UCLouvain

de Duve INSTITUTE

HELIOS is a marker of HLA-E-restricted CD8 T cells with an atypical cytokine production profile

Damien Neyens

September 2021

Thesis submitted for the degree of PhD in fulfillment of all the requirements in Biomedical and Pharmaceutical Sciences

Thesis supervisor:

Prof. Pierre van der Bruggen
de Duve Institute, Université catholique de Louvain

Jury members:

President: Prof. Laure Dumoutier
de Duve Institute, Université catholique de Louvain

Prof. Stefan Constantinescu
de Duve Institute, Université catholique de Louvain

Prof. Pierre Coulie
de Duve Institute, Université catholique de Louvain

Prof. Olivier Feron
Institut de recherche expérimentale et clinique, Université catholique de Louvain

Prof. Stanislas Goriely
Institute for Medical Immunology, Université libre de Bruxelles

Prof. Monika Wolkers
Sanquin Research, University of Amsterdam

Alors que cette thèse s'achève enfin, je souhaite remercier les personnes qui m'ont accompagné tout au long de celle-ci.

Je souhaite tout d'abord commencer par remercier mon promoteur, Pierre van der Bruggen, pour m'avoir accueilli dans son laboratoire ainsi que pour la confiance et l'autonomie qu'il m'a données durant ces 5 années.

Je tiens également à remercier les membres de mon comité d'accompagnement pour m'avoir suivi et conseillé tout au long de ma thèse. Je remercie également les Professeurs Wolkers et Goriely d'avoir accepté de faire partie de mon jury de thèse ainsi que pour leurs remarques pertinentes.

J'ai également eu la chance d'encadrer plusieurs étudiants durant ma thèse : Alessio, Francesca, Antoine, Achraqat et Assia. Vous encadrer m'a permis d'apprendre à adapter ma façon d'enseigner en fonction des qualités de chacun et chacune. Je vous souhaite le meilleur pour la suite.

Comment ne pas remercier mes collègues: AAA, ABD, AMB, CED, CVR, CWI, JVL, MAL, SVI et THH pour la bonne ambiance qui règne dans le laboratoire. Je tiens à remercier plus particulièrement THH, AAA, CED et CWI pour l'aide qu'ils m'ont apportée sur mon projet. Merci à CVR, alias le gland sans cœur, pour son aide avec les analyses bio-informatiques. Je tiens à remercier AMB pour l'aide qu'elle m'a apportée dans l'écriture avec son œil extérieur au projet ainsi que la relecture de mon anglais. Je souhaite également remercier d'anciens collègues que j'ai croisés durant mon mémoire ou ma thèse : AEP, MGA, QUD et REB. Plus particulièrement, je tiens à remercier REB qui m'a encadré et formé durant mon mémoire ainsi que QUD, alias la licorne, pour son aide précieuse au début de ce projet. Je tiens également à remercier les personnes extérieures au labo de PVDB qui m'ont apporté leur aide dont Nicolas et Davide pour les (nombreux) tris par cytométrie en flux ainsi que Gérald pour l'apprentissage de la construction de librairie scRNA-seq.

Je ne peux pas oublier de remercier les équipes informatiques, administratives et techniques qui nous permettent de travailler dans un environnement de qualité. Il m'est impossible de tous les citer mais parmi celles-ci, je tiens à remercier tout particulièrement Carine, Samira et Olivier.

Ce travail n'aurait pas pu être réalisé sans l'aide de financements du FNRS, WELBIO et la Fondation contre le Cancer. Il n'aurait pas non plus pu être réalisé sans la collaboration avec les équipes des Cliniques universitaires Saint-Luc pour l'accès aux échantillons humains.

Pour terminer, je souhaite remercier mes amis et ma famille, plus particulièrement mes parents et ma sœur (Porcinet/Gazoline) pour leur soutient durant cette thèse.

Abstract

The transcription factor HELIOS is best known for being expressed in CD4 regulatory T cells. In mice, HELIOS is also expressed by exhausted CD8 T cells. However, data on the nature of human HELIOS^{+} CD8 T cells are scarce and contradictory. Here, we characterized human HELIOS^{+} CD8 T cells, which represent about 21% of blood CD8 T cells. We found that the majority of HELIOS^{+} CD8 T cells are antigen-experienced and recognize peptides presented by MHC class Ib molecule HLA-E. In contrast to HELIOS^{-} T-BET^{high} CD8 T cells (Tc1 effectors), HELIOS^{+} Tc1 effectors produce low amounts of IFN-γ and TNF-α. HELIOS^{+} CD8 T cells express Th17/Tc17-related genes RORγt, RORα, PLZF, CD161, CCL20 and IL-17A. Thus, HELIOS is expressed by several CD8 T cell populations, including Tc1 effectors (that produce low amounts of cytokines) and Tc17. Exhausted (TOX^{+}) CD8 T cells isolated from ovarian carcinomas express HELIOS, suggesting the existence of either HLA-E-restricted anti-tumor CD8 T cells or two populations of exhausted T cells, namely TOX^{+} HELIOS^{-} and TOX^{+} HELIOS^{+}. It remains to be understood why so many individuals maintain high amounts of Tc1 effectors that secrete low amounts of cytokines upon activation in their blood and what the connection between HELIOS and HLA-E restriction means.

Table of contents

1. Introduction

1.1 Preamble.

Our defense against pathogens (bacteria, viruses, …) is ensured by the immune system, which is also able to recognize tumor cells but fails to eliminate them in cancer patients. Key players of this complex system are CD8 T cells, which are able to directly kill infected cells and tumor cells. In cancer patients, however, as well as in chronically infected patients, CD8 T cells fail to eradicate tumor cells or infected cells. Stimulating the immune system improves cancer patients' outcome (Hamid et al. 2013; Hodi et al. 2010; Wolchok et al. 2013). However, not all patients respond to immunotherapeutic strategies. It is important to keep studying these cells to increase our knowledge about how they function to potentially increase the efficacy of treatments.

In this introduction, I will first discuss T cell development in the thymus and how CD8 T cells get activated. I will briefly describe the different CD8 T cell subpopulations present in human blood. Then, I will describe a form of CD8 T cell dysfunction called T cell "exhaustion". Finally, I will focus on transcription factors and their role in T cell biology as well as T cell dysfunction.

1.2 T cell development in the thymus.

Lymphoid progenitors leave the bone marrow and migrate to the thymus where TCR rearrangement takes place. During maturation, T cell precursors undergo positive and negative selection (Kumar, Connors, and Farber 2018). Positive selection describes the survival of T cells with a functional $\alpha\beta$ TCR that weakly interact with self-HLA loaded with a self-antigen. T cells unable to interact with self-HLA loaded with a self-antigen die. Similarly, T cells undergo apoptosis if the interaction between $\alpha\beta$ TCR and self-HLA loaded with a self-antigen is too strong. This process is called negative selection and is a central

tolerance mechanism (Klein et al. 2014; Starr, Jameson, and Hogquist 2003). After positive and negative selection, the remaining T cells exit the thymus, migrate to the periphery and display naïve T cell markers: CCR7 and CD45RA (Kumar et al. 2018).

1.3 Regulation of T cell activation.

1.3.1 TCR signalling pathway.

1.3.1.1 Initial steps of TCR activation.

CD8 T cells recognize peptides presented by human leukocyte antigen (HLA) class I molecules. Some autoreactive T cells escape negative selection in the thymus. Under normal circumstances, such T cells do not provoke autoimmunity because of counteracting peripheral tolerance mechanisms, e.g. anergy or regulatory T cells (Bandyopadhyay, Soto-Nieves, and Macian 2007; Vignali, Collison, and Workman 2008). Under physiological conditions, most of human cells present peptides derived from self-proteins. But under pathological condition, e.g. an infection, peptides derived from non-self are presented. CD8 T cells recognize HLA class Ia-peptide complexes through the TCR. The TCR consists of alpha and beta chains that jointly recognize the antigen. However, neither TCR chain possesses intracellular signalling sequences. Instead, the TCR chains are non-covalently associated with six CD3 chains (two γ, ε, δ and two ζ) that contain one to three immunoreceptor tyrosine-based activation motifs (ITAMs) (Smith-Garvin, Koretzky, and Jordan 2009). Engagement of the TCR results in the activation of a cascade of signalling events that start with the exclusion of the phosphatase CD45 and the convergence of LCK towards immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3γ, CD3ε, CD3δ and CD3ζ. LCK phosphorylates the three ITAMs of

each CD3ζ, which recruits tyrosine kinase ZAP-70 to CD3ζ. Next, ZAP-70 is also phosphorylated and activated by LCK (Chan et al. 1992; Iwashima et al. 1994). Active ZAP-70 then phosphorylates adaptor protein SLP76 and four tyrosines on the scaffold protein LAT (Wardenburg et al. 1996; Zhang et al. 1998) (Figure 1). The Phosphorylated tyrosines of LAT serve as anchor points for SLP76, GRB2, ADAP, GADS, ITK, PLCγ1, NCK1 and VAV1. This complex of signalling molecules is called the LAT signalosome where the TCR signal is amplified and diversified. ITK, which is recruited to the LAT signalosome and activated there, phosphorylates PLCγ1, which cleaves PIP2 into DAG and IP3. The TCR signalling pathway separates into three main pathways downstream of ITK: Ca^{++}-NFAT, NF-κB and Ras-MAPKinase pathways (Figures 1 and 5) (Smith-Garvin, Koretzky, and Jordan 2009; Brownlie and Zamoyska 2013).

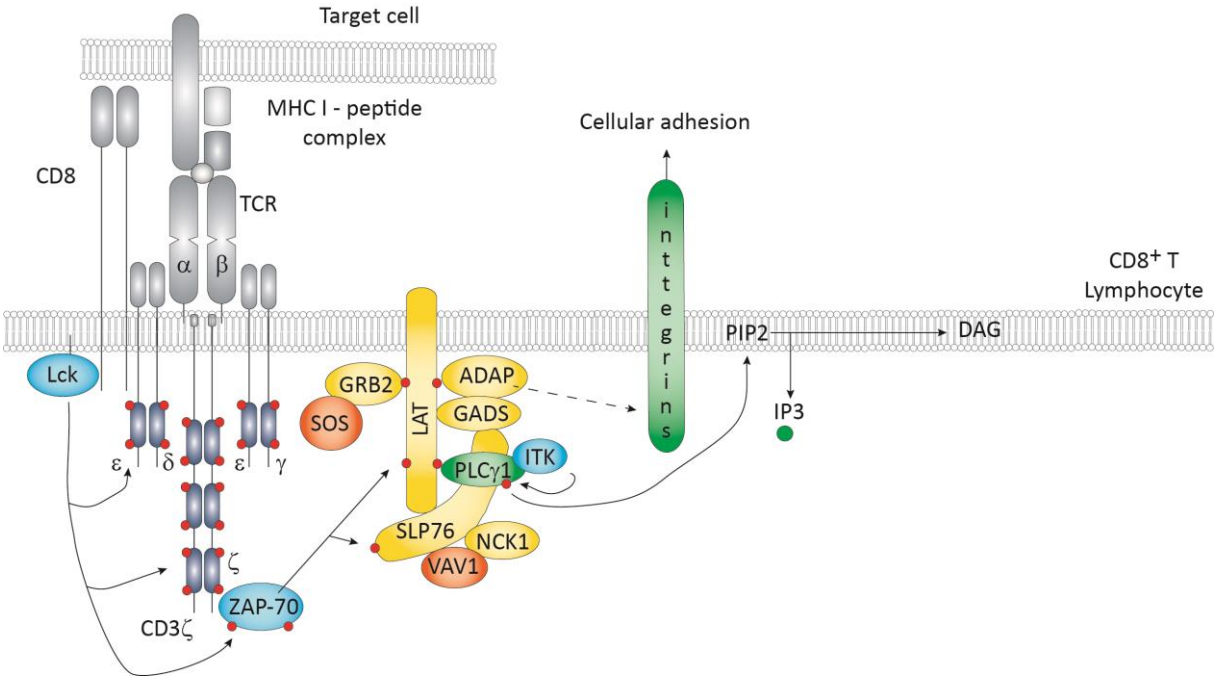


Figure 1. Early steps of the TCR signalling pathway. *Upon contact between TCR and the appropriate major histocompatibility complex class Ia (MHC I)-peptide complex, activation of the TCR signalling pathway is triggered. LCK phosphorylates ITAMs on CD3ζ. This recruits ZAP-70, which is then also phosphorylated by LCK. ZAP-70 phosphorylates SLP76 and LAT, which triggers the assembly of the LAT signalosome. ITK then phosphorylates PLCγ1, which cleaves PIP_2 in DAG and IP3. Kinases are in blue, phosphate groups are in red dots, adaptor proteins are in yellow and guanine nucleotide exchange factors (GEFs) are in orange (inspired from Brownlie and Zamoyska 2013).*

1.3.1.2 The Ca^{++}-NFAT pathway.

IP3 activates its receptor on the endoplasmic reticulum (ER), which triggers Ca^{++} release from the ER. Ca^{++} forms a complex with calmodulin and activates CaMK. CaMK in turn phosphorylates and activates calcineurin, which dephosphorylates NFAT, allowing NFAT to translocate into the nucleus and modulate the expression of its target genes (Smith-Garvin, Koretzky, and Jordan 2009). The Ca^{++}-NFAT pathway is involved in the induction of the expression of genes encoding T cell effector proteins (Figures 2 and 5).

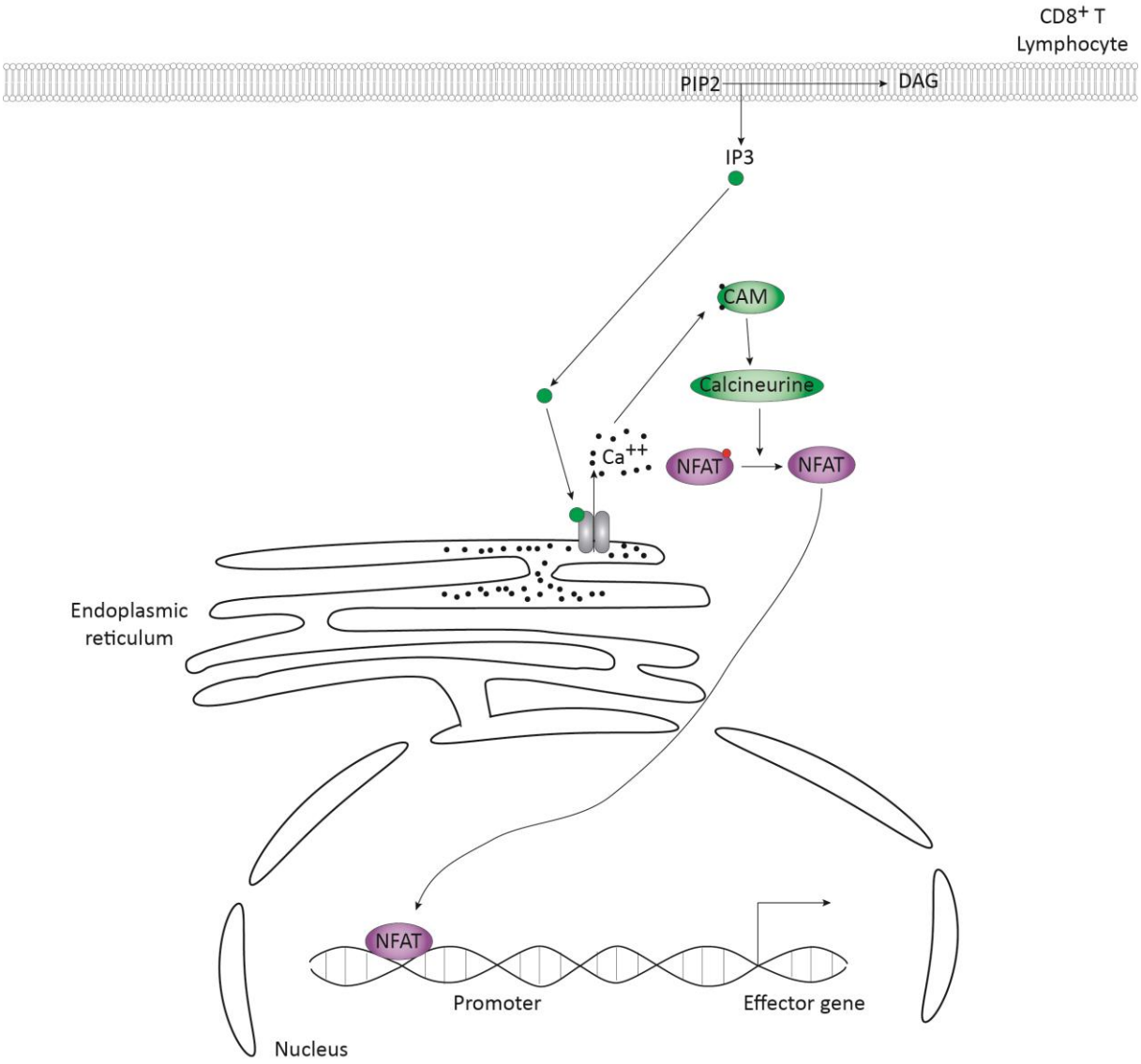


Figure 2. The Ca^{++}-NFAT pathway. *IP_3 initiates Ca^{++} efflux from the ER leading to NFAT dephosphorylation and translocation into the nucleus. This pathway is involved in the induction of the expression of genes coding effector proteins. Transcription factors are in purple (inspired from Brownlie and Zamoyska 2013).*

1.3.1.3 The NF-κB pathway.

DAG triggers PKCθ activation. PKCθ phosphorylates CARMA1, triggering the formation of the CARMA1/BCL10/MALT1 complex. This complex is responsible for the degradation of IKKγ, the regulatory subunit of the IKK complex, probably through its polyubuquitination by TRAF6 (Sun et al. 2004). Thus, activated IKK phosphorylates IκB, which is in turn polyubiquitinated and degraded (Smith-Garvin, Koretzky, and Jordan 2009; Brownlie and Zamoyska 2013). IκB binds to

NF-κB and sequesters it within the cytosol. The degradation of IκB releases NF-κB which translocates into the nucleus to modulate the expression of genes involved in T cell effector functions (Figures 3 and 5).

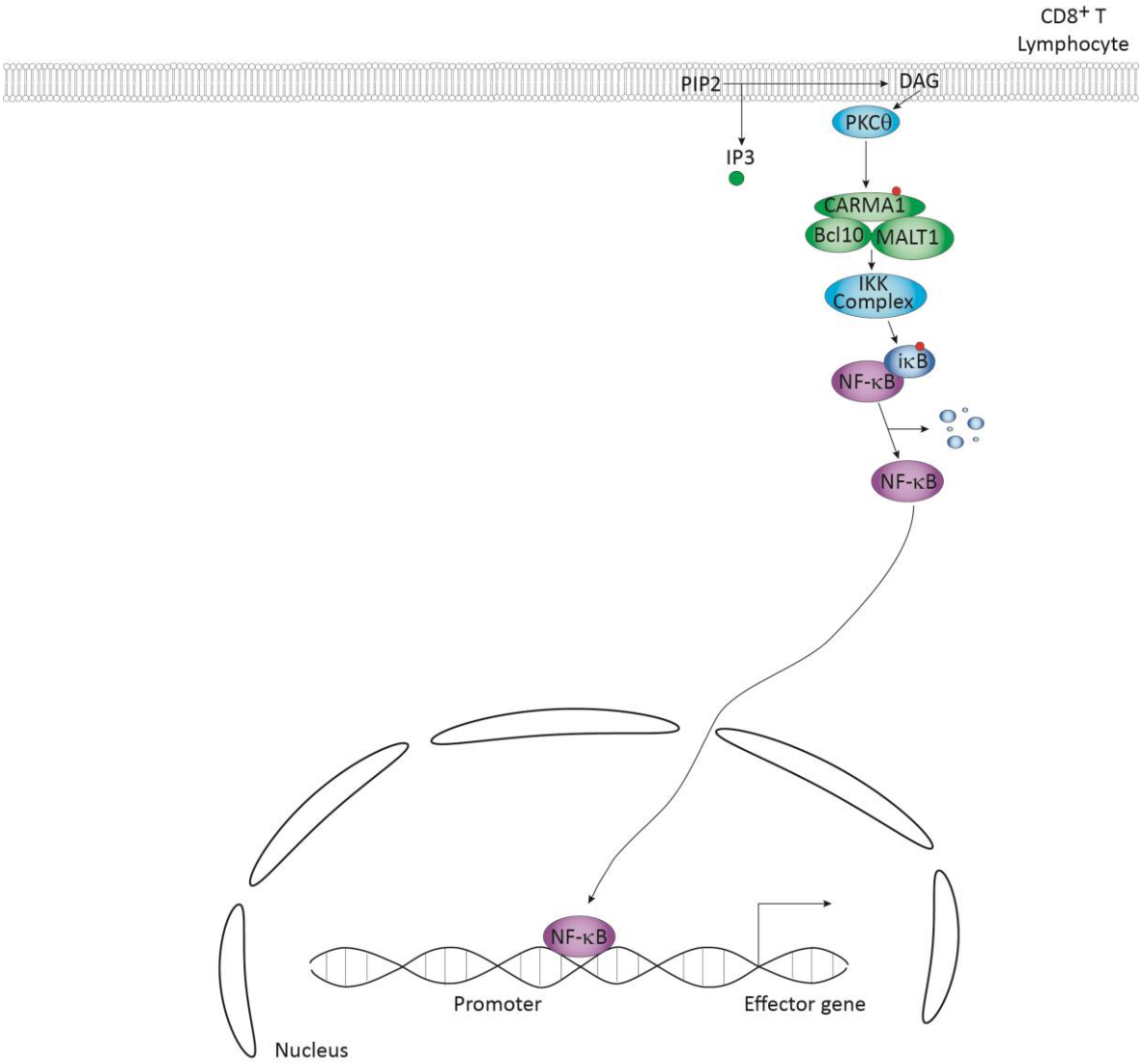


Figure 3. The NF-κB pathway. *DAG activates PKC, resulting in activation of the IKK complex, which phosphorylates IκB and targets it for degradation. NF-κB bound to IκB is thus released and translocates into the nucleus. This pathway is involved in inducing the expression of genes encoding effector proteins. Kinases are in blue, phosphate groups are represented by red dots and transcription factors are in purple (inspired from Brownlie and Zamoyska 2013).*

1.3.1.4 The Ras-MAP-kinase pathway.

Besides initiating the NF-κB pathway, DAG is also involved in the recruitment of RasGRP, a guanine nucleotide exchange factor (GEF) for Ras (Finco et al. 1998; Roose et al. 2005). Together with GRB2, another GEF for Ras also recruited to

the LAT signalosome, RasGRP1 activates Ras. Ras is responsible for activating the MAPKKK Raf-1, which in turn activates the MAPKKs MEK1 and MEK2. MEK1 and MEK2 phosphorylate and activate the MAPKs ERK1 and ERK2. ERK1 and ERK2 activate AP-1, which is a heterodimeric transcription factor. AP-1 cooperates with NFAT and modulates the expression of genes encoding effector proteins (Smith-Garvin, Koretzky, and Jordan 2009; Brownlie and Zamoyska 2013). The Ras-MAPK pathway is also involved in triggering T cell proliferation (Figures 4 and 5).

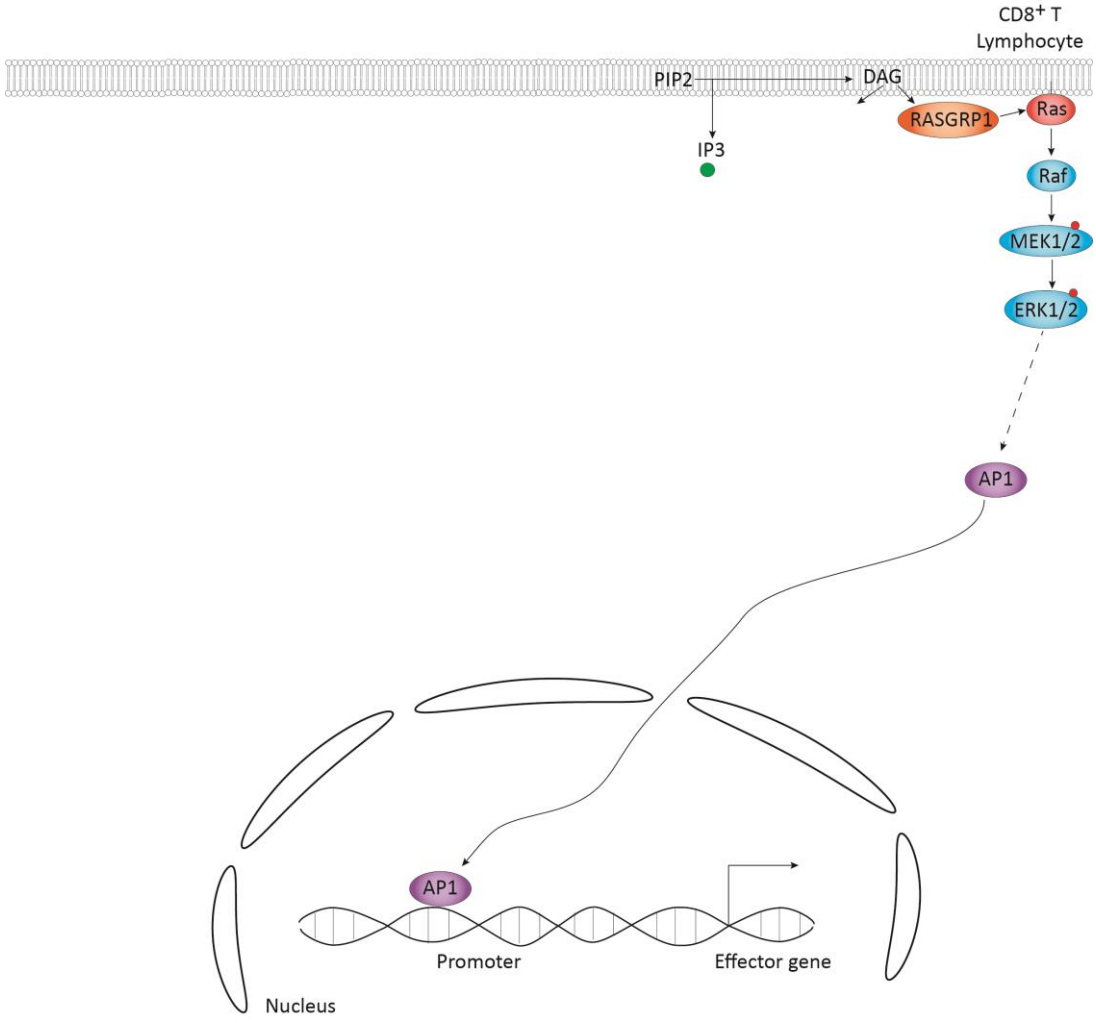
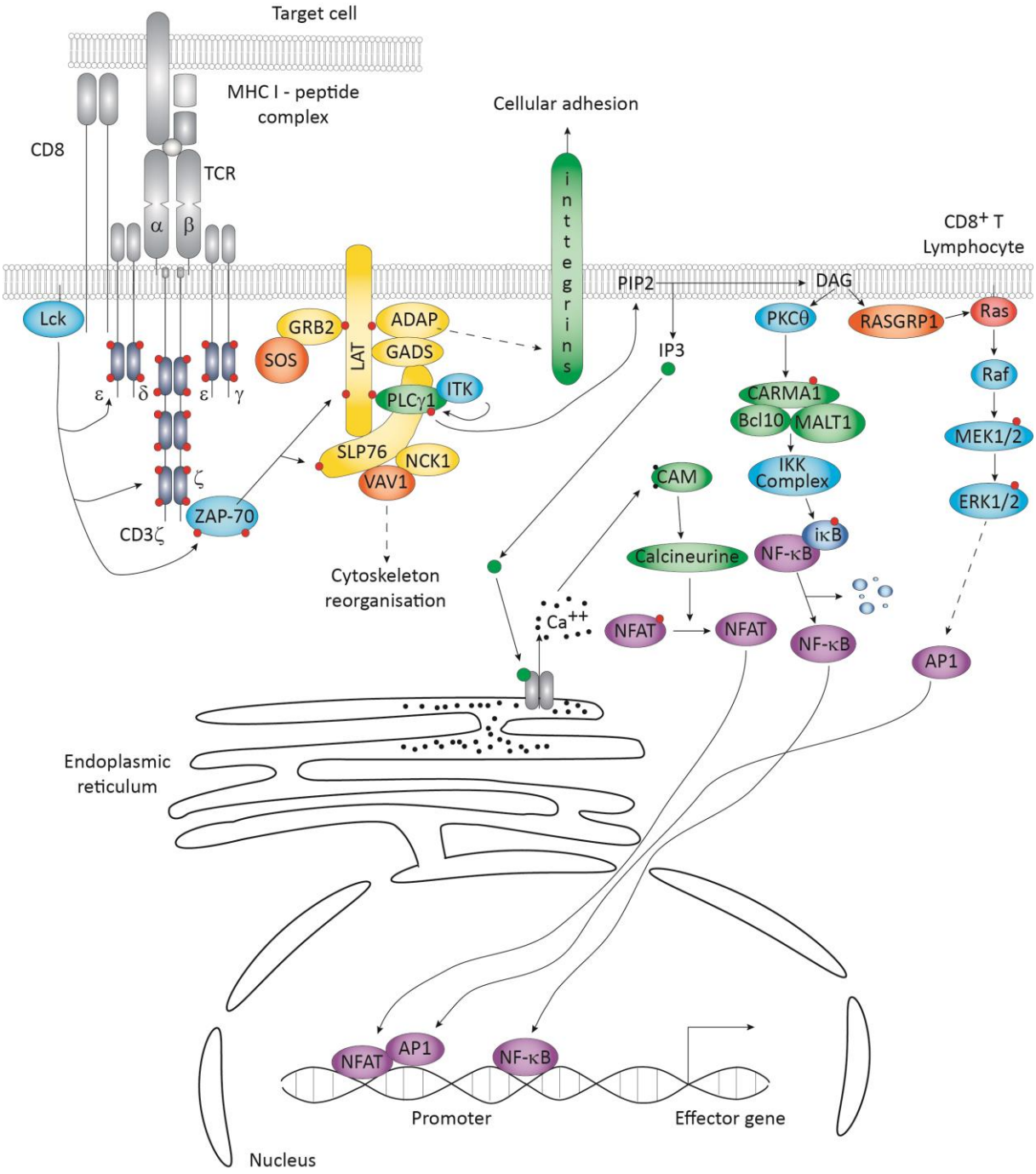


Figure 4. The Ras-MAPK pathway. *DAG recruits RasGRP1, a GEF for Ras, leading to Ras activation. Ras in turn initiates the MAP kinases cascade, resulting in the activation of the transcription factor AP-1. This pathway is involved in inducing the expression of genes encoding effector proteins and T cell expansion. Kinases are in blue, phosphate groups are represented by red dots, GEFs are in orange and transcription factors in purple (inspired from Brownlie and Zamoyska 2013).*

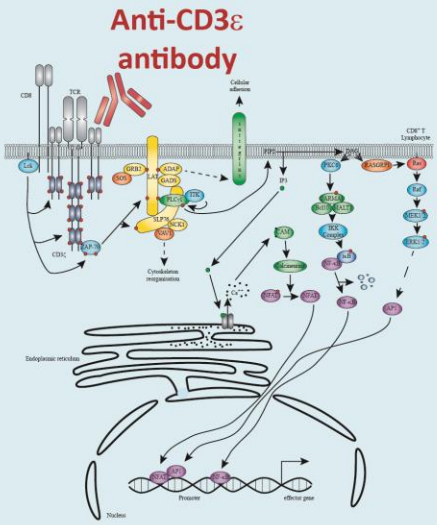
Although not discussed in detail here, engagement of the TCR complex also results in the activation of signalling pathways involved in cytoskeleton rearrangement. Other pathways modulate the affinity of integrins and control the anchoring of T cells to their targets (Figure 5) (Smith-Garvin, Koretzky, and Jordan 2009; Brownlie and Zamoyska 2013).

Figure 5. The TCR signalling pathway. *Upon prolonged contact between the TCR and the appropriate MHC class Ia-peptide complex, activation of the TCR signalling cascade is triggered. Phosphorylation of CD3ζ by LCK recruits ZAP-70 to the TCR, where it is also phosphorylated and activated by LCK. The activated ZAP-70 dissociates from the TCR and phosphorylates SLP-76 and LAT. This triggers the assembly of the LAT signalosome where the TCR signal is amplified and diversified. ITK, which is recruited to the LAT signalosome and activated there, phosphorylates PLCγ1, which cleaves PIP_2 into DAG and IP_3. The TCR signalling pathway separates into three main pathways downstream of ITK: The Ca^{++}-NFAT pathway: IP_3 triggers a Ca^{++} flux from the ER leading to NFAT dephosphorylation and translocation into the nucleus. This pathway is involved in inducing the expression of genes encoding effector proteins. The NF-κB pathway: DAG activates PKC resulting in activation of the IKK complex, which phosphorylates IκB, targeting it for degradation. This releases NF-κB from its complex with IκB and NF-κB translocates into the nucleus. This pathway is involved in inducing the expression of genes encoding effector proteins. The third main pathway is the one of Ras and MAP kinases: DAG activates RasGRP1, a GEF for Ras, leading to Ras activation. Ras in turn initiates the MAP kinases cascade, resulting in the activation of the transcription factor AP-1. This pathway is involved in inducing the expression of genes encoding effector proteins and T cell expansion. Kinases are in blue, phosphate groups are represented by red dots, adaptor proteins are in yellow, GEFs are in orange and transcription factors in purple (inspired from Brownlie and Zamoyska 2013).*

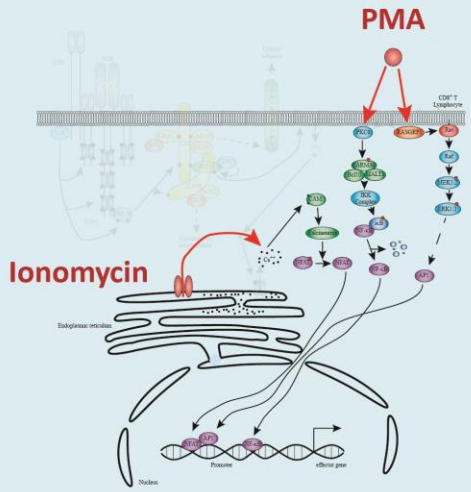

Target cell
MHC I - peptide complex
CD8
TCR
α
β
ε
δ
ε
γ
ζ
CD3ζ
Lck
ZAP-70
GRB2
SOS
LAT
ADAP
GADS
PLCγ1
ITK
SLP76
NCK1
VAV1
Cytoskeleton reorganisation
Cellular adhesion
integrins
PIP2
DAG
IP3
CD8+ T Lymphocyte
PKCθ
RASGRP1
Ras
Raf
MEK1/2
ERK1/2
CARMA1
Bcl10
MALT1
IKK Complex
iκB
NF-κB
CAM
Calcineurine
Ca++
NFAT
NFAT
NF-κB
AP1
Endoplasmic reticulum
NFAT
AP1
NF-κB
Promoter
Effector gene
Nucleus

BOX 1 How to activate T cells *in vitro*?

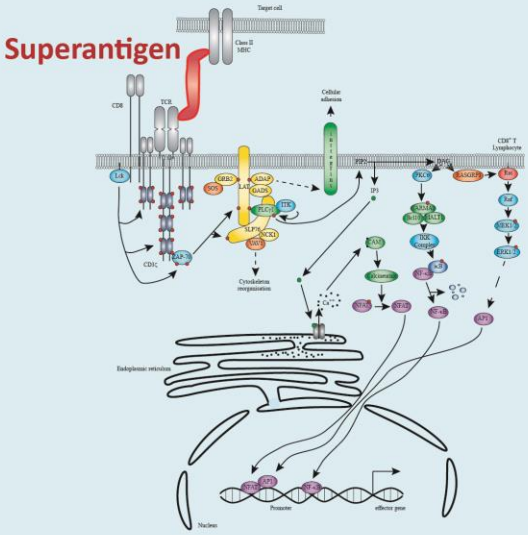
A Anti-CD3ε antibody



B PMA and ionomycin



C Bacterial toxins



T cell specificity is not always known. Different kinds of stimuli exist to ativate T cells regardless of their specificity. ***(A)*** Anti-CD3ε antibodies activate T cells through their TCR-CD3 complex. Agonist antibodies that activate costimulatory molecule such as CD28 can be added depending on the purpose of the activation. ***(B)*** PMA and ionomycin activate T cells downstream of the TCR-CD3 complex. If a decrease in IFN-γ production is observed with an anti-CD3 antibody and reproduced with PMA-ionomycin stimulation, it indicates a blockade in the late signalling part of the TCR pathway. ***(C)*** Bacterial toxins, e.g. superantigens, can also be used. They bind to MHC class II molecule on the target cell and the Vβ chain of the TCR on T cells. It allows to activate T cells with a target cell and therefore to have cell-cell contacts. Bacterial toxins also interact with costimulatory molecules such as CD28. Other ways to activate T cells *in vitro* exist: lectins , e.g. PHA, allogeneic cells,... each one of them with its advantages and disadvantages.

1.3.2 T cell co-stimulatory molecules.

1.3.2.1 CD28.

CD28 is a co-stimulation molecule present at the surface of T cells. Its activation is required during priming – first activation – of naïve T cells; otherwise they will undergo anergy. Anergy is a state in which T cells have lost the ability to respond to TCR stimulation. Upon interaction with one of its ligands, CD80 or CD86, on an antigen-presenting cell (APC), LCK phosphorylates tyrosines 191 and 218 of CD28, triggering the recruitment of GRB2 and PI3K via YMNM and PYAP motifs (Boomer and Green 2010). PI3K phosphorylates PIP2 into PIP3, which leads to the activation of PDK1. PDK1 phosphorylates and activates the kinase Akt, which is responsible for the activation of the mTOR pathway. Another target of Akt is GSK3, which is inactivated through phosphorylation by Akt. The role of GSK3 is to phosphorylate NFAT and thus sequester it in the cytosol. Thus, by inactivating GSK3, Akt favors NFAT activation (Boomer and Green 2010; Lenschow, Walunas, and Jeffrey 1996; Smith-Garvin et al. 2009).

PIP3 also increases the recruitment of TCR signalling proteins, including ITK and PLCγ1 via their PH domains (Figure 6). CD28 activation increases T cell survival, proliferation and interleukin-2 (IL-2) production. It is also involved in a metabolic reprogramming, for example, through the increased expression of glucose transporter 1 (GLUT1) (Boomer and Green 2010; Smith-Garvin et al. 2009).

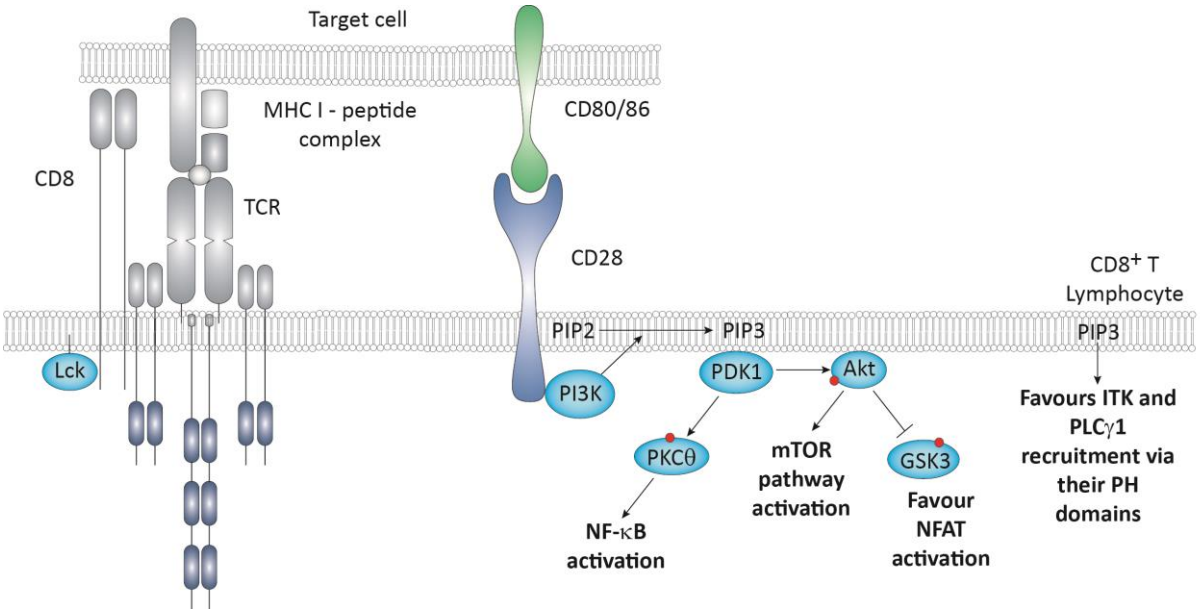


Figure 6. The CD28 signalling pathway. *CD28 increases the signal strength transmitted through the TCR signalling pathway in several ways: Upon ligand binding, CD28 recruits PI3K, which phosphorylates PIP2 to PIP3. This leads to PDK1 activation, which in turn activates PKCθ, increasing the signal through the NF-κB pathway. PDK1 also activates Akt, which activates mTOR, promoting T cell proliferation. Akt phosphorylates GSK3 and inactivates it. This prevents GSK3 from phosphorylating NFAT increasing therefore the signal through the NFAT pathway. Kinases are in blue and phosphate groups are represented by red dots.*

1.3.2.2 Inducible T-cell costimulator (ICOS).

ICOS contains an YMFM motif, like CD28, allowing the recruitment of PI3K and the activation of the Akt pathway. However unlike CD28, ICOS lacks a PYAP motif and is unable to recruit GRB2 through either the PYAP or YMFM motives. This could explain the distinct effects of ICOS and CD28 co-stimulation. For example ICOS induces lower levels of IL-2 production than CD28 (Chen and Flies 2013*)*.

