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The transcription factor TOX controls differentiation and maintenance of $CD8^+$ T-cells in chronic infections

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Table of Contents

1. Abstract 3
2. Introduction 6
   2.1 The Immune System 6
   2.2 $CD8^+$ T-cells are the army of adaptive immunity 8
   2.3 LCMV Model to study $CD8^+$ T-cell biology in viral infections 9
   2.4 $CD8^+$ T-cell response to acute infections 10
   2.5 $CD8^+$ T-cell response to chronic infections 11
   2.6 Intrinsic and extrinsic determinants of exhaustion 12
   2.7 Transcriptional and epigenetic control of exhaustion 16
   2.8 Antigen amount is critical for T-cell differentiation in chronic infections 17
   2.9 $CD8^+$ T-cell maintenance during chronic infections 18
   2.10 T-cell exhaustion as functional adaptation 19
   2.11 Thymocyte selection-associated high mobility group box protein 21
3. Aim of the study 24
4. Materials and methods 25
5. Results 33
   5.1 TOX is upregulated in chronically stimulated $CD8^+$ T-cells 33
   5.2 TOX correlates with the chronic phenotype 34
   5.3 The absence of TOX reduces $CD8^+$ T-cell dysfunction 38
   5.4 The absence of TOX alters gene expression profiles of dysfunctional $CD8^+$ T-cells 41
   5.5 The absence of TOX augments effector function of $CD8^+$ T-cells 43
   5.6 TOX selectively augments survival of dysfunctional $CD8^+$ T-cells 45
   5.7 TOX is dispensable for memory $CD8^+$ T-cell generation in acute infection 46
   5.8 TOX ensures the maintenance of Tcf-1^+ cells in dysfunctional T-cell populations 48
   5.9 The absence of TOX alters gene expression in Tcf-1^+ cells 48
   5.10 The absence of TOX diminishes PD-1 expression on Tcf-1^+ cells 51
6. Discussion 53
7. Conclusions and outlook 63
8. Abbreviations 64
9. References 66

1. Abstract

During chronic infections, virus-specific $CD8^+$ T-cells acquire a differentiation program that is distinct from the one acquired in acute infections. The persisting antigen stimulation causes T-cells to become dysfunctional which means that they show impaired effector functions and up-regulated inhibitory receptor expression. Antigen amount is a critical factor for the generation of the chronic phenotype, which is retained in persistent infections even after antigen removal. To investigate key determinants in the generation of the chronic phenotype, we use the LCMV mouse model with a newly developed experimental system that allowed us to compare T-cells with acute-functional and chronic-dysfunctional phenotype, and identify core genes responsible for the generation and maintenance of the chronic phenotype. The transcriptional profile analysis revealed the transcription factor TOX as one of the most upregulated genes in chronically stimulated T-cells. TOX expression correlates with the chronic phenotype and it is maintained after antigen withdrawal in chronically stimulated mice. TOX is induced by high antigen levels and its absence strongly affects dysfunctional T-cells, which are forced to acquire an effector phenotype, with decreased PD-1 but increased KLRG-1 expression and cytokines production. The induction of an acute phenotype in chronically stimulated T-cells reveals a deep alteration of the transcriptional program, and eventually results in impaired survival of dysfunctional $CD8^+$ T-cells, explained by a substantial loss of the critical Tcf-1^+ progenitor population. Interestingly, the enhanced effector phenotype is coupled with stronger effector function, which leads to a better viral clearance, but also to an increase in immunopathology.

TOX promotes the dysfunctional phenotype in $CD8^+$ T-cells, nonetheless ensures their fitness for long-term maintenance during chronic infections. Importantly, TOX prevents the acquisition of the effector phenotype and triggers a response in T-cells that protects the host form overwhelming immunopathology.

Therefore, TOX is a key regulator of the differentiation program that leads chronically stimulated T-cells to acquire a dysfunctional phenotype. This essential role in tuning T-cells function under persistent stimulation makes TOX a major target for future immunotherapeutic approaches.

Bei chronischen Infektionen erhalten virusspezifische $CD8^+$ T-Zellen ein Differenzierungsprogramm, das sich von dem bei akuten Infektionen erworbenen

unterscheidet. Die anhaltende Antigenstimulation bewirkt, dass T-Zellen dysfunktional werden, was bedeutet, dass sie beeinträchtigte Effektorfunktionen und eine hochregulierte inhibitorische Rezeptorexpression zeigen. Die Antigenmenge ist ein kritischer Faktor für die Entstehung des chronischen Phänotyps, der auch nach Antigenentfernung bei persistierenden Infektionen erhalten bleibt. Um wichtige Determinanten bei der Entstehung des chronischen Phänotyps zu untersuchen, verwenden wir das LCMV-Mausmodell mit einem neu entwickelten experimentellen System, mit dem wir T-Zellen mit akut-funktionellem und chronisch-dysfunktionalem Phänotyp vergleichen und Kerngene identifizieren können, die für die Generierung verantwortlich sind und Aufrechterhaltung des chronischen Phänotyps. Die Transkriptionsprofilanalyse zeigte, dass der Transkriptionsfaktor TOX eines der am meisten hochregulierten Gene in chronisch stimulierten T-Zellen ist. Die Tox-Expression korreliert mit dem chronischen Phänotyp und wird bei chronisch stimulierten Mäusen nach Antigen-Entzug aufrechterhalten. Diese Phänomene sind auch in menschlichen HCV-spezifischen $CD8^+$ -T-Zellen zu finden, da hohe PD-1-Spiegel mit einer hohen Tox-Expression korrelieren, die selbst nach einer antiviralen Behandlung erhalten bleibt. TOX wird durch hohe Antigenspiegel induziert und sein Fehlen beeinflusst stark dysfunktionale T-Zellen, die gezwungen sind, einen Effektorphänotyp zu erwerben, mit verminderter PD-1, aber erhöhter KLRG-1-Expression und Zytokinproduktion. Die Induktion eines akuten Phänotyps in chronisch stimulierten T-Zellen zeigt eine tiefgreifende Veränderung des Transkriptionsprogramms und führt schließlich zu einem beeinträchtigten Überleben von dysfunktionalen $CD8^+$ T-Zellen, was durch einen wesentlichen Verlust der kritischen Tcf-1^+ -Vorläuferpopulation erklärt wird. Interessanterweise ist der verstärkte Effektorphänotyp mit einer stärkeren Effektorfunktion gekoppelt, was zu einer besseren Virusclearance, aber auch zu einer erhöhten Immunpathologie führt.TOX fördert den dysfunktionalen Phänotyp von $CD8^+$ T-Zellen, sichert jedoch deren langfristige Erhaltung bei chronischen Infektionen. Wichtig ist, dass TOX den Erwerb des Effektor-Phänotyps verhindert und eine Antwort in T-Zellen auslöst,

die den Wirt vor einer überwältigenden Immunpathologie schützt.Daher ist TOX ein Schlüsselregulator des Differenzierungsprogramms, das chronisch stimulierte T-Zellen dazu bringt, einen dysfunktionalen Phänotyp zu erhalten. Diese essentielle Rolle beim Abstimmen der T-Zellen-Funktion unter anhaltender Stimulation macht TOX zu einem Hauptziel für zukünftige immuntherapeutische Ansätze.

2. Introduction

2.1 The Immune System

The immune system is the ensemble of effector cells and molecules that protect the body from infectious agents and from other foreign noninfectious substances.

The immune system is organized into two parts: the first line of defense is the innate immune system, which acts fast, but nonspecifically. The innate response involves neutrophils, macrophages, basophils and monocytes [1]. The second line of defense is represented by the adaptive immune system that raises a specific, stronger response and includes B and T lymphocytes (CD4 or CD8 positive) [2].

When an individual encounters an infectious agent, physical and chemical barriers are the initial defenses against infection. For example, antimicrobial proteins secreted at mucosal surfaces prevent microbes from entering the body [3]. If these barriers are overcome, other components of the immune system come into play. The complement system can immediately recognize and destroy foreign agents [4], and phagocytic white blood cells of the innate immunity, like macrophages and neutrophils, can ingest and kill microbes by producing toxic chemicals and degradative enzymes [5]. Moreover, they produce secreted proteins called cytokines and chemokines that initiate the process known as inflammation [6]. The inflammation process leads to the recruitment of cells to the infected tissues, from both innate and adaptive immunity. If the innate response fails to eliminate the infection, the adaptive immunity is initiated with the expansion of lymphocytes that can recognize and fight the foreign antigen [7]. Dendritic cells are the link between innate and adaptive immunity. Like macrophages and neutrophils, they degrade the pathogens they take up, but their main role is not their clearance [8]. Rather, the encounter with the pathogen stimulates them to mature and migrate through the lymphatic system to regional lymph nodes, where they present the antigen and express the costimulatory molecules necessary to activate antigen-specific T lymphocytes through their antigen receptor (TCR) [9].

2.2 $CD8^+$ T-cells are the army of adaptive immunity

Mature lymphocytes exit the thymus and re-circulate through blood and lymphoid organs (lymph nodes, spleen and mucosal lymphoid tissues) [10]. Recirculating T-cells are called naïve if they have not yet encountered the specific antigen. A naïve T cells must receive three signals to be activated and start an efficient immune response: the first one is the signals derived from the interaction of the specific peptide:MHC complex with the TCR, the second one is the co-stimulatory signal, driven by the molecule CD28 that interacts with CD80 and CD82 expressed by antigen presenting cells (APC) [11]. Costimulatory signals are necessary to T cells to become activated and to survive, otherwise T-cells get deleted or become anergic, a state of nonresponsiveness to antgens [12]. The third signal is represented by the cytokines produced by the APC that promote the development of effector functions on $CD8^+$ T-cells and differentiation of different subsets of $CD4^+$ T-cells [13]. $CD8^+$ T-cells interact with APC and are activated in the lymph nodes, where they proliferate strongly and then exit to reach the periphery, where they can recognized peptide:MHC class I complex on the infected cells and kill them by inducing apoptosis [14]. The mechanism of action of activated T-cells rely on the release of cytotoxic granules, modified lysosomes that contains perforin, granzymes and granulysin [15, 16]. Perforin is responsible for the delivery of the contents of granules into cytoplasm of target cells [17], while granzymes are serine proteases able to activate apoptosis of the target cell [18, 19] . Activated $CD8^+$ T-cells also release cytokines that are de-novo synthetized and act as effector molecules. Interferon γ (FNγ) functions as blocker of viral replication, is involved in activation of macrophages and induce MHC II expression on APC [20]. TNF has a role in activating macrophages and in killing target cells through their interaction with TNFR-I, which induces apoptosis [21]. Moreover, TNF it is an important cytokine that starts local restriction of the infection, but induces shock when it is systemically released [22].

2.3 LCMV Model to study CD8$^+$ T-cell biology in viral infections

Lymphocytic choriomeningitis virus (LCMV) is an enveloped ambisense RNA virus and was isolated from Charles Armstrong in 1933 from encephalitis-affected patients, from which he coined the name [23]. Even though it was isolated from humans, the primary reservoir of the LCMV is house mouse (*Mus musculus*) and the LCMV mouse model has quickly become a great system to broad the knowledge of the immune system and virus-induced pathology [24].

In 1973, Zinkernagel and Doherty used the LCMV mouse model to demonstrated that cytotoxic T lymphocytes (CTLs) from infected mice were able to specifically recognize and kill infected cells presenting the viral antigen on the major histocompatibility complex [25]. The phenomenon by which a T-cells can recognize and respond to a peptide only when it is bound to the MHC complex is called MHC restriction, and Zinkernagel and Doherty were awarded the Nobel Prize in 1996 for its discovery.

Another important milestone achieved with LCMV mouse model studies has been the understanding of immunological memory, the ability of an immune cell to survive for long time, recognize a previously encountered pathogen, and trigger an immune response. In fact, CD8$^+$ T-cells from LCMV immune mice were shown to survive in virus-free host and to protect the host from LCMV infection [26, 27]. In addition, LCMV studies led to the assessment of the frequency of a precursor virus specific T-cell and its expansion kinetics. In details, Blattman et. al. showed that, in a naïve mouse, LCMV specific CD8$^+$ T-cells are only one every in 1-2 x 10^5 T-cells, but after activation they increase by 1000 fold before contracting [28].

Furthermore, the LCMV mouse model was used to characterize the disease caused by an over-reacting immune system, known as immunopathology. LCMV is a non cytopathic virus, meaning that cells and tissues are not directly damaged by the virus itself, but rather

by the activated virus-specific T cells. In fact, has been shown that $CD8^+$ T-cells are responsible to cause meningitis that was not induced by the LCMV viral infection [24, 29-31].

There are several strains of LCMV, among which the most widely used in research are LCMV Armstrong and LCMV Clone 13. Armstrong is cleared rapidly by the host and it is referred to as acute (Armstrong) LCMV infection. Clone 13 is tropic for visceral organs and leads to persistent LCMV infection [32]. The Clone 13 strain has one nucleotide mutations that affect the process of glycoprotein formation and tropism, and a second base pair mutation that affects the polymerase which has an impact on replicative capacity [24, 33]. The mutation in the glycoprotein is responsible for the stronger affinity to its receptor, the α-dystroglycan, thought which the virus infect macrophages, fibroblastic reticular cells, dendritic cells, impeding antiviral function and promoting viral persistence [34-39]. The mutation in the polymerase, instead, induces an higher viral replication in dendritic cells and increased viremia [40]. Since LCMV Armstrong and LCMV Clone 13 differ by only three amino acids, the T-cell epitopes are preserved, and this allows a direct comparison between T-cell responses in acute or chronic infection. LCMV virus has three dominant epitopes (H-$2D^b$-restricted np396-404 (Db/NP396), H-$2D^b$-restricted gp33-41 (Db/GP33), H-$2K^b$-restricted gp34-43 (Kb/GP34) and one subdominant epitope (H-$2D^b$-restricted gp276-286 (Db/GP276), recognized by specific T-cells [41]. The introduction of the tetramer technology and the generation of TCR transgenic mice, which generate $CD8^+$ T-cells specific for the gp33-epitope (P14αβ T-cells), enabled the enrichment of the antigen specific population, leading to a more precise comparison of the same population in the two different infection, acute or chronic [42, 43].

2.4 $CD8^+$ T-cell response to acute infections

A viral infection is defined acute when the disease occurs rapidly and it is resolved within days, with complete elimination of the responsible virus. Following an acute infection, naïve antigen-specific $CD8^+$ T-cells are primed by APC within the lymphoid tissues and organs, through which they are constantly recirculating. The activation of a naïve $CD8^+$ T-cells leads to their proliferation and differentiation in two major specialized subpopulations: short-lived effector cells (SLECs), characterized by $KLRG1^{high}$ and $CD127^{low}$ expression and memory precursor effector cells (MPECs), characterized by $KLRG1^{low}$ and $CD127^{high}$ expression. Short-lived effector cells are responsible for the production of effector cytokines and for the killing of target cells, but, after the peak of the response and the antigen removal, they will die during the contraction phase [44, 45]. The memory precursor effector cells, on the other hand, form the population of T-cells that survive to form the long-lived memory population that persist in the absence of antigen and acquire the ability of ‘‘self-renewal’’ by undergoing homeostatic proliferation in response to IL-7 and IL-15 [46]. Memory cells are able to undergo rapid proliferation and can rapidly reactivate effector functions upon antigen encounter [47]. In addition, memory cells can be divided in central memory T-cells (Tcm), and in effector memory T-cells (Tem). Tcm are found in lymphoid tissues and express high amounts of CD62L, CD27, and CXCR3, produce IL-2 but express low amounts of CD43 and cytotoxic molecules, bearing an enhanced proliferative capacity and longevity. Tem, on the other hand, are maintained in peripheral tissues and express low amount of CD62L, CD127, CD27 and high amount of KLRG1 and CD34 [48-50].

