Molecular Regulation of Memory T Cell Differentiation and Function

Thesis

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MOLECULAR REGULATION OF MEMORY T CELL DIFFERENTIATION AND FUNCTIONS

For the Degree of
Doctor of Philosophy
International PhD Program in Immunology

HUMANITAS CLINICAL AND RESEARCH INSTITUTE
Milan, Italy
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Abstract

Memory T cells are largely heterogeneous. The molecular mechanisms at the basis of their effector functions are still under investigation. FACS-sorted memory T cell subsets exposed to TCR activation indicated that IRF8 is upregulated rapidly in all T cells, but its expression is rapidly lost, hence suggesting a role during the early stages of effector T cell programming. The aim of the first part of the work was to dissect the biological role of IRF8 in CD8+ T cells. I transferred Irf8-/- or Irf8+/+ CD8+ T cells in recipient WT mice, then infected those mice with LCMV. My results showed that Irf8 deficiency led to a faster transition towards memory phenotype after the acute phase. IRF8 silencing in human CD8+ T cells, followed by transcriptomic analysis, revealed a down regulation of effector related genes, accompanied by the upregulation of key memory TFs (FOXO1, MYB, TCF7). Accordingly, overexpressing IRF8 in primary human CD8+ T cells resulted in FOXO1 repression. ChIP sequencing identified an IRF8 binding on FOXO1 promoter region. Taken together, I conclude that IRF8 restrains FOXO1 expression by binding directly to the chromatin.

Adoptive T cell transfer immunotherapy benefits from early differentiated memory T cells capable to persist in the long term and to generate anti-tumour effectors. Cellular differentiation, metabolism and survival can be regulated by reactive oxygen species, but the mechanism is still poorly understood. I found that cellular antioxidant capacity is higher in human CD8+ T naïve (T_N) cells compared to more differentiated memory T cell subsets. I expanded human CD8+ T_N cells in vitro in the presence of ROS scavenger N-acetyl-cysteine (NAC) and found that
limiting global ROS metabolism during T\textsubscript{N} cell activation hindered effector differentiation and enabled the generation of stem-like memory T cell precursors. I thus define a novel mechanism to generate potent T cell memory based on limiting ROS metabolism for ACT immunotherapy.
Author’s contribution

E. Lugli and I conceived the study, analysed and interpreted the data of the first part of the thesis. K. Pilipow, E. Lugli and I conceived the study, analysed, interpreted the data and wrote the manuscripts of the second part of the thesis. Others members of the “Laboratory of Translational Immunology” supported the experiments. I generated RNA libraries for sequencing experiments. S. Puccio and E.M.C. Mazza performed bioinformatic analyses. P. Di Lucia, Mirela Kuka and M. Iannacone performed experiments with C57/BL6 mice. S. Gautam and L. Gattinoni performed tumour experiments with NSG mice.
List of abbreviations

ACT adoptive Cell Transfer
AICD Activation-Induced Cell Death
AICE AP1-IRF composite elements
Ag Antigen
ALL Acute Lymphoblastic Leukaemia
APC Antigen Presenting Cell
AP1 JUN/Activator Protein 1
BATF Basic leucine zipper Transcription Factor ATF-like
BET Bromodomain and Extra-Terminal motif
CAR Chimeric Antigen receptor
CART-19 anti-CD19 CAR T
CBP and CREB-binding protein
CCR CC chemokine receptor
CEA Carcinoembryonic Antigen
CFSE 5-(and 6)-Carboxyfluorescein diacetate Succinimidyl Ester
CMP Common Myeloid Progenitor
CPM Count Per Million
CPT1α Carnitine Palmitoyl Transferase 1α
CREs cyclic AMP response element
CTL Cytotoxic Lymphocyte
CTLA-4 Cytotoxic T-Lymphocyte Antigen-4
CTRL control
CXCR C-X-C Motif Chemokine Receptor
DAMP Damage Associated Molecular Pattern
DBD DNA Binding Domain
DC Dendritic Cell
DN Double Negative
DP double positive
DRP1 Dynamin Related Protein 1
EICE ETS-IRF Composite Element
EIRE ETS-IRF Response Element
ER Endoplasmic Reticulum
ETC Electron Transport Chain
FA Fatty acid
FACS Fluorescence-Activated Cell Sorting
FADH2 Flavin Adenine Dinucleotide
FAO Fatty Acid Oxidation
FOXO Forkhead Box O
GCL Glutamate-Cysteine Ligase
GCLC GCL catalytic subunit
GCLM GCL modifier subunit
GLUT1 Glucose Transporter 1
GMP granulocyte-monocyte progenitor
gp-100 glycoprotein-100
GS glutathione-synthase
GSEA Gene Set Enrichment Analysis
GSH Reduced Glutathione
GSK3β glycogen synthase kinase-3β
GSSG oxidised glutathione
GZMB Granzyme
HAT Histone Acetyltransferase
HIF1α Hypoxia Inducible Factor 1α
HSC Hematopoietic Stem Cell
IAD IRF-Associated Domain
ICAM-1 Intercellular Adhesion Molecule-1
IECS IRF-ETS Composite Sequence
IL-7Rα α-chain of IL-7 Receptor
IP Immunoprecipitation
IRF Interferon Regulatory Factor
ISRE IFN-stimulated response element
i.v. intravenous
Janus Kinase JAK
KLRG1 Killer cell Lectin-like Receptor-1
LAL Lysosomal Acid Lipase
LCMV Lymphocytic Choriomeningitis Mammarenavirus
Lck Lymphocyte Cell-Specific Protein-Tyrosine Kinase
LEF1 Lymphoid Enhancer-binding Factor 1
LFA Lymphocytes Function associate Antigen
mAbs monoclonal Antibodies
MAGEA-3 Melanoma-Associated Antigen 3
MAPK Mitogen-Activated Protein Kinase
MART-1 Melanoma Antigen Recognized by T-Cells 1
mBCI Monochlorobimane
MFN Mitofusin
MD Menadione sodium bisulfide
MDP Monocyte-DC Progenitor
MHC Major Histocompatibility Complex
MOI Multiplicity Of Infection
MnSOD Manganese Superoxide Dismutase
MPEC Memory Precursor Effector Cell
mTOR mammalian Target Of Rapamycin
NAC N-acetylcyesteine
NADH Nicotinamide Adenine Dinucleotide
NF-κB Nuclear Factor kappa B
NOX NAPDH Oxidase
NSG NOD/SCID/γ-chain knock out
NY-ESO-1 Cancer-testis antigen
OAP1 OSP-Associated Protein 1
OXPHOS Oxidative Phosphorylation
PBMC Peripheral Blood Mononuclear Cell
PD1 Programmed Death 1
p.i. post infection
PI3K Phosphoinositide 3-Kinase
PMA Phorbol 12-Myrisate 13-Acetate
p-mTOR Phosphorylated mTOR
RISP Rieske Iron Sulfur Protein
ROS Reactive Oxygen Species
RT Room Temperature
R10 RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine
Sh Short hairpin
SLEC Short Lived Effector Cell
SLO Secondary Lymphoid Organ
SP Single Positive
STAT Signal Transducer and Activator of Transcription
TAA Tumor Associated Antigen
TBS Tris Buffered Saline
TCA Tricarboxylic Acid
TCF7 T-Cell Factor 7

$T_{CM}$ T Central Memory

TCR T cell receptor

TEC Thymic Epithelial Cells

$T_{EM}$ T Effector Memory

TF Transcription Factor

TFBS Transcription Factor Binding Sites

TIL Tumour Infiltrating Lymphocyte

$T_H$ T Helper

$T_N$ T Naïve

TRE TPA response element

$T_{SCM}$ T Stem Cell Memory

TTE or TEMRA T Terminal Effector re-expressing CD45RA

VitC L-Ascorbic acid

WB Western Blot
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1 Introduction

1.1 Adaptive immunity: CD8+ T cell maturation, homeostasis and transcriptional regulation

1.1.1 Maturation of T cells

T lymphocytes are part of the adaptive immune system involved in cell-mediated immunity. They are characterised by the presence on the cell membrane of a specific receptor, the T cell receptor (TCR) (Germain, 2002) that recognises cognate antigens (Ag) presented by major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs) or target cells. T cells are classified in αβ T lymphocytes and γδ lymphocytes, where the TCR is composed by α and β or γ and δ chains, respectively (Robey and Fowlkes, 1994).

T cells mature from lymphoid precursors that in turn originate from hematopoietic stem cells (HSC). These precursors migrate from bone marrow to the thymus where they undergo a process of maturation and selection (Lai and Kondo, 2008). The thymus is divided into a central medulla and a peripheral cortex, the latter surrounded by an outer capsule. Here, thymocytes come into close contact with many cells, in particular, with thymic epithelial cells (TEC) that influence their maturation, proliferation and selection. In addition, stromal cells express MHC, that is required for negative and positive selection (Gameiro et al., 2010).

Four major subsets of thymocytes can be identified in the thymus according to the CD4 and CD8 coreceptor expression: double negative (DN), double positive (DP) and CD4 and CD8 single positive (SP). During the first steps of thymocyte differentiation, DN thymocytes recombine variable (V), diversity (D) and joining (J) segments of the TCR β gene.
locus (Spits, 2002), thereby resulting in the exposure of a pre-TCR complex constituted by β and invariant pre-α chains on cell surface. Subsequently, DP thymocytes express either CD4 and CD8 molecules and undergo rearrangement of α chain with the recombination of V and J segments (Spits, 2002). TCR rearrangement is followed by the so-called positive and negative selections. At this stage, many clonotypes with a unique TCR are generated due to the stochastic recombination of the V(D)J segments. In the thymus, cortex cells present self-antigen peptides mounted on MHC class I or II molecules (the p:MHC complex). Thymocytes that bind the p:MHC with sufficient affinity are positively selected, while those recognizing the p:MHC with low affinity undergo apoptosis. MHC class I favours the maturation of CD8+ T cells while MHC class II that of CD4+ T cells (Starr et al., 2003). Negative selection occurs on the border between the cortex and the medulla of the thymus, where thymocytes interact again with self p:MHC complexes on TECs: TCRs that bind p:MHCs too strongly cause the cell death of these self-reactive T cells (Starr et al., 2003, Moran and Hogquist, 2012).

Selected T cells are thus released in the periphery as mature naïve T (T\text{N}) cells, whose survival and “homeostatic” proliferation is governed by cytokines, mainly interleukin 7 (IL-7), in combination with self p:MHC complexes with low affinity for the TCR, which is insufficient to induce autoimmunity but essential for tonic stimulation. T\text{N} cells recirculate in secondary lymphoid organ (SLO; spleen and lymph nodes) via the blood and the lymphatic system where they continuously scan APCs in search of their cognate exogenous antigen (Schluns et al., 2000).
CD4+ and CD8+ cells differ substantially. CD4+ T lymphocytes mainly produce cytokines and provide “help” to other cell types, such as B cells for the production of class-switched antibodies, and are thus commonly referred to as T helper cells. In contrast, CD8+ T lymphocytes are mainly responsible for cell-mediated lysis of target cells and therefore are commonly referred to as cytotoxic lymphocytes (CTL) (Zhang and Bevan, 2011). Following TCR-p:MHCI interaction, CD8+ T cells and APCs or the target cell form the so-called immunological synapsis, constituted by reciprocal interactions between adhesion molecules, such as the intercellular adhesion molecule-1 (ICAM-1) expressed on the surface of APCs and lymphocytes function associate antigen 1 (LFA-1) expressed by CD8+ T cells (Bertoni et al., 2018). These interactions result in a cascade of intracellular signals leading to T cell activation, rapid cell proliferation (the so-called clonal expansion phase of the immune response) and the acquisition of effector functions, such as the production of anti-microbial cytokines and the release of intracellular granules containing cytotoxic molecules such as perforin, granulysin and granzymes (A, B, C, K), among others, both involved in target cell killing (Lieberman, 2003). When the pathogen or the tumour is cleared, the majority of effector T cells dies (contraction phase). However, a small proportion of antigen specific cells differentiates into a long-lived population of memory T cells (memory phase), which confers lifelong protection (Lanzavecchia and Sallusto, 2000). These cells ensure a faster and more robust reaction in response to secondary antigenic challenge, thus contributing to protection upon re-infection (Sallusto et al., 2004).
1.1.2 The human T cell memory compartment

The introduction of fluorescence activated cell sorting (FACS) and monoclonal antibody technologies allowed the analysis of markers, expressed at the single cell level, and ultimately led to define heterogeneity in the memory T cell compartment (Lugli et al., 2017, Lugli et al., 2013b, De Rosa et al., 2001). Analysis of surface and intracellular proteins by FACS, combined with gene expression profile by RNA sequencing or microarray technologies, revealed that CD8+ T cell subsets differ in phenotype, transcriptional profile and effector functions.

Sallusto and Lanzavecchia reported that the human memory CD8+ T lymphocytes can be roughly divided into two functionally distinct subsets, central memory (TCM) and effector memory T (TEM) cells, according to expression of the CC chemokine receptor-7 (CCR7), a receptor that mediates homing to the SLO (Sallusto et al., 2014). Moreover, the expression of the tyrosine phosphatase CD45 long isoform (CD45RA), involved in the T cell activation, discriminates four subsets that describe T cell maturation (LaSalle and Hafler, 1991, Sallusto et al., 1999): CCR7+CD45RA+ T_N, CCR7+CD45RA- T_CM, CCR7-CD45RA- T_EM cells and CCR7-CD45RA+ terminal effector cells re-expressing CD45RA (T_E or T_EMRA) (Tian et al., 2017) (Sallusto et al., 2004) (Fig. 1).

T_N and T_CM cells are also characterized by the expression of the tumour necrosis factor superfamily member receptor and costimulatory molecule CD27, the cell adhesion molecule L-selectin (CD62L), required for the homing into SLO, and cell adhesion molecules lymphocyte function-associated antigen 1 and 3, (LFA-1 or CD11a and LFA-3 or
CD58). Furthermore, T<sub>N</sub> cells express the α-chain of IL-7 Receptor (IL-7R<sub>α</sub> or CD127) (Lugli et al., 2017, Mahnke et al., 2013).

Differently from T<sub>N</sub>, homeostatic proliferation of memory T cells is self-antigen independent, but cytokine (mainly IL-15)-dependent and ensures maintenance of these cells in the long-term. In this regard, memory T cells are capable to self-renew while simultaneously giving rise to more-differentiated progeny. Indeed, T<sub>CM</sub> were considered stem cell-like cells similarly to stem cells of somatic tissues (Buchholz et al., 2016). Recently, a new, rare subset of antigen-experienced cells (nearly 3% of the total memory compartment) has been identified in humans and non-human primates, characterised by enhanced stem cell-like properties compared to T<sub>CM</sub>, and thus named stem cell memory (T<sub>SCM</sub>) cells (Gattinoni et al., 2011, Lugli et al., 2013b, Lugli et al., 2013a). T<sub>SCM</sub> cells show a naïve-like phenotype, being CCR7+, CD27+, CD45RA+, CD62L+, CD127+ but share multiple features with conventional memory cells, in particular the overexpression of the CD95 antigen and the β-chain of the interleukin (IL-2/IL-15 receptor complex (CD122) on the cell surface (Lugli et al., 2017), rapid production of effector cytokines and clonal expansion (Fig.1). Instead, T<sub>CM</sub> cells maintain the expression of naïve markers CD62L, CD127, CCR7 and CD27, but are CD45RA- while upregulating the CD45 short isoform, CD45RO. T<sub>N</sub>, T<sub>SCM</sub> and T<sub>CM</sub> produce mainly IL-2, while the CCR7- memory subset produces high levels of IFN<sub>γ</sub> and reduced levels of IL-2. The TNF production is high in all memory T cell subsets (Mahnke et al., 2013, Geginat et al., 2003, Sallusto et al., 1999). Despite T<sub>SCM</sub> have enhanced stem-like potential compared to T<sub>CM</sub> cells, both subsets show a gene expression profile associated with survival and with
long-term maintenance as suggested increased expression of lymphoid enhancer-binding factor 1 (LEF1) and T-Cell Factor 7 (TCF7) expression, increased level of IL-7R (favoring signaling mediated by IL-7) and lower levels of the pro-apoptotic gene BIM compared to more differentiated subsets such as TEM (Lugli et al., 2013a, Snow and Larsen, 2017). While TSCM and TCM cells home to secondary lymphoid tissues thanks to CCR7 expression, CCR7- TEM cells are mainly found in peripheral tissues thanks to the upregulation of receptors mediating their homing such as CC-chemokine receptor type 3 (CCR3), CCR5 and C-X-C Motif Chemokine Receptor-6 (CXCR6) (Nolz et al., 2011). Finally, CD45RA- CCR7- TTE express the senescence markers CD57 and killer cell lectin-like receptor-1 (KLRG-1), are characterized by replicative senescence but are still capable of producing copious amounts of proinflammatory cytokines IFN-γ and TNF (Ibegbu et al., 2005).

