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Novel understanding of $CD8^+$ T-cell regulation in Rheumatoid Arthritis
–
deciphering the close interplay between inflammation, epigenetics, and metabolism

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2 ABBREVIATIONS

1,3-BPG	1,3-Bisphosphoglycerate
2HG	2 hydroxyglutarate
2-PG	2-Phosphoglycerate
5Aza	5 Azacytidine
5HMC	5-Hydroxymethylcytosin
6PG	6-phosphogluconate
6-PGL	6-phosphogluconolactone
Ac-CoA	Acetyl-CoA
ACK	Ammonium, Chloride, Potassium
ACLY	ATP citrate lyase
ACN	Aconitase
ACPA	Anti-citrullinated protein antibody
AIRE	Autoimmune regulator
aKG	α-ketoglutarate
a-KGDH	α-ketoglutarate-dehydrogenase
AKT	Protein kinase B
ALDOL	Aldolase
AMPA	Anti-modified-protein antibody
AP-1	Activator Protein 1
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
APS	Autoimmune polyendocrine syndrome
AS	Ankylosing Spondylitis
ATAC seq	Assay for Transposase-Accessible Chromatin using sequencing
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAIA	Collagen antibody induced arthritis
CBA	Cytokine Bead Array
CCR	chemokine receptor
CD	Cluster of differentiation
CFA	Complete Freud's adjuvant
CIA	Collagen-induced arthritis
CII	Collagen type II
CI-V	Complex I-V
CoA	Coenzyme A
CoQ	Coenzyme Q
CRP	C reactive protein
CS	Citrate synthase
CTD	Connective Tissue Disease
CTLA1	Cytotoxic T-lymphocyte-associated Protein 4
CytC	Cytochrome C
D2HGDH	2Hydroxyglutarate dehydrogenase
DAS28-CRP	Disease activity score including 28 joints and the CRP
DC	Dendritic cell
DGK	Diacylglycerol kinase

DHAP	Dihydroxyacetone phosphate
DIA	Data independent acquisition
DKFZ	German Cancer Research Centre
DKFZ	German Cancer Research Centre
DN	Double negative
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
DNMT	DNA methyl transferase
DP	Soluble positive
EBV	Eppstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ENOL	Enolase
ESI	Electron spray ionisation
ESR	Erythrocyte sedimentation rate
ETC	Electron transport chain
F-1,6-BP	Fructose-,6-bisphosphate
F-6-P	Fructose-6-phosphate
FADH	Flavin adenine dinucleotide
FASN	Fatty acid synthase
FCS	Foetal calf serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FH	Fumarate hydratase
G3P	Glyceraldehyde-3-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GDH	Glutamate dehydrogenase
GLS	Glutaminase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glucose-6-phosphate isomerase
GPI	Glucose-6-phosphate-isomerase
GPR	G-protein coupled receptor
GSEA	Gene set enrichment analysis
HAT	Histone-acetyl-transferases
HDAC	Histone-deacetylases
HIF-1α	Hypoxia inducible factor-1α
HK	Hexokinase
HLA	Human leukocyte antigen
IDH	Isocitrate dehydrogenase
IFR1	Interferon regulatory factor
Ig	Immunoglobulin
IL	Interleukin
IL-xR	IL-x receptor
Ipex	The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus Kinase
LDH	Lactate dehydrogenase
LDHA	Lactate dehydrogenase A

LFQ	Label-free quantification
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MAGE	Melanoma antigen gene
M-CSF	Macrophage colony-stimulating factor,
MCT4	Monocarboxylate-Transporter 4
MDH	Malate dehydrogenase
MHC	Major Histocompatibility Complex
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass Spectrometry
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NMR	Nuclear Magnetic Resonance
NOS	Nitric oxide synthase
NOX4	NADPH oxidase 4
OA	Osteoarthritis
OAA	Oxaloacetate
OXPHOS	Oxidative Phosphorylation
PA	Phosphatidic acid
PBMC	Peripheral mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal Component analysis
PD1	Programmed cell death protein 1
PDC	Pyruvate-dehydrogenase-complex
PDH	Pyruvate dehydrogenase
PD-L1	Programmed cell death 1 ligand
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGM	Phosphosglyceratemutase
PIA	Pristane-Induced Arthritis
PK	Pyruvate kinase
PMA	Phorbol 12-myristate 13-acetate
PMT	Photomultiplier tube
PRR	Pattern recognition receptor
PsA	Psoriatic arthritis
PTM	Post-translational modification
Pyr	Pyruvate
qPCR	Quantitative real-time polymerase chain reaction
QRILC	Quantile Regression Imputation of Left-Censored data
R-5-P	Ribulose-5-phosphate
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor κB ligand

RF	Rheumatoid factor
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SAM	S-Adenosyl-Methionine
SCS	Succinyl-CoA-synthetase
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SLE	systemic lupus erythematosus
STAT	Signal Transducers and Activators of Transcription
TBS	Tris buffered Saline
TBST	Tris buffered saline with Tween
TCA	Tri-carboxylic acid
T_{CM}	Central Memory T-cells
TCR	T cell receptor
T_{EM}	Effector Memory T-cells
TET	Ten-eleven translocation
T_{H}	T helper cell
TNF	Tumour necrosis factor
T_{PH}	peripheral T-helper cells
TPI	Triose phosphate isomerase
T_{reg}	Regulatory T-cell
T_{EMRA}	Effector T-cells
UTR	Untranslated region
VAS	Visual analogue score
αCDx	anti CD x

3 ZUSAMMENFASSUNG

Rheumatoide Arthritis ist eine Autoimmunerkrankung, die etwa 1 % der erwachsenen Bevölkerung betrifft. Die Erkrankung äußert sich durch Schmerzen und Schwellungen in den Gelenken und bei unzureichender Behandlung kommt es zu schwerwiegenden, irreversiblen Schäden der Gelenke. Da der Ursprung der Krankheit weiterhin unbekannt ist, ist eine Heilung derzeit nicht möglich und verfügbare Therapien beschränken sich hauptsächlich auf symptomatische Behandlungen. Auf molekularer Ebene äußert sich die rheumatoide Arthritis durch eine Hyper-Inflammation. Diese wird durch eine Aktivierung von Fibroblasten in der Synovialmembran und die Infiltration der Gelenke durch autoreaktive Immunzellen verursacht. $CD8^+$ T-Zellen gehören aufgrund ihrer zytotoxischen und gewebezerstörenden Eigenschaften zu den Hauptakteuren rheumatoider Arthritis und machen einen Großteil der Gelenk-infiltrierenden Immunzellen aus. Zusätzlich zu ihrem pro-inflammatorischen Phänotyp greifen $CD8^+$ T-Zellen in rheumatoider Arthritis hauptsächlich auf die aerobe Glykolyse zur Energiegewinnung zurück, was wiederum die Immunaktivität (initiiert? und) aufrecht erhält. Krebszellen weisen einen ähnlichen Metabolismus auf, der sich durch epigenetische Aktivierung von pro-glykolytischen Enzymen und Deaktivierung von anti-glykolytischen Enzymen manifestiert. Außerdem sind Metabolismus und Epigenetik eng verflochten, da Zwischenprodukte des Zitrat Zyklus als Co-faktoren und Inhibitoren von epigenetischen Schlüsselenzymen („writers“ – Etablierung epigenetischer Marker und „erasers“ - Entfernung epigenetischer Marker) fungieren. Ziel der vorliegenden Thesis war es, die zugrunde liegenden Mechanismen der Stoffwechselumstellung von oxidativer Phosphorylierung zu aerober Glykolyse zu entschlüsseln und neue Ansätze zu ermitteln, die in den entzündlich-glykolytischen Teufelskreis der $CD8^+$ T-Zellen eingreifen. Hierzu wurden *ex vivo* Zellen von Patienten mit rheumatoider Arthritis und von gesunden Probanden mithilfe modernster -omics Methoden und Multivariate-Analysen in Kombination mit konfirmatorischen *in-vitro*-Tests untersucht. Die Analyse des Zellstoffwechsels und der Immunfunktion haben eine epigenetisch-metabolische Achse als wichtige Grundlage des $CD8^+$ T-Zell-Phänotyps in rheumatoider Arthritis identifiziert. Die Ergebnisse zeigen eine Akkumulierung von 2-Hydroxyglutarat und einen durch TET (DNA-Demethylase) und DNMT1 (DNA-Methyl-Transferase) vermittelten Anstieg der DNA-Methylierung in $CD8^+$ T-Zellen in rheumatoider Arthritis. Der Ansatz, diesen Phänotyp durch die Supplementierung mit dem TET-Co-faktor α-Ketoglutarat umzukehren, war erfolgreich. In Folge der Zugabe von α-Ketoglutarat wurden die inflammatorische Aktivität und die Effektor-Funktionen der Zellen reduziert. Somit ist es plausibel, dass der Autoimmunphänotyp der $CD8^+$ T-Zellen bei rheumatoider Arthritis auf einem veränderten epigenetischen Profil beruht, das sich in aerober Glykolyse und der Produktion von regulatorischen Metaboliten manifestiert, die einerseits pro-inflammatorische Signalwege und andererseits epigenetische Marker stabilisieren. Darüber hinaus haben sich in dieser Thesis α-Ketoglutarat als wirksamer Regulator der $CD8^+$ T-Zell Aktivität bei rheumatoider Arthritis und der zelluläre Stoffwechsel als vielversprechende therapeutisches Angriffspunkte für rheumatoide Arthritis herausgestellt. Diese Herangehensweise birgt das Potenzial, therapeutisch an den Grundlagen der Krankheit anzusetzen.

4 SUMMARY

Rheumatoid arthritis is an auto-immune disease affecting approximately 1 % of the adult population. It presents with stiffness and swelling of the joints, and can culminate into severe deformations if not treated early and adequately. Due to the unknown origin of rheumatoid arthritis, there is presently no cure and therapies are mainly symptomatic. On a molecular level, rheumatoid arthritis is manifested by hyper-inflammation and massive fibroblast activation and immune-cell infiltration. $CD8^+$ T-cells are major drivers of the disease due to their cytotoxic and tissue-destructive capacities. They also make up a large fraction of joint-infiltrating immune cells. Next to their pro-inflammatory phenotype, $CD8^+$ T-cells in rheumatoid arthritis mainly rely on aerobic glycolysis for their energy production to (initiate ? and) sustain their pro-inflammatory immune activity. Cancer cells are known to maintain a similar metabolism by epigenetically up-regulating the gene expression of pro-glycolytic enzymes and down-regulating the gene expression of TCA cycle and oxidative phosphorylation related enzymes. Further, metabolism and epigenetics are closely related as many TCA cycle intermediates are important co-factors or inhibitors of epigenetic writers and erasers. This thesis aimed to detangle the underlying mechanisms of the metabolic switch from oxidative phosphorylation to aerobic glycolysis and to introduce novel approaches to interrupt the inflammatory-glycolytic cycle by *ex vivo* analyses of $CD8^+$ T-cells from rheumatoid arthritis patients in comparison with cells from healthy donors. Using state-of-the-art -omics methods and multivariate analyses combined with confirmatory *in vitro* assays examining the cellular metabolism and immune function, I identified an epigenetic-metabolic axis as an important driver of the rheumatoid arthritis $CD8^+$ T-cell phenotype. The results show the accumulation of 2-hydroxyglutarate and a TET (DNA demethylase) and DNMT1 (DNA methyl-transferase) driven increase in DNA-methylation in RA $CD8^+$ T-cells. The approach to rescue this phenotype with the supplementation of the TET co-factor α-ketoglutarate was successful and rendered the cells less inflammatory and reduced their effector functions. Thus, it is plausible that the auto-immune phenotype of $CD8^+$ T-cells in rheumatoid arthritis is based on an altered epigenetic landscape that manifests aerobic glycolysis and the production of regulatory metabolites that stabilise on the one hand, the pro-inflammatory signalling and, on the other hand, the epigenetic remodelling. Further, this thesis introduces α-ketoglutarate as a potent modulator of $CD8^+$ T-cell activity in rheumatoid arthritis. It inaugurates cellular metabolism as a promising therapeutic target in rheumatoid arthritis that not only alleviates the symptoms but harbours the potential to act on the foundation of the disease.

5 INTRODUCTION

5.1 The Immune System

The immune system is an effective cluster of organs, tissues and sequential processes that come into action when external pathogens such as viruses, bacteria or parasites invade the host organism or internal threats such as cancer arise. The first line of defence is anatomical barriers, such as skin, mucosa, and epithelia, which work along with biochemical agents as defensins. Another auto-detect system is the complement proteins in the blood plasma, which can detect pathogen surface proteins and further activate a defence response. After this rather general but fast restraint (minutes upon pathogen intrusion), the cellular immune response is initiated. It can be divided into the innate immune system and the adaptive immunity, the latter being antigen/pathogen specific and able to form an immunological memory, however, intertwined and inevitably dependent on the innate immune players.

The eventual outcome of an activation of the immune system is inflammation. Inflammation is characterised by redness, swelling, heat and pain, all subsequent symptoms of a dilation of blood vessels in the affected area and the following infiltration by immune cells and their pro-inflammatory effector functions.

5.1.1 Innate Immune Response

Most innate immune cells derive from the common myeloid progenitor in the bone marrow (Kondo, 2010). It gives rise to granulocytes, mast cells, macrophages, and dendritic cells (DC).

Granulocytes can be further subclassified into neutrophils, eosinophils, and basophils and contain granules filled with anti-pathogenic mediators that can be released upon pathogen encounter. Neutrophils can also phagocyte microorganisms and destroy them efficiently, while eosinophils and basophils are mostly involved in fighting parasites by degranulation, as they are too large to be digested. Mast cells can be found in local tissues and are involved in inducing inflammation.

Dendritic cells and macrophages migrate from the peripheral blood into tissues and phagocyte pathogens, infected or dead cells and other kinds of debris. Both further orchestrate the immune response by releasing mediators as chemokines and cytokines to attract and activate further immune cells. This is mainly mediated by the activation of pattern recognition receptors (PRR), which recognize so called immunogens such as the bacterial cell wall component lipopolysaccharide (LPS) (Janeway, 1989). While macrophages are mainly involved in (pathogen) clearance, dendritic cells process the ingested microorganisms and proteins and present their peptides to cells of the adaptive immune system. Thus, they are an inevitable bridge between the innate and adaptive immune response.

5.1.2 Adaptive Immune Response

The common lymphoid progenitor gives rise to natural killer cells, innate lymphoid cells, which per definition belong to the innate immune system, T-, and B-lymphocytes (Kondo, 2010). B-cell development is completed in the bone marrow and T-cell precursors migrate to the thymus to

finish their maturation process. Both mature but still cognate antigen naïve B-cells and T-cells then enter the circulation of the peripheral blood.
The adaptive immune system is characterized by its specificity, the ability to recognize and react against distinct pathogen-structures, the production of antibodies, by forming memory cells, which can be reactivated by a repeated encounter with a pathogen. The recognized structures are proteins or other constituents of the pathogen, which are called antigens (historically based on the observation that they are "antibody-generating"). Recently, also an antigen-independent activation of T-cells was observed implicating that also players of the adaptive immune system can harbour innate functions (Lee et al., 2020).

Antibodies, also called immunoglobulins (Ig), are found in the plasma, and thus belong to the humoral immunity like the complement factors. However, they can recognize specific antigens. They consist of a variable part, which binds to the antigen and a constant part (Fc), which binds to Fc-receptors on other immune cells. The following opsonisation of the antigen draws the innate immune cells' attention to that antigen. Further, they can coat antigens or whole pathogens such as viruses by binding to them and neutralizing them. Bridging the innate and the adaptive immune system, immunoglobulins can also activate the complement system in a more efficient manner than a pathogen surface only.

Both B-cells and T-cells express cell surface receptors that can specifically recognize one antigen peptide, which premises a huge variety of these receptors. The B-cell receptor is a surface bound immunoglobulin, while the T-cell receptor (TCR) is similar to the variable region of antibodies. The high variability of both receptors (and the soluble antibodies) is due to a DNA (deoxyribonucleic acid) recombination process, that randomly selects the building blocks and thus, always creates a new receptor for a new epitope (Alt et al., 1992). While the common understanding of this process is the recognition of conserved DNA sequences by recombination processes, it was found that also the DNA structure can influence the binding of these enzymes (Hoolehan et al., 2022). This adds a new, less genome dependent aspect to the TCR repertoire generation.

Upon antigen recognition by or after presentation by antigen presenting cells (APC), as dendritic cells (DC), lymphocytes are activated and undergo proliferation. B-cells eventually differentiate into plasma cells, which produce one specific type of secreted antibody to fuel the antibody pool for a lifetime. T-cells also initiate clonal expansion and turn into distinct types of effector cells: T-helper cells interact with B-cells and support the antibody production, cytotoxic T-cells can eliminate infected cells or tumour cells, and regulatory T-cells can limit the immune response in order to prevent destructive inflammation. Eventually, the activated T-cells turn into memory T-cells that can be reactivated at a subsequent encounter with the respective antigen and are then able to initiate a much faster immune response.

As this thesis focuses on $CD8^+$ T-cells (CD – cluster of differentiation), T-lymphocytes will be introduced in further detail in the following section.

5.1.2.1 T-cell development and central tolerance

T-cells derive from the common lymphoid progenitor in the bone marrow and then migrate into the thymus via high endothelial venules. The thymus is organized in a cortical and a medulla compartment, whose local cells support and guide the T-cell maturation process. Eventually, 90 % of the T-cell pool will be αβ-T-cell, while 10 % express the γ and δ TCR chains and are separated early in the thymic development to become γδT-cells (not further discussed here) (Kenneth Murphy et al., 2022).

The premature T-cells arrive in the medulla, where the venules are located, and then transit to the subcapsular region(Lind et al., 2001). Here, the thymic epithelial cells activate transcription factors TCF1 and GATA3 via Notch signalling, which induces the first maturation process of the so far early thymic progenitors (double negative 1 (DN1) stage) to the DN2 stage (Seo & Taniuchi, 2016). The subsequent differentiation stages via DN3 and DN4 comprise the TCR rearrangement and the expression of a pre-TCR. DN3 cells are already committed to differentiate into αβ-T-cells and cannot fuel other hematopoietic lineages anymore. If the β chain rearrangement fails, these cells undergo apoptosis whereas successful DN4 cells proliferate in the cortex area (Seo & Taniuchi, 2016). Cells harbouring a pre-TCR induce the expression of CD4 and CD8. This developmental stage is called double-positive (DP) T-cell, which, upon proliferation, complete the α-chain rearrangement and express a complete αβ TCR, which can recognize a specific – the cognate - antigen. DP T-cells migrate to the cortex where they interact with thymic cortical epithelial cells and undergo positive selection (Klein et al., 2009): the cortical epithelial cells express MHC (major histocompatibility complex, in humans: HLA – human leukocyte antigen) class I and MHC class II molecules coupled to self-antigens, which can be recognized by the TCR and CD8 or CD4 respectively. Binding of a DP T-cell to an MHC II molecule is a survival signal for the cell and likewise induces maturation: the cell will stop expressing CD8 and will develop into a single positive CD4 T-cell. Accordingly, binding of a DP T-cell to an MHC I molecule, induces the maturation of single positive CD8 T-cell (Klein et al., 2014). This differentiation process limits the T-cell recognition to self-MHC molecules (self MHC-restriction) and DP T-cells, which do not bind at all undergo apoptosis. Further, it comprises a major aspect of central tolerance – negative selection: in order to prevent later, non-desired reactions with self-antigens, DP T-cells which bind with too high affinity or with too low affinity to the presented MHC molecules do not receive a survival signal, but also undergo apoptosis (Hogquist & Jameson, 2014).

The single positive T-cells migrate to the thymic medulla where they are presented self-antigens by medullary epithelial cells, DCs and macrophages (Takaba & Takayanagi, 2017). This is the second part of central tolerance and negative selection – auto-reactive T-cells undergo apoptosis to impede destructive auto-immune reactions. However, some self-antigen reactive cells can eventually develop into regulatory T-cells (T_{reg}), which are important in peripheral tolerance (Hogquist & Jameson, 2014). The variety of presented self-antigens is mediated by the gene *AIRE*, which is short for autoimmune regulator. *AIRE* expressing cells, are not limited to their local environment concerning their protein expression but can also express tissue-extrinsic proteins (Perniola, 2018). This secures an organism wide tolerance towards self-antigens.

Finally, mature, single positive $CD8^+$ and $CD4^+$ T-cells leave the thymus into the periphery - either

into the peripheral blood stream or the lymphatic system. These exported cells only make up around 2 % of the original DP pool (Kenneth Murphy et al., 2022).

5.1.2.2 T-cell activation

Naïve cells that enter the bloodstream migrate to secondary lymphoid tissues, where they potentially encounter antigens and get activated. The antigens are presented to T-cells by DCs, which reside in non-lymphatic tissues, such as barrier tissues like mucosa, where they ingest and digest antigens and then travel to lymph nodes to present the antigen peptides on MHC I and MCH II molecules. Cytokines like interleukin 1 (IL-1) and Tumour-necrosis-factor α (TNF-α) support the presentation of MHC:peptide complexes on the cell surface (Weaver & Unanue, 1990). Since APCs also digest cell debris, they can also present self-antigens to T-cells. However, their ingestion does not trigger PRRs as bacterial or viral molecules would and thus, they do not induce a danger signal. Instead, the activation of PRRs activates the expression of the co-stimulatory molecules CD80 and CD86 on the APC cell surface. The combination of an MHC-peptide complex, recognized by the TCR, and the binding of T-cell CD28 to the co-stimulatory proteins CD80 and CD86 is necessary to successfully activate T-cells (Lanzavecchia & Sallusto, 2001). Further, certain cytokines released by the APC are important in this process: IL-6, IL-12, IL-23, and IL-4 (Lanzavecchia & Sallusto, 2001). Next to the pro-inflammatory signalling, co-stimulation was also observed to play an important role in metabolic adaptation upon $CD8^+$ T-cell activation (Beckermann et al., 2020). These immuno-metabolic aspects will be introduced in section 5.3. Apart from professional APCs as DCs, all nucleated cells express and can present antigens on MHC I. Thus, cytotoxic T-cells can also interact with, recognize, and kill virus-infected cells or tumour cells from various tissues. The encounter of the cognate antigen and costimulatory molecules prime T-cells to active, so-called effector T-cells. While $CD4^+$ T-cells differentiate into several types of T-helper cells, dependent on the signals they receive during the priming, $CD8^+$ T-cells become cytolytic effector cells, capable of killing cells.
In order to guarantee a competent immune response, the effector T-cells undergo clonal expansion, creating a pool of identical cells by massive proliferation.

MOLECULAR PATHWAYS INVOLVED IN T-CELL ACTIVATION

The activation of a T-cell initiates a synchronized cascade of signalling pathways (described in further detail in section 5.1.2.3) and protein expression and presentation. After the first line of activation signalling described in the previous section, the expression of CD69, IL-2 and IL-2R (IL-2 receptor) are initiated. IL-2 supports the proliferation and activation of T-cells but was discovered to be more important for later regulatory T-cells, which cannot autonomously produce IL-2 (Malek & Bayer, 2004). It was even discovered that IL-2 signals were able to induce transplant tolerance in a murine adoptive transfer model (Hirai et al., 2021).
Apart from the MHC:TCR and the CD80/86:CD28 costimulatory binding, further interactions between T-cells and APCs take place such as via members of the TNF receptor family. Binding of T-cell bound CD40L to its receptor CD40 on the APC surface is further promoting the T-cell activation, serving as a signal amplifying mechanism (Elgueta et al., 2009).
Later in the cascade so called immune checkpoint inhibitors come into action: CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) and PD1 (Programmed cell death 1). CTLA-4 competes with

CD28 for CD80/86 binding and due to its higher affinity for the APC protein it is able to dampen the costimulatory signal. Thus, it inhibits the T-cell activity and prevents an overshooting immune response (Halliday et al., 2020). Once the CTLA-4:CD80/86 connection is established, the complex is trans-endocytosed by the T-cell, leaving less CD80/86 on the APC-surface available for costimulation (Qureshi et al., 2011). PD1, on the contrary, binds to its ligand PD-L1 (Programmed cell death 1 ligand) expressed by the APC, which impairs the intracellular CD28 and TCR signalling cascades within the T-cell (Freeman et al., 2000). Deficiencies in all three above mentioned proteins cause severe immune defects: CD40L deficiency results in immunodeficiency and a hyper IgM syndrome (Allen et al., 1993), CTLA-4 and PD1 deficiencies lead to autoimmune phenotypes (Nishimura et al., 1999; Schubert et al., 2014).

While naïve T-cells are quiescent, have low metabolic activity, and are in cell cycle arrest, their activation triggers a cascade of intracellular processes. Initially, the transcription factors NFAT (Nuclear factor of activated T-cells), NFκB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) and AP-1 (Activator Protein 1) are activated and re-induce gene – and protein expression. The metabolism is shifted via the Akt-mTOR axis (AKT: Protein kinase B; mTOR: mammalian target of rapamycin) from the resting phase oxidative phosphorylation (OXPHOS) towards aerobic glycolysis, whereby imported glucose is metabolized into pyruvate and then lactate (see section 5.3 for further details)(Bental & Deutsch, 1993a; Maciver et al., 2013). These processes enable the cell to gain energy and create building blocks for re-entering the cell cycle, cell division, and protein expression. The entire process of T-cell activation until clonal expansion takes several days.

5.1.2.3 T-cell receptor signalling in detail

A fully functional TCR-complex consists of a TCR (αβ), the CD3 complex and the mainly intracellular ζ chain – this whole complex is usually referred to as the TCR. The intracellular parts of the four CD3 chains contain one immunoreceptor tyrosine-based activation motif (ITAM) each and the two ζ chains three ITAMs each – 10 ITAMs in total per TCR. The binding of the TCR to the peptide:MHC complex and a simultaneous binding of the co-receptors to the respective MHC achieves to bring the intracellularly co-receptor bound lymphocyte cell-specific protein-tyrosine kinase (Lck) in close proximity to the ITAMS. Lck then phosphorylates the ITAMs, initiating the intracellular TCR signalling cascade. Zap-70 is recruited to the ITAM phosphorylation sites, activated by binding to them. Subsequently, a complex of Zap-70, SLP-76, and the transmembrane protein LAT (linker for activation of T-cells) is formed (Au-Yeung et al., 2009; Balagopalan et al., 2010; Bartelt & Houtman, 2013; Jordan & Koretzky, 2010; W. Zhang et al., 1998). Costimulation by binding of CD28 to CD80/86 phosphorylates the cytoplasmatic motif of CD28, which recruits and activates the PI3-kinase (phosphoinositide 3 kinase) (Fruman & Cantley, 2002). Together, they activate a variety of downstream processes for T-cell activation:
PI3K phosphorylates its target PIP2 (phosphatidylinositol 4,5-bisphosphate), leading to the formation of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate). PIP3 and the LAT:SLP-76 complex subsequently recruit ITK (IL-2 Inducible T-cell kinase), which phosphorylates PIP3-recruited PLCγ (Phospholipase Cγ) (Berg et al., 2005). PLCγ is the initiator of three major activation

pathways in the T-cell (which are also used in many other cell types), by cleaving PIP2 into DAG (diacylglycerol) and IP3 (inositol 1,4,5-triphosphate) (Y. R. Yang et al., 2012):

1. IP3 diffuses in the cytoplasm and binds to its receptor at the endoplasmatic reticulum, causing a Ca^{2+} release. Elevated Ca^{2+} plasma levels activate many processes including the dephosphorylation of the transcription factor NFAT, which translocates to the nucleus, where it acts as a transcription factor for multiple important T-cell activation genes, such as cytokines (Hogan et al., 2003).
2. The DAG released by PLCγ recruits RasGRP (RAS guanyl nucleotide-releasing protein), which activates Ras (Roose et al., 2005). Additionally, Ras can be activated via LAT:Grb2:SOS creating a positive feedback loop, amplifying the Ras signalling (Das et al., 2009; Downward et al., 1990). Downstream of Ras the MAPK (Mitogen activated protein kinase) pathway is activated, which phosphorylates AP-1, re-initiating the transcription of cytokines and cell cycle proteins.
3. DAG also recruits PKCθ (protein kinase C θ) to the plasma membrane, which initiates a signalling cascade finalizing in the release of NFκB from its inhibitor, allowing it to translocate to the nucleus and act as a transcription factor (Blonska & Lin, 2009; Matsumoto et al., 2005).

In addition to gene expression initiation by transcription factors, PI3K can also activate Akt and mTOR. Akt regulates the expression of proteins important for T-cell migration and homing upon activation, such as CD62L and CCR7 (Kerdiles et al., 2009). Further, Akt facilitates the metabolic switch towards aerobic glycolysis and the increased production of building blocks by increasing the expression of nutrient transporters and by activating mTOR and thus, biomolecule synthesis (Kane & Weiss, 2003).

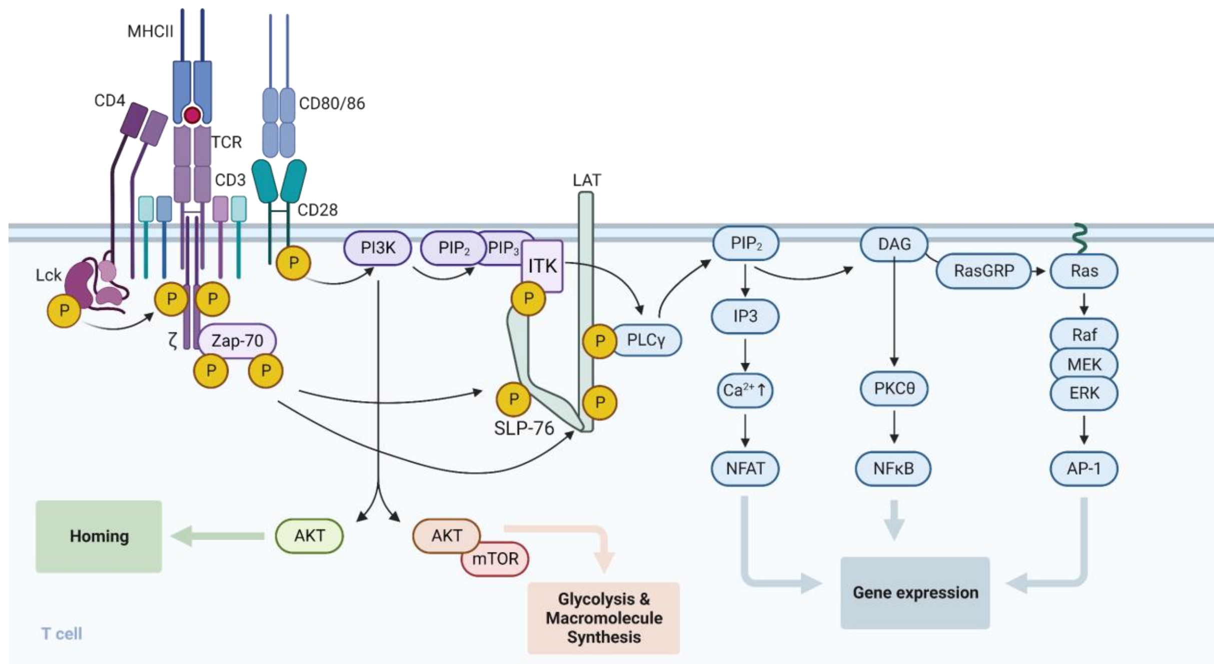

Figure 1: T-cell receptor signalling

Schematic overview of the signalling pathways downstream of TCR activation; exemplary for a CD4+ T-cell. Abbreviations are explained in the main text.

(Adapted from "TCR Signalling Network," created by Gaia Lugano using BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates)

In order to strengthen and stabilize the TCR activation an immunological synapse is formed between the APC and the T-cell (Delon, 2000). TCR activation initiates the activation of several GTPases, which induce actin polymerization in the surroundings and a conformational change in the integrin protein LFA-1 (Lymphocyte Function-Associated Antigen 1), increasing its affinity for the APC integrin ICAM-1 (Intercellular Adhesion Molecule 1) (Bezman & Koretzky, 2007; Monks et al., 1998; Mor et al., 2007). This connection forms a tight, circular synapse where the TCR is located in the central supramolecular activation complex (cSMAC) and the integrins in the surroundings, the peripheral SMAC (pSMAC) (Monks et al., 1998). Additionally, on the APC side, cytoskeleton remodelling is of equal importance to stabilise the synapse and TCR signalling (Leithner et al., 2021). Besides stabilising peripheral T-cell activation, the synapse formation was observed to already influence the T-cell development steps in the thymus (Allam et al., 2021). In CD8+ T-cells the secretion of cytotoxic molecules also takes place inside the immunological synapse (Stinchcombe et al., 2001).

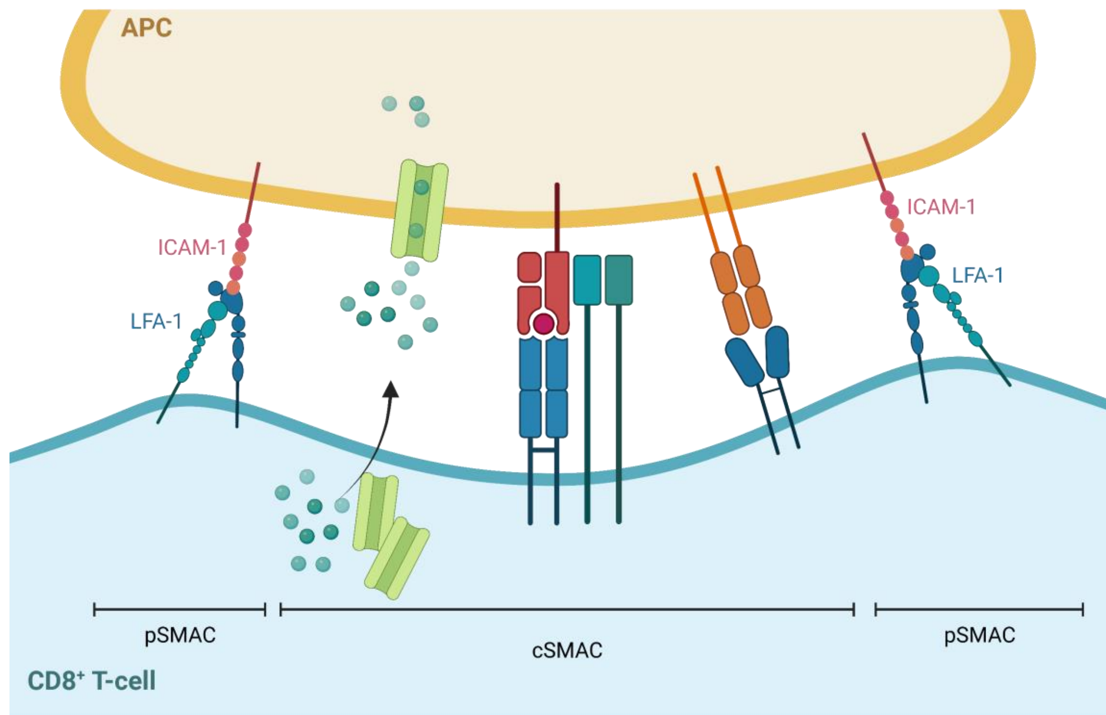

Figure 2: Schematic Representation of an immunological synapse between a CD8⁺ T-cell and an APC (abbreviations are explained in the main text) Created with BioRender.com

As mentioned in section 5.1.2.2 CTLA-4 and PD-1 can decrease the T-cell activation. In the case of CTLA-4the endocytosis of CD80/86 molecules reduces the available signalling molecules, while PD-1 probably recruits dephosphorylases at its cytoplasmatic motifs, which dephosphorylate various intracellular TCR signalling molecules described above and thus, limit the downstream pathway activation (Parry et al., 2005).

