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**Repetitive antigen stimulations
disarm specific human T lymphocytes**

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Enfin nous y voici ! Quelle aventure...

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ABSTRACT

In mouse models of chronic viral infection and cancer, T cells lose the ability to proliferate and exert effector functions, and are considered to be exhausted. It is difficult to establish whether these cells are dysfunctional because of antigen persistence, inflammatory conditions or a suppressive microenvironment. In humans, the corresponding T cells can only be tested *ex vivo*, which provides a momentary snapshot of their function only. To examine whether repetitive rounds of antigen exposure can cause human T cell dysfunction, we subjected human antigen-specific CD8⁺ T cells to repeated stimulations with either melanoma cells or antigen-presenting mature dendritic cells. Human T cells lost cytokine production and lytic activity while retaining proliferation. Antigen removal failed to restore function. Surface expression of inhibitory receptors cannot explain the dysfunction. We concluded that repetitive rounds of antigen stimulations are sufficient to render human CD8⁺ T cell dysfunctional. Consequently, therapeutical strategies should not only target the suppressive environment but also the mechanisms underlying this dysfunction.

INTRODUCTION

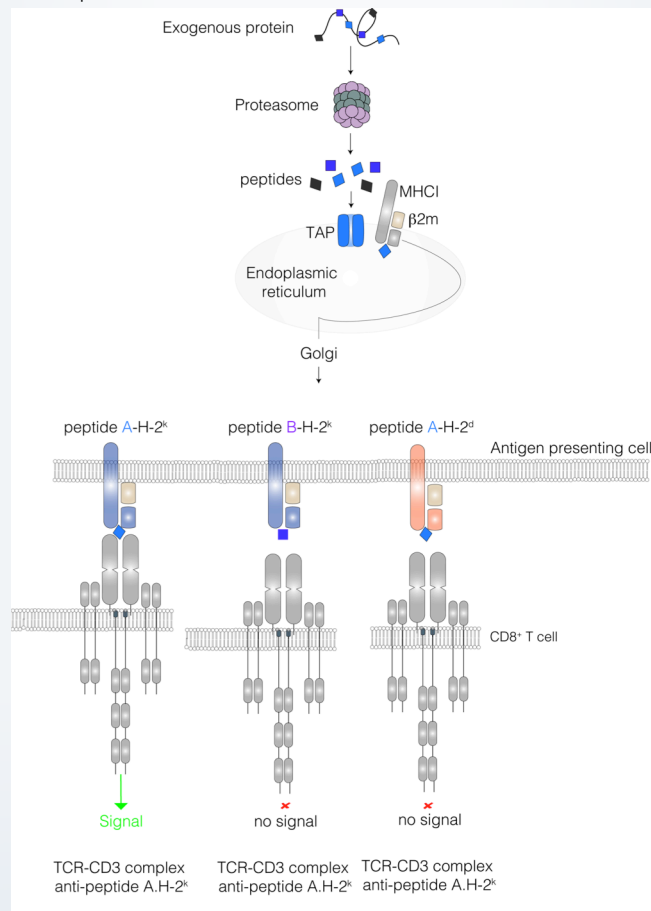
PREAMBLE

CD8⁺ T cells are essential for clearing viral, protozoan, and intracellular bacterial infections ^{5,6}. Multiple studies showed that CD8⁺ T cells are also a key component of antitumor immunity both in human and mice ⁷⁻¹⁰. However, many different mechanisms contribute to the failure of CD8⁺ T cells in pathological conditions. One of the mechanisms implies functional silencing of effector T cells. This is exploited by viruses and cancers to prevent destruction by the immune system. The discovery that dysfunctional T cells may be rejuvenated and that their functional defects can be partially reversed has been a major breakthrough. Despite the clinical opportunities to target dysfunctional T cells, the phenomenon remains poorly understood.

In this chapter, I will first introduce T cell differentiation in the context of a controlled infection. Secondly, I will discuss the known deregulation mechanisms governing T cell dysfunction during a persistent infection.

Box 1**Antigen processing and MHC I restriction**

Proteins are mainly degraded by the proteasome, which is a large proteolytic complex that is mainly located in the cytosol. The products of protein degradation, peptides, are translocated to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, peptides associate with MHC class I molecules (e.g. A-H-2^k haplotype) and the $\beta 2m$ microglobulin ($\beta 2m$). The complex migrates through the Golgi to the cell surface. A T cell clone bears a unique T cell receptor (TCR) specific for a peptide-MHC complex³.



CD8⁺ T cell differentiation in acute infection

During the initial encounter with a pathogen, naïve CD8⁺ T cells bearing T cell receptors (TCR) specific for cognate antigens undergo clonal expansion. The specificity of this selection is driven by the interaction on the one hand by short pathogen-derived peptides presented by major histocompatibility complex class I (MHC I) molecules on the surface of antigen presenting cells (APC) (*Box 1*). On the other hand by TCR activation on the surface of T cells resulting from peptide/MHC I ligation^{5,11}. It is now clear that TCR activation alone is not sufficient. Secondary stimuli delivered by co-signaling molecules are required for T cell activation.

TCR SIGNALING INITIATION

The TCR is a heterodimer composed of α and β subunits. These are unique to each individual T cell. The TCR α and β subunits themselves contain no signal transducing intracellular domains. However, they associate non-covalently to the non-polymorphic CD3 ϵ –CD3 δ , CD3 ϵ –CD3 γ , and CD3 ζ –CD3 ζ dimers^{1,12}. Each CD3 molecule contains at least one immunotyrosine activation motif (ITAM), YxxL/Ix, which is critical for signaling transduction. The entire TCR complex contains no catalytic activity. Precisely how the ligation of the TCR with peptide-MHC complex changes the signaling balance in favour of T cell activation is still debated and has been extensively discussed elsewhere^{12,13}. Following triggering of the TCR, lymphocyte-specific protein tyrosine kinase (LCK), associated to co-receptor CD8, is recruited to the TCR–CD3 complex and phosphorylates ITAMs of the intracellular CD3 chains^{12,14}. Phosphorylation of the ITAMs enables the recruitment of ζ -chain associated protein kinase of 70 kDa (ZAP70). It is in turn phosphorylated by LCK. This phosphorylation results in conformational changes in ZAP70 that promotes its kinase activity. Activated ZAP70 phosphorylates tyrosine residues of the key adaptor molecule linker for activation of T cells (LAT), which act as docking sites for kinases and other adaptor molecules to form a multiprotein complex, termed the LAT signalosome. Important molecules that constitute this complex include Phospholipase C γ 1 (PLC γ 1), GRB2 (Growth factor, Receptor-Bound protein 2), GRB2-related Adaptor protein

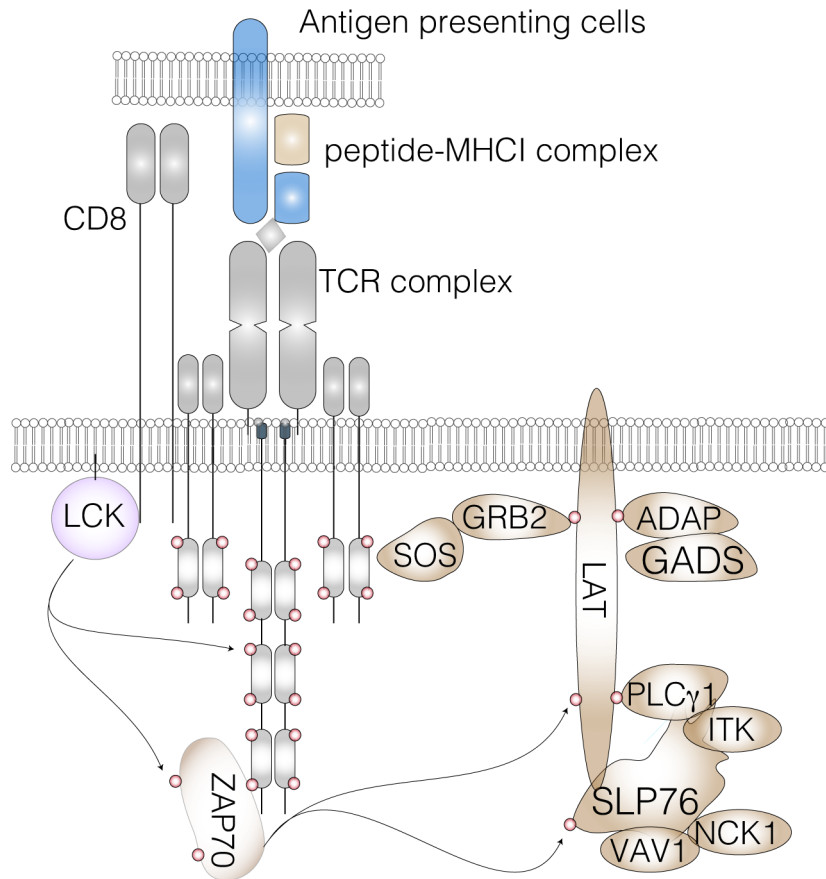


Figure 1: TCR signaling initiation. *The interaction between specific peptide-MHC complex and the TCR initiates a signaling cascade. LCK phosphorylates ITAMs of the TCR-CD3 complex. It induces the recruitment of ZAP70, which in turn is phosphorylated by LCK. Activated ZAP70 phosphorylates LAT, which acts as a docking site for kinases and other adaptor molecules to form a multiprotein complex, termed the LAT signalosome.*

(GRAP2) also known as GADS, SH2 domain-containing leukocyte protein of 76 kDa (SLP76), adhesion- and degranulation-promoting adaptor protein (ADAP), interleukin-2-inducible T cell kinase (ITK), non-catalytic region of tyrosine kinase adaptor protein 1 (NCK1) and Vav1^{12,14} (*Figure 1*).

TCR SIGNAL TRANSDUCTION

PLC γ 1 is found in the proximal signaling complex bound to SLP76, Vav1, and LAT. Activation of PLC γ 1 requires at least three classes of protein tyrosine kinases; LCK, ZAP70 and ITK in T cells^{15,16}. Activated PLC γ 1 hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to Inositol-1,4,5-trisphosphate (IP3), which induces the release of Ca⁺⁺ from endoplasmic reticulum, and diacylglycerol (DAG), which activates protein kinase C (PKC) and RasGRP-dependent signaling pathways^{12,15,17}. The signal will propagate through major signaling pathways that play a crucial role in the induction of numerous T-cell activation-associated responses.

The Nuclear factor activated of T cells (NFAT) pathway

IP3, through opening calcium-release-activated channels, induces the release of calcium from the endoplasmic reticulum, resulting in an increased calcium concentration in the cytoplasm. Increased intracytoplasmic Ca⁺⁺ levels trigger activation of calmodulin which in turn activates the calmodulin-dependent phosphatase calcineurin. Calcineurin controls intracellular localization of nuclear factor of activated T cells (NFAT) family members. The NFAT family consists of five members: NFAT1 (also known as NFATp or NFATc2), NFAT2 (also known as NFATc or NFATc1), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATx or NFATc3) and NFAT5^{18,19}. Only NFAT1, NFAT2 and NFAT4 are relevant in T cells^{18,19}. These are key regulators of cytokine gene expression²⁰. Constitutive phosphorylation of NFAT members induces cytoplasmic sequestration by binding to 14-3-3 proteins. This phosphorylation is mediated by NFAT kinases that include casein kinase 1 (CK1) and glycogen-synthase kinase 3 (GSK3)²¹⁻²³. Activated calcineurin dephosphorylates NFAT members, allowing their translocation to the nucleus^{19,24,25} (*Figure 2*).

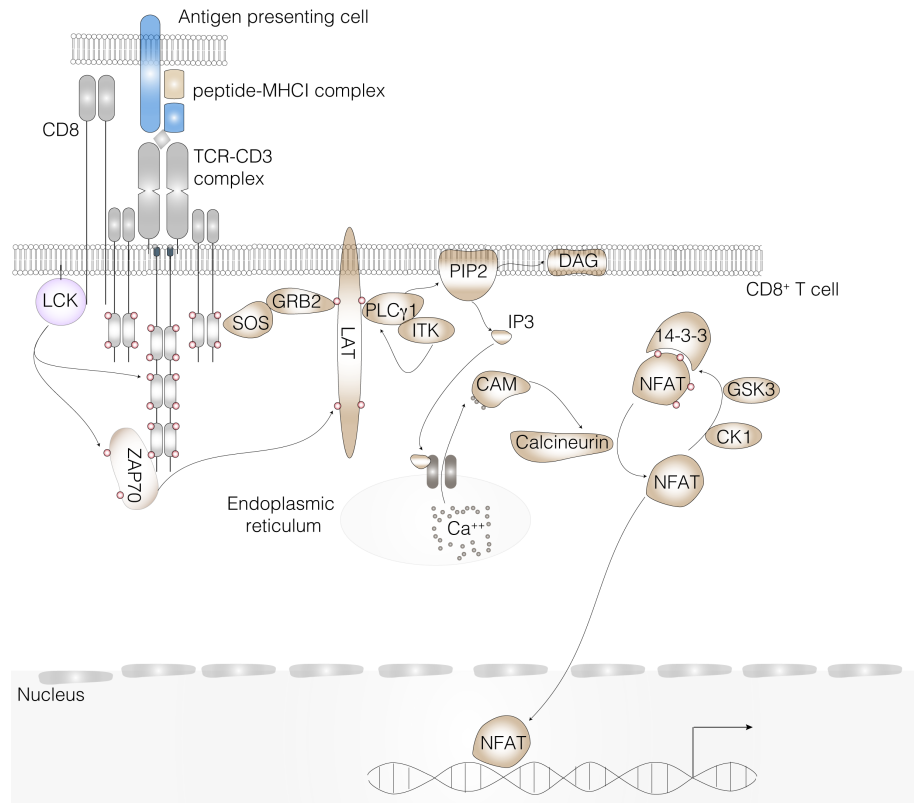


Figure 2: The NFAT pathway in T cells. *Phosphorylated NFAT is confined bound to 14-3-3 protein in the cytoplasm. This phosphorylation is mediated NFAT kinases including CK1 and GSK3. Ligation of the TCR triggers the activation of PLCγ1. Activated PLCγ1 hydrolyses PIP2 into IP3 and DAG. IP3 induces an increase in intracellular calcium concentrations. The calcium binds calmodulin (CAM) and activates calcineurin. Activated calcineurin dephosphorylates NFAT allowing its translocation to the nucleus.*

The DAG-mediated signaling pathways

DAG binds with high affinity to proteins that contain a conserved cysteine-rich domain (H-X₁₂-C-X₂-C-X_{13/14}-C-X₂-C-X₄-H-X₂-C-X₇-C). In lymphocytes, this motif is found for instance in Ras/Rap guanyl-releasing protein GRP family of guanine nucleotide exchange factors (GEFs), which activate Ras and Rap GTPases, and in serine/threonine kinases protein kinase C (PKC). Thus, TCR-induced DAG production results in the activation of two major pathways involving Ras and PKC (*Figure 3*).

GEF promote the transition of Ras from the inactive GDP-bound state to the active GTP-bound conformation. Two Ras GEFs are present in T cells: son of sevenless (SOS) and RasGRP. Activated Ras leads to the activation of the serine-threonine kinase Raf-1, a mitogen-associated protein kinase kinase kinase (MAPKKK). Raf-1 phosphorylates and activates MAPK kinases (MAPKKs also known as MEK), which in turn phosphorylate and activate the MAPK's extracellular signal-regulated kinase 1 (Erk1) and Erk2. Erk stimulates the activity of the transcription factor Elk1. Elk1 regulates the activity of c-Fos and thus contributes to the activation of the activator protein-1 (AP-1 = c-Jun&c-Fos) (*Figure 3*)^{14,26}.

The second major signaling pathway regulated by DAG is mediated by PKC. Multiple PKC isoforms are expressed in lymphocytes including α , β I, β II, δ , ϵ , η , and θ ¹⁶. PKC exerts multiple roles in T cells. The best-defined role is the control of transcription factor nuclear factor-kappa B (NF- κ B)^{27,28}. In T cells PKC ϵ and θ are essential for this role²⁹. PKC activation through its phosphorylation of CARMA1 induces the assembly of a trimolecular complex, CARMA1/Bcl10/MALT1 (CBM complex). CARMA1 phosphorylation induces conformational changes required for the oligomerization and association with Bcl10 and MALT1^{30,31}. CBM complex is polyubiquitinated on Bcl10 and MALT1, a process implicating TRAF6. This ubiquitination leads to the recruitment of IKK (I κ B kinase) via the IKK γ polyubiquitin binding motif. This association drives the polyubiquitination of IKK γ and phosphorylation of IKK β ^{32,33}. Activated IKK β phosphorylates I κ B α and lead to its degradation. I κ B α associates via Rel homology domain

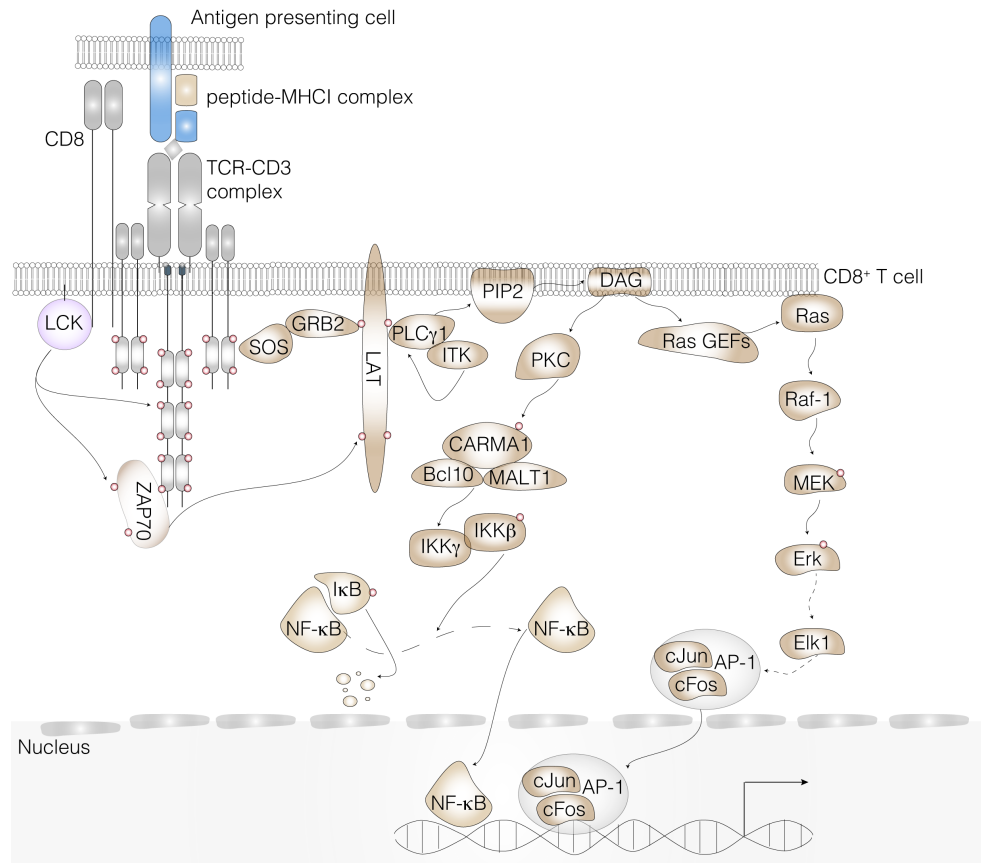


Figure 3: The DAG-mediated signaling pathways. *TCR ligation triggers the production of DAG, which induces two signaling pathways. DAG can activate PKC leading to the degradation of I κ B. I κ B degradation leads to “freeing” of NF- κ B that can translocate to the nucleus. DAG also activates Ras-GEF and thereby induces the activation of Erk1/2. Erk regulates the activity of AP-1 through Elk1.*

(RHD) to NF- κ B, keeping it latent in the cytosol. The degradation of I κ B α leads to “freeing” of nuclear localization motif and DNA-binding domains of NF- κ B. Unbound NF- κ B translocates to the nucleus. The NF- κ B family of transcription factors comprises of five related proteins, c-Rel, p65 (RelA), RelB, p50 (NF- κ B1) and p52 (NF- κ B2) that interact to form homodimers and heterodimers with distinct gene regulatory functions involved in T cell survival, proliferation, and effector functions (*Figure 3*)³⁴.

Immunological synapse

T cell activation leads to the formation of tight contacts between T cells and antigen-presenting cells. This structure is known as the immunological synapse (*Box 2*)³⁵. The establishment of this structure requires integrin activation, actin remodeling and microtubular organization.

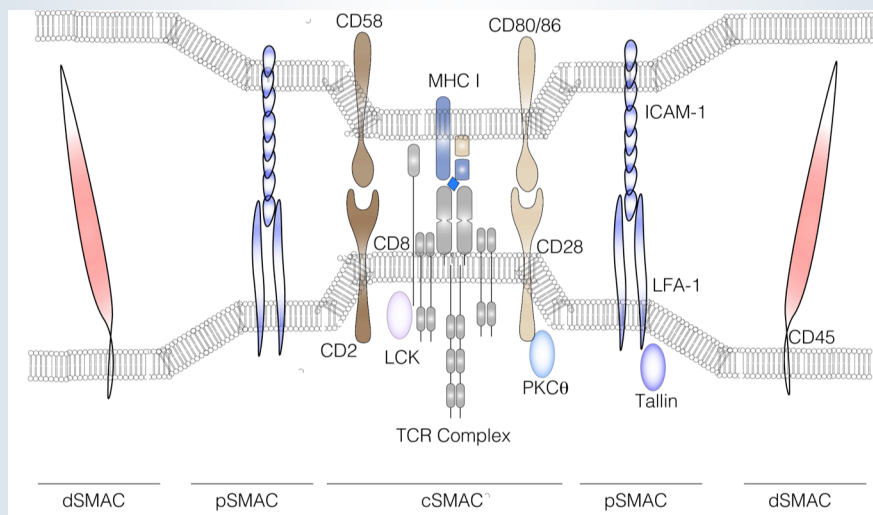
Actin oligomerization induced by TCR activation is a result of complex molecular interactions. The best-characterized pathway involves the Arp2/3 (actin-related proteins 2/3) complex, a member of the nucleation factors protein family². The Arp2/3 complex is thought to mimic the growing end of an actin filament. Once bound to F-actin, the Arp2/3 complex is presented with an actin monomer bound to nucleation-promoting factors (NPF), causing daughter filament elongation. Activation of Arp2/3 requires its interaction with NPF including Wiskott-Aldrich syndrome protein (WASp), WAVE2, and hematopoietic cell lineage-specific protein 1 (HS1). SLP76 acts as a docking site for WASp, Vav1, NCK Rho family GTPases nucleotide exchange factors (*Figure 1*). Vav1 mediates activation of Rho GTPases Rac1 and Cdc42. These GTPases will activate WASp and WAVE2.

Molecular mechanisms controlling the polarization of the MTOC (microtubular organizing center) remain poorly understood. Data suggest a major role of motor protein dynein through its interaction with ADAP, an adaptor protein linked to the SLP76 complex³⁶.

TCR signaling induces the activation of integrins, which are receptors responsible for mediating cell/cell or cell/matrix adhesion³⁷. This

Box 2**The immunological synapse**

The immunological synapse is a structure formed at the interface between T cell and antigen presenting cells. The synapse is organized into three supra-molecular activation clusters (SMAC): central (cSMAC), distal (dSMAC) and peripheral (pSMAC). TCR, CD2, CD28 and signaling molecules such as PKC θ and LCK are located in the cSMAC. The pSMAC is a ring that surrounds the TCR, containing LFA-1, ICAM-1 and Talin. The dSMAC is enriched in proteins with large ectodomains such as CD45. Because of this structure, the cSMAC was considered as the site of TCR signaling initiation. It is now largely accepted that TCR signaling occurs before the formation of the cSMAC. Later, it appears that the signaling at the cSMAC is even lower than in the pSMAC. This structure may rather play a role in TCR internalization/degradation. In addition, the immunological synapse plays a role in the polarized secretion of lytic granules and cytokines ^{1,2}.



phenomenon is known as “inside-out signaling”. The activation of integrins results in a conformational change that converts integrin affinity from low or intermediate levels to high affinity. Complex pathways regulate integrin activation. Conditional knock outs in T cells reveal the roles of several molecules including SLP76, Vav1, ADAP, SKAP-55, RapL, RIAM, Rap1, Talin and Kindlin ³⁷.

Remark

Here I present selected pathways to illustrate how T cells can induce molecular changes upon TCR ligation to MHC-peptide complex. Other signaling pathways may occur. Furthermore, I present these pathways as a set of linear events. That is not illustrating the complexity and the interconnections between the different signaling molecules which lie outside the scope of this thesis.

CO-STIMULATION

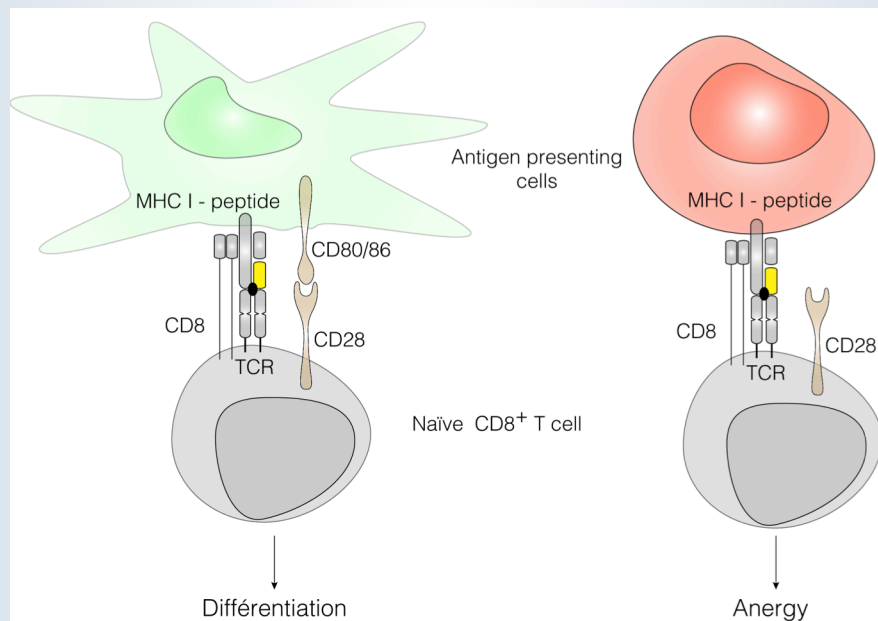
T cell activation, solely through the TCR, results in a nonresponsive state named anergy ⁶. Co-ligation of other cell surface receptors are required to fully activate T cells and avoid anergy (*Box 3*). This phenomenon is known as co-stimulation and refers to activation signals delivered to T cells by cell surface receptors other than the TCR itself. The signaling induced by the TCR and co-signaling molecules often overlap. It is thus difficult to identify a unique molecular program induced by co-stimulatory molecules. Most co-signaling molecules are members of the immunoglobulin superfamily (IgSF) and tumor necrosis factor receptor superfamily (TNFRSF).

Co-stimulatory signaling downstream induced by molecules of the IgSF

The regulatory mechanism of co-stimulatory receptors is determined by the structure of their intracellular domain. The best-characterized member of the IgSF is CD28 ³⁸. Its intracellular cytoplasmic tail contains YMN and PYAP motifs. Following the binding of CD28 to its ligands CD80 or CD86, the YMN motif interacts with the p85 subunit of PI3K (Phosphatidylinositol 3-kinase), which converts PIP2 to phosphatidylinositol (3,4,5) trisphosphate (PIP3).

Box 3**Anergy**

Anergy is defined as a dysfunctional T cell state occurring when naïve T cells are activated in absence of positive co-stimulation, such as CD28. T cell anergy plays a major role to prevent autoimmunity by silencing auto-reactive T cells. Anergic T cells are characterized by low production of IL2 and cell cycle arrest at the G1/S phase. Molecular mechanisms inducing T cell anergy are poorly understood. It has been proposed that TCR activation alone results in improper mTOR and Ras/MAPK signaling, thereby resulting in epigenetic changes. Transcription factor Erg2 seems to be a key player in anergy. Additionally, homodimerization of NFAT, IKAROS protein and Sirtuin I seems to promote T cell anergy ⁴.



PIP3 act as a docking site for PDK1 and its target AKT, also known as PKB (Protein kinase B). Activation of the AKT pathway affects numerous responses including T cell effector functions and survival, through activation of NF- κ B, NFAT, BCL-XL, mTOR (mammalian target of rapamycin), GLUT1 (glucose transporter type 1) and others ³⁹. In addition, CD28 favors the recruitment of PKC θ and RasGRP to the immunological synapse and its subsequent activation. RasGRP activation depends on the binding of GRB2 to both the YNMN and PYAP motifs. This induces the activation of Ras and its downstream signaling. Recruitment of GRB2 to CD28 is essential for IL2 production (*Figure 4*) ³⁸.

Another well known member of the IgSF family is ICOS. Its intracellular cytoplasmic tail contains a unique YMFM SH2-binding motif that recruits both p85 and p50a subunits of PI3K and subsequently AKT activation. As p50 subunit is more active than the p85, ICOS engagement induces a more potent AKT signal than CD28, which interacts with p85 only ⁴⁰. However the YMFM motif of ICOS is unable to bind GRB2. Thus ICOS cannot induce IL2 production, unlike CD28 ³⁸.

Co-stimulatory signaling downstream of TNFRSF receptors

TNFRSF co-signaling receptors synergize with TCR signaling to promote cell cycle progression, cytokine production and T cell survival, through TRAF (tumor necrosis factor [TNF] receptor-associated factor) adaptors. Recruitment of TRAF adaptors is induced by interaction of three TNF receptors with 3 ligands on antigen-presenting cells ⁴¹. The transduction of the signal through TRAF adaptors remains unclear. However it leads to the activation of NF- κ B, JNK, MAPK, AP1, Erk and NFAT. The variability of the response between TNFRSF co-signaling receptors can be partially explained by their association with specific TRAF adaptors. 4-1BB can bind TRAF1, TRAF2 and TRAF3. OX40 and CD27 can bind TRAF2, TRAF3 and TRAF5, and HVEM and CD30 can bind TRAF1, TRAF2, TRAF3 and TRAF5. GITR recruits only TRAF2 ³⁸.

