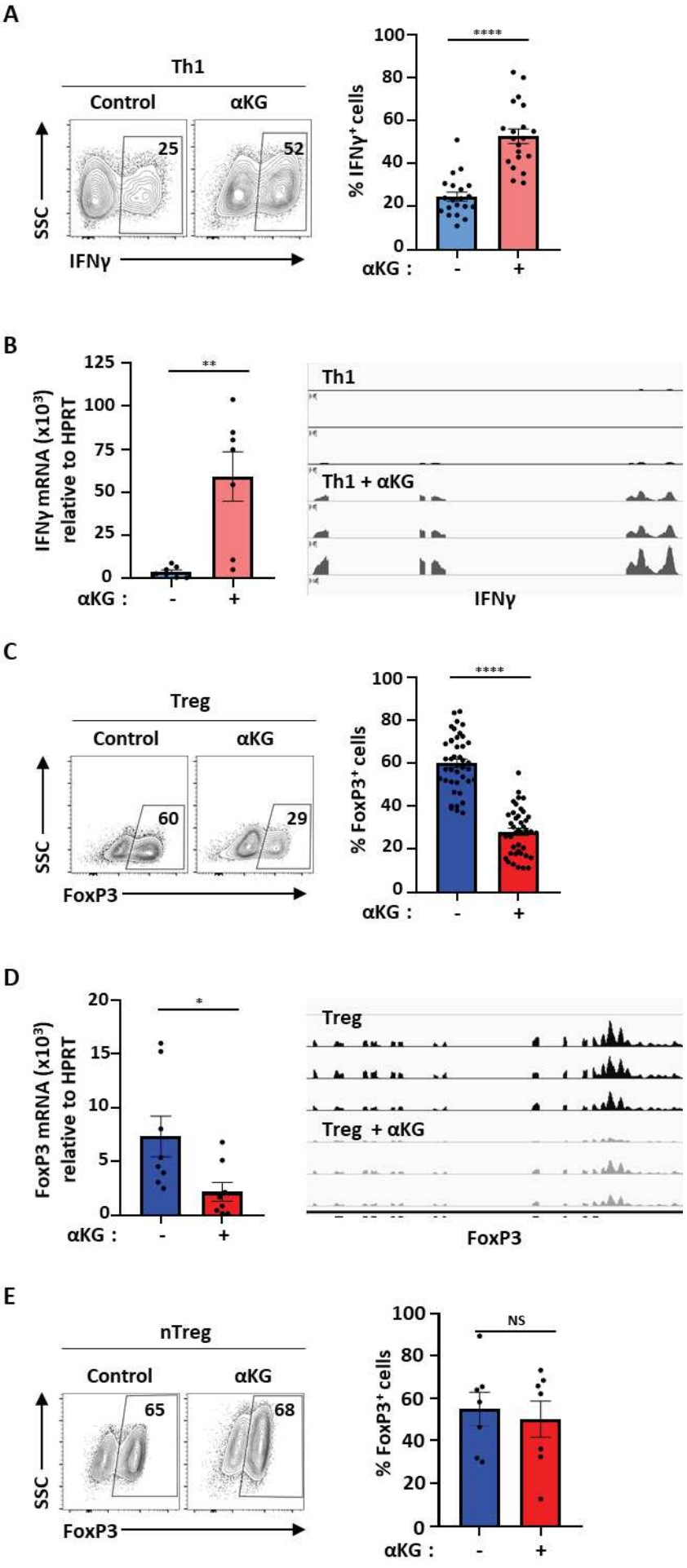
A
Th1
Control
αKG
25
52
SSC
IFNγ
% IFNγ⁺ cells
****
αKG :
B
IFNγ mRNA (x10³) relative to HPRT
**
αKG :
Th1
Th1 + αKG
IFNγ
C
Treg
Control
αKG
60
29
SSC
FoxP3
% FoxP3⁺ cells
****
αKG :
D
FoxP3 mRNA (x10³) relative to HPRT
*
αKG :
Treg
Treg + αKG
FoxP3
E
nTreg
Control
αKG
65
68
SSC
FoxP3
% FoxP3⁺ cells
NS
αKG :

Figure 2- αKG induces an inflammatory profile in Treg-polarized CD4 T cells

(A) Naïve CD4 T cells were activated under Treg polarizing conditions in the absence or presence of αKG and the percentages of IFNγ$^+$ T cells were assessed at day 4 by intracellular staining. Representative dot plots (left) as well as a quantifications of the means ± SEM of 40 independent experiments (right) are shown. Statistical significance was assessed by an unpaired 2-tailed t-test (****, p<0.0001). **(B)** Representative histograms of T-bet protein expression are shown (left) as well as a quantification of the MFIs ± SEM of 8 individual experiments (left, unpaired 2-tailed t-test; p=0.0006). **(C)** T-bet and Granzyme B (GrzB) transcripts were quantified by qRT-PCR and normalized to HPRT and each point shows an independent experiment with means ± SEM presented as horizontal lines (n=7 and n=8 independent experiments for T-bet and GrzB, respectively; unpaired 2-tailed t-test; p=0.0456 and p=0.0095 respectively). **(D)** IFNγ, TNFα and GM-CSF transcripts were assessed by qRT-PCR and each point represents data from an independent experiment (IFNγ, n=8, p=0.0006; TNFα n=8, p=0.6691; GM-CSF, n=4, p=0.714). **(E)** Cytokine secretion was quantified at day 3 of Treg polarization by cytometric bead array (before the addition of supplementary IL-2). IFNγ, IL-17, IL-2, TNFα, and GM-CSF levels in 10 independent experiments are presented with means ± SEM shown as horizontal lines (unpaired 2-tailed T-test; IFNγ, p<0.0001; IL-17, p<0.0001; IL-2, p=0.002; TNFα, p<0.0001; and GM-CSF, p<0.0001).

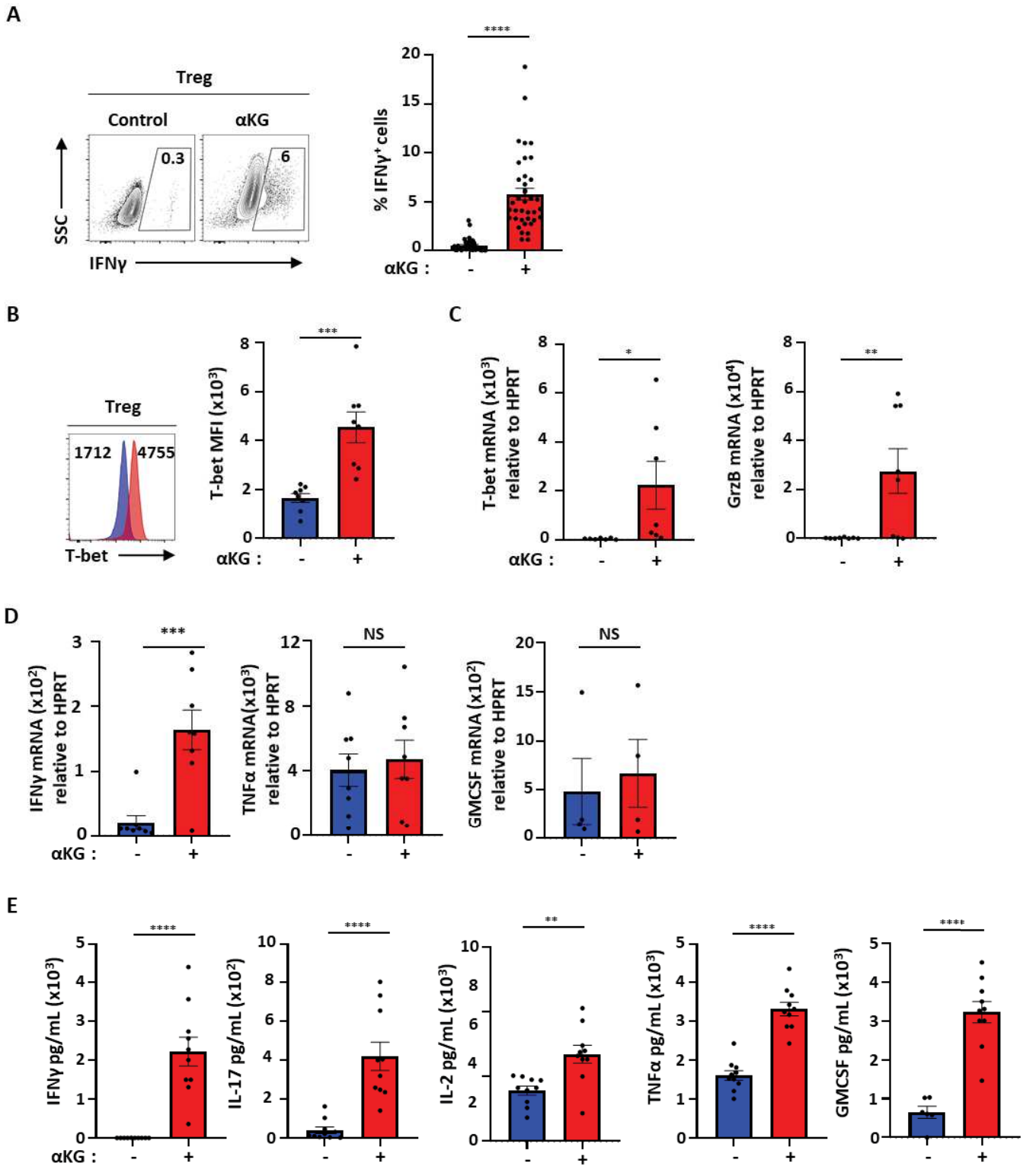
A
Treg
Control
αKG
0.3
6
SSC
IFNγ
% IFNγ+ cells
αKG :
B
Treg
1712
4755
T-bet
T-bet MFI (x10³)
C
T-bet mRNA (x10³) relative to HPRT
GrzB mRNA (x10⁴) relative to HPRT
D
IFNγ mRNA (x10²) relative to HPRT
TNFα mRNA(x10³) relative to HPRT
GMCSF mRNA (x10²) relative to HPRT
NS
E
IFNγ pg/mL (x10³)
IL-17 pg/mL (x10²)
IL-2 pg/mL (x10³)
TNFα pg/mL (x10³)
GMCSF pg/mL (x10³)

Figure 3- ErbB2-CAR^{+} T cells polarized ex vivo in the presence of αKG maintain an in vivo inflammatory profile following adoptive transfer into tumor-bearing mice

(A) Schematic of the tumor challenge protocol following the subcutaneous injection of the 24JK fibrosarcoma cell line expressing the ErbB2 antigen (24JK-ERB) into the flanks of RAG2$^{-/-}$ mice. Naïve CD4 T cells expressing the ErbB2-CAR were activated in Th1 or Treg polarizing conditions, in the absence of presence of αKG for 4 days, and then adoptively transferred into tumor-bearing mice at day 7. Mice were sacrificed 13 days later (day 20). Figure created with Biorender.com **(B)** The percentages of CD4^{+}, FoxP3^{+}, and IFNγ^{+} T cells recovered from lymph nodes of tumor-bearing mice were evaluated at day 20 and representative plots are shown (left). Quantifications of the percentages of T cells with the indicated phenotypes are presented with each point representing data from an individual mouse from 2 independent experiments (n=6-8). **(C)** The percentages of CD4^{+}, FoxP3^{+}, and IFNγ^{+} T cells recovered from mouse tumors were evaluated at day 20 and representative plots (left) and quantifications were assessed as above. Statistical differences were determined by a one-way ANOVA and Tukey test for multiple comparisons (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; NS, not-significant).

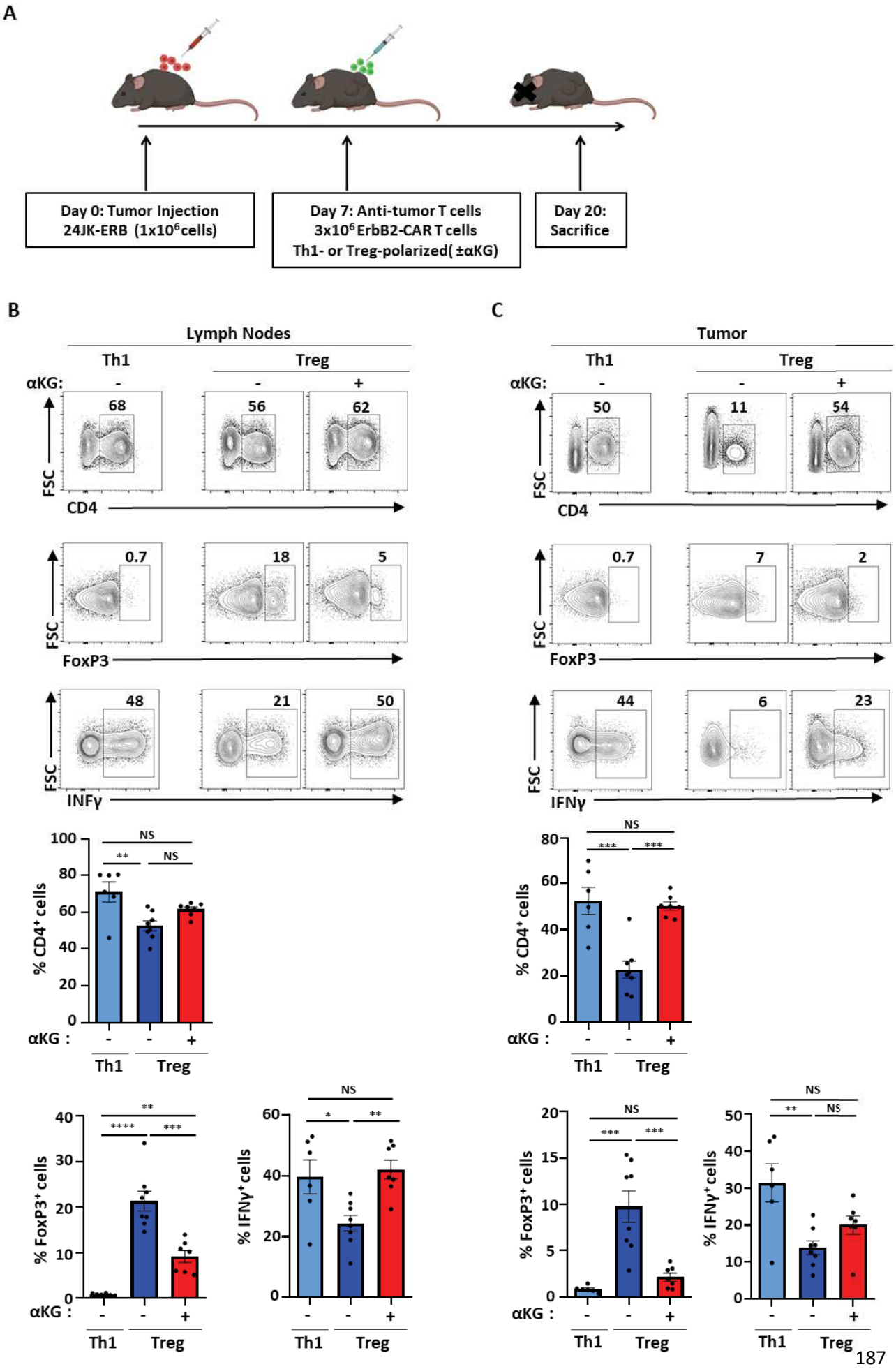
A
Day 0: Tumor Injection
24JK-ERB (1x10⁶cells)
Day 7: Anti-tumor T cells
3x10⁶ ErbB2-CAR T cells
Th1- or Treg-polarized(±αKG)
Day 20:
Sacrifice
B
Lymph Nodes
Th1
Treg
αKG:
68
56
62
CD4
FSC
0.7
18
5
FoxP3
48
21
50
INFγ
% CD4⁺ cells
% FoxP3⁺ cells
% IFNγ⁺ cells
C
Tumor
50
11
54
7
2
44
6
23
IFNγ
NS

Figure 4- Genes affecting membrane-related processes are strongly impacted by αKG in Treg polarizing conditions

Murine naïve CD4 T cells were stimulated with plate-bound αCD3/αCD28 and polarized to Th1 or iTreg conditions in the presence or absence of exogenous αKG. On day 4 following polarization, cells were collected and transcript analysed by RNAseq. **(A).** Volcano plot representations of the differential expression analyses of genes expressed in T cells activated in Th1 (left) and Treg (right) polarization conditions in the absence versus presence of αKG. The x-axis shows $\log_2$ fold-changes in expression and the y-axis presents the log odds of a gene being differentially expressed. Downregulated (left) and upregulated (right) genes with a 3-fold change threshold are shown as dotted lines. **(B)** Venn diagram representation of the RNAseq data showing the number of genes upregulated (left) and downregulated (right) in Th1- (dark grey) and Treg- (light grey) polarizing cells (as based on a 3-fold change), with the intersection of common genes. **(C)** Genes identified in the transcription factor term in Treg-polarizing conditions, in the absence or presence of αKG, are presented. Statistical significance is indicated in the color scheme. **(D)** Gene ontology analysis (DAVID 6.7) of RNA-seq data. Significantly enriched terms associated to differentially expressed transcripts that were either upregulated (left) or downregulated (right) in Treg polarizing conditions in the presence of αKG versus control Treg-polarizing conditions are presented. The percentages of hits identified in each pathway are depicted, with the highest percentages of genes clustered in membrane-related pathways.

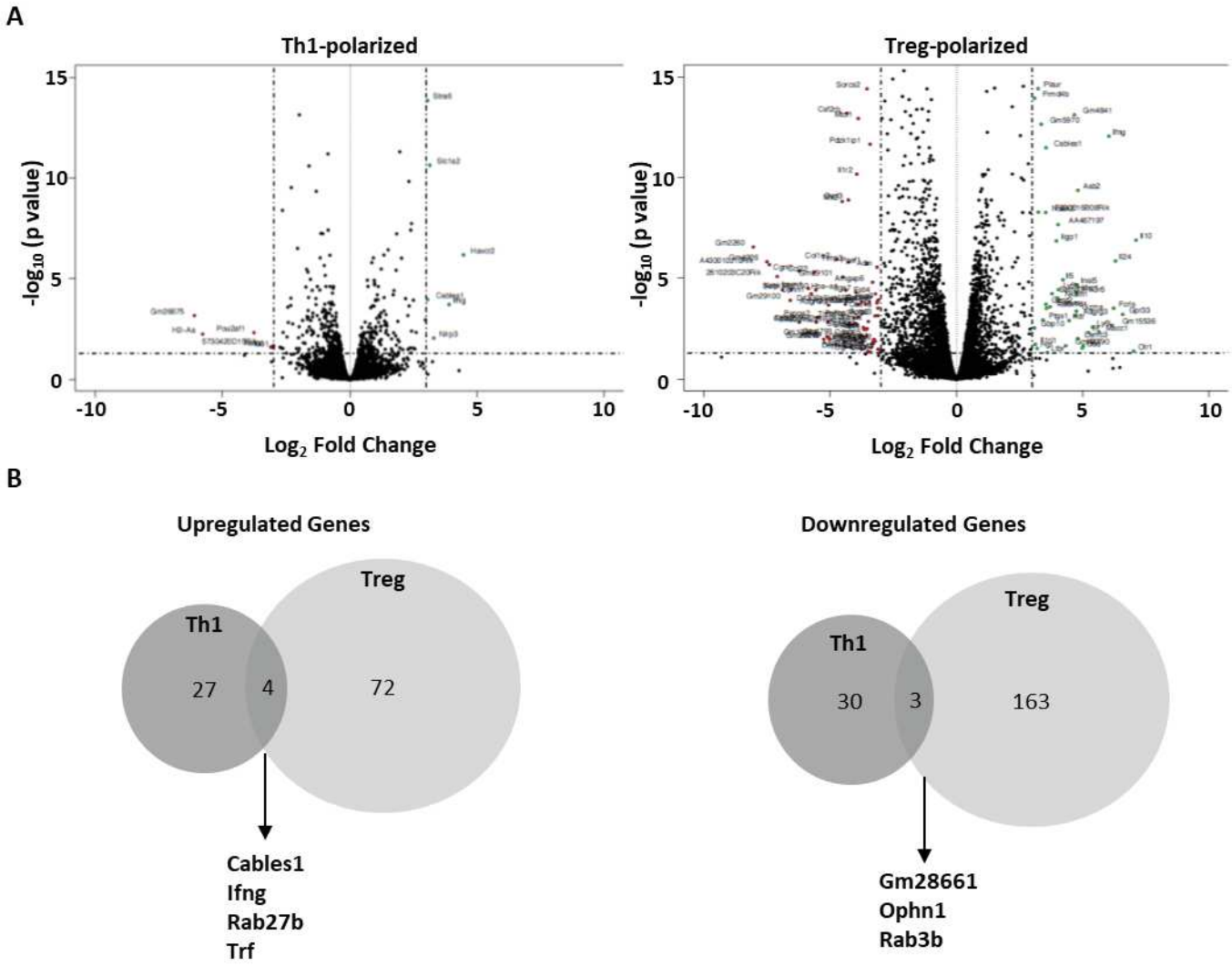
A
Th1-polarized
Treg-polarized
-log10 (p value)
Log2 Fold Change
B
Upregulated Genes
Th1
Treg
27
4
72
Cables1
Ifng
Rab27b
Trf
Downregulated Genes
Th1
Treg
30
3
163
Gm28661
Ophn1
Rab3b
C
Expression over mean
Significance
Treg
Treg + αKG
Irf1
Irf8
Tbx21
Stat1
Runx2
Hopx
Tfap2a
Sox4
Mycl
Klf4
Fos
Foxo4
Trerf1
Zfp361
Rere
Ar
Plagl1
Tead1
Rpl11
Ube3c
Eif3h
3
2.5
2
1.5
1
0.5
0
Significance
1e-15 to 1e-6
<1e-20
Not significant
D
Upregulated
Downregulated
Treg-polarized
17%
15%
Membrane
Glycoprotein
Signal Peptide
Nucleus
Metal binding

Figure 5- Lipid saturation and membrane order is altered by αKG under Treg polarization conditions

(A) Specific transcripts of genes implicated in cholesterol biosynthesis that are significantly decreased in Treg polarizing conditions in the presence of αKG and RNASeq data from 3 independent experiments are presented. **(B)** Abca1 and Abcg1 mRNA levels were evaluated by qRT-PCR and normalized to HPRT. Points from 6-7 independent experiments per condition are presented and means ± SEM are shown as horizontal lines (****, $p<0.0001$). **(C)** Lipidomic analysis of total lipid levels (Picomol) in $1x10^6$ CD4 T cells polarized to a Treg fate in the absence or presence of αKG in 2 independent experiments performed in triplicate (left). The percentage abundance of the different lipid classes was evaluated (right panel: CE, cholesterol esters; Cer, ceramides; CL, cardiolipin; DAG, diacylglycerols; TAG, triacylglycerol; HC, hexosyl ceramide; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamine; LP∗, LPC and LPE plasmalogens; PC, phosphatidylcholines; PCO, PC plasmalogens; PE, phosphatidylethanolamines; PEO, PE plasmalogens; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins). Data are presented as means ± SEM of 3 technical replicates from 2 independent experiments (Exp 1, Exp 2). **(D)** Naïve CD4 T cells were activated in Treg polarizing conditions in the absence or presence of αKG and following addition of cholesterol (water soluble, 50mM). FoxP3 expression was evaluated on day 4 by intracellular staining and representative dot plots are presented (left) as well as a quantification of the means ± SEM of 5 independent experiments. Statistical difference was determined by a one-way ANOVA and Tukey test for multiple comparisons (NS, not significant). **(E)** The mean abundance of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipid species with the indicated numbers of double bonds was assessed in conditions of Treg polarization in the presence of αKG and ratios for each level of unsaturation relative to control Treg polarization conditions (control) are presented. Statistical differences were assessed between the values of 0-2 and 3-12 double bonds. **(F)** Lipid packing, imaged by C-laurdan spectral microscopy and quantified by Generalized Polarization (GP), was evaluated following Treg polarization in the absence (129 cells) or presence of αKG (132 cells). Quantification, corresponding to increasing lipid order, is presented as means ± SEM from 3 individual experiments, and statistical difference was determined by determined by an unpaired 2-tailed t-test; $p<0.0001$.