5.1.2.4 CD4⁺ Effector T-cells

Activated CD4⁺ T-cells can develop into several effector cell types – T-helper cell types – dependent on the cytokines released by the APCs in a paracrine way, or by the T-cell itself in an autocrine way. The main CD4⁺ T-helper cell types, their functions, and the responsible cytokines and signalling pathways are depicted in the table below (Table 1). A special type of T-helper cells are follicular helper cells (T_{FH}), which stably express CXCR5 and localize in the germinal centres to interact with B-cells, supporting the antibody class switch and antibody production(Nurieva et al., 2008). Furthermore, T_{FH} cells can display characteristics of other T-helper cell types and manoeuvre the Ig class: for example, T_H1 like T_{FH} cells drive IgG2 production and T_H2 like T_{FH} drive IgE production, in correspondence with their functions (Nurieva et al., 2008).
In the absence of co-stimulatory signals CD4⁺ T-cells can become anergic.

Table 1: The different T-helper cell types and their characteristics (Luckheeram et al., 2012)

T-helper cell type	T_H1	T_H2	T_H9	T_H17	T_{FH}	pT_{reg}
Cytokine released by APC	IFN-γ IL-12	IL-4	TGF-β IL-9	TGF-β IL-6 IL-23	IL-6 IL-21	TGF-β IL-2
Activated signalling molecules	STAT1 STAT4	STAT6	STAT6	STAT3	STAT3	STAT5
Activated transcription factors	Tbet	GATA3	GATA3 IRF4	RORγt	Bcl-6	FoxP3
Cytokines released by T-helper cell	IFN-γ	IL-4 IL-5 IL-13	IL-9	IL-17 IL-22	IL-21	TGF-β IL-10
Function/Activity of T-helper cell	Fight microbial infections / infected phagocytes	Fight multicellular parasites and helping in tissue repair	Anti-tumour and autoimmune inflammation	Fight extracellular bacteria and fungi, activate neutrophils and monocytes and epithelial cells	Interact with B-cells to induce antibody production	Regulate immune responses in the periphery, preventing overshooting- and auto-immune responses

5.1.2.5 $CD8^+$ effector T-cells

In contrast to $CD4^+$ T-cells, $CD8^+$ T-cells bind to MHC I, which can be expressed by all nucleated cells and are not limited to professional APCs. Upon activation, $CD8^+$ T-cells differentiate in effector cells – cytotoxic T-lymphocytes (CTL), which harbour the capacity to induce apoptosis in their target cells. This feature is of major importance when it comes to intracellular infections, like viral infections, and to the elimination of tumour cells. Besides, CTL specifically attack their target cells thus, limiting the damage they are causing to single cells, and preventing global tissue destruction.

Similar to T_H1 cells, IL-2, IL-12, and IFN-γ prime $CD8^+$ effector cells, as well as the transcription factors Tbet and Blimp1, and induce the activation of mTOR for the metabolic support of clonal expansion (Cui & Kaech, 2010; Mescher et al., 2006). Without the secondary stimuli IL-12 or IFN-γ $CD8^+$ T-cells become anergic and do not develop into effector CTLs (Mescher et al., 2006).

Cytotoxic T-cells have several mechanisms to induce apoptosis in the target cells:

1. Fas ligand mediated: Fas-ligand is expressed on the $CD8^+$ T-cell surface and binds to CD95 (Fas) on the target cell. The Fas associated death domain can then activate the caspase cascade via caspase 8 and initiate apoptosis of the target cell (Krammer, 2000).
2. Granzyme mediated: $CD8^+$ T-cells express 5 granzyme variants, which are delivered into the target cell by membrane channels formed by perforin. However, Granzyme B was found to be able to enter the target cell by endocytosis independent of perforin (Motyka

et al., 2000). Granzyme B can induce apoptosis via two mechanisms. It harbours a proteolytic function, enabling the direct cleavage of pro-caspases, and activating the caspase cascade (Waugh et al., 2000). Furthermore, it can cause mitochondrial membrane permeability, leading to cytochrome C release, which again activates caspases and apoptosis (Heibein et al., 1999; MacDonald et al., 1999). Granzyme A also has proteolytic capacity and many targets, but its main effect might be the cleavage of DNA, supporting apoptosis by DNA fragmentation (Beresford et al., 1999; Lieberman, 2010). Other human granzymes are granzyme H and K. While granzyme K seems to be perforin dependent, granzyme H can enter the cell via endocytosis like granzyme B (Edwards et al., 1999; MacDonald et al., 1999). The entire granzyme functions, pathways, and targets remain to be fully understood.

3. Granulysin mediated: Granulysin is only found in humans and can initiate apoptosis in high concentrations via caspase activation (Gamen et al., 1998). More importantly, granulysin is anti-microbial, increasing the membrane permeability of intracellular bacteria und thus eliminating them (Ernst et al., 2000; Stenger et al., 1998). However, in contrast to some granzymes, granulysin is dependent on perforin to enter the cells (Stenger et al., 1998).

In response to activation CD8$^+$ T-cells upregulate the expression of cytotoxic molecules. Additionally, in order to ensure a fast reaction, “ready to use” cytotoxic molecules are stored in intracellular granules in their active forms together with serglycin to prevent premature activity (Grujic et al., 2005). More recently, so-called “supramolecule attack particles” were discovered as transmission complexes for cytotoxic molecules (Bálint et al., 2020). Next to their cytotoxic capacities, CD8$^+$ T-cells can increase the global immune response by releasing pro-inflammatory cytokines like IFN-γ, and TNF-α (Kristensen et al., 2004).

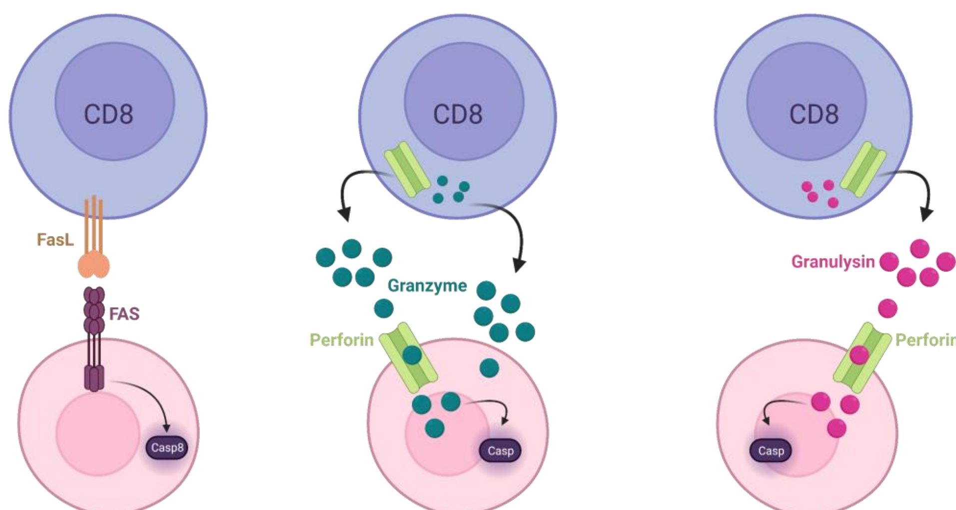

Figure 3: Schematic display of the cytotoxic functions of CD8$^+$ T-cells
Casp = caspase. Created with BioRender.com

5.1.2.6 Peripheral regulatory T-cells

As described in section 0 central tolerance is achieved during thymic development of T-cells by the presentation of self-antigens and selecting some T-cells to become T_{reg} cells. Another subset of T_{regs} develops from naïve T-cells in the periphery. If during the antigen encounter by MHC presentation costimulatory signals from IL-2 and TGF-β are present, FoxP3 expression is induced and the T-cell differentiates into a $CD4^+$ T_{reg} cell (Lohr et al., 2006). T_{reg}s are important for maintaining homeostasis and preventing autoimmunity. There are different mechanisms how this can be achieved (Vignali et al., 2008):

- The $CD4^+T_{reg}$ itself can release anti-inflammatory cytokines, such as TGF-β and IL-10.
- It is thought that $CD4^+T_{reg}$s express high affinity CD25 (IL-2R) and thus deprive the surroundings from activating IL-2.
- $CD4^+T_{reg}$s can induce apoptosis via granzyme release, and they can target APCs by expressing CTLA-4 and thus, limiting the available CD80/86 for costimulation.

Both loss of function and overactivity of regulatory T-cells can have deleterious outcomes: while for example a loss of FoxP3 results in autoimmunity, increased T_{reg} numbers or activity lead to insufficient pathogen clearance and tumour control (Josefowicz et al., 2012).

When it comes to $CD8^+$ T_{reg}s, the data are not that conclusive. Over the years many $CD8^+$ T_{reg} subsets were proposed. However, the definition of their phenotypes was not as straight forward as in $CD4^+$ T_{reg}s, and their *in vitro* vs. *in vivo* regulatory functions were not consistent, as well as the reproducibility in the human system. Niederlova et al. extensively reviewed the available data on “$CD8^+$ $FOXP3^+$ T-cells, $CD8^+$ $CD122^+$ T-cells, $CD8^+$ $CD28^{low/-}$ T-cells, $CD8^+$ $CD45RC^{low}$ T-cells, T-cells expressing CD8αα and Qa-1(HLA-E)-restricted $CD8^+$ T-cells” addressing their uniqueness, their suppressor functions, the proposed mechanisms, and their self-tolerance (Niederlova et al., 2021). They eventually came to the conclusion that regulatory $CD8^+$ subsets exist and harbour great therapeutic potential, however, their definition and characterisation remains difficult, and they fall short to the $CD4^+$ T_{reg}s concerning the detectable cell numbers *in vivo*.

5.1.2.7 Memory T-cells

A characterising aspect of the adaptive immune system is to form an immunological memory to be able to react faster to the same stimulus at a future encounter. Importantly, the principle of vaccination is based on immunological memory, being able to prepare the immune system for an encounter with a certain pathogen.

While T-cell activation is followed by clonal expansion, after a certain time clonal contraction takes places, leaving only a few clones of the antigen specific T-cell to develop into long lived memory cells. Only a small proportion of the clonal population survives the contraction phase and can be found in various organs and tissues and only a minor part remains in the peripheral blood, which is one reason, why the studies of human memory T-cells are difficult (Farber et al., 2014). Which factors decide which cell survives and which will undergo apoptosis, as well as the timepoint of this decision during T-cell differentiation remain incompletely understood. For $CD8^+$ T-cells it seems that already during clonal expansion, and thus, days prior to memory formation, the expression of IL-15 and IL-7 receptors marks the cells that differentiate into

memory cells, as they keep receiving survival signals via these cytokines(Ku et al., 2000; Prlic & Bevan, 2008). Likewise, the signalling mechanisms and changes in gene expression programs switching from proliferating and highly active behaviour, towards homeostasis, longevity, and maintenance metabolism are not yet fully understood. Epigenetic regulation as the fundamental process in memory cell differentiation is discussed (Ku et al., 2000; Tough et al., 2020).

Nonetheless, surface molecules to identify the final memory cell subclasses are well characterized. Memory T-cells can be subclassified into three groups: central memory cells (T_{CM}), effector memory cells (T_{EM}) and tissue resident memory cells (T_{RM}). Which tissues and regions they travel to is determined by the expression of surface molecules and chemokine receptors. The surface markers characterizing these subsets are CD45RA, CD45RO, and CCR7. $CD45RO^{+}CD45RA^{-}$ T-cells can be generally identified as memory cells. CCR7 induces homing in lymphatic tissues and is important for naïve T-cells but also for T_{CM} cells, as they reside in the lymphoid tissues (Sallusto et al., 1999). The combination of CCR7 and CD45RA expression can thus be consulted to identify the specialized subsets: T_{CM} – $CD45RA^{-}CCR7^{+}$; T_{EM} – $CD45RA^{-}CCR7^{-}$; T_{RM} – $CD45RA^{-}CCR7^{-}CD69^{+}$ (Farber et al., 2014; Sallusto et al., 2004).

A reactivation of the memory cells can be achieved by a second antigen encounter, which might be the key aspect of peripheral memory cells residing at the entry sides of pathogens. However, they can also be reactivated antigen-independently by pro-inflammatory cytokines (Woodland & Kohlmeier, 2009).

Similar to T-cells, also B-cells eventually develop into memory cells and antibody secreting plasma cells. In short, upon antigen encounter by APC presentation and the interaction with T_{FH} cells, clonal expansion and antibody production by B-cells take place in the dark zone of a germinal centre, and eventually few clones remain as short-lived plasma cells in the periphery or as long-lived plasma cells in the bone marrow (Akkaya et al., 2019). While plasma cells are residing in the bone marrow, memory B-cells can still travel the periphery and be re-activated upon a second encounter with their cognate antigen.

Data from smallpox vaccinations have shown, that the immunological memory is for life but degrades over time. While antibody and B-cell mediated memory is very stable, $CD8^{+}$ memory declines faster and $CD4^{+}$ mediated memory shows the fastest regression(Hammarlund et al., 2003, 2010).

5.2 Autoimmunity

5.2.1 General aspects of autoimmunity

As described above, the human immune system is a complex, well organized, and tightly controlled network. Nevertheless, there are several pathologies, genetic disorders, or other malfunctions, which disrupt signalling pathways and control mechanism, leading to the breakdown of self-tolerance and the immunological attack of the organism. The prevalence of autoimmune diseases is around 5 % of the general population, with lower prevalence of the single diseases, and they affect disproportionally more women.

Table 2: Simplified summary of common autoimmune diseases and their mechanisms
(Adapted from (Kenneth Murphy et al., 2022))

Diseases	Organ / Systemic	Reaction against / Autoantigen	Driven by	Consequence
Psoriasis	Organ specific	Skin-associated antigens	Autoreactive T-cells	Inflammation of skin with formation of scaly patches or plaques
Rheumatoid Arthritis	Systemic	Antigens localized to joint synovium, eventually other organs	Autoreactive T-cells, autoantibodies/immune complexes	Joint inflammation and destruction causing arthritis
Hashimoto's thyroiditis	Organ specific	Thyroid antigens	Autoantibodies and autoreactive T-cells	Destruction of thyroid tissue and hypothyroidism
Systemic lupus erythematosus	Systemic	DNA and chromatin proteins, ribonucleoproteins	Autoantibodies and autoreactive T-cells	Glomerulonephritis. Vasculitis, rash
Sjögren's syndrome	Systemic	Ribonucleoproteins	Autoantibodies and autoreactive T-cells	Lymphocyte infiltration of exocrine glands, leading to dry eyes/mouth, other organs also involved
Crohn's disease	Organ specific	Microbiome players	Autoreactive T-cells	Intestinal inflammation
Multiple Sclerosis	Organ specific	Antigens localized in the nervous system	Autoreactive T-cells	Destruction of myelin sheaths, leading to muscle weakness and ataxia
Type I diabetes mellitus	Organ specific	Pancreatic islet β cells	Autoreactive T-cells	Destruction of β cells – no production of insulin

Autoimmune diseases can be restricted to specific organs or affect the organism systemically. Although their exact initiation factors remain unknown, they are always driven by a break of tolerance in central as well as peripheral tolerance, induced by genetic and/or environmental factors.

5.2.1.1 Genetic susceptibility

The probably most extreme breaks of tolerance induced by genetic disorders are based on deficiencies in AIRE and FoxP3. AIRE is one of the main players in central tolerance, by enabling the presentation of self-antigens during thymic maturation of T-cells and eliminating autoreactive T-cells by negative selection. A mutation in AIRE causes autoimmune polyendocrine syndrome (APS), leading to symptoms ranging from candidiasis, hypoparathyroidism, affecting adrenal glands and many other organs (Aaltonen et al., 1997). While this mutation affects all T-cells undergoing thymic development, disorders in FoxP3 only affect T_{reg} cells, disturbing later time points of T-cell activation. Mutations in the FoxP3 gene, which is localized on the X chromosome, cause immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX), mainly affecting the intestine, the pancreas, and the skin, however, also other organs can be affected to variable extents (Bennett et al., 2001).

Various patients with autoimmune disorders express certain HLA (MHC) haplotypes, which are not restricted to the respective diseases but show at least a predisposition; for example, in rheumatoid arthritis the common HLA haplotype is HLA-DRB1 (Trowsdale, 2011; Van Der Helm-van Mil et al., 2007). How exactly HLA proteins contribute to autoimmune emergence or progression is not resolved yet. One proposed mechanism is that the simple over-presence of the respective HLA type increases its antigen expression and thus the chance of over-activation of the immune system, however, correlations between gene/protein expression levels and disease severity could not be found (Atassi & Casali, 2008). Nevertheless, although the genotype is no sufficient diagnostic or prognostic marker, it still provides information and can, when in doubt, help to find a differential diagnosis.

Another interesting candidate is CTLA-4, which was found to show a genetic polymorphism in type I diabetes, which resulted in less mRNA expression and protein expression (Hammarlund et al., 2003). The immunoregulatory function of CTLA-4 implies the immune-activating effect, if less or even no protein is expressed.

Cytokines and signalling pathways are also affected. Genetic polymorphisms, such as of the IL-23R were connected to some auto-immune diseases and the importance of IL-23 in T_H17 activity further supported these findings (Hammarlund et al., 2003). Furthermore, the success of the therapeutic antibody *Ustekinumab*, which neutralizes IL-12 and IL-23 and is applied in Psoriasis, Psoriatic arthritis, Colitis, and Crohn's disease, emphasizes the impact of these genotypes.

5.2.1.2 Broken tolerance

The major cause of autoimmunity is the disruption of immune tolerance, which can have different causes.

Despite the elaborate system of central tolerance induction, central tolerance is not inerrable. In a mouse model engineered to tissue-specifically express the protein Cre-recombinase, around 40 % of Cre-specific $CD4^+$T-cells escaped negative selection (Legoux et al., 2015). In a best-case-scenario, these escaped, auto-reactive cells can be controlled in the periphery by turning into T_{reg} cells, by inhibitory molecules like CTLA-4 and PD-1, or by resting in an anergic state.

Unfortunately, and in the worst case-scenario, also T_{reg} cells can fail, inhibitory molecules can be disposed by genetic polymorphisms as described above, and B- and T-cell anergy is known to be reversible under highly pro-inflammatory conditions (Akdis & Blaser, 1999; Goodnow et al., 1991).

In the periphery several aspects can lead to a break of tolerance. There is the possibility of altered protein expression or wrong protein folding and post translational modifications (PTM) that render antigens immune cell activating. The most popular PTM in autoimmunity might be citrullination: in rheumatoid arthritis (RA) autoantibodies against citrullinated proteins (ACPA) can be detected and are used for diagnostic purposes (Schellekens et al., 1998). The crux of PTMs is that they are recognized as foreign antigens, however, harbouring enough similarity with the self-antigen, so that an autoimmune reaction is initiated. The recognition of PTM proteins as foreign antigens is probably promoted in combination with other, environmental noxae, which will be addressed later.
Apoptosis is a quiet cell death compared to necrosis, as it follows a strict programme of DNA fragmentation, contraction of the cell, membrane blebbing and a controlled clearance of the debris by specialized phagocytes. If, however, this process fails or the following phagocytosis and degradation process is defective, parts of the apoptotic cell can be presented via MHC to immune cells and can potentially lead to the production of, for example, anti-nuclear antibodies, which are a major pathogenicity driver in systemic lupus erythematosus (SLE) (Arbuckle et al., 2003; Sisirak et al., 2016).
Some tissues in the organism are considered immunological sanctuaries, because they are sterile and successfully separated from the immune system. Such immunologically deprived areas are the nervous system, the testis, and the cornea. Certain triggers like trauma or infections can disrupt these barriers and make the hidden proteins available for auto-reactive cells, which otherwise would have stayed inactive (Kraus-Mackiw, 1990; Theofilopoulos et al., 2017).

5.2.1.3 Environmental triggers

Twin studies revealed that not only the genome influences the emergence of autoimmune diseases, but also environmental factors have a major influence (extensively reviewed by Bogdanos et al., 2012).
Infections are common external triggers for autoimmunity. Some epitopes on pathogen proteins are similar to those of self-proteins, serving as an immune-evasion mechanism. Inversely, this harbours the potential of immune cells recognizing these antigens as “foreign” but accidentally also reacting against the mimicked self-antigen. This so-called molecular mimicry is exemplified by EBV (Eppstein-Barr virus) infections, which are known to be associated with multiple sclerosis, RA and others, by *P. gingivalis* infections, which expresses an enolase similar to the human α-enolase that auto-reactive cells in RA react against, and by *C.jejunii* infections that are associated with immune reactions against ganglial proteins in Guillain-Barré syndrome (Rojas et al., 2018).
Also, commensal bacteria display a risk factor for autoimmunity. A shift in the microbiome composition is for example found in inflammatory bowel disease (Huttenhower et al., 2014). Furthermore, some metabolites like short chain fatty acids produced by gut-microbes were discovered to modulate T_{reg} responses (Smith et al., 2013).

Moreover, external factors can influence autoimmune diseases. UV radiation is a causative agent in photosensitive cutaneous Lupus: it causes major skin lesions by inducing apoptosis, which cannot be cleared sufficiently, and necrosis occurs. This drives the inflammatory processes and the auto-inflammation (Kuhn et al., 2014). Smoking is an environmental factor causing oxidative stress, apoptosis, and inflammation and it is highly associated with the production of ACPAs in RA (K. Chang et al., 2014). There is a correlation between vitamin D deficiency and RA, which indicates a protective role of vitamin D in the disease. The mechanisms, however, are a subject of ongoing discussion (Meena et al., 2018). Another immunotoxic agent is the anaesthetic halothane, which is known to induce protein alterations through radical stress and eventually causing autoimmune hepatitis (Habibollahi et al., 2011). Also other chemicals and noxae are probably able to induce such auto-inflammatory stress reactions.

5.2.1.4 Other influencing factors

Most autoimmune diseases have a sex bias, affecting women more than men. This bias has long been studied, however, it is still not possible to pinpoint the observation to one or several factors. Females generally display a higher immunocompetence and a stronger humoral response, which could be due to the immunostimulatory effect of oestrogens, which would also be in concert with the observation that autoimmune diseases commonly emerge after puberty entry (Habibollahi et al., 2011). Another interesting aspect is X-chromosome inactivation. In females one X-chromosome is randomly, epigenetically inactivated in cells, which leads to a mosaic expression of X-linked genes and prevents a dual expression of the genes. As some important immune related genes are expressed from the X-chromosome and expressing them twice would drastically increase the immune response, escape from X-inactivation harbours an important factor for the female bias in autoimmunity (Mousavi et al., 2020a). Additionally, changes in the ratio of X-inactivation, deviating from the general 50:50 ratio, were found in autoimmunity, emphasizing the possible role of epigenetic X-inactivation even more (Azzouz et al., 2011).

Another proposed regulatory mechanism in autoimmunity are non-coding, regulatory RNAs. miRNAs were found to increase lymphocyte proliferation in mice, to be associated with spontaneous auto immunity, and miRNA polymorphisms were found in several autoimmune disease related genome studies (Garo & Murugaiyan, 2016).

5.2.1.5 Autoimmune progression by epitope spreading

The break of tolerance is usually the initiation of autoimmunity and once established, other factors drive the progression of the reaction. A common driver of progression is epitope spreading.

Epitope spreading describes the process by which the initial immune response against the so-called dominant epitope of an antigen, spreads over to also non-dominant epitopes of the antigen or even to other epitopes. This can be observed in autoimmunity but also in other cases of tissue damage and in graft rejection (graft versus host disease): the initial immune reaction causes tissue damage and the release of cell debris, which revives and promotes the immune response and even expands it to new epitopes (Vanderlugt & Miller, 2002). The pro-inflammatory environment established by immune cells and cytokines further contributes to this process.

5.2.2 Rheumatoid Arthritis

The following section will focus on pathogenesis, diagnosis, treatment, and future perspectives of RA as the core subject of this thesis, while other autoimmune diseases will be introduced in less detail.

RA is a multifactorial, systemic, autoimmune disease, which typically manifests itself by chronic inflammation of the joints, inducing painful swelling and stiffness. The underlying pathogenic mechanisms are immune cell infiltration and hyperplasia of the synovium, neo-angiogenesis, and fibrosis, eventually leading to functional impairment, destruction, and a loss of function of the affected joint. Eventually also extraarticular organ manifestations may occur. The prevalence of RA is 0.5 % - 1 % in Europe and North America with a profound higher incidence for women then for men and a peak in incidence in the 6th life decade (Myasoedova et al., 2010; van der Woude & van der Helm-van Mil, 2018). However, a high number of undiagnosed and thus unreported cases of (autoantibody-negative) RA is suspected. Collectively, RA is characterized by high direct, indirect, and intangible costs, but also considerable gain of scientific insight and medical innovations in the past years.

5.2.2.1 Clinical manifestation and diagnosis

CLINICAL MANIFESTATION

The clinical spectrum of RA, as of other rheumatic diseases, encompasses a broad array of symptoms that can vary in intensity and presentation among individuals, implying the necessity of lifelong surveillance and management. The exact origin of the disease remains unknown; however, it probably requires a combination of a (genetic) predisposition responsible for the formation of auto-reactive cells and environmental factors that trigger disease onset.

Predominantly involving the small joints of the hands, wrists, and feet, RA is characterized by pain, soft swelling limited to the joint, warmth, and prolonged morning stiffness of more than 30 min up to several hours (Aletaha & Smolen, 2018). However, also larger joints can be affected. A cardinal feature is the symmetrical polyarthritis pattern, which involves multiple joints on bilateral sides of the body. The chronic joint inflammation promotes cartilage erosion, bone degradation, and joint deformities over time, if not treated early and sufficiently (Aletaha et al., 2011). A rare, but severe and potentially life-threatening complication is the involvement of the atlantoaxial joint.

Next to the joint-related symptoms, RA often exhibits extra articular and even systemic involvement. A common extra articular manifestation in autoantibody-positive patients are rheumatoid nodules, which are subcutaneous lumps, particularly common in pressure-prone regions like the elbows. Fatigue, general malaise, and low-grade fever are common and reflect the systemic inflammatory burden. Furthermore, vasculitis, involving inflammation of blood vessels, can lead to skin ulcers, peripheral neuropathy, and even potentially affect internal organs. A frequent, underrecognized complication particularly in male patients with history of smoking is an interstitial lung involvement with progressive lung fibrosis (Spagnolo et al., 2018). Less common but severe is RA-associated myocarditis, which can manifest as chest pain, shortness of

breath, palpitations, and, in severe cases, heart failure symptoms (Crowson et al., 2018).
Due to the autoimmunity autoantibodies occur frequently in RA patients. These can be the already mentioned ACPA or rheumatoid factor (RF) (discovered by Erik Waaler 1937), which are anti IgG antibodies that were originally found in RA patients but are now known to be rather unspecific, as they can also increase during for example pregnancy. Collectively, autoantibody-positive RA patients and particularly those with high autoantibody titres, early bone erosions and history of smoking are significantly more likely to experience an aggressive course of disease with joint destruction and extraarticular manifestations (Bugatti et al., 2018).

DIAGNOSIS

For RA diagnosis several criteria are consulted:
Anamnesis: Assessment of tender and swollen joints, as well as of other typical symptoms such as morning stiffness and fatigue and signs of extraarticular manifestations or important differential diagnoses (e.g., presence of psoriasis vulgaris as a hint of psoriatic disease). Also, the family history has to be taken into account regarding genetic predisposition, comorbidities, and medication.

Laboratory: Specific autoantibodies, including RF and ACPA, as well as acute phase reactants, such as ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein), corroborating the ongoing systemic inflammation, must be analysed. Further, the genotype for HLA-DRB1 can be assessed. Extended immune-serological analyses may be necessary in patients with extraarticular manifestations, to differentiate from other systemic inflammatory diseases such as vasculitis or connective tissue diseases.

Imaging: Imaging modalities, notably X-rays and magnetic resonance imaging (MRI), are valuable tools for assessing joint inflammation, damage, and tracking disease progression. X-rays reveal erosions, joint space narrowing, and periarticular osteopenia, while MRI provides intricate details of soft tissue inflammation and cartilage involvement.

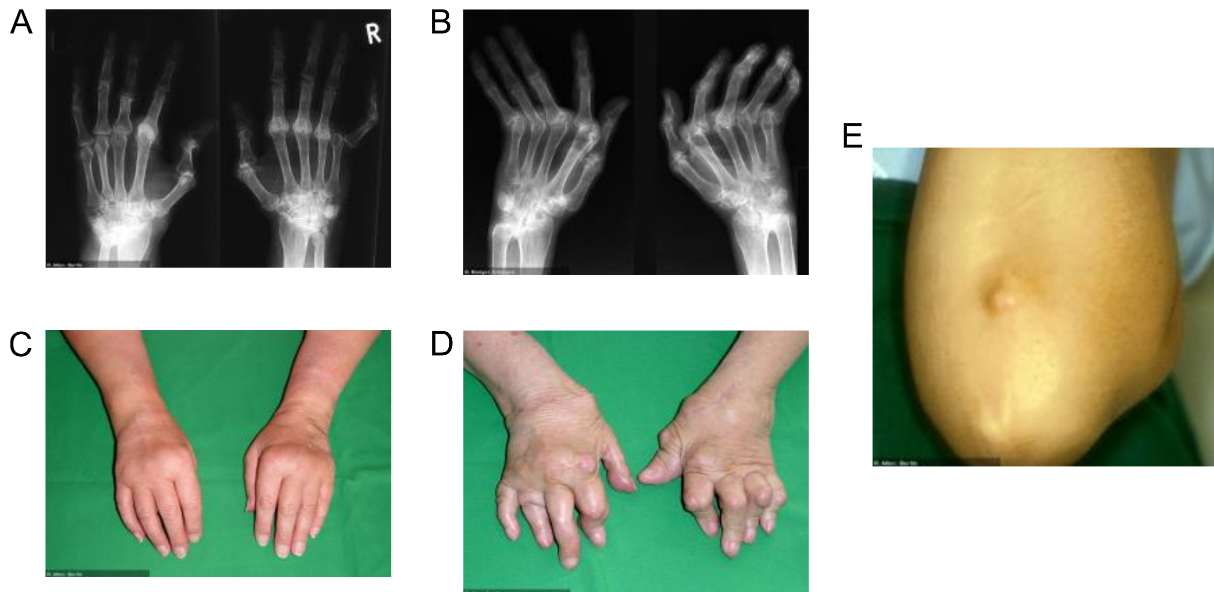

Figure 4: Photographs of typical clinical manifestations of RA

(A) Mutilating RA untreated. **(B)** Severely deforming RA of the hands. **(C)** Typical symmetrical swelling of the metacarpophalangeal joints. **(D)** RA insufficiently controlled for many years. **(E)** Extra articular rheumatoid nodules.

(Alten & Blaubeuren Merckle GmbH, 2004)

DIAGNOSTIC SCORES

There are no validated diagnostic criteria for the clinical routine for RA and most other rheumatic and musculoskeletal diseases. However, a collaborative effort between the American College of Rheumatology and the European League Against Rheumatism, lead to more comprehensible classification criteria, as a significant advancement in accurately defining RA primarily for clinical trials in 2010. Though not directly validated for clinical diagnosis of RA, these criteria nevertheless promote understanding of the core features of the disease and thus can provide lead also in clinical routine to recognize early-stage RA, facilitate timely intervention and improve patient outcomes(Aletaha et al., 2010).

The 2010 Rheumatoid Arthritis Classification Criteria are meant for patients with at least one swollen joint and no other possible explanation.

Table 3: The 2010 Rheumatoid Arthritis Classification Criteria (Aletaha et al., 2010)

1. 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
2. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
3. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or normal ESR	1
4. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

A score of 6 or more indicates a definitive RA.

Collectively, it may not be neglected that RA and other rheumatic diseases harbour a lot of subjectivity when it comes to the manifestations and severeness of the symptoms. Thus, a precise examination by an experienced rheumatologist beyond these criteria is essential.

In order to be able to scale the disease severity, the DAS28-CRP (Disease activity score including 28 joints and the CRP) score was introduced (Fransen et al., 2004). This score is calculated with the following formula, in which VAS depicts the visual analogue score about the global health of the patient:

$$DAS28 - CRP = 0.56 * \sqrt{\#of\ tender\ joints} + 0.28 * \sqrt{\#of\ swollen\ joints} + 0.36 * \ln(\mathrm{CRP} + 1) + 0.014 * \mathrm{VAS} + 0.96$$

A DAS28-CRP value between 0 and 2.6 indicates no disease activity (remission), > 2.6 to 3.2 corresponds to low disease activity and values > 3.2 describe high disease activity.

The DAS28 score can also be calculated without the CRP value or using the ESR value instead, which is subject to ongoing discussions (Greenmyer et al., 2020; Matsui et al., 2007). As in this thesis the DAS28-CRP score is used, these will not be further discussed here. However, it has to be noted that DAS28 score has some general shortcomings, which include lack of reporting of feet involvement leading to underestimation of disease activity and the high rating of the patient's self-assessment resulting in overestimation in chronic pain syndromes as comorbidity. Therefore, additional remission scores have been established in the past years for clinical trials, most importantly.

DIFFERENTIAL DIAGNOSIS

A major challenge in the clinical routine is to differentially diagnose rheumatic diseases as the symptoms are, as stated above, truly diverse. The following list provides some clues that can be consulted for discriminating some of the main diseases of the rheumatic cluster and osteoarthritis (van Schaardenburg & Breedveld, 1994):

Psoriatic Arthritis (PsA):

PsA is linked to psoriasis vulgaris. Its clinical presentation is diverse and, in some cases, when symmetrical polyarthritis of small joints without other symptoms is present, it may be indistinguishable from RA. However, other than RA, it can also have a predominantly asymmetrical pattern, involve distal joints not affected in RA, primarily large joints, spine, tendons, or tendon insertions (enthesitis) and/or all structures in a finger or toe (dactylitis). While psoriatic skin/nail changes are helpful to diagnose PsA, some patients only have a past or family history of psoriasis or may develop it after onset of musculoskeletal manifestation. Imaging may show typical erosions, nail region abnormalities, joint damage and "pencil-in-cup" deformities.