CD8⁺ T CELL SUBSETS

Classical CD8⁺ T cell differentiation occurs in acute viral infection, such as influenza or yellow fever. In this setting, a CD8⁺ T cell response is

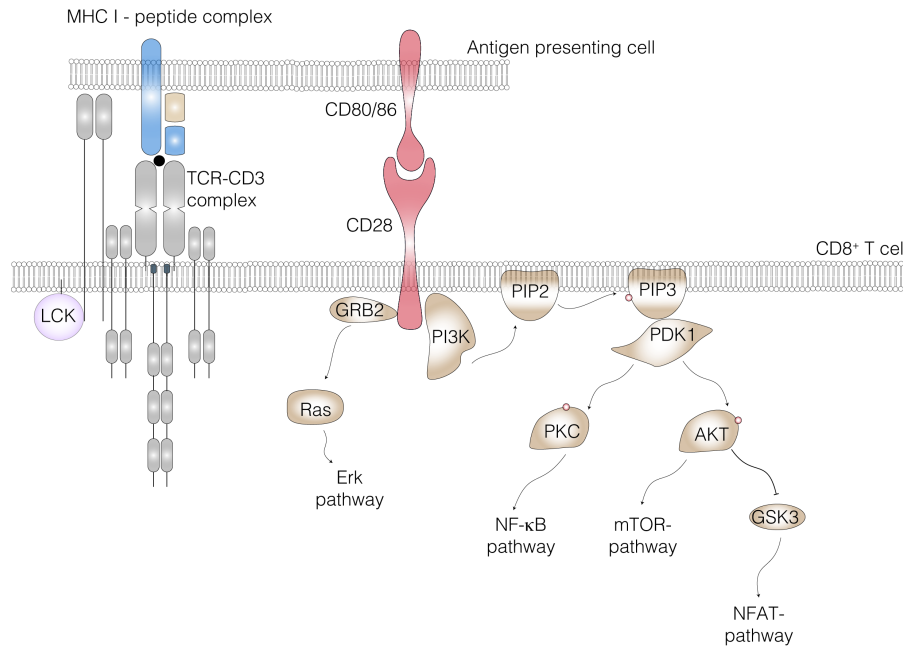


Figure 4: Co-signaling pathway downstream of CD28. Upon ligation to its ligands CD80/86, CD28 recruits PI3K. Through this molecule, CD28 can potentiate all major signaling pathways induced by TCR signaling.

characterized by three distinguishable phases, (i) the clonal expansion of naïve T cells into effector cells, (ii) the contraction phase of effector CD8⁺ T cells following elimination of the infecting pathogen and (iii) the maintenance of memory T cells. The timing and mechanisms underlying the differentiation of naïve cells into effector cells, and memory cells is not yet clear and is still a matter of debate. I will not address this topic here. Additional information can be found in Kaech *et al.* and Gerritsen *et al.* ^{42,43}. Instead, I will describe the three main subpopulations of CD8⁺ T cells, naïve, effector and memory T cells, and the transcription factors implicated in effector and memory T cell development.

Naïve CD8⁺ T cells

During an infection, priming of naïve CD8⁺ T cells by APCs occurs in secondary lymphoid organs such as lymph nodes and the spleen ^{43,44}. To find their antigen, naïve T cells alternate constantly between circulating in the blood and homing to secondary lymphoid organs. To migrate into the lymph nodes or the spleen, naïve T cells express several molecules such as CD62L and CCR7. CD62L is implicated in the attachment to the high endothelial venules (HEVs) whereas CCR7 binds to CCL21 expressed by HEVs and mediates the activation of LFA1. Activated LFA1 binds ICAM1. This induces the arrest and the migration of naïve T cell into the secondary lymphoid organs ⁴³.

Upon activation, CCR7 is transiently up-regulated to retain the activated T cell in the lymph node ⁴³. This up-regulation is mediated by TCR signaling. Naïve antigen specific CD8⁺ T cells undergo a massive clonal expansion and generate effector CD8⁺ T cells. Both in mice and humans, naïve CD8⁺ T cells specific for a given peptide/MHC I complex exist at a frequency of 10⁻⁵-10⁻⁷ T cells in the naïve CD8⁺ T-cell pool ⁴⁵⁻⁴⁷. During the clonal expansion phase, CD8⁺ T cells robustly proliferate with a 10⁴- to 10⁵- fold expansion ^{48,49}.

Effector CD8⁺ T cells

To eliminate the infection CD8⁺ T cells have to leave the lymph nodes and migrate to the site of infection. This migration is a complex and poorly understood process. It implicates the down-regulation of CD62L and CCR7,

allowing the exit of effector T cell from the secondary lymphoid organs^{43,50,51}. It has been hypothesized that homing of effector CD8⁺ T cells to the infection site is dictated by specific “area codes”. If T cells priming occurs in the skin-draining lymph nodes, for example, effector T cells express preferentially skin homing surface molecules e.g. CCR4, CCR10, E-selectin and P-selectin^{52,53}. T cell priming in the gastrointestinal associated lymphoid tissue (GALT) leads consistently to expression of small intestinal mucosa homing surface molecules, e.g a4b7 and CCR9^{52,54}.

Effector T cells acquire effector function to eradicate the infection. A major characteristic of CD8⁺ T cells is their ability to kill target cells expressing specific peptide/MHC I complexes. Upon TCR activation CD8⁺ T cells are able to kill infected cells by two distinct molecular pathways⁵⁵. (i) The first mechanism depends on perforin and granzymes proteins. Perforin is a protein present in the cytoplasmic granules of CD8⁺ T cells and is secreted to form pores on target cell membranes. Granzymes proteins can therefore move into the target cell and activate the caspase cascade in target cells and induce apoptosis^{5,56,57}. (ii) The second mechanism is mediated by the up-regulation of FasL (CD95L) on CD8⁺ T cells. The binding of FasL with Fas, which is expressed on target cells, instigates receptor oligomerization. This engages Fas-associated death domain (FADD), which can activate the caspase pathway and induce apoptosis of the target cells⁵⁸.

The CD8⁺ T cells can also exert antiviral functions via a non-cytolytic pathway through the secretion of chemokines and antiviral cytokines, such as gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α)⁵. These molecules can directly interfere with a pathogen by modifying its gene expression or, indirectly, by activating phagocytic cells such as macrophages or neutrophils⁵⁹.

A major difference between cytolytic and non-cytolytic antiviral functions of CD8⁺ T cells is the regulation of effector genes. Resting T cells contain lytic granules containing perforin and granzymes, which are inactive due to the acidic pH of the granules. Upon TCR activation, the lytic granules are secreted⁵⁷. On the other hand, cytokine secretion requires *de novo* protein synthesis following TCR activation⁴⁴.

After eradication of the pathogen, effector CD8⁺ T cells undergo a

contraction phase wherein the majority of antigen-specific effector CD8⁺ T cells die by apoptosis. A small percentage, 5–20%, of virus-specific memory T cells, survives in an antigen-independent manner for a long time^{42,43,60}.

Memory CD8⁺ T cells

The maintenance of memory CD8⁺ T cells is cytokine-dependent, mainly through the actions of IL-7 and IL-15^{42,43}. These cytokines promote memory CD8⁺ T cell survival and self-renewal through homeostatic proliferation⁶¹. The main characteristic of memory CD8⁺ T cells is their ability to rapidly generate secondary T cell response upon re-exposure to the cognate antigen. Memory T cells are heterogeneous but can typically be divided into two subpopulations according to their localization^{62,63}.

Central memory T cells (T_{CM}) reside in secondary lymphoid organs and are characterized by the expression of CD62L⁺ and CCR7⁺, which are required for re-circulating through the lymph nodes. T_{CM} cells are self-renewing and can rapidly expand and differentiate following re-challenge^{52,62,64,65}.

Effector memory T cells (T_{EM}) cells are located in non-lymphoid tissue. Unlike T_{CM}, they lack lymph node homing markers CD62L and CCR7. T_{EM} cells constitutively retain some effector functions such as cytokine production but have limited homeostatic turnover^{62,64,66,67}.

Additional memory subsets (T_{EMRA}, T_{RM} etc.) can be defined based on the tissue localization, homing markers recall ability, effector functions and resting state^{64,68,69}.

TRANSCRIPTIONAL REGULATION OF CD8⁺ T CELL DIFFERENTIATION

Multiple studies have examined the role of specific transcription factors in T cell differentiation. These studies were done with (i) conditional-deficient CD8⁺ T cells in mice model and (ii) gene expression profiles of naïve, effector, and memory CD8⁺ T cells. They indicated that T cell differentiation is controlled by competing sets of transcription factors. Here, I will describe the two main sets of transcription factors (*Figure 5*).

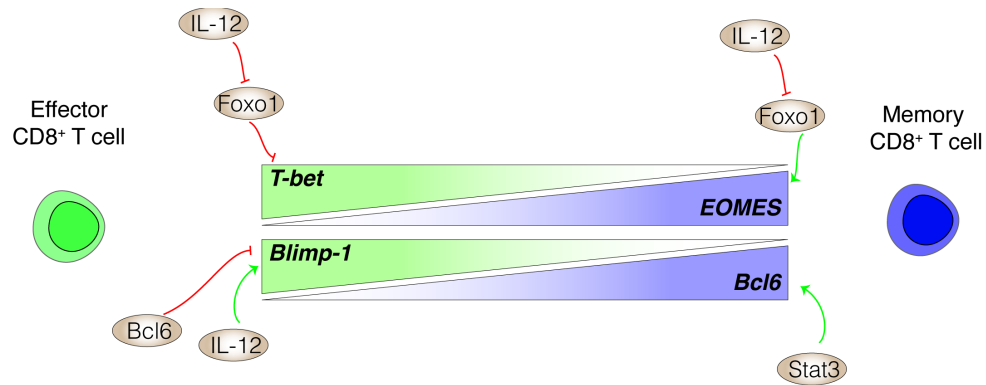


Figure 5: Transcription factors that control CD8⁺ T cell differentiation. Effector cell and memory cell subpopulations seem to be regulated by the activity of competing sets of transcription factors. *T-bet* and *Blimp-1* are more active in effector CD8⁺ T cells, whereas *EOMES* and *Bcl6* are active in memory CD8⁺ T cells. Adapted from Kaech et al. 2012⁴².

The T-bet-Eomes-Foxo1 axis

T-bet, a member of the T-box transcription factor family, plays a crucial role in the generation of effector T cells. Following priming, TCR signaling, IL-12-mediated signals and mTOR activity induce T-bet expression.⁷⁰⁻⁷³ Eomesodermin (Eomes) is also a member of the T-box transcription factor family. Eomes is induced in early effector CD8⁺ T cells by IL2⁷⁴. Foxo1 (Forkhead box O) belongs to the Foxo transcription factor family. In T cells, Foxo1 seems to be regulated by inflammatory cytokines through Stat3, since Stat3-deficient mice have low levels of Foxo1⁷⁵.

Eomes and T-bet cooperate in CD8⁺ T cell differentiation through partially redundant activities⁷². T-bet and Eomes enhance expression of effector function genes, such as IFN- γ , Gzmb, perforin, CXCR3 and CXCR4^{42,71,76}. In memory T cells, T-bet and Eomes collaborate to sustain the expression of IL2R β , a component of IL-15 receptor and therefore enhance the IL-15 signaling, which is required for the survival of memory T cells⁷¹. CD8⁺ T cells deficient in either T-bet or Eomes can still respond to viral infection and undergo normal differentiation. However, CD8⁺ T cells lacking both T-bet and Eomes lose CTL identity and abnormally differentiate into IL-17-producing CD8⁺ T cells. This causes excessive neutrophil infiltration and a lethal inflammatory syndrome during lymphocytic choriomeningitis virus (LCMV) infection⁷⁷.

Despite cooperation between Eomes and T-bet, their expression is somehow reciprocal and depends on Foxo1 activity (*Figure 5*). In the early phase of T cell differentiation, T-bet is highly expressed. This expression is promoted by IL-12 through the inhibition of Foxo1^{78,79}. In contrast, Eomes expression is attenuated by Foxo-1-inhibition through IL-12-mediated signals and mTOR activity^{72,79}. In the late phase of T cell activation, Eomes expression is increased while T-bet expression declines as memory cells form^{42,80}. Thus the ratio of T-bet to Eomes is high at effector T cell stages and low at memory T cell stages, indicating that T cell differentiation is dependent of the ratio of T-bet to Eomes expression, which is controlled by Foxo1 activity.

The Blimp1-Bcl6 axis

B lymphocyte-induced maturation protein 1 (Blimp-1; also known as PRDM-1) is a transcriptional repressor important for the maintenance of T cell homeostasis^{81,82}. Blimp-1 is induced upon TCR activation and its expression is regulated by IL2, IL-12 and IL21^{74,83,84}. Blimp-1 is preferentially expressed at effector stages and enhances CD8⁺ T cell functions, such as migration and IFN- γ and Gzmb expression. Deeper insight in the role of Blimp-1 in effector CD8⁺ T cells come from mice studies. A Conditional knockout of Blimp-1 in CD8⁺ T cells leads to aberrant CD8⁺ T cell differentiation. These cells fail to eradicate influenza virus due to a poor recruitment of virus-specific T cells to the lungs⁸⁵.

B-cell lymphoma 6 (Bcl-6) expression is increased and sustained by IL-10- and IL21-induced signaling through Stat3⁸⁶. Bcl-6 seems to play a role in the differentiation at memory CD8⁺ T cell stages. Bcl-6 over-expressing CD8⁺ T cells have increased numbers of memory cells, while the opposite is true for Bcl-6 knock-out CD8⁺ T cells⁸⁷. Bcl-6 represses Blimp-1 activity and their expression is inversely correlated (*Figure 5*).

CD8⁺ T cell exhaustion

The T cell differentiation model described above is the chart for most acute infections. Under certain conditions, especially in chronic infections where antigen persists, the immune system is susceptible to abnormal functioning, e.g. T cell function can be impaired. This phenomenon is known as T cell exhaustion. The lymphocytic choriomeningitis virus (LCMV) infection in mice has been very useful model for investigating the alteration of effector functions arising in chronic infections.

THE LCMV MODEL

Mice are the natural host of LCMV. The LCMV model relies on the Armstrong strain (Arm) and the Clone 13 strain (C13). Intra-peritoneal injection of the Arm strain into adult mice gives rise to acute infection in which the virus is cleared within 7-10 days^{88,89}. Intravenous inoculation of

C13 results in persistent infection.

The C13 strain is a variant of the Arm strain and was isolated from the spleen of mice infected with the Arm strain⁹⁰. The C13 and Arm strains differ by five nucleotide mutations resulting in three amino acid substitutions⁹¹⁻⁹³. The first coding change occurs in the L protein, an RNase-dependent RNA polymerase, from a lysine in the Arm strain to a glutamine in the C13 at position 1079 (K1079Q)⁹³. The two other mutations, N176D and F260L occur in the surface glycoprotein 1 (GP1)⁹³.

The importance of the 1079Q mutation in the induction of viral persistence is controversial. Using reverse LCMV genetics, Berghaler *et al.* showed that this mutation is essential for the viral persistence by increasing replicative capacity⁹⁴. The group of Oldstone, using phenotypic characterization of recombinant LCMV, showed that the mutation K1079Q results in increase viral titers in macrophages but seems not to be required for viral persistence^{93,95}. Additionally, the group of Oldstone reported that F260L and N176D mutations increase affinity of C13 for the LCMV receptor, α -dystroglycan, which is a cellular receptor for the extracellular matrix proteins⁹³. But only F260L mutation was essential for the induction of viral persistence and T cell exhaustion⁹³. The group of Oldstone re-enforced their argument reporting that F260L mutation was found in 22 out of 22 in LCMV mutants causing persisting infections. Whereas, the 1079Q mutation in L polymerase was only observed in 3 out of 16 persistent isolates examined at this position⁹³.

These mutations do not affect any known T cell epitopes. The two main characterized immuno-dominant epitopes are GP33-41 and NP396-404. It is thus possible to track the same virus-specific T cells during both acute and chronic viral infections.

THE LOSS OF EFFECTOR FUNCTIONS IN LCMV MODEL

Initial studies during LCMV C13 chronic infection described the T cell exhaustion phenomenon as a physical deletion of antigen specific-CD8⁺ T cells⁹⁶. The design of MHC multimers allowing the detection of antigen-specific T cells revealed that T cell are maintained in a poorly functional state^{97,98}.

CD8⁺ T cell exhaustion is progressive with cells loosing some properties before others⁹⁹⁻¹⁰¹. The first signs of T cell exhaustion are the

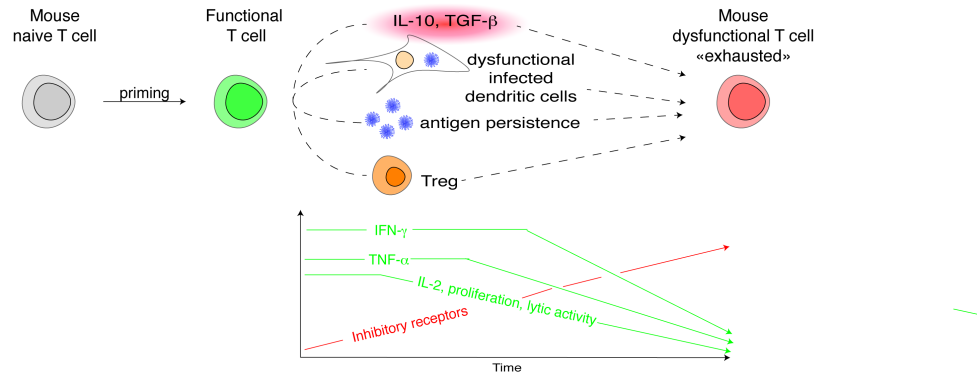


Figure 6: Development and functions of CD8⁺ T cells responding to chronic LCMV C13 infection. Upon infection with C13 LCMV, murine T cells become dysfunctional. Multiple factors play a role in the development of this dysfunction. The dysfunction is hierarchical with some effector functions lost before others.

loss of IL2 production and of high proliferative capacity and cytotoxicity. Subsequently, cells lose the ability to produce TNF- α . The loss of IFN- γ and β -chemokines are observed during severe stage of exhaustion (Figure 6).

The parameters that cause the development of T cell exhaustion

Although T cell exhaustion has been intensively studied during the past two decades, the cause of T cell exhaustion remains speculative. The parameters that influence establishment and maintenance of exhaustion include antigen levels, which are defined by viral loads, CD4⁺ T cells plasticity and immunosuppressive cytokines. I will not discuss the controversial interplay between type I IFN levels and NK cells.

Antigen persistence as a cause of CD8⁺ T cell exhaustion

The level and duration of antigen exposure seems to be a key feature of T cell exhaustion. This is well illustrated by adoptive transfer experiments. If CD8⁺ T cells are primed during infection with C13 but are transferred after 5-8 days into mice infected with the Arm strain, they are able to differentiate into functional CD8⁺ memory T cells ^{102,103}.

Furthermore, multiple studies have demonstrated the importance of antigen abundance. CD8⁺ T cell specific for abundant epitopes become more exhausted than those recognizing antigens with low expression levels, within the same chronically infected host ⁹⁹. Consistently, a mutation of a viral epitope prevents the exhaustion of the CD8⁺ T cell population specific for this epitope, while CD8⁺ T cell specific for unaltered epitopes progress to exhaustion ¹⁰⁴.

CD4⁺ T cells plasticity

CD4⁺ T cells are characterized by their plasticity to differentiate into functional subpopulations that exert different functions depending on cytokine profile, expression of co-signaling molecules and homing receptors ¹⁰⁵. The inflammatory environment governs this plasticity ¹⁰⁶. The CD4⁺ T helper subset plays a vital role in CD8⁺ T cell differentiation through the activation of dendritic cells. These promote CD8⁺ T cell priming, and secretion of IL2 resulting in CD8⁺ T cell expansion and function ⁶⁸. The

importance of CD4⁺ T cells is well illustrated by studies in chronic LCMV infection, in which CD4⁺ T cells have been depleted. This depletion results in a more severe CD8⁺ T cell exhaustion ¹⁰⁷.

During chronic LCMV infection, CD4⁺ T cells can also become exhausted and lose effector functions such as IL2 secretion ^{108,109}. IL2-induced signals are important for CD8⁺ T cell survival in chronic viral infection. Indeed CD8⁺ T cells lacking the expression of the high affinity IL2 receptor are depleted ¹¹⁰.

CD4⁺ regulatory T cells (Treg) are characterized in mice by the expression of the transcription factor forkhead box P3 (FoxP3). This subpopulation plays a crucial role in autoimmune responses ¹¹¹. Depletion of Tregs in chronic LCMV model results in increased effector functions of exhausted viral-specific CD8⁺ T cells ¹¹².

Immunosuppressive cytokines

IL-10 is a potent anti-inflammatory cytokine that plays a central role in limiting T cell proliferation, APCs functions, as well as cytokines and chemokines synthesis ¹¹³. In the early stages of acute and chronic LCMV infection, the levels of IL-10 are similar but remain high only in chronic infection ¹¹⁴. The predominant role of IL-10 in the chronic LCMV model is highlighted by the effect of IL-10 blockade, which promotes T cell function and improves viral control ^{114,115}. During LCMV infection, numerous cell types have been shown to produce IL-10, including CD4⁺ and CD8⁺ T cells, macrophages and dendritic cells. The main source of IL-10 remains a matter of debate ¹¹⁵⁻¹¹⁷. A recent report suggests that CD8 α ⁻ dendritic cells directly infected with C13 produce high amounts of IL-10 ¹¹⁸. In addition, infected dendritic cells are able to induce IL-10 expression by LCMV-specific CD4⁺ T cells ¹¹⁸. This study point out that infected CD8 α ⁻ dendritic cells could be key players of the IL-10 induction cascade.

The transforming growth factor- β (TGF- β) is an immunosuppressive cytokine that plays a crucial role in T cell development, homeostasis, tolerance, and differentiation ¹¹⁹. TGF- β signaling is primarily mediated through the Smad family of transcription factors ¹¹⁹. During chronic LCMV infection, CD8⁺ T cells show increased phosphorylation of Smad2 as

compared with T cells collected during acute infection, suggesting a role for TGF- β signaling^{120,121}. A selective inhibition of TGF- β signaling in CD8⁺ T cells through expression of a dominant-negative receptor improved the T cell response and is associated with viral clearance. However, systemic administration of TGF- β antagonists, inhibitors or blocking antibodies, increases T cell functions but has no significant impact on viral clearance¹²¹.

THE TRANSCRIPTIONAL PROGRAM OF EXHAUSTED CD8⁺ T CELLS

Genome-wide transcriptional analysis of exhausted T cells revealed a specific transcriptional program distinct from effector and memory T cells.

Inhibitory receptors

The identification of PD-1 in the LCMV model was a real breakthrough in the field of T cell exhaustion. Genome-wide analysis of CD8⁺ T cells specific for the GP276 LCMV-derived epitope obtained during chronic infection or acute infection (> 60 days) revealed that PD-1 is up-regulated in exhausted T cells¹²². Analysis of T cells specific for multiple epitopes confirms that PD-1 was up-regulated at the surface of all exhausted LCMV-specific T cells. Interestingly, blockade of the PD-1 pathway increased the CD8⁺ T cell response and enhanced viral clearance. The authors concluded that PD-1 is a marker of exhausted T cells¹²².

PD-1 expression is sustained by antigen persistence. Indeed, CD8⁺ T cells specific for the GP33-41 LCMV-derived peptide are PD-1^{low} and remain functional when an escape mutation within the GP33-41 epitope occurs in chronic LCMV infection¹⁰⁴.

Although PD-1 has been extensively studied in T cell exhaustion, its intracellular signaling pathway remains poorly understood. Information about how PD-1 inhibits T cell signaling come from *in vitro* studies of acutely activated T cells. During T cell activation, PD-1 is located in TCR microclusters and translocates to the cSMAC of the immunological synapse^{123,124}. The cytoplasmic tail of PD-1 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM)^{38,125}. Following interaction between PD-1 and its ligands B7-

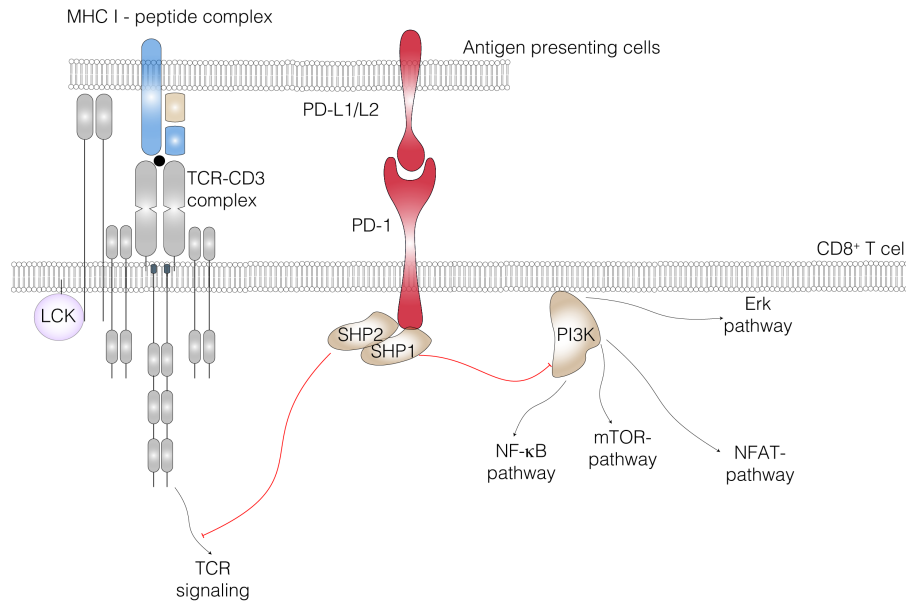


Figure 7: Inhibitory mechanisms of PD-1. Upon ligation to its ligands PD-L1/PD-L2, PD-1 recruits phosphatases SHP1 and SHP2. These mediate PD-1 inhibition.

H1 (PD-L1) or B7-DC (PD-L2), both motifs appear to be phosphorylated. However, only ITSM induces the recruitment of the tyrosine-protein phosphatases SHP1 (also known as PTPN6) and/or SHP2 (also known as PTPN11)^{38,126}. Subsequently, the phosphatases induce the dephosphorylation of CD3 ζ , ZAP70 and PKC θ . Other studies indicate that PD-1 can inhibit T cell functions through the PI3K, AKT and RAS pathways^{127,128}, and through cell cycle control (*Figure 7*)¹²⁹.

In vivo blockade of the PD-1/PD-L1 pathway with an anti-PD-L1 blocking antibody from day 23 to day 37 post-C13 infection enhances virus-specific CD8⁺ T cell responses both quantitatively and qualitatively¹²². Compared to untreated mice, treated mice have higher numbers of LCMV-specific CD8⁺ T cells, more virus-specific CD8⁺ T cells capable of producing both IFN- γ and TNF- α and have reduced virus levels in the serum, the spleen and the liver¹²². Surprisingly, PD-L1^{-/-} mice infected with LCMV C13 died due to immunopathologic damages¹²². Moreover, exhausted T cells treated *in vitro* for 18 h with an anti-PD-L1 blocking antibody remain poorly functional¹³⁰.

In the same genome-wide analysis that revealed the role of PD-1, a second inhibitory receptor was found to be up-regulated at the surface of exhausted T cells: cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)¹²². CTLA-4 is another member of the IgSF family. CTLA-4 binds to B7-1 (CD80) and B7-2 (CD86) like CD28³⁸. Unlike PD-1, the intracellular tail of CTLA-4 contains an YVKM motif¹³¹. Upon ligation, the YVKM motif associates with SHP2 and serine/threonine protein phosphatase 2A (PP2A). Like PD-1, phosphatase recruitment induces dephosphorylation of the CD3 ζ , LAT and ZAP70 resulting in inhibition of T cell functions^{38,132}. Additionally, CTLA-4 competition with CD28 for the binding to CD80/86 is sufficient to reduce T cell function¹³³. Surprisingly, treatment of chronically infected mice with anti-CTLA-4 blocking antibody had no effect on either virus-specific T-cell responses or viral control. Also, there were no synergistic effects of *in vivo* co-blockade with anti-PD-L1 and anti-CTLA-4¹²².

An additional transcriptional analysis revealed that PD-1 and CTLA-4 are not the only immunosuppressive molecule up-regulated in exhausted CD8⁺ T cells¹³⁴. The report of Wherry *et al.* mentioned 13 potential inhibitory receptors up-regulated in exhausted T cells. These inhibitory

receptors included 2B4, Ly49 family members (Klra7 also known as Ly49D-GE, Klra3 also known as Ly49c), GP49B, CD160, Ptger4, Ptger2, PirB, LAG-3, KLRG1 and BTLA. Exhausted CD8⁺ T cells co-express several of these inhibitory receptors. Their numbers and expression intensity correlate with the severity of chronic infection ¹³⁰.

Lymphocyte activating gene-3 (LAG-3) is a MHC II ligand, structurally homologous to CD4 ¹³⁵. LAG-3 binds MHC II with a higher affinity than CD4 ¹³⁶. The intracellular tail of LAG-3 has no ITIM or ITSM motif. It contains a KIEELE motif, which is unique among all known inhibitory receptors and essential for its inhibitory function ^{137,138}. LAG-3 negatively regulates T cell functions through the inhibition of the calcium flux that is induced by TCR signaling ^{138,139}. Despite the up-regulation of LAG-3 on exhausted CD8⁺ T cells, T cell functions are similar in mice treated with blocking anti-LAG-3 antibody compared to untreated mice ¹³⁰. Consistently, T cell exhaustion is observed in LAG-3 deficient mice during chronic LCMV infection ¹⁴⁰. However, *in vivo* blockade of LAG-3 in combination with PD-1 blockade led to substantially greater reversal of T cell exhaustion and viral control compared to either blockade alone ¹³⁰.