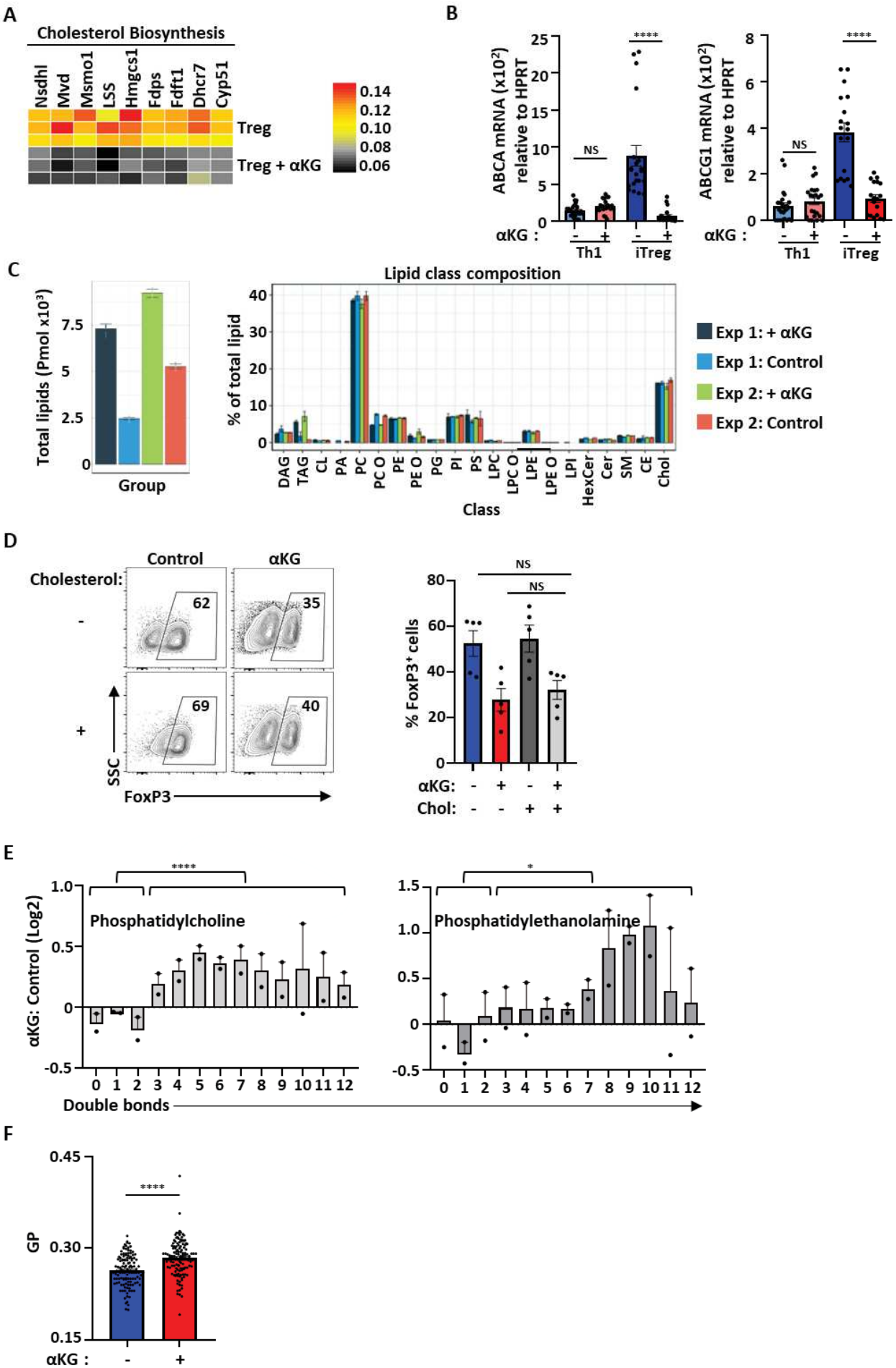
A
Cholesterol Biosynthesis
Nsdhl
Mvd
Msmo1
LSS
Hmgcs1
Fdps
Fdft1
Dhcr7
Cyp51
Treg
Treg + αKG
0.14
0.12
0.10
0.08
0.06
B
ABCA mRNA (x10²) relative to HPRT
ABCG1 mRNA (x10²) relative to HPRT
NS
****
αKG :
Th1
iTreg
C
Total lipids (Pmol x10³)
Group
Lipid class composition
% of total lipid
DAG
TAG
CL
PA
PC
PC O
PE
PE O
PG
PI
PS
LPC
LPC O
LPE
LPE O
LPI
HexCer
Cer
SM
CE
Chol
Class
Exp 1: + αKG
Exp 1: Control
Exp 2: + αKG
Exp 2: Control
D
Control
αKG
Cholesterol:
62
35
69
40
SSC
FoxP3
% FoxP3⁺ cells
αKG:
Chol:
E
Phosphatidylcholine
Phosphatidylethanolamine
αKG: Control (Log2)
Double bonds
F
GP
αKG :

Figure 6- Treg differentiation is enhanced by blocking the αKG-induced augmentation in oxidative phosphorylation

(A) Oxygen consumption (OCR), a measure of oxidative phosphorylation, was monitored on a Seahorse XFe96 analyzer at day 2 of polarization of naïve CD4 T cells to a Th1 versus Treg fate; means ± SEM of data from 9 independent experiments are shown (left panel). The impact of injecting αKG directly into the XFe96 flux analyzer at day 2 of Treg polarization was evaluated (middle panel; data are representative of 1 of 2 independent experiments). OCR of CD4 T cells polarized to a Treg fate at day 4 of stimulation, in the absence or presence of αKG, is presented (means ± SEM of 9 independent experiments). **(B)** The ATP/AMP ratio of CD4 T cells polarized to a Treg fate in control conditions or in the presence of αKG were evaluated by mass spectrometry and means ± SEM of triplicate technical replicates from 2 independent experiments are presented and statistical difference determined by an unpaired 2-tailed t-test. **(C)** The levels of αKG, succinate, malate, citrate and 2-hydroxyglutarate (2HG) were evaluated by mass spectrometry and the ratio of these metabolites in αKG-treated to control conditions are presented as means ± SEM of triplicate technical replicates from 2 independent experiments. Statistical significance was evaluated by an unpaired 2-tailed t-test. **(D)** To assess the impact of the αKG/succinate ratio on Treg differentiation, naïve CD4 T cells were stimulated in Treg polarization conditions in the presence or absence of αKG and succinate (7 mM). FoxP3 expression was evaluated a day 4 and representative plots are shown (left). Quantification of FoxP3 levels are presented as means ± SEM from 3 independent experiments and significance was evaluated by a one-way ANOVA and Tukey multiple comparison test (*, $p<0.05$; NS, not significant). **(E)** Oxygen consumption (OCR) was monitored in CD4 T cells stimulated in Treg-polarizing conditions in the presence of αKG and the impact of dimethyl malonate (DMM) was evaluated. Data are representative of means of 6 samples in 2 independent experiments. **(F)** The impact of malonate (10 mM) on Treg polarization was evaluated in the absence of presence of αKG. Representative FACS plots of FoxP3 expression are presented at day 4 (left) and quantifications of the means ± SEM of 19 independent experiments are presented (right). Significance was determined by a one-way ANOVA and Tukey multiple comparison test (**, $p<0.01$; ****, $p<0.0001$; NS, not significant). **(G)** IFNγ, IL-17, IL-2, TNFα, and GM-CSF secretion by CD4 T cells polarized to a Treg fate in the absence of presence of αKG and/or malonate was evaluated at day 4 and means ± SEM are presented (n=10 independent experiments for αKG conditions and n=5 for malonate conditions). Significance was determined by a one-way ANOVA and Tukey multiple comparison test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$; ****, $p<0.0001$; NS, not significant).

Figure 6

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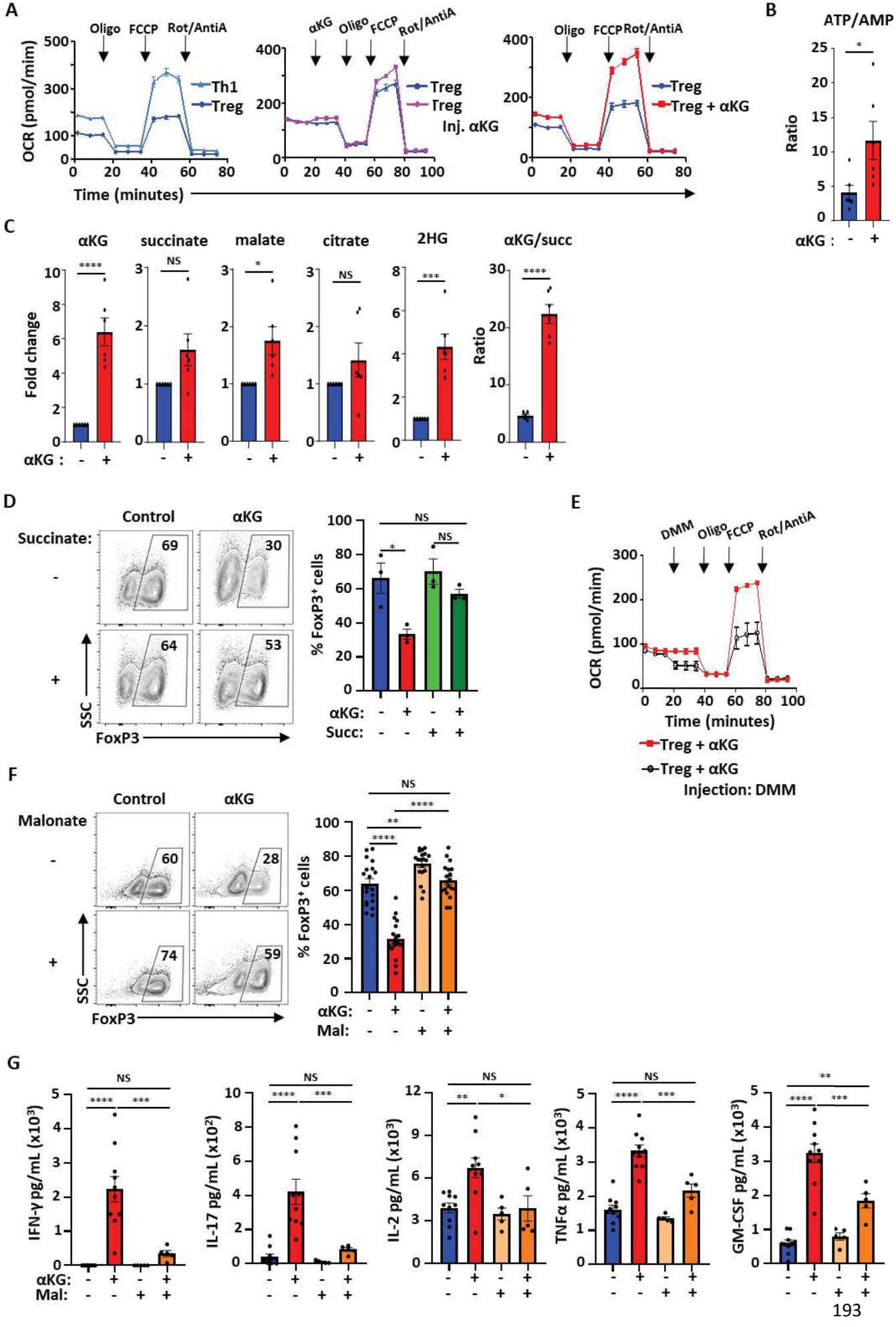
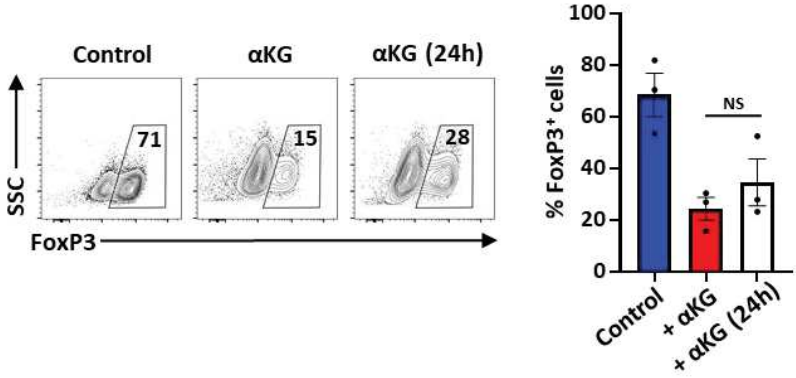
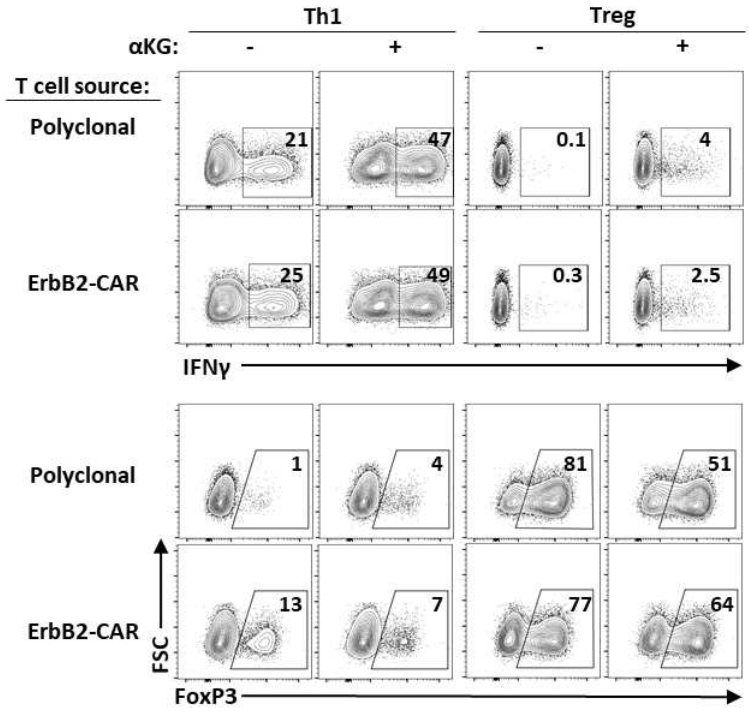




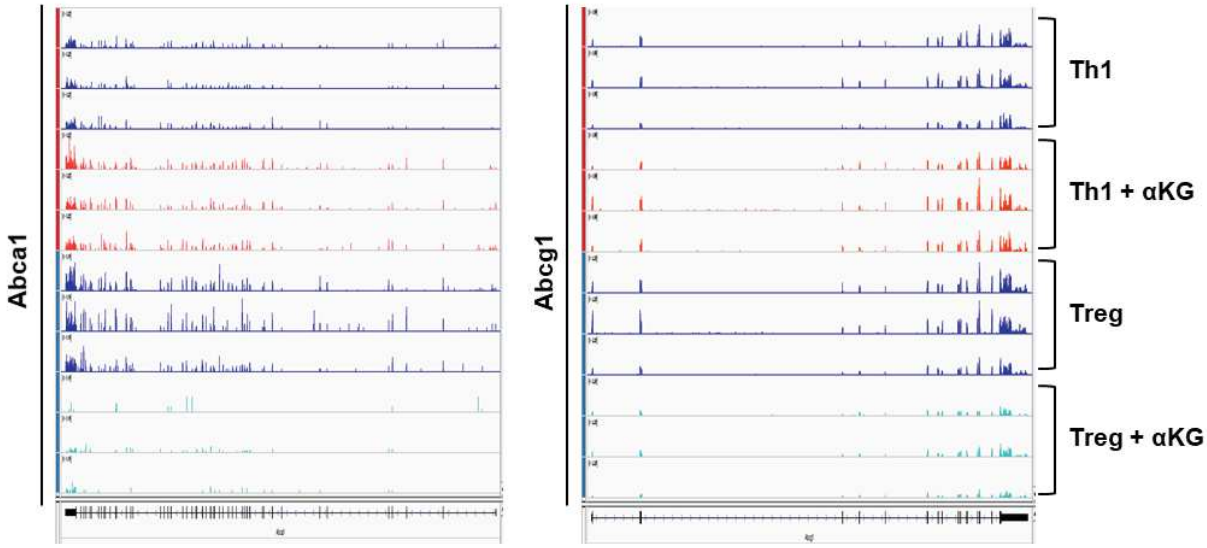
Supplemental Figure 1- Treg polarization is attenuated by αKG following TCR stimulation

Naïve CD4 T cells were activated under Treg-polarizing conditions and αKG was added either at the time of activation or at 24h post stimulation. FoxP3 expression was evaluated at day 4 and representative dot plots are shown (left) as well as a quantification of the means ± SEM of 3 independent experiments. Significance was assessed by a one-way ANOVA and Tukey multiple comparison test (NS, not significant).



Supplemental Figure 2- ErbB2-CAR T cells exhibit an attenuated Treg polarization in the presence of αKG

The potential of ErbB2-CAR T cells following stimulation in Treg-polarizing conditions in the absence or presence of αKG compared to polyclonal T cells to produce IFNγ was evaluated by intracellular staining and representative plots are shown (top plots). The induction of FoxP3 expression was also evaluated (bottom plots).



Supplemental Figure 3- Abca1 and Abcg1 levels are decreased by αKG treatment in Treg-polarizing conditions

RNAseq read densities of the *Abca1* and *Abcg1* genes in Th1- and Treg-polarizing conditions in the absence of presence of αKG are presented (n=3).

Supplemental Figure 4

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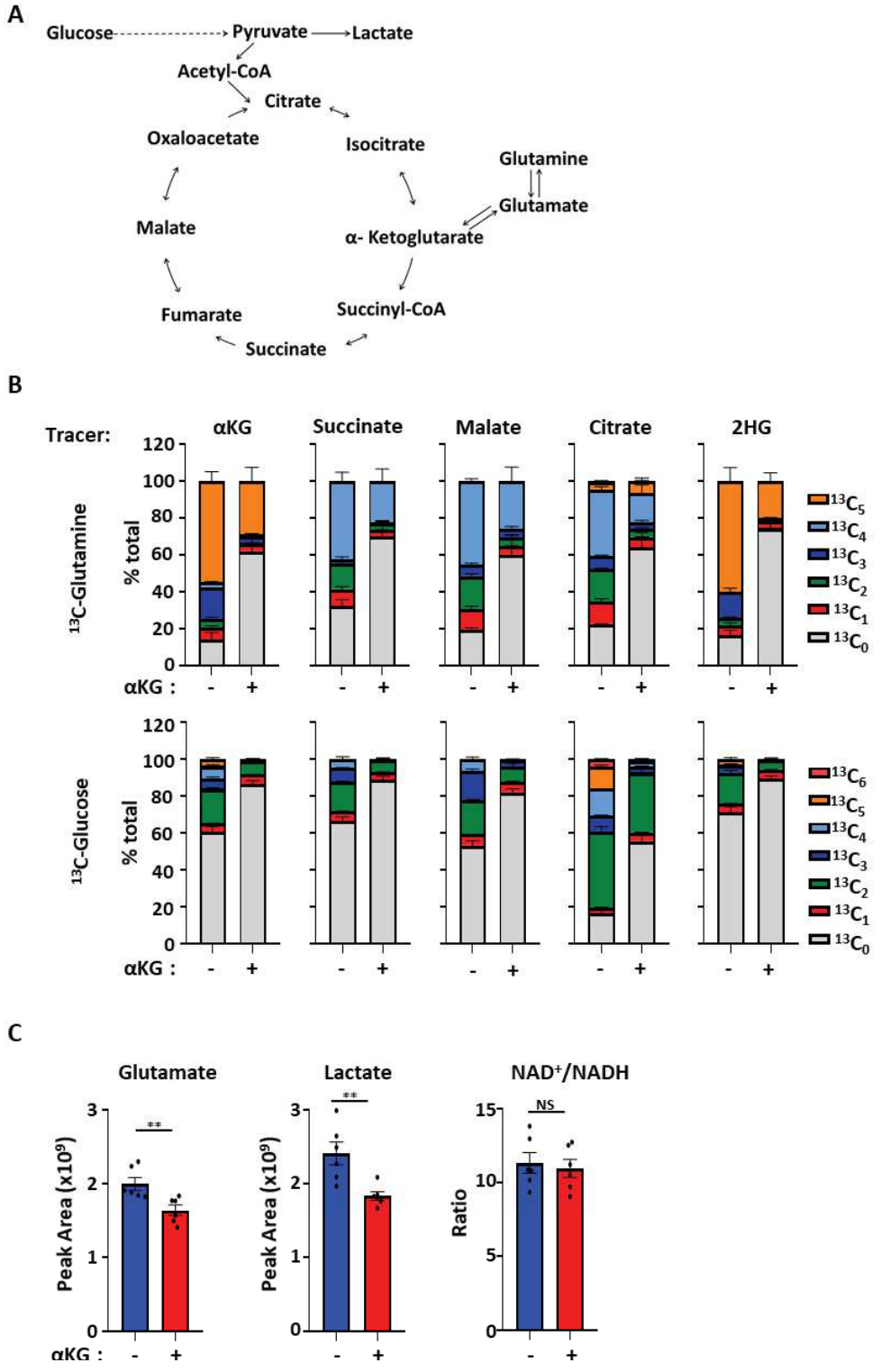
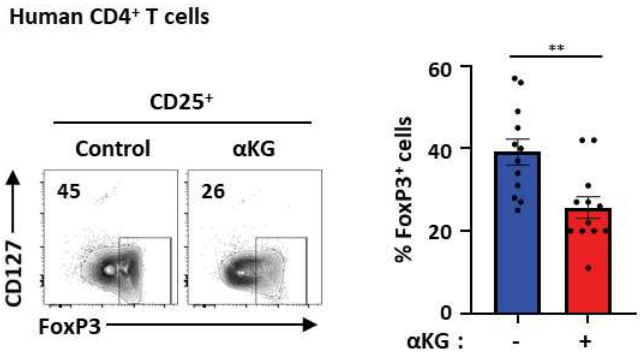


Supplemental Figure 4- Tracing of glucose and glutamine metabolism in Treg-polarizing conditions

(A) Schematic representation of the TCA cycle, showing the entry of glucose and glutamine. **(B)** The distribution of isotopologues from and [^{13}C]glutamine and [^{13}C]glucose into TCA cycle intermediates are shown. **(C)** The levels of glutamate and lactate as well as the NAD$^+$/NADH ratio were assessed in the indicated conditions by mass spectrometry (means ± SEM; n= 6 technical replicates in 2 biologically independent samples). Statistical differences were determined by an unpaired 2-tail t-test.