Ankylosing Spondylitis (AS):

AS affects primary the axial skeleton, causing back pain often starting at the lower back due to sacroiliitis, stiffness, and progressive impairment due to ankylosis. Additionally, peripheral joint or tendon involvement and/or ocular inflammation (uveitis) may occur. AS is associated with the HLA-B27 genetic marker, however, axial involvement may also occur in other spondyloarthritides such as PsA often without the presence of this marker. Imaging reveals sacroiliitis, syndesmophytes and spinal fusion can occur.

Sarcoidosis:

Sarcoidosis is a multi-organ disorder with granuloma formation. Musculoskeletal involvement apart from oligoarthritis of primarily ankle joints in acute manifestations is rare. Differentiating factors include other organ manifestations, presence of elevated angiotensin converting enzyme (ACE), soluble interleukin 2-receptor (sCD25) and/or neopterin and biopsy-proven granulomas.

Connective tissue diseases (CTD):

CTDs are multisystemic autoimmune disorders affecting connective tissues. They often have distinct clinical and serological patterns, e.g., systemic lupus erythematosus (SLE) presents with a wide array of symptoms, including skin rashes, joint, muscle and kidney involvement. Systemic sclerosis (scleroderma) involves skin tightening and internal organ fibrosis.

Osteoarthritis (OA):

OA is a degenerative disorder and affects weight-bearing joints, leading to pain mostly after activity. It lacks systemic symptoms and inflammatory markers but can lead to significant pain and suffering. It may involve similar joints as RA and coincide with RA on proximal interphalangeal joints with diagnostic difficulties. However, mostly also distal finger joints and joints of thumb and big toe typically spared by RA are involved, which is well detectable by imaging due to joint space narrowing, osteophytes, and typical morphology of degenerative joint destruction.

5.2.2.2 Pathogenesis

RA manifestation starts with a yet unknown activation of the macrophage-like type A synoviocytes (reviewed by Siouti & Andreakos, 2019). This triggers the release of pro-inflammatory cytokines and chemokines that initiate the infiltration of the joint synovial membrane by T-cells and B-cells. Moreover, endothelial cells are activated, and angiogenesis is initiated in the otherwise non-vascularized environment. The usually very thin synovial membrane thickens and deforms by the proliferation of fibroblasts-like synoviocytes, leading to the formation of the fibrotic "pannus" (Bartok & Firestein, 2010). A pro-inflammatory environment is generated by cytokines, which further promotes the attraction of immune cells and the expression of effector molecules and mediators. These also include metalloproteinases and RANKL (receptor activator of nuclear factor κB ligand), which eventually induce the destruction of bone and cartilage (Takeshita et al., 2021). The basis of RA pathogenesis is the intertwined network of immune cells and fibroblasts, their interactions, their numerous cytokines, and their mutual support, rendering it impossible to make out a single culprit (yet).

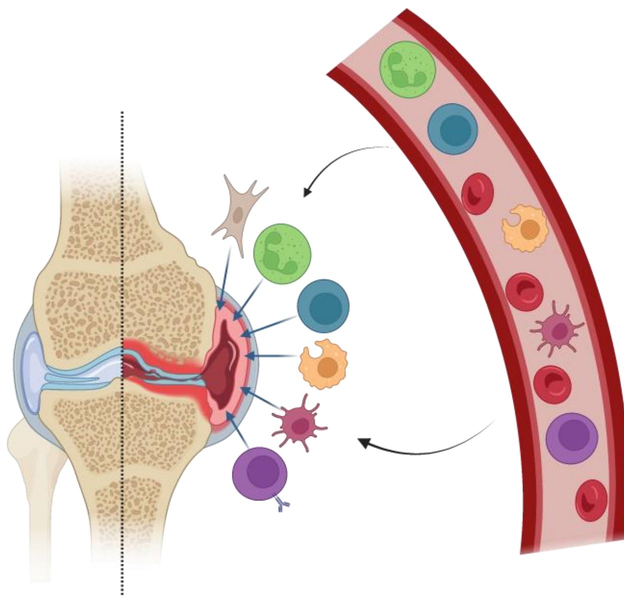

Figure 5: Simplified representation of the joint-related RA pathogenesis

The left part of the picture displays a healthy joint while the right part represents the pannus formation by immune-cell infiltration. Created with Biorender.com.

INNATE IMMUNE CELLS

DCs in RA express higher levels of CCR6 and thus migrate to the synovium, where they secrete TGF-β, IL-1β, IL-6, and IL-23, activating T_H17 cells and overall contribute to the pro-inflammatory environment by cytokine secretion (Segura et al., 2013; van Schaardenburg & Breedveld, 1994). Furthermore, they express BAFF (B-cell activating factor) increasing auto-antibody production, which is also reflected in the higher numbers of synovial DC in ACPA-seropositive patients compared to seronegative ones (Lebre et al., 2008). In addition to cytokines, they also release growth factors like M-CSF (Macrophage colony-stimulating factor) and FGF (Fibroblast growth factor), promoting fibrosis and osteoclast formation (Saferding & Blüml, 2020).

Type A synoviocytes and monocytes entering the joint differentiate into macrophages. In the synovium macrophages are major producers of TNF-α, a driver of inflammation, IL-1β, which activates fibroblasts, and several chemokines, attracting more immune cells to the joint (Saferding & Blüml, 2020). Their capacity to secrete reactive oxygen species (ROS) further drives tissue damage and the related epitope spreading. Interestingly, removing macrophages from a RA rat model significantly reduced the symptoms and improved inflammation (Saferding & Blüml, 2020). Next to their immune function, macrophages are the precursors of osteoclasts and thus, can majorly contribute to matrix destruction upon differentiation.

Due to their aggressive pro-inflammatory functions, neutrophils majorly contribute to tissue damage and destruction at the site of inflammation: they release ROS and radicals, and the ingestion of the resulting cell and tissue debris further fuels their pro-inflammatory profile (Cecchi et al., 2018). Additionally, they express peptidyl arginine deiminase type 4, an enzyme that citrullinates proteins, increasing the amount of ACPA targets. In a knockout mouse model of RA, the elimination of the enzyme decreased serum IgG and IgM and cytokine levels (Suzuki et al., 2016).

Natural killer cells were also found in synovial fluid and harbour a great destructive potential due to their cytotoxic capacity (Pridgeon et al., 2003). Literature on their actual role in RA is still controversial and it is not yet clear, if they have a protective or progressive effect (Kucuksezer et al., 2021).

B-CELLS

Serum and synovial fluid from RA patients contain higher levels of BAFF and APRIL (A proliferation-inducing ligand) compared to healthy controls. These B-cell activators can be produced by various other immune cells such as DC, neutrophils, macrophages, and synoviocytes. In combination with CD40L expressed by T-cells and IL-6, and IL-21, BAFF, and APRIL form a cocktail that massively induces B-cell proliferation and activation (Arkatkar et al., 2017; Moura et al., 2011; Peters et al., 2009). The importance of these cytokines is supported by studies showing that their inhibition in mouse models greatly improved the disease, as well as by the success of the anti-IL-6 therapy (tocilizumab) (H. Wu et al., 2020; D. A. Young et al., 2007). The high numbers of auto-reactive B-cells in RA indicate a malfunction of the checkpoints, which usually prevent the release of self-reactive cells.

Next to professional APCs, antigens can be presented to T-cells by B-cells to induce an immune

response. This ability was found to be a driver for pathogenicity in proteoglycan induced arthritis, as antigen-specific B-cells were necessary to prime T-cells (O'Neill et al., 2005). The synovial membrane in RA generates an ectopic lymphoid tissue, which resembles secondary lymphoid tissues (Bombardieri et al., 2017): there is ongoing B-cell antigen presentation, interaction with T-cells, and even somatic hypermutation and antibody formation (Gause et al., 1995). Moreover, plasma cells reside in the inflamed synovium, producing ACPA and RF directly at the site of inflammation (Humby et al., 2009). Generally, the autoantibody production by peripheral as well as synovial B-cells is probably their main and their most deleterious contribution to RA pathogenesis.

In addition to their immune-stimulatory capacities, there are certain subtypes of B-cells which also express RANKL and contribute to osteoclast formation (Meednu et al., 2016; Thorarinsdottir et al., 2019).

AUTOANTIBODIES

As already mentioned in the previous section, autoantibodies are important drivers of RA by directing and activating T-cells and other immune cells. However, there is also a heterogeneous population of seronegative patients, which points out that autoantibodies are not a prerequisite for disease emergence and maintenance.

RFs were the first autoantibodies detected in RA. They can be IgA, IgM, and IgG classes and are directed against the Fc part of IgG antibodies. This mainly induces the formation of immune-complexes, large conglomerates of antibodies and antigens, which induce pro-inflammatory immune activation.

In contrast to RF, ACPAs are a more specific marker for RA and elevated titres of ACPAs can be detected several years before appearance of the first clinical symptoms of RA (Hensvold et al., 2017; Kroot et al., 2000). Likewise, the downstream effects are rather targeted, as they induce immune responses against specific proteins rather than a general immune activation. Citrullination can occur on various proteins and might even be pathologically induced by environmental factors as smoking (K. Chang et al., 2014). A prominent representative of citrullinated proteins and the ACPA against it is collagen type II (CII), as anti-CII ACPAs can initiate the immune response in joints and thus explain, to some extent, the joint focus in RA (Haag et al., 2014).

Next to citrullination other PTMs can be targets for autoantibodies, such as carbamylation and acetylation. Furthermore, many of these autoantibodies display cross reactivity towards other PTMs. Thus, the more general term "anti-modified-protein antibody" (AMPA) was introduced and might better reflect the actual situation (Kissel et al., 2020).

$CD4^+$ T-CELLS

$CD4^+$ T-cells are implicated in both, the initiation and perpetuation of the autoimmune response targeting joint tissue. Due to the discovery of the HLA-DRB1 (an MHC II protein) genotype as a major risk factor for RA, a lot of focus was set on $CD4^+$ T-cells in the pathogenesis of RA. Further, the above mentioned CTLA-4 and FoxP3 polymorphisms, indicate a vital role for $CD4^+$ T_{reg}s in the break of tolerance. $CD4^+$ T-cells infiltrate the synovium and as they were found to secrete IFN-γ, T_H1 cells were considered drivers of RA (Schulze-Koops & Kalden, 2001). In correlation to ectopic

germinal centres in the synovium, the $CD4^+$ T-cells in the synovium express CD45RO, defining them as antigen-experienced cells that are capable of supporting autoantibody production by B-cells (Thomas et al., 1992). PD- 1^{hi} $CXCR5^-$ $CD4^+$ T-cells in the synovium were thus defined as peripheral T-helper cells (T_{PH}) (Rao et al., 2017). Interestingly, $CD4^+$ T-cells in RA were observed to have a senescent $CD28^-$ phenotype, which is usually associated with less responsiveness, however in this case they did not require costimulation for the expression and release of pro-inflammatory cytokines (Park et al., 1997). Additionally driving the pathogenesis, the $CD4^+$ T-cell pool in RA patients showed limited TCR diversity already in naïve cells, implying that the auto-reactivity is somehow predestined (U. G. Wagner et al., 1998).
Synovial fibroblasts also secrete cytokines and chemo attractants, recruiting and activating T-cells, mainly pro-inflammatory T_H17 cells via IL-6 (Cascão et al., 2010; Pène et al., 2008; Tu et al., 2022). Their importance for RA pathogenesis was demonstrated in a mouse model of RA, where the loss of IL-23 and thus T_H17 cells led to a disease resistance (Patel & Kuchroo, 2015). Next to their active phenotype, T_H17 cells can also promote osteoclastogenesis and hence bone resorption (Kotake et al., 1999). Nevertheless, anti IL-17 therapies are not as effective as expected in RA patients and turned out to be more successful in psoriasis and psoriatic arthritis patients.

$CD8^+$ T-CELLS

The role of $CD8^+$ T-cells in RA was rather underestimated for a long time, despite their cytotoxic effector functions and the destructive potential they harbour. However, by now we know that around 40 % of the synovium-infiltrating T-cells are $CD8^+$ T-cells (McInnes, 2003). Additionally, the frequency of $CD8^+$ T-cells, especially of $CD8^+$ T_{EM}-cells, is increased in the synovial fluid of RA patients (Cho et al., 2012). The same study could also show that RA $CD8^+$ T-cells had a less differentiated but more active phenotype and showed increased proliferation compared to healthy controls. Although $CD8^+$ T-cells from synovial fluid had a higher activity, the production of pro-inflammatory cytokines by synovial $CD8^+$ T-cells correlates with that from peripheral blood $CD8^+$ T-cells, demonstrating that also peripheral blood CD8 are eligible for investigation (Carvalheiro et al., 2015). Accordingly, the cytokine expression was positively correlated to the DAS28 of the patients, underlining the importance of $CD8^+$ T-cells in RA (Carvalheiro et al., 2015). Further, it was shown that the formation of ectopic germinal centres in the synovial membrane of RA patients is mainly induced by $CD8^+$ T-cells, emphasizing the role of $CD8^+$ T-cells for activity and interactions at the site of inflammation (Kang et al., 2002). The depletion of $CD8^+$ T-cells in the KBxN (see below) mouse model improved RA rapidly and cytokine levels were decreasing, illustrating that they are drivers of the disease in this model (Raposo et al., 2010).
A very recent study, which performed single cell TCR sequencing from RA patient samples found that there are large populations of granzyme B expressing $CD8^+$ T-cells with TCR against citrullinated proteins in the synovium, emphasizing their involvement in synovitis and joint destruction (Moon et al., 2023).
Further, $CD8^+$ T-cells express lower levels of PD-1 displaying a non-exhausted, prolonged active profile, which seems to be increasingly promoted by smoking and nicotine (S. Li et al., 2014; Wasén et al., 2017).
Taken together, the role of $CD8^+$ T-cells in promoting and maintaining inflammation in RA can and

should not be neglected and a more profound understanding of their activity and regulation might support the search for new therapeutic agents.

5.2.2.3 Animal models of RA

RA is not naturally occurring in rodents, thus, several animal models with transgene-dependent spontaneous or protein-induced polyarthritis were developed.
Collagen-Induced Arthritis (CIA) Model (Trentham et al., 1977):
Mice are immunised with rat CII and complete Freud's adjuvant (CFA) that induce an autoimmune response directed against the joints. Autoreactive T-cells simultaneously recognize collagen peptides as foreign and self, as there is only one amino acid difference, leading to the activation of inflammatory cascades, cytokine production, immune cell infiltration, synovial hyperplasia, and joint destruction. Involvement of T helper 17 (Th17) cells and regulatory T cells (Tregs) contributes to disease development. Collagen Antibody-Induced Arthritis (CAIA) can be induced by transferring the serum of CIA mice to healthy wild type mice.

K/BxN and K/BxN Serum Transfer Model (Kouskoff et al., 1996):
In this model, mice with the K/BxN TCR transgene produce T-cells that are specific for glucose-6-phosphate isomerase (GPI), a self-antigen present in joint tissues. Upon activation, these T-cells promote the production of anti-GPI antibodies. The autoantibodies form immune complexes inducing a wide-ranging immune response. Serum from diseased animals can be transferred to healthy wild type mice and will also induce RA there. The K/BxN model is helpful to study the antibody-based processes in RA.

TNF Transgenic Mouse Models (Keffer et al., 1991):
In the TNF transgenic mouse model, mice are genetically engineered to express elevated levels of TNF-α, often under the control of a promoter that drives its expression in immune cells. The excess TNF-α production leads to chronic systemic inflammation, resembling the inflammatory environment observed in RA, stimulating the activation of various immune cells, including macrophages, neutrophils, and T cells. The TNF model is a good means to study anti TNF therapies.

SKG Mouse Model (Sakaguchi et al., 2003):
In the SKG mouse model, mice carry a specific genetic mutation in the ZAP-gene, leading to a defect in TCR signalling, to the breakdown of immune tolerance, and allowing the activation of autoreactive T-cells. As a spontaneous model, SKG mice resemble many aspects of the human RA and allows their exploration.

Pristane-Induced Arthritis (PIA) Rat Model (Vingsbo et al., 1996):
In the PIA model, rats are injected intradermally with pristane, a hydrocarbon oil. Pristane serves as an adjuvant, activating APCs at the injection site. The activated APCs process and present self-antigens to T-cells, inducing a breakdown of immune tolerance and the initiation of an autoimmune response against joint tissue.

5.2.2.4 Therapeutic strategies

Numerous innovative therapeutic options for RA patients have emerged since the turn of the millennium. However, as previously stated, all aim at a symptomatic relief by curbing inflammation. A curative treatment is not yet available. Nevertheless, in contrast to the initial anti autoimmune therapies, which were mostly global immunosuppression with the respective adverse effects, targeted approaches were mainly developed in the past years. Due to the multifaceted nature of the disease and the very individual progressions it is important to involve patients in treatment decisions, considering their individual disease manifestations and courses, lifestyles, preferences, concerns, and expectations. Further, despite the new developments, the therapies at hand still harbour the potential for a variety of side effects, which is why individual comorbidities should be considered with regard to the individual patient, when choosing treatment options.

Evidence-based treatment algorithms have been formulated and updated by national and international societies to facilitate and standardize therapeutical decisions, most recently the “EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs” (Smolen et al., 2022).
Briefly, the treatment should be initiated as early as possible, ideally immediately after diagnosis. The first therapy phase is a combination of Methotrexate (MTX) and possibly glucocorticoids, or another conventional DMARD if there is a contraindication for MTX. The glucocorticoid therapy should be designed in a pulse therapy and be tapered and stopped as soon as possible. The subsequent assessments should be after three and six months and if the symptoms improved, a dose reduction can be initiated. If there is no or low improvement other conventional DMARD combinations can be tested. However, if the prognosis is rather disadvantageous (for example high autoantibody levels or high DAS) another biological DMARD can be added, or JAK inhibitors can be consulted. Again, after an assessment after three to six months the therapy can be sustained, or the dose even reduced, if there is improvement. Otherwise, the biological DMARD is to be changed. These guidelines should be followed until – ideally – a remission is achieved.
In the cited recommendation it was agreed that the choice of therapy should always be made based on the costs of the drug, if there are two or more options that achieve the same outcome. Costs in this case were understood as financial but also socially and practicably, for example should oral MTX administration be favoured over parenteral and biosimilar DMARDs (adalimumab, etanercept, infliximab, rituximab) over biological originator DMARDs.

Table 4: Available therapeutic options for RA

Adapted from (Smolen et al., 2022)

Class	Drug (example)	Mode of action (if known)
Glucocorticoids	Prednisolone	Anti-inflammatory by downregulation of intracellular pro-inflammatory signalling
DMARDs – Disease modifying anti-rheumatic drugs		
Synthetic DMARDs		
	Conventional	
	Methotrexate	Folate antagonist, thought to reduce cell division and proliferation (exact mechanism not known)
	Leflunomide	Inhibits de novo pyrimidine synthesis, decreases immune cell proliferation mainly T-cells
	Sulfasalazine	Anti-inflammatory by inhibiting prostaglandin synthesis
	Hdroxychloroquine	Thought to interfere with lysosome function and antigen processing
	Targeted	
	Baricitinib	JAK1 and JAK2 inhibitor, decreasing IL-6 and IFN-γ production
	Filgotinib	JAK1 inhibitor, mainly IL-6 reduction
	Tofacitinib	JAK1 and JAK3 inhibitor, IL-2, IL-4, IL-6, IL-15, and IL-21
	Upadacitinib	JAK1 inhibitor, mainly IL-2, IL-4, IL-6, IL-15, and IL-21
Biological DMARDs		
	Adalimumab	Monoclonal antibody against TNFα
	Certolizumab	Monoclonal antibody fragment against TNFα
	Etanercept	Human TNF receptor fused to the Fc-portion of anti TNFα, sequesters TNFα
	Golimumab	Monoclonal antibody against TNFα
	Infliximab	Monoclonal antibody against TNFα
	Sarilumab	Monoclonal antibody against IL-6R
	Tocilizumab	Monoclonal antibody against IL-6R
	Abatacept	Fusion of the extracellular domain of CTLA-4 and the Fc-portion of IgG, prevents T-cell costimulation
	Rituximab	Monoclonal antibody against CD20, induces B-cell death by antibody-dependent cellular cytotoxicity

5.3 Immunometabolism

A few years ago, immunometabolism was a rather new, emerging field. By now it can be considered a manifest part of immunology research. That metabolic processes might influence immune-cells and their effector functions, becomes evident if one considers the amount of energy and the required building blocks that are necessary to keep up with the high proliferative turnover and the massively increased production of cytokines, chemokines, and other immune modulators. The following sections will briefly introduce the general concepts of the basic metabolic pathways glycolysis, TCA-cycle and OXPHOS and then more detailed discuss the immunometabolic regulations in immune-cells and especially in $CD8^+$ T-cells in RA.

5.3.1 Metabolic Pathways

5.3.1.1 Glycolysis

Carbohydrates are the body's primary energy sources, followed by fatty acids and proteins. Following consumption, carbohydrates are broken down to glucose, which is then processed, generating adenosine triphosphate (ATP). The first catabolic reaction chain in this process is glycolysis. Glycolysis does not require oxygen and thus generates energy in a fast, anaerobic manner. Glucose is transformed into pyruvate by ten enzymatic reactions before being transported into the mitochondria. Under aerobic conditions the pyruvate dehydrogenase (PDH) transforms pyruvate into acetyl-Coenzyme A (CoA), which drives downstream catabolic activities. Under anaerobic conditions, lactate is produced from pyruvate by lactate dehydrogenase A (LDHA) and mainly transported out of the cell by MCT4 (Monocarboxylat-Transporter-4).
Intermediate metabolites of glycolysis can, however, also enter other metabolic pathways (see pentose phosphate pathway).
Per molecule of glucose, glycolysis produces two molecules of ATP and two molecules of reduced NADH (Nicotinamide adenine dinucleotide).

5.3.1.2 Pentose Phosphate Pathway

In the pentose phosphate pathway, glucose-6-phosphate is transferred into 5-carbon-sugars, which are used for nucleotide genesis. This reaction produces the antioxidant nicotinamide adenine dinucleotide phosphate (NADPH), which can be, amongst others, used to produce ROS for oxidative burst. The final product of the pentose phosphate pathway is fructose-1,6-bisphosphate, which can re-enter glycolysis.

5.3.1.3 Fatty acid oxidation

Next to glucose fatty acids are a major nutrient source. Upon addition of an acetyl-CoA chain to the fatty acids, β-oxidation produces huge quantities of acetyl-CoA, NADH, and $FADH_2$ (Flavin adenine dinucleotide), all of which are utilized to produce ATP in the TCA cycle and the electron transport chain. Fatty acid oxidation is an aerobic pathway. Eventually, one palmitate molecule can yield around 100 molecules of ATP, rendering fatty acid oxidation the most productive energetic pathway.

5.3.1.4 TCA-cycle

Acetyl-CoA molecules produced by glycolysis, fatty acid oxidation, and amino acid breakdown enter the mitochondria, where they are further processed by the TCA cycle. All the products of its reactions serve as the reactants for subsequent syntheses, creating a cyclic process. Acetyl-CoA degrades into two molecules of CO_2 during the cycle. Reduced $FADH_2$ and NADH are additional side products that are further processed as electrons in the electron transport chain (ETC) for the ultimate synthesis of ATP by OXPHOS. Only one ATP molecule is generated by the TCA cycle itself for every two molecules of glucose / for every acetyl-CoA molecule.

Next to acetyl-CoA, the TCA cycle can also be fuelled by anaplerotic reactions. Pyruvate can be converted into oxaloacetate by the pyruvate decarboxylase and glutaminolysis can produce α-ketoglutarate (aKG) from glutamine via glutamate.

The TCA cycle is an amphibolic process, which means that it can work in a catabolic, energy producing way, or in an anabolic way, only generating co-factors and building blocks for the homeostasis of biosynthetic processes (Baldwin & Krebs, 1981). Isocitrate dehydrogenase and aKG dehydrogenase are the control steps of the cycle and aKG dehydrogenase is also the rate limiting enzyme. Both proteins are inhibited by the products NADH and ATP, in order not to produce excess energy.

5.3.1.5 Oxidative Phosphorylation

OXPHOS, which is carried out by the fifth complex of the ETC, is the last stage of ATP synthesis. Four protein complexes make up the ETC, which is found at the inner mitochondrial membrane (see table 1). Electrons are moved from one complex to the next when $FADH_2$ and NADH from the TCA cycle are re-oxidized to FAD^+ and NAD^+. As a redox coenzyme, complex I and II convert ubiquinone to ubiquinol, whilst complex III and IV are reducing cytochrome C. It is interesting to note that succinate dehydrogenase (SDH), an enzyme that is also a component of the TCA cycle, is present in complex II. To create the mitochondrial membrane potential, all complexes—aside from complex II—transport protons into the intermembrane space. The ATP synthase, which is frequently referred to as complex V, utilizes this potential. The protons flow back into the mitochondrial matrix and pass the enzyme in order to drive OXPHOS, which is the process that produces ATP. By completing all metabolic processes, 38 molecules of ATP are generated for every molecule of glucose.

5.3.1.6 One carbon metabolism

One-carbon metabolism describes the metabolic processing of folate, which can not be produced *de novo* by humans and thus, is essential to be taken up by nutrition, for example in the form of vitamin B9 (folic acid). Folic acid is converted to dihydrofolate, which is further processed to tetrahydrofolate and finally to 5,10-methylene-tetrahydrofolate and further similar products. All species have a pteridine ring, which can carry 1C units that are important for other metabolic processes. Further downstream, the one-carbon pathway gives rise to amino acids as serine, thymidine, and methionine and also purines.

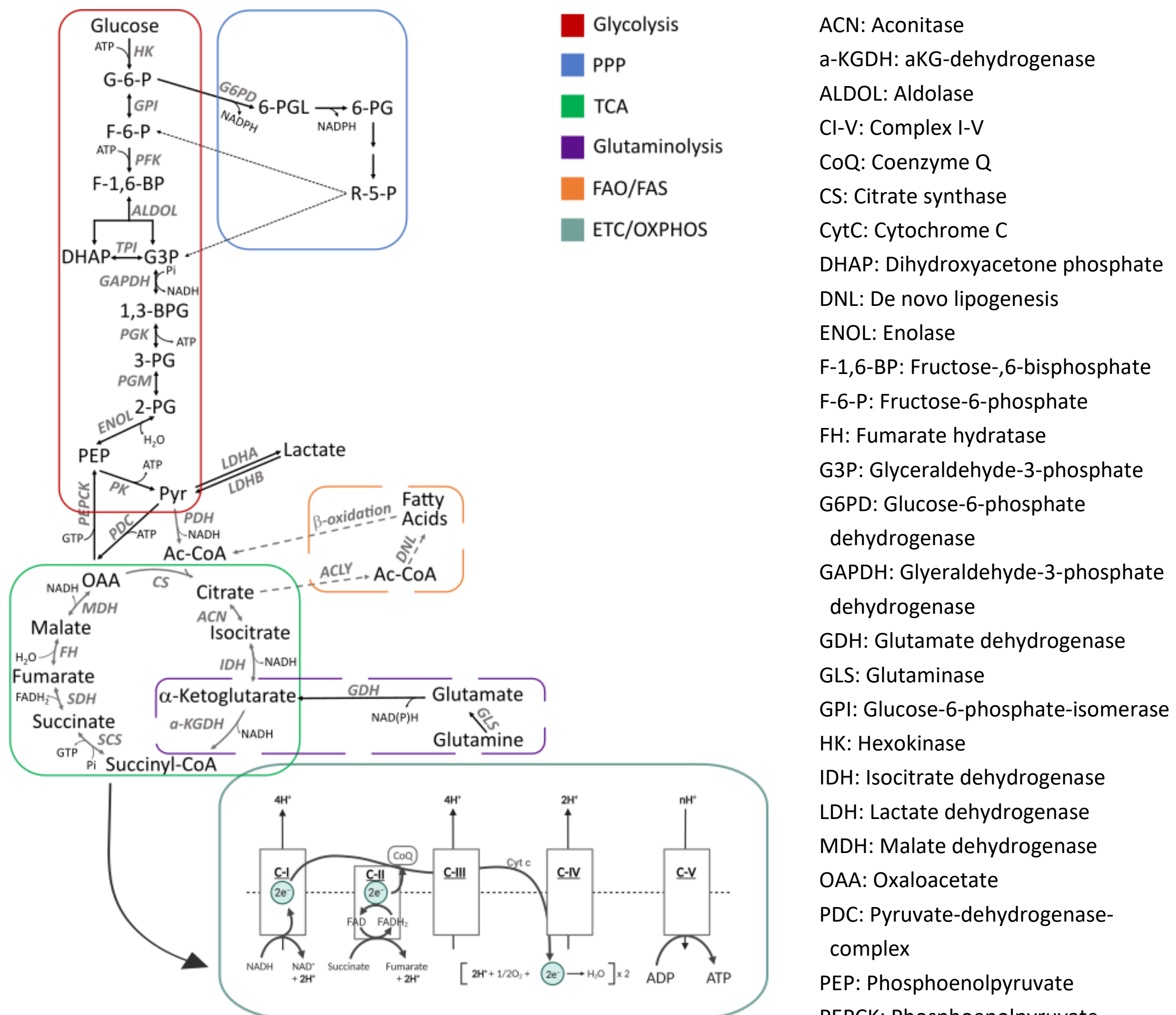

Figure 6: Schematic display of the main metabolic pathways and their connections

Adapted with authorization from learning materials created by Prof. Rui Carvalho.

Abbreviations:

1,3-BPG: 1,3-Bisphosphoglycerate
2-PG:2-Phosphoglycerate
6PG: 6-phosphogluconate
6-PGL: 6-phosphogluconolactone
Ac-CoA: Acetyl-CoA
ACLY: ATP citrate lyase
ACN: Aconitase
a-KGDH: aKG-dehydrogenase
ALDOL: Aldolase
CI-V: Complex I-V
CoQ: Coenzyme Q
CS: Citrate synthase
CytC: Cytochrome C
DHAP: Dihydroxyacetone phosphate
DNL: De novo lipogenesis
ENOL: Enolase
F-1,6-BP: Fructose-,6-bisphosphate
F-6-P: Fructose-6-phosphate
FH: Fumarate hydratase
G3P: Glyceraldehyde-3-phosphate
G6PD: Glucose-6-phosphate dehydrogenase
GAPDH: Glyeraldehyde-3-phosphate dehydrogenase
GDH: Glutamate dehydrogenase
GLS: Glutaminase
GPI: Glucose-6-phosphate-isomerase
HK: Hexokinase
IDH: Isocitrate dehydrogenase
LDH: Lactate dehydrogenase
MDH: Malate dehydrogenase
OAA: Oxaloacetate
PDC: Pyruvate-dehydrogenase-complex
PEP: Phosphoenolpyruvate
PEPCK: Phosphoenolpyruvate carboxykinase
PFK: Phosphofructokinase
PGM: Phosphosglyceratemutase
PK: Pyruvate kinase
Pyr: Pyruvate
R-5-P: Ribulose-5-phosphate
SCS: Succinyl-CoA-synthetase
SDH: Succinate dehydrogenase
TPI: Triose phosphate isomerase

5.3.2 Metabolism of immune cells

Although OXPHOS generates more energy per molecule of glucose, macrophages were discovered to primarily depend on glycolysis upon activation and inversely that when glycolysis was inhibited, they could not be activated (Michl et al., 1976). It was observed that also other immune cells mainly rely on glycolysis during their active/effector phase such as DCs, NK cells, B-cells, and T-cells (Donnelly et al., 2014; Doughty et al., 2006; Krawczyk et al., 2010; Souto-Carneiro et al., 2020). From a signalling perspective the increase in glycolysis makes sense due to the activation of the AKT/mTOR axis, which promotes glucose import and metabolism. From an energy

production perspective, glycolysis does not provide as much ATP as OXPHOS, but it is the faster pathway, which is necessary for the immediate switch to effector functions. Furthermore, glucose-6-phosphate from glycolysis can enter the PPP, providing necessary building blocks for macromolecule biosynthesis and proliferation, as well as NADPH for ROS production and oxidative burst.

High lactate concentrations at inflammation sites due to increased aerobic glycolysis were described to be sensed by $CD4^{+}$ and $CD8^{+}$ T-cells, which in reaction to the high lactate concentrations decreased their migratory activity, further promoting inflammation in these environments (Haas et al., 2015).

Upon pathogen clearance, T-cells enter the memory stage, which is accompanied by a switch back to TCA and OXPHOS, probably being more suitable for long term energy homeostasis. In macrophages it was found that M2 like macrophages, which are rather anti-inflammatory, also use the TCA cycle more than pro-inflammatory M1 like macrophages (Jha et al., 2015). Furthermore, DCs and macrophages seem to have a broken TCA cycle, indicated by the accumulation of intermediates as citrate and succinate (Tannahill et al., 2013). Citrate, if available in excess, can be transported to the cytosol and used for prostaglandin synthesis. Moreover, it can be converted into itaconate, a quite recently discovered anti-microbial metabolite. Succinate can induce prolonged IL-1β synthesis by stabilizing HIF-1α (hypoxia inducible factor-1α) (Tannahill et al., 2013).

HIF-1α is generally an interesting metabolic transcription factor. Under normoxic conditions it is hydroxylated and thus, degraded, while under hypoxic conditions the hydroxylation enzyme is inactive and HIF-1α can translocate into the nucleus, where it activates the transcription of glycolysis genes such as pyruvate dehydrogenase kinase, which prevents the formation of acetyl-CoA from pyruvate and thus further drives glycolysis (J. W. Kim et al., 2006; Schofield & Ratcliffe, 2004). This pathway is affected in physiological and pathophysiological situations where metabolites as succinate, fumarate and lactate accumulate and inhibit the hydroxylation of HIF-1α, also under aerobic conditions (Pollard et al., 2003; Végran et al., 2011).

Additionally, the metabolism of certain amino acids can prime immune cell functions. Glutamine is required for IL-1 production by macrophages, for nitric oxide synthesis, and for antigen-driven activation of T-cells and B-cells. Arginine is also necessary for nitric oxide production and arginine deprivation impairs T-cell activation and proliferation. Serine can be used by T-cells for glycine production and one carbon metabolism, which are important for effector cell expansion. Tryptophan is mainly broken down by the indoleamine 2,3-dioxygenase, which is upregulated in macrophages and DCs and overall tryptophan metabolism is associated with activation- and inflammation-suppression in various immune cells. (extensively reviewed by L. Yang et al., 2023).