T cell immunoglobulin and mucin domain containing molecule-3 (TIM3 also known as HAVCR2) is a cell surface molecule that binds to galectin-9, a soluble lectin ^{138,141}. The intracellular tail of TIM3 does not have ITIM or ITSM motifs. It contains five tyrosine residues that can be phosphorylated by Src kinases or ITK ^{138,142,143}. In the absence of galectin-9, HLA-B associated transcript 3 (Bat3) is associated to the cytoplasmic tail of TIM3 and represses TIM3 activity. Ligation of galectin-9 to TIM3 leads to the phosphorylation of the tyrosines in the intracellular tail of TIM3 and the release of Bat3, leading to T cell function inhibition ¹⁴³. Under some conditions, TIM3 expression has also been shown to enhance proximal T cell signaling ^{142,144}. A recent report solved these contradictory results showing that TIM3 has a suppressive activity when associated to carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), another well-known molecule involved in T-cell inhibition ¹⁴⁵. During LCMV chronic infection, PD-1⁺TIM3⁺ T cells are more exhausted than PD-1⁺TIM3⁻ in terms of proliferation and secretion of effector cytokines, such as IFN- γ , TNF- α , and IL2 ¹⁴⁶. PD-1⁺TIM3⁺ CD8⁺ T cell subpopulation also produces IL-10. Combined blockade of TIM3 and PD-1 pathways *in vivo* synergistically

improved CD8⁺ T cell responses and viral control in chronically infected mice ¹⁴⁶.

CD160 is a member of the IgSF. CD160 has two ligands, MHC I and Herpes Virus Entry Mediator (HVEM) ¹⁴⁷. The intracellular domain of CD160 contains a tyrosine motif and is able to mediate signaling events through LCK and Erk1/2 ¹⁴⁸. CD160 can both promote and inhibit T cell functions ^{149,150}. These dual effects may be dependent on the ligands. *In vitro* blocking of CD160 enhances the cytotoxicity of exhausted LCMV-specific CD8⁺ T cells ¹³⁰.

2B4 (also known as CD244), which binds to CD48, is a member of the IgSF. The intracellular tail of 2B4 contains ITSM motifs, suggesting an inhibitory mechanism similar to PD-1 ¹⁵¹. However, 2B4 can also have activating effects on TCR signaling ¹⁵². These divergent outcomes are also observed in chronic LCMV infection. *In vitro* blockade of 2B4 with an anti-CD48 blocking antibody decreased production of IFN- γ by 2B4^{int} T cells but enhanced IFN- γ production by 2B4^{hi} T cells isolated from C13 infected mice ¹³⁰.

T cell immunoreceptor with Ig and ITIM domains (TIGIT) is a member of the IgSF. TIGIT binds to CD155 (also known as PVR) ^{153,154}. TIGIT shares the same ligand than the co-stimulatory molecule CD226 (also known as DNAM-1). Similarly to the CD28:CTLA-4 axis, TIGIT may inhibit T cell function by competing for ligand binding with CD226 ¹⁵⁵. TIGIT can also directly interact with CD226 and this interaction impaired CD226 homodimerization and function. The intracellular tail of TIGIT contains an ITIM motif, suggesting an inhibitory mechanism similar to PD-1 ¹⁵⁴. However, there is no experimental evidence of T cell signaling inhibition by TIGIT. In transcriptomic analysis of exhausted T cells, TIGIT was not differentially expressed between exhausted, effector and memory T cells ¹³⁴. However, a recent study showed that TIGIT is highly expressed by PD-1^{high} exhausted T cells. Blocking of TIGIT alone does not significantly affect T cell functions and viral titers. But anti-TIGIT antibody acts synergistically with anti-PD-L1 antibody to enhance effector functions of CD8⁺ T cell and viral clearance ¹⁵³.

The previous paragraphs highlight the important role of inhibitory receptors on T cell functions in chronic viral infection. Inhibitory receptors cooperate to modulate T cell functions. To my knowledge, no publication

reported an effect of Klra7, Klra3, GP49B, Ptger4, Ptger2, PirB, LAG-3, KLRG1 and BTLA blockade in the LCMV C13 infection.

Transcription factors

The role of Blimp-1 (*Prdm1* gene) in CD8⁺ T cell exhaustion is highlighted by experiments with Blimp-1 deficient T cells in chronic LCMV infection. Complete deletion (*Prdm1^{fllox/fllox} X Gzmb-Cre*) of Blimp-1 in CD8⁺ T cells reduces expression of inhibitory receptors and enhances expression of both CD127 and CD62L, two molecules associated with memory CD8⁺ T cell differentiation ¹⁵⁶. Despite the lower expression of inhibitory receptors, CD8⁺ T cells completely deficient for Blimp-1 remain poorly functional and failed to control viremia. Surprisingly, partial deletion (*Prdm1^{fllox/wt} X Gzmb-Cre*) of Blimp-1 in CD8⁺ T cells reduces the expression of inhibitory receptors, similarly to the fully deficient CD8⁺ T cells. These heterozygous T cells retain residual cytotoxic activity ¹⁵⁶. These results suggest that Blimp-1 may act as a rheostat, balancing exhaustion and effector function.

The transcription factor T-bet is down-regulated in exhausted CD8⁺ T cells during chronic LCMV infection. T-bet expression is inversely correlated to the expression of a subset of inhibitory receptors, including PD-1, LAG-3, CD160, 2B4, TIM3 and BTLA. T-bet regulates especially PD-1 by directly binding to a regulatory region upstream of the transcriptional start site of its encoding gene ¹⁵⁷. Deletion of T-bet in CD8⁺ T cells during chronic LCMV infection results in higher expression of PD-1 and other inhibitory receptors, and higher viral load. Conversely, overexpression of T-bet improves T cell functions and results in lower expression of PD-1 and other inhibitory receptors ¹⁵⁷.

T-bet expression can be also used to delineate different subsets of exhausted T cells when combined with Eomes and PD-1 expression. The first subset is T-bet^{hi}PD-1^{int} with residual proliferative potential ¹⁵⁸. The second is Eomes^{hi}PD-1^{hi} with higher co-expression of inhibitory receptors and limited proliferative capacity. Antigen persistence causes T-bet^{hi}PD-1^{int} to convert to Eomes^{hi}PD-1^{hi} cells. This suggests that there is a hierarchy in the subpopulations with the T-bet^{hi} subset being the progenitor ¹⁵⁸. Interestingly, blocking the PD-1 pathway seems to only re-invigorate the T-bet^{hi}PD-1^{int} subset ¹⁵⁹.

Transcriptional profiling revealed that the mRNA encoding NFAT2 is

more highly expressed in exhausted CD8⁺ T cells compared to naïve, effector and memory CD8⁺ T cells. Experiments with cell lines have shown that NFAT1 can induce PD-1 expression by binding directly to a regulatory element of the PD-1-encoding gene ¹⁶⁰. A recent study used an engineered version of NFAT that is incapable to cooperate with AP-1 to show that CD8⁺ T cells expressing this engineered NFAT have impaired effector functions ¹⁶¹. Transcriptome analysis of these T cells shows a similar transcriptome profile than exhausted T cells, including up-regulation of inhibitory receptors. NFAT1- and NFAT2-deficient mice infected with LCMV C13 showed a decrease in the frequency of cells co-expressing PD-1 and TIM3 or LAG-3 ¹⁶¹.

Another transcription factor that plays a role in T cell exhaustion is Foxo1 ¹⁶². Foxo1 is a positive regulator of PD-1 through direct binding to the promotor of the PD-1-encoding gene. But it is also required for Bcl2 expression, which is crucial for the maintenance of CD8⁺ T cells during chronic LCMV infection. Thus, exhausted CD8⁺ T cells lacking Foxo1 fail to persist and control chronic viral infection ¹⁶².

Studies failed to identify a unique transcription factor or set of factors that drive CD8⁺ T cells to exhaustion. All previously described transcription factors play also critical roles in T cell differentiation. While additional factors (e.g. Pbx3, BATF, Klf4, Foxo3) could also contribute to T cell exhaustion, I am unaware of any studies confirming such a link in the persistent LCMV C13 model.

Epigenetic regulation of exhausted CD8⁺ T cells

The epigenetic landscape of a cell greatly influences on how and where transcription factors can bind. Accessibility of transcription factors to gene regulatory elements is controlled by covalent modifications to histones and DNA that affect chromatin structure, resulting in an “epigenetic signature” in cell type. Epigenetic changes are also informative in exhausted T cells. For example, a study showed decreased levels of histone 3 acetylated at lysines 9 and 14, in exhausted CD8⁺ T cells compared with Ag-specific CD8⁺ T cells from mice infected acutely with the LCMV Armstrong strain ¹⁶³. Consistent with this idea, treatment with histone deacetylase inhibitors resulted in increased cytokine production *in vitro* by the exhausted T cells. Adoptive transfer of these cells into mice infected with LCMV C13 led to the

generation of memory responses and increased cytokine production with reduced viral burden in spleen and liver¹⁶³. Recent publications used assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq approach) to compare the chromatin accessibility in exhausted CD8⁺ T cells to effector or memory CD8⁺ T cells. Notably, the most accessible chromatin regions present in CD8⁺ T exhausted cells were shared with effector CD8⁺ T cells responding to acute LCMV infection or with memory CD8⁺ T cells. For instance, chromatin accessibility near key effector-related genes including, *Ifng*, *Gzma*, *Gzmk*, *Fasl*, and *Prf1*, was similar between effector and exhausted cells. The accessibility was also similar for some genes coding for inhibitory receptors including *Havcr2*, *Lag3*, and *Ctla4*. However, a novel enhancer, located 22 kb 59 of the transcriptional start site of the *Pdcd1* gene (coding for PD-1), is only accessible in exhausted cells¹⁶⁴⁻¹⁶⁶.

Evidence supports the notion that DNA methylation is also a critical epigenetic mechanism for establishing exhausted CD8⁺ T cells. During chronic LCMV infection, the PD-1 gene locus remains unmethylated leading to sustain PD-1 expression¹⁶⁷. A recent report showed that it is critical to acquire *de novo* DNA methylation during and after the peak of an effector CD8⁺ T cell response to chronic LCMV infection to establish T cell exhaustion¹⁶⁸. Indeed, inhibition of Dnmt3a-mediated *de novo* DNA methylation in chronic LCMV infection protects CD8⁺ T cell from exhaustion¹⁶⁸. However, blocking the PD-1/PD-L1 pathway fails to reprogram the epigenetic landscape of exhausted CD8⁺ T cells. This limits the effect of the treatment^{168,169}.

Does CD8⁺ T cell exhaustion occur in humans?

Soon after the studies in the LCMV mouse model, exhaustion was also observed in humans. In the following chapters, I will use the term “exhaustion” only for dysfunctional T cell in the LCMV C13 mouse model. When referring to hypo-responsive human T cells, I will use the term “dysfunction”.

CD8⁺ T CELL DYSFUNCTION IN HUMAN CHRONIC VIRAL INFECTION

Several studies have identified CD8⁺ T cells partially or completely dysfunctional in patients with human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) ¹⁷⁰⁻¹⁷². Similarly to exhaustion, multiple parameters could influence T cell dysfunction in human chronic viral infections. Antigen persistence seems to be one key factor. In HIV and HCV infection, escape mutations are associated with higher effector functions in CD8⁺ T cells directed against epitopes that mutate ^{173,174}. In HIV, the severity of T cell dysfunction is correlated with high viral loads ^{175,176}. In HCV infection, anti-viral CD8⁺ T cells regain effector functions during spontaneous resolution of the infection and viral drop ¹⁷⁷.

IL-10 could influence CD8⁺ T cell dysfunction occurring in human chronic viral infections. Elevated levels of IL-10 have been reported in HBV, HCV and HIV infection ¹⁷⁸⁻¹⁸⁰. In HIV, IL-10 levels increase with disease progression ¹⁸¹. *In vitro* blockade of IL-10 results in increased effector function of HIV-specific CD8⁺ T cells ¹⁷⁸.

Transcriptomic analysis revealed that the signature of HIV-specific CD8⁺ T cells isolated from progressing patients was significantly correlated with the profiles of exhausted LCMV-specific CD8⁺ T cells from C13 chronic infection in mice ¹⁸². Virus-specific CD8⁺ T cells express several inhibitory receptors including PD-1, TIM3, CD160, and 2B4 ¹⁸³⁻¹⁸⁸. Interestingly *in vitro* blockade of the PD-1/PD-L1 pathway results in increased effector functions of the virus-specific CD8⁺ T cells ^{185,186,188,189}. A combination of blocking PD-1 and TIM3 enhances T cell function better than blocking either receptor on their own. This suggests a cooperation between inhibitory receptors in human chronic viral infection ¹⁸⁸. Similarly to exhausted T cells, expression of inhibitory receptors is inversely correlated to T-bet expression, but correlates with Eomes. Indeed, HIV-specific dysfunctional CD8⁺ T cells are T-bet^{int}/Eomes^{hi} ¹⁸³.

A protein that seems to play a major role in HIV-specific CD8⁺ T cells is the transcription factor Basic leucine zipper transcription factor, ATF-like (BATF). BATF belongs to a family of AP-1 transcription factors. By forming dimers with c-Jun, it negatively regulates AP-1 (c-Jun/c-Fos)-

induced genes ¹⁹⁰. In an elegant study with HIV-specific CD8⁺ T cells from progressive donors, BATF was identified as a transcription factor induced in T cells upon engagement of the PD-1/PD-L1 pathway ¹⁸². BATF was overexpressed in HIV-specific CD8⁺ T from progressive patients. Inhibition of BATF restored effector functions in these T cells. BATF expression is now considered as a marker of T cell dysfunction in human and in murine exhausted T cell while no study has assessed its role in a persisting LCMV C13 infection.

CD8⁺ T CELL DYSFUNCTION IN HUMAN TUMORS

Melanoma cells express specific antigens that can be recognized by CD8⁺ T lymphocytes ^{9,191}. Several reports including ours indicate that freshly isolated human tumor infiltrating lymphocytes (TIL) secrete low levels of cytokines and are not able to lyse target cells ¹⁹²⁻¹⁹⁵. It is nevertheless difficult to determine the driving mechanism of T cell dysfunction in cancer. The tumor microenvironment is immunosuppressive (reviewed in Wiëers *et al.* ¹⁰). Several mechanisms present at the tumor site have been reported to inhibit T cell function including, immunosuppressive cells such as Treg or MDSCs, inhibitory enzymes such as IDO or arginase, and soluble factors such as galectins, or TGF-β.

The transcriptome of cancer-specific CD8⁺ T cells isolated from blood was compared to the transcriptome of cancer-specific CD8⁺ T cells from lymph node metastases (TILN) of melanoma patients. This analysis showed that the gene set described for exhausted murine T cells was significantly enriched in cancer-specific human CD8⁺ T cells from metastases ¹⁹⁶. This set of genes includes inhibitory receptors such as, CTLA-4, LAG-3, CD160 and 2B4. Surprisingly, no transcription factors implicated in T cell exhaustion was differentially expressed between functional cancer-specific CD8⁺ T cells from blood versus dysfunctional cancer-specific CD8⁺ T cells from TILN. It was unexpected to not observe a differential expression of Blimp-1 and BATF because they are associated to the expression of inhibitory receptors. Although PD-1 was not differentially expressed in this transcriptome analysis, several reports have observed its expression at the surface of TIL isolated from different cancer including melanoma, breast, prostate, ovarian, RCC, hepatocellular

carcinoma, CLL, and NSCLC¹⁹⁷⁻²⁰⁰. The expression of PD-1 is often associated with poor effector functions, and *in vitro* blocking of the PD-1/PD-L1 pathway seems to increase T cell proliferation²⁰⁰. Similarly to murine exhausted T cells, overexpression of multiple inhibitory receptors is associated with a more severe dysfunctional stage of human tumor-specific CD8⁺ T cells. PD-1⁺TIM3⁺, PD-1⁺TIM3⁺BTLA⁺ and PD-1⁺LAG-3⁺ tumor-specific CD8⁺ T cells from melanoma and ovarian cancer patients produce fewer cytokines intracellularly than cells expressing only PD-1 or none of these receptors^{199,201,202}.

Inhibitory receptors as targets for cancer immunotherapy

MODELS IN MICE

Proof of efficacy of a therapeutical approach in tumor-bearing animals is a mandatory step before clinical trial. Early studies in mice have shown that P815 tumor mastocytoma overexpressing PD-L1 can suppress CD8⁺ T cell cytolytic activity. Blocking the PD-1/PD-L1 pathway in this tumor model reduces tumor growth²⁰³. Furthermore, other studies have highlighted the efficacy of a PD-1/PD-L1 blockade in mice models, either in syngeneic models, such as B16 melanoma and CT26 colon carcinoma, or in xenograft tumor models^{204,205}.

In contrast to the LCMV C13 infection where it has no effect, anti-CTLA-4 blocking antibodies enhance the rejection of transplanted colon carcinoma, fibrosarcoma prostate carcinoma, renal cell carcinoma, and lymphoma tumors in mice²⁰⁶. CTLA-4 blocking in mouse models does not result in widespread systemic autoimmunity. Combination of PD-1 and CTLA-4 blocking has demonstrated synergic effect²⁰⁶. Interestingly, blockade of inhibitory receptors seems to induce a durable adaptive anti-tumor response. Indeed, some mice models showed that treatment against inhibitory receptors could lead to the complete rejection of tumors and subsequent protection from developing new disease during re-challenges with homologous tumor cells^{204,206}.

However, vaccination strategies using irradiated tumor cells combined with CTLA-4 blocking led to immune-related adverse events (AEs). In the B16 melanoma model, mice from the experimental group developed vitiligo²⁰⁷. In a transgenic prostate adenocarcinoma mouse model, mice developed prostatitis²⁰⁸. Using a combined CTLA-4 and PD-1 blockade, no supplemental toxicity was reported²⁰⁶.

TRIALS IN PATIENTS

Blocking inhibitory receptors in human cancer patients has shown impressive clinical results. The first antibody targeting an inhibitory receptor that showed clinical efficacy in human patients was the anti-CTLA-4 antibody, ipilimumab. Pooling long-term survival data across several phase II and phase III trials showed three years survival rates of 22% in all patients, 26% in treatment-naïve patients, and 20% in previously treated patients²⁰⁹. Consistent with the preclinical models, durable responses, of more than 3 years, were observed in some patients treated with anti-CTLA-4 antibody²¹⁰.

Blocking the PD-1/PD-L1 pathway has shown impressive results in advanced melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC) and metastatic bladder cancer. In a phase III head-to-head trial comparing anti-CTLA-4 (ipilimumab) and anti-PD-1 (pembrolizumab), response rates with the PD-1 blocking antibody were higher: ~30% with pembrolizumab versus ~10% of patients with ipilimumab. The survival rates at 1-year was also higher with pembrolizumab versus ipilimumab: ~70% versus ~60%²¹⁰.

Combinations of anti-CTLA-4 and anti-PD-1 blocking antibodies are currently investigated in human cancer patients. In metastatic melanoma, the combination of ipilimumab and the anti-PD-1 nivolumab results in increased response rates (58% versus 19%) and improved progression-free survival (58% versus 19%; 11.5 versus 2.9 months)²¹¹.

In cancer patients, blocking inhibitory receptors is associated with AEs including rash or pruritus, gastrointestinal disorders, and endocrinopathies²¹². The severity of AEs is higher with anti-CTLA-4 ipilimumab (20%) than pembrolizumab (~10%)²¹⁰. Combining anti-CTLA-4 and anti-PD-1 in patients with previously untreated melanoma or recurrent

small cell lung cancer (SCLC), results in higher incidence of AEs: ~50% with the combination, ~25% with ipilimumab alone and ~15% with nivolumab alone²¹².

Concluding remarks and aim of the study

T cell exhaustion has been intensively studied in chronic LCMV infection. This mouse model is the source of major discoveries about T cell exhaustion. Nevertheless the main cause of T cell exhaustion remains unknown. A number of suppressive mechanisms are able to drive T cell exhaustion in chronic LCMV infection. Many reports suggest that antigen persistence is the main driver. However, it is difficult to establish if T cell exhaustion is the cause of the high antigen levels or whether antigen persistence is the cause of T cell exhaustion.

Some indications support the notion that exhausted T cells in LCMV chronic infection are not fully dysfunctional. Indeed, exhausted PD-1⁺ T cells showed robust proliferation upon transfer to naïve hosts which are subsequently exposed to acute LCMV infection ²¹³. The re-expanded T cells controlled the viral infection despite their exhausted phenotype.

Human T cells isolated from chronic viral infection or tumors are often dysfunctional upon *ex vivo* re-activation and they frequently express inhibitory receptors. This explains why the term “exhaustion” was extended to humans. However, the experiments performed *ex vivo* with human T cells provide a momentary snapshot of dysfunction. They do not provide the formal causal link between T cell dysfunction and the expression of inhibitory receptors. It is also impossible to distinguish whether human T cell dysfunction results from antigen persistence or from the tumor microenvironment, which includes hypoxia, regulatory cells and suppressive molecules. Gene signatures of tumor-specific human CD8⁺ T cells from melanoma metastasis showed some similarities with mouse exhausted program, as well as with human anergic and tolerant CD8⁺ T cells ²¹⁴.

Nevertheless, blocking inhibitory receptors has been used in clinical trials with impressive results. However, some patients failed to respond or relapsed. Other mechanisms could exist that suppress T cell function. These mechanisms could represent the next generation of cancer immunotherapy.

Studying how the hierarchy of dysfunction evolves in human tumor-specific TIL is challenging, as comparisons overtime for human patients are impossible. We reasoned that an experimental approach by which to address the link between antigen persistence and human T cell dysfunction would be to perform a longitudinal analysis overtime of effector functions and surface expression of inhibitory receptors on T cells submitted to frequent and repeated antigen stimulations.

First, we isolated naïve antigen-specific human CD8⁺ T cells to avoid including other T cells more prone to exhaustion. Second, the few isolated T cells were primed and expanded with mature dendritic cells to avoid anergy and to obtain *bona fide* effector cells. Third, we submitted the expanded effector T cells to repeated bi-weekly stimulations with tumor cells presenting the antigen to avoid non-specific *stimuli* such as anti-CD3 antibodies or superantigens. We monitored T cell proliferation, effector functions and inhibitory receptor expression during 10-13 cycles of these stimulations.

RESULTS

RESULTS

Isolation, priming and amplification of human antigen-specific CD8⁺ T cells

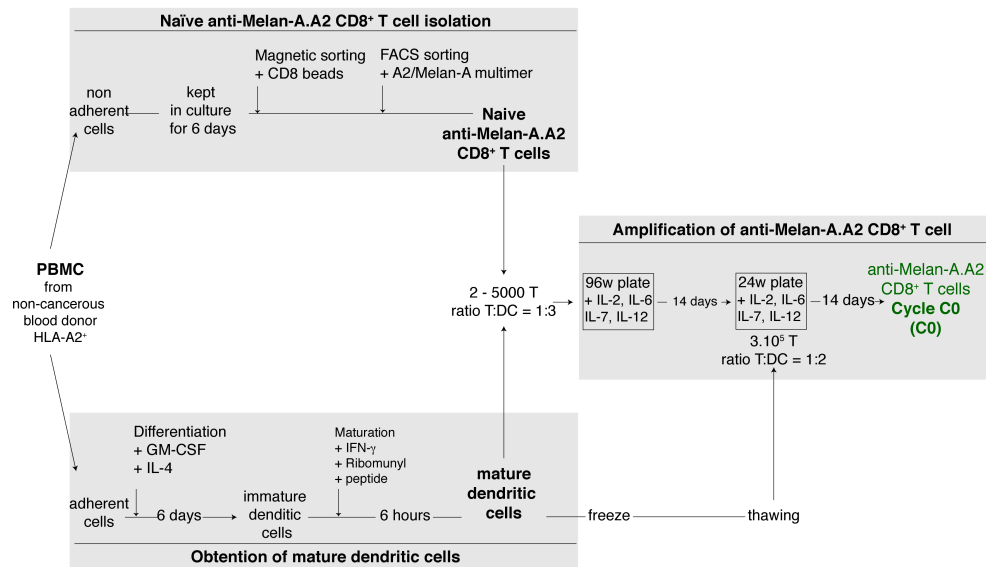
High frequencies of naive Melan-A-specific CD8⁺ T cells can be found in a large proportion of HLA-A2⁺ healthy individuals, about 1/10,000 of CD8⁺ T cells ²¹⁵. We used A2/Melan-A fluorescent tetramers to isolate anti-Melan-A.A2 CD8⁺ T cells by flow cytometry. Starting from 30 million CD8⁺ T cells for donor LB1801, we obtained 5,000 anti-Melan-A.A2 CD8⁺ T cells, which were primed for amplification with Melan-A peptide-pulsed mature autologous dendritic cells in the presence of IL2, IL-6, IL-7, and IL-12 (*overview of the procedure in Figure 8A*). After two cycles of amplification, we obtained 200 million T cells, which were analyzed for specificity and function.

A great majority, 84%, of the CD8⁺ T cells were positive for the A2/Melan-A fluorescent multimer. One week and one further stimulation later, 99% of the cells were multimer-positive (*Figure 9A*). These T cells secreted IFN- γ specifically when cultured in the presence of Melan-A peptide-loaded T2 cells, which are an HLA-A2⁺ antigen-presenting cell line derived from B cell lymphoblasts (*Figure 9B*).

We tested furthermore specific re-activation of the T cells through HLA-A2⁺Melan-A⁺ CP50-MEL.1 melanoma cells and non-specific *stimuli* including beads coated with anti-CD3/-CD28 antibodies and a mix of phorbol myristate acetate (PMA) and ionomycin. The T cells secreted IFN- γ in response to all specific and non-specific *stimuli* tested (*Figure 9B*). We obtained similar results with cells isolated from 7 different donors (*Figure 9C*).

We successfully primed and amplified naïve T cells and obtained sufficient functional effector cells for antigen chronic stimulation.

A. Isolation, priming and differentiation of human naïve anti-Melan-A.A2 CD8⁺ T cells into effector cells



B. Frequent and repeated antigen stimulations

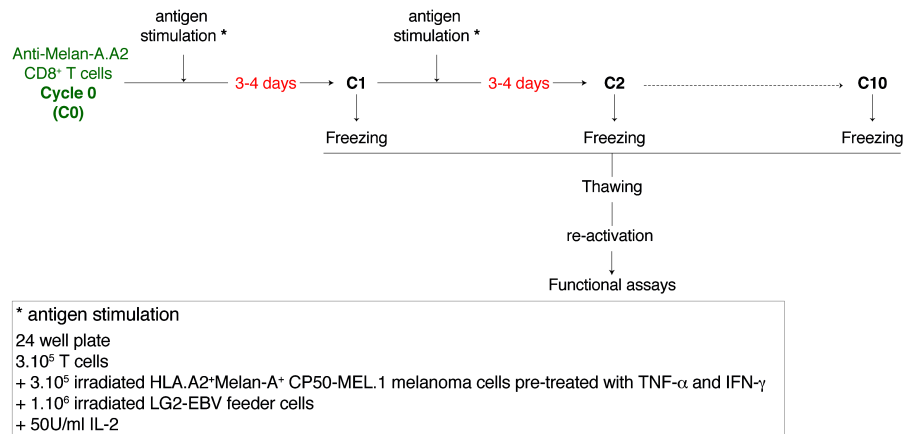


Figure 8: Overview of the experimental approach. (A) Procedure used to isolate and amplify specific human anti-Melan-A.A2 CD8⁺ T cells. **(B)** Procedure used for the stimulation cycles resulting in human CD8⁺ T cell dysfunction.

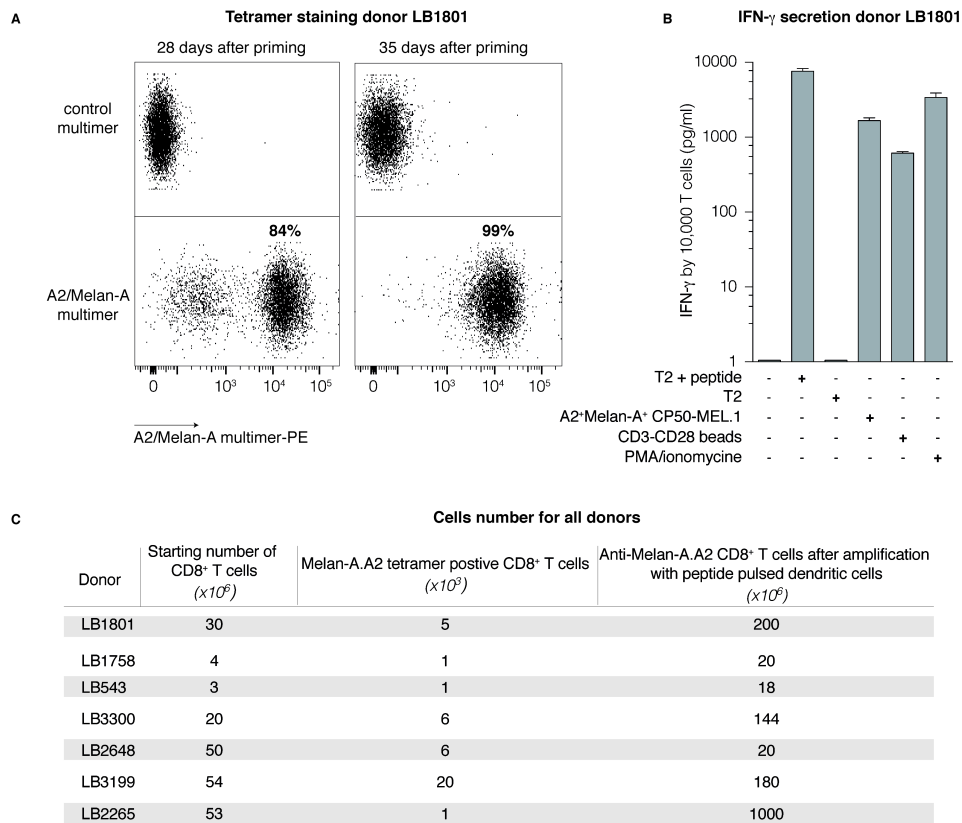


Figure 9: Specificity and functional phenotype of human anti-Melan-A2 CD8⁺ T cells. (A) T cells from donor LB1801 were stained with an A2/Melan-A multimer coupled to PE and analyzed by flow cytometry. (B) IFN- γ secretion was estimated by ELISA in the supernatant of 20 h co-culture of 10,000 T cells re-activated with different stimuli; means $\pm$ s.d. of triplicates. (C) Data obtained with other donors.

