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Investigating the lactate/SLC5A12-induced metabolic signalling network in inflammation

Submitted in partial fulfillment of the requirements of the Degree of Doctor of Philosophy

by

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“Your mind is everything.

What you think, you become” (Buddha)

“For my family….”

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STATEMENT OF ORIGINALITY

I, **Valentina Pucino**, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Details of collaboration and publications:

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2. **Pucino V**, Cucchi D, Mauro C. Lactate transporters as therapeutic targets in cancer and inflammatory diseases. Expert Opin Ther Targets. 2018; 22:735-743

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ABSTRACT

The tissue microenvironment is a key determinant of pathology in both inflammation and cancer. It is now understood that the tumour microenvironment is a niche that favours tumour growth over anti-tumour immune surveillance. Accumulation of lactate in the tumour microenvironment, largely due to the accelerated metabolism of cancer cells avidly consuming most of the scarcely available nutrients, has been shown to drive regulatory T cell responses favouring tumour growth.

Immune cells that infiltrate the tissue microenvironments in inflammatory disorders find similarly harsh conditions, including scarce nutrients and high levels of lactate and other metabolites. Yet opposite to the tumour microenvironment, the inflamed tissue features high levels of inflammatory cytokines with impairment of Treg in favour of Th1 and Th17 immune responses, contributing to the perpetuation of the chronic inflammatory disease process. Indeed, in inflammatory disorders lactate is an amplifier of the inflammatory response.

Here, I explored the response of $CD4^+$ T cells to lactate in the context of inflammation.

Specifically, the project aimed at characterizing whether the lactate/SLC5A12-induced metabolic signalling pathway modulates inflammatory immune responses.

I employed a combination of immunology, mass spectrometry and biochemistry approaches on human peripheral blood mononuclear cells from healthy controls (HC) and rheumatoid arthritis (RA) subjects, as well as mononuclear cells from inflamed arthritic synovia and tonsils. I also used RNA-sequencing and clinical

scores from a well-characterized early rheumatoid arthritis cohort and a murine model of $CD4^+$ T cell-driven arthritis.

I found that:

1. SLC5A12 is up-regulated by $CD4^+$ but not $CD8^+$ T cells upon T-cell-receptor (TCR) triggering. This expression is higher on $CD4^+$ T cells isolated from RA synovial joints, where lactate is more abundant, as compared to peripheral RA and HC $CD4^+$ T-cells.
2. Lactate-uptake by $CD4^+$ T cells through SLC5A12 causes a reprogramming of intracellular metabolism, including reduced glycolysis and enhanced TCA cycle and fatty acid synthesis substrates.
3. SLC5A12-mediated lactate influx into human $CD4^+$ T cells contributed to reshaping their effector phenotype, leading to increased IL-
4. 17 production via nuclear PKM2/Stat3 signalling and enhanced fatty acid synthesis.
5. SLC5A12-mediated lactate influx caused increased $CD4^+$ T cell retention at the inflamed tissue as a consequence of impaired cell motility caused by reduced glycolysis and enhanced fatty acid synthesis.
6. Antibody-mediated blockade of SLC5A12 ameliorates the clinical course of $CD4^+$ T cell-driven human glucose 6 phosphate isomerase (hG6PI)-induced arthritis.
7. Lactate/SLC5A12-induced metabolic reprogramming in $CD4^+$ T cells is a distinctive mechanism of lymphoid RA pathogenesis.

These findings establish lactate as an active signalling metabolite that contributes to the perpetuation of chronic inflammation and provide a novel therapeutic rationale to combat chronic inflammatory diseases.

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1 ABBREVIATIONS

1, 3-BPG	1,3-bisphosphoglycerate
2-DG	2-Deoxyglucose
AAV	ANCA-associated vasculitis
Ab	Antibody
ACC	acetyl-CoA Carboxylase
ADP	Adenosine bi phosphate
ADPGK	ADP-dependent glucokinase
AICAR	5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AID	Activation-induced cytidine deaminase
Akt / PkB	Protein kinase B
AMPK	5'-AMP activated protein kinase
ANLS	Astrocyte neuron lactate shuttle
ANT	Adenine nucleotide translocase
APC	Antigen Presenting Cell
Arg2	Arginase 2
AS	Ankylosing spondylitis
ATM	Ataxia telangiectasia mutated
ATP	Adenosine tri phosphate
BAX	BCL-2 associated X
BCL	B-cell lymphoma
BrPa	Bromopyruvate
BS	Blood serum
BSA	Bovine serum albumin
C1q	Complement component 1q
C3	Complement component 3
C75	4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid
Ca_2^+	Calcium ion
CAD	Coronary artery disease
cAMP	Cyclic Adenosine monophsophate
CCR/L	-CC- motif receptor / ligand
CD	Cluster of differentiation
CD62L	Leukocyte receptor L-selectin
cDNA	Complementary DNA

CFA	Complete freund adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen-induced arthritis
CNS	Central nervous system
CO2	Carbon dioxide
COX	Cytochrome c oxidase
CPT1a	Carntine palytoyl transferase 1a
CR2	Complement receptor 2
CRP	C reactive protein
CRTH2	Prostaglandin D2 receptor 2
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
CVD	Cardiovascular disease
CXCR/L	-CXC-motif receptor / ligand
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DASA	N,N'-diarylsulfonamide
DC	Dendritic cell
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's Modified Eagle Medium
DMS	Dimethyl fumarate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EC	Endothelial cell
EC50	Half maximal effective concentration
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence
ECSIT	Evolutionarily conserved signaling intermediate in toll pathway
ELISA	Enzyme linked immunosorbent assay
ELS	Ectopic lymphoid structures
ETC	Electron transport chain
FA	Fatty acid
FACS	Fluorescent activated cell sorting
FAO	Fatty acid oxidation

FAS	Fatty acid synthesis
FBP	Fructose-bisphosphatase
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FDC	Follicular dendritic cells
FLS	Fibroblast
FOXP3	Forkhead box P3
FSC	Forward scatter
G6P	Glucose-6-Phosphate
G6PD	Glucose-6-phosphate dehydrogenase
G6PI	Glucose-6-phosphate isomerase
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GATA3	GATA binding protein 3
GCK	Glucokinase
GLUT	Glucose transporter
GPCRs	G protein-coupled receptors
GPD2	Glycerol-3-phosphate dehydrogenase 2
GPR	G protein receptor
GSH	Glutathione (reduced)
GSK3b	Glycogen synthase kinase 3b
GSSH	Oxidized glutathione
GTP	Guanosin Tri Phosphate
H&E	Haematoxylin and eosin
H2DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
H2O2	Hydrogen peroxide
HC	Healthy controls
HCl	Hydrochloric acid
HDACs	Histone deacetylases
HEV	High endothelial venules
HIF1α	Hypoxia inducible factor 1α
HK	Hexokinase
IBD	Intestinal bowel disease
ICAM1	Intercellular adhesion molecule 1
ICOS	Inducible T-cell costimulator
Ig	Immunoglobulin
IL	Interleukin

IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine based activation motif
Itk	Inducible T cell kinase
JNK	c-Jun N-terminal kinases
KATs	Lysine acetyltransferases
KEAP	Kelch-like ECH-associated protein 1
LD	Lipid droplets
LDH	Lactate dehydrogenase
LDHA	LDH subunit A
LDHB	LDH subunit B
LFA1	Leukocyte function associated antigen 1
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MAM	Mitochondria-associated membranes
MAPK	Mitogen-activated protein kinase
MCs	Mononuclear cells
MCT	Monocarbocylate transporter
MCT	Monocarboxylic transporter
MDSCs	Myeloid-derived suppressor cells
MetS	Metabolic syndrome
MHC	Major Histocompatibility complex
MMP	Metalloproteinase
MnSOD	Manganese-dependent superoxide dismutase (SOD2)
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
$NAD^+(H)$	Nicotinamide dinucleotide oxidized (reduced)
$NADP^+(H)$	Nicotinamide dinucleotide phosphate (reduced)
NCK	Non-catalytic region of tyrosine kinase adaptor protein
NDRG3	N-Myc downstream-regulated gene 3
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
NK	Natural killer
NRF2	Nuclear factor erythroid 2–related factor 2
NSCLC	Non-small cell lung carcinomas
OA	Osteoarthritis
OCR	Oxygen consumption rate

OD	Optical density
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Perchloric acid
PD1	Programmed cell death 1
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PFKFB3	Phsophofructokinase / Fructose-bisphosphatase
PGK	Phosphoglyceryl-lysine
PHDs	Prolyl-hydroxylase containing enzymes
PI3K	Phosphoinositide 3 kinase
PK	Pyruvate kinase
PKM	Pyruvate kinase muscle
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PNAD	Peripheral node addressin
PPP	Pentose Phosphate Pathway
PRR	Pattern recognition receptors
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Rheumatoid arthritis
RASF	Rheumatoid arthritis synovial fluid
RA-ST	Rheumatoid arthritis synovial tissue
RIPA	Radio immunoprecipitation assay buffer
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RORγ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RPM	Revolutions per minute
S1P(R)	Sphingosine-1-phosphate (receptor)
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS – Polyacrylamide gel electrophoresis
SF	Synovial fluid
SFMCs	Synovial fluid mononuclear cells
SH3PXD2A	SH3 and PX domain-containing protein 2A

SIRT	Sirtuin
SLC	Solute Carrier
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
SLP76	SH2 domain containing leukocyte protein of 76kDa
SMCTs	Sodium-coupled transporters
SOD2	Superoxide dismutase 2
SSC	Side scatter
STAT	Signal transducer and activator of transcription proteins
SUCNR	Succinate receptor
TAM	Tumour associated macrophages
TBST	Tween containing tris buffered saline
TCA	Tri cyclic acid cycle
Tconv	Conventional T cells
TCR	T Cell Receptor
TDAG8	T cell death-associated gene 8
Tfh	Follicular helper T cells
TGF	Transforming grow factor
Th	Helper T cells
TLR	Toll like receptor
TMB	3,3,5,5’ Tetramethylbenzidine
TNFα	Tumour necrosis factor α
TOFA	5-(Tetradecyloxy)-2-furoic acid
TRAF	TNF receptor associated factors
Treg	Regulatory T cells
VDAC	Voltage-dependent anion-selective channel protein
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
ZAP70	Z-chain associated protein kinase

2 INTRODUCTION

2.1 The immune system

The immune system consists of the innate and the adaptive arms, which are closely interconnected. The innate arm includes physical, chemical, and microbiological barriers, immune cells such as neutrophils, monocytes, macrophages, molecules (i.e. complement, cytokines) and acute phase proteins, which provide immediate host defense.

Adaptive immunity consists of antigen-specific reactions through T and B-lymphocytes. The adaptive response has memory, so that subsequent exposure to the same antigen leads to a more vigorous and rapid response (Delves PJ, 2000, Delves PJ, 2000). More details on the adaptive immune system are described in the *section 2.1.2*.

2.1.1 *Cellular communication*

In order to work effectively cells need to be recruited to sites of inflammation where they can be appropriately activated. This is achieved via intracellular signal pathways induced by external factors, such as cytokines, adhesion molecules or chemokines, which are able to bind the receptors.

Cytokines are small molecules produced by immune cells. They share a wide variety of functions such as regulation of cell activation, division, apoptosis, or chemotaxis. Cytokines act as autocrine, paracrine, or endocrine messengers. Cytokines produced by leucocytes and having effects mainly on other white cells are termed interleukins.

Chemokines are members of the cytokine family that have a key role in leucocyte migration. Chemokines are classified according to the position of two cysteine (C)

residues compared with the other amino acids (X). The two main subgroups are CXC (-chemokines) and CC (-chemokines). Chemokines can address leucocytes to the inflammatory sites (inflammatory chemokines) or to the secondary lymphoid tissues such as the spleen or limph nodes (homing chemokines).

2.1.2 *Innate immunity*

The innate system, although not antigen specific, is able to recognize foreign pathogens via pattern-recognition receptors (PRR). These recognize pathogen-associated molecular pattern structure (i.e.lipopolysaccharide, lipotechoic acid, mannans etc.) present on microbes. The pattern-recognition receptor molecules include: 1) those inducing endocytosis and thus enhancing antigen presentation; 2) those initiating nuclear factor transduction and cell activation (toll-like receptors) and 3) those, which act as opsonins for example mannan-binding lectin.

The processed product is then presented by APCs favoring the adaptive immune response, phagocytosis, opsonisation, and complement-mediated lysis (Takeuchi O, 2010).

2.1.3 *The adaptive immune system*

The adaptive immune system includes T and B cells, which are specialized in protecting the body in an antigen-specific manner (Guermonprez P, 2002). The antigen is firstly presented to and then recognized by the antigen specific T or B cell. This leads to cell priming, activation, and differentiation, which usually occur within the specialized

environment of lymphoid tissues. Then the effector response takes place generally in the inflammatory sites.

2.1.3.1 Antigen presentation to T cells

The first contact with the antigen occurs via highly specialized antigen presenting cells (APCs), such as dendritic cells (DC), macrophages, NK and B cells (Guermonprez et al., 2002). These cells are generally located in areas like skin or mucosal surfaces where the availability of antigens is high (Steinman RM and Banchereau J, 2007). There they can interact with the antigen via surface receptor (i.e. lectins), which recognize pathogen carbohydrate recognition element. This allows the APCs to bind and internalize the pathogen. Processed antigens are presented to T cells in association with major histocompatibility complex (MHC) class I or class II molecules. When the antigen is produced endogenously within the cell (such as viral or tumour proteins) it is complexed with MHC class I through intracellular processing pathways. Alternatively, APCs can take up the antigen by endocytosis after receptor recognition. Exogenous antigen is processed via a different pathway to endogenous, and presented on the cell membrane in association with MHC class II molecules. The MHC ensures that only antigens derived from foreign molecules are recognized. Co-receptor stimulation, other than antigen-MHC complex, is required in order to obtain a correct T cell activation.

Generally, T lymphocytes meet the antigen in the secondary lymphoid organs (SLO) such as lymph nodes. The antigen is brought directly in the lymphatics, or within APCs that have endocytosed the antigen locally.

2.1.3.2 T-cell receptor signaling and cell activation

Responsible for the recognition of peptide MHC complexes by naïve T cells is the T-cell receptor (TCR). The TCR is a heterodimer consisting of a α- and β-chain that are each anchored to the cell membrane and in complex with the signaling molecule CD3 (Weiss A and Stobo JD, 1984). The majority of T cells express the α- and β-chain, in about 5% of T cells these are replaced by a γ- and δ-chain.

When antigen is bound to the TCR it creates a complex with the CD3 on the cell surface. The phosphorylation of tyrosines within the cytoplasmic tail of the CD3 complex called immunoreceptor tyrosine-based activation motifs (ITAMs), starts a downstream cascade of signal transduction leading to the activation of genes that coordinate T cell activation and proliferation programmes.

In brief, the phosphorylation of lymphocyte protein tyrosine kinase (Lck) in turn activates the ζ-chain associated protein kinase (Zap70), which promotes the recruitment and activation of the adaptor proteins Slp-76, Vav, NCK, GADS and inducible T cell kinase (Itk, **Figure I**).

Early studies on TCR transduction signals started with TCR-deficient Jurkat T cells that could be stimulated pharmacologically with the combination of phorbol esters and Ca_2^+ ionophores (Weiss A, 1987). This observation was followed by the findings of an increase in intracellular free Ca_2^+ upon TCR stimulation in both Jurkat cells and primary T cells. The rise of intracellular Ca_2^+ was demonstrated to be associated to inositol trisphosphate (IP3) production and to the influx of Ca_2^+ from the extracellular space (Imboden JB, 1985). The downstream target of TCR activation was then identified in the phospholipase C (PLC), which generates diacylglycerol (DAG) an IP3 leading to the accumulation of intracellular Ca_2^+ (Smith-Garvin JE, 2009; **Figure I**).

The activation of naïve T cells is a complex process that requires not only the TCR signal, but also the stimulation of several other costimulatory molecules (Chen L and Flies DB, 2013). Without these co-signals the cell will either become anergic (unreactive to TCR stimulation) or die by programmed cell death. The main co-receptors for T-cell activation are CD80 (B7-1), CD86 (B7-2) and CD40 expressed on APCs, that bind CD28, CTLA-4, and CD40 ligand on the T cell, respectively. Activated dendritic cells are the most potent stimulators of naïve T cells, producing large amounts of B7 and CD40.

Furthermore, CD28 signalling activation stabilizes TCR activation and IL2 expression, which in turn favors T cell survival (Rudd CE, 1996).

Inflammatory mediators also induce the upregulation of co-stimulatory molecules; therefore, a T cell is much more likely to be activated if it meets its specific antigen via an antigen-presenting cell, which has been previously exposed to an inflammatory environment. T cell receptor activation and downstream signaling lead to dramatic changes in gene expression, metabolic activity and macromolecule synthesis. This, in turn, enables the T cell to alter its morphology, grow and start to proliferate.

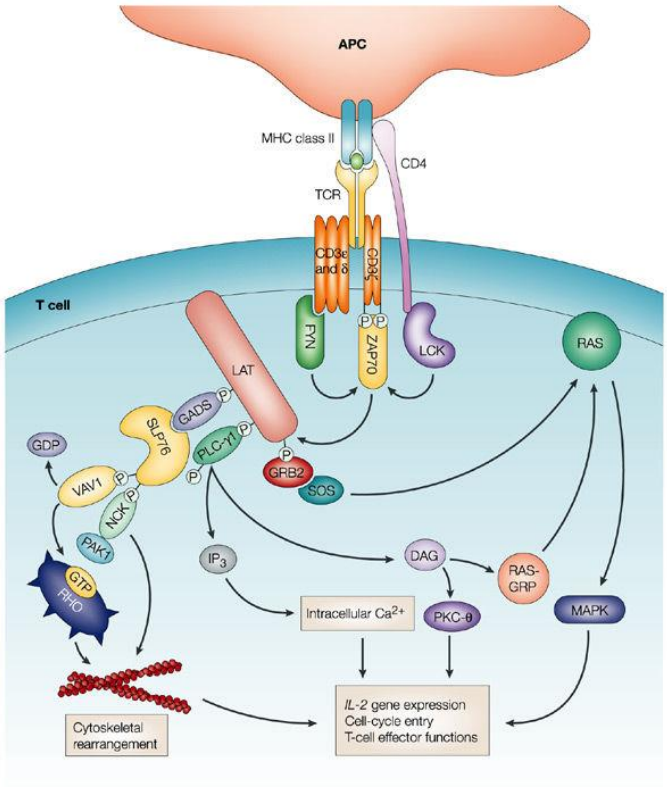


Figure I - TCR signaling model. Following antigen recognition, TCR induces multiple intracellular signals. This includes the activation of proteins, transcription factors that in turn modulate gene expression and immune homeostasis (Adapted from: Abraham RT, 2004).

2.1.3.3 Acquisition of effector functions

Naïve T cells are characterized by surface expression of CCR7, L-selectin (CD62L) and the IL-7R (CD127) that enable them to effectively patrol secondary lymphoid organs. Upon TCR activation naïve T cells undergo morphological changes that are fundamental to achieve an efficient adaptive immune response. More specifically, after the formation of the complex antigen-MHC-TCR, T cells increase their size, start to produce cytokines and express surface markers of T cell activation such as CD25, CD44 and CD69, integrins (i.e. LFA-1 and VLA-4), and chemokine receptors such as CCR5 and CXCR3 on the Th1 subset, CCR4 and CRTH2 on the type 2 helper subset as well as CCR6 on the Th17 subset (Islam SA and Luster AD, 2012). All these events enable T cells to egress from the lymph nodes and travel towards the site of inflammation.

2.1.3.4 T cell trafficking

T cells undergo four distinct steps during their migration through blood vessels. These include tethering, rolling, activation and arrest (Marelli-Berg F, 2008). Naïve T cells enter into secondary lymphoid organs (SLO) via high endothelial venules (HEV), which expresses PNAd – an O-linked carbohydrate moiety. PNAd binds the leukocyte receptor L-selectin (CD62L) based on the T cell membrane. Subsequently, T cell rolling is facilitated by the interaction between integrin leukocyte function-associated antigen 1 (LFA-1) with the endothelial inter-cellular adhesion molecule 1 (ICAM 1) (von Andrian and Mempel, 2003). Once in the lymph node, T cell upregulate LFA1 that interacts with ICAM1 on the endothelium favoring the T cell arrest. CCR7, the receptor for CCL19 or

CCL21 chemokines, is also up-regulated by naïve T cells thus reinforcing the T cell adhesion (Marelli-Berg F, 2008).

Recent studies also revealed the importance of sphingosine-1-phosphate (S1P) as a regulator of T cell trafficking. Sphingosine-1-phosphate (S1P) is a lipid second messenger that signals via five G protein-coupled receptors (S1P1–5R). Further studies established that conditional deletion of S1PR1 in T cells alone was sufficient to block their egress from the thymus (Chun J, 2010; Schwab SR, 2005).

The balance between CCR7 retention signals and S1P1 egress signals is also important for the modulation of T cell activation (Pham THM, 2008; Spiegel S, 2011).

Exposure to high concentrations of S1P results in S1P1 internalization, making cells unresponsive to migration cues in blood or lymph nodes whereas CCL19 can desensitize CCR7 signalling (Kohout TA, 2004).

Loss of CCR7 results in reduced T-lymphocyte dwell time in the lymph node, implying that CCR7 provides a signal to counter S1P1-mediated egress (Kohout TA, 2004)..

Recently it has been shown that during an inflammatory response, lymphocyte recruitment into tissue is tightly controlled. In particular, in response to adiponectin, B cells, which express adiponectin receptor (AdipoR1/2), are able to inhibit T cell trafficking by secreting a peptide (PEPITEM) proteolytically derived from 14.3.3 zeta delta (14.3.3.ζδ) protein. PEPITEM binds to cadherin-15 on endothelial cells, promoting synthesis and release of sphingosine-1 phosphate, which inhibits trafficking of T cells without affecting recruitment of other leukocytes (**Figure II**, Chimen M, 2015).

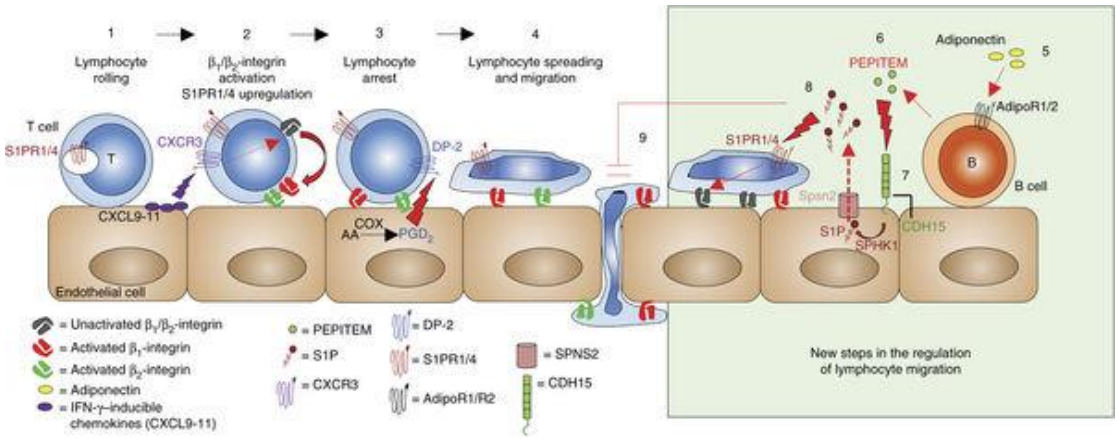


Figure II - T cell trafficking during inflammation. Inflammatory stimuli such as pro-inflammatory cytokines (i.e. TNF-α and IFN-γ) activate a cascade of signals including the expression of chemokine receptors, integrins, S1PR1-4 on T cell surface and endothelial cells respectively. This allows T cells to flow in the blood vessels and reach inflammatory sites (Adapted from: Chimen M, 2015).

2.1.3.5 T helper differentiation

In the SLO, T cells become efficiently activated and increased in number and can finally migrate to the inflamed tissues. This is due to pro-inflammatory chemokines and cytokines that are mainly secreted by endothelial cells, and local inflammatory immune cells in the inflammatory environment such as TNF-α, IL-1β, and IL-17 (Haas R, 2013).

A "cross talk" between the innate and adaptive immune systems is necessary in order to achieve an efficient defense against invading pathogens.

Presenting cells such as dendritic cells (DCs), upon antigen recognition, help CD4+ T cells to differentiate into a variety of effector subsets, Th1, Th2, Th17, follicular helper T (Tfh) cells, and induced Treg (iTreg) cells. Cytokines in the microenvironment and the strength of the interaction of the T cell antigen receptor with antigen orchestrate the differentiation pathway (Boyton RJ, 2002, Sethi A, 2013). Th1 cells are characterized by IFN production and are involved in cellular immunity against intracellular microorganisms. The signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet are activated during Th1 polarization (**Figure III**, Leung S, 2010).

Th2 cells produce IL-4, IL-5, and IL-13 and are required for humoral immunity to control helminths and other extracellular pathogens. GATA3 and Stat6 are needed for the differentiation of Th2 cells. Th17 cells produce IL-17A, IL-17F, and IL-22 and play important roles in the clearance of extracellular bacteria and fungi, especially at the level of mucosal surfaces. Th17 cell differentiation requires retinoid related orphan receptor (ROR)t, a transcription factor that is induced by TGFβ in combination with the pro-inflammatory cytokines IL-6, IL-21, and IL-23, all of which activate Stat3 phosphorylation (Chen Z, 2007). Tfh cells are a subset of helper T cells that regulate the

maturation of B cell responses. Differentiation of these cells requires the cytokine IL-21 (Vogelzang A, 2008; Nurieva RI, 2008) and the expression of the transcription factor BCL6 (Fazilleau N, 2009).

Regulation of effector T cell responses is required for effective control of infections and autoimmune diseases. Aberrant Th1 and Th17 cell responses play critical roles in organ-specific autoimmunity, whereas Th2 cells are mainly involved in allergy and asthma. Treg cells have essential roles in maintenance of immune homeostasis, regulating these effector T cell responses and thus preventing their potentially pathogenic effects through a variety of mechanisms (Liang B, 2008; Shevach EM, 2009; **Figure III**).

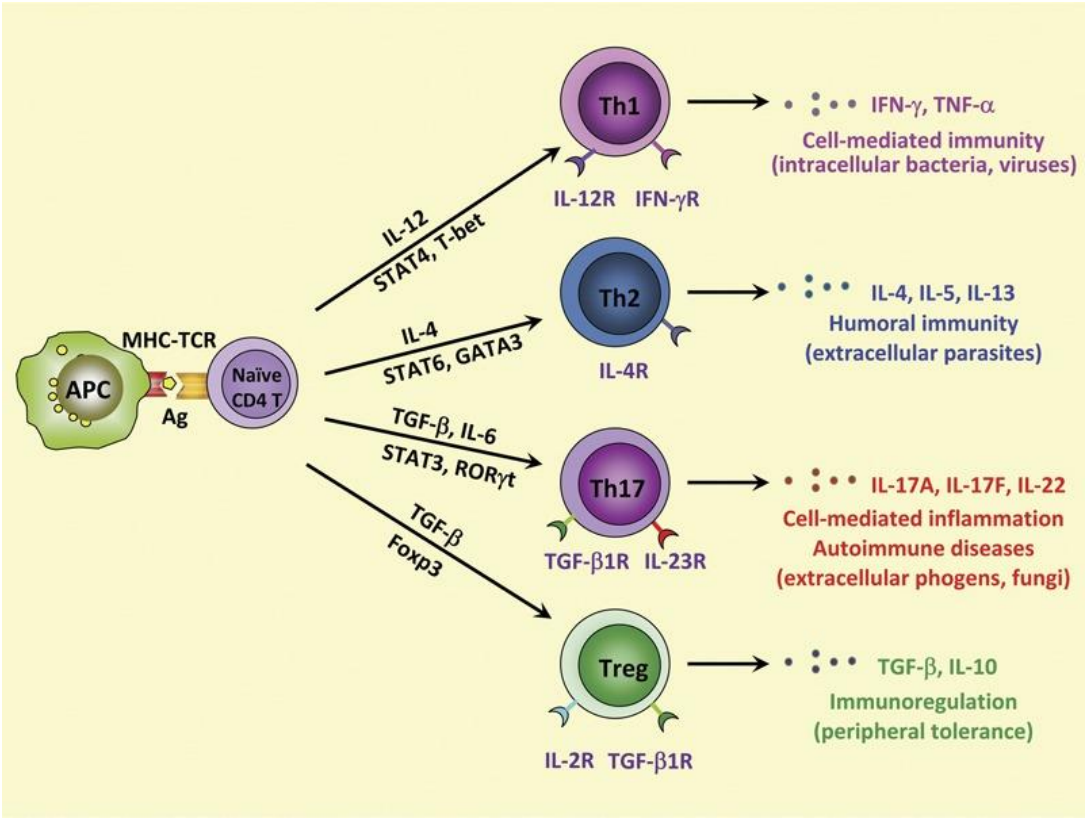


Figure III - CD4$^+$ T cell differentiation. Upon TCR activation triggered by antigen-presenting cells, naive CD4 T cells differentiate into distinct Th subsets producing specific cytokines. The differentiation process involves upregulation of master transcriptional regulators and activation of Stat proteins (Adapted from: Leung S, 2010).

2.2 The inflammatory environment

Once in the inflammatory site, T cells are exposed to numerous factors that might affect their functions i.e., low pH and oxygen, high lactate and other metabolites as well as reactive oxygen species. All these components modulate T cells as well as other immune cell responses and contribute to the establishment of chronic inflammation (Haas R, 2013).

2.2.1 *Hypoxia*

Hypoxia, low oxygen concentrations (<2%), is a prominent feature of the inflammatory microenvironment. The occurrence of hypoxia at sites of inflammation is due to a combination of increased oxygen demand and decreased supply. Oxygen consumption is elevated at inflammatory sites due to the high metabolic demand associated with active inflammation, which is necessary to sustain the synthesis of inflammatory mediators, enzymes, and cytokines (Haas R, 2013; Taylor CT 2016).

A number of recent studies have demonstrated that the microenvironment at sites of inflammation often becomes profoundly hypoxic. Inflammatory pathologies in which tissue hypoxia has been documented include atherosclerosis, rheumatoid arthritis, obesity, infection, ischemic disease, cancer, and inflammatory bowel disease (Eltzschig HK, 2011; Lalonde C, 1988)

Hypoxia can actively affect inflammatory processes through the regulation of oxygen-sensitive signalling pathways in multiple immune cell subtypes that are either resident within the inflamed tissue or have migrated from the oxygen-rich bloodstream to the hypoxic inflammatory milieu (Eltzschig HK, 2011; Scholz CC, 2013).

Hypoxia-inducible factor (HIF) is considered a master regulator of the cellular response to hypoxia (Semenza GL, 2012; Semenza GL, 1998). Under normoxic conditions (2-3% oxygen) HIF1α is hydroxylated by prolyl-hydroxy-domain (PHD) containing enzymes which is a signal for ubiquitinylation-dependent proteosomal degradation. Hypoxia on the other hand inactivates the PHDs and in turn activates the translocation of HIF1α into the nucleus and transcription of its more than 200 target genes responsible for energy metabolism, cell differentiation, migration and apoptosis (Ong SG and Hausenloy DJ, 2012). HIF1α activation promotes a metabolic shift to glycolysis as well as altered transcriptional responses in differentiating T cells (via regulation of the nuclear hormone receptor RORγt and the transcriptional regulator FOXP3), creating an environment favorable for the differentiation to Th17 cells rather than Tregs (Barbi J, 2013; Dang EV, 2011).

2.2.2 *Reactive oxygen species*

During mitochondrial electron transport chain (ETC) leakage of electrons occurs naturally reducing molecular oxygen only partially (one-electron reduction), thus generating the superoxide anion O^{2-}, mainly at complexes I, II and III. Superoxide is then converted to hydrogen peroxide H_2O_2 by superoxide dismutase 2 (SOD2) into the mitochondria or by SOD1 in the cytosol. Superoxide, H_2O_2 and hydroxyl radical (OH) are reactive oxygen species (ROS) able to oxidize many different targets (lipids, proteins and DNA) because of their unstable chemical nature. ROS have been thought for decades to be unwanted by-products of cellular metabolism only involved in inducing damages to cellular components. This idea is now rapidly changing, as evidence of physiological roles of ROS as signals in the regulation of cellular processes are growing, showing that

finely tuned levels of ROS is necessary for the proper activation of specific cellular responses.

Inflammation is one of those processes in which ROS production seems to have an important role. In 2011 West et al demonstrated that mitochondrial (mROS) production is positively regulated after triggering TLRs localized on the cytoplasmic membrane (TLR1/2/4) to enhance macrophages bactericidal activity, via the recruitment of mitochondria to phagosomes and increasing the production of mROS by the complex I of the ETC (West AP, 2011). The inhibition of the production of mROS results in decreased bactericidal activity of macrophages, both *in vitro* and *in vivo*. This effect is mediated via the interaction between TRAF6 (an adapter protein downstream of TLR signaling) and ECSIT (a protein implicated in assembly of complex I) (Vogel RO, 2007), leading to the production of ROS (West AP, 2011). In the same year, Bulua and co-workers showed that blocking mROS generation blunts the production of pro-inflammatory cytokines upon LPS treatment, thus decreasing LPS-induced MAPK activation (in MEFs cells and human PBMC), confirming a physiological role of mROS in orchestrating immune responses (Bulua AC, 2011). mROS play a role also in the adaptive immune system; indeed, Sena et al. have demonstrated that following TCR engagement there is a peak in mROS levels induced by the influx of calcium, and these ROS are necessary for the induction of IL-2 (Bulua AC, 2011). The authors showed that complex III is mainly responsible for the production of mROS in activated T cells. Indeed, T cells lacking a protein necessary for the functionality of complex III (Uqcrfs1) fail to induce IL-2 *in vitro* and are unable to expand antigen-specific T cells (both $CD4^+$ and $CD8^+$ T cells) *in vivo*. The induction of IL-2 was rescued upon providing exogenous H_2O_2. They provide a partial mechanism to explain this effect, showing that cells with impaired complex III activity fail to induce the activation of NFAT1, which is required for IL-2 induction (Sena LA, 2013; **Figure IV**).

Calcium stored in the endoplasmic reticulum regulates mROS generation. Once it is released upon TCR engagement, calcium is taken up by mitochondria to fuel the Kreb cycle (**Figure IV**), thereby increasing the amounts of NADH and Krebs cycle intermediates. Mitochondrial calcium influx contributes to mROS generation in T cells, and succinate drives mROS generation in macrophages. ROS production is related to mitochondrial membrane potential (Suski JM, 2012). A reduction of membrane potential in $CD8^+$ T cells decreases ROS production and increases the expression of the ROS detoxifiers catalase, glutathione peroxidase 4 and superoxide dismutase (SOD1 and SOD2). Th1 and Th17 cells with high membrane potential have increased expression of IFNγ or IL-17A and IL-17F, respectively (Sukumar M, 2016).

Another source of mROS in T cells is the inner mitochondrial membrane enzyme glycerol-3-phosphate dehydrogenase (GPD2; Kamiński MM, 2012; **Figure IV**).

Briefly, it was shown that TCR-triggered activation induces the glycolytic enzyme ADP-dependent glucokinase (ADPGK), which mediates the generation of the oxidative signal. This leads to excessive production of glycerol-3-phosphate, which is imported into mitochondria for biosynthesis of lipids or nucleotides. Here GPD2 oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate, providing electrons to hyper-reduce ubiquinone and subsequently support ROS release from mitochondria.

Downregulation of ADPGK or GPD2 by small interfering RNA inhibits oxidative signal generation and induction of NF-κB-dependent gene expression (Kamiński MM, 2012).

This evidence provides that physiological levels of mROS are necessary to activate specific responses, particularly in the immune system, and its abrogation results in impairment of both the innate and adaptive immune function. Our knowledge of the exact mechanism of action of ROS still needs to be further expanded, but it is becoming clear that ROS production and redox signalling play a crucial role in orchestrating and

regulating cellular biology, highlighting that anti-oxidant therapies, in some instances, might be detrimental if they were to completely blunt basal levels of ROS. The question remains as to whether any additional signaling modules in the ETC can indeed modulate cytoplasmic or even extracellular pathways resulting in specific biological outcomes.

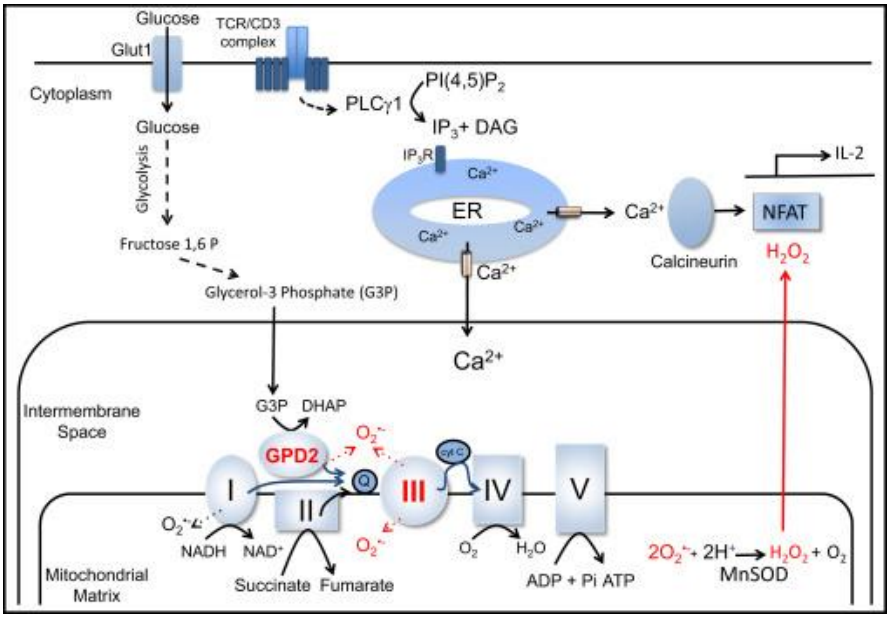


Figure IV - Mitochondrial ROS formation. TCR stimulation leads to Ca_2^+ release from endoplasmic reticulum, which in turn is taken up by mitochondria and stimulates the activity of TCA-cycle enzymes and flux through the ETC. Two are the possible sources of ROS: 1) mitochondrial complex III (Sena LA, 2013) and 2) mitochondrial GPD2 (Kamiński MM, 2012). Both processes can generate O^{2-} (Adapted from: Murphy MP, 2013).

2.2.3 *pH in inflammation*

pH is the molar concentration of protons in aqueous solution (pH = - log[H^+] where [H^+]). The pH scale, ranges from 0 (strongly acidic) to 14 (strongly basic) with a neutral pH in the middle of this scale. Blood is normally slightly basic, with a normal pH range of 7.35 to 7.45. The body uses different mechanisms to control the blood's acid-base balance. These mechanisms involve the lungs, kidney and buffer systems such as the production of bicarbonate. The importance of acid-base homeostasis in the regulation of physiological cellular responses has been recognized for many years (Lardner A, 2001).

The pH affects the ionization state the carboxylic acid and the amino group leading to a change of the confirmation and function of the enzyme. This phenomenon might affect most cellular functions, including cAMP and calcium signalling, DNA and protein/enzyme synthesis as well as cell function and viability (Busa WB, 1994).

A characteristic feature of the inflammatory site is local acidosis, which is partly due to the accumulation of lactate, the end product of glycolytic cells, and to the presence of bacterial metabolism products (Menkin V, 1956).

There are a number of studies, mostly done *in vitro*, investigating extracellular pH effects on immune cells (Lardner A, 2001). Interestingly there is much evidence of an impairment of chemotaxis of leukocytes as well as a reduced cytotoxicity in macrophages, neutrophils, NK and $CD8^+$ T cells at acidic pH. This phenomenon particularly takes place in the tumour microenvironment where the loss of immune cell effector functions, attributed to the low pH, would favor the tumour growth (Lardner A, 2001; Nahas GG, 1971; Gabig TG, Simchowitz L, 1985; Geffner JR, 1993).