1.3.2.3 CD27.

CD27, a TNF receptor superfamily (TNFRSF) member, plays an important role in T cell co-stimulation. Expression of its ligand CD70 is induced on APCs by interferon gamma (IFN-γ) and certain toll-like receptors (TLRs). CD27 activation leads to NF-κB activation, reinforcement of AP-1 activation and expression of

anti-apoptotic molecules (Grant, Nüssing, Sant, Clemens, & Kedzierska, 2017). CD27 is particularly important for the priming of naïve CD8 T cells as shown in KO mouse models. Disturbing the CD27/CD70 axis leads to decreased proliferation, which is only partially overcome by an increase in CD28 co-stimulation. In humans, increased CD27 co-stimulation *in vitro* enhanced CD8 T cell proliferation, IFN-γ release, and tumor necrosis factor alpha (TNF-α) secretion (Grant et al. 2017; Chen and Flies 2013).

1.3.2.4 The TNFR superfamily.

The TNFRSF contains several co-stimulatory molecules: e.g. CD27 (discussed before), 4-1BB (CD137), OX40, glucocorticoid-induced TNFR-related protein (GITR) and herpesvirus entry mediator (HVEM). These receptors have similar effects on T cell activation. Upon ligand binding, they recruit TRAFs adaptor proteins and will reinforce the activity of several signalling pathways such as the NF-κB, MAPK and NFAT pathways. OX40 and 4-1BB also promote T cell survival similarly to CD27 by enhancing the expression of anti-apoptotic molecules (Chen and Flies 2013).

Other co-stimulatory molecules including class I–restricted T cell–associated molecule (CRTAM or CD355), DNAX accessory molecule-1 (DNAM-1 or CD226), and certain activating natural killer (NK) receptors are also expressed by T cells and enhance their activity. Lymphocyte function-associated antigen 1 (LFA-1) and other adhesion molecules also function as co-stimulatory molecules (Petit et al. 2016).

Co-stimulatory receptors increase the activity of CD8 T cells. But their functions are balanced by inhibitory mechanisms to prevent deleterious effects of an uncontrolled or prolonged CD8 T cell response. Different kinds of regulatory mechanisms exist: e.g. immunosuppressive cells,

immunosuppressive enzymes, inhibitory receptors. Here, I will only discuss inhibitory receptors. Other inhibitory mechanisms are reviewed in detail elsewhere (Kitamura, Qian, and Pollard 2015; Vignali et al. 2008).

1.3.3 Inhibitory receptors.

1.3.3.1 Programmed cell death 1 (PD-1).

PD-1 is a surface receptor encoded by the *PDCD1* gene. Its ligands are programmed death-ligand 1 and 2 (PD-L1 and PD-L2). While PD-1 is absent on naïve T cells, its expression increases on activated T cells after TCR activation (Agata et al. 1996). PD-1 dampens the immune response to prevent immunopathology: PD-1 or PD-L1-KO mice infected with the LCMV virus die due to CD8 T cell-mediated immunopathology (Barber et al. 2006; Frebel et al. 2012; Mueller et al. 2010). Furthermore, PD-1 plays a role in self-tolerance as ageing PD-1-KO mice spontaneously develop a lupus-like disease. Moreover, in a lupus mouse model (Fas mutation lpr), Nishimura and colleagues showed that PD-1 deletion accelerated and increased the lupus-like disease (Nishimura et al. 1999).

PD-1 is a transmembrane protein belonging to the immunoglobulin superfamily. Its intracellular domain contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Upon binding of its ligand (PD-L1 or PD-L2), PD-1 is phosphorylated on tyrosines of ITIM and ITSM domains. This recruits protein tyrosine phosphatases like Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2. These phosphatases interfere with the TCR but also the CD28 signalling pathways by dephosphorylating proteins within the signalling cascade. This interferes with the PI3K/Akt/mTOR pathway. Additionally, PD-1 induces the expression of the transcription factor Basic

Leucine Zipper ATF-Like Transcription Factor (BATF), which reduces the expression of effector genes (Pauken and Wherry 2015) (Figure 7).

In chronic viral infections and cancer, PD-1 can be an obstacle to the elimination of the infected or cancerous cells. We will discuss this phenomenon in the chapter on T cell exhaustion.

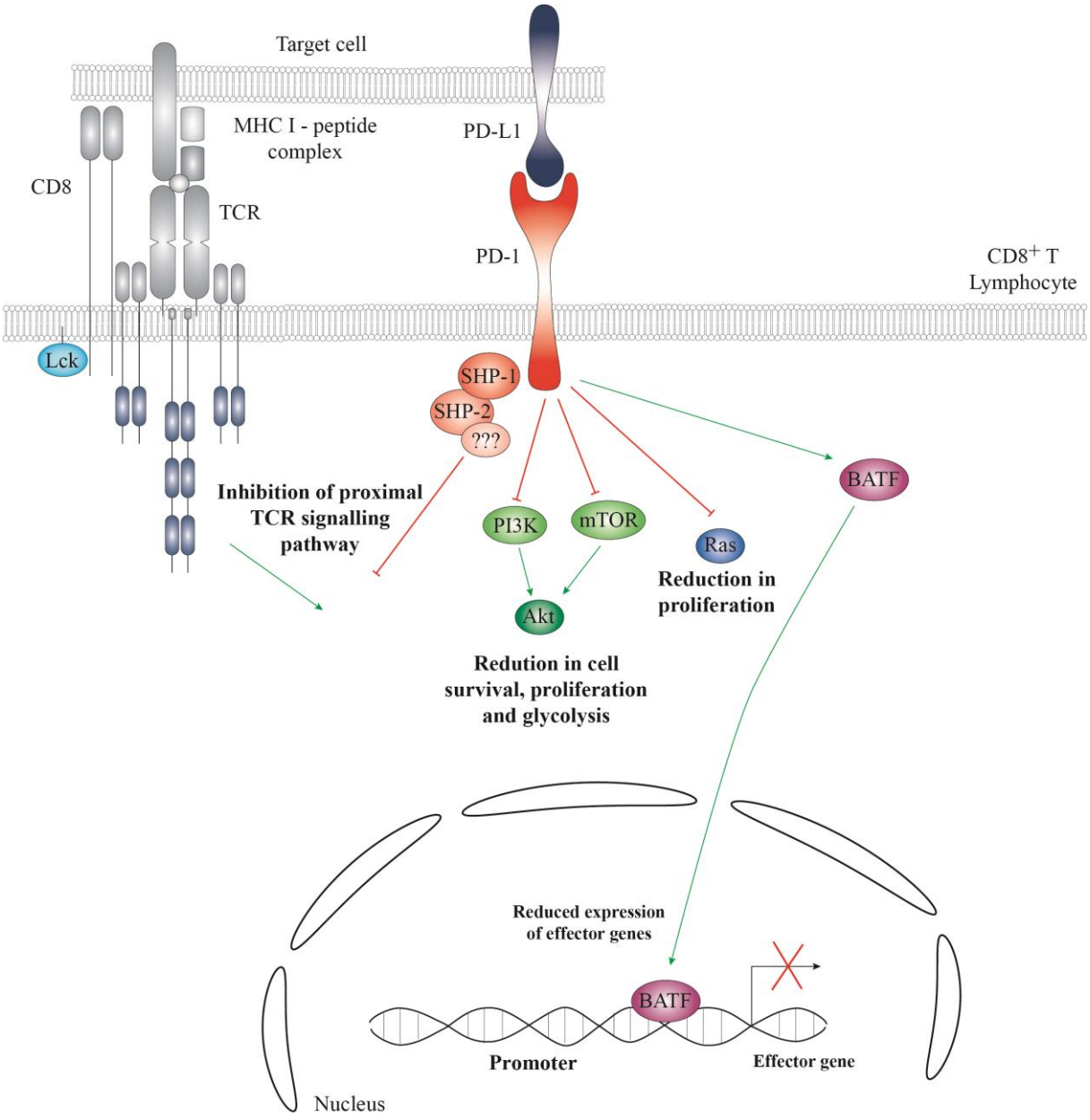


Figure 7. The PD-1 signalling pathway. *PD-1 inhibits T cells in multiple ways: it inhibits proximal TCR signalling by recruiting SHP-1/2; it interferes with the PI3K/Akt and Ras pathways, which impacts the proliferation of T cells; and it induces the expression of the transcription factor BATF, which in turn represses the expression of effector genes (inspired from Pauken and Wherry 2015).*

1.3.3.2 Other inhibitory receptors.

Other inhibitory receptors include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which competes with CD28 for its ligands CD80/CD86, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), Lymphocyte

activation gene 3 protein (LAG-3), T Cell immunoreceptor with Ig and ITIM domains (TIGIT), CD200R, B- and T-lymphocyte attenuator (BTLA) and CD160. These receptors will not be discussed here. Additional information can be found in these reviews (McLane, Abdel-Hakeem, and Wherry 2019; Pauken and Wherry 2015; Wherry 2011).

1.4 CD8 T cell differentiation.

During an acute infection, APCs prime naïve CD8 T cells in secondary lymphoid organs. After being activated through their TCR and co-stimulatory molecules, naïve CD8 T cells differentiate into effector cells. The pool of effector cells generated is heterogeneous. Most of activated CD8 T cells differentiate into short-lived effector cells (SLEC) ($CD127^-$ $KLRG1^+$) and the rest into memory precursor effector cells (MPEC) ($CD127^+$ $KLRG1^-$). SLECs are governed by T-box protein expressed in T cells (T-BET) and B lymphocyte-induced maturation protein-1 (BLIMP-1), which promotes effector functions. In contrast, MPECs are governed by B-cell lymphoma 6 protein (BCL-6), Eomesodermin (EOMES) and T cell factor 1 (TCF-1). After pathogen clearance, most SLECs die and MPECs differentiate into memory cells (Cui and Kaech 2010).

Several subsets of memory cells have been identified. In humans, they are defined as follows: central memory T cells (T_{CM}) are C-C chemokine receptor type 7^+ ($CCR7^+$) $CD27^+$ $CD45RA^-$; effector memory T cells (T_{EM}) are $CCR7^-$ $CD27^-$ and $CD45RA^-$; effector memory $CD45RA^+$ T cells (T_{EMRA}) are $CCR7^-$ $CD27^-$ $CD45RA^+$ (Figure 8). T_{CM} have good proliferative capacity and low effector functions. T_{EM} have lower proliferative capacity but higher effector functions than T_{CM}. Upon antigen re-exposure, T_{CM} require more time to re-express effector proteins compared to T_{EM}, which respond immediately. T_{EMRA} cells are called terminally differentiated, display short telomeres and express

senescence markers (Mahnke et al. 2013). However, these so called terminally differentiated CD8 T cells proliferate well *in vitro*. Moreover, the *in vitro* expression of CCR7 and CD45RA are activation-dependent with CD45RA being expressed on resting T cells (Carrasco et al. 2006). Thus, are $CCR7^-$ $CD45RA^+$ CD8 T cells truly terminally differentiated or are they simply T cells that have not encountered their antigen for some time (Carrasco et al. 2006)? The T_{EMRA} CD8 T cell population may be heterogeneous and only contain some terminally differentiated cells, as shown by Verma and colleagues. Using CD57, they sorted anti-cytomegalovirus (CMV) CD8 T_{EMRA} $CD57^-$ and $CD57^+$, showing that $CD57^-$ T_{EMRA} have longer telomeres, proliferate better and produce more IFN-γ (Verma et al. 2017). Moreover, T_{EMRA} $CD57^-$ lose CD45RA expression after stimulation, which is coherent with data from Carrasco and colleagues and indicates that $CD57^-$ T_{EMRA} are not terminally differentiated. This was not the case for $CD57^+$ T_{EMRA} (Carrasco et al. 2006; Verma et al. 2017).

A more refined classification of memory CD8 T cell subsets present in human blood has been described by Romero and colleagues. The authors used CD28 and CD27 in addition to CCR7 and CD45RA. In the T_{CM} compartment the vast majority of cells are CD28 and CD27 double positive. Four distinct T_{EM} subsets have been identified: EM1 ($CD27^+/CD28^+$), EM2 ($CD27^+/CD28^-$), EM3 ($CD27^-/CD28^-$) and EM4 ($CD27^-/CD28^+$). T_{EMRA} were separated into three subsets: E ($CD27^-/CD28^-$), pE1 ($CD27^+/CD28^+$) and pE2 ($CD27^+/CD28^-$) (Romero et al. 2007) (Figure 8). According to the authors the differentiation of CD8 T cells happens in a sequential manner from naïve to central memory and then to the sequential effector memory stages EM1, EM2 and EM3. After reaching EM3 stage of the effector memory compartment, cells arrive at the E stage of the T_{EMRA} compartment (Romero et al. 2007). This way of thinking in which T cell differentiation is linear and not circular is not in line with other studies also

conducted with human cells (Carrasco et al. 2006; Verma et al. 2017). It is unclear where EM4, pE1 and pE2 stage are located in this differentiation model. Nevertheless, the addition of these two markers (CD27 and CD28) distinguishes four functionally different subsets in the T_{EM} compartment. Of these, EM3 represents the population with the highest effector properties. For example, cytotoxicity is higher in EM3 than in the other T_{EM} subpopulations (Romero et al. 2007).

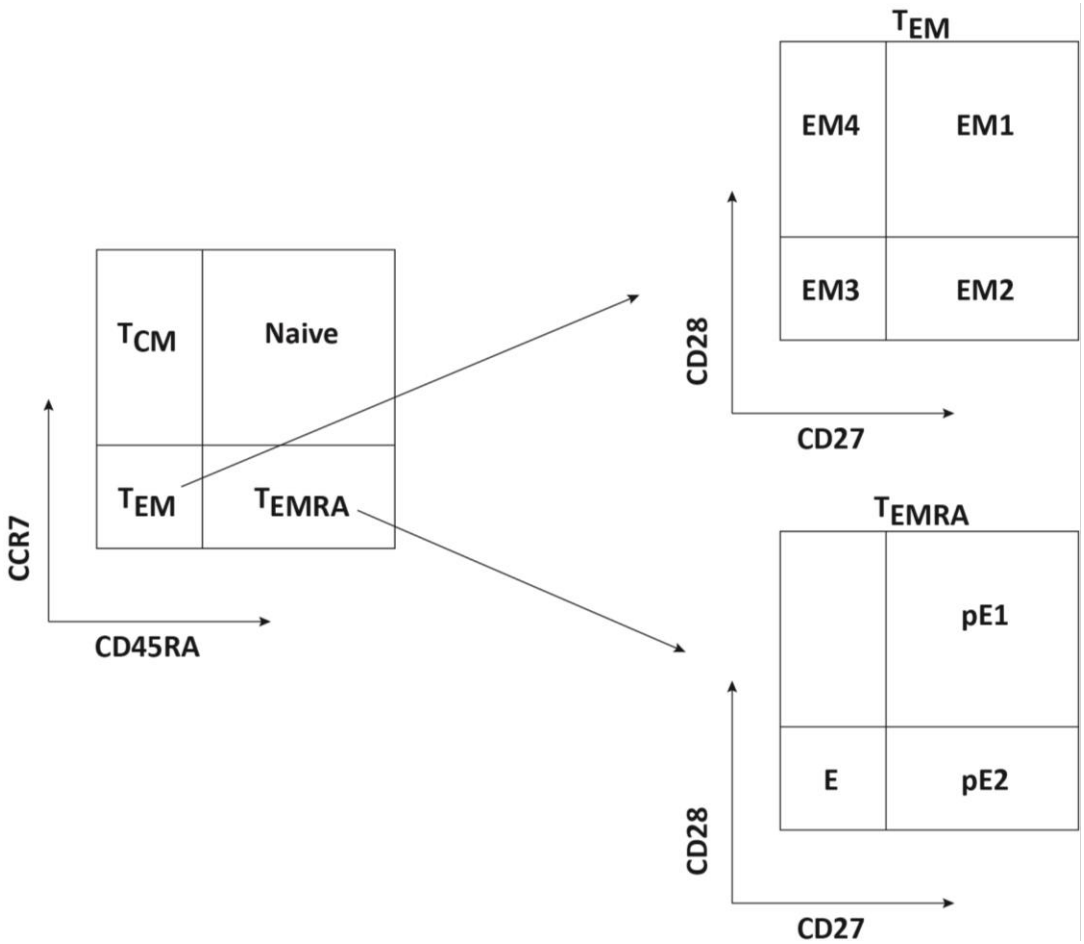


Figure 8. CD8 T cell subsets in human blood. *Scheme describing markers used to distinguish CD8 T cells subsets. On the left panel, CCR7 and CD45RA distinguish naïve T cells from T_{CM}, T_{EM} and T_{EMRA}. On the right panels, CD27 and CD28 identify T_{EM} and T_{EMRA} subpopulations.*

To conclude, CCR7 and CD45RA are useful tools to exclude naïve CD8 T cells from experimental analysis and distinguish T_{CM}, T_{EM} and T_{EMRA}. Additional markers, such as CD27 and CD28, enable a more refined distinction of CD8 T cell populations in human blood.

BOX 2 Resident memory T cells

Resident memory T cells (T_{RM}) are present in tissues. T_{RM} are present at potential infection sites (e.g. skin and gut) and can react promptly to infections. Mice lacking T_{RM} have impaired viral clearance (Jiang et al. 2012). CD8 T_{RM} produce IFN-γ and lyse target cells upon recognition of their antigen (Topham and Reilly 2018; Masopust and Soerens 2019; Schenkel and Masopust 2014; Mueller and Mackay 2016).

CD8 T_{RM} are characterized by the expression of CD69 and CD103. However, neither marker is specific or exclusive to T_{RM}. CD69 and CD103 are expressed only by a fraction of CD8 T_{RM} and CD69 is also expressed by activated T cells (Masopust and Soerens 2019; Schenkel and Masopust 2014). Interestingly, T_{RM} express low levels of CCR7 and S1PR1, which are associated with T cell egress from tissues (Mueller and Mackay 2016).

In mice, HOBIT expression by T_{RM} is important to repress the expression of genes involved in T cell egress. However, in humans the link between HOBIT and T_{RM} is not as evident as in mice. Instead, low expression of T-BET, EOMES and TCF-1 is characteristic of human T_{RM}. Other transcriptions factors have been shown to be expressed by T_{RM} in a tissue-dependent manner, e.g. RUNX3, AHR, BATF (Topham and Reilly 2018; Mueller and Mackay 2016).

1.5 CD8 T cell exhaustion and the LCMV model.

CD8 T cell exhaustion has been defined by John Wherry as follows (McLane et al. 2019; Pauken and Wherry 2015; Wherry 2011):

1) loss of function and proliferative capacity in a progressive or sequential way;
2) increased and sustained expression of inhibitory receptors;
3) altered response to homeostatic cytokines (IL-7/IL-15) involved in memory T cell maintenance;
4) metabolic reprogramming;
5) distinct expression pattern and use of transcription factors compared to effector and memory T cells, and;
6) epigenetic reprogramming characteristic of exhausted CD8 T cells.

This phenomenon occurs through prolonged contact between T cells and their antigen, e.g. during chronic viral infections or cancer. In mice, CD8 T cell exhaustion has been extensively studied in the LCMV model.

1.5.1 Chronic viral infection.

1.5.1.1 Lymphocytic choriomeningitis virus (LCMV) model.

LCMV infections provide an interesting model because the Armstrong strain of the virus causes acute infection, while the clone 13 strain results in chronic infection (Zajac et al. 1998). The mutations that characterize these two strains do not occur in major CD8 T cell epitopes. Thus, it is possible to track CD8 T cells directed against the same epitope in acute versus chronic infections (McLane et al. 2019; Pauken and Wherry 2015; Wherry 2011).

In this model, the loss of effector functions happens hierarchically. First, IL-2 production is lost. Then, TNF-α production and the capacity to kill infected cells are impaired. The capacity to produce IFN-γ is altered at later and more severe stages of T cell exhaustion. Exhausted CD8 T cells can still produce some chemokines, including CCL3, CCL4 and CCL5 (Wherry et al. 2003, 2007; Zajac et al. 1998). The severity of the exhausted phenotype is influenced by the antigen load and the presence of CD4 T cells. While activated T cells normally express inhibitory receptors - as inhibitory receptors provide negative feedback loops to avoid deleterious effects of sustained immune responses on the organism -, exhausted CD8 T cells express a multitude of inhibitory receptors at higher and more sustained levels (Barber et al. 2006; Wherry et al. 2007). Transcriptomic and epigenetic studies have shown that exhausted CD8 T cells have a transcriptional program and epigenetic landscape distinct from effector or memory CD8 T cells (Wherry et al. 2007).

Inhibitory receptors restrain CD8 T cell responses in the LCMV model. For example, the team of Rafi Ahmed showed that PD-1 is transiently expressed during acute infections and then returns to basal level. However, in chronic infections the level of PD-1 expression is increased and sustained over time (Barber et al. 2006; Wherry et al. 2007). Blocking PD-1 with anti-PD-L1 antibodies enhances CD8 T cell responses and viral clearance in chronic LCMV infections. PD-1 blockade also increases CD8 T cell proliferation and the number of CD8 T cells able to produce cytokines (IFN-γ and TNF-α) in chronic LCMV infections (Barber et al. 2006). Blocking TIM-3 alone, another inhibitory receptor, alone increases the proliferation of virus-specific CD8 T cells in chronic LCMV infections, but has little effect on viral clearance. Combining TIM-3 and PD-1 blockades results in more efficient viral clearance compared to PD-1 blockade alone, indicating that targeting multiple inhibitory receptors may be

an answer in such context of T cell exhaustion (Jin et al. 2010). The inhibitory receptor LAG-3 is also expressed at a high and sustained level during chronic LCMV infections. Blocking LAG-3 alone fails to affect the number of virus-specific CD8 T cells. However, combining LAG-3 and PD-1 blockade increases the number of virus-specific CD8 T cells and their ability to produce cytokines such as IFN-γ and TNF-α compared to PD-1 blockade alone. The combination also enhanced viral clearance compared to either treatment alone (Blackburn et al. 2009).

Although the pathways initiating T cell exhaustion still remain to be elucidated, persistent antigenic stimulation plays a key role in T cell exhaustion: antigen burden and exposure time aggravate the exhaustion. Higher antigen burden leads to a more severe exhaustion. If T cells from chronically infected mice are adoptively transferred into uninfected mice early enough (less than 3 weeks after infection), they can differentiate into memory cells. Negative co-stimulation through inhibitory receptors and exposure to chronic inflammation are also important factors. The last element is the absence of CD4 help as shown by depleting CD4 T cells prior to infection (Matloubian, Concepcion, and Ahmed 1994).

Using bioinformatics, Doering and colleagues defined networks of transcriptional regulation that govern T cell exhaustion. Their study revealed that the role of T-BET, EOMES and BLIMP-1 is altered in exhausted CD8 T cells and drive a distinct transcriptional program. They also identified transcription factors less studied in T cell exhaustion: TOX, HELIOS, TCF-1 and interferon regulatory factor 4 (IRF4) (Doering et al. 2012). Recently, TOX has been confirmed as a key transcription factor of T cell exhaustion (Alfei et al. 2019;

Khan et al. 2019; Scott et al. 2019). We will discuss in detail the role of TOX in a next chapter focused on T cell transcription factors.

Pitfalls of the LCMV model.

In experiments using the LCMV model, the time is a critical factor. Usually, in acute infections effector CD8 T cells are tested between days 6 and 8 after infection, and memory CD8 T cells between days 30 to 35. In chronic infections, exhausted CD8 T cells are collected around day 20. However, the viral load in acute and chronic infections differs dramatically over time. In acute infections, the viral load between days 6 and 8 has already greatly decreased compared to the initial titers or to the viral load in chronic LCMV infections at day 20 (Wherry et al. 2003). This induces a bias when comparing effector CD8 T cells from acute infections with exhausted T cells from chronic infections: the amount of antigen to which T cells are exposed (Figure 9). Thus, observed differences could be attributed to exhaustion or to the difference of antigen load at the time cells are collected. For example, in such an experiment, inhibitory receptors like PD-1 and TIM-3 will have higher expression profiles in exhausted CD8 T cells compared to effector CD8 T cells from the acute infection. But would this be the case if effector CD8 T cells in the acute infections were taken earlier when the antigen burden is similar to that of the chronic infection at day 20?

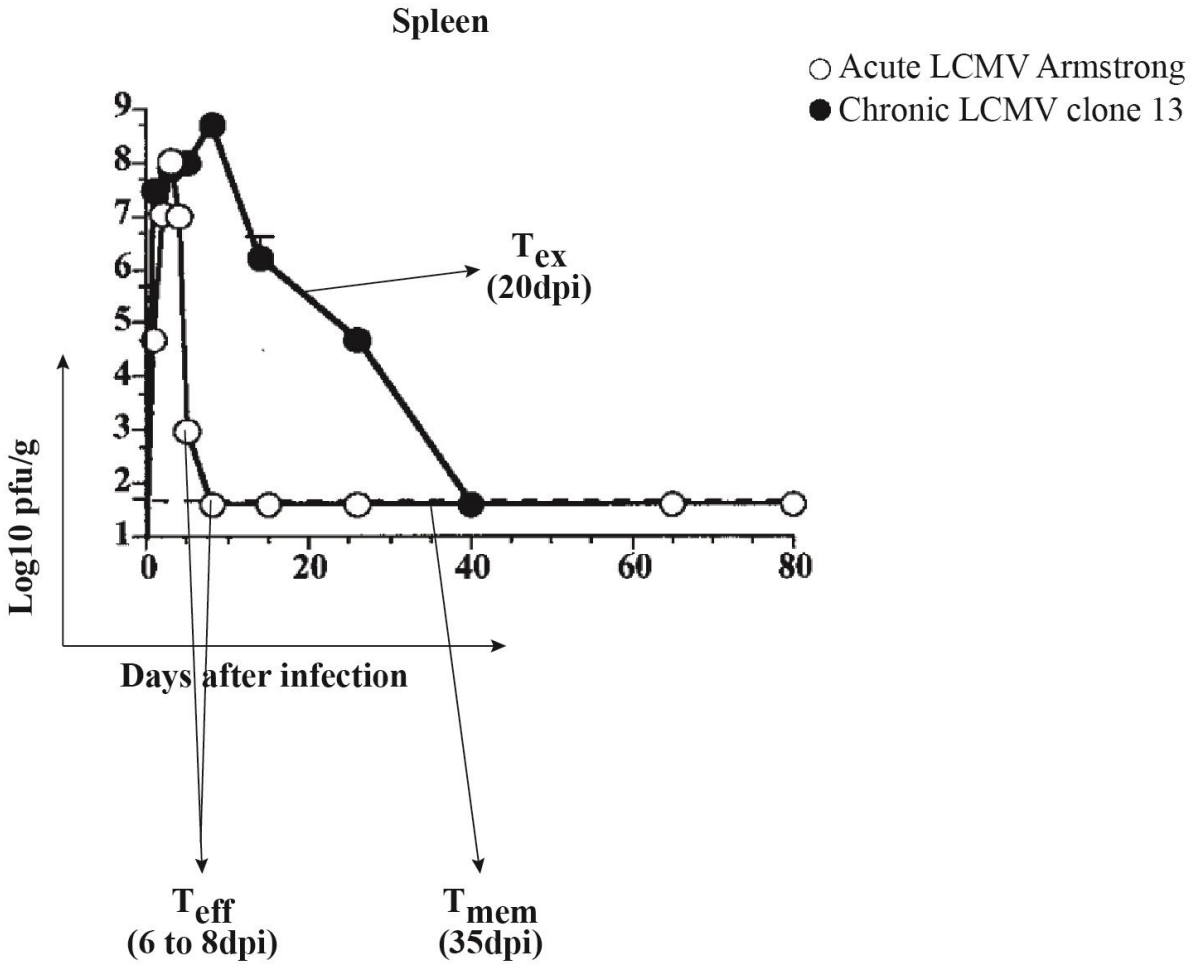


Figure 9. Evolution of LCMV viral load in spleen over time. *This figure illustrates the amount of antigen present at usual time-points of harvesting to compare CD8 T cells between the acute and the chronic LCMV infections. During acute infection, the antigen is already practically absent from the spleen on days 6 to 8 when CD8 T cells are called effector cells and harvested. Data are taken from Wherry et al. 2003.*

1.5.1.2 Human chronic viral infection.

Parallels have been drawn between chronic LCMV infection in mice and chronic viral infection in humans, such as human immunodeficiency virus (HIV) or chronic infections with hepatitis C virus (HCV) or hepatitis B virus (HBV). In these diseases, T cells have been described as exhausted as they express inhibitory receptors like PD-1, have poor functions and fail to clear the infection (Goepfert et al. 2000; Gruener et al. 2001; Kaufmann et al. 2007; Petrovas et al. 2006; Shankar et al. 2000; Urbani et al. 2006; Ye et al. 2015). In HCV-infected

patients, cytokine production (IFN-γ and TNF-α) and upregulation of the activation marker CD69 are impaired after stimulation in HCV-specific CD8 T cells but not in bystander CD8 T cells (anti-CMV and anti- Epstein-Barr virus (EBV)) (Gruener et al. 2001). HCV-specific CD8 T cells express more inhibitory receptors (PD-1, 2B4 and CD160) than bystander CD8 T cells (anti-influenza). This is not surprising because anti-HCV CD8 T cells are in contact with their antigen, which is not the case for anti-influenza CD8 T cells (Bengsch et al. 2010). *In vitro*, PD-1 blockade enhances the proliferation of HCV-specific CD8 T cells (Urbani et al. 2006).

1.5.2 T cell exhaustion and cancer.

In an analogy with chronic viral infections, tumor infiltrating lymphocytes (TILs) have often been described as exhausted due to their poor effector functions that include decreased IFN-γ production, and increased expression of a multitude of inhibitory receptors (PD-1, TIM-3, LAG-3, …) (Ahmadzadeh et al. 2009; Fourcade et al. 2010; Matsuzaki et al. 2010; Zarour 2016; Zhang et al. 2010). The two main exhausted CD8 T cell populations described in the LCMV model have also been described in murine tumor models by the team of Nicholas Haining. In similar experiments to those of Rafi Ahmed, they showed the existence of progenitor and terminally exhausted CD8 T cells in a B16-OVA melanoma model (Miller et al. 2019). Moreover, like in the LCMV model, blocking PD-1 reinvigorates progenitor exhausted CD8 T cells in cancer (Huang et al. 2017; Miller et al. 2019). In fact, blocking inhibitory receptors PD-1 or/and CTLA-4 have shown prominent clinical efficacy in cancer patients (Hamid et al. 2013; Hodi et al. 2010; Wolchok et al. 2013).

1.6 T cell Transcription factors and their role under physiological and pathological conditions.

1.6.1 T-BET and EOMES.

T-BET is encoded by the *TBX21* gene. Like EOMES, it belongs to the T-box transcription factor family. T-BET is well-known for its role in committing CD4 T cells to the Th1 lineage, where it is required for IFN-γ expression and to prevent IL-4 expression. IL-12 inversely regulates the expression of T-BET (increases) and EOMES (represses) (Takemoto et al. 2006).

In murine CD8 T cells, knocking-out T-BET has no effect on IFN-γ production. However, forcing the expression of dominant-negative T-BET dramatically reduces IFN-γ production by CD8 T cells. This experiment shows that another transcription factor exists, which is redundant to T-BET and ensures IFN-γ expression in CD8 T cells. This transcription factor is EOMES (Pearce et al. 2003). EOMES and T-BET also both regulate cytotoxic activity, albeit less stringently than IFN-γ production. Similarly, knocking-out T-BET alone results in a small decrease in granzyme B expression by CD8 T cells. But, when dominant-negative T-BET is expressed, a dramatic decrease in granzyme B expression is observed. Again, EOMES rescues granzyme B expression in CD8 T cells in the absence of T-BET. There is however a small proportion of granzyme B expression that is strictly T-BET-dependent (Pearce et al. 2003). Supporting these data, T cell-specific T-BET-KO mice (CD4-Cre *Tbx21*$^{fl/fl}$) managed to control LCMV acute infection. However, T cells in mice with the T cell-specific double-KO for T-BET and EOMES failed to control the infection. Additionally, CD8 T cells in the double-KO mice developed a Th17 phenotype,

typified by expressing RORγt, the Th17 lineage transcription factor, and producing IL-17 (Intlekofer et al. 2008).

Interestingly, during chronic LCMV infection the T-BET-KO mice display an altered phenotype. Viral titers are higher than in wild-type mice. PD-1 expression is also higher in the absence of T-BET. But overexpression of T-BET lowered the expression of PD-1 and other inhibitory receptors (LAG3, CD160 and BTLA). However, the authors do not mention whether T-BET overexpression impacts viral titers (Kao et al. 2011). Taylor and colleagues confirmed this link between T-BET and PD-1. In fact, T-BET is a repressor of PD-1 expression: increasing T-BET expression through GSK3 inhibition leads to a decrease in PD-1 expression by CD8 T cells. Inhibiting GSK3 accelerates the viral clearance during an acute infection with murine hepatitis virus 68 in mice (Taylor et al. 2016).

BOX 3 CD4 T cell subsets

Several CD4 T cell subpopulations have been described based on cytokines and transcription factors expression profile. Th1 differentiation requires the expression of the transcription factor T-BET which is induced by IL-12. Th1 secrete IFN-γ and are implicated in immune responses against intracellular pathogens (Ruterbusch, Pruner, Shehata, & Pepper, 2020; Szabo et al., 2000). Th2 secrete IL-4, IL-5 and IL-13 and express the transcription factor GATA3. IL-4 is required for Th2 differentiation. Th2 are implicated in immune responses against extracellular pathogens (Ruterbusch et al., 2020; Wei-ping & Flavell, 1997).

Th17 secrete IL-17A/F. They can also secrete IL-22. Cytokines involved in Th17 differentiation are: IL-1β, IL-6, TGF-β and IL-23. Transcription factors RORγt, RORα and PLZF are expressed by Th17 (Ivanov et al., 2006; Singh et al., 2015; Yang et al., 2008). AHR is also expressed by Th17. Th17 play a protective role against pathogens (bacteria, fungi) (Korn, Bettelli, Oukka, & Kuchroo, 2009).

CD4 Tregs secrete IL-10 and active TGF-β. CD4 Tregs express the transcription factor FOXP3. In humans, FOXP3 expression is not restricted to CD4 Tregs but can also be expressed by activated T cells (Baron et al. 2007). TGF-β can be used to differentiate CD4 T cells into Tregs *in vitro*. CD4 Tregs are implicated in peripheral tolerance. Their role is to prevent autoimmune responses as well as keeping the immune response against pathogens under control (Vignali, Collison, & Workman, 2008).

Th9, Th22 and follicular helper CD4 T cells have been described (Crotty, 2019; Schmitt & Bopp, 2017; Trifari, Kaplan, Tran, Crellin, & Spits, 2009).

1.6.2 BLIMP-1 is essential for effector CD8 T cell differentiation.

BLIMP-1 (gene *PRDM1*) is a transcription factor that belongs to the PRDM family, which is characterized by the presence of a PR domain and a variable number of zinc finger domains. BLIMP-1 contains five zinc finger domains.

To prove that BLIMP-1 is essential for the regulation of T cell activity, Martins and colleagues used T cell-specific KO mice (*Prdm1*$^{fl/fl}$ Lck-Cre). Deletion of BLIMP-1 resulted in severe colitis. BLIMP-1-deficient CD4 T cells produce more IL-2 and IFN-γ and less IL-10 compared to CD4 T cells from control mice (Martins et al. 2006). Similar results were obtained using T cell-deficient mice (Rag1-KO mice) reconstituted with fetal liver stem cells that express a BLIMP-1 mutant that lacks the DNA-binding domain. These mice rapidly developed a fatal autoimmune disease characterized by lymphocyte infiltration and tissue destruction of different organs (lung, liver and gut) (Kallies et al. 2006).

These observations suggest a role for BLIMP-1 in dampening T cell responses. However, Kallies and colleagues showed that BLIMP-1 is essential for an appropriate T cell response and the differentiation of short-lived effector cells (SLEC) (Kallies et al. 2009). Indeed, mice deficient for BLIMP-1 fail to control influenza infection. CD8 T cells lacking BLIMP-1 fail to fully develop their cytotoxic capacity with reduced expression of granzyme B, granzyme K and perforin (Kallies et al. 2006). Rutishauser and colleagues corroborated these observations by showing a decrease in granzyme B expression in BLIMP-1 KO CD8 T cells upon acute infection with the LCMV and a lack of SLEC differentiation. However, the killing capacity was unaffected in their model (Rutishauser et al. 2009). BLIMP-1 and T-BET are linked: in BLIMP-1-deficient CD8 T cells, the induction of T-BET expression was lower compared to control cells. In contrast, EOMES and BCL-6 expression were higher in BLIMP-1-

deficient CD8 T cells (Kallies et al. 2009). These data - BLIMP-1 repressing BCL-6 expression and inducing T-BET expression - has since been independently confirmed (Cimmino et al. 2008; Rutishauser et al. 2009).

However, Xin and colleagues failed to reproduce this BLIMP-1-dependent T-BET expression. They showed using both BLIMP-1 and T-BET-KO T cells that the expression of T-BET and BLIMP-1 are independent from each other. Instead, they showed that BLIMP-1 and T-BET are at the center of the regulation of effector CD8 T cell differentiation (Xin et al. 2016). T-BET and BLIMP-1 regulate a common set of genes in addition to their own distinct sets of genes, indicating that they are at least partially not redundant (Xin et al. 2016). Data from acute infections with influenza or LCMV in both T-BET and BLIMP-1-KO mice showed a reduction in the generation of $GZMB^+$ IFN-γ^+ CD8 T cells. The double deficient mice die very quickly after the onset of the infection. This is due to immunopathology, which is not the case for single deficient mice. CD8 T cells cause the immunopathology and depleting them rescues the double-deficient mice. This indicates that while BLIMP-1 and T-BET are essential for SLEC differentiation, they are also crucial for avoiding immunopathology (Xin et al. 2016).

During chronic LCMV infection, BLIMP-1 expression is increased in exhausted CD8 T cells compared to effector CD8 T cells from the acute infection. BLIMP-1 conditional KO using Gzmb-Cre *Prdm1*$^{fl/fl}$ had no effect on viral load. However, partial deletion of BLIMP-1 using Gzmb-Cre *Prdm1*$^{fl/+}$ mice reduced expression of inhibitory receptor PD-1, increased IL-2 production and cytotoxic activity. Gzmb-Cre *Prdm1*$^{fl/+}$ mice had a better control on viral replication (Shin et al. 2009).

Collectively, these results clearly show that BLIMP-1 is not only important for mounting a robust immune response with appropriate effector CD8 T cell differentiation but also for preventing T cell-mediated immune pathology. Moreover, the level of BLIMP-1 expression needs to be tightly regulated as its increased expression participates to T cell exhaustion.

1.6.3 TCF-1 is essential for memory CD8 T cell formation.

TCF-1, which is encoded by the *TCF7* gene, is downstream of the Wnt/β-catenin pathway, but can also be activated through NOTCH signalling. Besides thymocytes development, TCF-1 plays a crucial role in T cell differentiation. It is highly expressed in naïve CD8 T cells. But upon activation, inflammatory signals like IL-12 downregulate TCF-1. TCF-1 is then re-expressed by memory precursor effector cells (MPEC) cells and is required for proper memory CD8 T cell differentiation (Kim et al. 2020). The impact of TCF-1 on the formation of T cell memory was shown by Jeannet and colleagues: TCF-1-deficient mice initially respond normally during acute LCMV infection. Similarly to wild-type mice, some CD8 T cells are maintained after the contraction phase. However, after re-challenge with LCMV, CD8 T cells from TCF-1-deficient mice expand poorly compared to wild-type, indicating impaired memory differentiation in the absence of TCF-1. The poor formation of CD8 T cell memory was caused by the lack of MPECs formation during the primary immune response. The poor secondary immune response was correlated with poor control of viral replication (Jeannet et al. 2010).

However, controversy persists about the role of TCF-1 in primary T cell responses. Zhou and colleagues used TCF-1-deficient OT-1 CD8 T cells with a transgenic TCR that recognizes the ovalbumin SIINFEKL peptide. They transferred low numbers of T cells ($6x10^3$) into recipient mice and infected

them with ovalbumin-expressing *Listeria monocytogenes*. In this model, the expansion of CD8 T cells during the primary immune response was defective. Similarly to Jeannet et al, the secondary immune response was impaired due to poor T cell expansion after re-challenge (Zhou et al. 2010).

In absence of TCF-1, MPECs and memory CD8 T cell formation are impaired. These "MPECs" express transcription factors associated with SLECs, such as T-BET and BLIMP-1, instead of expressing MPECs-associated transcription factors (TCF-1, EOMES and BCL-6) (Kim et al. 2020).

In chronic viral infection, two main exhausted CD8 T cell populations have been identified: Progenitor-exhausted CD8 T cells are PD-1^{int}, TIM-3^{-}, $CXCR5^{+}$, TCF-1^{+} and $EOMES^{low}$, and terminally-exhausted CD8 T cells express PD-1^{high}, TIM-3^{high} and $EOMES^{high}$, T-BET^{low}, $CXCR5^{-}$ and $TCF1^{-}$ (Im et al. 2016). Progenitor-exhausted CD8 T cells retain memory-like properties, like self-renewal (Im et al. 2016). When progenitor-exhausted CD8 T cells are adoptively transferred into chronic LCMV-infected mice, these cells differentiate into terminally-exhausted. This is not the case when terminally-exhausted CD8 T cells are transferred (Im et al. 2016). In the absence of TCF-1, the formation of progenitor-exhausted CD8 T cells is impaired (Im et al. 2016; Jadhav et al. 2019).Terminally-exhausted CD8 T cells retain some cytotoxic activity. They continue to proliferate but fail to proliferate further upon additional stimulation (Im et al. 2016; McLane et al. 2019).

All these findings show that TCF-1 is important for T cell self-renewal and expansion. It is involved in memory CD8 T cell differentiation during acute infections. TCF-1 is also essential for "pseudo-memory" CD8 T cells during chronic viral infections.