RESULTS

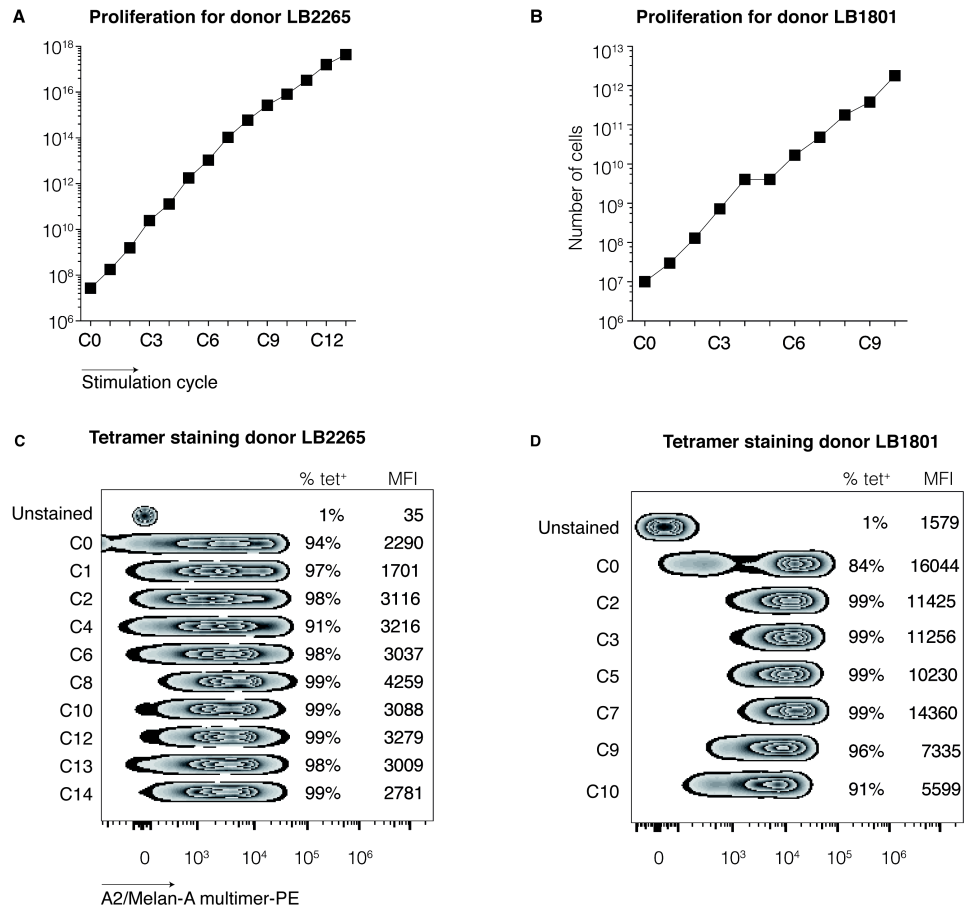


Figure 10: Human anti-Melan-A.A2 CD8⁺ T cells retain the ability to proliferate and to be stained with A2/Melan-A multimers. *T cells were stimulated every 3-4 days with irradiated A2⁺Melan-A⁺ CP50-MEL.1, feeder cells and IL2. C0-C10 indicate the stimulation cycles. (A, B) Cells were counted before each stimulation cycle. The Y axis represents the theoretical number of cells obtained if all the T cells were kept in culture. Monoplicate. (C, D) T cells were incubated with A2/Melan-A multimers coupled to PE and anti-CD8 antibody. Data represents results for CD8⁺ T cells.*

What is the effect of repetitive antigen stimulations on CD8⁺ T cell functions?

The expanded T cells were subjected to stimulation every 3-4 days with irradiated HLA-A2⁺Melan-A⁺ melanoma cells, which were previously treated with IFN- γ and TNF- α to up-regulate PD-L1, a ligand of inhibitory receptor PD-1 (*overview of the procedure in Figure 8B*). The T cells underwent several cycles of stimulation, and T cell samples were frozen at each cycle 3 days after the last stimulation. The functional phenotype of the different T cell samples was compared using T cells that were thawed and re-activated. In the course of this report, we will use stimulation to refer to antigen stimulation cycles, and re-activation to refer to co-culture for functional assays. C refers to the number of stimulation cycle. Thus C1 = T cells that underwent through 1 stimulation cycle.

REPETITIVE ANTIGEN STIMULATIONS DO NOT AFFECT T CELL PROLIFERATION AND THEIR CAPACITY TO RECOGNIZE MHC/PEPTIDES COMPLEXES

Anti-Melan-A.A2 CD8⁺ T cells were counted after each stimulation cycle and we established a proliferation curve. The cells retained the ability to proliferate during the stimulation cycles. *Figure 10A&B* shows data for 2 donors but similar data were obtained for 7 donors. We conclude that repetitive antigen stimulations alone do not affect T cell proliferation.

Anti-Melan-A.A2 CD8⁺ T cells submitted to stimulation cycles were stained with A2/Melan-A fluorescent multimers. Examples of data obtained from 2 donors are shown in *Figure 10C&D*. For the T cells of donors LB2265 (*Figure 10C*) and for 4 other donors (*data not shown*), we observed a steady tetramer staining. The T cells of donors LB1801 (*Figure 10D*) and LB1758 (*data not shown*) showed also a steady expression of the tetramer staining through stimulation cycles C2-C7. However, we observed a decrease in fluorescence of tetramer-stained cells of cycles 9-10 (*Figure 10D*). This suggests these cells express the

RESULTS

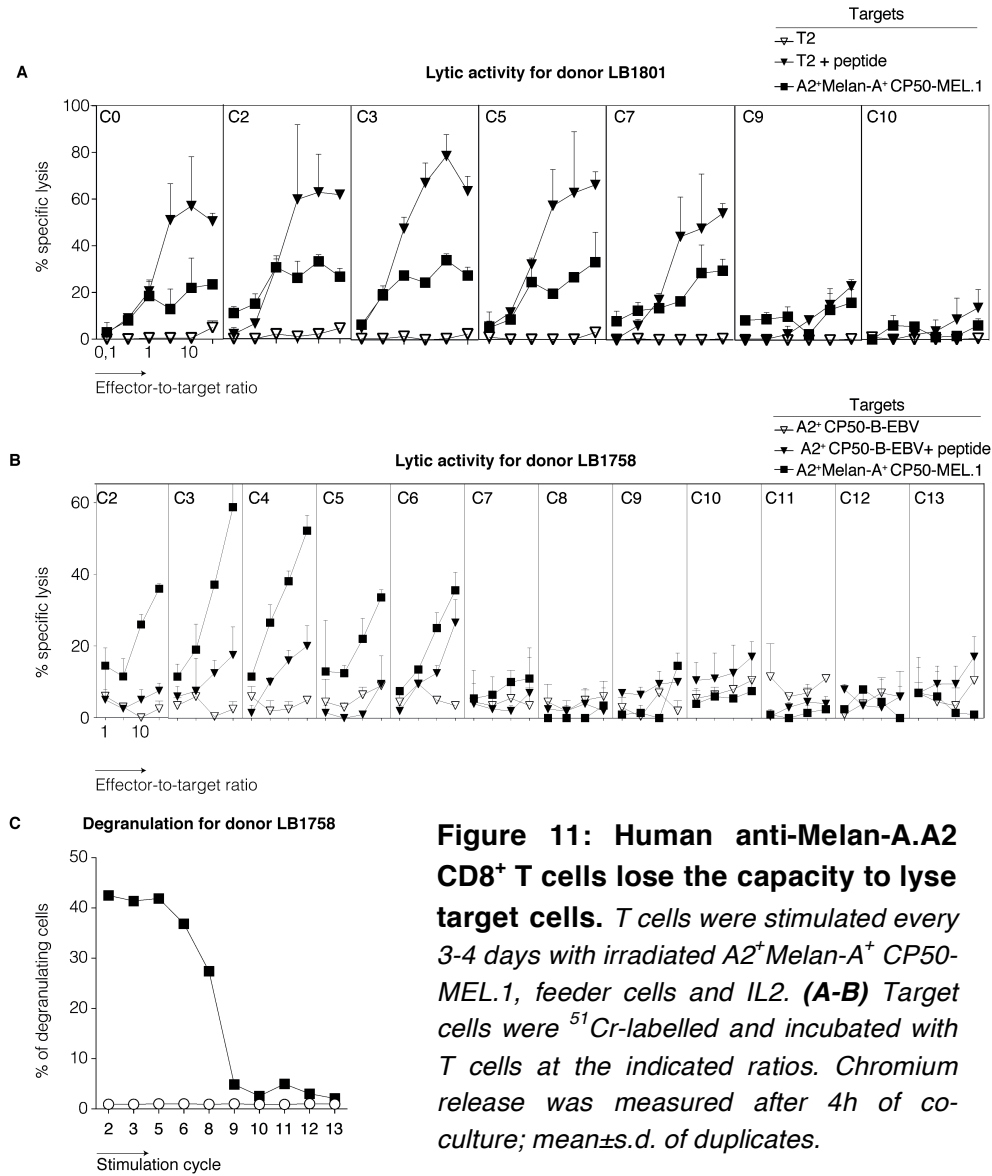


Figure 11: Human anti-Melan-A.A2 CD8⁺ T cells lose the capacity to lyse target cells. *T cells were stimulated every 3-4 days with irradiated A2⁺Melan-A⁺ CP50-MEL.1, feeder cells and IL2. (A-B) Target cells were ⁵¹Cr-labelled and incubated with T cells at the indicated ratios. Chromium release was measured after 4h of co-culture; mean±s.d. of duplicates.*

(C) *Cells were re-activated with A2⁺Melan-A⁺ CP50-MEL.1 melanoma cells previously treated with TNF- α and IFN- γ . Brefeldin-A and anti-CD107a and anti-CD107b antibodies were added simultaneously to the stimulators. After 5 h of stimulation, cells were labeled for CD8 β . Data represent the percentage of CD8⁺ T cells with detectable surface CD107.*

Melan-A-specific TCR at lower levels during later stimulation cycles or a clonal selection of T cell bearing a TCR with lower affinity for Melan-A peptide.

REPETITIVE ANTIGEN STIMULATIONS LEAD TO A LOSS OF LYTIC ACTIVITY

We used several targets to estimate the lytic activity of anti-Melan-A.A2 CD8⁺ T cells. Target cells were either HLA-A2⁺Melan-A⁺ melanoma cells or HLA-A2⁺ T2 cells or HLA-A2⁺ CP50-B-EBV cells pulsed with peptide. The targets were labeled with radioactive chromium. The T cells were co-cultured with the target cells for 4 hours. Lytic activity was determined by measuring radioactive chromium released from target cells into the supernatant. Cells collected after the first 2-5 cycles were able to lyse target cells presenting the Melan-A peptide (*Figure 11*). However, after 7 to 9 cycles of stimulation, the T cells lost the ability to lyse target cells. The particular time-point at which this occurred was donor-dependent (*Figure 11A&B*).

We also measured degranulation, a prerequisite for cytolysis (*Figure 11C*). The percentage of degranulating cells was estimated by the surface expression of CD107a and CD107b. These at rest reside in cytolytic granules and are mobilized to the cell surface by exocytosis upon activation. After re-activation by TNF- α and IFN- γ pre-treated HLA-A2⁺Melan-A⁺ CP50 melanoma cells, the percentage of CD107⁺ cells was high in cells submitted to 2-6 stimulation cycles. This percentage strongly decreased by cycle 9 (*Figure 11C*).

We concluded that frequent and repeated antigen stimulation leads to dysfunction of T cell cytotoxicity.

RESULTS

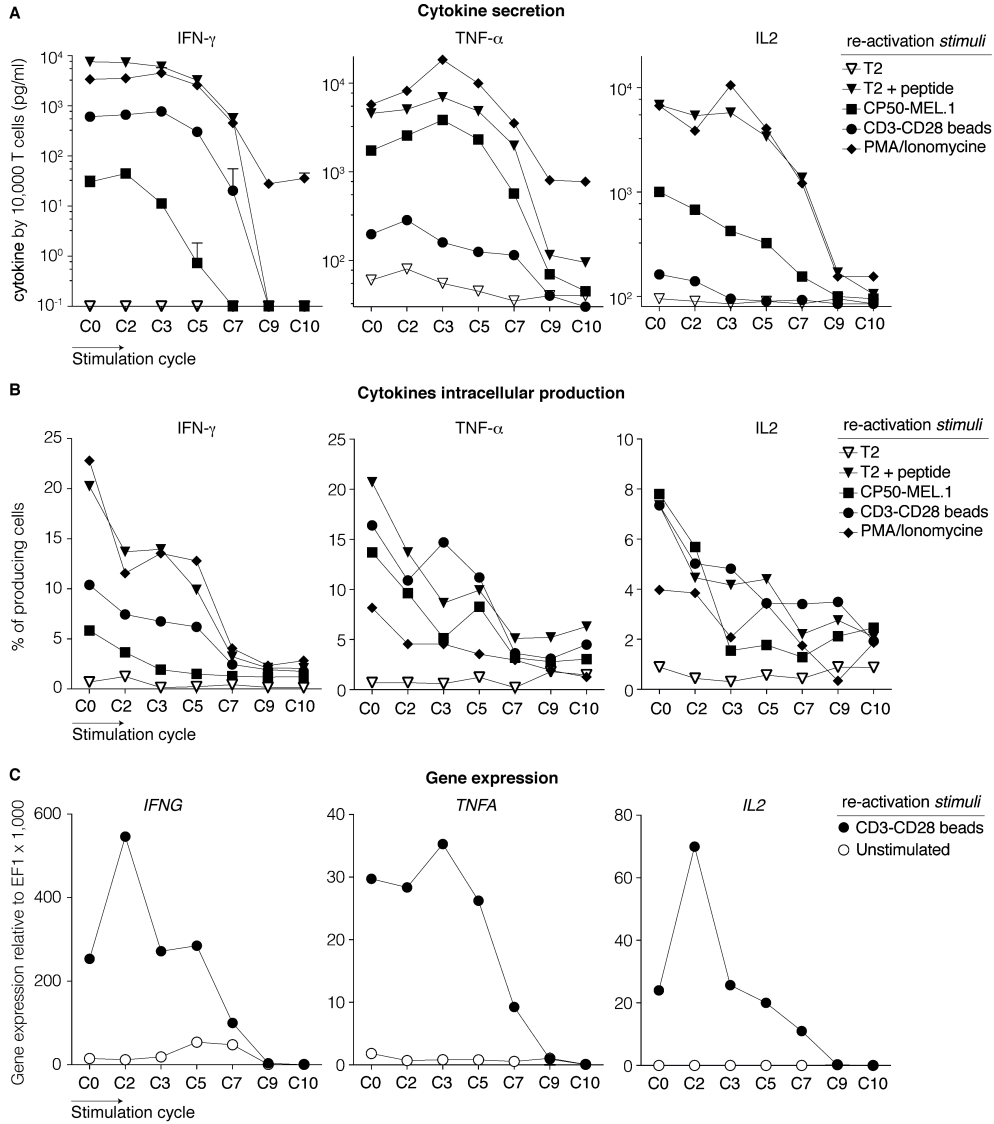


Figure 12: Human anti-Melan-A.A2 CD8⁺ T cells lose the ability to produce cytokines. *T* cells were stimulated every 3-4 days with irradiated A2⁺Melan-A⁺ CP50-MEL.1, feeder cells and IL2. **(A)** Data obtained with *T* cells isolated from donor LB1801. **(D-F)** Data obtained with *T* cells isolated from different donors. *T* cells were re-activated for 20 h **(A, B)** or 2 h **(C)** with different stimuli. **(A)** Cytokine secretion was measured by bioplex; means $\pm$ s.d. of triplicates. **(B)** Cytokine intracellular staining in the presence of brefeldin A. **(C)** Cytokine mRNA expression was measured by RT-qPCR.

REPETITIVE ANTIGEN STIMULATIONS LEAD TO A LOSS OF CYTOKINE SECRETION AND CYTOKINE PRODUCTION

To measure cytokine secretion, anti-Melan-A.A2 CD8⁺ T cells were re-activated with specific or non-specific *stimuli*. The specific *stimuli* consisted of HLA-A2⁺ T2 cells pulsed with the Melan-A peptide or HLA-A2⁺ CP50 melanoma cells pre-treated with TNF- α and IFN- γ . The following non-specific *stimuli* were used: beads covered with anti-CD3/-CD28 antibodies or a mix of the PKC (Protein kinase C) agonist PMA and the ionophore ionomycin. Cells from donor LB1801 collected after the first 3-4 cycles secreted high amounts of IFN- γ when re-activated with specific or non-specific *stimuli* (Figure 12A). IFN- γ secretion gradually decreased to zero by C9 for all *stimuli* except for PMA/ionomycin. In that case, the defect is only partial. This observation suggests that several molecular mechanisms are implicated in the T cell dysfunction: one mechanism upstream PKC and NFAT signaling and one mechanism downstream. Similar observations have been made for TNF- α and IL2 (Figure 12A).

We recently reported a type of T cell dysfunction that is characterized by impaired cytokine secretion but intact intracellular production of cytokines²¹⁶. We assessed whether the observed loss of cytokine secretion results from down-regulation of cytokine production. We monitored cytokine production by intracellular staining. Anti-Melan-A.A2 CD8⁺ T cells were re-activated with either specific *stimuli* or non-specific *stimuli* in the presence of Brefeldin A, an inhibitor of protein exocytosis. In agreement with the secretion data, we measured a decrease in intracellular production of IFN- γ , TNF- α and IL2 (Figure 12B).

To further determine the cause of the decreased production of cytokines, we also measured cytokine gene expression. T cells were re-activated with beads covered with anti-CD3/-CD28 antibodies. We used only this *stimulus* to avoid contamination of T cell RNA by RNA from melanoma cells or T2 cells. Cytokine gene expression was measured by RT-qPCR. We observed a decrease in the expression of cytokine-encoding genes (Figure 12C).

RESULTS

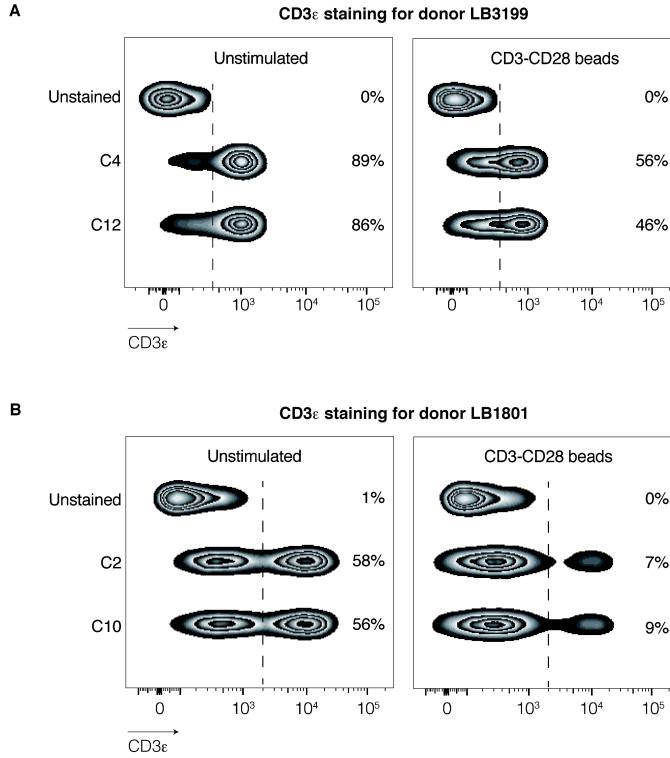


Figure 13: Dysfunctional human CD8⁺ T cells remain able to engage TCR receptor. *T cells obtained from multiple donors were stimulated every 3-4 days with irradiated A2⁺Melan-A⁺ CP50-MEL.1 melanoma cells, feeder cells and IL2. After 6h of re-activation with beads covered with anti-CD3/-CD28 antibodies, cells were stained with anti-CD3 ϵ and anti-CD8 β . Data represent the percentage of CD8⁺ T cells with detectable surface CD3 ϵ . (A) Data obtained with T cells isolated from donor LB3199. (B) Data obtained with T cells isolated from donor LB1801.*

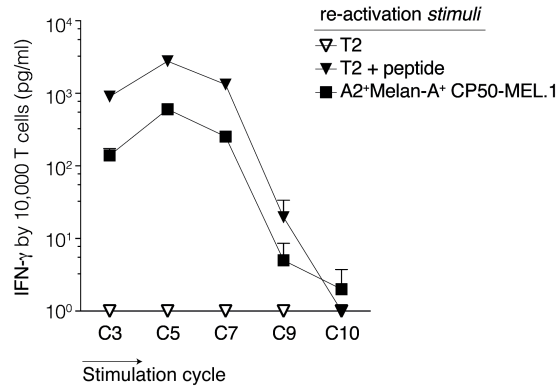
REPETITIVE ANTIGEN STIMULATIONS DO NOT AFFECT T CELL ACTIVATION

T cell function requires TCR signaling. We thus assessed whether the observed T cell dysfunction was due to a defect in the initiation of T cell signaling. To assess the capacity of the T cells to initiate TCR signaling, we measured the surface expression of CD3 ϵ by flow cytometry. We used functional and dysfunctional T cells from 2 donors. The functional T cells had undergone 2 to 4 cycles of stimulation, the dysfunctional cells 10 to 12 cycles. The expression of surface CD3 ϵ was measured before and after re-activation with beads covered with anti-CD3/-CD28 antibodies. Before re-activation, the functional C4 and dysfunctional C12 T cells of donor LB3199 expressed CD3 ϵ at similar levels at the cell surface (*Figure 13A*). Upon re-activation, both C4 and C12 cells down-regulated surface CD3 ϵ to a similar extent (*Figure 13A*).

For donor LB1801, only about 50% of T cells are CD3 ϵ ⁺ before re-activation. This is probably due to antigen maintenance as we renewed antigen-presenting cells every 3-4 days (*Figure 13B*). Nevertheless, after re-activation, we observed CD3 ϵ down-regulation at similar levels in functional C2 cells and dysfunctional C10 cells (*Figure 13B*). We conclude that dysfunctional cells are still able to initiate TCR signaling, suggesting that the molecular mechanism responsible for T cell dysfunction is located downstream the TCR engagement.

RESULTS

A IFN- γ secretion by donor LB3300's clone



B Lytic activity by donor LBB330's clone

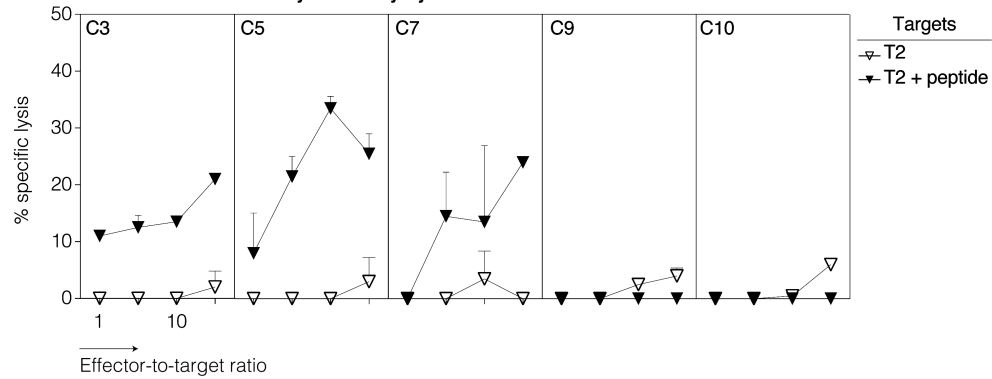


Figure 14: Dysfunctional human CD8⁺ T cells were also obtained with a human anti-Melan-A.A2 CD8⁺ T cell clone. A CD8⁺ T cell clone was derived from anti-Melan-A.A2 T cells obtained from donor LB3300. T cells were stimulated every 3-4 days, for 10 cycles, with irradiated A2⁺Melan-A⁺ CP50-MEL.1 melanoma cells, LG2-EBV feeder cells and IL2. **(A)** T cells were re-activated for 20 h with different stimuli. Mean of IFN- γ secretion measured by ELISA; means $\pm$ s.d. of triplicates. **(B)** Target cells were ⁵¹Cr-labelled and incubated with T cells at the indicated ratios. Chromium release was measured after 4 h of co-culture; mean $\pm$ s.d. of duplicates.

Is the loss of function observed due to antigen persistence?

DYSFUNCTION DOES NOT RESULT FROM CLONAL SELECTION OF POORLY FUNCTIONAL CD8⁺ T CELLS

Since the previous results were obtained with polyclonal anti-Melan-A.A2 CD8⁺ T cells, we could not exclude that the observed T cell dysfunction resulted from the gradual selection of a highly proliferative T cell subpopulation with low effector functions. We thus derived an anti-Melan-A.A2 CD8⁺ T cell clone from functional effector anti-Melan-A.A2 polyclonal CD8⁺ T cells obtained as described above (*Figure 8A*). The derived CTL clone was submitted to chronic antigen stimulations as before (*Figure 8B*). After several stimulations cycles, we again observed a gradual loss of IFN- γ secretion (*Figure 14A*) and of lytic activity (*Figure 14B*). We conclude that the loss of function is not due to a clonal selection of highly proliferative T cell subpopulations with low effector functions.

T CELL DYSFUNCTION IS INDEPENDENT FROM IN VITRO PRIMING PROTOCOL AND THE TCR SPECIFICITY

The previous data were obtained with anti-Melan-A.A2 CD8⁺ T cells obtained from naïve CD8⁺ T cells. We wanted to exclude that this subpopulation of naïve anti-Melan-A.A2 CD8⁺ T cells was more susceptible to T cell dysfunction than other T cell subpopulations. Furthermore, we wanted to exclude that our *in vitro* priming protocol would render the T cells prone to the dysfunctional state. To address this issue, we used polyclonal anti-MUM-3 CD8⁺ T cells and a CD8⁺ T cell clone directed against a mutated peptide expressed by breast cancer cells²¹⁷. The polyclonal cells were isolated from blood of cancerous donors using A68/MUM-3 fluorescent multimers. These cells were amplified and submitted every 3-4 days to stimulation cycles using HLA- A68⁺ melanoma cells previously treated with IFN- γ and TNF- α . We also observed a loss of IFN- γ secretion after 10 stimulation cycles (*Figure 15A*).

RESULTS

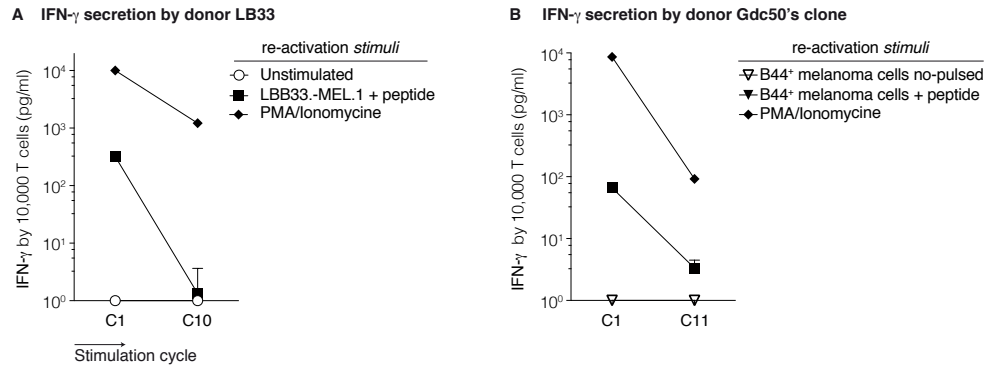


Figure 15: Dysfunctional human CD8⁺ T cells were obtained with CD8⁺ T cell not directed against Melan-A.A2. *T cells were stimulated every 3-4 days, for 10-11 cycles, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. T cells were re-activated for 20 h with different stimuli. Mean of IFN- γ secretion measured by ELISA; means $\pm$ s.d. of triplicates. (A) Donor LB33's polyclonal T cells were directed against MUM3 peptide presented by HLA-A68. (B) Donor Gdc50's clone is directed against a mutated tumor peptide presented by HLA-B44.*

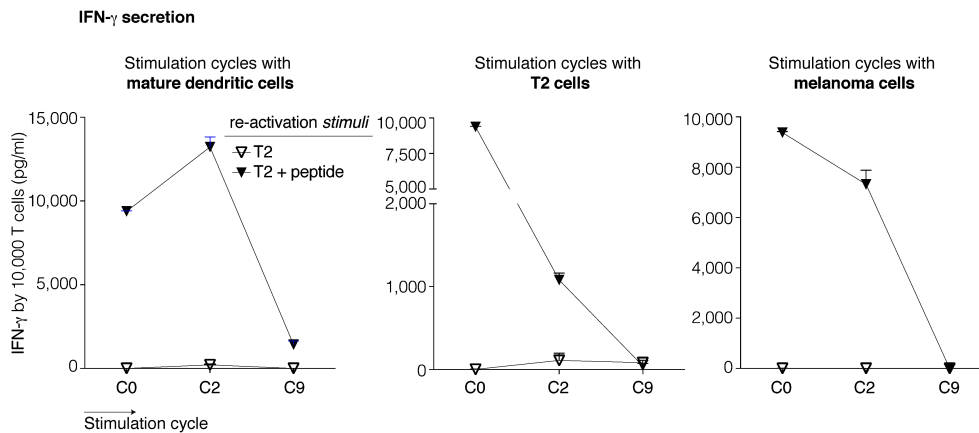


Figure 16: Human CD8⁺ T cell dysfunction is also observed using other stimulator cells. *T cells were stimulated nine times every 3-4 days with either irradiated A2⁺ cells (mature dendritic cells or T2) previously pulsed with Melan-A peptide EAAGIGILTV or irradiated A2⁺Melan-A⁺ melanoma cells. Feeders and IL2 were added to the co-culture with T2 cells and melanoma cells. IL2, IL-6, IL-7 and IL-12 were added to the co-culture with mature dendritic cells. For re-activation, 10,000 T cells were cultured for 20 h with T2 cells pulsed or not with the*

Melan-A peptide. IFN- γ secretion was measured by ELISA; means $\pm$ s.d. of triplicates.

Gdc50's T cell clone was isolated from TIL. The clonotype was repeated at the tumor site, indicating an *in vivo* priming (*unpublished data from Pierre Coulie's group*). We submitted this clone to repeated antigen stimulation and also observed a loss of function (*Figure 15B*). These data suggest that the dysfunctional state is independent of the priming protocol or T cell subpopulations.