2.5 $CD8^+$ T-cell response to chronic infections

While an acute infection is resolved within a relative short window of time, a chronic infection lasts for a long period, and occurs when the primary infection is not cleared by the adaptive immune response. The establishment of a chronic infection is due to the concomitance of two conditions: the persistent presence of the virus and the adjustment of the immune system to control viral replication but, at the same time, to avoid damage of persistently

infected tissues [51]. This is particularly true for the $CD8^+$ T-cell response that could cause massive tissue damage by killing infected cells and releasing inflammatory cytokines. In fact, during chronic infections, $CD8^+$ T-cells functions are tightly regulated by an interweaving network of factors, which results in the generation of a dysfunctional status, known as exhaustion. $CD8^+$ T-cell exhaustion was first described with chronic LCMV infection in mice during which virus-specific $CD8^+$ T-cells persist but lack effector functions [52]. Similar types of dysfunction have also been described in other experimental models of viral infection, during human chronic infections, such as HIV or HCV, and during cancer [53-55]. Key features of exhausted $CD8^+$ T-cells are the severe impairment in cytokine production, such as IL-2, TNF and IFNγ, and the expression of high level of inhibitory receptors, such as PD-1 [56-58]. However, T-cells with various levels of function appear during the course of a chronic infection. In a partial exhaustion stage, only few effector functions are lost, such as ability to produce IL-2 and TNF-α, and to exert cytotoxic functions, while IFN-γ production is only mildly affected. As long as the exhaustion process continues, other effector functions are lost, such as IFN-γ production. In a full exhaustion stage, the loss of all effector functions is complete, and T-cells are not able to produce cytokines or kill target cells in vitro peptide stimulation assays [41].

What causes T-cell exhaustion is still an open question of research. Different factors concur to the development of $CD8^+$ T-cell exhaustion, such as high virus load, strength of TCR stimulation and epitope abundance, signals from altered inflammatory and tissue microenvironments, other lymphocyte populations such as $CD4^+$ T-cells, B cells and regulatory T (TReg) cells, inhibitory signals from cytokines and cell surface inhibitory receptors [51, 56, 59].

2.6 Intrinsic and extrinsic determinants of exhaustion

One key feature in the establishment of exhaustion is the continuous exposure to antigen. An early study correlates the different exhaustion stage (fully functional, partially exhausted, fully exhausted, or physically deleted) with the epitope load and duration of infection [41, 60]. Indeed, epitopes that persist at higher levels during LCMV Clone 13 infection, such as np396 (nucleoprotein) and gp34 (glycoprotein), are responsible of the clonal deletion of their T-cell counterpart, while epitopes that induce a lower level of stimulation, such as gp33 and gp276, promote functional exhaustion of their specific T-cells [41]. This has been suggested to be the result of a higher avidity of T-cells for np396 than for the gp33-peptide, establishing a link between the magnitude of T-cell stimulation and the onset of T-cell dysfunction.

Beyond TCR avidity, cell-intrinsic and extrinsic negative regulating pathways are fundamental factors in the definition of exhaustion. Cell-intrinsic negative regulating pathways are the inhibitors receptors, which collectively operate to negatively regulate the functional and proliferative potential of the responding cells [61, 62]. In addition to PD-1 [63], many other cell surface inhibitory receptors are co-expressed by dysfunctional T-cell [64], such as LAG-3 [65, 66], CD244 (2B4) [67, 68], CD160 [69], Tim-3[70, 71] and CTLA-4 [72]. How the different inhibitor receptors orchestrate the T-cell response is not completely understood. However, the increased recovery of exhausted T-cells after the simultaneous blockade of PD-1 and LAG-3 compared to blockade of one receptor [64] leads to the hypothesis that inhibitor receptors have non-redundant functions. It is possible that each inhibitory receptor regulates a distinct cellular function. The PD-1 pathway affects survival and/or proliferation of exhausted $CD8^+$ T-cells [73, 74], and LAG-3 affects cell cycle progression and has less influence on cell survival or apoptosis [75]. CD244 (2B4) and CD160 also affect non-overlapping functions of exhausted $CD8^+$ T-cells [64]. Although the molecular mechanisms through which inhibitory receptors act to regulate T-cells are not completely defined yet, studies suggest that these mechanisms can be very different among the receptors. For example, CTLA-4 can act as negative regulator by competing with CD28

for costimulatory ligands [76]. In contrast, inhibitory motif–containing molecules such as PD-1 can attenuate signalling by recruiting phosphatases (such as SHP-1, SHP-2 or SHIP) to TCR-proximal signalling complexes [77, 78]. Moreover, PD-1 ligation can induce the expression of proteins involved in inhibiting T-cell function [79], which suggests a potential additional mechanism by which these receptors operate. Modern therapies against cancer include the blockade of different inhibitory receptor on T-cells to enhance their functionality and kill cancer cells. Since inhibitor receptors represent an immunological checkpoint that regulates T-cell function, their inhibition is called “checkpoint blockade” therapy. The therapeutic blockade of different inhibitor receptor is showing positive results in the treatment of cancer patients [80].

As part of cell-extrinsic regulating pathway, immunomodulatory cytokines, such as IL-10 and transforming growth factor-β (TGF-β), play an important role. Interleukin-10 production is early upregulated in mice infected with LCMV Clone 13 [81], moreover elevated levels of IL-10 have been associated with persistent infection by HCV [82] and HIV [83]. Increased IL-10 production in LCMV Clone 13 infected mice induces T-cell inactivation and results in viral persistence [81], while anti–IL-10R treatment of persistently infected mice restores antiviral $CD8^+$ T-cell responses [84], highlighting a key role of this cytokine in the maintenance of CD8 dysfunction.

The immunosuppressive cytokine TGF-β has been shown to inhibit T-cell differentiation and proliferation [85] and its disruption leads to a dysregulation of T-cell function with augmented inflammation and pathology [86]. Specifically in the context of chronic infection, cell-intrinsic TGF-β signalling is responsible for virus-specific-$CD8^+$ T-cell apoptosis via upregulation of the pro-apoptotic protein Bim and subsequent decreased cell numbers [87]. Moreover, when TGF-β signalling is attenuated, $CD8^+$ T-cells functions are increased and the LCMV Clone 13 clearance is accelerated [87].

While IL-10 and TGF-β foster T-cell exhaustion, other cytokines such as IL-2, IL-7 and IL-21, act like positive regulators of T-cell responses and can enhance immunity during chronic infection [88]. In fact, IL-2 therapy has been shown to increase the frequency of virus-specific $CD8^+$ T-cells in LCMV Clone 13 infected mice and their ability to control the virus [89]. With similar results, IL-7 therapy increases the number of virus-specific $CD8^+$ T-cells in LCMV Clone 13 infected mice, accompanied by their enhanced function, viral clearance, and PD-1 downregulation [90]. IL-21 is needed to sustain antiviral $CD8^+$ T-cell responses during chronic viral infections, preventing deletion of chronically stimulated $CD8^+$ T-cells, and therefore is involved in the resolution of the persistent infection [91-93]. Interestingly, administration of IL-21 to CD4-deficient mice infected with LCMV Clone 13 cause a reduction in virus levels, by enhancing the functions and suppressing the deletion of virus-specific $CD8^+$ T-cells. The enhanced $CD8^+$ T-cells effector function, however, induce a strong immunopathology in the mice [94]. In HIV-1 infected patients, the amount of IL-21 correlates with $CD4^+$ T-cell counts, and the higher levels of IL-21 co-exist with increased frequencies of HIV-specific $CD8^+$ T-cells [95]. Thus, during chronic infection, changes in both negative and positive regulatory cytokines are key mechanism to regulate virus-specific T-cell response.

As already alluded to above, $CD4^+$ T-cell help is critical for maintaining $CD8^+$ T-cell function during chronic infection [96]. In fact, $CD4^+$ T-cells depletion before infection with LCMV Clone 13 exacerbates $CD8^+$ T-cell exhaustion and causes higher viral titer compared with mice with an intact $CD4^+$ T-cell compartment [97]. Moreover, it has been shown that increasing HIV-1–specific CD4 T-helper cell function leads to the recovery of HIV-1–specific $CD8^+$ T-cell response [98] and that virus specific $CD8^+$ T-cell response can be reinvigorated with the adoptive transfer of $CD4^+$ T-cells in mice chronically infected with LCMV Clone 13 [99]. $CD4^+$ T-cells extent a positive role in chronically stimulated $CD8^+$T-cells during persistent infection via the production of IL-21 [92]; moreover, it has recently been reported

that IL-21 restrict the expansion of virus-specific Treg cells, and therefore enhancing $CD8^+$ T-cells response [100]. Thus, conventional $CD4^+$ T-cells have a positive impact on modulating $CD8^+$ T-cell function during persistent antigenic stimulation. In contrast, it has been described that Treg are associated with ineffective immune responses during chronic infections, including those by Friend leukemia virus [101], HIV [102], HCV [103], and HBV [104]. Treg cells are activated in LCMV Clone 13 infected mice and their ability to suppress virus-specific T-cell response is strengthened [105]. Functional LCMV-specific $CD8^+$ T-cells are expanded after Treg cell depletion, even though the molecule programmed cell death ligand-1 (PD-L1) is up-regulated, impeding killing of target cells and viral clearance. Treg depletion augment the reduction of viral titer seen with PD-L1 blockade [106] suggesting a role of Treg cells in maintaining $CD8^+$ T-cell exhaustion, but the need of PD-1 pathway blockade for eliminating infected cells.

2.7 Transcriptional and epigenetic control of exhaustion

The recent development of transcriptional profiling approaches combines the phenotypical and functional characterization of exhausted T-cells with the possibility to define the molecular network required for the generation of the exhaustion state. Different studies have identified changes in TCR and cytokine signaling pathways, chemokine expression and migratory potential, and metabolism [56] as pathway mostly involved in the generation of dysfunctional T-cells. Moreover, a number of key transcription factors were identified that control the degree of exhaustion during chronic infection, including *Irf4* [107], *T-bet* [108], *Blimp-1* [109], *Eomesodermin* (*Eomes*) [110], *von Hippel-Lindau tumor suppressor* (*VHL*) [111], and *Foxo1* [112]. Thus, a complex network of known and unknown factors is responsible for the transcriptional regulation of exhaustion. In addition to changes in gene transcription profile, exhausted T-cells are characterized by changes in the epigenetic

landscape. In fact, ATAC-sequencing and RNA-sequencing profiling of $CD8^+$ T-cells in acute or chronic infection have shown that exhausted T-cells bear unique changes in chromatin accessibility and gene expression compared to effector, memory and naïve T-cell [113].

In addition to transcription factors and epigenetic modifications, gene expression is critically regulated by the state of DNA methylation. A recent study has shown that de novo DNA methylation acquired during and after the peak of an effector $CD8^+$ T-cell response to a chronic viral infection or tumor challenge is critical for establishing T-cell exhaustion [114]. Moreover, the same study demonstrates that the epigenetic programs maintained in dysfunctional T-cell hamper their rejuvenation after PD-1 blockade therapy [114].

2.8 Antigen amount is critical for T-cell differentiation in chronic infections

$CD8^+$ T-cells during chronic infections show a heterogeneous phenotype, but so far it is unclear what determines their fate in acquiring a more or less marked dysfunctional phenotype. Nevertheless, different lines of evidence suggest that strength of TCR stimulation, epitope abundance, viral load and $CD4^+$ T-cell help are critical elements in determining the development of the chronic phenotype.

We address these fundamental questions by using a newly developed experimental system, which is based on recombinant strains of LCMV Clone 13. To assess the effect of the TCR affinity on the chronic phenotype, we analyzed APLs that bind similarly to the MHC complex but differ in their potency of stimulating the gp33 specific cells. A weaker variant virus, called C6 (KAV YNF CTC) because of the presence of a Cysteine instead of an Alanine in the 6^{th} amino acid of the gp33 sequence, shows virus persistence pattern in the blood and kidneys that resembles the one of wild-type LCMV Clone 13. Since the only mutation is restricted to the gp33 sequence, the C6 Clone 13 mutant strain induces similar numbers of gp276-

specific T-cells compared to wt Clone 13, with identical PD-1 expression and cytokine secretion profiles, meaning that both type of strains causes a chronic infection of comparable extent. Even if the C6 virus variant can induce a chronic infection, P14 T-cells developed in the lower affinity stimulation condition show a milder exhausted phenotype, but still acquire the cardinal feature of dysfunctional T-cells [115]. This indicate that the affinity of the TCR stimulation is responsible for the development of exhaustion.

To investigate how antigen quantity affects T-cell responses in chronic infections, we developed an experimental setup in which P14 TCR-transgenic $CD8^+$ T-cells are exposed to different amounts of the LCMV-derived gp33/Db epitope. We achieved this by generating an LCMV Clone 13 virus variant that expresses a known Valine to Alanine amino acid substitution (A3 LCMV mutant strain), which results in a non–H-2Db–binding peptide KAA YNF ATC [116]. By mixing this gp33-mutaded virus variant with wild-type Clone 13 virus, we could alter the amount of gp33 epitope, without affecting total virus burden or presentation of other epitopes. In fact, pure wild-type and mixed Clone 13 infections contained similar total virus titer at days 7 and 28 post-infection, confirming that the two conditions give rise to a persistent infection. Surprisingly, when exposed to a low amount of antigen, $CD8^+$ T-cells acquire an acute effector phenotype, even though they don't acquire feature of memory T-cells and remain $CD127^-$ and KLRG-1^+ [115]. This work highlights how the frequency of TCR stimulation more critically affects T-cell differentiation in chronic infections than the actual strength of the TCR signal.

2.9 $CD8^+$ T-cell maintenance during chronic infections

An earlier work from our group showed that T-cells, developed in chronic infection, with high levels of PD-1 and low levels of cytokines could undergo re−expansion in a secondary host mouse and protect it from acute viral infection, while retaining the exhausted phenotype

[117]. The secondary expansion is a quality of memory cells and it implies that a population with memory-like properties is present in chronic infection. In fact, following studies have identified a population of Tcf-1$^+$ CD8$^+$ T memory-like cells that is responsible for the maintenance of the immune response during chronic viral infection [118-120]. This memory-like population has at the same time the capacity of regenerating themselves and of giving rise to more terminally differentiated effector T-cells. Similarly to central memory T-cells, this memory-like T-cells do not show an effector signature. However, in contrast to conventional memory cells, Tcf-1$^+$ CD8$^+$ T-cells preserve the features of an exhausted phenotype [118]. Importantly, they and are able to respond to PD-1 blockade and this makes them a primary target for immunotherapy approaches [118]. A circulating memory-like subset of virus specific CD8$^+$ T-cells has been identified also in human HCV infection. In fact, a population of Tcf-1$^+$ CD127$^+$ PD-1$^+$ CD8$^+$ T-cells was shown to survive and to maintain recall potential after the clearance of the HCV infection [121]. The memory-like population represent a reservoir in chronic infections, ensuring the continuous generation of terminally differentiated short live effector cells that exert a considerable level of virus control and represent a promising target to boost CD8$^+$ T-cell responses in immune therapeutic interventions [122].

2.10 T-cell exhaustion as functional adaptation

Several clinical observations and experimental studies have led to the concept of exhaustion as a unique differentiation program, in which the functional adaptation of CD8$^+$ T-cells under persistent stimulation ensures a balance between virus control and immunopathology. In fact, the increase in viral load after CD8$^+$ T-cell depletion in SIV-infected rhesus macaques and the appearance of virus variants that express mutated T-cell epitopes indicate that T-cells retain partial viral control in chronic infections [123]. Moreover, the recovery of T-cell function after blockade of the interaction between PD-1 and its ligand PDL-1 indicates that

T-cell responses are downregulated rather than being terminated during chronic infections [63]. Exhausted T-cells represent a distinct population, defined by phenotypical, functional and metabolic features, co-ordinated by a transcriptional and epigenetic program that differs from the naïve, effector, memory or anergic one [56]. Importantly, exhausted T-cells maintain their dysfunctional phenotype even after re-expansion in a secondary host, implying a key level of stability of their differentiation program [117].