**Figure 1** Heterogeneity of the memory T cell compartment. The expression level of surface markers (CD45RA, CD45RO, CCR7, IL-7Rα, CD95 and CD122) identifies six major subsets of quiescent T cells. Transitional memory T (T<sub>T</sub>M) cells have been described in some instances, yet their identity remains poorly defined. Once activated, effector cells generally acquire a similar phenotypic identity. Proliferative potential differs among the different subsets and is highest in T<sub>SCM</sub> cells.
1.1.3 Models of T cell memory formation

After the resolution of the primary infection, effector cells undergo activation-induced cell death (AICD), a programmed cell death primed by Fas (CD95)/Fas-L interaction. Only a small subset of these activated cells forms long-lived memory T cells (Roberts et al., 2003). Models of viral infection in mice revealed that, at the peak of the clonal expansion (generally 7 days post infection), the vast majority of the effector CD8+ T cells displays a CD127- KLRG-1+ short lived effector cells (SLECs) phenotype that is committed to terminal differentiation following clearance of the infection. Instead, a small population displays a CD127+ KLRG-1- memory precursor effector cell (MPEC) phenotype, capable to survive and committed to give rise to long-lived memory T cells (Kaech et al., 2002).

The mechanisms regulating differentiation toward memory or effector cells have been in part elucidated during the last two decades. Currently, two models to explain the differentiation of memory T cells from activated T_N are accepted. According to the “linear model”, activated CD8+ T_N cells pass through an obligatory effector state before generating memory cells. This model is supported by the observation that MPECs upregulate multiple effector molecules such as granzyme B (GZMB) and display epigenetic modifications at effector loci that resemble those found in SLECs (Youngblood et al., 2017). Instead, the “developmental model” assumes that T_N cells can directly differentiate into memory cells (Restifo and Gattinoni, 2013). According to this model, the effector stage is the terminal phase of differentiation, after which the activated T cells undergo apoptosis. Evidence supporting this model emerged from stimulation of effector cells in vitro, according to which constitutive Ag stimulation of
melanoma gp100 TCR-transgenic T\textsubscript{N} cells led to terminal differentiated cells, thus preventing the development of anticancer memory cells and decreasing anti-tumour efficacy of adoptively transferred cells in melanoma-bearing mice (Gattinoni et al., 2005). Conversely, early differentiated memory CD8\textsuperscript{+} T cells were shown to generate both differentiated effector cells and a pool of cells maintaining their original phenotype, thus suggesting stem-like properties (Gattinoni et al., 2011). In vitro and in vivo, enhanced proliferation of CD8\textsuperscript{+} T cells following primary contact with the cognate Ag leads to short live effector cell differentiation and poor transition to the memory phase, while the opposite occurs for minimally expanded T cells (Buchholz et al., 2013) (Gerlach et al., 2013). In this context, the TCR signal strength is thought to play a major role, according to which overstimulation results in decreased memory formation (Restifo and Gattinoni, 2013).

In support of the “developmental model”, Restifo and colleagues also described an epigenetic mechanism that explains the differences in gene expression among T cell memory subsets. Taking advantage of chromatin immunoprecipitation followed by sequencing (ChIPseq) technology, the authors characterized H3K4me3 histone modifications, associated with transcriptional activation, and H3K27me3 associated with gene repression in T cell memory subsets compared to T\textsubscript{N} cells. In line with their gene expression profile, murine T\textsubscript{N} and T\textsubscript{SCM}, but not T\textsubscript{CM} and T\textsubscript{EM} cells, showed significant enrichment of H3K4me3 at the promoters of memory related genes (e.g. Tcf7, Lef1, Foxo1 and Klf2) and reduced chromatin accessibility at the loci of effector related genes (e.g. Prf1, Ifng, Gzmb, Tbx21 and Prdm1) (Crompton et al., 2016). These data can, at least in
part, explain why T_{SCM} cells have enhanced self-renewal capability compared to T_{CM} and T_{EM} upon TCR stimulation (Gattinoni et al., 2011). All together, these evidences suggest that T cell differentiation is hierarchically organized in steps according to the relationship:

$$T_N \rightarrow T_{SCM} \rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{TE}$$ (Lugli et al., 2013a).

### 1.1.4 Homeostasis of human T cells

In the absence of Ag stimulation, lymphocytes undergo slow turnover that tightly controls their abundance (Jameson, 2002, Jameson, 2005). The homeostasis of CD8+ T cells is mainly driven by $\gamma$-chain ($\gamma_c$) cytokines (IL-2, IL-7 and IL-15) and, in the case of T$_N$ cells, is also dependent on TCR-self p:MHC interactions (Surh and Sprent, 2008). T$_N$ cells show poor survival compared to memory cells when deprived of TCR-self p:MHC contact (Boymn et al., 2009, Boyman et al., 2007). In addition, the turnover of memory T cells is relatively higher compared to T$_N$ cells under steady-state conditions, because of their enhanced response to $\gamma_c$ cytokines.

It is known that T$_N$ cells depend on IL-7 to self-renew (Boymn et al., 2009). Non-hematopoietic stromal and epithelial cells of the bone marrow, thymus, secondary lymphoid tissues, liver and intestine are involved in producing IL-7 independently from external stimuli (Fry and Mackall, 2005). The IL-7 receptor (IL-7R) is composed of 2 subunits: the common $\gamma_c$ CD132 (shared with IL-2, IL-4, IL-9, IL-15 receptors), and the $\alpha$ chain (CD127), which confers cytokine specificity (Fry and Mackall, 2005). Binding of IL-7 to IL-7R induces the activation of Janus Kinase 1 (Jak1) and Janus Kinase 2 (Jak2), which are bound to CD127 and CD132...
intracellular domains, respectively (Fry and Mackall, 2005). This interaction leads to the recruitment of Signal Transducer and Activator of Transcription 5a/b (STAT5a/b), which in turn migrates to the nucleus and drives the upregulation of the anti-apoptotic molecule Bcl-2 (Fry and Mackall, 2005). All T cell subsets express CD127 except T_{TE} (Surh and Sprent, 2008, Sprent and Surh, 2011). In particular, T_{N} cells are highly dependent on IL-7/IL-7R signaling for their survival, as blocking IL-7 with a neutralizing monoclonal antibody or the adoptive transfer of CD8+ T cells into IL-7 deficient mice reduced T_{N} cell survival (Surh and Sprent, 2008).

Other cytokines play a nonredundant role in T cells homeostasis. For instance, IL-2 promotes the generation of effector cells, while IL-15 promotes memory T cell differentiation from T_{N} precursors. Importantly, the proliferative response of T_{N} and memory T cell subsets to these homeostatic cytokines is different (Geginat et al., 2003), indicating a different potential of these cells to persist in the long term. Notably, IL-2 and IL-15 cytokines are structurally and functionally related and share two of their receptor chains, the IL-2/15Rβ (CD122) and the common γc CD132. Indeed, IL-2 and IL-15 trigger common downstream signaling pathways (Tamzalit et al., 2014) that govern T cell maturation and proliferation (Ma et al., 2006). During peripheral memory T cell differentiation, CD122 and IL-2Rα (CD25) are gradually upregulated (Sallusto et al., 1999, Sallusto et al., 2014). Upon inflammatory signals, dendritic cells (DCs), monocytes and epithelial cells produce IL-15 that is mounted on the high affinity α-chain receptor (IL-15Rα), either present of the cell surface (the prevalent form found in vivo) or secreted. The IL-15/IL-15Rα heterodimer is a super agonist compared to IL-15 monomer,
and stimulates target cells such as CD8+ T cells via trans-presentation (Tamzalit et al., 2014). The IL-15Rβγ receptor of CD8+ T cells thus elicits activation of Src family molecules such as Lymphocyte Cell-Specific Protein-Tyrosine Kinase (Lck) and Proto-oncogene Tyrosine-protein Kinase Fyn, in turn driving Phosphoinositide 3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways (Ma et al., 2006).

1.1.5 IRF-BATF interaction mediates transcriptional regulation in immune cells

Interferon regulatory factors (IRFs) are a family of transcription factors involved in the regulation of immune cell development and activity. IRF family is composed by 9 members (IRF1-9), both in humans and mice, and is characterised by a conserved N-terminal DNA binding domain (DBD) and the C-terminal IRF-associated domain (IAD) that mediates the association with other proteins (Yanai et al., 2012). The DBD recognizes a sequence of four nucleotides 5’-GAAA-3’ contained into the IFN-stimulated response elements (ISREs, 5’-GAAANNGAAA-3’) (Yanai et al., 2012). Some IRF proteins can bind DNA in the absence of binding partners. However, factors like IRF4 and IRF8 require heterodimerization with other transcription factors to bind DNA efficiently (Li et al., 2012). The C-terminal IAD mediates the physical association with other proteins (Yanai et al., 2012). IRF partners were identified by the presence of known transcription factor binding sequences adjacent to the IRF-core binding motif. PU.1, a transcription factor of the ETS family, is one of the partners that cooperate with IRF4 and IRF8. Bioinformatic analyses identified the IRF binding motif associated with ETS consensus region (Li et al., 2012). The ETS-IRF composite element (EICE 5’-GGAANNGAAA-3’) (Shukla and Lu, 2014)
has been found at the promoter or enhancer regions of genes fundamental for B cell and myeloid cell development and functions. Alternatively, the IRF4/8-PU.1 complex recognises ETS-IRF response element (EIRE, 5’GGAAANNGAAA3’) and, at a lesser extent, the IRF-ETS composite sequence (IECS, 5’GAAANNGGAA3’) (Li et al., 2012). Moreover, IRF4 interacts with basic leucine zipper transcription factor ATF-like (BATF) 3 and the JUN/Activator Protein 1 (AP1) complex. ChIPseq experiments of IRF4 binding sites revealed the presence of AP1-IRF composite elements (AICE 5’-TGAC/GTCANNNNGAAA-3’) or IRF-AP1 sites (5’TTTCNNNNTCATGA-3’ or 5’GAAATGATCA-3’) in T helper (T_H) cells, B cells and DC (Glasmacher et al., 2012).

IRF proteins bind different loci according to microenvironmental changes and differentiation stage of the cells. As observed in macrophages, IRF8 constitutively binds the genomic region involved in lineage commitment in the absence of stimulation, while it regulates genes involved in effector functions after LPS stimulation (Mancino et al., 2015). Similar observations emerged by characterizing IRF4 binding region in B cells (Xu et al., 2015). Moreover, it has been suggested that IRF family members play a central role in promoting histone modifications and subsequent histone depletion, thereby facilitating transcription. In support of this, it has been shown that IRF factors directly interact with histone modifiers like histone acetyltransferases (HATs) p300 and CREB-binding protein (CBP) (Zhang et al., 2015).

IRF4 plays a central role in regulating gene expression, differentiation and function of multiple innate and adaptive immune populations. IRF4 was demonstrated to promote plasma cells
differentiation (Xu et al., 2015), as well as the development of CD4 T\textsubscript{H} subsets (Glasmacher et al., 2012). Moreover, IRF4 regulates CD8+ T cells metabolism in response to energy demand following activation. In particular, the activation of glycolysis is regulated by IRF4 expression levels that in turn depend on the TCR signal strength stimulation. Indeed, IRF4-deficient CD8+ T cells undergo initial activation, but cannot sustain long-term proliferation and effector functions, thereby suggesting a fundamental role of IRF4 in supporting full effector differentiation (Man et al., 2013).

IRF8 is homologous to IRF4 because it shows similar gene sequence and shares the molecular protein structure as well as DNA binding region (Singh et al., 2013). Thus, it was hypothesized that IRF4 and IRF8 play a redundant functions in the regulation of gene expression (Glasmacher et al., 2012, Li et al., 2012). However, it has been shown that these transcription factors can display an antithetic role by competition for the binding of the same loci in B cells, where IRF4 was shown to prime plasma cells differentiation while IRF8 to favour the development of germinal centre B cells (Xu et al., 2015). Instead, IRF8 directly interacts with IRF4, PU.1 and BATF to bind efficiently the DNA and promotes the differentiation of T\textsubscript{H}9 by inducing IL-9 and IL-21 expression in CD4+ T cells (Humblin et al., 2017).

Like other IRF elements, IRF8 is involved in the regulation of myeloid and lymphoid cell development and effector functions. Analysis of expression in hematopoietic cell populations revealed that IRF8 is absent in HSCs, while it is detected in common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs), showing high expression in
monocyte-DC progenitors (MDPs), plasmacytoid DC (pDC), CD8α+ classical DC (cDC) and CD103+ cDC. By contrast, IRF8 expression is low in CD8α- DCs and granulocytes (Yanez et al., 2017). Moreover, it is expressed by B cells and activated T cells. Among T cells, IRF8 is not expressed by quiescent T<sub>N</sub> cells, but its expression is rapidly induced upon TCR activation (Nelson et al., 1996), suggesting its role in T cell differentiation and/or their effector functions.

Mechanistically, IRF8 can act either as an activator or a repressor of gene transcription. Evidence suggests that IRF8 primarily regulates the epigenetic landscape of the enhancers rather than the transcription of the genes per se. In fact, wild type and IRF8-deficient myeloid cells (e.g. monocytes and DCs) show few differences in gene expression but significant modifications of epigenetic profiles. In particular, epigenetic profile of IRF8-deficient cells is more similar to that of myeloid progenitor cells than wild type, fully differentiated cells (Kurotaki et al., 2019).

As mentioned above, IRF4 and IRF8 require additional partners, such as BATF family members (BATF, BATF2 and BATF3), to efficiently bind DNA (Li et al., 2012). BATF proteins are highly conserved between humans and mice. BATF and BATF3 are expressed in hematopoietic cells, while BATF2 is also detected in non-hematopoietic tissues (Chang et al., 2018). BATF family members are characterized by the presence of basic leucine zipper domain that includes DNA binding domain and a leucine zipper motif. The leucine zipper motif is involved in the physical interaction with other transcription factors, such as JUN proteins. JUN and FOS proteins heterodimerize to form the AP1 complex involved in the transcriptional regulation of many genes in different tissues (Dorsey et al.,
These complexes recognize TPA response elements (TREs TGA(C/G)TCA) or cyclic AMP response elements (CREs TGACGTCA) (Tussiwand et al., 2012). It was demonstrated that BATF3 is required to CD8α+ cDC differentiation; however, it was shown that BATF compensates the absence of BATF3 by co-operating with IRF4 and IRF8 TFs (Tussiwand et al., 2012). Importantly, this interaction also regulates gene expression in T and B cells, suggesting a common mechanism of action among different populations of leukocytes. In fact, IRF4 binds AICE elements interacting with BATF. By contrast, BATF can bind AICE elements also in the absence of IRF4 (Tussiwand et al., 2012).

1.2 Immunotherapy

T cells play a key role in cancer progression and their regulation has been long known to regulate fundamental aspects of anti-tumour immune responses (Ostrooumov et al., 2018). In last decade, two immunotherapeutic approaches approved in the clinical practice have definitively shown that the immune system is directly involved in tumour regression, namely immune checkpoint blockade, capable of blocking inhibitory receptors expressed by T lymphocytes in the cancer milieu (Sharma and Allison, 2015), and adoptive T cell transfer (ACT), consisting in the reinfusion of T cells with tumour specificity following expansion in vitro to increased numbers.

Immunotherapy of cancer is in rapid development, and its importance is emphasized by the recent 2018 Nobel Prize in Physiology and Medicine recognized to James Allison and Tasuku Honjo for the discovery of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death 1
(PD1) receptors as inhibitory mechanisms of the anti-tumour immune response mediated by T cells.

1.2.1 Adoptive cell transfer

The first ACT-based immunotherapy was practised in the 1980s, employing lymphokine (IL-2)-activated killer (LAK) cells to treat tumours in mouse models and cancer patients (Rosenberg et al., 1985). Later, cytokine-induced killer (CIK), i.e., cells isolated from patient’s peripheral blood mononuclear cells (PBMCs) and expanded in vitro followed by reinfusion, were tested (Meng et al., 2017). Despite leading to some objective response, CIK and LAK-based therapies exhibited poor efficacy in vivo, possibly due to the low specificities of these cell products.