5.3.3 Immunometabolism in rheumatoid arthritis

Metabolism is known to play a role in several autoimmune diseases (N. Huang & Perl, 2018). Due to the focus of this thesis on RA, I will concentrate on immunometabolic modulation in RA in this section.
One of the earliest studies proposing a mechanistic correlation between metabolism and RA, observed that synovial cells exhibited an increased glycolytic activity (Henderson et al., 1979). Further, several studies analysed the serum and plasma metabolites in RA patients compared to healthy controls and found profound changes that even correlated with disease activity, as for example increased lactate and glucose levels (S. P. Young et al., 2013). In accordance, RA joints were discovered to create a hypoxic environment inducing HIF-1α, which promotes pro-inflammatory immunometabolism and increased glycolysis as described above (Guo & Chen, 2020). Synovial fibroblasts from RA patients produce higher levels of lactate than those from OA patients, which could be even increased by the stimulation with T-cell mediators (Kvacskay et al., 2021). In accordance, the inhibition of MCT4 that transports lactate out of the cells was discovered to decrease CIA (Fujii et al., 2015). In contrast to the upregulated glycolysis in RA macrophages and DCs, $CD4^+$ T-cells take up a lot of glucose but fuel the PPP with it, which leads to an altered ROS homeostasis (Z. Yang et al., 2016).
As stated above, succinate can increase the pro-inflammatory environment by stabilizing IL-1β. This effect was found in RA due to increased succinate levels, which also promoted angiogenesis, pannus formation, and additionally the binding of succinate to GPR91 (G-protein coupled receptor) increased DC migration and DC mediated TH1 activation in RA (Y. Li et al., 2018; Rubic et al., 2008). The fatty acid composition in RA synovia also differs from those from healthy controls (Bole & Peltier, 1962). In RA $CD4^+$ T-cells lipid droplets accumulate and influence the cellular metabolism, promoting an inflammatory profile (Shen et al., 2017).
Amino acid metabolism of serine, glycine, and phenylalanine was discovered to be linked to lymphoid gene expression levels and alanine, aspartate, glutamate metabolism to synovial TNF-α levels in RA (Narasimhan et al., 2018).
Finally, common therapeutic agents used for RA treatment have been found to modulate immune cell metabolism. Glucocorticoids or example can influence the respiratory rate in animal models, MTX probably influences the purine and pyrimidine synthesis, and JAK inhibitors were shown to reduce glycolysis in the synovial environment (Cronstein & Aune, 2020; McGarry et al., 2018; Moreno-Aurioles & Sobrino, 1991).

5.3.4 Immunometabolism of $CD8^+$ T-cells in rheumatoid arthritis

The utilisation of aerobic glycolysis was first described for - and for long thought to be restricted to – cancer cells by Otto Warburg in 1923, which is why this observation was named after him: Warburg effect. The altered metabolism of cancer cells even became a "hallmark of cancer" in the famous classification by Hanahan and Weinberg in 2000. By now we know that the switch to aerobic glycolysis is not a feature limited to cancer but in fact a necessary adjustment in highly proliferative cells – such as $CD8^+$ T-cells upon activation (Bental & Deutsch, 1993b). Eventually, by differentiating into memory cells, $CD8^+$ T-cells preferentially use OXPHOS again to keep up a long term energy homeostasis (van der Windt et al., 2012).

$CD8^+$ T-cells in RA however, seem to be stuck in a vicious circle between immune-activation and aerobic glycolysis, they produce large amounts of lactate, decrease the usage of downstream pathways like the TCA cycle and oxidative phosphorylation (OXPHOS), and they cannot reverse the metabolic switch (Souto-Carneiro et al., 2020). Most importantly, this observation was absent in $CD8^+$ T-cells from PsA and spondylitis patients. Partly, this might be explainable by the constant presence of autoantigens and pro-inflammatory triggers in RA. Interestingly however, already naïve stage RA $CD8^+$ T-cells display this hyper-glycolytic phenotype, rather suggesting a regulatory origin (Souto-Carneiro et al., 2020). The same study then further analysed the gene expression levels of glycolytic enzymes, revealing an increase in gene expression in RA $CD8^+$ T-cells, most prominently in LDHA expression. *In vitro* inhibition of LDHA with the small chemical compound FX11, was able to reduce the pro-inflammatory phenotype of the cells, thus, LDHA might be a linchpin of the metabolic phenotype in RA. LDHA catalyses the reaction from pyruvate to lactate, withdrawing pyruvate from the downstream TCA cycle reactions.
In addition to the glycolytic changes in RA $CD8^+$ T-cells, lipid metabolism is also altered. On the one hand, $CD8^+$ T-cells from RA patients seem to import and store more neutral lipids (Kraus et al., 2023; Souto-Carneiro et al., 2020). On the other hand, $CD8^+$ T-cells from MTX therapied patients displayed major changes in the gene expression profile of lipid metabolism related genes and further, the *in vitro* inhibition of fatty acid oxidation reduced the pro-inflammatory profile of $CD8^+$ T-cells (Kraus et al., 2023).
Intriguingly, the glycolytic profile could also be observed in $CD8^+$ T-cells from cancer patients undergoing immune-checkpoint-inhibitor treatment and developing RA-like immune-related adverse-events (Benesova et al., 2022). This further stresses that metabolic activity might be an underlying cause for the RA $CD8^+$ T-cell phenotype and thus, its regulation has to be further elucidated.

5.3.5 Moonlighting of metabolic enzymes

Apart from the so far described enzymes, their roles, reactions, substrates, and products, many enzymes can also perform distinct reactions or work in other subcellular locations. All glycolytic enzymes can for example also be found in the nucleus, although the exact roles are not yet evident (J. W. Kim & Dang, 2005). PKM2 for example can act as an transcription factor and increase HIF-1α expression and LDHA is translocated into the nucleus upon phosphorylation, binds to DNA, and produces nuclear lactate (Boukouris et al., 2016).
On a post transcriptional level, some metabolic enzymes can influence the protein expression by binding to mRNAs: GAPDH can bind the mRNA of IFN-γ and IL-2 if glucose concentrations are low, preventing their transcription, and LDHA binds AU-rich (adenine and uracile) elements in GM-CSF mRNA (granulocyte-macrophage colony-stimulating factor) and thus, may also be able to bind to other mRNAs with AU-rich elements (C. H. Chang et al., 2013; Pioli et al., 2002).

5.4 Epigenetics

To fit into the nucleus, DNA is tightly packed and wrapped around histone protein complexes, which, however, renders the compact regions inaccessible for for example transcription. Thus, chromatin remodelling is a constantly ongoing process, providing access to currently required areas. Histones are typically modified on their N-terminal residues and the nature of the modification determines their effect. Acetylation of lysine residues for example is associated with transcriptional activation. The modifications are dynamic and can be added or removed by so called *writers* or *erasers.* In the case of acetylation: histone-acetyl-transferases (HAT) using acetyl-CoA and histone-deacetylases (HDAC) (reviewed by Bannister & Kouzarides, 2011). Also other modifications are possible such as methylation, phosphorylation, ubiquitinylation and recently discovered lactlyation (D. Zhang et al., 2019).
These processes provide the possibility to regulate gene expression, can be bequeathed and are called "epigenetics". The common definition of epigenetics is: "The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al. 1996).

5.4.1 DNA Methylation

Next to histone modification, a major epigenetic mechanism is the methylation of DNA. DNA methylation occurs at cytosine residues – 5-methylcytosin (5-MC) – predominantly in cytosine and guanine rich clusters, called CpG islands. DNA methylation can be passed on during cell division and is also hereditary, accounting, next to histone-based epigenetics, for non-germline hereditary traits.
The *writers* of DNA methylation are DNA-methyl-transferases (DNMT) using S-Adenosyl-Methionine (SAM) as a methyl group donor. While DNMT3A and 3B are de novo methyl transferases that provide DNA methylation during embryonic development (Okano et al. 1998a, 1999; Hsieh 1999b), DNMT1 is understood as a maintainer of DNA methylation in somatic cells, mostly methylating a covalent strand symmetrically but it can also perform some *de novo* methylation ((Bestor 1992; Pradhan et al. 1999).
DNA demethylation can occur passively by not maintaining the methylation profile or actively by *erasers*. The removal of DNA methylation is performed by enzymes of the TET (ten-eleven translocation) family, which interestingly require aKG and Fe(II) as cofactors (Ito et al., 2010).
DNA methylation is an indispensable mechanism to maintain a healthy gene expression homeostasis, as a deletion of *DNMT1* results in the disruption of mono-allelic gene expression, as well a X chromosome inactivation, which is also mediated by DNA methylation (Li et al. 1993, Miniou et al. 1994; Hansen et al. 2000). The DNA methylation profile is altered in several tumours, which already became apparent with the discovery that MAGE genes (Melanoma Antigen Gene) that are completely silenced in somatic cells and were reactivated in tumours harbouring a lower methylation profile (De Smet et al. 1996, 1999)).
DNA methylation at promoters is generally associated with gene silencing, for example because transcription factors cannot bind anymore to methylated CpGs (Stadler et al., 2011; Weber et al., 2007). The role of gene body methylation is less defined, however, thought to promote gene expression. Methylated CpG occur at higher frequencies in exons than in introns and could

support RNA splicing by slowing down the polymerase and facilitation the exon exclusion (Shukla et al., 2011). Further, gene body methylation could mask gene intrinsic promoters and thus ensure a smooth run through of the polymerase (Maunakea et al., 2010).

5.4.2 Interplay of Epigenetics and Metabolism

Methionine is the precursor of SAM. As an essential amino acid, its intake and metabolization via the one carbon metabolism are crucial for the availability of SAM for DNMTs and also histone methyl transferases. TET enzymes need aKG as a co-factor and thus can be competitively inhibited by structural analogues like the TCA intermediates succinate, fumarate, and 2-hydroxyglutarate (2HG), the latter being produced from aKG by for example LDHA, MDH, and mutant IDH (W. Xu et al., 2011). Thus, the loss of FH or SDH can majorly influence the transcriptional programmes in cells and may contribute to for example cancer development (Sciacovelli et al., 2016; Zhao et al., 2020). In an oxygen deprived tumour environment, hypoxia was demonstrated to inhibit DNA and histone demethylases, thereby increasing methylation, and altering the epigenetic landscape (Batie et al., 2019; Thienpont et al., 2016). Mutant IDH1 and IDH2 are drivers of several cancer types by producing 2HG and thus, inhibiting demethylation reactions. The resulting new epigenetic structure and transcriptional profile favours cancer cell development and disease progression and IDH2 inhibitors have proven successful in AML so far (Cerchione et al., 2021).
Accordingly, the supply with acetyl-CoA is indispensable for histone acetylation. Citrate production as a substrate for acetyl-CoA and short chain fatty acid oxidation are thus vulnerable pathways when it comes to maintain the high acetylation turnover. As a matter of course, this also accounts for other modification products such as lactate for lactylation, or succinate for succiniylation. Glycolysis and the TCA cycle decrease the NAD^+/NADH ratio resulting in less NAD^+ as a co-factor for sirtuin-driven histone deacetylation. Thus, differences in the usage extent of these pathways can alter the chromatin structure (T. Zhang & Kraus, 2010). This regulation mechanism could potentially be interesting with regard to metabolic pathways moonlighting in the nucleus.

5.4.3 Epigenetics and T-cells

Many of the above-mentioned metabolites and pathways are also crucial in immunity. Thus, there is a close interaction and connection between epigenetic and immunometabolic processes. As fast as 20 min after T-cell activation, the IL-2 promoter is demethylated, allowing the transcription of the IL-2 gene (Bird, 2003). Similarly, $CD4^+$ T-cells express FoxP3 when its promoter is demethylated and 5-Azacytidine (5Aza) treatment inhibiting DNA methylation led to an increase of Fox3 in previously $CD4^+$ $CD25^-$ cells (Jones & Taylor, 1980; H.-P. Kim & Leonard, 2007). This implies a key role for DNA methylation in the development of T_{reg} cells. Further, T-cells upregulate the methionine influx upon TCR engagement and increase the amount of available SAM for DNA and histone methylation, which in turn conducts T-cells proliferation (Sinclair et al., 2019). In this regard, restricting methionine in the diet of multiple sclerosis mice downregulated the T_H17 activity and thus disease activity (Roy et al., 2020). Similarly, glycolysis and one carbon metabolism drive the SAM production in activated macrophages, which promotes their activity by epigenetically upregulating IL-1β (Yu et al., 2019). Accordingly, in DNMT1 deficient mice, which

could not maintain their methylation profile, T_H2 cytokine expression was increased, further supporting that DNA methylation is required for pro-inflammatory T-cells (Makar & Wilson, 2004).

In human $CD8^+$ T-cells, ATAC sequencing (Assay for Transposase-Accessible Chromatin using sequencing) revealed that their subsets have different chromatin structures, naïve and EM cells being rather divergent while EM and CM cells had a similar chromatin architecture (Moskowitz et al., 2017). Interestingly, in a mouse model the deletion of TET2 and the concomitant increase in DNA methylation, promoted a memory cell phenotype (Carty et al., 2018). Upon vaccination, memory $CD8^+$ T-cells retain demethylated promoter areas for INF-β and granzyme B, providing a fast accessibility, probably for future encounters (Akondy et al., 2017). Histone deacetylation by sirtuin 2, impaired the transcription of important genes for glycolysis and glutaminolysis and thus, decreased the tumour infiltration potential of $CD8^+$ T-cells (Hamaidi et al., 2020). As a reaction to long antigen exposure, $CD8^+$ T-cells can get exhausted and do not fulfil their effector functions anymore. This was shown to be mediated by *de novo* DNA methylation and inducing a demethylation even augmented the efficiency of checkpoint inhibition in mice (Ghoneim et al., 2017).

Taken together immune cell activation and subset differentiation are tightly connected to and controlled by the epigenetic landscape and its dynamics.

5.4.4 The role of epigenetics in rheumatoid arthritis

The observation that RA harbours a genetic predisposition but is not per se hereditary, hints towards an epigenetic influence. However, most of the research on epigenetic regulations has been made in cancer cells and models.

Global DNA hypomethylation was discovered in RA PBMCs compared to healthy controls, in combination with elevated DNMT1 expression, however, which was further negatively correlated to disease severity (C. C. Liu et al., 2011).

In RA synovial fibroblasts several differentially methylated regions could be found and associated to inflammatory activity (Nakano et al., 2013). Moreover, these methylation patterns could discriminate RA fibroblasts from OA fibroblast. In addition, the ratio of HATs to HDACs was shifted towards HAT expression in the synovium of RA patients, indicating transcriptional activation (Huber et al., 2007). In line with this, treatment of CIA mice with HDAC inhibitors prevented disease onset and could also reverse disease progression (Lin et al., 2007). Even human clinical trials have been performed, however, the ubiquitous character of HDAC inhibitor targets remains a major limitation of the approach (Vojinovic et al., 2011).

Promoter hypermethylation was discovered at the CTLA-4 locus in T-cells from RA patients compared to healthy controls, impairing the immune-suppressive function of CTLA-4:CD80/86 binding (Cribbs et al., 2014). Accordingly, upon *in vitro* MTX treatment of $CD4^+$ T-cells from RA patients, the FoxP3 promoter was hypomethylated, initiating regulatory functions (Cribbs et al., 2015).

Analogue to the HDAC inhibition approach, inhibition of DNA methylation by 5AZA administration in CIA mice achieved a reduction of the disease severity, lower anti-CII IgG, as well as a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory IL-10 production (Petralia et

al., 2019). Whether this global therapeutic approach is favourable over HDAC inhibition, has to be further elucidated.

An interesting aspect of the influence of epigenetics in RA is X-chromosome activation by DNA methylation. The higher prevalence of RA (and other autoimmune diseases) in women remains elusive. X-chromosome inactivation, which is only apparent in XX females, could thus provide a new explanation approach. Indeed, skewed X-chromosome inactivation (deviance from the common 50:50 ratio) was found to be more prevalent in women with RA than in healthy controls (Chabchoub et al., 2009). Additionally, many immune-related proteins are encoded on the X-chromosome and a twofold-expression or non-expression could have deleterious consequences (Mousavi et al., 2020b).

In summary, the investigation of epigenetic regulation of gene expression in RA harbours great potential. The diverse and sometimes even contradictory observations made so far, however, comprise the undeniable need for further examination.

5.5 Hypothesis

Based on the previous findings that CD8+ T-cells in RA display a hyper-glycolytic metabolic phenotype with increased (p)LDHA expression and high lactate levels and that T-cell activation, inflammation, and aerobic glycolysis are tightly connected, my aim for this thesis is to decipher the underlying regulatory mechanisms that perpetuate this immunometabolic phenotype. CD8+ T-cells seem to be trapped in a vicious circle between aerobic glycolysis and immune activation and inflammatory activity, with a, however, unknown initiation.

Since, these changes could be already observed in naïve cells, this suggests an antigen independent activation of the cells. Further, the lack of a clear identification of a genetic disposition related to metabolic processes in rheumatological diseases poses the possibility that the initiating factors of the metabolic switch might be regulated on an epigenetic level, which is more volatile but still inheritable. From cancer cells it is known that the Warburg-effect like metabolism is maintained by the epigenetic silencing of "anti-Warburg enzymes", such as the silencing of *LDHB* and the promotion of *LDHA* expression. These changes in the epigenetic landscape can be achieved by meddling with the expression levels of epigenetic enzymes due to i.e. mutations or by regulating their activity on a protein level.

Due to the metabolic remodelling towards glycolysis in RA CD8+ T-cells, resulting in increased conversion of pyruvate into lactate and thus in its withdrawal from the downstream TCA cycle, I expected that the TCA cycle (and the downstream ETC and OXPHOS) will have a reduced contribution to RA CD8+ T-cell metabolism and function. This decline of TCA cycle activity could decrease the availability of TCA intermediates such as aKG, an important co-factor for dioxygenases such as DNA-demethylating TET enzymes. Thus, the overall DNA methylation and the specific DNA methylation in "anti-Warburg enzymes" and pro-inflammatory mediators would be increased and promote the expression of genes required for aerobic glycolysis and inflammation. The persistence of aerobic glycolysis would maintain the imbalance of available metabolites, further promote the lack of aKG and retain the pro-inflammatory, pro-glycolytic, and pro-autoimmune epigenetic landscape.

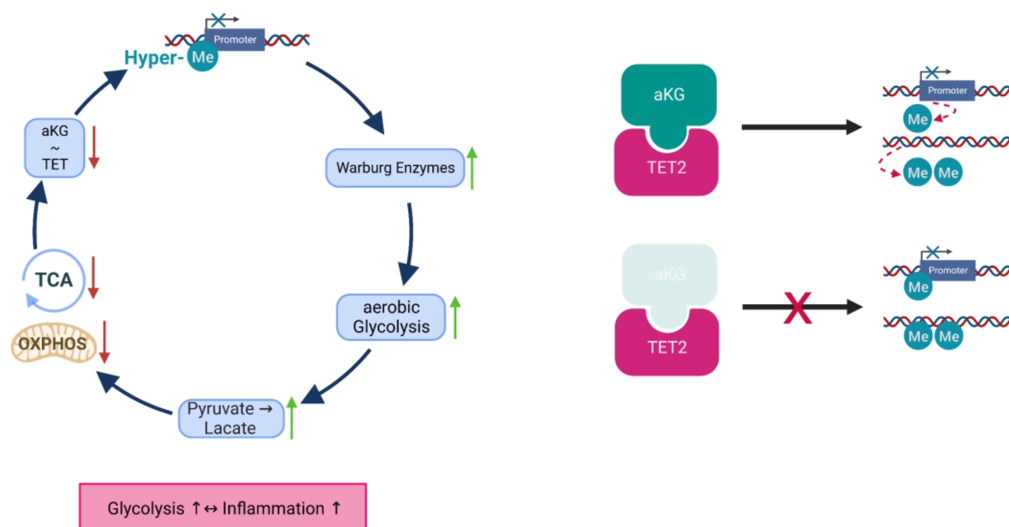

Figure 7: Schematic flowchart of the proposed vicious circle

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In order to confirm this hypothesis, I isolated peripheral blood CD8+ T-cells from RA patients and healthy donors and pursued to analyse the TCA metabolite concentrations in the *ex vivo* CD8+ T-cells, their proteome and phospho-proteome, and the DNA methylation of the cells. Finally, I examined the *in vitro* influence of 5-Azacytidine and aKG on the changes of the functional capacities of the CD8+ T-cells.

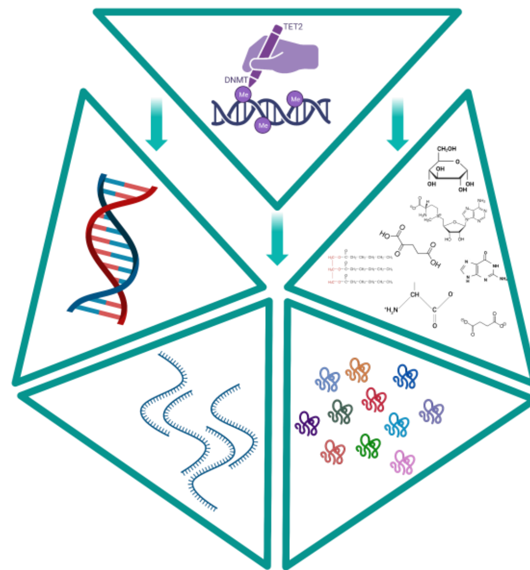

Figure 8: Representation of the interplay of the intended experimental processes
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6 MATERIAL & METHODS

6.1 Reagents

6.1.1 Consumables

Table 5: List of consumables

96 v bottom plates (FACS)	ThermoFisher (Langenselbold, Germany)
Cell culture plate 6-, 12-, 24- and 96-well, sterile	Greiner Bio-One GmbH (Frickenhausen, Germany)
Cell strainer 20 µm	pluriSelect (Leipzig, Germany)
Cell strainer 70 µm	pluriSelect (Leipzig, Germany)
FACS-tubes PS, 5 ml, 75x12 mm	Sarstedt AG & Co (Nümbrecht, Germany)
Gloves “Touch N Tuff”, Nitrile	Ansell Healthcare (Anderlecht, Belgium)
LS Columns	Miltenyi Biotec (Bergisch Gladbach, Germany)
Luer-Lok™ Tip, 20 ml Syringe	BD Biosciences (Heidelberg, Germany)
MicroAmp™ optical adhesive film	Applied Biosystems (Waltham, MA, USA)
MicroAmp™ qPCR plates 96	Applied Biosystems (Waltham, MA, USA)
Microcentrifuge Tubes, 1.5 ml, and 2 ml	Eppendorf AG (Hamburg, Germany)
Mini-PROTEAN® TGX™ Precast Protein Gels	Bio-Rad Laboratories GmbH (Feldkirchen, Germany)
Pipet tips 10 µl; 200 µl; 1000 µl	Sarstedt AG & Co (Nümbrecht, Germany)
PP PCR-Tubes, Nat. 0.2 ml	Greiner Bio-One GmbH (Frickenhausen, Germany)
PP Tube 50 ml / 15 ml	Greiner Bio-One GmbH (Frickenhausen, Germany)
Serological Pipette, 5 ml;10 ml;25 ml sterile	Sarstedt AG & Co (Nümbrecht, Germany)
Socorex Gel Loading Tips Qualitix®, 200µL	Neolab (Heidelberg, Germany)
Norell® Select Series™ 5 mm NMR tubes. frequency 800 MHz	Norell (Morganton, NC, USA)

6.1.2 Chemicals and reagents

Table 6: List of used chemicals and reagents

Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Biosciences (Heidelberg, Germany)
BlueBlock PF 10x blocking solution	SERVA Electrophoresis GmbH (Heidelberg, Germany)
BodiPy452	ThermoFisher (Langenselbold, Germany)
cOmplete™ ULTRA Tablets, Mini, EDTA-free, *EASY*pack Protease Inhibitor Cocktail	Roche Diagnostics Deutschland GmbH (Mannheim, Germany)
D_2O	Euriso-top (St.-Aubin Cedex, France)
d3-lactic acid	C/D/N Isotopes, Inc. (Quebec, Canada)

d4-3-hydroxy-glutaric acid	Amsterdam (UMC, Netherlands)
d4-citric acid	C/D/N Isotopes, Inc. (Quebec, Canada)
d4-nitrophenol	Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA)
EDTA (Ethylenediaminetetraacetic acid) disodium salt dehydrate p.A.	AppliChem GmbH (Darmstadt, Germany)
FACS Clean™	BD Biosciences (Heidelberg, Germany)
FACS Flow	BD Biosciences (Heidelberg, Germany)
Foetal Calf Serum (FCS)	Biochrom GmbH (Berlin, Germany)
Glucose ^{12}C	Sigma-Aldrich (Taufkirchen, Germany)
Glucose U^{13}C	Sigma-Aldrich (Taufkirchen, Germany)
Glutamine	Sigma-Aldrich (Taufkirchen, Germany)
Heparin	B. Braun SE (Melsungen, Germany)
Laemmli Buffer 10x, for SDS PAGE	SERVA Electrophoresis GmbH (Heidelberg, Germany)
NaCl	B. Braun SE (Melsungen, Germany)
N-methyl-N-(trimethylsilyl)heptafluorobutyramide	MSHFBA, Macherey-Nagel (Düren, Germany)
Nonidet p-40 (NP-40)	Sigma-Aldrich (Taufkirchen, Germany)
Oligos	Merck (Darmstadt, Germany)
Penicillin/Streptomycin (Pen/Strep) (10000 U/ml)	Sigma-Aldrich (Taufkirchen, Germany)
pentafluorobenzylhydroxylamine	Sigma-Aldrich (Taufkirchen, Germany)
Phosphate buffered saline (PBS)	ThermoFisher (Langenselbold, Germany)
PhosSTOP™ Inhibitor Tablets for Phosphatase	Roche Diagnostics Deutschland GmbH (Mannheim, Germany)
PMA (phorbol 12-myristate 13-acetate)	Cayman Chemical (Ann Arbor, USA)
Ponceau S solution	Sigma-Aldrich (Taufkirchen, Germany)
RPMI	ThermoFisher (Langenselbold, Germany)
Sodium orthovanadate (Na3VO4) BioChemica BC	AppliChem GmbH (Darmstadt, Germany)
ß-Mercapto-Ethanol	Bio-Rad Laboratories GmbH (Feldkirchen, Germany)
Tris for molecular biology BC	AppliChem GmbH (Darmstadt, Germany)
Triton® X-100	Merck Millipore (Darmstadt, Germany)
Trypan blue	Sigma-Aldrich (Taufkirchen, Germany)
Tween®20	Carl Roth GmbH (Karlsruhe, Germany)
Unstained Protein Standard, Broad Range (11-245 kDa)	New England BioLabs GmbH (Frankfurt, Germany)
WesternBright ECL HRP substrate	Advansta Inc. (Menlo Park, USA)
Zombie Violet L/D stain	Biolegend (San Diego, CA, USA)

6.1.3 Buffers and cell culture media

Table 7: List of used buffers and cell culture media

4 x SDS sample buffer for gel-electrophoresis	20 ml Tris 0.5 M pH 6.8 4 g SDS 20 ml Gylcerol 50 µl ß-mercaptoethanol Bromophenol blue
ACK buffer (Ammonium, Chloride, Potassium)	0.15 M Ammonium Chloride 0.01 M Potassium Bicarbonate 0.0001 M Disodium EDTA
MACS (Magnetic-activated cell sorting) buffer	0.5 % BSA 2 mM EDTA in PBS
Ponceau S solution	0.5% Ponceau S 3% TCA
RiPA Lysis buffer	1% NP-40 0.25% Deoxycholate 50 mM Tris (pH = 8) 150 mM NaCl 1 mM EDTA x 2 H_2O (pH = 8) 1 mM Na_3VO_4 1 mM NaF
TBS (10x) buffer	100 mM Tris 1.5 M NaCl In Aqua dd.
TBST washing buffer for Western Blot	100 ml 10x TBS 1 ml Tween20 900 ml Aqua dd.
Urea Lysis Buffer	100 mM Tris-HCl pH 8.5 7 M Urea 1 % Triton X-100 10 U/ml DNase I 1 mM magnesium chloride 1% benzonase (units) 1 mM sodium orthovanadate phosphoSTOP phosphatases inhibitors complete mini EDTA free protease inhibitors

6.1.4 Kits

Table 8: List of used Kits

Biozym cDNA Synthesis Kit	Biozym Scientific (Hessisch Oldendorf, Germany)
EpiQuik DNMT Activity/Inhibition ELISA Easy Kit (Colorimetric)	Epigentek (Farmingdale, NY, USA)
EpiQuik Nuclear Extraction Kit	Epigentek (Farmingdale, NY, USA)
LEGENDplex™ Human CD8/NK Panel (13-plex)	Biolegend (San Diego, CA, USA)
MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit (Colorimetric)	Epigentek (Farmingdale, NY, USA)
MojoSort™ Mouse CD8 T Cell Isolation Kit	Biolegend (San Diego, CA, USA)
Pierce™ BCA Protein Assay Kit	ThermoFisher (Langenselbold, Germany)
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems™ (Darmstadt, Germany)
Quick-DNA™ Miniprep Kit	Zymo Research (Freiburg, Germany)
Quick-RNA™ Miniprep Kit	Zymo Research (Freiburg, Germany)
TET Hydroxylase Activity Quantification Kit (Fluorometric)	Abcam (Cambridge, UK)
Trans-Blot Turbo RTA Midi 0.2 µm Nitrocellulose Transfer Kit	Bio-Rad Laboratories GmbH (Feldkirchen, Germany)
True-Nuclear™ Transcription Factor Buffer Set	Biolegend (San Diego, CA, USA)

6.1.5 Equipment and Software

Table 9: List of used instruments and software

Analysewaage ME104	Mettler Toledo (Columbus, OH, USA)
BD FACS Diva™ Software	BD Biosciences (Heidelberg, Germany)
Biorender	Biorender
Bruker Ascend™ 600	Bruker Corporation (Billerica, MA, USA)
Bruker TopSpin 4.3.0	Bruker Corporation (Billerica, MA, USA)
ChemoStar ECL & Fluorescence Imager	Intas Science Imaging Instruments GmbH (Göttingen, Germany)
CLARIOstar Plus Plate Reader	BMG Labtech (Ortenberg, Germany)
Data Analysis Software Suite for LEGENDplex	Biolegend (San Diego, CA, USA)
Easypet Pipet aid	Eppendorf AG (Hamburg, Germany)
Flow cytometer, BD FACSSymphony™	BD Biosciences (Heidelberg, Germany)
FlowJo™	BD Biosciences (Heidelberg, Germany)
GraphPad Prism version10 for Windows	GraphPad Software, San Diego, California USA
ImageJ	National Institutes of Health, Bethesda, Maryland, USA
Incubator, Heracell™ 240i, CO2, 37°C	Heraeus Instruments (Hanau, Germany)
Microscope Eclipse TE3000	Nikon (Minato, Japan)
Microsoft Office	Mircosoft (Redmont, USA)

Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad Laboratories GmbH (Feldkirchen, Germany)
Mr. Frosty™ Freezing Container	ThermoFisher (Langenselbold, Germany)
MSD 5977A - quadrupole mass spectrometer	Agilent (Santa Rosa, CA, USA)
NanoDrop 2000	ThermoFisher (Langenselbold, Germany)
Neubauer counting chamber	Carl Roth GmbH & Co.KG (Karlsruhe, Germany)
Orbitrap Exploris 480 mass spectrometer	ThermoFisher (Langenselbold, Germany)
OriginPro 2022 9.9.0.220	OriginLab Corporation (Northampton, MA, USA)
Pipet, Pipetman 10 µl, 20 µl, 200 µl, 1000 µl	Gilson Inc (Middleton, UK)
Spectra Max M2	Molecular Devices (San Jose, CA, USA)
StepOne Real-Time PCR System	Applied Biosystems (Darmstadt, Germany)
Tabletop centrifuge, Biofuge pico	Heraeus Instruments (Hanau, Germany)
Thermocycler, Mastercycler gradient	Eppendorf AG (Hamburg, Germany)
Trans-Blot® Turbo™ Transfer System	Bio-Rad Laboratories GmbH (Feldkirchen, Germany)
Vortex mixer, reax top	Heidolph Instruments GmbH & Co.KG (Schwabach, Germany)
Zentrifuge Labofuge 400R	Heraeus (Hanau, Germany)
Zentrifuge Rotofix 32	Hettich Zentrifugen (Tuttlingen, Germany)

6.1.6 Oligos

Table 10: List of used Oligonucleotides

Gene Name	Direction	Sequence (5' to 3')	Length	Annealing Temperature [°C]
D2HGDH	Forward	GTGTCTGGAATTCTGGTTTG	20	59.6
D2HGDH	Reverse	GTGAGCCATATCGAAGAAAC	20	58.8
DGKA	Forward	GAAGTGGATAATGTTCCCAG	20	58.4
DGKA	Reverse	ATTCTAACTTGTCTTCTGGC	20	55.1
DGKG	Forward	CTGAAAGACATTGAGCAGAG	20	57.7
DGKG	Reverse	CCACACCAATGGAGAAATAG	20	59.4
DGKZ	Forward	AGTCAGTGTCTCGAAGAAAG	20	56.1
DGKZ	Reverse	CGGAAATTTATCTTCTCCAGC	21	61.5
DNMT1	Forward	CGTAAAGAAGAATTATCCGAGG	22	60.5
DNMT1	Reverse	GTTTTCTAGACGTCCATTCAC	21	57.7
FGF2	Forward	TGGCTTCTAAATGTGTTACG	20	57.9
FGF2	Reverse	GTTTATACTGCCCAGTTCG	19	57.2
HIF1A	Forward	AAAATCTCATCCAAGAAGCC	20	59.7
HIF1A	Reverse	AATGTTCCAATTCCTACTGC	20	58.1
HK1	Forward	CTGTTTGAGAAGATGGTCAG	20	57.4
HK1	Reverse	CATCACTGGTGTTAAACTTCC	21	58.6
HK2	Forward	GAAAGCAACTGTTTGAGAAG	20	56.7
HK2	Reverse	CAATGTCTGAGATGTCTTTGG	21	59.8
IDH3B	Forward	CTGAACTGTACCCCAAAATC	20	58.5
IDH3B	Reverse	GTATTCTGCACTATAGCTCTC	21	52.4
IFNGR2	Forward	AGTAACATCTTTAGAGTCGGG	21	56.5
IFNGR2	Reverse	TCAGGCCTCTATATTTCAGG	20	58.4
IL10	Forward	GCCTTTAATAAGCTCCAAGAG	21	58.7
IL10	Reverse	ATCTTCATTGTCATGTAGGC	20	56.5
IL2	Forward	AGGGATCTGAAACAACATTC	20	58.0
IL2	Reverse	GCCTGATATGTTTTAAGTGGG	21	59.9
LDHA	Forward	CACCATGATTAAGGGTCTTTAC	22	58.9
LDHA	Reverse	AGGTCTGAGATTCCATTCTG	20	58.2
LDHB	Forward	CAACAATGGTAAAGGGGATG	20	61.6
LDHB	Reverse	TCACTAGTCACAGGTCTTTTAG	22	55.3
PKM2	Forward	ATGTTGATATGGTGTTTGCG	20	60.9
PKM2	Reverse	ATTTCATCAAACCTCCGAAC	20	60.4
SDHC	Forward	TATCTACAGTTGGTCTCTTCC	21	54.9
SDHC	Reverse	CATAGAGGACAACACAGTAAG	21	53.9
TET2	Forward	GGATCTAAAGGGAGATAGAGAC	22	56.6
TET2	Reverse	CTAGTTGAATTCAGCAGCTC	20	56.8

6.1.7 Antibodies

Table 11: List of used antibodies for Western Blot and Flow Cytometry

Target Protein	Tag	species	Reactivity	Dilution	Manufacturer
anti-rabbit	HRP	goat	rabbit	1:2000	Cell Signalling Technology (Danvers, MA, USA)
CD11a	BUV496	mouse	human	1:100	BD Biosciences (Heidelberg, Germany)
CD197 / CCR7	PE/Cy5	mouse	human	1:100	Biolegend (San Diego, CA, USA)
CD279 / PD-1	BV650	mouse	human	1:100	Biolegend (San Diego, CA, USA)
CD28	-	mouse	human	1:250	Biolegend (San Diego, CA, USA)
CD3	-	mouse	human	1:333	Biolegend (San Diego, CA, USA)
CD3	BV510	mouse	human	1:100	Biolegend (San Diego, CA, USA)
CD45RA	BUV805	mouse	human	1:100	BD Biosciences (Heidelberg, Germany)
CD69	BUV615	mouse	human	1:100	BD Biosciences (Heidelberg, Germany)
CD8	BUV395	mouse	human	1:100	BD Biosciences (Heidelberg, Germany)
CD95	BUV737	mouse	human	1:100	BD Biosciences (Heidelberg, Germany)
Cofilin	-	rabbit	Human	1:1000	Cell Signalling Technology (Danvers, MA, USA)
CTLA-4	PE-Dazzle	mouse	human	1:100	Biolegend (San Diego, CA, USA)
DNMT1		rabbit	human	1:1000	Cell Signalling Technology (Danvers, MA, USA)
Hsp90		rabbit	human	1:1000	Cell Signalling Technology (Danvers, MA, USA)
Ki67	AlexaFluor700	mouse	human	1:50	Biolegend (San Diego, CA, USA)
NFATc1	-	rabbit	Human	1:500	Cell Signalling Technology (Danvers, MA, USA)
pDNMT1		rabbit	human	1:1000	NEB

6.2 Methods

6.2.1 Patient Recruitment

All patients were recruited in the Rheumatology outpatient clinic of the Heidelberg University Hospital after they have been informed in detail by a doctor involved in the study. 80 ml of heparinized blood were collected by the central blood withdrawal team of the Heidelberg University Hospital upon informed, written consent and in the course of a routine blood withdrawal. Healthy donors must not have had any auto-immune disease or take immune modulatory drugs.