Epigenetic studies support the stability of the exhausted phenotype. In fact, has been shown that exhausted $CD8^+$ T-cells retain an unmethylated status in the promoter region of the gene encoding PD-1 (*Pdcd1*), even when the viral load is reduced to undetectable levels at the late phase of mouse LCMV Clone 13 infection [124]. In the same line, antigen-specific $CD8^+$ T-cells from antiviral treated HIV patients and from elite controller patients present lack of methylation in the *Pdcd1* promoter region [125], further indicating a fixation of the epigenetic imprint in the *Pdcd1* locus. Moreover, the stability of the exhaustion phenotype is conserved in spontaneously resolved HCV infection, where $CD8^+$ T-cells retain an exhausted phenotype even after pathogen clearance [121]. Interestingly, it has been shown that the epigenetic stability of exhausted T-cells restrict the efficacy of the PD-1 blockade, limiting the durability of the functional restoration of T-cell [126]. Thus, to develop successful therapeutic approaches for overcoming T-cell exhaustion is of fundamental importance to portray the epigenetic profiling that programs the stability of exhausted T-cells [127]. In addition, the epigenetic landscape of exhausted T-cells remodels the enhancer region and the binding of transcription factors, thus primarily controls the transcriptional state of T-cell exhaustion [127, 128]. Taken together, these data imply that exhausted $CD8^+$ T-cells occupy a distinct differentiation state. Thus, exhaustion does not seem to be neither a terminal differentiation state, nor an unresponsive T-cell state. Instead, exhaustion could represent an adaptation of chronically stimulated T-cells to a hyporesponsive state. In this way, even though the exhausted T-cells are not able to clear the pathogen, they still can control the

infection without causing immunopathology [122]. In fact, the elimination of keys factors responsible for the setting of the dysfunctional phenotype, such as PD-1 or HIFα, leads to a fatal disease [111, 129]. By inducing the exhaustion differentiation program, it is possible to reach the balance needed to maintain $CD8^+$ T-cells and to protect the whole organism from pathology.

2.11 Thymocyte selection-associated high mobility group box protein

TOX was first identified as a gene expressed during thymocyte maturation in the thymus, but not in the periphery [130]. TOX is a member of the HMG-box superfamily of proteins, and defines a small subfamily of proteins including Tox2, Tox3 and Tox4, all conserved in vertebrate species [131]. Looking at the key residues in the TOX box, TOX appears to fit into the sequence-independent DNA-binding family of HMG box proteins [131]. The TOX protein has 526 amino acids with an acidic N-terminal domain, a bipartite nuclear localization signal sequence and a single centrally located HMG box motif. The NCS (Nuclear Localization Sequence) and the HMG box sequence spann exon 5 and 6 [131].

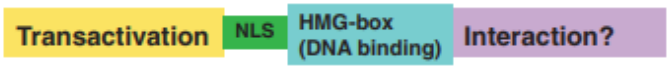

Figure 1. TOX protein structure. Amino acid sequence of TOX protein, with indicated exons. Color code represent different functional parts of the protein to which the exons belong.

In developing thymocytes, TOX is upregulated in a transient way during β-selection and positive selection. It has been shown that, during the positive selection, TCR-mediated calcineurin signaling is responsible for TOX upregulation in DP cells and that TOX plays a role in CD4 silencing and CD8 lineage commitment, possibly via up-regulation of Runx3 and/or changes in chromatin structure [132]. In fact, in mice that constitutively express TOX, the CD4 downregulation leads to CD8 single positive (CD8SP) cell formation, but these CD8SP cells are not able to mature or exit the thymus [132, 133]. On the other hand, mice deficient in TOX (KO) revealed a requirement for TOX in $CD4^+$ T-cell lineage development. In fact, in the absence of TOX, developing thymocytes initiated positive selection, however the significant loss of $CD4^+$ T-cells in KO animals results in T-cell lymphopenia [133]. Interestingly, TOX KO CD8SP thymocytes in the spleen could be activated to have cytolytic effector functions [132]. Moreover, splenic $CD8^+$ T-cell numbers were not reduced in TOX KO compared with wild-type animals [134]. However, the percentage of naïve $CD44^{hi}$ $CD8^+$

T-cells was also increased in TOX KO mice [134]. CD44 is an adhesion molecule that is expressed by most T-cells and mediates the binding to the extra cellular matrix and other cells via its ligand glycosaminoglycan hyaluronic acid [135]. CD44 expression is upregulated on naïve T-cells after activation via the T-cell receptor [136]. Nevertheless, even if TOX KO splenic $CD8^+$ T-cells expressed higher CD44, they showed normal proliferative responses to anti-CD3/CD28 mAb stimulation in the absence or presence of IL-2, and were able to produce IFNγ and other cytolytic effector functions upon stimulation, although at lower lever compared with wild-type effector cells [134]. In addition to its role in thymocyte development and differentiation, TOX has a role in the development of lymph nodes, Peyer's patches and NK cells [137].

TOX levels in thymocytes has been linked to Calcineurin activity [132]. Calcineurin is a $Ca2^+$-dependent phosphatase that acts by controlling nuclear trafficking of target proteins including several transcription factors, among which the NFAT family members, NFATc1–NFATc4. NFAT proteins interact with structurally unrelated Fos-Jun (AP-1) transcription factors to form NFAT:AP-1 complexes [138]. A recent study showed that a form of NFATc1 that is both constitutively active and unable to interact with AP-1 promotes the exhaustion program and upregulates TOX expression [139], linking for the first time TOX to the exhaustion program.

Recently, has been shown that TOX is a transcriptional regulator of CTLs in a mouse model of CNS autoimmune disease [140]. So far, there are no evidences yet of its role in chronic infections.

3. Aim of the study

T-cells arising in chronic infections are different form naïve, memory and effector cells and the main question is what are the determinants for the development of the exhausted phenotype. Different lines of evidence suggest that strength of TCR stimulation, epitope abundance, and viral load are critical elements in determining the development of the chronic phenotype. We recently showed that the antigen amount is a key determinant for a T-cells to be exhausted, while the affinity of the TCR has a smaller impact.

Our first aim was to address which molecular pathways and which molecules determine and regulate T-cell differentiation in chronic or acute infections. Therefore, to disclose the key features that differentiate nonfunctional from functional $CD8^+$ T-cells, we used two different experimental settings that allowed us to define genes tightly linked to the exhaustion phenotype and intrinsically determining dysfunctional T-cells. We merged our data with published data set [141], to identify major regulators of T-cell exhaustion.

Our second aim was to reveal the role of the transcription factor TOX in the network that drives the differentiation program of exhausted T-cells. The development of exhausted T-cells occurs in chronic infections and in cancer, and it is characterized by phenotypical and transcriptional programs. Identifying a new master regulator is of primary importance to disclose new therapeutic targets to restore functionality in exhausted T-cells.

4. Materials and methods

Mice: P14 TCRαβ transgenic mice were kindly provided by A. Oxenius (Zurich, Switzerland)[142] and Vβ5 TCRβ-only transgenic mice [143] by P. Fink (Seattle, USA). C57BL6/N_TOX$^{tm1a(KOMP)Wtsi}$ founder mice were purchased from the KOMP repository and crossed with a FLP deleter strain to eliminate the LacZ reporter and to convert them into TOX$^{tm1c(KOMP)Wtsi}$ mice. The progeny were crossed with Mx-Cre, Rosa26-stop-EYFP (Jackson laboratories), and P14 transgenic mice and afterwards intercrossed to generate TOX$^{tm1c(KOMP)Wtsi}$_x_Mx-Cre_x_Rosa26-stop-EYFP_x_P14 quadruple transgenic mice. Mx-Cre_x_Rosa26-stop-EYFP_x_ P14 or TOX hemizygous mice were used as controls. Donor mice were treated with 200µg of PolyIC (Sigma, Germany) 2-7 days before cell harvest. The elimination of the 5th exon in the EYFP^{+} P14s was confirmed by PCR. Given the mixed background, we obtained C57BL6/N and C57BL6/J from Charles River (France) and used F1 mice as hosts for adoptive T-cell transfers.

Mice were bred and maintained in SPF facilities and infected in modified-SPF animal facilities initially at the University of Lausanne in Switzerland and later at the Technical

University of Munich in Germany. Experiments were performed in at least six-week-old mice in compliance with the Institutional and governmental regulations in Switzerland and Germany and were approved by the responsible veterinarian authorities of the Swiss Canton Vaud and the “Regierung von Oberbayern” in Germany.

Infections LCMV stocks were produced in our laboratory and kept frozen. Viral stock were diluted in PBS before being inject4ed. LCMV Armstrong was intraperitoneally injected at the dose of $2x10^5$ PFU per mouse. LCMV Clone 13, including variant strains, was injected intravenously. The low affinity altered peptide ligand expressing LCMV Clone 13-C6 was described previously [115]. The mixed-mutant infections described in the paper attached [115] were performed by mixing the gp33 deficient LCMV Clone 13 mutant (A3 strain, encoding an H-2Db binding deficient gp33 altered peptide ligand) with wild-type LCMV Clone 13. The initial experiments were performed at the University of Lausanne in Switzerland, and $2x10^6$ PFU of LCMV Clone 13 was used per mouse. For the mixed infection 1.33x106 PFU gp-33 deficient (A3 APL) LCMV Clone 13 and 0.66x106 PFU wt LCMV Clone 13 was used per mouse. After the relocation of the laboratory to the Technical University of Munich, Germany, $5x10^6$ PFU of LCMV Clone 13 wild type and $4,5x10^6$ PFU gp-33 deficient LCMV Clone 13 and 0.5x106 PFU wt LCMV Clone 13 for the mixed infection were used per mouse [117]. The increased dose for the viral infection was due to the use of a different animal facility.

Purification of mouse T-cells, adoptive cell transfers: Single cell splenocyte suspensions were obtained by mashing total spleens through a 100 µm nylon cell strainer (BD Falcon) and red blood cells were lysed with a hypotonic ACK buffer. Transgenic $CD8^+$ T-cells were isolated using the mouse $CD8^+$ T-cell enrichment kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). $2\text{-}5x10^3$ $CD45.1^+$ congenic naïve wt or ΔTOX P14αβ were transferred into naïve $CD45.2^+$ C57BL/6 mice. P14αβ T-cells were re-isolated from infected mice through magnetic

cell separation, using anti-CD45.1 biotinylated antibody and anti-biotin microbeads (Miltenyl Biotech, Bergisch-Gladbach, Germany).

Surface and intracellular antibody staining and flow cytometry cell sorting of mouse cells: Surface staining was performed for 30 min at 4°C in FACS buffer (PBS supplemented with 2% FCS (Sigma Aldrich) and 0.01% azide (Sigma Aldrich) using the following antibodies: anti-CD8a (53-6.7), CD45.1 (A20), CD45.2 (104), PD-1 (RMP1-30), Klrg1 (2F1), CD127 (eBioSB/199), CD62L (MEL-14), Tim3 (RMT3-23), CD5 (53-7.3), CD69 (H1.2F3) from Biolegend, APC conjugated-gp33 tetramer (TCMetrix). For detection of EdU, the Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Thermo Fisher) was used. Cells were washed twice and fixed in PBS supplemented with 1% formaldehyde, 2% glucose and 0.03% azide for 20 min. Then the cells were washed again and re-suspended in FACS buffer. For cytokines production assay, splenocytes were re-stimulated in vitro with gp33-41 (gp33) peptide (5mM) for 5h in the presence of Brefeldin A (7 µg/ml) for the last 4.5 h. For intracellular cytokine staining, cells fixed and permeabilized, using the Cytofix/Cytoperm Kit (BD) and stained with mAbs for IFNγ (XMG1.2), TNF (MP6-XT22). For intracellular transcription factor staining, the Foxp3 / Transcription Factor Staining kit (eBioscience) was used and cells stained with anti-Tcf-1 (S33966, BD Pharmingen), TOX (TXRX10, eBioscience), Eomes (Dan11mag), and T-bet (eBio4B10, both from eBioscience). For flow cytometry sorting, living cells were stained in 2% FCS RPMI media and sorted on a FACS Aria Fusion instrument (BD). Flow cytometry measurements of cells were performed on an LSR-Fortessa flow cytometer (BD). All data were analyzed using FlowJo (TreeStar).

Next generation sequencing (NGS): At day 8 or 20 post infection, splenocytes were enriched for CD45.1 + P14 T-cells using biotin labelled ant-CD45.1 antibodies and anti-biotin conjugated microbeads in combination with magnetic MACS cell separation (Miltenyi Biotech, Bergisch-Gladbach, Germany). High purity (>95%) samples were then obtained by

flow cytometry based sorting for GFP^+ $CD45.1^+$ P14 cells. The cells were lysed and RNA was extracted using the Agencourt RNAdvance Cell v2 kit (A47942, Beckman Coulter). RNA integrity number (RIN) and yield were assessed using RNA 6000 Pico Kit (5067-1513, Agilent). Only samples with RIN>8 were used for downstream cDNA synthesis and library preparation. cDNA synthesis and PCR amplification using 1 ng of total RNA from each sample was performed using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (634891, Takara/Clontech). After cDNA synthesis, each sample was subjected to 12 cycles of PCR amplification. The generated amplicons were assessed and their concentration was determined with the use of Agilent High Sensitivity DNA Kit (5067-4626, Agilent). 150 pg of the resulting amplified cDNA were used for library preparation with the Illumina Nextera XT DNA Library reagents (FC-131-1024, Illumina). After PCR amplification of the fragmented libraries, the samples were purified with (0.6x) Agencourt AMPure XP beads and eluted in 10 µl of molecular grade water. The quality of the resulting library was assessed with the use of Agilent High Sensitivity DNA Kit (5067-4626, Agilent). The library quantification was performed based on the Illumina recommendations (SY-930-1010, Illumina) with the use of KAPA SYBR FAST qPCR Master Mix (KK4600, Kapa Biosystems). The samples were sequenced on Illumina HiSeq 2500 system at the following conditions - rapid run, 100 base pairs single-end read, dual-indexed sequencing resulting in 20 million reads per sample. The preparation of NGS was performed with the help of my colleague Kristiyan Kanev.

NGS data processing: Reads were processed using snakemake pipelines[144] as indicated under (https://gitlab.lrz.de/ImmunoPhysio/bulkSeqPipe). Sequencing quality was assessed with fastqc [145], filtering was performed by trimmomatic v0.36 [146] using, mapping by STAR v2.5.3a [147] with genome Mus_musculus.GRCm38, counting by htseq v0.9.1 [148] with annotation Mus_musculus.GRCm38.91. To supervise STAR and fastqc results we used multiqc v1.2[149]. All used parameters and adapters can be found at

(https://gitlab.lrz.de/ImmunoPhysio/alfei_TOX). The processing of NGS data was performed with the help of my colleagues Patrick Roelli and Ming Wu.

NGS data analysis: Genes with total reads lower than 10 across all the samples were removed before comparisons. Differential expression analysis was performed with methods based on the negative binomial distribution in DESeq2 (version 1.18.1)[150], using default parameters. Read counts were modeled as a negative binomial distribution with estimated mean values and gene-specific dispersion parameters; each gene was fitted as a generalized linear model (GLM); Wald statistics and Benjamini & Hochberg (BH) [151] were used for significance test and multiple comparisons with mean expression values greater than 50, absolute log2 fold change larger than 1 and adjusted p value smaller than 0.05 were considered significant. The analysis of NGS data was performed with the help of my colleagues Patrick Roelli and Ming Wu.

Gene expression plots: genes were selected from differentially expressed gene lists of ΔTOX Vs TOX Wt at different time points. Z-score derived from the normalized expression values were obtained from DESeq2 (version 1.18.1)[150].

Microarrays. P14 T-cells isolated from infected mice were sorted and frozen before being sent to IMGM Laboratories for the generation of Microarray gene expression profiles. Agilent mouse GE v2 Microarrays (4x44K) (**Figure 2B**) and Agilent SurePrint G3 Mouse Gene expression 8x60KK Microarrays (**Figure 2A**) were performed as described in the manufacturer's instructions. Microarrays signals were detected using the Agilent DNA Microarray Scanner (Scan Control A.8.4.1 Software, Agilent Technologies) and analyzed with Feature Extraction Software 10.7.3.1 (Agilent Technologies) with default parameters (protocol GE1-107_Sep09 and Grid: 028005_D_F_20130207. GeneSpring GX12 (for Supp. Fig 1A) and GeneSpring GX13.0 (for Supp. Fig 1B) (Agilent Technologies) were used for

normalization and analysis of the raw data as well for quality control. The preparation of Microarrays was performed with the help of my colleagues Daniel T. Utzschneider.

Microarray analysis: Limma (version 3.34.9) was used to perform statistical analysis of the microarrays data [152]. Values were corrected by 'saddle normexp' [153], normalized by 'Quantile' method [154] and log2-transformed for the downstream analysis. Genes that were detected to be differentially expressed were filtered by absolute log fold change >1 and adjusted p value < 0.05. The p-values were adjusted for multiple testing by the Benjamini & Hochberg (BH)[151] method. The analysis was performed with the help of my colleagues Patrick Roelli and Ming Wu.