Currently, ACT immunotherapy in cancer comprises the isolation, activation and in vitro expansion of patients’ T lymphocytes including tumour-specific T cells. Tumour infiltrating lymphocytes (TILs)-based therapy was developed by Rosenberg et al. in 1988 for the treatment of melanoma patients (Rosenberg et al., 1988). T cells recovered from patient’s tumour tissues are expanded in vitro for two weeks by stimulation via the TCR and in the presence of high-dose IL-2. TILs cultures capable to react against tumour cells are then selected, further expanded and finally infused back into patients (Fig. 2). Initial studies showed that TIL ACT caused cancer regression in a substantial fraction of treated patients; however, these effects were transient, possibly due to the limited capability of T cells to persist in the host (Rosenberg et al., 1988). In fact, isolated TILs are terminally differentiated, survive poorly and have decreased effector functions because of chronic antigen stimulation.
required for their expansion in vitro. Moreover, TILs recognise tumour associated antigens (TAAs) that are expressed also on the surface of healthy cells, thus possibly causing autoimmune reactions (Ruella and Kalos, 2014). New approaches meant to overcome these limitations by increasing persistence and ameliorating the specific recognition and elimination of cancer cells. These strategies include the expansion of autologous T cells by Ag-pulsed APCs and the redirection of T cell specificities with Ag-specific TCRs or chimeric antigen receptors (CARs) (Wang et al., 2014). Expansion of autologous cells consists in the recovery of T cells from TILs or PBMCs exposed to APCs previously pulsed with tumour-derived peptides. In this way, only the tumour-specific T cells are capable to proliferate. This method can theoretically be employed in nearly all patients since cancer-specific T cells can be derived by stimulating autologous PBMCs. Nevertheless, as mentioned above, many of such TAAs can also be expressed by normal cells, thus resulting in autoimmunity (Kato et al., 2018, Yossef et al., 2018).

Identification of tumour-specific antigens and their receptors emerged as an important step to optimize ACT-based therapies. In this context, autologous T cells are genetically modified with TAA-specific TCRs or CARs in order to redirect the specificity of T cells to recognize and eliminate cancer cells (Fig. 2). TCR-transduced T cells specific for Ags including cancer-testis antigen NY-ESO-1, glycoprotein-100 (gp100), carcinoembryonic antigen (CEA), Melanoma Antigen Recognized by T-Cells 1 (MART-1) and Melanoma-Associated Antigen 3 (MAGEA3) have been tested in many clinical trials (Hinrichs and Rosenberg, 2014). The first clinical trial employing the autologous T cells genetically modified to
express the MART-1 TCR was reported by Rosenberg et al. in 2002 (Dudley et al., 2002). Two of 17 patients displayed an objective response with partial tumour regression and with no significant toxicity. Although at very low numbers, the transferred T cells could be detected for more than one year. A subsequent trial utilizing T cells that expressed a high affinity MART-1 or gp100-specific TCRs showed an objective response, but autoimmune side effects were observed such as the killing of epidermal melanocytes that also express MART-1 and gp100. Loss of melanocytes was associated with the dermal and epidermal infiltration of lymphocytes as well as the manifestation of vitiligo in patients on later follow-up (Johnson et al., 2009). Notably, transduction of high affinity-TCR was demonstrated to enhance the anti-tumour response. However, clinical data indicate that TCRs with intermediate affinity reduced the severity of side effects, emphasising the importance of finely tuning the strength of anti-tumour response (Corse et al., 2010).

The first ACT treatment for cancer approved by the FDA in 2017 and the EMA in 2018 are Tisagenlecleucel and axicabtagene ciloleucel approved for the treatment of patients with acute lymphoblastic leukaemia (ALL) and diffuse large-B cells lymphoma respectively (Hopfinger et al., 2019). Both Tisagenlecleucel and axicabtagene-ciloleucel consist in CAR redirected lymphocytes recognizing the B cell/tumour cell antigen CD19.

Genetically-modified T lymphocytes expressing CARs are effective alternative to TCR-transduced T cells. CARs are hybrid transmembrane receptors consisting of the extracellular domain, which is a light chain immunoglobulin bound to a spacer element, combined with an intracellular domain that drives T cell activation. CAR expression redirects T cell
specificity independently of MHC recognition. New generation of CARs have been developed and include different types of signalling domains (Ramos and Dotti, 2011).

The intracellular domain of first-generation CARs contained the CD3ζ chain while second generation CARs included signalling domains derived from other co-stimulatory receptors, such as those from CD28, CD137 (also known as 4-1BB) or CD134 (also known as OX40). Third generation CARs now display a combination of multiple signalling domains, such as CD3ζ-CD28-OX40 or CD3ζ-CD28-41BB (Zhang et al., 2017).

CAR and TCR-transduced T cell production is a complex method characterized by multiple steps that include the recovery of T cells from the peripheral blood of the patient, T cell engineering via CAR or TCR-expressing lentiviral or retroviral vectors and in vitro expansion prior to re-infusion (Ruella and Kalos, 2014). CARs have different advantages over TCRs. First, CARs are not influenced by MHC restriction, therefore, they

Figure 2. Major approaches of ACT A) Collection and expansion of autologous TILs from disaggregated tumour biopsies. Tumour-reactive TILs are selected for tumour reactivity before the reinfusion in patients; B) Isolation of circulating autologous T cells from blood. Lymphocytes are genetically manipulated to express a given TCR or CAR, followed by expansion in vitro and reinfused into the patient.
can be used in patients independently of their MHC haplotype. Second, anti-tumour activity is not affected by HLA downregulation, a major mechanism of tumour-escape following infusion of TCR-modified T cells (although downregulation of CD19 targeted by CARs has been described as a mechanism of therapy resistance – see later). Third, CARs could theoretically target any protein, assuming antibodies against that antigen can be produced (Ruella and Kalos, 2014).

First promising results from CAR-based therapy were shown in patients affected by B cell malignancies treated with anti-CD19 CAR T cells (CART-19) in 2003 (Onea and Jazirehi, 2016, Kochenderfer and Rosenberg, 2013, Kochenderfer et al., 2010, Davila et al., 2014). The authors showed that CART-19 cells possess a good expansion rate and persistence after infusion, enabling both tumour eradication and long-term surveillance (Davila et al., 2014, Kochenderfer et al., 2010, Onea and Jazirehi, 2016, Kochenderfer and Rosenberg, 2013).

However, many CAR-based therapies have limited applicability due to severe side effects, the most relevant being the cytokine release syndrome (Lamers et al., 2013). Indeed, the rapid elimination of large numbers of tumour cells induces the release of damage associated molecular patterns (DAMPs) that trigger high production of pro-inflammatory cytokines (IL-6, IL-10, GM-CSF, TNFα, IFNγ) (Sentman, 2013). Steroids were shown to mitigate this side effect but, at the same time, could limit the efficacy of CAR treatment (Morgan et al., 2013). Tocilizumab, an anti-IL-6 antibody approved for the treatment of rheumatoid arthritis, is currently employed to mitigate the inflammatory burden induced by CAR T cells. Moreover, CARs can recognize molecules
shared by normal and tumour cells. For this reason, patients treated with CD19-redirected CAR T cells are characterized by long-term B cell aplasia. Finally, CARs can cross-react with Ags that are present on the surface of normal cells, potentially resulting in off-target toxicity (Sentman, 2013).

The use of CAR T cells has been recently tested for the treatment of solid tumours, although major challenges currently limit broad applicability, including the limited CD8+ T cells infiltration at the tumour site and the immunosuppressive activity exerted by the tumour microenvironment on CAR T cells. Clinical trials assessed, or are currently assessing the efficacy of CAR T cells in different solid tumours including glioblastoma (NCT02209376), neuroblastoma (NCT02311621), mesothelioma (NCT02414269), breast (NCT02547961) and ovarian cancer (NCT02498912) (Yeku et al., 2017). I will not discuss this topic in detail because not of major importance for this thesis.

1.2.2 T cell differentiation status and ACT

Limited persistence of the transferred T cells is the major barrier to effective ACT in the clinic. As mentioned in the previous paragraphs, T cells are organized in subsets, with different characteristics at the level of phenotype, gene expression, functional capacity and migratory potential towards inflamed tissues. It was originally thought that differentiated T cells characterized by a potent cytotoxic capacity were the best candidate for ACT and tumour eradication, based on their in vitro capacity to kill tumour cells. However, subsequent preclinical and clinical data indicated that less differentiated T cells, such as T_{CM}, display enhanced T cell
persistence and stronger tumoricidal potential compared to $T_{EM}$ cells (Klebanoff et al., 2012, Hinrichs et al., 2009, Gattinoni et al., 2005, Klebanoff et al., 2005). Similarly, effector cells derived from $T_{CM}$, had better persistence compared to those obtained from $T_{EM}$ cells, as suggested by adoptive transfer into immunodeficient mice or non-human primates (Berger et al., 2008, Wang et al., 2011b). More recently, $T_{SCM}$ cells have demonstrated superior self-renewal, capability to derive more differentiated effector progeny compared to $T_{CM}$ and $T_{EM}$ and enhanced anti-tumour potential upon specificity redirection with CARs (Gattinoni et al., 2011, Lugli et al., 2013a, Gattinoni and Restifo, 2013). Therefore, $T_{SCM}$ are considered a promising candidate population to be used in ACT. However, their paucity in the peripheral blood and at the tumour site impose limitations on their clinical application. Many laboratories are currently working on developing new methods to obtain CD8+ T populations with stem-like characteristics from $T_N$ precursors (Gattinoni et al., 2011, Lugli et al., 2013b, Cieri et al., 2013, Sabatino et al., 2016, Alvarez-Fernandez et al., 2016).

### 1.2.3 Methods to generate stem-like memory T cells in vitro

Immunotherapy approaches employ TCR or CAR-modified T cells recovered from TILs or PBMCs from patients. Unfortunately, this method suffers from the variability of T cell composition among patients, due to age of donors (Lugli et al., 2007), history of Ag exposure (Appay et al., 2002) and previous therapeutic treatments (Mackall et al., 1997).

Intense investigations over the past two decades led to the better understanding of the mechanisms involved in the origin of short-lived
effectors and long-lived memory T cells, including the development of new approaches to interfere with the T cell differentiation process. Blocking terminal differentiation and promoting long-term memory is particularly relevant to increase the efficacy of ACT immunotherapy for cancer. Notably, several protocols are now available to generate large numbers of CD8+ T cells with a stem-like memory phenotype.

Cognate antigen recognition via the TCR induces a signalling cascade involving the activation of PI3K and Protein Kinase B (AKT) pathway, which in turn activates the mammalian target of rapamycin (mTOR), Nuclear Factor kappa B (NF-κB), JAK/STAT and Forkhead Box O (FOXOs) family of transcription factors (Kim and Suresh, 2013). It has been demonstrated that sustained Akt signalling promotes effector cell development. In this regard, Kim et al. tested the effect of Akt blockade to favour CD8+ T cell memory formation (Kim et al., 2012). Treatment of mice with the pan-Akt inhibitor A-443654 reduced mTOR activity during the expansion phase post infection (p.i.). At day 35 p.i., the numbers of splenic CD8+ T cells were higher in A-443654-treated mice compared to untreated mice (Kim et al., 2012). Similarly, stimulation of human CD8+ T cell in the presence of Akt inhibitors impaired effector differentiation, increased persistence following ACT in immunodeficient mice and ameliorated anti-tumour responses in a xenogeneic model of myeloma (van der Waart et al., 2014) and melanoma (Crompton et al., 2015).

In addition, maintenance of CD8+ stem-like memory T cells was show to be regulated by the Wnt/β-catenin pathway (Kim and Suresh, 2013, Restifo et al., 2012). Taking advantage of a mouse model of β-catenin loss of function, the Restifo group demonstrated that the survival
of memory CD8+ T cells depends on Wnt/β-catenin signalling. In detail, it was shown that the increased activation of Wnt signalling, achieved by glycogen synthase kinase-3β (GSK3β) inhibitor TWS119, induced the generation of stem-like memory CD8+ T cells from T_N precursors (Gattinoni et al., 2011, Restifo et al., 2012). The in vitro generated T_{SCM} cells displayed increased antitumor response compared to T_{CM} or T_{EM} cells. Since TWS119 was previously shown to limit T cells expansion and viability (Gattinoni et al., 2011, Gattinoni et al., 2009), supplementation with homeostatic cytokines IL-7 and IL-21 led to an increased numbers of T_{SCM} cells. In addition, CD19 CAR-T_{SCM} cells exhibited a potent anti-tumour response in a xenogeneic model of leukaemia (Sabatino et al., 2016).

As an alternative to the protocol proposed by Restifo’s group, Cieri et al. obtained cells with a T_{SCM}-like phenotype by stimulating T_N precursors with αCD3/28 in the presence of the memory-supporting cytokines IL-7 and IL-15. These cells expanded considerably more and had a superior persistence in the long-term when adoptively transferred in NSG mice compared to T_{CM} and T_{EM} cells expanded in the same conditions (Cieri et al., 2013). My group has additionally shown that diminished TCR signal strength, obtained by titrating the amount of αCD3 in culture, plays a pivotal role in favouring the differentiation of CD8+ T_N cells towards a T_{SCM}-like phenotype (Zanon et al., 2017).

As previously discussed in paragraph 1.1.3, epigenetic modifications accompany CD8+ T cell differentiation. Recently, Kagoya et al. performed a comprehensive screening of chemical compounds directed to specific epigenetic targets and showed that growing CD8+ T cells in the presence
of JQ1, an inhibitor of bromodomain and extra-terminal motif (BET) proteins, resulted in the generation of stem-like T cells. The authors additionally found that BET protein BRD4 positively regulates the expression of BATF, an important inducer of CD8+ T cell effector and terminal differentiation (Kagoya et al., 2016). Compared to untreated cells, the JQ1-treated cells expanded to higher numbers in vitro and in vivo, infiltrated tissues more efficiently and displayed enhanced anti-tumour immunity in two different xenogeneic models of leukaemia and melanoma (Kagoya et al., 2016), thereby highlighting the importance of interfering with genetic modifications to alter CD8+ T cell differentiation.

1.2.4 CD8+ T cell metabolism

In the last decade, several reports have highlighted the central role of metabolism in regulating the differentiation and function of immune cells. In the context of CD8+ T cells, different metabolic processes govern cellular activation, clonal expansion, contraction and development of long-lived memory T cells, as well as terminal differentiation and dysfunction.

Quiescent T cells, such as naïve and memory cells isolated ex vivo, preferentially engage fatty acid oxidation (FAO), a metabolic process occurring in mitochondria and involving the catabolism of fatty acids (FAs) that are used as a substrate of oxidative phosphorylation (OXPHOS) (Raud et al., 2018).

Upon activation, CD8+ T cells require increased energy to support proliferation, effector functions and survival. To fulfil the rapid energetic demand, activated effector CD8+ T cells switch their metabolism from OXPHOS towards glucose consumption. This phenomenon, that occurs
regardless of the aerobic conditions, is collectively known as Warburg effect (Vander Heiden et al., 2009). TCR stimulation, combined with CD28 co-stimulus and IL-2, leads to PI3K-dependent AKT phosphorylation and activation. Phospho-AKT in turn triggers the phosphorylation of a key metabolic regulator, the mammalian target of rapamycin, or mTOR, a subunit of mTORC1 and mTORC2 complexes, that are intracellular sensors of cellular stress and nutrient availability. It is widely accepted that mTOR plays a fundamental role in orchestrating cell proliferation, growth and metabolism. Phosphorylated mTOR (p-mTOR) regulates many cellular mechanisms, including glucose metabolism. In particular, p-mTOR upregulates the expression of glucose transporter 1 (GLUT1) on the cell surface and of glycolytic enzymes in the cytoplasm (Raud et al., 2018). Due to its effects on cellular metabolism, mTOR largely affects CD8+ T cell differentiation. Indeed, mTOR inhibition leads to increased generation of memory cells (Araki et al., 2010), together with increased FAO by these cells (Pollizzi et al., 2015). Inversely, sustained activation of mTOR causes a potent effector response (Kim et al., 2012). Among mTOR targets, c-MYC (Wang et al., 2011a) and hypoxia inducible factor 1α (HIF1α) (Doedens et al., 2013) emerge as key TF involved in metabolic reprogramming of activated T cells. c-MYC stimulates the expression of glycolysis related enzymes, while HIF1α regulates glucose uptake and metabolism.