In conclusion, the inflammatory microenvironment harbors several physical and chemical factors that influence the activity and function of infiltrating immune cells and might contribute to the progression and outcome of chronic inflammatory diseases.

2.3 Metabolites as signalling molecules

The metabolic regulation of immune cells during health and disease has gained much attention as the active reconfiguration of immune cell metabolism enables these cells to sustain certain effector functions. The focus has been so far on the necessity of the main catabolic pathways glycolysis, fatty acid oxidation (FAO), the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation as well as amino acid metabolism during activation, proliferation, differentiation, and function as a response to extracellular signals.

It is now becoming increasingly evident that small-molecule intermediates of these metabolic pathways, besides their anabolic and catabolic function, can act as intra- and extracellular signals that influence the outcome of an immune response. The roles of metabolite signalling stretch from regulation of cytokine production via indirect effects on the cellular redox state (Kesarwani P, 2013) or direct interaction with transcription factors binding the specific cytokine promoter elements (Tannahill GM, 2013) and modulating the activity of transmembrane ion channels (Yu Y, 2014), to interference with cell migration and differentiation. Interestingly, a few G protein-coupled receptors, such as GPR81, that are activated by intermediates of metabolism have recently been identified, supporting a role for metabolites as extracellular signals (Cai TQ, 2008; He W, 2004).

Enhanced mitochondrial metabolism increases TCA intermediates and ATP, which can be used for posttranslational modification of proteins. In addition to the well-established phosphorylation (ATP) and acetylation of proteins (acetate), other TCA intermediates such as malate and succinate can be used to modify proteins through malonylation (Peng C, 2011) and succinylation (Zhang Z, 2011), respectively.

Succinate was also observed to stabilize HIF1α in LPS-treated macrophages and was associated with enhanced production of IL-1β (Tannahill GM, 2013). In addition, increased mitochondrial oxidation of succinate via succinate dehydrogenase (SDH) and an elevation of mitochondrial membrane potential combine to drive mitochondrial reactive oxygen species (ROS) production (Mills EL, 2016). Conversely, to succinate, itaconate exerts anti-inflammatory effects *in vitro* and *in vivo* by inhibiting SDH and regulating succinate levels in LPS activated macrophages. Itaconate was also found to limit the production of IL-1β, IL-18, IL-6, IL-12, NO, and HIF1α (Lampropoulou V, 2016). On the same line, a paper from the O'Neill lab, has recently described that itaconate is required for the activation of the anti-inflammatory transcription factor NRF2 by LPS in mouse and human macrophages. In particular the authors found that itaconate alkylates cysteine residues on the protein KEAP1, enabling NRF2 to increase the expression of downstream genes with anti-oxidant and anti-inflammatory capacities (Mills EL, 2018).

The tryptophan metabolite 6-formylindolo[3,2-b] carbazole (FICZ) was shown to have very high affinity for AhR. Exposure of CD4 T cells to FICZ under Th17-polarizing conditions enhances Th17 development and functions (Veldohen M, 2009).

The NAD^+-dependent lysine deacylase SIRT5, which removes both succinyl and malonyl groups, targets GAPDH and alters its enzymatic activity, directly tying metabolites generated in the TCA cycle to a key glycolytic mediator (Nishida Y, 2015). Also, the glycolytic metabolite 1,3-bisphosphoglycerate (1,3-BPG) reacts with selected lysine residues to form 3-phosphoglyceryl-lysine (PGK), affecting protein structure and function (Moellering RE, 2013). PGK modifications inhibit glycolytic enzymes, and when cells are exposed to high glucose levels these modifications accumulate, redirecting glycolytic intermediates to other biosynthetic pathways (Moellering RE, 2013). Similarly,

modification of metabolite transporters and cytokine receptors by sugar moieties generated in central carbon metabolism (N-linked glycosylation or O-GlcNAcylation) can modify subcellular localization and activity, thereby affecting T cell functions (Swamy M, 2016). Furthermore, amino acids such as hypusine and citrulline are generated through polyamine and arginine metabolism, respectively, contributing to posttranslational modifications of proteins that influence cell function (Mowen KA, 2014). How metabolites acting as signalling intermediates contribute to the indispensable role of mitochondrial metabolism during early T cell activation, and for the later engagement of glycolytic metabolism and effector functions, remains to be resolved. In the following *section 2.3.1*, I will describe in more details the role of lactate in inflammation.

2.3.1 *Lactate at the crossroad of metabolism, inflammation and cancer*

Lactate is a ubiquitous metabolite, described for the first time at the beginning of the 20th century (Baumann F, 2009). Lactate production occurs mainly in the cell cytoplasm in hypoxic conditions or as a consequence of high rates of glycolysis in proliferating cells via lactate dehydrogenase (LDH), an enzyme that also regenerates the reduced form of nicotinamide adenine dinucleotide (NADH) (Bonuccelli G, 2010). Activated immune cells as well as cancer cells rely on glycolysis more than oxidative phosphorylation (OXPHOS) as an ATP energy source even in presence of oxygen. This phenomenon is called the ‘Warburg effect’ (Haas R, 2016; Bonuccelli G, 2010; Colegio OR, 2014; Su S, 2014) and therefore lactate is produced and accumulates in the extracellular space. Lactate can be present in solution either in its undissociated form (i.e., lactic acid) at low pH or as the ion salt (i.e., sodium lactate) at higher pH.

Our lab has found that depending on the acidification of the microenvironment, lactate exerts different effects on T cells.

More specifically, lactic acid is able to modulate $CD8^+$ but not $CD4^+$ T cell functions (Haas R, 2015), whereas sodium lactate regulates $CD4^+$ T cell migration and cytokine production without affecting $CD8^+$ T cells. Notably, progressive acidification of medium containing sodium lactate (10 mM) with HCl, which increases the availability of lactic acid, reduced the motility of $CD8^+$ T cells. Importantly, neither the presence in the culture media of sodium lactate (10 mM) alone nor acidifying the culture media to pH 4.5 with HCl alone had any effects on the motility of $CD8^+$ T cells. These data suggest that the inhibition of $CD8^+$ T cell motility by lactic acid requires the simultaneous availability of lactate and H^+ in order to take place (Haas R, 2015).

Lactate uptake and secretion requires the presence of transporters on the cell plasma membrane. Four members of the solute carrier 16a family of 12-membrane pass, proton-linked monocarboxylic acid symporters i.e. monocarboxylic transporter 1 (MCT1, also known as SLC16A1), MCT2 (also known as SLC16A7), MCT3 (also known as SLC16A8), and MCT4 (also known as SLC16A3), and two sodium-coupled lactate cotransporters (SLC5A12, SLC5A8) have been described (Halestrap AP, 2012; Srinivas SR, 2005).

These channels, although sharing conserved sequence motifs, show different affinity for lactate and other monocarboxylates (Hirschhaeuser F, 2011; Doherty JR, 2013). The transport depends on pH, intra- and extracellular lactate concentration as well as other substrates such as pyruvate, butyrate, etc. (Hirschhaeuser F, 2011; Doherty JR, 2013). More details about lactate transporters are given in the following *section 2.3.2*.

Lactate accumulates in the tumour and inflammatory environment as a consequence of high glycolytic rate. Here lactate, flowing through specific transporters, can modify and redirect metabolic pathways. Indeed, a recent paper shows how lactate is taken up by

cells which use it to fuel the TCA cycle in human non-small-cell lung cancers (NSCLCs) (Faubert B, 2017). This allows biomass synthesis for tumour growth. Later in 2017, Hui et al. published another important piece of research in *Nature journal* demonstrating the role of lactate in fuelling the TCA cycle, both in normal and cancerous tissues. Analysis of the flux of circulating metabolites in mice showed that lactate is a major source of carbon for the TCA cycle. The infusion of [^{13}C]-lactate, in fed and fasted mice, showed extensive labelling of TCA cycle metabolites in all tissues, and in lung and pancreatic tumours the contribution of circulating lactate to the activity of TCA cycle was greater than that of glucose. These findings demonstrate that lactate is a main feeder of the TCA cycle both in normal and cancerous tissues, as well as that glycolysis and TCA cycle are uncoupled at the level of lactate, thus allowing the independent regulation of the two processes, which also happen in a tissue-specific manner (Hui S, 2017).

The effects of lactate are not only due to its ability to feed metabolic pathways, but also to its ability to trigger a signalling pathway via its receptor Gpr81. This is a surface lactate receptor involved in the regulation of lipolysis (Lafontan M, 2009) but also in cancer cell survival (Roland CL, 2014). Feng et al., recently showed that lactate, through Gpr81, is able to regulate the expression of PD-L1 in human lung cancer cells. PD-L1 is the ligand of PD1; a receptor expressed on the membrane of activated T cells, responsible for reduced proliferation and effector function of T cells, and a major target for cancer immunotherapy (Feng J, 2017). The authors show that lactate upregulates the expression of PD-L1 at a transcriptional level, in a Gpr81-dependent manner, and this upregulation leads to suppression of the effector function of T cells in co-culture experiments. Overall, the data point to a role for lactate in tumour escape from immune-surveillance.

Different subsets of T cells show different metabolic requirements, with cytotoxic and effector T cells relying more on glycolysis for proliferation and cytokine production (Macintyre AN, 2014) and, conversely, Tregs being more dependent on oxidative

phosphorylation (OXPHOS) (Michalek RD, 2011; Gerriets VA, 2015). Recently, Angelin et al, demonstrated that FOXP3 is able to reprogram the metabolism of Tregs, allowing them to cope with low-glucose, high-lactate microenvironments. The authors found that the transcription factor FOXP3 promotes an increase in the oxygen consumption rate in induced Tregs (iTregs) and the production of ROS. Moreover, in the same paper the authors show that FOXP3 is able to suppress the activity of Myc by binding to its promoter and reducing the expression of Myc-dependent transcripts, most of which are involved in the regulation of glycolysis. FOXP3 was also able to regulate the direction of the LDH reaction in favour of the oxidation of L-lactate to pyruvate, leading to a decreased production of lactate by Tregs (as compared to conventional T cells). The authors also conclude that Tregs can sustain exposure to lactate much better than effector and cytotoxic T cells, which are instead impaired by it. Indeed, effector T cells require NAD^+ replenishment to sustain the flux of glycolysis, but the excess of utilization of lactate by LDH depletes the available pool of NAD^+, leading to a decreased glycolytic flux; Tregs instead possess higher levels of NAD^+ and due to their intrinsic metabolism are less affected by reduced glycolysis. The authors discuss that this adaptation could be detrimental in the tumour microenvironment, where the high concentration of lactate may inhibit anti-tumour immunity without affecting the regulatory component, which can further dampen the immune response against the tumour (Angelin A, 2017).

The role of lactate in immune disorders has been studied at a lesser extent than in cancer biology. Nonetheless, recent evidence demonstrate how lactate has a crucial role also in orchestrating immune responses in inflammatory conditions. In 2016, Peng et al. formally demonstrated that LDHA activity is necessary in $CD4^+$ T cells to sustain aerobic glycolysis and express interferon-γ (IFNγ), thus allowing a proper differentiation in T helper 1 (Th1) cells. The authors found that genetic deletion of LDH isoform A (LDHA) in $CD4^+$ T cells, reduced significantly glucose consumption, promoting a shift towards an

oxidative metabolism, and, more importantly, led to a reduction of IFNγ expression at a transcriptional level. The reduction of INFγ transcript (along with many others), was due to an overall decrease of histone acetylation. Histone acetylation requires acetyl-coenzyme A (acetyl-CoA), but in the absence of LDHA, the consequent increased activity of the TCA cycle does not allow the export of acetyl-CoA from the mitochondria to the cytosol, reducing the pool of acetyl groups. These data demonstrate that LDHA regulates INFγ production in Th1 cells, through a fine-tuned epigenetic mechanism of histone acetylation coupled with the cellular metabolism (Peng C, 2011).

Lactate is also able, as previously mentioned, to orchestrate immune cells functions. Acid lactate, for example, can suppress the proliferation and cytokine production of human cytotoxic T lymphocytes (CTLs) in cancer patients (Fischer K, 2007) as well as their migratory abilities (Haas R, 2015). This effect can be at least in part explained by lactate inhibition of TCR-triggered phosphorylation of JNK, c-Jun and p38, which is implicated in IFNγ production (Mendler AN, 2012). An inhibitor of monocarboxylate transporters, alpha-cyano-4-hydroxycinnamic acid, was reported to block lactate-augmented inflammatory gene expression and NF-kappaB activity in human macrophages, indicating that lactate transport through monocarboxylate transporters is required for macrophage effector functions (Samuvel DJ, 2009).

On the same line, Colegio et al. show that tumour-associated macrophages (TAMs) ‘sense’ metabolic changes typical of the tumour microenvironment. Cancer cells produce high amount of lactic acid, which is extruded in the intercellular space via MCT4. Released lactic acid is then taken up by tumour-associated macrophages via MCT1, promoting in turn macrophage polarization toward a TAM phenotype via the induction of HIF1α and arginase 2. These effects induce vascular endothelial growth factor (VEGF) production and ultimately support tumour growth in a feed forward loop (Colegio OR, 2014). Notably, TNF secretion by human monocytes was found to be suppressed in the

presence of high lactate concentration and reduced pH in tumour microenvironment (Mendler AN, 2012). Neutralizing tumour acidity with bicarbonate monotherapy was able to impair the growth of some cancer types (Pilon-Thomas S, 2016). Similarly, another study pointed to the role of lactic acid in the TAM regulation of the epithelial-to-mesenchymal transition (Su S, 2014).

Besides MCTs, proton-sensing GPCRs such as T cell death-associated gene 8 (TDAG8) have been shown to be important for the modulation of T cells in an acidic tumour environment and during inflammation (Pilon-Thomas S, 2016; Ishii S, 2005).

Targeting lactate transporters has become a promising therapeutic avenue in oncology. A recent study found that blocking the lactate transporter MCT1 reduced the proliferation of breast cancer cells co-expressing MCT1 and MCT4 (Hong CS, 2016) and reduced HIF1α induced angiogenesis in cervix squamous carcinoma (Sonveaux P 2008; Sonveaux P, 2012). On the other hand, other authors showed that lactate mediated angiogenesis is independent of HIF1α but is due to direct binding to NDRG3 during hypoxia. This event leads to Raf/ERK1/2 signalling pathway activation and promotes proliferation and angiogenesis (Lee DC, 2015). LDH, the enzyme that converts pyruvate to lactate, is increased in tumour cells (Fantin VR, 2006; Husain Z, 2013). A decreased frequency of myeloid-derived suppressor cells (MDSCs) was observed in the spleens of mice carrying LDHA-depleted tumours. NK cells isolated from LDH-A-depleted tumours had improved cytolytic function. The addition of exogenous lactate increased the frequency of MDSCs generated from murine bone marrow cells and inhibited cytolytic function of both human and murine NK cells *in vitro*. This reduction of NK cytotoxic activity was accompanied by lower expression of perforin and granzyme. Therefore, tumour-derived lactate hinders NK cell-mediated responses directly via inhibition of cytolytic function as well as indirectly by increasing the numbers of MDSCs that inhibit NK cytotoxicity (Husain Z,

2013). This evidence is also supported by the recent paper by Brand et al showing that lactate leads to tumour immune escape by inhibiting the function and survival of T and NK cells (Brand A, 2016). On the same line, another study supports the role of LDHA in tumour maintenance (Fantin VR, 2006). Knocking down LDHA by short hairpin RNAs resulted in a reduced ability of tumour cells to proliferate under hypoxic conditions. This phenomenon was accompanied by increased mitochondrial respiration and decrease of mitochondrial membrane potential. The tumourigenicity of the LDHA-deficient cells was severely diminished, and this phenotype was reversed by complementation with the human LDHA (Fantin VR, 2006).

Lactate has been proven to be important also for the physiology of the brain, through the astrocyte-neuron lactate shuttle (ANLS). This lactate exchange was first described in 1994 and highlights the existence of lactate-producing cells (astrocytes) and lactate-consuming cells (neurons): in this model, the neurotransmitter glutamate released in the synapse triggers glucose uptake and therefore lactate production by astrocytes; lactate so produced is then utilized by neurons as a source of energy (Pellerin L, 1994; Magistretti PJ, 2018).

Taken together, it is clear that lactate is not just a metabolic end-product, but rather a proper source of carbon and, more importantly, a signal that affects the behavior and the differentiation of many different cell types.

2.3.2 *Lactate and autoimmunity*

Lactate regulates many homeostatic functions. For example, it fuels gluconeogenesis in the liver and oxidative metabolism in the brain (Cornell NW, 1973; Pellerin L, 1994). Interestingly, in T cells, lactate acts as an immunomodulatory molecule able to control

their migratory capacity and effector functions (Haas R, 2015). While in tumour cells, lactate plays a key role in promoting their migration and expansion, in activated T cells isolated from healthy volunteers lactate activates a stop migration signal inside the inflamed tissue. This is due to the interaction of sodium lactate and lactic acid with the transporters SLC5A12 and SLC16A1, respectively, which are selectively expressed on the surface of $CD4^+$ and $CD8^+$ subsets (Haas R, 2015). Sodium lactate-mediated inhibition of T cell migration is regulated via lactate interference with the glycolytic pathway (Haas R, 2015; Droge W, 1987). Indeed, $CD4^+$ T cells, in the presence of sodium lactate, display a downregulation of glycolytic enzymes such as HK1. Lactate is also involved in the production of IL-17 and plastic acquisition of Th17 features in the inflamed site. In 2011, Yabu et al. showed how lactic acid enhances the production of IL-23/IL-17 by $CD4^+$ T cells, acting as a pro-inflammatory signal (Yabu M, 2011, Shime H, 2008). More recently, in our lab Haas et al. demonstrated that sodium lactate is able to modulate effector T cells function, up-regulating the production of IL-17 in $CD4^+$ T cells. As mentioned at the beginning of this paragraph, lactate is also able to impair the migratory capabilities of $CD4^+$ T cells, thus causing their entrapment in the site of inflammation. This finding has an important impact on the understanding of the role of lactate in the inflammatory site, such as the inflamed synovium in rheumatoid arthritis, where lactate may act as an inflammatory signal leading to the entrapment of $CD4^+$ T cells and the stimulation of IL-17 production, thus sustaining inflammation. Interestingly, all these effects are mediated by a specific sodium lactate transporter, SLC5A12, expressed on the membrane of $CD4^+$ T cells (Haas R, 2015).

Therefore, it seems fair to conclude that lactate behaves as a pro-inflammatory signal in the context of inflammation, leading to the production of cytokines necessary for the differentiation of specific T cell subsets and regulating their ability to migrate.

Physiological lactate concentration is in the range of 1.5–3 mM in blood and tissues of

healthy individuals via specific absorption systems in the kidney operated by SLC5A8 and SLC5A12. However, lactate levels can rise up to10 mM in inflammatory pathologies (e.g. rheumatic synovial fluid) and even 30–40 mM in cancerous tissues (Haas R 2015; Colegio OR, 2014; Hirschhaeuser F, 2011). Accumulation of lactate in the synovial fluid of RA patients is the result of high synovial cells metabolic demand (Haas R, 2015). Even though there is not a clear correlation between lactate concentration in the synovial fluid and the disease activity, some authors found that synovial lactate measurement could be a reliable indicator for differentiating inflammatory arthritis (Gobelet C, 1984). On the same line, LDH isoenzymes were found higher in serum and synovial fluid of RA compared to osteoarthritis (OA) patients (Pejovic M, 1992) and LDH activity was found increased in RA synovial tissues compared to healthy controls (Lindy S, 1971). Moreover, a positive correlation between LDH-A, high lactate levels and tumour progression has been widely documented in various tumours.

Due to the 'emerging' role of lactate in the field of inflammation, lactate transporters are gaining much attention as novel therapeutic targets. In this regard, it has been recently found that monocarboxylate lactate transporter 4 (MCT4) is up-regulated by RA synovial fibroblast (RASFs) compared to osteoarthritis (OA) SF (Fujii W, 2015). The silencing of MCT4, by inducing apoptosis of synovial cells, inhibited the proliferation of RASFs and was able to reduce the severity of arthritis in mice with collagen-induced arthritis (CIA) (Fujii W, 2015).

RA synovitis is characterized by distinctive histological patterns. In a 40% of RA patient immune cells can be found spatially grouped into follicular structures which can acquire features of SLO with high-endothelial venules (HEV), T/B-cell segregation, autoantibody production and follicular-dendritic cell (FDC)-networks. These structures, which develop in the inflammatory sites outside the SLO, are called ectopic lymphoid-like structures (ELS) (more details in *section 2.5.1*).

Although the mechanisms that are responsible for the preferential accumulation of autoreactive B and T cells in ELS are not fully understood, a direct role has been proposed for Epstein–Barr virus (EBV) and IL-17 production by Th17 cells in the development of autoimmunity (Pitzalis C, 2013; Pitzalis C, 2014; Croia C, 2013; Humby F, 2009). The rheumatoid synovial environment is paradigmatic of all the lactate-induced changes in T cells, including entrapment, IL-17 secretion and loss of antigen responsiveness (Haas R, 2015). Our group has recently found that SLC5A12 is highly expressed in human RA synovial tissues (ST). Strikingly, its levels significantly increased in correlation with the T cell score and with the formation of ELSs which are rich in IL-17 (Haas R, 2015; Peters A, 2011; Jones GW, 2015; Jones GW, 2016), thus suggesting a possible role of lactate/SLC5A12-induced metabolic signalling network in promoting chronic inflammation in RA. I will discuss more in depth about the role of lactate in the pathogenesis of RA in the *section 2.5* and *chapter V*.

The abundance of lactate in inflammatory sites is responsible, at least in part, for their acidity and hypoxia. Indeed the lactate signalling and subsequent biologic responses appear to be functionally coupled to the HIF1 α -induced metabolic reprogramming, by employing NDRG3 as critical link (Lee DC, 2015). Indeed, it promotes the switch towards a Th17 phenotype while inhibits regulatory T cell differentiation (Shi LZ, 2011). This might explain a possible mechanism through which lactate enhances IL-17 production during hypoxia.

Taken together lactate plays an important role in inducing and promoting inflammation and in the modulation of immune cell functions. Targeting lactate transporters, LDH, and HIF1α may represent a novel therapeutic intervention in autoimmune and inflammatory diseases elicited by pro-inflammatory T cells.

2.3.3 *Classification of lactate transporters*

Lactate flux in and out of the cells is facilitated by transporters, which are classified in monocarboxylate transporters (MCTs) and sodium-coupled transporters (SMCTs). MCTs include a family of 14 transmembrane proteins encoded by the SLC16A gene family. According to the Milton Saier classification (http://www.tcdb.org), MCTs belong to the monocarboxylate porter (MCP) family, which in turn is part of the facilitator superfamily (MFS). MCTs have been identified in all eukaryotic organisms and can transport a wide variety of substrates (Perez-Escuredo J, 2016; **Table 1**).

MCT1-4 are proton-linked transporters responsible for transport of several monocarboxylate metabolites, such as pyruvate, L-lactate and ketone bodies (acetoacetate and D-β-hydroxybutyrate) together with a proton across the plasma membrane (Halestrap AP, 1999; Halestrap AP, 2004). Other identified MCTs are MCT8, which shows high affinity for the thyroid hormones T3 and T4, and MCT10/TAT1, a transporter of aromatic amino acids (Halestrap AP, 2004; Visser WE, 2011). MCT6 has been reported to facilitate the proton-linked transport of bumetanide (Murakami Y, 2005). MCT7 has been implicated in the export of ketone bodies by hepatocytes (Hugo SE, 2012).

MCT9 has been identified as a sodium- and pH-independent carnitine efflux transporter when it was expressed in Xenopus oocytes injected with [^{3}H]-carnitine (Suhre K, 2011). The substrates and functions of the other MCT family members are yet not known.

MCTs are expressed in a wide range of tissues (such as brain, skeletal muscle, heart, bowel and liver) and display many physiological functions. In particular, they play a pivotal role in the control of the central metabolism of glucose, gluconeogenesis, activation of T-lymphocytes, spermatogenesis, pancreatic β cell activity, thyroid hormone metabolism and drug transport (Perez-Escuredo J, 2016).

Among others, MCTs are important regulators of intracellular lactate and pH. Indeed, highly glycolytic cells, such as during inflammation or tumours, utilize MCT transporters to export lactate.

Lactate is one of the main substrates of MCT1–4. This metabolite is generated from pyruvate (produced from glycolysis and glutaminolysis) during lactic fermentation. In most normal tissues where lactate is produced, MCT1 is responsible for its export across the plasma membrane in to the extracellular space (Halestrap AP, 2004, Halestrap AP, 2012).

Lactate can be taken up from the extracellular space and used as a substrate to fuel metabolic pathways such as lipogenesis, gluconeogenesis, TCA cycle and oxidative phosphorylation (OXPHOS) (Perez-Escuredo, 2016; Faubert B, 2017). Cells that utilize lactate may express different MCTs depending on tissues and species (Halestrap AP, 2004; Bonen A, 2001; Sonveaux P, 2008; Van Hée VF, 2015; Huang CK, 2017).

In many cancer cells with an oxidative metabolic fingerprint, MCT1 is the most expressed MCT isoform (Sonveaux P, 2008; Kennedy KM, 2013). However, in glycolytic cancer cells and other specific tissues such as white muscle fibres and astrocytes, MCT4 is expressed at higher level than MCT1 and mediates lactate export (Ullah MS, 2006; Sonveaux P, 2008; Baltazar F, 2014). Accordingly, there is increasing evidence in support of the shuttling of this metabolite between cells with different metabolic behaviors within the same tissue. Such phenomenon has been described in the skeletal muscle where glycolytic/white fibres export lactate through MCT4 and oxidative/red fibers import lactate through MCT1 to fuel the TCA cycle (Juel C, 1999). A similar mechanism has been proposed to account for a metabolic symbiosis between glycolytic/hypoxic cancer cells and oxidative/oxygenated cancer cells in tumours (Sonveaux P, 2008). Notoriously in the brain, glycolytic oligodendrocytes and astrocytes

export lactate through MCT1 and MCT4 to fuel oxidative neurons expressing MCT2 (Brooks GA, 2009; Pellerin L, 2012; Funfschilling U, 2012; Saab AS, 2013).

In contrast to MCTs, which function as H^+-coupled electroneutral transporters, SMCTs function as Na^+-coupled electrogenic transporter. The transport process is electrogenic as more than 1 Na^+ is transported per transport cycle with a Na^+/monocarboxylate substrate ratio of ≥ 2. Two members of the sodium-coupled monocarboxylate transporter family (SMCT) have been identified so far, the high-affinity transporter SMCT1 (SLC5A8) and the low-affinity SMCT2 (SLC5A12) (Rodriguez AM, 2002; Srinivas SR, 2005). The SLC5A8 gene was originally identified from a library of kidney cDNA as a close structural relative of the human Na/I symporter (SLC5A5) (Rodriguez AM, 2002). Other than in the kidney, SMCT1 has been subsequently detected in intestine, salivary gland, thyroid gland, brain, and retina (Ganapathy V, 2008). Substrates of SMCT1 are similar to those of MCTs (Gopal E, 2005; Morris ME and Felmlee MA, 2008). SMCT1 mediates the transport of monocarboxylic acids such as lactate, pyruvate, propionate, butyrate, nicotinate, and short-chain fatty acids (**Table 2**).

The affinity of the transporter for these monocarboxylates is quite high, with a Michaelis constant in the range of 200–400 μM.

Less is known about SMCT2; mRNA expression of SMCT2 was detected in kidney, small intestine, and skeletal muscle and to a lesser level in brain and retina. Functional characterization of SMCT2 suggested substrate specificity similar to that of SMCT1. However, the affinities of SMCT2 for monocarboxylate substrates are approximately 35- to 80-fold lower than those of SMCT1 (Srinivas SR, 2005).

In the kidney, SMCT1 is expressed in the apical membrane of tubular epithelial cells in the S2-S3 proximal tubule segments. Here SMCT1 is involved in renal reabsorption of lactate and pyruvate in a sodium-dependent transport (Gopal E, 2007b; Ganapathy V, 2008; Barac-Nieto M, 1980; Yanase H, 2008). Indeed, SLC5A8-deficient or knockout

mice exhibit increased urinary excretion of lactate (Frank H, 2008). Renal SMCT2 is localized at the brush border with higher expression in the initial part of the proximal tubules and gradually decreasing toward the S3 segment. Thus, the proximal convoluted tubules provide low and high affinity transporters in the upper and lower proximal tubules, respectively.

In the brain, SMCT1 exhibits a neuron-specific distribution and may mediate cellular uptake of lactate and ketone bodies, the primary energy substrates of neurons (Martin PM, 2006), while SMCT2 is specifically expressed by astrocytes. Besides the physiological functions, several reports suggested a tumour-suppressing role for SMCT1. High frequency of aberrant methylation or down-regulation of the SLC5A8 gene has been observed in human colon cancer, papillary thyroid carcinomas, pancreatic cancer, prostate tumour, acute myeloid leukemia, and glioma formation (Ganapathy V, 2008; Park JY, 2008, Li H, 2003).

In the bowel, SLC5A8 is expressed in the lumen-facing apical membrane of colonic and intestinal epithelial cells, while SLC5A12 is expressed primarily in the small intestinal tract (Iwanaga T, 2006; Gopal E, 2007).

Very recently, these transporters have been also linked with the function of immune cells. Interestingly, SLC5A12 has been found expressed by human and mice lymphocytes. In particular, it was found that SLC5A12 is selectively expressed on the surface of $CD4^+$ but not of $CD8^+$ T cells (Haas R, 2015; Haas R, 2016). Whether or not immune cells express SLC5A8 is still unknown.

2.3.4 *Lactate transporters in cancer and autoimmunity*

MCTs, especially MCT1 and MCT4, are widely expressed in a variety of immune and cancer cells. In the tumour microenvironment, cancer cells produce high amount of lactate, which is extruded in the intercellular space via MCT4. Released lactate is then taken up by macrophages via MCT1, promoting their polarization toward a TAM phenotype via the induction of HIF1α and arginase 2. These effects induce neo-angiogenesis, via vascular endothelial growth factor (VEGF) production, and ultimately promote tumour growth (Colegio OR, 2014; **Figure V**).

MCT1 expression has been reported in a variety of human malignancies including head and neck, lung, stomach, colon, prostate and cervix cancers, as well as gliomas (Sonveaux P, 2008; Kennedy KM, 2010; Pinheiro C 2012; Miranda-Goncalves V, 2013; Afonso LL, 2015). MCT1 has also been proposed to be the most important isoform responsible for lactate transport across the plasma membrane in breast and bladder cancer, non-small cell lung carcinomas (NSCLC) and ovarian carcinomas (Afonso LL, 2015).

MCT4 is also widely distributed in different cancer types. Its expression has indeed been reported in breast, colon, bladder, and prostate cancers, as well as in cancers of the gynecologic tract and gliomas (Pinheiro C, 2012; Miranda-Goncalves V, 2013; Afonso LL, 2015).

Recently, Pertega-Gomes and Baltazar (Pertega-Gomes N, 2014) reported a correlation between the expression of MCT1, MCT2 and MCT4 and the different stages of prostate cancer progression. MCT1 and MCT2 would play a role in tumour maintenance, whereas MCT4 would increase tumour aggressiveness. MCT2 was also proposed as an interesting biomarker for prostate cancer (Pertega-Gomes N, 2014). In another study focused at NSCLC, Eilertsen et al. proposed MCT1 as a biomarker for prognostic and survival (Eilertsen M, 2014). The co-expression of GLUT1 and MCT1 and of GLUT1 and MCT4

was found to be a negative prognostic factor associated with poor disease-specific survival.

MCT2 and MCT4 show a high intracellular expression. This suggests a possible role of these transporters in mediating lactate and/or pyruvate transport across the membranes of intracellular vesicles or organelles (Baltazar F, 2014; Afonso LL, 2015).

Lactate transporters have been described in inflammatory sites such as rheumatoid arthritis (RA) synovium (Haas R, 2015; Pucino V, 2018; **Figure V**).

Unlike in tumours where lactate plays a key role in promoting cancer cell migration and growth, in inflammatory sites lactate activates a stop migration signal leading to local entrapment of T cells (Haas R, 2015; **Figure V**).

This phenomenon is due to the interaction of sodium lactate and lactic acid with the transporters SLC5A12 and SLC16A1, which are selectively expressed on the surface of $CD4^+$ and $CD8^+$ subsets, respectively (Haas R, 2015). Sodium lactate-mediated inhibition of $CD4^+$ T cell migration is regulated via lactate interference of metabolic pathways (Haas R, 2015; Droge W, 1987). Sodium lactate via SLC5A12 prompts $CD4^+$ T cells to start producing higher amounts of pro-inflammatory cytokines, in particular IL-17A, while lactic acid causes impairment of $CD8^+$ T cell-mediated killing through SLC16A1 (Haas, 2015; Fisher K, 2007; Mendler AN, 2012). All these events promote T cell retention in the inflammatory sites and exacerbate the process of chronic inflammation (Haas R, 2015).

Similar to cancer cells, highly proliferating RA synovial fibroblast (RASFs) express high levels of MCT4, thus promoting synovial fluid acidification (Fujii W, 2015). Silencing of MCT4 led to inhibited proliferation of RASFs and reduced the severity of arthritis in the mouse model of collagen-induced arthritis (CIA) (Fujii W, 2015). On the same line, other authors found that MCT4 is required for macrophage activation upon TLR2 and TLR4 stimulations. MCT4 knockdown led to enhanced intracellular accumulation of lactate and

decreased glycolysis in LPS-treated macrophages reducing their active response during inflammation (Tan Z, 2015). This evidence suggests a potential role of lactate transporter inhibitors in the therapy of RA. Lactate transporter tissue expression and function is shown in **Table 1** and **2**.

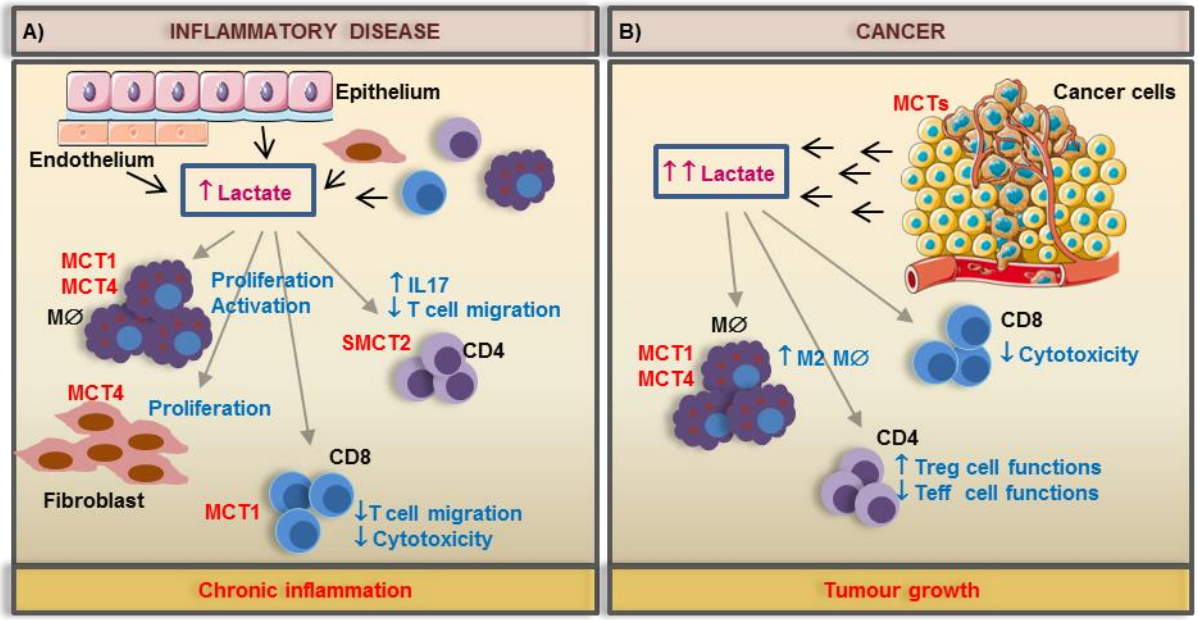


Figure V - Accumulation of lactate occurs in the inflamed tissue and in the tumour microenvironment where it promotes chronic inflammation and tumour growth. A) During the inflammatory process immune cells sense (via specific transporters) and respond to the high levels of lactate that is produced by endothelial and epithelial cells as well as by the infiltrating immune cells, which all consume the available glucose. The sodium lactate transporter SMCT2 (SLC5A12) and the lactic acid transporter MCT1 (SLC16A1) are expressed by $CD4^+$ and $CD8^+$ T cells, respectively. MCT4 is expressed by fibroblasts and macrophages; the latter also display high levels of MCT1. Through these transporters, lactate modulates immune cell functions. In particular, lactate promotes fibroblast proliferation as well as macrophage (MØ) proliferation and activation. Lactate also inhibits T cell motility thus causing T cell entrapment in the inflamed tissue. There, in response to lactate $CD4^+$ T cells produce higher amounts of IL-

17 and $CD8^+$ cytotoxic activity is affected. Overall, these events promote chronic inflammation. B) Cancer cells express high levels of MCTs and release high amount of lactate in the tumour microenvironment, as the product of their pronounced glycolytic metabolism, which leaves infiltrating immune cells reliant on carbon sources other than glucose for their metabolism. There, lactate has an effect on wide range immune cell functions. It induces the switch of macrophages to an M2 phenotype via the transporters MCT1 and MCT4. It also promotes T cell differentiation to the Treg phenotype while suppressing Teff functions (i.e. cytokine production, cytotoxicity), thus leading to enhanced tumour growth (Adapted from: Pucino V, 2018).

Table 1 - MCTs transporters (Adapted from: Pucino V, 2018)

MCT transporter	Common name	Substrate	Distribution
SLC16A1	MCT1	Lactate, pyruvate, ketone bodies	Ubiquitous
SLC16A2	MCT8	T2, rT3, T3, T4	Ubiquitous
SLC16A3	MCT4	Lactate, ketone bodies	Skeletal muscle, chondrocytes, leucocytes, testis, lung, brain, ovary, placenta, heart, leucocytes
SLC16A4	MCT5	N/A	Brain, muscle, liver, kidney, lung, ovary, placenta, heart
SLC16A5	MCT6	Bumetanide probenecid nateglinide	Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta
SLC16A6	MCT7	Ketone bodies	Liver, brain, pancreas, muscle, prostate
SLC16A7	MCT2	Pyruvate, lactate, ketone bodies	High expression in testis, moderate to low in spleen, heart, kidney, pancreas, skeletal muscle, brain and leucocytes
SLC16A8	MCT3	Lactate	Retinal pigment epithelium, choroid plexus
SLC16A9	MCT9	Carnitine	Endometrium, testis, ovary, breast, brain, kidney, spleen, retina
SLC16A10	MCT10	N/A	Kidney (basolateral), intestine, muscle, placenta, heart
SLC16A11	MCT11	N/A	Skin, lung, ovary, breast, lung, pancreas, retinal pigment epithelium, choroid plexus
SLC16A12	MCT12	N/A	Kidney, retina, lung testis
SLC16A13	MCT13	N/A	Breast, bone marrow stem cells
SLC16A14	MCT14	N/A	Brain, heart, muscle, ovary, prostate, breast, lung, pancreas liver, spleen, thymus

Table 2 - SMCTs transporters (Adapted from: Pucino V, 2018)

HUGO nomenclature	Common name	Function	Distribution
SLC5A1	SGLT1	Na^+/glucose or Na^+/galactose	Gastrointestinal tract, liver, kidney, male tissues
SLC5A2	SGLT2	Na^+/glucose	Kidney, male tissues
SLC5A3	SMIT1	Na+/myoinositol	Ubiquitous
SLC5A4	SGLT3	Glucose-sensitive Na^+-channel	Gastrointestinal tract
SLC5A5	NIS	Na^+/iodide	Gastrointestinal tract, endocrine tissues, female tissues
SLC5A6	SMVT	Na^+/biotin or Na^+/pantothenate	Ubiquitous
SLC5A7	CHT1	Na^+/Cl−/choline	Low expression in gastrointestinal tract, kidney, endocrine tissues, female and male tissues
SLC5A8	SMCT1	Na^+/monocarboxylate	Intestin, kidney (apical membrane of tubular epithelial cells), brain, salivary gland, thyroid gland
SLC5A9	SMT	Na^+/mannose	Gastrointestinal tract
SLC5A10	N/A	Unknown	Kidney
SLC5A11	SMIT2	Na^+/myoinositol	Gastrointestinal tract, kidney, female tissues, brain
SLC5A12	SMCT2	Na^+/monocarboxylate	Small intestin, kidney, brain, retina, male tissues, lymphoid organs, leucocytes

2.4 Crosstalk between immunity and metabolism

Immune cells are able to respond to inflammatory stimuli by switching from a quiescent to an activated status. This phenomenon is mostly regulated by receptors and transcription factors, which determine changes in the expression of large numbers of genes and results in the acquisition of new functions. Immune cells acquire the capability to produce cytokines, lipid mediators and metabolites as well as the ability to proliferate, differentiate into specialized subtypes and migrate to target tissues.