Genome-Wide and Loci-Specific Methylation Analysis: Naïve and P14 CD8 T-cells were sorted from the splenocytes of acutely or chronically infected mice. DNA was isolated by using the QIAGEN DNeasy kit. Genomic DNA was bisulfite treated using the EZ DNA methylation kit (Zymo Research). Bisulfite-induced deamination of cytosine allows for sequencing-based discrimination of methylated versus non-methylated cytosine [155]. The bisulfite-modified DNA was PCR amplified with locus-specific primers. The PCR amplicon was cloned into the pGEM-T TA cloning vector (Promega) and then transformed into XL10-Gold ultracompetent E. coli bacteria (Stratagene). Individual bacterial colonies were grown overnight over Luria-Bertani (LB) agar containing ampicillin (100 mg/L), X-gal (80 mg/L), and IPTG (20 mM). White colonies were selected and subcultured into LB broth with ampicillin (100 mg/L) overnight; the cloning vector was purified; and the genomic insert was sequenced. This experiment was performed in collaboration with Prof. Ben Youngblood and Dr. Hazem E. Ghoneim (St. Jude Children's Research Hospital).

Human HCV-specific CD8$^+$ T-cells. HLA-A*02-positive (HLA-A*02+) subjects with chronic HCV infections; spontaneously cleared HCV infections; HCV infections cleared through

Harvoni [Gilead Sciences], Sovaldi [Gilead Sciences]/Daklinza [BMS], or Viekirax/Exviera [AbbVie] treatment; and healthy control donors (attending the University Hospital of Freiburg were included in the study). In detail: 5 patients, all not in the acute phase and unknown time post infection were used as healthy control; 5 patients, of which 3 had HCV infection more than 5 years ago and 2 presumably more than 2 years ago, were chosen as spontaneously cleared donors; 6 patients, all 24 weeks after the end of therapy (36 weeks after therapy initiation); of which 5 have been chronically HCV-infected for more than 20 years and one is not precisely known but presumably at least for 10 years, were taken as cleared donors; 6 patients, of which 4 cleared the infection most probably more than 20 years ago and 2 unknown time but presumably at least since 10 years, were taken as spontaneously cleared donors.

Written informed consent was obtained in all cases, and the study was conducted in accordance with federal guidelines, local ethics committee regulations, and the Declaration of Helsinki (1975). Approval was obtained from the ethics committee of the Albert-Ludwigs-Universität, Freiburg, Germany (HBUF; 474/14). Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood by density gradient centrifugation. Peptide-MHC class I tetramer-based enrichment procedures were performed as described previously 12. $CD8^+$ T-cell subset in healthy donors were defined based on CD45RA and CCR7 surface (mAb clones: HI100 and G043H7, respectively). Peptides of HLA-A*02-restricted HCV-derived epitopes, influenza virus (FLU)-derived epitope were obtained from Genaxxon. Peptides were dissolved in dimethyl sulfoxide (Sigma, Germany) at 20 mg/ml and diluted in complete medium to 1mg/ml before usage. Major histocompatibility complex (MHC) class I epitope-specific tetramers were generated by conjugation of biotinylated peptide-MHC class I monomers with PE- or APC conjugated streptavidin at a MHCI:Strepatividin molar ratio of 5:1.

The following reagents were used for multi-parametric flow cytometry: anti-HLA-A*02 (BB7.2, BD), anti-CD45RA (HI100, BD), anti-TOX (TXRX10, eBioscience), anti-CCR7

(G043H7, BioLegend), anti-PD1 (EH12.2H7, BioLegend), anti-TCF1 (C63D9, Cell Signalling), anti-CD8 (RPA-T8, BD). 7-AAD (BD Biosciences) was used for live/dead discrimination. FoxP3/Transcription Factor Staining Buffer Set (eBioscience) was applied according to the manufacturer's instructions to stain for nuclear molecules. Cells were fixed with paraformaldehyde (2% PFA) before sample acquisition on LSRFortessa (BD Biosciences). This experiment was performed in collaboration with Dr. Maike Hofmann and Prof. Robert Thimme (Universitätsklinikum Freiburg).

General data Analyses: Bar graphs depict the mean ± SEM or ± SD as indicated. Statistical analyses were performed with Prism 7.0 (Graphpad Software). Non-paired t tests (two-tailed) were used according to the type of experiments. p values < 0.05 were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); p values > 0.05 were non-significant (ns).

5. Results

5.1 TOX is upregulated in chronically stimulated CD8$^+$ T-cells

To identify genes responsible for the generation and maintenance of the chronic phenotype, we generated and compared gene expression profiling of CD8$^+$ T-cells bearing acute/functional or chronic/dysfunctional phenotype. We have previously shown that modifying the gp-33 amount in chronic infection leads to the development of CD8$^+$ T-cells with an acute phenotype, without affecting the overall chronic infection features [115]. Therefore, we compared gene expression profiles of P14 T-cells generate in high (LCMV Clone 13 pure wt) or low (mixing LCMV Clone 13 wt and the A3 mutant variant) antigen chronic infection to identify genes that determine the acquisition of the dysfunctional phenotype in a chronic infection. Moreover, we compared gene expression profile of P14 T-cells isolated from acute or chronic infection and re-expanded in acute condition to identify genes stably imprinted in the chronic phenotype. The volcano plots in **Figure 2** show TOX as one of the most upregulated gene in the dysfunctional population in both experimental settings.

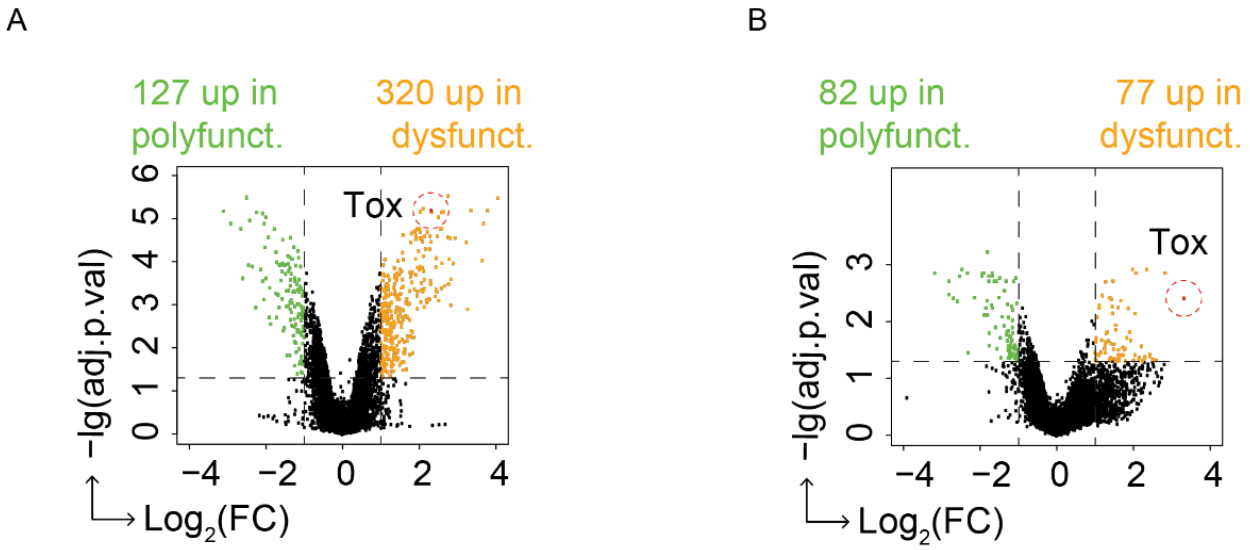

Figure 2. TOX is a signature gene of dysfunctional T-cells. A) $2x10^3$ P14 T-cells were transferred into C57BL/6 mice then infected with LCMV Clone 13 wt or Clone13 wt mixed with gp33-deficient LCMV Clone 13 mutant strain. P14 were re-isolated on day 28, sorted by flow cytometry and analyzed for gene expression profile using RNA microarrays. The volcano plot graphs Log_2 fold-changes against the adjusted p-values. Dashed vertical and horizontal lines indicate the filters used (Log_2 FC = ±1.0 and adjusted p-value < 0.05). The number of genes that are above the filter criteria are written on top. **B)** $2x10^3$ P14 T-cells were transferred into C57BL/6 mice engrafted with $2x10^3$ P14 T-cells and subjected to a primary LCMV Armstrong or Clone 13 infection. 28 days later,P14 were re-isolated and transferred into naïve host mice, infected then with LCMV Armstrong. 8 days later, cells were harvested, analyzed, and graphically illustrated as explained in A. Cells were sorted by D. Utzschneider. Data processing and analysis was done by M. Wu and P. Roelli. (modified from Alfei et al., 2019 [156]).

The upregulation of TOX exclusively in high antigen dose chronic infection and the maintenance of its expression even after re-expansion from a chronic to an acute infection highlight the importance of TOX in the development and stability of the chronic phenotype. Thus, TOX can be defined as a signature gene of dysfunctional T-cells.

5.2 TOX correlates with the chronic phenotype

We confirmed the expression of TOX at a protein level on day 8, 30 p.i. and after re-expansion of P14 in acute and chronic LCMV infection. For the acute infection, we used LCMV strain Armstrong, while for the chronic infection we used the different P14 stimulation conditions obtained with the LCMV Clone 13 variant C6 and a mix of Clone 13 wt and gp-33 deficient Clone 13 mutant strain (named mix infection), as described above [115]. At day

8 post infection, TOX expression is upregulated in P14 in LCMV Clone 13 pure infection and at lower level in LCMV C6 low affinity variant and in the mix infection. The degree of TOX upregulation correlates with the strength of the chronic phenotype, and TOX expression is antigen-dose but not antigen-affinity dependent, as shown in **Figure 3**. In fact, the low affinity LCMV Clone 13 variant C6 evokes a stronger chronic phenotype compared to the mix Clone 13 infection setting [115]. TOX failed to be upregulated during an acute infection, both at early or later time point.

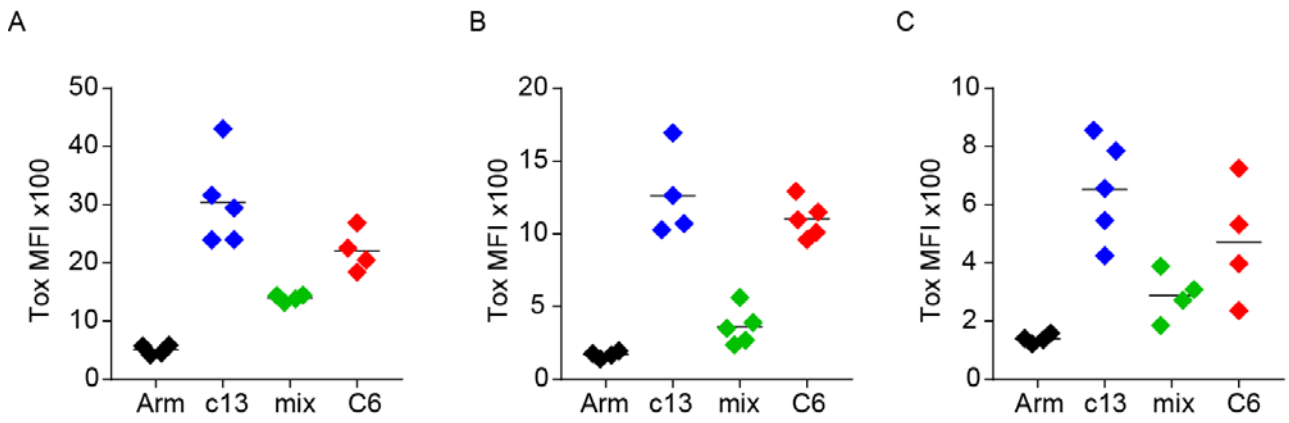

Figure 3. TOX expression is stable in chronic phenotype. $2x10^3$ P14 are adoptively transfer in CBL57/6 naïve host, infected then with LCMV Armstrong (Arm), Clone 13 wt (c13), Clone 13 wt + Clone 13 A3 (mix), Clone 13 C6 (C6). The graphs represent MFI (Mean Fluorescence Intensity) of TOX at day 8 **(A)**, day 28 **(B)** after the first infection or day 8 after transfer in a secondary host and Armstrong re-challenge **(C)**. Symbols represent individual mice, the mean is shown.

The maintenance of TOX expression in P14 T-cells developed in chronic infections but re-expanded in an acute infection is a mark of stability of its expression program. In fact, the promoter of TOX becomes progressively de-methylated over time, as depicted in **Figure 4** confirming that TOX expression is fixed with the enforcement of the dysfunctional status of T-cells.

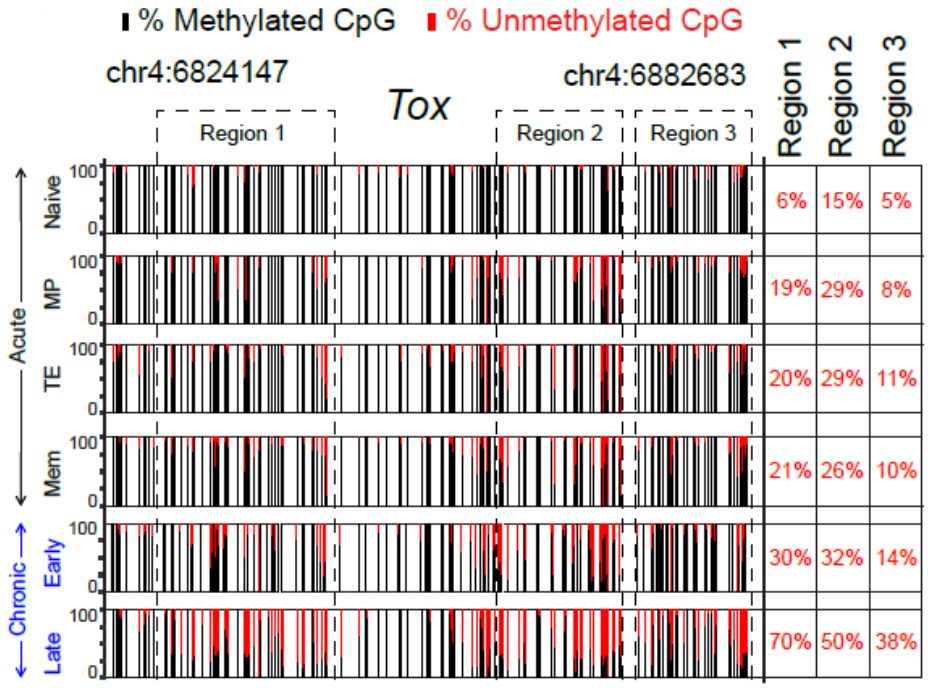

Figure 4. TOX promoter is progressively de-methylated over time in chronic infection. DNA-methylation analysis in the TOX locus among naïve, LCMV Armstrong expanded KLRG1^{high}CD127^{low} (TE), KLRG1^{low}CD127^{high} (MP), and day 30 memory P14 T-cells versus LCMV Clone 13 primed day 8 (early) or 30 (late) P14 cells. Vertical lines indicate CpG positions and the red to black ratio the percentage of unmethylated versus methylated reads. This result was obtained with the help of Prof. B. Youngblood and Dr. H. Ghoneim (Immunology department, St. Jude Children's Research Hospital, TN). (modified from Alfei et al., 2019 [156]).

The analysis of CD8$^+$ T-cells early during chronic infection shows a negative correlation in both total CD8$^+$ T-cells from the host (upper row) and transferred P14s T-cells (lower row) of TOX expression with KLGR1, expressed in effector cells [56] (**Fig. 5A**), while a positive correlation with the exhaustion marker PD-1 (**Fig. 5B**).