Patients and healthy donors were not included in the study if one of the following applied:
1. Malignant or neoplastic diseases
2. Viral or bacterial infection in the last 4 weeks
3. Vaccination in the last 4 weeks
4. Pregnancy
5. Intake of anticoagulants
6. Absence of consent

Table 12: Demographic and clinical parameters

The average dosage of each medication was calculated only for the patients taking that medication. SD = standard deviation.

	Healthy controls	RA patients
Total	24	50
Female	13	41
Age *(SD)*	37 *(11)*	59 *(15)*
Male	11	9
Age *(SD)*	43 *(19)*	62 *(10)*
Clinical Parameters		
DAS28-CRP mean *(SD)*		2.3 *(1.1)*
CRP mean *(SD)* [mg / l]		5.6 *(6.5)*
Seropositive		30
RF positive		17
Anti-CCP positive		28
HLA-B27 negative		10
HLA-B27 no data		40
BMI mean *(SD)*		25.4 *(3.9)*
Medication		
MTX		25
Mean *(SD)* [mg]		13.4 *(3.7)*
Glucocorticoids		18
Mean *(SD)* [mg]		4.9 *(1.7)*
Leflunomide		10
Mean *(SD)* [mg]		17.3 *(3.9)*
Sulfasalazine		6
Mean *(SD)* [mg]		500 *(0)*
Hydroxychloroquine		2
Mean *(SD)* [mg]		200 *(0)*
Allopurinol		1
Mean *(SD)* [mg]		300
COX-2 inhibitors		2
Mean *(SD)* [mg]		95 *(5)*
Colchicine		3
Mean *(SD)* [mg]		7 *(9.2)*
bDMARDs		
Anti-TNF		1
Anti-IL-6R		1
Anti-CD20		1
Anti-IL-6		1
JAK-blockade		3

6.2.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from the donated, heparinized blood in the course of 4 h after the blood withdrawal by density gradient isolation. 16 ml of Pancoll solution were pipetted into a 50 ml separation tubes and centrifuged at 1000 x g for 1 min without break. Afterwards the blood was diluted 1:2 with PBS and layered on top of the Pancoll, followed by centrifugation for 10 min at 1000 x g without break. The top layer containing serum, Pancoll and the PBMC fraction was transferred to a fresh 50 ml tube. PBS was added ad. 50 ml. To pellet the cells, the tube was centrifuged for 10 min at 250 x g. In the case of a contamination of the pellet with erythrocytes, a red blood cell lysis with ACK buffer was performed: the pellet was resuspended in 5 – 10 ml ACK buffer and incubated for 2 min, followed by centrifugation for 10 min at 250 x g.

6.2.3 Isolation of $CD8^+$ T-cells

$CD8^+$ T-cells were isolated from the PBMC by magnetic bead isolation. A negative selection approach was chosen. The isolation was performed according to the manufacturer's protocol. Briefly, the PBMC were resuspended in 1 ml MACS buffer and 35 µl biotin-antibody cocktail were added and incubated for 10 min at 4 °C. Afterwards, 35 µl streptavidin nanobeads were added and incubated for 10 min at 4 °C. In the meantime, a LS column was flushed with 3 ml MACS buffer. The cell-nanobead mixture was filtered through a 20 µm cell strainer and then transferred on the column. The original tube was flushed with another 1 ml of MACS buffer, which was also added to the column. Finally, the column was flushed with 3 ml of MACS buffer and the cells were counted in a 1:10 dilution in trypan blue with a Neubauer counting chamber.

6.2.4 Cell culture

In the case of culturing experiments the cells were cultured in RPMI medium with 5 mM or 10 mM of glucose (either ^{12}C-glucose or 1,6- or $U^{13}C$-glucose for NMR experiments), 10 % FCS, 1 % Penicillin/Streptomycin, and 1 % glutamine at 37 °C and under 5 % CO_2. If not stated otherwise, the cells were cultured for 3 days unstimulated or stimulated with αCD3 and αCD28 and/or other stimuli.

6.2.5 Flow Cytometry

For the flow cytometric analysis of cell surface markers and intracellular or functional markers, the following protocols were performed, if not stated otherwise:

6.2.5.1 Antibody Staining

The cells were washed with PBS (resuspension in 500 µl of PBS and centrifugation at 7500 rpm for 4 min) and then resuspended in the antibody cocktails (1 µl antibody per 100 µl PBS). Incubation was performed for 40 min at room temperature (RT). After another washing step with PBS, the cells were analysed by flow cytometry or further treated for intracellular staining. For intracellular staining the cells were fixed with 100 µl of the True-Nuclear™ fixation buffer for 45 min at RT or overnight at 4 °C. Afterwards the cells were washed with 500 µl of the True-Nuclear™ perm/wash buffer and finally incubated with the antibodies for the intracellular markers (diluted 1:50 in True-Nuclear™ perm/wash buffer) overnight at 4 °C. Analysis by flow cytometry was then performed at the next day.

6.2.5.2 Instrument Setup

To setup the PMT (photo multiplier tube) voltages and for compensation of fluorescence spillover, single staining with latex beads were performed and fluorescence-minus-one controls were used.

6.2.6 Proteome

To analyse the proteome of the $CD8^+$ T-cells, $5x10^6$ ex vivo cells per sample were lysed and the protein content was determined by Pierce™ BCA Protein Assay Kit according to the manufacturer's protocol. The lysis was performed with 300 µl of the Urea Lysis buffer in a 4 °C ultrasound water bath for 1 h. For some samples previously produced lysates were used, these lysates were made with RIPA buffer with phosphatase and protease inhibitors. The samples were then handed over to Dr Dominic Helm's team of the MS (Mass Spectrometry)-based Protein Analysis Unit, GPCF at the German Cancer Research Centre (DKFZ) Heidelberg. Briefly, they were digested with Lyc-C and trypsin and in an 8 M Urea buffer and subsequently desalted using Sep-Pak cartridges. The resulting peptides have been loaded on a cartridge trap column, packed with Acclaim PepMap300 C18, 5µm, 300Å wide pore (Thermo Scientific) and separated via a gradient from 3% to 40% ACN on a nanoEase MZ Peptide analytical column (300Å, 1.7µm, 75µm x 200 mm, Waters) using a 150min MS-method. The eluting peptides have been analysed by an online coupled Orbitrap Exploris 480 mass spectrometer.

6.2.7 Analysis of intracellular metabolites

Intracellular metabolites of $CD8^+$ T-cells were analysed by GC-MS and ESI (Electron spray ionisation)-MS/MS mass spectrometry. For each sample $1x10^6$ ex vivo cells were handed over to the team of Dr Jürgen Okun's team of the department of General Paediatrics, Division of Neuropediatrics and Metabolic Medicine, Centre for Paediatric and Adolescent Medicine, University Hospital Heidelberg. Another $1x10^6$ cells were lysed with RIPA lysis buffer supplemented with phosphatase and protease inhibitors and the protein concentrations were analysed with the Pierce™ BCA Protein Assay Kit according to the manufacturer's protocol. Briefly, the acids in the samples were oximated using pentafluorobenzylhydroxylamine in hydrochloric acid solution, extracted with ethyl acetate, dried and silylated with N-methyl-N-trimethylsilylheptafluorobutyramide, which can then be analysed using gas chromatography. For quantitative determination, a mass spectrometer in EI mode was used as a detector. The quantitative evaluation was carried out by comparing the peak areas using an internal standard.

For the mass spectrometry analysis, the cell pellet was lysed in 100 µl water. The following internal standards were added to 85 µl of the lysate:

100 µl d4-nitrophenol (1.25 mM)

200 µl d3-lactic acid (1mM)

100 µl d4-citric acid (1 mM)

50 µl d4-3-hydroxy-glutaric acid

Oximation was performed by the addition of 100 µl pentafluorobenzylhydroxylamine (100 mM) in an acidic environment, adding 300 µl of 5 M HCl (room temperature for 1 h). To improve phase separation, saturated ammonium chloride solution was added. The extraction was performed two times with 5 ml ethyl acetate each. The ethyl acetate fractions were dried with sodium sulphate and then under a steady nitrogen stream at 40 °C. Derivatisation of the samples was performed with 50 µl N-methyl-N-(trimethylsilyl)heptafluorobutyramide at 60 °C for 1 h.
Gas chromatographic (GC) separation was achieved using a capillary column and helium as the carrier gas: DB-5MS, 30 m × 0.25 mm; film thickness: 0.25; Agilent J&W Scientific, Folsom, CA, USA. The downstream MS analysis was performed on an MSD 5977A quadrupole mass spectrometer in the selective ion-monitoring mode with electron impact ionisation. A sample volume of 1 µl of the derivatised sample was injected in pulsed split mode: injection pulse pressure 2.5 bar until 0.5 min, Split Ratio 50:1, Split-Flow 93 ml/min.
GC parameters:

oven: 80 °C for 2 min, ramp 50 °C/min to 150 °C, ramp 10 °C/min to 300 °C, and hold for 2 min at 300 °C.

Injector temperature: 260 °C constant pressure 1.25 bar

Interface temperature: 260 °C.

MS parameters:

Source temperature: 230 °C
Dwell time: 25 ms
SIM:

Table 13: Fragment ions for quantification (SIM)

Metabolite	m/z
citric acid	183
3-hydroxy-glutaric acid	217
d3-lactic acid	222
malic acid	233
fumaric acid	245
isocitric acid	245
succinic acid	247
d4-3-hydroxy-glutaric acid	262
d4-citric acid	276
pyruvic acid	340
oxaloacetic acid	456
oxoglutaric acid	470

The response factors were determined using a one-point calibration.

The analysis of acylcarnitines and amino acids was performed with the MassChrom® Amino Acids and acylcarnitines from Dried Blood / Non Derivatised kit from chromosystems according to the manufacturer's protocol.

The quantified concentrations were normalized to the respective protein contents.

6.2.8 Nuclear Magnetic Resonance (NMR)

Glucose and lactate levels were determined in cell culture supernatants upon 72 h of cultivation. The supernatants were diluted 1:1.25 with D_2O and a fumarate standard (10 mM fumarate in D_2O) was added at a final concentration of 2mM. 600 µl of the sample were transferred to a 5 mm NMR tube. NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5-mm 1H{13C/15N/31P} z-axis gradient cryogenic probe. For the NMR spectral acquisition of 1D 1H NOESY-presat spectra the Bruker standard pulse program noesygppr1d were used in conjunction with published acquisition parameters (Vignoli et al., 2019). 32 scans were acquired per sample with a total repetition time of 6.6 seconds for each scan. The NMR data were processed and analysed with Bruker TopSpin 4.3.0 NMR software. 1H chemical shifts were referenced to the resonance of fumarate at 6.5 ppm.

6.2.9 DNA Methylation array

The DNA methylation profile was analysed with the Infinium MethylationEPIC v2.0 array from Illumina. The DNA of $5x10^6$ ex vivo $CD8^+$ T-cells was isolated with the Quick-DNA™ Miniprep Kit from Zymo Research according to the manufacturer's protocol and eluted in water. Subsequently the DNA concentrations of the samples was measured by NanoDrop and 20 µl of a 60 ng/µl dilution was sent to the Life&Brain GmbH in Bonn where the bisulfite conversion and the analysis were performed.

6.2.10 qPCR (Quantitative real time polymerase chain reaction)

To analyse gene expression levels, mRNA was isolated from $1\text{-}2x10^6$ $CD8^+$ T-cells with the Quick-RNA Miniprep Kit™ accordingly to the manufacturer's instructions. The RNA concentrations were measured by NanoDrop™ and 180 ng were used for cDNA transcription with the Biozym cDNA Synthesis Kit according to the manufacturer's instructions.
For the qPCR reaction 5 µl of cDNA, 5µl of a mixture of forward and reverse primers at a 600 nM concentration and 10 µl of PowerUp™ SYBR™ Green Master Mix were used.
The following cycler programme was run:

Step	Temperature	Time	Cycles
UDG activation	50 °C	2 min	1
Polymerase activation	95 °C	2 min	1
Denature	95 °C	3 s	40
Anneal	60 °C	30 s	

For quality control a melt curve was generated after the qPCR. Primers and sequences can be found in Table 10. Gene expression levels were normalized to RPL13A and the $2^{-\Delta CT}$ value was used for differential expression analysis.

6.2.11 Western Blot

Protein expression was analysed by Western Blot. Around $2x10^6$ $CD8^+$ T-cells were lysed using RIPA lysis buffer complemented with protease and phosphatase inhibitors. The cells were resuspended in around 70 µl of complete lysis buffer and incubated on ice for 45 min with occasional vortexing in between. After centrifugation at 13000 rpm for 30 min at 4 °C the supernatant was transferred to a new reaction tube and the concentrations were measured by BCA according to the manufacturer's protocol.

10-20 µg of protein were supplemented with 4x sample buffer, incubated at 95 °C for 2 min, cooled and loaded onto a precast TGX gel. The gels were run for 30 min at 200 V and subsequently blotted onto a 2 µm nitrocellulose membrane with the recommended TurboBlot™ programme by BioRad. Upon blotting, the membrane was incubated in a ponceau red solution to stain the protein bands to improve cutting precision in the next steps.

The membrane was blocked using 1x BlueBlock solution from SERVA Electrophoresis GmbH for 30 min at RT and afterwards the single membrane parts were incubated in the respective antibody solutions (1:1000 in 1x BlueBlock) over night at 4 °C. The following day the membranes were washed three times with TBS-T for 10 min at RT and then incubated with the secondary antibody (1:1000 in 1x BlueBlock) for 1 h at RT. Upon another three times of washing with TBS-T the membranes were incubated shortly with HRP-substrate ECL, and signal detection was performed with a camera. Quantification of the band intensities was performed using the gel analysis tool of ImageJ.

6.2.12 Enzyme activity assays and global DNA methylation

To analyse the activities of DNMTs and TET enzymes, nuclear extracts were made from $CD8^+$ T-cells with the EpiQuik Nuclear Extraction Kit from Epigentek according to the manufacturer's protocol. The protein concentration was determined using the Pierce BCA kit, also according to the manufacturer's protocol. The enzyme activities were measured using the respective kits from Epigentek, according to the manufacturer's protocol. I used 4 µl of nuclear extracts and then normalized to the protein concentrations.

The global DNA methylation was assessed in DNA isolated with the Quick-DNA™ Miniprep Kit from Zymo Research and the MethylFlash Hydroxymethylated DNA 5-hmC Quantification Kit from Epigentek according to the manufacturers' protocols.

6.2.13 Cytokine Bead Array

To determine the concentrations of the secreted cytokines in the supernatants, the cells were incubated and stimulated as stated respectively for 72 h. Afterwards, the supernatants were harvested and analysed using the LEGENDplex™ Human CD8/NK Panel by Biolegend according to the manufacturers protocol. The incubation of the supernatants with the beads was performed over night at 4 °C. The measurements were performed using the BD FACS Symphony with the HTS plate reader system. Data analysis was performed using the provided software.

6.2.14 Data analysis

All complex bioinformatic analysis were performed with the help of Dr Junyan Lu, indicated in the following section by J.L. in the headlines.

6.2.14.1 Flow Cytometry

Flow cytometry data was acquired, and compensation was performed with the BD FACSDiva™ Software, while the data analysis was performed with FlowJo™. Further data and statistical analysis were performed using GraphPad Prism version 9.

6.2.14.2 Proteome and Phospho Proteome analysis (J.L.)

The data analysis and the statistical analysis were performed in cooperation with Martin Schneider:

Data analysis was carried out by MaxQuant (version 1.6.14.0). In total 68905 peptides and 6339 proteins could have been identified by MSMS based on an FDR (False discovery rate) cutoff of 0.01 on peptide level and 0.01 on protein level. Identified in all samples were 5726 peptides and 2771 proteins. Match between runs option was enabled to transfer peptide identifications across Raw files based on accurate retention time and m/z. Quantification was done using a label free quantification (LFQ) approach based on the MaxLFQ algorithm (Cox et al., 2014). Further, Limma (R) (Smyth, 2005), ProDA (*Bioconductor - ProDA*, n.d.), GraphPadPrism, and OriginPro 2022 9.9.0.220 were used. The MS/MS identified 68905 peptides and 6340 proteins.

Preprocessing was started by identifying the number of missing values in the single samples. Due to the large number of missing values RA62 was removed from the data set, harbouring a too large unreliability. Protein abundance in at least 50 % had to be given to be included in the analysis. Missing values were imputed with the QRILC (Quantile Regression Imputation of Left-Censored data) algorithm. To achieve a better distribution, the data was normalized using log2 transformation for a good visualisation and variance stabilisation for the further analysis. A principal component analysis (PCA) was performed to identify batch effects or other external influences. The compositions of the two used lysis buffers turned out to be a string confounder, which was corrected by regression in a later analysis step.

The downstream hypothesis testing was performed with proDA, which, in contrast to Limma, also uses the low abundance information and corrects for biases and dies not even need imputation. Due to the low cohort size, p-values were used to identify possible targets. The box plots were created using the variance stabilisation normalized data and thus display the relative expression of the proteins. Pathway analyses were performed with the GSEA (gene set enrichment analysis) software (Subramanian et al., 2005).

6.2.14.3 Metabolites (J.L.)

The output data of the targeted MS approach were first normalized. Generalized log transformation was used, as log transformation would not consider zero values, which carry necessary information in this experiment type. The PCA analysis revealed the experiment-carry-out date as a confounder, which was corrected for using the ComBat package, which also removed the variance caused by the observed batch effect (Y. Zhang et al., 2020). Differential expression

was tested using limma, a linear regression model. The presented box plots show the log2 transformed data for better visualization.

6.2.14.4 DNA methylation data (J.L.)

The current state of the art analysis tool for DNA methylation data sets is minifi, which was used as described in: (Aryee et al., 2014). In short, the readout CpG sites were annotated to the respective genes, single nucleotide polymorphisms were removed from the data set. Due to low quality and probes, which showed cross reactions around 43000 probes were excluded from the analysis, leaving around 800000 probes. Further the data was normalized to achieve a better resolution.

A PCA was performed to identify possible confounders characterizing the groups. Commonly used to visualize data is the beta value, displaying the ratio of methylated vs. unmethylated sites. For hypothesis testing the beta values were transformed into m values, which display a better normal distribution.

6.2.14.5 Data Integration – all -omics datasets together (J.L.)

Due to the small sample size we intended to integrate all data sets to find a common tendency in all data sets. While a PCA tries to find a confounder, which explains a variance in a single data set, the mofa model was used for this approach, as it can identify factors that explain variances in several data sets (Argelaguet et al., 2018). Thus, an additional preprocessing and batch effect correction was not necessary, as they are limited to the single data sets. Importantly, this analysis usually needs a sample size of > 100 samples, which is why the presented analysis can only be used for exploratory approaches.

7 RESULTS

7.1 The metabolome and proteome of CD8$^+$ T-cells from RA patients reveal an anabolic TCA cycle usage and the accumulation of regulatory metabolites compared to healthy controls

Due to the previous observations that CD8$^+$ T-cells from RA primarily use aerobic glycolysis in order to meet their energetic requirements and do not resort to OXPHOS as much anymore (Souto-Carneiro et al., 2020), I wanted to examine the consequences for the metabolic pathways downstream of glycolysis. Thus, I analysed a targeted set of intracellular metabolites and the whole proteome of *ex vivo* CD8$^+$ T-cells from RA patients and healthy controls in cooperation with Prof. Dr Jürgen Okun, head of the metabolic centre Heidelberg – division of inborn errors of metabolism, and Dr Dominic Helm, head of the proteomics core facility of the DKFZ, respectively. I was supported by Martin Schneider (DKFZ) and Dr Junyan Lu (UK HD) with the analysis of the complex, multivariate datasets.

The metabolite analysis was focused on TCA intermediates, fatty acids, and amino acids (see Figure 9). While there were no significant changes in the amino acid and fatty acid composition between RA and healthy control CD8$^+$ T-cells, some TCA intermediates were significantly, differentially expressed. Intriguingly, the metabolites that were significantly increased in RA CD8$^+$ T-cells were aKG, succinate, malate and 2HG, which are important cofactors and/or competitive inhibitors of several enzymes involved in cell signalling and DNA-methylation. Also the intracellular concentrations of the other TCA intermediates and lactate were tendentially increased in RA CD8$^+$ T-cells, however, not reaching statistical significance. It must be noted that the concentrations of oxaloacetate and aKG were close to or did not reach the detection limits in some samples.

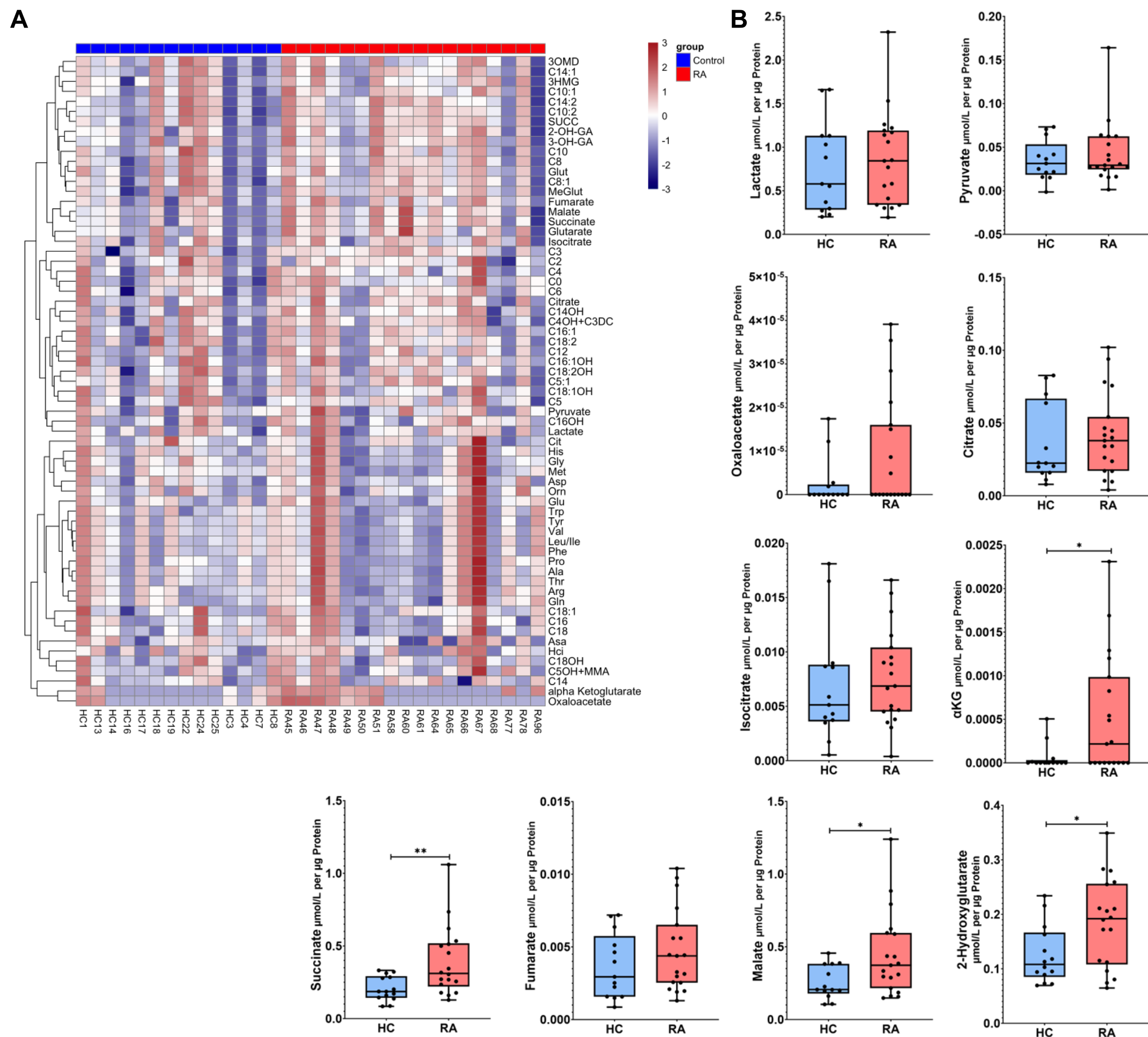
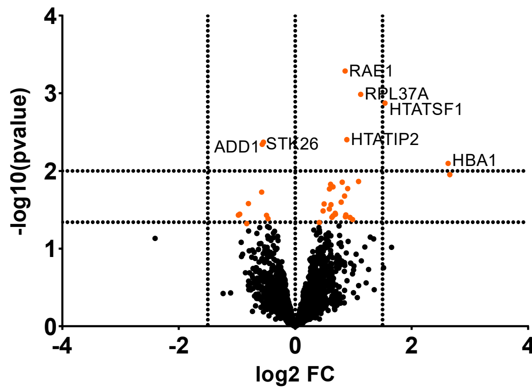
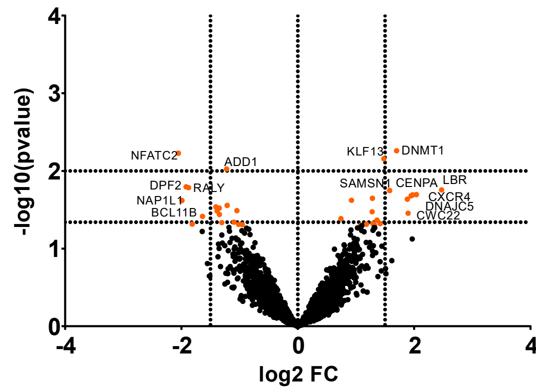


Figure 9: Targeted analysis of CD8⁺ T-cells intracellular metabolites

The intracellular metabolites were analysed from lysates from *ex vivo* CD8⁺ T-cells from 14 healthy donors (HC) and 18 RA patients (RA). (**A**) Heatmap of all analysed metabolites. (**B**) Lactate, pyruvate, and all TCA intermediates as metabolites of interest and the only significantly, differentially expressed metabolites: aKG, succinate, malate and 2HG. Box plots show min to max distribution, statistics were calculated using limma; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

The proteome and phospho-proteome data-analysis performed by Dr Junyan Lu using proDA resulted in 36 p-value significant differentially expressed proteins (see Figure 10). I further performed a pre-ranked pathway analysis with the significantly up- and downregulated proteins using the GSEA software to obtain information about the biological processes that are probably altered based on the protein expression profile. Due to the small sample size of 36 the resulting pathways did not reach statistical significance and can be found in Supplementary Table 3 and Supplementary Table 4. Thus, I decided to include all proteins with a fold change ≥ 0.5 and ≤ - 0.5. I proceeded likewise with the phospho-proteome dataset. Based on this analysis the pathways in Figure 10 were up- or down regulated in RA $CD8^+$ T-cells compared to $CD8^+$ T-cells from healthy controls (top 30 regulated pathways can be found in Supplementary Table 1 and Supplementary Table 2). As expected, in RA the upregulated pathways mainly related to immune- and cell activation, in accordance with the pro-inflammatory autoimmune phenotype of the cells. Nicely confirming the previous observations, the main downregulated pathways in RA were metabolic pathways, more precisely metabolic processes dealing with macromolecules like proteins and fatty acids that are usually processed by OXPHOS. More importantly, these results indicate that the metabolic switch in RA $CD8^+$ T-cells is not only a by-product but a characterising feature of these cells and their pathogenic role in the disease.
The analysis of the phospho-proteome revealed the increase of phosphorylation of the DNA methylating enzyme DNMT1 in RA $CD8^+$ T-cells compared to healthy controls as the most significant hit. Further, the pathway analysis identified cytoskeleton and thus, probably cell cycle and proliferation associated proteins to be less phosphorylated and an increase in phosphorylation of proteins involved in transcription and mRNA modification, as well as chromatin remodelling (top 30 regulated pathways can be found in Supplementary Table 5 and Supplementary Table 6). Unfortunately, the data set was too small to analyse the possible kinase-signatures that are underlying the phosphorylation events and could thus, give insights into the signalling pathways involved.





downregulated in RA ← → upregulated in RA

less phos. in RA ← → more phos. in RA

Enrichment plot: GOBP_GLUTAMINE_FAMILY_AMINO_ACID_METABOLIC_PROCESS

Enrichment plot: GOBP_ANTIMICROBIAL_HUMORAL_RESPONSE

Enrichment plot: GOBP_REGULATION_OF_ACTIN_FILAMENT_BASED_PROCESS

Enrichment plot: GOBP_MYELOID_CELL_DIFFERENTIATION

Enrichment plot: GOBP_TRNA_METABOLIC_PROCESS

Enrichment plot: GOBP_HUMORAL_IMMUNE_RESPONSE

Enrichment plot: GOBP_REGULATION_OF_CELLULAR_COMPONENT_BIOGENESIS

Enrichment plot: GOBP_REGULATION_OF_MRNA_PROCESSING

Enrichment plot: GOBP_TRNA_PROCESSING

Enrichment plot: GOBP_CELLULAR_OXIDANT_DETOXIFICATION

Enrichment plot: GOBP_REGULATION_OF_CYTOSKELETON_ORGANIZATION

Enrichment plot: GOBP_REGULATION_OF_RNA_SPLICING

Enrichment plot: GOBP_RNA_METHYLATION

Enrichment plot: GOBP_CELLULAR_RESPONSE_TO_TOXIC_SUBSTANCE

Enrichment plot: GOBP_REGULATION_OF_ORGANELLE_ORGANIZATION

Enrichment plot: GOBP_REGULATION_OF_MRNA_SPLICING_VIA_SPLICEOSOME

Enrichment plot: GOBP_NEGATIVE_REGULATION_OF_UBIQUITIN_DEPENDENT_PROTEIN_CATABOLIC_PROCESS

Enrichment plot: GOBP_DETOXIFICATION

Enrichment plot: GOBP_REGULATION_OF_ACTIN_FILAMENT_LENGTH

Enrichment plot: GOBP_CHROMATIN_REMODELING

Figure 10: Proteome and phospho-proteome analysis (p. 71)

The proteome and phospho-proteome were analysed by MS/MS from lysates from *ex vivo* CD8$^+$ T-cells from 8 RA patients (RA) and 5 healthy donors (HC). All differentially expressed proteins with a fold change of higher than 0.5 and lower than -0.5, respectively, were analysed via GSEA to identify GOPB pathways. In case of the phospho-proteome the most significant phosphorylation site was chosen for the analysis if several phosphorylation sites of the protein were present in the selected dataset. The top five up- and downregulated pathways for each dataset are displayed. The lists of the top 30 pathways can be found in section 13.

The metabolite concentrations and the protein expression levels already indicated an altered regulation of the TCA cycle. In order to better comprehend this modified activity, I correlated the paired metabolite concentrations and the respective enzyme expression levels from the CD8$^+$ T-cells of the samples that were analysed in both experiments (see Figure 11). In the CD8$^+$ T-cells from healthy controls I observed several strong spearman correlations with high significances, many of which reaching correlation coefficients of 1 or -1. For example, citrate concentrations and ACO1 expression levels had a significant positive correlation and lactate and pyruvate a strong negative correlation, representing their positions and activities in the TCA. Overall, the correlation matrix for the healthy control CD8$^+$ T-cells displays a running, catabolic TCA cycle, where substrates, products, and enzymes are tightly connected and regulated by each other. The number of strong correlations decreased massively in the RA CD8$^+$ T-cells. Overall, the interconnections seemed to be disrupted. This observation indicates that the TCA cycle is rather used for anabolic biomolecule homeostasis than for energy production and downstream OXPHOS. Furter, this is in line with the observed accumulation of TCA metabolites in RA CD8$^+$ T-cells compared to healthy controls, which indicates that the usual hand in hand "substrate-to-enzyme-to-product" cycle process is corrupted.