HUMAN CD8⁺ T CELL DYSFUNCTION IS ALSO OBSERVED AFTER STIMULATION WITH PEPTIDE-LOADED MATURE DENDRITIC CELLS OR T2 CELLS

We then analyzed whether the antigen-presenting cells used during the stimulation cycles influence T cell dysfunction. Tumors cells can produce several immunosuppressive factors²¹⁸. We used both HLA-A2⁺ Melan-A⁺ melanoma cells and two different Melan-A peptide-loaded HLA-A2⁺ cells: mature dendritic cells and T2 cells. Dysfunction was observed after 9 stimulation cycles, independently of the stimulator cells used (*Figure 16*). We conclude that loss of function is not due to an inhibitory factor secreted by melanoma cells. Even stimulation with professional antigen-presenting cells, such as dendritic cells, leads to a loss of function.

ANTIGEN REMOVAL DOES NOT RESTORE HUMAN CD8⁺ T CELL FUNCTIONS

When cultured in absence of antigen, hypo-responsiveness T cells, such as anergic T cells, often recover their functions^{6,102,219,220}. This indicates that their impairment is transient and reversible. To examine whether *in vitro*-induced T cell dysfunction is also transient and reversible, we kept human T cells of different donors and stimulation cycles in medium and IL2 for up to 24 days after the last stimulation. After this resting period, T cells were re-activated with melanoma cells to measure their function. C2-derived T cells, whether rested or not, secreted IFN- γ (*Figure 17*). C9-derived T cells did not secrete IFN- γ , even after three weeks of rest. Therefore, dysfunction was not reversed by the absence of antigen in our experimental conditions.

RESULTS

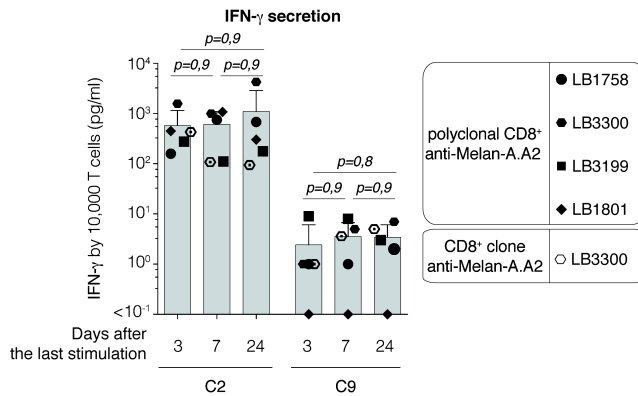


Figure 17: Antigen removal does not restore human T cell function. *T* cells were kept in culture in the presence of IL2. For re-activation, 10,000 *T* cells were cultured for 20 h with A2⁺Melan-A⁺ melanoma cells. IFN- γ secretion was measured by ELISA; means $\pm$ s.d. of triplicates. Wilcoxon signed-rank test.

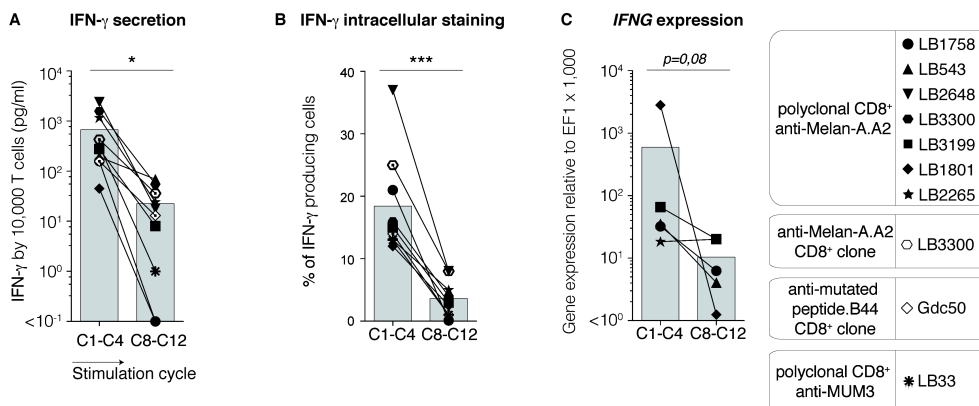


Figure 18: Dysfunctional human CD8⁺ T cells were obtained from multiple donors. *T* cells obtained from multiple donors were stimulated every 3-4 days for 8-12 stimulation cycles with irradiated A2⁺Melan-A⁺ CP50-MEL.1 melanoma cells, feeder cells and IL2. *T* cells were re-activated for 20 h with A2⁺Melan-A⁺ CP50-MEL.1. **(A)** Mean of IFN- γ secretion measured by ELISA from each donor. Paired *t*-test, **P*<0,05. **(B)** Mean of intracellular staining for IFN- γ in the presence of brefeldin A. Paired *t*-test, ****P*<0,001. **(C)** Mean of IFNG gene expression. Paired *t*-test. Data obtained with *T* cells isolated from different donors.

All preceding data suggest that the observed loss of function is driven by antigen persistence. Considering that antigen removal did not restore T cell function, we postulate that the dysfunctional state is permanently maintained through the expression by T cells of yet unidentified gene(s). Thus, antigen persistence could induce transcriptional changes imprinted in T cells.

In summary, we observed after 8-14 stimulation cycles, a clear decrease in the secretion of IFN- γ , intracellular IFN- γ production and IFN- γ gene expression in 7 out of 8 polyclonal CD8⁺ T cells and in all (n=2) CD8⁺ T cell clones. (*Figure 18*). But one, the T cells of donor LB2265, showed dysfunctional intracellular IFN- γ production when re-activated with melanoma cells but not with peptide-pulsed T2 or non-specific *stimuli*. This donor's T cells present a different dysfunctional phenotype to the others.

We tentatively conclude that antigen persistence leads to a loss of cytokine secretion due to the absence of cytokine production.

Is T cell dysfunction mediated by a previously described mechanism?

HUMAN CD8⁺ T CELL DYSFUNCTION IS NOT DUE TO INHIBITORY RECEPTORS

In chronic LCMV C13 infection, exhausted T cells up-regulate inhibitory receptors. *In vivo* blockade of inhibitory receptors can restore functions of these exhausted T cells¹²². Therefore, we examined whether anti-PD-1 and/or anti-CTLA-4 blocking antibodies rescue effector functions of human dysfunctional CD8⁺ T cells. To re-activate the T cells, we used PD-L1⁺ melanoma cells or PD-L2⁺ CD80⁺ CD86⁺ T2 cells. We failed to observe any increase in IFN- γ secretion, whether the T cells were pre-

RESULTS

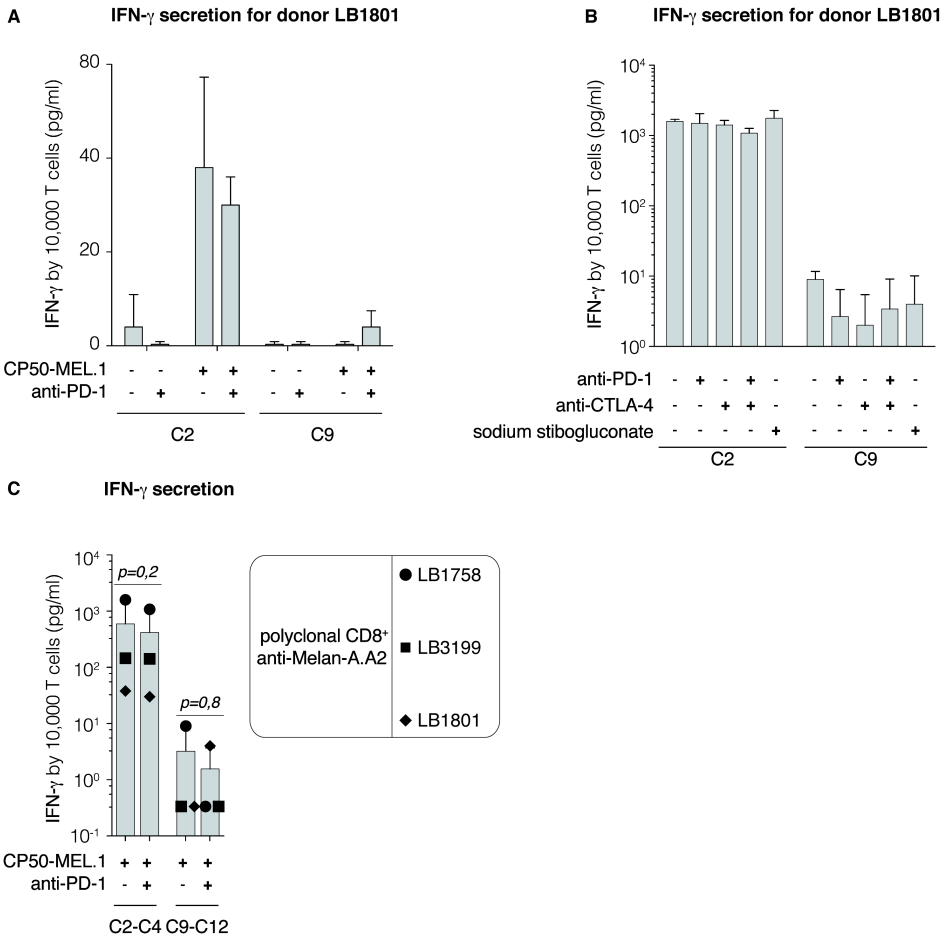


Figure 19: Inhibitory receptors antagonists do not rescue T cell function. T cells were treated with different inhibitors for 20 h. T cells were re-activated with A2⁺ Melan-A⁺ PD-L1⁺ melanoma cells (**A**, **C**) or with A2⁺ PD-L2⁺ CD80⁺ CD86⁺ T2 cells (**B**). IFN- γ secretion was measured by ELISA after overnight co-culture. (**A**, **B**) Representative histograms obtained with donor LB1801's T cells. (**C**) Data obtained with T cells isolated from 3 donors. Wilcoxon signed-rank test.

treated with anti-PD-1 and/or anti-CTLA-4 antibodies for 20 h (*Figure 19A&B*) or for 5 days (*data not shown*). As PD-1 ligation mediates intracellular signaling via phosphatase SHP-1/-2^{124,221}, we also treated the T cells with sodium stibogluconate a phosphatase inhibitor. Again no increased IFN- γ secretion was observed (*Figure 19B*).

Because multiple inhibitory receptors can lead to T cell dysfunction, we then examined if the dysfunction correlated with increased surface expression of other inhibitory receptors (*Figure 20*)^{100,130,196}. We performed a longitudinal expression analysis over time. Human anti-Melan-A.A2 CD8⁺ T cells obtained from 4 donors were analyzed 4-5 days after the last stimulation (unstimulated) and after overnight re-activation with beads coated with anti-CD3/CD28 antibodies. We analyzed the expression of 18 inhibitory receptors namely PD-1, CTLA-4, TIM3, BTLA, LAG3, CD244, NKG2A, CD160, CD200R, CD38, CD39, CD73, KLRG1, KLRD1, CD66a, CD85j, CD161 and CD158. Examples of data obtained for one donor are shown for PD-1, TIM3, CTLA-4, and BTLA in concatenated zebra plot using Flowjo® software (*Figure 20A&B*). PD-1 surface expression was not higher on dysfunctional T cells (C7-C10) than on functional T cells (C2-C5). CTLA-4 and TIM3 were not detected at the surface of donor LB1801's CD8⁺ T cells. For BTLA, we observed a slight increase at the surface of dysfunctional T cells.

In *Figure 20C*, we draw a heatmap using fold change of MFI of surface expression of inhibitory receptors compared to the unstained condition for 4 donors. We could not identify a single common inhibitory receptor for all donors that had higher surface expression on dysfunctional T cells (C8-C13) than on functional T cells (C2-C5). For example, up-regulation of BTLA observed with donor LB1801's T cells, was not seen with T cells from the 3 other donors. Considering that we are looking for a common inhibitory mechanism, we tentatively conclude that inhibitory receptors, (at least the 18 we have analyzed) are not responsible of T cell dysfunction.

We also analyzed expression of inhibitory receptors on human CD8⁺ T cells kept in absence of antigen for 24 days. Examples of data obtained for one donor are shown for PD-1, TIM3 and BTLA in *Figure 21*.

RESULTS

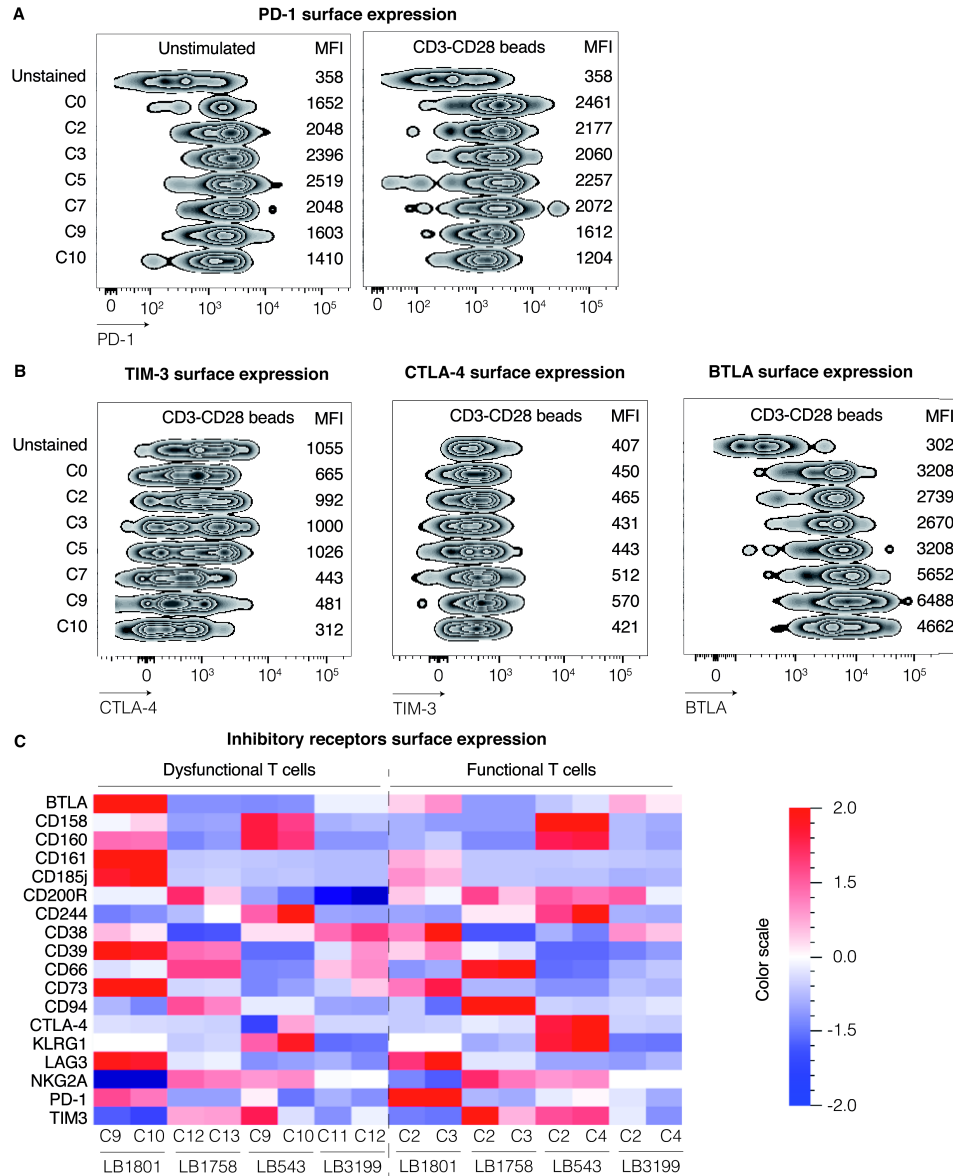


Figure 20: Surface expression of inhibitory receptors. *T* cells were stimulated every 3-4 days, for 10-13 cycles, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. *T* cells were re-activated or not with beads covered with anti-CD3/-CD28 antibodies. *T* cells were stained with an anti-inhibitory receptor mAb. **(A, B)** MFI for donor LB1801. **(C)** Heatmap of fold change of MFI of inhibitory receptors surface expression compared to the unstained condition. Data obtained with *T* cells isolated from 4 donors.

Four to five days after the last stimulation, inhibitory receptors were expressed at the surface of C3-C10-derived T cells. By day 24 after the last stimulation cycle, these receptors have disappeared from the surface of the T cells (*Figure 21*). This suggests that in our experimental system these receptors are rather activation markers.

T CELL DYSFUNCTION CAN BE REVERSED AT AN EARLY PHASE BY REMOVING SURFACE GALECTIN

We previously described that the presence of galectin on the surface of TILs can block their ability to secrete cytokines. TILs recovered their ability to secrete high amounts of IFN- γ after a short treatment with a galectin competitive binder, N-acetyllactosamine (LacNAc)^{193-195,216}. Here, we examined the role of galectin in the dysfunction of our T cells by treating functional and dysfunctional T cells from donor LB1801 with galectin antagonists. Treatment with LacNAc (*Figure 22A*) or an anti-galectin-3 antibody (*Figure 22B*) increased IFN- γ secretion of C2-C5-derived cells. However, C7-C10-derived cells did not respond to the treatment (*Figure 22A&B*). The dysfunction of C7-C10-derived cells is independent of galectin-3 as we observed that treating the T cells with an anti-galectin-3 antibody detaches surface galectin-3 (*Figure 22C*). T cells from two other donors treated with LacNAc showed also an increase in IFN- γ secretion by C2-derived cells but not C10-derived cells (*Figure 22D*). Thus, galectin antagonists boost the function of human T cells only during an early phase of antigen-induced T cell dysfunction. However, an advanced stage of T cell dysfunction is not reversed by galectin removal.

THE LATE PHASE OF T CELL DYSFUNCTION IS NEITHER DUE TO SUSTAINED CD3 ζ DOWNREGULATION NOR TO SENESENCE

Engagement of the T cell receptor results first in CD3 ζ internalization and degradation, and subsequently in expression of newly synthesized CD3 ζ ²²². It has been reported that human T cells with a sustained CD3 ζ down-regulation are dysfunctional and secrete fewer cytokines^{223,224}. Before re-activation, CD3 ζ is expressed at the same level in C2 and C10 T cells, excluding sustained CD3 ζ down-regulation (*Figure 23A*).

RESULTS

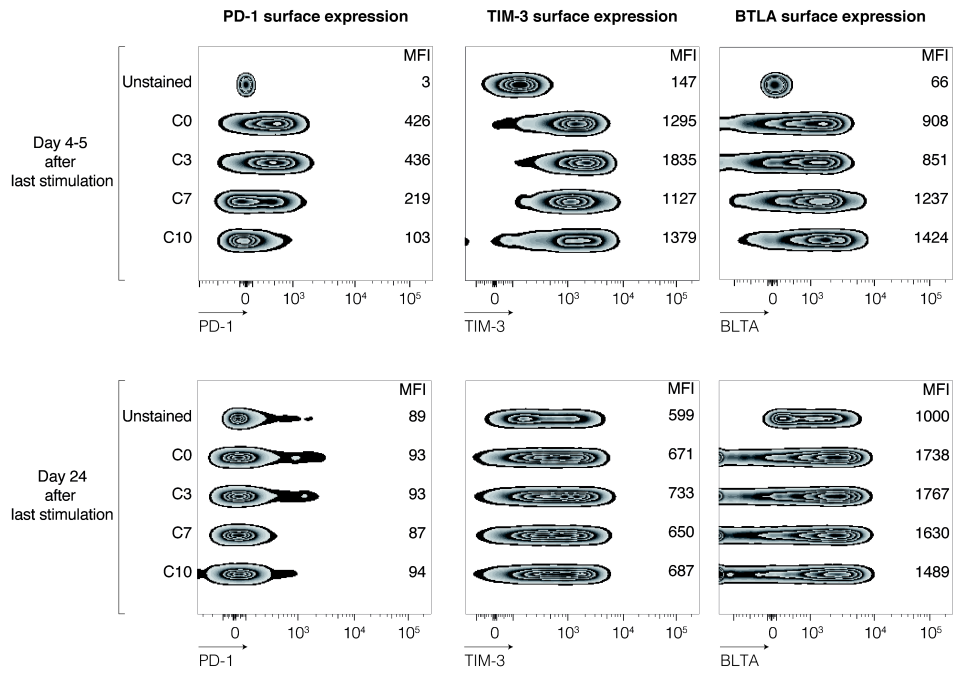


Figure 21: Human CD8⁺ T cells rested for 24 days do not express inhibitory receptors. *T cells were kept in culture in the presence of IL2. Cells were stained with anti-PD-1, anti-TIM3, anti-BTLA mAb.*

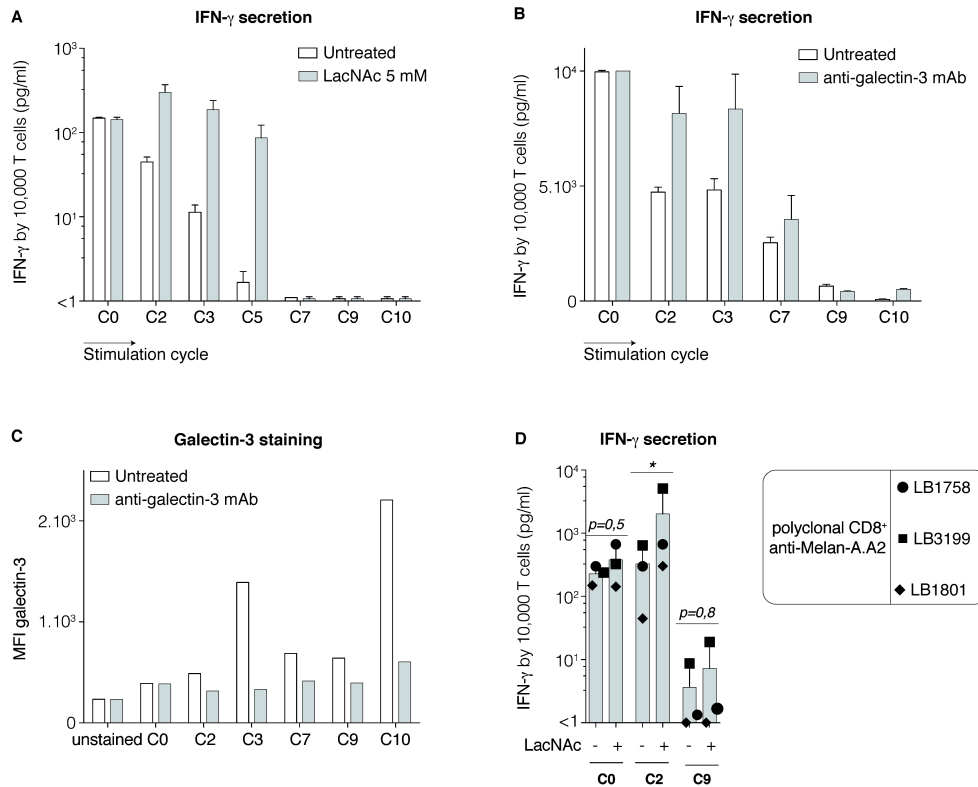


Figure 22: An early phase of human CD8⁺ T cell dysfunction can be reversed by removing surface galectin. (A, D) T cells were first treated for 2 h with 5 mM LacNAc, then re-activated for 20 h with A2⁺Melan-A⁺ melanoma cells. IFN- γ secretion was measured by ELISA; means $\pm$ s.d. of triplicates. **(A)** Data obtained for donor LB1801. **(B, C)** T cells were treated for 2 h with a rat IgG anti-galectin-3 antibody which was subsequently removed magnetically using anti-rat IgG-coated magnetic beads. This procedure was repeated twice before using the T cells either for functional assays **(B)** or for galectin-3 staining **(C)**. **(B)** T cells were re-activated with A2⁺ T2 cells pulsed with Melan-A peptide. IFN- γ secretion was measured by ELISA after overnight co-culture; means $\pm$ s.d. of triplicates. **(C)** T cells were stained with an anti-galectin-3 mAb coupled to biotin followed by secondary phycoerythrin-coupled Neutravidin. Galectin-3 MFI for CD8⁺ T cell. **(D)** IFN- γ secretion data obtained from 3 donors. Wilcoxon signed-rank test.

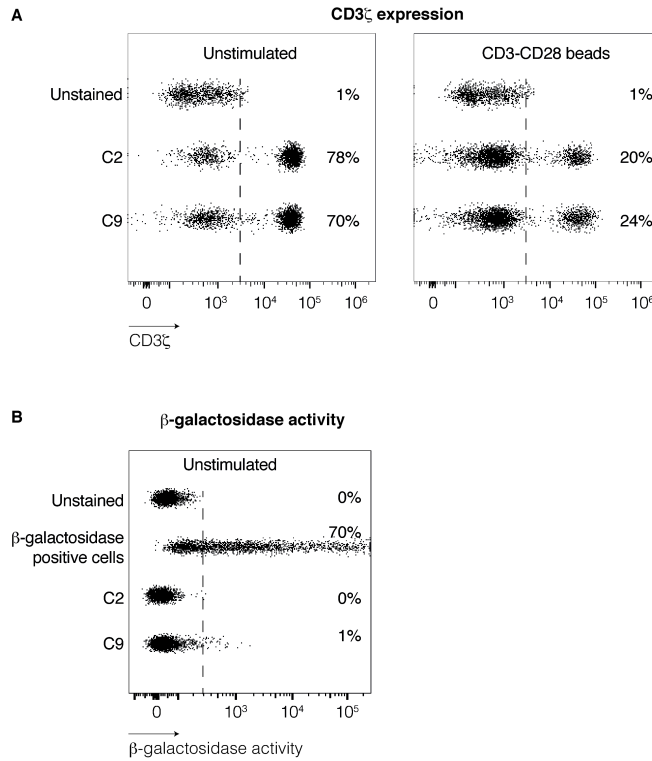


Figure 23: Human T cell dysfunction is neither due to sustained CD3 ζ down-regulation nor senescence. (A) Intracellular staining with anti-CD3 ζ antibody. Data represent the percentage of positive cells. (B) A total of 10^6 T cells were incubated for 10 min at 37°C. A solution of fluorescein di-V-galactoside was added to the cells for 1 min at 37°C. The reaction was stopped by addition of cold propidium iodide. Data represent the percentage of positive cells. These results were obtained with cells from donor LB1758 and are representative of data obtained with 3 other donors.

Upon re-activation, both functional and dysfunctional T cells lose CD3 ζ surface expression to a similar extent (*Figure 23A*). This suggests that dysfunctional T cells are able to initiate TCR signaling as well as functional ones.

Senescent CD8⁺ T cells with defective cytolytic activity accumulate in aging individuals, in younger persons with chronic viral infections, and in patients with cancer^{131,225,226}. Senescence is associated with an increased β -galactosidase activity²²⁷. Human dysfunctional T cells did not exhibit increased β -galactosidase activity indicating that they do not have a senescent phenotype (*Figure 23B*).

Taken together, we conclude that antigen-driven human T cell dysfunction was caused by surface galectin during an early phase (C2-C5-derived T cells). However, the cause of the dysfunction phenomenon of the late phase (C7-C10) remains to be understood.

Transcriptome analysis of dysfunctional human CD8⁺ T cells

To identify the molecular pathways underlying antigen-driven human T cell dysfunction, we performed comparative whole-genome transcriptome analyses of functional C4-C5-derived T cells and dysfunctional C9-C10-derived T cells. We used functional and dysfunctional cells obtained from two anti-Melan-A.A2 polyclonal CD8⁺ T cells and one anti-Melan-A.A2 CD8⁺ T cell clone. Cells were re-activated for 20 h with beads covered with anti-CD3/-CD28 antibodies. We checked RNA integrity using bioanalyzer agilent. We sequenced only samples with a RIN (RNA Integrity Number) higher than 8 (*data not shown*). Library preparation and sequencing were performed by Beckman Coulter Genomics.

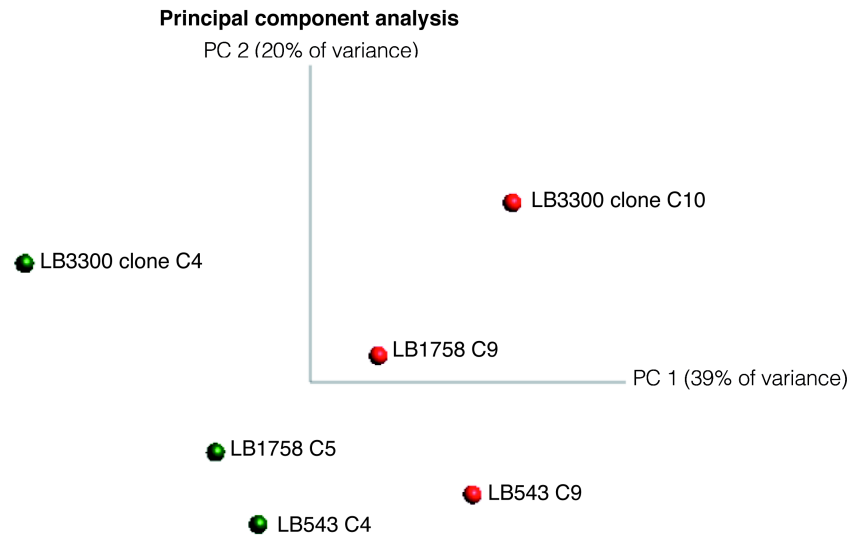


Figure 24: Principal component analysis revealed distinct gene signature between functional and dysfunctional T cells. *Principal component analysis of RNA-seq expression data from all biological replicates functional versus dysfunctional T cells ($n = 3$).*

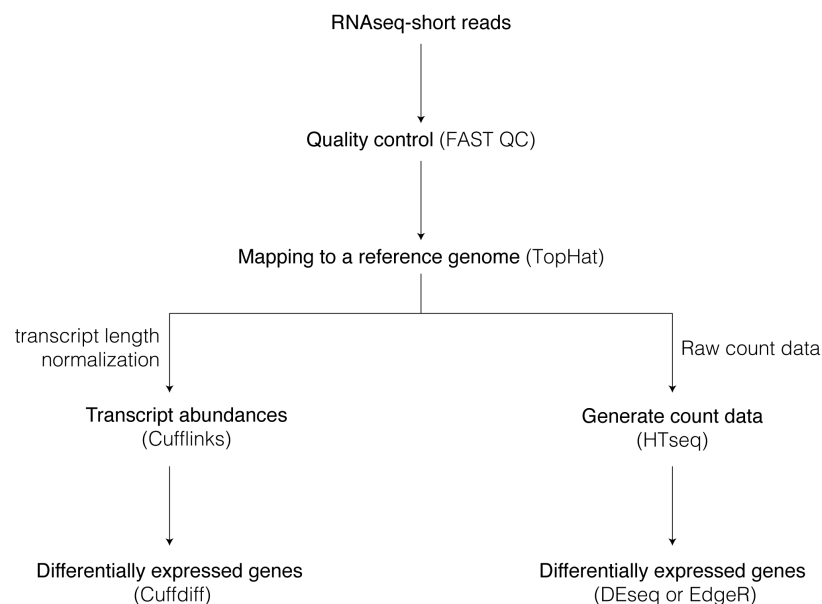


Figure 25: Overview of the procedure for differential expression analysis of RNAseq data. Adapted from Zhang *et al.*, 2014²²⁸.