All these functions require the involvement of metabolic pathways. In the last few years, there has been an increasing appreciation of how metabolic pathways can modulate immune cell responses and be responsible of T cell fate (Pearce EL and Pearce EJ, 2013).

2.4.1 *T cell metabolism in health and autoimmunity*

Metabolic alterations have been shown to be associated with inflammatory disorders such as tumours and autoimmune diseases. Cancer, as well as immune cells, can switch their metabolic status according to the surrounding inflammatory environment.

Effector T cells are key players of adaptive immunity and require high metabolic demand in order to activate, proliferate, migrate and differentiate. More specifically, they increase the glycolytic flux via the glucose transporter GLUT1, GLUT3 and the biosynthesis of proteins, nucleic acids and lipids that are essential for their functions (Palmer CS, 2015; Macintyre AN, 2014). On the other side quiescent T cells mainly rely upon fatty acid oxidation and mitochondrial respiration to fuel their energy demand (Buck MD, 2016; Pearce EL, 2013).

Memory T cells and/or chronically activated T cells do not proliferate and do not require high-energy demand. Indeed, they mainly utilize oxidative phosphorylation (OXPHOS) to produce the required amounts of ATP (Buck MD, 2016; Pearce EL, 2013).

$CD4^+$ T cells can undergo a metabolic reprogramming according to their differentiation state. While effector T cells such as Th1, Th2 and Th17 increase glycolysis over mitochondrial metabolism, Tregs and memory T cells display a metabolic program involving mainly lipid oxidation and OXPHOS (Gerriets VA, 2012).

Alterations of this delicate balance might lead to the establishment of a non-resolving-inflammatory environment, chronic inflammation and autoimmunity (Nathan C, 2010).

Molecular mechanisms underlining metabolic pathways in the immune system have mainly been studied in tumours; but their role in autoimmunity is still under investigation (Li Z, 2016). Evidence of metabolic alterations have been shown in mouse model and patients affected by autoimmune disorders.

Splenocytes isolated from lupus prone mice preferentially convert glucose to CO2 increasing mitochondrial metabolism for ATP synthesis (Wahl DR, 2010). Clinical, epidemiological and experimental data in humans have suggested that the pathogenesis of several immune-mediated disorders might involve factors, hormones and neural mediators that link metabolism and immune system (Procaccini C, 2014; Cantarini L, 2016). For example, leptin, an adipocyte-derived hormone that control food intake and metabolism has been suggested to play an important role in the modulation of T cell functions (De Rosa V, 2015; Cassano S, 2015). Leptin production is altered in many autoimmune diseases such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), etc. (Procaccini C, 2014).

Recently, also metabolites such as succinate, citrate, lactate, etc. - i.e. intermediate and end-products of metabolic pathways - have been shown to activate signalling resulting in the modulation of immune functions (**Figure VI**, Haas R, 2016; Haas R, 2015; O'Neill

LA, 2014).

Metabolic pathway defects have been found in several autoimmune disorders. For instance, $CD4^+$ T cells from patients with active SLE display a metabolic dysregulation in both humans and mice. Specifically $CD4^+$ T cells from SLE patients exhibit enhanced glycolysis and mitochondrial metabolism (Yin Y, 2015; Mehta MM, 2015). Inhibition of these pathways with 2-deoxyglucose (2-DG, glycolysis inhibitor) and metformin (mitochondrial respiration inhibitor), respectively resulted in a reduced IFNγ production *in vitro* and normalized T cell metabolism and disease phenotypes *in vivo*. $CD4^+$ T cells from SLE patients also display increased activity of the mammalian target of rapamycin (mTOR) (Yin Y, 2015). Rapamycin treatment ameliorated T cell function and prolonged survival in lupus-prone MRL/lpr mice and reduced disease manifestations and diseases activity in SLE patients (Fernandez DR, 2006; Fernandez D, 2009). There is evidence for a strong association between SLE and complement C1q deficiency. Interestingly, a recent study revealed that C1q, but not C3, restrains the response to self-antigens by modulating the mitochondrial metabolism of $CD8^+$ T cells thus supporting the hypothesis that metabolic abnormalities in these cells play a key role in the pathogenesis of the disease (Ling GS, 2018). Conversely, another study carried on SLE, revealed reduced glycolysis, Krebs cycle, fatty acid β oxidation and amino acid metabolism in these patients compared to healthy controls. This dysregulation associates with dampened energy generation, high oxidative stress, inflammation, altered lipid profiles and a prothrombotic state (Wu T, 2012). Furthermore, serum levels of total LDH, LDH1 and LDH2, were significantly higher in patients with diffuse proliferative lupus nephritis. Specifically the study showed a positive correlation between LDH isoforms with the SLE activity index, the renal pathologic score, and with the renal glomerular hyper-cellularity thus suggesting a possible role of LDH as novel biomarker for disease progression (Inoue T, 1986).

A bioenergetic deregulation has also been associated to MS, a chronic inflammatory

disease, which leads to focal plaques of demyelination and tissue injury in the CNS. Studies on relapsing remitting MS patients have identified defects of mitochondrial respiratory chain complex IV [cytochrome c oxidase (COX)] in some active lesions (Mahad DJ, 2009). Furthermore, a metabolomic study has revealed changes in MS patient serum levels of different metabolites such as glutamine and glutamate and oxidants/antioxidants unbalance compared to healthy controls. Glutamate concentration correlated with disease severity and glutamine is critically required for naive $CD4^+$ T cell differentiation toward Th1 and Th17 pro-inflammatory T cells (Tavazzi B, 2011; Nakaya M, 2014). Studies on MS patients carried by Matarese's group also revealed metabolic alterations (De Rosa V, 2015). A recent study from the same group shows how glycolysis is required for the expression of FOXP3, a master regulator in the development and suppressive function of regulatory T cells. MS subjects display impaired rates of glycolysis, FOXP3 expression deficiency that in turn determines altered peripheral Treg generation and function (De Rosa V, 2015). Conversely, other authors found increased glucose metabolism in the cerebrospinal fluid (CSF) as well as in the serum of MS patients with higher levels of lactate as compared to healthy controls. These parameters positively correlated and associated with disease progression and activity (Albanese M, 2016; Regenold WT, 2008; Amorini AM, 2014). Metabolic dysregulation in RA will be discussed in the *paragraph 2.5.3.*

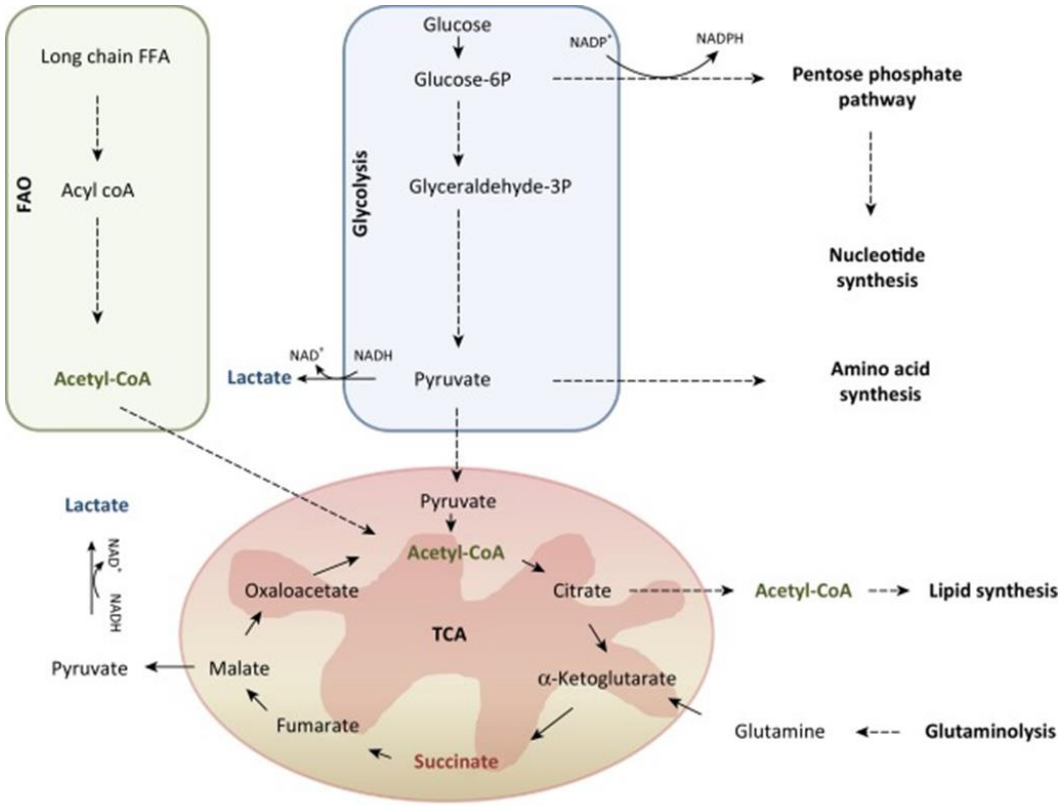


Figure VI - Metabolic pathways. Immune cells, i.e. T cells, use metabolic pathways [glycolysis, blue; tricarboxylic acid (TCA) cycle, red; fatty acid oxidation (FAO), green] in order to produce energy for their effector functions. Metabolic intermediates such as lactate and succinate are also produced. These are substrates for anabolic processes including lipid and nucleotide synthesis, but can also act as regulatory signalling molecules (Adapted from: Haas R, 2016).

2.4.2 *Translational immunometabolism: metabolic comorbidities in autoimmune/rheumatic disorders*

Alteration of metabolic pathways in immune cells may in part explain the increase rate of metabolic complications in patients affected by autoimmune/rheumatic disorders especially cardiovascular diseases (CVD) and metabolic syndrome (MetS) compared to the healthy population. These events have a great effect on mortality (Medina G, 2018; Nurmohamed MT, 2015).

The pathogenic factors involved are not yet fully understood. Metabolic syndrome (MetS), which includes insulin resistance, central obesity, dyslipidemia, and hypertension, may provide a link between accelerated atherosclerosis and inflammation in these diseases. Oxidative stress markers, thrombogenic or pro-inflammatory mediators released during the course of autoimmune rheumatic diseases may play a key role in the development in atherosclerosis, or metabolic disturbances frequently observed in these patients (Medina G, 2018; Nurmohamed MT, 2015). On the other side, pro-inflammatory molecules release by adipose tissue in the course of obesity can also potentiate the risk and the poor prognosis of rheumatic disorders (Nurmohamed MT, 2018; Procaccini C, 2015).

Obesity is linked to an increased risk of osteoarthritis (OA). Although mechanical factors can promote OA progression, the primary risk factors for OA onset are supposed to be body mass index (BMI) and fat tissue mass (Francisco V, 2018). Cytokines and adipokines, such as leptin, released by the adipose tissue have a pivotal role in the joint destruction in OA (Rosenbaum M 1996; J Ku JH, 2009).

A link between SLE and MetS also exists (Katz P, 2011). The results are somehow controversial. In renal lupus, active inflammatory disease and damage are SLE-related factors that drive MetS development. The use of antimalarial agents appeared to be

protective in MetS development if started at early onset of the disease (Parker B, 2015). In another study, the frequency of MS was not significantly different from that of the control group, but almost half of the patients were found with this syndrome (Mobini M, 2018). High incidence and variability of CVD risk factors was found in female lupus patients (Monção CSA, 2018).

Ankylosing spondylitis (AS) has been associated with a risk of MetS. It has been reported that AS patients have higher risk of MetS and CVDs than in healthy controls. In particular, systolic blood pressure, triglyceride and LDL cholesterol were found significantly higher in AS pts than in controls; on the contrary, HDL cholesterol was lower (Malesci D, 2017). Overall MetS was found in higher in AS than controls, even after receiving anti-tumour necrosis factor (anti-TNF) therapy (Papadakis JA, 2009). Similar result were found in psoriatic arthritis patients (Haroon M, 2016).

Several features of MetS, such as dyslipidemia, diabetes, hyperuricemia have also been reported, with higher prevalence in Sjögren's syndrome. Hypertriglyceridemia and diabetes were associated with a higher prevalence of extraglandular features, especially renal, liver, and vasculitic involvement (Ramos-Casals M, 2007).

MetS has been shown in ANCA-associated vasculitis (AAV). MetS is associated with a more pro-inflammatory state in AAV and might increase the risk of developing a relapse of AAV (Petermann Smits DR, 2013).

Finally, CVD is reported to be the primary cause of death in RA, which higher prevalence in RA than in the general population (Turesson C, 2004; Liang KP, 2010). High risk of metabolic syndrome has also been detected (Hallajzadeh J, 2017) thus suggesting that a metabolic dysregulation is present in these patients as discussed in the following *section 2.5*.

2.5 Rheumatoid arthritis

RA is an autoimmune disease characterized by chronic synovial inflammation and hyperplasia ("swelling"), autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction ("deformity"), and systemic complications such as cardiovascular, pulmonary, psychological, and skeletal disorders. Progressive disability, systemic complications and early death are still a reality leading to socioeconomic costs and several unmet needs. Indeed, current conventional and biologic disease modifying therapies sometimes fail or produce only partial responses. Reliable predictive biomarkers of prognosis, therapeutic response, and toxicity are lacking (McInnes IB, 2011; Dennis GJr, 2014).

Rheumatoid arthritis is a multifactorial disease involving a complex interplay among genotype and environmental triggers. Twin studies implicate genetic factors in rheumatoid arthritis, with concordance rates of 15 to 30% among monozygotic twins and 5% among dizygotic twins. Patients who are positive for rheumatoid factor or ACPA also show an association with the human leukocyte antigen (HLA)–DRB1 locus. ACPA, are often detected in patients before the development of arthritis (Rantapää-Dahlqvist S, 2003).

Infectious agents (e.g., Epstein–Barr virus, cytomegalovirus, proteus species, and Escherichia coli) and their products (e.g., heat-shock proteins) as well as periodontal disease due to Porphyromonas gingivalis have been linked with rheumatoid arthritis (Kamphuis S, 2005; Sato K, 2017).

Moreover, the gastrointestinal microbiome is now recognized to influence autoimmunity in mouse models of arthritis (Horta-Baas G, 2017). In this context, gut commensal segmented filamentous bacterium (SFB) was shown to have a role in promoting Th17

development and functions and promote autoimmunity in K/BxN mouse model of arthritis (Bradley CP, 2017).

Why the loss of tolerance is associated with the onset of inflammation in the joint is still unclear (McInnes IB, 2011).

RA synovitis is characterized by leukocyte infiltration in the synovial compartment and neo-angiogenesis due to the hypoxic environment. The increasing expression on vessels of adhesion molecules (including integrins, selectins, and members of the immunoglobulin superfamily) and chemokines enables cells to migrate in the inflamed site. Here, the insufficient lymphangiogenesis, which limits cellular egress, cytokines and local fibroblast activation, allows the buildup of synovial inflammation (Szekanecz Z, 2009; Polzer K, 2008).

There are several key players contributing to the arthritis pathobiology. T cells play a pivotal role in the pathogenesis of rheumatoid arthritis however direct targeting of T cells i.e. cyclosporine or T-cell–depleting therapeutics as well as abatacept (a fusion protein containing cytotoxic T-lymphocyte–associated antigen 4 and the FC fragment of IgG1 to disrupt antigen presentation by blocking T-cell costimulation) has shown limited or no efficacy (Panayi GS, 2006).

There is an increasing interest in the role of type 17 helper T cells (Th17), a T cell subset that produces interleukin 17A, 17F, 21, and 22 and TNFα (Chabaud M, 1998; Miossec P, 2009). This pro-inflammatory milieu sustains Th17 differentiation and suppresses differentiation and functionality of regulatory T cells, thus leading to activation of tissue-resident fibroblasts and chondrocytes leading to bone and cartilage destruction (Genovese MC, 2010).

Humoral adaptive immunity also displays an important role in rheumatoid arthritis. Synovial B cells are mainly localized in T-cell–B-cell aggregates; indeed, some tissues

develop ectopic lymphoid follicles (Seyler TM, 2005) whose function is still under investigation.

2.5.1 *Ectopic lymphoid structures*

Lymphoid tissues are classified into primary and secondary lymphoid organs. Primary lymphoid organs include the bone marrow and the thymus and are primarily responsible for the selection of naive B and T lymphocytes bearing highly diverse B and T cell receptors in their germline sequences. Secondary lymphoid organs (lymph nodes, spleen, mucosal-associated lymphoid tissues such as Peyer's patches) regulate the recirculation of leukocytes and are critical for mounting high-affinity adaptive immune responses upon antigenic challenge (Pitzalis C, 2013; Pitzalis C, 2014).

Tertiary lymphoid organs are defined as clusters of lymphomonocytic cells that form at sites of chronic inflammation. Hence, they are also known as ectopic lymphoid structures (ELSs) and they can acquire phenotypic features that are characteristic of secondary lymphoid organs (Humby F, 2009).

Indeed, these structures show T and B cell segregation (B cell follicles surrounded by T cell rich areas). Furthermore, vascular structures with the appearance of high endothelial venules (HEVs) develop, and networks of stromal-derived follicular dendritic cells (FDCs) differentiate; these FDCs express the long isoform of complement receptor type 2 (Cr2, also known as CD21), which contributes to the presentation of immune complexes necessary to generate activated B cells. Plasmablasts and plasma cells subsequently accumulate around the follicles of T cells and B cells. A considerable proportion of ELSs also display functional features of ectopic germinal centers, such as expression of the enzyme activation-induced cytidine deaminase (AID), also known as single-stranded

DNA cytosine deaminase and evidence of both in situ B cell affinity maturation and clonal selection (Bombardieri M, 2017).

Immune cells recruited to the site of inflammation, in cross-talk with resident cells which are tissue-specific, exert an active role in the initiation of ELS development via the release of several chemokines such as CXCL13, CCL19, CCL21, and CXCL12 and their receptors CXCR5 (for CXCL13), CCR7 (for CCL19 and CCL21), and CXCR4 (for CXCL12) (**Figure VII**, Corsiero E, 2016).

Cytokines such as IL-17, IL-21, IL-22, IL-23, and TNF, produced at the site of the inflammation, are also directly involved in ELSs formation (**Figure VII**, Jones GW, 2016). In particular, IL-17 produced by a subset of podoplanin-expressing CD4 T cells has been associated with ELS formation in animal models of inducible ELS and the IL-23/IL-17 axis has been recently linked with ELS formation in RA (Cañete JD, 2015). Another cytokine involved in ELSs formation is IL-22. Indeed, it was recently found that IL-22 is able to induce CXCL-13 by a subset of resident stromal cells expressing gp38 (Barone F, 2015).

Among CD4 T cell subsets, Tfh play a pivotal role in the germinal centre reaction, regulating B cell activation, antibody affinity maturation, and the expression of surface receptors such as inducible T-cell costimulator (ICOS) and programed cell death protein 1 (PD1). They also express the transcription factor B cell lymphoma protein 6 (BCL6), which promotes the expression of CXC-R5 and represses other T-cell subset-specific transcription factors (Shulman Z, 2014; Liu D, 2015). IL-21 is the main cytokine released by Tfh. On B cells, IL-21/IL-21R interactions provide potent signalling for B-cell survival, proliferation, and differentiation (Zotos D, 2010). In addition, IL-21 has been shown to play a fundamental role in the function of ELSs formation (Linterman MA, 2010).

Opposite to IL-17, IL-21 and IL-22, IL-27 exerts a negative regulation on ELSs formation. Indeed, it has been recently shown that IL27Rα−/− mice developed a more severe form of arthritis after immunization and multiple lymphoid aggregates forming in the inflamed synovial tissue (Jones GW, 2015). IL-27 can also restrict the expansion of Th17 cells and suppress secretion of IL-17, a cytokine associated also with survival and proliferation of B cells (Stumhofer JS, 2006; Jones GW, 2015). Concerning ELS development in human synovium, it has been observed that IL27 is inversely correlated with the degree of lymphocytic infiltration in the inflamed tissue as well as with the expression of IL17 and IL21 at mRNA level (Jones GW, 2015). Conversely a paper published by Batten et al who found that IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells (Batten M, 2010).

The formation of ELSs has been described in organ-specific autoimmune conditions, during solid tumourigenesis, in chronic infections and in graft rejection (Bombardieri M, 2017).

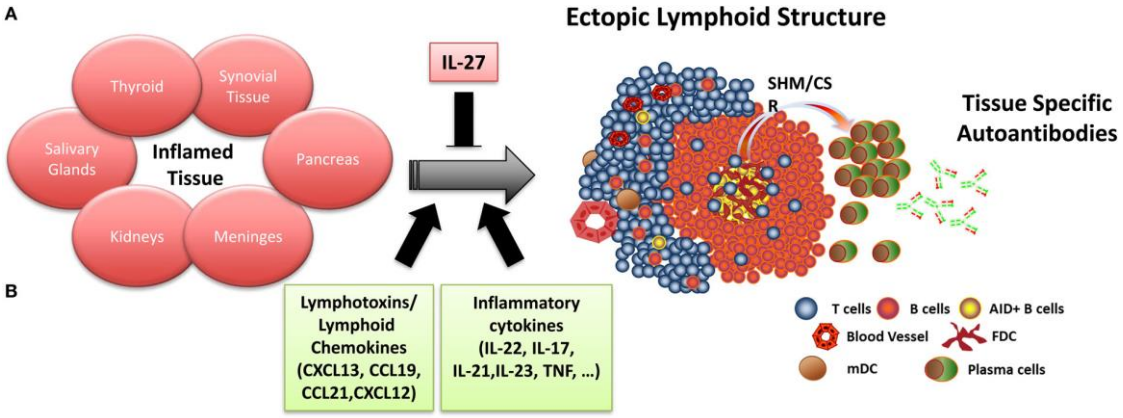
ELSs have been extensively studied in the synovium of patients with RA, salivary glands of patients with Sjögren syndrome as well as in the kidneys of patients with SLE and in the muscle of patients with dermatomyositis or polymyositis (Bombardieri M, 2017).

In RA, the activation of resident synovial cells and the synovial microvasculature, together with the infiltration of immune cells from the peripheral pool, leads to chronic proliferative synovitis with the formation of the so called pannus, which directly damages cartilage and subchondral bone (McInnes IB, 2011). Advances in our understanding of the complex heterogeneity of the histopathology and the underlying molecular signatures have enabled the definition of at least three microstructural levels of organization (pathotypes) of synovial tissue in RA: follicular synovitis with the presence of aggregates of B cells and T cells that form ELSs (lymphoid pathotype, 40% of RA patients); a

diffuse pattern of infiltration with prevalent monocyte–macrophage infiltration (myeloid pathotype, 40% of RA patients); and a pauci-immune synovitis with scarce or no immune cell infiltration (fibroid pathotype, 20% of RA patients) (**Figure VIII**, Klimiuk PA, 1997; Manzo A, 2005; Dennis GJr, 2014; Pitzalis C, 2013; Takemura S, 2001).

Although ELSs primarily develop within the sub-lining of the synovial tissue in patients with RA, they can also be detected at extra-articular sites (i.e. the lung and bone marrow) (Bugatti S, 2005; Rangel-Moreno J, 2006). ELSs in RA are characterized by the formation, by T cells and B cells, of discrete perivascular clusters, which can also be graded (G1, G2 or G3) according to the aggregate radial cell count (a central blood vessel surrounded by cell layers; Yanni G, 1992; Thurlings RM, 2008; Vos K, 2007). It is now generally accepted that ELSs form in approximately 40% of patients with RA, but this prevalence varies considerably depending on the site of the biopsy, the sampling technique (joint replacement versus arthroscopy versus ultrasound-guided biopsy), the stage of the disease, the treatments received by an individual patient, and the staining and scoring procedure (Manzo A, 2010).

Taken together there is clear evidence that, in autoimmune diseases, ELS directly contribute to the maintenance of the immune response at the site of inflammation, hence participating in the local development of autoimmunity and tissue damage. The recognition that ELS development may play a key pathogenic role in autoimmunity and may be exploited as potential biomarker for disease evolution and response to therapy is gaining much attention (Pitzalis C, 2013).



Disease Specific Autoantigens	Disease/Tissue-related ELS
Citrullinated proteins	Rheumatoid Arthritis/Synovial Tissue
Ribonucleoproteins Ro/SSA and La/SSB	Sjogren's syndrome/Salivary Glands
Thyroglobulin/Thyroperoxidase	Hashimoto's thyroiditis/Thyroids
Insulin ?	Type 1 Diabetis/Pancreas
Acetylcholine receptor	Myasthenia Gravis/Thymus
Double stranded DNA	Systemic Lupus Erythematosus/Kidneys
?	Multiple sclerosis/Meninges

Figure VII - Ectopic lymphoid structure (ELS) in autoimmune diseases. (A) Chemokines and cytokines involved in the formation and maintenance of ELS where the affinity maturation and clonal selection of autoantibodies toward specific autoantigens takes place. (B) Autoimmune diseases and the specific autoantigenes found associated with ELS formation (Adapted from: Corsiero E, 2015).

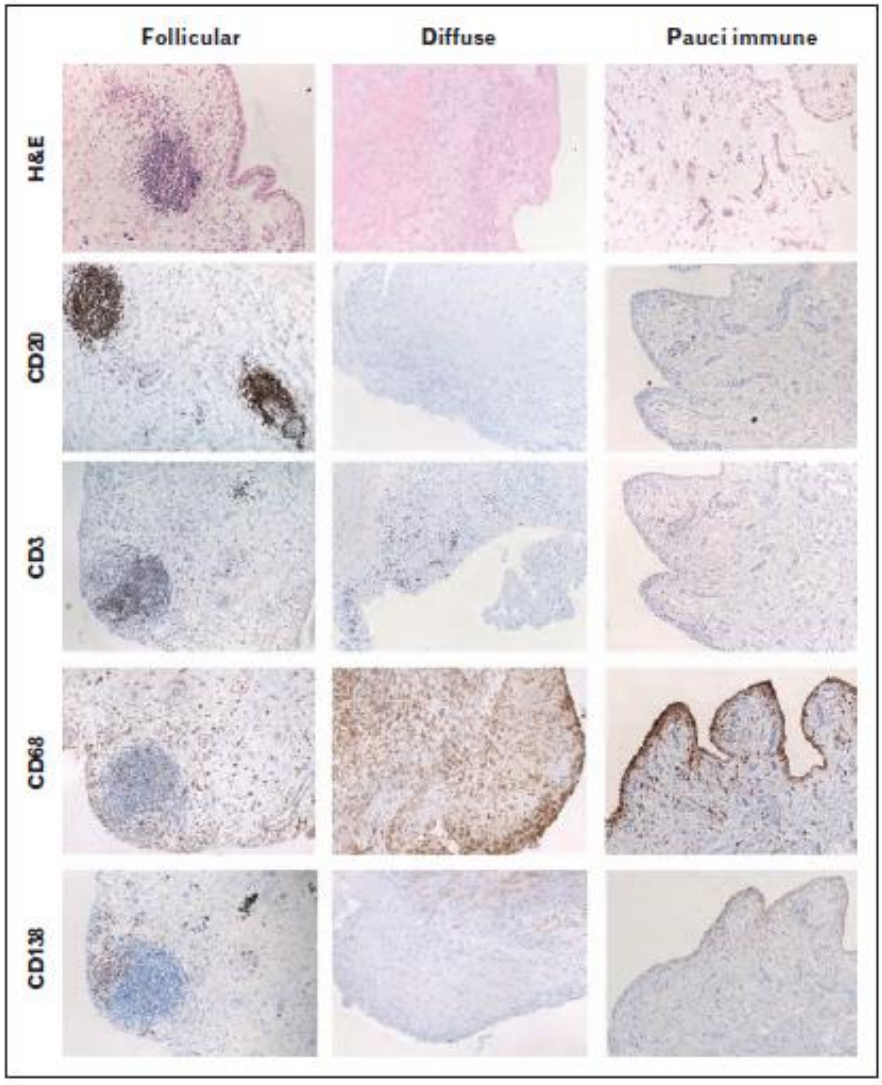


Figure VIII - Histological pattern of RA synovitis. Histomorphological features of RA synovitis. Three mm sections of paraffin embedded RA synovial tissues were stained with haematoxylin and eosin (H&E) and by immunohistochemisty (IHC) for B cells (CD20), T cells (CD3), macrophages (CD68) and plasma cells (CD138) (Adapted from: Pitzalis C, 2013).

2.5.2 *Diagnosis of RA*

RA has a wide clinical spectrum ranging from mild joint symptoms to severe inflammation and joint erosion with systemic comorbidities which include include cardiovascular disease, osteoporosis, interstitial lung disease, infection, cancer, fatigue, depression, mental difficulties, and trouble working. The first classification criteria was proposed by the American College of Rheumatology (ACR) in 1956 and then revised in 1958 and 1987, and more recently a new classification criteria has been developed by the ACR and the European League Against Rheumatism (EULAR) in 2010 (**Table 4**, Aletha D, 2010). For classification, diagnosis and treatments purposes, a patient with a score ≥ 6 is indicative of the presence of definite RA. This classification, compared to the earlier ACR 1987, includes also autoantibody status and acute phase response, showing a higher sensitivity but also a lower specificity, especially in patients aged ≥ 60 years (Berglin E, 2013). Once the diagnosis of RA is given, patients start treatment with conventional DMARDs such as metothrexate and/or sulphasalazine and/or hydroxychloroquine and/or corticosteroids.

Disease Activity Score of 28 joints (DAS28) is widely used as an indicator of RA disease activity and response to treatment (Fransen J, 2009). Joints included are (bilaterally): proximal interphalangeal joints (10 joints), metacarpophalangeal joints (10), wrists (2), elbows (2), shoulders (2) and knees. When looking at these joints, both the number of joints with tenderness upon touching (TEN28) and swelling (SW28) are counted. The erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) are measured and the affected person makes a subjective assessment of disease activity on a scale between 0 and 100, where 0 is "no activity" and 100 is "highest activity possible" (Fransen J, 2009). Based on DAS28 a poor, moderate or good response to treatment is calculated with severe disease activity defined as a DAS28 of

> 5.1) and moderate disease activity (defined as a DAS28 of 3.2 to ≤ 5.1) (**Table 5**, Fransen J, 2009). The aim of the treatment is to reach a DAS28 ≤ 3.2, which is considered low disease activity and the therapy can be escalated accordingly. Patients with persistent high disease activity (DAS28 > 5.1) at 6 months from the disgnosis are commenced on biologic DMARDs according to National Institute for Clinical Excellemce (NICE) prescribing guidelines (https: //www.nice.org.uk/Recommendations/treat-to-target-strategy). Biologic DMARDs include etanercept, adalimumab, infliximab, certolizumab pegol, and golimumab, which are all part of a class of drugs called tumour necrosis factor (TNF) inhibitors. Biologic DMARDs also include other agents with different targets, suc as anakinra (anti IL1 receptor), abatacept (fusion protein composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA4), rituximab (anti CD20), and tocilizumab (anti IL6). Another group of DMARDs, called kinase inhibitors, includes tofacitinib. A biologic DMARD or a kinase inhibitor is often combined with methotrexate or other DMARDs to improve efficacy.

Although the outcome and the obvious success of the current therapeutic approaches has significantly improved in recent years, RA remains a condition with large unmet clinical needs. Clinical studies have shown that achievement of treatment-free remission can be reached only by 9.4-15% of patients (van der Woude D, 2009). Additionally, 20-40% of the patients do not respond to the available therapeutics (Kremer JM, 2001; Taylor PC, 2009).

Table 4 - 2010 ACR/EULAR classification criteria for rheumatoid arthritis

	Score
Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
Serology (at least 1 test result is needed for classification)	
Negative rheumatoid factor (RF) and anti citrullinated protein antibody (ACPA)	0
Low-positive RF or low-positive ACPA	2
High-positive RF or High-positive ACPA	3
Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

Table 5 - EULAR response criteria based on DAS28

Current DAS28	DAS28 decrease from initial value		
	> 1.2	> 0.6 and ≤ 1.2	≤ 0.6
≤ 3.2	Good response	Moderate response	No response
> 3.2 and ≤ 5.1	Moderate response	Moderate response	No response
> 5.1	Moderate response	No response	No response

2.5.3 *Metabolic dysregulation in RA*

Metabolic alterations have been observed in several autoimmune diseases (Pucino V, 2017; Cantarini L, 2017). A new field of study that investigates the interface and the link among immune response, nutrition, and metabolism has recently developed and many of the interactions between metabolic pathways and the immune system seem to be orchestrated by a complex network of soluble mediators derived from immune cells and adipocytes such as leptin and adiponectin (Procaccini C, 2015). An increasing number of human studies showed that adipocytokines could play a key role in the pathogenesis of RA but the results are still controversial.

In this context, leptin, at the crossroad between metabolism and immunity, has been identified as a potential trigger for the breakdown of immune self-tolerance. Indeed, leptin is able to induce cell activation, pro-inflammatory cytokine secretion as well as production of autoantibodies and angiogenesis leading to autoimmunity and chronic inflammation (Procaccini C, 2015; Pucino V, 2014). In addition to leptin, other neuroendocrine factors are involved in the pathogenesis of autoimmune disorders (Procaccini C, 2014).

Studies on RA patients have shown metabolic pathway alterations in these patients. Interestingly, some metabolism related enzymes such as enolase, aldolase and glucose-6-phosphate isomerase have been described as autoantigens promoting autoimmunity in RA (Chang X, 2011).

Proliferating cells, including lymphocytes, use aerobic glycolysis (Warburg effect) to quickly generate energy in the form of ATP. In particular, aerobic glycolysis is preferred over oxidative phosphorylation for its expedited production of energy and for the generation of necessary building materials to accommodate the increasing cell mass (Tsokos GC, 2016).

RA naive $CD4^+$ T cells display a deficit in starting glycolysis in comparison to healthy T cells. In particular, these cells have a deficiency of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), a glycolytic regulator enzyme. This leads to a state of energy deprivation and a tendency towards senescence and defective autophagy. This glycolytic delay prompts RA $CD4^+$ T cells to up-regulate glucose-6-phosphate dehydrogenase (G6PD) and shift their metabolic pathway towards the pentose phosphate pathway (PPP). These events lead to in the production of high amounts of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), ROS consumption and altered activation of ataxia telangiectasia mutated (ATM), a key enzyme involved in cell cycle. All these alterations result in a high cellular proliferation, a switch toward pro-inflammatory $CD4^+$ T cell subsets such as Th1 and Th17 and chronic inflammation (Yang Z, 2013; Yang Z, 2016). These abnormalities were reversible via replenishment of the ROS pool with the naphthoquinone menadione, inhibition of the synthesis of the ROS quencher glutathione, or blockade of glucose shunting into the PPP (Yang Z, 2013; Yang Z, 2016).

ATP^{low} $pyruvate^{low}$ $NADPH^{high}$ RA $CD4^+$ T cells also display an excess synthesis of FAs (fatty acids) and cytoplasmic lipid droplet (LD) deposition. This resulted in an upregulated T cell locomotion program, high production of the podosome scaffolding protein TKS5, and ultimately a functional phenotype of hyper-motility.

$TKS5^{hi}$ RA T cells spontaneously formed actin- and cortactin-rich membrane ruffles, which empowered them to penetrate into non-lymphoid tissue and establish lasting inflammatory infiltrates. All these effects were restored after FA synthesis inhibitor treatment (Shen Y, 2017).

FAS is essential for Th17 differentiation and functions in contrast of Treg cells, which rely on FA oxidation. Pharmacologic inhibition of acetyl-CoA carboxylase (ACC), a crucial FAS enzyme, was able to delay the disease and to reduce the severity of

experimental autoimmune encephalomyelitis (EAE). This suggests a potential role for FAS targets for immunomodulation (Berod L, 2014).

If RA T cells display a deficit of glycolyis, synovial RA fibroblasts develop an intrinsic ability to employ glycolytic metabolism under metabolic stress. Indeed, glucose deprivation or glycolytic inhibitors such as such as 2-deoxy-D-glucose (2-DG), bromopyruvate (BrPa) and 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one, impaired synovial fibroblast cytokine secretion, proliferation, and migration as well as the disease severity in a mouse model of arthritis (Garcia-Carbonell R, 2010). In line with this evidence, the inducible isoform of hexokinase, HK2, which catalyzes the phosphorylation of glucose to G6P, the first committed step in glucose metabolism, was found highly expressed by RA synovial FLS in comparison to OA. Interestingly, after HK2 silencing, RA FLS were less invasive while the overexpression of HK2 enhanced the levels of MMP, IL6, and IL8 other then their migratory rate (Bustamante MF, 2018). These data were further confirmed *in vivo* in a mouse model of arthritis. The authors showed that the HK2 deletion in murine FLS ameliorates disease severity of arthritis. These data suggest a novel potential therapeutic target for RA (Bustamante MF, 2018). Altered metabolism in RA fibroblasts has also been associated to the hypoxic microenvironment typical of the inflamed sites. Specifically, it was found that hypoxia induced a downregulation of mitochondrial respiration and an increase of glycolysis in RA fibroblasts, which in turn promoted synovial invasive mechanisms (Biniecka M, 2016). This should support abnormal angiogenesis, cellular invasion and pannus formation (Biniecka M, 2014; Biniecka M, 2016). Altered mitochondrial respiration correlated with higher mtDNA mutations and ROS in RA compared to OA FLS (Da Sylva TR, 2005), and with elevated matrix metalloproteinase (MMP) expression and an invasive phenotype (Harty LC, 2012). Another study, this time on macrophages, showed that RA mitochondria consume more oxygen, generated more ATP than healthy macrophages. This is due to

interorganelle connections with the endoplasmic reticulum, forming mitochondria-associated membranes (MAM), which consist of some proteins and a region on ER containing lipid biosynthetic enzyme connected reversibly to mitochondria. This leads to 1) the calcium transfer through MAM sites which was able to sustain mitochondrial hyperactivity, and 2) to the inactivation of glycogen synthase kinase 3b (GSK3b), a serine/threonine kinase functioning as a metabolic switch.

Furthermore, MAM formation and GSK3b inactivation sustained production of the collagenase cathepsin K, a macrophage effector function closely correlated with clinical disease activity in RA (Zeisbrich M, 2018).

2.5.4 *Metabolites in RA*

The study of metabolic intermediates is becoming a new emerging field. More than intermediate bystanders, succinate, lactate, fumarate etc. function as signalling molecule to link metabolic reprograming with immune and inflammatory responses in RA and other conditions. A gas chromatography–mass spectrometry (GC–MS) study, carried on RA sera samples, showed perturbations in different bioenergetic pathways such as glycolysis, fatty acid and amino acid metabolism and TCA cycle. In particular they found decreased levels of several amino acids (leucine, phenylalanine, pyroglutamate, serine, isoleucine, methionine, threonine, proline and valine) and glucose alongside with increased levels of fatty acids such as palmitelaidate, oleate, trans-9-octadecenoate,cis-5,8,11-eicosatrienoate, docosahexaenoate, 2-ketoisocaproateand 3-methyl-2-oxovalerate and cholesterol (Zhou J, 2016). On the same line, another very recent study describes a metabolite signature in serum that correlate with gene expression profiling in synovial tissue from patients with active RA. Specifically the authors observed an association of

serine/glycine/phenylalanine metabolism and aminoacyl-tRNA biosynthesis with lymphoid cell gene signature. Alanine/aspartate/glutamate metabolism and choline-derived metabolites correlated with TNFα synovial expression. Circulating ketone bodies were associated with gene expression of synovial metalloproteinases. These data pointed out the relationship between serum metabolite profiles and synovial biomarker profiling suggesting that the 1H-nuclear magnetic resonance (NMR) may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium of patients with RA (Narasimhan R, 2018).