1.6.4 TOX, the exhausted CD8 T cell-specific transcription factor.

TOX belongs to the high mobility group (HMG) box proteins and, like TCF-1 and lymphoid enhancer-binding factor 1 (LEF-1), possesses a single DNA-binding HMG box motif. TOX has been predicted to bind in a sequence-independent, structure-dependent manner (O'Flaherty and Kaye 2003).

During chronic LCMV infection, TOX is rapidly expressed at high levels, whereas during acute infection it is only transiently expressed at low levels (Alfei et al. 2019; Khan et al. 2019; Yao et al. 2019). *In vitro*, peptide-stimulated CD4 T cells or concanavalin A-stimulated splenocytes do not express TOX (Wilkinson et al. 2002). In concordance with models of what influence T cell exhaustion, TOX expression depends on antigen quantity rather than antigen affinity (Alfei et al. 2019). NFAT2 induces the expression of TOX in a calcineurin-dependent manner. But sustained TOX expression requires neither calcineurin nor NFAT2 (Khan et al. 2019; Scott et al. 2019).

Dietmar Zhen and colleagues examined the impact of deleting TOX in clone 13 chronic LCMV infections using T cells with a truncated version of TOX (TOX^{Δ}). At early stages of infection, TOX^{Δ} CD8 T cells present a more polyfunctionnal phenotype (increased IFN-γ and TNF-α production) compared to TOX WT CD8 T cells. However, a dramatic decrease of both progenitor- and terminally-exhausted cells is observed in TOX^{Δ} CD8 T cells during chronic LCMV infection compared to WT CD8 T cells. The authors suggest that the decrease of terminally-exhausted CD8 T cells results from the loss of progenitor-exhausted CD8 T cells, indicating that TOX is required to maintain progenitor-exhausted CD8 T cells. Interestingly, when TOX is deleted 20 days after infection, no phenotypic differences were observed. It suggests that TOX is required for the

induction of the exhaustion program but not for its immediate maintenance (Alfei et al. 2019).

Forcing TOX expression in CD8 T cells drives key features of T cell exhaustion, e.g. little IFN-γ/TNF-α production and increased PD-1/LAG-3 expression, indicating that TOX is sufficient to establish the exhaustion program. Moreover, expressing TOX in a fibroblast cell line induced a transcriptional signature that resembles the one of exhausted T cells (Alfei et al. 2019; Khan et al. 2019).

In tumor models, TOX expression in TILs correlated with PD-1 expression (Khan et al. 2019; Scott et al. 2019; Yao et al. 2019). Schietinger and colleagues showed that TOX expression is dependent on chronic TCR stimulation rather than the immunosuppressive tumor microenvironment. Indeed, TOX expression is only detected in tumor-specific TILs and not in bystander TILs. In line with data from the LCMV model, complete deletion of TOX alters CD8 T cell survival (Scott et al. 2019). However, CD8 T cells heterozygous for TOX control tumor growth better than WT CD8 T cells (Khan et al. 2019; Yao et al. 2019).

In humans, TOX expression has been detected in anti-HCV CD8 T cells of patients with chronic but not resolved HCV infection, but not in influenza-specific memory CD8 T cells (Alfei et al. 2019). In cancer, TOX expression is detected in TILs from melanoma, lung, breast and ovarian tumors (Scott et al. 2019).

Collectively, these findings strongly suggest that TOX is one of the pioneer factors establishing the exhaustion transcriptional program. Additionally, TOX appears to be a better marker of exhausted CD8 T cells than inhibitory receptors.

1.6.5 IRF4 is essential for CD4 and CD8 T cell responses.

IRF4 is a transcription factor that is upregulated in T cells after activation. This upregulation is dependent on signal strength. IRF4 regulates the expression of key T cell transcription factors including T-BET, BLIMP-1 and TCF-1 (Mahnke et al. 2016; Man et al. 2013; Wu et al. 2017).

1.6.5.1 IRF4 is required for CD4 T cell responses.

In CD4 T cells, IRF4 is required for successful Th1 responses. In a *Listeria monocytogenes* infection model, IRF4-KO mice fail to develop an appropriate Th1 response. CD4 T cells from IRF4-KO mice produce less IFN-γ, TNF-α and CD40L. Not surprisingly, T-BET, the Th1 lineage transcription factor, is less expressed by CD4 T cells in IRF4-KO mice. CD4 T cells from IRF4-KO mice proliferate less, reduce glycolysis and have decreased maximal respiratory capacity compared to CD4 T cells from WT mice (Mahnke et al. 2016).

In an allogeneic heart transplantation model, absence of IRF4 protects from graft rejection (Wu et al. 2017). Allograft rejection is mediated by CD4 T cells and is impaired in IRF4-KO mice. IRF4-deficient CD4 T cells have a decreased IFN-γ and IL-17 production and an increase in PD-1 expression. This increased PD-1 expression is due to HELIOS expression in absence of IRF4. Blocking PD-1 in IRF4-KO mice prevented allograft acceptance (Wu et al. 2017).

1.6.5.2 IRF4 is essential for CD8 T cell responses but participates to T cell exhaustion.

Deleting IRF4 impairs CD8 T cell responses to acute LCMV infection (Man et al. 2013). Compared to WT, IRF4-KO CD8 T cells express less granzyme B, and produce less IFN-γ and TNF-α. In IRF4-KO mice, the frequency of virus-specific CD8 T cells is dramatically reduced due to increased cell death. Regarding

metabolic reprogramming, IRF4-KO CD8 T cells have a reduced glycolysis (Man et al. 2013).

Impaired CD8 T cell response was also reported in acute influenza infection. IRF4 was dispensable to trigger T cell activation and proliferation but was essential for sustaining CD8 T cell expansion because IRF4-KO CD8 T cells die at higher rates. IRF4 was responsible for the repression of CDK inhibitors expression and the pro-apoptotic molecule Bim (Yao et al. 2013).

A third research group showed also an impaired CD8 T cell response in a *Listeria monocytogenes* infection model. IRF4-KO CD8 T cells fail to mount a protective immune response with similar defects as IRF4-KO CD8 T cells during acute viral infection: expression of effector molecules (Granzyme B and K, perforin, IFN-γ and TNF-α) is downregulated in IRF4-KO CD8 T cells compared to WT ones. They also showed that IRF4 controls the expression of key transcription factors for CD8 T cell differentiation. IRF4-KO CD8 T cells express less T-BET and BLIMP-1 and more EOMES and BCL6 compared to WT CD8 T cells (Raczkowski et al. 2013).

Although IRF4 is essential for mounting effective CD8 T cell responses, IRF4 also seems important in initiating CD8 T cell exhaustion in the LCMV model. CD8 T cells from mice with chronic LCMV infections express higher level of IRF4 than CD8 T cells from mice with acute LCMV infections. To examine the impact of IRF4 in chronic LCMV infections, $IRF4^{+/fl}$ CD4-Cre mice were used. This partial IRF4-KO avoids excessive expansion issues of anti-LCMV CD8 T cells as seen in full KO. Partial deletion of IRF4 decreased the expression of inhibitory receptors PD-1, TIM-3, LAG-3, 2B4, TGIT and CTLA-4. It increased IFN-γ, TNF-α and IL-2 production. Exhausted CD8 T cells have metabolic defects such as decreased membrane potential of mitochondria and decreased mitochondrial

mass. The partial deletion of IRF4 rescues metabolic defects: increased glucose uptake, expression of CD98 (an amino acid transporter chain) and expression of CD71 (a transferrin receptor). IRF4 participates in establishing the exhaustion transcriptional profile and represses memory differentiation by blocking TCF-1 expression (Man et al. 2017).

The example of IRF4 clearly shows that while a transcription factor can be essential for mounting a robust immune response; its overexpression can result in T cell exhaustion in certain conditions, such as chronic viral infection.

1.6.6 HELIOS.

HELIOS is encoded by the IKAROS Zinc finger protein 2 gene (*IKZF2*). It belongs to the IKAROS family alongside IKAROS (*IKZF1*), HELIOS (*IKZF2*), AIOLOS (*IKZF3*), EOS (*IKZF4*) and PEGASUS (*IKZF5*). All IKAROS family members share a common structure characterized by one Zinc finger group at the N-terminal and another at the C-terminal. Members of the IKZF family form homo or heterodimers (McCarty et al. 2003). HELIOS has been shown to interact with components or the chromatin remodeling complex NuRD (Sridharan and Smale 2007). NuRD possesses a histone deacetylase activity and thus is associated with repression of gene expression.

1.6.6.1 HELIOS stabilizes regulatory T cell phenotype.

As shown by co-staining FOXP3 and HELIOS, around 70% of murine and human CD4 Tregs express HELIOS (Thornton et al. 2010). For humans, this is an approximation as FOXP3 is expressed by Tregs and some activated CD4 T cells (Baron et al. 2007). The authors claim that HELIOS is a marker that distinguishes thymic-derived from peripherally induced CD4 Tregs because over time $FOXP3^{+}$ $HELIOS^{-}$ CD4 Tregs appear in the periphery. Supporting this theory, *in vitro*

differentiated human and mouse CD4 Tregs do not express HELIOS. Similar results were obtained with an *in vivo* mouse model in which peripheral CD4 T cells are converted into Tregs using oral exposure to the antigen (Mucida et al. 2005; Sun et al. 2007; Thornton et al. 2010).

HELIOS is required to maintain the Treg phenotype, but to a lesser extent than FOXP3. *Ikzf2*-KO mice develop autoimmune symptoms after 6 to 8 months compared to FOXP3-deficient (scurfy) mice which die within weeks (Ju et al. 2012; Kim et al. 2015). The role of HELIOS in T cells was confirmed using *Rag2* KO mice (mice without T cells) adoptively transferred with *Ikzf2*-KO T cells: these mice developed autoimmunity. Direct implication of Tregs was shown using *Foxp3*-Cre *Ikzf2*$^{fl/fl}$ mice as they developed autoimmune symptoms after 5 months and bone marrow (BM) chimeras reconstituted with *Ikzf2*$^{-/-}$ BM and Scurfy BM rapidly developed autoimmunity (Kim et al. 2015). However, controversy still exists: CD4-Cre *Ikzf2*$^{fl/fl}$ mice lacked autoimmune symptoms, which is not coherent with the findings discussed above (Skadow et al. 2019; Thornton et al. 2010).

The activity of both CD4 and CD8 Tregs are disturbed in the absence of HELIOS (Kim et al. 2015). HELIOS^{+} CD4 Tregs suppress T cell functions more effectively than HELIOS^{-} CD4 Tregs (Sugita et al. 2015; Thornton et al. 2019). HELIOS binds directly to the IL-2 promoter and represses IL-2 expression in CD4 Tregs (Baine et al. 2013). Additionally, it has been shown that engineered Tregs - which overexpress FOXP3 - that also overexpress HELIOS have better immunosuppressive properties (Seng et al. 2020).

Although the term Tregs usually refers to CD4 Tregs, CD8 Tregs have been described. In a murine model of experimental autoimmune encephalitis (EAE), CD8 Tregs recognize and eliminate pathogenic CD4 T cells. Anti-MOG

(Myelin Oligodendrocyte Glycoprotein) TCR Vβ8.2 CD4 T cells mediate EAE development. Immunizing mice with a non-self-antigen, which triggers a TCR Vβ8.2 CD4 T cell response, protects mice from developing EAE. This protection is mediated by Qa-1-restricted CD8 Tregs that recognize peptides from TCR Vβ leader sequences presented by Qa-1 (murine homolog of HLA-E) and lyse them (Hu et al. 2004; Jiang et al. 1998; Tang et al. 2006; Varthaman et al. 2010, 2011)(Varthaman et al. 2011). CD8 Tregs are also able to inhibit lupus-like autoimmune disease through the specific recognition of Qa-1/peptide complexes on follicular helper CD4 T cells. HELIOS-deficient CD8 Tregs failed to protect mice against autoimmune disease underlying a similar role for HELIOS in CD8 Tregs and CD4 Tregs (Kim et al. 2015).

BOX 4 **HLA-E**

HLA-E is a MHC class Ib molecule. It is less polymorphic than MHC class Ia molecule (HLA-A/B/C). Only two functional alleles have been described: HLA-E*01:01 and HLA-E*01:03 (Kraemer, Blasczyk, & Bade-Doeding, 2014).

Under physiological conditions, HLA-E form a trimeric complex with the β2-microglobuline (β2m) and a peptide derived from the leader sequence of MHC class Ia or HLA-G. It binds to CD94/NKG2A and gives an inhibitory signal to NK cells to prevent their cytotoxic activity towards healthy cells (Kraemer et al., 2014).

During an infection, if a pathogen interferes with MHC class Ia expression, there is no more peptide loaded on HLA-E which is then sequestered inside the cell. This removes the inhibitory signal to NK cells allowing them to lyse the infected cell that cannot be recognized by CD8 T cells (Kraemer et al., 2014).

Some peptides derived from pathogens proteins bind to HLA-E. In this case, viruses like CMV lower MHC class Ia expression without compromising the presence of HLA-E at the cell surface. But, NK cells have been shown to recognize changes in peptide sequence loaded on HLA-E leading to CD94/NKG2C activation which triggers cytotoxic activity and cytokine secretion (IFN-γ, TNF-α and CCL3) (Hammer et al., 2018). In addition, some CD8 T cells recognize pathogens-derived peptides presented by HLA-E and lyse their target (Pietra et al., 2003; Romagnani et al., 2004).

1.6.6.2 HELIOS role in effector CD8 T cells: activation, exhaustion marker or neither?

In mice, HELIOS expression is upregulated in exhausted CD8 T cells during chronic LCMV infection (Doering et al. 2012; Khan et al. 2019; Pereira et al. 2017). CD8 T cells from mice with dysregulated NFAT1-AP1 signalling share common characteristics with exhausted CD8 T cells (increased expression of PD-1, TIM-3 and LAG-3 and TOX) and upregulate HELIOS expression (Martinez et al. 2015).

Akimova et al suggested that HELIOS is a marker of T cell activation because they observed increased HELIOS levels in proliferating T cells *in vitro* (Akimova et al. 2011). However, other authors failed to reproduce this observation: HELIOS was not expressed by CD8 T cells directed against the HLA-A2-restricted epitope of the tick-borne encephalitis virus (TBEV) during the immune response (Blom et al. 2015). In fact, in complete contradiction to Akimova et al, activated CD8 T cells (Ki-67$^+$ and CD38$^+$) expressed less HELIOS than resting CD8 T cells (Ki-67$^-$ CD38$^-$) (Blom et al. 2015).

The precise role of HELIOS in CD8 T cells remains elusive. It is claimed to be essential for CD8 Tregs and upregulated in exhausted CD8 T cells in mice. In parallel, it is claimed as an activation marker for murine and human CD8 T cells *in vitro* which could not be corroborated *in vivo* in humans.

1.7 Aim of the study.

1.7.1 Global objective: Study exhaustion-related transcription factors (IRF4, HELIOS and TOX) in CD8 T cells.

The literature on transcription factors implicated in CD8 T cell exhaustion reports contradictory data obtained mostly in mice. One of the best examples is IRF4 which is required for initiating CD8 T cell responses but also involved in CD8 T cell exhaustion initiation. Another one is HELIOS which is reported as expressed by murine exhausted CD8 T cells but is already expressed by CD8 T cells from human blood donors.

Our goal is to better understand the role of exhaustion-related transcription factors in human CD8 T cells. Long term, the results of this study could help improve chimeric antigen receptor (CAR) T cells against solid tumors by avoiding CAR T cell exhaustion.

To achieve this goal, we used ovarian cancer TILs to correlate the expression of exhaustion-related transcription factors with CD8 T cell function, e.g. IFN-γ production. We also planned to perform scRNA-seq analysis on CD8 TILs to have an unbiased approach. In parallel we tried to modulate *in vitro* the expression of these transcription factors and assess the impact on T cell functions (Figure 10).

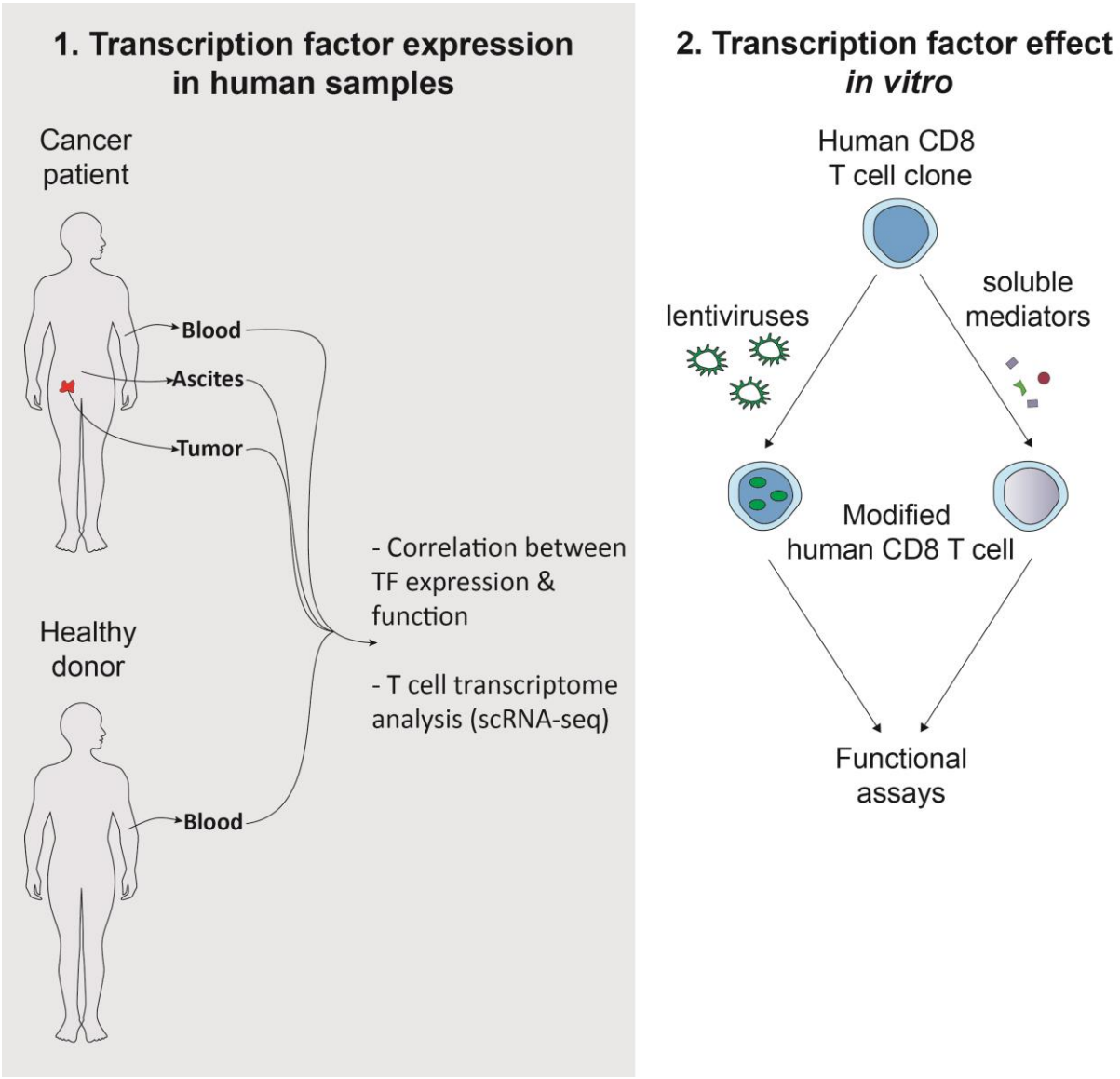


Figure 10. Project plan. *Step 1: Correlation between transcription factors expression and CD8 T cell function (IFN-γ) in tumor samples. Step 2: Modulation of transcription factors expression and impact on T cell function.*

1.7.2 Thesis objective: Characterize HELIOS+ CD8 T cells.

During a preliminary experiment, we noticed in the blood of non-cancerous donors the presence of HELIOS$^+$ CD8 T cells that did not produce high amounts of IFN-γ (Figure 11). This arouse our curiosity because HELIOS is expressed by murine exhausted CD8 T cells, by murine CD4 and CD8 Tregs and by human CD4 Tregs (Doering et al. 2012; Khan et al. 2019; Kim et al. 2015; Pereira et al. 2017; Thornton et al. 2010). Moreover, data on the nature of human HELIOS$^+$ CD8 T cells are scarce and contradictory.

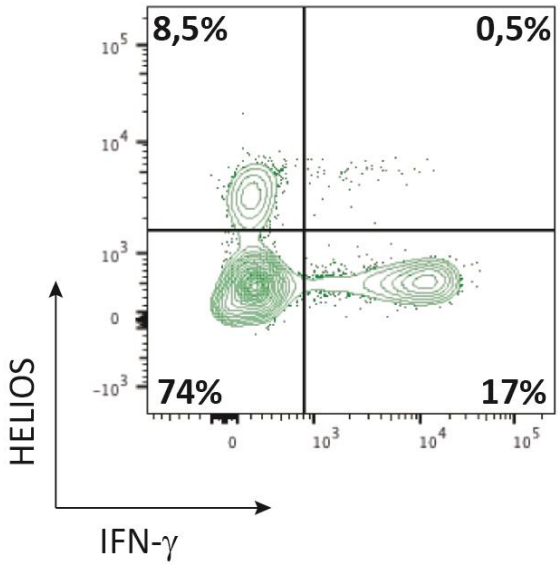


Figure 11. Preliminary observation: HELIOS$^+$ CD8 T cells produce low amounts of IFN-γ. *PBMCs were thawed for 2h at 37°C with 5U/ml DNase. Cells were stimulated with coated anti-CD3 (1μg/ml) for 5h at 37°C. After the first hour of stimulation, 5μg/ml of Brefeldin A was added to block cytokine secretion. At the end of the 5h stimulation, cells were stained for viability, CD2, CD8β and CD45RA. Cells were fixed and permeabilized overnight, and stained intracellularly for HELIOS and IFN-γ. Samples were analysed by flow cytometry. The graph shows IFN-γ production by HELIOS$^+$ and HELIOS$^-$ CD45RA$^-$ CD8 T cells for one donor (naïve and T_{EMRA} excluded).*

We decided to study this HELIOS$^+$ CD8 T cell population in the blood of donors to better define its characteristics and potentially understand its role. First, we decided to determine the distribution of HELIOS$^+$ CD8 T cells among naïve, T_{CM}, T_{EM} and T_{EMRA} and tried to identify their cognate antigens. Then, we used transcriptomic analysis to have an overview of HELIOS$^+$ CD8 T cells characteristics. We evaluated their ability to produce and secrete cytokines and to proliferate. Finally, we looked at the activation status of HELIOS$^+$ CD8 T cells in the blood of SARS-CoV2 patients and in TILs from ovarian cancer patients.

2 Results

2.1 HELIOS$^+$ CD8$^+$ T cells are antigen-experienced.

To determine the frequency and differentiation status of HELIOS$^+$ CD8 T cells in the blood of donors, we analysed peripheral blood mononuclear cells (PBMCs) by flow cytometry. HELIOS$^+$ CD8 T cells were present in every donor (mean of 21% of CD8 T cells) (Figure 12A and B). This observation is comparable to previous works (mean of 15% of CD8 T cells, 5 donors) (Blom et al. 2015).

Next, we analysed the expression of HELIOS by different CD8 T cell subpopulations (naïve, T_{CM}, T_{EM} and T_{EMRA}) by co-staining the cells with CCR7 and CD45RA. We observed that most HELIOS$^+$ CD8 T cells have already encountered their antigen and are either T_{EM} (CCR7$^-$ CD45RA$^-$) or T_{EMRA} (CCR7$^-$ CD45RA$^+$) (Figure 12C and D).

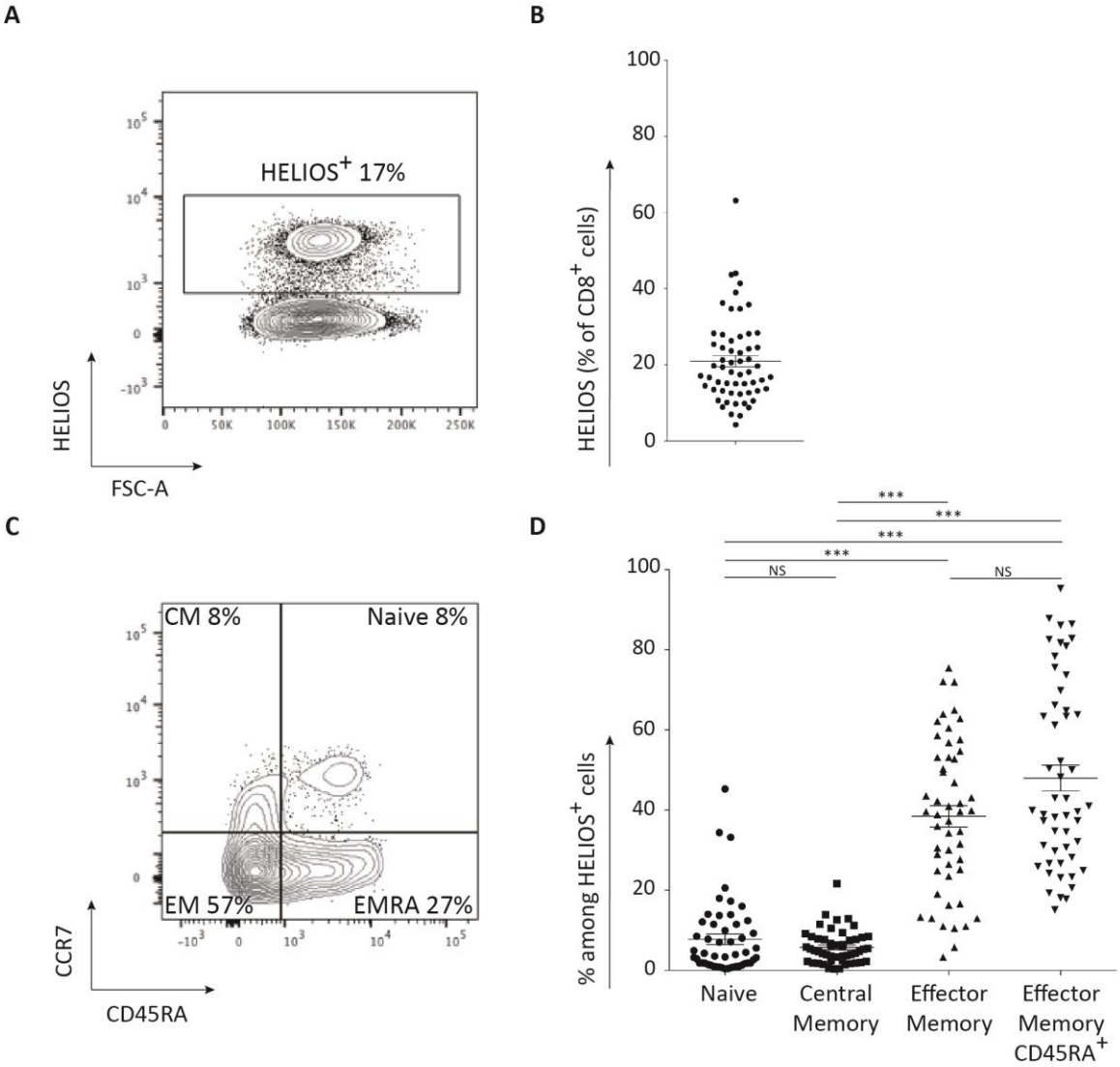


Figure 12. The majority of HELIOS$^+$ CD8 T cells are antigen-experienced. *PBMCs were thawed and rested for 2h at 37°C. The cells were stained for viability, CD2, CD8β, CCR7 and CD45RA. Next, the T cells were fixed and permeabilized overnight and stained intracellularly for HELIOS at 4°C. The samples were analysed by flow cytometry.* ***(A)*** *The prevalence of HELIOS$^+$ cells in living CD2$^+$ CD8β^+ cells is shown for one representative donor and* ***(B)*** *for 58 donors.* ***(C)*** *Distribution of living CD2$^+$ CD8β^+ HELIOS$^+$ cells among T cell subsets is shown for one representative donor and* ***(D)*** *for 51 donors. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (one-way ANOVA).*

2.2 HELIOS$^+$ CD8 T cells are mostly HLA-E-restricted.

As the majority of HELIOS$^+$ CD8 T cells are antigen-experienced, we tested if HELIOS$^+$ CD8 T cells could be stained by HLA class Ia (HLA-A/B/C) multimers loaded with either CMV, EBV or influenza peptides on HLA-A2 or by CMV peptides on HLA-B7. Strikingly, none of these multimers stained the HELIOS$^+$ CD8 T cells (Figure 13A).

Next, we examined if HELIOS$^+$ CD8 T cells recognize a CMV peptide presented by the HLA class Ib molecule HLA-E. As CD94/NKG2 receptors bind to HLA-E, CD94 was blocked with an antibody during the staining. An HLA-E multimer loaded with a *Mycobacterium tuberculosis* (*Mtb*) peptide was used as a negative control for staining specificity. HELIOS was expressed by some HLA-E-restricted anti-CMV CD8 T cells (between 5% and 90%) (Figure 13A and B). This HLA-E-restricted anti-CMV CD8 T cell population represents about 17% of HELIOS$^+$ CD8 T cells and 6% of CD8 T cells (Figure 13C and D). Using an HLA-E multimer loaded with an EBV peptide, we only found one donor in whom multimer$^+$ CD8 T cells were detected. In this donor, the majority of HLA-E-restricted CD8 T cells expressed HELIOS (Figure 13A and C).

These results suggest that HELIOS expression is rather HLA-dependent than virus-dependent because CD8 T cells that recognized peptides on HLA-E expressed HELIOS at significantly higher frequency than CD8 T cells that recognized peptides of the same virus presented on HLA class Ia.

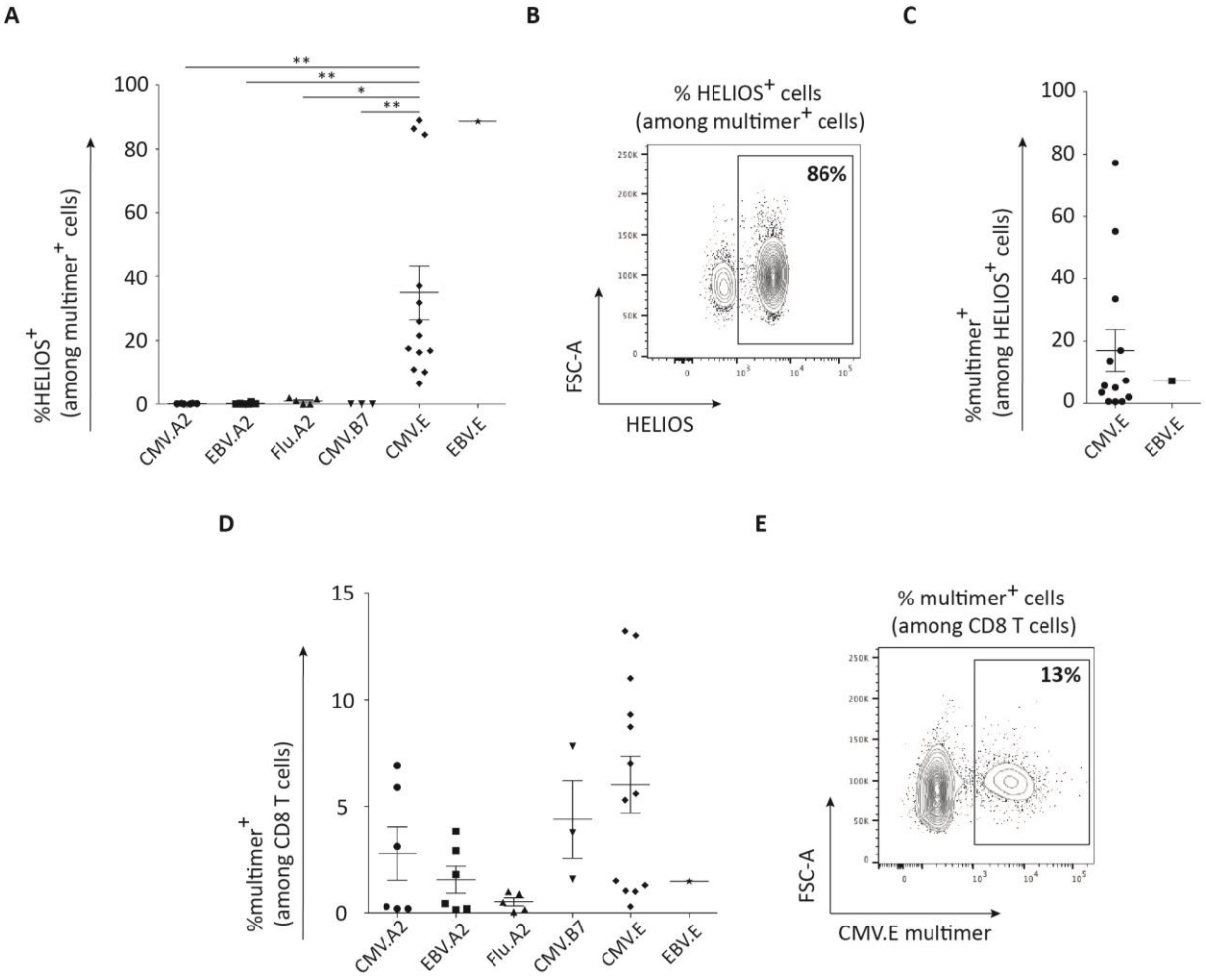


Figure 13. HELIOS$^+$ CD8 T cells are found in HLA-E-restricted T cells. *PBMCs were thawed and rested for 2h at 37°C. The cells were labelled with a multimer and stained for viability, CD2, CD8β, CCR7, CD45RA. Next, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS at 4°C. Samples were analysed by flow cytometry.* ***(A)*** *Frequency of HELIOS$^+$ cells in living CD2$^+$ CD8β$^+$ multimer$^+$ cells are shown for virus-derived peptides presented by HLA-A2, HLA-B7 and HLA-E. CMV.A2 (pp65: NLVPMVATV) and EBV.A2 (BMF-L1: GLCTLVAML) multimer$^+$ CD8 T cells were detected in 6 out of 11 HLA-A2 donors and 5 out of 11 for Flu.A2 (M1: GILGFVFTL) multimer. CMV.B7 (pp65: RPHERNGFTVL or TPRVTGGGAM) multimer$^+$ CD8 T cells were detected in 2 out 4 HLA-B7 donors for peptide RPHERNGFVTL and 1 out of 4 HLA-B7 donors for peptide TPRVTGGGAM. CMV.E (UL40: VMAPRTLIL) multimer$^+$ CD8 T cells were detected in 13 out of 20 non-typed donors. EBV.E (BZF-L1: SQAPLPCVL) multimer$^+$ CD8 T cells were detected in 1 out of 20 non-typed donors.* ***(B)*** *Percentage of HELIOS$^+$ cells among multimer$^+$ cells are shown for one donor with the CMV.E multimer.* ***(C)*** *Percentage of multimer$^+$ cells among HELIOS$^+$ CD8 T cells are shown for CMV.E and EBV.E multimers.* ***(D)*** *Frequency of multimer$^+$ cells for patients with multimer$^+$ cells are shown.* ***(E)*** *Percentage of multimer$^+$ cells among CD8 T cells are shown for one patient with the CMV.E multimer. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (Kruskal-Wallis test).*

2.3 Transcriptomes of HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}.

To better understand their characteristics, we compared HELIOS$^+$ with HELIOS$^-$ CD8 T cells using RNA-sequencing. Freshly isolated CD8 peripheral blood lymphocytes (PBLs) from 3 donors were activated or not for 5h with plate-bound anti-CD3 at 37°C. Activation was used to detect the whole panel of cytokines differentially expressed between both populations. After activation, cells were separated by flow cytometry into HELIOS$^+$ and HELIOS$^-$ populations. Fixing and permeabilising cells for intracellular antibody staining, as is required for HELIOS, degrades RNA. To avoid RNA degradation, we added RNase inhibitors to the buffers until RNA extraction could be performed (Hrvatin et al. 2014) (Figure 14). We only analysed the transcriptomes of T_{EM} because in the donors we used, we isolated more T_{EM} than T_{EMRA} and were able to extract sufficient amounts of RNA only from T_{EM}. Library construction and RNA-sequencing were performed by Genewiz.

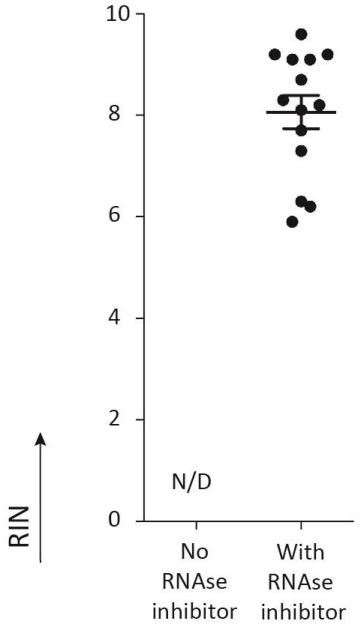
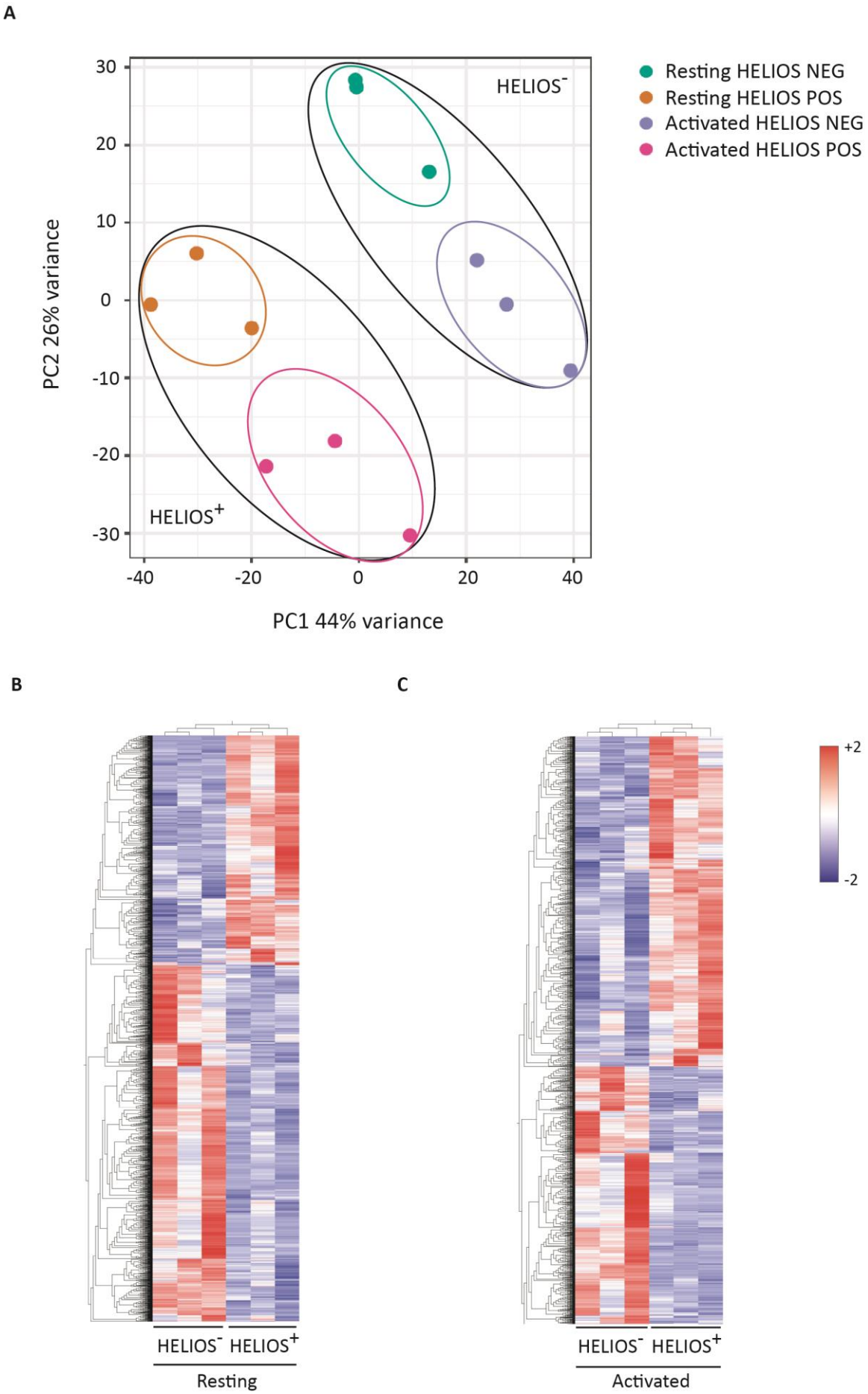


Figure 14. RNase inhibitors improve extraction of good quality RNA from sorted fixed and permeabilized cells. *PBMCs were thawed and rested for 2h at 37°C. Next, cells were submitted to the intracellular staining procedure in presence or absence of RNase inhibitors. RNA was then extracted and RNA integrity number (RIN) was measured using the bioanalyzer from Agilent. N/D means not determined: RNA was too degraded to determine a RIN score.*

2.3.1 Transcriptomic differences between CD8 T_{EM} expressing HELIOS or not.

The principal component analysis (PCA) plot shows that HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} have distinct transcriptional programs. Within each group, samples are separated based on activation (Figure 15A). We analysed the differentially expressed genes (DEGs) between resting HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}, and activated HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. DESeq2 was used for DEGs analysis. We identified 1822 DEGs in resting and 1179 DEGs in activated conditions (adjusted p-value < 0,05) (Love, Huber, and Anders 2014) (Figure 15B and C).