PRINCIPAL COMPONENT ANALYSIS

Principal component analysis (PCA) is a mathematical algorithm that reduces the number of dimensions in multidimensional data sets to fewer dimensions, while retaining the data sets' variability. It accomplishes this reduction by identifying directions, called principal components. In PCA, each sample has scores associated with each principal component (PC). By using a few components (transcripts for transcriptomic data), each sample can be represented by relatively few numbers of transcripts instead of by values for thousands of variables. In a score plot, the closer the distance between samples, the more similar they are with respect to the features represented by the PCs selected for the score plot^{229,230}.

Functional C4-C5-derived T cells did not cluster with dysfunctional C9-C10-derived T cells (*Figure 24*). This observation suggests that these cell populations have a distinct gene signature.

DIFFERENTIAL GENE EXPRESSION ANALYSIS

To quantify gene expression, RNA-Seq reads need to be aligned to a reference genome. The number of mapped (successfully aligned) reads allows an estimation of the relative expression level of genes. Subsequently, statistical methods are applied to test the significance of differences between groups. Differentially expressed genes are selected based on a combination of expression change threshold and score cutoff, which are usually generated by statistical modeling. There is no consensus on which analysis method is optimal for differentially expressed gene analysis. A number of tools have been developed (*Figure 25*). The most popular RNA-Seq tools are Cufflinks, DEseq and edgeR. The statistical method of these softwares is based on the negative binomial distribution. This distribution is more appropriate for biological replicates because it has two parameters, encoding the mean and the

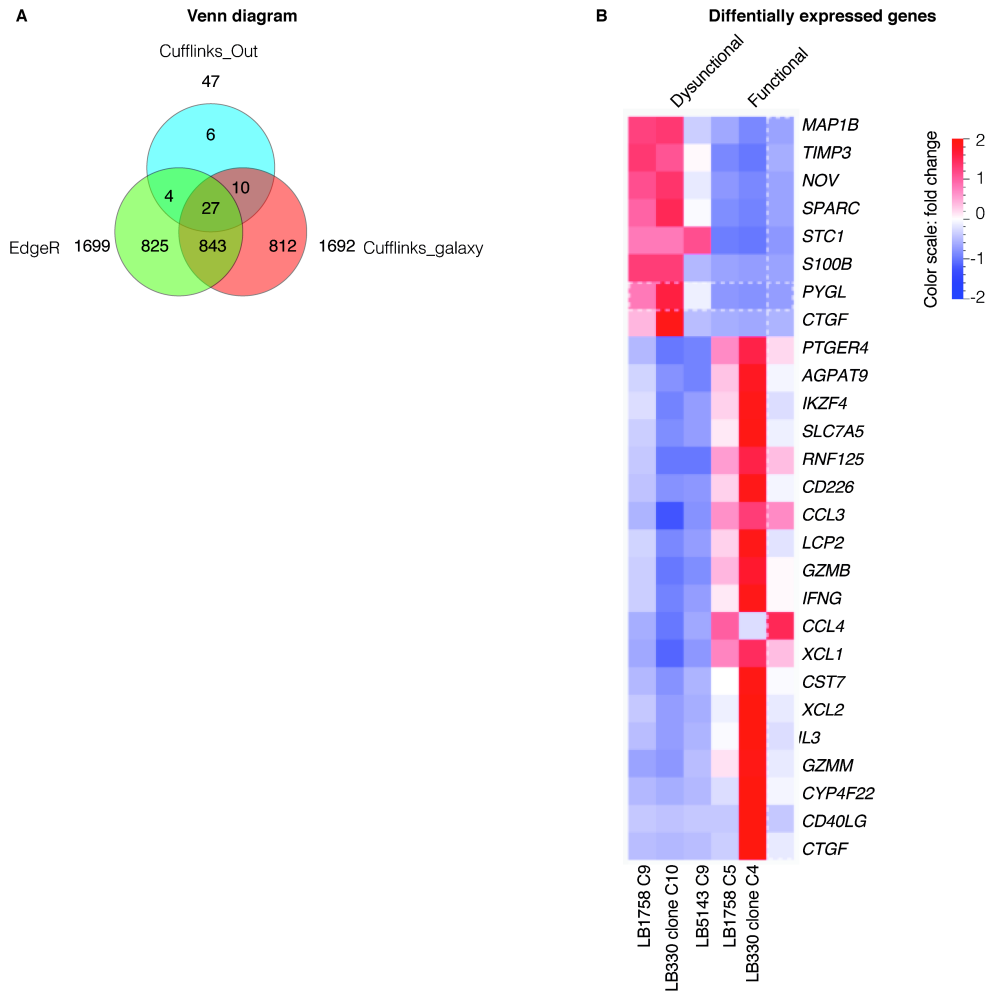


Figure 26: Genes differentially expressed between functional and dysfunctional T cells. (A) Venn diagram of differential gene expression analysis from Cufflinks-galaxy (red), Cufflinks_out (blue) and edgeR (green). **(B)** Heatmap of gene levels for all 27 differentially expressed genes in common between the three analysis methods.

dispersion. Using a parameter assuming the mean is equal to the variance would generate more false positive results because of the high variability of biological replicates²³¹. Cufflinks adopts an algorithm that assembles the mapped reads into possible transcripts and then generates a final transcriptome for each condition (*i.e.* functional and dysfunctional). Next, it can identify differentially expressed transcripts and genes, differential splicing, and promoter-preference changes^{228,232}. DESeq and edgeR are very similar since they use mapped reads count matrices as input and identify the difference between the count reads for a transcript. The difference between DESeq and edgeR is in the mathematic algorithm. In DESeq, the method assume that variance and mean are linked by local regression to detect differentially expressed genes²³³. In EdgeR, empirical Bayes methods are used to moderate the degree of dispersion across transcripts²³⁴. No single method is clearly superior for differential expression analysis for RNA-Seq datasets²²⁸. Cufflinks is not recommended for differential expression if sequencing coverage is low (*i.e.* <10 million reads per individual sample). In our case, coverage was ± 33 million reads per sample²²⁸. DESeq is not recommended if sequencing coverage is unbalanced between groups²²⁸. This phenomenon often occurs with multiplexed samples, which is the case in our experiment. EdgeR is more susceptible to generate false positive results²²⁸.

We decided to use Cufflinks and edgeR for differential gene expression analysis. The analysis with Cufflinks was performed lab-internally using galaxy (Cufflinks-galaxy) and lab-externally (Cufflinks-out) with the help of a bioinformatician (Ahmed Essaghir). We generated two lists of differentially expressed genes between functional and dysfunctional genes. Significant differential gene expression was defined as $|\log_2(\text{ratio})| \geq 1$ (2-fold) and false discovery rate (FDR) $\leq 0,0.5$. We identified 1692 differentially expressed genes with the “Cufflinks-galaxy” list and 47 with the “Cufflinks-out”. This difference was probably due to different versions of Cufflinks used. It has been shown that versions of cufflinks perform differently²²⁸. Using the edgeR software, we identified 1699 differentially expressed genes. Venn diagram shows the intersection

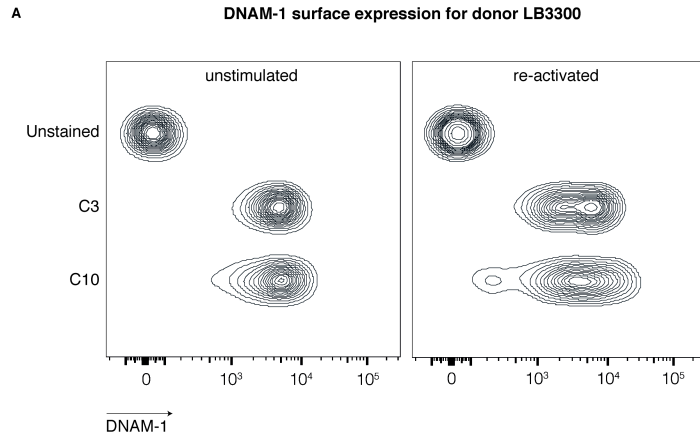


Figure 27: Expression of DNAM-1 at the surface of anti-Melan-A.A2 T cells. T cells were stimulated every 3-4 days, for 10-13 cycles, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. Cells were stained with anti-DNAM-1 mAb.

of the numbers of the differentially expressed genes identified from the three lists (Figure 26A). We decided to focus on the 27 differentially expressed genes common in all three lists (Figure 26B).

A list of genes potentially implicated in T cell dysfunction

EFFECTOR FUNCTION GENES

Among the 27 differentially expressed genes, 19 were down-regulated in dysfunctional T cells. Among these, we observed low expression of effector function genes (e.g. *GZMB*, *IFNG*, *IL3*, *GRZMM*, *CCL3*, *CCL4*, *XCL1*, *XCL2*, *CD40LG*) consistent with the loss of function seen. We postulate that these genes are a consequence rather than the cause of T cell dysfunction.

ITK and *LCP2* (coding for SLP76) code for proteins implicated in early TCR signaling¹⁴. However, we observed that a mix of PMA and ionomycin, did not restore T cell function (Figure 12). PMA is an agonist of PKC and ionomycin is an ionophore that induces calcium flux. A mix of PMA/ionomycin induces a signaling cascade downstream of ITK and SLP76. If the dysfunction was due to ITK and SLP76 down-regulation, we should not have observed any difference in cytokine secretion after re-activation with the PMA/ionomycin-cocktail. We conclude that down-regulation of these genes was not the cause of T cell dysfunction.

EXPERIMENTAL VALIDATION OF RNASEQ DATA

Among the 16 remaining genes, we decided to focus our analysis on *CD226*, *SLC7A5*, *IKZF4*, *RNF125*, *AGAPT9*, *TIMP3* and *SPARC*.

CD226 gene codes for DNAM-1 protein, a co-stimulatory molecule. The ligand of DNAM-1 is CD155²³⁵. DNAM-1 shares ligand specificity with TIGIT, an inhibitory receptor on the T cell surface. TIGIT inhibits T

RESULTS

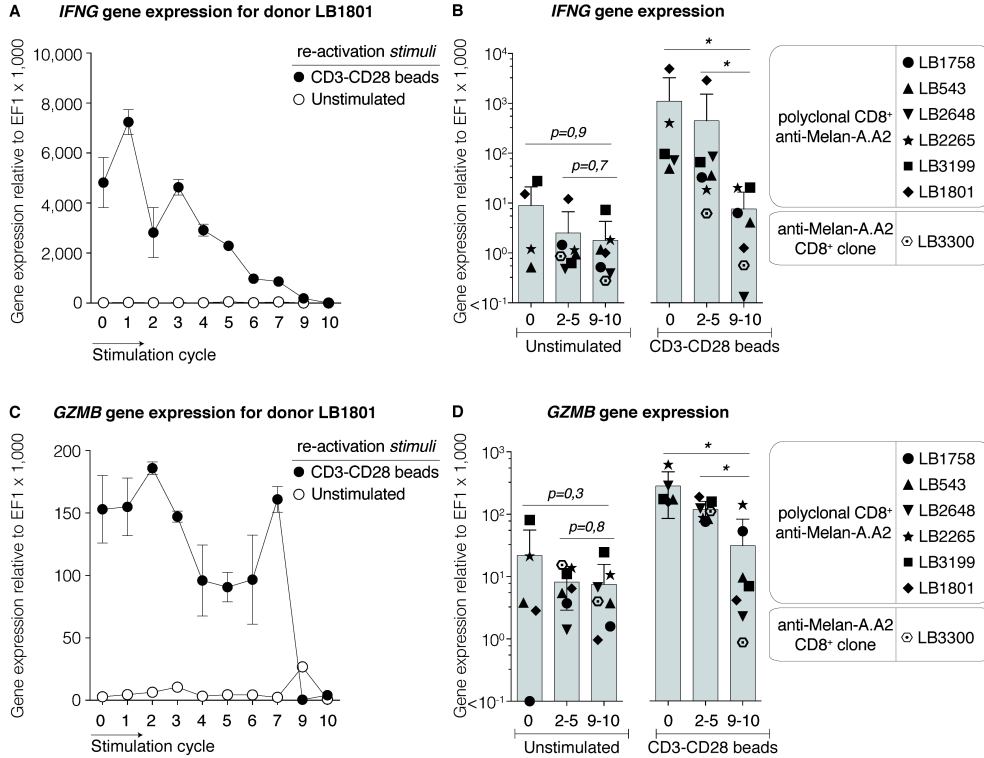


Figure 28: IFNG and GZMB gene expression. T cells were stimulated 10 times, every 3-4 days, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. The T cell samples were re-activated for 20 h with beads covered with anti-CD3/CD28 antibodies. mRNA expression was measured by RT-qPCR; means $\pm$ s.d. of triplicates. **(A, C)** Data obtained with donor LB1801's T cells. **(B, D)** Data obtained with T cells isolated from multiple donors. **(B)** Wilcoxon signed-rank test. * $P < 0,05$. **(D)** Paired t-test. * $P < 0,05$.

cell proliferation and cytokine production through competition with CD226 for ligand binding²³⁵. A reduced expression of DNAM-1 would increase intracellular inhibitory signals by TIGIT. We stained dysfunctional T cells with an anti-DNAM-1 antibody to confirm that surface reflects the RNAseq data at the protein level. However, DNAM-1 expression was not decreased at the surface of dysfunctional T cells (*Figure 27*).

False positive results in RNAseq, long half-life of the protein or recycling of cell surface receptors are possible explanations for the differences observed between the RNAseq data and the protein expression analysis. We conclude that down-regulation of DNAM-1 is not responsible of antigen-driven human T cell dysfunction.

We performed RT-qPCR analysis to confirm the RNAseq data for *SLC7A5*, *IKZF4*, *RNF125*, *AGAPT9*, *TIMP3* and *SPARC*. We also measured the expression of *GZMB* and *IFNG* as positive controls. Cells were re-activated or not for 20 h with beads covered with anti-CD3/-CD28 antibodies. We observed a decrease in *IFNG* and *GRZMB* gene expression in agreement with the RNAseq data (*Figure 28A&B*).

In *Figure 18* we observed that the T cells isolated from donor LB2265 present a different dysfunctional state. After 14 stimulations cycles, cells have lost the capacity to secrete cytokines but retain the ability to produce intracellular cytokine. Interestingly donor LB2265 did not present a decrease in *IFNG* and *GRZMB* expression consistent with this previous result (*Figure 28*).

We also reproduced RNAseq data for *SLC7A5*, *IKZF4* and *AGAPT9*. Examples of data for *SLC7A5* are shown in *Figure 29A&B*. However we observed no differences for these three genes between functional and dysfunctional states before re-activation. We hypothesize that the inhibitory mechanism should already be present in dysfunctional T cells before re-activation since dysfunctional T cells do not recover their functions in the absence of antigen (*Figure 29*). Therefore, *SLC7A5*, *IKZF4*, *AGPAT9* were disregarded at this point of the investigation. The down-regulation of these genes is a consequence of reduced T cell activation rather than the cause of T cell dysfunction.

RESULTS

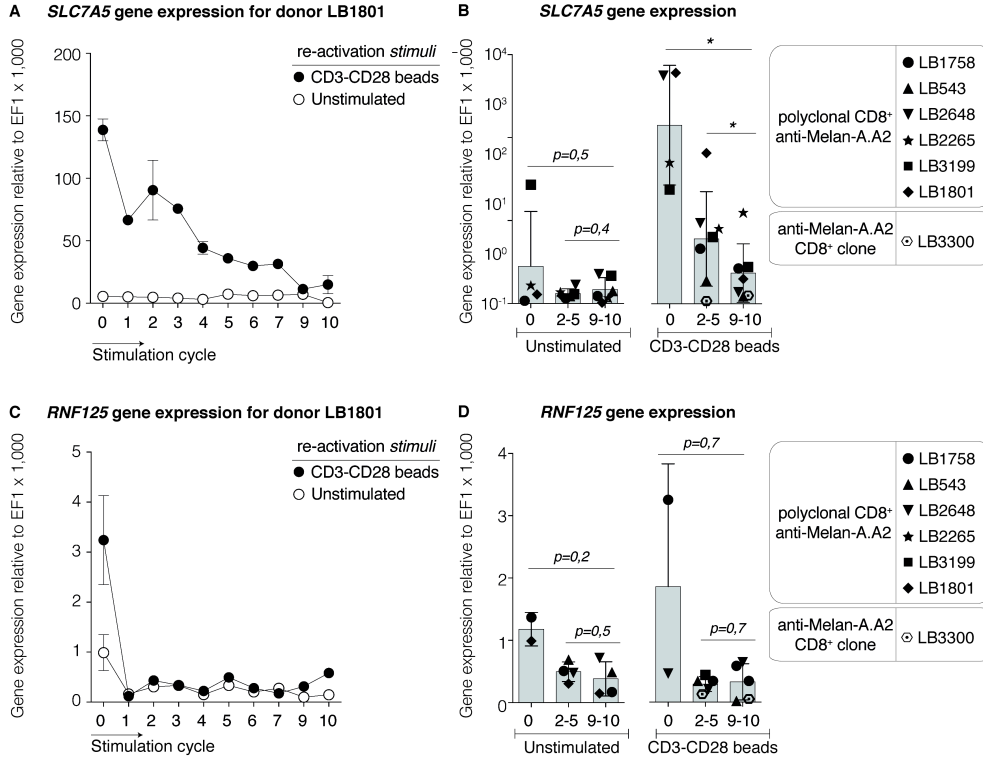


Figure 29: SLC7A5 and RNF125 gene expression. T cells were stimulated 10 times, every 3-4 days, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. The different T cell samples were re-activated for 20 h with beads covered with anti-CD3/-CD28 antibodies. mRNA expression was measured by RT-qPCR; means $\pm$ s.d. of triplicates. **(A, C)** Data obtained with donor LB1801's T cells. **(B, D)** Data obtained with T cells isolated from multiple donors. **(B)** Paired t-test. * $P < 0,05$ **(C)** Wilcoxon signed-rank test.

Regarding *RNF125* we could not reproduce the differences observed in the RNAseq data for all donors. We observed no difference in functional and dysfunctional T cells for donor LB1801 (*Figure 29C&D*). We conclude that *RNF125* is not the cause of the loss of function.

RT-qPCR data for *TIMP3* and *SPARC* were in line with the RNAseq data. The expression of these genes was inversely correlated to *IFNG* and *GRZMB* (*Figure 30A&B*). Moreover, we observed a high expression of these genes in dysfunctional T cells compared to functional ones before re-activation (*Figure 30C&D*).

Interestingly, C14-derived T cells of donor LB2265, which display the particular phenotype of not losing IFN- γ intracellular production and gene synthesis did not up-regulate these genes. We concluded that these two genes are potential mediators of antigen-driven human T cell dysfunction.

TIMP3 AND SPARC ARE DIFFERENTIALLY EXPRESSED AT THE PROTEIN LEVEL

We analyzed whether the differences of *TIMP3* and *SPARC* above described at the mRNA level were observed at the protein level. *TIMP3* and *SPARC* are secreted proteins. We used ELISA to detect the secretion of these proteins in the supernatant of cells re-activated or not for 20 h with beads covered with anti-CD3/-CD28 beads. Consistent with the up-regulation of *TIMP3* and *SPARC* expression in dysfunctional T cells at the RNA level, we observed a gradual increase of *TIMP3* and *SPARC* at the protein level with increasing dysfunction (*Figure 31*).

DO DYSFUNCTIONAL T CELLS ISOLATE FROM TUMOR PRESENTS HALLMARKS OF IN VITRO-INDUCED DYSFUNCTIONAL T CELLS?

We analyzed *TIMP3* and *SPARC* gene expression in T cells isolated from cancerous patients. From total cell samples, we isolated CD2⁺ cells containing T lymphocytes, NK cells and a subpopulation of B cells. We used T cells from nine ovarian cancer patients for which we had blood, ascites and solid tumor from the same patient. We compared the

RESULTS

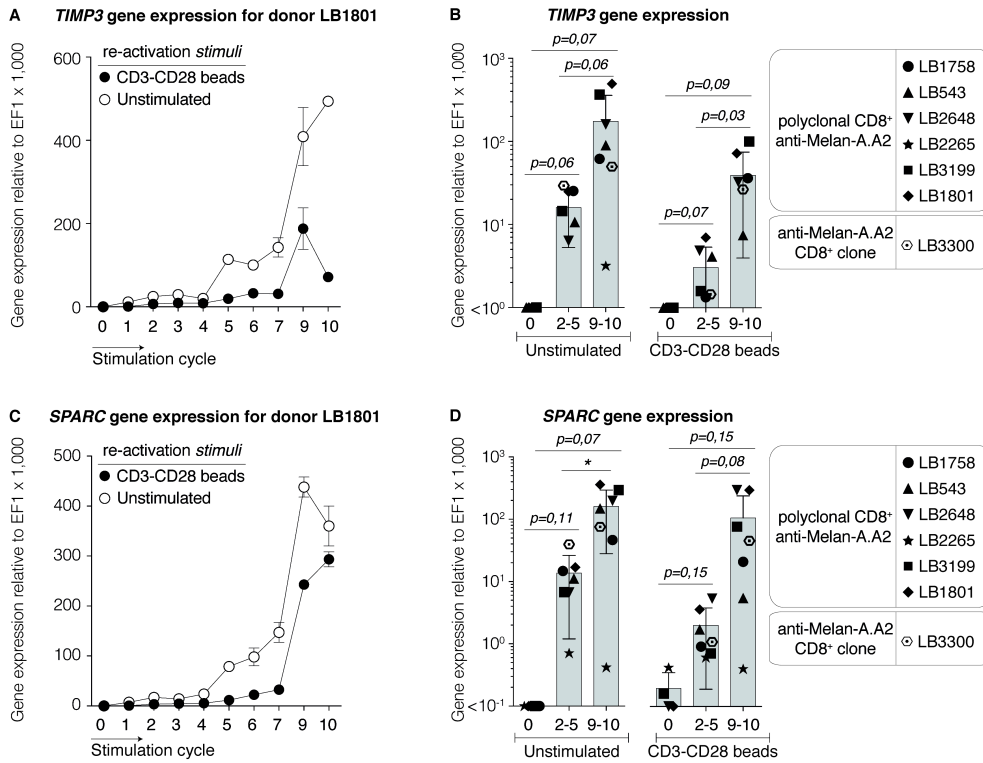


Figure 30: TIMP3 and SPARC gene expression. T cells were stimulated 10 times, every 3-4 days, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. The different T cell samples were re-activated for 20 h with beads covered with anti-CD3/-CD28 antibodies. mRNA expression was measured by RT-qPCR; means $\pm$ s.d. of triplicates. **(A, C)** Data obtained with donor LB1801's T cells. **(B, D)** Data obtained with T cells isolated from multiple donors. **(B, C)** Paired t-test. * $P < 0,05$.

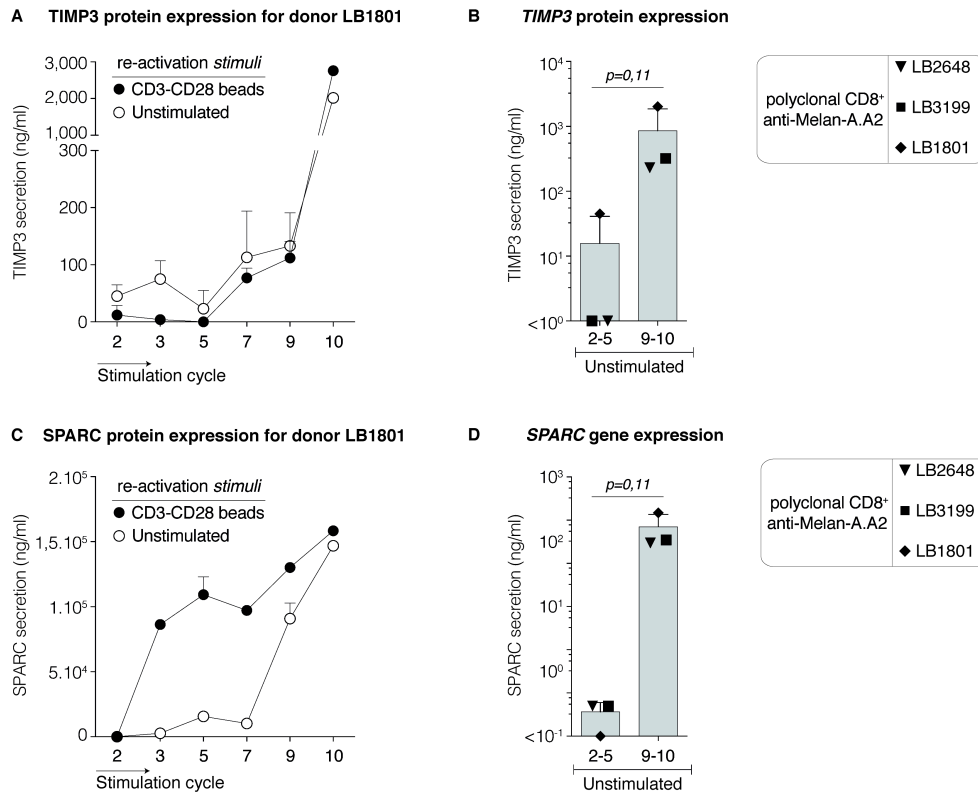


Figure 31: TIMP3 and SPARC protein expression. *T* cells were stimulated 10 times, every 3-4 days, with irradiated melanoma cells, LG2-EBV feeder cells and IL2. The different *T* cell samples were re-activated for 20 h with beads covered with anti-CD3/-CD28 antibodies. Protein secretion was measured by ELISA; means $\pm$ s.d. of triplicates. **(A, C)** Data obtained with donor LB1801's *T* cells. **(B, D)** Data obtained with *T* cells isolated from multiple donors.

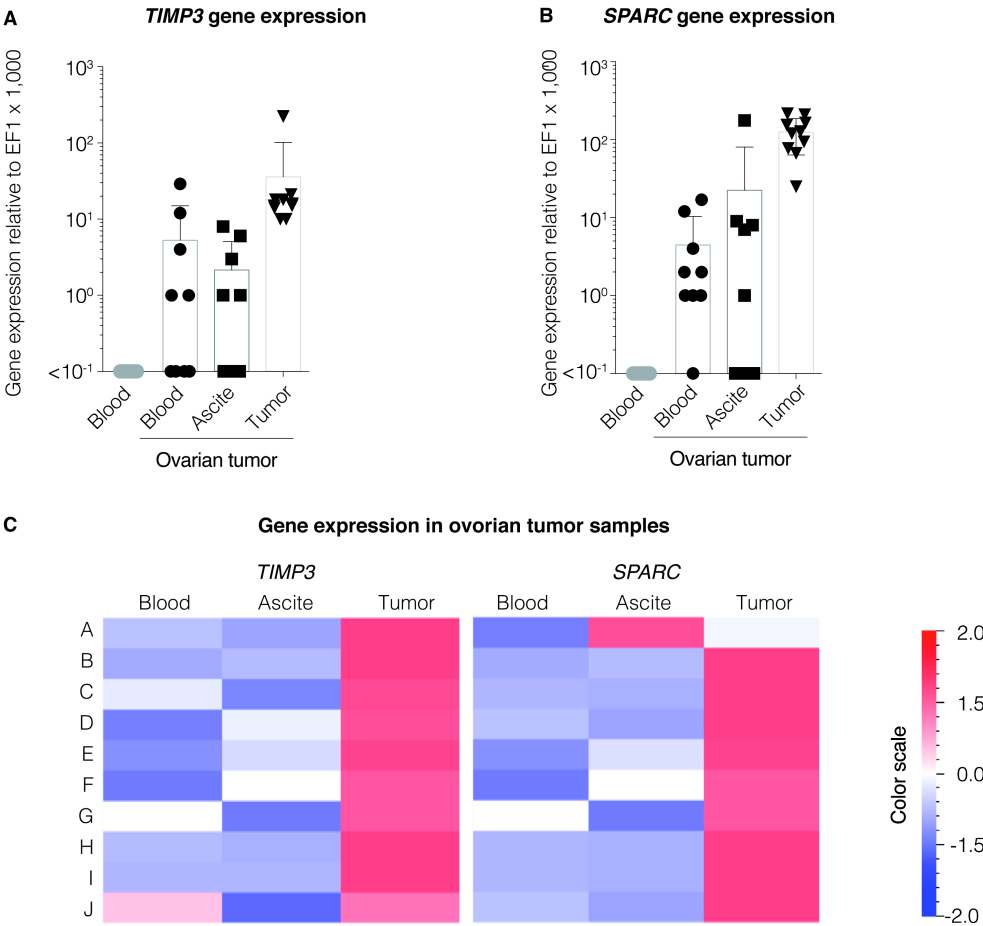


Figure 32: *TIMP3* and *SPARC* gene expression in T cells isolated from human samples. From total cells, *CD2*⁺ cells were sorted by rosetting. mRNA expression was measured by RT-qPCR; means±s.d. of triplicates. **(A)** Data for healthy donors or cancer patient. **(B)** Heatmap of ovarian cancer patient. A-J represents individual patients.

expression of TIMP3 and SPARC in these samples to cells isolated from non-cancerous blood donors. CD2⁺ cells isolated from non-cancerous donors did not express neither *TIMP3* nor *SPARC* (*Figure 32A&B*). We detected *TIMP3* and *SPARC* gene expression in CD2⁺ cells isolated from blood, ascites and solid tumors of ovarian cancer patients, with the respective expression of these genes being highest in CD2⁺ isolated from solid tumors (*Figure 32A&B*). We represented the data in a heatmap to analyze the expression of *TIMP3* and *SPARC* within the same sample. However, for the same donor, higher expression in the blood is not correlated with higher expression in the ascites or in the tumor (*Figure 32C*). We conclude that although the expression of TIMP3 and SPARC is not a proof of T cell dysfunction, these genes could be used as marker of T cells submitted to repetitive antigen stimulations.