In vitro studies further highlighted the role of metabolites as signalling molecules. Studies on succinate have shown that LPS-activated inflammatory (M1) macrophages present an intracellular accumulation of this metabolite due to an altered Krebs cycle (Jha AK, 2015). Furthermore, intracellular succinate promoted the stabilization of HIF1α and enhanced pro-inflammatory IL-1β production (Tannahill GM, 2013). In addition, when activated by inflammatory stimuli, macrophages release succinate into the extracellular milieu and simultaneously up-regulate Gpr91, which functions as an autocrine and paracrine sensor for extracellular succinate to enhance IL-1β production. Gpr91-deficient mice lack this metabolic sensor and show reduced macrophage activation and production of IL1β during antigen-induced arthritis. Succinate is abundant in synovial fluids from RA patients, and these fluids elicit IL-1β release from macrophages in a Gpr91-dependent manner. In this paper, the authors propose a model of Gpr91/succinate-dependent feed-forward loop of macrophage activation and suggest Gpr91 antagonists as novel therapeutic principles to treat RA (Littlewood-Evans A, 2016). Notably, Sucnr1−/− knockout mice display a reduced arthritis by reducing dendritic cell traffic and expansion of Th17 cells in the lymph nodes (Saraiva AL, 2018).

On the same line, it was found that intracellular succinate induced angiogenesis through HIF1α induction, while extracellular succinate acted on Gpr91 activation, working

together to disturb energy metabolism and exacerbate inflammation and angiogenesis in arthritis synovium. Suppression of SDH could prevent succinate accumulation and inhibit angiogenesis via blocking HIF1α/VEGF axis. This finding not only provides a novel insight into angiogenesis, but also reveals a potential therapeutic strategy to attenuate revascularization in arthritis (Li Y, 2018).

If succinate exhibits pro-inflammatory activity, other metabolites such as fumarate and itaconate were found to have an anti-inflammatory function. With regard to fumarate, the methyl ester dimethyl fumarate (DMF) that is already approved for the treatment for the relapsing MS, inhibited RANKL-mediated osteoclastogenesis and bone destruction *via* induction of NRF2-mediated transcription of antioxidant genes and consequent decrease in intracellular ROS levels (Yamaguchi Y, 2018).

3 RATIONALE AND AIMS

Inflammatory sites, including RA synovitis, are characterized by the accumulation of lactate, which is partly responsible for their acidity. For many years, lactate has been considered a "waste" glycolytic metabolite; only recently, lactate has been recognized as immunomodulatory molecule.

In particular, lactate can affect $CD4^+$ T cell migration and function, via the SLC5A12 lactate transporter, leading to and perpetuating chronic inflammation. The project aimed at characterizing whether the lactate/SLC5A12-induced metabolic signalling pathway modulates inflammatory immune responses. In particular, I assessed the impact of this signalling pathway *in vitro* and *ex vivo*, in healthy and RA $CD4^+$ T cells, in RA tissues and *in vivo* in a murine model of arthritis.

Based on previous evidence, I further elucidated the underlying mechanism through which lactate ties metabolic reprogramming to the modulation of T cell functions (IL-17A production and T cell entrapment in the inflammatory sites).

These findings can open new therapeutic perspectives for the treatment of chronic inflammatory diseases.

4 MATERIALS AND METHODS

4.1 Patients

Blood, synovial fluid and synovial tissues were obtained from RA patients diagnosed according to the revised American College of Rheumatology (ACR) criteria (Aletaha D, 2010). Demographic and clinical characteristics are presented in **Table 6** (**Appendix**). Healthy controls (HC) were recruited through the NHS blood and transplant service. Individuals with cancer, infections or other inflammatory comorbidities were excluded. Written informed consent was obtained by all participants, HC and RA, according to ethics approval from National Research Ethics Service Committee London (EMR Biobank, LREC07/Q0605/129).

For messenger RNA (mRNA) sequencing, mRNA was extracted from synovial tissue samples obtained by ultrasound-guided biopsy from patients with early active RA (< 12 months' duration) who were naïve-to treatment with disease modifying antirheumatic drugs (DMARDs) (n = 87). Patients were enrolled in the Pathobiology of Early Arthritis Cohort (PEAC, details at http://www.peac-mrc.mds.qmul.ac.uk) at the Centre for Experimental Medicine and Rheumatology of Queen Mary University (REC 05/Q0703/198, London, UK). All patients belonging to this cohort underwent to a baseline ultrasound-guided biopsy on the most inflamed accessible joint as previously described (Kelly S, 2015); synovial fluid and blood were also collected. Afterward, patients started treatment with conventional DMARDs (methotrexate, leflunomide and/or sulphasalazine and/or hydroxychloroquine and/or corticosteroids). The response to treatment was evaluated at six months according to DAS28-CRP (*see paragraph 2.5.2*).

4.2 T cell isolation and *in vitro* activation

Peripheral blood mononuclear cells (PBMCs) from HC and paired PBMCs and synovial fluid mononuclear cells (SFMCs) were obtained from RA patients. PBMCs and SFMCs were isolated by density gradient centrifugation [lymphoprep (GE Healthcare) and histopaque H-1070 (Sigma-Aldrich) respectively].

Cell were counted using a Neubauer counting chamber under light microscopy; cells were pre-diluted in RPMI 1640, then 1:1 in trypan blue (a cell viability dye that stains nuclei of dead cells). The number of cells was determined using the following formula: $N = M \times D \times 10^4$ where N = number of cells, M = number of cells in the grid, D = diluition factor.

Cells (2×10^6/mL) were cultured in 48-well flat-bottomed plates in medium (RPMI 1640, ThernoFisher) supplemented with 100 U/mL penicillin/streptomycin (Invitrogen), 10% FBS (ThernoFisher) or 10% autologous blood serum or 10% RA synovial fluid. Cells were then stimulated, or not with 0.2 μg/mL anti-CD3 monoclonal antibody (CD3 mAb, eBioscience) in order to achieve an efficient T cell activation. I did not use CD28 mAb for the activation of T cells when using the whole PBMCs or MCs since the co-stimulation for T cell activation is genearally given by other APC cells such as B cells, DC and monocytes (Kurrle R, 1986).

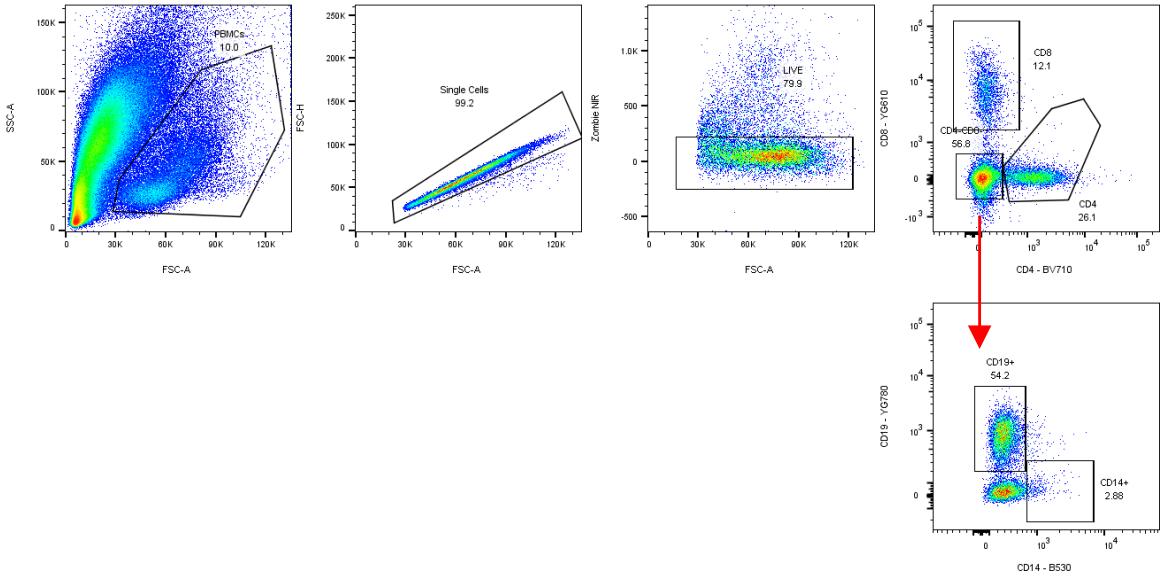
For the experiments on human tonsils, tissues were mashed through a cell strainer and mononuclear cells (MCs) were isolated. Cells were washed twice with RPMI 1640 and re-suspended in pre-warmed RPMI 1640 plus 10% FBS and activated or not with 0.2 μg/mL CD3 mAb (eBioscience).

$CD4^+$ T cells were purified from freshly isolated PBMCs and MCs by negative selection using a magnetic cell separation (EasySep, Stem Cell Technology) and then stimulated for 3 days in the presence of anti-CD3 and anti-CD28 mAbs coated dynabeads (0.1 beads

per cell; Invitrogen). For cytokine detection in the final 4 hours of culture, cells were treated with 50 ng/mL PMA and 500 ng/mL ionomycin and 1:1.000 brefeldin A (Sigma-Aldrich), followed by surface staining for CD4 (RPA-T4, Biolegend).

4.3 Flow cytometry

In order to assess SLC5A12 expression on different immune cell types, RA/HC PBMCs, RA SFMCs or tonsil MCs were initially stained with Live/Dead dye (1:1000, ZOMBIE/NIR, fixable viability dye, Biologend) for 15 minutes at room temperature protected from light to allow detection and removal of dead cells from the analysis. Without washing, cells were then stained with BV711–labeled anti–CD4 (1:100, Biologend), Pedazzle–labeled anti–CD8 (0.25:100, Biologend), FITC–labeled anti–CD14 (1:100, Biologend), PeCy7–labeled anti–CD19 (1:100, Biologend), (*see gating strategy below*). Rabbit SLC5A12 unconjugated primary antibody (0.4:100, HPA060904 - Atlas Antibodies) was added after fixing and permeabilizing cells (fixation-permeabilization buffer; eBioscience) for 30 min followed by AF555 goat anti-rabbit (1:1000, Invitrogen) secondary antibody. Rabbit IgG was used as isotype control (Dako). To assess the impact of SLC5A12 blockade on cytokine production and $CD4^+$ T cell subsets we stained tonsil MCs, treated with or without SLC5A12 Ab (48 hours), with BV711–labeled anti–CD4 (1:100), BV-450–labeled anti–PD1 (1:100) and PE/Cy5.5-labeled anti–CXCR5 (4:100). Thereafter, I washed, fixed, and permeabilized cells (fixation-permeabilization buffer; eBioscience) and stained with BV450-labeled anti-IL-17A, APC-labeled anti-FOXP3, PE-labeled anti-IL-10, FITC-labeled IFNγ. Intracellular staining was assessed by flow cytometry using a LSR Fortessa II (BD Biosciences) and FlowJo version 7.6.5 software. All monoclonal antibodies were from Biolegend.



Gating strategy for SLC5A12 expression by immune cells

Sequential gating strategy used for SLC5A12 expression by $CD4^+$, $CD8^+$ T cells and $CD19^+/CD14^+$ cells. $CD19^+/CD14^+$ cells were gated on $CD4^-CD8^-$ double negative cells.

4.4 Immunofluorescence and confocal microscopy

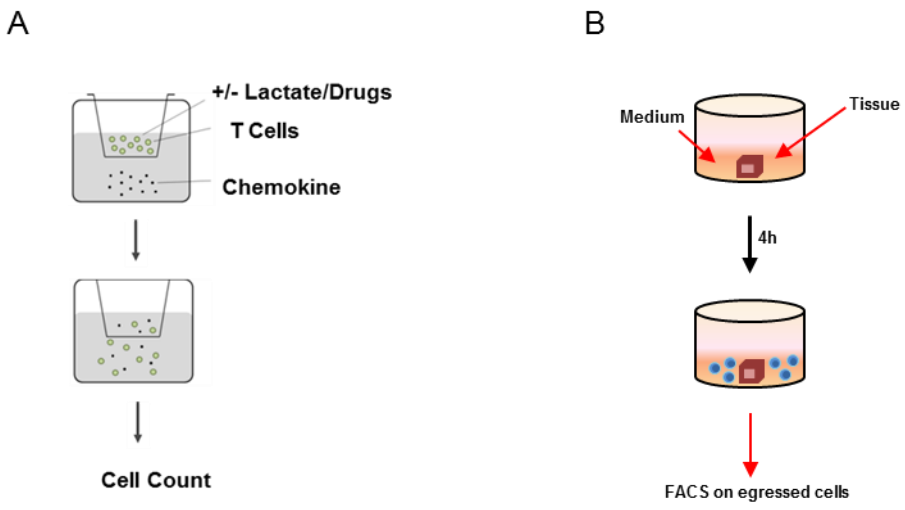
For SLC5A12 single and double (with CD4, CD8, CD20, CD68) immunofluorescence, after antigen retrieval (S2367, Dako) (45 minutes) and block of non-specific binding (1 hour), paraffin-embedded tonsil tissue sections were incubated for 1 hour with anti-SLC5A12 Ab (1:50, Novus Biologicals) and then overnight at 4°C with anti-CD4, anti-CD8, anti-CD20 or anti-CD68 (1:50, Dako). The day after were washed in PBS and incubated with fluorochrome-conjugated secondary antibodies (1:300, Invitrogen). The slides were washed in PBS for up to 5 minutes, mounted in fluorescence mounting medium (DakoCytomation) containing 1 μg/mL DAPI, and examined by Olympus IX81 fluorescence microscope. The list of primary and secondary antibodies is shown in **Table 6** (**Appendix**).

For the intracellular detection of HK2, human $CD4^+$ T cells cultured on glass coverslips were incubated for 5 minutes with 300 nM mitotracker deep red FM (Molecular Probes) at 37°C in 5% CO_2 atmosphere. After the incubation period, cells were washed twice and fixed/permeabilized in permeabilization/fixation buffer (eBioscience) overnight at 4°C. After washing with PBS, the cells were incubated with the primary antibodies against anti-HK2 (1:200, Cell Signalling Technology) for 1 hour at room temperature followed by 30 minutes incubation with 1:200 secondary Alexa Fluor 555 conjugated goat anti-rabbit (Invitrogen). Alexa Fluor 488 phalloidin (ThermoFisher) for the actin staining was also added at this stage. One million cells resuspeded in 100 uL PBS were counterstained with DAPI to detect nuclei, spun in the cytospin (250 rpm for 5 min) to allow the attachment to the coverslips and then mounted for microscopy. All images were acquired using a confocal microscope LSM880 (Zeiss).

4.5 Chemokinesis assays and tissue organ culture

Chemotaxis assays were performed in 7µm trans-well inlays (Corning). One h before the assay cells were incubated with sodium lactate (10 mM) and pre-treated or not (1 hour) with SLC5A12 polyclonal Ab (2.5 ug/mL, HPA060904 - Atlas antibodies) or monoclonal mAbs (1:50; SLC5A12 mAb clones: 3C7, 4G2, 6E1, 7C1, 9G4, 9G7 and 10E11) purified from sera of rat immunized with SLC5A12 recombinant peptide (*see paragraph 4.8*). In some experiments sodium lactate treated cells were pre-treated (2 hours) or not with metabolic drugs: C75 (10 uM), TOFA (20 uM), DHEA (20 uM), DASA (20 uM), AICAR (1 mM). In all the assays $3x10^5$ lymphocytes suspended in migration medium (RPMI 1640 2% FBS), were seeded in the upper trans-well chamber; CXCL10 chemokine (300 ng/mL) was added to the lower chamber. Migrated T cells were counted in cell counting chambers 4h after seeding, then percent of migrated cells was calculated (Schematic A).

For the analysis of egressed MCs, small pieces (1-3 mm^2) of tonsil or synovial tissue were placed in 48-well plate in RPMI 1640 10% FBS. The amount of cell egressed from the tissue in the cell culture supernatant was analyzed by FACS after 4 hours (Schematic B).



4.6 Molecular signalling and western blot analyses

For western blot analyses, protein were extracted by lysing $CD4^+$ T cells (2×10^6) in RIPA lysis buffer (65 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail tablets (#04693132001, Roche). Equivalent amounts of protein (30 µg of total), as determined by standard Bradford assay (Bio-Rad), were loaded, fractionated by SDS-PAGE and transferred to polyvinyildene difluoride membranes using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 minutes, membranes were washed once with TBST and incubated overnight at 4°C with a 1:1000 dilution of primary antibodies against pStat3, Stat3, pStat1, Stat1, PKM1/2, pACC, ACC, pAMPK, AMPK, HK1, HK2, Enolase 1 alpha, GCK, aldolase, acetyl-lysine, histone H3, VDAC (Cell Signalling Technology), PFK (Novus Biologicals), SLC5A12 (Sigma) and ß-Actin (Santa Cruz Biotechnology). Membranes were then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:2000). Blots were washed twice with TBST and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols. Density of bands was calculated via the use of ImageJ software.

4.7 Cell organelle fractionation

Fractionation of nuclear, mitochondrial and cytosolic extracts was performed by using nuclear extraction kit (Abcam) and mitochondria isolation kit for cultured cells (Thermo Scientific) according to the manufacturer's instructions. Cell compartments were enriched

according to the manufacturer's instructions. Here, 6-20x10^6 $CD4^+$ T cells per condition were subjected to a series of lysis and centrifugation steps to yield cytosolic, mitochondrial and nuclear fractions.

4.8 Measurement of extra- and intracellular metabolites and ROS

Intracellular metabolites (NAD^+/NADH, acetyl-CoA, citrate) were measured with colorimetric or fluorescent kits (Biovision) according to the instructions. $NADP^+$/NADPH were measured using a colorimetric assay kit (Abcam).

Samples were collected after washing in RPMI 1640 culture media and quenched in liquid nitrogen to stop metabolism. For acetyl-CoA, citrate measurements, samples were lysed with RIPA buffer and deproteinized with perchloric acid (PCA) and sodium hydroxide prior to the assay. For NAD^+/NADH and $NADP^+$/NADPH, after a series of spinning and washing, pellet cells were extracted with a specific buffer provided. After spinning, supernatants were transferred in labelled tubes and kept on ice. An aliquot of 200 uL was transferred in micro centrifuge tubes and heated at 60°C in a heating block to allow all $NADP^+$ or NAD^+ to be decomposed and NADPH and NADH to remain intact. Reaction mix was then added to samples labelled as: total NAD, NADH, total NADP and NADPH followed by developer for 1-4 hours incubation. The optical density (OD) of each well was then determined using a microplate reader set to 450 nm taking multiple readings.

Reactive oxygen species were measured using the fluorescent probe carboxy-H2DCFDA (Invitrogen, Molecular Probes). Briefly, $CD4^+$ T cells were incubated with 1 μM H2DCFDA/PBS for 30 minutes in the dark. The excess dye was removed with three washes in warm PBS and 2.5x10^5 cells /100 μL PBS were plated in a black 96 well plate.

After addition of 10 mM sodium lactate or 1 mM H_2O_2 fluorescence was recorded in a time wise manner at Ex 492 nm / Em 520 nm.

4.9 Lactate mesurement in human samples

Lactate concentration was measured in the synovial fluid of RA or OA patients using the lactate colorimetric assay kit (Biovision). Briefly, for the standard curve preparation lactate standard 1 nmol/µL was prepared by adding 10 µL of the 100 nmol/µL lactate standard (stock solution) to 990 µL of lactate assay buffer. After mixing well 0, 2, 4, 6, 8 and 10 µL were added into a series of wells and the volume was adjusted to 50 µL/well with lactate assay buffer to generate 0, 2, 4, 6, 8 and 10 nmol/well of the lactate standard. Samples (1 µL/well) were added in other wells and the volume adjusted to 50 µL/well with lactate assay buffer. A reaction mix (50 µL/well) containing the enzyme, the probe and the buffer was then added. After an incubation for 30 minutes at room temperature, protected from light I measured the absorbance (OD 570 nm) in a microplate reader.

4.10 Metabolomics, stable isotype tracing and fatty acid labelling

Following extraction, $CD4^+$ T cells were activated in media with anti-CD3 and anti-CD28 mAbs for 24 hours followed by further 48 hour culture with lactate alone or in the presence of SLC5A12 Ab in medium containing low glucose (5 mM) and 5% FBS. Spent medium was collected and processed for metabolite extraction as described (Mackay GM, 2015). Briefly, medium from each condition is diluted 50 fold with cold extraction solvent consisting of 50% methanol, 30% acetonitrile and 20% water. Polar metabolites were extracted by vortexing the tubes for 10 minutes and then centrifugation at 16.000 ×

g for 10 minutes at 4 °C. The supernatants are transferred to glass vials and kept at -75 °C prior to LC-MS analysis. Glucose, lactate and glutamine concentrations in the spent medium were quantified using external calibration curves generated by spiking in different concentrations of ^{13}C-glucose, ^{13}C-lactate, and ^{13}C-glutamine in the medium and extraction into extraction solvent. Peak areas from the samples were extrapolated to the standard curve peak areas and absolute concentrations of glucose, lactate and glutamine were obtained. Uptake rates of metabolites were calculated as difference in concentrations normalized to the area under the growth curve of cells.

For ^{13}C-lactate tracing into polar metabolites, CD4^{+} T cells were activated for 48h in low glucose medium with 5% FBS in a 24-well cell culture plate in triplicate, with or without SLC5A12 Ab. After 48 hours, the medium was replaced with fresh medium containing 10 mM ^{13}C-U-lactate (Sigma) with or without SLC5A12 Ab for 2 hours. Medium was removed by centrifugation and metabolites were extracted from the cell pellets with cold extraction solvent (50% methanol, 30% acetonitrile and 20% water). The LC-MS parameters for data acquisition were kept the same as previously described (Mackay GM, 2015).

For ^{13}C lactate tracing into palmitate, CD4^{+} T cells were activated in low glucose medium (5 mM) and 10% FBS in a 24-well cell culture plate in duplicate in presence of 10 mM ^{13}C-U-lactate (Sigma) with or without SLC5A12 Ab for 0, 24, 48 and 72 hours. At the end of each time point, medium was aspirated and cell pellets were treated with 750 µL of 1:1 cold PBS: methanol and total lipid fraction was extracted in 500 µL of chloroform. This extract was dried under inert nitrogen, reconstituted in 90 µL chloroform and total fatty acids were derivatized using the transesterification reagent MethPrep II (Thermofisher Scientific, UK) before analysis by GC-MS as described (Tumanov S, 2015).

4.11 Seahorse

Glycolytic and mitochondrial metabolism was evaluated by measuring the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) respectively with a Seahorse XF96e Extracellular Flux Analyzer. Briefly, T cells were activated in RPMI 1640 supplemented with 10% FBS for 12 hours. One hour before the experiment, $3x10^5$ T cells were seeded in a 96-well microplate in XF Assay Dulbecco's Modified Eagle Medium (DMEM), in presence of 10 mM of glucose, and incubated in a non-CO2 incubator.

Fatty acid oxidation was analyzed by measuring the OCR with a Seahorse XF96e Extracellular Flux Analyzer according to the manufacturer protocol. Briefly, $2.5x10^5$ T cells were seeded in a 96 well micro plate in XF-DMEM-medium containing 2.5 mM glucose and incubated for 45 minutes in a CO2 free incubator. 15 minutes prior to the assay, control cells were treated with 40 μM etomoxir to block CPT1a. Just before starting the assay, the cells were treated with 167 μM BSA-palmitate or BSA alone. During the assay 1 μM oligomycin and 1 μM FCCP were injected. Experiments with the seahorse system were done with the following assay conditions: 3 minutes of mixture; 3 minutes waiting; and 3 minutes of measurement. Sodium lactate (10 mM) metabolic drugs or PBS, was injected during the assay at the indicated time points.

4.12 RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated from 10^6 $CD4^+$ T cells or 10 mg RA synovial tissue using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and assessed for quality and quantity using absorption ratios of 260/280 nm and 260/230 nm. Hereby cells

were lysed in lysis buffer RLT, nucleic acids were precipitated with 70% ethanol and RNA bound to spin columns. Following several washing steps, RNA was eluted in dH_2O. The isolated RNA was reverse transcribed to complementary DNA (cDNA) using commercially available kits according to the manufacturer's instructions (Applied Biosystems). Briefly, 1 ug of total RNA was mixed with buffer, deoxy-nucleotides (dNTPs) and reverse transcriptase and incubated for 2 hours at 30°C, followed by a 5 minutes heat inactivation step at 85°C. cDNA was diluted to 10 ng/μL and stored at -20°C or -80°C for subsequent use.

Quantitative gene expression analysis was performed using SYBR Green Supermix (Biorad) in CFX connect light cycler (Biorad), according to the manufacturer's instructions. Gene relative expression was calculated using the ΔΔct method (Livak KJ, Schmittgen TD, 2001) and normalized to a reference control (GAPDH or Actin). A complete list of primers used is available in the supporting information (**Table 7, Appendix**).

4.13 Tissue biopsy procedure

For the RNA sequencing data, 87 synovial samples from patients presenting with early inflammatory arthritis were analyzed. The immune-histological features of rheumatoid synovitis in each patient were assessed by ultrasound (US) guided synovial biopsy of clinically involved (swollen) joints. US examination (in grey scale and power Doppler) and US-guided synovial biopsy were performed using a General Electric (GE Healthcare, Fairfield, Connecticut, USA) Logiq 9 ultrasound machine with a two-dimensional M12L transducer, grey-scale frequency 12MHz. Synovial thickening and degree of power

Doppler signal were scored using a previously reported semi-quantitative score (0–3) (Pitzalis C, 2013).

4.14 Tissue RNA extraction

All tissue samples were maintained on ice and homogenized in a fume hood using a Polytron benchtop laboratory homogenizer. Samples were homogenized at short five-second intervals until all the tissue had been sheared/homogenized. The probe of the homogenizer was cleaned thoroughly in between samples by washing initially in RNase Away solution, followed by four washes in sterile/RNase-free Baxter water. RNA was extracted from a minimum of 10 mg of synovial tissue homogenized at 4°C in Trizol reagent (Life Technologies, Invitrogen Division, UK). Chloroform was mixed with the lysate and following centrifugation the aqueous RNA layer was transferred to a new microcentrifuge tube. Isopropanol at 4°C was mixed with the RNA layer. Following incubation and centrifugation, the isopropanol was removed and the RNA pellet washed with 70% ethanol. The pellet was re-dissolved in RNase-free water. The concentration/purity of the RNA sample(s) were measured using the NanoDrop 2000C (Lab Tech, UK) and the quality (RIN) was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies, UK).

4.15 RNA sequencing, read alignment, and transcript assembly

Metabolic genes were chosen by using KEGG pathway database. The sequencing workflow was conducted by Illumina TruSeq platform. Raw data FASTQ files were first screened for quality using FastQC (http: // www.bioinformatics.babraham.ac.uk /projects

/fastqc/), inspecting multiple aspects of sequencing quality, including GC content, overall read quality, individual base qualities and read length distribution. The quality-checked FASTQ files were aligned to the human genome reference (GRCh37/hg19) using The Genomic Short-read Nucleotide Alignment Program (GSNAP) with an associated transcriptome map (GTF file, http://support.illumina.com/sequencing/sequencing_software/igenome.html) used to guide transcriptome assembly. To determine transcript abundances, i.e., read counts, the 'multicov' function of BEDTools 2.15.0 was used and counts were computed as the total number of reads that aligned to a transcript region ($50x10^6$ reads per tissue, pair-end). Additionally counts were computed over individual exons in each transcript. All subsequent bioinformatics analyses were conducted in the R Programming Language environment. RNA-seq data are uploaded to ArrayExpress and are accessible via accession E-MTAB-6141 at: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6141.

4.16 H&E procedure

FFPE tissue blocks were sectioned at a thickness of 3 μm using a Leica RM1235 microtome. Sections were mounted on to Superfrost plus (+) slides and left to dry in a slide rack at room temperature for 30 minutes. Slides were heated (60ºC) for a minimum of 30 minutes prior to staining. Tissue sections were stained with haemotoxylin and eosin (H&E), mounted with DePex and left to dry overnight. Slides were imaged using an Olympus BX61 microscope for initial grading. 0 = normal; 1 = minimal infiltration of inflammatory cells in synovium and periarticular tissue of affected joints; 2 = mild

infiltration; 3 = moderate infiltration with moderate edema; 4 = marked infiltration affecting most area with marked edema.

The histological assessment of synovial tissues is carried out in order to define the predominant histological pattern of synovitis as either diffuse or aggregate. In addition, each section underwent semi-quantitative assessment for three synovial membrane features: (i) thickness of the synovial lining cell layer, (ii) stroma cell density and (iii) inflammatory cell infiltrate. The ranking of alterations is on a scale from none (0), slight (1) and moderate (2) to strong (3) very strong (4) for each feature. Max score of 4 was given.

4.17 Enzyme Linked Immunosorbent Assay

Secreted IL-17A and IFNγ were measured in cell culture supernatants from 2-4x10^6 $CD4^+$ T cells/well with a human IL-17A (homodimer) or IFNγ ELISA Ready-SET-Go Assay (eBioscience) according to the instructions. Briefly after coating a 96-well plate with a capture antibody (overnight at - 4°C), I washed (3 times) with wash buffer (PBS, 0.05% tween -20) and then blocked the wells with ELISA/ELISPOT diluent (eBioscence) for 1 hour. Once the incubation with blocking solution was complete, after washing, samples and curve were added at the final volume of 100 uL/well and incubated for 2 hours. The curve was built doing seven 2-fold serial dilutions from a standard stock solution. Afterward a detection antibody (1 hour) was added, following by avidin-HRP (30 minutes) and TMB (3,3,5,5' tetramethylbenzidine, 15 minutes) solution. Upon color development (usually 15-30 minutes), the reaction was stopped with 50 uL/well of 2N sulfuric acid solution (H_2SO_4). The OD of each well was then determined using a microplate reader set to 450 nm. The standard curve is then constructed by plotting the

mean absorbance for each standard on the y-axis against the concentration (known) on the x-axis and draw the best-fit curve through the points on the graph.

4.18 Human glucose 6 phosphate isomerase-induced mouse model of arthritis

DBA/1 mice purchased from Charles River were immunized s.c. with 20 μg human hG6PI synthetic peptide (hG6PI325-339; ThermoFisher Scientific) in CFA (Sigma-Aldrich). The indicated amount of peptide was mixed with CFA in a 1:1 ratio (v/v) and emulsified by sonication. For induction of arthritis, 100 μL of the emulsion was given s.c. at the base of the tail. On day 7 – a time point at the onset of disease – and 11 post induction (as indicated by the arrows in the **Figure 11A**), mice were left untreated or treated sub-plantar into the rear paws with 20 uL of 0.1 mg/mL antibody; infliximab (Remicade, Janssen Biologics), TNF mAb (TN3-19.12, BD bioscience), SLC5A12 Ab (Abcam), iso-TNF Ab (BD biosciences) and iso-SLC5A12 Ab (Abcam). Antibody and the corresponding isotype control was injected into the right or left back paw respectively of the same mouse. The development of disease was monitored daily by visually assessing the clinical score. A score of 0 indicates no clinical signs of arthritis; a score of 1 for each of the fingers, pad and ankle indicates swelling and redness. Maximum score for each paw is 7. A trained observer who was blinded to the immunization status of the mice performed the scoring. The grade of inflammation was assessed by H&E as described in *section 4.5*.

4.19 Generation of neutralizing monoclonal antibodies (mAbs) targeting SLC5A12

The generation of neutralizing monoclonal antibodies (mAbs) targeting SLC5A12 was performed by Aldevron.

Rats were immunized with SLC5A12 peptide (sequence of the peptide: QGSTH - AGGFH - NVLEQ - STNGS - RLHIF - DFDVD - PLRRH - C). After 45 days of immunization and 4 applications, very strong reactivity of the immune sera could be detected in the animals immunized with the peptide when tested by an enzyme - linked immunosorbent assay (ELISA) on plates coated with the target antigen (peptide SLC5A12 - hum - KLH). No reactivity could be detected in any of the immune sera against the protein used as control (BSA). In view of these very encouraging results animals were sacrificed, the lymphocytes isolated and cell fusion and ~400 clones were screened via ELISA/IQue. In the first round of screening 4 clones were selected based on IQue (FACS) results. These supernatants bound to the full - length cell surface protein and are thus strong candidates for binding to the native protein. Additional 50 clones were selected based on the good results from ELISA.

In the last round of the screening the top 7 candidates (3C7, 4G2, 6E1, 7C1, 9G4, 9G7 and 10E11 mAbs clones) were selected based on ELISA and FACS-measured binding on cells expressing the full - length protein.

Finally three clones were chosen and were propagated into T25 flasks; BTK-3B10-C7 (IgG2a / kappa), BTK-3C7-C7 (IgG2a / kappa), BTK-10E11-G8-G8 (IgG1/IgG2a/c kappa/lambda) were successfully sub-cloned.

FACS-measured binding analysis of these 3 clones on human activated CD4+ T cells expressing the full-length protein in presence or not of SLC5A12 peptide in the culture was further performed.

4.20 Statistical analysis

In all experiments, data are expressed as mean ± SEM unless stated otherwise. Statistical tests were selected based on appropriate assumptions with respect to data distribution and variance characteristics. Student's *t*-test (two-tailed) or ANOVA test (one-way or two-ways) were used for the statistical analysis as appropriate. Statistical significance was defined as $P \leq 0.05$. GraphPad Prism software (GraphPad Software) was used for the analysis.

5 RESULTS

5.1 CHAPTER I – SLC5A12 EXPRESSION IN HEALTH AND DISEASE

5.1.1 *The expression of the lactate transporter SLC5A12 by immune cells is regulated by activation and inflammatory stimuli*

Lactate modulates $CD4^+$ T cell migratory abilities and cytokine production via the sodium-coupled lactate transporter SLC5A12, which is selectively expressed by $CD4^+$ but not by $CD8^+$ T cells (Haas R, 2015). To assess whether the expression of SLC5A12 may be regulated by activation stimuli, peripheral blood mononuclear cells (PBMCs) from healthy control (HC) subjects were treated for 48 hours with anti-CD3 monoclonal antibody (mAb) or left untreated. TCR activation led to upregulation of SLC5A12 by peripheral $CD4^+$ but not $CD8^+$ T cells (**Figure 1A-C**). In the same experiment, activation led to up-regulation of SLC5A12 also in peripheral $CD14^+$ and $CD19^+$ cells (monocytes and B cells, respectively) (**Figure 1D-F**). In comparison, the same immune cell subsets were negative for SLC5A12 or expressed it at low levels in untreated PBMCs (**Figure 1A, C, Figure 1D, F**). Interestingly, when I analysed mononuclear cells (MCs) from inflamed tonsils excised from patients subjected to tonsillectomy, $CD4^+$ T cells were $SLC5A12^+$, irrespective of any activation stimuli (**Figure 2A-C**). Likewise, $CD14^+/CD68^+$ and $CD19^+/CD20^+$ cells were $SLC5A12^+$, independent of any activation (**Figure 2D-F**). In contrast, $CD8^+$ T cells were mostly negative for SLC5A12 (**Figure 2 A-C, G**), which was consistent with data in **Figure 1A-C**.

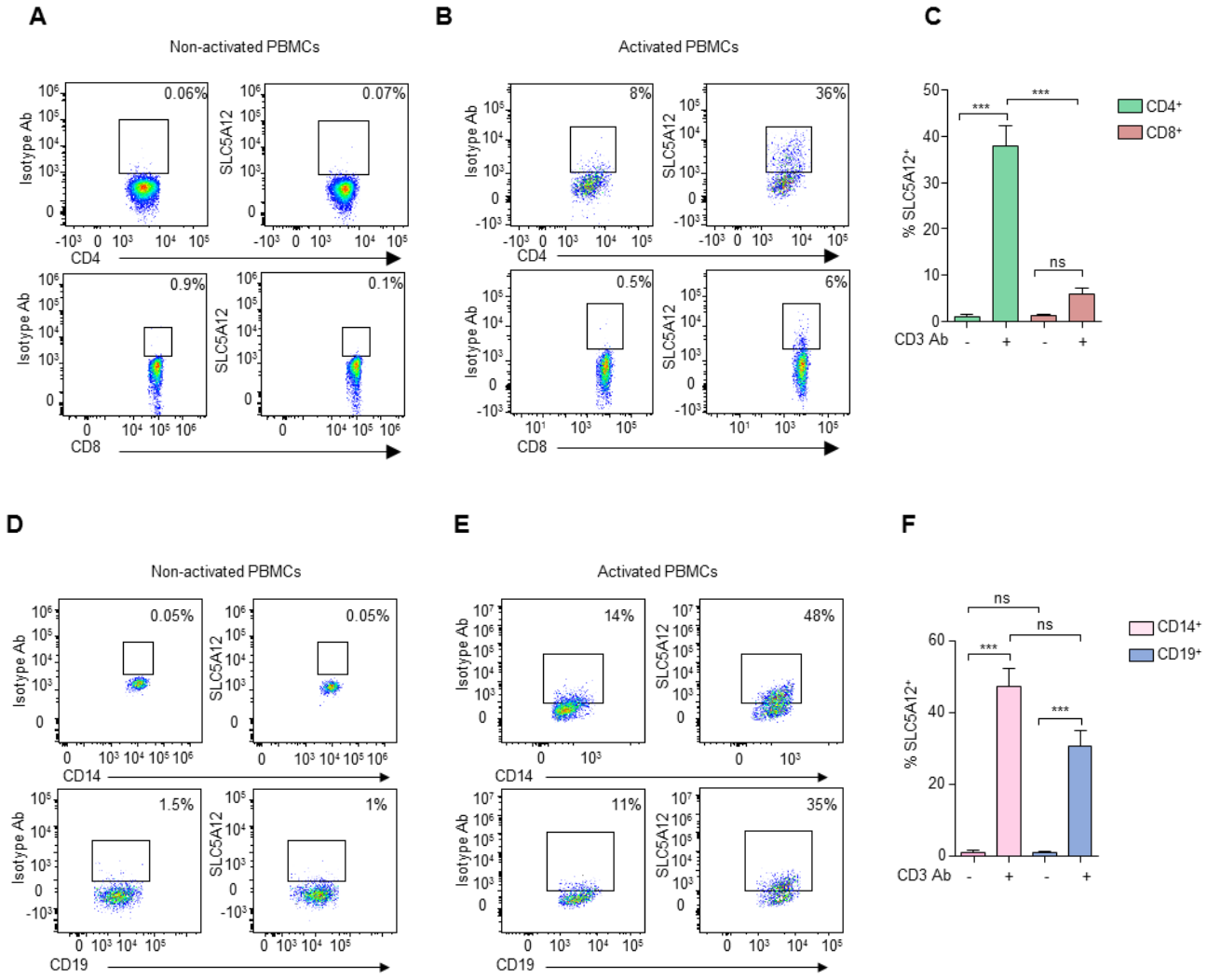


Figure 1 - SLC5A12 up-regulation by T cells requires TCR engagement. (A, B, D, E) Representative flow cytometry plots of SLC5A12 expression and (C, F) quantification of percentage of $CD4^+SLC5A12^+$, $CD8^+SLC5A12^+$, $CD14^+SLC5A12^+$ and $CD19^+SLC5A12^+$ cells in non-activated (n = 3) (A, D) and anti-CD3 mAb activated (n = 6) (B, E) PBMCs gated for $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ freshly isolated from HC. Comparisons were evaluated using one-way ANOVA. Data are expressed as mean ± s.e.m. *P≤0.05; **P≤0.01; ***P≤0.001; ns=not significant.