Figure 15. HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} have distinct transcriptional programs. *Freshly isolated CD8 PBLs from 3 donors were activated or not with plate-bound anti-CD3 antibody (1μg/ml) for 5h at 37°C. Cells were labelled for viability, CD2, CD8β, CCR7 and CD45RA. Next, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS in the presence of RNase inhibitors at 4°C. HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) were sorted by flow cytometry. RNA was extracted to perform paired-end RNA-sequencing. Data normalization was done with DESeq2.* ***(A)*** *Principal component analysis of RNA-seq expression data is shown. Each dot represents one sample. (B) Heatmap of differentially expressed genes between resting HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. (C) Heatmap of differentially expressed genes between activated HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. Analysis was performed with DESeq2 (differentially expressed gene: adjusted p-value < 0,05).*


A
Resting HELIOS NEG
Resting HELIOS POS
Activated HELIOS NEG
Activated HELIOS POS
HELIOS-
HELIOS+
PC2 26% variance
PC1 44% variance
30
20
10
0
-10
-20
-30
-40
-20
0
20
40
B
C
+2
-2
HELIOS- HELIOS+
Resting
HELIOS- HELIOS+
Activated

2.3.2 HELIOS+ CD8 T_{EM} react to anti-CD3 activation.

We compared resting versus activated HELIOS$^+$ CD8 T_{EM} and resting versus activated HELIOS$^-$ CD8 T_{EM}. Activation caused the differential expression of 2857 genes in HELIOS$^+$ CD8 T_{EM} and 3447 genes in HELIOS$^-$ CD8 T_{EM}. 1936 genes are differentially expressed upon activation in both HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} (Figure 16). Yet, 1511 genes are differentially expressed upon activation uniquely in HELIOS$^-$ CD8 T_{EM} and 921 genes in HELIOS$^+$ CD8 T_{EM} (Figure 16).

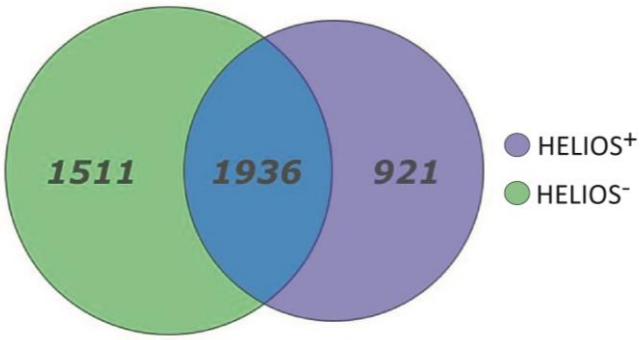


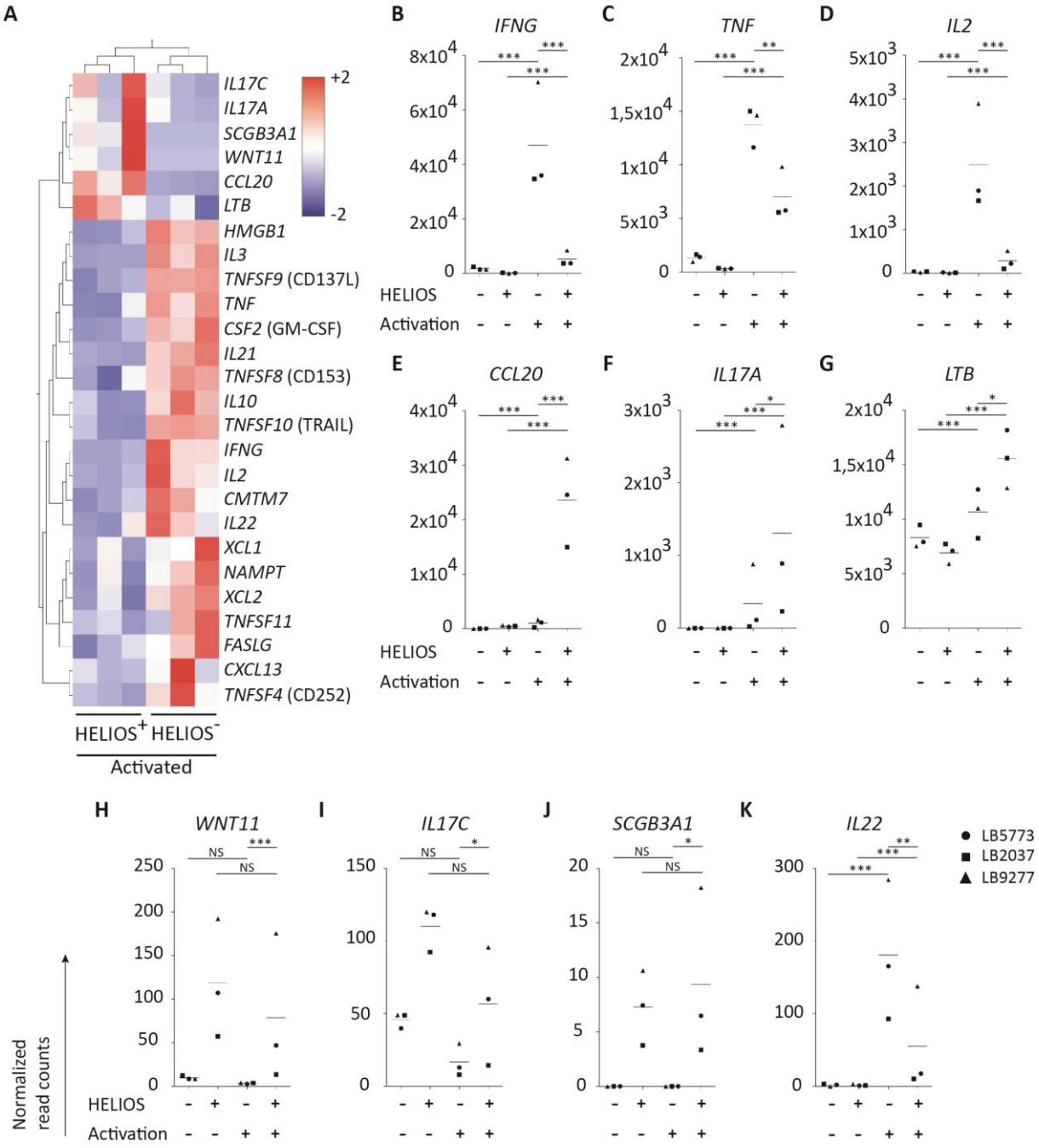
Figure 16. Different gene sets are differentially expressed in HELIOS$^+$ and HELIOS$^-$ CD8 T cells upon T cell activation. *Freshly isolated CD8 PBLs from 3 donors were activated with plate-bound anti-CD3 antibody (1μg/ml) for 5h at 37°C. Cells were labelled for viability, CD2, CD8β, CCR7 and CD45RA. Cells were then fixed and permeabilized overnight and stained intracellularly for HELIOS in presence of RNase inhibitors at 4°C. HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) were sorted by flow cytometry. RNA was then extracted to perform paired-end RNA-sequencing. Data normalization and analysis was done with DESeq2. Activation-induced DEGs in both HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} were compared using a Venn diagram.*

2.3.3 HELIOS+ CD8 T_{EM} express more CCL20 and IL-17A but less IFN-γ, TNF-α and IL-2.

We selected differentially expressed cytokines in HELIOS$^+$ CD8 T_{EM} based on gene ontology annotations. Most of the differentially expressed cytokines are less expressed in HELIOS$^+$ CD8 T_{EM} (Figure 17A). They express less *IFNG*, *TNF* and *IL2*; three cytokines associated with effector CD8 T cell functions (Figure 17A, B, C and D). In contrast, *LTB*, *IL17A* and *CCL20* are expressed at higher levels by HELIOS$^+$ CD8 T_{EM} (Figure 17A, E, F and G). *IL17A* and *CCL20* are two

cytokines expressed by Th17/Tc17. The expression of *WNT11*, *SCGB3A1* and *IL17C* is increased in HELIOS$^+$ CD8 T_{EM} and activation independent (Figure 17H, I and J). *IL22*, a cytokine expressed by Th22/Tc22 that can also be expressed by Th17/Tc17, is expressed at lower levels in HELIOS$^+$ CD8 T_{EM} (Figure 17K). Although DESeq2 does not allow to compare expression levels between genes, TPM normalisation shows that *WNT11*, *SCGB3A1*, *IL17C* and *IL22* are expressed at much lower levels compared to the other cytokines mentioned above (data not shown). Thus our results suggest the presence of Tc17 among HELIOS$^+$ CD8 T cells.

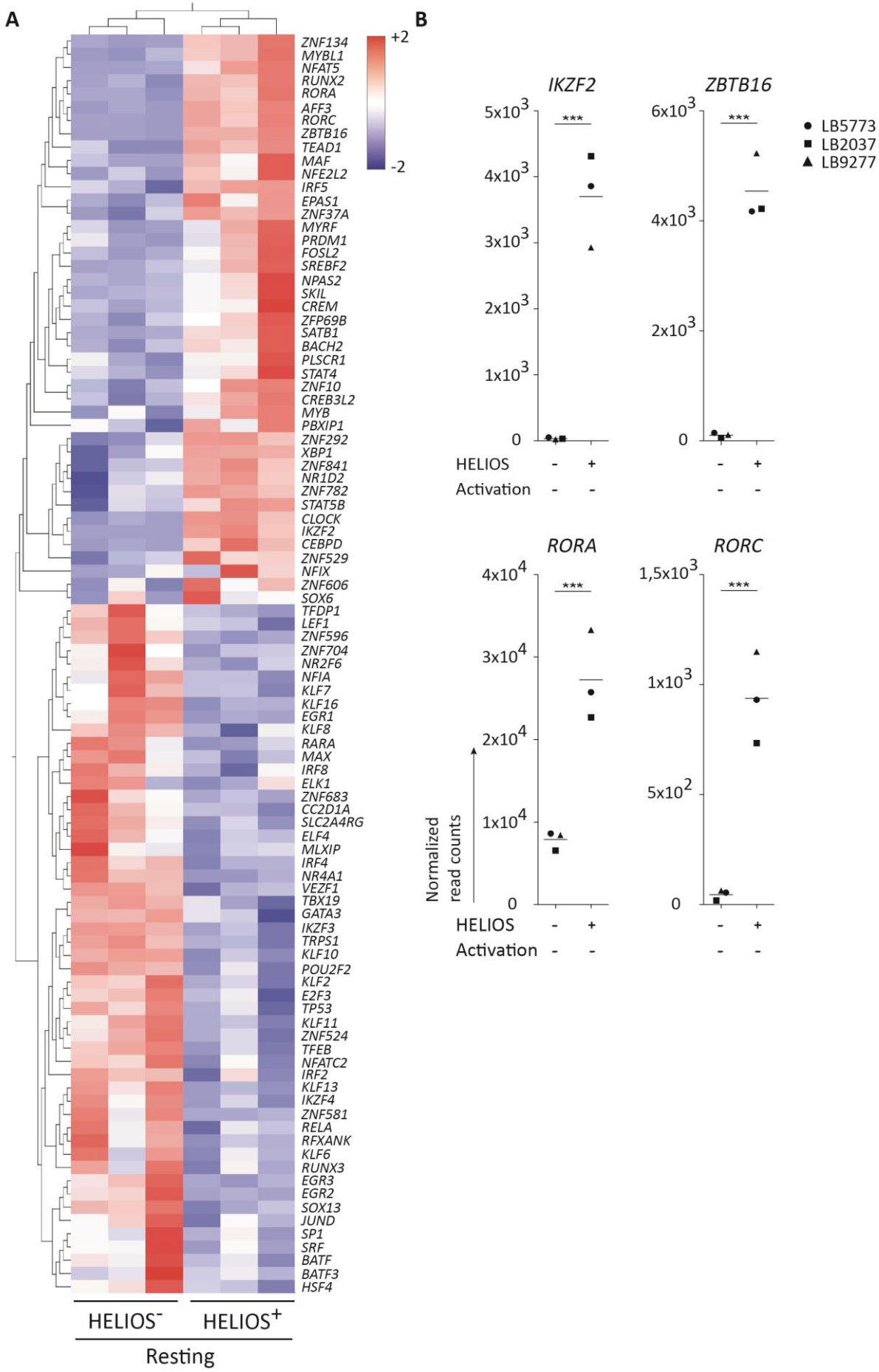
Figure 17. Heatmap of differentially expressed cytokines between activated HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. *Freshly isolated CD8 PBLs were activated or not with plate-bound anti-CD3 antibody (1μg/ml) for 5h at 37°C. Cells were labelled for viability, CD2, CD8β, CCR7 and CD45RA. Next, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS in presence of RNase inhibitors at 4°C. HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) were sorted by flow cytometry. RNA was extracted to perform paired-end RNA-sequencing. Data normalization and analysis was done with DESeq2* ***(A)*** *Heatmap of differentially expressed cytokines between activated HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. Cytokines were selected based on gene ontology annotations GO:0005125 (cytokine activity) and GO:0008009 (chemokine activity). Gene expression values for* ***(B)*** *IFNG,* ***(C)*** *TNF,* ***(D)*** *IL2,* ***(E)*** *CCL20,* ***(F)*** *IL17A,* ***(G)*** *LTB,* ***(H)*** *WNT11,* ***(I)*** *IL17C,* ***(J)*** *SCGB3A1 and* ***(K)*** *IL22 are shown in normalized read counts. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (DESeq2 paired samples analysis).*


A
IL17C
IL17A
SCGB3A1
WNT11
CCL20
LTB
HMGB1
IL3
TNFSF9 (CD137L)
TNF
CSF2 (GM-CSF)
IL21
TNFSF8 (CD153)
IL10
TNFSF10 (TRAIL)
IFNG
IL2
CMTM7
IL22
XCL1
NAMPT
XCL2
TNFSF11
FASLG
CXCL13
TNFSF4 (CD252)
+2
-2
HELIOS+ HELIOS-
Activated
B IFNG
C TNF
D IL2
E CCL20
F IL17A
G LTB
HELIOS
Activation
H WNT11
I IL17C
J SCGB3A1
K IL22
Normalized read counts
LB5773
LB2037
LB9277

2.3.4 HELIOS$^+$ CD8 T_{EM} express Th17-related transcription factors.

IKZF2 encodes HELIOS. Accordingly, *IKZF2* is expressed at higher levels by HELIOS$^+$ than HELIOS$^-$ CD8 T_{EM} (Figure 18A and B). We found that other members of the *IKZF* family were differentially expressed: *IKZF3* and *IKZF4*. We observed 1,5 fold higher expression in HELIOS$^-$ CD8 T cells of *IKZF3* and *IKZF4*. *IRF4* was twice as highly expressed by HELIOS$^-$ CD8 T_{EM} (Figure 18A). Surprisingly, Th17-lineage transcription factors or transcription factors related to Th17 are more highly expressed by HELIOS$^+$ CD8 T_{EM}: *RORA* (RORα) 3,3x, *RORC* (RORγt) 18,8x and *ZBTB16* (PLZF) 41,5x (Figure 18A and B). These results suggest again the presence of Tc17 among HELIOS$^+$ CD8 T cells.

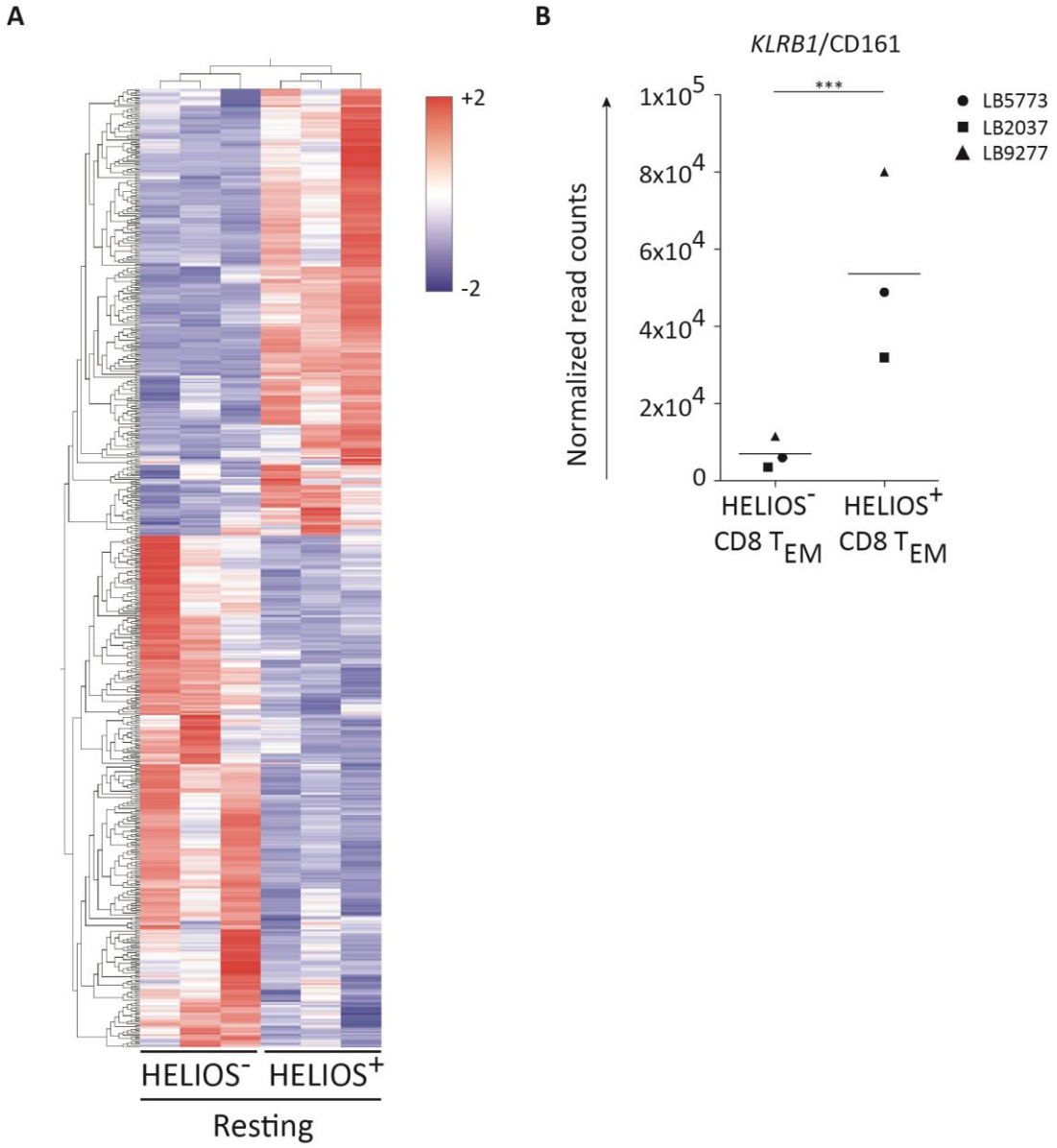
Figure 18. HELIOS$^+$ CD8 T_{EM} express Th17/Tc17-related transcription factors. *Freshly isolated CD8 PBLs from 3 donors were activated with plate-bound anti-CD3 antibody (1μg/ml) for 5h at 37°C. Cells were labelled for viability, CD2, CD8β, CCR7 and CD45RA. Then, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS in presence of RNase inhibitors at 4°C. HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) were sorted by flow cytometry. RNA was then extracted to perform paired-end RNA-sequencing. Data normalization and analysis was done with DESeq2* ***(A)*** *Heatmap of differentially expressed transcription factors between resting HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. Transcription factors were selected based on gene ontology annotations GO:0003700, GO:0001228 and GO:0001227.* ***(B)*** *Expression values for IKZF2, ZBTB16, RORC and RORA are shown for the 3 donors in resting state. Expression values after 5h of activation are not relevant in this case. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (DESeq 2 paired samples analysis).*


A
B
+2
-2
ZNF134
MYBL1
NFAT5
RUNX2
RORA
AFF3
RORC
ZBTB16
TEAD1
MAF
NFE2L2
IRF5
EPAS1
ZNF37A
MYRF
PRDM1
FOSL2
SREBF2
NPAS2
SKIL
CREM
ZFP69B
SATB1
BACH2
PLSCR1
STAT4
ZNF10
CREB3L2
MYB
PBXIP1
ZNF292
XBP1
ZNF841
NR1D2
ZNF782
STAT5B
CLOCK
IKZF2
CEBPD
ZNF529
NFIX
ZNF606
SOX6
TFDP1
LEF1
ZNF596
ZNF704
NR2F6
NFIA
KLF7
KLF16
EGR1
KLF8
RARA
MAX
IRF8
ELK1
ZNF683
CC2D1A
SLC2A4RG
ELF4
MLXIP
IRF4
NR4A1
VEZF1
TBX19
GATA3
IKZF3
TRPS1
KLF10
POU2F2
KLF2
E2F3
TP53
KLF11
ZNF524
TFEB
NFATC2
IRF2
KLF13
IKZF4
ZNF581
RELA
RFXANK
KLF6
RUNX3
EGR3
EGR2
SOX13
JUND
SP1
SRF
BATF
BATF3
HSF4
HELIOS- HELIOS+
Resting
IKZF2
ZBTB16
RORA
RORC
***
LB5773
LB2037
LB9277
HELIOS
Activation
Normalized read counts

2.3.5 CD161 is a surface marker specific to HELIOS+ CD8 T cells.

We retrieved a list of potential surface markers based on gene ontology annotations (Figure 19A). We observed that CD161 (*KLRB1*), a NK cell receptor and Tc17 marker, is expressed by HELIOS$^+$ but not HELIOS$^-$ CD8 T_{EM} (Figure 19B) (Maggi et al. 2010).

Figure 19. Heatmap of differentially expressed surface markers between resting HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. *Freshly isolated CD8 PBLs from 3 donors were stimulated with plate-bound anti-CD3 antibody (1μg/ml) for 5h at 37°C. Cells were stained for viability, CD2, CD8β, CCR7 and CD45RA. Cells were then fixed and permeabilized overnight and stained intracellularly for HELIOS at 4°C. Cells were then FACS sorted. RNA was then extracted to perform paired-end RNA-sequencing.* ***(A)*** *Heatmap of differentially expressed surface markers between resting HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM}. Potential surface markers were selected base on the following gene ontology annotations: GO:0005886, GO:0004872 and GO:0009897.* ***(B)*** *Expression values for CD161/KLRB1 are shown for the 3 donors in resting state. Expression values after 5h of activation are not relevant in this case. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (DESeq 2 paired samples analysis).*



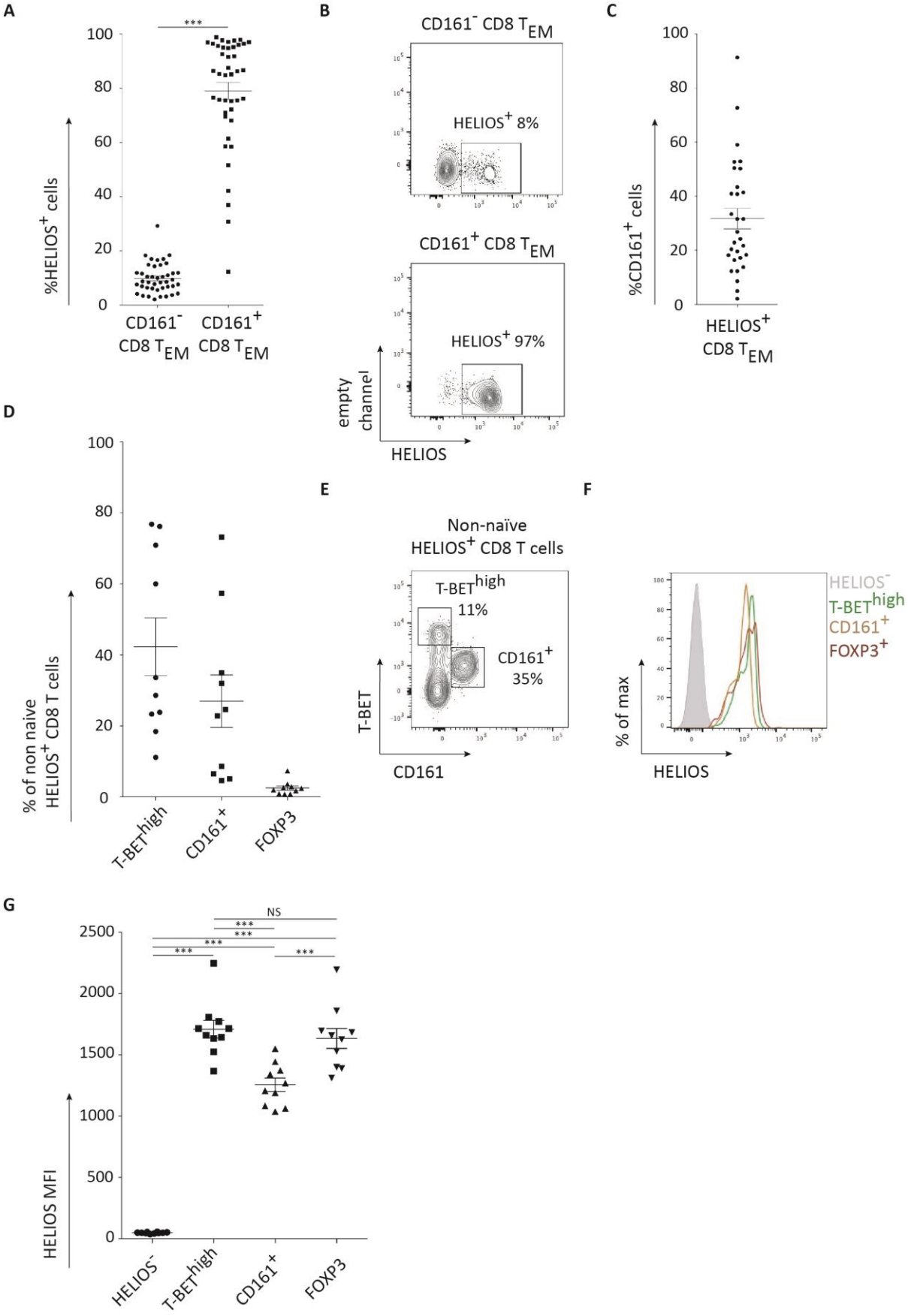
To confirm this observation, we analysed by flow cytometry CD8 PBLs labelled with anti-HELIOS and anti-CD161 antibodies. However, CD161 is not sufficient as a surface marker for HELIOS$^+$ CD8 T_{EM} to reliably separate pure HELIOS$^+$ and HELIOS$^-$ CD8 T_{EM} from each other by flow cytometry (Figure 20A, B and C). In some donors, HELIOS$^+$ CD8 T_{EM} and HELIOS$^-$ CD8 T_{EM} are separable with over 90% purity by using CD161 (Figure 20A and B). But in other donors less than 90% of CD161$^+$ CD8 T cells or more than 10% of CD161$^-$ CD8 T cells are HELIOS$^+$. Furthermore, only about one third of HELIOS$^+$ CD8 T_{EM} express CD161 (Figure 20C). However, while imperfect, it is the best surface marker that correlates to HELIOS$^+$ CD8 T_{EM} in some donors.

CD161 (*KLRB1*/NKRP-1A) is a C-type lectin that is expressed by a subset of NK cells. Upon LLT1 ligation, CD161 inhibits NK cell cytotoxicity (Aldemir et al. 2005; Rosen et al. 2005). CD161 is also expressed by a subset of CD8 T cells identified as T_{C17} (Maggi et al. 2010).

About a third of HELIOS$^+$ CD8 T_{EM} express CD161 and around a quarter of HELIOS$^+$ CD8 T cells (naïve excluded) (Figure 20C and D). By co-staining CD161, T-BET and FOXP3, we showed that HELIOS$^+$ CD8 T cells (naïve excluded) contain around 40% T-BET^{high} cells (Tc1 effectors), 25% CD161$^+$ cells (Tc17) and 2,5% FOXP3$^+$ cells (potentially Tregs) (Figure 20D and E). Additionally, we showed that HELIOS$^+$ Tc1 effectors and HELIOS$^+$ FOXP3$^+$ CD8 T cells express slightly more HELIOS than HELIOS$^+$ Tc17 (Figure 20F and G).

Our results suggest that HELIOS$^+$ CD8 T cells are heterogeneous and composed of several subpopulations. In the two next chapters, we will first focus on the cytokines produced and secreted by HELIOS$^+$ T-BET^{high} CD8 T cells (Tc1 effectors) and then on the cytokines secreted and proliferation capacity of CD161$^+$ HELIOS$^+$ CD8 T cells (Tc17).

Figure 20. CD161 is selectively expressed by HELIOS$^+$ CD8 T_{EM}. *PBMCs were thawed for 2h at 37°C. Cells were labelled for viability, CD2, CD8β, CCR7, CD45RA and CD161. Then, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS. Samples were analysed by flow cytometry.* ***(A)*** *Percentage of HELIOS$^+$ cells among CD161$^-$ and CD161$^+$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) are shown for 42 donors and* ***(B)*** *one donor.* ***(C)*** *Frequency of CD161$^+$ cells among HELIOS$^+$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$).* ***(D)*** *Frequency of Tc1 (T-BET^{high}), Tc17 (CD161$^+$) and FOXP3$^+$ cells among HELIOS$^+$ CD8 T cells are shown for 10 donors and* ***(E)*** *for one donor.* ***(F)*** *Intensity (MFI) of HELIOS staining is shown for one donor and* ***(G)*** *for 10 donors. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (****(B)*** *Wilcoxon signed-rank test and* ***(G)*** *one-way ANOVA).*


A
%HELIOS+ cells
***
CD161- CD8 TEM
CD161+ CD8 TEM
B
CD161- CD8 TEM
HELIOS+ 8%
CD161+ CD8 TEM
HELIOS+ 97%
empty channel
HELIOS
C
%CD161+ cells
HELIOS+ CD8 TEM
D
% of non naive HELIOS+ CD8 T cells
T-BEThigh
CD161+
FOXP3
E
Non-naïve HELIOS+ CD8 T cells
T-BEThigh 11%
CD161+ 35%
T-BET
CD161
F
HELIOS-
T-BEThigh
CD161+
FOXP3+
% of max
HELIOS
G
NS
***
HELIOS MFI
HELIOS-
T-BEThigh
CD161+
FOXP3

2.4 HELIOS$^+$ Tc1 effectors produce low amounts of Tc1-related cytokines.

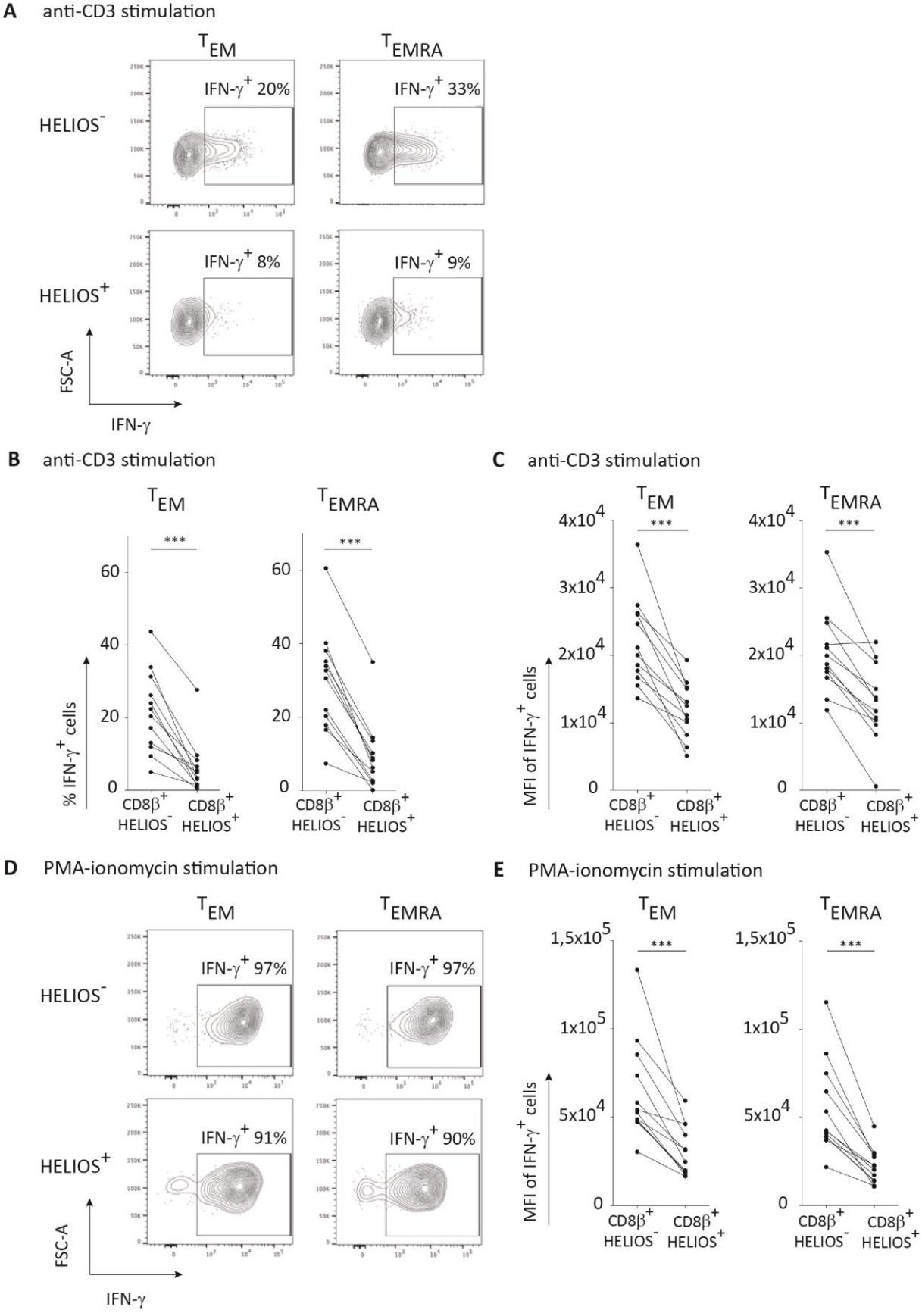
2.4.1 HELIOS$^+$ Tc1 effectors produce low amounts of IFN-γ.

To confirm whether the low expression of *IFNG* results in reduced production of IFN-γ by HELIOS$^+$ T-BET^{high} CD8 T cells (HELIOS$^+$ Tc1 effectors), we performed IFN-γ intracellular production assays with HELIOS$^+$ and HELIOS$^-$ CD8 T cells. PBMCs were activated for 5h with plate-bound anti-CD3 at 37°C. We only analysed IFN-γ production by T_{EM} and T_{EMRA} because naïve CD8 T cells and T_{CM} do not produce IFN-γ within 5h of activation by anti-CD3.

We compared the IFN-γ production in HELIOS$^+$ versus HELIOS$^-$ Tc1 effectors T_{EM} and T_{EMRA}. The data shows that HELIOS$^+$ CD8 T_{EM} and T_{EMRA} produce less IFN-γ than HELIOS$^-$ cells both in frequency and intensity (Figure 21A, B and C). HELIOS$^+$ CD8 T_{EM} and T_{EMRA} activated with PMA-ionomycin, which bypasses early TCR signalling events, produce also less IFN-γ than HELIOS$^-$ CD8 T cells (Figure 21D and E).

This result suggests the presence of Tc1 effectors characterized by low Tc1-related cytokines production capacity and HELIOS expression. Moreover, results obtained with PMA-ionomycin indicate that late TCR signalling events or even *IFNG* gene accessibility differ between HELIOS$^+$ and HELIOS$^-$ CD8 T cells.

Figure 21. HELIOS$^+$ CD8 T cells produce less IFN-γ than HELIOS$^-$ CD8$^+$ T cells. *PBMCs were thawed for 2h at 37°C with 5U/ml DNase. Cells were stimulated with coated anti-CD3 (1μg/ml) or PMA (1ng/ml) and ionomycin (1μg/ml) for 5h at 37°C. After the first hour of stimulation 5μg/ml of Brefeldin A was added to block cytokine secretion. At the end of the 5h stimulation cells were stained for viability, CD2, CD8β, CCR7 and CD45RA. Cells were then fixed and permeabilized overnight and stained intracellularly for T-BET, HELIOS and IFN-γ. Sample were analysed by flow cytometry.* ***(A)*** *Representative plots for anti-CD3 activation are shown for T_{EM} and T_{EMRA} for one donor.* ***(B)*** *Percentage of IFN-γ^+ cells and* ***(C)*** *median of fluorescence intensity are shown for T_{EM} and T_{EMRA} of 12 donors.* ***(D)*** *Representative plots for PMA-ionomycin activation are shown for T_{EM} and T_{EMRA} for one donor.* ***(E)*** *Median of fluorescence intensity are shown for T_{EM} and T_{EMRA} of 12 donors P values * = < 0,05, ** = < 0,01, *** = < 0,001 (paired t test).*


A
anti-CD3 stimulation
T EM
T EMRA
IFN-γ+ 20%
IFN-γ+ 33%
HELIOS-
IFN-γ+ 8%
IFN-γ+ 9%
HELIOS+
FSC-A
IFN-γ
B
anti-CD3 stimulation
T EM
T EMRA
***
% IFN-γ+ cells
CD8β+ HELIOS-
CD8β+ HELIOS+
C
anti-CD3 stimulation
MFI of IFN-γ+ cells
D
PMA-ionomycin stimulation
IFN-γ+ 97%
IFN-γ+ 97%
IFN-γ+ 91%
IFN-γ+ 90%
E
PMA-ionomycin stimulation

2.4.2 HELIOS$^+$ Tc1 effectors secrete low amounts of TNF-α.

To confirm that the HELIOS$^+$ CD8 T cells that express low levels of the gene TNF secrete no or low levels of TNF-α, we assessed TNF-α secretion at the single cell level. We wished to analyse secretion rather than production (intracellular accumulation of the cytokine) because production does not always correlate with secretion (Petit et al. 2016).

We took advantage of the unique mechanism of secretion of TNF-α. TNF-α is produced as a membrane-bound precursor that is later released from the plasma membrane by the enzyme ADAM17/TACE. Blocking ADAM17/TACE with TAPI-0 sequesters TNF-α on the surface of TNF-α-producing cells. Cells with membrane-sequestered TNF-α can be identified by flow cytometry (Figure 22A). As membrane-bound TNF-α is internalised by the cells, cells have to be stained for TNF-α during the stimulation (Figure 22B) (Bueno et al. 2002; Haney et al. 2011; Huang et al. 2013).