Compared to Tn, memory T cells show higher expression of the carnitine palmitoyl transferase 1α (CPT1α) on the mitochondrial membrane, a key enzyme involved in the transportation of FAs from the cytoplasm to mitochondria (Raud et al., 2018). In particular, it has been
shown that CD8+ T cells do not enhance FA uptake from the extracellular milieu, rather they mobilize FAs stored in cytosolic lipid droplets thanks to the activity of lysosomal acid lipase (LAL) (O'Sullivan et al., 2014). Studies in mice infected with the intracellular pathogen Listeria monocytogenes genetically modified to express ovalbumin have shown that mitochondrial shape and content change during effector and memory CD8+ T cell differentiation. In this model, memory cells showed increased mitochondrial mass compared to effector T cells, and have elongated mitochondria, that are fused and are characterized by tight cristae, possibly reflecting efficient OXPHOS. Instead, effector T cells are characterized by fragmented mitochondria with loose cristae, (Buck et al., 2016). Likewise, TCR-transgenic CD8+ T N cells activated by the cognate peptide and differentiated in vitro to effector cells by IL-2 have fragmented mitochondria, while those differentiated to memory cells by IL-15 show fused mitochondria. These differences depend on fission factor Dynamin related protein 1 (Drp1), which was also demonstrated to influence the cellular metabolism. In fact, the upregulation of glycolysis in consequence of T cells activation is largely affected by the chemical inactivation of Drp1 with Mdivi-1, a chemical compound that blocks fission. Several proteins involved in the mitochondrial fusion, such as OSP-associated protein 1 (OAP1), Mitofusin-1 and 2 (MFN1 and MFN2) also regulate metabolism. In this regard, conditional deletion of Opa1 in T cells resulted in the alteration in cristae structure in cells cultured in vitro in the presence of IL-2 or IL-15, while OPA1 overexpression sustained FAO metabolism and increased the number of elongated mitochondria in effector T cells. Similar results were
obtained by treating effector cells with the fission inhibitor Mdivi-1 or by favouring fusion of mitochondria with M1 (Buck et al., 2016).

### 1.2.5 Reactive oxygen species (ROS) generation in CD8+ T cells

Mitochondrial OXPHOS is the main source of reactive oxygen species (ROS) in T cells (Zorov et al., 2014). TCR stimulation with the cognate antigen leads to a Ca\(^{2+}\) release from the endoplasmic reticulum (ER) to promoting tricarboxylic acid (TCA) cycle in the mitochondria (Trebak and Kinet, 2019). The nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) are TCA products required as substrates to generate ATP during the electron transport chain (ETC). ETC is composed by 5 complexes localized in the inner mitochondrial membrane. NADH transfers electrons to complex I while FADH\(_2\) to complex II; electrons are so transferred to complex III through ubiquinone and then to complex IV through cytochrome c. Simultaneously, protons are actively pumped in the intermembrane space, generating a proton gradient within the intermembrane space. This gradient is exploited by complex V of ETC, the ATP synthase, to synthesize ATP. The final acceptor of electrons during the aerobic respiration is O\(_2\), which generates H\(_2\)O as a product of ETC reaction (Ahmad and Kahwaji, 2019). Oxygen consumption results in the production of radicals such as oxygen superoxide (O\(_2^-\)) that is rapidly converted to H\(_2\)O\(_2\) by the manganese superoxide dismutase (MnSOD). ROS are also generated as consequence of NAPDH oxidase (NOX) activity, because electrons transferred from NADH to O\(_2\) generate O\(_2^-\) that is subsequently converted into H\(_2\)O\(_2\) (Lambeth, 2004). While intracellular enzymes such as
peroxiredoxins, glutathione peroxidase and catalase are responsible for its metabolism to H₂O, H₂O₂ is relatively more stable than O₂⁻, and is involved in several biochemical and signalling reactions, as explained below.

1.2.6 ROS signalling in CD8+ T cells

ROS do not represent waste products of biochemical reactions, but are actively involved in cellular signalling by oxidizing cysteine thiols in proteins (Zhang et al., 2016). Among ROS, H₂O₂ was shown to efficiently cross the cellular membranes thus reacting with cysteine residues (Gough and Cotter, 2011). In particular, H₂O₂ promotes the sulfenylation reaction consisting in the oxidation of thiolate group (R-S⁻) to sulfenic acid (R-SOH) (Poole et al., 2004). In turn, this post-translational modification was demonstrated to favour further modifications such as sulfinylation, disulfide bond formation, and glutathionylation (Poole et al., 2004), which regulate the activity of proteins including TF, kinases, phosphatases and histone deacetylases (Holmstrom and Finkel, 2014). For instance, ROS regulate the activity of many proteins involved in stem cell vitality, stemness and pluripotency such as OCT4, HIF1, NRF2, FOXOs and ATM, just to mention a few (Wang et al., 2013). ROS were shown to inactivate OCT4 by the oxidation of cysteines, thus promoting ESC maturation (Marsboom et al., 2016).

The proteins directly modified by ROS play a fundamental role as redox sensors and their activity has consequences in T cell activation and differentiation (Bigarella et al., 2014) (Yarosz and Chang, 2018). In this regard, it has been recently shown that CD4+ T cells lacking Rieske Iron
Sulfur Protein (RISP), a complex III subunit codified by the *Uqcrfs* gene have reduced ROS production associated to the inability to generate effector cells after stimulation; the deficient T cells failed to produce IL-2 and showed low level of activation markers as CD69 and CD25, suggesting that T cell activation depends, at least in part, on ROS (Sena et al., 2013)

ROS homeostasis is also essential to maintain cellular integrity. Indeed, low levels of ROS increase T cells activation, but excessive amounts of ROS determine DNA damage and consequently cell death (Yarosz and Chang, 2018). Reduced glutathione (GSH), a tripeptide formed of glutamine, cysteine and glycine, is the most abundant antioxidant molecule inside the cell capable of buffering ROS levels (Yarosz and Chang, 2018). GSH can be generated de novo by glutamate-cysteine ligase (GCL), composed by a catalytic subunit (GCLC), a modifier subunit (GCLM) and glutathione-synthase (GS), or by a recycling pathway in which the oxidised form of glutathione (GSSG) is converted into the reduced form by glutathione-disulphide reductase (Lu, 2013). Antioxidant mechanisms also involve enzymes belonging to the thioredoxin family (Ren et al., 2017). This family is not discussed in this thesis as poorly relevant for the work I performed.

Evidence suggests that the buffering of ROS inhibits senescence and sustains cellular stemness (Shaban et al., 2017, Jeong et al., 2018). For instance, exogenous GSH, preserves the multidirectional differentiation of adipose tissue-derived mesenchymal stem cells cultured in vitro (Liao et al., 2019).
In T cells, cellular activation is associated to increased levels of ROS and the upregulation of genes involved in glutathione synthesis. The loss of function of the enzyme GCL was demonstrated to affect T cell homeostasis and proliferation, without a significant effect on T cell activation, at least in mice (Rashida Gnanaprakasam et al., 2018). Moreover, it was reported that Gclc-deficiency impaired IL-17 production by T_h17 cells and sustained FOXP3+ expression in CD4+ regulatory T cells differentiated in vitro from T_N (Lian et al., 2018). Reduced production of ATP levels and glycolytic activity were linked to GSH downregulation in activated Gclc-deficient T cells. In line with this observation, the glycolic activity was at least in part rescued by treating Gclc-deficient T cell with N-acetyl cysteine (NAC) and exogenous GSH, thus demonstrating that GSH is required for metabolic switch upon cellular activation (Mak et al., 2017).
2 Aim of the project

Preclinical data and clinical trials demonstrated that the differentiation status of adoptively-transferred T cells at the time of infusion is a major correlate of anti-tumour T cell responses. This is ascribed to the presence of stem-like memory T cells with enhanced persistence and effector differentiation potential compared to terminally-differentiated T cells. The aim of this work was to identify the molecular regulators at the basis of human memory CD8+ T cell differentiation from naïve precursors.

In the first part, I tested whether modulation of specific TFs expression led to altered memory development in terms of effector functions and capacity to persist in the long-term. Recent data in the literature indicate that IRF8 is a part of the complex of TFs involving BATF, IRF4 and members of the AP-1 family. How these molecules, especially IRF8, regulate effector and memory T cell differentiation in humans is poorly known.

In the second part, I focused on CD8+ T cell ROS metabolism and its role in memory T cell differentiation, starting from the hypothesis that enhanced antioxidant metabolism favours stem-like memory T cell development. In this regard, I optimized a CAR-T cell expansion protocol to obtain highly proliferating cells with a stem-like memory phenotype and enhanced anti-tumour potential.

3 Materials and methods

3.1 Cell culture and treatment

PBMCs were isolated from buffy coats from healthy donors and were stored in liquid nitrogen according to standard procedures. CD8+ T cells were cultured in a complete RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine (R10). In all experiments, the concentrations of the chemical compounds were as follow (determined by preliminary titration experiments), unless otherwise specified: 20 mM N-acetylcysteine (NAC) (MilliporeSigma), 5 μM menadione sodium bisulfide (MD) (MilliporeSigma), 4 mM reduced glutathione (GSH) (MilliporeSigma), 100 μM L-Ascorbic acid (vitC) (MilliporeSigma), 25 μM Orlistat (Cayman). Compounds were added to the culture medium at the time of cell seeding.

Cell proliferation was determined by the analysis of the 2 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies) dilution. In all experiments, CFSE-stained cells cultured in the presence of IL-7 and IL-15, both at 1 ng/ml, served as non-proliferating control.

To detect S6 phosphorylation (pS6), cells were fixed with 4% formaldehyde for 15 minutes at room temperature, washed with 1% BSA in PBS without Ca²⁺ and Mg²⁺ (PBS-/-), incubated with 0,1% Triton X-100 for 30 minutes at room temperature, washed twice with 1% BSA in PBS-/-, and subsequently permeabilized for 10 minutes on ice with ice-cold methanol (50% in PBS-/-). Cells were washed twice with 1% BSA in PBS-/-.
/- and stained with monoclonal antibodies (mAbs) specific for CD3, CD8, and pS6 (Ser240/244).

### 3.2 Western Blot (WB)

Cells pellet was lysed with lysis buffer (150 mM NaCl, 1% NP-40 100%, 0.1 gr/100mL Deoxicolate-Na, 0.1% SDS, 50 mM Tris-HCl 5mM EDTA) in ice for 30-45 min and centrifuged in a minicentrifuge at 4°C for 20 min at 14000 rpm. The supernatant was transferred in a fresh tube kept in ice. Protein concentration was quantified with the Lowry method (Lowry et al., 1951). Generally, 30-50 µg of protein samples were mixed with the loading buffer (glycerol, water, SDS, blue of bromophenol, βmercaptoethanol), vortexed and boiled at 95°C for 5 minutes. SDS-PAGE electrophoresis was run at 100V.

Wet transfer of the proteins from the acrylamide gel into the nitrocellulose membrane was run at 4°C 100V for 1 hour. The membrane was saturated with a blocking solution [5% BSA in tris buffered saline (TBS) + 0.1% Tween (TBST)] for 1 hour at room temperature (RT). Subsequently, the membrane was incubated overnight at 4°C in a shaker with pre-titrated concentration of primary Abs in 10mL of 5% BSA in TBST. After incubation, primary Abs were washed 3 times with TBST for 10 minutes and incubated with secondary Abs (αrabbit; 1:5000) diluted in 5% milk in TBST for 1 hour at RT. After incubation, secondary Abs were washed 3 times with TBST for 10 minutes. The membrane was developed with two solutions of luminol enhancer and peroxide buffer solution (Millipore) for 5 min at RT, followed by the acquisition with Chemidoc (BIORAD). Band density was analysed with ImageJ.
### Table 1 Antibodies used for Western blot and immunoprecipitation experiments.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Ab</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<tr>
<td>human</td>
<td>BATF</td>
<td>DTC5</td>
</tr>
<tr>
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<td>αrabbit</td>
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### 3.3 Immunoprecipitation (IP)

The ideal lysis buffer for IP minimizes the protein denaturation while releasing an adequate amount of proteins from the sample.

A non-denaturing lysis buffer (for antigens that are detergent-soluble and are recognized in their native form by the antibody) was used: 20 mM Tris HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 (NP-40), 2 mM EDTA, protease inhibitor cocktail (Merck). Washing buffer was prepared as indicated: 10mM Tris; adjust to pH 7.4, 1 mM EDTA, 1 mM EGTA; pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2 mM sodium orthovanadate, protease inhibitor cocktail. Blocking solution was PBS/- and 0.5% BSA (w/v).

Cells were collected into pre-cooled 1.5 mL Eppendorf tubes on ice, washed with ice-cold PBS/- and subsequently lysed with ice-cold lysis buffer, left on ice for 30 minutes, followed by centrifugation at 4°C at 13,000 rpm for 20 minutes. The supernatant was then collected into a fresh Eppendorf tube kept in ice.

To bind Ab to beads, 50 µl of magnetic Dynabeads (Invitrogen) were washed with 1.5 ml blocking solution using a magnet and re-suspend in 125 µl of blocking solution containing 5 µg of Abs. Beads were incubated with Abs overnight on a rotating platform at 4°C; subsequently cell lysate was incubated with bead-antibody conjugate mixture at 4°C under rotary agitation overnight. At the end of incubation, beads were washed with
blocking solution using a magnetic stand. The supernatant (unbound fraction) was removed from the beads and stored at 4°C. The protein complex was removed from the beads by acidification using a buffer containing 0.1–0.2 M glycine, pH 2.0–3.0 (weakening the interaction between the antibody and the beads). The eluted sample was neutralized immediately with Tris, pH 8.0–8.5.

### 3.4 Flow cytometry and cell sorting

Samples were analysed or sorted using the following anti-human or anti-mouse fluorochrome-conjugated mAbs (Table 2):

<table>
<thead>
<tr>
<th>Specificity</th>
<th>mAb</th>
<th>Clone</th>
</tr>
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<td>D57.2.2E</td>
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</table>

**Table 2 Fluorochrome conjugated antibodies used for flow cytometry analysis and cell sorting**

Fluorochrome-conjugated mAbs were purchased from BD, BioLegend and eBiosciences. All antibodies were titrated on human PBMCs or mouse splenocytes and used at the concentration giving the best signal-to-noise ratio, as described (Lugli et al., 2017).
In all experiments, cells were stained for 15 minutes at RT with a live/dead fixable dead cell stain kit (MIX 1, Zombie, Life Technologies) to exclude dead cells, which may bind mAbs non-specifically and thus result in false positive signals. Cells were then stained for 20 minutes at RT with a combination of surface mAbs (MIX 3, Table 1), as described (Lugli et al., 2017). Chemokine receptors were revealed by incubating cells at 37°C for 20 minutes to favour their recycling through the plasma membrane, as demonstrated in our laboratory (data not shown) (MIX 2). The Cytofix/Cytoperm kit (BD Biosciences) allowed to detect intracellular Ki-67 and cytokines. Intracellular TFs were detected following fixation of cells with the FoxP3/transcription factor staining buffer set (eBioscience). Fatty acid uptake was measured by incubating cells in a culture medium containing 16 nM Bodipy FL C16 (Invitrogen) at 37°C for 30 minutes. Glucose uptake was measured by incubating cells in R10 containing 150 μM 2-NBDG (Cayman) at 37°C for 5 minutes. GSH was measured by incubating cells with 10 μM mBCI (Invitrogen) at 37°C for 10 minutes. Total cellular ROS were detected by staining cells in R10 containing 1 μM CellROX (Invitrogen) at 37°C for 30 minutes. In the case of peripheral blood samples collected from mice, red blood cells were lysed with ACK solution (NH₄Cl, KHCO₃, EDTA) for 5 minutes at RT, washed twice with PBS-/-, and stained with mAbs (Table 1) with the same procedures mentioned above. All samples were fixed in 1% formalin for 20 minutes at RT before acquisition.
Samples were acquired on a Fortessa flow cytometer or on A5 Symphony or separated via a flow cytometry Aria III cell sorter (all from BD Biosciences). Single-stained controls, prepared with antibody-capture beads (BD), were used for compensation. T cell subsets were defined as shown in Fig. 3: first gating on singlets (FSC-H versus FSC-A), live CD3+ T cells (CD3 versus AQUA) and lymphocytes (SSC versus FSC). The gating strategy included unstimulated and stimulated lymphocytes; for CD8+ T cells, T_N were defined as CD45RO- CCR7+ CD27+ CD95-; T_SCM: CD45RO- CD45RA+ CCR7+ CD27+ CD95+; T_CM are CD45RO+ CCR7+; T_EM are CD45RO+ CCR7--; T_TE are identified as CD45RO- CCR7-.