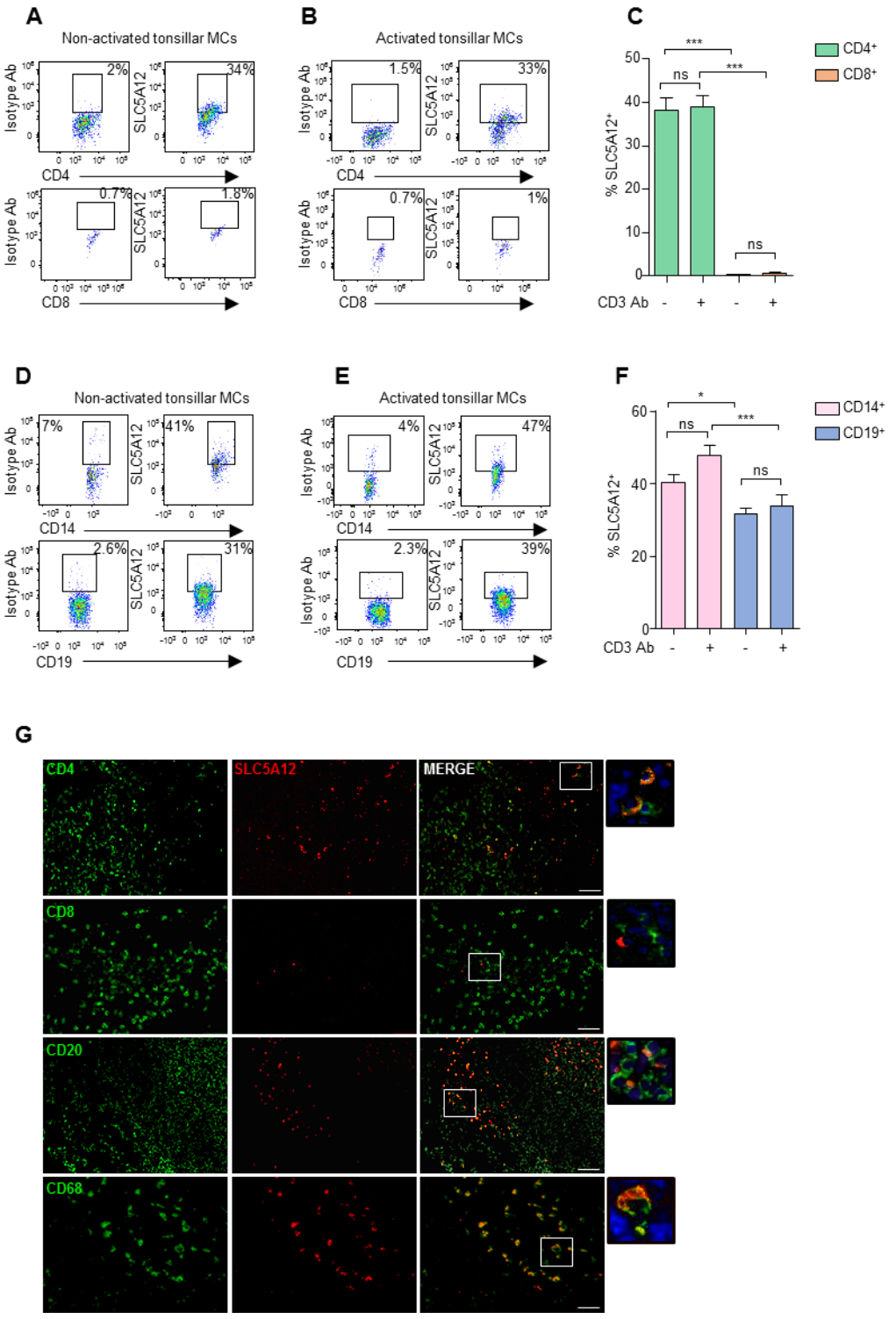


Figure 2 – SLC5A12 expression by peripheral and tissue-resident immune cells. (A-E) Representative flow cytometry plots of SLC5A12 expression in non-activated (n = 4, A, D) and anti-CD3 mAb activated (n = 4, B, E) tonsil MCs, gated for $CD4^+$, $CD8^+$,

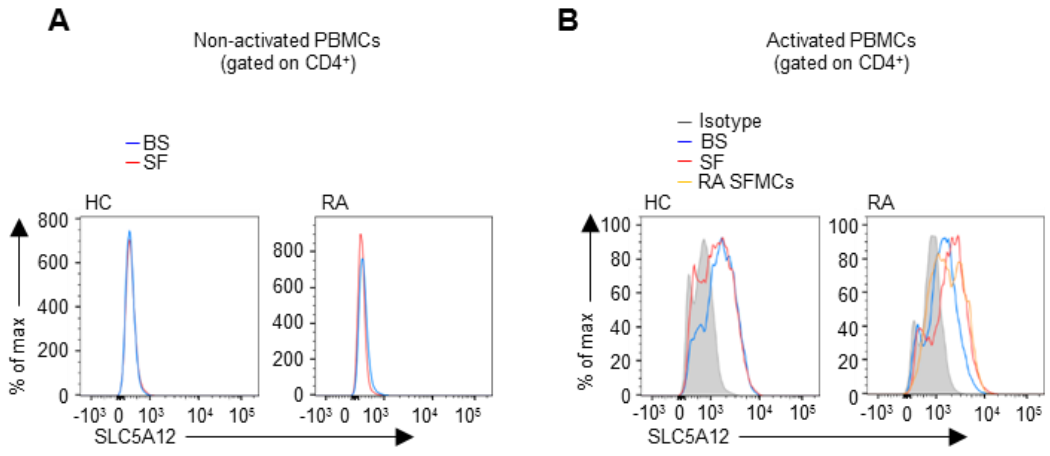
$CD14^+$, $CD19^+$ and quantification of the percentage (C, F). (G) Double immunofluorescence staining for SLC5A12 (red) and CD4, CD8, CD20 or CD68 (green). Merging (orange\yellow) of green and red channels demonstrates that SLC5A12 is expressed by $CD3^+CD4^+$ (T cells), $CD20^+$ (B cells) and $CD68^+$ (macrophages). Scale bars 50 uM. Comparisons were evaluated using one-way ANOVA. Data are expressed as mean ± s.e.m. *P≤0.05; **P≤0.01; ***P≤0.001; ns=not significant.

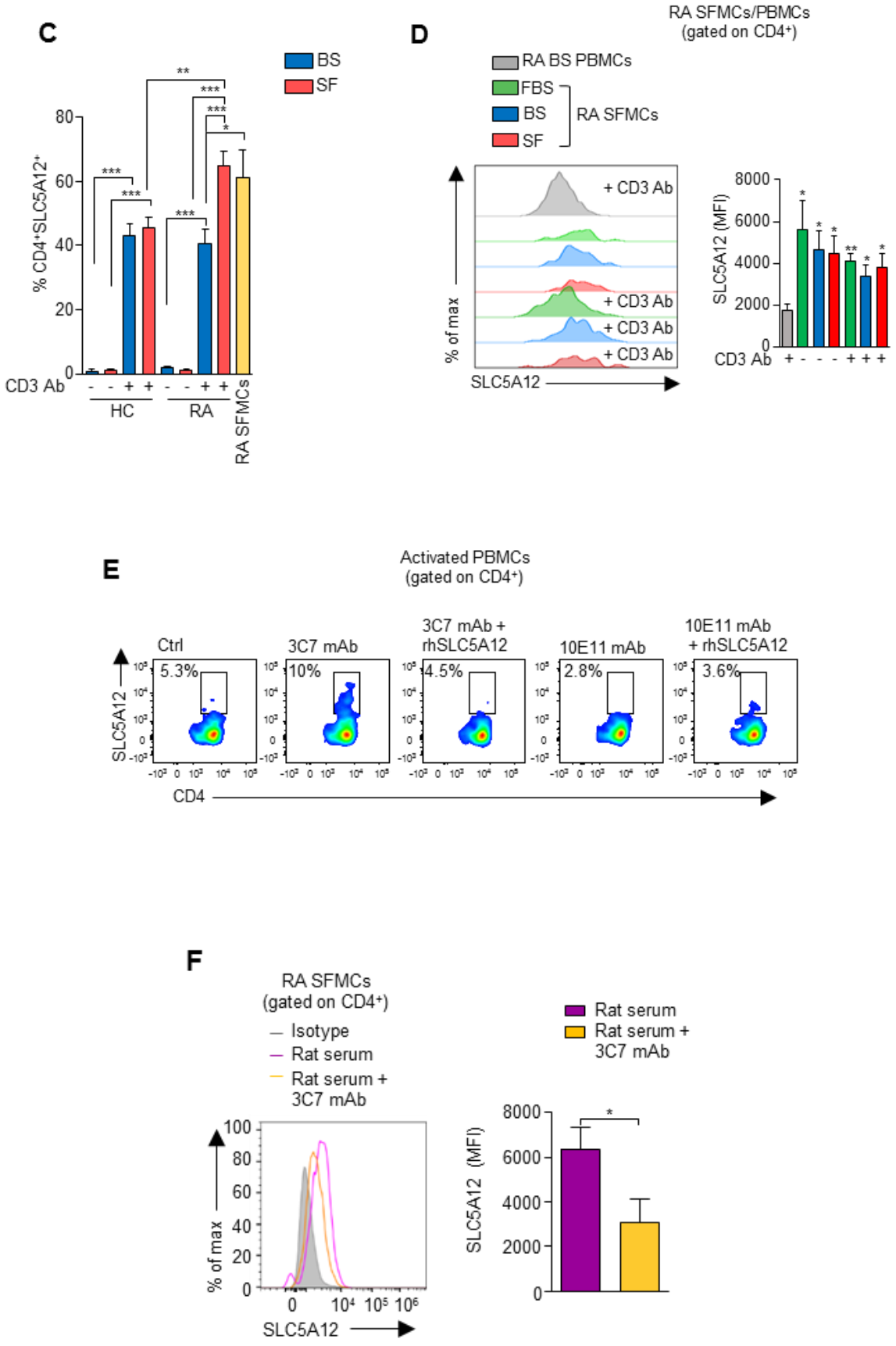
5.1.2 *SLC5A12 is upregulated in activated peripheral RA CD4$^+$ T cells cultured in autologous synovial fluid*

To confirm whether the inflammatory environment has a role in the regulation of SLC5A12 expression by CD4$^+$ T cells, I next compared the SLC5A12 expression in HC versus RA CD4$^+$ T cells. I used RA patients as model of autoimmune/inflammatory disorder. Furthermore, during these years I have had the opportunity to use RA patient samples as part of the HTA-approved EMR Early Arthritis Biobank. Age-matched HC and RA PBMCs were defrosted, washed two times and seeded in 24-well plates at the concentration of 2x10^6 cells/mL. Cells were treated or not with anti-CD3 mAb and cultured in medium supplemented with matched 5% autologous blood serum (BS) or 5% RA synovial fluid (RA SF).

Surprisingly, no difference was observed in the percentage of CD4$^+$SLC5A12$^+$ T cells when we compared non-activated HC and RA PBMCs cultured in medium containing autologous BS or RA SF (**Figure 3A-C**). Even though anti-CD3 mAb-mediated activation led to up-regulation of SLC5A12 by CD4$^+$ T cells, again no difference was observed in the percentage of CD4$^+$SLC5A12$^+$ T cells when we compared HC and RA PBMCs activated in medium containing autologous BS (**Figure 3A-C**). In contrast, anti-CD3 mAb-mediated activation of RA but not of HC PBMCs in the presence of 5% RA SF led to a robust further upregulation of SLC5A12 by CD4$^+$ T cells as compared to HC or RA activated CD4$^+$ T cells in the presence of BS (**Figure 3A-C**). Importantly, I observed that SLC5A12 expression levels by CD4$^+$ T cells in RA PBMCs, activated in the presence of RA SF, were comparable to those expressed by CD4$^+$ T cells in synovial fluid mononuclear cells (SFMCs) from RA joints (**Figure 3C**) consistent with the data in **Figure 2**. I also found that CD4$^+$ T cells in RA SFMCs presented high levels of SLC5A12 irrespective of any activation or inflammatory stimuli, as compared to CD4$^+$ T

cells in RA PBMCs activated in the presence of autologous BS (**Figure 3D**). In a further experiment, I tested a novel mAb targeting SLC5A12. This antibody was generated by immunizing rats with a peptide comprising the predicted extracellular loop 3 of SLC5A12 (*see section 4.8*). Out of ~400 screened clones, 3C7 was selected for its ability to specifically recognize SLC5A12 (**Figure 3E**). Treatment of freshly isolated RA SFMCs with 3C7 mAb led to a reduced expression of the transporter itself by $CD4^+$ T cells (**Figure 3F**). I then wondered whether lactate, at concentrations similar to that measured in RA SF (Haas R, 2015) might play a direct role in the regulation of the expression of SLC5A12. Incubation of activated peripheral $CD4^+$ T cells – isolated from HC PBMCs with negative selection magnetic beads – with 10 mM sodium lactate contributed to the induction of SLC5A12 expression both at mRNA and protein level. Furthermore, pre-incubation with an anti-SLC5A12 polyclonal antibody (SLC5A12 Ab) prevented lactate-induced upregulation of SLC5A12 (**Figure 3G, H**). These data suggested that accumulation of extracellular lactate, such as at inflammatory sites, may cause a feedforward signal leading to the upregulation of the lactate transporter SLC5A12 by $CD4^+$ T cells. This effect is counteracted by SLC5A12 blockade.




C
BS
SF
% CD4+SLC5A12+
CD3 Ab
HC
RA
RA SFMCs
D
RA SFMCs/PBMCs
(gated on CD4+)
RA BS PBMCs
FBS
BS
SF
RA SFMCs
+ CD3 Ab
% of max
SLC5A12
SLC5A12 (MFI)
E
Activated PBMCs
(gated on CD4+)
Ctrl
3C7 mAb
3C7 mAb +
rhSLC5A12
10E11 mAb
10E11 mAb
+ rhSLC5A12
5.3%
10%
4.5%
2.8%
3.6%
SLC5A12
CD4
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RA SFMCs
(gated on CD4+)
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SLC5A12 (MFI)

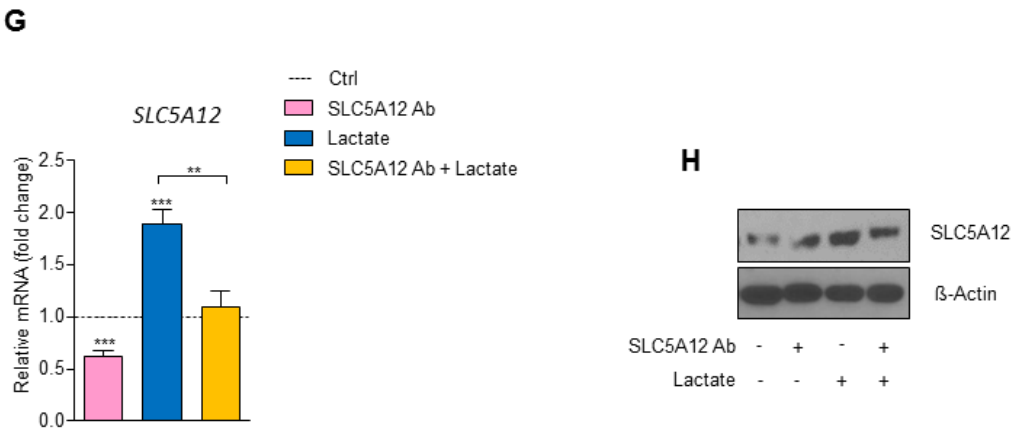


Figure 3 - SLC5A12 expression is regulated by inflammation. (A-C) Representative histograms (A, B) and graph bars (C) of SLC5A12 expression by $CD4^+$ T cells in unstimulated (A) and CD3 mAb stimulated HC (n = 4, B) and RA (n = 5, C) PBMCs. Cells were cultured in RPMI 1640 medium supplemented with 5% RA or HC autologous blood serum (BS) or 5% RA synovial fluid (SF) (A-C). (D) Representative plots (left) and quantifications (right) showing the expression of SLC5A12 in anti-CD3 mAb activated or not-activated RA SFMCs (n = 8) gated for $CD4^+$ T cells. MFI, mean fluorescent intensity. Cells were cultured in RPMI 1640 medium supplemented with 5% FBS (n = 3), 5% autologous BS (n = 8) or 5% RA SF (n = 8). Anti-CD3 mAb activated BS RA PBMCs (n = 5) were used as control. (E) Representative flow cytometry plots of SLC5A12 expression in anti-CD3 mAb stimulated PBMCs from HC gated for $CD4^+$ T cells. Cells were pre-incubated for 1 hour in the presence or absence of SLC5A12 recombinant peptide (rhSLC5A12, 1:100) before a further incubation with anti-SLC5A12 mAbs (3C7 IgG or 10E11 IgG). Goat anti-rat IgG ALEXA-555 (Invitrogen) (1:200) was used as secondary antibody and rat serum was used as control. (F) Representative histogram (left) and quantifications (right) analyzing the expression of SLC5A12 in RA SF $CD4^+$ T cells treated or not with 3C7 mAb (n = 5) or control rat sera. (G, H) SLC5A12 mRNA expression (n = 5, G) and protein (representative, H) in anti-CD3/CD28 mAbs activated $CD4^+$ T cells isolated from HC treated or not with sodium lactate (10 mM) in presence or not of SLC5A12 blocking Ab (2.5 ug/mL, HPA060904 - Atlas Antibodies). Comparisons were evaluated using one-way ANOVA (C) or two-tailed Student's *t*-test (D, F-G). Data are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.

5.2 CHAPTER II – LACTATE MODULATES INTRA- AND EXTRACELLULAR METABOLITES AND METABOLIC PATHWAYS

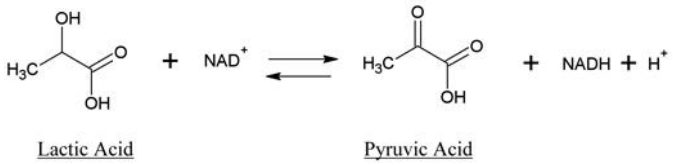
5.2.1 *Lactate influx into CD4$^+$ T cells via SLC5A12 causes reduced glycolysis and increased TCA cycle-derived citrate but no major changes in mitochondrial oxygen consumption*

Having observed both an increase in the expression levels of the lactate transporter SLC5A12 in response to activation and inflammatory (including lactate itself) stimuli, and the absence of other lactate transporters, i.e. MCT1, in CD4$^+$ T cells (Haas R, 2015; **Figure 1-3**), I wondered whether SLC5A12 may serve as a main carrier for an influx of lactate in to CD4$^+$ T cells that have reached the inflamed tissue and hence are exposed to high concentration of extracellular lactate. To address this question, several extracellular and intracellular metabolites were measured via mass spectrometry analysis (**Figure 4A**), cellular metabolism assay kits (**Figure 4B, F-G**), seahorse flux analyser (**Figure 4C**) and mass spec-based metabolic tracer analysis (**Figure 4D, E**).

Peripheral CD4$^+$ T cells were isolated from HC PBMCs with negative selection magnetic beads and then activated with anti-CD3 and anti-CD28 mAbs. After 24 hours, cells were switched to a medium containing low glucose (5 mM) and 5% FBS in which they were incubated for additional 48 hours with lactate in the presence or absence of SLC5A12 Ab or were left untreated. Finally, culture media were collected and analysed by mass spectrometry. Exposure to lactate caused a decrease in glucose uptake along with a reduction in lactate release and a non-significant reduction in glutamine uptake by activated CD4$^+$ T cells (**Figure 4A**). All these effects were reversed by cell incubation with anti-SLC5A12 Ab (**Figure 4A**).

I then asked whether a reduced glucose flux, due to lactate exposure, could lead to a decreased glycolysis. To address this question, I looked at the levels of NAD^+ and NADH in $CD4^+$ T cells treated with lactate.

NAD^+ is a key co-factor of the sixth reaction of the glycolytic cascade catalysed by glyceraldehyde 3-phosphate dehydrogenase. As a consequence of this reaction, NAD^+ is reduced to NADH, which acts as an inhibitory feedback on glycolysis. NADH can be re-oxidised to NAD^+ via the lactate dehydrogenase reaction converting pyruvate to lactate. This reaction is key to maintain a steady flow of glycolysis (*reaction below*).



Lactic Acid Pyruvic Acid

However, lactate dehydrogenase can perform the reverse reaction when cells are exposed to high levels of extracellular lactate as it happens at the site of inflammation, with reduction of NAD^+ to NADH and consequent inhibitory feedback on glycolysis. Indeed, upon exposure to lactate I observed a drop in the NAD^+/NADH ratio in $CD4^+$ T cells, indicating a relative increase of intracellular NADH over NAD^+ (**Figure 4B**). NADH has many biological functions in the cell, including acting as a cofactor in the NADH dehydrogenase / complex I of the mitochondrial electron transport chain (ETC). The ETC generates ATP and ROS during its activation (Finkel T and Holbrook NJ, 2000).

Prompted by this finding, I then tested the effects of lactate on both the glycolytic energy metabolism and the mitochondrial respiration of 12 hours anti-CD3/CD28 mAbs activated $CD4^+$ T by measuring the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) via the use of the seahorse analyzer. ECAR was decreased upon lactate treatment. The drop had an average of 47 mpH/min in the

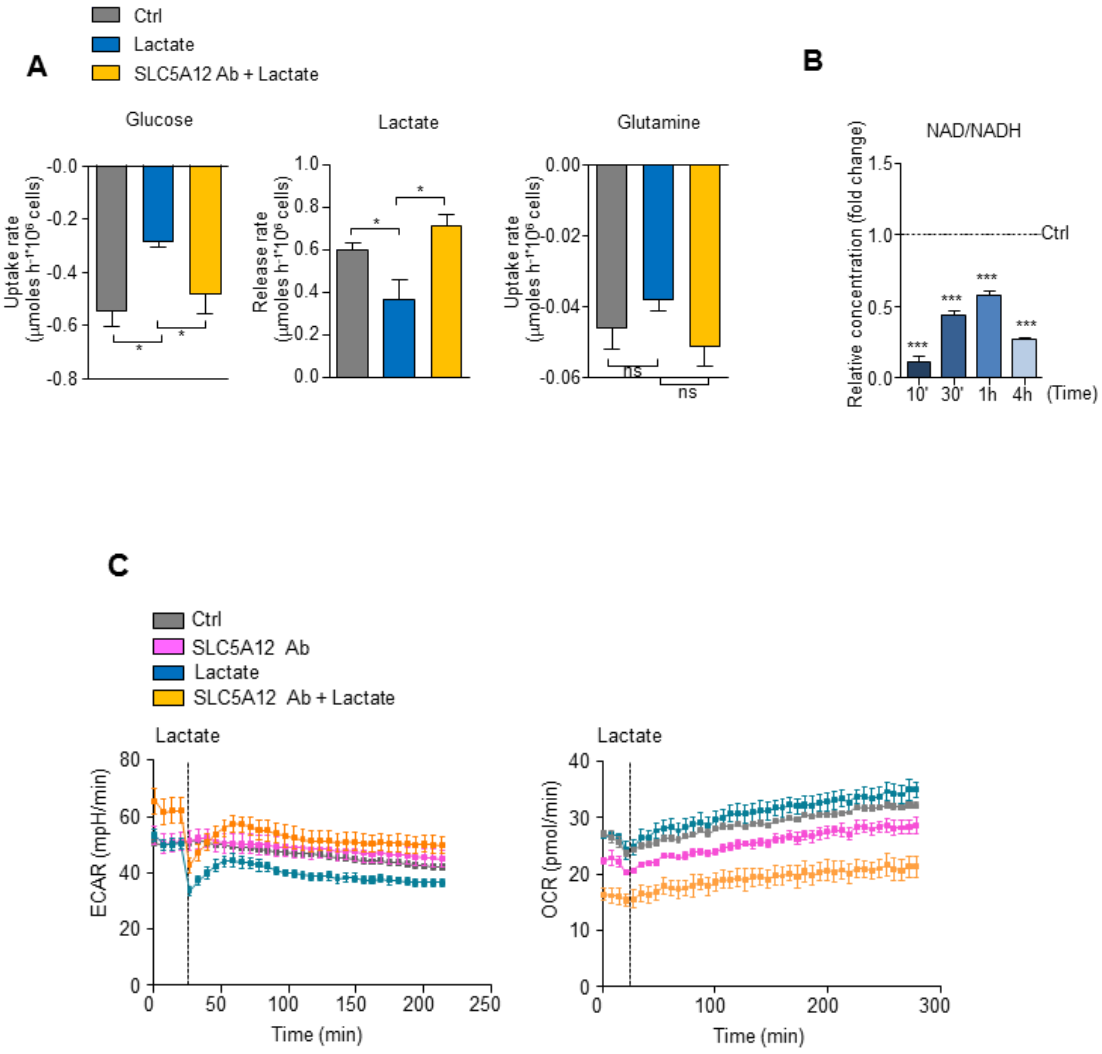
untreated control to a level of less than 40 mpH/min with lactate exposure. This effect was reverted by the treatment with the SLC5A12 Ab where ECAR increased from 40 mpH/min to an average of 53 mpH/min (**Figure 4C, left**). An increase of ECAR, from 51 mpH/min to 63 mpH/min was observed before lactate injection in $CD4^+$ T cells pre-treated with anti-SLC5A12 Ab.

As lactate has been shown to fuel OXPHOS in a number of cells, including neurons and muscle cells (Cornell, NW 1973; Kompanje EJ, 2007; Pellerin L, 1998), OCR, which is an indicator of OXPHOS or mitochondrial respiration, was also measured. Surprisingly, OCR levels were not affected by sodium lactate. Conversely, $CD4^+$ T cells, pre-treated with SLC5A12 Ab showed a drop in OCR before and after lactate injection (**Figure 4C, right**) an effect that will require further investigations.

I reasoned that these findings might be explained by an influx of lactate in to $CD4^+$ T cells when they are in a lactate-rich environment with consequent conversion to pyruvate and reduction of NAD^+ to NADH slowing down the glycolytic flow. Pyruvate may then enter the tricarboxylic (TCA) cycle, but considering that I have not observed an increase in mitochondrial oxygen consumption rate, I wondered what the fate of the lactate-derived carbons was. To test our rationale in a direct fashion, mass spectrometry-based tracer analysis of [^{13}C]-lactate was performed. Specifically, $CD4^+$ T cells were activated and incubated with [^{13}C]-lactate in the presence or absence of anti-SLC5A12 Ab. Intracellular metabolites were then exctracted and analysed by mass-spectrometry. I found that a significant proportion of [^{13}C]-lactate was converted to pyruvate and citrate, and that this effect was abrogated by incubation with SLC5A12 Ab (**Figure 4D, E**). Consistent with these data, by using commercial cellular metabolism assay kits, an increase in acetyl-CoA and citrate levels was also found in $CD4^+$ T cells exposed to lactate at different time points (**Figure 4F, G**). These data suggest that when exposed to

high levels of lactate, such as in an inflamed tissue, $CD4^+$ T cells can take up lactate via the specific carrier SLC5A12 and convert it to citrate.

This process promotes an inhibitory feedback on glycolysis without affecting mitochondrial oxygen consumption rate, leaving with the question of what the fate of citrate would be.



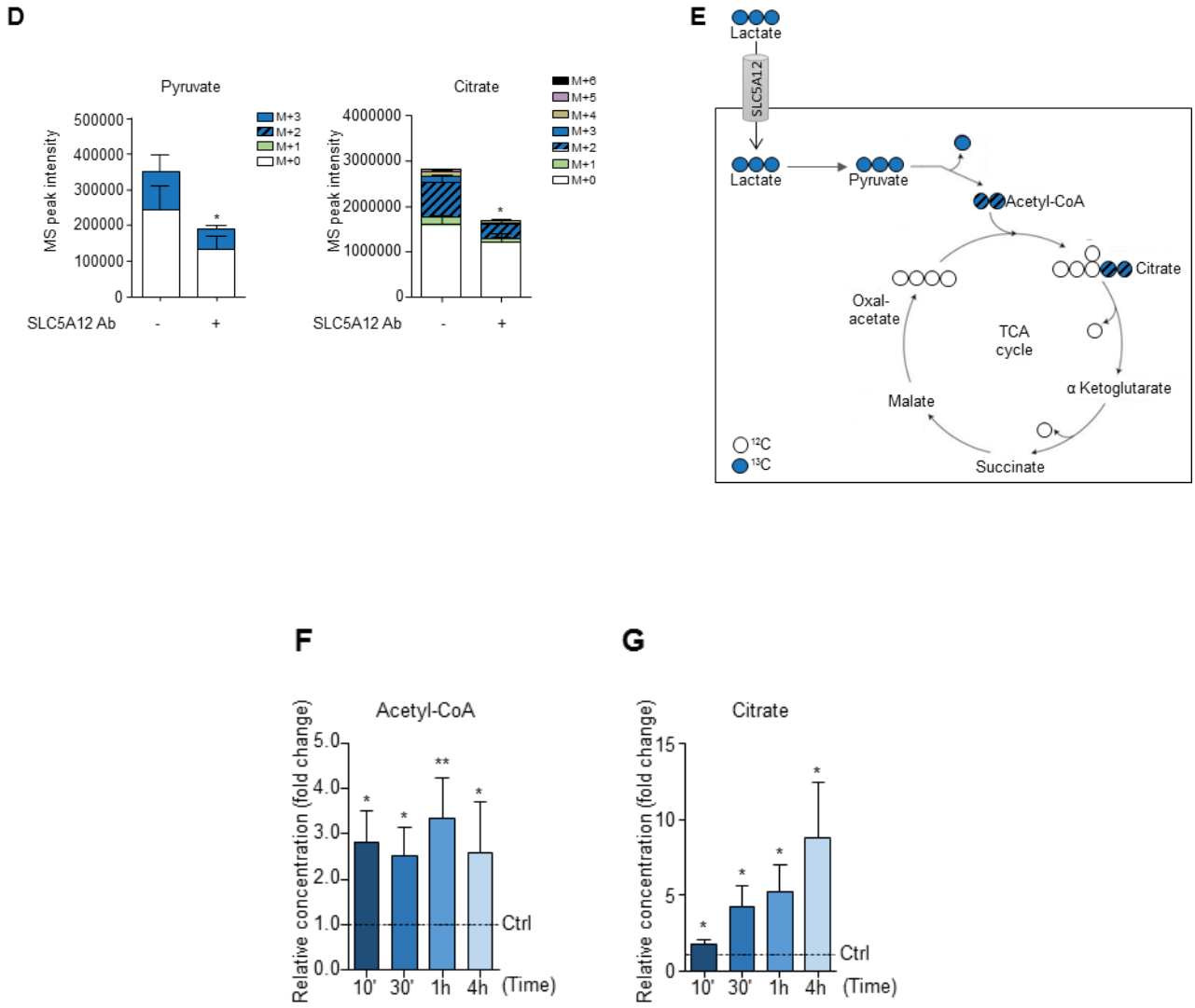


Figure 4 - Lactate uptake by CD4$^+$ T cells impacts intracellular utilization of central carbon metabolic pathways. (A) Glucose, and glutamine uptake rates and lactate release rate for activated CD4$^+$ T cells isolated from HC PBMCs, then activated with anti-CD3 and anti-CD28 mAbs for 24 hours followed by further 48-hour culture with lactate alone or in the presence of SLC5A12 Ab, or left untreated, in medium containing low glucose (5 mM) and 5% FB. (B) NAD$^+$ and NADH intracellular levels in CD4$^+$ T cells treated with sodium lactate (10 mM) for the indicated time points after 72-hour activation and shown as NAD$^+$/NADH ratio. Lactate-untreated CD4$^+$ T cells (Ctrl - dotted line) set to 1. (C) Seahorse measurements of extracellular acidification (left) and oxygen consumption (right) rates (ECAR and OCR, respectively) by CD4$^+$ T cells treated with sodium lactate (10 mM) and/or SLC5A12 Ab, or left untreated after 72-hour activation. (D-E)^{13}C tracing of ^{13}C-U-lactate into pyruvate and citrate. Activated CD4$^+$ T cells were incubated for 48 hours with ^{13}C-U-lactate in the presence or absence of SLC5A12 Ab (2.5 ug/mL,

HPA060904 - Atlas Antibodies) in medium containing low glucose (5 mM) and 5% FBS. Polar metabolites were extracted, analysed by LC-MS and peak areas of mass isotopologues normalized to cell number are represented. (F-G) acetyl-CoA (F) and citrate (G) intracellular levels in $CD4^+$ T cells treated with sodium lactate (10 mM) for the indicated time points after 72-hour activation. Lactate-untreated $CD4^+$ T cells (Ctrl - dotted line) set to 1. Data is representative of n = 3 biological replicates (A, C, G), n = 2 biological replicates (B, D F). Two-tailed Student's *t*-test (A-G) was used for comparisons. Data are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.

5.3 CHAPTER III – LACTATE MODULATES IL-17A PRODUCTION BY HUMAN CD4$^+$ T CELLS

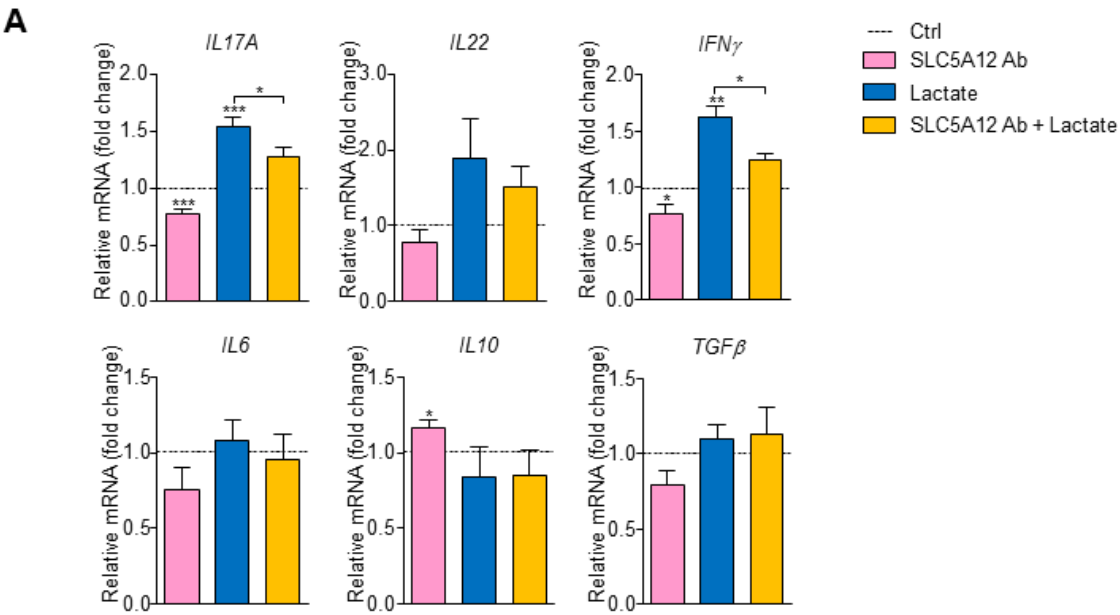
5.3.1 *Lactate shapes the effector phenotype of CD4$^+$ T cells at the site of inflammation via SLC5A12*

Based on the findings showing that lactate modulates SLC5A12 expression by CD4$^+$ T cells, I then tested the effects of exposure to high levels of lactate in the presence or absence of SLC5A12 Ab on the effector phenotype of activated tissue-resident CD4$^+$ T cells isolated from inflamed human tonsils. I observed an up-regulation of *IL17A* and *IFNγ* mRNAs in response to lactate, which was reversed by incubation with SLC5A12 Ab. IL17-family member *IL22* also showed a tendency to upregulation in response to lactate. I did not observe any significant modulation in other cytokines (i.e. inflammatory *IL6* or immunosuppressive *IL10* and *TGFβ*; **Figure 5A**). However, the observed upregulations at the mRNA level resulted in only *IL17A* but not *IFNγ* upregulation at the protein level upon treatment with lactate and again this response was abolished by incubation with SLC5A12 Ab (**Figure 5B**).

Supporting the findings of IL17 upregulation, also the mRNA of *RORγT*, the signature transcription factor of the Th17 T cell subset, was elevated as a consequence of exposure to lactate and again this response was abolished by incubation with SLC5A12 Ab (**Figure 5C**). Interestingly, the expression of the transcription factor *FOXO1*, which limits the differentiation of CD4$^+$ T cells to the Th17 subset and the consequent production of IL-17 (Ouyang W, 2009), was reduced by treatment with lactate, even though incubation with anti-SLC5A12 Ab did not have any effects on its expression (**Figure 5C**). Consistent with data in **Figure 5A**, expression of *FOXP3*, the signature

transcription factor of regulatory T (Treg) cells producing *TGFβ* and *IL10* was not modified by lactate treatment (**Figure 5C**). Furthermore, I observed a lactate-dependent regulation of *PD1* but not of the transcription factor *BCL6* or the chemokine receptor *CXCR5* (**Figure 5C**).

To gain direct insights on the impact of lactate on the effector phenotype of $CD4^+$ T cells at the site of inflammation, we conducted intracellular staining of $CD4^+IL17^+$ (Th17), CD4+IFNγ^+ (Th1), $CD4^+PD1^+CXCR5^+$ (Tfh) and $CD4^+FOXP3^+$ or $CD4^+IL10^+$ (Treg) subsets isolated from highly inflamed tonsils (**Figure 5D**). Incubation with SLC5A12 Ab resulted in a reduction in the Th17 and Tfh T cell subsets with a less pronounced but still significant reduction in the Th1 and no modulation of the Treg subsets (**Figure 5D**).