Supplemental Figure 5- Human CD4$^+$ T cells exhibit attenuated Treg polarization in the presence of αKG

Naïve human CD4$^+$ T cells were activated in Treg-polarizing conditions and at day 4 of stimulation, Treg polarization was evaluated as a function of a CD25$^+$/CD127$^-$/FoxP3$^+$ phenotype and representative dot plots are shown (left). Quantification of the percentages of FoxP3$^+$ cells is shown for 12 independent experiments (12 distinct donors) and significance was determined by an unpaired t-test (**, p=0.0034).

Materials and Methods

Mice

Ly5.1 or Ly5.2 C57BL/6 mice were crossed with Foxp3-GFP reporter mice and mice harboring an ErbB2-CAR (Yong et al., 2015) were crossed with C57BL/6 FOXP3GFP mice. Mice were housed in a conventional, pathogen-free facility at the Institut de Génétique Moléculaire de Montpellier. Animal care and experiments All experiments were approved by the local animal facility institutional review board in accordance with French national guidelines.

T cell isolation, activation and culture

Murine $CD4^+$ T cells from the indicated mice strains were purified using the MACS $CD4^+$ T cell negative selection kit (Miltenyi Biotec) and naïve $CD4^+$ T cells were then sorted on the basis of a $CD4^+$ $CD8^-$ $CD62L^{hi}$ $CD44^-$ GFP^- expression profile on a FACSAria flow cytometer (BD Biosciences). Natural Tregs were sorted as $CD4^+$ $CD8^-$ $CD62L^{hi}$ $CD44^-$ $CD25^+GFP^+$. T cell activation was performed using plate-bound α-CD3 (clone 145-2C11, 1 mg/ml) and α-CD28 (clone PV-1, 1 mg/ml) monoclonal antibodies in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco-Life technologies). For Th1 and iTreg-polarizing conditions, IL-12 (15 ng/ml) and αIL-4 antibody (5 ug/ml) or hTGF-β (3 ng/ml) and hIL-2 (100 U/ml), respectively, were added to the cultures. When indicated, cell permeable αKG (dimethyl ketoglutarate, 3.5 mm; Sigma), malonate (dimethyl malonate, 10mM; Sigma), succinate (diethyl succinate, 10mM; Sigma) and water soluble cholesterol (50mM, C4951, Sigma) were added. Cells were maintained in a standard tissue culture incubator containing atmospheric (20%) O2 and 5% CO_2.

Human $CD4^+$ T cells were isolated from healthy adult donors. All experiments using primary human cells were conducted in accordance with the Declaration of Helsinki and IRB approval to the French Blood Bank (Etablissement Francais du Sang). T lymphocytes were purified by negative-selection using Rosette tetramers (StemCell Technologies) and the purity was monitored by flow cytometry. For sorting of Human $CD4^+$ Naïve T cells, $CD4^+$ T cells were sorted on the basis of $CD4^+$ $CD8^-$ $CD45RA^+$ $CD45RO^-$ $CCR7^+$ $CD62L^+$ $CD127^{low}$ and $CD25^-$ on a BD FACSAria flow cytometer. For Th1 and iTreg-polarizing conditions, IL-12 (10 ng/ml) and α–IL-4 antibody (10 mg/ml) or human TGF-β (3 ng/ml) and rhIL-2 (100 U/ml) respectively, were

added to the cultures. T cell activation was performed using plate-bound α-CD3 (clone OKT3, Biolegend) and α-CD28 (clone 9.3, kindly provided by Carl June) mAbs at a concentration of 1 μg/ml in RPMI medium 1640 (Life Technologies) supplemented with 10% FCS and 1% penicillin/streptomycin (Gibco-Life technologies).

Immunophenotyping and flow cytometric analysis

Immunophenotyping of cells was performed with fluorochrome-conjugated antibodies, and intracellular staining was performed after the fixation and permeabilization of the cells (intracellular staining kit from ThermoFisher or BD Bioscience). Analysis of cells collected from in vivo experiments were performed following Fc block treatment (2.4G2, BioXcell) for 10 minutes prior to incubation with conjugated antibodies for FACS analysis. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 ug/ml; all from Sigma) for 3.5–4h at 37 °C. Cells were labeled with fixable viability dye prior to fixation, followed by at least 3 hours of intracellular staining with the following antibodies; Foxp3-PeCy7, T-Bet-Pe and IFNγ-APC. Cytokine production (IFNγ, TNFα, IL-2, IL-17 and IL-10) was also assessed by Cytometric Bead Array (CBA) Kit (BD Biosciences) using supernatants at day 3 of polarization. For human cells; immunophenotyping was performed using fluorochrome-conjugated mAbs against CD4, CD8, CD45RA, CD45RO, CCR7 and CD62L (BD Bioscience, Beckman Coulter or eBioscience). Cells were analyzed on a FACSCanto or LSRII-Fortessa flow cytometer (BD Biosciences). Data analysis was performed using FlowJo (Tree Star software) and FCAPArray Software (CBA analysis). Antibodies are presented in **Table 1**.

Tumour model and adoptive T cell transfer

The murine 24JK fibrosarcoma cell line expressing the human ErbB2/Her2 antigen was generated as described Yong, 2016 #3110}. 24JK-ErbB2 (1×10^6) were subcutaneously injected into $RAG2^{-/-}$ mice and allowed to establish over a 7 days period. On day 7, $3\text{-}5 \times 10^6$ *in vitro* ErbB2-CAR T cells, activated as indicated, were injected retroorbitally into tumor-bearing mice. For endpoint analyses,: tumors were excised, mechanically digested in T cell culture media (as above) using collagenase IV (1 mg/ml, Sigma) and DNASE I (500μg/ml, Roche), incubated for 25 minutes at 37°C and processed into single cell suspensions for FACS analysis.

Lymph nodes and spleens of tumor bearing mice were isolated and dissociated into single cell suspensions in PBS + 2% FCS and analyzed by flow cytometry.

RNA-seq analysis

T cells (5x 10^6) were lysed in triplicates in TRIzol (Thermofisher). Total RNA was isolated using the mirVana (Applied Biosystems) isolation kit. RNA-seq libraries were prepared from 4 µg of total RNA using the TruSeq RNA sample preparation kit following manufacturer's protocol (Illumina). Briefly, oligo-dT purified mRNA was fragmented and subjected to first and second strand cDNA synthesis. cDNA fragments were blunt-ended, ligated to Illumina adaptors, and PCR amplified to enrich for the fragments ligated to adaptors. The resulting cDNA libraries were verified and quantified on Agilent Bioanalyzer and RNA-seq was conducted using the GAIIx Genome Analyzer (Illumina). Reads from RNA-seq were mapped to the human genome (GRCh37, hg19) using the splice-aware aligner TopHat with option – mate-inner-dist 160 – coverage-search – microexon-search – max-multi hits. Aligned reads were then visualized on a local mirror of the UCSC Genome Browser. Quantification of gene expression was performed by counting aligned reads on each gene, for each condition. DEGseq was then applied to identify differentially expressed genes with *p value* $\leqslant 1e^{-5}$.

Gene expression analysis

RNA was isolated from T cells as indicated using the RNeasy Micro Kit and then reverse-transcribed to produce cDNA using oligonucleotide priming with the QuantiTect Reverse Transcription Kit (both Qiagen). Quantitative PCR was performed using the Quantitect SYBR green PCR Master mix (Roche) with 10 ng of DNA and 0.5 µM primers in a final volume of 10 µl. Amplification of DNAs was performed using the LightCycler 480 (Roche). Each sample was amplified in triplicate. Relative expression was calculated by normalization to HPRT and primer sequences are presented in **Table 2**.

Lipid analyses

Mass spectrometry-based lipid analysis was performed at Lipotype GmbH (Dresden, Germany) as previously described (Sampaio et al., 2011). Lipids were extracted using a two-step chloroform/methanol procedure (Ejsing et al., 2009). Samples were analyzed by direct infusion on a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa

NanoMate ion source (Advion Biosciences) as described (Mitroulis et al., 2018). Phospholipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid quadrupole linear ion trap mass spectrometer (4000 QTRAP system, AB SCIEX) equipped with a TriVersa NanoMate robotic nanosource (Advion Biosciences) as previously described (Talebi et al., 2018).

Generalized polarization (GP) was calculated to determine molecular order of the plasma membrane. Cells were stained with Laurdan (ThermoFisher) and fixed with 4% paraformadehyde before super-resolution STED microscopy with scanning fluorescence correlation spectroscopy (scanning STED-FCS, sSTED-FCS) to characterize the spatial and temporal heterogeneity of lipid interactions as previously described (Sezgin et al., 2019). Quantification of fluorescence intensity was performed using NIH ImageJ.

Mass spectrometry (LC-MS)

For profiling of intracellular metabolites, cells ($1x10^6$) were treated as previously described (Oburoglu et al., 2014). Briefly, cells were incubated in RPMI medium without glucose or glutamine and supplemented with uniformly labeled [U-$^{13}C_6$]glucose or [U-$^{13}C_5$-$^{15}N_2$]glutamine, respectively, for 24 hours. Cells were rapidly washed in ice-cold PBS and metabolites were extracted in a 50% methanol / 30% acetonitrile/ 20% water solution. The volume of extraction solution was adjusted to 2 x 10^6 cells / ml for all samples and were normalized to the number of cells obtained at the end of the 24-hour incubation period. All cultures were performed in triplicate. Both cell extracts and media were centrifuged at 16,000g for 10min at 4°C and supernatants were analyzed by HPLC-MS. Samples were separated on a Thermo Scientific Accela liquid chromatography system coupled with a guard column (Hichrom) and a ZIC-pHILIC column (Merck). The separation was performed using a gradient elution in a solution of 20mM ammonium carbonate, with 0.1% ammonium hydroxide and acetonitrile. Metabolites were detected using a Thermo Scientific Exactive Orbitrap mass spectrometer with electrospray ionization, operating in a polarity switching mode. Raw data were analyzed with Xcalibur 2.2 and LCquan 2.7 Thermo scientific software.

Extracellular flux analysis

OCR and ECAR were measured using the XFe-96 Extracellular Flux Analyzer (Seahorse Biosciences, Agilent). Cells (1.7-2x10^5/well) were resuspended in XF media (buffered RPMI) in the presence of glucose (11 mM) and L-glutamine (2 mM), added to poly-D-lysine-coated wells (0.1mg/ml, Millipore) and monitored in basal conditions and in response to oligomycin (1 µM), FCCP (1 µM), rotenone (100 nM) and antimycin A (1 µM; Sigma). Metabolites were added as indicated.

Statistical analyses

Data were analyzed using GraphPad software version 8 (Graph Pad Prism, La Jolla, CA) and p values were calculated using a one-way ANOVA (Tukey's post-hoc test) or paired t-tests as indicated.

Table 1: Antibody List

Antigen	Fluorochrome	Company	Clone	Catalog #	Lot
CD25	AAF	EBIO	PC61.5	47-0251.82	4277960
TCRB	AF594	BIOLEGEND	H57-597	109238	B216283
CD62L	APC	BD	MEL-14	553152	7116619
IFNY	APC	EBIO	XMG1.2	17-7311-82	4332526
CD62L	APC	BD	MEL-14	553152	8218563
CD8A	APC	BD	53-6.7	553035	5230918
CD8	APC-R700	BD	53-6.7	564983	6137644
CD8	APCEF780	INVITRO	53-6.7	47-0081-82	1911422
CD45	APCEF780	EBIO	30-F11	47-0451-80	4297796
CD44	APCEF780	EBIO	IM7	47-0441-82	4329926
CD8A	APCEF780	EBIO	53-6.7	47-0081-82	E08443-1638
CD4	BV711	BD	RM4-5	563726	8086836
CD45.2	BV711	BD	104	563685	7123514
IL17A	BV786	BD	TC11-18H10	564171	4177831
CD45.2	FITC	BD	104	553772	24533
CD8	FITC	BD	53-6.7	553031	38608
CD86	FITC	BD	GL1	553691	85425
CD62L	PE	BD	MEL-14	553151	74060

TBET	PE	EBIO	EBIO4B10	12-5825-82	4313524
TNFA	PE	EBIO	MP6-XT22	12-7321-71	E028998
CD4	PE	BD	RM4-5	553049	16834
CD45RB	PE	INVITRO	16A	MA5-17886	SE2385342
CD45.2	PE	BD	104	560695	6028599
IL17A	PE	EBIO	EBIO17B7	12-7177-81	4276914
CD11C	PE	BD	HL3	557401	64082
CD45.1	PE	BD	A20	553776	81093
CD4	PE	BD	RM4-5	553049	4189673
CCR7	PECF594	BD	4B12	563596	5070605
CD25	PECY7	BD	PC61	552880	7341665
FOXP3	PECY7	EBIO	FJK-16S	25-5773-82	1988695
CD25	PECY7	BD	PC61	552880	7341665
CD44	PECY7	EBIO	IM7	25-0441-82	4290712
IL-2	PECY7	BD	JES6-5H4	560538	2104623
F4/80	PECY7	EBIO	BM8	25-4801-82	E07631-1630
CD4	PERCPCY5.5	BD	RM4-5	550954	7047561
CD4	PERCPCY5.5	BD	RM4-5	550954	7047561
TBET	PERCPCY5.5	EBIO	EBIO4B10	45-5825-82	4305907
CCR3	PERCPCY5.5	BD	83103	742079	7317604
NK1.1	PERCPCY5.5	BD	PK136	551114	32032
CD8A	PERCPCY5.5	BD	53-6.7	551162	7051685
CD71	PERCPEF710	EBIO	R17217	46-0711-82	E14768-103
CD4	V450	BD	RM4-5	560468	560468
CD44	V450	BD	IM7	560451	4059830
CD45.1	V450	BD	RM4-5	560468	7048677
GR1	V450	BD	RB6-8C5	560453	6308706
CD44	V500	BD	IM7	560780	3291621
IL17A	V605	BD	TC11-18H10	564169	6182616
CD11B	V605	BD	M1/70	563015	7096987
CD45.1	V650	BD	A20	563754	7075831
CD4	V650	BD	RM4-5	563747	7282983
TBET	V650	BD	O4-46	564142	4091574
PD1	V650	BD	J43	744546	8183502
IL17A	V650				
CD19	V650	BD	1D3	563235	8122612
CD3	V650	BD	145-2C11	564378	4135556
RORYT	V650	BD	Q31-378	564722	7226869

Table 2: Primer Sequences

Gene	Forward 5'-3'	Reverse 5'-3'
FOXP3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
IFNγ	TGGCTCTGCAGGATTTTCATG	TCAAGTGGCATAGATGTGGAAGAA
TBET	CAACAACCCCTTTGCCAAAG (S)	TCCCCCAAGCAGTTGACAGT
GMCSF	TTTACTTTTCCTGGGCATTG	TAGCTGGCTGTCATGTTCAA
IL10	ACCTGCTCCACTGCCTTGCT	GGTTGCCAAGCCTTATCGGA
TNFα	CATCTTCTCAAAATTCGAGTGACAA	GGGAGTAGACAAGGTACAACCC
HPRT	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA
ACTIN	CAATAGTGATGACCTGGCCGT	AGAGGGAAATCGTGCGTGAC
GRZB	TGCTGACCTTGTCTCTGGCC	TAGTCTGGGTGGGGAATGCA
ABCA1	AAAACCGCAGACATCCTTCAG	CATACCGAAACTCGTTCACCC
ABCG1	CTTTCCTACTCTGTACCCGAGG	CGGGGCATTCCATTGATAAGG
ACAT1	GAAACCGGCTGTCAAAATCTGG	TGTGACCATTTCTGTATGTGTCC
CPT1	TTGGGCCGGTTGCTGAT	GTCTCAGGGCTAGAGAACTTGGAA
LSS	CGACATCACTGCTCAGGAGA	TGGCACAGGACTTGTTGAAG
CYP51	TCTCCAATTCCATTCCTTGG	GCCCACCATGGTAAAGCTAA
LDLR	TGACTCAGACGAACAAGGCTG	ATCTAGGCAATCTCGGTCTCC
HMGCR	AGCTTGCCCGAATTGTATGTG	TCTGTTGTGAACCATGTGACTTC
HMGCS	AACTGGTGCAGAAATCTCTAGC	GGTTGAATAGCTCAGAACTAGCC
SREBP1	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
SREBP2	GCAGCAACGGGACCATTCT	CCCCATGACTAAGTCCTTCAACT
IRF1	CAGAGGAAAGAGAGAAAGTCC	CACACGGTGACAGTGCTGG
IRF8	GGGCTGATCTGGGAAAATGATGAGA	CCCAGGCCTTGAAGATGGAAGCA
ACACA	CATCACCATCAGCCTGGTTACA	ACTGTGTACGCTCTTCGGCAT
LXRA	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT

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Discussion

Cancer is considered as the “Pathology of the Century” (Falzone et al., 2018). Despite the improvement in cancer treatment, in 2015 it was estimated that over 8.7 million of people died from cancer neoplasia and near 17.5 million of new cases were detected (GBD Mortality and Causes of Death Collaborators Collaborators, 2016). Radiotherapy and chemotherapy have been extensively used, however the limited efficiency on refractory and metastatic cancers as well as the off-side target effects revealed the necessity to develop other therapies. In the last decades, cancer immunotherapy has demonstrated to be a new promising solution: In 2013, it was acknowledged as the breakthrough of the year by Science due to the concept of targeting directly the immune system (and not the tumor itself) and to the fantastic success in the treatment of certain cancers such as leukemia and melanomas (Couzin-Frankel, 2013; Sambi et al., 2019). Manipulating and improving anti-tumor immune responses, instead of using pharmacological/chemical anti-tumor treatments, was indeed an ingenious approach.

One of the major innovations on cancer immunotherapy is CAR T Cells. Until now, CAR T cell therapies have been associated with high success rate for the treatment of hematological malignancies (Cao et al., 2018; Wei et al., 2017). CAR T cells were accepted for clinical use in the European Union for the first time last year. The two first CAR constructs (Kymriah and Yescarta) were approved to treat several cancers such as ALL, CLL, DLBCL (diffuse large B-cell lymphoma), SLL (small lymphocytic lymphoma), PMBCL (primary mediastinal large B-cell lymphoma) and FL (follicular lymphoma) (European Medicines Agency.2018). Despite the high success of CAR T cells in blood cancers, the treatment of solid tumors has been less fruitful (Yong et al., 2017) and one of the reasons is the TME. Firstly, the existence of physical barriers (stromal cells) which negatively impact T cell infiltration (Newick et al., 2017; Yong et al., 2017). Secondly, the existence of an adverse metabolic environment that can impact T cell anti-tumor functions (Anderson et al., 2017; Buck et al., 2017). As described in the **section IV** of this thesis literature review, the metabolic activity of tumor cells as well as myeloid and stromal cells resident in the TME can induce a deprivation of nutrients and as a result a competition for resources with T cells. Furthermore, from these metabolic reactions, by-products such as lactate are produced as well, creating a hostile T cell surrounding environment. Thus, it becomes important to evaluate the impact of these metabolic conditions on T cell differentiation and effector function in order to optimize CAR T cell

immunotherapies. To that purpose, during my PhD, I evaluated how the metabolic environment can impact T cell functions.

Nutrient deprivation and T cell function

One of the approaches used during my thesis to assess the potential impact of the TME on T cells, was to stimulate T cells in nutrient depleted conditions. Glutamine, the most abundant amino acid in the human plasma (Roth, 2008), was one of the nutrients I focused on during my thesis. The study of glutamine metabolism on T cell functions is particularly relevant in this context as is was demonstrated that some malignant cells highly consume and metabolize glutamine to sustain their grow and survival (Choi and Park, 2018; Kovacevic and Morris, 1972). More specifically, it has been demonstrated that cancers as NSCLC, breast and brain are particularly dependent on glutamine and upregulate the transporter ASCT2 in order to enhance glutamine uptake (Marquez et al., 2017; Mohamed et al., 2014).

In our lab, we demonstrated that glutamine availability is important for the differentiation of naïve $CD4^+$ T cells towards a Th1 fate **(Chapter 2, Article II)** (Klysz et al., 2015). In glutamine-deprived conditions, naïve T cells stimulated under Th1 polarizing conditions were not able to differentiate into Th1 cells and consequently to secrete IFNγ. However, Th2 and iTreg differentiations were not impacted, showing the glutamine is not required for Th2 and iTreg differentiation program. Furthermore, lower glutamine availability pushed naïve $CD4^+$ T cells toward a Treg phenotype even under Th1 polarizing conditions in a TFG-β dependent manner. Thus, absence of glutamine favored iTreg and blocked Th1 differentiation. To understand what was the rate limiting step, we decided to look specifically at the glutamine metabolic pathway. As described in **section II** of this thesis literature review, αKG is one of the crucial metabolites that results from glutamine catabolism. Based on this knowledge, we decided to evaluate if αKG levels were a rate-limiting step. Indeed, when extracellular αKG was added in glutamine deprived conditions, naïve $CD4^+$ T cells were able to differentiate towards a Th1 fate and T-bet expression as well as IFNγ secretion was restored. These results were translational to the humans. Metzler and colleagues showed that glutamine restriction induce high level of FoxP3 expression as well in human T cells (Metzler et al., 2016). Also, similar results were obtain when glutamine-dependent purine and pyrimidine synthesis was blocked (Metzler et al., 2016). However, in this study exogenous αKG was not able to restore Th1

differentiation, demonstrating the existence of differences between murine and human T cells.