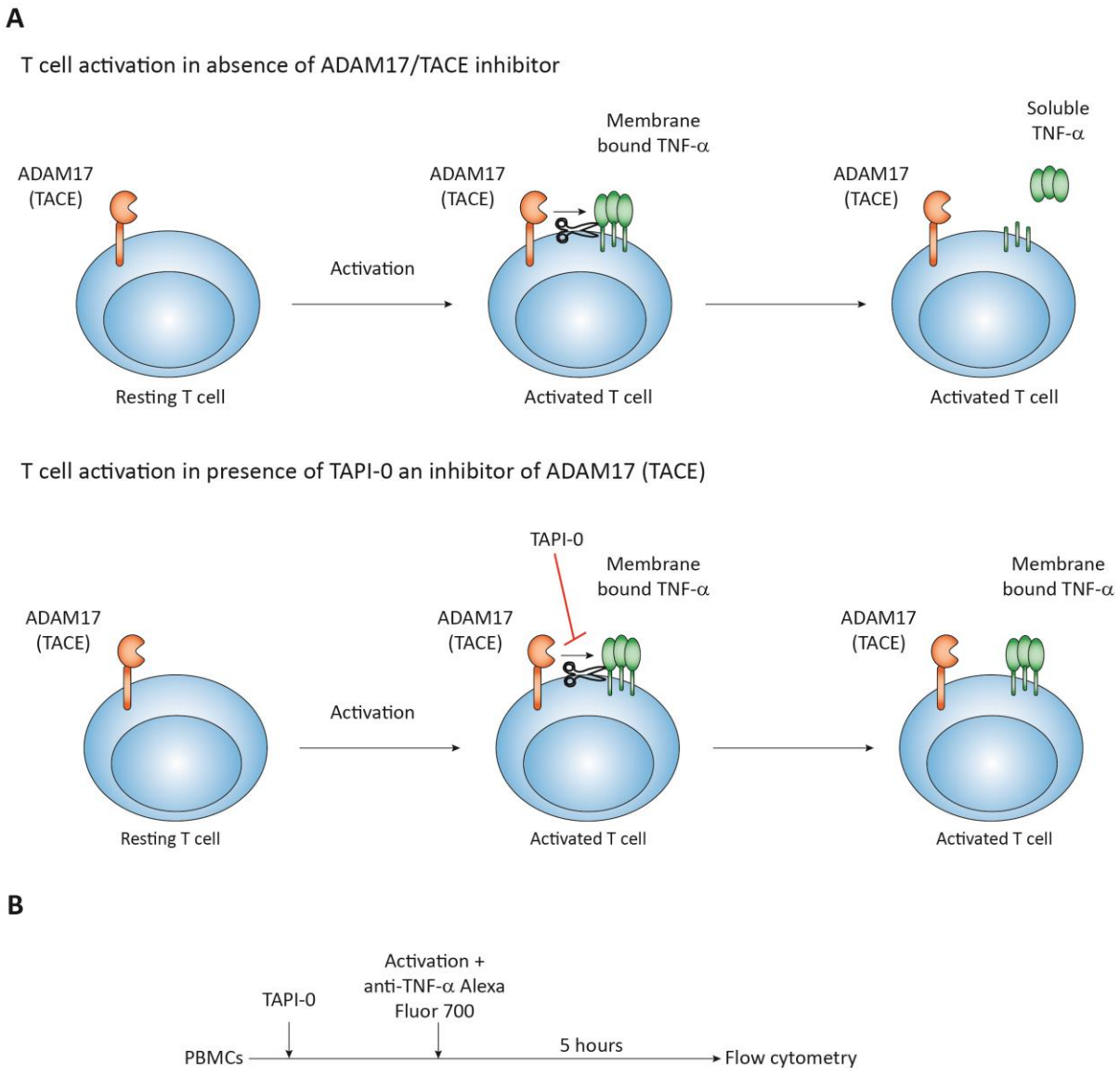


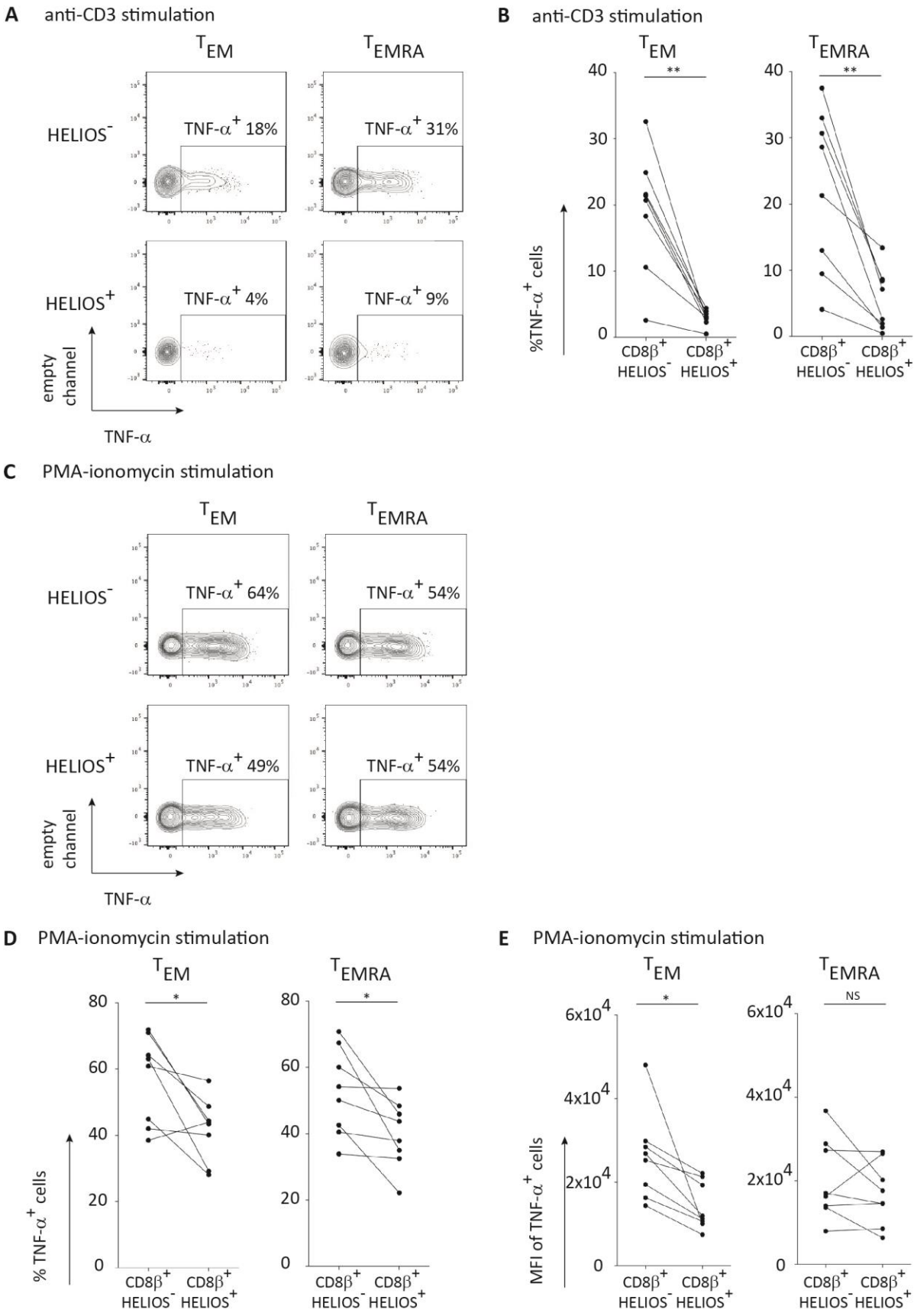
Figure 22. ADAM17/TACE inhibitors to assess TNF-α secretion by T cells. (A) *When T cells are activated, TNF-α is produced as a membrane-bound trimer. ADAM17/TACE releases TNF-α from the plasma membrane. In the presence of TAPI-0 an ADAM17/TACE inhibitor, TNF-α is not released from the plasma membrane, allowing for TNF-α to be stained with an antibody. When TNF-α is not released from the plasma membrane, TNF-α is internalized.* ***(B)*** *Anti-TNF-α antibodies have to be present during activation. Activation time has to be short, e.g. not 20h, to avoid fluorophore degradation after internalisation of TNF-α-antibody complexes.*

PBMCs were activated for 5h with plate-bound anti-CD3 at 37°C. We only analysed TNF-α production by T_{EM} and T_{EMRA} because naïve CD8 T cells and T_{CM} do not produce TNF-α within 5h of anti-CD3 activation.

We compared TNF-α secretion by HELIOS$^+$ versus HELIOS$^-$ Tc1 effectors T_{EM} and T_{EMRA}. The data shows that HELIOS$^+$ CD8 T_{EM} and T_{EMRA} secrete less TNF-α than HELIOS$^-$ (Figure 23A and B). Using a PMA-ionomycin cocktail, we observed less striking differences (Figure 23C, D and E).

Taken together, the TNF-α secretion and IFN-γ production assays suggest the presence of HELIOS$^+$ Tc1 effectors (T-BET^{high}) with low Tc1-related cytokines production capacity.

Figure 23. HELIOS$^+$ CD8 T cells secrete less TNF-α than HELIOS$^-$ CD8 T cells. *PBMCs were thawed for 2h at 37°C with 5U/ml DNase. Cells were stimulated with coated anti-CD3 (1μg/ml) or PMA (1ng/ml) and ionomycin (1μg/ml) for 5h at 37°C. During the stimulation 10nM of TAPI-0 (TACE inhibitor) and anti-TNF-α antibody at 2μg/ml was added to the cells. At the end of the 5h stimulation cells were stained for viability, CD2, CD8β, CCR7 and CD45RA. Cells were then fixed and permeabilized and stained intracellularly for T-BET and HELIOS at 4°C. Cells were analysed by flow cytometry.* ***(A)*** *Representative plots for anti-CD3 stimulation are shown for T_{EM} and T_{EMRA} for one donor.* ***(B)*** *Percentage of TNF-α$^+$ cells are shown for T_{EM} and T_{EMRA} of 8 donors.* ***(C)*** *Representative plots for PMA-ionomycin activation are shown for T_{EM} and T_{EMRA} for one donor.* ***(D)*** *Percentage of TNF-α$^+$ cells and* ***(E)*** *median of fluorescence intensity are shown for T_{EM} and T_{EMRA} of 8 donors. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (paired t test).*


A
anti-CD3 stimulation
T EM
T EMRA
HELIOS-
HELIOS+
TNF-α+ 18%
TNF-α+ 31%
TNF-α+ 4%
TNF-α+ 9%
empty channel
TNF-α
B
anti-CD3 stimulation
T EM
T EMRA
%TNF-α+ cells
**
**
CD8β+ HELIOS-
CD8β+ HELIOS+
C
PMA-ionomycin stimulation
T EM
T EMRA
HELIOS-
HELIOS+
TNF-α+ 64%
TNF-α+ 54%
TNF-α+ 49%
TNF-α+ 54%
empty channel
TNF-α
D
PMA-ionomycin stimulation
T EM
T EMRA
% TNF-α+ cells
*
*
CD8β+ HELIOS-
CD8β+ HELIOS+
E
PMA-ionomycin stimulation
T EM
T EMRA
MFI of TNF-α+ cells
*
NS
6x10^4
4x10^4
2x10^4
CD8β+ HELIOS-
CD8β+ HELIOS+

2.5 HELIOS$^+$ CD161$^+$ CD8 T cells secrete Tc17-related cytokines and have less proliferative capacity.

2.5.1 CD161$^+$ CD8 T_{EM} secrete Th17- and Tc17-related cytokines.

Human CD161$^+$ CD8 T cells have been described as Tc17 (Maggi et al. 2010). To confirm whether the increased expression of *IL17A* results in increased IL17-A secretion by HELIOS$^+$ CD8 T cells, we performed cytokine secretion assays. We sorted HELIOS$^+$ (CD161$^+$) and HELIOS$^-$ (CD161$^-$) CD8 T_{EM} from PBMCs by CD161. Sorted CD8 T cells were activated with anti-CD3-CD28 beads or PMA-ionomycin for 20h at 37°C.

We showed that CD161$^+$ CD8 T_{EM} secrete more IL-17A in response to PMA-ionomycin compared to CD161$^-$ CD8 T_{EM}. However, T cell activation with anti-CD3-CD28 beads failed to elicit IL-17A secretion (Figure 24).

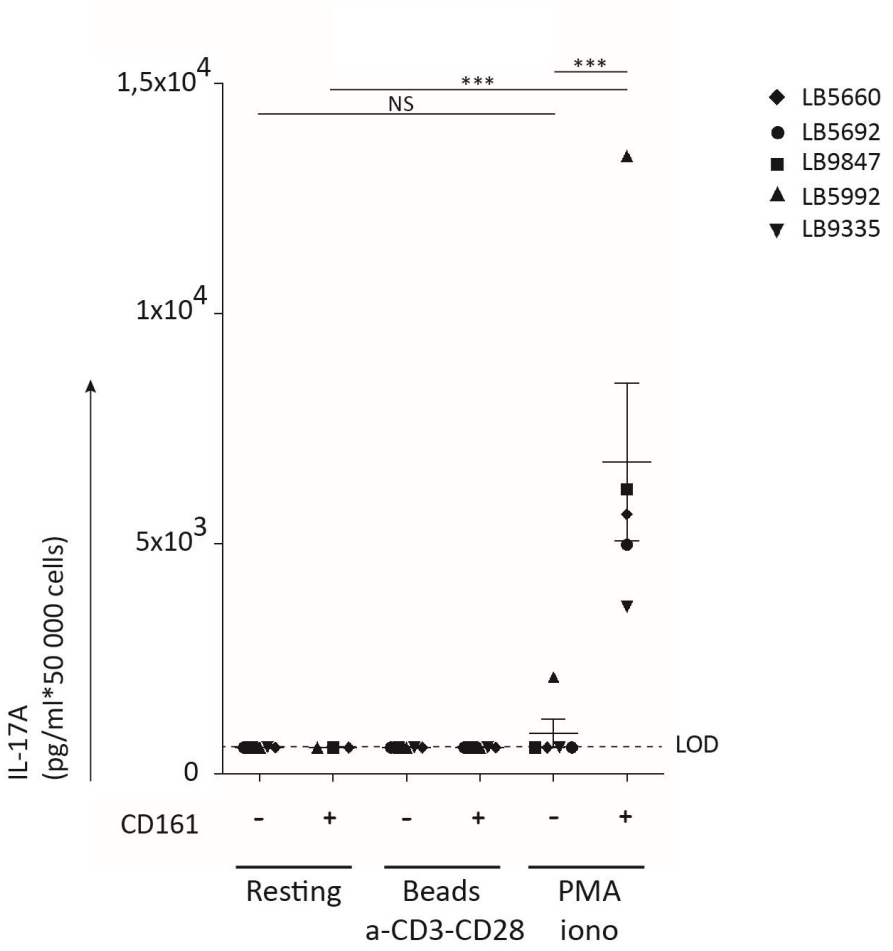


Figure 24. CD161$^+$ CD8 T_{EM} secrete IL-17A. *CD8 T cells were thawed for 2h at 37°C with 5U/ml of DNase. Cells were labelled at 4°C with antibodies against CD8β, CCR7, CD45RA and CD161. CD161$^+$ and CD161$^-$ CD8 T_{EM} (CCR7$^-$ CD45RA$^-$) were sorted by flow cytometry. T cells were activated with either anti-CD3-CD28 beads (1b:3T) or 1ng/ml PMA and 1μg/ml ionomycin at 37°C for 20h. IL-17A was measured in the supernatant using LEGENDplex (Biolegend). The experiment was performed on 5 donors. LOD = limit of detection. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (one-way ANOVA).*

2.5.2 CD161$^+$ HELIOS$^+$ CD8 T_{EM} have less proliferative capacity.

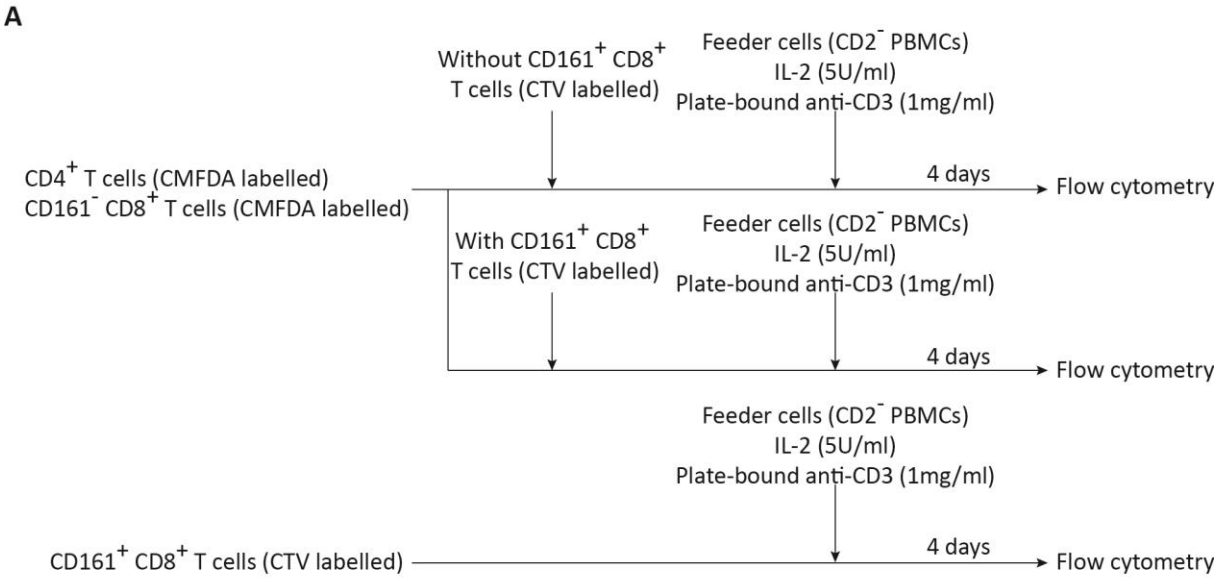
We looked at the proliferative capacity of CD161$^+$ HELIOS$^+$ CD8 T cells. At the same time, we looked at the impact these cells could have on proliferation of other T cells. Indeed, HELIOS expression has been connected to CD4 and CD8 Tregs in mice which are known to suppress T cell activity at least in part through inhibition of proliferation. We performed a co-culture experiment between CD4 T cells and CD161$^+$ and CD161$^-$ CD8 T_{EM}. To do so, Autologous

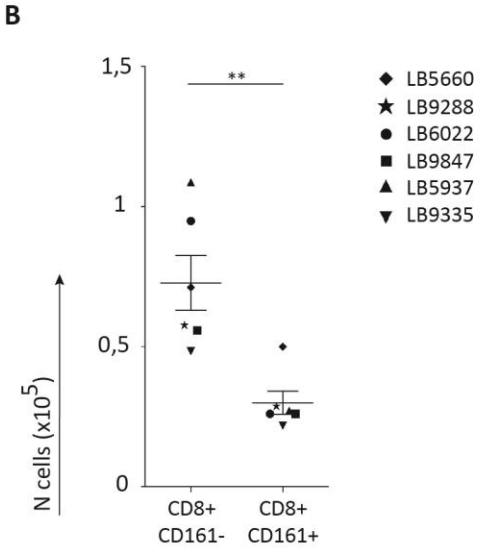
$CD2^-$ PBMCS were isolated by rosetting and γ-irradiated to serve as feeder cells to support T cell proliferation. CD4 T cells were sorted by MACS and labelled with cell tracker CMFDA. $CD161^+$ ($HELIOS^+$) and $CD161^-$ ($HELIOS^-$) CD8 T_{EM} were sorted from PBMCs by flow cytometry and labelled with distinct cell trackers (CTV and CMFDA). CD4 T cells, $CD161^+$ and $CD161^-$ CD8 T_{EM} were activated with plate-bound anti-CD3 in the presence of feeder cells (γ-irradiated autologous $CD2^-$ PBMCs) and 5U/ml IL-2 (Figure 25A). After 4 days, the total number of CD4, $CD161^-$ CD8 and $CD161^+$ CD8 T cells was determined by flow cytometry.

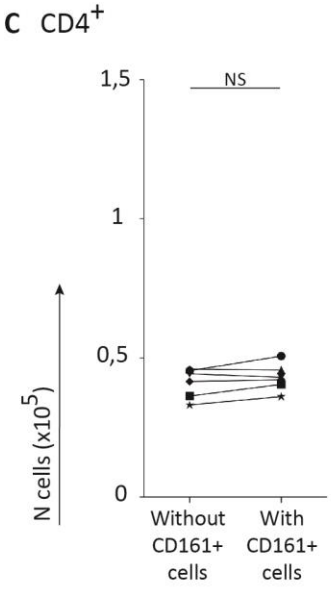
$CD161^+$ CD8 T_{EM} proliferated less than $CD161^-$ CD8 T_{EM} (Figure 25B). This lower proliferative capacity could not be transferred to co-cultured CD4 or $CD161^-$ CD8 T cells (Figure 25C and D). The proliferation of $CD161^+$ CD8 T_{EM} was not affected by the presence of CD4 T cells and $CD161^-$ CD8 T_{EM} (Figure 25E)

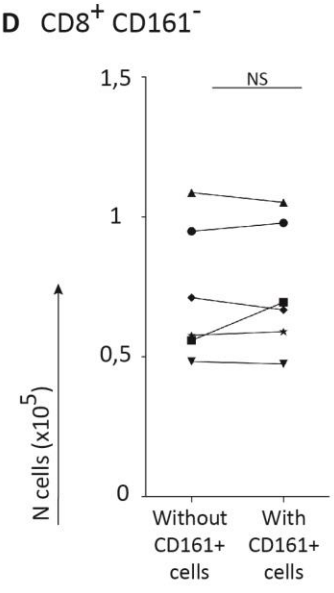
These results show that $CD161^+$ ($HELIOS^+$) CD8 T_{EM} do not display regulatory properties - at least concerning the ability to suppress T cell proliferation.

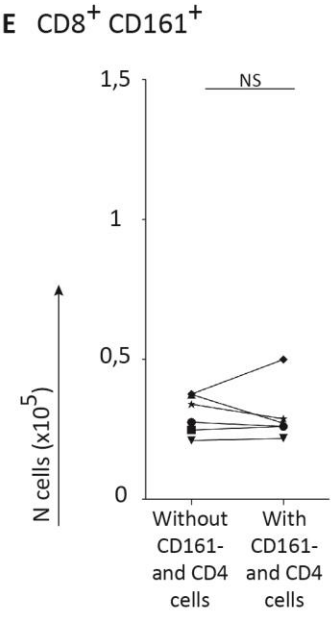
Figure 25. $CD161^+$ CD8 T_{EM} have less proliferative capacity than $CD161^-$ CD8 T_{EM}. *CD4 and CD8 T cells were thawed for 2h at 37°C with 5U/ml of DNase. Cells were then labelled at 4°C with antibodies against CD8β, CCR7, CD45RA and CD161. $CD161^+$ and $CD161^-$ CD8 T_{EM} were FACS sorted. CD4 T cells and $CD161^-$ CD8 T_{EM} were labelled with cell tracker 5-chloromethylfluorescein diacetate (CMFDA) and $CD161^+$ CD8 T_{EM} with cell trace violet (CTV). CD4 T cells, $CD161^+$ and $CD161^-$ CD8 T_{EM} were activated with plate-bound anti-CD3 (1μg/ml) in the presence of γ-irradiated autologous $CD2^-$ PBMCs (ratio 1:1) and 5U/ml IL-2 for 4 days at 37C. The total number of cells for each T cell population was determined.* ***(A)*** *Plan of co-culture conditions.* ***(B)*** *Number of CD4 T cells,* ***(C)*** *$CD161^-$ CD8 T_{EM} and* ***(D)*** *$CD161^+$ CD8 T_{EM} after at the end of co-culture.* ***(E)*** *Comparison of $CD161^+$ and $CD161^-$ CD8 T_{EM} proliferation. The experiment was performed on 6 donors. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (paired t test).*


A
Without CD161+ CD8+ T cells (CTV labelled)
Feeder cells (CD2- PBMCs)
IL-2 (5U/ml)
Plate-bound anti-CD3 (1mg/ml)
CD4+ T cells (CMFDA labelled)
CD161- CD8+ T cells (CMFDA labelled)
4 days
Flow cytometry
With CD161+ CD8+ T cells (CTV labelled)
Feeder cells (CD2- PBMCs)
IL-2 (5U/ml)
Plate-bound anti-CD3 (1mg/ml)
4 days
Flow cytometry
Feeder cells (CD2- PBMCs)
IL-2 (5U/ml)
Plate-bound anti-CD3 (1mg/ml)
CD161+ CD8+ T cells (CTV labelled)
4 days
Flow cytometry


B
1,5
1
0,5
0
**
N cells (x10^5)
CD8+ CD161-
CD8+ CD161+
LB5660
LB9288
LB6022
LB9847
LB5937
LB9335


C CD4+
1,5
1
0,5
0
NS
N cells (x10^5)
Without CD161+ cells
With CD161+ cells


D CD8+ CD161-
1,5
1
0,5
0
NS
N cells (x10^5)
Without CD161+ cells
With CD161+ cells


E CD8+ CD161+
1,5
1
0,5
0
NS
N cells (x10^5)
Without CD161- and CD4 cells
With CD161- and CD4 cells

2.5.3 Knocking out HELIOS increases CD161+ CD8 T_{EM} proliferation.

We used CRISPR-Cas9 to knock out HELIOS in primary $CD161^+$ CD8 T cells. $CD161^+$ ($HELIOS^+$) and $CD161^-$ ($HELIOS^-$) CD8 T_{EM} were sorted by flow cytometry from PBMCs of blood donors. Cells were electroporated with guide RNA-Cas9 complexes. Then, cells were activated with anti-CD3-CD28 beads in the presence of 50U/ml IL-2 at 37°C, and counted 7 days later. A fraction of the cells were reactivated for 5h with plate-bound anti-CD3 at 37°C and sent for RNA-sequencing. RNA-sequencing is ongoing.

As shown in Figure 26, we confirmed the reduced proliferative capacity of $CD161^+$ CD8 TEM compared to $CD161^-$ ones. Knocking out HELIOS increased the proliferation of $CD161^+$ CD8 T_{EM} to levels comparable to those of $CD161^-$ CD8 T_{EM} (Figure 26). The density of cells is shown rather than an amplification fold because of the high mortality of cells during electroporation. To confirm the impact of HELIOS-KO on $CD161^+$ CD8 T_{EM} proliferation, this experiment should be performed again with the addition of a cell tracker.

This observation indicates that knocking out HELIOS impacts HELIOS-expressing CD8 T cells. Details about how HELIOS regulates CD8 T cells will be revealed by the transcriptomic analyses.

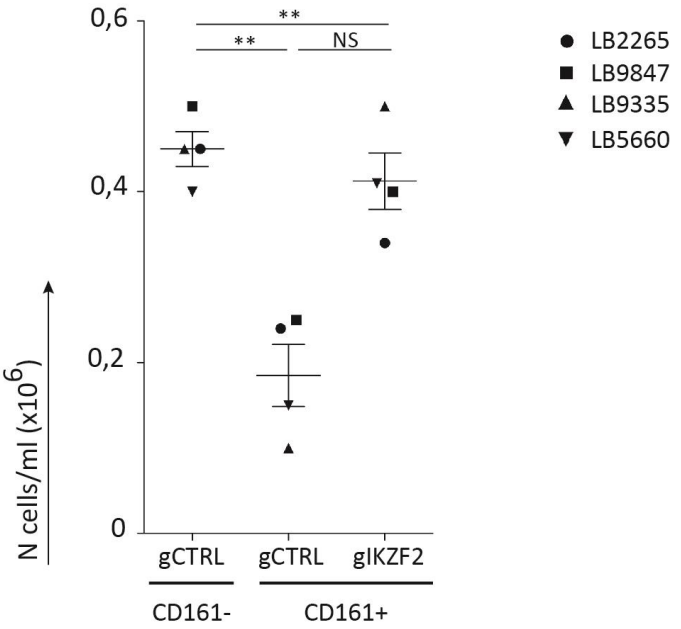


Figure 26. **Impact of knocking out HELIOS on HELIOS$^+$ CD8 T cell proliferation.** CD8 T cells were thawed for 2h at 37°C with 5U/ml of DNase. Cells were then labelled at 4°C with antibodies against CD8β, CCR7, CD45RA and CD161. CD161$^+$ and CD161$^-$ CD8 T_{EM} were FACS sorted. Cells were then electroporated with g*IKZF2*-Cas9 or gCTRL-Cas9 complexes. After 5h of resting, cells were activated with beads anti-CD3-CD28 in presence of 50U/ml IL-2 for a week at 37°C. Cells were then counted with a haemocytometer and trypan blue. The density of cells is shown. Mean +/- SEM are shown for 4 donors. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (paired t test).

2.6 HELIOS expression and activation status in pathology.

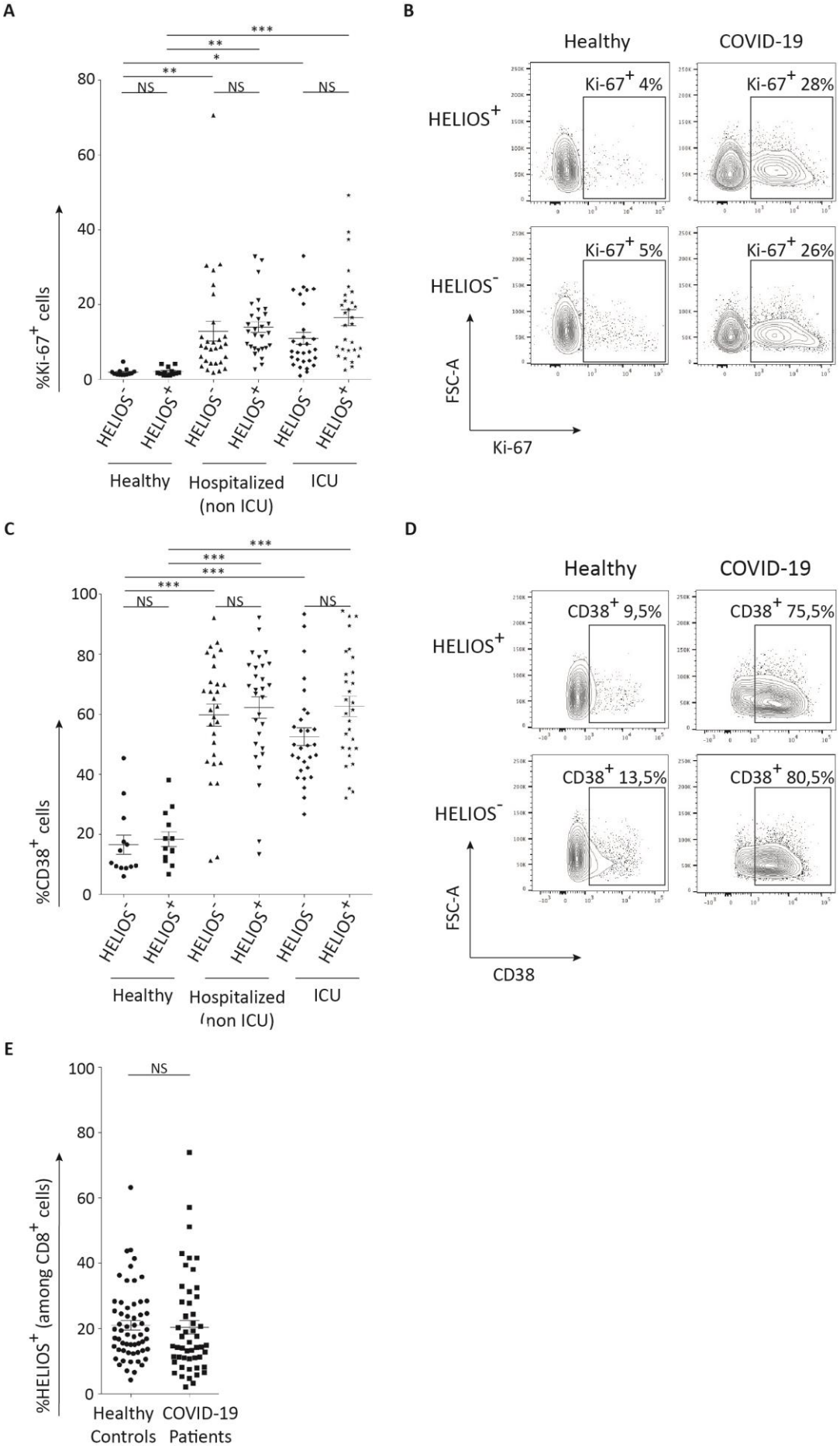
2.6.1 HELIOS+ CD8 T cells are activated during SARS-CoV2 viral infection.

We showed with the transcriptomic analysis that HELIOS$^+$ CD8 T cells could be stimulated *in vitro*. To confirm that HELIOS$^+$ CD8 T cells are reactive *in vivo*, we collected blood samples from COVID-19 patients hospitalized at the Cliniques universitaires Saint-Luc (Brussels) during the SARS-CoV2 pandemic. We analysed the expression of the T cell activation marker CD38 by flow cytometry. CD38 and the proliferation marker Ki-67 are upregulated by CD8 T cells during acute viral infections (Agrati et al. 2016; Mathew et al. 2020).

More CD8 T cells of COVID-19 patients than of healthy controls express Ki-67 or CD38 (Figure 27A, B, C and D). This is true for HELIOS$^-$ as well as for HELIOS$^+$ CD8 T cells (Figure 27A, B, C and D). No significant difference was observed between HELIOS$^-$ and HELIOS$^+$ CD8 T cells (Figure 27A, B, C and D). The proportion of HELIOS$^+$ cells among the CD8 T cells did not differ between blood of healthy donors and COVID-19 patients (Figure 27E).

We conclude that HELIOS$^+$ CD8 T cells are activated and proliferate during acute viral infection. Despite their low ability to produce cytokines in general in blood donors, they are not anergic but respond to infection.

Figure 27. HELIOS$^+$ CD8 T cells are activated during acute SARS-CoV2 infection. *PBMCs were thawed for 2h at 37°C. Cells were labelled for viability, CD19, CD20, CD33, CD8β, CCR7, CD45RA and CD38. Cells were fixed, permeabilized and stained intracellularly for HELIOS and Ki-67. Samples were analysed by flow cytometry.* ***(A)*** *Frequency of Ki-67$^+$ cells in living non-naïve CD8β^+ cells for several donors and* ***(B)*** *for one healthy and one COVID-19 patient.* ***(C)*** *Frequency of CD38$^+$ cells in living non-naïve CD8β^+ cells. and* ***(D)*** *for one healthy and one COVID-19 patient.* ***(E)*** *Frequency of HELIOS$^+$ cells in living CD2$^+$ CD8β^+ cells are shown for healthy controls and SARS-CoV2 infected patients. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (**(A)** and **(B)** one-way ANOVA, **(C)** t-test).*


A
B
C
D
E
%Ki-67+ cells
HELIOS-
HELIOS+
Healthy
Hospitalized (non ICU)
ICU
NS
Healthy
COVID-19
Ki-67+ 4%
Ki-67+ 28%
Ki-67+ 5%
Ki-67+ 26%
FSC-A
Ki-67
%CD38+ cells
CD38+ 9,5%
CD38+ 75,5%
CD38+ 13,5%
CD38+ 80,5%
CD38
%HELIOS+ (among CD8+ cells)
Healthy Controls
COVID-19 Patients

2.6.2 HELIOS+ CD8 T cells are activated in ovarian carcinoma.

To assess if HELIOS$^+$ CD8 T cells are activated and/or exhausted in tumors, we collected blood and tumor samples from patients with ovarian carcinoma at the Cliniques universitaires Saint-Luc (Brussels). Tumor tissues underwent mechanical and enzymatic dissociation. Then, the expression of the proliferation marker Ki-67 and the exhaustion marker TOX were analysed in CD8 TILs.

HELIOS$^+$ CD8 T cells are less frequent among non-naïve CD8 T cells within tumors than in the blood of ovarian cancer patients (12,5% versus 24%) (Figure 28A). HELIOS$^+$ and HELIOS$^-$ CD8 T cells express Ki-67 equally but more HELIOS$^+$ CD8 T cells express TOX compared to HELIOS$^-$ CD8 T cells (Figure 28B, C, D and E).

These observations suggest that HELIOS$^+$ CD8 T cells are activated within tumors similarly to HELIOS$^-$ CD8 T cells. Some intratumoral HELIOS$^+$ CD8 T cells probably recognize tumor antigens, based on knowledge about TOX in murine models. Indeed, TOX is selectively expressed in anti-tumor TILs but not in bystander TILs (Scott et al. 2019).

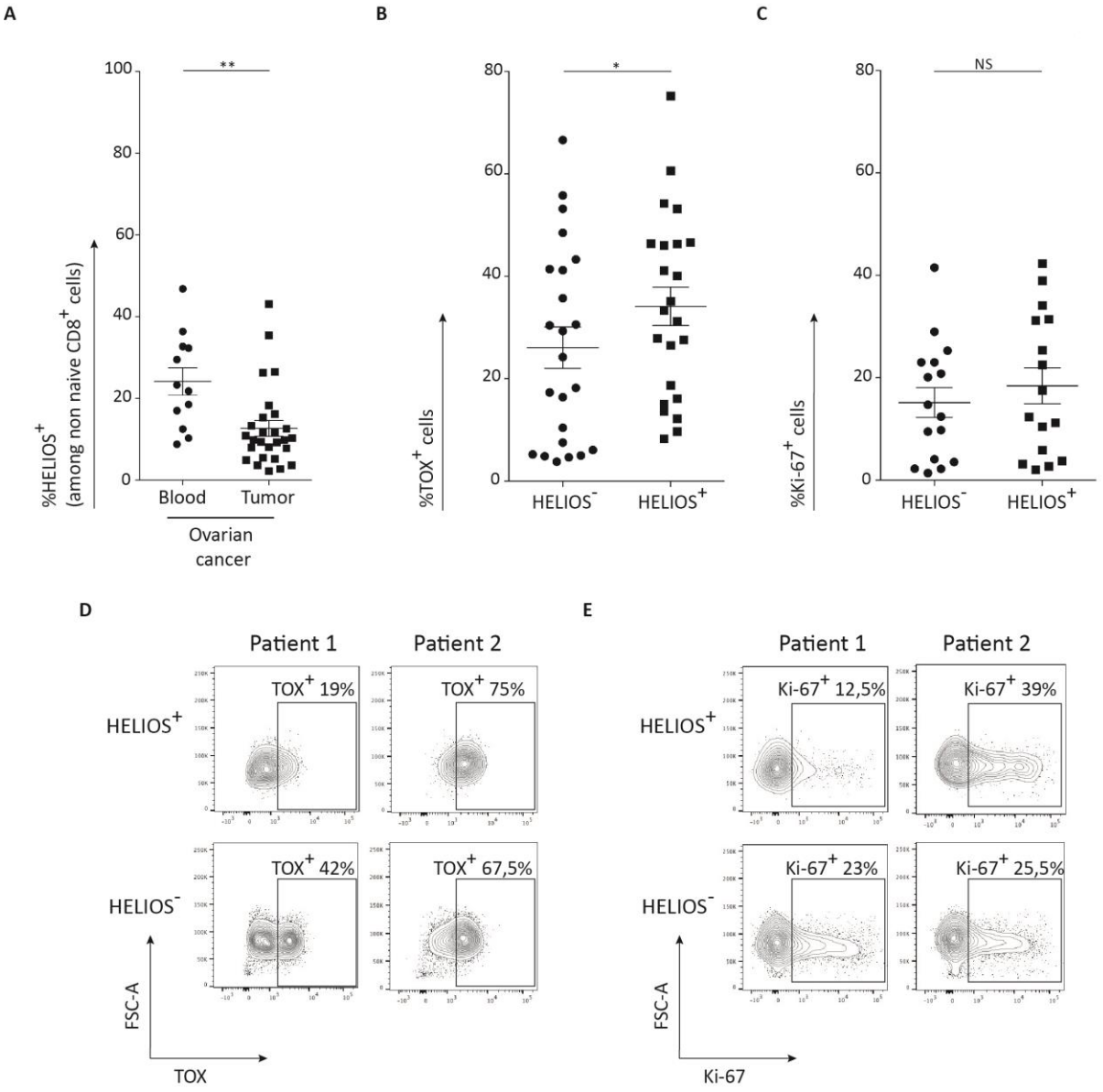


Figure 28. HELIOS$^+$ CD8 T cells are activated in ovarian carcinoma. *Cells isolated from ovarian tumors were thawed for 2h at 37°C. Cells were labelled for viability, CD19, CD20, CD33, CD326, CD2, CD8β, CCR7, CD45RA. Then, cells were fixed and permeabilized overnight and stained intracellularly for HELIOS, Ki-67 and TOX at 4°C. Samples were analysed by flow cytometry.* ***(A)*** *Frequency of HELIOS$^+$ cells in living non naïve CD2$^+$ CD8β$^+$ cells are shown for blood and tumors from ovarian cancer patients.* ***(B)*** *Frequency of Ki-67$^+$ cells in living non-naïve CD8β$^+$ cells for multiple patients and* ***(D)*** *for two patients.* ***(C)*** *Frequency of CD38$^+$ cells in living non-naïve CD2$^+$ CD8β$^+$ cells for multiple patients and* ***(E)*** *for two patients. Mean +/- SEM are shown. P values * = < 0,05, ** = < 0,01, *** = < 0,001 (****(A)*** *t-test,* ***(B)*** *and* ***(C)*** *paired t-test).*

3 Discussion

3.1 What drives HELIOS expression?

Before discussing what signal(s) drive HELIOS expression, it is important to consider where HELIOS expression is acquired: in the thymus or in the periphery. The majority of HELIOS$^+$ CD8 T cells are T_{EM} and T_{EMRA}, thus are antigen-experienced T cells. However, few HELIOS$^+$ CD8 T cells express CCR7 and CD45RA, which identify naïve T cells. This suggests that HELIOS expression is acquired during T cell maturation in the thymus and is not induced during or after CD8 T cell priming in the periphery. In accordance, HELIOS is only expressed by murine thymic-derived CD4 Tregs and not by peripherally-induced CD4 Tregs (Thornton et al. 2010).

Our data, as well as data from the literature, suggest that HELIOS$^+$ CD8 T cells are HLA-E-restricted (Kim et al. 2015). Is there a link between HLA-E restriction and HELIOS expression? If this is the case, why is HELIOS expressed by only a fraction of HLA-E-restricted CD8 T cells? Interestingly, Qa-1 (HLA-E homolog)-restricted CD8 T cells in mice are positively selected in the thymus by different cell types compared to MHC class Ia-restricted CD8 T cells. Briefly, positive selection is the process selecting CD8 T cells able to interact moderately with self-MHC molecules. While MHC class Ia-restricted CD8 T cells undergo positive selection on thymic epithelial cells, Qa-1-restricted CD8 T cells do so on either thymic epithelial cells or hematopoietic cells (Anderson and Brossay 2016; Sullivan et al. 2002). Thus, MHC class Ib-restricted CD8 T cells certainly receive different signals during positive selection. If some HLA-E-restricted CD8 T cells are positively selected by hematopoietic cells (dendritic cells), this could explain why a fraction of HLA-E-restricted CD8 T cells express HELIOS.

HELIOS expression can be induced in the periphery in a context of antigen persistence. It has been shown that HELIOS mRNA is upregulated in exhausted CD8 T cells compared to naïve, effector or memory CD8 T cells in mice (Doering et al. 2012; Khan et al. 2019; Pereira et al. 2017). However, these data are only based on gene expression and not protein level.

Based on current knowledge about HELIOS and CD8 T cells, it is likely that part of HELIOS-expressing CD8 T cells acquire HELIOS expression in the thymus. However, in certain contexts HELIOS expression is likely acquired in the periphery.

3.2 Is there a causal link between HELIOS expression and low IFN-γ, TNF-α and IL-2 production?

We established a correlation between HELIOS expression and low expression of IFN-γ, TNF-α and IL-2, but is HELIOS the cause? To answer if HELIOS binds directly to the promoter of these cytokines encoding genes, ChIP-Seq for HELIOS should be performed. As HELIOS binds to the *IL2* promoter in CD4 Tregs in mice (Baine et al. 2013), it is highly probable that this is also the case in CD8 T cells. We think it is likely that HELIOS also binds to the promoter of *IFNG* because the *IFNG* promoter contains core sequence motifs (GGGA and GGAAA) that HELIOS recognizes (Hahm et al. 1998). While confirmatory data are lacking it is likely that HELIOS repress the transcription of effector cytokines including IL-2 and IFN-γ (Figure 29).