RESULTS

DISCUSSION

DISCUSSION

Do previous models formally establish a link between antigen persistence and T cell dysfunction?

The phenotype and functional deficit of exhausted CD8⁺ T cells have been studied extensively. Although the persistence of the antigen seems to be at the root of T cell dysfunction, the driving force behind exhaustion remains speculative. Previous reports attempted to address this issue.

MOUSE MODEL

In the mouse model of LCMV infection, the Armstrong 53b parental strain induces an acute viral infection, whereas a naturally arising C13 mutant strain induces a chronic viral infection. LCMV Armstrong and clone 13 exhibit differences in their tropism within the spleen. C13 causes a higher level of infection of antigen-presenting cells²³⁶. Consequently, infected dendritic cells are functionally impaired and represent targets for destruction by CD8⁺ T cells. The importance of this was highlighted by the inability of LCMV-infected dendritic cells to stimulate T cell proliferation in assays with allogeneic T cells²³⁶. Furthermore LCMV-infected dendritic cells express higher levels of IL-10 and can induce in turn IL-10 production in LCMV-specific CD4⁺ T cells^{117,118}. In addition to dendritic cell modification, C13-infected mice exhibit a generalized immunodeficiency and are unable to mount efficient immune responses to other viruses²³⁷⁻²³⁹, parasites²⁴⁰, or tumors²⁴¹. It is therefore difficult to formally conclude in this mouse model that the dysfunction of exhausted T cells is mediated by antigen persistence.

In an influenza mouse model, control mice receive a single injection of the virus H1N1 strain PR8. The other mice receive two injections per week for four consecutive weeks²²⁰. Upon *in vivo* re-challenge with influenza virus CD8⁺ T cells display defects in expansion and intracellular cytokine production. This model is devoid of altered dendritic cells and

DISCUSSION

inflammatory cytokines. But the tropism of the PR8 strain for macrophages and the presence of antigen-specific Treg in the PR8 influenza mouse model could have contributed to the observed T cell dysfunction^{242,243}.

In an inducible tumor mouse model, expression of an oncogenic driver, the SV40 large T antigen, is induced in hepatocytes. SV40-specific T cells gradually become unable to produce IFN- γ and TNF- α upon *ex vivo* peptide stimulation. However, antigen-presenting hepatocytes lack co-stimulation molecules, such as CD80 and CD86. It is thus possible that the hepatocytes contribute to the exhaustion process by inducing T cell anergy. Indeed, transcriptomic studies show that SV40 antigen-specific T cells analyzed 8 days after the induction of the SV40 antigen do not express CD28. In contrast SV40 antigen-specific T cells still expressed CD28 8 days after infection with an SV40-expressing *Listeria monocytogenes*²⁴⁴.

EX VIVO ANALYSIS OF HUMAN T CELLS

Human T cells freshly isolated from patients with a chronic infection, a solid tumor or tumor ascites are often dysfunctional upon *ex vivo* re-activation^{192,195,245,246}. They frequently express several inhibitory receptors such as PD-1, CTLA-4, KLRG1, 2B4, and TIM3^{100,196}. Hence the idea was born that these human T cells are exhausted. However, existing correlations between the expression of inhibitory receptors and T cell dysfunction do not demonstrate a causative role of these receptors in T cell dysfunction. The experiments performed *ex vivo* with human T cells provide a momentary snapshot of dysfunction. It is impossible to distinguish whether human T cell dysfunction results from antigen persistence or from the tumor microenvironment, which includes hypoxia, regulatory cells and suppressive molecules.

Taken together neither the mouse models nor the *ex vivo* analysis of human T cells could establish with certainty that antigen persistence causes T cell dysfunction.

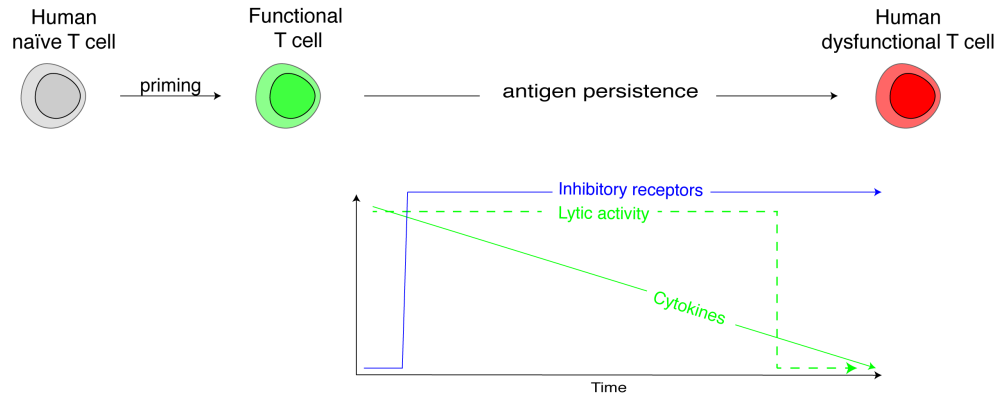


Figure 33: Hierarchical loss of function by human CD8⁺ T cells submitted *in vitro* to repetitive antigen stimulations. Human CD8⁺ T cell dysfunction is characterized by the step-wise loss of functions. Cytokine secretion is lost progressively and it is the first sign of the dysfunctional state. Lytic activity is lost at later stages of T cell dysfunction. The dysfunction is not correlated to the expression of inhibitory receptors.

A human system to study antigen persistence and T cell dysfunction

To address this issue, we have developed an experimental approach that relies on frequent and repeated antigen stimulation of antigen-specific T cells. Importantly, our experimental system is devoid of pro-inflammatory cytokines or a suppressive microenvironment. We used T cells specific for the Melan-A.A2 antigen expressed by melanoma cells. This allowed us to perform the antigenic stimulations with antigen-presenting cells instead of non-specific *stimuli*, such as beads covered with anti-CD3/-CD28 antibodies. The dysfunctional state that we observed is characterized by a loss of lytic activity and the loss of intracellular cytokine production. The dysfunction is sequential. CD8⁺ T cells first lose the capacity to produce and secrete IFN- γ , TNF- α and IL2. The Lytic activity is lost at a later stage. Interestingly, fully dysfunctional T cells retain the ability to initiate TCR signaling and to proliferate (*Figure 33*).

UNCOUPLING OF CYTOTOXICITY AND INTRACELLULAR CYTOKINE PRODUCTION

Valitutti *et al.* describe cytotoxicity and cytokine secretion as uncoupled in T cells based on the different amounts of stimulation required to trigger either process²⁴⁷. Using various antigen concentrations, they observed T cells exhibiting cytotoxicity at antigen concentrations at which intracellular cytokine production is undetectable. This observation raised the concept that T cells exhibit two activation thresholds: one for the formation of the lytic synapse and the polarized secretion of lytic granules and another for the stimulatory synapse formation and cytokine production²⁴⁸. Indeed, cytotoxicity can be triggered rapidly and by extremely low peptide concentrations (10^{-12} to 10^{-15} M)²⁴⁷⁻²⁴⁹. Induction of intracellular cytokine production was observed at peptide concentrations higher than 10^{-9} M and required a prolonged contact with the antigen presenting cells^{247,248}.

In our model of antigen stimulations, the T cells first lose the production of cytokines and at later stages lose lytic activity. If this was an effect of uncoupling, it could be due to insufficient peptide concentrations

or lower levels of surface TCR/CD3 complexes. However, peptide concentrations were steady. Furthermore, the peptide was supplied in various ways, including T2 antigen-presenting cells pulsed with 10^{-6} M of Melan-A.A2 peptide. Moreover the levels of CD3 ϵ , required for TCR/CD3 complex surface expression were identical on C0 to C10-derived T cells (Figure 13)²⁵⁰.

We thus tentatively conclude that the dysfunctional state to persistent antigen develops in a step-wise and progressive manner. The earliest sign of human T cell dysfunction that we observed is the loss of cytokine secretion, which is the result of a defect in intracellular cytokine production. The cytolytic activity was only observed in a severe dysfunctional state.

THE MAINTENANCE OF T CELL PROLIFERATION IS PROBABLY DUE TO EXOGENOUS IL2

The uncoupling of intracellular cytokine production and T cell proliferation has been engineered in murine T cells²⁵¹. The CD4⁺ T cells were derived from transgenic mice expressing various combinations of wild-type CD3 ITAMs (with 10 functional ITAMs) and mutant CD3 ITAMs (with 2-4 functional ITAMs). After ligation of the TCR-CD3, the complexes with only 2-4 functional ITAMs had impaired proliferative ability, keeping however the capacity to induce cytokine secretion. Thus higher multiplicities of TCR-CD3 ITAMs are required for TCR-driven proliferation, as compared to intracellular cytokine production. According to this study, we expect to observe a loss of proliferation before cytokine production. We thus hypothesize that proliferation was maintained by the exogenous addition of 50U/ml of IL2 in our culture conditions.

In a single preliminary experiment, we performed the antigen stimulations every 3-4 days in the presence of 50, 25 or 5 U/ml of IL2. The CD8⁺ T cells progressively lost IFN- γ secretion, independently of the concentration of IL2 (*data not shown*). Only with 5U/ml of IL2, we observed a decreased proliferation concurrent to the loss of IFN- γ secretion (*data not shown*), confirming our hypothesis. It also suggests that the dysfunctional CD8⁺ T cells retain the capacity to respond to IL2-induced proliferation²⁵². It raised up the following question: would

dysfunctional T cells be detectable *in vivo* (see below). Indeed CD8⁺ T cells that lack expression of the IL2 receptor are rapidly lost in mice chronic infection¹¹⁰. Noteworthy CD4⁺ T helper cells can provide exogenous IL2 *in vivo* and maintain human dysfunctional T cell survival. Indeed, *in vivo*, CD4⁺ T cells are major producers of IL2 and play a vital role in supporting CD8⁺ T cell response^{252,253}.

REPRODUCIBILITY

Our experimental approach is highly reproducible as we observed a dysfunction in 7 out of 8 polyclonal CD8⁺ T cells populations submitted to repeated antigen stimulations and all (n=2) clonal CD8⁺ T cell populations. It is important to notice an exception. The T cells from donor LB2265 had lost the ability to secrete cytokines after 12 stimulation cycles. In contrast with other donors, these T cells retain the ability to produce intracellularly cytokines upon re-activation. We are currently repeating expansion and stimulation cycles with other T cells isolated from the same donor. Moreover these T cells are used as control cells for gene expression experiments (see below). Indeed, the particular phenotype of these T cells could help us to decipher the human T cell dysfunction.

HUMAN CD4⁺ T CELL DYSFUNCTION

Murine CD8⁺ T cells are the primary model for T cell exhaustion. During LCMV C13 chronic infection, CD4⁺ T cells also lose effector function such as TNF- α and IFN- γ intracellular production^{108,254}. CD4⁺ T cell exhaustion seems to be distinct from CD8⁺ T cell exhaustion in some aspects. For example, CD4⁺ T cells lose effector functions earlier than CD8⁺ T cells^{101,102}.

We also submitted human polyclonal antigen-specific CD4⁺ T cells to several frequent stimulations. After 10 stimulation cycles, we observed dysfunctional human CD4⁺ T cells unable to produce intracellular TNF- α (*data not shown*). Thus, similarly to human CD8⁺ T cells, persistence of the antigen seems to induce human CD4⁺ T cell dysfunction. Nevertheless, additional experiments are needed to further assess the characteristics of human CD4⁺ T cells dysfunction compared to CD8⁺ T cells dysfunction.

The dysfunction is driven by antigen persistence

THE DYSFUNCTION CAN BE OBTAINED AFTER REPEATED STIMULATIONS WITH PEPTIDE-PULSED MATURE DENDRITIC CELLS

Tumors cells can evade immune surveillance by impeding T cell function by producing several immunosuppressive factors ²¹⁸, *i.e.* Indoleamine Dioxygenase (IDO), an enzyme that catalyzes the first step in tryptophan catabolism along the kynurenine pathway. IDO-expressing tumor cells consume available extracellular tryptophan depriving T cells of this essential amino acid ²⁵⁵. The metabolites generated by IDO, such as L-kynurenine, also can induce T cell dysfunction ²⁵⁶. Another inhibitory mechanism of tumors cells could be their metabolism that might generate high amounts of lactic acid, due to the up-regulation of glycolytic enzymes ²⁵⁷. *In vitro* experiments have shown the capacity of lactic acid to block intracellular cytokine production and diminish cytotoxic activity by T cells ²⁵⁸.

To exclude that intrinsic immunosuppressive characteristics of melanoma cells could interfere with the observed T cell dysfunction, we performed stimulation cycles of CD8⁺ T cells with peptide-pulsed mature dendritic cells. We also observed a loss of function. We conclude that T cell dysfunction was not due to a factor provided by melanoma cells.

THE DYSFUNCTIONAL STATE DOES NOT RESULT FROM CLONAL SELECTION

In a polyclonal CD8⁺ T cell population, the T cells that express a TCR with a high antigen avidity are preferentially driven to expansion ²⁵⁹. In our experimental approach, we first performed repeated and stimulation cycles with polyclonal anti-Melan-A.A2 CD8⁺ T cell. The dysfunctional state could thus result from clonal selection of highly proliferative T cell population with low effector functions. But human T lymphocyte clones derived from the same progenitor with a unique TCR have the same avidity for peptide-MHC. As we observed CD8⁺ T cell dysfunction with clonal T cell populations submitted to repetitive stimulation cycles, it indicates that human T cell dysfunction is not due to clonal selection.

THE DYSFUNCTIONAL CD8⁺ T CELLS ARE NOT ANERGIC

Naïve T cells primed in the absence of CD28-mediated signals have been shown to enter in a dysfunctional state, named anergy ⁶. Anergy can be induced in T cell clones in about 6-12 h by delivering a strong TCR signal in the absence of co-stimulation or by stimulating with a low-affinity ligand in the presence of co-stimulation. Naïve anti-Melan-A.A2 CD8⁺ T cells were primed with autologous mature dendritic cells 2 times every 14 days and the amplified anti-Melan-A.A2 T cells were functional (*Figure 9*), indicating that T cells did not enter in an anergic state.

Anergy was reported to require antigen persistence to maintain the unresponsive state and can be reversed by the addition of IL2 ^{4,6,260,261}. We did not observe any recovery of T cell functions after 3 weeks of antigen removal in the presence of IL2. We conclude that the dysfunctional state is distinct from the classical *in vivo* anergy that can be reversed in absence of antigen ⁶.

THE DYSFUNCTION DOES NOT RESULT FROM A CONVERSION OF FUNCTIONAL CELLS INTO REGULATORY CELLS

The group of Suzuki described a regulatory T cell population with a CD8⁺CD122⁺ phenotype in mice ²⁶². Murine CD8⁺CD122⁺ Tregs are CD122⁺ (IL2Rb), CXCR3⁺ CD44^{high}, CD62L^{high} CCR7⁺, and largely CD127⁻ ²⁶³⁻²⁶⁵. This population seems to be involved in the maintenance of T cell homeostasis ²⁶², in the control of conventional T cell responses ²⁶⁶⁻²⁶⁸ and in the regulation of autoimmune diseases ²⁶⁹. This population also has the capacity to inhibit murine allograft rejection ²⁷⁰. Suppressive activity of this regulatory T cell population is mediated by IL-10 ²⁶⁶. A human equivalent of murine CD8⁺CD122⁺ Treg has also been described. This population has CD8⁺CXCR3⁺CD45RA⁻CD62L⁺CCR7⁺ phenotype, but does not express CD122. These human cells also produce IL-10 and exhibit an *in vitro* suppressive activity by inhibiting IFN- γ production from CD8⁺CXCR3⁻ cells ²⁷¹.

The dysfunctional T cells derived from our experimental approach did not produce increased levels of the immune-suppressive cytokine IL-10 (*RNAseq data*), suggesting that the dysfunction does not reflect a conversion of functional T cells to a regulatory T cell phenotype.

Collectively, previous observations suggest that human T cell dysfunction is driven by antigen persistence. However, we cannot exclude that (i) a crucial factor for maintaining CD8⁺ T cell function is missing or (ii) both melanoma cells and mature dendritic cells provide immunosuppressive signals.

Antigen-driven human T cell dysfunction is distinct from T cell exhaustion

As mentioned before, I employ the term “T cell exhaustion” to define the T cell dysfunction that has been reported during chronic LCMV C13 infection in mice. Genome-wide transcriptional studies revealed a transcriptional program specific to these exhausted T cells. We thus compared the signature of these cells to the antigen-driven human T cell dysfunction studied during this thesis.

EFFECTOR FUNCTIONS

Loss of effector functions in murine exhausted T cells is hierarchical. Loss of IL2 production, high proliferative capacity and cytotoxicity are the first signs of T cell exhaustion. Subsequently, TNF- α intracellular production is lost at intermediate stages, and IFN- γ production and degranulation are lost in more severe stage of exhaustion^{99,272}.

Similarly to murine T cells, human CD8⁺ T cells lose effectors functions in a hierarchical, however distinct, manner. We observed that human CD8⁺ T cells lose the capacity to produce all cytokines (IFN- γ , TNF- α and IL2) simultaneously while cytotoxicity was lost by human CD8⁺ T cells later.

TRANSCRIPTION FACTORS

A transcription factor specific for exhausted T cell has not been identified. But several transcription factors have been proposed as important for the coordination of gene expression networks in murine exhausted CD8⁺ T cells. These include *T-bet*, *Eomes*, *Prdm1* and *NFAT*.

However, we did not observe a differential expression of these transcription factors between functional and dysfunctional human T cells in the transcriptomic analysis.

INHIBITORY RECEPTORS

Transcriptional analysis of murine exhausted T cells identified the inhibitory receptor PD-1 as negative regulatory pathway operating in T cell exhaustion¹²². *In vivo* blockade of the PD-1/PD-L1 pathway during chronic LCMV C13 infection was shown to increase the functions of virus-specific CD8⁺ T cells and result in a lower viral load¹²². In addition to PD-1, several inhibitory receptors are expressed at the surface of exhausted T cells, such as CTLA-4, LAG-3, TIM3, CD160 and 2B4^{100,130}. Inhibitory receptors are considered by many authors as a hallmark of T cell exhaustion.

Many of these inhibitory receptors were up-regulated at the surface of human chronically stimulated CD8⁺ T cells, but their expression level was similar on functional and dysfunctional T cells (*Figure 33*). *In vitro* blockade of PD-1/PD-L1 axis with antibodies did not increase the ability of dysfunctional T cells to secrete IFN- γ upon stimulation in our hands.

In conclusion, these results indicate that the dysfunctional state induced *in vitro* by repetitive antigen stimulations is distinct from the exhaustion.

Should we reconsider the role of inhibitory receptors in T cell dysfunction

SURFACE EXPRESSION OF INHIBITORY RECEPTORS IS NOT SUFFICIENT TO DEFINE T CELL DYSFUNCTION

After several publications on the role of inhibitory receptors in murine T cell exhaustion, expression of these receptors was used as a marker of human T cell dysfunction in chronic viral infection and cancer^{184,189,196,273-275}. However, both in mice and human, some studies have already shown that the expression of inhibitory receptors is not enough to define a T cell

as dysfunctional. In the context of Acute Friend retrovirus infection in mice, CD8⁺ T cells that have up-regulated PD-1 are nevertheless highly cytotoxic and able to control the infection ²⁷⁶. In the context of cancer, human TIL express PD-1⁺, BTLA⁺, TIM3⁺, but proliferate *ex vivo* and can mediate potent antitumor responses upon adoptive transfer into patients ²⁷⁷.

In our experimental approach, C2-C5 T cells express inhibitory receptors but are functional. Therefore, together with other reports, our study supports the notion that the expression of inhibitory receptors is not sufficient to define a T cell as dysfunctional.

ARE INHIBITORY RECEPTORS MARKERS OF T CELL ACTIVATION?

PD-1 was discovered in 1996 as a protein up-regulated in T and B lymphocytes following *in vitro* activation ²⁷⁸. This pre-dated the association of PD-1 to T cell exhaustion. Several reports have linked expression of inhibitory receptors to T cell activation ²⁷⁹⁻²⁸². Indeed, in the LCMV mouse model of T cell exhaustion, inhibitory receptors are expressed in the early phases of both acute and chronic LCMV infection and the expression was not correlated with dysfunction ¹³⁴. Sustained expression of inhibitory receptors is maintained only in chronic infection. Perhaps this is mediated by antigen persistence, which maintains CD8⁺ T cells in an activated state. In accordance, CD8⁺ T cells revert into a resting state after antigen clearance during which the expression of CTLA-4 and PD-1 is down-regulated in the acute infection model.

In agreement with this hypothesis, we observed a down-regulation of inhibitory receptors at the surface of human dysfunctional T cells rested for three weeks in absence of antigen, as compared to T cells examined 3-4 days after the last stimulation.

MOLECULAR MECHANISM DOWNSTREAM OF INHIBITORY RECEPTOR SIGNALING

The understanding of molecular mechanism downstream inhibitory receptor signaling is incomplete. For example, the PD-1 mechanism of action is defined as follows: (i) PD-1 possesses an ITIM motif, (ii) upon

ligation with PD-L1, phosphatases SHP1 and/or SHP2 are recruited to PD-1, (iii) SHP1 and/or SHP2 inhibit early T cell signaling and functions by inducing Zap70 dephosphorylation and de-activation^{123,124,221}. According to this view, blocking the PD-1/PD-L1 pathway *in vitro* in PD-1⁺ T cells should rapidly increase T cell function. Also, the engagement of PD-1 on PD-1⁺ T cells should rapidly inhibit T cell function. However, it was reported that beads coated with PD-L1 fail to inhibit activation of PD-1⁺ T cells²⁸². We also failed to increase IFN- γ secretion by PD-1⁺ TILs after treating them for a few hours with anti-PD-1 and anti-CTLA-4 blocking antibodies (*our unpublished data*).

Interestingly, *in vitro* studies that showed overcome of PD-1/PD-L1 inhibition on T cell functions were performed on 5-days T cell co-cultures with anti-PD-1 or -PD-L1 antibody. If the engagement of PD-1 and PD-L1 block T cell functions, we would expect to see this effect after an overnight treatment at the latest. However, if five days are needed to show the inhibitory functions of the PD-1/PD-L1-axis, we tentatively suggest that this is rather due to an effect on T cell proliferation than effector functions^{184,189,201,274,275}.

A recent report showed that PD-1 does not inhibit TCR signaling but CD28-induced signaling. The study used *in vitro* a cell-free reconstitution system in which the cytoplasmic domain of PD-1, CD3 ζ , CD3 ϵ , CD28, ICOS, DAP10, CD226, CD96, TIGIT, and CTLA-4 were bound to the surface of large unilamellar vesicles that mimic the plasma membrane of T cells²⁸³. They observed that the phosphatases SHP2 has a binding specificity for phosphorylated PD-1 (superior than CTLA-4 and TIGIT) and dephosphorylates CD28 with a higher sensitivity compared to CD3 ζ and CD3 ϵ ²⁸³. These observations were confirmed in Jurkat cells. In agreement with these data, anti-PD-L1/PD-1 therapy to restore antiviral and antitumor T cell responses depends on CD28/B7.1 and/or B7.2 pathway²⁸⁴. Indeed, antagonists of CD28/B7 pathway and conditional deletion of CD28 in virus-specific T cells annihilate the effect of anti-PD-L1 in chronic LCMV infection. In mice, B7-costimulation was also necessary for effective anti-PD-L1 treatment in tumor-bearing mice since anti-B7 antibody induce tumor progression in anti-PD-L1 treated mice. These data have to be considered carefully. Indeed, CD28 is

expressed by close to 100% of mouse CD4⁺ and CD8⁺ T cells. In contrast, only 80% of human CD4⁺ and 50% of human CD8⁺ T cells express CD28²⁸⁵.

Although PD-1 is expressed on human T cells in our experimental approach, blocking PD-1/PD-L1 or inhibiting phosphatases for 20 h or 5 days yielded no significant effect on the ability of T cell function or proliferation. This observation strengthens the notion that additional studies are needed to decipher molecular pathways downstream of PD-1, and all inhibitory receptors.

The early phase of dysfunction can be reverted with galectin antagonists

Since the presence of inhibitory receptors did not explain antigen-driven human CD8⁺ T cell dysfunction, we examined a possible role of galectin-3. Galectins belong to a family of lectins that share the ability to bind b-galactosides and contain homologous carbohydrate recognition domains. Galectins bind to natural glycoconjugate ligands expressed on cell surfaces. We reported that treatment of TIL with galectin ligand LacNAc boosted secretion of cytokines by TIL upon *in vitro* re-activation^{193-195,216}. Why do galectin-3 ligands improve TIL function? Compared to recently activated T cells resting T cells harbor a set of glycans that are more numerous and better ligands for galectin-3²⁸⁶. Galectins participate in the formation of galectin-glycoprotein lattices. This restricts the mobility of surface T cell receptors which is indispensable for efficient T cell activation^{216,286}.

We observed the presence of surface galectin-3 on T cells after several stimulation cycles. Treating the cells with a galectin antagonist, LacNAc or an anti-galectin-3 antibody, reversed the gradual loss of cytokine secretion observed during the first cycles of antigen stimulation.

Could inhibiting the formation of galectins-glycoprotein lattices formation prevent the later phase of antigen-driven human T cell dysfunction? A recent report points out that galectin lattices play a role in

the signaling mediated by co-stimulatory molecules. Glycoprotein-galectin lattices trap LFA-1 resulting in the lack of its recruitment to the immunological synapse ²¹⁶. The costimulatory molecule CD28 is glycosylated and could potentially be impeded by galectins. Costimulatory signals are essential to induce AP1 transcription factor. NFAT, in the absence of cooperation with the unrelated transcription factor AP-1 can drive the expression of genes associated with T cell dysfunction ¹⁶¹. It would therefore be interesting to perform our chronic antigen stimulation protocol in the presence of galectin antagonists to examine whether the late phase of T cell dysfunction is prevented.

The molecular signature of human dysfunctional CD8⁺ T cells

TRANSCRIPTOME ANALYSIS AND CANDIDATE GENES

The main causes of T cell dysfunction reported in the literature could not explain the *in vitro*-induced T cell dysfunction. It raised the hypothesis that a new inhibitory mechanism could mediate this dysfunction. From the transcriptome analysis of dysfunctional versus functional human CD8⁺ T cells 27 candidate genes were identified. Genes coding for effector function proteins (*i.e.* *GZMB*, *IFNG*) were excluded from further analysis because their lower expression levels in dysfunctional T cells probably arises from an altered T cell activation state. Genes that were differentially expressed in re-activated cells but showed no differential expression before re-activation were not selected either, as the observed difference could result from a less efficient activation of the dysfunctional T cells. Genes that showed differential expression patterns at the RNA level but not in regard to the protein level, such as DNAM-1 were also disregarded at this point of the investigation. Genes that were not differentially expressed between functional and dysfunctional T cells for all donors, such as *RNF125*, were temporary excluded for further analysis.

In the transcriptome analysis, we focused our attention on *TIMP3* and *SPARC* mainly because of their high expression in dysfunctional T cells. We confirmed by RT-qPCRs that *TIMP3* and *SPARC* were more

highly expressed in dysfunctional T cells compared to functional T cells before re-activation (*Figure 30*). The release of TIMP3 and SPARC in the supernatant of T cells cultures was inversely correlated to the secretion of IFN- γ (*Figure 31*). Interestingly, expression of TIMP3 and SPARC was very low at C0. Both genes were silent in blood T cells.

TIMP3

TIMP3 is a secreted protein, which inhibits matrix metalloproteinases (MMPs). Those are responsible for the degradation of the extracellular matrix. Among its targets are gelatinases, MMP2 and MMP9, which respectively digest gelatin and several molecules of the extracellular matrix such as collagens, laminin and aggrecan. TIMP3 expression is observed in most normal tissues. It is down-regulated in some types of tumors. Its expression reduces tumor growth, angiogenesis and macrophage infiltration. While its inhibition increases tumor growth and angiogenesis in human melanoma cells lines in xenograft mice models²⁸⁷.

The impact of TIMP3 on the immune system is poorly understood. MMP2 and MMP9 are produced by murine T cells and regulate T cell proliferation. Inhibition of MMP2 and 9 leads to a 80% decrease in the proliferation of CD4⁺ and CD8⁺ T cells and a 25% decrease of IL2 secretion²⁸⁸. It is possible that MMPs act on lymphocyte functions as NFAT expression is decreased in their absence²⁸⁸. Finally, the recruitment of T cells was significantly disrupted in mice deficient for MMP9²⁸⁸.

TIMP3 also inhibits A Disintegrin and Metalloproteases (ADAM) 10 and 17. Both proteases are responsible for the first cleavage of Notch, which is then further cleaved by a γ -secretase. This cleavage induces the release of the intracellular domain of Notch (NICD), which can then translocate to the nucleus. Notch1 is also activated by MT1-MMP in a non-canonical fashion²⁸⁹. Similarly to ADAMs 10 and 17, TIMP3 inhibits MT1-MMP and thus could inhibit Notch1 non-canonical activation. The Notch signaling pathway appears to be essential for the differentiation and the effector functions of CD8⁺ T lymphocytes²⁹⁰. Notch acts positively on the regulation of cytolytic effector functions by direct binding of NICD on

the *GZMB* promoter ²⁹¹. Interestingly, we observed a decrease in *GZMB* expression in our dysfunctional T-cells.

SPARC

SPARC, also known as osteonectin, is another secreted protein. SPARC is able to bind calcium and participates in the reorganization of the extracellular matrix. It inhibits cell adhesion, which has a direct impact on cell migration, either positive or negative depending on the level of SPARC secreted ²⁹². SPARC expression is known to be up-regulated in many different types of tumors ²⁹³. It can be produced by both reactive stromal cells and cancer cells. It was reported to be induced by hypoxia and acidity at the tumor-stroma interface ²⁹⁴.