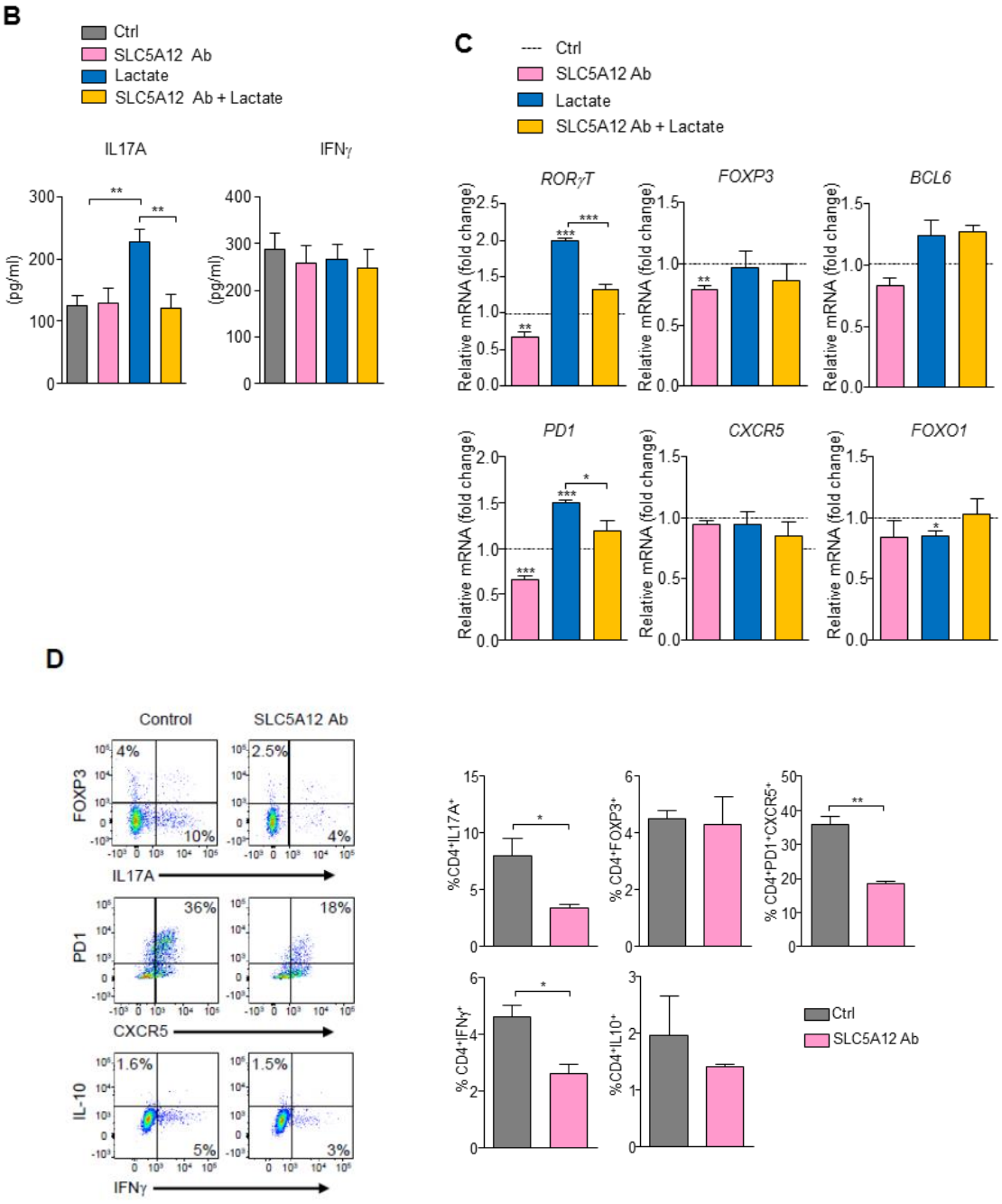


Figure 5 - Lactate modulates T cell effector functions and these effects are counteracted by SLC5A12 blockade. (A) Relative mRNA expression levels of the cytokines *IL17A*, *IL22*, *IFNγ*, *IL10*, *IL6*, *TGFβ* as assessed by qRT-PCR in 12h anti-CD3/CD28 mAbs activated $CD4^+$ T cells treated with sodium lactate (10 mM) +/- anti-SLC5A12 Ab (2.5 ug/mL, HPA060904 - Atlas Antibodies); mRNA levels of each cytokine expressed by untreated $CD4^+$ T cells were set to 1 (Ctrl - dotted line). (B) IL-

17A and IFNγ ELISAs (pg/mL), from supernatants of 24h activated $CD4^+$ T cells. Cells were treated with sodium lactate +/- SLC5A12 Ab or left untreated. (C) Relative mRNA expression levels of *RORγT*, *FOXP3*, *FOXO1*, *BCL6*, *PD1*, *CXCR5*, as assessed by qRT-PCR in 12h anti-CD3/CD28 mAbs activated $CD4^+$ T cells treated with sodium lactate in presence or not of anti-SLC5A12 Ab. mRNA levels in untreated $CD4^+$ T cells (Ctrl - dotted line) were set to 1. (D) Representative flow cytometry plots (left) and percentage (right) of $CD4^+IL17^+$ (Th17), $CD4^+IFN\gamma^+$ (Th1), $CD4^+PD1^+CXCR5^+$ (Tfh) and $CD4^+FOXP3^+$/$CD4^+IL10^+$ (Tregs) tonsil-resident $CD4^+$ T cells. Cells were treated or not with anti-SLC5A12 Ab. Data is representative of n = 5 biological replicates (A, C), n = 5 biological replicates with technical duplicates (B, left), n = 5 biological replicates (B, right) and n = 3 biological replicates (D). Comparisons were evaluated Student's *t*-test. Data are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.

5.3.2 *Lactate induces IL-17 expression via nuclear PKM2-mediated Stat3 phosphorylation and enhanced fatty acid synthesis (FAS)*

I next asked how lactate may promote IL-17 expression and whether the lactate influx via SLC5A12 and its intracellular conversion to citrate may play a role in this response of $CD4^+$ T cells. Exposure of activated $CD4^+$ T cells to inflamed tissue levels of lactate caused a rapid, marked elevation of intracellular reactive oxygen species (ROS; **Figure 6A**) in line with increased NADH levels described in **Figure 4B**. PKM2 molecules normally function as homo-tetramers in the cytosol converting phosphorenolpyruvate to pyruvate in the last reaction of glycolysis. ROS can promote the dimerization of the glycolytic enzyme pyruvate kinase M2 (PKM2). PKM2 dimers are found in the nucleus where they can phosphorylate transcription factors, including signal transducer and activator of transcription 3 (Stat3), a known transcriptional regulator of IL-17 (Shirai T, 2016; Yang XO, 2007). Indeed, I found that lactate also promoted the translocation of PKM2 in the nucleus and the phosphorylation of Stat3 (**Figure 6B**). Activation of Stat3 occurred as early as 1 hour after cell treatment with lactate (**Figure 6B**) and could still be observed at 12 hours (**Figure 6C**). Stat1, another Stat family-member implicated in Th17 differentiation (Peters A, 2015), was also phosphorylated at the same time point (**Figure 6C**). Phosphorylation of Stat1/3 was ablated by incubation with SLC5A12 Ab (**Figure 6C**).

The involvement of glycolysis in the lactate-mediated IL-17A production has been ruled out previously (Haas R, 2016). In particular, neither glycolysis activators nor inhibitors were able to reverse lactate-induced IL-17 (Haas R, 2016).

For this reason, I investigated other possible metabolic pathways that could be involved in this phenomenon. In this context, *de novo* fatty acid synthesis (FAS) is a biological process that is implicated in the differentiation of the Th17 T cell subset (Berod L, 2014).

In addition, I observed that activated $CD4^+$ T cells can take up lactate and convert it to citrate and acetyl-CoA (**Figure 4D-G**), which are the substrates of FAS.

I therefore asked whether exposure to lactate may induce FAS in these cells by assessing the activation levels of A5'-AMP activated protein kinase (AMPK) and acetyl-CoA-Carboxylase (ACC) key enzymes in the regulation of cellular fatty acid metabolism. I found that lactate caused a decrease in phosphorylated ACC at Serine 79 indicating increased ACC enzymatic activity (**Figure 6D**). Moreover, I also detected a decrease in phosphorylated AMPKα at Threonine 172 (**Figure 6D**), indicating reduced AMPK enzymatic activity.

ACC is a key enzyme in the regulation of cellular fatty acid metabolism and exists in humans and other mammals in two isoforms, ACC1 and ACC2. Both enzymes catalyse the ATP-dependent carboxylation of CoA. Whereas ACC1 is present in the cytosol and is crucial for *de novo* synthesis of fatty acids (Chirala SS, 2004), ACC2 is associated with the outer mitochondrial membrane and functions as an important regulator of mitochondrial fatty acid oxidation (FAO, Abu-Elheiga L, 2001; Abu-Elheiga L, 2009). It has been recently found that the inhibition of ACC1 restrains the formation of human and mouse Th17 and promotes Tregs development (Berod L, 2014). Opposite to Th17, Treg cells rely on mitochondrial lipid oxidation in order to proliferate following the activation of AMP-activated kinase (AMPK) (Michalek RD, 2011). However, AMPK does not only regulate FAO but it also inhibits *de novo* FAS (Everts B, 2014; Lee J, 2014).

ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the main molecular precursor for fatty acid synthesis. When FAS is activated, malonyl-CoA also acts as an inhibitor for mitochondrial β-oxidation as it inhibits carnitine palmitoyl transferase 1a (CPT1a) by direct interaction and thus the transport of fatty acids into the mitochondrial matrix (Foster DW, 2012).

In line with this, I found a marked decrease in phosphorylated ACC in the cytosol of activated, lactate-treated $CD4^+$ T cells but no major change in phosphorylated ACC in the mitochondria (**Figure 6E**). Taken together these data suggest that lactate may not have major effects on fatty acid oxidation (FAO) but that lactate-derived citrate and acetyl-CoA may serve as substrates for *de novo* synthesis of fatty acids, in activated $CD4^+$ T cells.

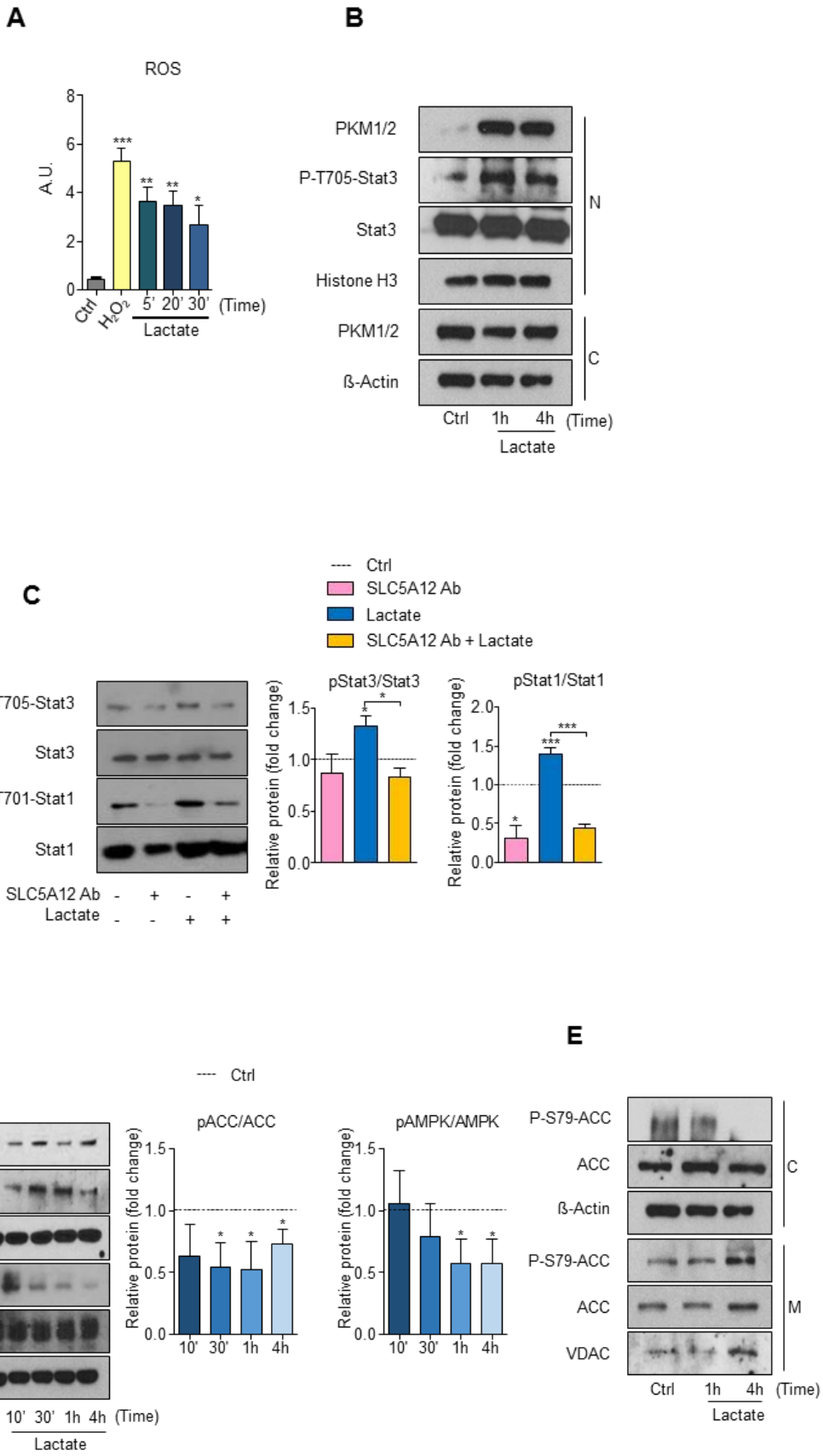
To test any effects of lactate on FAO in a more direct fashion, OCR was measured in activated $CD4^+$ T cells cultured in 2.5 mM glucose and 1 μM BSA-palmitate that served as a substrate for FAO, or BSA alone. BSA-palmitate raised OCR as compared to BSA alone, but lactate did not affect either condition. Addition of etomoxir, an inhibitor of the key enzyme carnitine palmitoyltransferase-1 (CPT1) in the initiation of FAO, reduced BSA-palmitate OCR to the levels observed in the BSA alone control and again this effect was not affected by lactate (**Figure 6F**).

To then test whether lactate-derived citrate and acetyl-CoA maybe utilized for *de novo* synthesis of fatty acids, activated $CD4^+$ T were incubated with [^{13}C]-U-lactate and traced [^{13}C] labelling in palmitate. As shown in **Figure 6G**, lactate [^{13}C] labels nearly 50% of newly synthesized palmitate. This effect was inhibited in cells that were incubated with SLC5A12 Ab indicating incorporation of lactate derived carbons in palmitate backbone (**Figure 6G**).

Given the importance of both Stat3 and FAS in the differentiation of the Th17 T cell subset (Berod L, 2014; Shi, LZ 2011), I finally asked whether lactate may modulate the expression of IL-17 via either or both pathways. Activated $CD4^+$ T cells were treated with: 5-(tetradecyloxy)-2-furoic acid (TOFA), a competitive inhibitor of acetyl-CoA carboxylase (ACC; Berod L, 2014); 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid, inhibitor of fatty acid synthase (C75; Shen Yi, 2017). dehydroepiandrosterone (DHEA), an inhibitor of glucose-6-phosphate dehydrogenase

(G6PDH, Raineri R and Levy HR, 1970), key step in the pentose phosphate pathway (PPP) providing NADPH equivalents for FAS (Schwartz AG, 2004; Dugan RE and Porter JW, 1969) was also added. As expected, all three inhibitors increased phosphorylated ACC levels, indicating an inhibitory effect on FAS. DHEA also increased phosphorylated AMPK (**Figure 6H**). With these three compounds, I then tested the impact of lactate-induced FAS on Stat3 activation and IL-17 synthesis. All three drugs reduced lactate-induced phosphorylation of Stat3 (**Figure 6I**) and expression of IL-17A (**Figure 6J**). Also the AMPK activator 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR; Corton JM, 1995) and the potent and selective PKM2 activator N,N'-diarylsulfonamide (DASA), which stabilizes cytosolic PKM2 homo-tetramers and prevents PKM2 dimer translocation in the nucleus (Anastasiou D, 2011), markedly reduced the expression of IL-17A (**Figure 6J**). Interestingly, a combination treatment with DASA and C75 or TOFA resulted in an additional reduction of lactate-induced IL-17A production as compared to each compound alone (**Figure 6J**).

Taken together these data indicate that lactate modulates IL-17 expression by activating two pathways, PKM2 translocation in the nucleus and enhanced FAS, converging on Stat3-induced transcription of IL-17 (**Figure 6K**).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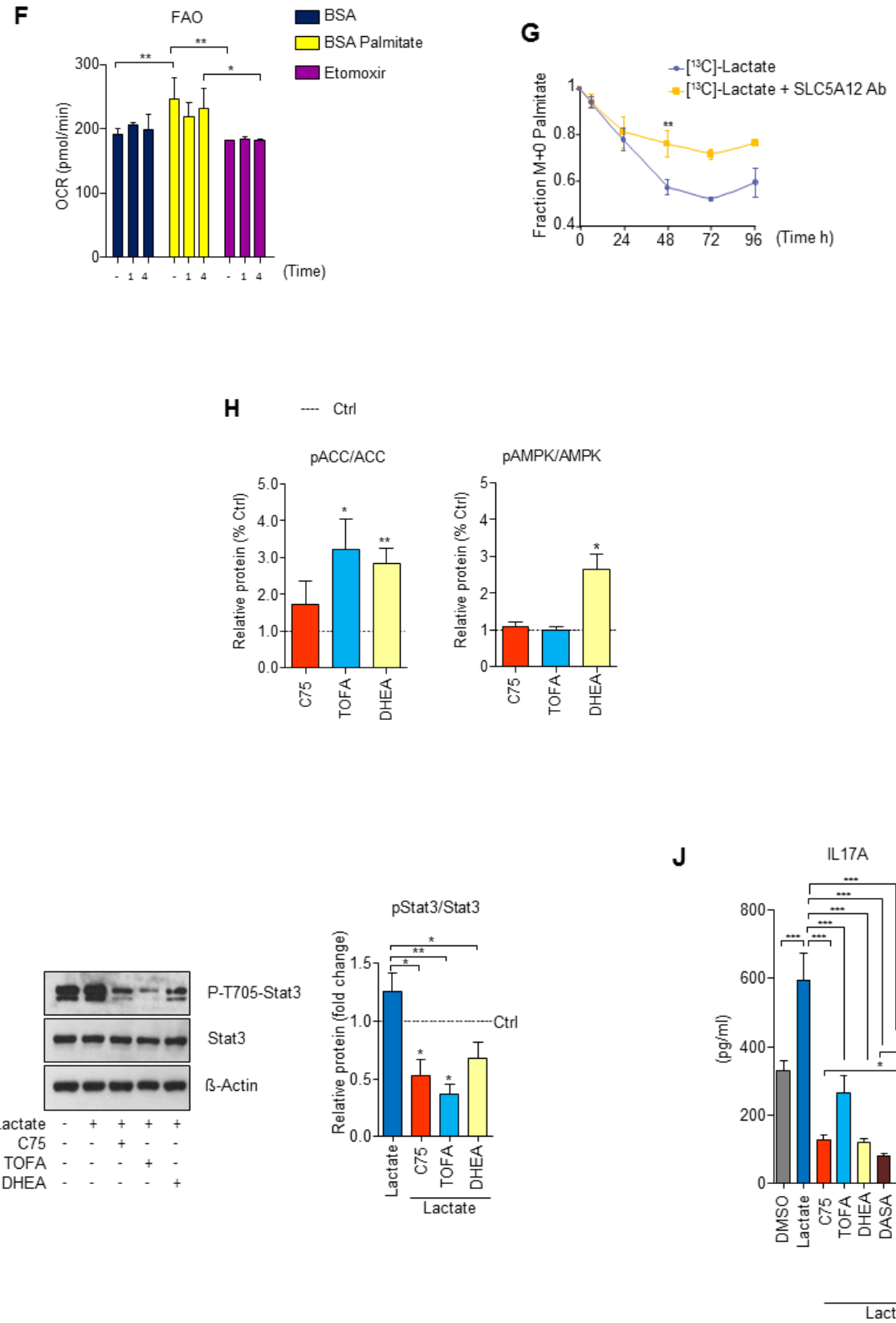
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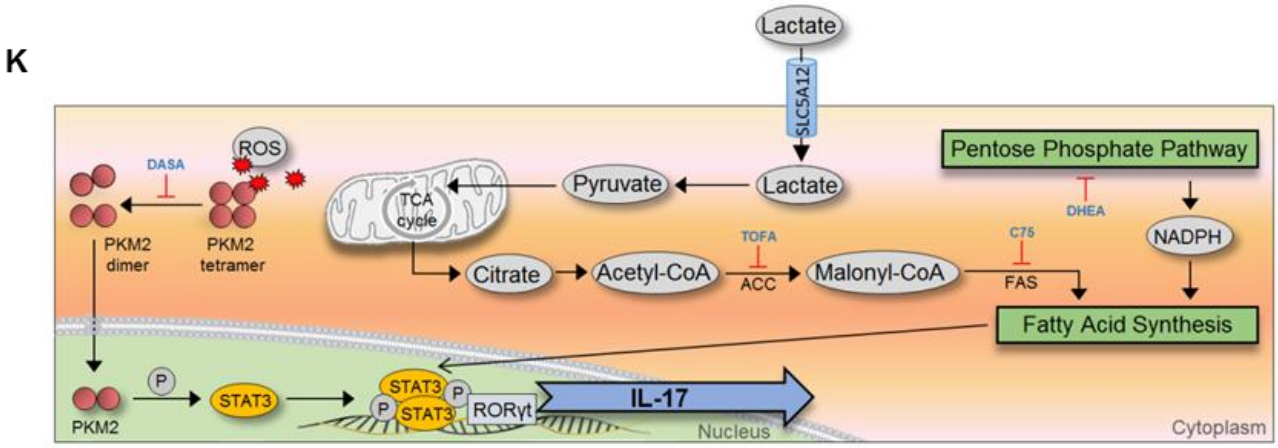


Figure 6 - Lactate induces IL-17A via PKM2-mediated STAT3 phosphorylation and FAS. (A) Reactive oxygen species (ROS) in activated $CD4^+$ T cells in presence of sodium lactate (10 mM, time course). PBS only or H_2O_2 were used as negative and positive control respectively. (B) Western blots of cytosolic or nuclear fractions of anti-CD3/CD28 mAbs (TCR)-activated $CD4^+$ T cells untreated or treated with sodium lactate (10 mM) for 1h and 4h, detected with antibodies against PKM1/2, P-Stat3 (P-T705-Stat3), Stat3, β-actin and Histone H3. (C) Western blot and densitometric analysis of P-Stat3 (P-T705-Stat3), Stat3, P-Stat1 (P-T701-Stat1), Stat1 in activated human $CD4^+$ T cells in presence or not of sodium lactate. Cells were pre-treated (1 hour) with anti-SLC5A12 Ab or left untreated. (D) Western blot analysis and densitometric quantifications of P-ACC (P-S79-ACC), ACC, P-AMPK (P-T172-AMPK) and AMPK in activated $CD4^+$ T cells in presence of sodium lactate (time course). Beta-actin was used as control. (E) Western blots of cytosolic or mitochondrial fractions of TCR-activated $CD4^+$ T cells untreated or treated with sodium lactate (10 mM) for 1 and 4 hours, detected with antibodies against P-ACC (P-S79-ACC) and ACC. Beta-actin and voltage-dependent anion-selective channel protein (VDAC) were used as control. (F) Fatty acid oxidation (FAO) in TCR-activated $CD4^+$ T cells treated or not with sodium lactate (10 mM) for 1h and 4h in the presence of 2.5 mM Glucose, BSA-palmitate (167 μM) or BSA alone or CPT1a inhibitor Etomoxir (40 uM). (G) Quantification of [^{13}C] labelled palmitate in TCR-activated $CD4^+$ T cells treated or not with [^{13}C]-lactate (10 mM) in presence or not of anti-SLC5A12 Ab. (H) Densitometric quantifications of P-ACC (P-S79-ACC), ACC, P-AMPK (P-T172-AMPK) and AMPK in TCR-activated $CD4^+$ T cells treated with metabolic drugs C75 (10 uM), TOFA (20 uM) and DHEA (20 uM). (I) Western blots (left panel) and densitometric analysis (right panel) of P-Stat3 (P-T705-Stat3) and Stat3 in activated human $CD4^+$ T cells in presence or not of sodium lactate and

metabolic drugs C75 (10 uM), TOFA (20 uM) and DHEA (20 uM). (J) IL-17A production (pg/mL) from supernatants of TCR-activated $CD4^+$ T cells treated overnight with sodium lactate and metabolic drugs C75 (10 uM), TOFA (20 uM), DHEA (20 uM), DASA (20 uM), AICAR (1 mM). (K) Schematic depicting selected intracellular metabolic pathways and signalling modulated by lactate. Data is representative of n = 3 biological replicates (A, C, D, F), n = 2 biological replicates with technical duplicates (G-I), n = 5 biological replicates with technical duplicates (J). Comparisons were evaluated using two-tailed Student's *t*-test (A-D, G), two-way ANOVA (F) or one-way ANOVA (I, J). Data are expressed as mean ± s.e.m.*P<0.05; **P<0.01; ***P<0.001.

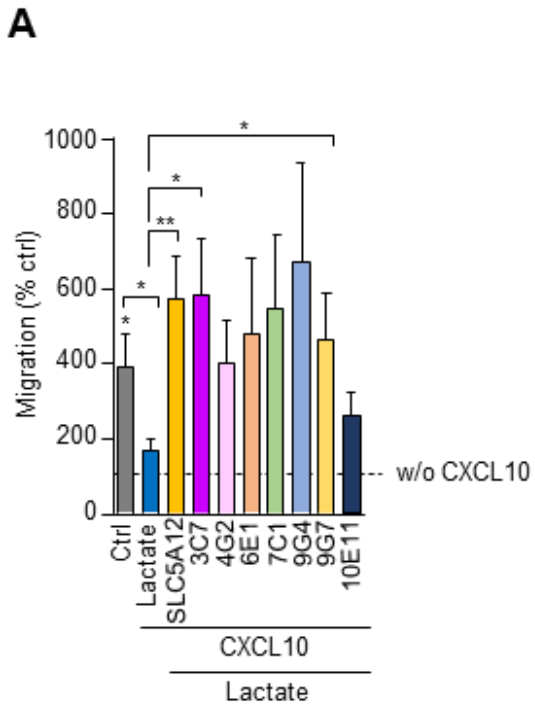
5.4 CHAPTER IV – LACTATE INDUCES T CELL ENTRAPMENT IN THE INFLAMMATORY SITE

5.4.1 *SLC5A12 blockade reverses lactate inhibition of CD4$^+$ T cell locomotion*

Building upon our previous findings that inflamed tissue levels of lactate induce a 'stop migration signal' in activated CD4$^+$ (Haas R, 2015; Pucino V, 2017), I then assessed the impact of interfering with SLC5A12 function on human CD4$^+$ T cell migration *in vitro* and ability to egress from the inflamed tissue *ex vivo*. HC CD4$^+$ T cells were isolated using negative selection beads, activated with anti-CD3 and anti-CD28 antibodies for 3 days and the chemotactic response was induced by the pro-inflammatory chemokine CXCL10. Isolated activated CD4$^+$ T cells were treated with sodium lactate (10 mM) in presence or not of anti-SLC5A12 Abs. I used in parallel the polyclonal Ab (2.5 ug/mL, HPA060904 - Atlas Antibodies) and the monoclonal anti-SLC5A12 mAb (1:50) generated by our group (*see paragraph 4.8*). Chemokinesis was evaluated after 4 hours in a trans-well (**Figure 7A**). As expected, SLC5A12 Ab reversed the 'stop migration signal' induced by lactate (**Figure 7A**; Haas et al., 2015). Furthermore, the mAb clones 3C7 and 9G7 were able to significantly restore the lactate-mediated inhibition of T cell locomotion (**Figure 7A**).

Prompted by these results, I sought to assess the impact of SLC5A12 blockade, on T cell egress, *ex vivo*. I started by culturing equal size tissue sections (1-3 mm^2) from juxtaposing areas of tonsil biopsies – isolated from patients who had been subjected to tonsillectomy – in the presence or absence of lactate and/or anti-SLC5A12 Ab. I then assessed by flow cytometry the type and number of immune cells released in the culture media in each condition (**Figure 7B**). Lactate reduced the egress of CD4$^+$ T cells as

compared to the control condition and this effect was reversed by anti-SLC5A12 Ab. Interestingly, SLC5A12 Ab blockade did not significantly reverse lactate mediated inhibition of cell egress in $CD8^+$, $CD19^+$ or $CD14^+$ cells (**Figure 7B**). I therefore tested 3C7 mAb alongside SLC5A12 Ab in the *ex vivo* egress model in synovial tissues. Again SLC5A12 Ab was able to reverse the lactate-mediated retention of $CD4^+$ T cell in the tissue and a similar effect was obtained with 3C7 mAb (**Figure 7C, D**).



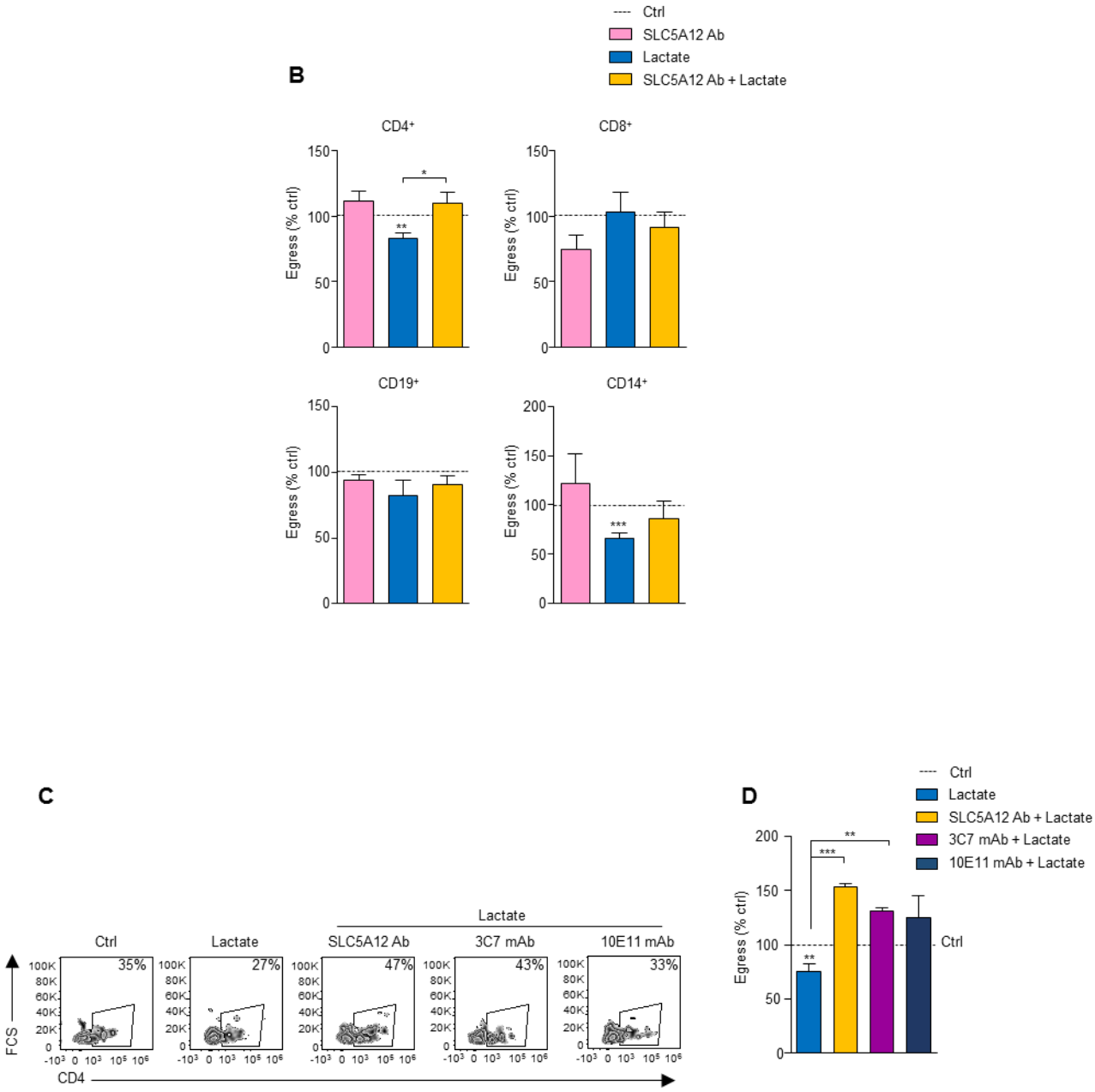


Figure 7 - SLC5A12 blockade promotes the egress of CD4$^+$ T cell from the inflamed tissue. (A) *In vitro* chemotaxis (4 hours) of activated human CD4$^+$ T cells towards CXCL10 (300 ng/mL) in the presence or not of sodium lactate (10 mM) +/- anti-SLC5A12 polyclonal Ab (2.5 ug/mL) or monoclonal mAbs (1:50; 3C7, 4G2, 6E1, 7C1, 9G4, 9G7 and 10E11 mAb clones), as shown. CXCL10 untreated CD4$^+$ T cells (dotted line) were set to 100. (B) Analysis of MCs egress (4 hours) from human tonsil tissues treated with anti-SLC5A12 Ab, sodium lactate or the combination of both. Cells were stained for CD4$^+$, CD8$^+$, CD19$^+$ and CD14$^+$. (C, D) Representative flow cytometry plots

(C) and quantification (D) of egressed $CD4^+$ T cells from RA synovial tissue culture in the shown experimental conditions. Data is representative of n = 3 biological replicates with technical duplicates (A, B), n = 3 biological replicates (D). Graphs show mean ± s.e.m. Comparisons were evaluated using two-tailed Student's *t*-test. Data are expressed as mean ± s.e.m. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

5.4.2 *Lactate limits T cell motility via reducing glycolysis and potentiating FAS*

Next, I asked how the metabolic adaptation of activated $CD4^+$ T cells to inflamed tissue levels of lactate impacted on their response to migratory stimuli. Glycolysis is required for the motility of activated, murine $CD4^+$ T cells (Haas R, 2015) and FAS supports invasiveness of inflamed tissues by peripheral $CD4^+$ T cells (Shen Y, 2017). Based on these findings, I started to investigate how T cells adapt their metabolism in response to the exposure to extracellular lactate. I first analysed the effect of exposure of activated, human $CD4^+$ T cells to lactate on several glycolytic enzymes in a time-course experiment. I observed reduced levels of hexokinase 1 (HK1), HK2, phosphofructokinase (PFK), enolase 1α and pyruvate kinase (PKM1/2; **Figure 8A**), indicating reduced rates of glycolysis, consistent with data in **Figure 4A-C**. However, lactate-induced downregulation of HK1 and enolase1α but not of HK2 and PKM1/2 was impeded by cell incubation with SLC5A12 Ab (**Figure 8B**). These data suggest specific checkpoints of lactate-mediated control of glycolysis, in addition to the observed increased conversion of NAD^+ to NADH, as shown in **Figure 4B**.

Based on the observation that lactate is converted to citrate and increases acetyl-CoA (**Figure 4D-G**), I began to investigate whether the effect of reduction of glycolysis could be related to lactate-induced acetylation followed by protein degradation (**Figure 8C**). Acetylation is mediated by sirtuins, a family of histone deacetylases (HDACs) that are involved in mitochondrial metabolism, aging, and mediate the effect of calorie restriction (Canto C, 2009). Sirtuins catalyze the deacetylation reaction and NAD^+ is the coenzyme. The expression of sirtuins increases when the NAD^+/NADH ratio rises (Gambini J, 2011). When lactate levels increase and this metabolite is re-converted to pyruvate, the NAD^+/NADH ratio decreases as shown in **Figure 4B**, lowering sirtuin activity. The

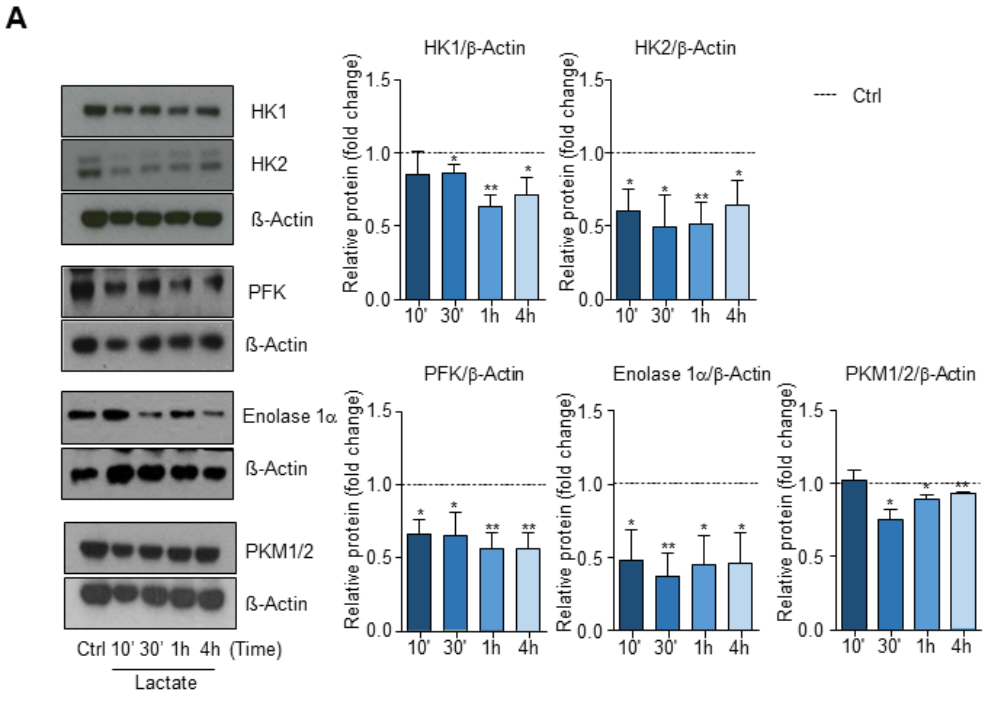
regulation of protein acetylation is considered to be of great importance for mitochondria, which contain high levels of NAD and low number of acetylated proteins, which in turn regulate mitochondrial function (Kim SC, 2006). In the absence of mitochondrial Sirt3, mitochondrial proteins become hyperacetylated, leading in turn to mitochondrial dysfunction, which is a common phenomenon of age and -related diseases (Anderson KA, 2013). On the same line, another study shows that acetylation is also important to regulate glycolysis. The authors found that HK2 binding to the mitochondria is decreased in muscle from high fat diet-fed SIRT3 KO mice, suggesting decreased HK2 activity (Lantier L, 2015).

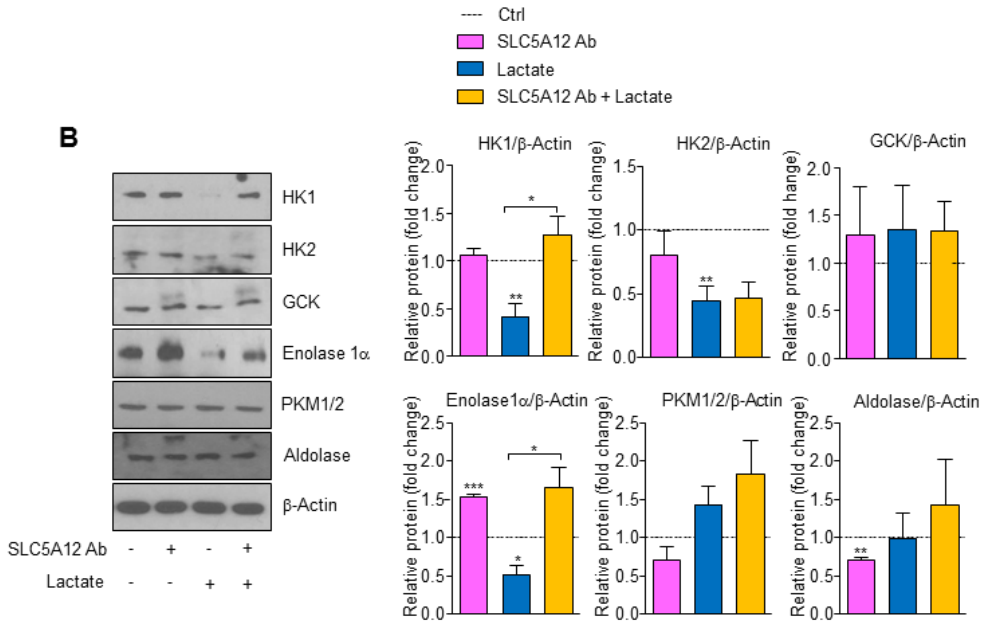
To test sirtuin activity I probed whole cell protein lysates with an antibody that detects acetylated lysine residues. Interestingly lactate treatment did not change the level of protein acetylation in a time course experiment (**Figure 8C**) but this checkpoint control will require further investigations.

Another important checkpoint for the glucose fate is HK2 cellular localization. While cytosolic HK2 mediates glycolysis, mitochondrial HK2 promotes anabolic pathways. More specifically, HK2 localization on to the outer membrane of the mitochondria diverts glucose-6-phosphate in to the PPP producing NADPH equivalents and anabolic intermediates rather than being used in glycolysis to produce energy (Mathupala SP, 2009). Based on this evidence and on the data in **Figure 8A** showing a marked modulation of HK2 upon lactate, I then focused on HK2 and its intracellular localization.

I found an increase in mitochondrial HK2 after 4 hour lactate treatment compared to untreated cells (**Figure 8D**). This observation was supported by confocal microscopy data showing co-localization of HK2 with mitochondria upon 4-hour treatment with lactate (**Figure 8E**). We also found a reduction in the $NADP^+$/NADPH ratio at 1 hour and 4 hours after cell treatment with lactate (**Figure 8F**), consistent with an induced shunt of glucose-6-phosphate into the PPP and with the observed induction in FAS.