Figure 3 Gating strategy used to identify T cells at different stages of maturation by polychromatic flow cytometry. CD8+ T cells are identified by gating on CD3+ and Aqua Live/Dead- cells. Gating on CD8+ T cells, T_N are CD45RO- CCR7+ CD27+ CD95-; T_SCM are CD45RO- CCR7+ CD27+ CD95+; T_CM are CD45RO+ CCR7+; T_EM are CD45RO+ CCR7--; T_TE are identified as CD45RO- CCR7-. (Fig. 3).
3.5 Magnetic isolation of CD8+ T cells

Human PBMCs, cryopreserved in FBS + 10% DMSO, were thawed in culture medium warmed at 37°C. After centrifugation, cells were re-suspended in HBSS-/- (final volume: 50ml) and counted in a 0.004% Trypan blue solution with a Burker counting chamber. 100*10^6 cells in 1 ml of separation buffer were filtered with a 40 μm cell strainer in Falcon ‘U bottom’ 15 ml tubes and negatively separated with the Mojosort human CD8+ naïve T cell kit (Biolegend). Briefly, cells were incubated on ice for 15 minutes with a cocktail of Biotin-conjugated Abs (αCD4, αCD16, αCD19, αCD20, αCD36, αCD45RO, αCD56, αCD57, αCD235ab, αCD244, αTCR γ/δ) followed by the addition of Streptavidin-coated Nanobeads and incubated on ice for an additional 15 minutes. The supernatant containing the CD8+ T_N suspension was recovered using a magnetic stand. The purity of the separation was subsequently analyzed by flow cytometry with a panel of mAbs directed to recognize the T cell differentiation antigens CCR7, CD45RA, CD45RO, CD27 and CD95. A product of at least ~95% CD8+ T_N cells was considered pure and used for experiments.

3.6 T cell stimulation in vitro

Purified CD8+ T_N cells (0.25x10^6 cells/mL, unless otherwise indicated) were cultured in R10 and stimulated with αCD3/28 antibody-coated beads (Dynabeads, Invitrogen) with a 2:1 bead:cell ratio in 96 U-bottomed plate. Human cytokines (IL-7, IL-15, IL-2, IL-12; Peprotech) were pre-titrated, then used at 10 ng/mL each, unless otherwise indicated. Cells
were cultured for 7 days. Medium was replaced every 2-3 days, on average.

### 3.6.1 PMA/Ionomycin stimulation

To induce cytokine production, cells were plated in U-bottom 96-well plate and stimulated with 10 ng/mL phorbol 12-myrisate 13-acetate (PMA; Sigma Aldrich) and 1 µg/mL Ionomycin (Sigma Aldrich) or left unstimulated, at 37°C, in presence of 1 µg/mL Golgi Plug (BD Bioscences), for 3h. Subsequently, cells were harvested and analysed by FACS. Background cytokine production of the unstimulated condition was subtracted from stimulated cells.

### 3.6.2 CD3/CD28 stimulation

Flat-bottomed 96-well plates were coated with 1µg/ml αCD3 mAb (eBiolegend, clone OKT3) in PBS/- of 100 µl/well and incubated at 37°C for 3h. Excess of antibody was removed after the incubation period by washing with PBS/-.. Immediately after, human CD8+ T cells resuspended in R10 containing the desired stimuli and soluble αCD28 mAb (eBiolegend) at 1µg/ml were plated and incubated at 37°C overnight.

### 3.7 Lentiviral transduction

The following lentiviral vectors were used to perturb IRF8 expression: backbone psi-LVRU6MP bearing a short hairpin (sh) specific for IRF8 transcript, together with scramble control, both expressing the reporter mCherry (GeneCopoeia), or backbone PIXTRC312 bearing an ORF encoding IRF8, together with an empty control, both expressing GFP (Sigma-Aldrich).
Lentiviral particles were produced as described (Tiscornia et al., 2006), stored at -80°C and thawed at RT shortly before transduction to avoid virus inactivation. Lentiviral particles were used at the multiplicity of infection (MOI=5), previously determined following transduction of Jurkat cells.

CD8+ T cells were activated with αCD3/CD28 beads in the presence of IL-7/15 concurrently, cells were transduced by incubation of viral particles (resuspended in 50 μl) for 24h at 37°C. A second transduction was repeated the next day. Transduction efficiency was monitored by the analysis of the reporter protein expression by FACS (mCherry or GFP, depending on the construct).

3.8 Real-time PCR (qPCR)

Total RNA was isolated from cells with RNeasy Micro Kit (Qiagen) and retro-transcribed using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed with hydrolysis probes: IRF8 (Hs00175238_m1), 18S (Hs99999901_s1) and B2M (Hs00187842_m1) as reference genes (Applied Biosystems). I used Universal PCR Master Mix, No AmpErase® UNG (Roche) in MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Expression levels were normalized (ΔCt) with B2M or 18S endogenous reference control (ref) and the mRNA expression was calculated using 2⁻⁰·⁵⁽ΔCt sample - ΔCt ref⁾ formula.
3.9 Mice

C57BL/6J were purchased from Charles River. LCMV-P14 CD45.1 TCR transgenic mice were obtained through the Swiss Immunological Mouse Repository (SwImMR, Zurich, Switzerland).

Irf8 wild type (Irf8+/+) CD45.1, and deficient (Irf8−/−) CD54.2 mice were purchased from Jackson Laboratories. To obtain Irf8+/+ P14 CD45.1 mice and Irf8+/+ CD45.1 C57BL/6J mice were crossed with P14 CD45.1 mice and to obtain Irf8−/− P14 CD45.1/2 mice Irf8−/− CD45.2 mice were crossed with P14 CD45.1 mice.

NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratories), bred in SPF conditions, were used for adoptive transfer experiments.

3.9.1 CD8+ T cell isolation, adoptive transfer, infection and tumor models

CD8+ T_N cells from spleens of LCMV-P14 WT CD45.1 (P14 Ctrl) and LCMV-P14 Irf8−/− CD45.1/2 (P14 Irf8−/−) mice were isolated by cell sorting and subsequently expanded in vitro. Cells were activated for 1h at 37° C with GP33-41 peptide in presence of 20ng/mL IL-2. At day 7-8 cells were harvested and their phenotype was analyzed by FACS.

For adoptive transfer, 1 x 10⁴ P14 Ctrl and 1 x 10⁴ P14 IRF8−/− T cells were co-injected intravenously into C57BL/6J recipients one day prior to intravenous (i.v.) infection with 2x10⁵ focus-forming units (ffu) of LCMV-WE virus. At day 7, 21, and 65 p.i., mice were retro-orbitally bled and sacrificed to perform flow cytometry analysis.

In re-challenge experiment, CD8+ T cells from spleens of C57BL/6J
host injected with a mix of P14 Ctrl and P14 Irf8-/- cells 65 days before, were isolated by cell sorting based on the expression of CD45.1 and CD45.1/2 congenic markers, respectively. Memory CD8+ T cells were subsequently transferred into secondary C57BL/6J naïve host, infected a day after as described above.

All infectious procedure was performed in designated Biosafety Level 2 (BSL-2) and BSL-3 workspaces in accordance with institutional guidelines.

In experiments with NSG mice, 1 × 10^8 NAC-treated or control CD8+ T cells were co-transferred by retro-orbital injection with 5-6 × 10^6 autologous PBMCs, previously depleted of CD8+ T cells. Mice were sacrificed 15 days after transfer.

Spleens and lymph nodes were dissected to obtain single-cell suspensions according to standard protocols. Single-cell suspensions of spleens were obtained after smashing through a 40 μm cell strainer. For tumor experiments and ACT, female NSG mice were intravenously injected with 2.5 × 10^5 NALM6-GL cells, followed 7 days later by 2.5 × 10^5 CD19-CAR+CD8+ T cells expanded in vitro in the presence or absence of 20 mM NAC. Recombinant human IL-15 (obtained from the National Cancer Institute) was injected intraperitoneally every other day (1 μg per mouse). Tumor burden was measured using the Xenogen IVIS Lumina.

### 3.9.2 Generation of murine effector CD8+ T cells and adoptive transfer.

Splenocytes from 9-week-old male Cor93 TCR-transgenic mice (bearing a transgenic TCR specific for the HBV virus epitope Cor93) were incubated with 10 μg/ml Cor93-100 peptide at 37°C for 1 hour, washed,
and cultured in complete RPMI 1640 (10% FBS, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 10 mM HEPES, 100 mM nonessential amino acids, and 1% penicillin/streptomycin). In this case, NAC was used at 10 mM to avoid inhibition of proliferation. Two days later, cells were cultured in fresh medium supplemented with 20 ng/ml of recombinant IL-2.

5 × 10^6 CD8+ T cells were injected i.v. into wild-type recipient animals (C57BL/6). At day 5 or day 35 after transfer, mice were sacrificed and spleens and lymph nodes were dissected to obtain single-cell suspension according to standard protocols. Cells were then prepared for flow cytometry as described above.

3.10 RNA library preparation

Procedure for low INPUT (0.5-5 ng of total RNA) with RIN ≥7.5 was used. A mixture of 1 μl of 10 μM Oligo-dT30VN and 1 μl of 10 mM dNTP was added to 2 μL of RNA and incubated at 72°C for 3 minutes. Retrotranscription and subsequently, a pre-amplification reaction were performed. Obtained cDNA was purified by adding Ampure XP beads (1:1 ratio) to each sample and placed on the compatible magnet stand. The supernatant was eliminated, and beads were washed with 80% EtOH. The attached material was eluted from the beads with H₂O. The size distribution was checked on Agilent high-sensitivity DNA chip. A good library should be free of short (<500bp) fragments and should show a peak at ~1.5-2kb (Picelli et al., 2013). Subsequently, a tagmentation reaction was performed by using the Illumina Nextera XT DNA sample preparation kit; the amplification of the fragments was done by using the Illumina INDEX primers. Obtained fragments were purified by AMPure XP beads in
a ratio of 0.6:1 and using magnet stand. The concentration of each library was measured by Glowmax according to manufacturer’s instructions. The size distribution was checked on Agilent high sensitivity DNA chip. A good library should show a broad peak at ~300-800bp.

**3.11 RNAseq**

Before sequencing each library was diluted to a concentration of 2 nM and pooled (final concentration 1.2 pM), followed by sequencing on Illumina Nextseq 500 using the NextSeq 500/550 High Output v2 kit (75 × 1 cycles, Illumina).

**3.12 ChIP-seq**

Jurkat cells were transduced with a lentiviral vector bearing a cDNA for IRF8 overexpression. After 10 days, 10 million of cells were fixed with methanol-free formaldehyde at a final concentration of 1%; incubated for 10 minutes followed by quenching with glycine at a final concentration of 0.125 M and washed with cold PBS. I incubated cells with lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40) for 10 minutes in ice. After lysis, intact nuclei were collected by centrifugation and incubated with Sonication Buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 0.1% SDS) in ice for 10 minutes. I sonicated the samples with BiorupotorPico for 50 cycles of 30 sec ON /30 sec OFF. Sonicated chromatin was immune precipitated with Dynabeads magnetic beads (Life Technologies) coated with 5 μg αV5 TAG antibody. Crosslink of this IP DNA was reversed by incubating at 65°C overnight in the presence of elution buffer (TE+SDS). DNA was purified with qiaquick kit (Qiagen).
3.13 Statistical analysis

Analyses were performed using GraphPad PRISM 7 software. Data distribution was analysed by 3 tests (D’Agostino & Pearson normality test, Shapiro-Wilk normality test, KS normality test). Parametric paired Student t-test, non-parametric paired Wilcoxon rank test and unpaired MannWhitney test were employed to compare two groups. For more than three groups, One-Way Analysis of Variance (ANOVA) was used. P values are two-sided and were considered significant when ≤ 0.05.

3.14 RNAseq analysis

Libraries were sequenced to generate 76-bp single-end reads using Illumina NextSeq-500 sequencing machines. After quality control, raw reads were aligned to the Human genome (GRCh38.p12) and mouse genome (GRCm38.p6) using the STAR aligner with default parameters (version 2.7.0). Gene-based read counts were then obtained using HTSeq count module (version 0.11) and GENCODE annotation. The read counts were imported into R statistical software (http://www.r-project.org/) and differential gene expression analysis was performed using the edgeR package (version 3.5.1). For pair-wise comparisons, raw read counts were normalized using the TMM method (trimmed mean of log-ratio values) and genes that failed to achieve a count per million (CPM) mapped reads value greater than 1 in at least two libraries were not considered. P-values were adjusted using the Benjamini-Hochberg method. Genes were considered differentially expressed when p-value<0.05. The heatmap representing the log2 of CPM was obtained using pheatmap R package.
(version 1.0.12) with the distance method "correlation" for both rows and columns.

### 3.15 Gene Signature Identification (RNAseq)

Gene Set Enrichment Analysis (GSEA) was performed using GSEA (version 3.0) software (Broad Institute, MIT) and gene list ranked based on log2 fold changes. The gene set enrichment analysis was conducted in pre-ranked mode with scoring scheme “classic” and 1,000 permutations. The maximum gene set size was fixed at 5,000 genes, and the minimum size fixed at 10 genes. The gene signature was retrieved from the Molecular Signatures Database (MSigDB v6.2).

### 3.16 Motif enrichment analysis

The PScan software tool (version 1.5) was used to perform the in silico computational analysis of over-represented transcription factor binding sites (TFBS) within the 5′-promoter regions of differentially expressed genes. PScan was ran on [-950, +50] bp upstream regions onto the Homo Sapiens JASPAR 2018_NR database.

### 3.17 Microarray bioinformatics analysis

Microarray probe fluorescence signals were converted to expression values using robust multiarray average procedure RMA of Bioconductor Affy package. All data analyses were performed in R version 3.4.4 using Bioconductor libraries and R statistical packages.

### 3.18 Bioinformatics analysis of ChIP-sequencing data

Reads were aligned to human genome (GRCh38.p12) using Bowtie2 (version 2.1.0) in local alignment mode. After alignment to the reference
genome, mitochondrial and ambiguously mapped reads were discarded
with Samtools, further used for sorting and indexing mapping files. Bigwig
files for IGV genome coverage visualization were generated with the
multiBamSummary module from the deepTools suite (version 3.2.0). To
call peaks, we used MACS2 (version 2.1.2) with these parameters:
callpeak gsize mm nomodel extsize 147 and Qvalue 1e-3. Peaks within 30
kb upstream and 10kb downstream of the TSS or within intragenic regions
were annotated with the closest TSS using ChIPSeeker (version 1.18.0)
and GENCODE gene annotation.
4 Results

4.1 Molecular regulation of memory T cell differentiation and function

4.1.1 IRF8 is preferentially upregulated by activated naïve-like CD8+ T cells

Characterization of T cells could be achieved in vitro by stimulating FACS-sorted cells with a combination of antibodies activating the TCR and co-stimulatory molecules. In particular, it was shown that CD3 (part of the TCR signalling complex) and the co-stimulatory receptor CD28 could be targeted to trigger the polyclonal activation of lymphocytes. Additional targeting of CD2 can be used to amplify TCR-mediated signalling. Since a prolonged stimulation of TCR favours T cell exhaustion and reduces cellular viability, only short-term stimulations included co-stimulation of CD2. Microarray analysis of ex vivo sorted CD8+ T_N, T_SCM, T_CTM and T_EM cells from 3 healthy individuals stimulated with αCD3/2/28 for 3 and 18 hours identified IRF8 as preferentially upregulated in T_SCM cells (Fig. 4A). I next performed a validation by qPCR, and I found that, overall, IRF8 is not detected in unstimulated cells, and its expression peaks at 11 hours post stimulation followed by subsequent downregulation (Fig. 4B). Moreover, I confirmed that IRF8 is preferentially expressed by T_SCM cells. Since T_SCM represents a rare subset belonging to CD8+ T cell compartment (about 3%) (Table 3), the quantification in T_SCM could be done only at 0h, 11h and 18h post stimulation in vitro (Fig. 4B).

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<tbody>
<tr>
<td>T_N</td>
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<td>1500060</td>
<td>2937145</td>
<td>1400574</td>
<td>1155566</td>
<td>1438873</td>
</tr>
<tr>
<td>T_SCM</td>
<td>307123</td>
<td>155350</td>
<td>108533</td>
<td>52032</td>
<td>67137</td>
<td>64597</td>
</tr>
</tbody>
</table>
Table 3 Absolute number of ex-vivo FACS-sorted memory T cell subpopulations collected.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>T_CM</th>
<th>T_EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_N</td>
<td>1324990</td>
<td>1097476</td>
</tr>
<tr>
<td>T_SCM</td>
<td>470165</td>
<td>1282745</td>
</tr>
<tr>
<td>T_CM</td>
<td>581487</td>
<td>1070883</td>
</tr>
<tr>
<td>T_EM</td>
<td>540362</td>
<td>533698</td>
</tr>
<tr>
<td>T_Naive-like</td>
<td>226482</td>
<td>137126</td>
</tr>
<tr>
<td>T_EM/TE</td>
<td>314475</td>
<td>187088</td>
</tr>
</tbody>
</table>

Since T_N cells acquire a T_SCM phenotype following a short stimulation with αCD3/2/28 and T_N cells were previously shown to be similar to T_SCM cells as far as transcriptomic analysis is concerned (Gattinoni et al., 2011), I combined subsets of T_N and T_SCM (referred to as T_Naive-like) via FACS sorting and determined IRF8 protein expression in these cells and compared it to that of terminally differentiated T_EM/TE cells.