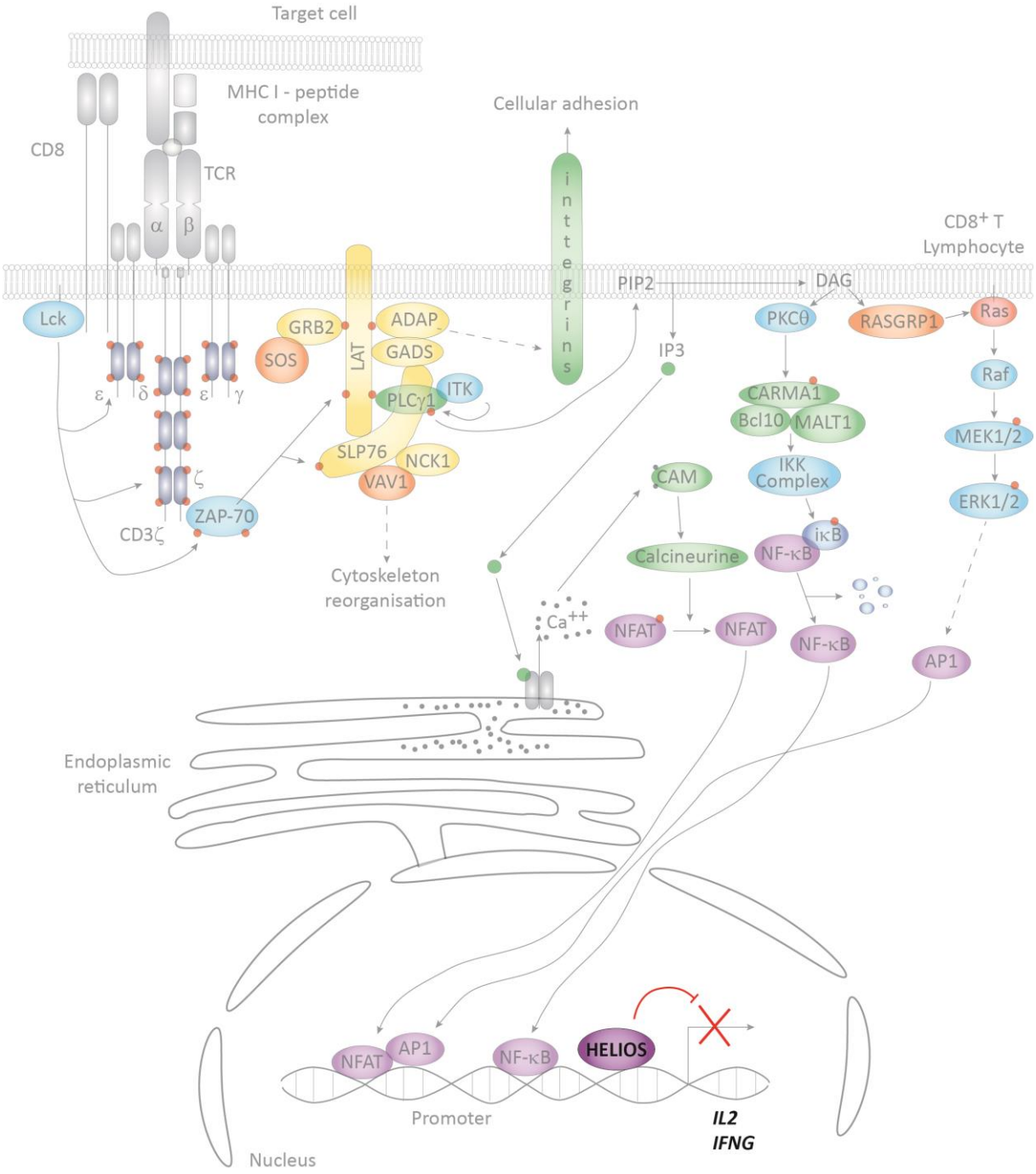


Figure 29. Does HELIOS prevent Tc1-related cytokines expression in human CD8 T cells? *As shown in Tregs, HELIOS binds directly to the IL2 promoter to prevent its expression. HELIOS likely suppresses IL-2 expression in human CD8 T cells by the same mechanism. Moreover, the IFNG promoter contains core sequences recognized by HELIOS. Although this requires confirmation, it is likely that HELIOS represses IFN-γ expression in human CD8 T cells.*

3.3 Are HELIOS+ CD8 T cells anergic?

T cell anergy participate to peripheral tolerance. It occurs when T cells encounter their antigen in the absence of co-stimulatory signals, e.g. CD80/CD86. This leads to low IL-2 production and cell cycle arrest at the G1/S phase (Crespo et al. 2013). HELIOS$^+$ CD8 T cells are not anergic: while they produce low amounts of IL-2, they do proliferate *in vitro* and express the proliferation marker Ki-67 in response to SARS-CoV2 infection and in tumors. Moreover, *EGR2* and *EGR3*, two anergy-related transcription factors, are not upregulated in HELIOS$^+$ CD8 T cells (Safford et al. 2005). In fact, EGR2 and EGR3 are upregulated in HELIOS$^-$ CD8 T cells compared to HELIOS$^+$ CD8 T cells.

As HELIOS$^+$ CD8 T cells aren't anergic, what type of CD8 T cells are they?

3.4 Do HELIOS+ CD8 T cells share characteristics with Tregs?

Very few HELIOS$^+$ CD8 T cells express FOXP3 (2,5% of non-naïve HELIOS$^+$ CD8 T cells), which is the lineage-defining transcription factor of CD4 Tregs. This could suggest that most HELIOS$^+$ CD8 T cells are not Tregs although FOXP3 is not an exclusive marker of Tregs in humans (Baron et al. 2007). CD4 Tregs produce TGF-β1 which is activated by GARP (Cuende et al. 2015; Liénart et al. 2018; Stockis et al. 2009; Stockis, Dedobbeleer, and Lucas 2017; Vignali et al. 2008). HELIOS$^+$ CD8 T cells do not express more TGF-β1 or GARP than HELIOS$^-$ CD8 T cells in our transcriptomic analysis. HELIOS$^+$ CD8 T cells produced even less IL-10 than HELIOS$^-$ CD8 T cells. Furthermore, CD161$^+$ CD8 T cells (HELIOS$^+$) failed to suppress the proliferation of other T cells (CD161$^-$ CD8 T cells and CD4 T cells). We did not observe suppressive activity at least for CD161 expressing HELIOS$^+$ CD8 T cells.

Murine CD8 Tregs lyse CD4 T cells following the recognition of Qa-1/peptide complex (Hu et al. 2004; Jiang et al. 1998; Varthaman et al. 2010, 2011). Human T cell clones that recognize TCR-Vβ peptides bound to HLA-E have been identified (Li et al. 2001; Ware et al. 1995). Here, we showed that HELIOS$^+$ CD8 T cells recognized a CMV peptide and an EBV peptide (1 donor) presented by HLA-E (Qa-1 homolog). It yet remains to be determined if HLA-E-restricted HELIOS$^+$ CD8 T cells recognize TCR-Vβ-derived peptides, and thus lyse CD4 T cells.

In conclusion, our data suggests that HELIOS$^+$ CD8 T cells differ from CD8 Tregs. Although this hypothesis deserves further investigation, the majority of HELIOS$^+$ CD8 T cells lacked the expression of transcription factors or of cytokines typical to Tregs. However, as HLA-E-restriction seems to correlate with HELIOS expression, it remains possible that a fraction of HELIOS$^+$ CD8 T cells recognize and lyse other T cells and thus portray regulatory capacities.

3.5 Are HELIOS$^+$ CD8 T cells Tc17?

In our transcriptomic data, we noticed the expression of several genes characteristic of Th17 cells: RORγt, RORα, PLZF and CCL20. RORγt and RORα are the two lineage-determining Th17 transcription factors. PLZF is a transcription factor involved in Th17 differentiation and stability. It binds to the RORγt and CCR6 promoters and positively regulates their expression (Singh et al. 2015). CCL20 is a chemokine selectively expressed by Th17.

We observed that the surface marker CD161 was selectively expressed by about 25% of non-naïve HELIOS$^+$ CD8 T cells. CD161^{high} CD8 T cells have been described to display Th17-like features such as RORγt, RORα and PLZF expression, IL-17A secretion and low IFN-γ production (Billerbeck et al. 2010;

Fergusson, Fleming, and Klenerman 2011; Konduri et al. 2021; Maggi et al. 2010). The $CD161^{+}$ $HELIOS^{+}$ CD8 T cells that we analysed seem to correspond to this previously described $CD161^{high}$ CD8 T cell population.

The $ROR\gamma t^{+}$ CD8 T cells that produce IL-17A and IL-22 are called Tc17. Cytokines involved in Tc17/Th17 differentiation are the same, e.g. IL-6 and TGF-β1 (Liang, Pan, and Ye 2015; Mittrücker, Visekruna, and Huber 2014; Yen et al. 2009). $HELIOS^{+}$ CD8 T cells expressed more IL-17A and CCL20 upon activation than $HELIOS^{-}$ CD8 T cells. However, IL-22 was expressed more by $HELIOS^{-}$ CD8 T cells. Tc17 are known to produce IL-17A and can also produce IL-22. CD8 T cells that produce IL-22 but not IL-17A and have been described and called Tc22 (Nograles et al. 2009). If there is an enrichment of Tc22 in $HELIOS^{-}$ CD8 T cells, this would explain the opposite observations for IL-17A and IL-22 expression.

We failed to detect IL-17A secretion with anti-CD3-CD28 beads. Upon PMA-ionomycin stimulation, $CD161^{+}$ ($HELIOS^{+}$) CD8 T cells did secrete IL-17A but $CD161^{-}$ ($HELIOS^{-}$) CD8 T cells did not. PMA-ionomycin is usually used to trigger cytokine production by Tc17 (Flores-Santibáñez et al. 2018; Yen et al. 2009). However, it is a strong stimulus, bypassing early TCR signalling events and thus not at all physiological. While anti-CD3 antibodies mimic physiological T cell activation mechanisms better because it activates the TCR-CD3 complex, they fail to form immunological synapses or provide co-stimulatory signals. In our experiments, CD28 co-stimulation signal was present but many other signals were lacking. The use of non-physiological methods of T cell activation through anti-CD3-CD28 antibodies could explain the lack of IL-17A secretion in this case.

Some but not all $HELIOS^{+}$ CD8 T cells are certainly Tc17. In fact, some $HELIOS^{+}$ CD8 T cells express high levels of T-BET, which is not the case for

Th17/Tc17 (Loyal et al. 2020). To assess if HELIOS^{+} CD8 T cells present a transcriptomic signature typical to Th17/Tc17 by gene set enrichment analysis (GSEA), we first compiled four Th17 versus Th1 gene signatures from the MSigDB data base (Subramanian et al. 2005). However, the four retrieved Th17 gene signatures strikingly lacked overlap (Figure 30). We at least expected higher similarity especially between the GSE11924 and GSE14308 gene sets because the T cells used for these signatures were treated similarly. Given the dissimilarity between Th17 gene signatures we did not perform GSEA on our data. This observation underlines the need for better quality and more consistent data sets, and the need to critically assess GSEA comparisons.

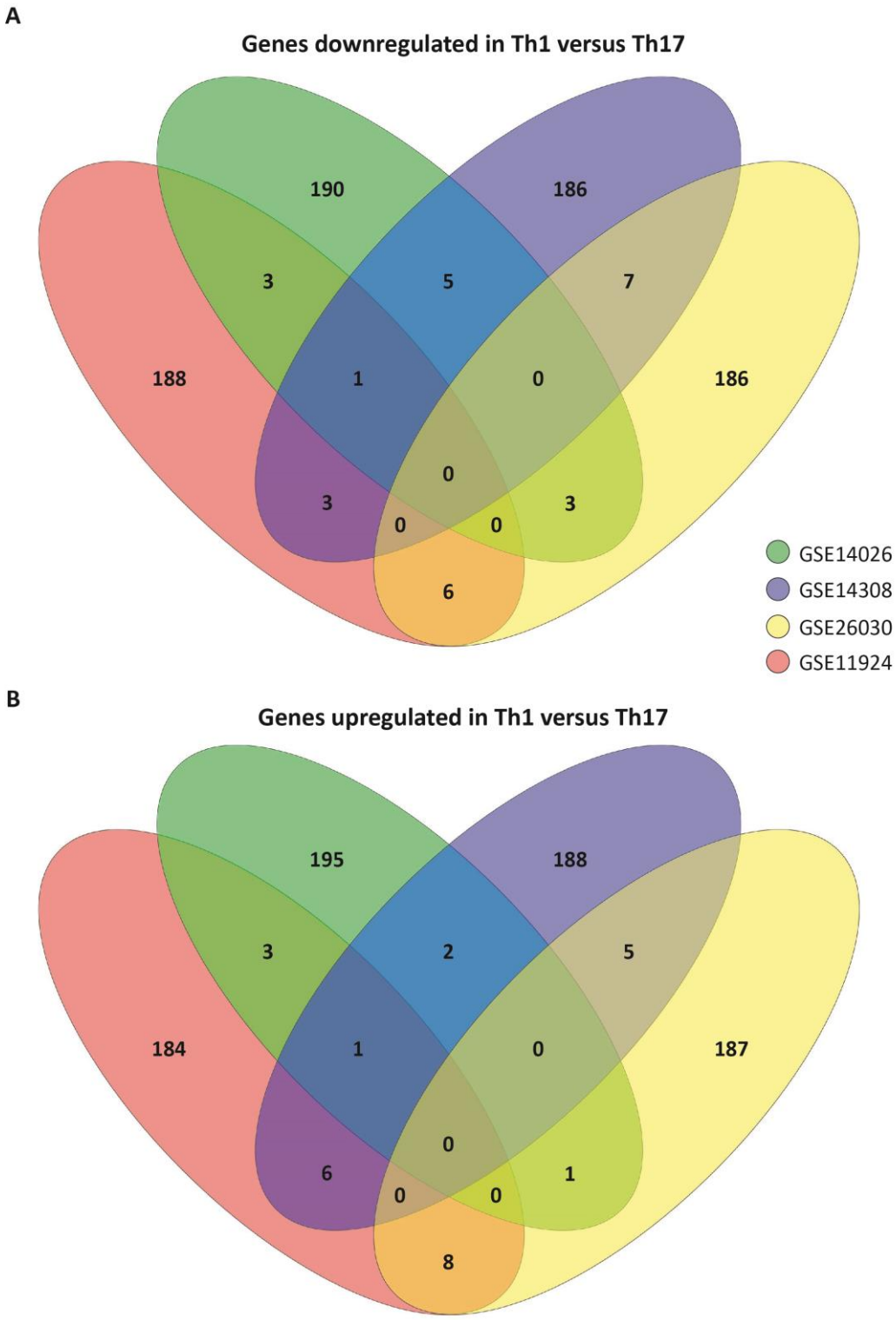


Figure 30. Known Th17 gene signatures differ dramatically. *We compared four Th17 versus Th1 gene signatures: GSE11924 and GSE14308 signatures compare in vitro polarized Th1 and Th17* (Nurieva et al. 2008; Wei et al. 2009)*, GSE14026 represents in vitro polarized Th1 and Th17 that were transferred back into mice and then sorted 15 days after transfer* (Lee et al. 2009)*, and GSE26030 compares in vitro polarized Th1 and Th17 that were further enriched in IFN-γ^{+} and IFN-γ^{-} cells respectively* (Muranski et al. 2011)*.* ***(A)*** *Downregulated and* ***(B)*** *upregulated genes in Th1 versus Th17.*

Tc17 help control fungal and viral infections (Hamada et al. 2009; Nanjappa et al. 2017, 2018). *In vitro* polarized Tc17 protect mice against lethal influenza infection comparably to *in vitro* polarized Tc1 (Hamada et al. 2009). MHC class Ib-restricted Tc17 also maintain skin homeostasis by controlling skin-specific microbiota (Linehan et al. 2018; Naik et al. 2015). Tc17 cells are also involved in inflammatory diseases such as psoriasis ; their accumulation is observed in skin psoriatic lesions (Res et al. 2010).

Tc17 functions are mediated by cytokines that include the pro-inflammatory IL-17A. IL-17A induces the expression of chemokines CXCL1, CXCL2 and CXCL8 by keratinocytes, which attract neutrophils (Furue et al. 2020; McGeachy, Cua, and Gaffen 2019). Furthermore, IL-17A induces the expression by keratinocytes of antimicrobial peptides, e.g. β-defensins, and thus protects against extracellular pathogens (*Candida albicans*, *Staphylococcus*, …) (Furue et al. 2020; McGeachy et al. 2019). Although IL-17A contributes to the wound healing process of the skin, it drives inflammation and keratinocytes hyperproliferation in psoriasis (Liang et al. 2015; McGeachy et al. 2019). Accordingly, targeting IL-17A in psoriasis has shown clinical efficacy in phase III studies (Langley et al. 2014).

We noticed high expression of CCL20 in our HELIOS^{+} CD8 T cells. CCR6, the CCL20 receptor, is expressed by Tc17, Th17 and Tregs cells. The CCL20/CCR6 axis is crucial for the recruitment of both pro-inflammatory (Tc17/Th17) and anti-inflammatory T cells (Tregs) (Schutyser, Struyf, and Van Damme 2003). Although both types of T cells are recruited through the CCL20/CCR6 axis, disruption of this axis leads to amelioration of symptoms in autoimmune disease models (EAE, psoriasis, rheumatoid arthritis) (Ranasinghe and Eri 2018).

It would be interesting to see if HELIOS is expressed by Th17. Based on the literature, it does not seem to be the case. In mice, IL-17A-producing T cells lack HELIOS (Mercer et al. 2014). In humans, HELIOS expression has not been detected in Th17 from either healthy individuals or rheumatoid arthritis and osteoarthritis patients. These data combined with our observations suggest that HELIOS is expressed by Tc17 but not Th17 (Paradowska-Gorycka et al. 2020). What is the role of HELIOS in Tc17? T cells that produce both IL-17A and IFN-γ have been described (Duhen et al. 2013). Would HELIOS be expressed in Tc17 to prevent IFN-γ an IL-2 production and stabilize their phenotype as suggested for CD4 Tregs (Baine et al. 2013; Kim et al. 2015)? It would be interesting to see if the frequencies of IFN-γ^+ IL-17A$^+$ are higher in Th17 compared to Tc17. Additionally, forcing HELIOS expression in Th17 or deleting HELIOS from Tc17 and performing a transcriptomic analysis, as well as IFN-γ and IL-17A production assays, would provide interesting information.

3.6 Are HELIOS$^+$ CD8 T cells Tc1 effectors?

T-BET is a transcription factor expressed in Th1 and Tc1 effectors that produce type 1 cytokines, e.g. IFN-γ (Cerwenka et al. 1998; Croft et al. 1994; Sad, Marcotte, and Mosmann 1995). T-BET is highly expressed by a fraction of HELIOS$^+$ CD8 T cells. However, HELIOS$^+$ T-BET^{high} CD8 T cells produce low amounts of the Th1/Tc1 effector-related cytokine IFN-γ.

We identified CD161 as a marker that is selectively expressed by a fraction of HELIOS$^+$ CD8 T cells, which correspond to Tc17. To date, we lack specific surface markers for HELIOS$^+$ Tc1 effectors. Thus, HELIOS$^+$ CD8 T cell populations cannot be studied specifically or entirely. To identify markers specific to HELIOS$^+$ CD8 T cell subpopulations, RNA-seq on fixed and

permeabilized cells, previously labelled for HELIOS, T-BET and CD161, should be performed to compare all subpopulations.

HELIOS$^+$ CD8 T cells contain Tc1 effectors that produce low amounts of IFN-γ and TNF-α. But are HELIOS$^+$ Tc1 effectors lytic? Having lytic cells that produce little pro-inflammatory cytokines might be beneficial for lysing infected target cells while avoiding potentially damaging cytokine-induced inflammation. This would increase the pool of lytic cells without increasing too much the amount of cytokines released. To answer this question, we would need a surface marker to sort HELIOS$^+$ Tc1 effectors to compare their lytic activity with that of HELIOS$^-$ Tc1 effectors. If HELIOS$^+$ Tc1 effectors fail to lyse their targets, the role of these CD8 T cells would still remain elusive.

3.7 Heterogeneity within HELIOS$^+$ CD8 T cell population.

As discussed in previous chapters, HELIOS$^+$ CD8 T cells are heterogeneous and composed of several subpopulations. Identify selective markers for each subpopulation would enable more detailed analysis of each subpopulation. A summary of the potential CD8 T cell subpopulations among HELIOS$^+$ CD8 T cells is shown in Figure 31.

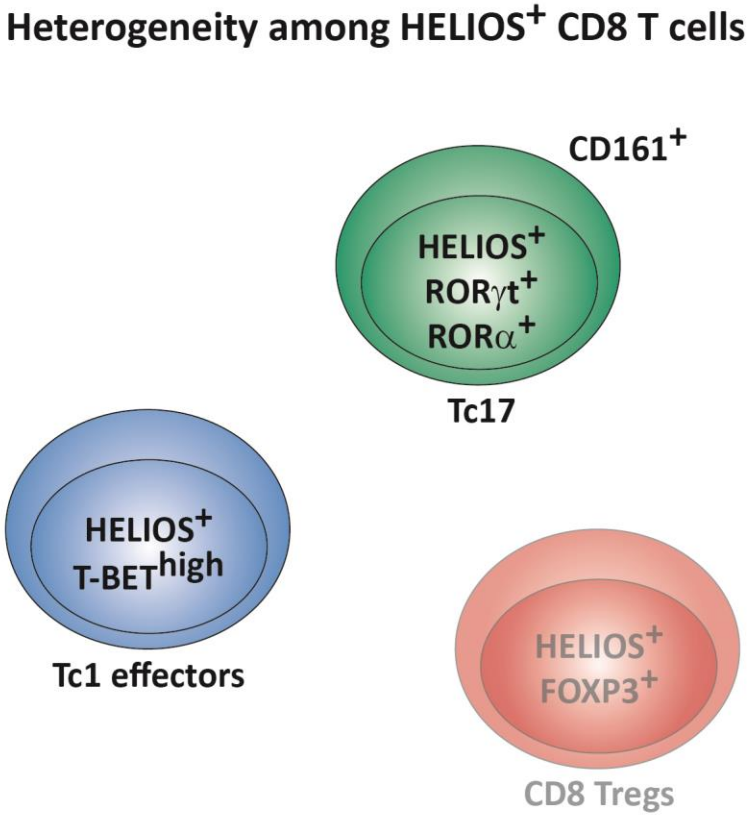


Figure 31. Heterogeneity among HELIOS$^+$ CD8 T cell population. *Our data suggest that HELIOS$^+$ CD8 T cells contain at least Tc17 (CD161$^+$, RORγt$^+$ and RORα^+) and Tc1 effectors (T-BET^{high}). In addition, based on the literature, HELIOS$^+$ CD8 T cells may contain CD8 Tregs that recognize TCR Vβ peptides.*

3.8 Is there a link between HLA-E restriction and the immunological role of HELIOS$^+$ CD8 T cells?

HLA-E-restricted CD8 T cells are directed against various pathogens (e.g. CMV, HCV, EBV, *Mycobacterium tuberculosis* (*Mtb*), and *Salmonella enterica serovar typhi* (typhoid fever)) (Joosten, Sullivan, and Ottenhoff 2016). Depending on the context, these cells have distinct properties. HLA-E restricted anti-CMV CD8 T cells are able to lyse target cells and produce IFN-γ upon stimulation (Joosten et al. 2016; Mazzarino et al. 2005; Romagnani et al. 2004). In response to *Mtb*-derived peptides presented on HLA-E, CD8 T cells show limited TRAIL-dependent cytotoxicity and produce TNF-α, IL-4 and IL-13 but not IFN-γ (Caccamo et al. 2015). Harriff *et al.* reported that CD8 T cells directed against a

glycosylated peptide from *Mtb* presented by HLA-E secreted IFN-γ (Harriff et al. 2017). Interestingly, the frequency of HLA-E-restricted anti-*Mtb* CD8 T cells decreases during antibiotic treatment while HLA-A2-restricted CD8 T cells frequency increases (Caccamo et al. 2015).

Taken together, these observations show that HLA-E-restricted CD8 T cells develop pathogen-specific functions. Thus, it is difficult to speculate what role HELIOS$^+$ CD8 T cells fulfill based only on the observed HLA-E restriction – particularly because not all HLA-E-restricted CD8 T cells express HELIOS. Comparing HLA class Ia-restricted, e.g. HLA-A2 or HLA-B7, anti-CMV CD8 T cells with HLA-E-restricted HELIOS$^+$ and HELIOS$^-$ anti-CMV CD8 T cells might define their functions more clearly.

Interestingly, CD8 T cells restricted by HLA-G, another MHC class Ib, exist in transgenic mice (expressing HLA-G, hβ2m and hCD8α): these mice, when infected with hCMV, develop HLA-G-restricted anti-CMV CD8 T cells (Lenfant et al. 2003). However, the existence of HLA-G-restricted anti-CMV CD8 T cells in humans has not been shown. If HLA-G-restricted CD8 T cells are identified in humans, determining whether these cells express HELIOS could strengthen the hypothesis that the expression of HELIOS depends on MHC class Ib restriction.

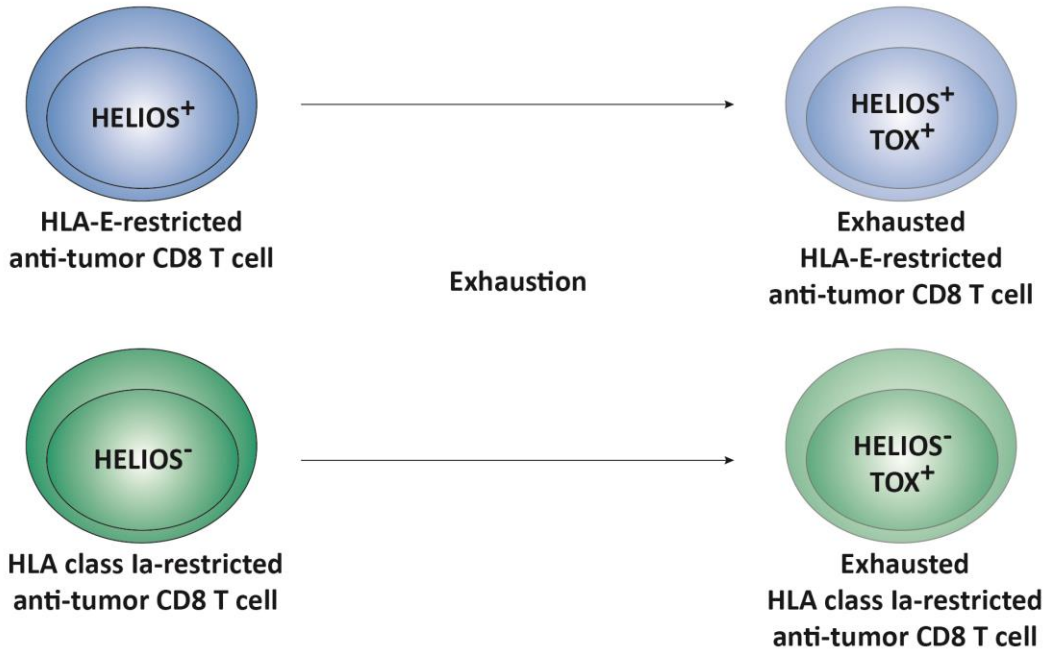
3.9 Significance of HELIOS$^+$ CD8 T cells in tumors

As HELIOS expression is upregulated in exhausted T cells in mice, we analysed HELIOS expression in tumors (Doering et al. 2012; Khan et al. 2019; Pereira et al. 2017). HELIOS$^+$ CD8 T cells are present in ovarian tumors. Using the T cell exhaustion marker TOX, we showed that some HELIOS$^+$ and HELIOS$^-$ CD8 T cells express TOX. In mice, it has clearly been shown that only anti-tumor CD8 TILs express TOX. Bystander TILs do not express TOX (Scott et al. 2019). This leads to

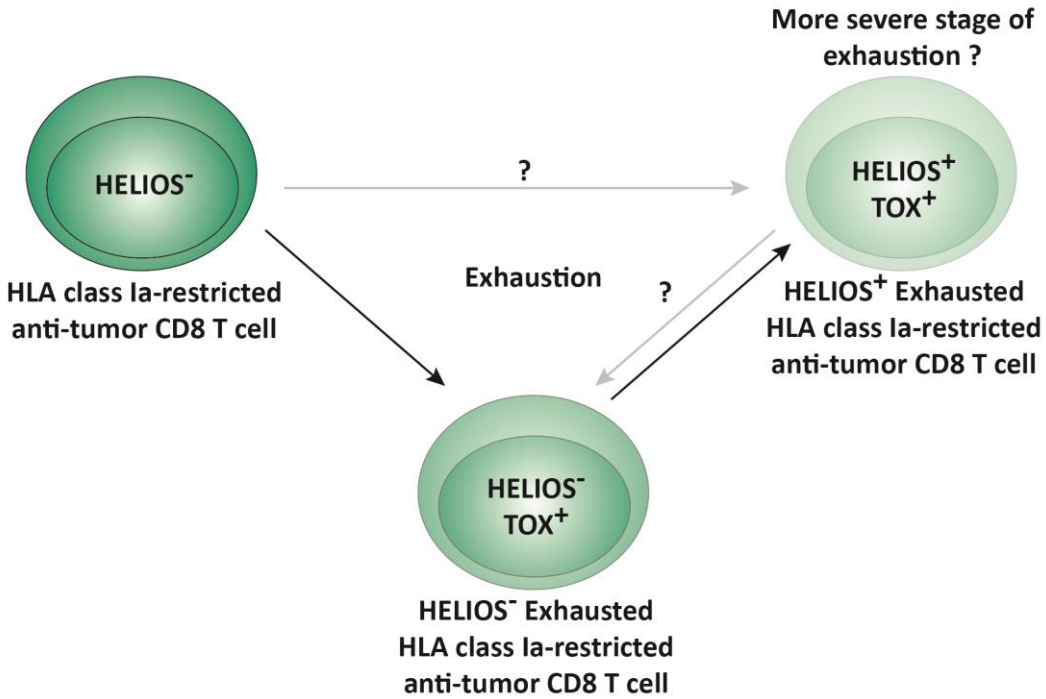
a tentative conclusion – the existence of anti-tumor HELIOS$^+$ CD8 T cells – and three hypotheses: (1) HLA-E-restricted anti-tumor CD8 T cells exist because we showed that only HLA-E-restricted CD8 T cells express HELIOS (Figure 32A); (2) HELIOS is expressed by some exhausted TOX$^+$ CD8 TILs irrespective of HLA-E restriction (Figure 32B); (3) a combination of both (Figure 32A and B). Although TOX is transiently expressed in non-exhausted CD8 T cells after activation, in our tumor samples, TOX expression correlated with poor IFN-γ production and therefore exhaustion (data not shown) (Maurice et al. 2021).

Figure 32. Why is HELIOS expressed by CD8 T cells in tumors? *HELIOS$^+$ CD8 T cells are present within tumors. Three reasons are conceivable:* ***(A)*** *HELIOS is expressed by HLA-E-restricted anti-tumor CD8 T cells;* ***(B)*** *Exhaustion induces HELIOS expression in HLA class Ia-restricted TOX$^+$ CD8 T cells and possibly indicates a more severe stage of exhaustion; the situation could be a combination of* ***(A)*** *and* ***(B)****.*

A TOX$^+$ HELIOS$^+$ CD8 T cells are HLA-E-restricted anti-tumor CD8 T cells



B HELIOS expression in tumors is due to T cell exhaustion



3.10 Concluding remarks.

Our data suggest that HELIOS should not just be considered as a Tregs or an exhausted CD8 T cell transcription factor. Indeed, we have shown that HELIOS$^+$ CD8 T cells are present in healthy individuals and the vast majority of them are certainly not Tregs. On the contrary, HELIOS$^+$ CD8 T cells comprise Tc1 effectors and Tc17.

We found HELIOS expressed by some HLA-E-restricted anti-viral CD8 T cells but not by MHC class Ia-restricted ones. This is in line with data on murine CD8 Tregs which express HELIOS and are Qa-1 (HLA-E homolog)-restricted. Interestingly, we found that some HELIOS$^+$ CD8 T cells are Tc17, which have been described as MHC class Ib-restricted in mice.

HELIOS has been shown to prevent IL-2 expression in Tregs. We found lower expression of IL-2, as well as other cytokines (IFN-γ, TNF-α), in HELIOS$^+$ CD8 T cells. As discussed in a previous chapter, if HELIOS$^+$ Tc1 effectors are able to lyse target cells and HELIOS is responsible of the lower expression of IFN-γ and/or TNF-α in CD8 T cells, this may be interesting for example in CAR T cell therapy. Indeed, adverse events due to inflammation are often met with CAR T cells (Brudno and Kochenderfer 2016; Gust et al. 2020; June et al. 2018; Santomasso et al. 2019; Wei et al. 2020). Being able to increase the number of lytic CAR T cells without increasing too much the amount of pro-inflammatory cytokines produced might be interesting.

4 Materials and methods

4.1 Cell lines and media.

LG2-EBV were cultured in Iscove's Modified Dulbecco's Medium (IMDM) *(Thermo Fischer Scientific, Waltham, Massachusets, 21980065)* supplemented with 10% fetal bovine serum (FBS), GlutaMAX *(Thermo Fischer Scientific, 35050061)* and penicillin (100U.ml^{-1}) streptomycin (100µg.ml^{-1}) *(Sigma Aldrich, St Louis, Missouri, P4333)*. T cells were cultured with IMDM supplemented with 10% human serum (HS) *(Ludwig Institute for Cancer Research (LICR), Brussels branch)*, GlutaMAX and penicillin (100U.ml^{-1}) streptomycin (100µg.ml^{-1}) referred as T cell medium. When indicated IL-2 *(Novartis, Bâle, Switzerland)* was added.

4.2 Isolation of cells from blood and tumors.

PBMCs were isolated from blood from healthy, hemochromatosis, COVID-19 and ovarian cancer patients using density gradient. Blood was added on top of lymphoprep *(Alere Technologies, Oslo, Norway, 1114547)* and then centrifuged at 870g for 20min at room temperature (RT) with minimum acceleration and brake. The cellular ring was recovered and then washed thrice with PBS/EDTA 1mM to remove platelets. First centrifugation was done at 400g for 10min at RT and the two following at 300g for 7min at RT. PBMCs were frozen in 50% IMDM 40% HS and 10% DMSO *(Santa Cruz Biotechnology, Dallas, Texas, sc-358801)*. Cells from ovarian tumors were isolated by mechanical dissociation of small tumor pieces using MACS dissociator *(Miltenyi Biotec, Paris, France)* followed by a 45min enzymatic digestion at 37°C using liberase low dispase (*Sigma Aldrich, 5466202001*) and low thermolysin (*Sigma Aldrich, 5401020001*) at recommended dilutions. A second mechanical dissociation using MACS dissociator *(Miltenyi, Bergisch Gladbach, Germany)* has been performed and the subsequent cell suspension has been 40µm filtered to remove remaining

aggregates. Cells were then washed thrice with PBS/EDTA 1mM and spun down at 300g for 7min at RT and frozen in 50% IMDM 40% HS and 10% DMSO.

4.3 $CD2^+$ and $CD2^-$ PBMCs isolation by Rosetting.

PBMCs were resuspended at $1x10^7$ cells.ml^{-1} in Roswell Park Memorial Institute medium (RPMI) *(Life technologies, 52400041)*. 2 –aminoethyl-isothiouronium bromide pre-treated sheep red blood cells *(Labormerk, Ochsenhausen, Germany, E-410)* were added (1ml for $1x10^8$ PBMCs). Cell suspension was added on top of lymphoprep and spun down at 870g for 20min at RT with minimum acceleration and brake. The cellular ring containing $CD2^-$ PBMCs was recovered and frozen in 50% IMDM 40% HS and 10% DMSO. The cell pellet containing $CD2^+$ PBMCs was resuspended in lysis buffer composed of 10% RPMI, 10% FBS and 80% NH_4Cl 160mM pH7,4. After one minute of incubation, cells were spun down at 800g for 1min at RT. Supernatant was removed and cells were washed with RPMI.

4.4 CD8 magnetic-activated cell sorting (MACS).

PBMCs were resuspended at $2x10^8$ cells/ml in staining buffer (PBS, 1mM EDTA and 1% HS) containing FCR blocking reagent *(Miltenyi, 130-059-901)* and CD8 microbeads *(Miltenyi, 130-045-201)* for 15 minutes at 4°C. Then, cells were passed on a 40μm filter and sorted with the AutoMACS *(Miltenyi)*. CD8 T cells were frozen in 50% IMDM 40%HS and 10% DMSO.

4.5 Cytokine production assay.

High binding 96-well flat bottom plates *(Greiner bio-one, 655061)* were coated with 100μl of 1μg/ml anti-CD3 antibody *(Biolegend, San Diego, California, clone OKT3, 317326)* diluted in PBS overnight at 4°C. Human PBMCs were thawed for

2h at 37°C in T cell medium with 5U/ml DNAse. Anti-CD3 coated plates were brought at 37°C for half an hour before stimulation. A total of $2x10^5$ cells were added per well in T cell medium. Cells were spun down 1min at 400g, and incubated at 37°C 8% CO_2. After 1 hour, 5μg/ml brefeldin A *(Sigma Aldrich, B7651)* were added and cells were incubated for an additional 4h at 37°C 8 % CO_2. After a total of 5h of stimulation, cells were brought to 4°C and stained as described in the flow cytometry section.

4.6 TNF-α secretion assay.

High binding 96-well flat bottom plates were coated with 100μl of 1μg/ml anti-CD3 antibody diluted in PBS overnight at 4°C. Human PBMCs were thawed for 2h at 37°C in T cell medium with 5U/ml DNAse. Anti-CD3 coated plates were brought at 37°C for half an hour before stimulation. A total of $2x10^5$ cells were added per well in T cell medium containing 10μg/ml TAPI-0 *(Sigma, SML1292*) and 2μg/ml anti-TNF-α Alexa Fluor 700 antibody *(Biolegend, clone Mab11, 502928)* and spun down 400g for 1min before incubation at 37°C 8% CO_2 for 5h. Next, cells were brought to 4°C and stained as described in the flow cytometry section.

4.7 Flow cytometry.

4.7.1 Multimer staining.

Cells were harvested, washed once with staining buffer and then labelled with 10nM PE-multimer for 10min at RT (Table 1). After, cells were diluted with one volume of staining buffer an incubated 15min at RT. Cells were washed twice with staining buffer at 4°C and spun down at 400g. Next, extracellular and intracellular staining with antibodies and fixable viability dye were performed as described below.

Origin	Protein	Peptide	HLA	Fluorochrome
CMV	pp65	NLVPMVATV	HLA-A*02:01	PE
CMV	pp65	RPHERNGFTVL	HLA-B7*07:02	PE
CMV	pp65	TPRVTGGGAM	HLA-B7*07:02	PE
CMV	UL-40	VMAPRTLIL	HLA-E*01:01	PE
EBV	BMFL-1	GLCTLVAML	HLA-A*02:01	PE
EBV	BZLF-1	SQAPLPCVL	HLA-E*01:03	PE
Influenza	M1	GILGFVFTL	HLA-A*02:01	PE
Mycobacterium tuberculosis	Rv1508	VMATRRNVL	HLA-E*01:01	PE

Table 1. List of multimers used.

4.7.2 Extracellular and intracellular staining.

Cells were harvested, washed once with staining buffer (PBS, 1mM EDTA and 1% HS) and then labelled extracellularly for 20min at 4°C with antibodies and fixable viability dye eFluor780 *(Thermo Fischer Scientific, 65-0865-18)* to exclude dead cells (Table 2). Cells were washed twice with staining buffer and once with PBS. Then, cells were fixed and permeabilized overnight at 4°C using eBioscience Foxp3 / Transcription Factor kit *(Thermo Fisher Scientific, 00-5523-00)*. Cells were washed thrice with permeabilisation buffer at 4°C, resuspended

in blocking solution (permeabilisation buffer 8% HS) and incubated for 15min at 4°C. Next, antibodies, diluted in permeabilisation buffer, were added for 2h at 4°C (Table 3). Cells were washed thrice with permeabilisation buffer. Finally, cells were resuspended in PBS 1% PFA and analyzed by flow cytometry using BD LSRII Fortessa *(Becton Disckinson (BD) Biosciences, Franklin lakes, New Jersey)*. Data were analyzed using Flowjo *(BD Biosciences)*.

Target	Fluorochrome	Clone	Dilution	Reference
CD19	APC-Cy7	SJ25C1	1/50	*Biolegend, 363010*
CD20	APC-Cy7	2H7	1/200	*Biolegend, 302314*
CD33	APC-Cy7	WM53	1/400	*Biolegend, 303442*
CD326	APC-Cy7	9C4	1/200	*Biolegend, 324246*
CD2	BV480	RPA-2.10	1/500	*BD Biosciences, 746538*
CD8β	PerCP-Cy5.5	2ST8.5H7	1/100	*BD Biosciences, custom antibody*
CCR7	PE	G043H7	1/80	*Biolegend, 353204*
CCR7	BV650	G043H7	1/40	*Biolegend, 353234*
CD45RA	FITC	HI100	1/200	*Biolegend, 304148*
PD-1	BV786	EH12.2H7	1/40	*Biolegend, 329930*
CD38	BV480	HIT2	1/30	*BD Biosciences, 566137*

Table 2. List of antibodies used for extracellular staining.

Target	Fluorochrome	Clone	Dilution	Reference
Ki-67	BV421	B56	1/50	*BD Biosciences, 562899*
HELIOS	PE-Cy7	22F6	1/25	*Biolegend, 137236*
HELIOS	PE	22F6	1/25	*Biolegend, 137216*
HELIOS	APC	22F6	1/25	*Biolegend, 137222*
T-BET	BV421	4B10	1/200	*Biolegend, 644816*
T-BET	APC	4B10	1/200	*Biolegend, 644814*
IFN-γ	Alexa-Fluor 700	B27	1/167	*Biolegend, 506516*
TNF-α	Alexa-Fluor 700	Mab11	1/200	*Biolegend, 502928*
IL-2	PE	MQ1-17H12	1/25	*Biolegend, 500307*
Histone H1	Alexa Fluor 488	AE-4	1/20	*Santa Cruz Biotechnology, sc-8030 AF488*
Histone H1	Alexa Fluor 647	AE-4	1/20	*Santa Cruz Biotechnology, sc-8030 AF647*

Table 3. List of antibodies used for intracellular staining.

4.7.3 Particularities for intracellular staining followed by RNA extraction.

When cells were stained intracellularly and FACS sorted for RNA extraction, RNAse inhibitor RNAsin Plus *(Promega, Madison, Wisconsin, N2615)* was added during the fixation/permeabilisation overnight at 2U/µl. During washes, RNAsin Plus was added at 0,04U/µl. During the staining, RNAsin Plus was at 2U/µl and at the end, cells were not resuspended in PFA 1% in this case but in the permeabilisation buffer containing 0,4U/µl RNAsin Plus. During the FACS sorting, cells were recovered in permeabilisation buffer containing 0,04U/µl RNAsin Plus.