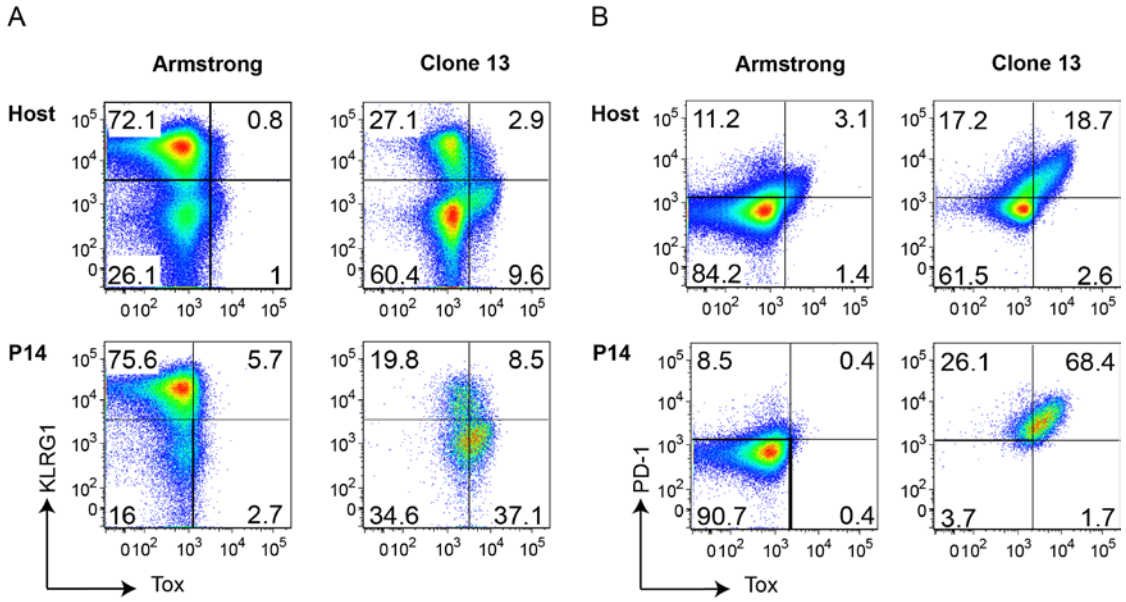

Figure 5. TOX correlates with the chronic phenotype. 2x10^3 P14 are adoptively transfer in CBL57/6 naïve host, infected then with LCMV Armstrong or Clone 13. Splenocytes are collected 8 days post infection. Representative FACS plot showing the expression of TOX (x axis) and KLRG1 **(A)** or PD-1 **(B)** (y axis) 8 days post LCMV Armstrong (Arm) or LCMV Clone 13 (c13) infection as indicated on top in total host CD8$^+$ T-cells (top) or P14 (bottom).

The correlation of TOX expression with PD-1, one of the key molecules linked to the chronic phenotype, is confirmed also in CD8$^+$ T-cells from chronic HCV infected patients, but not in influenza resolved patients. As shown in **Figure 6**, TOX MFI positively correlates with PD-1 MFI, decreasing from chronic ongoing to resolved HCV infection. Interestingly, the expression of TOX is detectable also in patients with antiviral therapy-resolved HCV infection, supporting the idea that its regulation is stably imprinted during the development of the chronic phenotype.

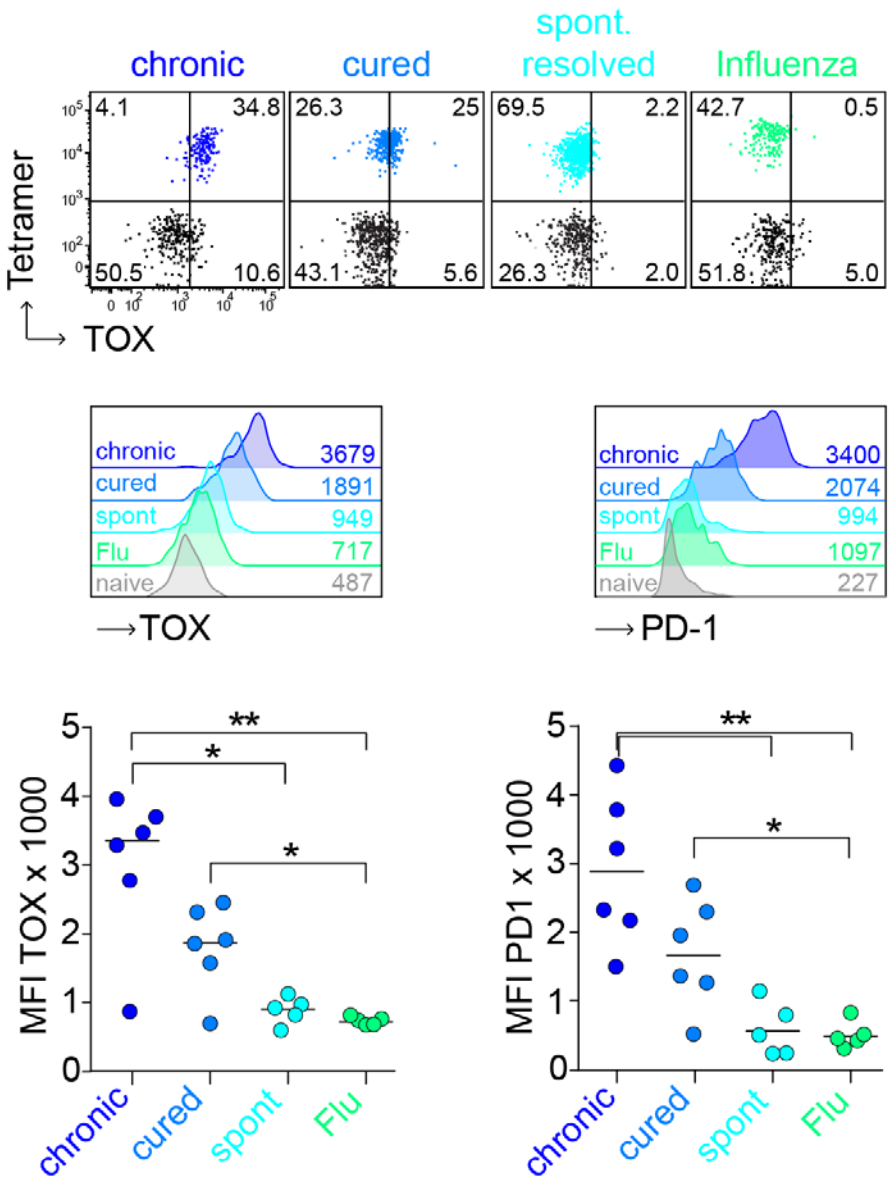

Figure 6. TOX correlates with the chronic phenotype in HCV patients. TOX and PD-1 expression among virus specific (Tetramer$^+$) T-cells in ongoing, treated, spontaneously resolved Human Hepatitis C infections and among influenza-specific (Tetramer$^+$) memory T-cells. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood by density gradient centrifugation; CD8$^+$ T-cell subset in healthy donors were defined based on CD45RA and CCR7 surface. Shown are representative dot plots and histograms for selected donors, and TOX and PD-1 MFI for multiple donors. Unpaired t tests were performed with *<0.05; **p < 0.01; ns = not significant (p > 0.05). This result was obtained with the help of Dr. M. Hofmann and Prof. R. Thimme (Universitätsklinikum Freiburg). (modified from Alfei et al., 2019 [156]).

5.3 The absence of TOX reduces CD8$^+$ T-cell dysfunction

To analyze in depth the role of TOX in the differentiation of $CD8^+$ T-cells during chronic infection, we generated a conditional mouse in which the depletion of the functional part of *TOX* gene is selectively eliminated in peripheral P14 T-cells, as described in the methods section.

ΔTOX P14 T-cells developed in a chronic infection show a less dysfunctional phenotype compared to wt P14, as shown by the decrease of PD-1 expression on the ΔTOX cells at day 8 post infection, which increases with time (d20, **Fig. 7A**). The percentage of ΔTOX P14 T-cells expressing KLRG1 is higher than those which have TOX, peaking on day 13 post infection (**Fig. 7B**). In addition, a higher percentage of ΔTOX P14 T-cells produce the cytokines IFNγ and TNF (**Fig. 7C**). Moreover, in ΔTOX cells, the ratio of the transcription factors Tbet and Eomes is altered (**Fig. 7D)**. The exhausted profile following LCMV Clone 13 infection is associated with an inverse relationship between T-bet and Eomes, with Eomes being overexpressed in exhausted cells [108, 110]. ΔTOX P14 T-cells show a decrease in Eomes expression, in line with their augmented effector phenotype. The group of Wherry has shown that T-bet represses the expression of the inhibitory receptor PD-1 and sustains virus-specific $CD8^+$ T-cell responses during chronic infection [108]. Since we could not find any difference in T-bet expression in ΔTOX P14 T-cells early during the infection, when instead PD-1 expression is affected, we can exclude that TOX acts via T-bet in repressing PD-1.

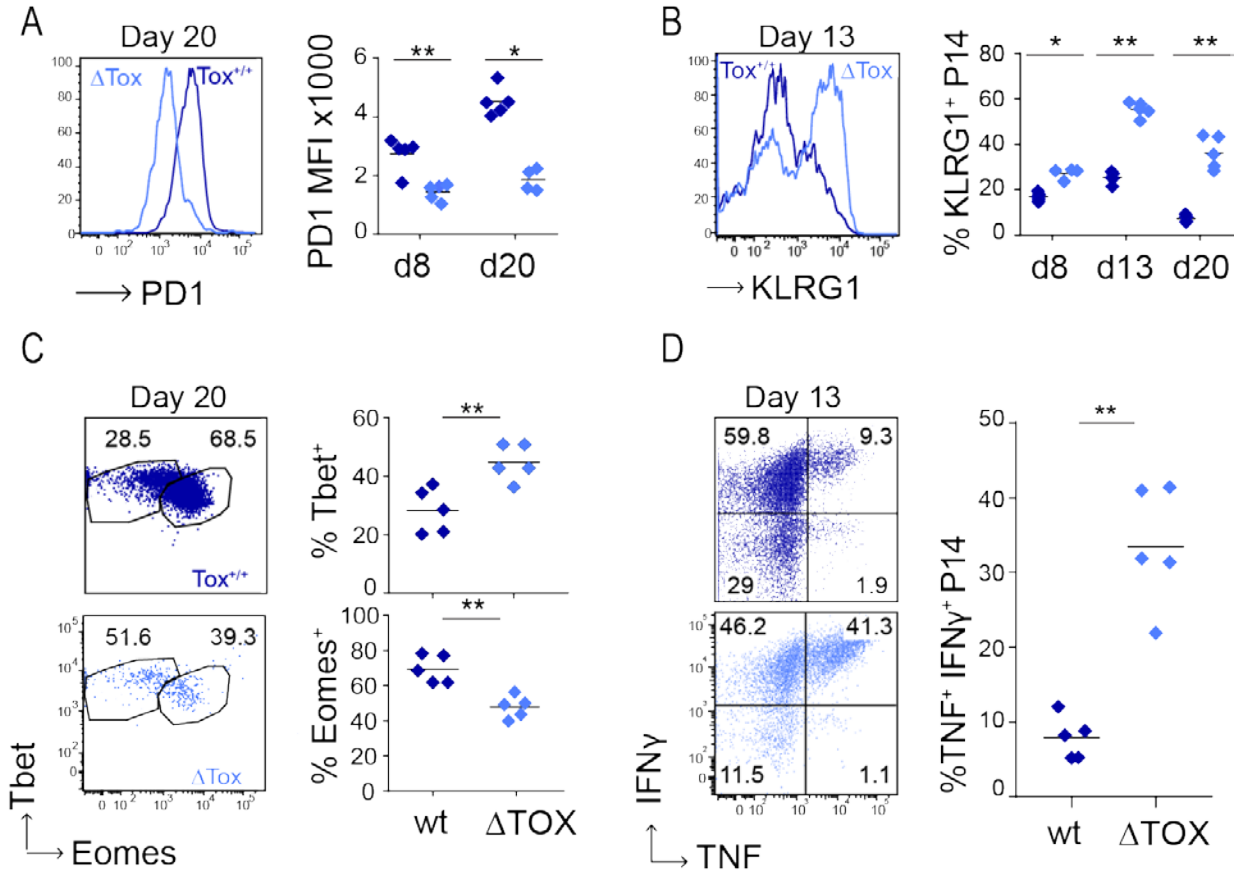

Figure 7. Absence of TOX reduces T-cell dysfunction. C57BL/6 mice received 2x10^3 wt or ΔTOX P14 T-cells and infected with 5x10^6 PFU LCMV Clone 13. PD-1 levels **(A)**, KLRG-1 expression **(B)**, cytokine production following in vitro gp33 re-stimulation in the presence of Brefeldin A **(C)**, Eomes and T-bet expression **(D)**. Shown are representative flow plots for selected time-points and data for all analyzed time-points in the adjacent graphs. (modified from Alfei et al., 2019 [156]).

In addition to phenotypic changes, we could observe also significant alteration on the opening status of the chromatin in ΔTOX compare to wt P14 T-cells, that increases with time of the infection, as depicted in Extended Figure 10 of our paper [156]. In detail, Pdcd1 locus (encoding for PD-1) has reduced accessibility, while the Tnf locus has increased accessibility in ΔTOX P14 T-cells.

These results show that the absence of TOX induces not only phenotypical changes, but has a significant impact at the epigenetic level. This imply a main role of TOX in the regulation of the differentiation program driving T-cell dysfunction.

5.4 The absence of TOX alters gene expression profiles of dysfunctional CD8$^+$ T-cells

To extend the analysis of the effect of TOX in the dysfunctional T-cells, we performed RNA sequencing on wt and ΔTOX P14 T-cells on day 8 and on day 20 post LCMV Clone 13 infection. The genes upregulated in the absence of TOX are link to T-cell activation, such as *Cd81*, *Cd109*, *Klrc2*, *Klrc3* and *Il2ra* (**Fig.8 A**). Moreover, the genes upregulated in wT-cells on day 20 post LCMV infection are linked to the exhaustion phenotype (i.e. *Cd160* and *Cd244*). What it is interesting to notice is that the amount of genes regulated by TOX increase over time, meaning that TOX initiates the program that gets enforced along with the chronic phase of the infection. Of note, the ΔTOX P14 population shows downregulation of a gene involved in T-cell memory, like *Sell, Bcl-2, Id3, Ccr7* and *Il7r* on day 8 post LCMV Clone 13 infection. This evidence suggests a primary role of TOX in survival of antigen specific CD8$^+$ T-cells.