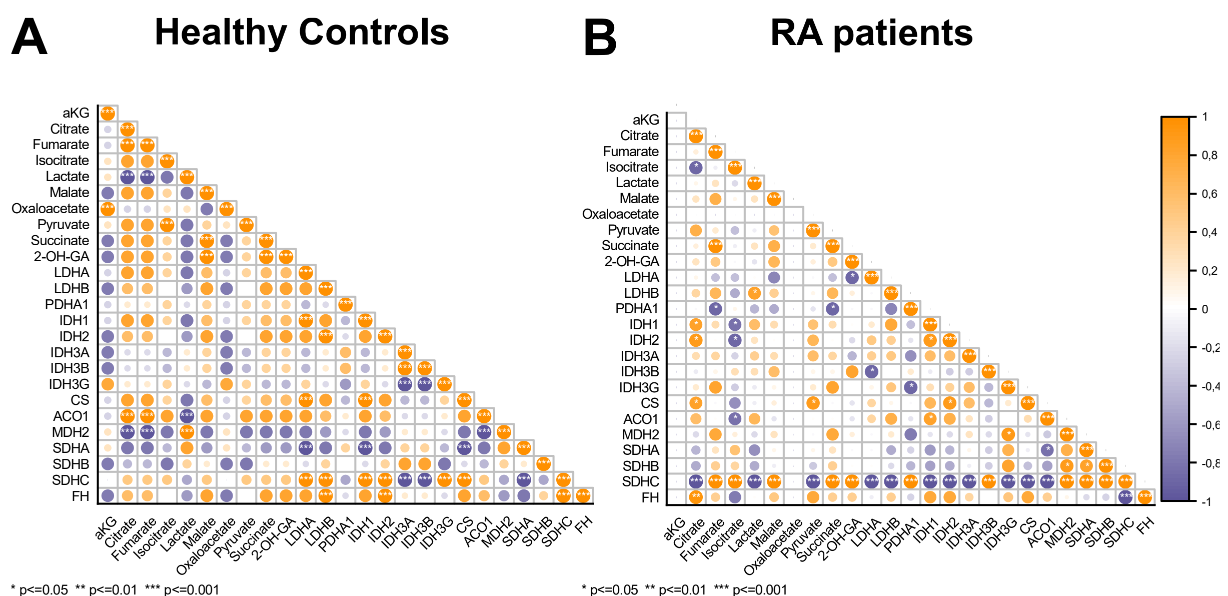
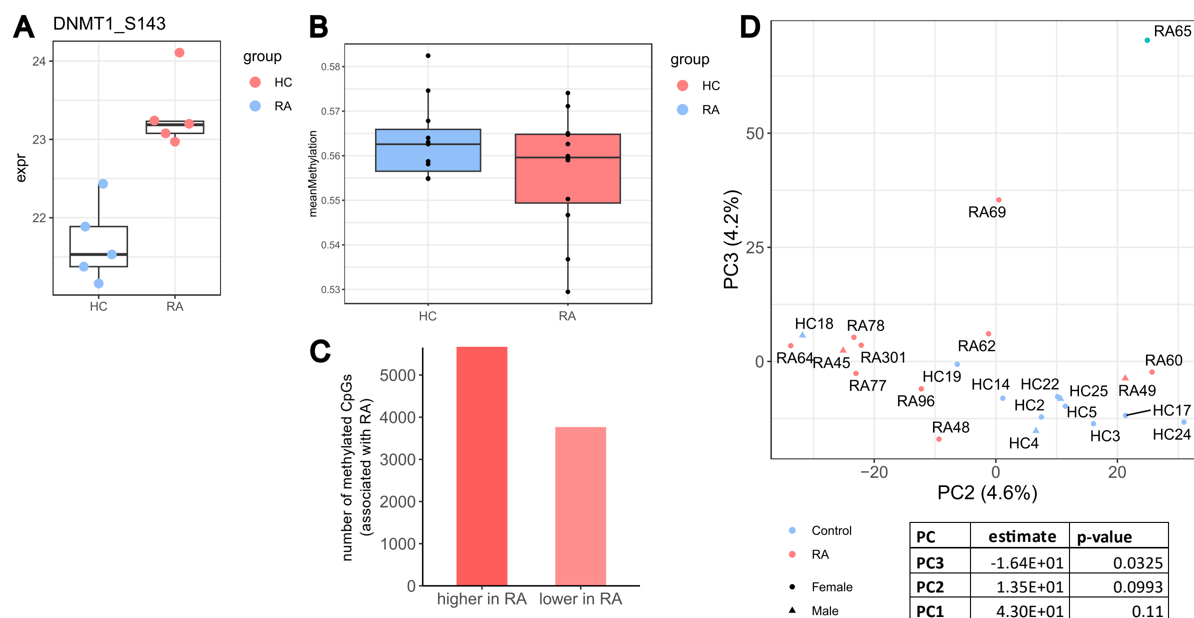


Figure 11: Correlation of intracellular metabolite concentrations and the respective enzyme expression levels

The involved TCA enzymes were selected from the proteome analysis and correlated by spearman correlation the concentrations of the related metabolites for matched samples: 6 RA patients and 4 healthy controls. Colours represent the spearman correlation coefficient r and circle size the p-values.

7.2 CD8$^+$ T-cells from RA patients display a distinct DNA methylation profile

The accumulation of 2HG, succinate, malate and fumarate harbours the potential of a deregulation of target enzymes, such as competitively inhibiting TET enzymes and altering the epigenetic landscape. In accordance with these findings, I examined the phospho-proteome, which was analysed in combination with the proteome, and found an increase of pDNMT1 at serine 143 in CD8$^+$ T-cells from RA patients (see Figure 12A). This phosphorylation was reported to increase DNMT1 stability, probably prolonging its activity and thus increases the overall DNTM1 activity in the cells (Estéve et al., 2011) . Due to these indications of a regulation in CD8$^+$ T-cells in RA by DNA methylation, I assessed the global DNA methylation in *ex vivo* CD8$^+$ T-cells from RA patient and healthy controls, some of which had paired data to the metabolites and the proteome and phospho-proteome analyses. The experimental procedure using the Illumina Infinium MethylationEPIC v2.0 Kit was performed by the Life&Brain GmbH (Bonn) and the final data analysis was performed with the help of Dr Junyan Lu. Two analyses were conducted: one global analysis of all detected CpG sites and one targeted with a gene list I provided based on inflammation and metabolism related genes.



PC	estimate	p-value
PC3	-1.64E+01	0.0325
PC2	1.35E+01	0.0993
PC1	4.30E+01	0.11

Figure 12: Analysis of the global DNA methylation

DNA methylation was assessed in DNA isolated from *ex vivo* CD8$^+$ T-cells from 12 RA patients (RA) and 11 healthy donors (HC) using the Infinium MethylationEPIC v2.0 kit from Illumina. **(A)** Box plot displaying the increase in DNMT1 phosphorylation derived from the phospho-proteome analysis (p-value = 0.005). **(B)** Box plot displaying the global DNA methylation in both donor groups. **(C)** Bar graph showing the methylated CpGs associated with RA and their methylation level compared to the respective healthy control CpGs. **(D)** PCA analysis of RA and healthy control DNA methylation displaying PC2 and PC3, which had the lowest p-values.

The overall analysis of the data showed no significant difference between the total DNA methylation of RA and healthy $CD8^+$ T-cells (see Figure 12B). Interestingly, a PCA revealed that $CD8^+$ T-cells from RA patients and healthy controls could be separated to a certain extent based on their DNA methylation profile, even though the model quality parameters Q^2 and R^2 were below the 0.5 threshold (potentially model overfitting due to moderate sample size) (see Figure 12D). The CpGs which were associated with RA and could be used to characterize the group where hypermethylated, which aligns with the previous observations and my hypothesis (see Figure 12C).

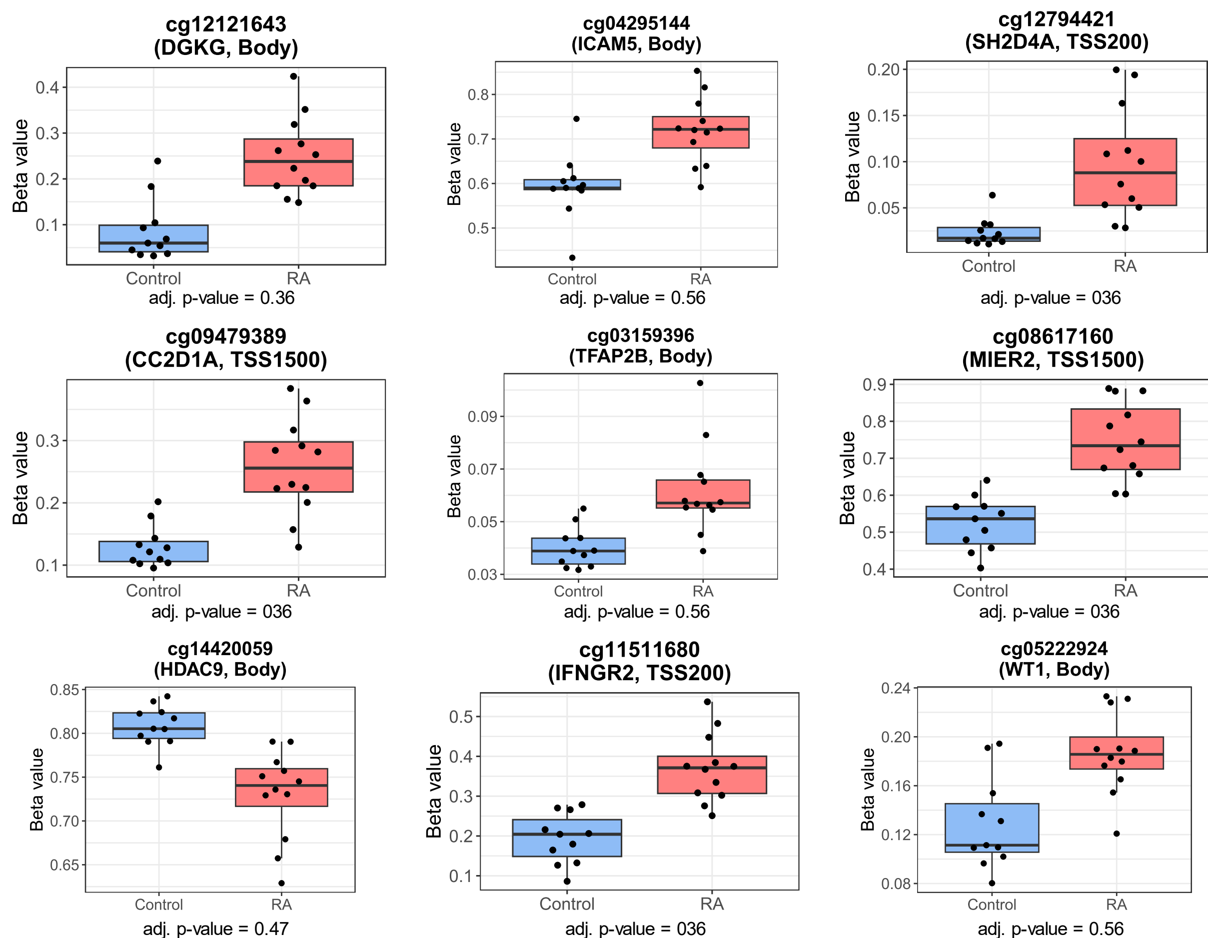


Figure 13: Selection of the top significant hits from the global DNA methylation analysis

The DNA methylation was analysed for DNA isolated from ex vivo $CD8^+$ T-cells from 12 RA patients (RA) and 11 healthy donors (control) using the Infinium MethylationEPIC v2.0 kit from Illumina. Displayed are box plots showing the beta value, which indicates the methylation level of the respective CpG, where 0 means completely unmethylated in all samples and 1 means completely methylated in every copy analysed.

The global DNA methylation analysis (see Figure 13) revealed many significantly, differentially methylated genes, which are involved in TCR signalling such as *DGK* (diacylglycerol kinase), *ICAM5*, and *SH2 domain 4A*. Further, transcription regulating factors were top hits such as *CC2D1A* and AP-2(*TFAP2B*), as well as genes involved in histone modifications *MIER2* and *HDAC9* (histone deacetylation). The high ranking of signalling molecule gene methylation is especially interesting since next to pDNMT1, also pNFATc2 was a top hit in the phospho-proteome analysis (see Figure 10), indicating regulations on TCR signalling level. Also *IFNGR2* was significantly promoter-

hypermethylated in RA CD8+ T-cells. *WT1* CpG sites were hypermethylated in the gene body at 10 sites in RA and in none in healthy controls, which is the most outstanding hit concerning the associated CpG sites. WT1 is considered a tumour suppressor and found mutated in leukaemia but mutans were also already found in SLE (Su et al., 2021).

To obtain a clearer picture of the DNA methylation of inflammatory and metabolic genes, an additional targeted analysis was performed (see Figure 14). Due to the findings of the global analysis I decided to also include the DGK isotypes in the targeted analysis. While *DKG* was hypermethylated in the gene body in RA CD8+ T-cells compared to healthy controls, *DGKA*, *DGKZ*, *DGKI*, and *DGKZ* were hypomethylated in the gene bodies in RA CD8+ T-cells. Interesting significantly differentially methylated metabolic genes were: *IDH3B*, *D2HGDH* (D2-hydroxyglutarate dehydrogenase), genes of the ETC complex I, and *NOS1* (Nitric Oxide Synthase), which were hypermethylated in the promoter region and *FASN* (fatty acid synthase), *LDHB*, *G6P*, and *LDHA* (p-value 0.08), which were hypomethylated in the promoter region compared to healthy controls. *IL2* displayed less DNA methylation in the promoter region of RA CD8+ T-cells than in healthy control CD8+ T-cells. Hypermethylation in the gene body of RA CD8+ T-cells could be observed for *G6PD*, *NOX4* (NADPH oxidase 4), *IRF1* (interferon regulatory factor), and *HIF1α*. While for the *NOS1* according to the promoter hypermethylation a gene body hypomethylation was detected, for *LDHB* gene body and promoter regions were both hypomethylated. *IDH1* and *LDHA* displayed hypomethylated CpGs in the 5'UTRs (untranslated region) and *IL7* in increased DNA methylation in the 1st exon.

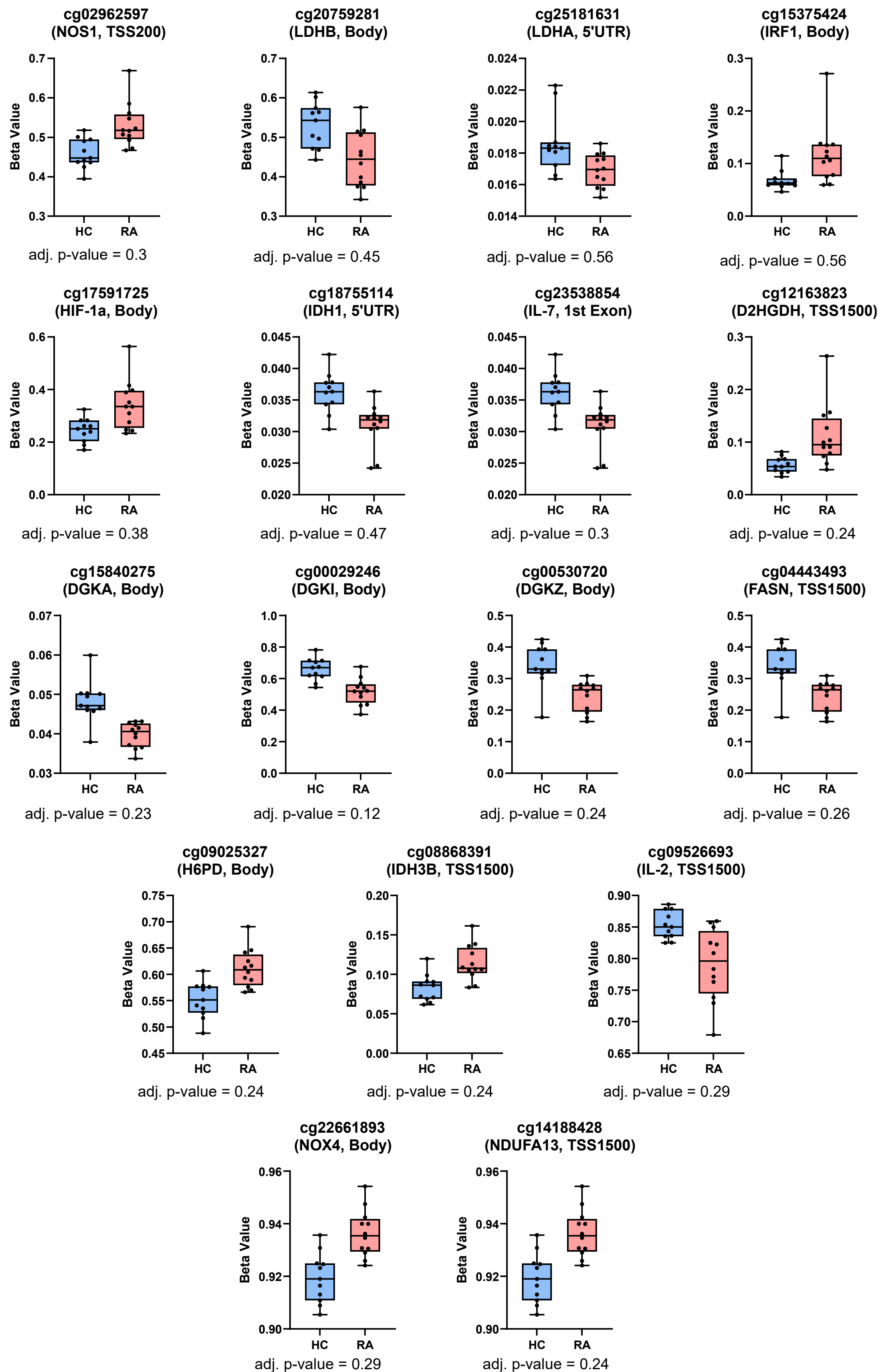


Figure 14: Selection of the top significant hits from the targeted DNA methylation analysis

The DNA methylation was analysed for DNA isolated from ex vivo $CD8^+$ T-cells from 12 RA patients (RA, red) and 11 healthy donors (healthy control, blue) using the Infinium MethylationEPIC v2.0 kit from Illumina. Displayed are box plots showing the median beta value, which indicates the methylation level of the respective CpG, where 0 means completely unmethylated in all samples and 1 means completely methylated in every copy analysed, whiskers display min and max.

7.3 No translations from DNA methylation into mRNA expression but increased DNMT1 phosphorylation could be found in a confirmation cohort

Due to its probable connection to the DNA-methylation profile, I wanted to confirm the results of the phospho-proteome analysis and examined the protein abundance of pDNMT1 in the confirmation cohort. DNMT1 in CD8+ T-cells from RA patients was significantly more phosphorylated than in healthy control (see Figure 15).

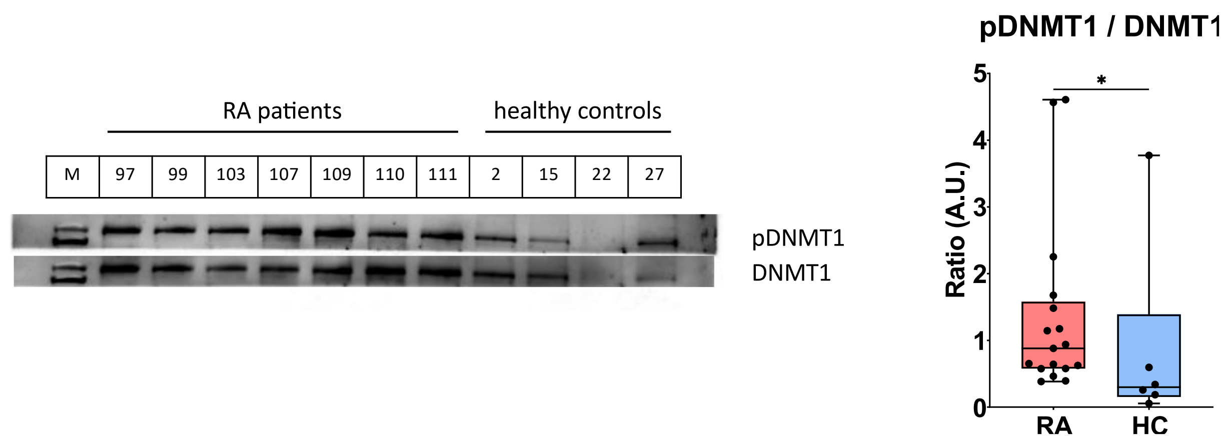


Figure 15: DNMT1 phosphorylation is increased in CD8+ T-cells from RA patients compared to healthy controls.
Protein expression of phosphorylated DNMT1 and total DNMT1 were assessed by Western Blot in whole cell lysates from 21 RA patients and 9 healthy controls. Representative blot is depicted. Quantification of the signals was performed using ImageJ.

To obtain a more immediate readout of the DNA methylation, CD8+ T-cells from patients and healthy control of a confirmatory cohort, distinct from the original sample group, were analysed for mRNA and protein expression (see Figure 16). A set of hits from the global and the target DNA methylation analysis was selected to examine the respective mRNA expression levels by qPCR. I analysed 21 RA samples and 10 healthy controls. For *DGKG* and *TET2* I did not obtain any signal. Significant differences could be observed in mRNA expression levels of *SDHC*, *DGKA*, *DGKZ*, and *PKM2*. *DGKA* and *DGKZ* both were hypomethylated in the gene body of RA CD8+ T-cells and their mRNAs were significantly lesser expressed. This supports the hypothesis that body methylation increases mRNA expression and vice versa. Unfortunately, all other analysed mRNAs did not allow any conclusions concerning the effects of the respective DNA methylation statuses.
SDHC expression was increased in healthy control cells, which might reflect the accumulation of succinate in RA CD8+ T-cells. Also *PKM2* mRNA expression levels were higher in healthy controls. In order to obtain more precises answers on how the DNA methylation affected the gene expression levels, especially for the rather under investigated non-promoter CpGs, large scale gene expression studies would need to be performed, such as RNAsequencing or ATAC sequencing.

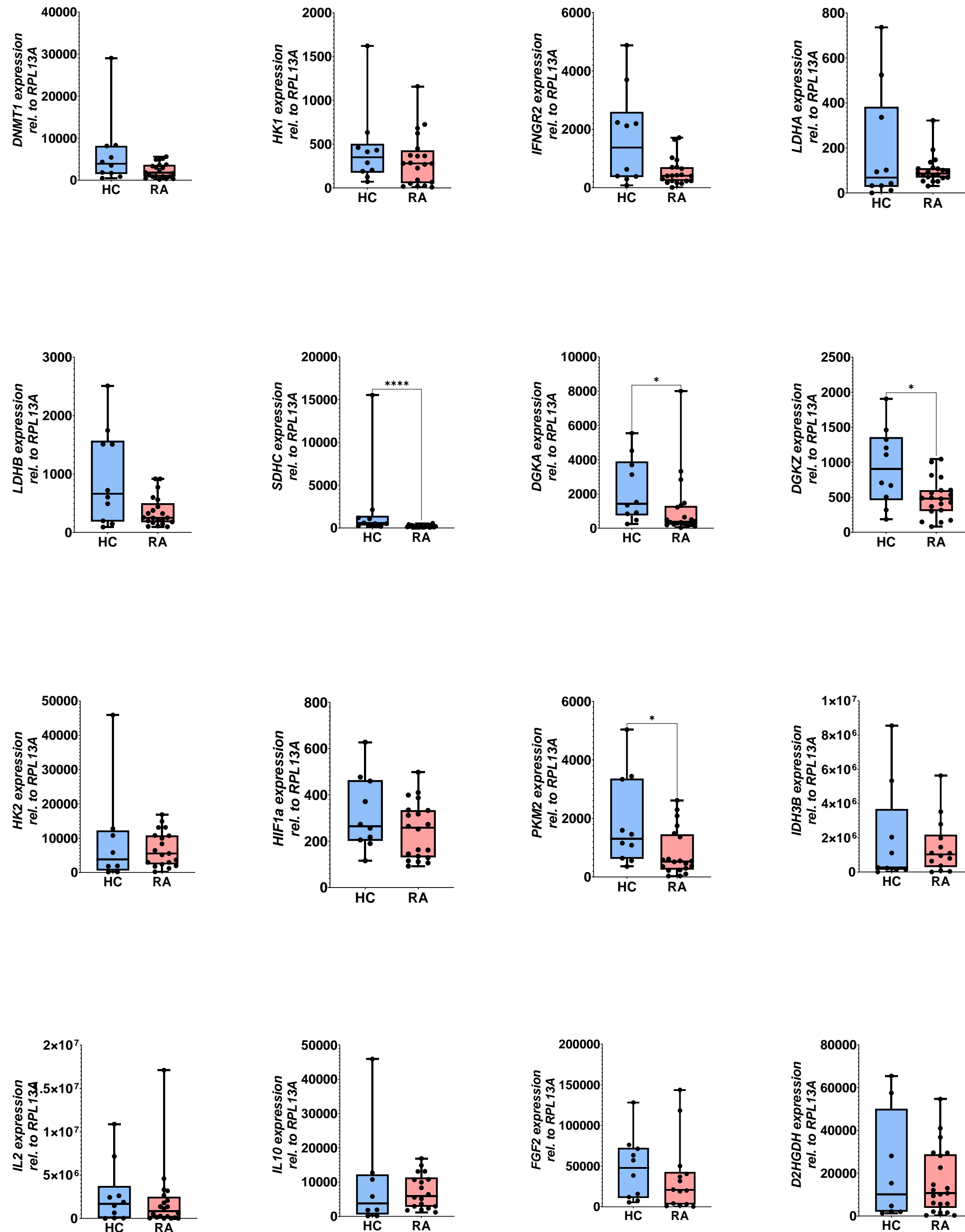


Figure 16: Gene expression analysis of a selection of genes from the DNA methylation array

Gene expression was analysed by qPCR for the selected genes from CD8⁺ T-cells from 21 RA patients (RA) and 10 healthy controls (HC) and normalized to *RPL13A* expression. Box plots display median, whiskers min and max, statistics were calculated using an unpaired Man-Whitney test; * p ≤ 0.05, ** p ≤ 0.005, *** p ≤ 0.001, **** p ≤ 0.0001.

7.4 Multi-omics data integration was not possible due to low sample size but identified confounders and non-confounders in clinical and demographic patient data

In order to identify more significant pathways and characteristics of the cells, I aimed to perform an integrative analysis that would incorporate the proteome, phospho-proteome, metabolite, DNA methylome, and multi-colour flow cytometry data (data not shown here). This approach would identify common features that do not reach significance in the single datasets but their appearance in all or several data sets would increase their impact. Together with Dr Junyan Lu we aimed to perform this analysis using the MOFA model, however, the number of samples, which we had all information of was too small to perform the analysis in a reliable manner (total n in this study = 32, n necessary for MOFA > 100).

Nonetheless, the integrative approach could be used to identify, if any clinical or demographic parameters did influence the results. A great advantage of analysing this in an integrative manner is that it automatically excludes batch effects or other influences (as the buffer composition in the proteome data (see section 6.2.14.2)) as it only accounts for similarities.

The MOFA model calculates so called factors which aim to explain as much of the variance in the datasets as possible. Factors that explain a lot of the variances in several data sets are capable to separate the groups analysed, in this case RA and healthy controls. As depicted in Figure 17B, factors 1 and 2 explained a lot of the variances in all data sets. In a next step the calculated factors were correlated with covariates such as gender, age, medication, and disease activity to rule out that they had any influence on the data. None of the factors correlated with the gender of the donors, however, the age correlated with factor 2 and factor 5. The medications seemed not to have global influences, which is especially of interest concerning the metabolome. Factors 2 and 4 were outstanding in explaining the variance in the metabolic data set but did not correlate with any medication type. Next to age as a strongly correlating covariate in factor 2, the disease activity score was equally strong correlated, however, with factor 7, which did not explain much variance and might be neglectable here.

Overall and regarding the low informative value due to the low sample size, the model indicated that demographic and clinical parameters did not influence the data in a way that has to be accounted for and that the effects I observed are independent of these environmental aspects.

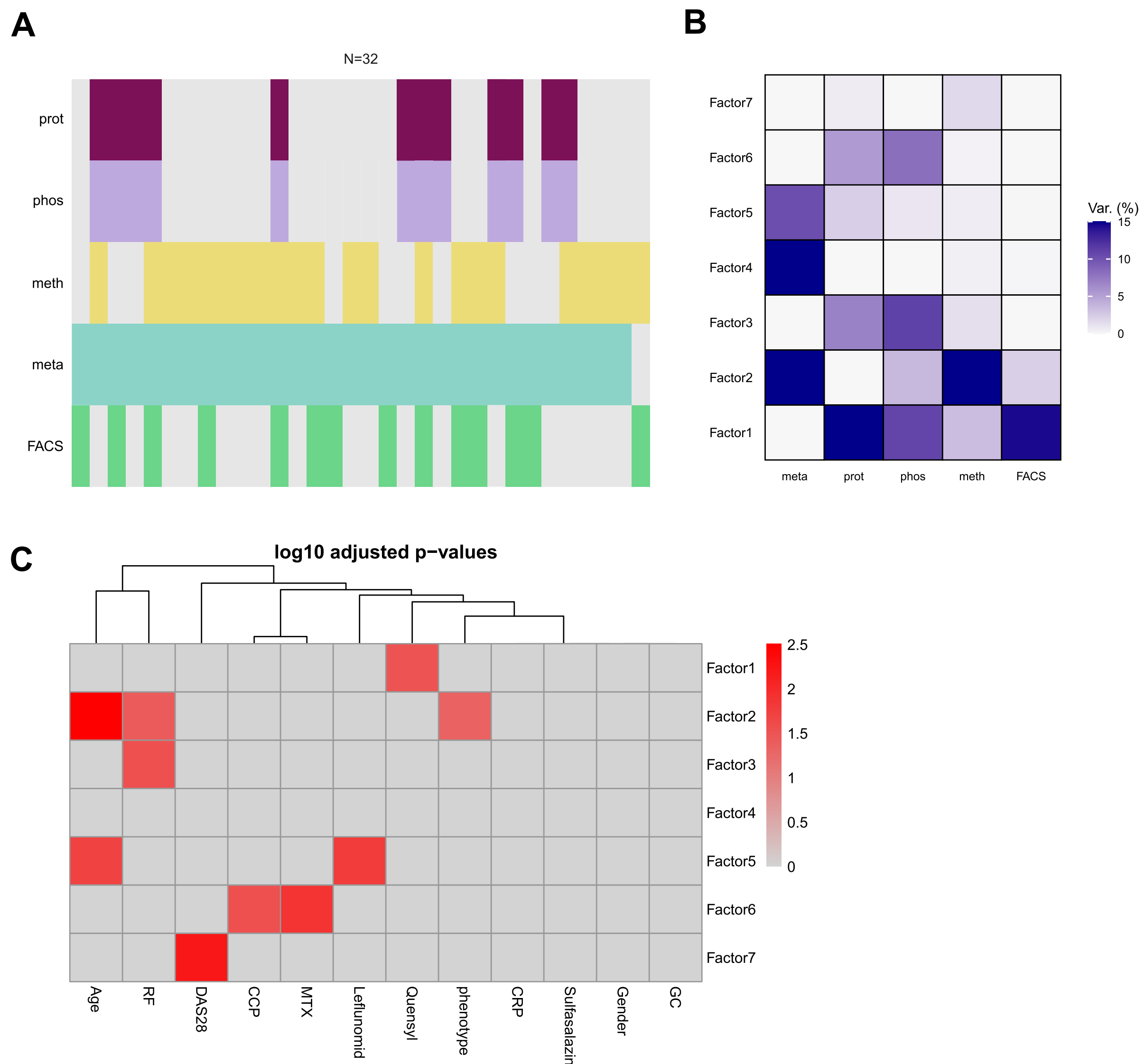


Figure 17: Integrative analysis of all datasets

(A) Schematic display of all datasets and the matched samples. **(B)** Factors identified by the MOFA analysis and the % of how much variance of the respective dataset they explain. **(C)** Demographic and clinical parameters influencing the factors. Colour range displays the log10 adjusted p-values. Prot = proteome, phos = phospho-proteome, meth = DNA methylome, meta = metabolite analysis, FACS = flow cytometry data (not shown here), GC = glucocorticoids

7.5 In CD8$^+$ T-cells from RA patients DNMT activity is increased while TET activity is decreased

Due to the observed phosphorylation of DNMT1, which might be increasing or at least prolonging enzyme activity, and the differences in DNA methylation levels in CD8$^+$ T-cells from RA patients compared to healthy controls, I analysed the enzyme activities of DNMT1 and TET. The enzyme activities were determined in nuclear protein extracts from *ex vivo* CD8$^+$ T-cells from 21 RA patients and 10 healthy controls by a luminometric (DNMT) and a fluorometric (TET enzymes) assay. In the DNMT1 assay one healthy control was detected as an outlier (ROUT test, Q = 0.5 %), which was thus excluded from the analysis. To maintain comparability, I also excluded this sample from the TET activity assay. While the DNMT methylation activity was significantly increased in RA patient derived CD8$^+$ T-cells, the TET hydroxylase activity was decreased in these samples (see Figure 18). These results are in accordance with the previous observation that DNMT1 is phosphorylated in RA cells and with the accumulation of TET inhibiting metabolites succinate, fumarate and 2HG.

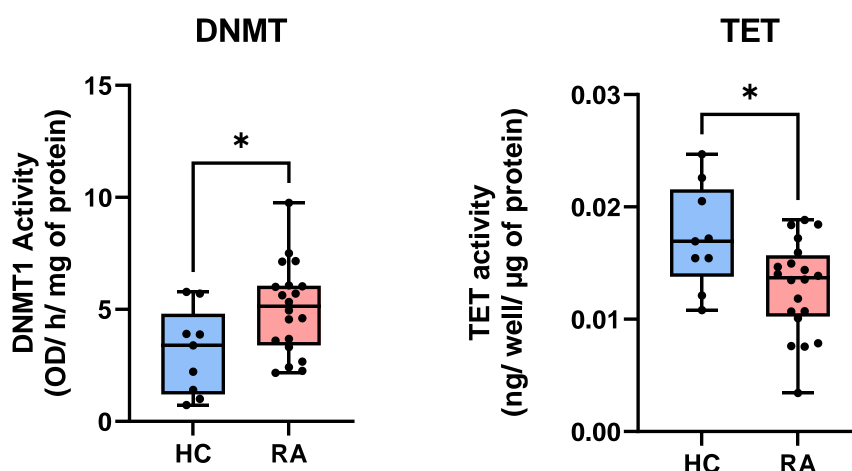


Figure 18: Activities of DNMT and TET enzymes

The enzyme activities of both key player enzymes in DNA methylation were analysed using a fluorometric and luminometric arrays with nuclear extracts from *ex vivo* CD8$^+$ T-cells 20 RA patients (RA) and 9 healthy controls (HC). Box plots display median, whiskers min and max, statistics were calculated using an unpaired Man-Whitney test; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

7.6 aKG but not 5Aza stimulation of CD8$^+$ T-cells harbours the capacity to dampen the pro-inflammatory phenotype and activity

In order to confirm the above findings on a functional level, CD8$^+$ T-cells from 14 RA patients and 10 healthy controls were *in vitro* stimulated for 72 h with anti CD3 (αCD3) and anti CD28 (αCD28), in combination with aKG and 5Aza or left unstimulated. Subsequently, the cells and the

supernatants were analysed for cell surface markers, global protein expression and ATP production, cytokine release, and metabolic activity.