These data brought important clues about Th1 and Treg metabolic requirements. The fact that glutamine is not equally required for pro-inflammatory Th1 cells and anti-inflammatory iTreg cells indicates that the metabolic requirements of both subsets are not the same. Indeed, it was demonstrated that T_{EFF} rely more in aerobic glycolysis while Treg cells are more dependent on fatty acid metabolism (Geltink et al., 2018; Michalek et al., 2011; Shi et al., 2011).

Another elegant study goes in line with the results from our lab as they demonstrated that ASCT2 knockout impeached the differentiation of Th1 and Th17 cells without affecting the differentiation of Th2 and Treg cells (Nakaya et al., 2014). Our results together with other groups data allow to conclude that Treg cells do not require glutamine, contrary to Th1 cells. Indeed, it has been demonstrated that Treg cells metabolic program is not dependent on amino acid metabolism. For instance, degradation of tryptophan in the cell environment by DCs (which can negatively impact T cell metabolism) results in a byproduct product formation (kynurenine) which was shown to promote iTreg differentiation (Mezrich et al., 2010). Deficiency in the neutral amino acid transporter LAT1 was demonstrated as well to impair CD4 and CD8 effector function without impacting Treg differentiation (Hayashi et al., 2013; Sinclair et al., 2013). Interestingly, it was as well shown that Treg cells induce, in skin grafts and dendritic cells, the expression of at least 5 enzymes that consume different essential amino acids (Cobbold et al., 2009). This work is curious as it highlights the capacity of Treg cells to induce amino acid depletion in the surrounding environment and it would be interesting to see if similar mechanisms take place in the TME. The fact the amino acid metabolism is not crucial for Treg cells, give to this subset a selective advantage in amino acid-deprived environments. However, Treg cells have other mechanisms to survive in hostile metabolic environments. For instance, it was shown that FoxP3 reprograms Treg cell metabolism to function in high-lactate and low-glucose environments, suppressing glycolysis, enhancing OXPHOS and increasing NAD oxidation (Angelin et al., 2017). Moreover, another elegant study demonstrated that FoxP3 induces FAO, which gives a selective advantage to Treg cells in FA-rich environments and protect them from lipotoxicity (Howie et al., 2017a).Thus, Treg cells metabolic activity is able to shape the environment, impacting other

cells function and, at the same time, contributes to its own differentiation program in hostile environments. Indeed, Treg metabolic characteristics can clearly favor their cell development and function in a TME that is characterized by low amino acids and glucose availability as well as high levels of lactate and FAs. It is known that Treg cells have a pro-tumor function and its presence on the TME is associated with a poor prognosis (Bates et al., 2006; Curiel et al., 2004; Hiraoka et al., 2006; Hou et al., 2017; Ormandy et al., 2005; Wolf et al., 2003). Our group has found that glutamine-deprive environments, such as the TME, benefit to iTreg differentiation.

The fact that Tregs seem to be less dependent of amino acids to sustain their metabolic activity, highlight the importance to study the potential impact of other amino acids deprivation on T_{EFF} and Treg cell functions. As such, during my PhD I addressed the role of another amino acid important to T cells: arginine. Arginine availability is also reduced in the TME as it is reviewed in the **section IV 2 A c**. The direct link between arginine deprivation on the TME and T cell function was already described. Rodriguez and colleagues revealed that reduced arginine availability by myeloid cells activity in the TME induced T cell receptor CD3ζ chain surface expression inhibition and antigen-specific T cell proliferation impairment (Rodriguez et al., 2004). As such, studying how reduced arginine availability impact on T cell functions becomes highly relevant. During my PhD, I evaluated the role of arginine on human T cell function using 3 different angles: by reducing arginine availability in the medium, by using drugs inhibiting different arginine metabolic pathways and finally through CAT-1 surface expression downregulation by a Sh-RNA approach. Our results demonstrated that arginine deprivation impacted T cell activation and proliferation **(Chapter 2, Addendum II, Figure 1).** These effects on T cell activation and proliferation were as well documented in few other publications (Rodriguez et al., 2007; Taheri et al., 2001; Zea et al., 2004). However, the CD4:CD8 ratio and CD45RA vs CD45RO percentages (naïve versus memory/effector cells) seemed not to be affected **(Chapter 2, Addendum II, Figure 2A and 2B)**. Regarding the effector function of those cells, we observed a diminished IFNγ secretion from $CD4^+$ T cells, however it was not significant based on our statistical analysis **(Chapter 2, Addendum II, Figure 2C)**. Only one group demonstrated a link between arginine availability, T cell phenotype and anti-tumor function, although using an opposite strategy. Geiger and colleagues showed that increased arginine extracellular levels enhanced the survival and anti-tumor activity by promoting a generation of a memory-like T cells (Geiger et al., 2016). Based

on this study, we would expect to see a decrease in the percentage of RO^+ (memory) cells in deprived arginine conditions, as increased arginine availability induced the generation of memory T cells. However, we observed no significant differences. As T cell memory differentiation is highly dependent on T cell metabolic program and memory T cells rely mainly on OXPHOS and FAO (Almeida et al., 2016; Verbist et al., 2012), to evaluate how T cell metabolism is impacted in arginine deprived conditions could be interesting approach angle to understand the T cell phenotype observed in arginine-deprived conditions. At which extend are OXPHOS and glycolysis impacted in the absence of arginine? Therefore, it would be interesting to pursue with this project and investigate the impact of arginine deprivation on $CD4^+$ T cell phenotype and differentiation. Notably, the impact of arginine availability on memory T cells as well as on iTregs and Th1 cells.

Arginine can fuel different metabolic axis of its own pathway **(Figure 17)**, what makes complex to analyze which is indeed important for T cell metabolic fitness. From arginine catabolism can result the production of citrulline and ornithine. Citrulline is an important precursor related with the TCA cycle while ornithine is an important precursor for polyamines synthesis. Thus, arginine deprivation conditions can have several and different implications on T cell metabolism. In order to understand more in detail for what for is arginine required in a $CD4^+$ T cell context, we used in culture three different drugs able to block different reactions **(Chapter 2, Addendum II, Figure 4A).** Preliminary data, indicates that while blocking citrulline synthesis (ADMA) or hyposination (GC7) does not impact T cell activation and proliferation, DFMO treatment, which block ornithine and polyamine synthesis, has a negative impact **(Chapter 2, Addendum II, Figure 4C and 4D)**. It was described that in human naïve T cells the activity levels of polyamine synthesis enzymes are low (Kay and Lindsay, 1973; Kay and Pegg, 1973) and increase upon TCR activation (notably ODC and S-adenosylmethionine decarboxylase) (Fidelus et al., 1984; Kay and Lindsay, 1973; Kay and Pegg, 1973; Scott et al., 1985). A more recent study demonstrates now, that polyamine levels in T cells are highly increased upon activation (Wang et al., 2011). Moreover, and in line with our data, they demonstrated as well that DFMO treatment inhibited activation-induced T cell proliferation (Wang et al., 2011). Interestingly, DFMO has been tested (phase II) to treat high risk neoblastoma (Sholler et al., 2018). Several studies demonstrated that some tumors have a high content on polyamines and the treatment with DFMO reduces tumor proliferation in

vitro and in xenograft models (Hixson et al., 1993; Hogarty et al., 2008; Koomoa et al., 2008; Rounbehler et al., 2009; Samal et al., 2013). However, based on our and others data, we could hypothesize that DFMO treatment could have an impact in TILs proliferation and, consequently, in anti-tumor function. Therefore, it would be interesting to evaluate the effect of DFMO on TILs in order to weight the impact of DFMO treatment in the immune system. Furthermore, it would be of interest to assess the impact on $CD4^+$ T cell differentiation in order to evaluate if DFMO treatment affects differently for instance Th1 and Treg cells and consequently creates a disequilibrium in T cell effector responses.

Finally, I also evaluated the potential impact of CAT-1 surface expression downregulation (an essential arginine transporter) **(Chapter 2, Addendum II, Figure 3)** during T cell activation. Decreased CAT-1 surface expression did not significantly impact CD4: CD8 ratio, T cell activation and proliferation. These results were surprising considering than in arginine deprived conditions T cell proliferation and activation were highly impacted. Therefore, by downregulating CAT-1 surface expression, in theory we should have reduced entry of arginine into the cell and therefore to have similar results as in arginine deprived culture conditions. However, the results did not go in the same direction. These results can be potentially explained by a compensatory mechanism. Apart of CAT-1, other nutrient transporters can mediate arginine entry into the cells such as CAT-2 and CAT-3 (Closs et al., 2006; Fotiadis et al., 2013). Regardless the fact that CAT-1 mRNA is selectively upregulated upon T cell activation, we could hypothesize that T cells can upregulate CAT2 and CAT3 in response to CAT-1 downregulation. Looking at the mRNA levels of these three transporters simultaneity in Sh transduced cells could give us important clues about this hypothesis. Moreover, it would be important to look at the arginine uptake level in Sh transduced cells (sh control vs shCAT-1) to measure the impact of CAT-1 downregulation in arginine entry into the cells.

Altogether, these data highlight the impact of nutrient deprivation on T cell activation, differentiation and effector function. As previous discussed, the TME is characterized by low nutrient-availability, notably glutamine and arginine. Therefore, when CAR T cell therapies are designed it would be important to evaluate the impact of the nutrient availability on CAR T cell anti-tumor function. Analyze CAR T cell differentiation and cytokine secretion in a nutrient deprived context could help to predict CAR T cell anti-tumor efficiency and help in the selection of T cell subsets with a better survival and/or enhanced functions in a hostile

environment. Moreover, understanding how nutrient availability impacts on CAR T cell differentiation and function, could help in the development of new tools to regulate and modulate CAR T cells metabolic program to fit them to the TME.

Selection of T cells based on nutrient transporters

In addition of using pharmacological treatments to enhance and modulate T cell anti-tumor function, we could think about select CAR T cells based on their nutrient transporters surface expression and on their metabolic characteristics, before inject them into the patients. During my PhD, I showed that sorting T cells based on GLUT1 surface expression allows the selection of T cells with different phenotypes and effector functions (Cretenet et al., 2016) (**Chapter 1, Article I)**. GLUT1^{High} cells have an increased percentage of CD8^{+} T cells, an increased acquisition of an effector phenotype and secrete higher amounts of IFNγ (Cretenet et al., 2016). Previous studies demonstrated that increased T cell glycolysis boosted anti-tumor effector functions, notably by promoting IFNγ secretion (Cham et al., 2008; Ho et al., 2015). Thus, our work together with others, demonstrate that GLUT1 surface expression and as well as increased glycolysis are highly linked to T cell effector function. Furthermore, our investigation shows that GLUT1^{High} cells also have increased effector function in hypoxia conditions. These results are particularly relevant in a context of anti-tumor therapy, as the TME is highly hypoxic (Vaupel and Mayer, 2007). Therefore, it would be interesting to evaluate *in vivo* the efficacy/fate of T cells sorted based on nutrient transporter expression, to address notably which subset has a better anti-tumor function. As GLUT1^{High} cells have an increased percentage of effector memory cells and secrete higher amounts of IFNγ it would be tempting to speculate that these cells will have a better anti-tumor function. Indeed, several studies demonstrated that T_{EFF}/ T_{EM} and consequent cytokine secretion induce tumor regression and maintain long-term tumor protection in preclinical models (Perret and Ronchese, 2008; Powell et al., 2005; Rosenberg and Dudley, 2004). Nonetheless, other studies showed that these cells persist poorly *in vivo*, which is not optimal for anti-tumor responses (Dudley et al., 2002; Gattinoni et al., 2005; Yee et al., 2002). Additionally, other works demonstrated that T_{CM} have also an effective tumor function changing the paradigm that only T_{EFF}/ T_{EM} are effective and should be used in anti-tumor immunotherapies (Beckhove et al., 2004; Busch et al., 2016; Klebanoff et al., 2005). Therefore, and even if GLUT1^{High} subset is enriched in effector T cell with higher proliferative capacity and effector function, it remains

to prove that GLUT1^{High} cells offers *in vivo* a better and persistent anti-tumor response than GLUT1^{Low} cells. With this propose, *in vivo* experiments on tumor-bearing mice are ongoing to evaluate the fate and anti-tumor function of these sorted subsets.

During my PhD I also had the opportunity to initiate studies evaluating the potential of CAT-1 nutrient transporter as a T cell selection tool. Preliminary data demonstrates that, as GLUT1^{High} cells, CAT-1^{High} cells proliferate faster and have a decreased CD4:CD8 ratio **(Chapter I, addendum I, Figure 3).** However, the cytokine prolife appeared to be slightly different between these two T cell subsets. CD8^{+} GLUT1^{High} cells secrete more IFNγ than CD8^{+} GLUT1^{Low}. However, these differences are not observed between CD8^{+} CAT-1^{high} and CD8^{+} CAT-1^{Low} subsets **(Chapter I, addendum I, Figure 5)**. Moreover, one major difference between these different subsets is that CD4^{+} GLUT1^{High} and CD4^{+} GLUT1^{Low} produce different levels of IFNγ whereas CD4^{+} CAT-1^{High} differ mainly from CD4^{+} CAT-1^{Low} on IL-17 secretion levels with IFNγ secretion only slightly affected **(Chapter I, addendum I, Figure 5)**. Therefore, these preliminary results suggest that the level of GLUT1 and CAT-1 surface expression allow the selection of T cell subsets with distinct effector functions, notably characterized by different IFNγ and IL-17 secretion levels. As a result, it would be interesting to further evaluate and characterize T cells selected based on CAT-1 surface expression to confirm this preliminary result. Moreover, it would be interesting to evaluate and compare the fate and function of these subsets in tumor-bearing mice. The fact that CD4^{+} CAT-1^{High} cells are associated with an increased IL-17 production and minimal differences on IFNγ secretion would be interesting to explore. The role of IL-17 on tumor development has been controversial so far and its pro- or anti-tumor functions remain under debate. For instance, in ovarian cancer, IL-17 secretion was demonstrated to be associated with a good prognosis, whereas in cancers such as colon and pancreatic cancer, IL-17 secretion was identified as a bad prognosis as reviewed in (Bailey et al., 2014). Therefore, the role of IL-17 on tumor growth remains to be elucidated and in this context, it would be important to address and compare the anti-tumor efficiency of T cells selected based on nutrient transporter expression characterized by distinct IL-17 vs IFNγ secretion pattern. Finally, it could be of interest to evaluate CAT-1 expression in hypoxia conditions and assess the phenotype and effector of T cells selected based on CAT-1 surface expression of T cell maintained in hypoxia.

αKG as a metabolic regulator of the Th1/iTreg differentiation.

As previously discussed, adding exogenous αKG in glutamine deprived conditions restored Th1 differentiation by rescuing T-bet expression and IFNγ secretion and by reducing FoxP3 expression **(Chapter 2, Article II)**. Nevertheless, the mechanisms by which αKG modulates Th1/Treg differentiation were not characterized. To answer this question, during my PhD I studied the impact of exogenous αKG added in nutrient replete conditions during both Th1 and iTreg polarizations.

Our data shows that when exogenous αKG is added, a higher IFNγ secretion in Th1-polarizing conditions is observed, which goes in line with our previous study which demonstrates that exogenous αKG restores IFNγ secretion in glutamine-deprived conditions. Moreover, in iTreg-polarizing conditions, FoxP3 expression decreases. This decrease in FoxP3 expression is associated by an increase in T-bet expression as well as IFNγ secretion and other inflammatory cytokines such IL-17, TNF-α and GM-CSF. Thus, αKG is able to enhance Th1 differentiation and significantly attenuate iTreg differentiation, shifting these cells towards an inflammatory phenotype **(Chapter 3, Article III, Figure 1)**. Interestingly, αKG treatment increased iTreg OXPHOS levels compared to the control condition **(Chapter 3, Article III, Figure 6)**. Furthermore, we also noticed an increase in the αKG/succinate ratio **(Chapter 3, Article III, Figure 6)**. As succinate is highly linked and required to mitochondrial complex II function, we selectively inhibited this complex by using malonate, a SDH competitive inhibitor, which leads to decrease OXPHOS capacity. Malonate treatment restored iTreg differentiation, which highlights the potential impact of OXPHOS modulation in iTreg differentiation and the possible role of the αKG/succinate ratio (**Chapter 3, Article III, Figure 6)**.

In order to evaluate if the phenotype observed upon αKG treatment was due an imbalance between αKG and succinate levels, we used two strategies to alter the αKG/succinate ratio: by adding exogenous succinate or by inhibiting SDH with malonate treatment (which should decrease the αKG/succinate ratio). Adding succinate to αKG -treated iTreg polarization rescued at least partially, the percentage of FoxP3 expressing cells as compared to control, however not statistically significant (**Chapter 3, Article III, Figure 6)**. On the other hand, adding malonate rescued significantly the generation of FoxP3-expressing cells. Thus, we can hypothesize that balancing the αKG/succinate ratio might be important to

rescue the percentage of FoxP3 expressing cells but we also need to take in consideration that SDH functioning itself might as well have an important role in iTreg differentiation **(Discussion, Figure 1)**. Importantly, SDH is the only enzyme that participates in both TCA cycle and ETC as part integrant of the mitochondrial complex II. Therefore, an increase in the TCA cycle metabolic rate mediated by αKG treatment could potentially lead to an increase of OXPHOS, notably through SDH enzymatic activity. Recently, a study demonstrated that mitochondrial complex II (SDH) could dictate T cell differentiation (Bailis et al., 2019). More specifically, they revealed that, upon malonate treatment or in the absence of SDH function (SDH KO mice), complex II was important for Th1 differentiation and IFNγ secretion in Th1 polarized conditions (Bailis et al., 2019). These results are in line with our data. Upon malonate treatment, we not only restore the percentage of FoxP3-expressing cells but we also decrease IFNγ secretion. In this work they also revealed that the ETC is differentially recruited in the different $CD4^+$ Th subsets. For instance, blocking separately each one of the five mitochondrial complexes had minimal effect on Th2 effector cells. Moreover, selective and individual blocking of both complex I and complex II impaired Th17 function (Bailis et al., 2019). Furthermore, the role of mitochondrial complexes on Treg cells was recently addressed by another group (Weinberg et al., 2019). In this study, using a murine model lacking specifically the mitochondrial complex III in Treg cells, they demonstrate the importance of this complex for Treg suppressive function. Nonetheless, Treg cell numbers, T cell maintenance and FoxP3 expression were not altered (Weinberg et al., 2019). Yet, the role of mitochondrial complex II on Treg cells remain to be elucidated. Based on these works and our data, we could therefore hypothesize that not all mitochondrial complexes, notably the complex II, function would be required for iTreg cells differentiation and probably complex II works as regulator of the Th1/iTreg cell differentiation. Increased SDH activity would therefore push the cells towards a Th1 phenotype whereas blocking it would favor iTreg cell differentiation.

Additional experiments would be required to test these hypotheses. It would be important to assess the TCA cycle metabolite levels in succinate- and malonate-treated conditions to evaluate its impact on TCA cycle metabolism and function. Also, it would be relevant to look at OCR levels in succinate- treated conditions to measure the impact of this treatment on OXPHOS. Does exogenous succinate significantly impact OXPHOS as observed

upon malonate treatment? Moreover, it would be as well important to further evaluate the potential role of SDH in iTreg differentiation. For instance, it was demonstrated that oxaloacetate (another TCA cycle metabolite) is a potent inhibitor of SDH activity (Gutman and Silman, 1975; Rutter et al., 2010). Therefore, adding exogenous oxaloacetate to αKG-treated cells could be a complementary approach to evaluate the role of the mitochondrial complex II. Moreover, as described in (Bailis et al., 2019), we could use an SDH knockout mice model to further evaluate the role of this complex in iTreg differentiation. Finally, it would be essential to look at SDH enzymatic activity: first, to see if there are differences between Th1 and iTreg cells (as these two types of cells rely on distinct OXPHOS levels); secondly, to see if αKG is able to directly impact SDH activity (which could explain the increase in OXPHOS). Finally, it would be as well essential to address SDH protein levels in both Th1, iTreg and iTreg αKG -treated cells.

Consequences of αKG treatment on epigenetic regulation

Notwithstanding the hypothesis discussed above, it is also important to take into account the important role of αKG in epigenetic regulation. Changes in the αKG/succinate ratio have been already demonstrated to have an impact in stem cell differentiation by epigenetic regulation, notably through TET proteins (Koivunen et al., 2007). As reviewed in the literature review of this thesis (**section II 3 C a**), TET proteins require αKG as a co-factor (Kaelin and McKnight, 2013). Furthermore, it is also known that succinate and fumarate are competitive inhibitors of αKG, which ultimately can block TET demethylation activity (Vatrinet et al., 2017). Thus, another hypothesis is that modulation of the αKG/succinate ratio could impact on T cell differentiation through epigenetic mechanisms. It is known that FoxP3 and IFNγ can be epigenetically regulated (Aune et al., 2013; Kitagawa et al., 2015; Peng et al., 2016; Polansky et al., 2008; Sekiya et al., 2016). Moreover, several recent studies highlighted the role of TET activity in the regulation of T cell effector function and differentiation. It was demonstrated that TET2-mediated epigenetic modifications where important for the production of Th1 (IFNγ) and Th17 (IL-17) cytokines but not for Th2 differentiation (Ichiyama et al., 2015). Furthermore, TET1, TET2 and TET3 were shown to be important for FoxP3 stability trough epigenetic regulation of the CNS promoter (Nair et al., 2016; Yue et al., 2016). Though, based on the literature previously mentioned, we could expect to detect an increased FoxP3 stability in αKG- treated conditions, as αKG is directly related with TET proteins function. Nonetheless,

and considering that we have a decreased percentage of FoxP3 expressing cells, we could hypothesize that, in our conditions, αKG is not promoting FoxP3 stability. However, it is important to take in consideration that 2HG levels are as well increased by 4 fold in iTreg αKG conditions **(Chapter 3, Article III, Figure 6).** As reviewed in the introduction (**section II 3 C a**), IDH mutations or LDH/MDH activity in specific conditions result in the conversion of αKG into D-2HG and L-2HG respectively. Furthermore, increased levels of 2HG (both enantiomers) can inhibit demethylation by blocking TET and JmjC activity **(Discussion, Figure 1)** (Chowdhury et al., 2011; Lu et al., 2012; Xu et al., 2011). Indeed, a recent study done in Th17-polarizing conditions demonstrated that increased levels of 2HG leaded in hypermethylation of the FoxP3 gene, consequently inhibiting FoxP3 transcription and increasing IL-17 secretion (Xu et al., 2017). Furthermore, by blocking conversion of glutamate to αKG, they demonstrated that FoxP3 expression increased and IL-17 secretion diminished due a reduction of 2HG production (Xu et al., 2017). Thus, αKG and 2HG levels are important for the balance between Th17 and iTreg cells and are important for FoxP3 gene regulation. In our work, in iTreg-polarized conditions in the presence of αKG, we observed as well an increase of IL-17 secretion, which goes along with this cited work. However, and despite the similarities between our data, the potential mechanisms involved could be distinct: In their study, the regulation appears to be dependent on GOT1 transamination activity and, at least at mRNA level (our RNA-sequencing data), we did not detect any significant difference generated by αKG treatment for this enzyme **(data not shown)**. Therefore, our data demonstrates that αKG treatment in iTreg conditions induce an increase of both αKG and 2HG intracellular levels **(Discussion, Figure 1)**. Both metabolites have a crucial role in TET proteins-mediated epigenetic regulation and several studies previously mentioned highlight and describe the role of TET protein specifically on FoxP3, IFNγ and IL-17 regulation **(Discussion, Figure 1)**. Therefore, it would be interesting to address if TET activity is increased or decreased in αKG treated iTreg cells and check the methylation levels of FoxP3, IFNγ and IL-17.