As expected from transcriptional data, IRF8 protein could not be detected in ex vivo sorted CD8+ T cell subsets (Fig. 4C). Upon stimulation with
αCD3/2/28, T\textsubscript{Naive-like} cells expressed ~10 times more IRF8 protein than T\textsubscript{EM/TE} cells as reported by Western blot analysis (Fig. 4C-D).

I next designed a polychromatic flow cytometry panel to better characterize the phenotype of IRF8-expressing CD8+ T cells from healthy individuals following activation with αCD3/28. Unstimulated T cells were used as a control. FACS data analysis by t-SNE showed that IRF8 is co-expressed with other markers involved in the regulation of T cells differentiation and effector functions (Fig. 5A). IRF8 was present only in CD69+ activated cells expressing the naïve-associated markers CD127, CCR7, CD27, and CD45RA. Moreover, IRF8+ cells also expressed additional TFs involved in effector differentiation, namely IRF4 and T-bet, as well as inhibitory receptors such as LAG3, PD1 and TIM3, but not TIGIT. The frequency of IRF8+ cells in different CD8+ T cell subsets at different time points after activation is reported in Fig. 5B. These results confirmed that IRF8 is preferentially expressed by less differentiated cells, and its expression decreased after 48h of stimulation. Preliminary data not shown here revealed that the IRF8 locus is progressively methylated with peripheral CD8 T cell differentiation, thereby providing a molecular explanation of the loss of IRF8 expression.
I next investigated the signals that might regulate IRF8 expression in activated CD8+ T cells. To this aim, I adopted a strategy optimized in my laboratory to obtain large numbers of T_{SCM} or effector T (T_{Effector}) cells from T_N precursors by stimulating cells for 7 days with αCD3/28 and IL-7/IL-15 or IL-2/IL-12, respectively, (Zanon et al., 2017). I could not detect differences in terms of IRF8 expression between in vitro-generated T_{SCM} and T_{Effector} cells re-stimulated with αCD3/28, suggesting that these cytokines are not involved in IRF8 regulation (Fig. 6 A).
Rather, _IRF8_ expression was enhanced by the potency of the co-stimulation, _i.e._, by adding CD2 to CD28, while it was little affected by the additional presence of the inflammatory cytokines IL-2 and IL-12 (Fig. 6 B).

### 4.1.2 IRF8 promotes full effector functions while blocking memory differentiation in CD8+ T cells

In order to better understand the molecular mechanisms downstream IRF8 in primary CD8+ T cells, I downregulated and overexpressed _IRF8_ by means of lentiviral vectors encoding _IRF8_ short hairpin RNA (shIRF8) or _IRF8_ cDNA, respectively. To test the efficacy of IRF8 silencing, I transduced activated T_N cells with shIRF8 or scrambled control (CTRL) shRNA lentivirus and subsequently isolated transduced cells by FACS via the expression of a reporter gene. Western blot analysis of these cells re-stimulated with αCD3/28 antibodies overnight confirmed the shRNA-
mediated downregulation of IRF8 expression (Fig. 7A). I was successful in collecting RNA from 3 different individuals, from which I prepared RNA libraries to investigate the effect of IRF8 downregulation at the level of the whole transcriptome by RNAseq. I identified 760 differentially expressed genes (DEGs) between CTRL and IRF8 silenced cells, of which 495 were downregulated while 265 were upregulated (Fig. 7B).

Figure 7. IRF8 controls memory-related gene expression in activated T cells. CD8+ T_N cells were transduced with a lentivirus vector delivering scrambled control shRNA (CTRL) or IRF8-directed shRNA (shIRF8). A) WB analysis of IRF8 protein expression in cells left unstimulated (-) or re-stimulated (+) with αCD3/28 antibodies after IRF8 silencing. B) Volcano plot representation of differentially expressed genes (DEGs) between control (CTRL) and IRF8 downregulated (shIRF8) CD8+ T cells with p-values <0.05 (n=3). Genes of interest are labelled.

In CD8+ T cells, it has been shown that effector to memory transition requires a metabolic switch from glycolysis to fatty acid oxidation (FAO) (Buck et al., 2016, Raud et al., 2018). In this regard, I found that gene expression of HK2 and PFKFB3, both encoding for enzymes involved in glycolysis, was affected by IRF8 downregulation. In line with these data, MYC, a TF regulating the expression of glycolytic genes, was also downregulated after IRF8 silencing (Fig. 7B).
Furthermore, several critical genes related to effector functions and activation such as \textit{TNFRSF18}, \textit{TNFSF9} and \textit{IL-2RB} (data not shown) were downregulated in shRNA-treated cells, thereby suggesting that IRF8 is required for the optimal activation of effector program in CD8+ T cells. It is currently unclear why \textit{IRF4}, a TF involved in effector differentiation, was instead upregulated upon IRF8 downregulation. IRF4 has been shown to be repressed by IRF8 in B cells (Xu et al., 2015), therefore, it is possible that the same occurs in human CD8+ T cells. At the same time, I found that memory-related TFs were upregulated with \textit{IRF8} silencing, namely \textit{TCF7} (Zhou et al., 2010), \textit{MYB} (Gautam et al., 2019) and \textit{FOXO1} (Hess Michelini et al., 2013) (Fig. 7B).

Gene set enrichment analysis (GSEA) revealed a positive association of \textit{IRF8} silencing with the oxidative phosphorylation pathway, as well as with gene sets upregulated in precursors of memory cells, such as T\textsubscript{N} cells, KLRG1\textsuperscript{low} or KLRG1\textsuperscript{int} compared to terminally differentiated KLRG1\textsuperscript{high} CD8+ T cells. At the same time, signature genes of glycolysis and glucose metabolism together with genes associated with effector cells were positively enriched in the control condition. The figure 8 shows in red the pathways that are enriched in IRF8 silencing samples, while in blue the pathways enriched in the CTRL conditions, following the analysis of \textit{shIRF8} versus CTRL. Pathways are selected from those with known biological functions that are particularly relevant to T cells (Fig. 8).
To further understand the role of IRF8, I transduced CD8+ T<sub>N</sub> cells, treated as in Fig. 7, with a lentiviral construct overexpressing the *IRF8* cDNA (overIRF8) or an empty vector control (CTRL) (Fig. 9A).

**Figure 8.** *IRF8* downregulation is associated with gene signatures of oxidative phosphorylation and T<sub>N</sub> cells. Bar plot shows Normalized Enrichment Score (NES) generated by GSEA tools of manually selected gene sets statistically significant for FDR<0.20.
I was successful in collecting RNA from 4 different donors that I used to prepare RNA libraries for deep sequencing (one donor later excluded by bioinformatics analysis due to low quality of the reads). I identified 562 DEGs between CTRL and overIRF8 CD8+ T cells, 299 of which were downregulated while 263 were upregulated. Fig. 9B shows that FOXO1 and IRF4, previously found up-regulated following IRF8 silencing, were downregulated with overIRF8. Other memory-related genes, like IL-2 and IL-7R were also downregulated (Fig. 9B).

In line with previous data of IRF8 downregulation, GSEA revealed that pathways associated with effector functions like cell cycle, glycolysis and cytokines pathways are associated with IRF8 upregulation, while oxidative phosphorylation and naïve and memory genes were instead enriched in the CTRL condition (Fig. 10).
IRF8 cooperates with BATF to bind DNA in human CD8+ T cells

IRF8 has been previously shown to interact with a network of TFs including BATF and JUN, and that the IRF8-BATF-JUN complex regulates gene expression that is responsible for murine T helper cell differentiation (Humblin et al., 2017, Murphy et al., 2013). We therefore tested whether the same scenario occurs in human CD8+ T cells.

By optimizing chromatin immunoprecipitation followed by sequencing (ChIPseq) in Jurkat cells overexpressing a tagged version of IRF8, I...
determined the genes that are regulated by IRF8 at the level of the whole genome (Fig. 11).

**Figure 11. IRF8 binds FOXO1 promoter in Jurkat cells.** ChIPseq assay in Jurkat cells transduced with IRF8 overexpression vector. The DNA is immunoprecipitated with an anti-IRF8-specific Ab. Homer analysis result showing an enrichment of the IRF8 motif MA0652.1 at the promoter region of FOXO1 transcription start site. IP: immunoprecipitation

Bioinformatics analysis of integrated ChIPseq and RNAseq data identified those genes that were bound by IRF8 and that were differentially expressed (thus putatively regulated by IRF8 directly). Among these, FOXO1 harboured an IRF8 binding motif in the promoter region that corresponded to a ChIPseq peak, indicating direct binding to DNA (Fig. 11). Notably, FOXO1 represents the only gene affected in IRF8-silenced and IRF8-overexpressing cells. Moreover, FOXO1 promoter harbours a binding site of IRF8, as revealed by a ChIPseq experiment in Jurkat cells.

To further confirm ChIPseq data, I performed a ChIP-PCR experiment on primary human CD8+ T cells. T_N cells were activated in the presence of IL7/15 and αCD3/28 beads and subsequently transduced with an lentiviral vector overexpressing IRF8 fused to the influenza V5 peptide, or an empty vector as a control. Cell lysates were immunoprecipitated with αV5 antibody. Then, I performed qPCR with three pairs of primers
mapping to the putative FOXO1 promoter region upstream the TSS. Results show an enrichment in IRF8-overexpressing compared to control cells, thus demonstrating the direct bind of IRF8 on the FOXO1 regulatory region. Experiment was performed on two donors at the time of writing the thesis (Fig. 12).

![Graph showing ChIP-PCR results](image)

**Figure 12.** ChIP-PCR on human activated CD8 T cells. T cells were transduced with IRF8-overexpressing lentiviral vector (IRF8 over) or empty vector as control (CTRL). The immunoprecipitated DNA with an αV5 antibody was detected by quantitative PCR using three couple of primers for genomic regions of FOXO1 promoter. Values are calculated as relative percentage on total DNA content.

I next used ChIPseq data to identify the transcriptional partners of IRF8 in T cells, reasoning that the DNA binding site of these TF(s) should be juxtaposed with the IRF motif, as previously described in murine CD4+ T cells (Schraml et al., 2009) and DCs (Tussiwand et al., 2012). Motif enrichment analysis indeed identified a significant association between IRF8 and BATF motifs, suggesting that these TFs co-bind to DNA, at least in some regions.
Recent data have shown that BATF operates an important checkpoint required for effector differentiation (Kurachi et al., 2014). In the absence of BATF, CD8+ T cells undergo initial activation, but fail to differentiate and survive. As revealed by GSEA, I determined that the IRF8 DNA binding motif was preferentially found in gene sets related to metabolism and inflammation, while the IRF8-BATF combined motif at the level of gene sets related to effector and memory differentiation and to the FoxO signalling pathway, in line with previous data (Fig. 13).

**Figure 13. In silico analysis displays BATF binding motif associated to the IRF8 motif.** Summary of statistically significant KEGG gene sets with IRF8 motif or IRF8_BATF-combined motif in the promoter. Features are ordered and colored according to their adjusted p value (p.adjust)< 0.20. Circle size indicates the ratio between the genes enriched and the total number of those genes in the pathway.

Further experiments of IRF8 co-IP followed by BATF immunoblotting determined that BATF was indeed physically associated with IRF8 in
activated CD8+ T cells. I found similar results when I first performed BATF co-IP followed by IRF8 immunoblotting (Fig. 14). On the left side of fig. 14, IRF8-BATF interaction occurred poorly in unstimulated TSCM cells due to low expression of IRF8 in this condition.

![Image of IRF8 binding BATF in in vitro-differentiated TSCM cells]

**Figure 14. IRF8 binds BATF in in vitro-differentiated TSCM cells.** TSCM cells were generated from CD8+ Tn for 10 days, then stimulated for 24h with αCD3/2/28 Milteny beads or left unstimulated (ustim). IRF8 and BATF were subsequently immunoprecipitated (IP), then blotted for BATF or IRF8, respectively. Unb: unbound IP fraction.

### 4.1.4 IRF8 restrains memory formation in vivo

Murine studies have shown that *Irf8* knock out mice (*Irf8-/-*) have enhanced susceptibility to viral infections and defective effector differentiation of CD8+ T cells (Holtschke et al., 1996), probably due to the lack of immature CD8α conventional dendritic cells in these mice that favour the maintenance of peripheral CD8+ T cells (Grajales-Reyes et al., 2015). Otherwise, the CD8+ T cell-intrinsic molecular mechanism of defective effector differentiation in these mice remains unexplored. In this regard, I first analysed the frequency and the phenotype of CD3+, CD4+ and CD8+ T cells obtained from *Irf8* wild type (*Irf8+/+*), heterozygous (*Irf8+/−*) and knockout (*Irf8-/-*) mice (Fig. 15).
As shown in Fig. 1, the frequencies and total numbers of CD3+ T cells were not altered among the different genotypes. Moreover, frequencies of CD4+ nor CD8+ lymphocytes were not altered in Irf8+/−, while they were significantly deregulated in Irf8−/− mice compared to Irf8+/+ littermates. In addition, CD8+ but not CD4+ lymphocyte cell numbers were significantly lower in Irf8−/− mice compared to the Irf8+/+ and Irf8+/− mice.

I next intended to dissect the specific role of IRF8 in CD8+ T cells during a viral infection. To this end, I bred Irf8−/− mice with P14 mice, bearing a transgenic TCR that recognizes the P14 lymphocytic choriomeningitis mammarenavirus (LCMV) epitope, to obtain P14 Irf8−/− mice. Next, I FACS-sorted CD8+ T cells from the spleen of these P14 as well as from Irf8+/+ P14 based on the expression of congenic markers and expanded them in vitro following priming with the GP33-41 cognate peptide.

Figure 15. Decreased CD8+ lymphocytes in Irf8−/− mice. Analyses of splenocytes from 10 week-old Irf8+/+, Irf8+/− and Irf8−/− mice by flow cytometry. A) Percentage of CD3+, CD4+ and CD8+ T cells in the spleen. B) Total numbers of CD3+, CD4+ and CD8+ T cells of individual age-matched Irf8+/+, Irf8+/− and Irf8−/− mice (n=6 per group). Horizontal bars indicate the mean±SEM * p<0.05 ANOVA test and Dunn’s multiple comparisons test.
peptide in the presence of 20 ng/mL IL-2 for 7 days. Ex vivo, the
distribution of naïve and memory subsets of CD8+ T cells, as revealed by
the analysis of CD44 and CD62L by flow cytometry, was relatively similar
between Irf8+/+ and Irf8-/- CD8+ T cells. However, Irf8-/- CD8+ T cells had
a defective capacity to generate CD62L-CD44+ T_{EM} cells following
activation, suggesting that Irf8 plays a role in this regard (Fig. 16A-B).

I next evaluated the role of Irf8 in the antiviral response of CD8+ T
cells in vivo. To this end, I FACS-sorted CD8+ T_N splenocytes from Irf8+/+
(CD45.1) and Irf8-/- (CD45.1/2) P14 mice. Cells were mixed at 1:1 ratio
and co-transferred to naïve C57BL/6 (CD45.2) recipient mice. The day
after transfer, mice were challenged with LCMV Armstrong clone (Fig. 17).

Figure 16. Delayed effector-phenotype differentiation in Irf8-/- P14 CD8+
T cells. A) FACS analysis of CD62L and CD44 surface markers at day 0 and
7 days after in vitro differentiation with GP33-41 peptide stimulation in the
presence of IL-2. Cells were gated on CD8+ cells B) Cumulative frequency of
CD8+ T cell subpopulations at day 7 of in vitro-expansion from A (n=2 per
group). Horizontal bars indicate the mean±SEM.
Expansion and differentiation of CD8+ T cells was monitored over time (Fig. 17, left panel). In terms of memory differentiation phenotypes, I observed a higher frequency of CD62L+ CD44+ cells (and in turn a lower frequency of CD62L- CD44+ effectors) within transferred Irf8-/- P14 CD8+ T cells compared to P14 Irf8+/+ CD8+ T cells at 7 days p.i. This difference tended to increase at day 21 and day 65 p.i. (Fig. 18A-B).