First, I confirmed the activity of 5Aza in a smaller cohort of cells. I stimulated *ex vivo* $CD8^+$ T-cells for 72 h with αCD3 / αCD28 and 1.5 µM 5Aza or left them unstimulated. 5Aza restimulation was performed every day. Following, I determined the 5HMC content in the DNA with a luminometric assay (see Figure 19A). The analysis confirmed the decrease of DNA methylation in healthy controls and RA cells under the given conditions. Thus, the experimental setup was also used for the functional readouts.

For the functional readouts, 5Aza (1.5 µM) and aKG (octyl aKG, d0: 500 µM, d1&2: 125 µM) were added to the αCD3 / αCD28 stimulated wells. Upon 72 h of stimulation the cells and supernatants were harvested and used for the subsequent experiments.
The cell surface marker expressions were analysed by flow cytometry. First, I analysed the $CD8^+$ T-cell subsets, in order to exclude that any of the following findings are based on differential distributions (see Figure 19B&C). I neither observed differences in the numbers of $CD8_{naive}$, $CD8_{TEMRA}$, $CD8_{EM}$, $CD8_{CM}$ cells comparing healthy control cells with RA patient derived cells, nor comparing the stimulation conditions. Thus, a for example more active phenotype in RA $CD8^+$ T-cells did not arise from a higher $CD8_{TEMRA}$ population in that cohort.
Further, I analysed live cells with a live/dead staining, cell surface markers for activation (CD69, CD95), CD11a for T-cell homing, markers for regulation and exhaustion (PD1, CTLA-4), and the intracellular proliferation marker Ki-67 (see Figure 19D-F).
The αCD3 / αCD28 stimulation induced a decrease in live cells, which is probably due to increased proliferation, activity and thus, also apoptosis in these cells. The 5Aza and aKG stimulation did not further alter the apoptosis rates in the cells, implying that the observed effect is mediated by the αCD3 / αCD28 stimulation and not the other stimuli. CD69 and CD95 expressions were significantly increased upon stimulation in both cohorts, as expected. The addition of aKG but not 5Aza, significantly reduced the activation of the cells in the healthy control cohort almost back to ns conditions. The same tendency was observed for RA $CD8^+$ T-cells, however not reaching statistical significance. CD11a surface expression was not increased upon stimulation in both cohorts, however, it was significantly reduced upon addition of 5Aza and aKG in RA and healthy control $CD8^+$ T-cells. The expression of CTLA-4 was decreased in RA $CD8^+$ T-cells compared to healthy control, however the stimulation with 5Aza and aKG did not further alter its expression in either cohort. PD1 expression showed the same tendency. The proliferation of unstimulated cells already was increased in RA $CD8^+$ T-cells compared to healthy controls. A slight increase in proliferation in healthy control $CD8^+$ T-cells was observed upon 5Aza stimulation but in no other condition in either cohort.
Overall, these results suggest, that the impact of DNMT1 stimulation is not as strong as the capacity of aKG to downregulate the activity of $CD8^+$ T-cells. The increased proliferation of healthy control $CD8^+$ T-cells upon 5Aza addition could have increased the 5Aza effect, which is only prominent upon cell division.

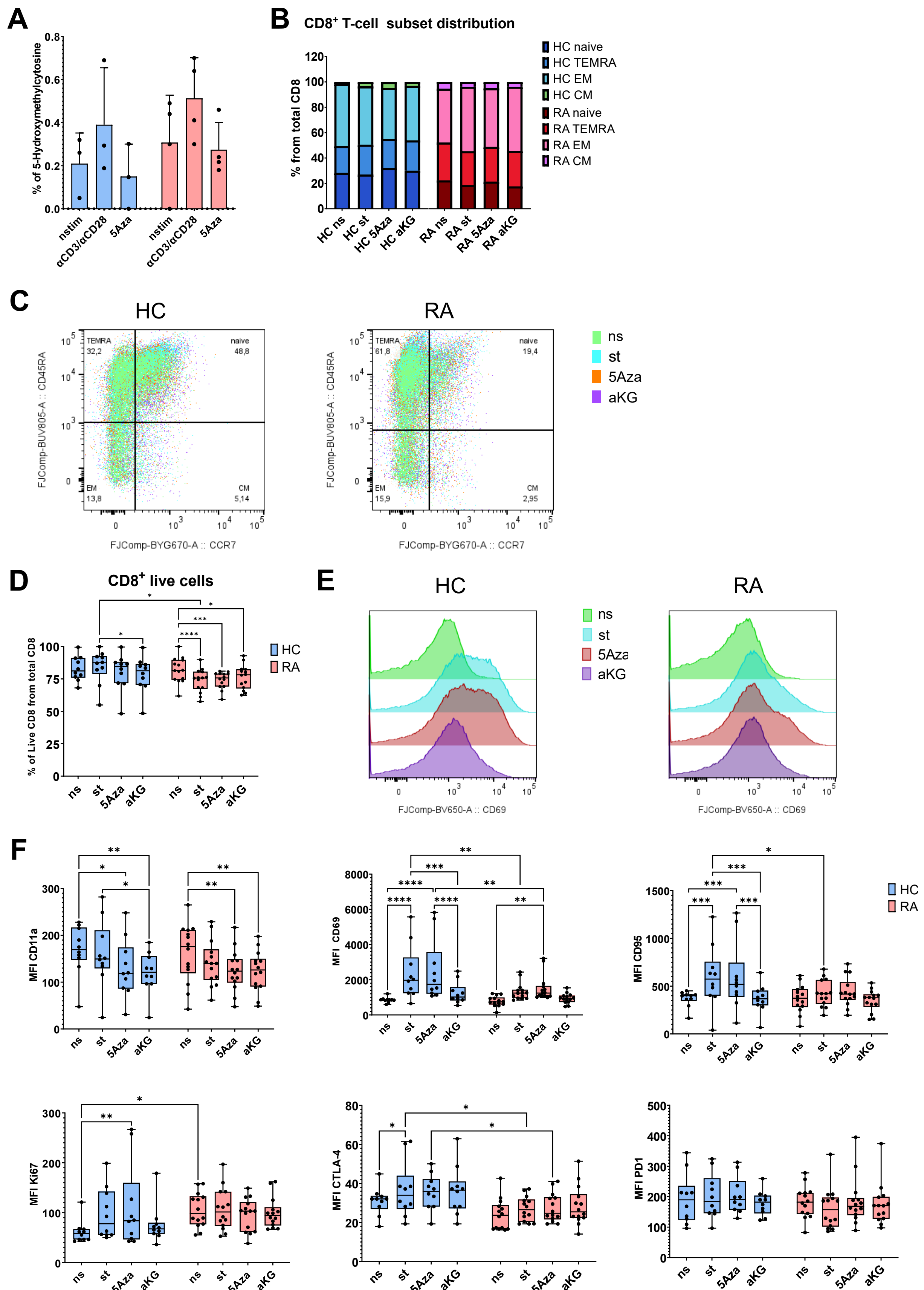


Figure 19: Flow Cytometric analysis CD8⁺ T-cells stimulated with 5Aza and aKG

(A) Assessment of the working concentration of 5Aza. CD8⁺ T-cells from 3 RA patients (red) and 3 healthy controls (blue) were stimulated for 3 days with αCD3 / αCD28, 1.5 µM 5Aza (%Aza), or left untreated (nstim). Subsequently the global DNA methylation was assessed by a luminometric assay. **(B-E)** CD8⁺ T-cells from 14 RA patients (RA, red) and 10 healthy controls

(HC, blue) were stimulated *in vitro* for 72 h with αCD3 / αCD28 (st), αCD3 / αCD28 + 1.5 µM 5Aza (5Aza), αCD3 / αCD28 + octyl aKG (d0: 500 µM, d1&2: 125 µM) (aKG) or left untreated (ns). Subsequently the expression of cell surface and intracellular markers and a live/dead staining **(D)** was analysed by flow cytometry. CD8⁺ T-cell subsets $CD8_{naive}$, $CD8T_{EMRA}$, $CD8_{EM}$, $CD8_{CM}$ were identified by the expression of CCR7 an CD45RA (see section 5.1.2) – summary bar graph **(B)** and dot plot overlays of the concatenated original fcs files **(C)**. Surface marker expression is displayed as summary data **(F)** and an exemplary histogram overlay of the concatenated fcs files **(E)**. Statistical analysis was performed using a 2way ANOVA with multiple comparisons corrected for by tukey, box plots display median, whiskers min and max; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

Secreted Cytokines in the supernatants were analysed by a cytokine bead array (see Figure 20). The analysed cytokines were assigned to the following groups:

Pro-inflammatory cytokines: TNF-α, IFN-γ, IL-2, IL-6, and IL-17a.
Anti-inflammatory cytokines: IL-4, and IL-10.
Cytotoxicity mediators: Granzyme A, granzyme B, perforin, sFas, and sFasL.

No significant differences in IL-6 and IL-2 secretion were found, which is, in the latter case, in accordance with the mRNA expression levels, although cytokine mRNA expression not reliably translates into protein concentrations. IFN-γ and TNF-α secretion increased in both RA and healthy control $CD8^+$ T-cells upon stimulation. Upon the addition of 5Aza to the cells the secretion levels remained, except from a slight decrease in healthy control TNF-α. Very prominent and in accordance with the surface molecule expression levels, aKG stimulation of the cells decreased the release of both pro-inflammatory cytokines back to a non-stimulated level. These observations were not made for IL-17a secretion. Anti-inflammatory IL-10 and IL-4 displayed an identical secretion pattern as IFN-γ and TNF-α, with the additional notice that the secretion levels under stimulated conditions were higher in RA $CD8^+$ T-cells compared to healthy controls. This indicates that the counteracting mechanism in RA cells is stronger, very likely, since the baseline activity is already elevated.
Also the cytolytic and cytotoxic molecules sFasL, granzyme B, and perforin were increasingly secreted upon αCD3 / αCD28 stimulation, the supernatant concentrations decreased upon addition of aKG to the culture and were unaffected by 5Aza in both RA and healthy control $CD8^+$ T-cells. Granzyme A, sFas and granulysin have not undergone a change as pronounced in comparison, but they follow the same trend.
Overall, these observations imply, that the passive demethylation process of the DNMT1 inhibition does not have large effects on the effector functions of $CD8^+$ T-cells but the stimulation with aKG and thus stimulation of aKG dependent enzymes like TET2 mediates an anti-inflammatory and anti-cytotoxic effect, which might be even more pronounced in RA $CD8^+$ T-cells compared to healthy controls. These findings are further supported by the clustering in the heatmap (bidirectional, complete clustering with euclidean distance method). In both cases, RA, and healthy controls, the αCD3 / αCD28 stimulated only and 5Aza stimulated conditions cluster together, while the aKG stimulated cells are more similar to the unstimulated controls.

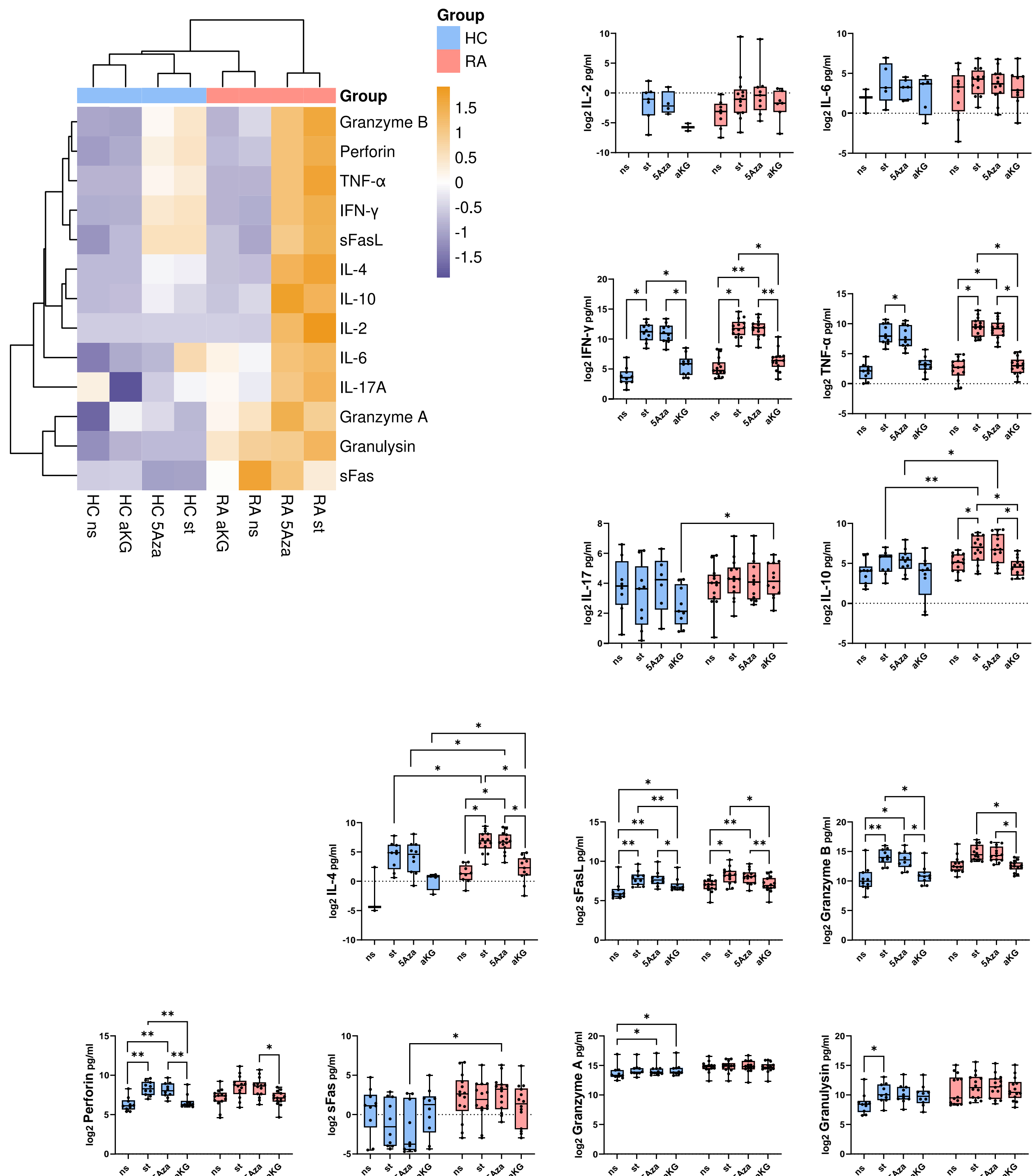


Figure 20: Cytokine Bead array of CD8+ T-cells upon 5Aza and aKG stimulation

The cytokine release of CD8+ T-cells of 10 healthy controls (HC, blue) and 14 RA patients (RA, red) was analysed in the supernatants upon 72h *in vitro* stimulation with αCD3 / αCD28 (st), αCD3 / αCD28 + 1.5 µM 5Aza (5Aza), αCD3 / αCD28 + octyl aKG (d0: 500 µM, d1&2: 125 µM) (aKG) or left unstimulated (ns). Cytokine concentrations were measured using a CBA array. The heatmap displays the normalized mean concentrations; clustering was performed by bidirectional, complete clustering with euclidean distance method. Statistical analysis was performed using a 2way ANOVA with multiple comparisons corrected for by tukey; box plots display the log2 transformed data for better visualisation, median, whiskers min and max; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

7.7 The *in vitro* treatment with aKG reduces the glycolytic rate and lactate production

Next to the functional readouts, I was interested how the treatment with 5Aza and aKG might affect the metabolic activity of the cells and if the glycolytic profile could be reverted. To assess whether the changes in the expression of the surface markers and immune mediators was connected to a change in metabolism, I quantified the glucose and lactate concentrations in the cell culture supernatants of the same samples. By adding uniformly labelled ^{13}C glucose to the cell culture medium (w/o glucose), the consumption of glucose and the directly related production of lactate can be quantified in the supernatants by ^{1}H-NMR analysis (see Figure 21). Using a fumarate standard, I was able to calculate the absolute concentrations of the metabolites. Further, the lactate enrichment as the percent of ^{13}C labelled lactate of the total (^{13}C and ^{12}C) lactate and the glycolytic rate as the percent of ^{13}C lactate production per consumed ^{13}C glucose could be calculated (see Figure 21B). Due to the low cell numbers in some samples, the respective ^{13}C lactate peaks were very low and difficult to quantify. Thus, the calculated metabolic rates may not reflect the full extent of the induced metabolic shifts.

As expected, upon stimulation of the $CD8^+$ T-cells the glycolytic rate and lactate production increased due to the shift to aerobic glycolysis. This was observed in RA and healthy control cells. In accordance with the cellular activity and the cytokine profile, aKG reduced the glycolytic activity. In the RA cells the reduction reached the ns levels, while in healthy control cells the lactate enrichment as well as the glycolytic rate were still significantly higher than in the ns conditions. 5Aza did not have a comparable effect. However, 5Aza addition to RA $CD8^+$ T-cells induced a small but statistically insignificant decrease in lactate enrichment and the glycolytic rate, which was absent in cells derived from healthy control cells. The overall ^{13}C glucose consumption was not altered significantly. However, they followed a similar tendency as the lactate enrichment in healthy control cells but not in RA patient-derived samples. This indicates that glucose is not necessarily imported at higher rates but seems to be used differently in RA $CD8^+$ T-cells.

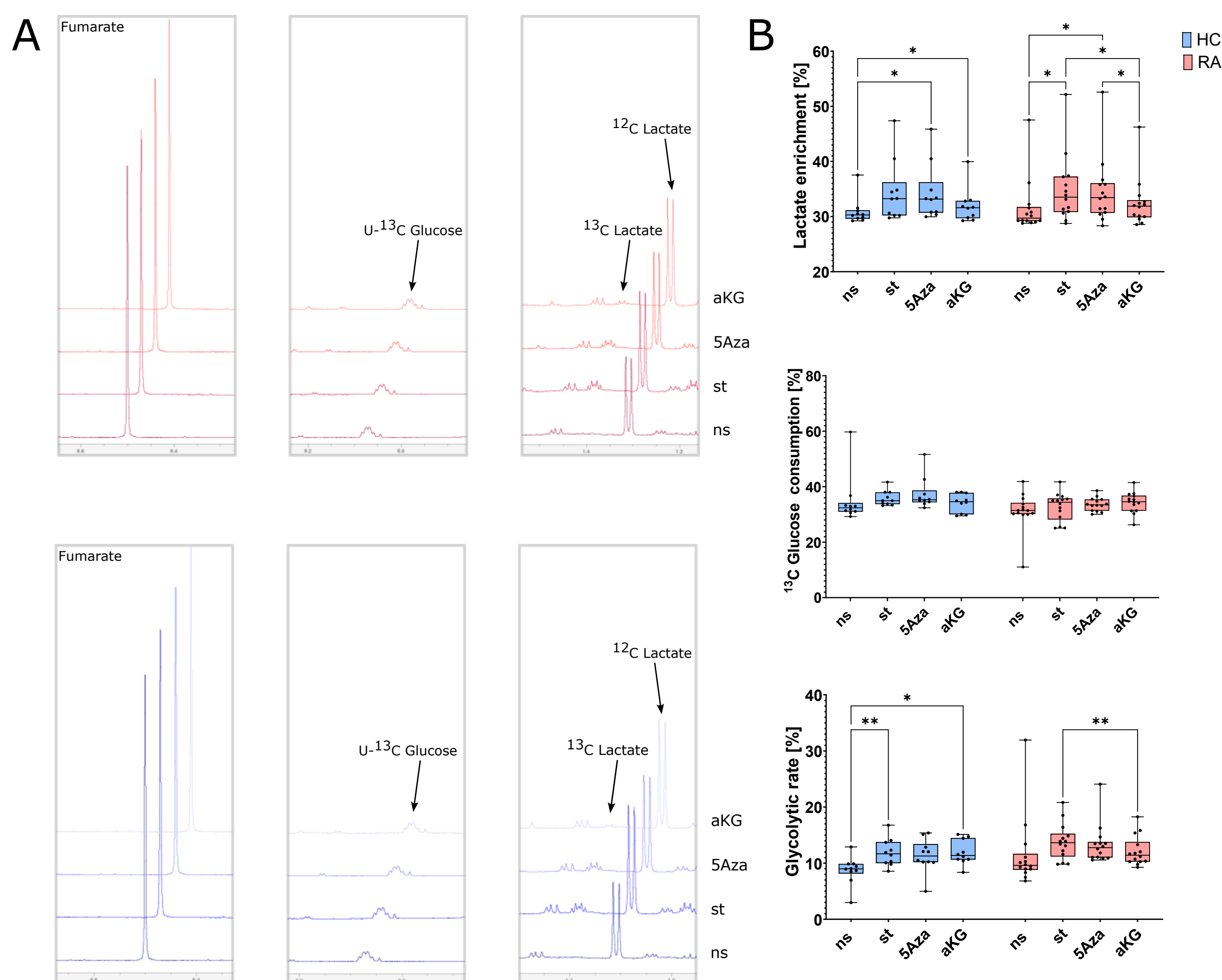


Figure 21: NMR analysis of extracellular metabolites upon *in vitro* stimulation

CD8+ T-cells of 14 RA patients and 10 healthy controls were stimulated for 72h *in vitro* with αCD3 / αCD28 (st), αCD3 / αCD28 + 1.5 µM 5Aza (5Aza), αCD3 / αCD28 + octyl aKG (d0: 500 µM, d1&2: 125 µM) (aKG) or left unstimulated (ns) in cell culture medium containing U-^{13}C glucose. The cell culture supernatants were collected and analysed by H^1 NMR to examine the concentrations of ^{13}C glucose, ^{12}C lactate and ^{13}C lactate. **(A)** Representative sections of the spectra of one RA patient (red, upper panel) and one healthy donor (blue, lower panel) showing the peaks used for quantification of the respective molecules. **(B)** Boxplots displaying the calculated glucose consumption, lactate enrichment (% of ^{13}C lactate of total lactate), and derived glycolytic rate (% of ^{13}C lactate produced from ^{13}C glucose). Statistical analysis was performed using a 2way ANOVA with multiple comparisons corrected for by tukey, box plots display median, whiskers min and max; * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.

8 DISCUSSION

The aim of this thesis was to examine the underlying signalling and regulatory pathways of the hyper-glycolytic and auto-inflammatory phenotype of CD8+ T-cells derived from RA patients. All experiments were performed *in vitro* with either directly derived *ex vivo* cells or upon 72h of cell culture with TCR-mediated stimulation. Due to the complexity of the analyses and the rather detail-oriented expectable changes between healthy controls and patients, I refrained from including other disease controls, such as PsA or AS. Nevertheless, this harbours the risk that the observed changes are rather inflammation/arthritis-specific than RA-specific. However, the previous studies identified the metabolic switch as unique for RA CD8+ T-cells (Souto-Carneiro et al., 2020). In order to adress any doubts, follow up-experiments, as discussed in the following passages and chapters, that follow more directed approaches than exploratory ones, can be performed including cells from disease-control groups.

8.1 2-Hydroxyglutarate stands out as a possible key-regulator in maintaining a pro-inflammatory phenotype

In contrast to the first part of my hypothesis that the focus on aerobic glycolysis in RA CD8+ T-cells shuts down the TCA cycle and thus, decreases the concentrations of its intermediates, I observed an increase in these metabolites. Due to the already mentioned amphibolic character of the TCA cycle, this implies an anabolic usage of the TCA cycle and the anaplerotic processes that can fuel TCA intermediates in RA CD8+ T-cells. Further, the pathway analyses indicated that rather the downstream processes like ETC, OXPHOS, and connected to these the metabolism of macromolecules as proteins and fatty acids, are those that might be downregulated in these cells. Nevertheless, scuccinate, fumarate, lactate, and 2-hydroxyglutarate can act as competitive inhibitors at several stages of aKG driven pathways. Thus, it is plausible that not a decrease in aKG is resposible for the metabolic switch and the connected hyper-glycolytic phenotype, but rather the accumulation of these other, structurally related, ibhibitory metabolites.
A common indicator for metabolic activity are the ratios bewteen aKG:succinate and aKG:citrate, which give insights into the TCA usage. Unfortunately, in my analyses, the detected aKG concentrations were very close to the detection limit and in the majority of samples did not even reach it. Thus, the interpretation of these results has to be handled with caution and the calculation of these ratios was not possible.
Especially interesting, is the increase of the “oncometabolite” 2HG in RA CD8+ T-cells, which is derived from aKG (see Figure 9). There are two enantiomers of 2HG – D- and L-2HG. While D2HG is predominantly produced by mutant IDH in acute myeloid leukaemia, L2HG can be produced under physiological conditions by LDHA and, to a lesser extent, by MDH and PHGDH, which is even increased in hypoxia and acidic environments (Intlekofer et al., 2015, 2017). The increase in 2HG in CD8+ T-cells under hypoxic conditions was not dependent on IDH polymorphisms and genome wide studies of RA patients did not reveal such mutations (Tyrakis et al., 2016). Thus, I exclude the possibility that the rises in 2HG levels, I observed in RA CD8+ T-cells, is due to RA-linked mutations in IDH, as it is in cancer. The mass spectrometry analysis method, I used to examine the

metabolite concentrations, is not able to distingiush between enantiomers. Nevertheless, I consider the L-enantiomer to be the more plausible species in the context of this project.

In 2016 Karlstaedt and colleagues investigated the role of 2HG in rodent heart tissue and discovered that 2HG impairs the aKGDH and ATP5B, reducing their acitivty (Karlstaedt et al., 2016). Further they concluded that 2HG promotes glycolysis and the usage of short chain fatty acids fo energy production, which feed the TCA directly via succinate, fumarate, and oxaloactetae and impair OXPHOS activity. These findings are in accordance with my presented results and support my hypothesis. The increase in TCA intermediates I have observed, is similar to their findings and the increase in 2HG ind RA $CD8^+$ T-cells could explain the decrease in aKG co-factor dependent enzyme activites. Further, adding aKG in excess might have overruled the impact of 2HG. It may not be neglegted though, that the accumulation of aKG to a certain extent, might also backfire by stabilising HIF-1α, which was observed in breast cancer, or by being even increasingly converted into 2HG again (Kuo et al., 2016).

Due to its structural similarity, 2HG is a competitive inhibitor of dioxygenases that require aKG as a co-factor (W. Xu et al., 2011). Amongst this group of enzymes are prolyl-hydroxylases and DNA- and histone.demethylases.
Prolyl-hydroxylase acitivty is for example necessary to target HIF-1α for degradation. Thus, high 2HG concentrations could prolongue HIF-1α activity in $CD8^+$ T-cells. HIF1-1α can further be stabilized by succinate and fumarate and is activating a transcriptional program that shifts the cellular metabolism from OXPHOS to glycolysis by incresing the transcription of glucose transporters, HK1 and HK2, LDHA, PDH, PDK1, and many more (W. Xu et al., 2011). Further, in the same study, aKG was able to rescue the metabolic phenotype. These data resemble my observations that the addition of aKG had an anti-inflammatory effect on the $CD8^+$ T-cell functionality, which might indicate a competition for binding sites between aKG and 2HG in RA $CD8^+$ T-cells (see discussion section 8.4).
Equally of interest is the capacity of 2HG to dose dependently inhibit TET dioxygenases and the DNA-demethylation. Again, L-2HG was found to have a higher potency to inhibit the enzmyes than D-2HG had (W. Xu et al., 2011). The epigenetic effect could be demonstrated to play a significant role in T_H17 cell fate, as high levels of 2HG increased DNA mehtlyation, thus, decreased FoxP3 expression levels and impaired the differentiation into T_{reg} cells (T. Xu et al., 2017). The same study could even detect a decrease in experimental autoimmune encephalitis (a multiple sclerosis animal model) severity in mice by inhibiting the 2HG poduction. Further emphasizing the pro-inflammatory role of 2HG, it was found to be an imporant inducer of T_H17 differentiation by altering the epigenetic landscape imporant for IL-17 expression, both *in vitro* and *in vivo* in colitis and asthma models (Miao et al., 2021).

8.2 The proteomic analyses identify the metabolic switch as a characterisitc of RA CD8$^+$ T-cells and an increase in DNA methylation by DNMT1

In combination with the analysis of the proteome, the idea to also investigate the phospho-proteome initially arose from the observation that LDHA is phosphorylated in RA CD8$^+$ T-cells, as a matter of enzyme activity regulation (data from MMSC, not published). It has to be ackknowleged that the sample size of the proteome analysis only allowed for a primary, exploratory approach. Further, the applied experimental method, data dependent acquistion, allows a global analysis of the proteome but fails to detect proteins with a low sequence coverage (such as for example TET2). By now a novel analysis method was established, data independet acquistion (DIA), which also improved the detection of the previously non-detected proteins. Thus, a future repetition of the experiment using DIA, with samples that match the other -omics data sets, would improve the value and the significance of the data.
While the proteome analysis supported my hypothesis that the metabolic pathways downstream of glycolysis are utilized to a lesser extent, it more importantly emphasized that the altered metabolism of CD8$^+$ T-cells in RA patients is a definite and stand-alone characteristic of the cells and not only a mere byproduct of their immune activation. Most interestingly, the phospho-proteome analysis identified an augmented phosphorylation of DNMT1 in RA CD8$^+$ T-cells, which could be confirmed by Western Blot in another confirmation cohort. As already stated above, the phosphorylation of DNMT1 via AKT increases its stability and probably induces a proglonged activity (Estéve et al., 2011). Regarding my hypothesis that a decreased TET activty would increase the overall DNA methylation, the increased activty of DNMT1 upon phosphorylation could amplify this effect even further. Supporting this idea, the epiegentic remodelling via the AKT-DNMT1 axis has been found to majorly influence the metabolic reprogramming in cancer cells, favouring glycolysis and lactate production and in turn to be reinforced by high lactate levels (T. Huang et al., 2020; W. Li et al., 2019; Luo et al., 2018). Luo et al. analysed the influence of DNMT1 in EBV associated cancer and found that the EBV protein LMP1 (latent membrane protein 1) can upregulate DNMT1 expression and activtiy. The result is metabolic reprogramming by epigenetic downregulation of OXPHOS. These findings are especially interesting, as EBV infections are considered to be triggers for the pathogenesis of RA and other auto-immune diseases and could contribute to the observed metabolic reprogramming in CD8$^+$ T-cells from RA patients.

Upon TCR activation, proteins of the NFAT transcription factor family get dephosphorylated, translocate to the nucelus, and activate the expression of target genes. The phospho-proteome analysis revealed a decreased phosphorylation level of NFATc2 in CD8$^+$ T-cells from RA patients, implying a higher acitivity of the transcription factor. NFAT has various downstream target, as for example *DGK*s (Zheng et al., 2008). Due to the decreased mRNA levels of *DKGA* and *DGKZ* in the presented samples, however, it is rather unlikely that the increased NFATc2 activity majorly influences their expression in the present context. Actually, NFATc2 is known to auto-reactively activate its own expression and to protect activated T-cells from apoptosis (Mognol et al., 2016).

Additionally, the knock out of *NFATc2* in a SLE mouse model decreased the disease severity and implied that NFATc2 is necessary for the development of autoimmunity by driving T-cell proliferation and activation (Seth et al., 2023). Therefore, the pro-inflammatory role of NFAT seems to predominate and its decreased phosphorylation is another piece adding to the autoimmunoty driving signalling cascade in RA CD8^{+} T-cells .

Taken together, the presented proteome analyses confirm the hypothesis that the metabolic switch towards aerobic glycoylsis in RA patient derived CD8^{+} T-cells is regulated on a transcriptional and epigentic level.

8.3 Epigenetic remodelling dertermines cell signalling and metabolic pathway usage in CD8^{+} T-cells from RA patients

The impact of epigenetic remodeling could further be emphasized by the observation that the TET enzyme activies were decreased and inversely the DNMT acitivies were increased in CD8^{+} T-cells from RA patients compared to healthy controls. These results are in accordance with the remaining results, the literature, and my hypothesis. Thus, I decided to acquire the global and a targeted DNA methylation profile of CD8^{+} T-cells which includedc cells used for the the proteome and metabolite analyses.
Consistent with the TET and DNMT activities, the CpG islands associated with the RA phenotype, identified by a PCA, were found to be hypermethylated. This adds hyper-DNA-methylation to the list of RA CD8^{+} T-cell characterising aspects. The detailed exploration of the single, differentially methylated CpGs revealed highly significant changes in the methylation profiles of genes cofing for thr DGK signalling proteins. Furthermore, WT1 stood out, as all gene body CpGs analysed were methylated in RA CD8^{+} T-cells, but none in the healthy control cohort. Unfortunately, FoxP3 could not be detected in the analysis although being a known canidate for regulation by methylation (see section 5.4.3).

DGKs catalyse the reaction from the active signalling molecule DAG into PA (phosphatidic acid). For instance, PMA, a common *in vitro* T-cell activating agent, is an anlogue to DAG. Thus, withdrawing DAG by DGK limits the signalling cascades of the TCR and T-cell activation (see Figure 1). There are several isoforms, of which DGKα, DGKδ, and DGKζ seem to be the predominant ones in T-cells. By the phosphorylation of DAG, they abrogate TCR signalling and T-cell activation. However, in early T-cell development they can impair Treg formation as they decrease the signal from the TCR:self-antigen binding (Joshi et al., 2013). DGKζ overexpression inhibits signalling pathways downstream of the TCR and IL-2 production, while cells deficient for DGKζ and DGKα were observed to have augmented proliferative capacity and decreased anergy induction, which, to a certain extent, was even co-stimulation independent (Olenchock et al., 2006; Zhong et al., 2002). Further, the anti-tumour activity of CD8^{+} T-cells was enhanced by DGKζ deficiency (and to a lesser extent DGKα), supporting PD1 checkpoint inhibition (Gu et al., 2021). Consistent, another group discovered decreased PD1 expression and increased IL-2 expression and cytotoxic activity in DGKζ deficient CD8^{+} T-cells (Arranz-Nicolas et al., 2020). Similarly, the anti-viral response of CD8^{+} T-cells was increased in mice deficient for these DGKs, their capacity

to establish immunological memory, however, was impaired (Shin et al., 2012; Zhong et al., 2003). Inversely, CD8+ T-cells in tumour tissues were found to express higher levels of DGKα, which rendered them tumour tolerating, while cells from non-tumour tissues did not show differences in DGKα expression (Prinz et al., 2012).
Although there are some studies on how DGK expression might be regulated, the mechanisms in T-cells in physological as well as in pahtological conditions, remain to be fully determined.