Few reports have described the function of SPARC in the context of immunity. However, its influence on the migration of antigen presenting cells, organization of the tumor stroma, and the presence of growth factors is proven. Its impact on cell mobility allows easier migration of Langerhans cells in SPARC^{KO} mice ²⁹⁵. Moreover, SPARC deletion has a direct impact on tumor development in mice. It results in the disruption of the surrounding stroma, which allows the tumor mass to expand. The stroma appears to have a protective role, creating a shield around the tumor mass. It also hampers the penetration of leukocytes to the tumor site. The presence of SPARC could therefore alter the contact between tumor cells and immune cells ²⁹⁶. Cells lacking the SPARC gene were reported to produce less TGF- β . It has also been reported that SPARC^{KO} mice exhibit accelerated activation of naive T cells suggesting that SPARC is a negative regulator of T cell function ²⁹⁶.

Future perspectives

HYPOTHESIS 1: TIMP3 AND/OR SPARC CAUSE OF T CELL DYSFUNCTION

We take into consideration that the two proteins, SPARC and TIMP3, could interact in the culture medium to induce T cell dysfunction, or that secreted protein A (*i.e.* SPARC) could induce a signaling pathway to

induce the expression of protein B (*i.e.* TIMP3). The following approaches will be considered for these genes independently or in combination in future studies.

To further examine the role of TIMP3 and SPARC, we will force the expression of TIMP3 and/or SPARC to induce the dysfunctional phenotype in functional T cells. We will use recombinant lentiviruses containing either a TIMP3-IRES-GFP and/or a SPARC-IRES-mCherry construct, and select single or double positive T cells by flow cytometry followed by functional assays. Functional assays will include cytokine secretion, intracellular cytokine production, and cytolytic activity.

In a second approach, we will inhibit TIMP3 and/or SPARC in dysfunctional T cells to block the expression of these two genes and examine if T cell functions are restored. Since TIMP3 and SPARC are secreted proteins, we could use blocking antibodies. However, no described blocking antibodies are available from commercial sources. In a preliminary experiment, polyclonal antibodies anti-TIMP3 and –SPARC failed to increase IFN- γ secretion by dysfunctional T cells. We therefore decided to produce monoclonal antibodies against TIMP3 and SPARC lab-internally. Alternatively, we will inhibit TIMP3 and/or SPARC expression by using siRNA, shRNA or CRISP/CAS9 approaches in dysfunctional T cells.

Previously, we assumed that T cell dysfunction is reversible. However, the dysfunctional cells are perhaps in an irreversible terminal differentiation state. Therefore, we will examine whether inhibition of SPARC and/or TIMP3 can prevent T cell dysfunction. We will inhibit these genes by CRISPR/Cas9 technologies in C0 functional T cells and perform repeated antigen stimulations. The limitation of this approach will be the selection of the modified CD8⁺ T cells. C0 functional T cells do not express TIMP3 and SPARC. Therefore, the deletion of the gene cannot be assessed by ELISA. To select genetically modified T cells we will clone infected T cells with a CRISPR/Cas9 construct and sequence the target region of the guide RNA.

Epigenetic regulations may provide a better understanding of the gradual up-regulation of TIMP3 and SPARC observed in T cells submitted

to antigen stimulation cycles. In a preliminary experiment, DNA was extracted from the same samples that have been used for the transcriptomic approach and from the “non-conventional” donor that does not lose the cytokine production. Samples were analyzed for genome-wide methylation, using the “Infinium Methylation” approach. The Infinium Methylation Assay provides quantitative methylation measurement at the single-CpG-site level^{297,298}. The methylation status of more than 480,000 cytosines distributed over the whole genome is assessed. The Infinium Methylation Assay detects cytosine methylation at CpG islands based on multiplexed genotyping of bisulfite-converted genomic DNA (gDNA). Upon treatment with bisulfite, unmethylated cytosine bases are converted to uracil, while methylated cytosine bases remain unchanged. The assay interrogates these chemically differentiated loci using two site-specific probes; one designed for the methylated locus (M bead type) and another for the unmethylated locus (U bead type). Single-base extension of the probes incorporates a labeled ddNTP, which is subsequently stained with a fluorescent reagent. The level of methylation for the interrogated locus can be determined by calculating the ratio of the fluorescent signals from the methylated versus unmethylated sites. PCA analysis showed a different signature between functional and dysfunctional CD8⁺ T cells for the same donor except for the “non-conventional” donor (*data not shown*). However, the methylation levels were not statistically different at any CpG site, after multi-testing correction, probably due to the low number of samples. We therefore decided to analyze the probes associated to TIMP3 and SPARC. No probes associated to SPARC were differentially methylated. However, four cytosines associated to TIMP3 were differentially methylated between functional and dysfunctional cells. These data have to be confirmed by bisulfite sequencing of the TIMP3 promoter.

Our two candidate genes, TIMP3 and SPARC, are silent in blood T cells, whether they are re-activated or resting. The two genes are both expressed/up-regulated in TILs suggesting that they could be therapeutic targets for immunotherapy. A mandatory step before clinical trials is to prove the efficacy of a therapeutical approach in tumor-bearing animals. First, we will examine whether our T cell dysfunction phenomenon takes place in murine T cells. In line with our experiments with human T cells,

we will isolate murine tumor-infiltrating lymphocytes from tumor ascites and solid tumors and test the expression of *Timp3* and *Sparc* by RT-qPCR. An alternative approach would be to analyze the effect of deleting *Sparc* or *Timp3* in CD8⁺ T cell completely (*Gzmb*-Cre X *Timp3*^{flox/flox} or *Sparc*^{flox/flox}) in tumor bearing mice or in chronic viral infection. Addition of a fluorescent protein under the control of *Timp3* and *Sparc* promoter would allow to study the expression kinetics of these genes.

HYPOTHESIS 2: TIMP3 AND/OR SPARC DO NOT CAUSE THE T CELL DYSFUNCTION

To reduce the number of candidate genes, we first decided to focus on the 27 genes differentially expressed and commonly identified by three analysis methods used. We will therefore consider other genes differentially expressed. We will focus first on the list of candidates genes observed with the edgeR method. Indeed, edgeR introduces potentially more false positives²²⁸. We take therefore less risk to miss an interesting gene.

Candidate genes up-regulated in dysfunctional cells will be screened in Jurkat cells, which are an immortalized cell line of T lymphocyte cells. A research group recently designed a triple parameter reporter based on a human Jurkat T cell line²⁹⁹. In this cell line, upon T cell activation, response elements for NF-κB, NFAT and AP-1 drive the expression of the fluorescent proteins CFP, eGFP and mCherry, respectively. In addition to allow a screening of candidates genes, this cell line will provide additional information about the signaling pathway implicated. The cDNAs of candidate genes will be introduced in a XX-IRES-puromycin construct and introduced in this cell line. As the Jurkat cell line is easily transducible, it will allow a rapid screening of candidate genes.

We could also use a wide approach with shRNA libraries. Short hairpin RNA (shRNA) are artificial RNA sequences that can be used to silence genes via RNA interference. shRNA expression vectors can be stably integrated into a target cells genome via retro- or lenti-viral gene transfer. These vectors contain a selection marker, puromycin or GFP. A number of pooled screens have been designed, with viral pools containing

up to 95,000 different shRNA³⁰⁰. The difficulty of this approach is to find a method to enrich shRNA that favors the phenotype of interest. In our experimental approach, the phenotype of interest is CD8⁺ T cells that will recover effector functions. Thus, shRNA libraries will be transfected in C7 partially dysfunctional T cells. Transfected cells will be subjected to five additional antigen stimulation cycles. After each stimulation cycle, cells will be enriched according to the selection marker and the function (IFN- γ secretion by flow cytometry). The modulator shRNA will be identified by sequencing.

Relevance of the model *in vivo*

We were concerned about the relevance of our *in vitro* observations about T cell dysfunction to *in vivo* conditions. Since we have not yet identified the cause of T cell dysfunction, we cannot assess whether this mechanisms inhibit T cells in chronic viral infection or in cancer. Nevertheless, we have a gene signature of our *in vitro*-induced dysfunctional T cells. Gene Set Enrichment Analysis (GSEA) is a method that determines whether two biological processes show statistically significant concordant differences within groups of genes³⁰¹. We therefore compared the gene signature of our dysfunctional T cells with that of T cells from human tumors using the transcriptional profiles of tumor-reactive human Mart-1/Melan-A-specific CD8⁺ T cells isolated from lymph node metastases (TILN) from melanoma patients¹⁹⁶. Interestingly, some genes down-regulated in TILN were also down-regulated in our dysfunctional T cells (*data not shown*). However, we found also that a set of genes up-regulated in TILN were more expressed in functional T cells. This set of genes up-regulated in TILN include *IFNG*, *GZMB* and *SLC7A5*. This observation was unexpected considering that TILN are supposed to be dysfunctional. We are not convinced that TILN signature is representative of a TIL signature.

We thus decided to isolate TIL from solid tumor and to analyze the expression of TIMP3 and SPARC. We observed that freshly isolated TIL express these genes. This observation indicates that the molecular signature of antigen-driven human T cell dysfunction can be found *in vivo*

DISCUSSION

in T cells under persistent antigen exposure. However, their expression in TIL does not demonstrate the causative role of TIMP3 and/or SPARC in TIL dysfunction.

Concluding remarks

The data presented in this thesis suggest reconsidering the surface expression of inhibitory receptors as markers of T cell dysfunction. Indeed, we show a functional T cell population expressing several inhibitory receptors as activation markers. This T cell population becomes gradually dysfunctional upon sequential antigen stimulation cycles, without any correlation with the expression of inhibitory receptors. Our preliminary results strongly suggest that we are unraveling a new mechanism of T cell dysfunction. The phenotype of our dysfunctional human T cells does not correspond to the phenotype of the T cells reported to be exhausted/dysfunctional in animal models. Why is this dysfunctional mechanism unknown until now? One reason could be that many research groups are searching for T cell regulatory mechanism using animal models. Although mice are the preferential animal model for *in vivo* immunological experimentation, significant differences between the murine and human immune system exist. In our research group, we mainly focus our work on human T cells. In the past this strategy allowed us to identify the role of galectin-3 in the dysfunction of TILs; a mechanism that had never been identified before in animal models and is not yet formally proven to take place in tumor-bearing mice. We hope to identify target molecules for the next generation of immunotherapy using the approach described in this thesis. Indeed, phase-I trials in lung cancer patients with anti-PD-1 antibody nivolumab show excellent durable overall radiological response rates in the 25% range. However, this example also shows that room for improvement exists.

DISCUSSION

MATERIALS AND METHODS

Cell lines, media, and reagents

LG2-EBV cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Life Technologies) supplemented with 10% fetal calf serum (FCS), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml Penicillin and 100 µg/ml streptomycin (P/S), hereafter referred to FCS complete medium. T2 cells were cultured in FCS complete medium supplemented with 0.075 mM β-mercaptoethanol. CP50-MEL.1 melanoma cells were cultured in FCS complete medium supplemented with 100 nM hydrocortisone, 50 µg/ml insulin, 1 mg/ml transferrin, 100 nM 17β-oestradiol and 300 nM Sodium selenite. Human recombinant interleukin (hIL)-2 was from Novartis, hIL-7 from Miltenyi Biotec, hIL-6 from Peprotech and hIL-12 from Wyeth-Ayerst. Melan-A multimer and MUM-3 multimer were obtained in the laboratory by folding respectively HLA-A*0201 with Melan-A peptide EAAGIGILTV or HLA-A68 with MUM-3 peptide EAFIQPITR. Biotinylated complexes were mixed with either streptavidin-phycoerythrin (PE) or streptavidin-allophycocyanin (APC).

CTL isolation and amplification

Samples of patient-derived blood and solid tumor were collected after approval by the institutional review boards of all collaborating institutions. Mononuclear cells were isolated from blood of donors without cancer, more precisely hemochromatosis patients, by lymphoprep (*Axis-Shield PoCAS*). Mature dendritic cells (DCs) were obtained as previously described in Ottaviani, *Cancer Immunol Immunother*, 2005³⁰². CD8⁺ T cells were sorted by a positive selection strategy (Miltenyi Biotec) from non-adherent cells. CD8⁺ T cells were incubated at room temperature (RT) with 20 nM A2/Melan-A/tetramer-APC. After 10 min, 1 µg/ml of anti-CD8α-FITC (LICR Brussels) was added to the solution for 15 min at RT. Cells were washed, sorted on a FACS ARIA III (BD Biosciences) and were stimulated in complete medium with 10% human serum (HS complete medium) supplemented 50 U/ml of hIL2, 10 ng/ml of hIL-7, 10 ng/ml of hIL-12 and

1,000 U/ml of hIL-6. CTLs were cultured in 96-well ultra low attachment plates round bottom with DCs, previously incubated for 1 h at 37°C with 1 µg/ml of Melan-A peptide and washed, at a ratio of 1 T cells: 3 DCs. After 14 days, anti-Melan-A.A2 CD8⁺ T cells were stimulated at a ratio of 1 T cells: 2 DCs.

LB33 CD8⁺ T cells were sorted by a positive selection strategy (Miltenyi Biotec) from peripheral blood mononuclear cells (PBMCs) of a melanoma patient. CD8⁺ T cells were incubated at RT with 20nM A68/MUM-3/tetramer-APC (LICR Brussels). After 10min, 1µg/ml of anti-CD8α-FITC (LICR Brussels) was added to the solution for 15min at RT. Cells were washed, sorted on a FACS ARIA III (BD biosciences) and stimulated in HS complete medium supplemented with 50 U/ml of hIL2 and 10 ng/ml of hIL-7, 10 ng/ml. CTLs were cultured in 96-well ultra low attachment plates round bottom with TNF-α and IFN-γ pre-treated LB33-MEL.1, at a ratio of 1 T cells: 2 melanoma cells. After 14 days, anti-Melan-A CD8⁺ T cells were stimulated at a ratio of 1 T cell: 1 melanoma cell.

Donor Gdc50's clone was isolated from the solid tumor of patient with breast cancer. Cells were extracted by mechanical disruption and by enzymatic digestion according to the MACS human tumor dissociation kit protocol (Miltenyi Biotec). The resulting cell suspension was washed with RPMI (Life Technologies) 1640 medium supplemented with 2% of HS, stained for cell sorting of CD8⁺ T cells with a mix of 1 µg/ml anti-CD3 (UCHT1; Biolegend), 1 µg/ml of anti-CD8α-FITC (LICR Brussels) and 0,5 µg/ml anti-CD4-PE (RPA-T4; Biolegend) during 30 min at 4°C in 1 mL of locally produced Hanks' balanced salt solution supplemented with 1% of HS (staining medium), and washed once. Living CD3⁺ CD8⁺ CD4⁻ cells were sorted on a FACS Aria III (BD Biosciences) as one cell per well in a 96-well plate (Costar). These clones were expanded in HS complete medium with IL2 (50 U/ml), IL-7 (10 ng/ml) gentamycin (50 µg/ml). Stimulations of the clone was performed by a cocktail of 40 ng/ml monoclonal anti-CD3 antibodies (OKT3; Janssen-Cilag), irradiated allogenic feeder cells (6 x 10⁴ LG2-EBV-B cells/well and 3 x 10⁵ PBMCs from different donors/well). Stimulations were repeated every 10 days.

Multimer labeling

Cells were incubated at RT with 10 nM A2/Melan-A/multimer-PE. After 10 min, 1/40 of anti-CD8 β -APC (clone 2ST8.5H7; BD PharMingen) was added to the solution for 15 min at RT. Cells were washed and fixed with 1% formaldehyde (w/v in PBS). Data were acquired on a FACS Fortessa (BD Biosciences) and analyzed using software FlowJo.

Stimulation cycles

Anti-Melan-A.A2 CD8⁺ T cells were stimulated in 2 ml HS complete medium supplemented with 50 U/ml hIL2. CTLs (3×10^5) were cultured in 24-well plates with 10^6 irradiated allogeneic LG2-EBV feeder cells and stimulated either with irradiated tumor cells (3×10^5) or irradiated T2 cells (3×10^5), which were previously incubated for 1 h at 37°C with 1 μ g/ml of Melan-A peptide and washed. For frequent and repeated stimulation with dendritic cells, CTLs (6×10^5) were cultured in 24-well plates in presence of hIL-7, hIL-12 and hIL-6, with irradiated mature dendritic cells (6×10^5), previously incubated for 1 h at 37°C with 1 μ g/ml of Melan-A peptide and washed. Twice a week, T cells were counted. One part was frozen and the other part was stimulated in the same conditions, as indicate above.

Secretion assays

A total of 10^4 T cells were plated in round-bottom microwells with either 10^4 T2 cells previously incubated at 37°C for 1 h with Melan-A peptide, with 3,333 beads coated with anti-CD3 and anti-CD28 antibodies (Dynabeads, Invitrogen), or with a cocktail of 1 ng/ml Phorbol-12-Myristate-13-Acetate (LC laboratories) and 1 μ g/ml ionomycin (Sigma-Aldrich). Stimulation with CP50-MEL.1 was performed in flat bottom microwells with 10^4 T cells and 10^4 CP50-MEL.1. Cytokines were measured by enzyme-linked

immunosorbent assay in the supernatants of 20 h co-culture using Biosource Cytoset reagents (IFN- γ ; Life Technologies) or by multiplex assay using the Bio-Rad Bioplex system (TNF- α and IL2).

For TIMP3 and SPARC secretion, a total of 5×10^4 T cells were plated in round-bottom microwells for 20 h. Protein secretion was measured by enzyme-linked immunosorbent assay in the supernatants using DuoSet reagents (R&D system).

Immunofluorescence and Flow cytometry

A total of 5×10^4 T cells were plated in round-bottom microwells with 16,666 beads coated with anti-CD3 and anti-CD28 antibodies (Dynabeads, Invitrogen). For intracellular cytokine detection: after 1 h, 5 μ g/ml of brefeldinA were added to the co-culture. After an additional 17 h of incubation, cells were fixed in 1% PFA, permeabilized with 0.1% saponin and stained with anti-IFN- γ -PE (clone B27), anti-IL2-APC (clone MQ1-17H12) and anti-TNF α -AlexaFluor 700 (clone MAb11; all from BD Pharmingen) for 30 min at RT.

For inhibitory receptor analyses, T cells were stained for 20 min at 4°C with a mix of antibodies (listed below). Data were acquired on a FACS Fortessa and analyzed using software FlowJo.

	Antibodies used for surface expression of inhibitory receptors
Mix 1	anti- PD-1 PE-Cy7 1/5 (<i>Biolegend, clone EH12.2H7, 329918</i>) anti- CTLA-4 APC 1/5 (<i>Becton Dickinson, clone BNI3, 555855</i>) anti- BTLA PE 1/5 (<i>Biolegend, clone MIH26, 344506</i>) anti- TIM3 FITC 1/20 (<i>R&D Systems, clone 344823, FAB2365G</i>) anti- NKG2A PerCP 1/10 (<i>R&D Systems, clone 131411, FAB1059C</i>) anti- CD25 AlexaFluor 700 1/20 (<i>Biolegend, clone BC96, 302622</i>) anti- CD8 APC-Cy7 1/20 (<i>Becton Dickinson, clone SK1, 557834</i>) anti- CD2 PE-Texas Red 1/540 (<i>Invitrogen, clone S5.5, CD0217</i>)
Mix 2	anti- CD160 AlexaFluor 488 1/20 (<i>Becton Dickinson, clone BY55, 562351</i>) anti- LAG3 biotinylated 1/20 (<i>R&D Systems, polyclonal, BAF2319</i>) anti- CD200R PE 1/30 (<i>Biolegend, clone 0X-108, 329306</i>) anti- CD244 (2B4) APC 1/20 (<i>Biolegend, clone C1.7, 329512</i>) anti- CD38 AlexaFluor 700 1/150 (<i>Biolegend, clone HIT2, 303524</i>) anti- CD39 PerCP-eFluor 710 1/20 (<i>eBioscience, San Diego, California, clone A1, 46-0399-42</i>) anti- CD73 PE-Cy7 1/60 (<i>Biolegend, clone AD2, 344010</i>) anti- CD2 PE-Texas Red 1/540 (<i>Invitrogen, clone S5.5, CD0217</i>)
Mix 3	anti- KIR APC 1/20 (<i>R&D systems, clone 180704, FAB1848A</i>) anti- CEACAM1 PE 1/50 (<i>Becton Dickinson, clone B1.1, 562361</i>) anti- KLRD1 (<i>Becton Dickinson, clone HP-3D9, 333706</i>) anti- ILT2 biotinylated 1/25 (<i>Biolegend, clone GHI/75, 333706</i>) anti- NKRP1A PE-Cy7 1/20 (<i>Biolegend, clone HP-3G10, 339918</i>) anti- CD2 PE-Texas Red 1/540 (<i>Invitrogen, clone S5.5, CD0217</i>)

For CD3 ζ expression, T cells were fixed in 1% PFA, permeabilized with 0.1% saponin and stained with anti-CD3 ζ (Clone 2H2D9; Beckman Coulter) for 30 min at RT.

β -galactosidase activity

A total of 10^6 T cells were incubated 10 min at 37°C. A solution of 2 mM fluorescein di-V-galactoside (ThermoFisher Scientific) was added to the cells for 1 min at 37°C. Reaction is stopped by addition of 10 μ g/ml of

Propidium Iodide (Sigma Aldrich). Data were acquired on a FACS Fortessa and analyzed using software FlowJo.

Quantitative PCR with reverse transcription

T cells were activated with beads coated with anti-CD3 and anti-CD28 antibodies (ratio 1 bead:10 T) by 1 min centrifugation and cocultured for 20 h. Then total RNA was extracted from cells, prepared with Macherey-Nagel Nucleospin RNA kit, and reversed transcribed with Superscript II (Invitrogen). PCR amplifications were performed in a final volume of 20 µl with TaqMan gene expression Mastermix (ThermoFisher Scientific), primers, probes and cDNA. Reaction was run on a StepOnePlus Real-Time PCR System (ThermoFisher) under standard conditions: 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were performed in technical triplicates. Primers (Eurogentec) and probes (Eurogentec) can be found hereunder.

Gene	Primers and probes
<i>IFNG</i>	Sens primer : CTAATTATTCGGTAACTGACTTGA (<i>Eurogentec, Liège, Belgium</i>) Antisens primer : CGAAACAGCATCTGACTCCTT (<i>Eurogentec</i>) Probe : TCCAACGCAAAGCAATGAACTCATCC (<i>Eurogentec</i>)
<i>GZMB</i>	Sens primer : AGTGCGAATCTGACTTACGCCA (<i>Eurogentec</i>) Antisens primer : GCAGGCTCGTGGAGGCAT (<i>Eurogentec</i>) Probe : CATTGTCTCCTATGGACGAA (<i>Eurogentec</i>)
<i>EEF1a</i>	Sens primer : GCTTCACTGCTCAGGTGAT (<i>Eurogentec</i>) Antisens primer : GCCGTGTGGCAATCCAAT (<i>Eurogentec</i>) Probe : AAATAAGCGCCGGCTATGCCCTG (<i>Eurogentec</i>)
<i>ITK</i>	Primers and probe (<i>Life Technologies, Carlsbad, California, hs00950634-m1</i>)
<i>LCP2</i>	Primers and probe (<i>Life Technologies, hs01092642_m1</i>)
<i>SLC7A5</i>	Primers and probe (<i>Life Technologies, hs01001190-m1</i>)
<i>AGPAT9</i>	Primers and probe (<i>Life Technologies, hs00262010-m1</i>)
<i>IKZF4</i>	Primers and probe (<i>Life Technologies, hs01042455-m1</i>)
<i>RNF125</i>	Primers and probe (<i>Life Technologies, hs01563016-m1</i>)

<i>TIMP3</i>	Primers and probe (<i>Life Technologies, hs00927214_m1</i>)
<i>SPARC</i>	Primers and probe (<i>Life Technologies, hs00277762-m1</i>)

Cytotoxicity assay

Target cells, T2 cells and CP50-MEL.1 cells were labeled for 1 h with 50 μ Ci of Na[⁵¹Cr]O₄ (Perkin Elmer LifeSciences) and washed. T2 cells were incubated with Melan-A peptide for 15 min at room temperature. T cells were added to the targets, and chromium release was measured after 4 h of incubation.

Treatment of T cells with antagonists

A total of 10⁴ T cells were plated in round bottom microwells in HS complete medium and incubated 2 h with 5 mM LacNAc (Carbosynth). For inhibitory receptor blockade, T cells were treated 20 h with anti-PD-1 (Pembrolizumab, Merck, 10 μ g/ml), and/or anti-CTLA-4 (Ipilimumab, Bristol-Myers Squibb, 10 μ g/ml), or sodium stibogluconate (Santa Cruz, 50 μ g/ml).

Labelling of surface galectins

Anti-Melan-A.A2 CD8⁺ T cells were treated for 15 min at 4 °C with an FcR blocking reagent (Miltenyi Biotec) and were incubated for 6h at 37 °C under agitation with 30 μ g/ml of an anti-galectin-3 antibody (Mabtech). Cells were washed before being stained with 5 μ g/ml of an anti-galectin-3 coupled to biotin (clone M3/38; Biolegend) followed by 1.25 μ g/ml NeutrAvidin-PE (Life Technologies) and with an anti-CD8 β -APC (clone 2ST8.5H7, BD Pharmingen). Cells were acquired on a FACS Fortessa.

RNAseq sample preparation and data analysis

A total of 1.2×10^6 T cells were plated in 24 well plates with 1.2×10^5 beads coated with anti-CD3 and anti-CD28 antibodies (Dynabeads, Invitrogen). After 20 h of co-culture, cells were washed twice with cold PBS and cell pellets were frozen and stored at -80°C .

RNA was isolated using Kit Nucleospin RNA (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Samples were subsequently analyzed for RNA integrity using an Agilent 2200 TapeStation (Agilent Technologies, Inc., Santa Clara, CA). RNA that was determined to be of high quality ($\text{RIN} \geq 8$) was sent for sequencing to Beckman Coulter Genomics (Danvers, Massachusetts).

Differential gene expression was determined using Cufflinks and edgeR^{234,303}. For Cufflinks software, expression levels were extracted as a FPKM value for each gene of each sample using. Genes with FPKM values of 0 across all samples were excluded. . For edgeR software, expression levels were extracted as a cpm value for each gene of each sample using. Genes with FPKM or cpm values of 0 across all samples were excluded. A false discovery rate (FDR) method was used to correct for multiple testing³⁰⁴. Significant differential gene expression was defined as $|\log_2(\text{ratio})| \geq 1$ (2-fold) and $\text{FDR} \leq 0.05$.

Statistic analysis

Statistic analysis was performed using SPSS software (IBM). The statistical method that was used is indicated in the figure legend.

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADAP	Adhesion and degranulation-promoting adapter protein
AEs	Immune-related adverse events
APC	Antigen presenting cells
Arm	Armstrong strain of the LCMV virus
Arp2/3	Actin-related protein 2/3 complex
BCL-XL	B-cell lymphoma-extra large
BCL10	B-cell lymphoma 10
BTLA	B and T-lymphocyte Associated
C13	Clone 13 of the LCMV virus
CARMA1	Caspase recruitment domain-containing protein 11
CCL ...	Chemokine (C-C motif) ligand ...
CCR7	C-C chemokine receptor type 7
CD ...	Cluster of differentiation ...
CK1	Casein kinase 1
cSMAC	Central supramolecular activation cluster
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DAG	DiAcylGlycerol
DNAM-1	DNAX accessory protein-1
dSMAC	Distal supramolecular activation cluster
Eomes	Eomesodermin
FADD	Fas-Associated protein with Death Domain
Foxo	Forkhead box O
GADS	Grb2-related adaptor downstream of Shc
GALT	Galactose-1-Phosphate Uridyltransferase
GEF	Guanine nucleotide exchange factors
GITR	glucocorticoid-induced TNFR-related protein
GLUT1	Glucose transporter 1
GRAP2	GRB2-related adapter protein 2
GRB2	Growth factor receptor-bound protein 2
GRP	Guanyl-releasing protein

GSK3	Glycogen synthase kinase 3
GZMB	Granzyme B
HVEM	Herpesvirus entry mediator
ICOS	Inducible T-cell costimulator
IFN	Interferon
IgSF	Immunoglobulin super family
IL	Interleukin
IP3	Inositol trisphosphate
ITAM	Immunotyrosine activation motif
ITK	interleukin-2-inducible T cell Kinase
JNK	c-Jun N-terminal kinases
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
LAT	Linker for Activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LCP2	Lymphocyte cytosolic protein 2
LTA	Lymphotoxin alpha
LTB	Lymphotoxin beta
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin
NCK1	Non-catalytic region of tyrosine kinase adaptor protein 1
NF- κ B	nuclear factor-kappa
NFAT	Nuclear factor of activated T-cells
NICD	NOTCH Intracellular Domain
NPF	Nucleation promoting factor

PD-1	Programmed cell Death 1
PD-L	Programmed death-ligand
PDK1	Phosphoinositide-dependent kinase-1
PI3K	phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisPhosphate
PIP3	Phosphatidylinositol 3,4,5-trisPhosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PKD	Protein Kinase D
PLC γ 1	PhosphoLipase C gamma 1
PMA	Phorbol-12-Myristate-13-Acetate
pSMAC	Peripheral supramolecular activation cluster
Ptger	Prostaglandin E2 receptor
Rap	Ras-related proteins
RasGRP	Ras Guanyl nucleotide-Releasing Protein
RHD	Rel homology domain
RIAM	Rap1-GTP-interacting adaptor molecul
RNF125	RiNg Finger protein 125
SH2	Src Homology 2
SKAP-55	Src kinase associated protein of 55 kDa
SLP76	SH2 domain-containing Leukocyte Protein of 76 kDa
SOS	Son of Sevenless
SPARC	Secreted Protein Acidic and Rich in Cysteine
T-bet	T-box transcription factor
TCM	Central memory T cell
TCR	T Cell Receptor
TEM	Effector memory T cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM3	T cell immunoglobulin and mucin domain 3
TIMP3	Tissue Inhibitor of MetalloProteinases 3
TNF	Tumor Necrosis Factor
TNFRSF	Tumor necrosis factor receptor superfamily

TRAF	TNF receptor associated factors
Treg	Regulatory T cell
WASp	Wiskott–Aldrich Syndrome protein
WAVE2	WASp Family Verprolin-Homologous Protein-2
XCL	Chemokine C motif ligand
ZAP70	Zeta-chain-Associated Protein kinase 70

ABBREVIATIONS

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