4.8 Cytokine secretion assay.

CD8 T cells were resuspended in T cell medium. Cells were seeded at 50 000 cells per well in a 96-well round bottom plate. Three conditions were set up. T cells were either kept unstimulated, stimulated with anti-CD3-CD28 beads (1b:3T) *(Thermo Fisher Scientific, 11132D)* or a cocktail of 1ng/ml PMA *(LC laboratories, Woburn, Massachusetts, P-1680)* and 1μg/ml Ionomycin (Sigma Aldrich, I0634). After 20h incubation at 37°C 8% CO_2, supernatants were recovered and stored at -80°C until cytokines quantification.

4.9 IL-17A quantification by LEGENDPlex.

LEGENDplex™ Human CD8/NK Panel kit *(Biolegend, 740267)* was used. The manufacturer protocol was followed. In brief, supernatants were diluted two times in assay buffer. Samples and standards were incubated with beads coated with capture antibodies for 2h at RT. Plates were washed twice with wash buffer and spun down at 250g 5min at RT. Beads were incubated with detection antibodies for 1h at RT. Without washing, streptavidin-PE was added during 30min at RT out of light. Plates were washed twice with wash buffer and spun down at 250g 5min at RT. Beads were resuspended in wash buffer and analysed by flow cytometry with a BD LSR II Fortessa. Data were analysed with the manufacturer software.

4.10 T cell co-culture.

γ-irradiated (100Gy) $CD2^-$ PBMCs, CD8 and CD4 PBLs from hemochromatosis patients were thawed for 2h at 37°C. $CD161^+$ and $CD161^-$ CD8 T_{EM} were sorted by flow cytometry. After sorting, cells were left for 1h at 37°C 8% CO_2. Cells were labelled with following cell trackers for 20min at 37°C: CD4 T cells and $CD161^-$ CD8 T_{EM} were labelled with CFMDA at 1μM *(Thermo Fischer Scientific,*

C2925) and CD161$^+$ CD8 T_{EM} with cell trace violet at 5μM *(Thermo Fischer Scientific, C34571)*. Then, cells were washed twice with T cell medium. Cells were resuspended and added in 96-well round bottom plate coated with anti-CD3 antibody (1μg/ml). Details regarding co-culture conditions are shown in Figure 33. CD2$^-$ PBMCs were used as feeder cells and added at 1:1 ratio with 5U/ml IL-2. After 4 days, cells were labelled extracellularly as described and analysed by flow cytometry on a FACS ARIA III *(BD Biosciences)*.

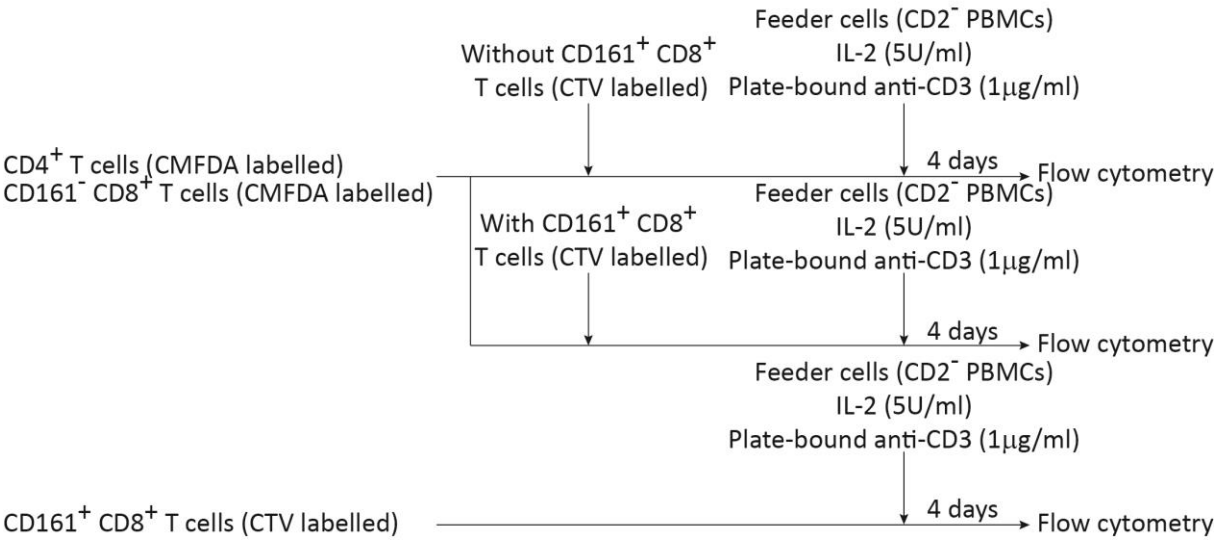


Figure 33. Co-culture conditions. *CD4 and CD8 T cells were thawed for 2h at 37°C with 5U/ml of Dnase. Cells were then labelled at 4°C with antibodies against CD8β, CCR7, CD45RA and CD161. CD161$^+$ and CD161$^-$ CD8 T_{EM} were FACS sorted. CD4 T cells and CD161$^-$ CD8 T_{EM} were labelled with cell tracker 5-chloromethylfluorescein diacetate (CMFDA) and CD161$^+$ CD8 T_{EM} with cell trace violet (CTV). CD4 T cells, CD161$^+$ and CD161$^-$ CD8 T_{EM} were activated with plate-bound anti-CD3 (1μg/ml) in the presence of γ-irradiated autologous CD2$^-$ PBMCs (ratio 1:1) and 5U/ml IL-2 for 4 days at 37C*

4.11 CRISPR-Cas9.

CD8 PBLs from hemochromatosis patients were thawed for 2h at 37°C. After thawing, CD161$^+$ and CD161$^-$ CD8 T_{EM} were sorted from blood of hemochromatosis patients by flow cytometry. After sorting, cells were left for 1h at 37°C. Cas9/gRNA complexes were prepared in RNAse free water *(Ambion, AM9906)*. A total of 5μg of Cas9 *(Thermo Fischer Scientific, A36499)* and

150pmol of gRNA (table 2) were incubated for 5min at RT. Then, cells were washed and medium was completely removed. Cells were resuspended in P3 primary cell electroporation buffer *(Lonza, Bâle, Switzerland, V4XP-3024)*. Cas9/gRNA complexes were added directly and incubated 2min with T cells in electroporation buffer. Next, cells were electroporated using "T cell human unstim high efficiency" program (FI115) of the 4D-nucleofector *(Lonza)*. Directly after electroporation, warm T cell medium containing 50U/ml IL-2 was added. Cells were left for 5h at 37°C 8% CO_2. Cells were washed once with T cell medium and resuspended in fresh T cell medium with 50U/ml IL-2. Feeder cells (LG2-EBV) were added at 3 feeder cells for 1 T cell. Cells were activated using anti-CD3-CD28 beads *(Thermo Fischer Scientific, 111.32D)* (1b:3T) during 7 days at 37°C 8% CO_2. After 7 days, cells were counted using trypan blue *(Sigma, T8154)*.

4.12 RNA extraction from fixed and permeabilized cells.

Recover all total nucleic acid isolation kit *(Thermo Fisher Scientific, AM1975)* was used. The reverse crosslinking with protease was done for 1h at 60°C. Then, RNA was extracted using the manufacturer protocol. After elution in water, RNA was precipitated by adding 0,1 volume sodium acetate 3M *(Thermo Fischer Scientific, AM9740)*, 3 volumes ice cold ethanol 100% and 1μg glycogen *(Thermo Fisher Scientific, R0551)*. Samples were incubated at -20°C overnight and spun down at 14000g 30min at 4°C. Samples were then washed 2 times with ice cold ethanol 75% and spun down at 14000g 10min at 4°C. After supernatant removal, samples were dried at RT and then resuspended in RNAse free water.

4.13 RNA-sequencing and transcriptomic analysis.

RNA integrity was checked using Agilent RNA 6000 pico kit *(Agilent, Santa Clara, California, 5067-1513)* and sent for ultra-low input RNA-sequencing *(Genewiz, Leipzig, Germany)*. Library construction is unstranded and poly-A-based. Between 20 and 30 million paired-reads were sequenced per sample on an Illumina HiSeq 2x150 platform. Data were analysed with the help of Christophe Vanhaver. In brief, after an initial quality control of FASTQ files, reads were trimmed using Trimmomatic and checked again for quality (Bolger, Lohse, and Usadel 2014). Next, trimmed reads were aligned to GRCh38 human genome with HISAT2 (Kim et al. 2019). Read counts were determined with featuresCount (Liao, Smyth, and Shi 2014). Normalization of read counts and analysis of differentially expressed genes were performed using DESeq2 (Love et al. 2014). Data visualization was done with Qlucore *(Qlucore, Lund, Sweden)*.

4.14 Statistical analysis.

Statistical analysis were made with GraphPad Prism v5 *(GraphPad, San Diego, California)* with the exception of RNA-sequencing for which paired samples analysis with DESeq2 was done. Statistical tests are indicated in figure legend.

5 Abbreviations

ADAP	Adhesion and degranulation-promoting adapter protein
AP-1	Activating protein 1
APC	Antigen presenting cell
Bat3	HLA-B-associated transcript 3
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
Bcl10	B-cell lymphoma/leukemia 10
BCL6	B-cell lymphoma 6 protein
BLIMP1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
BTLA	B- and T-lymphocyte attenuator
CAMK	Calcium/calmodulin-dependent protein kinase
CAR	Chimeric antigen receptor
CARMA1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1
CCL…	(C-C motif) ligand …
CCR…	C-C chemokine receptor type …
CD…	Cluster of differentiation …
CMFDA	5-chloromethylfluorescein diacetate
CMV	Cytomegalovirus
CRTAM	Cytotoxic And Regulatory T Cell Molecule
CSK	C-Terminal Src Kinase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTV	Cell trace violet
DAG	Diacylglycerol
DEG	Differentially expressed genes
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAM-1	DNAX Accessory Molecule-1
EAE	Experimental autoimmune encephalitis
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra-acetic acid
EOMES	Eomesodermin
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FBS	Extracellular signal regulated kinase
FOXP3	Forkhead box P3
GADS	GRB2-related adaptor downstream of Shc
GEF	Guanine nucleotide exchange factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor

GLUT1	Glucose transporter 1
GRB2	Growth factor receptor-bound protein 2
GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase-3
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMG	High–mobility group
HMGB1	High–mobility group box 1
HS	Human serum
HVEM	Herpes Virus Entry Mediator
ICOS	Inducible T-cell COStimulator
ICU	Intensive care unit
IFN-γ	Interferon gamma
IκB	Inhibitor of NF-kB
IKK	Inhibitor of nuclear factor kappa-B kinase
IKZF	Ikaros family zinc finger protein
IL-…	Interleukin …
IMDM	Iscove's Modified Dulbecco's Medium
IP3	Inositol 1,4,5-trisphosphate
IRF…	Interferon regulatory factor …
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITK	IL-2 inducible T-cell kinase
ITSM	Immunoreceptor Tyrosine-Based Switch Motif
LAG-3	Lymphocyte-Activation Gene 3
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LSECTIN	Liver Sinusoidal Endothelial Cell Lectin
LTB	Lymphotoxin beta
MACS	Magnetic-activated cell sorting
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEK	Mitogen-activated protein kinase, extracellular signal-regulated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MPECs	Memory precursor effector cells

Mtb	Mycobacterium tuberculosis
mTOR	Mammalian target of rapamycin
NCK1	Non-catalytic region of tyrosine kinase adaptor protein 1
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa b
NK	Natural killer
NKG2	Natural killer group 2
OVA	Ovalbumin
PAG	Glycosphingolipid-enriched microdomains
PBLs	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphobuffered saline
PCA	Principal component analysis
PD-1	Programmed cell death 1
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	Programmed death-ligand 1
PFA	Paraformaldehyde
PH	Pleckstrin homology domain
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphospahte
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKCθ	Protein kinase C theta
PLCγ1	Phospholypase C gamma 1
PLZF	Promyelocytic leukaemia zinc finger protein
PMA	Phorbol 12-myristate 13-acetate
RasGRP1	RAS guanyl nucleotide-releasing protein 1
RIN	RNA integrity number
RNA	Ribonucleic acid
RORα	Receptor-related orphan receptor alpha
RORγt	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
SHP1/2	Src homology region 2 domain-containing phosphatase-1
SLECs	Short lived effector cells
SLP76	SH2 domain containing leukocyte protein of 76kDa
T-BET	T-box protein expressed in T cells
TBEV	Tick-borne encephalitis virus
Tc1	Type 1 cytotoxic CD8 T cells
Tc17	IL-17-secreting CD8 T cells
Tc22	IL-22-secreting CD8 T cells

TCF-1	T cell factor 1
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cells
T_{EMRA}	Effector memory $CD45RA^{+}$ T cells
Th1	T helper cells type 1
Th17	T helper celsl type 17
Th2	T helper cells type 2
Th22	T helper cells type 22
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	Tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TNFRS	Tumor necrosis factor receptor superfamily
TOX	Thymocyte selection-associated high mobility group box protein
TRAF…	Tumor necrosis factor receptor–associated factor …
Tregs	Regulatory T cells
T_{RM}	Resident memory T cells
ZAP-70	Zeta-chain-associated protein kinase 70
ZBTB16	Zinc finger and BTB domain-containing 16

6 References

Agata, Yasutoshi, Akemi Kawasaki, Hiroyuki Nishimura, Yasumasa Ishida, Takeshi Tsubata, Hideo Yagita, and Tasuku Honjo. 1996. “Expression of the PD-1 Antigen on the Surface of Stimulated Mouse T and B Lymphocytes.” International Immunology 8(5):765–72.

Agrati, C., C. Castilletti, R. Casetti, A. Sacchi, L. Falasca, F. Turchi, N. Tumino, V. Bordoni, E. Cimini, D. Viola, E. Lalle, L. Bordi, S. Lanini, F. Martini, E. Nicastri, N. Petrosillo, V. Puro, M. Piacentini, A. Di Caro, G. P. Kobinger, A. Zumla, G. Ippolito, and M. R. Capobianchi. 2016. “Longitudinal Characterization of Dysfunctional T Cell-Activation during Human Acute Ebola Infection.” Cell Death and Disease 7(3).

Ahmadzadeh, Mojgan, Laura A. Johnson, Bianca Heemskerk, John R. Wunderlich, Mark E. Dudley, Donald E. White, and Steven A. Rosenberg. 2009. “Tumor Antigen-Specific CD8 T Cells Infiltrating the Tumor Express High Levels of PD-1 and Are Functionally Impaired.” Blood 114(8):1537–44.

Akimova, Tatiana, Ulf H. Beier, Liqing Wang, Matthew H. Levine, and Wayne W. Hancock. 2011. “Helios Expression Is a Marker of T Cell Activation and Proliferation.” PLoS ONE 6(8).

Aldemir, Hatice, Virginie Prod’homme, Marie-Jeanne Dumaurier, Christelle Retiere, Gwenola Poupon, Julie Cazareth, Franck Bihl, and Veronique M. Braud. 2005. “Cutting Edge: Lectin-Like Transcript 1 Is a Ligand for the CD161 Receptor.” The Journal of Immunology 175(12):7791–95.

Alfei, Francesca, Kristiyan Kanev, Maike Hofmann, Ming Wu, Hazem E. Ghoneim, Patrick Roelli, Daniel T. Utzschneider, Madlaina von Hoesslin, Jolie G. Cullen, Yiping Fan, Vasyl Eisenberg, Dirk Wohlleber, Katja Steiger, Doron Merkler, Mauro Delorenzi, Percy A. Knolle, Cyrille J. Cohen, Robert Thimme, Benjamin Youngblood, and Dietmar Zehn. 2019. “TOX Reinforces the Phenotype and Longevity of Exhausted T Cells in Chronic Viral Infection.” Nature.

Anderson, Courtney K. and Laurent Brossay. 2016. “The Role of MHC Class Ib-Restricted T Cells during Infection.” Immunogenetics 68(8):677–91.

Baine, Ian, Samik Basu, Rachel Ames, Rani S. Sellers, and Fernando Macian. 2013. “ Helios Induces Epigenetic Silencing of Il2 Gene Expression in Regulatory T Cells .” The Journal of Immunology 190(3):1008–16.

Bandyopadhyay, S., N. Soto-Nieves, and F. Macian. 2007. “Transcriptional Regulation of T Cell Tolerance.” Semin.Immunol. 19(1044-5323 (Print)):180–87.

Barber, Daniel L., E. Joh. Wherry, David Masopust, Baogong Zhu, James P. Allison, Arlene H. Sharpe, Gordon J. Freeman, and Rafi Ahmed. 2006. “Restoring Function in Exhausted CD8 T Cells during Chronic Viral Infection.” Nature 439(7077):682–87.

Baron, Udo, Stefan Floess, Georg Wieczorek, Katrin Baumann, Andreas Grützkau, Jun Dong, Andreas Thiel, Tina J. Boeld, Petra Hoffmann, Matthias Edinger, Ivana Türbachova, Alf Hamann, Sven Olek, and Jochen Huehn. 2007. “DNA Demethylation in the Human FOXP3 Locus Discriminates Regulatory T Cells from Activated FOXP3+ Conventional T Cells.” European Journal of Immunology 37(9):2378–89.

Bengsch, Bertram, Bianca Seigel, Marianne Ruhl, Jörg Timm, Martin Kuntz, Hubert E. Blum, Hanspeter Pircher, and Robert Thimme. 2010. “Coexpression of PD-1, 2B4, CD160 and KLRG1 on Exhausted HCV-Specific CD8+ T Cells Is Linked to Antigen Recognition and T Cell Differentiation.” PLoS Pathogens 6(6).

Billerbeck, Eva, Yu Hoi Kang, Lucy Walker, Helen Lockstone, Stefanie Grafmueller, Vicki Fleming, Jonathan Flint, Chris B. Willberg, Bertram Bengsch, Bianca Seigel, Narayan Ramamurthy, Nicole Zitzmann, Eleanor J. Barnes, Jonarthan Thevanayagam, Anisha Bhagwanani, Alasdair Leslie, Ye H. Oo, Simon Kollnberger, Paul Bowness, Oliver Drognitz, David H. Adams, Hubert E. Blum, Robert Thimme, and Paul Klenerman. 2010. “Analysis of CD161 Expression on Human CD8+ T Cells Defines a Distinct Functional Subset with Tissue-Homing Properties.” Proceedings of the National Academy of Sciences of the United States of America 107(7):3006–11.

Blackburn, Shawn D., Haina Shin, W. Nicholas Haining, Tao Zou, Creg J. Workman, Antonio Polley, Michael R. Betts, Gordon J. Freeman, Dario A. A. Vignali, and E. John Wherry. 2009. “Coregulation of CD8+ T Cell Exhaustion by

Multiple Inhibitory Receptors during Chronic Viral Infection." Nature Immunology 10(1):29–37.

Blom, Kim, Monika Braun, Jolita Pakalniene, Laura Dailidyte, Vivien Béziat, Margit H. Lampen, Jonas Klingström, Nina Lagerqvist, Torbjörn Kjerstadius, Jakob Michaëlsson, Lars Lindquist, Hans Gustaf Ljunggren, Johan K. Sandberg, Aukse Mickiene, and Sara Gredmark-Russ. 2015. "Specificity and Dynamics of Effector and Memory CD8 T Cell Responses in Human Tick-Borne Encephalitis Virus Infection." PLoS Pathogens 11(1):1–20.

Bolger, Anthony M., Marc Lohse, and Bjoern Usadel. 2014. "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data." Bioinformatics 30(15):2114–20.

Boomer, Jonathan S. and Jonathan M. Green. 2010. "An Enigmatic Tail of CD28 Signaling." Cold Spring Harbor Perspectives in Biology 2(8):1–21.

Brownlie, Rebecca J. and Rose Zamoyska. 2013. "T Cell Receptor Signalling Networks: Branched, Diversified and Bounded." Nature Reviews. Immunology 13(4):257–69.

Brudno, Jennifer N. and James N. Kochenderfer. 2016. "Toxicities of Chimeric Antigen Receptor T Cells: Recognition and Management." Blood 127(26):3321–30.

Bueno, Clara, Arancha Rodriguez-Caballero, Andrés García-Montero, Atanasio Pandiella, Julia Almeida, and Alberto Orfao. 2002. "A New Method for Detecting TNF-α-Secreting Cells Using Direct-Immunofluorescence Surface Membrane Stainings." Journal of Immunological Methods 264(1–2):77–87.

Caccamo, Nadia, Gabriella Pietra, Lucy C. Sullivan, Andrew G. Brooks, Teresa Prezzemolo, Marco P. La Manna, Diana Di liberto, Simone A. Joosten, Krista E. van Meijgaarden, Paola Di Carlo, Lucina Titone, Lorenzo Moretta, Maria C. Mingari, Tom H. M. Ottenhoff, and Francesco Dieli. 2015. "Human CD8 T Lymphocytes Recognize Mycobacterium Tuberculosis Antigens Presented by HLA-E during Active Tuberculosis and Express Type 2 Cytokines." European Journal of Immunology 45(4):1069–81.

Carrasco, Javier, Danièle Godelaine, Aline Van Pel, Thierry Boon, and Pierre Van Der Bruggen. 2006. “CD45RA on Human CD8 T Cells Is Sensitive to the Time Elapsed since the Last Antigenic Stimulation.” Blood 108(9):2897–2905.

Cerwenka, Adelheid, Laura L. Carter, Joyce B. Reome, Susan L. Swain, and Richard W. Dutton. 1998. “In Vivo Persistence of CD8 Polarized T Cell Subsets Producing Type 1 or Type 2 Cytokines.” Journal of Immunology (Baltimore, Md. : 1950) 161(1):97–105.

Chan, a C., M. Iwashima, C. W. Turck, and a Weiss. 1992. “ZAP-70: A 70 Kd Protein-Tyrosine Kinase That Associates with the TCR Zeta Chain.” Cell 71(4):649–62.

Chen, Lieping and Dallas B. Flies. 2013. “Molecular Mechanisms of T Cell Co-Stimulation and Co-Inhibition.” Nature Reviews Immunology 13(4):227–42.

Cimmino, Luisa, Gislaine A. Martins, Jerry Liao, Erna Magnusdottir, Gabriele Grunig, Rocio K. Perez, and Kathryn L. Calame. 2008. “ Blimp-1 Attenuates Th1 Differentiation by Repression of Ifng , Tbx21 , and Bcl6 Gene Expression .” The Journal of Immunology 181(4):2338–47.

Crespo, Joel, Haoyu Sun, Theodore H. Welling, Zhigang Tian, and Weiping Zou. 2013. “T Cell Anergy, Exhaustion, Senescence, and Stemness in the Tumor Microenvironment.” Current Opinion in Immunology 25(2):214–21.

Croft, By Michael, Laura Carter, Susan L. Swain, and Richard W. Dutton. 1994. “Generation of Polarized Antigen-Specific CD8 Effector Populations: Reciprocal Action of Interleukin (IL)-4 and IL-12 in Promoting Type 2 versus Type 1 Cytokine Profiles.” 180(November).

Crotty, Shane. 2019. “T Follicular Helper Cell Biology: A Decade of Discovery and Diseases.” *Immunity* 50(5):1132–48.

Cuende, J., S. Lienart, O. Dedobbeleer, B. van der Woning, G. De Boeck, J. Stockis, C. Huygens, D. Colau, J. Somja, P. Delvenne, M. Hannon, F. Baron, L. Dumoutier, J. C. Renauld, H. De Haard, M. Saunders, P. G. Coulie, and S. Lucas. 2015. “Monoclonal Antibodies against GARP/TGF- 1 Complexes Inhibit the Immunosuppressive Activity of Human Regulatory T Cells in Vivo.” Science Translational Medicine 7(284):284ra56-284ra56.

Cui, Weiguo and Susan M. Kaech. 2010. “Generation of Effector CD8+ T Cells and Their Conversion to Memory T Cells.” Immunological Reviews 236(1):151–66.

Doering, Travis A., Alison Crawford, Jill M. Angelosanto, Michael A. Paley, Carly G. Ziegler, and E. John Wherry. 2012. “Network Analysis Reveals Centrally Connected Genes and Pathways Involved in CD8+ T Cell Exhaustion versus Memory.” Immunity 37(6):1130–44.

Duhen, Rebekka, Simon Glatigny, Carlos A. Arbelaez, Tiffany C. Blair, Mohamed Oukka, and Estelle Bettelli. 2013. “Cutting Edge: The Pathogenicity of IFN-γ–Producing Th17 Cells Is Independent of T-Bet.” The Journal of Immunology 190(9):4478–82.

Fergusson, Joannah R., Vicki M. Fleming, and Paul Klenerman. 2011. “CD161-Expressing Human T Cells.” Frontiers in Immunology 2(AUG):1–7.

Finco, Timothy S., Theresa Kadlecek, Weiguo Zhang, Lawrence E. Samelson, and Arthur Weiss. 1998. “LAT Is Required for TCR-Mediated Activation of PLCγ1 and the Ras Pathway.” Immunity 9(5):617–26.

Flores-Santibáñez, Felipe, Bárbara Cuadra, Dominique Fernández, Mariana V. Rosemblatt, Sarah Núñez, Pablo Cruz, Felipe Gálvez-Cancino, J. César Cárdenas, Alvaro Lladser, Mario Rosemblatt, María Rosa Bono, and Daniela Sauma. 2018. “In Vitro-Generated Tc17 Cells Present a Memory Phenotype and Serve as a Reservoir of Tc1 Cells in Vivo.” Frontiers in Immunology 9(FEB):1–12.

Fourcade, Julien, Zhaojun Sun, Mourad Benallaoua, Philippe Guillaume, Immanuel F. Luescher, Cindy Sander, John M. Kirkwood, Vijay Kuchroo, and Hassane M. Zarour. 2010. “Upregulation of Tim-3 and PD-1 Expression Is Associated with Tumor Antigen-Specific CD8+ T Cell Dysfunction in Melanoma Patients.” Journal of Experimental Medicine 207(10):2175–86.

Frebel, Helge, Veronika Nindl, Reto A. Schuepbach, Thomas Braunschweiler, Kirsten Richter, Johannes Vogel, Carsten A. Wagner, Dominique Loffing-Cueni, Michael Kurrer, Burkhard Ludewig, and Annette Oxenius. 2012. “Programmed Death 1 Protects from Fatal Circulatory Failure during Systemic Virus Infection of Mice.” Journal of Experimental Medicine 209(13):2485–99.

Furue, Masutaka, Kazuhisa Furue, Gaku Tsuji, and Takeshi Nakahara. 2020. “Interleukin-17A and Keratinocytes in Psoriasis.” International Journal of Molecular Sciences 21(4):1–21.

Goepfert, Paul A., Anju Bansal, Bradley H. Edwards, G. Douglas Ritter, Ildefonso Tellez, Sylvia A. McPherson, Steffanie Sabbaj, and Mark J. Mulligan. 2000. “A Significant Number of Human Immunodeficiency Virus Epitope-Specific Cytotoxic T Lymphocytes Detected by Tetramer Binding Do Not Produce Gamma Interferon.” Journal of Virology 74(21):10249–55.

Grant, Emma J., Simone Nüssing, Sneha Sant, E. Bridie Clemens, and Katherine Kedzierska. 2017. “The Role of CD27 in Anti-Viral T-Cell Immunity.” Current Opinion in Virology 22:77–88.

Gruener, Norbert H., Franziska Lechner, Maria-Christina Jung, Helmut Diepolder, Tilman Gerlach, Georg Lauer, Bruce Walker, John Sullivan, Rodney Phillips, Gerd R. Pape, and Paul Klenerman. 2001. “ Sustained Dysfunction of Antiviral CD8 + T Lymphocytes after Infection with Hepatitis C Virus .” Journal of Virology 75(12):5550–58.

Gust, Juliane, Rafael Ponce, W. Conrad Liles, Gwenn A. Garden, and Cameron J. Turtle. 2020. “Cytokines in CAR T Cell–Associated Neurotoxicity.” Frontiers in Immunology 11(December):1–23.

Hahm, Kyungmin, Bradley S. Cobb, Aaron S. McCarty, Karen E. Brown, Christopher A. Klug, Robert Lee, Koichi Akashi, Irving L. Weissman, Amanda G. Fisher, and Stephen T. Smale. 1998. “Helios, a T Cell-Restricted Ikaros Family Member That Quantitatively Associates with Ikaros at Centromeric Heterochromatin.” Genes and Development 12(6):782–96.

Hamada, Hiromasa, Maria de la Luz Garcia-Hernandez, Joyce B. Reome, Sara K. Misra, Tara M. Strutt, Kai K. McKinstry, Andrea M. Cooper, Susan L. Swain, and Richard W. Dutton. 2009. “Tc17, a Unique Subset of CD8 T Cells That Can Protect against Lethal Influenza Challenge.” The Journal of Immunology 182(6):3469–81.

Hamid, Omid, Caroline Robert, Adil Daud, F. Stephen Hodi, Wen-Jen Hwu, Richard Kefford, Jedd D. Wolchok, Peter Hersey, Richard W. Joseph, Jeffrey S. Weber, Roxana Dronca, Tara C. Gangadhar, Amita Patnaik, Hassane Zarour,

Anthony M. Joshua, Kevin Gergich, Jeroen Elassaiss-Schaap, Alain Algazi, Christine Mateus, Peter Boasberg, Paul C. Tumeh, Bartosz Chmielowski, Scot W. Ebbinghaus, Xiaoyun Nicole Li, S. Peter Kang, and Antoni Ribas. 2013. “Safety and Tumor Responses with Lambrolizumab (Anti–PD-1) in Melanoma.” New England Journal of Medicine 369(2):134–44.

Hammer, Quirin, Timo Rückert, Eva Maria Borst, Josefine Dunst, André Haubner, Pawel Durek, Frederik Heinrich, Gilles Gasparoni, Marina Babic, Adriana Tomic, Gabriella Pietra, Mikalai Nienen, Igor Wolfgang Blau, Jörg Hofmann, Il Kang Na, Immo Prinz, Christian Koenecke, Philipp Hemmati, Nina Babel, Renate Arnold, Jörn Walter, Kevin Thurley, Mir Farzin Mashreghi, Martin Messerle, and Chiara Romagnani. 2018. “Peptide-Specific Recognition of Human Cytomegalovirus Strains Controls Adaptive Natural Killer Cells Article.” Nature Immunology 19(5):453–63.

Haney, Danielle, Máire F. Quigley, Tedi E. Asher, David R. Ambrozak, Emma Gostick, David A. Price, Daniel C. Douek, and Michael R. Betts. 2011. “Isolation of Viable Antigen-Specific CD8+ T Cells Based on Membrane-Bound Tumor Necrosis Factor (TNF)-α Expression.” Journal of Immunological Methods 369(1–2):33–41.

Harriff, Melanie J., Lisa M. Wolfe, Gwendolyn Swarbrick, Megan Null, Meghan E. Cansler, Elizabeth T. Canfield, Todd Vogt, Katelynne Gardner Toren, Wei Li, Mary Jackson, Deborah A. Lewinsohn, Karen M. Dobos, and David M. Lewinsohn. 2017. “HLA-E Presents Glycopeptides from the Mycobacterium Tuberculosis Protein MPT32 to Human CD8+ T Cells.” Scientific Reports 7(1):1–11.

Hodi, F. Stephen, Steven J. O’Day, David F. McDermott, Robert W. Weber, Jeffrey A. Sosman, John B. Haanen, Rene Gonzalez, Caroline Robert, Dirk Schadendorf, Jessica C. Hassel, Wallace Akerley, Alfons J. M. van den Eertwegh Jose Lutzky, Paul Lorigan, Julia M. Vaubel, Gerald P. Linette, David Hogg, Christian H. Ottensmeier Celeste Lebbé, Christian Peschel, Ian Quirt, Joseph I. Clark, Jedd D. Wolchok, Jeffrey S. Weber, Jason Tian, Michael J. Yellin, Geoffrey M. Nichol, Axel Hoos Ch.B., and Walter J. Urba. 2010. “New England Journal.” 711–23.

Hrvatin, Siniša, Francis Deng, Charles W. O'Donnell, David K. Gifford, and Douglas A. Melton. 2014. "MARIS: Method for Analyzing RNA Following Intracellular Sorting." PLoS ONE 9(3):1–6.

Hu, Dan, Koichi Ikizawa, Linrong Lu, Marie E. Sanchirico, Mari L. Shinohara, and Harvey Cantor. 2004. "Analysis of Regulatory CD8 T Cells in Qa-1-Deficient Mice." Nature Immunology 5(5):516–23.

Huang, Alexander C., Michael A. Postow, Robert J. Orlowski, Rosemarie Mick, Bertram Bengsch, Sasikanth Manne, Wei Xu, Shannon Harmon, Josephine R. Giles, Brandon Wenz, Matthew Adamow, Deborah Kuk, Katherine S. Panageas, Cristina Carrera, Phillip Wong, Felix Quagliarello, Bradley Wubbenhorst, Kurt D'Andrea, Kristen E. Pauken, Ramin S. Herati, Ryan P. Staupe, Jason M. Schenkel, Suzanne McGettigan, Shawn Kothari, Sangeeth M. George, Robert H. Vonderheide, Ravi K. Amaravadi, Giorgos C. Karakousis, Lynn M. Schuchter, Xiaowei Xu, Katherine L. Nathanson, Jedd D. Wolchok, Tara C. Gangadhar, and E. John Wherry. 2017. "T-Cell Invigoration to Tumour Burden Ratio Associated with Anti-PD-1 Response." Nature 545(7652):60–65.

Huang, Jun, Mario Brameshuber, Xun Zeng, Jianming Xie, Qi jing Li, Yueh hsiu Chien, Salvatore Valitutti, and Mark M. Davis. 2013. "A Single Peptide-Major Histocompatibility Complex Ligand Triggers Digital Cytokine Secretion in CD4+ T Cells." Immunity 39(5):846–57.

Im, Se Jin, Masao Hashimoto, Michael Y. Gerner, Junghwa Lee, Haydn T. Kissick, Matheus C. Burger, Qiang Shan, J. Scott Hale, Judong Lee, Tahseen H. Nasti, Arlene H. Sharpe, Gordon J. Freeman, Ronald N. Germain, Helder I. Nakaya, Hai Hui Xue, and Rafi Ahmed. 2016. "Defining CD8 + T Cells That Provide the Proliferative Burst after PD-1 Therapy." Nature 537(7620):417–21.

Intlekofer, Andrew M., Arnob Banerjee, Naofumi Takemoto, Scott M. Gordon, Caitlin S. DeJong, Haina Shin, Christopher A. Hunter, E. John Wherry, Tullia Lindsten, and Steven L. Reiner. 2008. "Anomalous Type 17 Response to Viral Infection by CD8+ T Cells Lacking T-Bet and Eomesodermin." Science 321(5887):408–11.

Ivanov, Ivaylo I., Brent S. McKenzie, Liang Zhou, Carlos E. Tadokoro, Alice Lepelley, Juan J. Lafaille, Daniel J. Cua, and Dan R. Littman. 2006. "The Orphan

Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells." Cell 126(6):1121–33.

Iwashima, Makio, Bryan A. Irving, Nicolai S. C. Van Oers, Andrew C. Chan, and Arthur Weissf. 1994. "Tc D." 263(February):1136–39.

Jadhav, Rohit R., Se Jin Im, Bin Hu, Masao Hashimoto, Peng Li, Jian Xin Lin, Warren J. Leonard, William J. Greenleaf, Rafi Ahmed, and Jorg J. Goronzy. 2019. "Epigenetic Signature of PD-1+ TCF1+ CD8 T Cells That Act as Resource Cells during Chronic Viral Infection and Respond to PD-1 Blockade." Proceedings of the National Academy of Sciences of the United States of America 116(28):14113–18.

Jeannet, Grégoire, Caroline Boudousquié, Noémie Gardiol, Joonsoo Kang, Joerg Huelsken, and Werner Held. 2010. "Essential Role of the Wnt Pathway Effector Tcf-1 for the Establishment of Functional CD8 T Cell Memory." Proceedings of the National Academy of Sciences of the United States of America 107(21):9777–82.

Jiang, Hong, Helena Kashleva, Li Xing Xu, James Forman, Lorraine Flaherty, Benvenuto Pernis, Ned S. Braunstein, and Leonard Chess. 1998. "T Cell Vaccination Induces T Cell Receptor Vβ-Specific Qa-1-Restricted Regulatory CD8+ T Cells." Proceedings of the National Academy of Sciences of the United States of America 95(8):4533–37.

Jiang, Xiaodong, Rachael A. Clark, Luzheng Liu, Amy J. Wagers, Robert C. Fuhlbrigge, and Thomas S. Kupper. 2012. "Skin Infection Generates Non-Migratory Memory CD8 + T RM Cells Providing Global Skin Immunity." Nature 483 (7388): 227–31. https://doi.org/10.1038/nature10851.

Jin, Hyun Tak, Ana C. Anderson, Wendy G. Tan, Erin E. West, Sang Jun Ha, Koichi Araki, Gordon J. Freeman, Vijay K. Kuchroo, and Rafi Ahmed. 2010. "Cooperation of Tim-3 and PD-1 in CD8 T-Cell Exhaustion during Chronic Viral Infection." Proceedings of the National Academy of Sciences of the United States of America 107(33):14733–38.

Joosten, Simone A., Lucy C. Sullivan, and Tom H. M. Ottenhoff. 2016. "Characteristics of HLA-E Restricted T-Cell Responses and Their Role in Infectious Diseases." Journal of Immunology Research 2016.

Ju, Shyr Te, Rahul Sharma, Felicia Gaskin, John T. Kung, and Shu Man Fu. 2012. “The Biology of Autoimmune Response in the Scurfy Mice That Lack the CD4+Foxp3+ Regulatory T-Cells.” Biology 1(1):18–42.

June, Carl H., Roddy S. O’Connor, Omkar U. Kawalekar, Saba Ghassemi, and Michael C. Milone. 2018. “CAR T Cell Immunotherapy for Human Cancer.” Science 359(6382):1361–65.

Kallies, Axel, Edwin D. Hawkins, Gabrielle T. Belz, Donald Metcalf, Mirja Hommel, Lynn M. Corcoran, Philip D. Hodgkin, and Stephen L. Nutt. 2006. “Transcriptional Repressor Blimp-1 Is Essential for T Cell Homeostasis and Self-Tolerance.” Nature Immunology 7(5):466–74.

Kallies, Axel, Annie Xin, Gabrielle T. Belz, and Stephen L. Nutt. 2009. “Blimp-1 Transcription Factor Is Required for the Differentiation of Effector CD8+ T Cells and Memory Responses.” Immunity 31(2):283–95.

Kao, Charlly, Kenneth J. Oestreich, Michael A. Paley, Alison Crawford, Jill M. Angelosanto, Mohammed Alkhatim A. Ali, Andrew M. Intlekofer, Jeremy M. Boss, Steven L. Reiner, Amy S. Weinmann, and E. John Wherry. 2011. “Transcription Factor T-Bet Represses Expression of the Inhibitory Receptor PD-1 and Sustains Virus-Specific CD8+ T Cell Responses during Chronic Infection.” Nature Immunology 12(7):663–71.

Kaufmann, Daniel E., Daniel G. Kavanagh, Florencia Pereyra, John J. Zaunders, Elizabeth W. Mackey, Toshiyuki Miura, Sarah Palmer, Mark Brockman, Almas Rathod, Alicja Piechocka-Trocha, Brett Baker, Baogong Zhu, Sylvie Le Gall, Michael T. Waring, Ryan Ahern, Kristin Moss, Anthony D. Kelleher, John M. Coffin, Gordon J. Freeman, Eric S. Rosenberg, and Bruce D. Walker. 2007. “Upregulation of CTLA-4 by HIV-Specific CD4+ T Cells Correlates with Disease Progression and Defines a Reversible Immune Dysfunction.” Nature Immunology 8(11):1246–54.

Khan, Omar, Josephine R. Giles, Sierra McDonald, Sasikanth Manne, Shin Foong Ngiow, Kunal P. Patel, Michael T. Werner, Alexander C. Huang, Katherine A. Alexander, Jennifer E. Wu, John Attanasio, Patrick Yan, Sangeeth M. George, Bertram Bengsch, Ryan P. Staupe, Greg Donahue, Wei Xu, Ravi K. Amaravadi, Xiaowei Xu, Giorgos C. Karakousis, Tara C. Mitchell, Lynn M. Schuchter,

Jonathan Kaye, Shelley L. Berger, and E. John Wherry. 2019. “TOX Transcriptionally and Epigenetically Programs CD8+ T Cell Exhaustion.” Nature (May).

Kim, Chulwoo, Jun Jin, Cornelia M. Weyand, and Jörg J. Goronzy. 2020. “The Transcription Factor Tcf1 in t Cell Differentiation and Aging.” International Journal of Molecular Sciences 21(18):1–16.

Kim, Daehwan, Joseph M. Paggi, Chanhee Park, Christopher Bennett, and Steven L. Salzberg. 2019. “Graph-Based Genome Alignment and Genotyping with HISAT2 and HISAT-Genotype.” Nature Biotechnology 37(8):907–15.

Kim, Hye Jung, R. Anthony Barnitz, Taras Kreslavsky, Flavian D. Brown, Howell Moffett, Madeleine E. Lemieux, Yasemin Kaygusuz, Torsten Meissner, Tobias A. W. Holderried, Susan Chan, Philippe Kastner, W. Nicholas Haining, and Harvey Cantor. 2015. “Stable Inhibitory Activity of Regulatory T Cells Requires the Transcription Factor Helios.” Science 350(6258):334–39.

Kitamura, Takanori, Bin-Zhi Qian, and Jeffrey W. Pollard. 2015. “Immune Cell Promotion of Metastasis.” Nature Reviews Immunology 15(2):73–86.

Klein, Ludger, Bruno Kyewski, Paul M. Allen, and Kristin A. Hogquist. 2014. “Positive and Negative Selection of the T Cell Repertoire: What Thymocytes See (and Don’t See).” Nature Reviews Immunology 14(6):377–91.