Figure 17. Experimental design of murine infection model. Naive CD8+ T cells isolated from splenocytes of P14 Irf8-/- CD45.1/2 and P14 Irf8+/+ CD45.1 mice were adoptively co-transferred at a ratio 1:1 into wild type (WT) recipient mice (CD45.2). The day after, host mice were infected with LCMV (primary infection). At day 7, 21 and 65 post infection (p.i.) blood and spleens were collected and CD8+ T cells were analyzed by FACS. At day 65 p.i., total CD8+ T cells were isolated from recipients splenocytes and subsequently FACS-sorted for CD45.1 (Irf8+/+) and CD45.1/2 (Irf8/-) and adoptively co-transferred in a ratio 1:1 into naive WT recipient mice for secondary challenge with LCMV the day after transfer. At day 5 p.i., blood and spleens were collected and CD8+ T cells were analyzed by FACS (n=4 or 3 mice per group).
In line with this finding, I found lower frequencies of KLRG1+ cells within *Irf8*−/− P14 CD8+ T cells compared to P14 *Irf8*+/+ cells at the same time points p.i. (Fig. 18C-D), indicating that *Irf8* is required for full effector differentiation.

Figure 18. *Irf8*−/− P14 CD8+ T cells show a faster memory transition compared to *Irf8*+/+ P14 CD8+ T cells. A) FACS analysis of CD62L and CD44 expression at 7, 21 and 65 days post infection (p.i.). Cells are gated on CD8+ congenic marker-positive T cells B) Cumulative frequencies of CD62L+ cells form A. C) FACS analysis of KLRG1 and CD127 expression at 7, 21 and 65 days p.i. Cells are gated as in A D) Cumulative frequencies of KLRG1+ cells from C (n=4 or 3 mice per group). Horizontal bars indicate the mean±SEM. * p<0.05 Multiple t test.
To gain more insights into the molecular mechanisms at the basis of the role of *Irf8* in CD8+ T cells during the response to LCMV infection, I isolated CD8+ T cells from spleens of mice at day 7 p.i. and FACS-sorted congenically marked *Irf8*+/+ and P14 *Irf8*−/− P14 CD8+ T cells for transcriptomic analysis by RNAseq. The heatmap in Fig. 19 reports the log2 fold change in gene expression of P14 *Irf8*+/+ vs P14 *Irf8*−/− CD8+ T cells and some key genes are highlighted. In line with the analysis of memory and effector subsets by flow cytometry, genes encoding for TFs involved in effector differentiation and function, such as *Batf* and *Tbx21*, were downregulated in IRF8-deficient cells. Effector markers such as granzymes (*Gzma*, *Gzmb*, and *Gzmm*) and *Klrg1* were similarly downregulated. Likewise, I observed elevated levels of genes associated with the development of long-lived memory T cells, including the master
regulators of T cell persistence and memory maintenance Tcf7 (encoding TCF-1), Lef1 and Foxo1, Sell (encoding CD62L) and Il7r. This transcriptional signature was accompanied by the decreased expression of genes codifying for enzymes involved in glycolysis (Eno1, Gapdh, Aldo, Pgam1, Pkm, Ldha), in line with data from human CD8+ T cells where IRF8 was downregulated.

Memory CD8+ T cells metabolize lipid stored in vesicular droplets of cytoplasm by activating the lipase LAL. In this regard, I found the upregulation of its codifying mRNA Lipa and Cpt1a, the main rate-limiting transporter of FAs from cytoplasm into mitochondria, in Irf8/- compared with Irf8+/+ cells, further supporting the hypothesis that IRF8 restrain memory differentiation.

We next evaluated the role of Irf8 in the response of memory CD8+ T cells to secondary infection. To this aim, total Irf8+/+ and Irf8/- P14 CD8+ T cells were FACS-separated at day 65 p.i. (memory phase) based on the expression of congenic markers and subsequently co-transferred in 1:1 ratio into naïve recipients, followed by LCMV infection. At day 5 p.i. spleens and blood were collected, and the phenotype was evaluated by FACS (Fig. 17, right panel). Irf8/- expanded considerably more than Irf8+/+ CD8+ T cells in this condition (Fig. 20A). In line with previous data, the former maintained higher level of CD62L expression compared to the latter (Fig. 20B). Altogether, I conclude that Irf8 deficiency in the context of a viral infection in a mouse model leads to a faster transition versus memory development during the contraction phase, and in turn provides a proliferative advantage in response to a secondary infection.
4.2 Generation of proliferating CD8+ T cells with early differentiated phenotype suitable for ACT

4.2.1 CD8+ T cells lose their antioxidant capacity during the differentiation process.

As mentioned previously, the success of the ACT immunotherapy correlates with the stem-like capacity of the transferred T cells (Berger et al., 2008). Since the number of stem-like T cells in healthy donors are too low for translational applications, protocols to generate these cells from uncommitted precursors in vitro are warranted.

In order to identify the gene signatures characterizing different subsets of CD8+ T cells, we retrospectively analyzed gene set profiles of public microarray data (Gattinoni et al., 2011). I found the glutathione-derived metabolic process to be significantly enriched in TN and TSCM cells compared to more differentiated TEM cells (Fig. 21A). Some of the DEGs included in this gene set had a role in the detoxification of products of oxidative stress, such as peroxiredoxin 1 (PRDX1) and PRDX5, nuclear
factor erythroid 2 like 1 (NFE2L1), and different members of glutathione S-transferase family, including GSTA1, GSTM3, GSTM2, and GSTM5 (Fig. 21B). Next, I verified whether this differential gene expression profile was associated with a diverse content of glutathione (GSH) among human memory T cell subsets isolated ex vivo.

**Figure 21.** T\(_N\) and T\(_{SCM}\) CD8\(^+\) cells have higher antioxidant capacity compared to more differentiated subsets. A) Gene set enrichment analysis (GSEA) of glutathione-derived metabolic process signature in T\(_N\) versus T\(_{EM}\) and T\(_{SCM}\) versus T\(_{EM}\) CD8\(^+\) T cells. Net enrichment score (NES) values are shown. B) Relative gene expression level of manually selected transcripts involved in the antioxidant response in T\(_N\), T\(_{SCM}\), T\(_{CM}\), and T\(_{EM}\) CD8\(^+\) T cell subsets (n=3 donor/subset). C) Representative FACS analysis of GSH levels (mBCI staining) in CD8\(^+\) T cell subsets from PBMCs. D) Mean ± SEM of mean fluorescence intensity (MFI) data, obtained as in C (n=10). mBCI, monochlorobimane; FMO, fluorescence minus one control. **p < 0.01, ***p < 0.001 parametric 1-way ANOVA test with Bonferroni post-test.

I stained PBMCs from healthy individuals with different fluorescently conjugated mAbs directed to discriminate the major subsets of naïve and memory CD8\(^+\) T cells, together with the fluorescent probe
monochlorobimane (mBCI), which reacts with the reduced form of glutathione (GSH). I discovered that GSH is mostly abundant in T<sub>N</sub> cells ex vivo, while it is progressively diminished with peripheral CD8+ T cell differentiation. Among the different memory T cell subsets identified, CD8+ T<sub>TE</sub> had the lowest amount (Fig. 21C-D).

**4.2.2 Manipulation of ROS amount impacts on the differentiation process of CD8+ T cells.**

The observation that the metabolic switch is associated with different stages of cell differentiation suggests that metabolic manipulation could be useful to obtain expanded CD8+ T cells with an early differentiated phenotype suitable for ACT. I thus hypothesized that promoting antioxidant metabolism could as well promote a naïve-like state in activated T cells. ROS levels can be manipulated by supplementing the culture medium with antioxidants compound such as apocynin (Sun et al., 2015), reduced glutathione (Ozawa et al., 2006), and the N-acetyl cysteine (NAC), the last widely employed in the clinic as a mucolytic agent (Mokhtari et al., 2017). When added to cell culture in vitro, NAC acts as antioxidant with direct effect in scavenging radicals as OH\(^-\), NO\(_2\)\(^-\) and CO\(_3\)\(^-\), but also as a source of cysteine, a GSH precursor (Aldini et al., 2018).

I activated T<sub>N</sub> CD8+ T cells with αCD3/28 Ab-coated beads in the presence of IL-2 and IL-12 for 8 days to induce effector differentiation in the presence of different antioxidant compounds, including NAC, reduced GSH, vitamin C (vitC), and Apocynin (Apo). Cells grown in the absence of these molecules were used as a control (CTRL). After 8 days of culture I analyzed the phenotype by testing the expression levels of CCR7 and
CD45RO by FACS analysis (Fig. 22A), and the proliferative rate by CFSE dilution assay (Fig. 21B). In the CTRL condition, T cells differentiated to a *bona fide* CCR7- CD45RO+ effector phenotype, as previously defined in my laboratory (Zanon et al., 2017).

Instead, the supplementation of NAC, GSH, and Apo but not vitC to culture favored the generation of cells with an early differentiated, CCR7+ phenotype (Fig. 22A), while arresting effector differentiation. Among all antioxidants tested, only NAC used at 20 mM generated T cells with a

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**Figure 22.** Modulating ROS levels impacts effector and memory CD8+ T cell differentiation in vitro. (A) Representative FACS analysis of CCR7 and CD45RO expression in human CD8+ T<sub>N</sub> cells activated with αCD3/28 + IL-2 and IL-12 and cultured in the presence of N-acetylcysteine (NAC), reduced glutathione (GSH), vitamin C (vitC), or apocynin (Apo) for 8 days. Treatments were supplemented daily. Additional DMSO control for Apo is shown. (NAC n=8; GSH, vitC, Apo n=3). (B) Representative histogram of CFSE dilution of cells cultured as in A. NS, CFSE-stained, non-proliferating control cells; CTRL, cells grown in the absence of antioxidants (C) Representative histogram of CFSE dilution of NAC-treated and CTRL CD8+ T cells evaluated at day 4 when cultured with different concentrations of NAC. NS, CFSE-stained, non-
CCR7+CD45RO- phenotype compatible with a \( T_N \) or \( T_{SCM} \) differentiation stages (Fig. 22A). In this condition, the proliferation of activated CD8+ \( T_N \) cells was comparable to that of CTRL cells, while it was in part or completely blocked at a concentration of 40 mM and 80 mM, respectively (Fig. 22B-C). Giving the potential to induce proliferating T cells with a naïve-like phenotype, I employed the 20 mM NAC concentration for the rest of the study.

I next tested whether the different antioxidant compounds could scavenge ROS by evaluating the levels of total cellular ROS and GSH after \( T_N \) activation in vitro. I found increased cellular GSH levels associated to a reduction of ROS in T cells treated with NAC or exogenous GSH (Fig. 23A and C). Instead, treatment with Apo caused the reduction of ROS, but I did not observe increased levels of GSH in the treated cells (Fig. 23 B). Conversely, the stimulation with vitC increased total cellular ROS (Fig. 23 D). Since vitC accelerated the effector differentiation (Fig. 22A), these results also suggest that augmenting ROS amount may favor terminal differentiation.
Figure 23. Reactive oxygen species (ROS) and reduced glutathione (GSH) levels in CD8+ T<sub>N</sub> cells treated with antioxidants molecules A-D) Mean±SEM of MFI values of CellROX and mBCI in CD8+ T<sub>N</sub> cells activated with αCD3/CD28 Dynabeads and IL-2, IL-12 in the absence or presence of NAC (A; 18h), Apo (B; 18h), GSH (C; 2h), vitC (D; 48h) (n=3-7). Horizontal bars indicate the mean±SEM. * p<0.05, ** p<0.01 paired Student’s t-test in all graphs except of mBCI staining of NAC where non-parametric Wilcoxon test was used.

To test this hypothesis in cell culture, I employed menadione (MD), a well-known inducer of oxidative stress. Adding MD to activated CD8+ T<sub>N</sub> cells in vitro for 2h led to a significant increase in intracellular ROS levels (Fig. 24A). In line with our hypothesis, prolonged treatment of these cells with MD led to a higher frequency of T<sub>EM</sub> cells compared to CTRL cells (Fig. 24B).

A comprehensive immunophenotypic analysis further revealed that NAC-treated cells had a decreased but detectable surface expression of CD95 and increased expression of CD45RA, CD27, and CXCR3
compared with CTRL cells, a phenotype reminiscent of T\textsubscript{SCM} cells. Instead, MD-treated cells had a uniform CD45RA\textsuperscript{-} CD27\textsuperscript{-} CD95\textsuperscript{bright} CXCR3\textsuperscript{int} phenotype, indicating enhanced effector differentiation (Fig. 24C).

Figure 24. Increasing ROS levels exacerbates effector differentiation. A) Mean±SEM of MFI of CellROX and mBCI in CD8\textsuperscript{+} T\textsubscript{N} as in Fig. 22 in the absence or presence of menadione (MD) for 2h. B) Representative CCR7 and CD45RO expression, as detected by FACS, of T\textsubscript{N} cells activated as in Fig. 22A. Medium was replaced every 3 days. C) FACS analysis of CD45RA, CD27, CD95, and CXCR3 by cells cultured as in B. PBMCs from a healthy donor are depicted as a staining control.

As I reported in section 1.1.2, CD8\textsuperscript{+} T cells produce different cytokine profiles during the different stages of differentiation, in particular less differentiated cells release mainly IL-2 and TNF, conversely more differentiated cells produce mainly IFN\textgamma (Mahnke et al., 2013). To verify if NAC impacts also the production profile of these cytokines, I re-stimulated CD8\textsuperscript{+} T cell cultures with phorbol 12-myristate 13-acetate (PMA) and ionomycin, followed by FACS analysis of intracellular cytokine production.
In line with their early-differentiated phenotype (Fig. 25A), NAC-treated CD8+ T cells had lower levels of IFNγ production and higher levels of IL-2 production compared to CTRL condition (Fig. 25B). While exacerbating effector differentiation at the phenotypic level (Fig. 25A), MD-treated cells had impaired effector functional capacity, as revealed by decreased IFNγ and IL-2 secretion (Fig. 25B).

Taken together, these data indicate that limiting ROS production by antioxidant compounds during CD8+ T cells expansion limits effector differentiation while allowing them to proliferate. On the contrary, increased ROS levels accelerate terminal differentiation.

### 4.2.3 NAC promotes long-lived memory T cell gene expression in CD8+ T cells

I next employed transcriptomic analysis to elucidate the molecular mechanisms at the basis of NAC action. With this approach, I found 1,997
DEGs between NAC-treated and CTRL CD8+ T cells, of which 902 were downregulated and 1,095 were upregulated (data not shown).

Fig. 26A shows genes grouped according to their biological functions and whose expression was affected by NAC treatment: genes encoding TFs associated with effector differentiation, such as *IRF4*, *BATF*, *EOMES*, *ID2*, *PRDM1*, and components of the AP-1 complex (i.e., FOS and JUN family members) were downregulated by NAC (Fig. 26A). Of note, NAC repressed *TBX21*, encoding T-bet, a TF involved in *IFNG* gene induction. Thus, these results were coherent with the finding that NAC-treated cells decreased the production of IFN-γ (Fig. 25B).
Moreover, multiple genes encoding molecules involved in cellular effector functions, such as chemokines (CCL1, CCL3, CCL4, and CCL22), granzymes (GZMA, GZMB, GZMH, and GZMK), and cytokines (IFNB1 and IFNG), were also downregulated (Fig. 26C).

In general, the transcriptomic profile showed a global inhibition of activation and exhaustion as a consequence of NAC treatment, as I observed lower levels of IL-2RA, CD40 and ICOS as well as HAVCR2 (TIM-3), LAG3, and CTLA4 genes expression in this treatment condition (Fig. 26B). Along the same line, several transcripts of adhesion molecules and chemokine receptors, usually expressed by effector cells, were downregulated by NAC treatment: ITGA6, ITGA3, CCR1, CCR3, CCR5, ITGAE, CCR4, and CXCR6. Conversely, PECAM1 (encoding CD31), CXCR3, and CCR7, that are associated with early-differentiated memory cells, were up regulated (Fig. 26D). Similarly, key TFs associated to memory cell formation and maintenance, such as TCF7 (codifying TCF-1), LEF1 and ID3 had increased expression in the presence of NAC (Fig. 26A). FACS analysis confirmed the differential expression of TCF-1, TIM-3, T-bet, and GZMB also at the protein level in NAC-treated vs. CTRL cells (Fig. 27).
CD8+ T cells cultured in the presence of NAC undergo metabolic reprogramming

As reported by Araki et al., increased mTOR activity favours effector differentiation, while its inhibition with rapamycin favours long-lived memory cell formation (Araki et al., 2010).

Since GSEA unveiled that the mTOR signaling pathway was inhibited by NAC treatment compared with CTRL (Fig. 28A), I investigated the effect of this antioxidant on the mTOR signaling cascade. To this aim, I checked the phosphorylation of S6 protein on mTOR-dependent sites Ser240/244 and Ser235/236 by FACS and found that phosphorylation at
both these was inhibited by NAC compared to CTRL (Fig. 28B and data not shown).