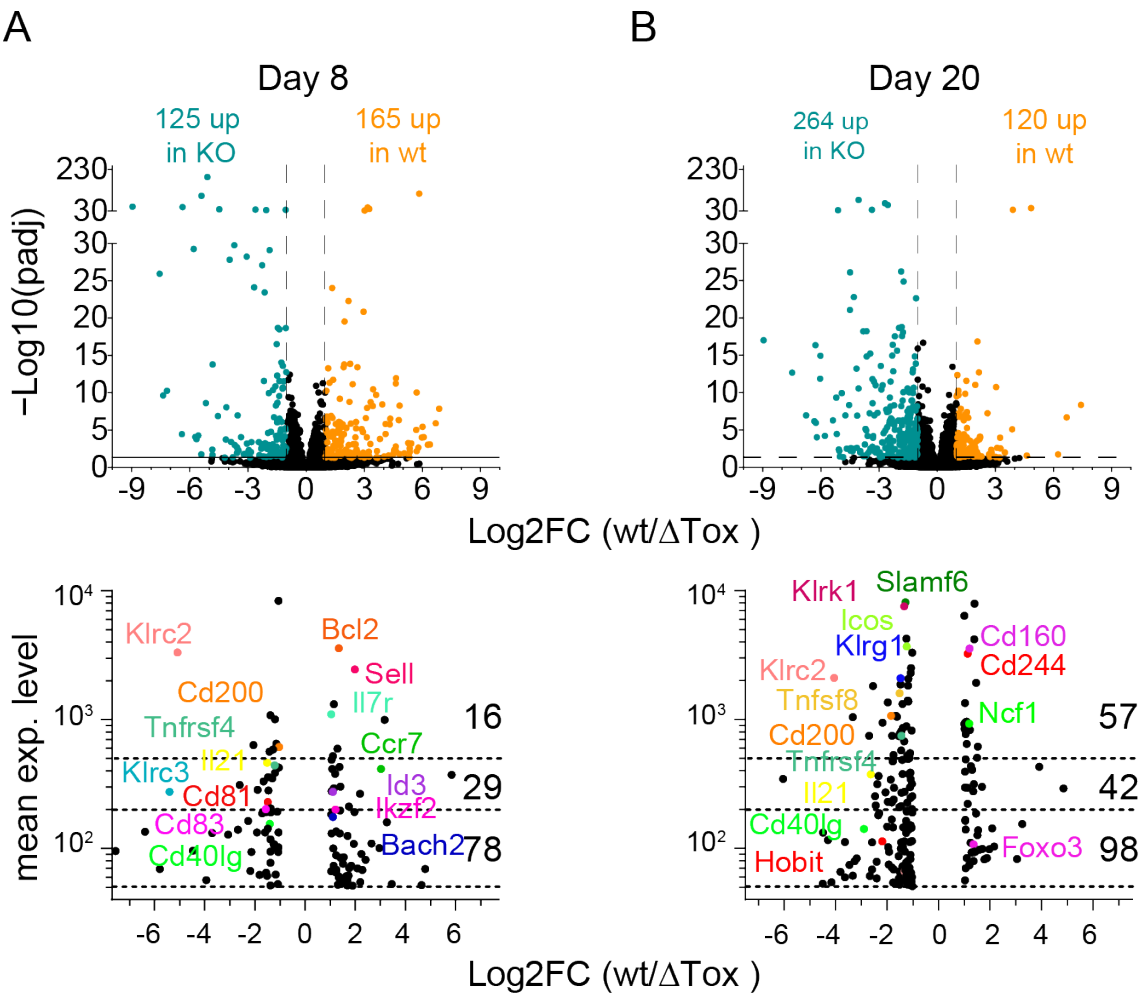

Figure 8. TOX regulates genes over time. wt or ΔTOX P14 T cells were transferred into C57BL/6 host mice, infected with 5x10^6 PFU LCMV Clone 13. On day on days 8 (**A**) and 20 p.i. (**B**) P14 T-cells were isolated for RNA sequencing. **Upper row**, unfiltered Volcano plots showing Log2FC of wt/ΔTOX against negative Log10 of the adj. p-value. Genes upregulated in wt are in orange and genes up-regulated in ΔTOX P14 T-cells in green. Dotted lines are set to Log2FC of 1 and -1 and adj. p-value of 0.05. **Lower row plots**, genes were filtered for mean expression levels ≥ 50 and adj. p-value ≤ 0.05 and Log2FC ≥ 1 or ≤ -1 and wt/ΔTOX Log2FC are plotted against mean expression values. Lines represents mean expression values of 50, 200, 500, the number of genes above the lines are annotated, gene of interest are highlighted in color with annotated names. RNA processing and acquisition of primary data was done by K. Kanev. Sequencing analysis were done with the help of M. Wu. (modified from Alfei et al., 2019 [156]).

What it is interesting to notice is that the amount of genes regulated by TOX increase over time, meaning that TOX initiates the program that get enforced along with the chronic phase of the infection. Of note, the ΔTOX P14 population shows downregulation of a gene involved in T-cell memory, like Sell, Bcl-2, Id3, Ccr7 and Il7r on day 8 post LCMV Clone 13 infection. This evidence suggests a primary role of TOX in survival of antigen specific CD8$^+$ T-cells. Of great interest, as shown if Figure 10 of our paper [156], in the absence of TOX the

accessibility of chromatin is progressively altered, inferring an additional role of TOX in epigenetic regulation.

5.5 The absence of TOX augments effector function of CD8$^+$ T-cells

To investigate if the less dysfunctional phenotype of the ΔTOX P14 T-cells correlates with augmented effector function of T-cells, ΔTOX and wt P14 T-cells are transferred into Vβ5 mice, in which the response to LCMV is severely impaired. As shown in **Figure 9**, Vβ5 mice engrafted with ΔTOX P14 T-cells show better viral clearance in the blood on day 7 post Interestingly, the Vβ5 mice engrafted with ΔTOX P14 T-cells show a higher weight loss, one of the signs recognized as indicator of chronic pain and disease, together with reduced grooming and motility [157]. While mice infected with LCMV strain Armstrong clear the infection in about one week, chronic infection caused by LCMV Clone 13 induces different levels of sickness that is not directly caused by the virus, but by the immune reaction. It has been shown that interferon is responsible for the weight loss, liver cell necrosis and death in LCMV infected animals. Given the increased effector signature and cytokines production of ΔTOX P14 T-cells, these acquired features might be responsible for T-cell driven pathology in the host.infection, and lower viral titer in the spleen, with the same number of P14 in the organ.

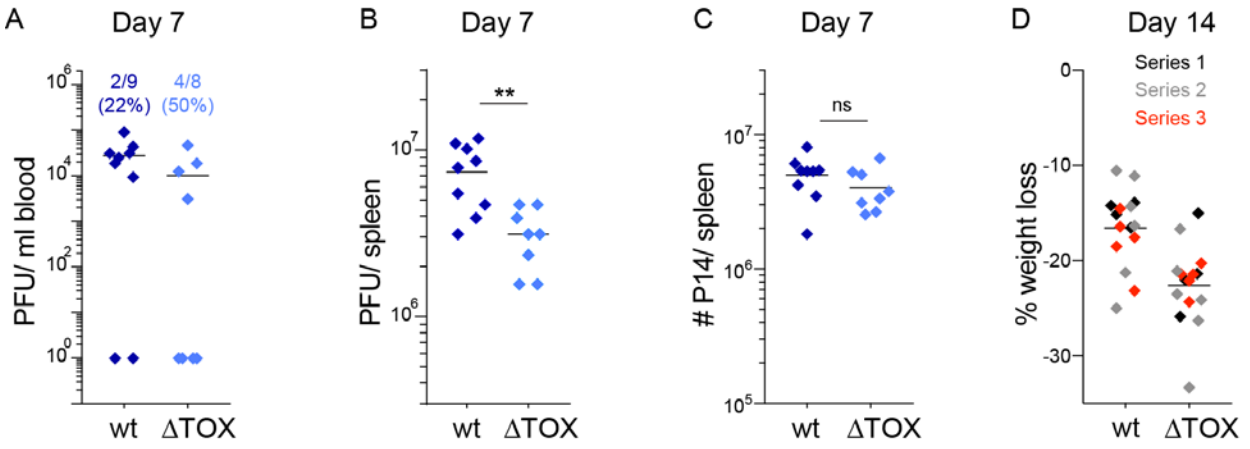

Figure 9. Absence of TOX augments effector function of T-cells, but causes host disease. Vβ5 mice, which carry the TCR-beta chain of OT-1 T-cells and whose T-cells are impaired in responding to LCMV, were engrafted with 10^4 wt or ΔTOX P14 T-cells and infected with 5x10^6 PFU LCMV Clone 13. Mice were analyzed for viral titers in the blood (day 7, **A**) and spleen (day 7, **B**), for numbers of P14s in the spleen (day 7, **C**), and for day 14 bodyweight decrease **(D)**. Data are representative for three independently performed experiments, with at least 4 mice per group. Symbols represent individual mice, the mean is shown. Unpaired t-tests were performed with with *<0.05; **p < 0.01; ns = not significant (p > 0.05). (modified from Alfei et al., 2019 [156]).

It has been shown that during LCMV Clone 13 persistent infection there is a selective migration of lymphocyte towards non lymphoid organs such as liver, lungs, brain and also bone marrow [41]. Thus, to further investigate the causes of the sickness of the mice, we decided to analyze lungs and liver from mice that received wt or ΔTOX P14 T-cells. As shown in Supplementary Figure 4 in our paper [156], mice that received ΔTOX P14 T-cells showed significantly more tissues damage and edemas in the lungs and they manifested parenchymal infiltration with formation of necrotic foci in the liver. The cause of the organs damage could be found in the feature of T-cells lacking Tox. An augmented effector phenotype can make the T-cells more effective in clearing the virus, but, at the same time, more predisposed to attack infected cells in organs and cause damage. Thus, these results indicate that TOX is needed to balance the effector activity of chronically stimulated cells and to avoid immunopathology.

5.6 TOX selectively augments survival of dysfunctional CD8⁺ T-cells

We next aim to verify if the down regulation of memory genes in ΔTOX P14 cells on day 8 post infection seen in **Figure 8** has an impact on the maintenance of ΔTOX P14 T-cells. Therefore, we determined the kinetics of wt and ΔTOX P14 cells by adoptively co-transferring similar amount of the two into the same host, in the context of an acute LCMV Armstrong infection, a pure LCMV Clone13 wt infection or a mix LCMV Clone13 infection. In this way, both type of P14 T-cells are exposed to the same inflammatory environment. As shown in **Figure 10**, ΔTOX P14 T-cells expand to the same extent than the wt in all three infections, but they start to decline after the second week of the infection selectively in the pure LCMV Clone13 infection, when the chronic phenotype is well established.

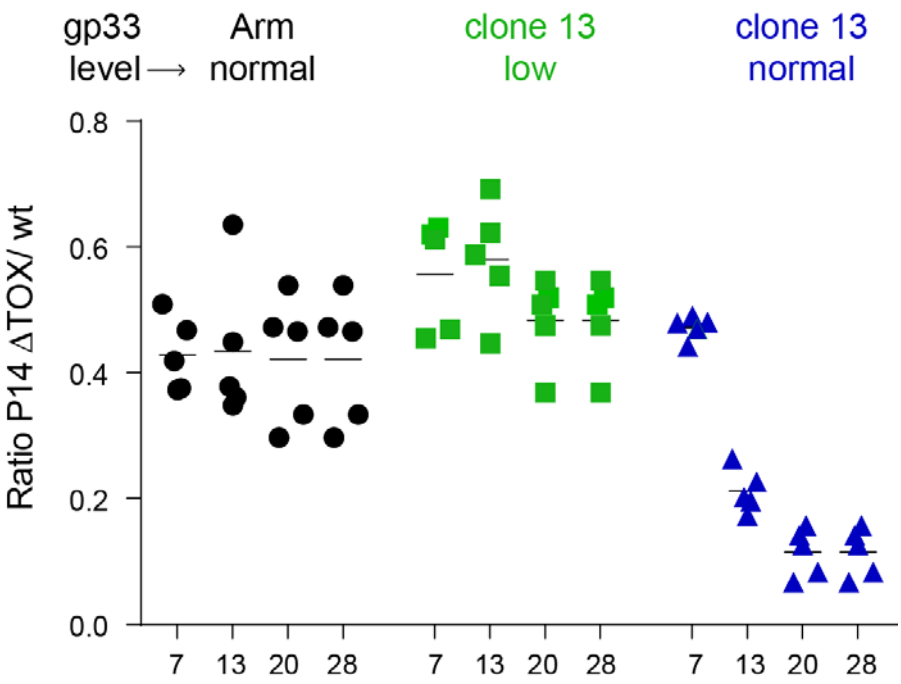

Figure 10. The survival of ΔTOX P14 is impaired selectively under chronic stimulation. 2000 CD45.1/2 ΔTOX and CD45.1/1 wt P14 were adoptively co-transferred in the same CD45.2/2 host, infected then with LCMV Armstrong, Clone13 with reduced gp33 antigen amount or wt Clone 13. Blood samples are collected at the indicated time points and ratio between ΔTOX and wt P14 calculated. Each dot indicates a mouse; mean is indicated. (modified from Alfei et al., 2019 [156]).

The survival defect is found only in dysfunctional P14s, as when the antigen amount is reduced, ΔTOX P14 cells are maintained as the wt P14s.

5.7 TOX is dispensable for memory CD8$^+$ T-cell generation in acute infection

To prove that the absence of TOX does not affect the generation of memory cells in acute infection, wt and ΔTOX P14 CD8$^+$ T-cells were adoptively co-transferred and the host mouse infected with LCMV Armstrong. At the memory phase (i.e. 28 days post- infection), P14 CD8$^+$ T-cells are re-isolated from the infected spleens and transfer to a secondary naïve host, infected then with LCMV Armstrong. As explained above, the marker CD127, KLRG1 and CD62L can separate different memory populations. The absence of TOX does not alter the generation of the Tcm or Tem populations in acute infection, as can been see from the percentage of CD127 and CD62L positive populations at the memory time point (day 28) of the infection (**Fig. 11 A -B**). Moreover, the number of P14 expressing Tcf-1 is similar (**Fig. 11 C**). One week after the infection of the secondary host, wt and ΔTOX P14 T-cells display similar phenotype (data not shown), and they were able to expand at a similar level (**Fig. 11 D**).

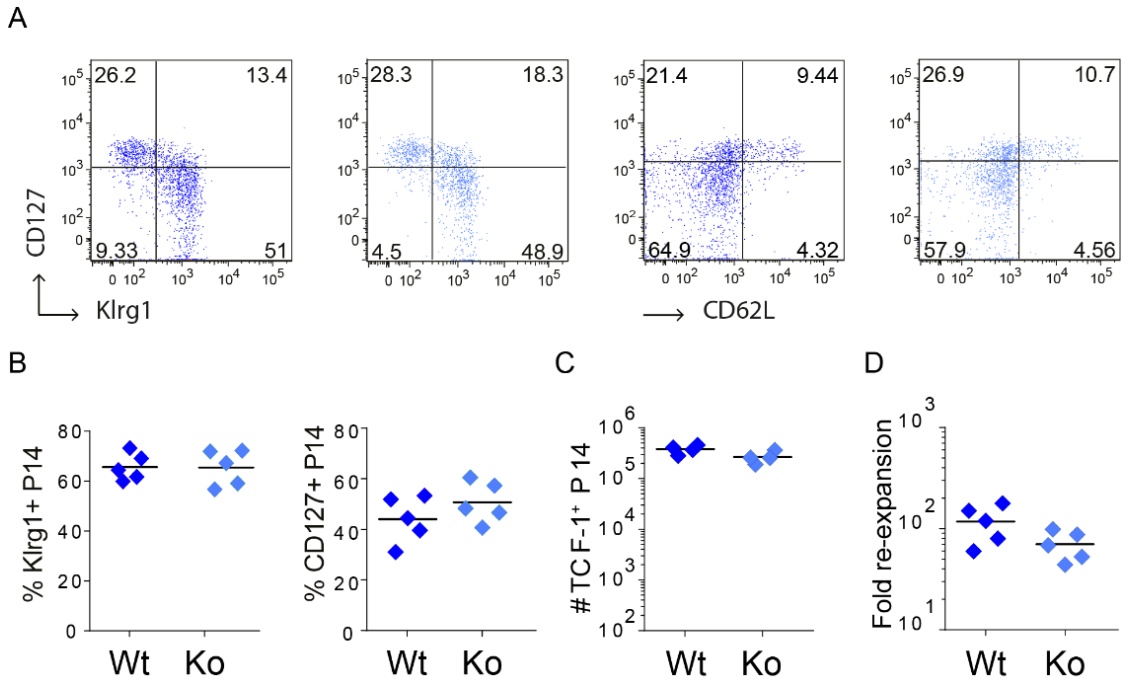

Figure 11. Absence of TOX does not alter phenotype of memory CD8⁺ T-cells. 2x10^3 wt and ΔTOX P14 were adoptively co-transfer in the same host, infected then with 2x10^5 PFU LCMV Armstrong. 28 days post-infection, splenocytes were isolated and their phenotype analyzed for memory markers. **A)** Representative FACS dot plot showing the percentage of Tem (KLRG1+ CD127-) or Tcm (CD62L+ CD127+) of wt and ΔTOX P14 T-cells. **B)** Percentage of wt and ΔTOX P14 T-cells expressing KLRG1 (left) and CD127 (right). **C)** Absolute number of P14s expressing Tcf-1. **D)** Expansion fold of ΔTOX and wt P14 T-cells in the spleen of the secondary host mice 8 days after challenge. Expansion was calculated assuming a 10% transfer efficacy. (modified from Alfei et al., 2019 [156]).

In addition to phenotypic data, we performed RNA sequencing in wt and ΔTOX P14 T-cells on day 8 post Armstrong infection. As shown in Extended data 3 in our paper [156], we found few genes that are differently regulated in absence of Tox. Interestingly, CD62L gene (Sell) results downregulated in the absence of TOX on day, while at later time point, we could not discern this difference at the protein level. One possible reason could be that TOX is temporary involved in the regulation some gene even in Armstrong infection at an early time point, but it is not needed for the development of an efficient memory compartment.