Impact of cholesterol metabolism and polyunsaturated fatty acid in iTreg differentiation

During our investigation, and using the RNA-seq approach, we observed that one of the most impacted metabolic pathways in αKG-treated iTreg cells was the cholesterol biosynthesis pathway which was highly downregulated **(Chapter 3, Article III, Figure 5A)**. In order to understand if this was directly impacting FoxP3 expression, we used two different

strategies: one, using inhibitor drugs of the cholesterol biosynthesis pathway (for instance, lovastatin); another adding exogenous cholesterol **(Chapter 3, Article III, Figure 5B)**. As a result, when cholesterol pathway was blocked, the percentage of FoxP3^{+} cells was maintained at equal levels as the control condition. Furthermore, when exogenous cholesterol was added in αKG treated conditions, we did not witness a rescue of the percentage of FoxP3 expressing cells **(Chapter 3, Article III, Figure 5B).** These results did not go in the direction that we were expecting. First of all, several studies reported that blocking cholesterol synthesis by statins treatment induces increase of FoxP3 expression and increase Treg cell numbers in mice (Forero-Pena and Gutierrez, 2013). Second of all, it would be logical that, if cholesterol synthesis is downregulated and consequently intracellular cholesterol levels are possibly reduced, adding exogenous cholesterol could be possibly a way to rescue iTreg differentiation. Then, as this was not observed and based on the literature, we hypothesize that the downregulation of cholesterol biosynthesis related genes could be due to an increase of intracellular cholesterol content as it was already shown that high cholesterol intracellular levels block cholesterol biosynthesis (Bietz et al., 2017; Horton et al., 2002; Ye and DeBose-Boyd, 2011). Furthermore, RNA-sequencing data also revealed that cholesterol efflux transporters ABCG1 and ABCA1 were downregulated, which could have as consequence an increase in intracellular cholesterol levels **(data not shown)**. However, and despite the overall increased lipids levels in αKG -treated iTreg, the intracellular cholesterol levels were equal on both conditions **(Chapter 3, Article III, Figure 5C)** and we did not detect differences in cholesterol efflux **(data not shown)**. Moreover, upregulation of ABCG1 and ABCA1 through LXR activation did not impact the percentage of FoxP3 expressing cells **(data not shown)**. As a conclusion, we did not observe any evidences that the cholesterol biosynthesis pathway was directly involved in FoxP3 regulation. However, it is important to highlight that the levels of PUFAs were increased in αKG-treated iTregs **(Chapter 3, Article III, Figure 5D)**. This is particularly interesting in this context as it was already shown that polyunsaturated fatty acids can inhibit SREBP1 function (Hannah et al., 2001; Takeuchi et al., 2010; Xu et al., 1999; Xu et al., 2001) and, therefore, negatively impact cholesterol biosynthesis. Thus, downregulation of cholesterol synthesis observed by RNA-seq analysis could be directly linked with the increased PUFAs intracellular levels in αKG treated conditions **(Discussion, Figure 1)**. Moreover, it is important to take in consideration the potential role of PUFAs on T cell membrane. So far, it has been described that treatment of human T cells with PUFAs diminished TCR/CD3-induced

calcium response and proliferation (Rossetti et al., 1997; Stulnig et al., 1998; Zeyda et al., 2002; Zurier et al., 1999). This is particularly interesting in this context, as several studies reported a role of TCR signaling in the regulation of Th and Treg cell differentiation (Li and Rudensky, 2016; van Panhuys, 2016). The role of TCR signaling in tTreg cell differentiation is well known (Li and Rudensky, 2016) (discussed in **section I**). The role of TCR signaling in iTreg differentiation was as well described in several studies: while IKK and calcium signaling promotes iTreg cell differentiation (Long et al., 2009; Ruan et al., 2009; Vaeth et al., 2012), strong AKT activation negatively impacts FoxP3 expression (Haxhinasto et al., 2008; Sauer et al., 2008). These results are not surprising because, as discussed in the introduction of this manuscript, AKT activation is crucial for mTOR (energy sensor pathway) function **(Figure 18)** and balanced mTOR activity **(Figure 24)** is required to maintained Treg stability and function. Therefore, it would be interesting to evaluate the potential role of polyunsaturated fatty acids in membrane structure and TCR signaling and consequently in T cell differentiation (treated or not by αKG) **(Discussion, Figure 1)**.

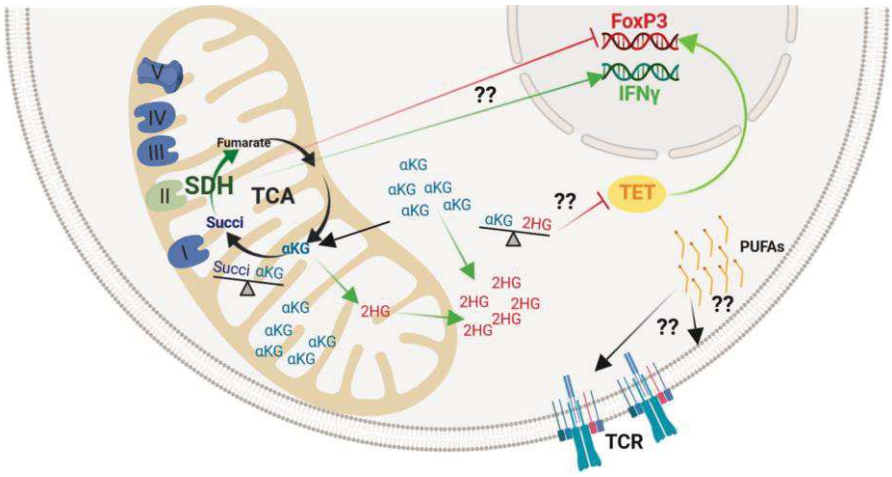

Figure 1 – Potential mechanisms involved in the α-KG-dependent inhibition of iTreg polarization

αKG is a glutamine-derived metabolite that can fulfill the TCA cycle and therefore increase its metabolic functioning rate. Therefore, we hypothesize that the high amount of extracellular αKG and changes in the αKG/succinate ratio might impact the SDH activity, resulting in increased OXPHOS levels, reduced percentage of FoxP3 expressing cells and increased IFNγ secretion. Furthermore, high αKG intracellular levels is also associated with an augmentation of 2HG intracellular levels which could lead to an altered αKG/2HG ratio and as a result could impact TET-mediated epigenetic control of the FoxP3 and IFNγ gene. It would be also interesting to evaluate the potential impact of PUFAs increased levels in T cell membrane and TCR signaling.

2HG (2-hydroxyglutarate), αKG (α-ketoglutarate), FoxP3 (forkhead box P3), IFNγ (interferon γ), SDH (succinate dehydrogenase), Succi (succinate), PUFAs (polyunsaturated fatty acids), TCA (tricarboxylic acid cycle), TCR (T cell receptor). Created with Biorender.com

Translation from the lab to a clinical application

To assess whether αKG effects were potentially translatable to the clinic, we assessed as well the impact of exogenous αKG in human iTreg conditions. Interestingly, we observed, as in murine Treg cells, that FoxP3 levels decreased upon αKG treatment **(Chapter 3, Article III, Supplemental Figure 5)**. However, in the same conditions we did not observe an increase in IFNγ secretion. These results suggest that even if FoxP3 regulation by αKG seems to be similar between species, IFNγ gene regulation might be different. It is important to note that an increase of other inflammatory cytokines such as TNF-α and GM-CSF was detected in αKG-treated human iTreg cells as well as an increase of granzyme B secretion **(data not shown)**. Therefore, it would be interesting to evaluate the effector function of αKG-treated human iTreg cells in a humanized mice model in order to study their potential anti-tumor function and to compare with the results already obtained in our murine model. Moreover, it would be interesting to study whether malonate treatment is able to restore FoxP3 expression in human iTreg cells generated in presence of αKG and assess if αKG treatment has the same metabolic impact. Therefore, to pursue the experiments on human iTreg cells is crucial and necessary to evaluate the translational potential of our work.

During my PhD I demonstrated that adding exogenous αKG in culture can impact T cell fate and effector function, potentiating Th1 differentiation and negatively impacting Treg differentiation. Therefore, it could be of interest to add αKG during the *ex vivo* generation of CAR T cells and before adoptive transfer to the patient. CAR T cells with increased IFNγ and TNFα secretory potential and reduced FoxP3 expression, would be interesting in a TME context. Moreover, manipulation of αKG levels in the TME could be as well an interesting strategy for the reduction of Treg cell numbers in the TME. The utilization of αKG in the clinic has been already done with other different proposes. For instance, the accumulation of 2HG in IDH1 and IDH2 mutant cells can be rescued by the addition of exogenous αKG (Xu et al., 2011). αKG has also been studied as treatment for certain cancers, principally in hypoxic conditions. Indeed, in pre-clinical models, αKG was able to restrict angiogenesis and to reduce expression of VEGF, resulting in tumor regression (Matsumoto et al., 2009). In another elegant study it was proved that αKG role in epigenetic modulation of tumor cells was important to facilitate anti-tumor treatments (Pan et al., 2016). Hence, these studies

demonstrate that the use of αKG in a clinical context is possible and associated with high anti-tumor efficiency.

During my thesis I also demonstrated that GLUT1 and potentially CAT-1 surface expression could be used to select T cells with different phenotypes, proliferation capacity and effector functions. These studies have been performed in human T cells opening therefore a direct path towards a translational application. However, these experiments were performed in non-engineered T cells from healthy donors. As a first step (and already on going for GLUT1) it is important to assess whether CAR T cells can be selected based on the nutrient transporter surface expression. Furthermore, we will then evaluate their anti-tumor function in a tumor-bearing humanized mouse model. If the results on murine models are encouraging, it would become exciting to evaluate the feasibility to include this strategy to on-going clinical protocols. CAR T cell manufacture protocols used in clinic are quite standardized with well-established procedures. Sorting T cells based on nutrient transporters surface expression would add an extra procedure to include. Thus, it would be interesting to evaluate the efficiency of the CAR T cell selection based on nutrient transporters surface expression and the stability/phenotype of sorted CAR T cells during the expansion phase and before injection into the patient.

Conclusion

For the last years, anti-tumor immunotherapies, notably CAR T cells, have been advancing at a rapid rate. However, there are still many challenges to overcome in order to make CAR T cell therapy efficient not only in blood malignancies, but also in solid tumors. During my thesis I demonstrated that we can target T cell metabolism to modulate T cell effector function. Pre-selecting CAR T cells based on their nutrient transporters surface expression and "priming" CAR T cell differentiation and function *in vitro* under certain metabolic conditions could be interesting approaches to overcome the metabolic environment found in the tumor tissue. Moreover, we demonstrated that metabolic changes imposed *in vitro* can be maintained *in vivo*, therefore showing that the T cell metabolism modulation could be a potential strategy to adopt in clinical trials. Altogether, we consider that T cell metabolism modulation can be used as tool to improve CAR T cells anti-tumor function and overcome the low successful rate in solid tumor treatment.

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Annexes

IMMUNOLOGY

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Resveratrol stimulates the metabolic reprogramming of human CD4$^+$ T cells to enhance effector function

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The polyphenol resveratrol activates the deacetylase Sirt1, resulting in various antioxidant, chemoprotectant, neuroprotective, cardioprotective, and anti-inflammatory properties. We found that at high concentrations of resveratrol, human CD4$^+$ T cells showed defective antigen receptor signaling and arrest at the G_1 stage of the cell cycle, whereas at low concentrations, cells were readily activated and exhibited enhanced Sirt1 deacetylase activity. Nevertheless, low-dose resveratrol rapidly stimulated genotoxic stress in the T cells, which resulted in engagement of a DNA damage response pathway that depended on the kinase ATR [ataxia telangiectasia–mutated (ATM) and Rad3-related], but not ATM, and subsequently in premitotic cell cycle arrest. The concomitant activation of p53 was coupled to the expression of gene products that regulate cell metabolism, leading to a metabolic reprogramming that was characterized by decreased glycolysis, increased glutamine consumption, and a shift to oxidative phosphorylation. These alterations in the bioenergetic homeostasis of CD4$^+$ T cells resulted in enhanced effector function, with both naïve and memory CD4$^+$ T cells secreting increased amounts of the inflammatory cytokine interferon-γ. Thus, our data highlight the wide range of metabolic adaptations that CD4$^+$ T lymphocytes undergo in response to genomic stress.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a natural polyphenolic compound that is produced by plants in response to environmental stress, providing them with protection from microbial infections (*1*–*4*). Resveratrol appears to mimic the effects of caloric restriction, increasing life span in lower organisms (*5*). Furthermore, this pharmacological agent has elicited much interest because of its potential to modulate a diverse array of pathological conditions, and it is associated with anticancer, antiaging, and anti-inflammatory properties (*6*–*10*). On the basis of the promising data emerging from ex vivo studies and preclinical animal models, resveratrol has been tested in more than 30 clinical trials involving more than 1000 individuals. Nevertheless, the specific pathologies in which resveratrol has substantial clinical benefits are not yet clear (*11*–*16*).

The pharmacological properties of resveratrol have been attributed, at least in part, to its activation of the nicotinamide adenine dinucleotide (NAD^+)–dependent silent information regulator 2 (Sir2) deacetylase (*17*) both in vitro (*18*, *19*) and in vivo (*20*). Overexpression of the mammalian Sir2 homolog sirtuin-1 (Sirt1) in mice extends their life span (*21*, *22*) and protects them from a diverse array of diseases (*23*–*28*). Conversely, knocking out Sirt1 is associated with autoimmunity (*29*–*32*). However, the effects of Sirt1 are likely to be complex. Although Sirt1 attenuates murine T cell signaling and effector function (*29*, *30*, *33*–*35*), it also promotes the differentiation of naïve CD4$^+$ T cells into T helper 17 (T_H17) effector cells in mice (*36*). Furthermore, physiological modifications of Sirt1 function in human T cell subsets have thus far not been evaluated.

T cell activity is of great importance in a wide range of pathophysiological conditions for which resveratrol activity is being clinically evaluated. Hence, elucidating the potential on-target and off-target effects of resveratrol on T lymphocytes is critical. T cells present a complex target because their cellular metabolism is altered after activation by a cognate antigen. The capacity of T lymphocytes to respond to stimulation by antigen depends on an extensive proliferative response, a process that requires new energetic and biosynthetic components that are supplied, at least in part, through a metabolic shift from oxidative phosphorylation (OXPHOS) toward glycolytic and glutaminolytic pathways (*37*–*39*). This shift from OXPHOS contrasts with the activity of resveratrol, a compound that generally increases mitochondrial activity and associated OXPHOS (*40*–*42*). However, note that resveratrol leads to a wide range of effects, including decreased, stabilized, and enhanced T cell effector functions (*43*–*46*).

Disparate effects of resveratrol on genomic stability have also been reported. In some studies, resveratrol contributes to genomic stability and reduces tumorigenesis by reducing the amount of reactive oxygen species (ROS), which leads to oxidative damage (*47*–*51*). However, in other studies, resveratrol mediates DNA damage, facilitating antitumor treatments (*47*, *52*–*59*). One possible reason for these discrepancies could be that resveratrol has distinct effects on quiescent cells versus proliferating cells. In this regard, T lymphocytes present a challenging target. Although they are generally quiescent, exposure to foreign antigen rapidly stimulates cell cycle entry and cellular proliferation. A coordinated response to genotoxic stress is regulated by the kinases ATM (ataxia telangiectasia–mutated) and ATR (ATM and Rad3-related) (*60*, *61*). Of interest are reports that resveratrol activates one or both of these kinases in different cellular contexts (*53*–*55*). Here, we report that resveratrol rapidly stimulates the ATR-dependent damage pathway in antigen-stimulated human CD4$^+$ T cells, with activation of the tumor suppressor p53. This genotoxic stress response links a metabolic reprogramming to an enhanced CD4 T cell effector function characterized by increased production of the cytokine interferon-γ (IFN-γ).

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RESULTS

Low-dose resveratrol increases the activity of the NAD$^+$-dependent deacetylase Sirt1 in primary human CD4$^+$ T cells

To gain insight into the role of resveratrol in modulating Sirt1 function in human CD4$^+$ T lymphocytes, we first examined its expression profile in response to T cell receptor (TCR) stimulation. We found that TCR engagement resulted in a substantial increase in Sirt1 abundance, with augmented nuclear localization and aggregation (Fig. 1A). Low-dose resveratrol (20 μM) further increased the mean fluorescence intensity (MFI) of Sirt1 staining by about twofold (Fig. 1A and fig. S1A). However, high-dose resveratrol (100 μM) attenuated the TCR-mediated increase in Sirt1 abundance, and these CD4$^+$ lymphocytes did not undergo blast formation (Fig. 1A and fig. S2A). This differed markedly from treatment with low-dose resveratrol, which augmented blast size (fig. S2A). Separating subsets of TCR-stimulated CD4$^+$ T cells based on their forward scatter (FSC) and side scatter (SSC) profiles demonstrated that Sirt1 abundance paralleled increases in cell size and granularity (Fig. 1B). As expected from these data, low-dose resveratrol substantially increased Sirt1 activity in TCR-stimulated lymphocytes, as monitored by the generation of *O*-acetyl–adenosine diphosphate–ribose (OAADPr), a reaction product of the Sirt-catalyzed, NAD$^+$-dependent deacetylation of target proteins (Fig. 1C). Thus, TCR stimulation combined with low-dose, but not high-dose, resveratrol augments Sirt1 activity in human T lymphocytes.

Low- and high-dose resveratrol stimulate distinct types of G_1 and premitotic cell cycle arrest in TCR-stimulated CD4$^+$ T cells

In light of our findings that high-dose resveratrol inhibited TCR-mediated increases in T cell size and granularity, it was of interest to determine how different doses of resveratrol affected T cell proliferation and cell cycle progression. Although cell viability was not affected by resveratrol (fig. S2A), high-dose resveratrol inhibited the entry of T lymphocytes into the G_1 phase of the cell cycle, as assessed by the detection of reduced amounts of total RNA (Fig. 2A and fig. S1B). On the other hand, T cells exposed to low-dose resveratrol exhibited a cell cycle entry and progression that was equivalent to that observed in control TCR-stimulated cells, with about 40% of cells having entered into S phase by day 3 of stimulation (Fig. 2A and fig. S1B). However, note that low-dose resveratrol almost completely abrogated TCR-mediated cellular proliferation (Fig. 2B and fig. S1C), an effect that was not ameliorated by the addition of exogenous interleukin-2 (IL-2) nor by extended time in culture (fig. S2, B and C).