**Figure 28. NAC treatment leads to a transient inhibition of mTOR pathway**

A) Gene set enrichment analysis (GSEA) of mTOR signaling pathway between CTRL and NAC-treated CD8+ T cells from data as in Fig. 25. B) CD8+ T cells were activated in the absence (CTRL) or presence of NAC as in Fig. 22 or subsequently left unstimulated or re-stimulated with αCD3/28 for 30 minutes in the absence of NAC. Bar graphs show the frequencies of Ser240/244 pS6+ T cells as detected by flow cytometry (n= 6 donors from 2 exp.). Horizontal bars indicate the mean±SEM. Statistical analyses were performed by parametric paired Student’s t test. **P < 0.01.

As mTOR signaling is nevertheless required for effector differentiation, it was thus crucial to subsequently assess whether NAC-mediated mTOR inhibition by NAC was transient, thereby not impacting CD8+ T cells activation following ACT. Therefore, I re-stimulated CD8+ T cells, generated after 8 days of NAC-treatment, with plate-bound αCD3 and soluble αCD28 Abs for 30 minutes in the absence of NAC, followed by FACS analysis of S6 protein phosphorylation, and found that removal of NAC restored S6 phosphorylation at comparable level with CTRL (Fig. 28B, anti-CD3/28). This experiment demonstrated that NAC-dependent mTOR inhibition is a reversible phenomenon.
As reported above, the mTOR pathway is involved in regulation of different metabolic processes including glycolysis (Salmond, 2018). Thus, it was reasonable to hypothesize that NAC-mediated mTOR inhibition also caused a block in glucose metabolism. GSEA revealed a decrease in glycolysis-related gene expression with NAC compared to CTRL treatment (Fig. 29A), specifically a downregulation of GAPDH, ENO2, PKM, and LDHA transcripts (Fig. 29B).

**Figure 29. Glycolytic pathway is inhibited by NAC treatment.** A) Gene set enrichment analysis (GSEA) of glycolysis signaling pathway between CTRL and NAC-treated CD8+ T from Fig. 25. B) Transcripts per million (TPM) of differentially expressed glycolytic genes are depicted (n=6). Horizontal bars indicate the mean±SEM. ****p < 0.0001 parametric paired Student’s t test.

As mentioned in the first chapter, one of the most important changes that occur during memory formation is the switch from glycolysis toward fatty acid oxidation (Buck et al., 2016). In line with these data, I found a increased expression of carnitine palmitoyltransferase1a (CPT1a) gene, a fatty acids (FAs) transporter from cytoplasm to mitochondria (Fig. 30A). In order to support their energy demand, memory CD8+ T cells use FAs from intracellular lipid droplets. The LAL enzyme plays a central role in mobilizing these FA stocks (O'Sullivan et al., 2014). Thus, I used 2-NBDG (a fluorescent glucose analog) and Bodipy FL C16 (fluorescent fatty acid synthetic precursor) measured by FACS analysis to respectively verify the
rate of glucose and FA uptake from extracellular milieu in cells cultured in the presence of NAC. In this regard, I found that both glucose and FA uptake were reduced in NAC-treated vs CTRL cells (Fig. 30B).

**Figure 30.** NAC-treatment causes a metabolic switch toward fatty acids utilization. A) CPT1A TPM values in CTRL and NAC-treated CD8+ T cells, activated for 8 days, obtained from Fig. 26 (n= 6). B) Cumulative data (n= 9 HD, 5 exp.) of 2-NBDG and Bodipy FL C16 uptake by CTRL and NAC-treated CD8+ T cells, activated for 8 days, as in Fig. 22A analyzed by FACS. Horizontal bars indicate the mean±SEM. *p < 0.05, ****p < 0.0001, parametric paired Student’s t test.

In line with the memory-like metabolism of NAC-generated CD8+ T cells, LIPA transcript (codifying LAL) was increased under this condition compared to CTRL (Fig. 31A). As it has been shown that memory CD8+ T cells rely on intracellular stores of FAs mobilized by LAL rather than from extracellular uptake, I verified whether orlistat, a lipase inhibitor, could arrest memory T cell differentiation induced by NAC. However, LAL inhibition had no effect in this regard, as evaluated by FACS analysis of memory-related markers (Fig. 31B).
Rather, the presence of orlistat reduced IFNγ and TNF production by NAC-induced memory CD8+ T cells (Fig. 31C), suggesting that intracellular FA metabolism supports effector functions rather than memory formation in this model of differentiation.

4.2.5 NAC promotes stem-like memory formation and potent antitumor immunity in vivo

Since I have demonstrated that T cells stimulated in the presence of NAC show similarities with the naturally-occurring T_{SCM} cells at the
phenotypic, transcriptional and functional level, I further characterized NAC-induced T cells *in vivo*.

In particular, I transferred NAC-treated cells obtained as in fig.26 in NOD/SCID/γ-chain knock out (NSG) mice to evaluate their engraftment, expansion and differentiation compared to CTRL cells. Mice were sacrificed 15 days after the adoptive cell transfer. NAC-treated cells and CTRL cells showed similar levels of CCR7, CD45RO, PD-1 and TIM-3 expression. However, lymphocytes pretreated with NAC showed an higher percentage of Ki-67+ cells, indicating an increased proliferative capacity. In addition, I observed a higher frequency of TCF1+ Ki67+ positive cells, thus suggesting a superior self-renewal potential in vivo (Fig.32).

![Figure 32. NAC promotes stem-like memory formation in vivo](image)

Bar graphs showing the ex vivo frequencies of Ki-67+ CD8+ T cells **A**; CTRL: n=10; NAC, n=9; n=3 exp.) and TCF-1+ cells within the Ki-67+ CD8+ T cell fraction **B**; CTRL and NAC: n=7; n=2 exp.) as obtained in spleens and lungs of mice treated as in A. Data refer to day 15 post transfer.
To consolidate our data in an immunocompetent mouse model, I isolated CD8+ T cells expressing a transgenic TCR specific for the hepatitis B viral epitope Cor93. Then, I stimulated the collected lymphocytes with the cognate antigen in the presence of IL-2 (to favor cellular expansion) in the presence or absence of NAC. Finally, stimulated CD8+ T cells were adoptively transferred in recipient mice. 35 days after transfer, NAC-treated CD8+ T cells showed a higher frequency of T_{SCM} compared to the control group in both the spleen and lymph nodes. These data suggest that the antioxidant activity of NAC, supplemented in the culture medium during the in vitro expansion, favors the generation of antigen specific T_{SCM} cells following adoptive transfer in vivo (Fig 33).

![Bar chart showing T_{SCM}, T_{CM}, and CD44^high CD62L^low T_{EM} frequencies in spleen and lymph nodes (LN) for CTRL and NAC groups.]

**Figure 33. NAC promotes stem-like memory formation in vivo**
Mean±SEM frequencies of T_{SCM}, T_{CM} and CD44^high CD62L^low T_{EM} within the Cor93-specific CD8+ T cells previously activated. Data refer to day 35 post transfer.

As mentioned in the paragraph 1.2.2, CD8+ T cells with a naive-like phenotype are advantageous for ACT therapy. To test whether NAC-
treated cells have enhanced anti-tumor potential in vivo, I generated CART-19 cells in the presence or absence of NAC, and subsequently transferred the cell products in NSG mice bearing established CD19+ acute lymphoblastic leukaemia. Figure 34 shows that CTRL cells provided only modest benefit compared to untreated mice, instead NAC-treated cells showed an improved capability to control tumor growth, associated with a prolonged survival. These results show that treatment with NAC improves the antitumor potential of CAR-T cells.

Figure 34 NAC promotes potent antitumor immunity in vivo A) Bioluminescence imaging of NALM-6 leukemic cells in NSG mice left untreated or adoptively-transferred with human CAR T cells generated as described in 22A. (n=5 mice/treatment group n=2 experiment). D) Kaplan-Meier survival curve of mice treated as in C. Statistical analyses were performed with Mantel-Cox analysis. ** p<0.01.
5 Discussion and conclusions

Elucidating the molecular mechanisms of effector and memory T cell differentiation is not only important in those conditions where T cells can mount robust anti-microbial (vaccines) or anti-tumour (ACT, immune checkpoint blockade) responses, but also in those pathological conditions where T cells play a major role in tissue destruction and chronic inflammation. In this thesis, I attempted to elucidate transcriptional and signalling mechanisms at the basis of effector vs. memory decision.

I discovered that IRF8 TF is mainly expressed by early differentiated memory CD8+ T cells. I demonstrated that IRF8 promotes effector differentiation by directly inhibiting FOXO1 expression. In particular, I found that IRF8 binds the promoter of FOXO1 gene in activated CD8+ T cells.

In the second part of my thesis, I reported that stimulation of activated naïve T cells with the antioxidant compound NAC promotes a naïve-like phenotype in differentiated T cell in vitro. Indeed, NAC-treated lymphocytes showed a superior self-renewal capacity (as demonstrated by the expression of Ki67 and TCF1) and consequently acquired more potent anti-tumour activity compared to CTRL cells when adoptively transferred in vivo.

IRF8 plays a critical role in the functional maturation of immune cells (Humblin et al., 2017, Shukla and Lu, 2014, Kurotaki et al., 2013, Kurotaki et al., 2019), but its role in CD8+ T cells was poorly understood. Herein, I demonstrated that IRF8 favours effector differentiation by repressing a key molecule involved in memory formation, FOXO1, and by facilitating glycolysis-related gene expression. IRF8 is preferentially expressed by T_N
and early differentiated memory CD8+ T cells in response to TCR-mediated activation and is rapidly lost afterwards. This finding together with the observation that memory CD8+ T cells lacking IRF8 expand considerably more in recall responses compared to WT cells, suggest that IRF8 function is particularly important during the early stages of T cell activation of T_N but not of memory T cells.

Several reports have shown that BATF interacts with JUN, IRF4 and IRF8 and that such macromolecular complex of transcription factors (TFs) regulates gene expression in effector CD4+ T cells (Chang et al., 2018, Li et al., 2012, Kurachi et al., 2014). Similarly, I have found that IRF8 interacts with BATF also in human CD8+ T cells.

BATF is required for CD4+ T\textsubscript{H}17 (Schraml et al., 2009), CD4+ T\textsubscript{FH} (Wu et al., 2018) and CD8+ T (Kurachi et al., 2014) cell differentiation. It has been shown that Batf\textsuperscript{-/-} mice are unable to contain a viral infection due to impaired clonal expansion and effector differentiation of CD8+ T cells (Kurachi et al., 2014). Similar observations emerged in Irf4\textsuperscript{-/-} CD8+ T cells. In particular, Irf4-deficient CD8+ T cells showed poor effector function potential accompanied to their incapability to clonally expand, both associated to metabolic catastrophe (Man et al., 2013). Notably, BATF and IRF4 share 80% of binding sites on the murine genome (Kurachi et al., 2014), thus explaining why mice lacking either BATF or IRF4 have similar phenotypes. Despite IRF8 and IRF4 are homologous proteins (Yanai et al., 2012) and both bind BATF in activated CD8+ T cells, IRF8 deficiency influences specific characteristic of CD8+ T cells that cannot be superimposed with those reported in Irf4\textsuperscript{-/-} or Batf\textsuperscript{-/-} CD8+ T cells. First, IRF8 deficiency slightly affected T cells survival and effector
functions compared to IRF4 or BATF. My hypothesis is that this is due to lower glycolytic capacity observed in \textit{Irf8}\textsuperscript{-/-} vs WT mice, as suggested by decreased expression of glycolytic enzymes in CD8+ T cells lacking, or downregulating, IRF8 in mice and humans, respectively. Second, IRF8 deficiency enhanced transition toward memory formation, likely explained by the direct inhibitory effect of IRF8 on \textit{FOXO1} transcription, as revealed by binding of IRF8 at the \textit{FOXO1} promoter, 800 bp upstream of its transcription start site. In line with this observation, \textit{Foxo1} is highly expressed by MPECs and promotes memory T cell generation and maintenance, while its poor expression promoted T\textsubscript{TE} cell differentiation (Utzschneider et al., 2018). Increased FOXO1 expression would thereby result in the increased expression of additional memory-related genes such as \textit{Il7r}, \textit{Sell} and \textit{Tcf7}, the last being a master regulator of memory T cell persistence. Collectively, our data indicate that IRF8 facilitates full effector differentiation by restraining the memory program. Experiments conducted on human memory CD8+ T cells confirmed that IRF8 is preferentially expressed by early differentiated cells. Overall, we hypothesize that IRF8 is required to adequately exploit the effector functions of memory T cells included T\textsubscript{SCM} cells.

However, \textit{Irf8} deficiency resulted in a mild phenotype in vivo, possibly due to the functional compensation by IRF4, which binds genomic regions similar to those bound by IRF8. This hypothesis is also corroborated by the finding that IRF8 deficiency results in increased IRF4 expression.
Profiling by transcriptomics technologies have recently shown the upregulation of \textit{IRF8} by exhausted CD8\(^+\) T cells in the tumour microenvironment (Mognol et al., 2017). Despite members of my lab failed to detect IRF8 in these cells from lung tumours ex vivo (data not shown), IRF8 could still play a role in favouring effector differentiation at the time of cognate antigen encounter or in response to immune checkpoint blockade. Testing this hypothesis will require further investigations.

In the second part of this work, I have demonstrated that antioxidants, added to cell culture at the time of T\(_N\) cell stimulation, uncouple differentiation from proliferation, thereby significantly sustaining the expansion of T\(_{SCM}\) cells with a naturally-occurring CD45RO\(^-\) CD45RA\(^{+/int}\)CC7\(^+\)CD95\(^{dim}\)CXCR3\(^{bright}\) phenotype. This is important...
because preclinical and clinical evidence reported that early differentiated cells show more potent anti-tumour activity compared with late differentiated cells, owing to their capacity to better persist in patients following ACT (Sabatino et al., 2016, Singh et al., 2016, Busch et al., 2016)

The enhanced self-renewal capacity of NAC-induced T\textsubscript{SCM} cells observed following transfer in NSG mice is ascribed to the presence of elevated levels of TCF-1, which favours long-term repopulation of stem-like CD8+ T cell precursors, also when persistent inflammation and antigen stimulation are present. Thus, the generation of TCF-1+ progenitors seems a prerequisite for successful ACT, as we observed in a preclinical model of CAR-redirected T cells.

It has been recently shown that mitochondrial ROS are needed to support clonal expansion and effector functions of CD4+ T cells (Sena et al., 2013, Yarosz and Chang, 2018). How is therefore possible that ROS scavenging, as shown in this work, promotes the superior effector functions of anti-tumour CD8+ T cells once transferred in vivo? T cell expansion in vitro to obtain enough amounts of cells for ACT inevitably results in terminal differentiation, loss of persistence potential and decreased effector functions following adoptive transfer. This is due to excessive stimulation via the TCR and inclusion of IL-2, a cytokine inducing terminal differentiation, in such expansion protocols. Antioxidants, first and foremost NAC, enabled expansion while transiently limiting differentiation in vitro. I have shown that NAC switched metabolism of T cells from glycolysis to FA oxidation, a profile generally associated to non-activated memory T cells. This is thought to be mediated by decreased
mTOR activity as long as NAC is present in culture. Once transferred in vivo, mTOR inhibition by NAC is unleashed, thereby favouring the full effector differentiation of CD8+ T cells which can persist more than CTRL cells. My data thus support a model where memory and effector maturation are affected by controlling ROS levels, and where excessive or very low levels result in apoptosis and incapability to derive effector T cells, respectively (Figure 31).

NAC has been reported to scavenge ROS directly (Aruoma et al., 1989), but I have also shown that NAC treatment increases the content of intracellular GSH, thereby leading to hypothesize that GSH is the main mediator of the antioxidant effect (Aldini et al., 2018). Previous reports have shown that several cellular proteins might be reduced following NAC treatment, and their activity can be either induced or inhibited. Although the direct targets of NAC remain to be identified, I have shown that NAC exerts a generalized inhibitory effect on several mechanisms regulated by ROS, including glycolysis, induction of TFs mediating terminal differentiation, and exhaustion.

In summary, NAC emerges as a novel, inexpensive tool to optimize current protocols of T cell expansion, preserving the long-lived memory T cells while allowing sufficient expansion. Clinical-grade NAC is available, thus favouring rapid translation. Future studies will have to compare the method I have described with existing methods to generate T_{SCM}-like cells in terms of anti-tumour activity in vivo.
Figure 36. Proposed model integrating the role of cellular ROS amount in the differentiation of CD8+ T cells. Low levels of ROS lead to quiescence/immunosuppression as CD8+ T cells are incapable to be activated. Extremely high amounts of cellular ROS lead to a rapid terminal differentiation and apoptosis due to the increased DNA damage. The use of antioxidants like NAC in cell culture at optimal concentrations (10-20 mM) inhibits effector differentiation while allowing proliferation and memory differentiation.
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7 Relevant papers