5.8 TOX ensures the maintenance of Tcf-1$^+$ cells in dysfunctional T-cell populations

Previous studies have shown that the maintenance of antigen specific population during the course of chronic infection is dependent of the Tcf-1 expressing population [118, 119]. Since we assessed a long-term maintenance defect in the ΔTOX P14 T-cells, we decided to investigate the Tcf-1 expression in the transferred P14s. The analysis of the spleens at day 8 post LCMV Clone 13 infection highlights a decrease in the Tcf-1$^+$ population in the ΔTOX P14 T-cells (**Fig. 12A**). However, the absolute number of Tcf-1$^+$ P14 is similar in the two conditions. This could be explained by an initial better expansion of the ΔTOX P14 T-cells bearing a lower PD-1 expression. In fact, it has been shown that P14s KO for PD-1 expand better [158]. However, at a later stage of the infection, an 80 fold reduction can be assessed in the number of ΔTOX P14 T-cells compared to wt (**Fig. 12B**).

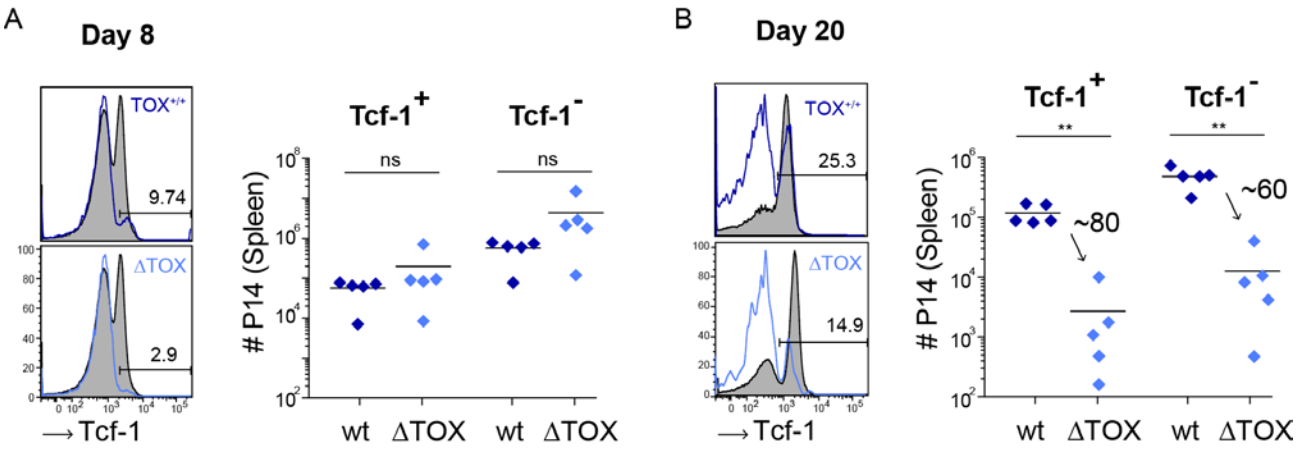

Figure 12. Absence of TOX impairs the maintenance of the Tcf-1 expressing population. C57BL/6 mice received 2x10^3 wt or ΔTOX P14 T-cells, infected with LCMV Clone 13. Representative histograms of P14s (solid colored lines) and data graphs showing the frequency and total numbers of Tcf-1$^+$ P14 T-cells on day 8 **(A)** and 20 **(B)** post infection. Gray shaded reference curves are endogenous CD8$^+$ T-cells. Symbols represent individual mice, mean is shown. Unpaired t test was performed with *<0.05; **p < 0.01; ns (p > 0.05). (modified from Alfei et al., 2019 [156]).

5.9 The absence of TOX alters gene expression in Tcf-1$^+$ cells

Previous studies have shown that the Tcf-1^{+} population is responsible for the maintenance of the CD8^{+} T-cell response during chronic infection is due to it having stemness properties [118, 119]. Tcf-1^{+} CD8^{+} T-cells, in fact, give rise to both Tcf-1^{+} and Tcf-1^{-} populations, assuring a prolonged CD8^{+} T-cell response over time during chronic infection. In light of these data, we linked the defect in the CD8^{+} T-cell maintenance in absence of TOX to a defect in the Tcf-1^{+} progenitors. To investigate further the role of TOX in the Tcf-1^{+} and Tcf-1^{-} compartment, we generated transcriptional profile analysis of the two populations on day 8 post LCMV Cone13 infection. Here, we used the extracellular inhibitor receptor Tim3 as a surrogate marker to identify the memory precursor cells, as it has been previously shown that the Tcf-1^{high} population expresses low level of Tim3 and the Tcf-1^{low} population expresses high level of Tim3 [119]. As shown in **Figure 12**, the number of genes differentially expressed between ΔTOX and wt P14s in the Tim3^{-} Tcf-1^{+} population is higher compared to the Tim3^{+}Tcf-1^{-} population, suggesting a primary role of TOX in the progenitor cells.

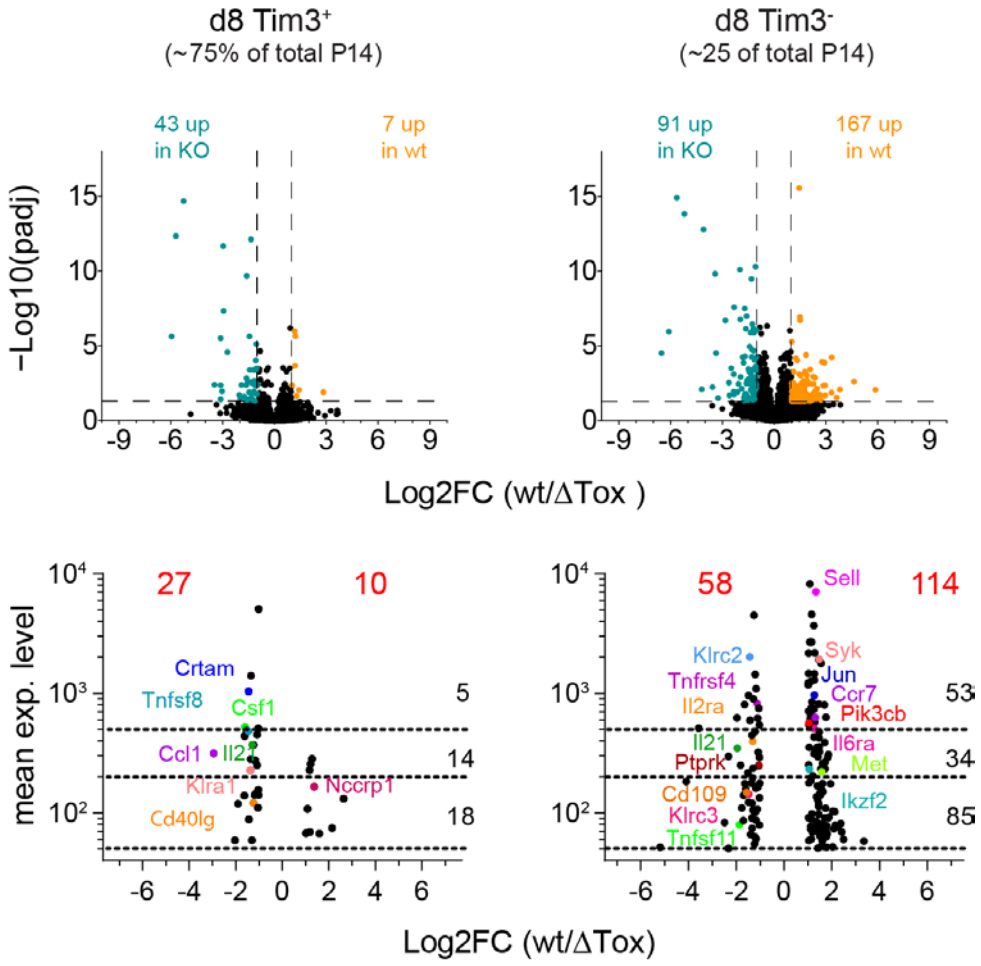

Figure 13. Proliferating precursor cells are predominantly impacted by the absence of TOX expression. wt or ΔTOX P14 T cells were transferred into C57BL/6 host mice, infected with $5x10^6$ PFU LCMV Clone 13. Spleens were harvested on day 8 and wt and ΔTOX P14 T-cells were sorted into Tim-3^+ and Tim-3^- T-cells and RNA sequencing performed. **Upper row**, unfiltered Volcano plots showing Log2FC of wt/ΔTOX plotted against negative Log10 of the adjusted p-value. Orange dots are genes upregulated in wt and green dots are up-regulated in ΔTox. Dotted lines are set to Log2FC of 1 and -1 and adj. p-value of 0.05. **Lower row plots**, shows genes filtered for mean expression levels ≥ 50, Log2FC ≥ 1 or ≤ -1, and adj. p-value ≤0.05 and wt/ΔTOX Log2FC are plotted against mean expression values. Lines are set to mean expression values of 50, 200, 500. Annotated are the number of genes above the lines. RNA processing and acquisition of primary data was done by K. Kanev. Sequencing analysis were done with the help of M. Wu. (modified from Alfei et al., 2019 [156]).

Of note, as shown in the Extended data 10 of our paper [156], the absence of TOX has an impact also in the methylation profile of Tim3^+, with increase methylated DNA regions compared to wt. Interestingly, the methylation profile of ΔTOX P14 is closed to the naïve T-cells, speculating a role of TOX in the demethylation of exhaustion associated genes.

5.10 The absence of TOX diminishes PD-1 expression on Tcf-1^{+} cells

On Day 8 post LCMV Clone13 infection, we found that the Tcf-1^{+} population is characterized by high expression level of TOX and PD-1, as shown in **Figure 14**. The Tcf-1^{+} population was previously shown to retain the exhastion signature [118, 120]. Here it is interestingly to notice that at en eraly time point the memory-like cells precursor express a stronger dysfunctional phenotype, coupled with decreased ability to produce cytokines (data not shown).

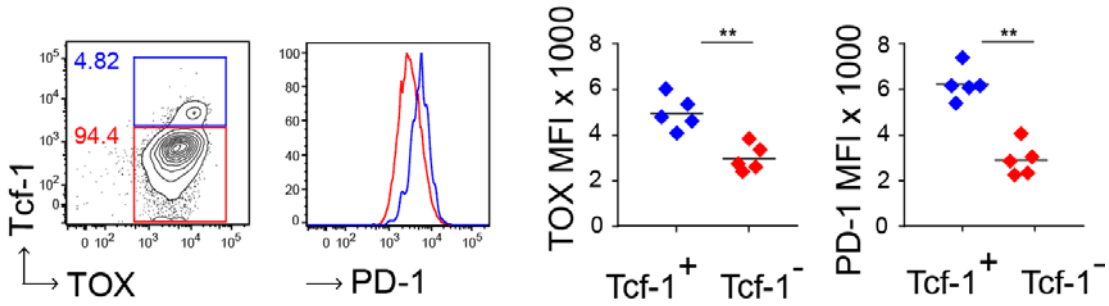

Figure 14. Tcf-1^{+} cells express high level of TOX and PD-1. C57BL/6 mice received 2x10^{3} wt P14 T-cells and infected with 5x10^{6} PFU LCMV Clone 13. Representative dot plot for TOX and Tcf-1 expression (up left), representative histograms of PD-1 expression (bottom left) and calculated MFI for TOX and PD-1 on Tcf-1^{+} (Blue) and Tcf-1^{-} (red) P14 T-cells (Top right and top left, respectively). Symbols are data for individual mice, mean is shown. Unpaired t test were performed with *<0.05; **p < 0.01; ns (p > 0.05). (modified from Alfei et al., 2019 [156]).

Interestingly, the expression of TOX was found increased also in Tcf-1^{+} tetramer^{+} CD8^{+} T-cells of HCV patients with ongoing chronic infection, but not in resolved HCV or influenza infection, further validating a strong correlation between TOX and memory-like cells compartment. This data highlight a possible implication of TOX in regulting this important subset of virus specific CD8^{+} T-cells, tuning their proliferative potential or recall re-expansion.

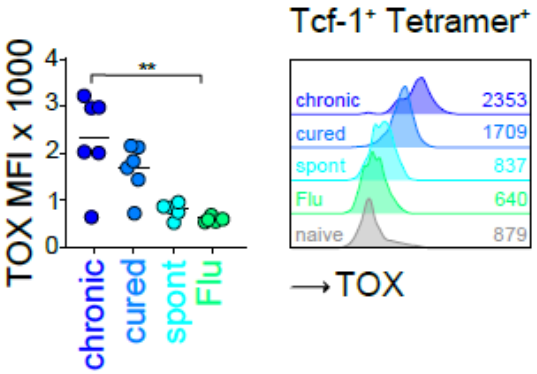

Figure 15. TOX expression in Tcf-1^{high} cells in human chronic HCV specific T-cells. Shown are representative dot plots and histograms for selected donors and TOX MFI for multiple donors Symbols are data for individual patient, solid horizontal lines represent the mean. Unpaired t test were performed with *<0.05; **p < 0.01; ns = not significant (p > 0.05). This result was obtained with the help of Dr. M. Hofmann and Prof. R. Thimme (Universitätsklinikum Freiburg). (modified from Alfei et al., 2019 [156]).

To better characterize phenotypically the Tcf-1$^+$ cells in absence of TOX, we analyzed the expression of exhaustion markers early in the infection (day 8). A co-staining of Tcf-1 and PD-1 showed that the expression of the inhibitor receptor is downregulated strongly in ΔTOX P14 Tcf-1$^+$ population, as depicted in **Figure 16**. Other inhibitory receptors, as 2B4 and Lag3, are not altered by TOX deficiency at this time point (data not shown). The significant effect on PD-1 expression in absence of TOX suggests a link between the level of PD-1 expression and T-cells maintenance in chronic infections.

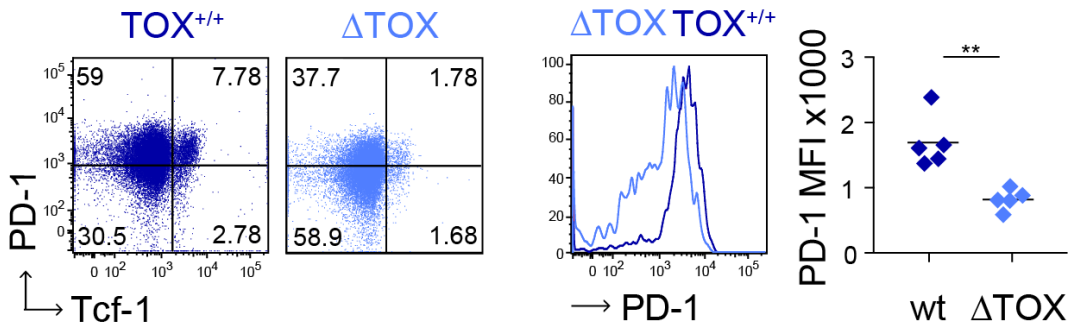

Figure 16. Absence of TOX diminishes PD-1 expression on Tcf-1⁺ cells. Dot plots show Tcf-1 and PD-1 co-expression levels on day 8 post infection for wt or ΔTOX P14 T-cells. Histograms indicate representative PD-1 expression levels in Tcf-1⁺ wt and Tcf-1⁺ ΔTOX P14 T-cells and corresponding PD-1 MFI data graphs for all animals. Symbols are data for individual mouse; solid horizontal lines represent the mean. Unpaired t test were performed with *<0.05; **p < 0.01; ns = not significant (p > 0.05). (modified from Alfei et al., 2019 [156]).

It has been previously shown that the Tcf-1⁺ memory like cells keep feature of exhausted cells [118]. This could lead to the hypothesis of a strong correlation between chronic phenotype and survival of dysfunctional T-cells, and due to the alteration of the chronic phenotype in absence of TOX, the overall fitness of the memory-like population is compromised.