Cell cycle entry and progression are tightly controlled processes involving the action of cyclin-dependent kinases (Cdks) and cyclins (Fig. 2C). To understand the molecular bases underlying the cell cycle arrest caused by different doses of resveratrol, we investigated the regulation of components of the cell cycle machinery. Cyclins D2, E1 and A2, and B1, as well as their cognate kinases, Cdk4/Cdk6, Cdk2, and Cdk1, respectively, were increased in abundance upon T cell activation. Although this increased abundance was not altered by low-dose resveratrol, it was significantly attenuated by high-dose resveratrol under conditions in which high amounts of the Cdk inhibitor p27^{Kip1} were maintained ($P < 0.05$ at day 1 and $P < 0.005$ at day 3; Fig. 2, D and E, and fig. S1D). However, by day 3 of activation, high-dose resveratrol-treated cells showed increased cyclin D2 and cyclin E1, but these cells did not progress into S phase (Fig. 2A). Cdk2, cyclin A2, and Cdk1 were not detectable, and phosphorylation of the

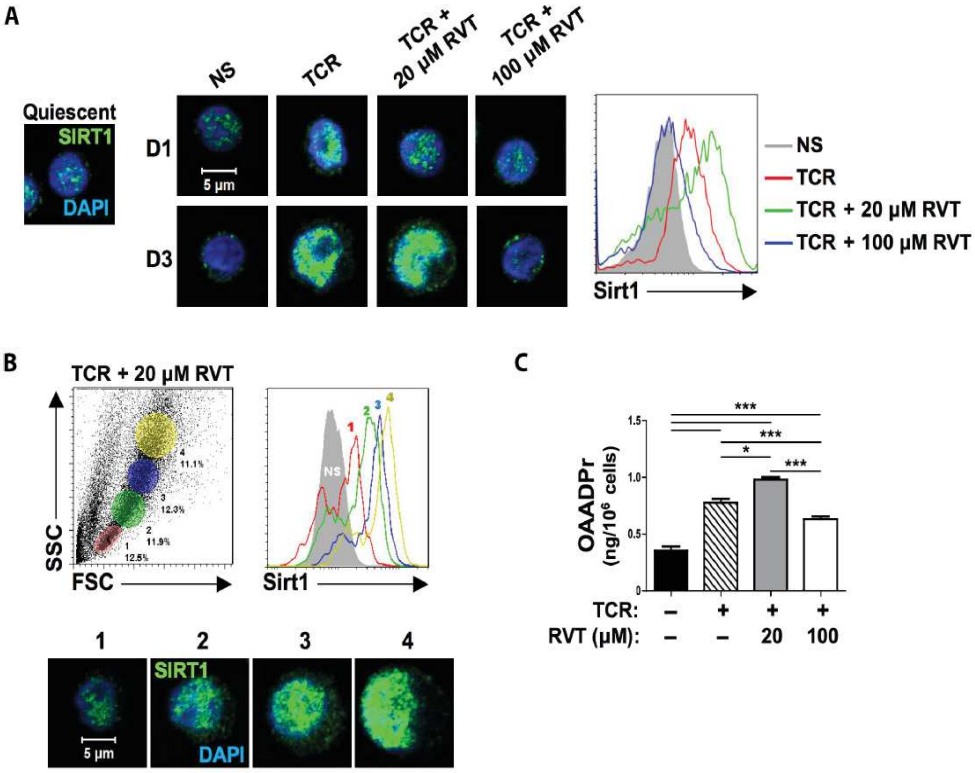

Fig. 1. Resveratrol modulates the TCR-stimulated activity of the NAD$^+$-dependent deacetylase Sirt1 and blast formation in human CD4$^+$ T cells. (**A**) Left: The presence of Sirt1 in freshly isolated quiescent human CD4$^+$ T cells was assessed by staining with a Sirt1 polyclonal antibody (green) and the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) (blue). The presence and localization of Sirt1 were also assessed after 1 (D1) or 3 (D3) days under nonstimulating conditions (NS) or after TCR stimulation (TCR) in the absence or presence of 20 or 100 μM resveratrol (RVT). Images are representative of 50 cells in three independent experiments. Right: Sirt1 was also monitored by flow cytometric analysis of quiescent human CD4$^+$ T cells and cells that were stimulated for 3 days through the TCR in the absence or presence of 20 or 100 μM resveratrol. Data are representative of four independent experiments. (**B**) Left: Sirt1 abundance in CD4$^+$ T cells treated with 20 μM resveratrol was monitored by flow cytometry as a function of both FSC and SSC. The four numbered populations of cells were distinguished on the basis of their FSC-SSC characteristics (dot plot), and Sirt1 abundance in the indicated populations was further analyzed. Right: Histograms are representative of 10 independent experiments. Bottom: Sirt1 abundance and localization in cells from the indicated populations were also analyzed by immunofluorescence staining. Images are representative of three experiments. (**C**) Sirt1 deacetylase activity was monitored as a function of the generation of OAADPr generation, a reaction product of Sirt1-catalyzed NAD$^+$-dependent protein deacetylation. CD4$^+$ T cells were untreated or were stimulated through the TCR in the absence or presence of 20 or 100 μM resveratrol for 3 days before the amount of OAADPr in each sample was determined. Data are means ± SEM of six independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ by one-way analysis of variance (ANOVA) and Tukey's post hoc test.

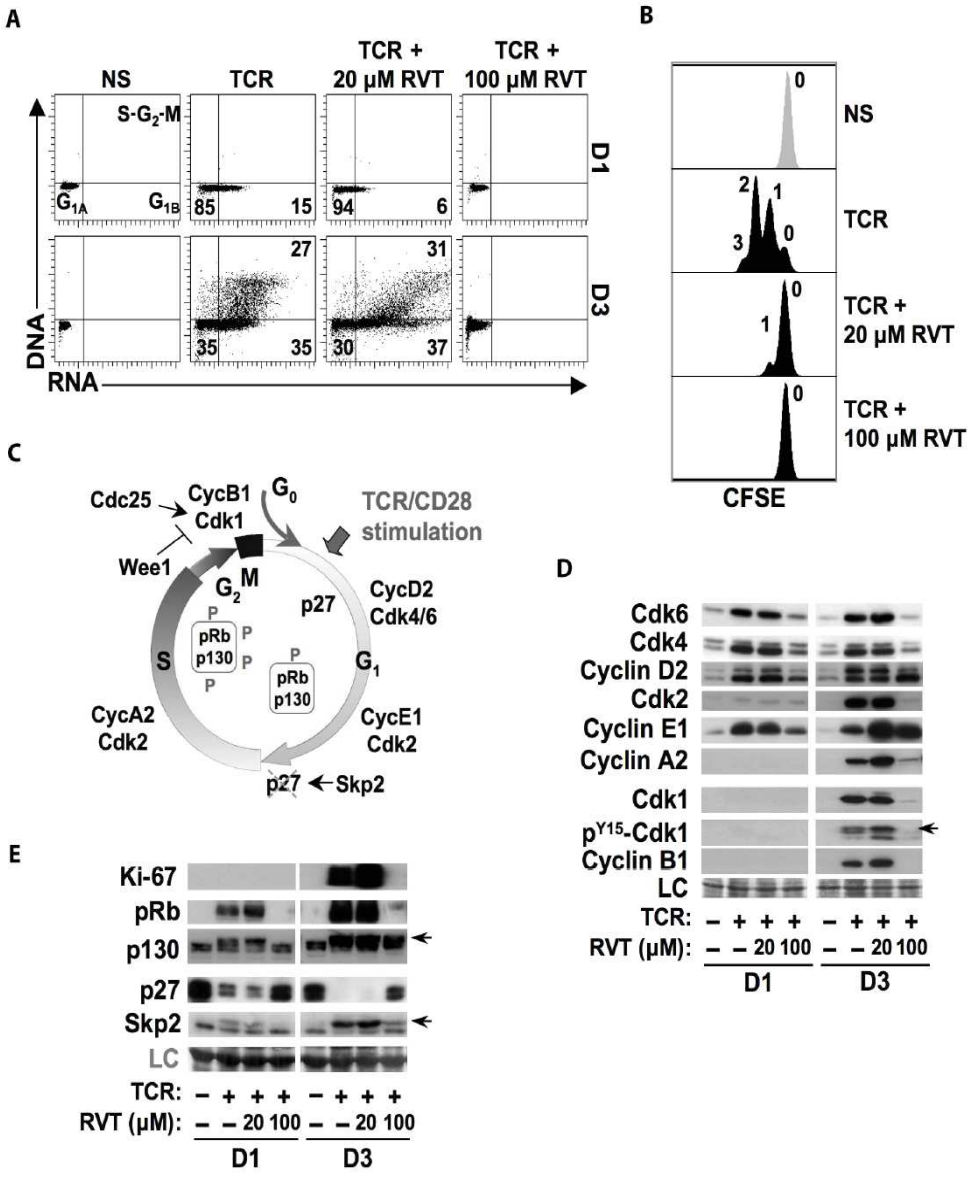

Fig. 2. Low- and high-dose resveratrol block TCR-mediated cell cycle progression at distinct stages of the cell cycle. (**A**) Cell cycle entry after TCR stimulation at day 1 (D1; top) and day 3 (D3; bottom) in the presence or absence of resveratrol was monitored by simultaneous staining of DNA and RNA with 7-aminoactinomycin D and pyronin Y, respectively. Representative dot plots from five experiments of nonstimulated and TCR-activated CD4 T cells, in the absence or presence of resveratrol, are shown. The percentages of cells in G_0-G_{1A} phase (lower left quadrant), G_{1B} phase (lower right quadrant), and S, G_2, and M phases (upper right quadrant) are indicated. (**B**) T cell proliferation under the indicated conditions was monitored by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, and dilution of the fluorescent dye was assessed at 72 hours. The number of division peaks is indicated in each histogram. Data are representative of six experiments. (**C**) Schematic representation of cell cycle regulators that are altered upon TCR-mediated cell cycle entry. Cell cycle progression requires the expression of cyclins and Cdks, the F-box protein Skp2-dependent and ubiquitin-mediated degradation of the $p27^{Kip1}$ Cdk inhibitor, and Cdk-mediated hyperphosphorylation of the pRb tumor suppressor and the related p130 pocket protein. Cdk1 activity and mitotic entry are regulated by the kinase Wee1 and the phosphatase of Cdc25. (**D**) The abundances of the cyclins-Cdks that regulate cell cycle entry, including cyclins D2, E1, A2, and B1, and Cdk4, Cdk6, Cdk2, and Cdk1 were monitored by Western blotting analysis on days 1 and 3 of activation. Data are representative of three independent experiments. The arrow indicates the hyperphosphorylated Cdk1 isoform. (**E**) The abundances of the Ki-67 proliferation marker, cell division inhibitors (pRb, p130, and p27), and the p27 regulator Skp2 under the indicated conditions were monitored by Western blotting analysis. Data are representative of three independent experiments. Arrows indicated hyperphosphorylated p130 and phosphorylated Skp2. Quantification of all panels is shown in fig. S1.

pocket proteins pRb (retinoblastoma protein) and p130, hallmarks of S-phase progression, was also not observed (Fig. 2, D and E, and fig. S1E). Furthermore, the Cdks regulating the G_1-S phase progression (Cdk4, Cdk6, Cdk2, and Cdk1) were not increased in abundance (Fig. 2D). We found that this was likely because of the reduced abundance and phosphorylation of the F-box protein Skp2 (Fig. 2E, upper band, and fig. S1E), the rate-limiting component responsible for $p27^{Kip1}$ ubiquitination and degradation (*62*, *63*). The premitotic cell cycle arrest that was triggered by low-dose resveratrol did not result in senescence, as shown by the enhanced phosphorylation of pocket proteins, increased amounts of cyclins A2 and B1 and the proliferation marker Ki-67, as well as decreased $p27^{Kip1}$ abundance (Fig. 2, D and E, and fig. S1, D and E). Furthermore, accumulation of hyperphosphorylated Cdk1 (Fig. 2D, arrow) suggests that resveratrol blocks the G_2-M transition of the cell cycle by abrogating the Cdc25-mediated activation of Cdk1 (Fig. 2C).

Low-dose resveratrol does not alter TCR-stimulated proximal and distal signaling cascades

To determine whether the cell cycle blockade mediated by low-dose resveratrol was due to defective initiation of the TCR signaling cascade, we first assessed proximal signaling intermediates. The kinase ZAP-70, which is associated with the TCRζ chain in activated lymphocytes, was phosphorylated by 1 min after TCR engagement and was not affected by either low- or high-dose resveratrol (Fig. 3A). Further downstream signaling was monitored as a function of extracellular signal–regulated kinase 1/2 (ERK1/2) and AKT phosphorylation. Neither ERK1/2 nor AKT phosphorylation was altered by low-dose resveratrol, and phosphorylation was only marginally decreased in the presence of high-dose resveratrol (Fig. 3, A and B, and fig. S1F).

TCR signaling also activates mammalian target of rapamycin (mTOR) (*64*–*66*), a serine/threonine protein kinase that integrates environmental cues such as nutrients, growth factors, and stress signals into an "optimal" cellular response (*67*, *68*). Sirt1 activity generally inhibits mTOR signaling (*69*–*71*), but in murine T cells, ectopic Sirt1 has not been shown to alter this cascade (*35*). mTOR complex 1 (mTORC1) activity is negatively regulated by tuberous sclerosis complex 1/2 (TSC1/2), which serves as a hub for both positive and negative cues for signaling kinases. Phosphorylation of TSC2 at T1462 by Akt leads to the activation of mTORC1, and in human $CD4^+$ T cells, low-dose resveratrol did not affect the TCR-mediated phosphorylation of this signaling molecule.

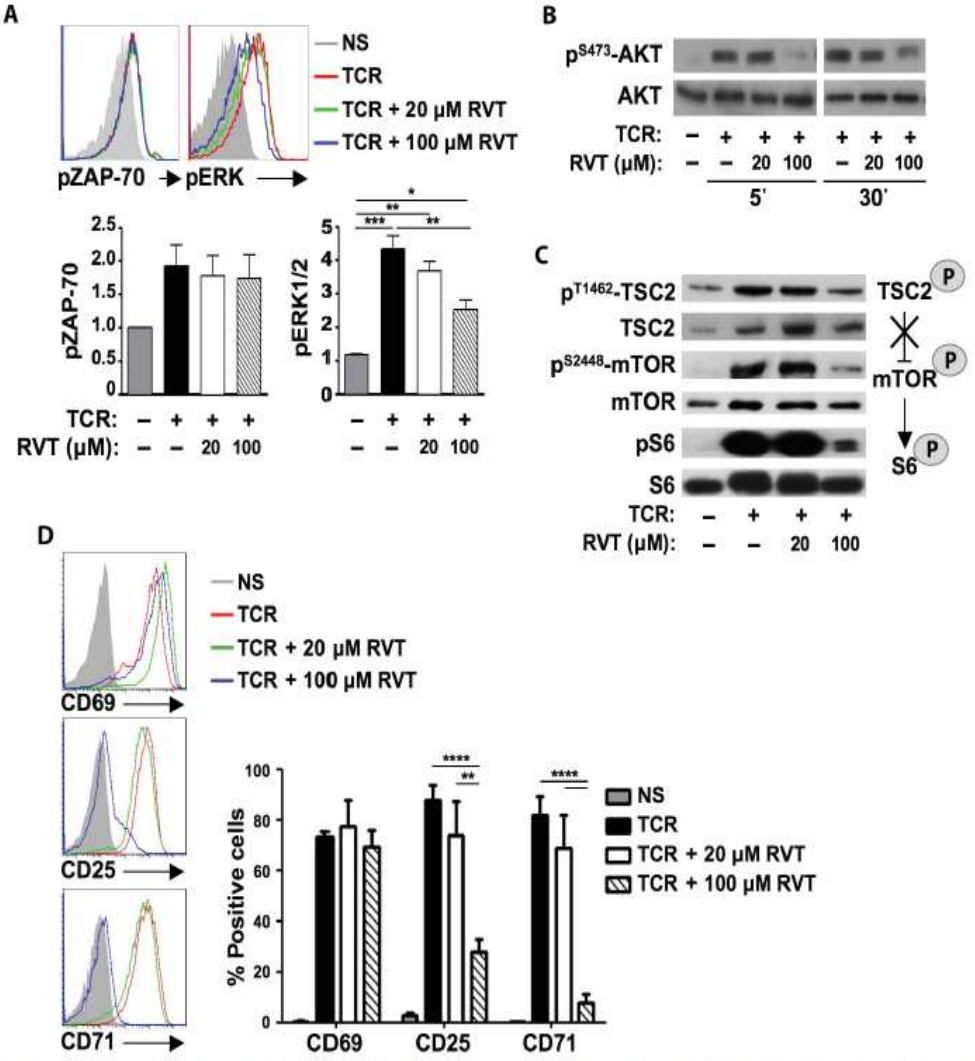

Fig. 3. TCR signaling is attenuated by high-dose, but not low-dose, resveratrol. (**A**) Top: Phosphorylation of ZAP-70 and ERK after TCR stimulation of human CD4$^+$ T cells in the presence of 20 or 100 μM resveratrol was monitored by flow cytometry. Representative histograms at 1 min after stimulation are presented. Bottom: Quantification of the fold increase in MFIs of the indicated proteins in stimulated relative to nonstimulated CD4$^+$ T cells. Data are means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.005 by one-way ANOVA and Tukey's post hoc test. (**B**) The relative amounts of total and phosphorylated Akt in T cells 5 and 30 min after activation under the indicated conditions were determined by Western blotting analysis. Data are representative of three experiments. (**C**) The extent of phosphorylation of TSC2, mTOR, and S6 in CD4$^+$ T cells 24 hours after stimulation under the indicated conditions was monitored by Western blotting analysis. Data are representative of three experiments. (**D**) Left: The cell surface expression of the CD69, IL-2Rα (CD25), and transferrin receptor (CD71) activation markers on CD4$^+$ T cells stimulated under the indicated conditions were assessed by flow cytometry. Histograms are representative of three independent experiments. Right: Quantification of the percentages of positive cells under each condition. **P < 0.01 and ****P < 0.0001 by two-way ANOVA with Bonferroni's post hoc test. Quantification data are shown in fig. S1.

Furthermore, neither phosphorylation of mTOR itself nor S6 ribosomal protein, a downstream mTOR substrate, was altered by low-dose resveratrol. Note that phosphorylation was significantly decreased in the presence of 100 μM resveratrol (P < 0.01; Fig. 3C and fig. S1G). Thus, mTOR signaling in TCR-stimulated T cells is attenuated by high-dose resveratrol, whereas at low doses, the activity resulting from TCR engagement is maintained.

We next assessed whether distal TCR signaling was altered by resveratrol, monitored as a function of the cell surface abundance of the CD69, CD25 (IL-2Rα subunit), and CD71 (transferrin receptor) activation markers. Surface abundance of CD69, due to the translocation of intracellular stores to the cell membrane without a requirement for protein synthesis (*72*, *73*), was increased in most of the activated cells, irrespective of the presence of resveratrol (Fig. 3D). In marked contrast, induction of CD25 and CD71, both of which are dependent on de novo protein synthesis, was significantly attenuated by high-dose resveratrol but was unaffected by low doses of the polyphenol (P < 0.001; Fig. 3D). Thus, only high-dose resveratrol impedes mTOR and distal TCR signaling cascades.

Low-dose resveratrol stimulates a replication stress response in TCR-stimulated CD4$^+$ T cells

The experiments performed thus far demonstrated that low-dose resveratrol inhibits CD4$^+$ T cell division under conditions in which TCR and mTOR signaling responses are maintained. To further explore this phenomenon and to determine the origin of the cell cycle arrest, we focused on the effects of low-dose resveratrol on genomic integrity. Resveratrol has been found to both positively and negatively affect genome integrity in cancer cells (*57*, *74*–*78*), but its function in primary human T cells has not been elucidated. To specifically address this point in T lymphocytes, we monitored histone H2AX phosphorylation (γH2AX). This modification identifies DNA damage foci as well as stalled replication forks that promote the concentration of repair proteins (*79*, *80*).

TCR engagement of CD4$^+$ T cells did not result in the augmentation of γH2AX (Fig. 2, A and D), at least at time points before entry into S phase (2 to 24 hours; Fig. 4A). However, in low-dose resveratrol, γH2AX$^+$ cells reached significantly higher percentages by 24 hours (45%, P < 0.005; Fig. 4A); these percentages were similar to those detected in the presence of aphidicolin, an inhibitor of replication polymerases that stalls replication forks and results in a late G_1-phase arrest (*81*). High-dose resveratrol had a distinct effect, increasing the abundance of H2AX foci in 7 to 10% of cells, irrespective of the kinetics or TCR stimulation. The lower amount of H2AX phosphorylation in cells treated with high-dose resveratrol may be due to their attenuated response to TCR stimulation (Fig. 3). Notably, stimulation with the homeostatic cytokine IL-7 significantly increased γH2AX in cells treated with high, but not low, doses of resveratrol (P < 0.005; Fig. 4B), suggesting that resveratrol effects on genomic integrity are likely to be dependent on the nature of the activation signal. Whereas it is not known how IL-7 signaling affects the potential of resveratrol to alter genomic integrity or its response to this stress, note that the addition of IL-2 did not alter resveratrol-driven H2AX phosphorylation in TCR-stimulated T cells (Fig. 4C).

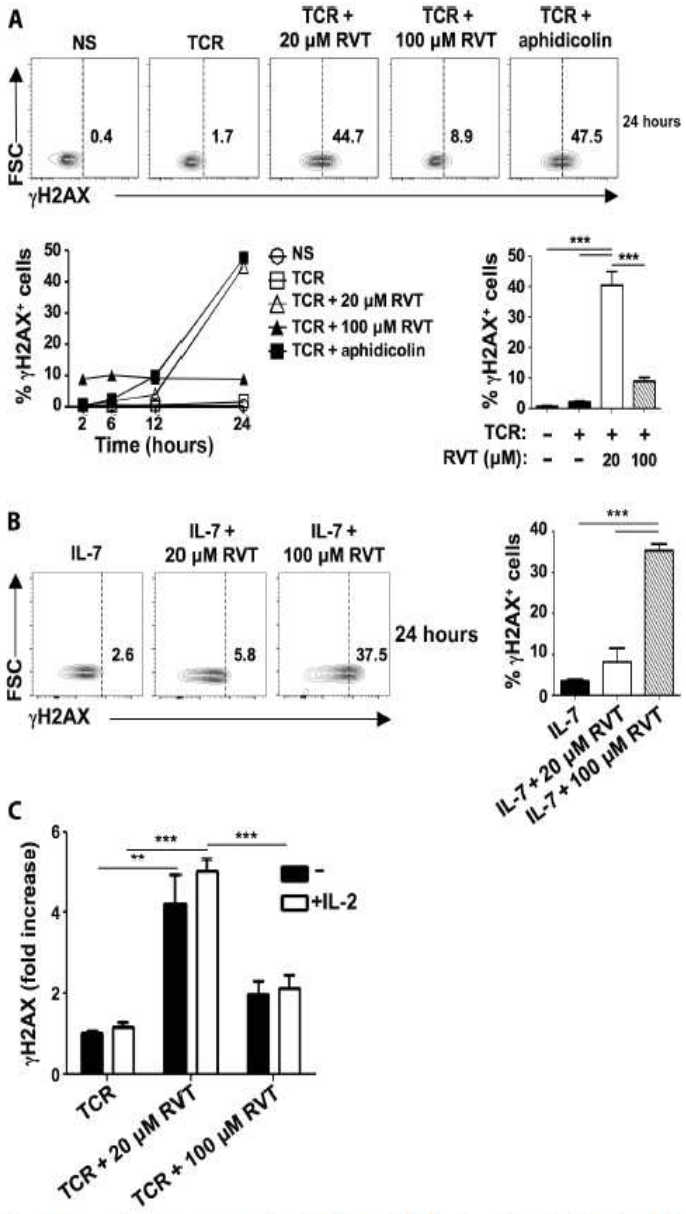

Fig. 4. Low-dose resveratrol stimulates H2AX phosphorylation in TCR-stimulated CD4$^+$ T lymphocytes. (**A**) Top: Freshly isolated quiescent CD4$^+$ T cells were either left nonstimulated or were TCR-stimulated in the presence of resveratrol (20 and 100 μM) or aphidicolin. The amount of H2AX phosphorylation (γH2AX) was assessed at 24 hours by flow cytometry. Data are representative of four independent experiments. Bottom left: The percentages of γH2AX-positive cells were quantified after 2, 6, 12, and 24 hours of stimulation. Data are representative of four independent experiments. Bottom right: Means ± SEM of γH2AX-positive cells from three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's post hoc test. ***P < 0.005. (**B**) Left: CD4$^+$ T cells were cultured with the homeostatic cytokine IL-7 (10 ng/ml) in the absence or presence of resveratrol (20 and 100 μM). H2AX phosphorylation (γH2AX) was assessed at 24 hours by flow cytometry. Dot plots are representative of three experiments. Right: Means ± SEM of three independent experiments. Data were analyzed by one-way ANOVA with Tukey's post hoc test. (**C**) CD4$^+$ T cells were TCR-stimulated in the presence or absence of resveratrol (20 and 100 μM) and in the presence or absence of IL-2 (50 U/ml). H2AX phosphorylation was assessed by flow cytometry as described earlier, and the increase in phosphorylation relative to that in cells stimulated by TCR engagement alone is shown. Data are means ± SEM of three experiments with statistical significance determined by one-way ANOVA with Tukey's post hoc test. **P < 0.01 and ***P < 0.005.