Therefore, I asked whether induction of FAS mediated by lactate is required to limit T cell locomotion in the inflamed tissue. Specifically, activated $CD4^+$ T cells were treated with lactate (10 mM) with or without DHEA, TOFA and C75 or left untreated and then subjected to chemokinesis in response to CXCL10. All compounds blocking FAS at different key steps unblocked lactate-induced inhibition of $CD4^+$ T cell motility (**Figure 8G**). Overall, these observations indicate that lactate-induced inhibition of $CD4^+$ T cell response to migratory stimuli and retention in the inflamed tissue is due to a metabolic adaptation which entails reduced glycolysis and translocation of HK2 to the outer membrane of mitochondria, which in turn supports NADPH-dependent de novo FAS (**Figure 8H**).




B
Ctrl
SLC5A12 Ab
Lactate
SLC5A12 Ab + Lactate
HK1
HK2
GCK
Enolase 1α
PKM1/2
Aldolase
β-Actin
SLC5A12 Ab - + - +
Lactate - - + +
HK1/β-Actin
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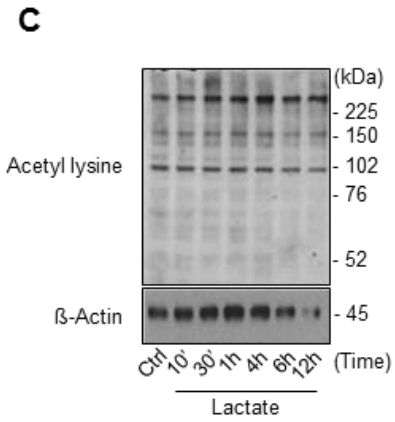
Aldolase/β-Actin
Relative protein (fold change)


C
Acetyl lysine
ß-Actin
(kDa)
225
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102
76
52
45
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10'
30'
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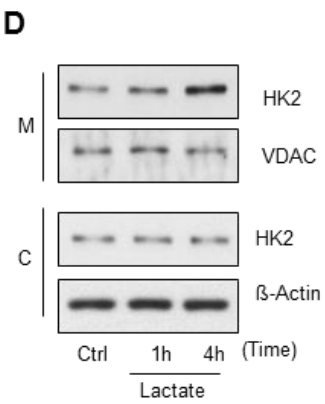
4h
6h
12h
(Time)
Lactate


D
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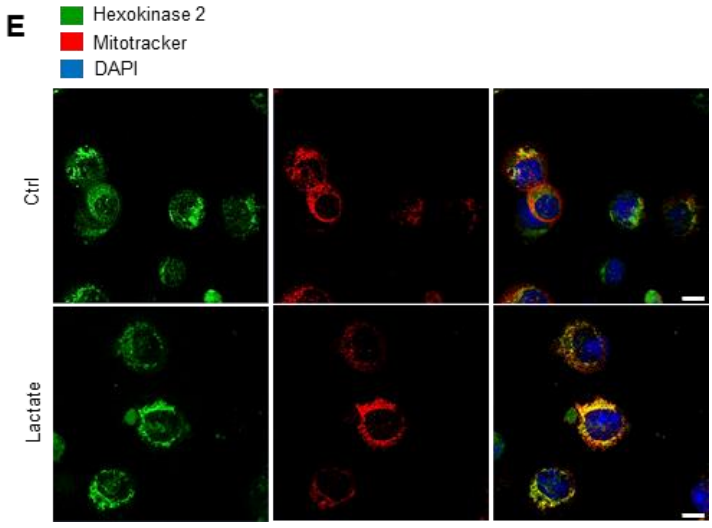
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Hexokinase 2
Mitotracker
DAPI
Ctrl
Lactate

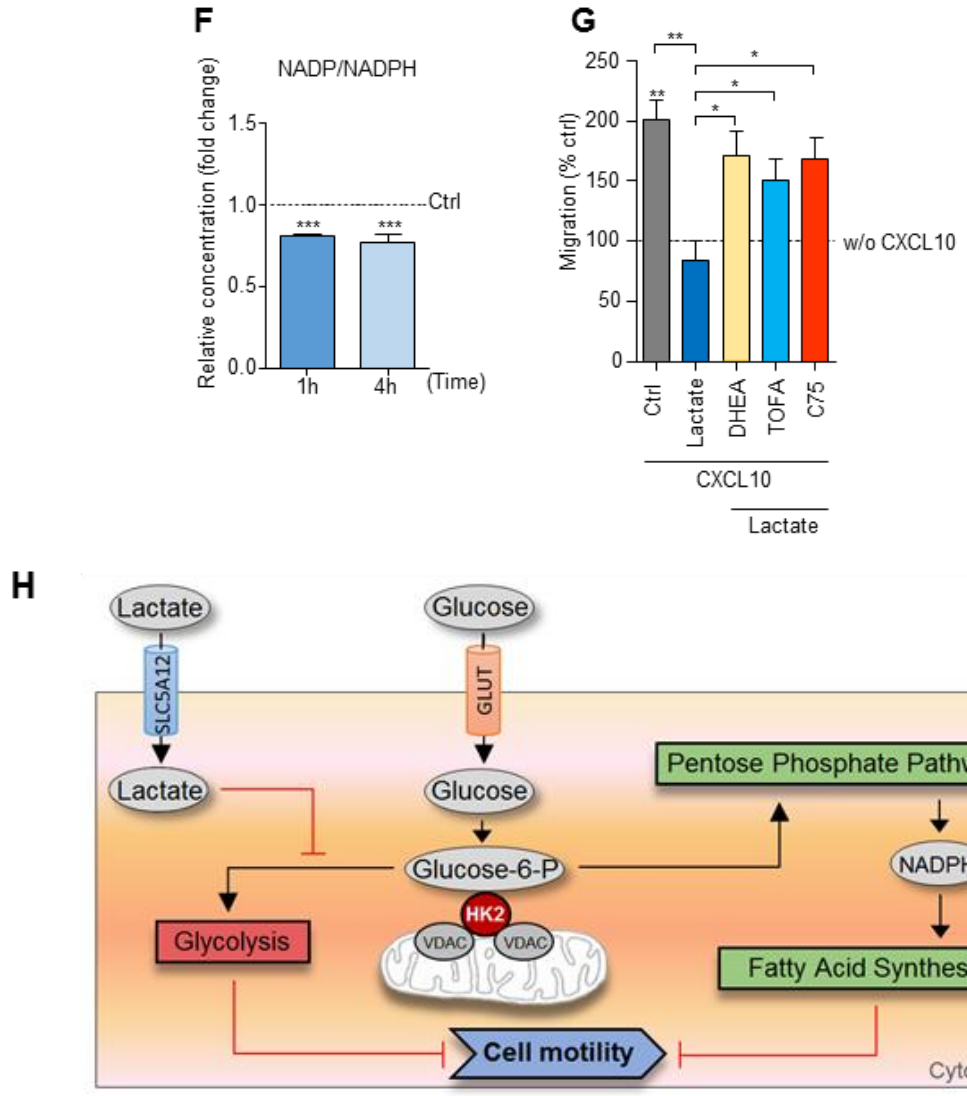


Figure 8 - Lactate reduces expression of glycolytic enzymes and modulates the activation state of enzymes regulating fatty acid metabolism in CD4$^+$ T cells. (A) Time course western blot (left panel) and densitometric analysis (right panel) of HK1, HK2, phosphofructokinase (PFK), enolase 1 alpha and pyruvate kinase M1/2 (PKM1/2) in activated CD4$^+$ T cells treated with sodium lactate (10 mM) for 10', 30', 1h and 4h. Densitometric quantification of western blots denotes mean ± s.e.m. (B) Western blot analysis of HK1, HK2, glucokinase (GCK), enolase 1 alpha, PKM1/2, aldolase; β-actin was used as control (left panel). Activated CD4$^+$ T cells were treated or not with lactate +/- SLC5A12 Ab for 12-24h. Densitometric quantification of western blots (right panel) denotes mean ± s.e.m. (C) Representative western blot of whole CD4$^+$ T cell lysates exposed for different times to 10 mM sodium lactate and probed with antibodies against total Lysine-Acetylation (K-Ac). (D) Western blot analysis of cytosolic (C),

mitochondrial (M) or nuclear (N) fractions of activated $CD4^+$ T cells, treated with sodium lactate (10 mM) for 1h and 4h. Antibodies against HK2, β-actin, VDAC, and Histone H3 were used. (E) Immunofluorescence images of $CD4^+$ T cells untreated or exposed to 4h sodium lactate (10 mM), stained with DAPI, anti-HK2 antibody or MitoTracker Deep Red FM (300 nM). Scale bars 10 uM. (F) $NADP^+$, NADPH, were measured in activated $CD4^+$ T cells (n = 5 with technical duplicates) treated with sodium lactate (10 mM) at different time points and shown as ratio. Untreated $CD4^+$ T cells (Ctrl - dotted line) were set to 1. (G) *In vitro* chemotaxis of activated $CD4^+$ T cells toward CXCL10 (300 ng/mL) in the presence of sodium lactate (10 mM) and metabolic drugs C75 (10 uM), TOFA (20 uM) and DHEA (20 uM). CXCL10 untreated $CD4^+$ T cells (without CXCL10 - dotted line) were set to 100. (H) Schematic of the proposed mechanism of lactate modulation of pentose phosphate pathway and fatty acid synthesis via mitochondrial localization of HK2.

Data is representative of n = 3 biological replicates (A-D), n = 2 biological replicates (E), n = 5 biological replicates with technical duplicates (F), n = 3 biological replicates with technical duplicates (G). Comparisons were evaluated using two tailed Student's *t*-test [A (right panel), B (right panel), F] and one-way Anova (G). Data are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.

5.5 CHAPTER V - CLINICAL RELEVANCE AND THERAPEUTIC ASPECTS: A FOCUS ON RHEUMATOID ARTHRITIS

5.5.1 *Lactate signalling in RA*

For more than 50 years, the inflamed joint has been recognized as a site of low glucose and high lactate (Goetzl EJ, 1971; Treuhaft and McCarty, 1971), reflective of the intense cellular turnover in the rheumatoid pannus. Accumulation of lactate in RA synovial fluid is in part responsible of the acidic environment of RA synovitis. It is well established that synovial fluid pH was significantly lower in active rheumatoids with inflammatory joints than in normal (7.2 vs 7.48) (Cummings NA and Nordby GL, 1966).

The rheumatoid synovial environment is paradigmatic of some of the lactate-induced changes seen in T cells, including cell entrapment, IL-17 secretion and loss of antigen responsiveness (Croia C, 2013). In particular, our group pioneered the discovery that lactate modulates specific T cell subsets via the interaction of lactate transporters. Sodium lactate selectively affects $CD4^+$ T cell functions via SLC5A12, while lactic acid was found to have an impact on $CD8^+$ T cell motility and cytolytic capability via its influx through SLC16A1 (Haas R, 2015; Pucino V, 2017; **Figure IX and Figure X**).

It has also been found by our group that lactate transporter SLC5A12 is highly expressed, at protein and molecular level, in RA synovial tissues and this expression significantly increased in correlation with the inflammatory T cell score of the samples tested (**Figure IX**, Haas R, 2015). Furthermore, lactate in the RA synovial fluid directly promotes an upregulation of the lactate transporter SLC5A12 by $CD4^+$ T cells (*see paragraph 5.1.2*). In line with this evidence, a positive correlation between synovium SLC5A12 and disease activity (measured as the difference between DAS28-CRP at baseline and DAS28-CRP at

6 months), was also found (**Figure 9A, B**). In addition, synovium SLC5A12 was less expressed in patients with a good response to treatment, according the EULAR response (**Table 5**), than in the one with a poor response (**Figure 9A, B**). These findings established lactate signalling as integral feature of RA and open up the possibility of a new biomarker for diseases progression and response to treatment and a novel target for therapeutic intervention.

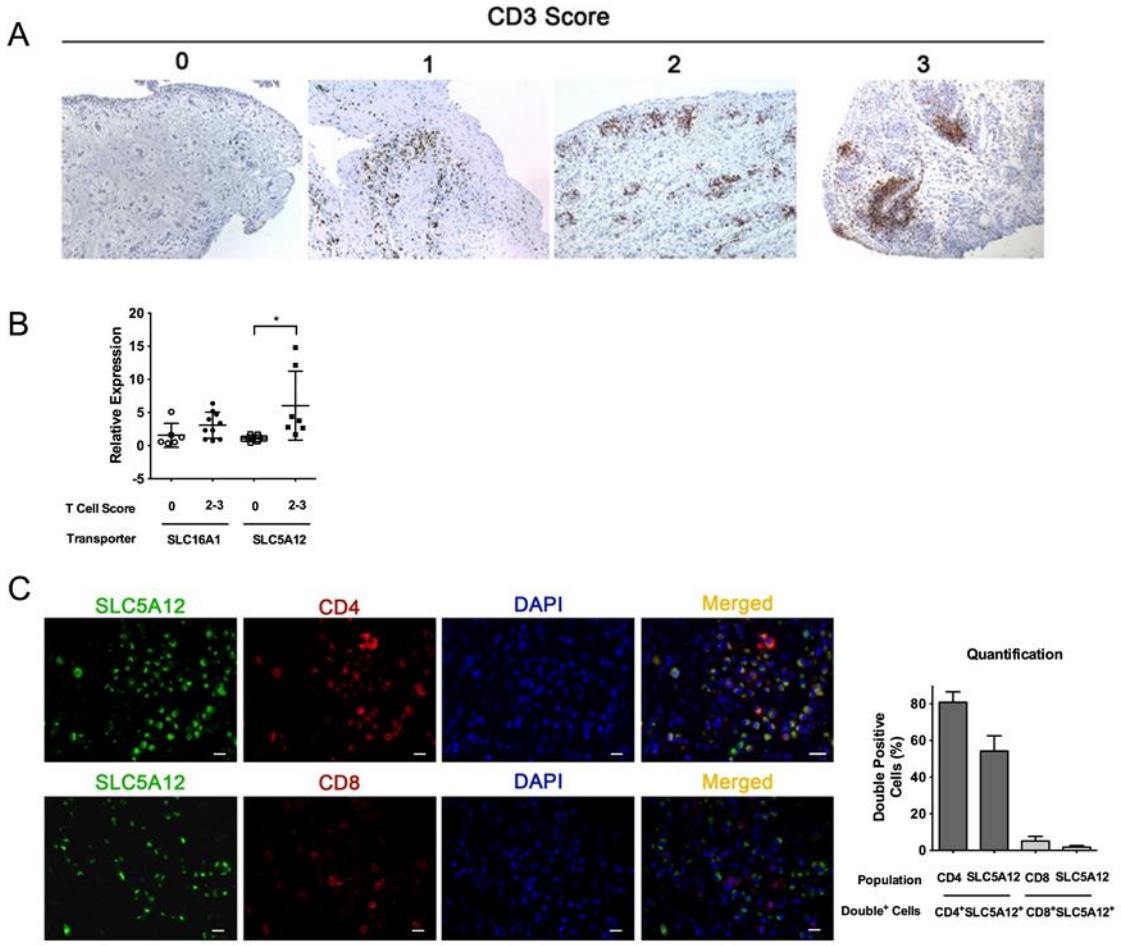


Figure IX - SLC5A12 expression in RA synovium. Representative images of RA synovial tissues stained for CD3 displaying progressively higher degree of T cell infiltration as quantified using a semi-quantitative score from T0 (absence of infiltrating T cells) to T3 (large number of infiltrating T cells organizing in ectopic follicles). (B) Relative mRNA expression levels of SLC16A1 and SlC5A12 in the synovial fluid isolated from the joints of RA patients. (C) Double immunofluorescence staining for

SLC5A12 and CD4 or CD8 in the synovial tissue of RA patients. SLC5A12 (green) is highly expressed within the RA synovium in the presence of a high degree of $CD4^+$ (red) T cell infiltration. Merging (yellow) of the green and red channels demonstrates that SLC5A12 is selectively expressed by $CD4^+$ but not $CD8^+$ infiltrating T cells. Quantification of the percentage of double positive cells is provided upon counting positive cells (single and double positive for each marker) in 6 images per condition. Columns represent % of double positive $CD4^+SLC5A12^+$ population within the $CD4^+$ or $SLC5A12^+$ cells and % of double positive $CD8^+SLC5A12^+$ population within the $CD8^+$ or $SLC5A12^+$ cells. Scale bars: 50 μm (Adapted from: Haas R, 2015).

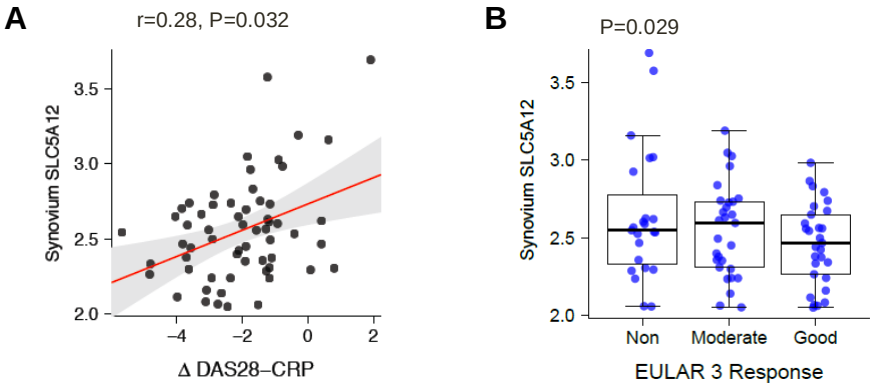


Figure 9 - SLC5A12 correlates with disease activity and response to treatment. (A) Synovium SLC5A12 transcript positively correlates with delta disease activity score (DAS28-CRP) calculated as the difference between DAS28-CRP at baseline and DAS28-CRP at 6 months. (B) Synovium SLC5A12 levels are reduced in patients with good EULAR response to treatment in comparison to non responder patients. Analysis was performed by using R program. Correlation analyses were performed using Spearman's correlation coefficients. One-way ANOVA test was used for comparisons in B; P=0.029 refers to the comparison between non and good response. $P \leq 0.05$ was considered significant.

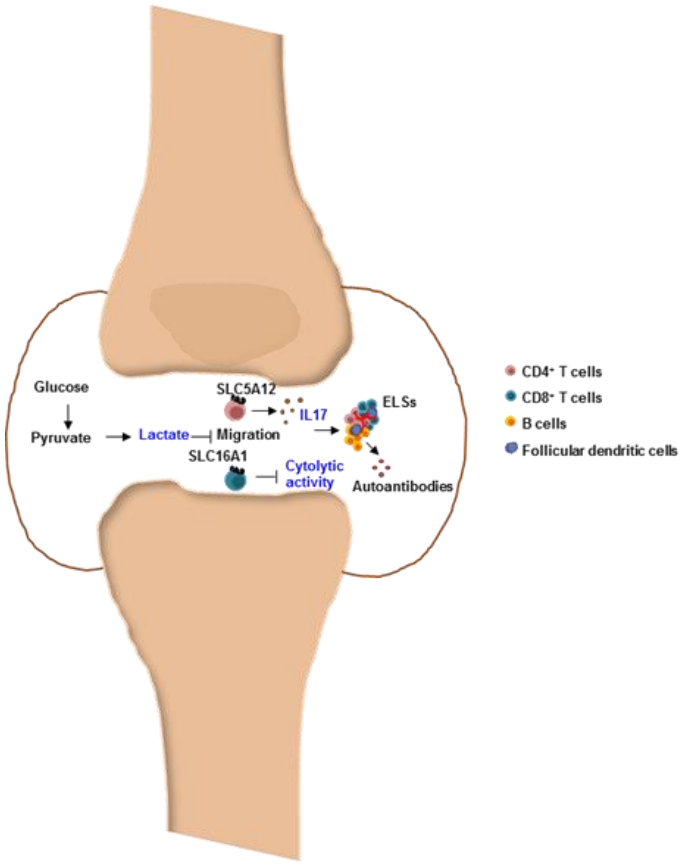


Figure X - Lactate modulates T cell functions in the RA joint. Lactate accumulates in synovial joints of RA patients and modulates T cell functions. Lactate accumulates in the synovial joints during inflammation, where it is in part responsible of the acidic environment. Here, T cells sense high concentrations of lactate via their specific carriers, the sodium lactate transporter SLC5A12 in $CD4^+$ T cells and the lactic acid transporter SLC16A1 in $CD8^+$ T cells. Via this interaction, lactate inhibits T cell motility by interfering with the glycolytic pathway, which is important for T cell migration. Loss of motility might lead to T cell entrapment in the synovial joint, where $CD4^+$ T cells produce high amounts of IL-17 and $CD8^+$ T cells lose their cytolytic activity. Increased retention of $CD4^+$ T cells accompanied by increased local levels of IL-17 might in turn be responsible of the formation of ELSs (Adapted from: Pucino V, 2017).

5.5.2 *Lactate/SLC5A12-induced metabolic signalling network is impaired in RA patients developing ELSs*

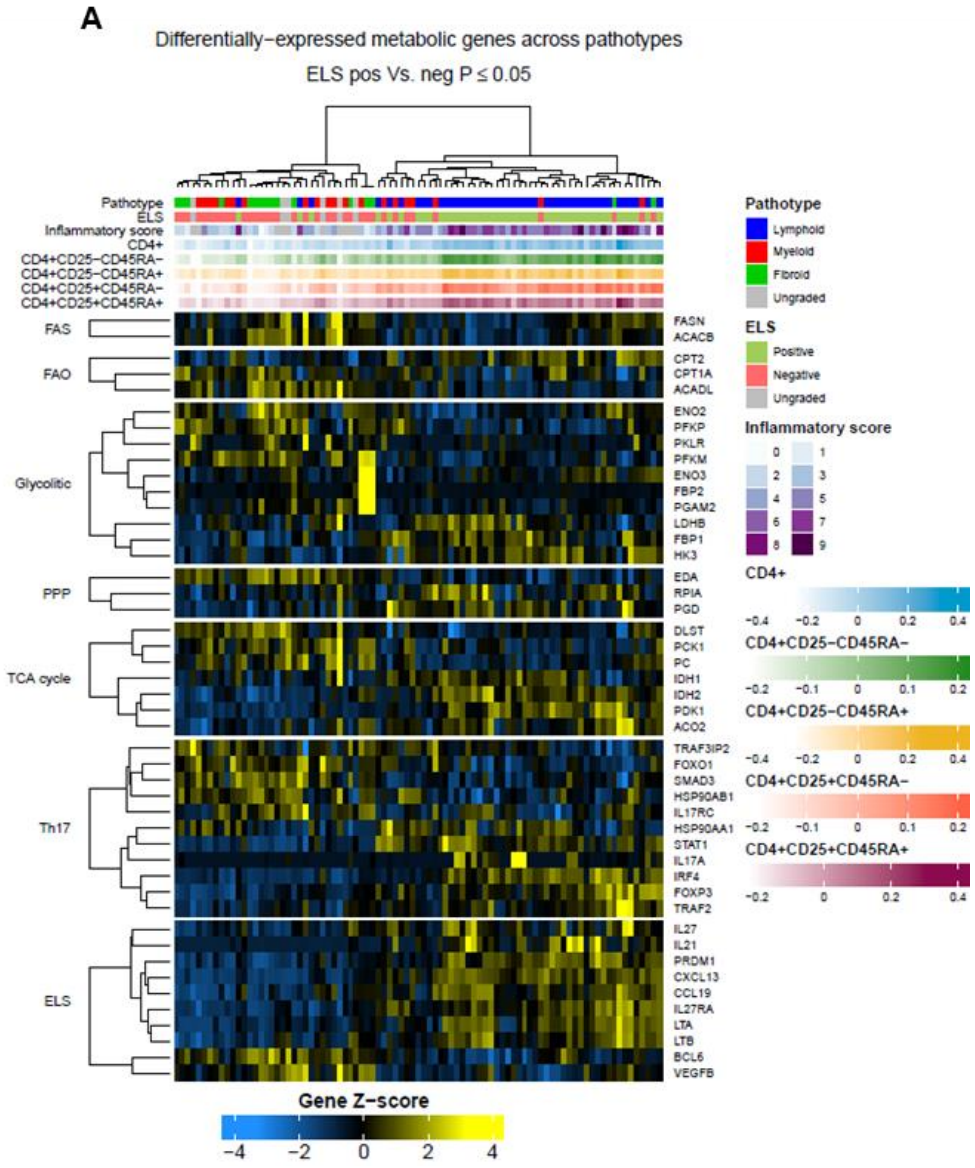
Recently there has been increasing interest in synovial biopsies to obtain inflamed synovial tissue from joints and thereby gain better understanding of the pathogenic events in these diseases. Histopathotype and pathological pathways-based patient stratification prior to therapeutic intervention is becoming an emergent field to identify biomarker predictors of clinical outcomes and responses to therapy (Dennis GJr, 2014; Pitzalis C, 2013)

During these years I have had the opportunity to work on a cohort of naïve-to-treatment RA patients belonging to the Pathobiology of Early Arthritis Cohort (PEAC) available in Prof. Pitzalis' lab. This includes 300 DMARD-naïve early arthritis patients with linked detailed pathobiological data, including gene expression profiling, serum and synovial fluid cytokine and chemokine analysis, biological tissue characterization, state-of-the-art ultrasound imaging and clinical phenotyping (*see paragraphs 2.5.2 and 4.1*).

Synovial biopsies collected for this cohort were classified by histological analysis according to pathotype (i.e. lymphoid, myeloid or fibroid) and presence of ectopic lymphoid structures (ELSs) (Pitzalis C, 2013). These are organized aggregates of T and B cells that develop at sites of chronic inflammation and are associated with more severe disease course and autoimmune responses, as well as reduced response to therapy (Pitzalis C, 2013; Cañete JD, 2009). ELSs are rich in $CD4^{+}IL17A^{+}$ cells which play a pivotal role in ELS formation and maintenance (Jones G, 2016). Synovial biopsies were also classified by histological analysis according to inflammatory score (Krenn score; Krenn V, 2002) and expression of cell-lineage $CD4^{+}$ T cell gene modules. As expected, the synovial biopsies with a lymphoid pathotype were also ELS positive and showed the highest inflammatory score and degree of infiltration by $CD4^{+}$ T cells (**Figure 10A**). In

this cohort I analysed by RNA-sequencing the expression of groups of metabolic genes by n = 87 synovial biopsies. In the lymphoid pathotype, I found evidence of expected patterns of Th17 differentiation genes, i.e. reduced FOXO1 and increased IL17A, as well as of ELS genes. When metabolic genes were analyzed, I found a downregulation of glycolytic genes concurrent with an upregulation of PPP and TCA cycle genes in the lymphoid pathotype as compared to the other pathotypes (**Figure 10A**). These data support a role for lactate/SLC5A12-induced metabolic reprogramming in $CD4^+$ T cells as a distinctive mechanism of lymphoid RA pathogenesis.

Overall, lactate levels in the synovial fluid did not differ between ELS+ and ELS- RA patients (**Figure 10B**).


A
Differentially−expressed metabolic genes across pathotypes
ELS pos Vs. neg P ≤ 0.05
Pathotype
ELS
Inflammatory score
CD4+
CD4+CD25−CD45RA−
CD4+CD25−CD45RA+
CD4+CD25+CD45RA−
CD4+CD25+CD45RA+
FAS
FAO
Glycolitic
PPP
TCA cycle
Th17
ELS
FASN
ACACB
CPT2
CPT1A
ACADL
ENO2
PFKP
PKLR
PFKM
ENO3
FBP2
PGAM2
LDHB
FBP1
HK3
EDA
RPIA
PGD
DLST
PCK1
PC
IDH1
IDH2
PDK1
ACO2
TRAF3IP2
FOXO1
SMAD3
HSP90AB1
IL17RC
HSP90AA1
STAT1
IL17A
IRF4
FOXP3
TRAF2
IL27
IL21
PRDM1
CXCL13
CCL19
IL27RA
LTA
LTB
BCL6
VEGFB
Gene Z−score
−4 −2 0 2 4
Pathotype
Lymphoid
Myeloid
Fibroid
Ungraded
ELS
Positive
Negative
Ungraded
Inflammatory score
0 1 2 3 4 5 6 7 8 9
CD4+
−0.4 −0.2 0 0.2 0.4
CD4+CD25−CD45RA−
−0.2 −0.1 0 0.1 0.2
CD4+CD25−CD45RA+
−0.4 −0.2 0 0.2 0.4
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CD4+CD25+CD45RA+
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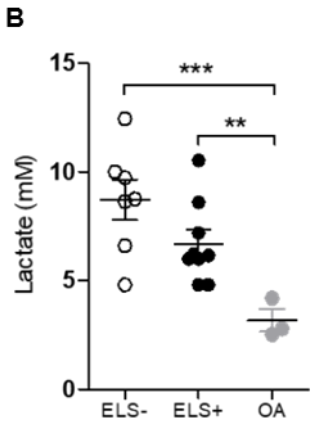
B
Lactate (mM)
15
10
5
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**
ELS-
ELS+
OA

Figure 10 - Synovial RNA-Sequencing in early rheumatoid arthritis shows differential expression of metabolic genes according to the histological patterns of synovitis. (A) Heatmap showing synovial RNA-seq expression of groups of metabolic genes differentially expressed (FDR < 0.05) between synovial biopsies (n = 87) from early rheumatoid arthritis. Synovial biopsies were classified as positive or negative for ELS by histological analysis. Upper tracks show synovial histology inflammatory score (Krenn score), expression level of cell-lineage $CD4^+$ T cell gene modules, ELS histology grouping and overall histology pathotype (Lymphoid, Myeloid or Fibroid). (B) Lactate levels (mM) in the synovial fluid of ELS- (n = 7) and ELS+ (n = 9) RA patients (B). Comparisons were evaluated using one-way ANOVA. Data are expressed as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.

5.5.3 *Antibody-mediated blockade of SLC5A12 ameliorates the clinical course of $CD4^+$ T cell-driven human-glucose-6-phosphate-isomerase (hG6PI)-induced arthritis*

I next interrogated the possible therapeutic implications of my findings. To evaluate the impact of SLC5A12 blockade on arthritis severity, a human glucose 6 phosphate isomerase (G6PI) mouse model was used. This model was chosen because of its similarity with human disease; indeed, G6PI in adjuvant induces symmetric polyarthritis of the small distal joints in genetically susceptible normal mice. Furthermore, $CD4^+$ T cells were the major drivers of arthritis (Schubert D, 2004) hence representing an as close a model as possible to human lymphoid RA. In brief, DBA/1 mice were immunized s.c. with 20 μg human hG6PI synthetic peptide. At the onset of disease (10 days), mice were untreated or treated sub-plantar into the rear paws with infliximab (Remicade, Janssen Biologics), TNF mAb (TN3-19.12, BD bioscience), SLC5A12 Ab, iso-TNF Ab and iso-SLC5A12 Ab. Antibody and the corresponding isotype control was injected into the right or left back paw respectively of the same mouse.

SLC5A12 Ab treatment reduced the arthritis score after 18 days from the onset of the disease as well as the histological score compared the isotype control (**Figure 11A-D**). This effect was even more remarkable than treatment with TNF mAb and Infliximab (**Figure 11A, C**).

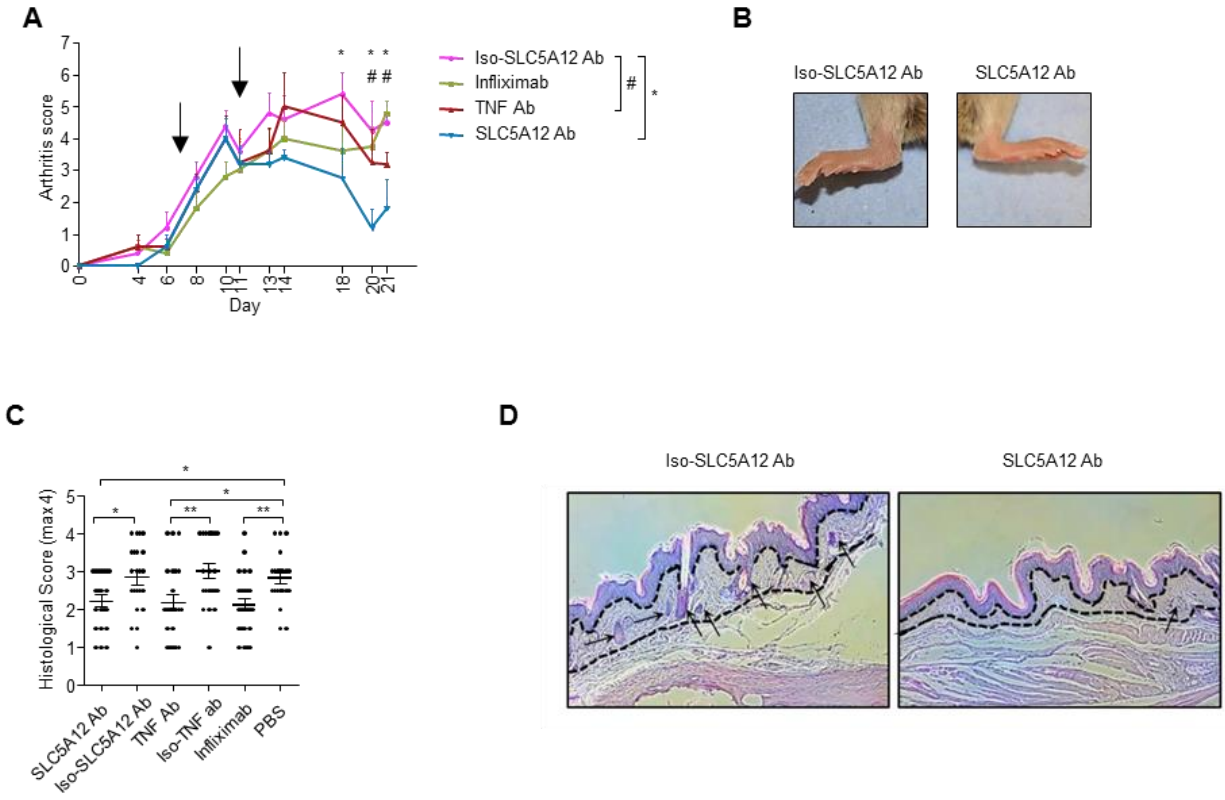

Figure 11 - Antibody-mediated blockade of SLC5A12 ameliorates the clinical course of CD4$^+$ T cell-driven human-glucose-6-phosphate-isomerase (hG6PI)-induced arthritis. (A) Arthritis score in mice treated with SLC5A12 Ab, infliximab, TNF mAb and an isotype control at day 7 and 11. (B) Representative images of the paws at day 21 post-immunization with hG6PI showing the effects of treatment of SLC5A12 Ab as compared to an SLC5A12 isotype control antibody (i.e. swelling, redness). (C) Histological score (max 4) and (D) representative H&E staining of ankles and pads sections showing reduced immune infiltrate in the SLC5A12 Ab treatment group (D, right panel) as compared to PBS and isotype control antibodies (D, left panel). Values denote mean ± SD, A (number of mice per group, n = 6), C (each dot corresponds to an H&E slide acquired from the representative group, n = 19-24 slides/group). Comparisons were evaluated using two tailed Student's *t*-test *P<0.05; **P<0.01.

6 DISCUSSION

For a long time after its discovery at the beginning of the 20th century, lactate has been considered a bystander molecule produced at the end of glycolysis. Only in the 90's, lactate has begun to be recognized as an active metabolite able to regulate immune cell responses. RA is characterized by the accumulation of lactate in the inflamed synovium, which is partly responsible for the establishment of an acidic and hypoxic environment. Here lactate inhibits T cell motility. Furthermore, sodium lactate promotes a plastic switch toward a Th17 subset by $CD4^+$ T cells while lactic acid reduces the cytotoxic capacity of $CD8^+$ T cells. Interestingly, all these events are dependent upon lactate's interference with intracellular metabolic pathways, specifically glycolysis and fatty acid synthesis. These phenomena contribute to the exacerbation of chronic inflammation and might be responsible for the breach of tolerance and the formation of long-lasting ELS at the site of inflammation such as in RA synovitis.

Lactate modulates T cell functions via carriers that belong to two families, the proton-linked-monocarboxylate-transporters (MCTs) and the Na^+-coupled- electrogenic-transporters that are expressed by several tissues including immune system, endothelium and epithelium. Their expression can increase during inflammation or in the tumor site. For this reason, targeting lactate transporters is becoming a new attractive area to develop new therapeutics for inflammation and cancer.

6.1 Lactate promotes metabolic rewiring in human CD4$^+$ T cells

Lactate has been shown to have a variety of effects on cellular metabolism, including the the increase of mitochondrial OXPHOS in neurons (Pellerin L and Magistretti PJ, 1994; Pellerin L, 1998; Magistretti PJ, 2018), the induction of gluconeogenesis during the Cori cycle in the liver and the synthesis of glycogen in skeletal muscle cells (Cornell NW, 1973). Furthermore, our group has recently discovered that lactate directly regulates glycolysis (Haas R, 2015). This takes place via different mechanisms I have investigated throughout these years that are in line with previous evidence showing that lactate acts as a potents glycolysis inhibitor affecting the rate limiting glycolytic enzyme phosphofructokinase (PFK; Leite TC, 2011). In particular, I have observed a decrease in the glucose flux as well as a reduced ECAR upon lactate treatment. Consistent with this observation, also the ratio NAD/NADH was dropped within the first 10 minutes up to 4h of lactate treatment, which is most likely due to the reversal of the LDH reaction due to lactate abundance (**Figure 4B**). NADH is used as an essential cofactor to allow the conversion of pyruvate to lactate by LDH. NADH is oxidized to NAD^+, which allows the glycolysis to persist. Excreting lactate through MCTs/SMCTs eliminates protons arising from the glyceraldehyde 3-phosphate dehydrogenase reaction in glycolysis, thus maintaining the pH homeostasis inside the cells and acidifying the extracellular space. Interestingly, together with reduced glucose flux in the presence of lactate I also observed a decreased lactate release that may suggest that T cells continue to use lactate as a carbon source during glucose deprivation.

This evidence is in line with a recent publication of Faubert et al, where the authors showed that lactate is a TCA cycle carbon source for cancer cells (Faubert B, 2017). Using intra-operative infusions of [^{13}C]-labeled nutrients, they were able to analyze the pattern of enrichment of labelled metabolites directly within the tumour (i.e. lung

tumours). The infusion of [U-^{13}C] glucose showed a different pattern of enrichment in the cancer tissue as compared to the benign lung, with an excess of labelled lactate (rather than other upstream glycolytic metabolites) within the tumour. Interestingly, the infusion of [^{13}C]-lactate caused not only the accumulation of labelled lactate itself in the tumour, but also of labelled pyruvate, alanine and TCA cycle intermediates, showing that lactate was actively being used to supply the TCA cycle. Lactate behaves as a major fuel for TCA cycle intermediates, not only for cancer cells but also for healthy activated T cells as confirmed by flux analysis data labelling lactate with [^{13}C] (**Figure 4D**). An increase of pyruvate and citrate was observed in the presence of [^{13}C]-lactate while a decrease was shown when cells were pre-treated with SLC5A12 Ab, confirming a reduced lactate flux in the TCA cycle by blocking this lactate transporter. These experiments were conducted in low glucose environment (5 mM) thus suggesting that in conditions of glucose deprivation and lactate excess, such as during inflammation, $CD4^+$ T cells utilize lactate as a fuel to sustain their metabolism.

NADH generated upon lactate treatment is taken up by electron transport chain for the generation of ROS (Ying W, 2008). In line with this, the levels of ROS in whole cell extracts after lactate exposure increased rapidly (**Figure 6A**). A possible mechanism for ROS generation can be the presence of flavin-dependent lactate oxidase in the intermembrane space of hepatic mitochondria (de Bari L, 2010). In this paper, the authors demonstrated that exposure of isolated rat mitochondria to lactate produced pyruvate in a 1:1 stoichiometry with the concomitant release of H_2O_2 that was independent of the respiratory chain. The reaction was subsequently inhibited by NAD^+ in a competitive manner. It is possible that a similar mechanism also led to the transient increase in ROS levels in $CD4^+$ T cells. ROS production is regulated by antioxidants such as manganese-dependent superoxide dismutase (MnSOD or SOD2) or glutathione (GSH). How lactate

affects these molecules is still unknown. It is possible that a direct or indirect inhibition of antioxidant systems mediated by lactate might explain the increased levels of ROS.