Konduri, Vanaja, Damilola Oyewole-Said, Jonathan Vazquez-Perez, Scott A. Weldon, Matthew M. Halpert, Jonathan M. Levitt, and William K. Decker. 2021. “CD8+CD161+ T-Cells: Cytotoxic Memory Cells With High Therapeutic Potential.” Frontiers in Immunology 11(February):1–10.

Korn, Thomas, Estelle Bettelli, Mohamed Oukka, and Vijay K. Kuchroo. 2009. “IL-17 and Th17 Cells.” Annual Review of Immunology 27:485–517.

Kraemer, Thomas, Rainer Blasczyk, and Christina Bade-Doeding. 2014. “HLA-E: A Novel Player for Histocompatibility.” Journal of Immunology Research 2014.

Kumar, Brahma V., Thomas J. Connors, and Donna L. Farber. 2018. “Human T Cell Development, Localization, and Function throughout Life.” Immunity 48(2):202–13.

Langley, Richard G., Boni E. Elewski, Mark Lebwohl, Kristian Reich, Christopher E. M. Griffiths, Kim Papp, Lluís Puig, Hidemi Nakagawa, Lynda Spelman, Bárður Sigurgeirsson, Enrique Rivas, Tsen-Fang Tsai, Norman Wasel, Stephen Tyring, Thomas Salko, Isabelle Hampele, Marianne Notter, Alexander Karpov, Silvia Helou, and Charis Papavassilis. 2014. “Secukinumab in Plaque Psoriasis — Results of Two Phase 3 Trials.” New England Journal of Medicine 371(4):326–38.

Lee, Yun Kyung, Henrietta Turner, Craig L. Maynard, James R. Oliver, Dongquan Chen, Charles O. Elson, and Casey T. Weaver. 2009. “Late Developmental Plasticity in the T Helper 17 Lineage.” Immunity 30(1):92–107.

Lenfant, Françoise, Nathalie Pizzato, Siyuan Liang, Christian Davrinche, Philippe Le Bouteiller, and Anatolij Horuzsko. 2003. “Induction of HLA-G-Restricted Human Cytomegalovirus Pp65 (UL83)-Specific Cytotoxic T Lymphocytes in HLA-G Transgenic Mice.” Journal of General Virology 84(2):307–17.

Lenschow, Deborah J., Theresa L. Walunas, and A. Jeffrey. 1996. “CD28 / B7 System of T Cell Costimulation.” Annual Review of Immunology 14:233–58.

Li, Jianfeng, Itamar Goldstein, Eva Glickman-Nir, Hong Jiang, and Leonard Chess. 2001. “ Induction of TCR Vβ-Specific CD8 + CTLs by TCR Vβ-Derived Peptides Bound to HLA-E .” The Journal of Immunology 167(7):3800–3808.

Liang, Yan, Hai Feng Pan, and Dong Qing Ye. 2015. “Tc17 Cells in Immunity and Systemic Autoimmunity.” International Reviews of Immunology 34(4):318–31.

Liao, Yang, Gordon K. Smyth, and Wei Shi. 2014. “FeatureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features.” Bioinformatics 30(7):923–30.

Liénart, Stéphanie, Romain Merceron, Christophe Vanderaa, Fanny Lambert, Didier Colau, Julie Stockis, Bas van der Woning, Hans De Haard, Michael Saunders, Pierre G. Coulie, Savvas N. Savvides, and Sophie Lucas. 2018. “Structural Basis of Latent TGF-B1 Presentation and Activation by GARP on Human Regulatory T Cells.” Science 956(November):952–56.

Linehan, Jonathan L., Oliver J. Harrison, Seong Ji Han, Allyson L. Byrd, Ivan Vujkovic-Cvijin, Alejandro V. Villarino, Shurjo K. Sen, Jahangheer Shaik, Margery

Smelkinson, Samira Tamoutounour, Nicholas Collins, Nicolas Bouladoux, Amiran Dzutsev, Stephan P. Rosshart, Jesse H. Arbuckle, Chyung Ru Wang, Thomas M. Kristie, Barbara Rehermann, Giorgio Trinchieri, Jason M. Brenchley, John J. O'Shea, and Yasmine Belkaid. 2018. "Non-Classical Immunity Controls Microbiota Impact on Skin Immunity and Tissue Repair." Cell 172(4):784-796.e18.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2." Genome Biology 15(12):1–21.

Loyal, Lucie, Sarah Warth, Karsten Jürchott, Felix Mölder, Christos Nikolaou, Nina Babel, Mikalai Nienen, Sibel Durlanik, Regina Stark, Beate Kruse, Marco Frentsch, Robert Sabat, Kerstin Wolk, and Andreas Thiel. 2020. "SLAMF7 and IL-6R Define Distinct Cytotoxic versus Helper Memory CD8+ T Cells." Nature Communications 11(1).

Maggi, Laura, Veronica Santarlasci, Manuela Capone, Anna Peired, Francesca Frosali, Sarah Q. Crome, Valentina Querci, Massimiliano Fambrini, Francesco Liotta, Megan K. Levings, Enrico Maggi, Lorenzo Cosmi, Sergio Romagnani, and Francesco Annunziato. 2010. "CD161 Is a Marker of All Human IL-17-Producing T-Cell Subsets and Is Induced by RORC." European Journal of Immunology 40(8):2174–81.

Mahnke, Justus, Valeá Schumacher, Stefanie Ahrens, Nadja Käding, Lea Marie Feldhoff, Magdalena Huber, Jan Rupp, Friederike Raczkowski, and Hans Willi Mittrücker. 2016. "Interferon Regulatory Factor 4 Controls T H1 Cell Effector Function and Metabolism." Scientific Reports 6(October 2015):1–12.

Mahnke, Yolanda D., Tess M. Brodie, Federica Sallusto, Mario Roederer, and Enrico Lugli. 2013. "The Who's Who of T-Cell Differentiation: Human Memory T-Cell Subsets." European Journal of Immunology 43(11):2797–2809.

Man, Kevin, Sarah S. Gabriel, Yang Liao, Renee Gloury, Simon Preston, Darren C. Henstridge, Marc Pellegrini, Dietmar Zehn, Friederike Berberich-Siebelt, Mark A. Febbraio, Wei Shi, and Axel Kallies. 2017. "Transcription Factor IRF4 Promotes CD8+ T Cell Exhaustion and Limits the Development of Memory-like T Cells during Chronic Infection." Immunity 47(6):1129-1141.e5.

Man, Kevin, Maria Miasari, Wei Shi, Annie Xin, Darren C. Henstridge, Simon Preston, Marc Pellegrini, Gabrielle T. Belz, Gordon K. Smyth, Mark A. Febbraio, Stephen L. Nutt, and Axel Kallies. 2013. “The Transcription Factor IRF4 Is Essential for TCR Affinity-Mediated Metabolic Programming and Clonal Expansion of T Cells.” Nature Immunology 14(11):1155–65.

Martinez, Gustavo J., Renata M. Pereira, Tarmo Äijö, Edward Y. Kim, Francesco Marangoni, Matthew E. Pipkin, Susan Togher, Vigo Heissmeyer, YiChen Zhang, Shane Crotty, Edward D. Lamperti, K. Mark Ansel, Thorsten R. Mempel, Harri Lähdesmäki, Patrick G. Hogan, and Anjana Rao. 2015. “The Transcription Factor NFAT Promotes Exhaustion of Activated CD8+ T Cells.” Immunity 42(2):265–78.

Martins, Gislâine A., Luisa Cimmino, Miriam Shapiro-Shelef, Matthias Szabolcs, Alan Herron, Erna Magnusdottir, and Kathryn Calame. 2006. “Transcriptional Repressor Blimp-1 Regulates T Cell Homeostasis and Function.” Nature Immunology 7(5):457–65.

Masopust, David, and Andrew G. Soerens. 2019. “Tissue-Resident T Cells and Other Resident Leukocytes.” Annual Review of Immunology 37: 521–46. https://doi.org/10.1146/annurev-immunol-042617-053214.

Mathew, Divij, Josephine R. Giles, Amy E. Baxter, Derek A. Oldridge, Allison R. Greenplate, Jennifer E. Wu, Cécile Alanio, Leticia Kuri-Cervantes, M. Betina Pampena, Kurt D’Andrea, Sasikanth Manne, Zeyu Chen, Yinghui Jane Huang, John P. Reilly, Ariel R. Weisman, Caroline A. G. Ittner, Oliva Kuthuru, Jeanette Dougherty, Kito Nzingha, Nicholas Han, Justin Kim, Ajinkya Pattekar, Eileen C. Goodwin, Elizabeth M. Anderson, Madison E. Weirick, Sigrid Gouma, Claudia P. Arevalo, Marcus J. Bolton, Fang Chen, Simon F. Lacey, Holly Ramage, Sara Cherry, Scott E. Hensley, Sokratis A. Apostolidis, Alexander C. Huang, Laura A. Vella, Michael R. Betts, Nuala J. Meyer, E. John Wherry, Zahidul Alam, Mary M. Addison, Katelyn T. Byrne, Aditi Chandra, Hélène C. Descamps, Yaroslav Kaminskiy, Jacob T. Hamilton, Julia Han Noll, Dalia K. Omran, Eric Perkey, Elizabeth M. Prager, Dana Pueschl, Jennifer B. Shah, Jake S. Shilan, and Ashley N. Vanderbeck. 2020. “Deep Immune Profiling of COVID-19 Patients Reveals Distinct Immunotypes with Therapeutic Implications.” Science 369(6508).

Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. “CD4+ T Cells Are Required to Sustain CD8+ Cytotoxic T-Cell Responses during Chronic Viral Infection.” Journal of Virology 68(12):8056–63.

Matsuzaki, Junko, Sacha Gnjatic, Paulette Mhawech-Fauceglia, Amy Beck, Austin Miller, Takemasa Tsuji, Cheryl Eppolito, Feng Qian, Shashikant Lele, Protul Shrikant, Lloyd J. Old, and Kunle Odunsi. 2010. “Tumor-Infiltrating NY-ESO-1-Specific CD8+ T Cells Are Negatively Regulated by LAG-3 and PD-1 in Human Ovarian Cancer.” Proceedings of the National Academy of Sciences of the United States of America 107(17):7875–80.

Maurice, Nicholas J., Jacqueline Berner, Alexis K. Taber, Dietmar Zehn, and Martin Prlic. 2021. “Inflammatory Signals Are Sufficient to Elicit TOX Expression in Mouse and Human CD8+ T Cells.” JCI Insight 6(13).

Mazzarino, Paola, Gabriella Pietra, Paola Vacca, Michela Falco, Didier Colau, Pierre Coulie, Lorenzo Moretta, and Maria Cristina Mingari. 2005. “Identification of Effector-Memory CMV-Specific T Lymphocytes That Kill CMV-Infected Target Cells in an HLA-E-Restricted Fashion.” European Journal of Immunology 35(11):3240–47.

McCarty, Aaron S., Gary Kleiger, David Eisenberg, and Stephen T. Smale. 2003. “Selective Dimerization of a C2H2 Zinc Finger Subfamily.” Molecular Cell 11(2):459–70.

McGeachy, Mandy J., Daniel J. Cua, and Sarah L. Gaffen. 2019. “The IL-17 Family of Cytokines in Health and Disease.” Immunity 50(4):892–906.

McLane, Laura M., Mohamed S. Abdel-Hakeem, and E. John Wherry. 2019. “CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer.” Annual Review of Immunology 37(1):457–95.

Mercer, Frances, Alka Khaitan, Lina Kozhaya, Judith A. Aberg, and Derya Unutmaz. 2014. “Differentiation of IL-17–Producing Effector and Regulatory Human T Cells from Lineage-Committed Naive Precursors.” The Journal of Immunology 193(3):1047–54.

Miller, Brian C., Debattama R. Sen, Rose Al Abosy, Kevin Bi, Yamini V. Virkud, Martin W. LaFleur, Kathleen B. Yates, Ana Lako, Kristen Felt, Girish S. Naik,

Michael Manos, Evisa Gjini, Juhi R. Kuchroo, Jeffrey J. Ishizuka, Jenna L. Collier, Gabriel K. Griffin, Seth Maleri, Dawn E. Comstock, Sarah A. Weiss, Flavian D. Brown, Arpit Panda, Margaret D. Zimmer, Robert T. Manguso, F. Stephen Hodi, Scott J. Rodig, Arlene H. Sharpe, and W. Nicholas Haining. 2019. “Subsets of Exhausted CD8 + T Cells Differentially Mediate Tumor Control and Respond to Checkpoint Blockade.” Nature Immunology 20(3):326–36.

Mittrücker, Hans Willi, Alexander Visekruna, and Magdalena Huber. 2014. “Heterogeneity in the Differentiation and Function of CD8+ T Cells.” Archivum Immunologiae et Therapiae Experimentalis 62(6):449–58.

Mucida, Daniel, Nino Kutchukhidze, Agustin Erazo, Momtchilo Russo, Juan J. Lafaille, and Maria A. Curotto De Lafaille. 2005. “Oral Tolerance in the Absence of Naturally Occurring Tregs.” Journal of Clinical Investigation 115(7):1923–33.

Mueller, Scott N., and Laura K. Mackay. 2016. “Tissue-Resident Memory T Cells: Local Specialists in Immune Defence.” Nature Reviews Immunology 16 (2): 79–89. https://doi.org/10.1038/nri.2015.3.

Mueller, Scott N., Vijay K. Vanguri, Sang Jun Ha, Erin E. West, Mary E. Keir, Jonathan N. Glickman, Arlene H. Sharpe, and Rafi Ahmed. 2010. “PD-L1 Has Distinct Functions in Hematopoietic and Nonhematopoietic Cells in Regulating T Cell Responses during Chronic Infection in Mice.” Journal of Clinical Investigation 120(7):2508–15.

Muranski, Pawel, Zachary A. Borman, Sid P. Kerkar, Christopher A. Klebanoff, Yun Ji, Luis Sanchez-Perez, Madhusudhanan Sukumar, Robert N. Reger, Zhiya Yu, Steven J. Kern, Rahul Roychoudhuri, Gabriela A. Ferreyra, Wei Shen, Scott K. Durum, Lionel Feigenbaum, Douglas C. Palmer, Paul A. Antony, Chi Chao Chan, Arian Laurence, Robert L. Danner, Luca Gattinoni, and Nicholas P. Restifo. 2011. “Th17 Cells Are Long Lived and Retain a Stem Cell-like Molecular Signature.” Immunity 35(6):972–85.

Naik, Shruti, Nicolas Bouladoux, Jonathan L. Linehan, Seong Ji Han, Oliver J. Harrison, Christoph Wilhelm, Sean Conlan, Sarah Himmelfarb, Allyson L. Byrd, Clayton Deming, Mariam Quinones, Jason M. Brenchley, Heidi H. Kong, Roxanne Tussiwand, Kenneth M. Murphy, Miriam Merad, Julia A. Segre, and

Yasmine Belkaid. 2015. “Commensal-Dendritic-Cell Interaction Specifies a Unique Protective Skin Immune Signature.” Nature 520(7545):104–8.

Nanjappa, Som G., Srinivasu Mudalagiriyappa, J. Scott Fites, M. Suresh, and Bruce S. Klein. 2018. “ CBLB Constrains Inactivated Vaccine–Induced CD8 + T Cell Responses and Immunity against Lethal Fungal Pneumonia .” The Journal of Immunology 201(6):1717–26.

Nanjappa, Som Gowda, Andrew J. McDermott, J. Scott Fites, Kevin Galles, Marcel Wüthrich, George S. Deepe, and Bruce S. Klein. 2017. “Antifungal Tc17 Cells Are Durable and Stable, Persisting as Long-Lasting Vaccine Memory without Plasticity towards IFNγ Cells.” PLoS Pathogens 13(5):1–25.

Nishimura, Hiroyuki, Masato Nose, Hiroshi Hiai, Nagahiro Minato, and Tasuku Honjo. 1999. “Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor.” Immunity 11(2):141–51.

Nograles, Kristine E., Lisa C. Zaba, Avner Shemer, Judilyn Fuentes-Duculan, Irma Cardinale, Toyoko Kikuchi, Michal Ramon, Reuven Bergman, James G. Krueger, and Emma Guttman-Yassky. 2009. “IL-22-Producing ‘T22’ T Cells Account for Upregulated IL-22 in Atopic Dermatitis despite Reduced IL-17-Producing TH17 T Cells.” Journal of Allergy and Clinical Immunology 123(6):1244-1252.e2.

Nurieva, Roza I., Yeonseok Chung, Daehee Hwang, Xuexian O. Yang, Hong Soon Kang, Li Ma, Yi hong Wang, Stephanie S. Watowich, Anton M. Jetten, Qiang Tian, and Chen Dong. 2008. “Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages.” Immunity 29(1):138–49.

O’Flaherty, Emmett and Jonathan Kaye. 2003. “TOX Defines a Conserved Subfamily of HMG-Box Proteins.” BMC Genomics 4:1–10.

Paradowska-Gorycka, Agnieszka, Anna Wajda, Katarzyna Romanowska-Próchnicka, Ewa Walczuk, Ewa Kuca-Warnawin, Tomasz Kmiolek, Barbara Stypinska, Ewa Rzeszotarska, Dominik Majewski, Pawel Piotr Jagodzinski, and Andrzej Pawlik. 2020. “Th17/Treg-Related Transcriptional Factor Expression and Cytokine Profile in Patients With Rheumatoid Arthritis.” Frontiers in Immunology 11(December):1–14.

Pauken, Kristen E. and E. John Wherry. 2015. “Overcoming T Cell Exhaustion in Infection and Cancer.” Trends in Immunology 36(4):265–76.

Pearce, Erika L., Alan C. Mullen, Gislâine A. Martins, Connie M. Krawczyk, Anne S. Hutchins, Valerie P. Zediak, Monica Banica, Catherine B. DiCioccio, Darrick A. Gross, Chai An Mao, Hao Shen, Nezih Cereb, Soo Y. Yang, Tullia Lindsten, Janet Rossant, Christopher A. Hunter, and Steven L. Reiner. 2003. “Control of Effector CD8+ T Cell Function by the Transcription Factor Eomesodermin.” Science 302(5647):1041–43.

Pereira, Renata M., Patrick G. Hogan, Anjana Rao, and Gustavo J. Martinez. 2017. “Transcriptional and Epigenetic Regulation of T Cell Hyporesponsiveness.” Journal of Leukocyte Biology 102(3):601–15.

Petit, Anne Elisabeth, Nathalie Demotte, Benoît Scheid, Claude Wildmann, Rene Bigirimana, Monica Gordon-Alonso, Javier Carrasco, Salvatore Valitutti, Daniele Godelaine, and Pierre Van Der Bruggen. 2016. “A Major Secretory Defect of Tumour-Infiltrating T Lymphocytes Due to Galectin Impairing LFA-1-Mediated Synapse Completion.” Nature Communications 7:1–15.

Petrovas, Constantinos, Joseph P. Casazza, Jason M. Brenchley, David A. Price, Emma Gostick, William C. Adams, Melissa L. Precopio, Timothy Schacker, Mario Roederer, Daniel C. Douek, and Richard A. Koup. 2006. “PD-1 Is a Regulator of Virus-Specific CD8+ T Cell Survival in HIV Infection.” Journal of Experimental Medicine 203(10):2281–92.

Pietra, Gabriella, Chiara Romagnani, Paola Mazzarino, Michela Falco, Enrico Millo, Alessandro Moretta, Lorenzo Moretta, and Maria Cristina Mingari. 2003. “HLA-E-Restricted Recognition of Cytomegalovirus-Derived Peptides by Human CD8+ Cytolytic T Lymphocytes.” Proceedings of the National Academy of Sciences of the United States of America 100(19):10896–901.

Raczkowski, Friederike, Josephine Ritter, Kira Heesch, Valéa Schumacher, Anna Guralnik, Lena Höcker, Hartmann Raifer, Matthias Klein, Tobias Bopp, Hani Harb, Dörthe A. Kesper, Petra I. Pfefferle, Melanie Grusdat, Philipp A. Lang, Hans Willi Mittrücker, and Magdalena Huber. 2013. “The Transcription Factor Interferon Regulatory Factor 4 Is Required for the Generation of Protective

Effector CD8+ T Cells.” Proceedings of the National Academy of Sciences of the United States of America 110(37):15019–24.

Ramirez, Jean Marie, Nicolò C. Brembilla, Olivier Sorg, Rachel Chicheportiche, Thomas Matthes, Jean Michel Dayer, Jean Hilaire Saurat, Eddy Roosnek, and Carlo Chizzolini. 2010. “Activation of the Aryl Hydrocarbon Receptor Reveals Distinct Requirements for IL-22 and IL-17 Production by Human T Helper Cells.” European Journal of Immunology 40(9):2450–59.

Ranasinghe, Ranmali and Rajaraman Eri. 2018. “Modulation of the CCR6-CCl20 Axis: A Potential Therapeutic Target in Inflammation and Cancer.” Medicina (Lithuania) 54(5).

Res, Pieter C. M., Gamze Piskin, Onno J. de Boer, Chris M. van der Loos, Peter Teeling, Jan D. Bos, and Marcel B. M. Teunissen. 2010. “Overrepresentation of IL-17A and IL-22 Producing CD8 T Cells in Lesional Skin Suggests Their Involvement in the Pathogenesis of Psoriasis.” PLoS ONE 5(11).

Romagnani, Chiara, Gabriella Pietra, Michela Falco, Paola Mazzarino, Lorenzo Moretta, and Maria Cristina Mingari. 2004. “HLA-E-Restricted Recognition of Human Cytomegalovirus by a Subset of Cytolytic T Lymphocytes.” Human Immunology 65(5):437–45.

Romero, Pedro, Alfred Zippelius, Isabel Kurth, Mikaël J. Pittet, Cédric Touvrey, Emanuela M. Iancu, Patricia Corthesy, Estelle Devevre, Daniel E. Speiser, and Nathalie Rufer. 2007. “ Four Functionally Distinct Populations of Human Effector-Memory CD8 + T Lymphocytes .” The Journal of Immunology 178(7):4112–19.

Roose, Jeroen P., Marianne Mollenauer, Vikas A. Gupta, James Stone, and Arthur Weiss. 2005. “A Diacylglycerol-Protein Kinase C-RasGRP1 Pathway Directs Ras Activation upon Antigen Receptor Stimulation of T Cells.” Molecular and Cellular Biology 25(11):4426–41.

Rosen, David B., Jayaram Bettadapura, Mohammed Alsharifi, Porunelloor A. Mathew, Hilary S. Warren, and Lewis L. Lanier. 2005. “Cutting Edge: Lectin-Like Transcript-1 Is a Ligand for the Inhibitory Human NKR-P1A Receptor.” The Journal of Immunology 175(12):7796–99.

Ruterbusch, Mikel, Kurt B. Pruner, Laila Shehata, and Marion Pepper. 2020. “In Vivo CD4+ T Cell Differentiation and Function: Revisiting the Th1/Th2 Paradigm.” Annual Review of Immunology 38:705–25.

Rutishauser, Rachel L., Gislâine A. Martins, Sergey Kalachikov, Anmol Chandele, Ian A. Parish, Eric Meffre, Joshy Jacob, Kathryn Calame, and Susan M. Kaech. 2009. “Transcriptional Repressor Blimp-1 Promotes CD8+ T Cell Terminal Differentiation and Represses the Acquisition of Central Memory T Cell Properties.” Immunity 31(2):296–308.

Sad, Subash, Rita Marcotte, and Tim R. Mosmann. 1995. “Cytokine-Induced Differentiation of Precursor Mouse CD8+ T Cells into Cytotoxic CD8+ T Cells Secreting Th1 or Th2 Cytokines.” Immunity 2(3):271–79.

Safford, Meredith, Samuel Collins, Michael A. Lutz, Amy Allen, Ching Tai Huang, Jeanne Kowalski, Amanda Blackford, Maureen R. Horton, Charles Drake, Ronald H. Schwartz, and Jonathan D. Powell. 2005. “Egr-2 and Egr-3 Are Negative Regulators of T Cell Activation.” Nature Immunology 6(5):472–80.

Santomasso, Bianca, Carlos Bachier, Jason Westin, Katayoun Rezvani, and Elizabeth J. Shpall. 2019. “The Other Side of CAR T-Cell Therapy: Cytokine Release Syndrome, Neurologic Toxicity, and Financial Burden.” American Society of Clinical Oncology Educational Book (39):433–44.

Schenkel, Jason M., and David Masopust. 2014. “Tissue-Resident Memory T Cells.” Immunity 41 (6): 886–97. https://doi.org/10.1016/j.immuni.2014.12.007.

Schmitt, Edgar and Tobias Bopp. 2017. “Discovery and Initial Characterization of Th9 Cells: The Early Years.” Seminars in Immunopathology 39(1):5–10.

Schutyser, Evemie, Sofie Struyf, and Jo Van Damme. 2003. “The CC Chemokine CCL20 and Its Receptor CCR6.” Cytokine and Growth Factor Reviews 14(5):409–26.

Scott, Andrew C., Friederike Dündar, Paul Zumbo, Smita S. Chandran, Christopher A. Klebanoff, Mojdeh Shakiba, Prerak Trivedi, Laura Menocal, Heather Appleby, Steven Camara, Dmitriy Zamarin, Tyler Walther, Alexandra Snyder, Matthew R. Femia, Elizabeth A. Comen, Hannah Y. Wen, Matthew D.

Hellmann, Niroshana Anandasabapathy, Yong Liu, Nasser K. Altorki, Peter Lauer, Olivier Levy, Michael S. Glickman, Jonathan Kaye, Doron Betel, Mary Philip, and Andrea Schietinger. 2019. “TOX Is a Critical Regulator of Tumour-Specific T Cell Differentiation.” Nature.

Seng, Amara, Kelsey L. Krausz, Dong Pei, Devin C. Koestler, Ryan T. Fischer, Thomas M. Yankee, and Mary A. Markiewicz. 2020. “Coexpression of FOXP3 and a Helios Isoform Enhances the Effectiveness of Human Engineered Regulatory T Cells.” Blood Advances 4(7):1325–39.

Shankar, P., M. Russo, B. Harnisch, M. Patterson, P. Skolnik, and J. Lieberman. 2000. “Impaired Function of Circulating HIV-Specific CD8+ T Cells in Chronic Human Immunodeficiency Virus Infection.” Blood 96(9):3094–3101.

Shin, Haina, Shawn D. Blackburn, Andrew M. Intlekofer, Charlly Kao, Jill M. Angelosanto, Steven L. Reiner, and E. John Wherry. 2009. “A Role for the Transcriptional Repressor Blimp-1 in CD8+ T Cell Exhaustion during Chronic Viral Infection.” Immunity 31(2):309–20.

Singh, Satya P., Hongwei H. Zhang, Hsinyi Tsang, Paul J. Gardina, Timothy G. Myers, Vijayaraj Nagarajan, Chang Hoon Lee, and Joshua M. Farber. 2015. “ PLZF Regulates CCR6 and Is Critical for the Acquisition and Maintenance of the Th17 Phenotype in Human Cells .” The Journal of Immunology 194(9):4350–61.

Skadow, Mathias, Vinay R. Penna, Jessica Galant-Swafford, Ethan M. Shevach, and Angela M. Thornton. 2019. “ Helios Deficiency Predisposes the Differentiation of CD4 + Foxp3 − T Cells into Peripherally Derived Regulatory T Cells .” The Journal of Immunology 203(2):370–78.

Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. “T Cell Activation.” Annual Review of Immunology 27:591–619.

Sridharan, Rupa and Stephen T. Smale. 2007. “Predominant Interaction of Both Ikaros and Helios with the NuRD Complex in Immature Thymocytes.” Journal of Biological Chemistry 282(41):30227–38.

Starr, Timothy K., Stephen C. Jameson, and Kristin A. Hogquist. 2003. “Positive and Negative Selection of T Cells.” Annual Review of Immunology 21:139–76.

Stockis, Julie, Didier Colau, Pierre G. Coulie, and Sophie Lucas. 2009. “Membrane Protein GARP Is a Receptor for Latent TGF-β on the Surface of Activated Human Treg.” European Journal of Immunology 39(12):3315–22.

Stockis, Julie, Olivier Dedobbeleer, and Sophie Lucas. 2017. “Role of GARP in the Activation of Latent TGF-B1.” Molecular BioSystems 13(10):1925–35.

Subramanian, Aravind, Pablo Tamayo, Vamsi K. Mootha, Sayan Mukherjee, Benjamin L. Ebert, Michael A. Gillette, Amanda Paulovich, Scott L. Pomeroy, Todd R. Golub, Eric S. Lander, and Jill P. Mesirov. 2005. “Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles.” Proceedings of the National Academy of Sciences of the United States of America 102(43):15545–50.

Sugita, Kazunari, Sho Hanakawa, Tetsuya Honda, Gen Kondoh, Yoshiki Miyachi, Kenji Kabashima, and Takashi Nomura. 2015. “Generation of Helios Reporter Mice and an Evaluation of the Suppressive Capacity of Helios+ Regulatory T Cells in Vitro.” Experimental Dermatology 24(7):554–56.

Sullivan, Barbara A., Piotr Kraj, Dominique A. Weber, Leszek Ignatowicz, and Peter E. Jensen. 2002. “Positive Selection of a Qa-1-Restricted T Cell Receptor with Specificity for Insulin.” Immunity 17(1):95–105.

Sun, Cheng Ming, Jason A. Hall, Rebecca B. Blank, Nicolas Bouladoux, Mohamed Oukka, J. Rodrigo Mora, and Yasmine Belkaid. 2007. “Small Intestine Lamina Propria Dendritic Cells Promote de Novo Generation of Foxp3 T Reg Cells via Retinoic Acid.” Journal of Experimental Medicine 204(8):1775–85.

Sun, Lijun, Li Deng, Chee Kwee Ea, Zong Ping Xia, and Zhijian J. Chen. 2004. “The TRAF6 Ubiquitin Ligase and TAK1 Kinase Mediate IKK Activation by BCL10 and MALT1 in T Lymphocytes.” Molecular Cell 14(3):289–301.

Szabo, Susanne J., Sean T. Kim, Gina L. Costa, Xiankui Zhang, C. Garrison Fathman, and Laurie H. Glimcher. 2000. “A Novel Transcription Factor, T-Bet, Directs Th1 Lineage Commitment.” Cell 100(6):655–69.

Takemoto, Naofumi, Andrew M. Intlekofer, John T. Northrup, E. John Wherry, and Steven L. Reiner. 2006. “ Cutting Edge: IL-12 Inversely Regulates T-Bet and

Eomesodermin Expression during Pathogen-Induced CD8 + T Cell Differentiation .” The Journal of Immunology 177(11):7515–19.

Tang, Xiaolei, Igor Maricic, Nikunj Purohit, Berge Bakamjian, Lisa M. Reed-Loisel, Tara Beeston, Peter Jensen, and Vipin Kumar. 2006. “ Regulation of Immunity by a Novel Population of Qa-1-Restricted CD8αα + TCRαβ + T Cells .” The Journal of Immunology 177(11):7645–55.

Taylor, Alison, James A. Harker, Kittiphat Chanthong, Philip G. Stevenson, Elina I. Zuniga, and Christopher E. Rudd. 2016. “Glycogen Synthase Kinase 3 Inactivation Drives T-Bet-Mediated Downregulation of Co-Receptor PD-to Enhance CD8+ Cytolytic T Cell Responses.” Immunity 44(2):274–86.

Thornton, Angela M., Patricia E. Korty, Dat Q. Tran, Elizabeth A. Wohlfert, Patrick E. Murray, Yasmine Belkaid, and Ethan M. Shevach. 2010. “ Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced Foxp3 + T Regulatory Cells .” The Journal of Immunology 184(7):3433–41.

Thornton, Angela M., Jinghua Lu, Patricia E. Korty, Yong Chan Kim, Craig Martens, Peter D. Sun, and Ethan M. Shevach. 2019. “Helios + and Helios − Treg Subpopulations Are Phenotypically and Functionally Distinct and Express Dissimilar TCR Repertoires.” European Journal of Immunology 49(3):398–412.

Topham, David J., and Emma C. Reilly. 2018. “Tissue-Resident Memory CD8+ T Cells: From Phenotype to Function.” Frontiers in Immunology 9 (MAR). https://doi.org/10.3389/fimmu.2018.00515.

Trifari, Sara, Charles D. Kaplan, Elise H. Tran, Natasha K. Crellin, and Hergen Spits. 2009. “Identification of a Human Helper T Cell Population That Has Abundant Production of Interleukin 22 and Is Distinct from TH-17, TH1 and TH2 Cells.” Nature Immunology 10(8):864–71.

Urbani, Simona, Barbara Amadei, Daniela Tola, Marco Massari, Simona Schivazappa, Gabriele Missale, and Carlo Ferrari. 2006. “PD-1 Expression in Acute Hepatitis C Virus (HCV) Infection Is Associated with HCV-Specific CD8 Exhaustion.” Journal of Virology 80(22):11398–403.

Varthaman, Aditi, Marc Clement, Jamila Khallou-Laschet, Giulia Fornasa, Anh Thu Gaston, Michael Dussiot, Giuseppina Caligiuri, Harvey Cantor, Srinivas Kaveri, and Antonino Nicoletti. 2011. “Physiological Induction of Regulatory Qa-1-Restricted CD8+ T Cells Triggered by Endogenous CD4+ T Cell Responses.” PLoS ONE 6(6):2–8.

Varthaman, Aditi, Jamila Khallou-Laschet, Marc Clement, Giulia Fornasa, Hye-Jung Kim, Anh-Thu Gaston, Michael Dussiot, Giuseppina Caligiuri, André Herbelin, Srinivas Kaveri, Harvey Cantor, and Antonino Nicoletti. 2010. “ Control of T Cell Reactivation by Regulatory Qa-1–Restricted CD8 + T Cells .” The Journal of Immunology 184(12):6585–91.

Verma, Kriti, Justyna Ogonek, Pavankumar Reddy Varanasi, Susanne Luther, Ivonne Bünting, Katrin Thomay, Yvonne Lisa Behrens, Eva Mischak-Weissinger, and Lothar Hambach. 2017. “Human CD8+ CD57- TEMRA Cells: Too Young to Be Called ‘Old.’” PLoS ONE 12(5):1–14.

Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. “How Regulatory T Cells Work.” Nat Rev Immunol 8(7):523–32.

Wardenburg, Juliane Bubeck, Chong Fu, Janet K. Jackman, Horst Flotow, Sandra E. Wilkinson, David H. Williams, Robin Johnson, Guanghui Kong, Andrew C. Chan, and Paul R. Findell. 1996. “Phosphorylation of SLP-76 by Kinase Is Required for T-Cell Receptor Function.” The Journal of Biological Chemistry (23):19641–44.

Ware, Randle, Hong Jiang, Ned Braunstein, Jennifer Kent, Ethan Wiener, Benvenuto Pernis, and Leonard Chess. 1995. “Human CD8+ T Lymphocyte Clones Specific for T Cell Receptor Vβ Families Expressed on Autologous CD4+ T Cells.” Immunity 2(2):177–84.

Wei, Gang, Lai Wei, Jinfang Zhu, Chongzhi Zang, Jane Hu-Li, Zhengju Yao, Kairong Cui, Yuka Kanno, Tae Young Roh, Wendy T. Watford, Dustin E. Schones, Weiqun Peng, Hong wei Sun, William E. Paul, John J. O’Shea, and Keji Zhao. 2009. “Global Mapping of H3K4me3 and H3K27me3 Reveals Specificity and Plasticity in Lineage Fate Determination of Differentiating CD4+ T Cells.” Immunity 30(1):155–67.

Wei, Jianshu, Yang Liu, Chunmeng Wang, Yajing Zhang, Chuan Tong, Guanghai Dai, Wei Wang, John E. J. Rasko, J. Joseph Melenhorst, Wenbin Qian, Aibin Liang, and Weidong Han. 2020. “The Model of Cytokine Release Syndrome in CAR T-Cell Treatment for B-Cell Non-Hodgkin Lymphoma.” Signal Transduction and Targeted Therapy 5(1).

Wei-ping, Zheng and Richard A. Flavell. 1997. “The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells.” Cell 89(1):587–96.

Wherry, E. John. 2011. “T Cell Exhaustion.” Nature Immunology 12(6):492–99.

Wherry, E. John, Joseph N. Blattman, Kaja Murali-Krishna, Robbert van der Most, and Rafi Ahmed. 2003. “Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment.” Journal of Virology 77(8):4911–27.

Wherry, E. John, Sang Jun Ha, Susan M. Kaech, W. Nicholas Haining, Surojit Sarkar, Vandana Kalia, Shruti Subramaniam, Joseph N. Blattman, Daniel L. Barber, and Rafi Ahmed. 2007. “Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection.” Immunity 27(4):670–84.

Wilkinson, Beveley, Jeff Y. F. Chen, Peggy Han, Kevin M. Rufner, Olivia D. Goularte, and Jonathan Kaye. 2002. “TOX: An HMG Box Protein Implicated in the Regulation of Thymocyte Selection.” Nature Immunology 3(3):272–80.

Wolchok, Jedd D., Harriet Kluger, Margaret K. Callahan, Michael A. Postow, Naiyer A. Rizvi, Alexander M. Lesokhin, Neil H. Segal, Charlotte E. Ariyan, Ruth-Ann Gordon, Kathleen Reed, Matthew M. Burke, Anne Caldwell, Stephanie A. Kronenberg, Blessing U. Agunwamba, Xiaoling Zhang, Israel Lowy, Hector David Inzunza, William Feely, Christine E. Horak, Quan Hong, Alan J. Korman, Jon M. Wigginton, Ashok Gupta, and Mario Sznol. 2013. “Nivolumab plus Ipilimumab in Advanced Melanoma.” New England Journal of Medicine 369(2):122–33.

Wu, Jie, Hedong Zhang, Xiaomin Shi, Xiang Xiao, Yihui Fan, Laurie J. Minze, Jin Wang, Rafik M. Ghobrial, Jiahong Xia, Roger Sciammas, Xian C. Li, and Wenhao Chen. 2017. “Ablation of Transcription Factor IRF4 Promotes Transplant Acceptance by Driving Allogenic CD4 + T Cell Dysfunction.” Immunity 47(6):1114-1128.e6.

Xin, Annie, Frederick Masson, Yang Liao, Simon Preston, Tianxia Guan, Renee Gloury, Moshe Olshansky, Jian-Xin Lin, Peng Li, Terence P. Speed, Gordon K. Smyth, Matthias Ernst, Warren J. Leonard, Marc Pellegrini, Susan M. Kaech, Stephen L. Nutt, Axel Kallies, Wei Shi, and Gabrielle T. Belz. 2016. “A Molecular Threshold for Effector CD8+ T Cell Differentiation Controlled by Transcription Factors Blimp-1 and T-Bet.” Nature Immunology 17(4):422–32.

Yang, Xuexian O., Bhanu P. Pappu, Roza Nurieva, Askar Akimzhanov, Hong Soon Kang, Yeonseok Chung, Li Ma, Bhavin Shah, Athanasia D. Panopoulos, Kimberly S. Schluns, Stephanie S. Watowich, Qiang Tian, Anton M. Jetten, and Chen Dong. 2008. “T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ.” Immunity 28(1):29–39.

Yao, Chen, Hong-Wei Sun, Neal E. Lacey, Yun Ji, E. Ashley Moseman, Han-Yu Shih, Elisabeth F. Heuston, Martha Kirby, Stacie Anderson, Jun Cheng, Omar Khan, Robin Handon, Julie Reilley, Jessica Fioravanti, Jinhui Hu, Selamawit Gossa, E. John Wherry, Luca Gattinoni, Dorian B. McGavern, John J. O’Shea, Pamela L. Schwartzberg, and Tuoqi Wu. 2019. “Single-Cell RNA-Seq Reveals TOX as a Key Regulator of CD8+ T Cell Persistence in Chronic Infection.” Nature Immunology 20(July).

Yao, Shuyu, Bruno Fernando Buzo, Duy Pham, Li Jiang, Elizabeth J. Taparowsky, Mark H. Kaplan, and Jie Sun. 2013. “Interferon Regulatory Factor 4 Sustains CD8+ T Cell Expansion and Effector Differentiation.” Immunity 39(5):833–45.

Ye, B., X. Liu, X. Li, H. Kong, L. Tian, and Y. Chen. 2015. “T-Cell Exhaustion in Chronic Hepatitis B Infection: Current Knowledge and Clinical Significance.” Cell Death & Disease 6:e1694.

Yen, Hung-Rong, Timothy J. Harris, Satoshi Wada, Joseph F. Grosso, Derese Getnet, Monica V. Goldberg, Kai-Li Liang, Tullia C. Bruno, Kristin J. Pyle, Siaw-Li Chan, Robert A. Anders, Cornelia L. Trimble, Adam J. Adler, Tzou-Yien Lin, Drew M. Pardoll, Ching-Tai Huang, and Charles G. Drake. 2009. “Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity.” The Journal of Immunology 183(11):7161–68.

Zajac, a J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. “Viral Immune Evasion Due to Persistence of

Activated T Cells without Effector Function." The Journal of Experimental Medicine 188(12):2205–13.

Zarour, Hassane M. 2016. "Reversing T-Cell Dysfunction and Exhaustion in Cancer." Clinical Cancer Research 22(8):1856–64.

Zhang, Weiguo, Joanne Sloan-Lancaster, Jason Kitchen, Ronald P. Trible, and Lawrence E. Samelson. 1998. "LAT: The ZAP-70 Tyrosine Kinase Substrate That Links T Cell Receptor to Cellular Activation." Cell 92(1):83–92.

Zhang, Yan, Shengdong Huang, Dejun Gong, Yanghua Qin, and Qian Shen. 2010. "Programmed Death-1 Upregulation Is Correlated with Dysfunction of Tumor-Infiltrating CD8+ T Lymphocytes in Human Non-Small Cell Lung Cancer." Cellular and Molecular Immunology 7(5):389–95.

Zhou, Xinyuan, Shuyang Yu, Dong Mei Zhao, John T. Harty, Vladimir P. Badovinac, and Hai Hui Xue. 2010. "Differentiation and Persistence of Memory CD8+ T Cells Depend on T Cell Factor 1." Immunity 33(2):229–40.