Resveratrol stimulates p53 phosphorylation and ATR-mediated cell cycle arrest

In light of the data shown earlier, it was critical to monitor activation of the p53 tumor suppressor, a protein that is phosphorylated in response to a wide range of genotoxic insults (*60*). At day 1 of TCR stimulation, low-dose resveratrol resulted in the accumulation and phosphorylation of p53, similar to that detected under conditions of aphidicolin-mediated stalled replication. Furthermore, by day 3, p53 phosphorylation was also increased under high-dose resveratrol conditions (P < 0.005; Fig. 5A and fig. S1H). Several hypotheses could account for the phosphorylation of p53: first, activation of the adenosine monophosphate–activated protein kinase (AMPK) pathway (*82*–*84*); second, activation of the ATM pathway in response to double-stranded DNA breaks (*60*, *85*); and third, activation of the ATR pathway in response to single-stranded breaks and replication stress due to stalled fork progression or DNA synthesis (*61*). Regarding the first hypothesis, glucose deprivation and AMPK activation have been specifically shown to stimulate p53 phosphorylation (*82*, *83*). However, an AMPK inhibitor (compound C) did not significantly alter resveratrol-stimulated p53 phosphorylation, although it decreased AMPK phosphorylation by a mean of 75% (Fig. 5B and fig. S1I). These data suggest that AMPK signaling does not drive p53 phosphorylation in resveratrol-treated CD4$^+$ T lymphocytes.

To address the second and third hypotheses, we assessed the implication of the ATM and ATR pathways in the response of CD4$^+$ T cells to resveratrol. Classically, ATR is stimulated by replication stress or single-stranded DNA breaks, resulting in the activation of checkpoint kinase 1 (Chk1) (*86*) and its downstream target, the kinase Wee1, a negative mitotic regulator (Fig. 5C) (*85*). Although Chk1 activation is often underestimated because of its transient phosphorylation (*87*), resveratrol stimulated both Chk1 and Wee1 phosphorylation (Fig. 5D and fig. S1J). Chk1 and Wee1 block Cdk1 activation by inhibiting Cdc25 phosphatase and hyperphosphorylating Cdk1, respectively (Fig. 5C). Accordingly, ATR signaling in response to low-dose resveratrol was associated with a defective dephosphorylation of Cdk1 that is required for Cdk1 activation and mitotic entry (Fig. 5D, arrow). In contrast, the ATM pathway was not activated by low-dose resveratrol because neither phosphorylation of Chk2 nor the downstream Cdk inhibitor p21$^{Waf1/Cip1}$ (p21) was detected (Fig. 5D and fig. S1J). As positive controls, we assessed the capacity of bleomycin and aphidicolin to efficiently activate ATM/ATR and ATR pathway intermediates, respectively (Fig. 5, C and D).

These data suggested that the early H2AX phosphorylation in resveratrol-treated CD4$^+$ T cells was due to a replication stress–like insult rather than an ATM signaling cascade stimulated by double-stranded DNA breaks. To test this possibility, we assessed the phosphorylation of the minichromosome maintenance (MCM) helicase complex, a key component of the prereplication complex that is specifically phosphorylated by ATR at Ser^{108} in response to multiple forms of DNA damage (*88*–*90*). Before the onset of S phase (24 hours), Mcm2 phosphorylation at S108 was augmented by low-dose resveratrol to a similar extent to that detected in response to aphidicolin and bleomycin (Fig. 5D and fig. S1J). Furthermore, although T cells exposed to high-dose resveratrol never progressed to S phase (Fig. 2, A and D), p53, Chk1, Wee1, and Mcm2 phosphorylation were also detected in these cells by 72 hours after stimulation (Fig. 5D). Together, these data suggest that resveratrol activates the ATR, but not ATM, signaling cascade in stimulated CD4$^+$ T cells.

We therefore assessed whether the resveratrol-stimulated phosphorylation of p53 was directly regulated by ATR signaling in resveratrol-treated

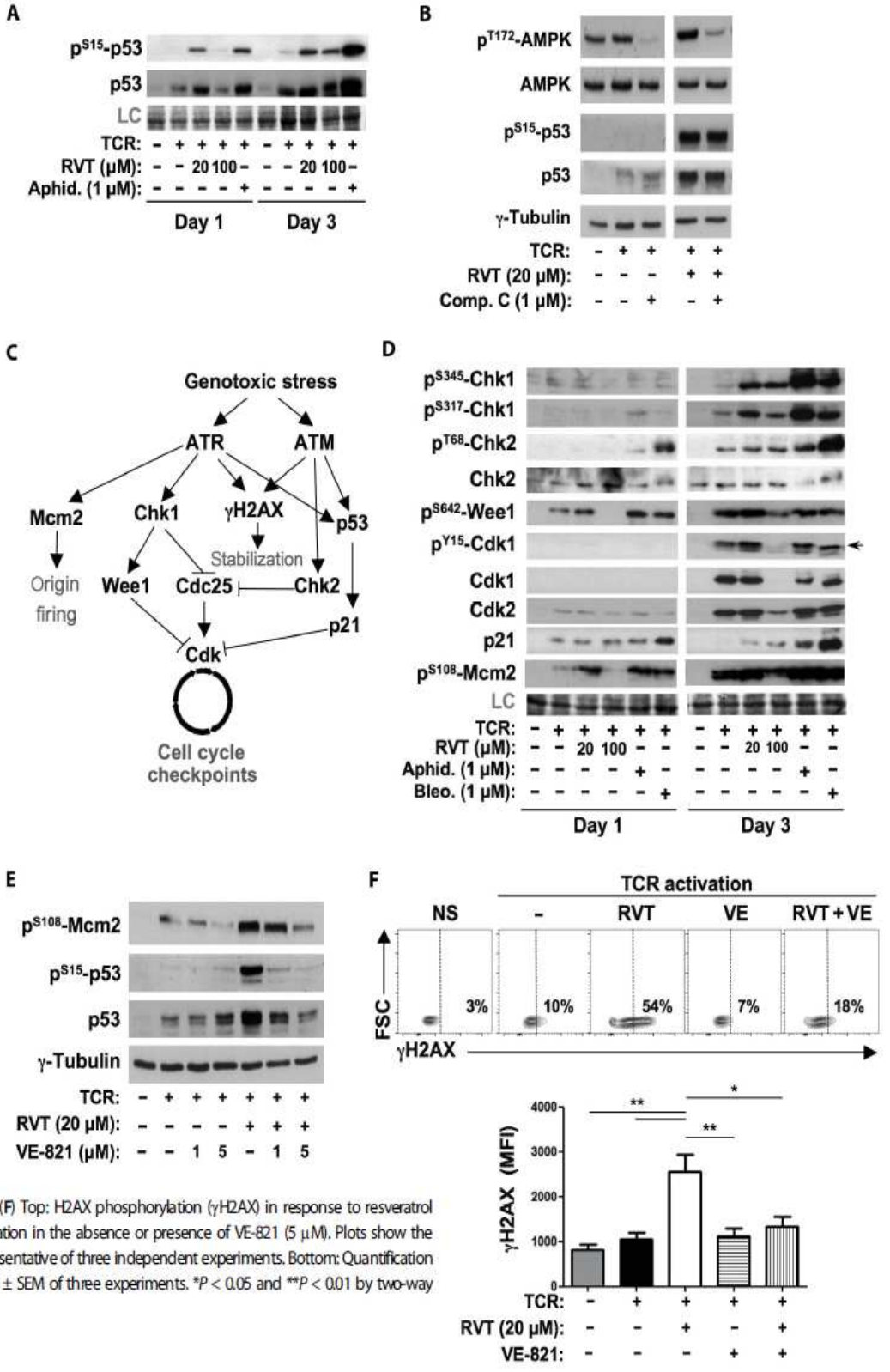

Fig. 5. Resveratrol stimulates p53 phosphorylation and the ATR-mediated arrest of the cell cycle. (**A**) Phosphorylation of p53 at Ser^{15} and total p53 were monitored by Western blotting analysis of $CD4^+$ T cells activated in the absence or presence of resveratrol (20 and 100 μM) or aphidicolin (1 μM). Representative blots at days 1 and 3 of activation and loading controls (LC) are shown. Data are representative of three experiments; quantification data are shown in fig. S1. (**B**) Phosphorylation of AMPK and p53 was assessed by Western blotting analysis of $CD4^+$ T cells at day 1 after treatment as indicated with resveratrol and the AMPK inhibitor (compound C; 1 μM). Representative blots showing phosphorylated and total proteins under the indicated conditions are shown. Data are representative of three experiments; quantification data are shown in fig. S1. (**C**) Schematic model of the ATR and ATM signaling cascades culminating in cell cycle arrest. The former results in Chk1 activation, whereas the latter proceeds through Chk2 activation and the p53-mediated expression of p21. p21 directly inhibits Cdks, whereas Chk1 blocks cell cycle progression by activating Wee1 and preventing the Cdc25-mediated dephosphorylation of Cdk1. (**D**) The abundance and phosphorylation of Chk1 and Chk2 and the cell cycle regulators Wee1, Cdk1, Cdk2, p21, and Mcm2 were monitored by Western blotting analysis of cells treated under the indicated conditions. Blots of samples at days 1 and 3 are representative of three experiments. The arrow indicates hyperphosphorylated Cdk1. Quantification data are shown in fig. S1. (**E**) Phosphorylation of Mcm2 and p53 in cells was assessed by Western blotting analysis with the appropriate phosphospecific antibodies at day 1 after treatment with resveratrol and the ATR inhibitor (VE-821; 1 and 5 μM). The amounts of total p53 and γ-tubulin were assessed. Data are representative of three independent experiments. Quantification data are shown in fig. S1. (**F**) Top: H2AX phosphorylation (γH2AX) in response to resveratrol treatment was assessed at 24 hours after stimulation in the absence or presence of VE-821 (5 μM). Plots show the percentages of γH2AX-positive cells and are representative of three independent experiments. Bottom: Quantification of the MFI of γH2AX staining is shown as means ± SEM of three experiments. $*P < 0.05$ and $**P < 0.01$ by two-way ANOVA with Bonferroni's post hoc test.

T cells. To this end, we tested the effects of VE-821, a potent ATR inhibitor (*91*, *92*). As expected, VE-821 decreased Mcm2 phosphorylation (Fig. 5E and fig. S1K). Moreover, p53 phosphorylation was attenuated by a mean of 90% in the presence of VE-821, and the global amount of p53 decreased ($P < 0.001$; Fig. 5E and fig. S1K), demonstrating that the ATR stress response pathway regulates the resveratrol activation of p53 in TCR-stimulated $CD4^+$ T cells. We also found that the ATR pathway is directly implicated in the formation of γH2AX foci (*61*) as VE-821 inhibited resveratrol activation of H2AX phosphorylation ($P < 0.05$; Fig. 5F). Together, these data reveal a critical role for the ATR cascade in mediating a p53-associated stress response in response to resveratrol.

$CD4^+$ T cells exhibit increased expression of p53-dependent target genes and undergo a metabolic switch in response to low-dose resveratrol

To elucidate potential molecular mechanisms associated with resveratrol treatment of $CD4^+$ T cells, we performed an array analysis of genes involved in DNA damage signaling responses. Whereas the expression

of multiple genes was altered after treatment with high-dose resveratrol or aphidicolin, only 1 of 84 assessed genes, *Bbc3* [*PUMA* (p53 upregulated modulator of apoptosis)], was consistently increased in expression in low-dose resveratrol–treated CD4 T cells (4.1- to 6.4-fold, $n = 3$; Fig. 6A and fig. S3). Furthermore, it was even more highly expressed at high-dose resveratrol (8- to 22-fold, $n = 3$; fig. S3). Notably, *PUMA* is a proapoptotic gene whose transcription is directly regulated by p53 (*93*). Because the p53 pathway has also been linked to a metabolic reprogramming (*94*–*96*), at least in part through the regulation of metabolic genes, we assessed whether expression of these genes is altered by resveratrol. Notably, expression of *TIGAR* (TP53-induced glycolysis and apoptosis regulator), *PGM* (phosphoglycerate mutase), *GLS2* (glutaminase 2), and *SCO2* (synthesis of cytochrome c oxidase 2) was significantly altered by resveratrol (Fig. 6B). p53-mediated induction of TIGAR, a fructose-2,6-bisphosphatase, and attenuated amounts of *PGM*, would both be expected to decrease glycolysis (*97*–*99*). Moreover, we found that whereas TCR engagement significantly increased the cell surface abundance of the glucose transporter Glut1, the amount was significantly lower in the presence of low-dose resveratrol and was abrogated by high-dose resveratrol ($P < 0.05$ and $P < 0.001$; Fig. 6C and fig. S1L). The changes correlated directly with glucose uptake and glycolysis, as monitored by the production of lactate and extracellular acidification ($P < 0.001$; Fig. 6D).

Glucose metabolism was decreased in the presence of resveratrol, but lymphocyte metabolism can also be fueled by other nutrients such as glutamine. Notably, the ASCT2 glutamine transporter, recently shown to be critical for T cell activation (*100*), was augmented in the presence of low-dose, but not high-dose, resveratrol. Moreover, the ratio of surface ASCT2/Glut1 was significantly increased by low-dose resveratrol as compared to TCR activation alone ($P < 0.01$; Fig. 6D). The potential importance of resveratrol-linked glutaminolysis in $CD4^+$ T cells was also suggested by the induction of the p53-dependent *GLS2* gene (Fig. 6B), catalyzing the hydrolysis of glutamine to glutamate (*101*–*103*). This link was corroborated by an increased glutamine uptake in activated T cells treated with low-dose resveratrol (Fig. 6E).

The increase in glutamine entry and the enzymes involved in the first steps of glutaminolysis suggested that p53 activation might augment OXPHOS in these T lymphocytes. In tumor cells, p53 decreases glycolysis while enhancing OXPHOS (*104*, *105*). One of the mechanisms through which p53 augments tricarboxylic acid (TCA) cycling from glutamine intermediates (anaplerosis) is through the expression of *SCO2*, a protein that catalyzes the transfer of reducing equivalents from cytochrome c to molecular oxygen in mitochondria (*103*). To assess the extent of OXPHOS in human $CD4^+$ T cells, we directly monitored the oxygen consumption rate (OCR) and found that TCR stimulation significantly increased the basal cellular respiration of $CD4^+$ T cells ($P < 0.005$; Fig. 6F). Low-dose resveratrol further enhanced respiration, but TCR-stimulated respiration was almost completely abrogated by high-dose resveratrol ($P < 0.05$; Fig. 6F). Increased oxygen consumption would also be expected to result in increased ROS, with mitochondria serving as the major intracellular source of ROS. Superoxide anion—the predominant ROS in mitochondria—was significantly augmented by resveratrol, as monitored by MitoSOX staining ($P < 0.05$; Fig. 6F). These metabolic parameters correlated directly with the bioenergetic profile of the $CD4^+$ T cells; intracellular adenosine triphosphate (ATP) was significantly increased in low-dose resveratrol–treated cells but was attenuated in lymphocytes treated with high-dose resveratrol ($P < 0.005$; Fig. 6F). Thus, resveratrol significantly modulated the expression of p53 target genes, concordant with changes in the metabolic state of these lymphocytes.

Altered metabolism in resveratrol-treated naïve and memory $CD4^+$ T cells enhances their effector function

In recent years, the critical importance of metabolism (and most specifically glucose metabolism) in T cell effector function has been established (*37*, *106*–*108*). However, entry of glutamine via the ASCT2 amino acid transporter (*SLC1A5*) has been shown to be a sine qua non for the effector function of murine $CD4^+$ T cells (*100*, *109*). Furthermore, although it has not been specifically shown for T cells, a heightened need for biosynthetic intermediates is often associated with a metabolic switch resulting in a disproportionate dependency on glutamine, undergoing anaplerotic reactions to form α-ketoglutarate for use in the TCA cycle (*110*–*112*). It was therefore of interest to assess whether $CD4^+$ T cell effector function is altered in the presence of resveratrol wherein biosynthetic intermediates were not required for proliferation, due to the block in cell cycle, and a switch to an oxidative metabolism was associated with increased intracellular ATP stores (Fig. 6).

T cell effector function is tightly linked to the differentiation state of the lymphocyte, and different subsets of memory T helper cells can be distinguished on the basis of chemokine receptor markers [reviewed in (*113*)]. It was therefore important to determine whether resveratrol potentially changed the survival of sorted naïve and memory T cell subsets (fig. S4A) or, rather, whether it altered the effector profile of memory T helper cells. We therefore monitored the surface abundance of CXCR3, CCR4, and CCR6 on memory $CD4^+$ T cells (fig. S4B). Although $CXCR3^+$ and $CCR4^+CCR6^+$ profiles are associated with T_H1 and T_H17 subsets, respectively, we found that CXCR3 and CCR4 were increased in most of the $CD4^+$ T cells after TCR stimulation (fig. S4B). Notably, the relative percentage of $CXCR3^+CCR6^+$ cells within the $CD4^+$ T cell subset was 1.7 ± 0.2–fold higher in the presence of low-dose resveratrol ($P < 0.01$; fig. S4B), suggesting a higher cytokine secretion potential. The percentages of naïve and memory $CD4^+$ T cells secreting IFN-γ after TCR stimulation (day 6) was greater than four- and twofold higher in the presence of low-dose resveratrol, respectively ($P < 0.005$; Fig. 7A). Furthermore, most of the T cells that secreted IFN-γ also produced IL-2 ($P < 0.005$; Fig. 7A). The increased capacity of resveratrol-treated T cells to secrete cytokines was also detected in the total $CD4^+$ population (fig. S4C). Thus, low-dose resveratrol stimulates an ATR-mediated cell cycle arrest in antigen receptor–stimulated $CD4^+$ T lymphocytes that is coupled to a metabolic reprogramming and augmented effector function.

DISCUSSION

Our study demonstrates that resveratrol modulates the potential of human $CD4^+$ T lymphocytes to respond to antigen receptor stimulation. Although resveratrol exerts effects in both Sirt1-dependent and Sirt1-independent manners (*20*, *114*), we found that high-dose resveratrol (100 μM) inhibited the TCR-induced expression of Sirt1 in T lymphocytes. In this condition, this was likely caused by an attenuation of mTOR and distal TCR signaling. In contrast, low-dose resveratrol (20 μM) markedly increased TCR-stimulated Sirt1 activity. Furthermore, p53—a tumor suppressor whose activity is coordinated by and coordinates that of Sirt1 (*115*)—was highly phosphorylated in response to low-dose resveratrol. We determined that this p53 phosphorylation was mediated by the kinase ATR, a key regulator of the genotoxic stress response pathway. Concordant with ATR and p53 signaling, T cells exposed to low-dose resveratrol underwent Chk1- and Wee1-mediated premitotic cell cycle arrest and induced expression of p53-dependent metabolic target genes, resulting in a metabolic shift with increased OXPHOS. Note that these conditions,