The results of the DNA methylation array revealed for *DGKG* a higher and for *DGKA,* and *DGKZ* a lower CpG methylation in the gene body. DGKγ was not yet found to play a role in T-cells and accordingly the subsequent qPCR did not return any signal for *DGKG* expression for both RA and healthy control CD8+ T-cells. This is, however, rather contradictory to the common opinion that gene body methylation increases transcription. On the contrary, *DGKA* and *DGKZ* gene expression were decreased in RA CD8+ T-cells, as to be expected based on the decrease in gene body methylation. Hence, the gene expression profile matches the increased pro-inflammatory phenotype in the cells, as it may be considered that a lower DGK activity is unable to abrogate TCR signalling. This further endorses the hypothesis of the modulation of the immune-activity by epigenetic means.

WT1 is a transcription factor that was originally found to play a role in renal tumors but by now is known to be also mutated in many leukemias (Rivera & Haber, 2005; L. Yang et al., 2007). It is physiologically involved in developmental processes of vascularisation, renal and adrenal tissue, splenic tissue, neuronal development, and angiogenesis. Moreover its expression was found in the bone marrow and immune cell development. Interestingly, its overexpression is correlated with DNA hyper-methylation and it can recruit DNMT1 to gene regions and support local DNA methylation, indicating that its transcriptional regulation might also be mediated on an epigenetic level (Szemes et al., 2013; B. Xu et al., 2011). Further, the *WT1* promoter itself was found to be acitvated in hypoxic conditions in a HIF1α dependent manner, which could potentiate the above-discussed effect of 2HG on HIF1α stability (K.-D. Wagner et al., 2003).
As WT1 is hypermethylated in the gene body in RA CD8+ T-cells, indicating active gene expression, it might be another player in the epigentic landscape of RA. Nevertheless, also hypomethylation in Intron1 was found to be transcriptionally activating in cancer (Malik et al., 2000). Thus, a gene expression analysis in CD8+ T-cells is necessary to reliably interpret this finding. Apart from that, WT1 positive vascularisation was found in synovitis from systemic sclerosis patients . It would thus be interesting, if it also plays a role in the neovascularitsation of the synovium in RA joints, which majorly contributes to the local inflammation.

The 2HG-dehydrogenase (D2HGDH), which was found to be promoter hypermethylated in RA CD8+ T-cells in this study, is converting 2HG back into aKG. Under hypoxic conditions the enzmye activity is reduced by half and thus, hypoxia might favour 2HG (Vatrinet et al., 2017). This is of interest in the context of RA in the joints, the site of local inflammation, which is a physiologically oxygen poor environment. The promoter hypermethylation, indicating less gene expression, is in line with the increased 2HG concentrations in RA CD8+ T-cells. Thus, the epigenetic landscape in RA CD8+ T-cells might favour high 2HG:aKG ratios. Unfortunately, *L2HGDH*, which is probably more relevant in non-cancer non-hypoxia environments, was less significantly methylated and

additionally mixed hyper- and hypomethylated in promtoter and gene body regions, so no conclusions could be drawn.

Generally it has to be considered that the direct link between DNA methylation and gene expression was only analysed for selected genes and that protein expression cannot be correlated to epigenetic regulation, as it is also regulated at various other stages. To fully elucidate the ongoing processes mRNA sequencing could be performed or ATAC sequencing as a more targeted approach.

8.4 Supplementation with aKG reduces the pro-inflammatory phenotype and function of CD8+ T-cells in the RA and the healthy control group to almost unstimulated levels probably in a two-stage mode of action

Based on the hypothesis that the autoimmune characterising metabolic switch in $CD8^+$ T-cells in RA is mediated via the aKG-2HG-epigenetic axis, I wanted to asses the functional consequences of counteracting this axis *in vitro*. Therefore, I treated cells of a confirmation donor cohort with either 5Aza to inhibitb the DNA-methylation maintenance by DNMT1 or with cell permaeable octyl-aKG in order to compensate and override the increased 2HG, succinate, and fumarate concentrations.
The functional assesment of the cells revealed that the addition of aKG but not DNMT1 inhibition had an anti-inflammatory effect. This was supported by changes in surface molecule expression, reduced cytokine/cytotoxic mediatos release, and by a decrease in the glycolytic rate of the cells. The decrease in live cell numbers upon CD3 and CD28 stimulation is probably to be attributed to activation induced apoptosis. There was no difference observable in both in Ki67 and in subset distribution, which stresses that the cell viability was not affected by the cell culture conditions. CD11a was decreased upon *in* vitro stimulation, which is not necessarily contradictory to the otherwise activated phenotype, as T-cells rather increase the affinity of homing markers upon activaion (see section 5.1.2.3). CD95 and CTLA-4 were generally less expressed on RA $CD8^+$ T-cells, which reinforces the notion that they possess a malfunctioning negative regulation capacity. While T-cell activation related CD95 and CD69 were downregulated upon aKG addition, inhibitory molecules PD1 and CTLA-4 intensities remained unaltered by the stimulation. This might imply a stronger effect on fastly upregulated molecules, than on molecules important at later timepoints.

The effect of aKG was even clearer in the cytokine/cytotoxic mediator release analysis. The overall cytokine expression was higher in RA $CD8^+$ T-cells than in healthy controls, which underlines their higher immune-activity. The high levels of IL-4 and IL-10 in RA cells might reflect a negative feedback loop, trying to dampen the pro-inflammatory response. Also these cytokines were decreased by aKG supplementation, however, to a lesser extent that for example TNFα and IFNγ. Noteworthy is the significant effect on the cytolytic and cytotoxic molecules, as they display the function of the $CD8^+$ T-cells and indicate that not only their pro-inflammatory phenotype and signalling could be reduced but also their cytotoxic effector functions. The release of cytokines is

not primarily regualted on gene expression level but by storage and fast, targeted release when necessary. This is mirrored in the presented qPCR results, where the gene expression of some cytokines was not different between healthy control and RA derived cells. Thus, it is unlikely that the observed strong effects on cytokine release are mediated by transcriptional activation or deactivation due to epigenteic changes.

Next to pro-inflammatory activity, a characteristic of CD8+ T-cells in RA is their altered metabolism compared to healthy control cells, mainly utilising small molecules for their energy production by aerobic glycolysis. This could be confirmed by the proteome analysis. Therefore, it was obvious to analyse the effect of 5Aza and aKG on this aspect. There where slight but non-significant reductions in the lactate enrichment and glycolytic rate in RA CD8+ T-cells upon 5Aza stimulation but not in healthy control CD8+ T-cells. Consistent with the findings of an anti-inflammatory effect of aKG on the T-cells, the metabolic effect was significant upon aKG supplementation, even in the healthy control cells. Due to the rather consistent glucose consumption, I suggest that these differences derive from an altered pathway usage in the respective groups.

It was observed before that supplementation of aKG could decrease pro-inflammatory signalling and cytokine production via the PHD and the impairment HIF-1α, AKT and NFκB signalling (He et al., 2017; Shrimali et al., 2021). The anti-inflammatory effect was shown *in vivo* in infection models in carp and pig and in a mouse model of type II diabetes (Agarwal et al., 2023; G. M. Liu et al., 2023; D. Wu et al., 2021). Further, AKT deficicency was observed to promote CD8+ memory T-cells formation and to impair effector functions (Eid et al., 2015). With regard to the presented results, it is plausible that the observed consequences of aKG supplementation are the sequential sum of two modes of action. The faster and immediate effect of aKG would be the promotion of PHD activity and thus the abrogation of important pro-inflammatory signalling cascades and transcriptional programms. Accordingly, AKT and LDHA activity are cis-influencing each other, which could amplify the pro-inflammatory and glycolytic effect but also the anti-inflammatory aKG signalling effect (Dai et al., 2023; K. Xu et al., 2021). Building on this, phase two follows with the activation of TET2 as a cofactor and the successive DNA demethylation, inducing a different transcriptional programm. This scenario would likewise throw light on the not or less pronounced impact of 5Aza presence, which can only be manifested after some rounds of cell division. Thus, it rather affects later stages of transcriptional reprogramming, which might be apparent to a minimal extent in the induced metabolic switch.

One of the pitfalls of the experimental setup of the thesis is the isolated analysis of CD8+ T-cells. Regarding the stabilisation and signalling of HIF-1α, it was for example found that lactate induced HIF-1α signalling in DCs dampens T-cell activity (Sanmarco et al., 2023). Thus, it has always to be considered that RA is a systemic diseases with multiple players interacting with each other.

The supplementation with aKG also affected healthy control cells, which is to be expected with regard to the probable effect on the signalling cascade. It is of positive note that both 5Aza and aKG did not decrease the cell viability. This was obseved with the small molecule inhibotor FX11, which inhibits LDHA and had a similar anti-inflammatory effect, however, at the cost of cell viability especially in healthy control cells (Souto-Carneiro et al., 2020). Furthermore, the tendencial changes upon 5Aza treatment were only apparent in RA CD8+ T-cells, opening the

possibility that the later epigenetic changes do not affect healthy control cells to the same extent. In order to elucidate this further, the *in vitro* treatment could pre pronlonged to 5 days and in addition to the functional readouts, gene expression and maybe chromatin remodeling should be analysed upon 5Aza and aKG treatment.

Taken all together, the data imply a close interconection between a influence on signalling pathways and epigeneitc regulation of gene expression programms by regulatory metabolites, mainly the shifted ratio between 2HG and aKG.

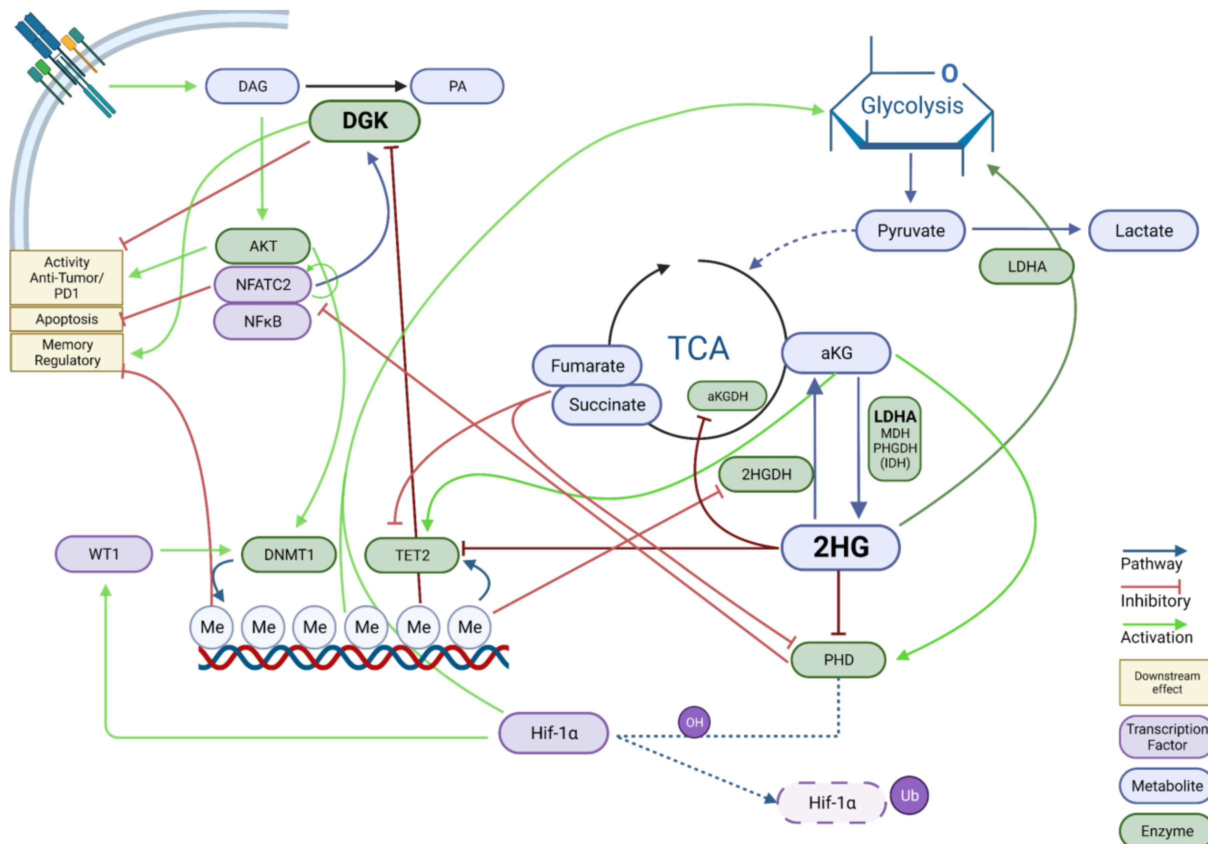


Figure 22: Schematic overview of the probably involved activating and inhibitory interactions in CD8⁺ T-cells from RA patients

The intracellular interplay of metabolic, cell signalling, and epigenetic pathways proposedly causing the observed phenotype in CD8+ T-cells from RA patients. 2HG and DGK seem to be linchpins in the metabolic and cell signalling areas, respectively. Their main regulation pathways are highlights by darker colours Abbreviations are explained in the main text. OH = hydroxylation, Ub = ubiquitinylation.

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9 CONCLUSION

Regarding the collected data and the literature as a whole, I can predict a series of consequential reactions that can venture the underlying concepts of the metabolic and inflammatory switch in $CD8^+$ T-cells in RA.
The cells display a high glycolytic phenotype and high lactate production based on an increase in glycolytic enzyme activity (Souto-Carneiro et al., 2020). The stagnated, anabolically used TCA cycle results in the accumulation of intermediates such as succinate, fumarate, and aKG. Due to augmented LDHA activity, the aKG is transformed into 2HG. The production of 2HG is also favoured by the epigenetic silencing of 2HGDH, which would catalyse the reaction back into aKG. Together and in combination with reduced aKG, LDHA, 2HG, succinate, and fumarate inhibit the DNA demethylation by TET2 and stabilise HIF-1α, AKT, and NFκB by inhibiting PHD. The transcriptional program initiated, results in a markedly pro-inflammatory / cytotoxic effector function and a glycolytic metabolism. Adding to this, the activation through phosphorylation and translocation of DNMT1 promotes DNA methylation. Further upstream, the (epigenetic) decrease in *DGK* transcription abrogates TCR-signalling limitation, and thus maintains a nearly endless T-cell activity.
This conglomerate of pro-inflammatory, effector-function promoting mediators and the maintenance of a supporting epigenetic landscape promotes the hyper-glycolytic and auto-immune phenotype of the $CD8^+$ T-cells in RA.

The supplementation of aKG counteracts this ensemble probably in two stages and at two points of attack. First and fast, the excess of aKG acts on the signalling and gene expression program by re-activating PHD and thus, interrupting HIF-1α, NFκB, and AKT signalling. The second stage is mediated by the re-activation of TET2 and the initiation of DNA-demethylation. Probably this is combined with a decrease in DNMT1 activity, mediated by the earlier signalling interference. The altered epigenetic pattern could then allow a return to OXPHOS, regulatory functions, and pathways that limit the pro-inflammatory signalling.

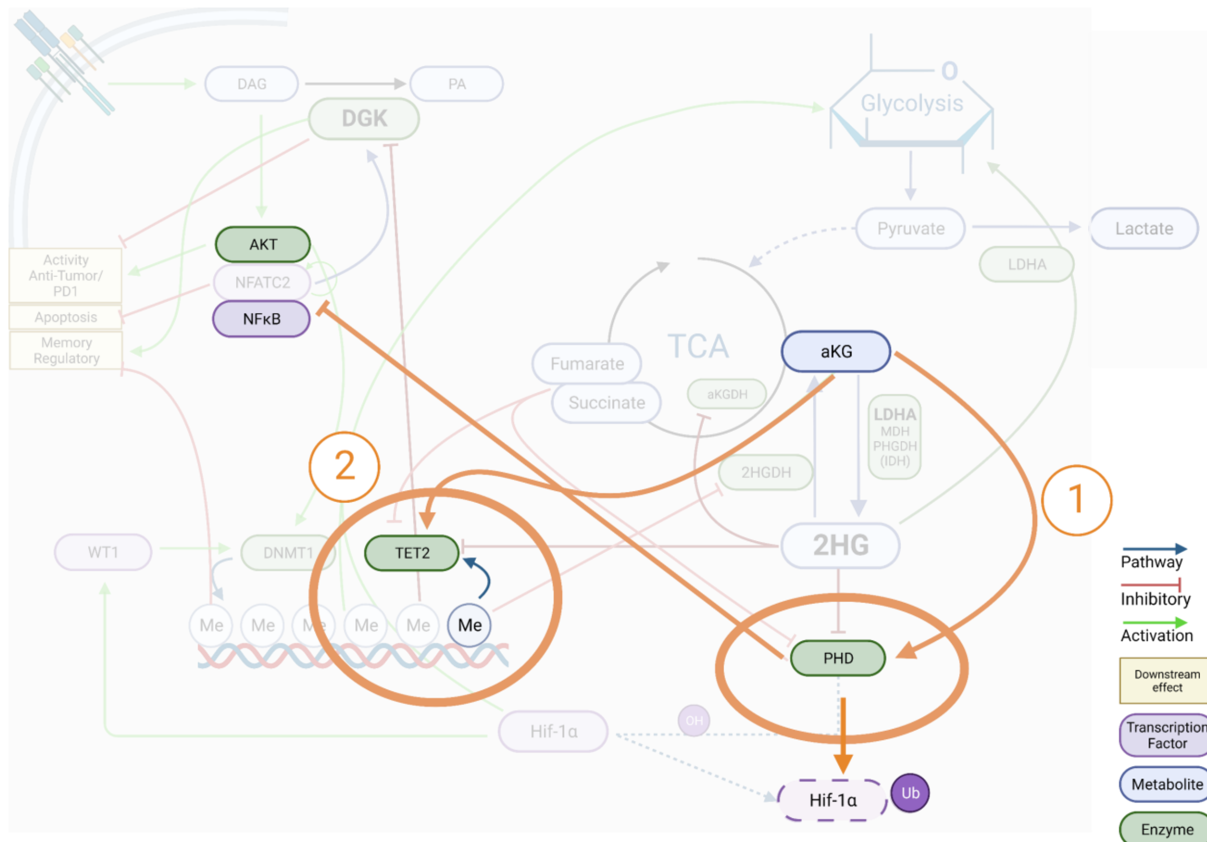

Figure 23: Schematic display of the proposed two stages of the aKG supplementation effects.
The treatment with cell permeable aKG has probably two sequential modes of actions based on the hypothesis displayed in Figure 22. **(1)** The fast influence on signalling by re-activating PHD and thus inhibiting the downstream signalling cascades of AKT and the gene expression activation by the transcription factors NFκB and HIF-1α. **(2)** By displacing competitive inhibitors, aKG acts as a co-factor for TET2 again and activates DNA demethylation. The effects on epigenetic remodelling and gene expression get apparent later than the effects of (1). Abbreviations are explained in the main text. OH = hydroxylation, Ub = ubiquitinylation.
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10 OUTLOOK

Based on the above findings and discussion, some follow up data should be acquired.
It would be a great asset to the data situation to repeat the proteomics analyses with DIA technology and an even better matched cohort of RA and healthy donors. This would enable me to also identify the expression levels of, so far, undetected proteins. In this regard, it might also be of interest to evaluate the enzymatic activities of other enzymes. For example, the catalysis of aKG into 2HG by LDHA. Adding to this it would be helpful to be able to assess the intracellular aKG concentrations more reliably by either increasing the sensitivity of the method or by trying to collect more cells.

Furthermore, the discovered anti-inflammatory effect of aKG should be examined in greater detail. Interesting would be the analysis of the DNA methylation or the chromatin accessibility upon stimulation with aKG and also to analyse a time course of these changes. This could either be achieved by repeating the global methylation array or by using a targeted ATAC sequencing

approach, which would be more time and cost efficient. By using the ATAC sequencing technique it would be possible to analyse a targeted set of genes, based on the here presented results and conclusions.

RA is a systemic disease and influenced by various internal and environmental factors. Although $CD8^+$ T-cells play a significant role in the pathogenesis of RA, this thesis presents an isolated view on the cell type in an *in vitro* setting. The next steps in order to further establish the presented findings should thus include the integration of cell-cell interactions, maybe cells derived from centres of inflammation such as the synovium, and the inclusion of *in vivo* data. Considerably, the *in vivo* data would be the most straightforward approach to reliably evaluate the effect of metabolic and epigenetic changes in RA. Moreover, it is necessary in order to validate the potential of aKG or related molecules as therapeutic agents. As stated above, the supplementation of aKG has already been performed in different animal models (Agarwal et al., 2023; G. M. Liu et al., 2023; D. Wu et al., 2021). Thus, it seems to be a feasible, ethical, and promising approach to for example examine the effects of an aKG supplemented diet on KBxN mice.
Ketogenic diet is a well-known dietary strategy that was already introduced to treat epilepsy in the 1920s. The diet focuses on high fat and low carbohydrate food intake and thus increases the concentrations of ketone bodies and decreases the blood glucose levels (reviewed by Dąbek et al., 2020). Studies investigating the molecular effects of the diet in rodents discovered a decrease in DNA methylation (Lusardi et al., 2015). Of the utmost interest, are the observations that a ketogenic diet was able to reduce the RA disease severity in a randomized, double-blind study including 61 patients (Heidt et al., 2023). Investigating the underlying molecular processes in the context of the findings of this thesis in PBMCs from a keto diet patient cohort would thus represent the best possible model to analyse the *in vivo* significance and its therapeutic strength.

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13 SUPPLEMENTARY MATERIAL

Supplementary Table 1: Proteome analysis: Top 30 pathways downregulated in RA based on proteins with a fold change from ≤ -0.5

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Glutamine family amino acid metabolic process	21	-0.55	0.008	1.000
2	tRNA metabolic process	23	-0.54	0.017	1.000
3	tRNA processing	17	-0.57	0.021	1.000
4	RNA methylation	17	-0.56	0.013	0.883
5	Negative regulation of ubiquitin dependent protein catabolic process	18	-0.53	0.026	1.000
6	Negative regulation of proteasomal protein catabolic process	15	-0.56	0.021	1.000
7	Negative regulation of proteolysis involved in protein catabolic process	20	-0.52	0.025	0.997
8	Formation of cytoplasmic translation initiation complex	15	-0.55	0.032	1.000
9	Mitotic cell cycle checkpoint signaling	18	-0.52	0.036	1.000
10	Regulation of alternative mRNA splicing via spliceosome	20	-0.49	0.040	1.000
11	Nucleoside bisphosphate metabolic process	36	-0.44	0.022	1.000
12	Nucleoside bisphosphate biosynthetic process	22	-0.47	0.040	1.000
13	Regulation of microtubule based process	51	-0.39	0.016	1.000
14	Cell cycle checkpoint signaling	27	-0.45	0.033	1.000
15	Organelle transport along microtubule	23	-0.44	0.053	1.000
16	Regulation of axonogenesis	16	-0.49	0.090	1.000
17	Regulation of mRNA processing	59	-0.35	0.034	1.000
18	Alpha amino acid metabolic process	50	-0.37	0.041	1.000
19	Regulation of circadian rhythm	21	-0.44	0.058	1.000
20	Regulation of mRNA splicing via spliceosome	48	-0.37	0.049	1.000
21	Spindle assembly	33	-0.40	0.079	1.000
22	Rhythmic process	46	-0.36	0.041	1.000
23	Cell cycle G2 M phase transition	23	-0.43	0.104	1.000
24	Regulation of proteasomal ubiquitin dependent protein catabolic process	32	-0.38	0.083	1.000
25	Regulation of ubiquitin dependent protein catabolic process	43	-0.36	0.053	1.000
26	Thioester metabolic process	29	-0.40	0.083	1.000
27	Lipid modification	46	-0.36	0.074	1.000
28	Ribonucleoprotein complex biogenesis	146	-0.29	0.019	1.000
29	Positive regulation of cell cycle process	35	-0.37	0.072	1.000
30	Regulation of microtubule cytoskeleton organization	39	-0.37	0.089	1.000

Supplementary Table 2: Proteome analysis: Top 30 pathways upregulated in RA based on proteins with a fold change ≥ 0.5

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Antimicrobial humoral response	10	0.76	0.000	0.011
2	Humoral immune response	11	0.70	0.000	0.016
3	Regulation of t cell activation	8	0.73	0.000	0.060
4	Leukocyte cell cell adhesion	11	0.64	0.000	0.066
5	Positive regulation of cell adhesion	17	0.53	0.002	0.060
6	Cellular response to toxic substance	7	0.75	0.003	0.055
7	Cellular oxidant detoxification	7	0.75	0.000	0.053
8	Detoxification	7	0.75	0.000	0.051
9	Response to oxidative stress	13	0.59	0.003	0.046
10	Response to toxic substance	9	0.66	0.000	0.050
11	Biological process involved in symbiotic interaction	9	0.66	0.000	0.048
12	Positive regulation of immune system process	27	0.45	0.001	0.047
13	Regulation of immune system process	30	0.42	0.000	0.043
14	Hydrogen peroxide metabolic process	7	0.75	0.000	0.040
15	Regulation of lymphocyte activation	9	0.65	0.000	0.045
16	Response to reactive oxygen species	10	0.65	0.000	0.045
17	B cell receptor signaling pathway	6	0.74	0.000	0.048
18	Antigen receptor mediated signaling pathway	9	0.66	0.005	0.048
19	Defense response to gram positive bacterium	5	0.82	0.000	0.054
20	Taxis	12	0.57	0.000	0.053
21	Reactive oxygen species metabolic process	9	0.64	0.002	0.053
22	Cell adhesion	37	0.38	0.004	0.052
23	Cell killing	7	0.70	0.000	0.050
24	Antibacterial humoral response	6	0.74	0.003	0.057
25	Positive regulation of lymphocyte activation	7	0.70	0.004	0.069
26	T cell activation	10	0.59	0.003	0.069
27	Response to bacterium	18	0.48	0.007	0.068
28	Defense response to bacterium	12	0.55	0.008	0.066
29	Response to wounding	27	0.41	0.004	0.071
30	Positive regulation of leukocyte cell cell adhesion	8	0.64	0.002	0.078

Supplementary Table 3: Proteome analysis: Pathways downregulated in RA based on p-value significant differentially expressed proteins

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Positive regulation of cell communication	8	-0.321	0.388	1.000
2	Cytoskeleton organization	6	-0.367	0.371	1.000
3	Regulation of organelle organizationale	5	-0.355	0.501	1.000
4	Cellular response to oxygen containing compound	6	-0.333	0.512	1.000
5	Phosphorylation	8	-0.286	0.510	1.000
6	Response to endogenous stimulus	6	-0.333	0.528	1.000
7	Positive regulation of protein metabolic process	6	-0.300	0.605	1.000
8	Regulation of intracellular signal transduction	6	-0.300	0.615	1.000
9	Regulation of protein modification process	6	-0.300	0.640	1.000
10	Regulation of phosphorylation	6	-0.300	0.623	1.000
11	Response to nitrogen compound	5	-0.323	0.622	1.000
12	Regulation of phosphorus metabolic process	6	-0.300	0.664	1.000
13	Locomotion	5	-0.290	0.735	1.000
14	Positive regulation of multicellular organismal process	5	-0.290	0.682	1.000
15	Fatty acid metabolic process	5	-0.290	0.737	1.000
16	Cellular lipid metabolic process	5	-0.290	0.737	1.000
17	Peptidyl amino acid modification	5	-0.290	0.731	1.000
18	Monocarboxylic acid metabolic process	5	-0.290	0.710	1.000
19	Lipid metabolic process	5	-0.290	0.716	0.965
20	Regulation of cell differentiation	5	-0.290	0.771	0.927
21	Positive regulation of developmental process	5	-0.290	0.743	0.894
22	Positive regulation of cell differentiation	5	-0.290	0.752	0.860
23	Cell motility	5	-0.290	0.744	0.823
24	Positive regulation of intracellular signal transduction	5	-0.290	0.773	0.792

Supplementary Table 4: Proteome analysis: Pathways upregulated in RA based on p-value significant differentially expressed proteins

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Response to oxygen containing compound	9	0.546	0.099	1.000
2	Organonitrogen compound catabolic process	5	0.645	0.107	1.000
3	Regulation of cell death	8	0.550	0.146	1.000
4	Regulation of catalytic activity	6	0.449	0.449	1.000
5	Negative regulation of cell death	5	0.448	0.592	1.000
6	Positive regulation of catalytic activity	5	0.408	0.653	1.000
7	Small molecule catabolic process	5	0.402	0.650	1.000
8	Small molecule biosynthetic process	5	0.388	0.671	1.000
9	Organonitrogen compound biosynthetic process	5	0.382	0.708	1.000
10	Positive regulation of molecular function	7	0.341	0.751	1.000
11	Biological process involved in interspecies interaction between organisms	5	0.382	0.733	1.000
12	Intracellular transport	6	0.356	0.740	1.000
13	Regulation of transport	5	0.369	0.726	1.000
14	Protein containing complex organization	6	0.351	0.741	1.000
15	Regulation of multicellular organismal development	5	0.357	0.744	1.000
16	Peptide metabolic process	5	0.355	0.784	1.000
17	Amide metabolic process	5	0.355	0.767	1.000
18	Small molecule metabolic process	9	0.291	0.806	1.000
19	Apoptotic process	6	0.320	0.798	0.960
20	Secretion	5	0.324	0.804	0.936
21	Positive regulation of rna metabolic process	5	0.293	0.871	0.966
22	Positive regulation of macromolecule biosynthetic process	5	0.293	0.889	0.928
23	Organic acid metabolic process	8	0.216	0.951	0.959
24	Response to oxygen containing compound	9	0.546	0.099	1.000

Supplementary Table 5: Phospho-Proteome analysis: Top 30 pathways upregulated in RA based on proteins with a fold change ≥ 0.5

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Myeloid cell differentiation	19	0.528	0.000	0.026
2	Regulation of mRNA processing	23	0.422	0.004	0.252
3	Regulation of RNA splicing	27	0.376	0.009	0.527
4	Regulation of mRNA splicing via spliceosome	17	0.447	0.021	0.551
5	Chromatin remodeling	31	0.300	0.044	1.000
6	Regulation of mRNA metabolic process	34	0.292	0.043	1.000
7	Animal organ morphogenesis	27	0.304	0.063	1.000
8	Regulation of hemopoiesis	20	0.333	0.081	1.000
9	Hemopoiesis	50	0.229	0.081	1.000
10	Peptidyl tyrosine modification	23	0.289	0.147	1.000
11	Rna processing	70	0.203	0.120	1.000
12	Carbohydrate metabolic process	21	0.295	0.164	1.000
13	Transmembrane receptor protein tyrosine kinase signaling pathway	34	0.243	0.170	1.000
14	Actomyosin structure organization	16	0.316	0.172	1.000
15	Regulation of cell development	39	0.231	0.170	1.000
16	RNA splicing	50	0.210	0.150	1.000
17	Negative regulation of nucleobase containing compound metabolic process	72	0.188	0.169	1.000
18	Inflammatory response	34	0.234	0.214	1.000
19	Response to lipid	27	0.243	0.229	1.000
20	Cell division	30	0.234	0.237	1.000
21	Small molecule metabolic process	40	0.212	0.229	1.000
22	Actin filament bundle organization	18	0.283	0.228	1.000
23	Regulation of response to wounding	18	0.271	0.287	1.000
24	Positive regulation of kinase activity	17	0.276	0.279	1.000
25	Regulation of multicellular organismal development	60	0.179	0.261	1.000
26	Positive regulation of transcription by rna polymerase ii	52	0.184	0.277	1.000
27	Positive regulation of establishment of protein localization	21	0.256	0.274	1.000
28	Developmental growth	24	0.244	0.273	1.000
29	Myeloid cell differentiation	19	0.528	0.000	0.026
30	Regulation of mRNA processing	23	0.422	0.004	0.252

Supplementary Table 6: Phospho-Proteome analysis: Top 30 pathways downregulated in RA based on proteins with a fold change from ≤ -0.5

	GOBP Pathway	Size	Enrichment Score	p-value	q-value
1	Regulation of actin filament based process	32	-0.403	0.000	0.389
2	Regulation of cellular component biogenesis	67	-0.308	0.002	0.280
3	Regulation of cytoskeleton organization	46	-0.340	0.002	0.204
4	Regulation of organelle organization	85	-0.281	0.004	0.216
5	Regulation of actin filament length	17	-0.475	0.008	0.193
6	Regulation of protein containing complex assembly	33	-0.342	0.006	0.410
7	Regulation of anatomical structure size	33	-0.337	0.004	0.388
8	Regulation of actin filament organization	27	-0.359	0.017	0.519
9	Regulation of cellular component size	26	-0.355	0.018	0.504
10	Cell cell adhesion	52	-0.281	0.009	0.463
11	Regulation of monoatomic ion transmembrane transport	18	-0.400	0.022	0.521
12	Regulation of monoatomic cation transmembrane transport	18	-0.400	0.022	0.479
13	Regulation of leukocyte proliferation	16	-0.407	0.033	0.581
14	Regulation of transmembrane transport	21	-0.360	0.021	0.559
15	Regulation of system process	19	-0.379	0.032	0.524
16	Actin filament polymerization	20	-0.359	0.036	0.536
17	Cell junction assembly	30	-0.307	0.043	0.602
18	Regulation of protein polymerization	23	-0.336	0.048	0.584
19	Alpha beta t cell activation	15	-0.403	0.044	0.567
20	Negative regulation of transport	18	-0.371	0.025	0.547
21	Regulation of supramolecular fiber organization	35	-0.289	0.040	0.535
22	Tissue homeostasis	16	-0.391	0.063	0.538
23	Negative regulation of organelle organization	26	-0.323	0.057	0.536
24	Leukocyte proliferation	23	-0.331	0.061	0.555
25	Regulation of cell junction assembly	18	-0.375	0.067	0.536
26	Cell adhesion	75	-0.226	0.031	0.519
27	Regulation of t cell activation	23	-0.322	0.047	0.535
28	Epithelial cell development	15	-0.386	0.063	0.542
29	Sexual reproduction	15	-0.381	0.073	0.535
30	Regulation of actin filament based process	32	-0.403	0.000	0.389