It is worth mentioning that, although lactate is a carbon fuel for TCA cycle and is able to increase OXPHOS in the brain (Pellerin L, 1994; Pellerin L, 2012; Magistretti PJ, 2018) as well as ROS production, $CD4^+$ T cell mitochondrial respiration, measured as OCR, was not influenced by lactate when compared to untreated cells (**Figure 4C**). Conversely, OCR was decreased by SLC5A12 Ab treatment, a finding that will require further investigations. A possible explanation is that SLC5A12 mediates the transport of other monocarboxylic acids such as pyruvate, propionate, butyrate, nicotinate, and short-chain fatty acids which, differently from lactate, might affect T cell mitochondrial respiration.

In support of the data showing that lactate is a feeder for the TCA cycle, an increase in acetyl-CoA and citrate levels under lactate exposure was also observed.

Acetyl-CoA is produced by the breakdown of both carbohydrates (by glycolysis) and lipids (by β-oxidation). It then enters the TCA cycle in the mitochondrion by combining with oxaloacetate to form citrate. In the cytosol, citrate can be reconverted to acetyl-CoA and then be used to synthesize fatty acids through carboxylation by acetyl-CoA carboxylase into malonyl-CoA, the first committed step in the synthesis of fatty acids. Notably, after lactate treatment I detected activation of the rate limiting enzyme for fatty acid synthesis acetyl-CoA carboxylase (ACC) (**Figure 6D**), which has been shown to be indispensable for IL-17 production in $CD4^+$ T cells (Berod L, 2014). This mechanism will be discussed in detail in the following *paragraph 6.2*.

Acetyl-CoA serves as a substrate for lysine acetyltransferases (KATs), which catalyze the transfer of acetyl groups to the epsilon-amino groups of lysine in histones and many other proteins. Fluctuations in the concentration of acetyl-CoA, reflecting the metabolic state of the cell, are translated into dynamic protein acetylation that regulate a variety of cell functions, including transcription, replication, DNA repair, cell cycle progression, and

aging (Shahbazian MD, 2007). Acetylation of cellular proteins creates specific degradation signals (Hwang CS, 2010) which can in turn modulate the expression of metabolic enzymes, including glycolytic ones, inducing a metabolic reprogramming (Cha Y, 2017). Interestingly, I did not observe an increased acetylation upon lactate treatment (**Figure 8C**) thus suggesting that the reduction of glycolytic enzymes may not be ascribed to protein degradation following acetylation but further investigation to understand this phenomenon is needed.

Along acetyl-coA, citrate levels were also found increased upon lactate treatment (**Figure 4G**). Citrate has been recently shown associated to drive an inflammatory response in M1 macrophages (Jha AK, 2015). Citrate is required for fatty acid biosynthesis, and once in the cytosol it is cleaved by citrate lyase into acetyl-CoA and oxaloacetate. Fatty acids derived from citrate and other metabolic pathways are essential for cellular growth and proliferation, and have been also implicated in DC activation and the production of pro-inflammatory mediators in those cells (Everts B, 2014). The incorporation of glucose-derived carbon into lipids in LPS-stimulated DCs is prevented by C75, an inhibitor of fatty acid synthase. Treatment with C75 impairs the expansion of endoplasmic reticulum and golgi in response to LPS, and subsequently the ability of DCs to present antigen and to generate pro-inflammatory cytokines such as IL-6, IL-12 and TNF, which suggests that the generation of fatty acids is essential for an appropriate immune response in these cells (Michelucci A, 2013). Moreover, citrate is a known inhibitor of glycolysis and its accumulation might be responsible for the prolonged glycolysis inhibition observed (Newsholme EA, 1977).

Notably, these data obtained *in vitro*, showing an increase in TCA cycle substrate availabilty and reduced glycolysis upon lactate treatment, were further confirmed in RA joints that, it is known, are rich in lactate. In particular, this metabolic signature, is characteristic of a specific subset of RA patients (ELS+ or lymphoid "pathotype")

characterized by high number of $CD4^+$ T cells. This evidence is also in line with the findings made by Weyand's group, showing a glycolytic deficiency along with increased PPP and FAS displayed by peripheral RA T cells (Yang Z, 2013; Yang Z, 2016; Shen Y, 2017). This is a very important point, which highlights the strong metabolic correlation between the peripheral and tissue compartment in a disease like RA. Correlation studies between serum/synovial fluid, blood and synovial biomarker profiling may be a promising tool for predicting specific pathogenic pathways in the inflamed synovium of patients with RA.

6.2 Lactate modulation of IL-17 production by human T cells

The mechanisms underpinning the crosstalk between the immune system and metabolism have been intensively studied for the last few years. As a metabolite produced at the end of glycolysis, lactate is emerging as an important signalling molecule promoting specific immune-inflammatory responses. The discovery of specific lactate transporters on the surface of immune cells has opened novel and important perspectives for the understanding of the pathogenesis of inflammatory and autoimmune disorders. Indeed, the effect of lactate on T cells recapitulates key features of T lymphocytes found in chronic inflammatory infiltrates, including their entrapment and production of high levels of IL-17 and loss of cytolytic activity by $CD4^+$ and $CD8^+$ T cells, respectively (Haas R, 2015). These events lead to the perpetuation of chronic inflammation such as in RA synovitis.

It became clear that this pro-inflammatory cytokine plays a key role in autoimmunity and more specifically in RA. The human IL-17A was first cloned in 1996 and it was found to be produced by activated $CD45(RO)^+$ memory helper T cells (Chabaud M, 1999). It was

shown in the same study that IL-17A induces the production of IL-6, IL-8, PGE2, and GCSF in a dose-dependent manner in cultures of RA synovial fibroblasts. The IL-17A effect was blocked with anti-IL-17 antibodies (Chabaud M, 1999). Interestingly, TNF-α had an additive effect on IL-17 induced secretion of IL6. Soon after this observation, it was found that synovial fluids of RA patients have high IL-17A levels compared to those with osteoarthritis. IL-4 or IL-13 completely inhibited the IL-17 production of cultured RA synovium tissue (Chabaud M, 1999) whereas exogenous IL-17 increased IL-6 production in synovial tissue cultures. These observations led to the conclusion that through the production of other pro-inflammatory cytokines, IL-17 has a significant, if not a central, role in the pathogenesis of RA (Chabaud M, 1999). IL-17, in particular, is also considered to be osteoclastogenic and involved in RA bone reabsorption and marginal erosions (Kotake S, 1999). Several mouse models of arthritis show the central role of IL-17 in the pathogenesis of the disease (Kugyelka R, 2016).

Several cell functions, including cytokine production, require energy from metabolic pathways. In this context, glycolysis fuels the energetic demands of $CD4^+$ T cell activation, growth and differentiation. Previous reports have shown that HIF induced glycolysis is necessary to promote the differentiation of naïve $CD4^+$ T cells toward the Th17 subset (Dang EV, 2011). Glycolysis is also necessary for the generation of inducible Treg cells from Tconv; indeed, it was found that the binding of the glycolytic enzyme enolase-1 to the FOXP3 promoter and to the conserved non-coding sequence 2 (CNS2) of the FOXP3 locus directly affected the expression of *FOXP3* splicing variant containing exon 2 (FOXP3-E2) which is related to Treg suppressive activity. Patients with multiple sclerosis or type 1 diabetes, show an altered expression of FOXP3-E2 along with impaired glycolysis and IL-2 signalling. This suggests a close link between metabolism and immune tolerance (De Rosa V, 2015).

As emphasized in this paper by De Rosa et al., metabolic enzymes can, in certain circumstances, translocate in to the nucleus, a phenomenon called “moonlighting”, where, as RNA-binding proteins (RBPs), in some instances, they regulate the expression of their target mRNAs and immune responses (De Rosa V, 2015; Boukouris AE, 2016). This metabolism-epigenetics axis facilitates adaptation to a changing environment in normal (e.g., development, stem cell differentiation) and disease states (e.g., cancer and autoimmunity), providing a potential novel therapeutic target.

In this context, the glycolytic enzyme PKM1/2 plays a crucial role in the regulation of transcription factors and cytokine production (Shirai T, 2016). PKM catalyzes the last step within glycolysis, the dephosphorylation of phosphoenolpyruvate to pyruvate, and consists of two isoforms PKM1 and 2.

In contrast to its splice variant PKM1, which is expressed in many adult tissues, PKM2 is allosterically activated in a feed-forward regulatory loop by an upstream glycolytic metabolite, fructose-1,6-bisphosphate (FBP) and is susceptible to inhibition by growth factor signalling through interaction with phosphotyrosine containing proteins (Anastasiou D, 2011).

These properties of PKM2 allow proliferating cells to divert glucose into anabolic pathways emanating from glycolysis in order to meet the increased biosynthetic demands of proliferation.

Association of PKM2 subunits into homotetramers is required for optimal enzymatic activity (Eigenbrodt E, 1992). Exposure of A549 human lung cancer cells to 1 mM H_2O_2, 250 mM diamide (a thiol-oxidizing compound), or hypoxia (1% O2) [which causes increased ROS production by mitochondrial complex III (Brunelle JK, 2005)] has been shown to increase the intracellular concentrations of ROS with a concomitant decrease in PKM2 activity (Anastasiou D, 2011).

This inhibition of PKM2 is able to divert glucose into the pentose phosphate pathway and thereby generate NADPH (Le Goffe C, 2002), which in turn provides sufficient reducing potential for detoxification of ROS by increasing reduced glutathione (GSH) allowing cells to withstand oxidative stress. It maybe possible that during excess lactate (i.e. inflammation or tumour), all the NADPH is used for a rapid antioxidative response determining GSH exhaustion.

Of note, treatment of purified recombinant PKM2 with the small-molecule PKM2 activator DASA-10 (NCGC00181061, a substituted N,N′- diarylsulfonamide) prevented inhibition of PKM2 by H_2O_2 (Anastasiou D, 2011).

The PKM2 potential has also been investigated in atherosclerotic coronary artery disease (CAD) and RA patient-derived macrophages. Here, the increased glucose uptake and glycolytic flux due to inflammation fuel the generation of mitochondrial ROS, which in turn promote dimerization of the glycolytic enzyme pyruvate kinase M2 (PKM2) and enable its nuclear translocation. Nuclear PKM2 functions as a protein kinase that phosphorylates the transcription factor Stat3, thus boosting IL-6 and IL-1β production (Shirai T, 2016; Weyand CM, 2017). Reducing glycolysis, scavenging superoxide and enforcing PKM2 tetramerization corrected the proinflammatory phenotype of CAD macrophages (Shirai T, 2016).

In line with these findings, an increase of PKM2 nuclear translocation with concomitant enhanced Stat3 phosphorylation upon lactate treatment was observed. Stat3 is involved in the Th17 differentiation (Yang XO, 2007) and interestingly, the inhibition of PKM2 nuclear translocation with DASA was able to reduce lactate mediated IL-17 production supporting the important role of this signalling pathway in the regulation of IL-17 by lactate.

Naive T cells mature and exit from the thymus primarily relying on OXPHOS for their metabolic needs, although they augment with glycolytic metabolism during times of

proliferation that follow TCR gene rearrangements. In secondary lymphoid organs, TCR ligation, co-stimulation, and growth factor cytokine signals induce clonal expansion and metabolic reprogramming of an antigen-specific T cell. This conversion to an activated effector T cell is marked by the engagement of aerobic glycolysis and increased OXPHOS activity. Glycolytic metabolism differentiates CD4 Th1, Th2, and Th17 effector cells (and possibly Tfh cells) from Treg cells. Promoting FAO and catabolic metabolism enhances Treg and memory T cell development (Buck MD, 2015) while FAS mainly sustains Th17 differentiation (Berod L, 2014).

In this project, I have analyzed total $CD4^+$ T cells, to understand the impact of sodium lactate on different $CD4^+$ T cell subsets during a TCR natural activation. Interestingly, *in vitro* incubation with sodium lactate, increased the expression of RORγT and IL-17 without affecting Treg population within the total CD4 population. This is in line with our previous findings performed in mouse showing that sodium lactate was able to induce a Th17 signature in different T cell subsets (Th0, Th1, Th2, and Th17) under polarized conditions (Haas R, 2015). In this context, it has been suggested that increased salt (sodium chloride, NaCl) concentrations found locally under physiological conditions *in vivo* markedly boost the induction of murine and human Th17 cells in autoimmune conditions (Kleinewietfeld M, 2013). The results presented in this thesis expand this observation to a broader range of salts, including sodium lactate. Th17 differentiation requires the engagement of glycolysis but also relies on *de novo* FAS (Berod L, 2014). In particular, FAS rate limiting enzyme, ACC1, is crucial for the formation of human and mouse Th17 (Berod L, 2014). Acetyl-CoA carboxylase (ACC) is rapidly regulated by reversible phosphorylation; phosphorylation inactivates ACC, whereas dephosphorylation activates the enzyme. ACC is AMPK downstream target (Winder WW, 1996). In this context, I found a reduced phosphorylation of ACC along with reduced pAMPK levels upon lactate exposure. This is quite surprising considering that

they are normally inversely regulated: when AMPK is active (phosphorylated), it negatively regulates ACC activation and *vice versa*. This suggests that lactate modulation of ACC does not depend on AMPK activation. Other mechanisms might be involved in the regulation of AMPK. It is reported that an excess of nutrients (glucose, amino acids), hormones (i.e. leptin) and cytokines such as TNFα can downregulate AMPK (Viollet B, 2010). Some of these factors might be directly regulated by lactate and could be worthwhile to investigate in the future.

De novo FAS requires the cofactor NADPH, produced in the PPP, in order to take place. In this regard, I found a reduced glycolysis and decreased NADP/NADPH ratio in lactate treated compared to untreated $CD4^+$ T cells. This is in line with a previous publication from Weyand's lab where they showed that RA T cells have an intrinsic deficit in the glycolysis breakdown due to the up-regulation of glucose-6-phosphate dehydrogenase (G6PD). Excess G6PD shunted glucose into the PPP, resulting in NADPH accumulation. NADPH serves as the ultimate donor of reductive power for the large majority of ROS-detoxifying enzymes (Fernandez-Marcos PJ, 2016). The increase of FAS induced by lactate may decrease the NADPH availability for the convertion of GSSG to GSH. This could lead to elevated intracellular reactive oxygen species, (**Figure 6A**), as a result of a perturbation of the glutathione pathway. These observations suggest that lactate promotes, on activated human healthy $CD4^+$ T cells, a shunt toward a lipid-biosynthesis program. Blocking lactate flux on $CD4^+$ T cells via SLC5A12 Ab was sufficient to reduce FAS and consequently the Stat3-IL-17 signalling pathway. These findings highlight a new mechanism consisting in the nuclear PKM2-Stat3 signalling and enhanced FAS through which lactate modulates IL-17 synthesis (**Figure 12**).

6.3 Lactate promotes T cell entrapment in the inflamed site

T lymphocyte motility is directly regulated by aerobic glycolysis (Haas R, 2015; Kishore M, 2017). Several studies show how chemokine treated activated T cells increase glucose uptake via the upregulation of specific glucose transporters and the engagement of intracellular signalling pathway (i.e. mTOR, AMPK). Glycolysis and AMPK signalling are required for efficient T cell migration. Indeed, CCL5 treatment of *ex vivo* activated human T cells induced the activation of the nutrient-sensing kinase AMPK and downstream substrates ACC1, PFKFB2, and GSK3. Treatment with 2-DG, an inhibitor of glycolysis, and an inhibitor of AMPK, reduced CCL5-mediated chemotaxis in a dose-dependent manner (Chan O, 2012). A similar investigation has shown that CXCL10 treatment was able to further up-regulate glycolytic enzymes as well as glucose transporters in activated $CD4^+$ T cells. This effect was inhibited by lactate treatment. These data pointed to lactate-mediated inhibition of $CD4^+$ T cell motility being due to an interference with glycolysis activated upon engagement of the chemokine receptor CXCR3 with the chemokine CXCL10 (Haas R, 2015). These findings were further confirmed in a mouse model of zymosan-induced peritonitis in which T cells are recruited to the inflamed site 5 days after zymosan injection (Haas R, 2015). Intraperitoneal injection of anti-SLC5A12 antibody caused a significant reduction of CFSE-labelled $CD4^+$ T cells in the peritoneum and their accumulation in the spleen in comparison to an isotype-matched control antibody (Haas R, 2015).

Lactate modulation of glycolysis has also been investigated in basal conditions in absence of a chemotactic stimulus. In particular, I obseved that in addition to a reduced glucose flux, several glycolytic enzymes, including HK, where decreased upon lactate exposure in a time dependent manner (**Figure 8A**).

HK catalyze the first committed step of glucose metabolism. Glucose transported through glucose transporters (GLUTs) on the plasma membrane is phosphorylated by HKs to produce glucose-6-phosphate (G6P). The two most common isoforms, HK1 and HK2, have overlapping tissue expression, but different subcellular distributions, with HK1 associated mainly with mitochondria and HK2 associated with both mitochondrial and cytoplasmic compartments. HK2 binds to voltage-dependent anion channel 1 (VDAC1), the outer mitochondrial membrane protein, which interacts with the adenine nucleotide translocase (ANT), forming a contact site between the outer and inner membranes (Fiek C, 1982; Vyssokikh MY, 2003). The mitochondrial bound HKs, has been shown to provide facilitation of coupling between glycolysis and oxidative phosphorylation through privileged/preferential access of HKs to ATP generated by mitochondria. The ADP generated by mitochondrial HK catalytic activity is shuttled back into mitochondria for re-phosphorylation conferring metabolic advantage (Arora KK, 1988). HK plays important roles in the regulation of anabolic and catabolic processes (Wilson JE, 1995; Wilson JE, 2003). Indeed, it has been shown that different subcellular distributions are associated with different metabolic roles (John S, 2011). In particular, HK2 is thought to play a key role in promoting anabolic pathways when bound to mitochondria (Matuphala SP, 2006).

In line with this evidence, I found an increased mitochondrial localization of HK2 after 4 hour lactate treatment in comparison to untreated cells as well as a reduction of $NADP^+$/NADPH ratio which suggests a shunt toward PPP and anabolic metabolism (**Figure 8D-F**).

HK2 acts as a proper glucose/G6P sensor, adjusting the balance between glycolysis-/mTORC1-mediated growth and autophagy-mediated preservation of energy homeostasis (Roberts DJ, 2014). Moreover, HK2 has pro-survival function antagonizing apoptotic BCL2 family proteins and thereby protects cells against apoptotic stimuli and

competitively inhibits Bax binding to mitochondria HK2 (Pastorino JG, 2002). Whether lactate regulates cell survival and apoptosis is still unknown and it might be very important to investigate this aspect in the future. The reduced locomotion observed under lactate treatment may be an adaptive response of T cells to stress in order to facilitate other processes such as cell survival.

HK2 was found expressed at high levels in RA synovitis where it regulates fibroblast aggressive function including tissue invasiveness and cytokine production (Bustamante MF, 2018). Interestingly these effects associated with an increased HK2 mitochondrial localization (Bustamante MF, 2018).

On the same line, HK2 was shown to be expressed at high levels by some cancer cells where it is necessary for their growth and immortalization (Mathupala SP, 2009).

Based on this evidence HK2 might be an attractive selective metabolic target for cancer and autoimmune disorders.

Glycolysis is also instrumental for regulatory T cell (Treg) migration. However, unlike in conventional T cells, in which migration is dependent upon mTORC1-induced glycolysis, Treg motility is sustained by a PI3K-mTORC2-mediated pathway leading to the induction of the enzyme GCK. GCK promoted cytoskeletal rearrangements by associating with actin, allowong Treg cells to migrate. Treg cells lacking this pathway were functionally suppressive but failed to migrate to skin allografts, prolong their survival and inhibit organ rejection (Kishore M, 2017).

Lymphocyte traffic is required to maintain homeostasis and perform appropriate immunological reactions (Marelli-Berg FM, 2018). To migrate into inflamed tissues, lymphocytes acquire spatial and functional asymmetries. Motile T cells display a polarized morphology with two distinct cell compartments: the leading edge and the uropod. During cell polarization, chemoattractant receptors, cell-adhesion molecules and cytoskeletal proteins are re-distributed within these cellular compartments. In this

context, mitochondria are highly dynamic organelles that distribute in the cytoplasm to meet specific cellular needs, and specifically concentrate at the uropod during lymphocyte migration by a process involving rearrangements of their shape. In particular, mitochondrial fission facilitates relocation of the organelles and promotes lymphocyte chemotaxis, whereas mitochondrial fusion inhibits both processes. Mitochondrial ATP from OXPHOS fuels this process (Marelli-Berg FM, 2018; Campello S, 2006).

In addition, the cytoskeleton rearranges during T cell migration (Serrador JM, 1999). Movement along microtubules, mediated by kinesin and dyneinmotors, controls intracellular distribution of mitochondria. It is possible that the close interactions of these organelles with microtubules might provide a convenient source of ATP and GTP, the latter used for tubulin polymerization, for cytoskeleton reorganization, at the uropod (Morris RL, 1993; Li Z, 2004). Whether or not lactate or other metabolites modulate T cell motility via cytoskeleton rearrangement is still under investigation.

Leukocyte recruitment is a pivotal process in the regulation of an inflammatory episode. Leukocyte subsets continuously traffic into, through and out of the stromal compartments of the bone marrow, thymus, lymphnodes and peripheral tissues throughout life. Growing evidence indicates that leukocyte trafficking through these compartments is altered to some extent in patients with chronic inflammatory arthritis and that this trafficking can vary between different patients and across the course of the disease (for example, between each phase of disease or following therapeutic intervention). Indeed, it is now recognized that patients with RA have defects in at least one, if not multiple, checkpoints that regulate the exit of leukocytes from primary and secondary lymphoid tissues and the entry of these cells into the joint itself (Buckley CD, 2018). However, attempts to target leukocyte accumulation within the joint by modulating the recruitment and retention of these cells (for example, with chemokine receptor inhibitors) have not been successful to date (Asquith DL, 2015). This lack of success is possibly explained by the multifactorial

nature of the leukocyte adhesion cascade and by the current lack of understanding of the molecular processes that are dysregulated at given disease phases in an individual patient. The increased 'stickiness' of T cells expressing PTPN22 (R620W) could potentially be responsible for reduced cell migration speeds in synovial tissue and increased tissue residency times (Burn GL, 2016). The bioactive lipid sphingosine-1-phosphate (S1P) reportedly promoted the persistence of activated $CD4^+$ T cells in a murine ear model of acute inflammation (Jaigirdar SA, 2017). Synovial tissue from patients with RA had increased levels of the enzyme sphingosine kinase 1, which is necessary for S1P synthesis, suggesting that S1P also contributes to the survival and persistence of T cells in the joint (Jaigirdar SA, 2017).

Finally, the results of a 2017 study demonstrated that the cellular metabolism of T cells from patients with RA is rewired in such a way that impairs their locomotion programme. Reduced glycolytic flux induced the upregulation and overexpression of the podosome scaffold adapter protein TKS5 (also known as SH3PXD2A), which is critically involved in the localization of membrane protrusions in migrating cells (Shen Y, 2017). As a result, T cells from patients with RA are much more hypermotile and tissue-penetrating than HC (Shen Y, 2017). This 'hypermotility' was caused by insufficient glycolytic breakdown due to a PFKFB3 deficiency, pyruvate reduction and a shunt toward anabolic glucose utilization (increased PPP and FAS). Enhanced FAS led to cytoplasmic lipid droplets accumulation, which are requisites for cell growth, proliferation and naïve to memory T cell conversion. Interestingly, the replenishment of pyruvate was sufficient to restore T cell locomotion, tissue-invasiveness and inflammation in a NOD scid gamma mice (NSG mice) engrafted with human synovial tissue.

In line with this finding, lactate treated $CD4^+$ T cells, like RA T cells, are also able to shunt toward anabolic pathways such as PPP and FAS. Blocking FAS and NADPH production with specific compounds was sufficient to restore T cell locomotion even in

the presence of lactate. This suggests that glycolytically impaired RA $CD4^+$ T cells utilize anabolic PPP and FAS to infiltrate the synovium and remain on the site. The same metabolic signature is displayed by healthy T cells exposed to high concentration of lactate.

The impairment of T cell motility along with a Th17 signature displayed by T cells, due to lactate excess, may lead to the establishment of long-lasting, highly efficient tertiary lymphoid structures in RA synovium as previously shown (Shen Y, 2017). ELSs structures develop in 40% of RA patients and their role is still poorly understood. In this subgroup of patients (ELS/lymphoid), a high percentage of $CD4^+$ T cells expressing SLC5A12 in the tissue was found (Haas R, 2015). Moreover, the synovial expression of SLC5A12 correlated with disease activity and response to therapy, thus supporting the role of lactate/SLC5A12 signalling as a possible biomarker of disease progression and therapeutic response.

The enrichment of SLC5A12 in the inflammatory sites raised the question of whether this lactate transporter may be involved in the entrapment of $CD4^+$ T cells within the chronically inflamed "milieu" of the RA synovia. Blocking SLC5A12 transporter with both polyclonal and monoclonal antibodies resulted in an increased T cell egress from the inflamed synovial tissue, in a reduced IL-17 production and in a decreased inflammation in a mouse model of arthritis. These observations make SLC5A12 a possible novel candidate as therapeutic target in inflammatory disorders.

6.4 Therapeutic potential

For many years, lactate has been considered a bystander product of glycolysis, produced during hypoxia or by highly proliferating cells. Only recently, lactate has received a well-

deserved consideration as a signalling metabolite. Its role in cancer biology and immunity is getting into the spotlight nowadays. The breakthrough that lactate plays a very important role in the interplay between inflammation, cancer, metabolism and immunity together with the discovery of lactate transporters expressed by different variety of cells (i.e. immune, stromal and cancer cells) has opened a new area of research and novel potential therapeutics. The emerging evidence that solid cancers can deprive the tumour environment of glucose and enrich it with lactate which in turn depresses effector and cytotoxic T cell functions and promotes suppressive Treg cells, has also shed lights on new immunosuppressive therapy approaches, based on potentiating Treg cell activity, in conditions where Treg cell functionality is overthrown (i.e. autoimmunity, transplantation). On the other side, in cancer this can offer a new potential therapeutic approach based on targeting cancer metabolism that can reduce detrimental Treg cells in favor of effector T cells thus enhancing anti-tumour response (Angelin A, 2017). Targeting lactate signalling via lactate transporters is becoming a new promising field in cancer. However, little is currently known about the regulation of MCT/SMTCs expression and activity in different tissues and peripheral blood and their regulation during inflammation or tumours. The discovery that MCTs can transport anticancer agents (i.e. 3-bromopyruvate, dichloroacetate and iodoacetate) across cell membranes (Baltazar F, 2014) suggests that those substrates can also act as anticancer compounds. Moreover, in some tumours MCT activity is being studied to identify new biomarkers that would allow predicting a therapeutic response.

If in cancer lactate suppresses T cell effector functions enabling the tumour to grow in the context of inflammatory disorders, such as RA, lactate behaves as a pro-inflammatory signal. Indeed, in the presence of high concentration of lactate, such as in the RA synovium, T cells are unable to egress ending up "entrapped" in the inflammatory site

(Haas R, 2015; Pucino V, 2017). Here, T cells produce a high amount of pro-inflammatory cytokines contributing to the establishment of chronic inflammation.

Importantly, the expression of lactate transporters positively correlated with the extent of T cell infiltrates in the synovium of RA patients (Haas R, 2015) suggesting a role of lactate in driving inflammation via these specific transporters. The clinical relevance of this study was further emphasized by the finding that pharmacological or antibody-mediated blockade of subtype-specific lactate transporters on T cells resulted in their release from the inflammatory site in an *in vivo* model of peritonitis. This evidence was confirmed *in vitro* on isolated activated human T cells and *ex vivo* evaluating the effect of SLC5A12 blockade on cell egress from an RA synovial tissue. Moreover, a much higher expression of SLC5A12 was found in tissue-resident compared to peripheral $CD4^+$ T cells that may correspond to a higher concentration of lactate in the inflamed tissue than in the peripheral blood. This aspect can be useful for the construction of a tissue specific antibody against SLC5A12, able to reduce the inflammation locally, minimizing the collateral effects of a systemic therapy. In support of this hypothesis, local injection of a commercially available SLC5A12 Ab was able to reduce the clinical and histological score of the disease in a mouse model of arthritis.

Taken together, targeting the lactate/SLC5A12 signalling pathway re-established T cell migration away from the inflammatory site and reduced the production of high amounts of IL-17, with potential therapeutic implications in the management of inflammatory disorders.

The distinct lactate transporter expression by $CD4^+$ and $CD8^+$ T cells (Haas R, 2015) may orchestrate their differential distribution in the inflamed tissues as well as affect their functional and migratory responses depending, for example, on the nature of the inflammatory exudate (i.e., more lactic acid versus sodium lactate). Targeting selected T cell subtypes via different lactate transporters might prove crucial in the modulation of

immune cell functions and to achieve beneficial therapeutic effects in diseases that are driven by these specific T cell subsets.

In this context, Th17 play an important role in RA, IBD, MS/EAE (Oukka M, 2008). Rising levels of circulating Th17 cells and IL-17 were observed in patients with an inadequate response to anti-TNF-α therapy (Chen DY, 2011). Despite several studies revealed the importance of IL-17 in the pathogenesis of RA, clinical trials with IL-17 blocking agents in RA have not reached striking results so far (Kugyelka R, 2016) thus other targets are needed.

This can be in part due to the heterogeneity of RA in terms of pathogenesis and histological patterns of synovitis (Pitzalis C, 2013) as well as the high variability of cytokine pathways that are activated can differ from patients to patients.

IL-17 and IL-17R family members show a high variability in the expression in individual patients (van Baarsen LG, 2014). Moreover, IL-17 is a complex system by itself. Indeed, it consists of 6 members with 5 known receptors. Therefore, it is not surprising that the blockade of IL17A or its receptor with monoclonal antibodies did not lead to complete disease remission so far. Currently it is more likely that IL-17 targeting agents could be used to in association with current therapies (Kugyelka R, 2016). For this reason, targeting the IL-17 axis in RA at different levels (i.e. Th17 differentiation, signalling, etc.), such as blocking SLC5A12, may provide better therapeutic results than the currently available monoclonal antibodies, in some selected patients, such as RA limphoid (ELS+), who have failed previous treatments (i.e. anti-TNF non responders).

7 CONCLUSIONS

Overall, in this thesis I have provided a summary of the potential role of lactate that, via a distinctive metabolic signalling network, may promote pathogenic characteristic typical of the inflammatory "milieu" (**Figure 12**). Thus, targeting specific metabolic pathways via lactate transporter-blocking agents may provide a promising therapeutic approach to reduce inflammation and induce immunosuppressive responses. Modulating selective T cell subsets via targeting specific lactate transporters may furnish a novel tool to reduce inflammation and may help to better understand the pathogenesis of inflammatory disorders.

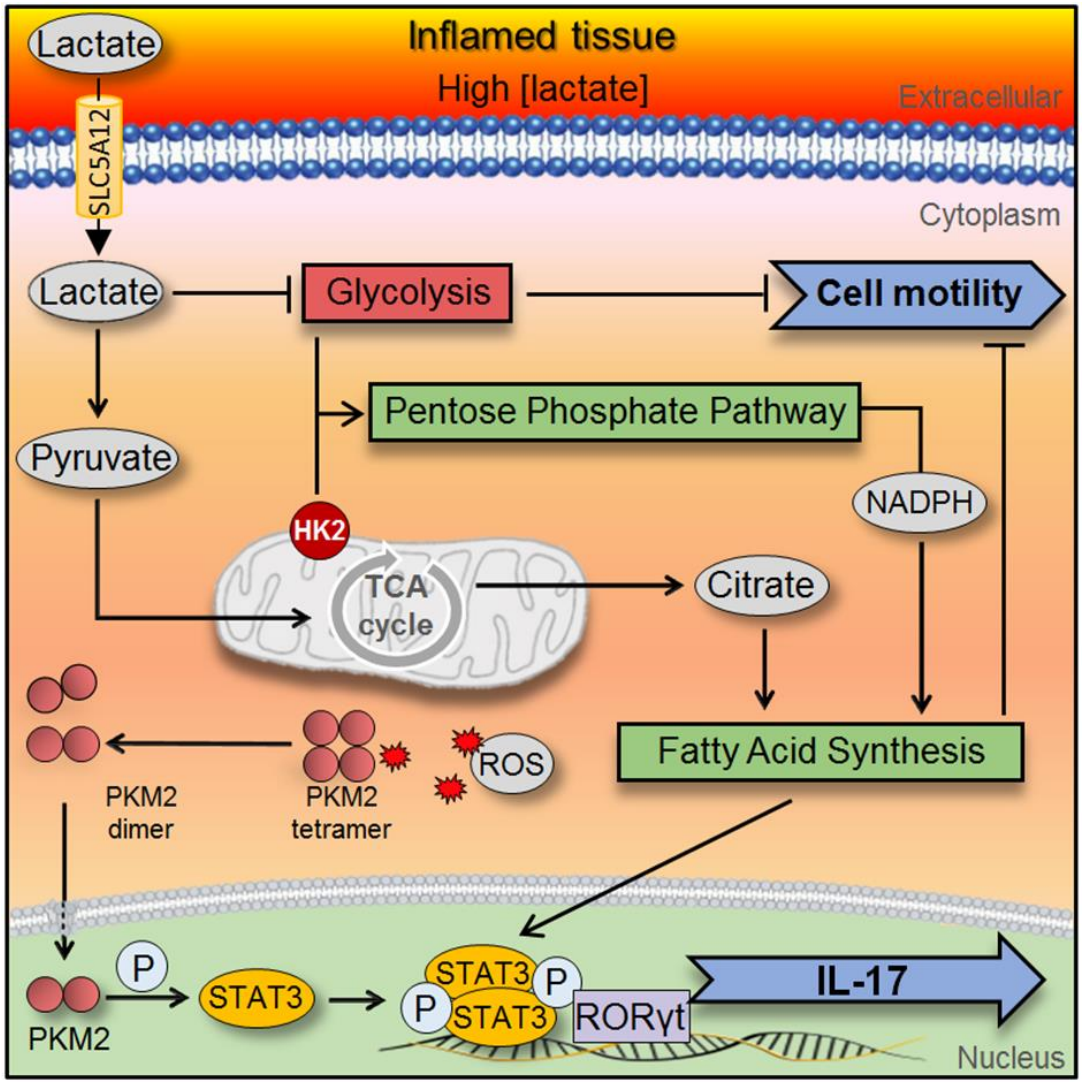

Figure 12 – Schematic summary. Lactate accumulation at the site of inflammation contributes, together with activation and inflammatory stimuli, to the up-regulation of the sodium-coupled lactate transporter SLC5A12 by human $CD4^+$ T cells. SLC5A12-mediated lactate influx in to human $CD4^+$ T cells initiates a nuclear PKM2/Stat3 signalling and enhances fatty acid synthesis leading to increased IL-17 production. SLC5A12-mediated lactate influx into human $CD4^+$ T cells also causes their damaging retention at the inflamed tissue as a consequence of reduced glycolysis and enhanced fatty acid synthesis. These events promote the perpetuation of chronic inflammation in diseases such as RA. Targeting SLC5A12 may constitute a novel therapeutic option to reduce inflammation.

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9 APPENDIX

Table 6 - Demographical patient data

Parameter	Study Population (n = 17)
Age (range)	35-76
Gender (%)	
Female	80
Male	20
ESR (range)	2-113
CRP (range)	5-26
DAS28 (%) < 2.1 > 5.2	 50 50
Erosive (%)	63
Treatment (%)	
Naïve to treatment	53
DMARDs	35
Steroids	5
Biologics	29
RF^+ and/or CCP^+ (%)	95

Table 7 - Primary and secondary antibodies used for immunofluorescence

Antigen	Specificity	Clone	Host	Source	Diluition
CD20	B lymphocytes	L-26	Mouse	DAKO	1:20
CD8	Cytotoxic/suppressor T cells subset	C8/144B	Mouse	DAKO	1:50
CD4	T-helper cells	4 B12	Mouse	DAKO	1:50
CD68	Monocytes/macrophages	M0876	Mouse	DAKO	1:50
SLC5A12	N/A	HPA045181	Rabbit	NOVUS	1:50

- Secondary antibodies used for immunofluorescence

Primary antibodies	Secondary antibodies immunofluorescence
CD20	Goat anti-mouse IgG2a ALEXA-488 (Invitrogen)
CD8	Goat anti-mouse IgG1 ALEXA-488 (Invitrogen)
CD4	Goat anti-mouse IgG1 ALEXA-488 (Invitrogen)
CD68	Goat anti-mouse IgG1 ALEXA-488 (Invitrogen)
SLC5A12	Goat anti-rabbit IgG ALEXA-555 (Invitrogen)

Table 8 - Primers used for qRT-PCR

Name	Sequence	Species	Description
BCL6_F	CGAATCCACACAGGAGAGAAA	human	B-cell lymphoma 6 protein
BCL6_R	ACGCGGTATTGCACCTTG	human	B-cell lymphoma 6 protein
CXCR5_F	GCTAACGCTGGAAATGGA	human	C-X-C chemokine receptor type 5
CXCR5_R	GCAGGGCAGAGATGATTT	human	C-X-C chemokine receptor type 5
Foxo1_F	AGGGTTAGTGAGCAGGTTACAC	human	Forkhead box protein O1
Foxo1_R	TGCTGCCAAGTCTGACGAAA	human	Forkhead box protein O1
Foxp3_F	CTGACCAAGGCTTCATCTGTG	human	Forkhead box P3
Foxp3_R	ACTCTGGGAATGTGCTGTTTC	human	Forkhead box P3
GAPDH_F	TCCTCTGACTTCAACAGCGA	human	Glyceraldehyde 3-phosphate dehydrogenase
GAPDH_R	GGGTCTTACTCCTTGGAGGC	human	Glyceraldehyde 3-phosphate dehydrogenase
IFNγ_F	GGCATTTTGAAGAATTGGAAAG	human	Interferon gamma
IFNγ_R	TTTGGATGCTCTGGTCATCTT	human	Interferon gamma
IL10_F	ACCTGCCTAACATGCTTCGAG	human	Interleukin 10
IL10_R	CCAGCTGATCCTTCATTTGAAAG	human	Interleukin 10
IL17A_F	TGTCCACCATGTGGCCTAAGAG	human	Interleukin 17A
IL17A_R	GTCCGAAATGAGGCTGTCTTTGA	human	Interleukin 17A
IL22_F	TCCAGAGGAATGTGCAAAAG	human	Interleukin 22
IL22_R	ACAGCAAATCCAGTTCTCCAA	human	Interleukin 22
IL6_F	AGTGAGGAACAAGCCAGAGC	human	Interleukin 6
IL6_R	GTCAGGGGTGGTTATTGCAT	human	Interleukin 6
PD1_F	ACCTGGGTGTTGGGAGGGCA	human	Programmed cell death protein 1
PD1_R	GGAGTGGATAGGCCACGGCG	human	Programmed cell death protein 1
RORγt_F	CCTGGGCTCCTCGCCTGACC	human	RAR-related orphan receptor gamma
RORγt_R	TCTCTCTGCCCTCAGCCTTGCC	human	RAR-related orphan receptor gamma
SLC5A12_F	GTGTGCTGTCTTCTCTGGCT	human	sodium-coupled monocarboxylate transporter 2
SLC5A12_R	GCCACAAAAAGTCCTGGCAG	human	sodium-coupled monocarboxylate transporter 2
TGFβ_F	AGCGACTCGCCAGAGTGGTTA	human	transforming growth factor beta
TGFβ_R	GCAGTGTGTTATCCCTGCTGTCA	human	transforming growth factor beta
βActin_F	AGTTGCGTTACACCCTTTCTTG	human	Actin
βActin_R	TCACCTTCACCGTTCCAGTTT	human	Actin