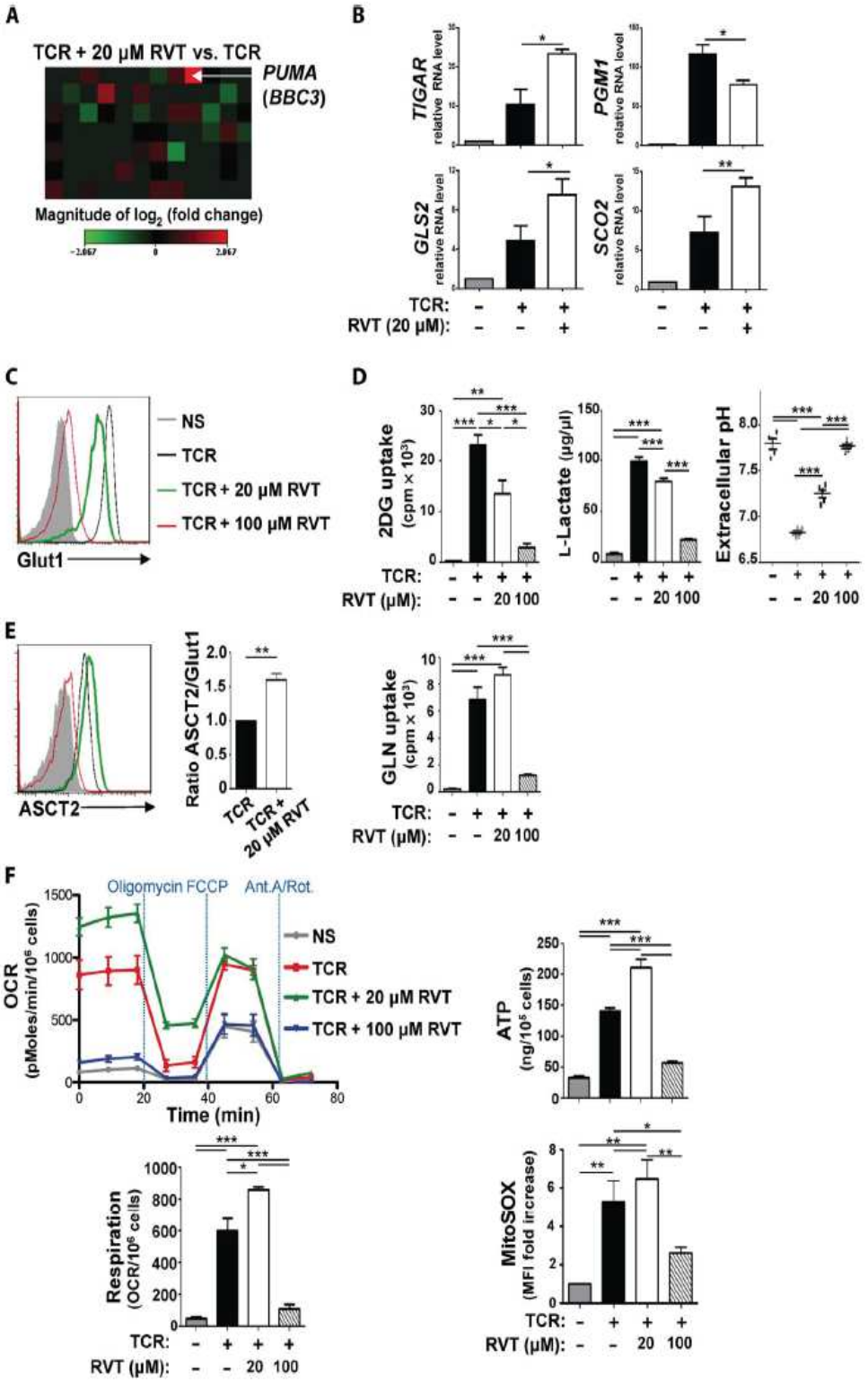

Fig. 6. TCR-stimulated CD4$^+$ T cells exhibit increased transcription of p53-dependent metabolic target genes and an altered T cell metabolism after exposure to resveratrol. (**A**) The expression of 84 genes involved in DNA damage signaling pathways was evaluated in CD4$^+$ T cells activated by TCR engagement alone as compared to TCR engagement in the presence of resveratrol (20 μM) using a polymerase chain reaction (PCR) array profile (Qiagen; fig. S3). Representative data in one of three samples at day 1 of stimulation are shown with only *Bbc3* (PUMA) significantly induced in the latter conditions (4.2- to 6.4-fold induction, $n = 3$). (**B**) Transcripts of p53 metabolic target genes including *TIGAR*, *PGM*, *GLS2*, and *SCO2* were monitored by quantitative reverse transcription PCR analysis of cells under the indicated conditions (day 3) and were normalized against the abundance of 18S ribosomal RNA. Data are means ± SEM of four independent experiments. *$P < 0.05$ and **$P < 0.01$ by paired *t* test. (**C**) Glut1 surface expression was monitored by flow cytometry, and representative histograms from three experiments under the indicated conditions (day 3) are shown, with quantifications shown in fig. S1. (**D**) 2-Deoxy-D[1-^{3}H] glucose (2DG) uptake ($n = 3$), lactate production ($n = 5$), and extracellular acidification ($n = 5$) were monitored in cells under the indicated conditions at day 3. Data are means ± SEM of the indicated number of experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ by one-way ANOVA with Tukey's post hoc test. (**E**) Left: ASCT2 surface expression was monitored by flow cytometry, and representative histograms are shown. Middle: The ratio ± SEM of ASCT2 to Glut1 abundance in cells stimulated through the TCR in the presence or absence of resveratrol are presented as a function of the MFI of both transporters ($n = 3$; paired *t* test). Right: Uptake of L-2,3,4-[^{3}H]glutamine was performed for 10 min, and mean counts per minute ± SEM for triplicate samples from three independent experiments at day 3 are presented. Data were analyzed by one-way ANOVA with Tukey's post hoc test. **$P < 0.01$. (**F**) Left: Cellular respiration was monitored on a Seahorse XF-24 analyzer, and OCRs of triplicate samples under basal conditions and in response to the indicated mitochondrial inhibitors are presented for cells on day 3 of activation. Mean basal consumption rates (OCR; picomoles/min per 10^6 cells) ± SEM of triplicate samples from three independent experiments are shown (upper right). Right: ATP was measured in cells under the same conditions by luminescent detection, and mean intracellular amounts ± SEM from data obtained in 10 independent experiments are presented. Data were analyzed by one-way ANOVA with Tukey's post hoc test. Mitochondrial superoxide anion was monitored using MitoSOX Red reagent, and the MFI ± SEM of triplicate samples are presented. Data were analyzed by paired *t* test. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Ant.A, antimycin A; Rot., rotenone. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$.

which promoted an enhanced bioenergetic profile, endowed CD4$^+$ T cells with a substantially enhanced cytokine secretion potential (Fig. 7B).

The role of resveratrol in protecting against carcinogenesis has been the subject of intense study, and multiple reports showed that resveratrol functions by preventing DNA damage formation as well as by improving DNA damage repair (*47*, *50*, *116*). Resveratrol affects multiple aspects of DNA metabolism, including DNA replication, recombination, repair, and telomere maintenance, as well as the redox state, thereby promoting the integrity of genomic DNA. However, in vitro, resveratrol mediates DNA cleavage in a process requiring DNA-bound copper [Cu(II)] ions (*49*, *57*, *59*, *117*–*119*). On the basis of diverse studies, it is nearly impossible to draw clear-cut conclusions about the effects of sirtuins or resveratrol on genomic stability. Both reduction of DNA breaks and an inhibition of replicative senescence (*57*, *74*–*78*), as well as the generation

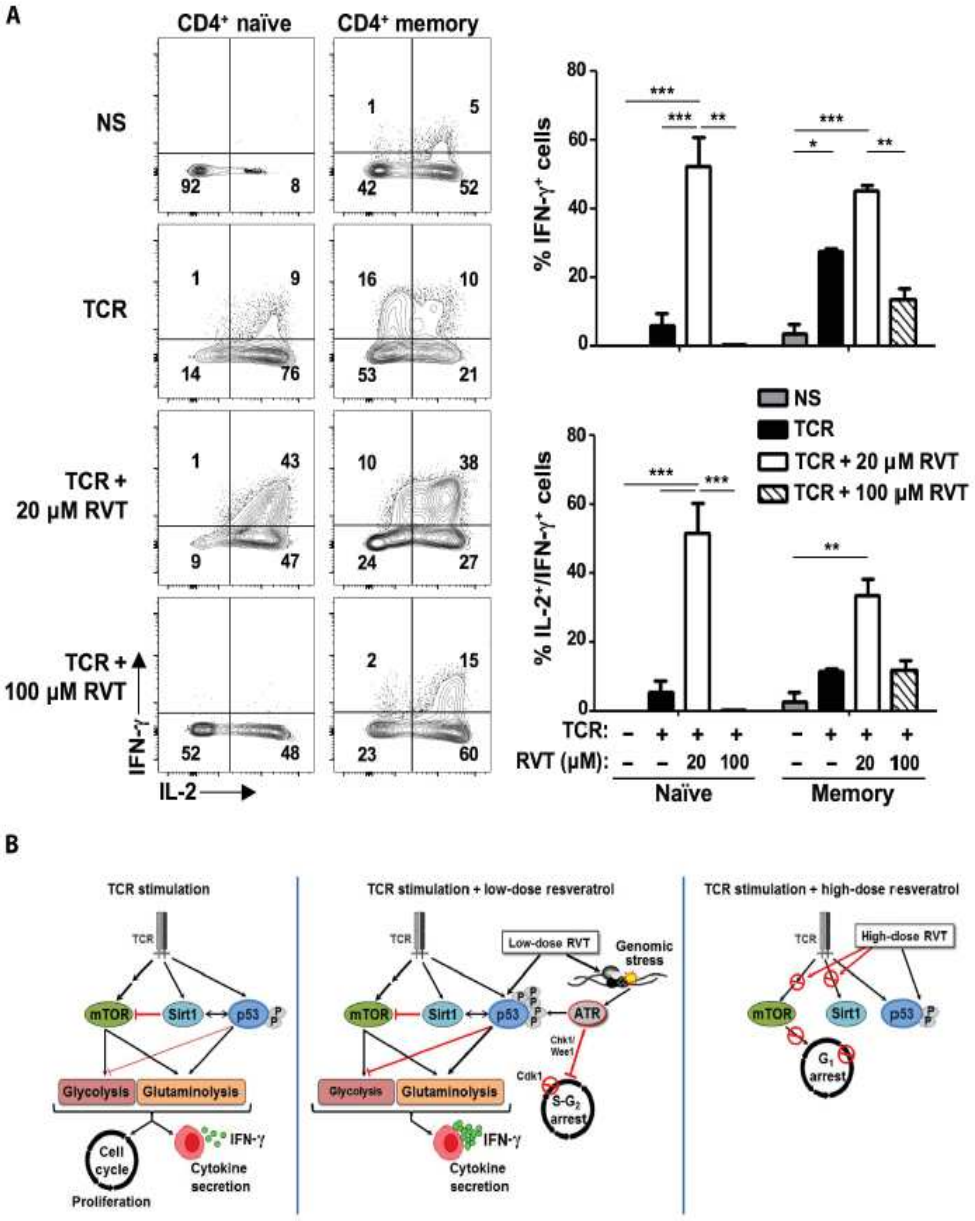

Fig. 7. IFN-γ secretion by TCR-stimulated naïve and memory CD4+ T cells is markedly enhanced by low-dose resveratrol. (**A**) Left: Naïve and memory CD4+ T cells were isolated, as described in fig. S4A, and were stimulated through the TCR in the presence or absence of resveratrol (20 and 100 μM). Secretion of IL-2 and IFN-γ was monitored by intracellular staining at day 6, and representative dot plots of three representative experiments are shown. Right: Quantification of the mean percentages ± SEM of IFN-γ–secreting and double IL-2/IFN-γ–secreting cells are shown. Data were analyzed by two-way ANOVA with Bonferroni's post hoc test. (**B**) Proposed model showing the effects of resveratrol on TCR-stimulated CD4+ T cells. Left: Sirt1 and p53 are interrelated, regulating mTOR signals and metabolic networks (*115*), and both are activated in response to TCR stimulation of human CD4+ T cells. The integration of TCR signals stimulates intracellular glycolysis and glutaminolysis, resulting in proliferation and effector function. Middle: In response to low-dose resveratrol, TCR-engaged CD4+ T cells undergo a genomic stress response, which results in an ATR- and Chk1-mediated S-G_2 cell cycle arrest. Moreover, ATR-mediated p53 signaling decreases glycolysis and increases glutaminolysis. Under these conditions, wherein cell cycle progression is blocked and OXPHOS is augmented, there is a substantial increase in IFN-γ secretion. Right: In response to high-dose resveratrol, TCR-mediated mTOR and Sirt1 signaling pathways are markedly attenuated, leading to p27-mediated G_1 cell cycle arrest.

of DNA breaks with associated senescence (*57*, *76*, *77*), have been reported (*120*). Here, we found that low-dose resveratrol triggered a marked DNA damage response in TCR-stimulated T cells, as shown by the presence of γH2AX in 20 to 50% of cells. Furthermore, DNA damage, or more precisely the response to genotoxic stress, was linked to the activation state of the T cell. Low-dose resveratrol was associated with H2AX phosphorylation in TCR-stimulated cells, whereas high-dose resveratrol resulted in H2AX phosphorylation in T cells exposed to the homeostatic cytokine IL-7.

The ATR pathway is activated under conditions of single-stranded DNA breaks or instability of replication forks (*85*). We find that low-dose resveratrol activated the ATR pathway within 24 hours of the treatment of stimulated CD4+ T cells, well before S-phase entry. Under these conditions, ATR rapidly phosphorylated histone H2AX and Mcm2 at Ser^{108}, with the latter potentially stabilizing prereplication complexes in response to DNA damage (*121*). In addition, at later time points, we found that resveratrol stimulated the ATR-mediated phosphorylation of Chk1 and Wee1, blocking Cdc25-mediated Cdk1 activation and mitotic entry (Fig. 7B). Although both ATR and ATM phosphorylate Ser^{15} of p53 (*60*), we found no evidence that resveratrol activated an ATM-Chk2-p53-p21 pathway, a pathway that is generally activated in response to double-stranded DNA breaks. Thus, our data suggest that resveratrol triggers a replication stress–like response rather than classical DNA damage. In this state, p53 appears to serve as a node between upstream stress signaling cascades and downstream DNA repair pathways (Fig. 7B) (*122*).

In CD4+ T cells, both low- and high-dose resveratrol induced transcription of the p53 proapoptotic target, *PUMA*. This was the only gene in an 84-gene DNA damage signaling pathway array to exhibit increased expression in response to low-dose resveratrol (fig. S3), suggesting that many of the effects of low-dose resveratrol occur at a posttranscriptional level. However, low-dose resveratrol altered the expression of all p53-directed metabolic gene targets that we assessed. Although other transcription factors, such as c-Myc and HIF-1α, also regulate cell metabolism and are produced in TCR-stimulated human CD4+ T cells, their abundance was not substantially modulated by resveratrol (fig. S5). Thus, we focused on p53, a tumor suppressor whose function has paradoxically been found to protect tumor cells from modest amounts of stress through metabolic reprogramming (*95*, *123*). The p53-mediated decreases in Glut1 and PGM abundance, together with increased TIGAR, attenuate aerobic glycolysis, whereas increased SCO2 and GLS2 abundance drives glutamine-driven OXPHOS (*97*, *101*–*103*, *124*), which was the case that we observed in resveratrol-treated CD4+ T cells. Note that cell cycle arrest and senescence act as signals for a cell to undergo metabolic reprogramming, decreasing glycolysis and increasing TCA cycle usage (*96*, *125*–*127*). Thus, our data suggest that the ATR-mediated cell cycle arrest initiated by low-dose resveratrol in CD4+ T cells was coupled to a

metabolic shift, adjusting the balance between glycolysis and OXPHOS (Fig. 7B).

The bioenergetic profile of resveratrol-treated T cells was altered by this skewing of metabolism away from glycolysis and toward a setting characterized by an increased ASCT2-to-Glut1 ratio, with an augmented glutamine transport, substantially increased mitochondrial ROS production, and increased OXPHOS. How this would affect T cell effector function is unclear because glycolysis can increase and decrease the potential of T cells to secrete effector cytokines, such as IFN-γ and IL-17 (*128*–*133*). Note that amino acid metabolism is essential for effector T cell differentiation (*100*, *109*, *134*, *135*), and memory cells rely more on OXPHOS than on glycolysis (*38*, *130*). In both naïve and memory $CD4^+$ T cells, low-dose resveratrol markedly augmented the amount of IFN-γ secreted, but this increase was even higher for naïve cells than for memory cells (means of 10- and 2-fold, respectively). Furthermore, in both cell types, resveratrol substantially increased the number of cells that produced both IL-2 and IFN-γ. Thus, our data suggest that resveratrol is an agent that, by altering the metabolic fitness of T lymphocytes, enhances their cytokine effector potential.

Adjusting the balance between glycolysis and OXPHOS can also have substantial effects in other cell types. Decreasing OXPHOS in mice expressing a mutant p53 markedly attenuates tumorigenesis (*136*). Thus, generating a context that is the converse of that shaped by resveratrol, that is, inhibiting a p53-mediated shift to mitochondrial metabolism, may be beneficial for individuals with an increased risk of developing cancers, such as Li-Fraumeni syndrome patients with germline mutations in the *TP53* gene. The potential use of resveratrol as a therapy for the treatment of neurological, cardiovascular, hepatic, and metabolic pathologies therefore necessitates a critical evaluation of its effect on T lymphocytes in vivo, especially in an autoimmune setting. The data shown here reveal a complex network of resveratrol-stimulated changes in cell cycle progression and metabolism, altering the potential of T lymphocytes to respond to foreign antigens.

MATERIALS AND METHODS

T cell isolation and culture

$CD4^+$ T cells were isolated from adult peripheral blood, obtained from healthy donors after informed consent. Cells were purified using negative-selection Rosette tetramers (STEMCELL Technologies), and the purity of the cell population was monitored on a FACSCanto II (BD Biosciences). Purities were always greater than 94%. Naïve and memory $CD4^+$ T cells were sorted on a FACSAria after staining with anti-CD4, anti-CD45RA, anti-CD45RO, CD62L, CD127, and CD25 antibodies (fig. S4A). Lymphocytes (1×10^6 per well in a 24-well plate) were cultured in RPMI 1640 + GlutaMAX (Gibco, Life Technologies) supplemented with 10% fetal calf serum (FCS) and 2% penicillin/streptomycin (Gibco, Life Technologies). For TCR stimulation, 24-well plates were coated with anti-CD3 (clone OKT3, BioLegend) and anti-CD28 (done 9.3, provided by C. June) monoclonal antibodies (mAbs) at a concentration of 1 μg/ml, and recombinant IL-2 (rIL-2) (50 U/ml) was added as indicated. T cells were also cultured in the presence of rIL-7 (10 ng/ml). As indicated, resveratrol (20 or 100 μM; Sigma-Aldrich), compound C1 (1 μM; Sigma-Aldrich), VE-821 (1 and 5 μM; Euromedex), aphidicolin (1 μM), and bleomycin (1 μM) were added to T cell cultures 1 hour before TCR stimulation.

Immunofluorescence

Cells were collected and coated on poly-L-lysine–treated slides. Cells were fixed in a 4% paraformaldehyde (PFA) solution [phosphate-buffered saline (PBS), 4% PFA] at 37°C for 15 min, permeabilized in PBS containing 3% bovine serum albumin (BSA)/0.1% saponin for 10 min, and blocked for nonspecific protein binding with 10% FCS. Staining with primary anti-Sirt1 antibody (Ab) (Santa Cruz Biotechnology) and a secondary Alexa Fluor 488–coupled anti-rabbit immunoglobulin G (Invitrogen) was performed in PBS containing 3% BSA for 1 hour at room temperature. Nuclei were then labeled by DAPI staining for 10 min at room temperature.

Flow cytometric analyses

To detect cell surface markers, cells were incubated with the appropriate fluorochrome-conjugated mAbs, and expression was monitored in comparison with isotype controls. Antibodies against CD4, CD25, CD69, and CD71 were from Beckman Coulter. Y319-phosphorylated ZAP-70 (BD Biosciences), T202/Y204-phosphorylated ERK1/2 (BD Biosciences), and phosphorylated H2AX (BioLegend) were detected after cell fixation and permeabilization. Surface Glut1 and ASCT2 were detected by binding to their respective retroviral envelope ligands fused to enhanced green fluorescent protein or recombinant rabbit fragment crystallizable (rFc) (Metafora Biosystems), as previously described (*72*, *137*–*139*). The presence of mitochondrial superoxide was assessed by staining with MitoSOX Red indicator (1 μM; Invitrogen). Proliferation was monitored as a function of carboxyfluorescein diacetate succinimidyl ester (Invitrogen) or violet proliferation dye (Invitrogen) dilution. Before staining for intracellular IFN-γ and IL-2 (BD Biosciences), cells were activated with phorbol 12-myristate 13-acetate (100 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) for 3.5 to 4 hours at 37°C. Cell cycle analysis was performed by simultaneous staining for DNA and RNA using 7-aminoactinomycin D (20 μM; Sigma-Aldrich) and pyronin Y (5 μM; Sigma-Aldrich), respectively. Cells were assessed on a FACSCanto II or BD LSR II Fortessa (BD Biosciences), and data were analyzed using FACSDiva (BD Biosciences) or FlowJo (Tree Star) software.

Metabolic assays

OCRs were measured on an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). TCR-stimulated T cells with and without low-dose resveratrol (20 μM) were seeded at a concentration of 1.5×10^6 cells, whereas nonstimulated and high-dose resveratrol-stimulated cells were seeded at a concentration of 2.0×10^6 cells in XF medium (nonbuffered Dulbecco's modified Eagle's medium containing 2.5 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate). Oxygen consumption was monitored under basal conditions and in response to oligomycin (1 μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1.5 μM), rotenone (100 nM), and antimycin A (1 μM; Sigma-Aldrich). The basal respiration rate was calculated as the difference between basal OCR and the OCR after inhibition of mitochondrial complexes 1 and 3 with rotenone and antimycin A, respectively. ATP and L-lactate were measured according to the standard procedures of the ATPlite kit (PerkinElmer) and L-lactate kit (Eton Bioscience), respectively. Extracellular pH was measured immediately after harvesting of medium using a standard pH meter.

Glucose and glutamine uptake assays

Cells (2×10^6) were starved by incubation at 37°C in serum and glucose- or glutamine-free RPMI 1640 for 30 min. Radiolabeled 2-deoxy-D-[1-^{3}H] glucose or glutamine-L-[3,4-^{3}H(N)] (PerkinElmer) was added to a final concentration of 0.1 mM (2 μCi/ml). Cells were incubated for 10 min at room temperature, washed in cold serum/glucose/glutamine-free RPMI 1640, and solubilized in 500 μl of 0.1% SDS. Radioactivity was measured by liquid scintillation.

Total protein extraction and analyses

Cells were lysed in lysis buffer containing 20 mM Hepes (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, and a protease inhibitor cocktail. After a 30-min incubation on ice, extracts were centrifuged, and supernatants were harvested. Extracts (20 μg) were resolved on SDS–polyacrylamide gel electrophoresis gels (8.5 to 12%) and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were incubated with the indicated antibodies (table S1) for 1 hour at room temperature or overnight at 4°C and with horseradish peroxidase–conjugated anti-goat, anti-rabbit, or anti-mouse secondary Abs, and immunoreactive proteins were visualized using enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions. Proteins were quantified with ImageJ software and normalized to amido black–stained proteins (loading controls) or tubulin as indicated.

Gene expression analysis by PCR array

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) and was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen). SYBR Green–based (SYBR Green I Master, Roche) real-time quantitative PCR (qPCR) for *TIGAR*, *PGM*, *GLS2*, SCO2, *HIF1A*, and *RNA18S* was performed with the LightCycler 480 Real-Time PCR System (Roche), and all primers are shown in table S2. To determine relative expression, samples for each experimental condition were run in duplicate and were normalized to 18S. Primer sequences used for amplification were designed with Primer3 and NetPrimer (PREMIER Biosoft) software packages. The expression of 84 genes involved in DNA damage signaling pathways was analyzed with the RT^2 Profiler PCR Array (SABiosciences, Qiagen) according to the manufacturer's instructions. Briefly, RNA was prepared from 5 million cells after 24 hours in culture under the indicated conditions using the Qiagen RNeasy kit. First-strand complementary DNA was then prepared and used in the PCR array in combination with SYBR Green qPCR master mixes on a Roche Light Cyder 480. Data were normalized to *ACTB* (actin beta), *B2M* (β_2-microglobulin), and *RPLP0* using the SABiosciences DNA template analysis software.

Statistical analyses

Data were analyzed with GraphPad Software (GraphPad Prism), and *P* values were calculated by one-way ANOVA (Tukey's post hoc test), two-way ANOVA (with Tukey's post hoc or Bonferroni's tests), or paired *t* tests, as indicated.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/10/501/eaal3024/DC1

Fig. S1. Quantification and statistical analyses of main data panels.

Fig. S2. Effects of resveratrol on the formation of CD4$^+$ T cell blasts and cell counts in response to TCR engagement.

Fig. S3. Effect of resveratrol treatment on the expression of DNA damage signaling pathway genes.

Fig. S4. Sorting of naïve and memory CD4$^+$ T cells for the assessment of phenotype and cytokine secretion profiles.

Fig. S5. TCR-mediated induction of *HIF1A* and c-Myc in human CD4$^+$ cells is not altered by low-dose resveratrol.

Table S1. Antibody list.

Table S2. Primer sequences.

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