6. Discussion

Chronic viral infections are a major public health issue. The WHO annual report reveals that an estimated 36.7 million people are living with HIV and that 325 million people with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. Most of the infected people don't have access to treatment. The lack of medical care results in increased risk of developing chronic liver disease, cancer, and death. Persistent viral infections are characterized by the presence of T-cells that progressively develop a dysfunctional phenotype with loss of effector functions, resulting in impairment of virus clearance. The key to develop immunological therapies against persisting infections is to increase the understanding of molecular mechanisms linked to the differentiation of functional and nonfunctional T-cell subsets and to find new ways to restore a potent T-cell response. Dysfunctional T-cells are the results of a complex molecular network that orchestrates the gene expression profile responsible of the execution of the exhaustion program. A main strategy to better characterize exhausted T-cells is to find which transcriptional regulators control their development. The current approaches for identifying genes differentially expressed between functional and nonfunctional T-cells compare gene expression profiles of T-cells recovered from week 4 Armstrong with T-cells from week 4 Clone 13 infection, juxtaposing resting memory T-cells with activated T-cells from a persistent infection [141]. This strategy hides an inherent weakness because these two populations are different in the level of activation (resting memory vs chronically stimulated). Moreover, acute or chronic LCMV strains differ in the level of inflammation they cause, the level of tissue destruction, antigen presentation kinetics, and tissue tropism of the virus [35, 39, 159], making the comparison difficult to interpret. Thus, to compare acute and chronic T-cells in a more suited way, we established an innovative set up by comparing gene expression profile of $CD8^+$ T-cells primarily developed during chronic or acute infection and re-expanded in an acute infection [117]. In this way, both types of cells are subjected to the same environment and level of stimulation and therefore the differences found are due to an epigenetic status imprinted during the

primary infection. Not only, we were able to detect genes that represent the core signature of the chronic phenotype by comparing $CD8^+$ T-cells with acute or chronic phenotype developed both in a chronic environment [115]. In this setting, the detected genes were not influenced by chronic inflammation. These two experimental approaches used to generate gene transcriptional profiles represent an important strategy to refine and complement published data set of exhausted T-cells. The identification of genes that stably fixed in dysfunctional T-cells and not merely induced by the persistent presence of an infection, is of high importance as they would be a major target for immunotherapeutic approaches. The collections of different transcriptional profiles and genomic studies generated by different groups has led to the identification of key molecular regulators of the exhaustion program. This differentiation program is defined by molecular circuits and transcriptional networks that are still only partially known. Up to now, a number of important transcription factors including *T-bet*, *Eomes*, *Blimp-1*, *Nfat*, *Batf*, *Vhl* and *Irf4* [107-109, 111] have been implicated in T-cell exhaustion, highlighting the existence of a complex net under the establishment of functional and metabolic exhaustion of T-cells. How this network is organized and how the different transcriptional regulators interact between each other is not defined yet. The progression of exhaustion could results from the generation of novel interactions or/and by dysregulation of established ones. Of note, many environmental factor are playing a role in the regulation of transcriptional factor activity and network, not surprising when considering the role of cytokines in the differentiation of naïve T-cells [160-162]. A work from Vijay K. Kuchroo and Aviv Regev groups has investigate the transcriptional network of Th17 differentiation, combining different approaches, from transcriptional profiling to computational methods [163]. They revealed the complexity of connections at the temporal and spatial level, identifying a large number of regulators organized in two different and antagonist functional modules for the Th17 differentiation, with both direct and indirect interactions [163]. This study is an example of how complex is the dynamic of the network of factors regulating cellular identity and function. Even though the definition of molecular interactions in a defined

cell is the final goal to eventually develop efficient target therapy, the first step is the discovery of which factors are involved in the differentiation program. Here we show that the transcription factor TOX is a novel important regulator of the exhaustion program, as its absence reprograms chronically stimulated T-cells to acquire an acute phenotype. An early and fundamental evidence was the stability of TOX expression in chronically stimulated, dysfunctional $CD8^+$ T-cells. The transcription and expression of a gene is regulated by the epigenetic status of its promoter, as methylation and demethylation usually correlate with gene inactivation and activation, respectively [164]. Noteworthy, we could show that the demethylation of the TOX promoter is reinforced along with the course of the chronic infection and its expression is maintained after re-expansion is an acute environment. These evidences confirm the stability of the exhausted phenotype, which is a key point in defining the differentiation program of exhausted T-cells. Many recent assorted studies have proven how T-cells maintain phenotypical, transcriptional and epigenetic features of dysfunctional cell even after antigen withdrawal [117, 124, 165]. In this context, the methylation profile of dysfunctional T-cells is the signature of their epigenetic stability [113, 114]. Indeed, the promoter of PD-1, one of the strongest markers for exhaustion, is maintained demetilated in LCMV Clone 13 infection [117, 124] and in antiretroviral therapy treated or elite controller HIV patients [125].

The potential of epigenetic manipulation as a molecular therapy to restore immune functions is of great importance, as was recently shown that PD-1 treated reinvigorated T-cells will come back to the exhaustion stage after cessation of the treatment [113]. In fact, exhausted T-cells are not able to respond to the PD-1 blockade due to the role of the de novo methyltransferase Dnmt3, which enforces the methylation of genes involved in T-cell dysfunction, restricting the efficacy of the immune-checkpoint inhibitor blockade therapy [114]. The importance of epigenetic programming in combination with the transcriptional regulatory networks in T-cell differentiation sketches the idea that transcription factors may be directing epigenetic programs. In fact, TOX belongs to the HMG-box protein [131], a

superfamily of non-histone chromosomal proteins with a DNA-binding domain that allows them to produce specific changes in target DNA structure. Other groups have investigated the downstream targets to which TOX binds in CNS-infiltrating $CD8^+$ T-cells in a model of experimental autoimmune encephalomyelitis and in HEK-293T-cell line [140, 166], using the conventional ChIP-sequencing technique or the DNA adenine methyltransferase identification (DamID) -Sequencing approach, respectively. The latter relies on the low-level *in vivo* expression of a fusion protein consisting of TOX and Dam enzyme in the HEK-293 cell line that does not express TOX. When TOX binds to the DNA, Dam methylates the nearby GATC sequences, which are selectively amplified and sequenced. Our list of differentially expressed genes between $Tox\Delta^{Ex5}$ and wt P14 T-cells on day 8 post Clone 13 infection was compared to the list of downstream targets identified in $CD8^+$ T-cells infiltrating the CNS, but no molecule in common was found. Instead, when our gene list was compared to the genes identified by Tox-Dam peaks, we found some interesting genes in common, among which *Bcl2, Ikzf2, Bach2* and *Id3*. Interestingly, these genes have a fundamental function in T-cells survival and are involved in the regulation of exhaustion. Thus, TOX might have a direct effect in controlling chronically stimulated $CD8^+$ T-cells by coordinating their survival and memory properties within the exhaustion program. Indeed, *Bcl-2* plays a critical role in regulating cell survival: while naive $CD8^+$ T-cells express a basal level of *Bcl-2*, in effector $CD8^+$ T-cells it is down-regulated in just before the death phase, but upregulated in surviving memory $CD8^+$ T-cells [167]. Moreover, *Foxo1*, a positive regulator of *Bcl-2* [168], has been recently shown to be fundamental for $CD8^+$ T-cells survival in chronic infection [169]. The transcriptional regulator Id3 promotes the survival of virus-specific $CD8^+$ T-cells in chronic infection [170] and loss of *Id3* leads to defective long-lived memory formation [171]. *Bach2* is required for maintenance of $CD8^+$ T-cell responses and restrains effector differentiation of $CD8^+$ T-cells [172]; in addition it was shown to negatively regulate the expression of Blimp-1, a promoter of exhaustion [173]. *Ikzf2*, alias *Helios*, has been identified as a “hub” gene in CD8 exhaustion and it is highly expressed in exhausted CD4 T-cells [107,

139, 141, 174]. However, the function of Helios in chronically stimulated T-cells has not been identified yet.

The identification of TOX as a central factor of the exhaustion program raises the question about its upstream regulators, to better define the position of TOX in the molecular circuits driving T-cell exhaustion. Here we show that its induction is strictly linked to the development of the chronic phenotype; in fact, TOX is not expressed by $CD8^+$ T-cells subjected to low antigen stimulation. In detail, its expression is dependent on how many times the TCR is engaged in respect to the affinity of the TCR stimulation [115], linking its expression to the amount of TCR signaling. In line with our evidences, the involvement of an ongoing TCR stimulation and the quality of its signaling in the generation of T-cell exhaustion has been investigated by several other studies. A work form the laboratory of Prof. Oxenius, has shown that the defect in cytokines production shown by dysfunctional T-cells it is not due to impairment in the proximal TCR signaling neither to the Ca^+ influx, but selectively to altered Nfatc1 nuclear translocation [175]. Nfatc1 is regulated by Ca/Calcineurin signaling after TCR engagement, and, upon T-cell activation, is dephosphorylated and translocates to the nucleus where it can activate target genes [176]. A recent study has provided a more detailed link between Nfatc1 and T-cell exhaustion. In fact, Nfatc1 can drive two parallel programs of $CD8^+$ T-cell, activation or exhaustion [139], depending on whether its cooperation with AP-1 and calcium mobilization is accompanied by concomitant MAP kinase activation or not [177]. When Nfatc1 can bind the DNA but it is not able to interact with the transcription factor AP-1, a transcriptional program that overlaps with that of exhausted T-cells is induced [139]. Of note, TOX is upregulated when this form of Nfatc1 is expressed in $CD8^+$ T-cells [139], but it is also induce by Ca/Calcineurin/Nfatc1 signaling during CD8 T-cell lineage commitment, encephalitogenic potential of autoreactive CD8 T-cells, and corticogenesis [132, 140, 166]. All these evidences from different studies confirm that the upstream regulators of TOX reside in the TCR stimulation. Moreover, the central role of TCR-Nfatc1 signaling during chronic infection has been recently confirmed by the

identification of a transcription factor circuit that regulates the exhaustion program. In fact, TCR-dependent transcription factors consisting of *Irf4*, *Batf*, and *Nfatc1* make up a central network for establishing T-cell exhaustion during chronic infection [107]. Interestingly in the Gene Transcription Regulation Database (GTRD) binding of *Irf4* and *Batf* are found both in the promoter and inside the TOX gene in ChIP sequencing experiments on $CD8^+$ T-cells (*gtrd.biouml.org*). Further studies will address how these factors and others members of this TCR-dependent molecular network promote exhaustion and regulate TOX.

The absence of TOX leads chronically stimulated cells to acquire an acute phenotype, but their long-term maintenance is severely impaired. This important evidence could be the proof of the selective survival of T-cells that stably display an exhausted phenotype [56]. Cells with an acute or chronic phenotype are formed in parallel in the early phase of an infection, and then the progression of the viral infection will favor T-cells with an acute or chronic phenotype [122]. Interestingly, cells that exhibit a dysfunctional cytokine profile are found also in the early face of an acute infection [56], thus the progressive appearance of exhausted T-cells during a chronic infection induces to believe that inflammation and persistent antigen stimulation are the main drivers of the acquisition of the chronic phenotype [41]. The dynamic of T-cell populations during the course of an infection has been analyzed in detail by two recent in vivo fate mapping studies. Work from Buchholz and Gerlach's groups have revealed that the dynamics of a single $CD8^+$ T-cell response during an acute infection is not uniform, with difference in the size and phenotype of the clones, from which memory or effector population arise [178, 179]. The different subset of clones are regulated by intrinsic and extrinsic signals, such as TCR signaling and differences in antigen-presenting cells or cytokines [178-180]. Such dynamics could be present also in chronic infection, with the persistent TCR stimulation and inflammation driving the preferential outgrowth of cells bearing chronic phenotype over the cells with an acute phenotype [122]. By preventing T-cells to acquire the dysfunctional phenotype, the absence

of TOX could result in the negative selection of cells in which the acute phenotype is enforcedly express in a condition of persistent stimulation.

The defective maintenance of ΔTOX $CD8^+$ T-cells is explained by a progressive reduction of the memory like population expressing Tcf-1. As revealed by recent publications, a small Tcf-1^+ population is responsible for the maintenance of $CD8^+$ T-cells in chronic infections [118, 119, 121] and it has the capacity of self-renewal and of giving rise to more exhausted effector cells. Interestingly, in the absence of Tcf-1, the initial expansion and the development of dysfunctional phenotype is unaltered while T-cell maintenance is severely compromised [118]. In contrast, the absence of TOX causes not only a survival defect of $CD8^+$ T-cells, but also a major alteration of their phenotype. The question if TOX drives two different and parallel programs, one for the maintenance and one for the phenotype, is hard to answer. At an early time point of the infection, absolute numbers of $CD8^+$ T-cells with or without TOX are similar, but the phenotype starts to diverge. However, a direct binding site of TOX on the *Tcf-7* promoter has not been identified yet [140, 166]. These data lead to the hypothesis that TOX drives a program responsible for the development of the chronic phenotype, ensuring the survival of only dysfunctional T-cells. Similarly, *Foxo1* is necessary for the differentiation of PD-1^{high} $Eomes^{high}$ terminally exhausted CTLs, but also to sustain virus-specific $CD8^+$ T-cells during chronic infection [112], confirming the requirement of a dysfunctional phenotype for $CD8^+$ T-cell long-term fitness. Opposing this, absence of *Tbet* exacerbate $CD8^+$ T-cell exhaustion, with consequent loss of antigen specific cells over the course of the chronic infection [108], what similarly happens to np396 epitope-specific T-cells [41] and to PD-1 KO cells [158]. These evidences highlight a necessary balance in which a regulated level of exhaustion ensures long-term survival of antigen specific, chronically stimulated T-cells. In fact, it is intriguing to notice that a decreased antigen load leads to the generation of T-cells with an acute and functional phenotype that are perfectly fit in a chronic infection, while the development of functional cells under persistent

stimulation is coupled to detrimental consequences for their survival. Since low antigen stimulation-induced acute phenotype ensures a normal proliferative capacity in $CD8^+$ T-cells [115], the survival of T-cells appears to be closely linked to their dysfunctional phenotype in high antigen condition. Thus, the amount of TCR stimulation and downstream regulation have a primary impact on $CD8^+$ T-cell differentiation during chronic infection. Here we show that TOX represents a key modulator, connecting chronic TCR stimulation with the development of the exhaustion phenotype and, at the same time, the maintenance of continuously stimulated T-cells.

The progressive loss of ΔTOX $CD8^+$ T-cells during the course of the infection could be due to a defect in the Tcf-1^- population, more terminally differentiated, or on the Tcf-1^+ progenitor population, that fails to renovate itself and to give rise to the Tcf-1^- cells [118, 119]. Our transcriptional and phenotypical analysis suggests that TOX has a more critical impact on the survival of Tcf-1^+ progenitor cells. In fact, at day 8 post infection, the absolute number of Tcf-1^+ $CD8^+$ T-cells is similar between KO and wt but the significant differentially expressed genes in Tcf-1^+ compartment outnumber the ones in the Tcf-1^- compartment. The primary effect of TOX absence on the Tcf-1^+ population could be explained by the fact that at an early time point Tcf-1^+ cells expressed higher amount of TOX and PD-1 compared to the Tcf-1^- counterpart. This means that they might be more dependent on the chronic phenotype. Later on, instead, the Tcf-1^- population is strongly affected because it is made up of more terminally PD-1^{high} differentiated T-cells. Since the Tcf-1^+ cells are responsible for CD8 response to PD-1 blockade therapy [118, 120], the presence of TOX in maintaining the Tcf-1^+ compartment results essential for the effectiveness of this therapeutic intervention.

Cytotoxic $CD8^+$ T-cells ensure that pathogens are efficiently eliminated. When the pathogen persist for longer time, as in chronic infection, continuously activated $CD8^+$ T-cells could eventually cause collateral tissue damage [51]. By ensuring a hypo-responsive state, TOX

can balance the presence of a critical number of virus specific T-cells with the protection of the host organs from an excessive effector response, and eventually pathology. In fact, we could ascertain that P14 T-cells lacking functional TOX can elicit a better viral control, but cause a more severe level of illness in the host mice. A similar outcome is achieved with the lack of the VHL protein, negative regulator of the HIFα [111]. Of note, the increased effector function in absence of TOX is counterbalanced by the loss of antigen specific $CD8^+$ T-cells. This data point out another important mechanism of regulation that characterizes the exhaustion program. Indeed, when chronically stimulated cells are not able to differentiate into the proper dysfunctional status, their survival is deeply impaired, protecting the host from unrestrained effector T-cells.

One key point of our study was the identification of a strong correlation of TOX with PD-1 expression in human HCV-specific $CD8^+$ T-cells. Interestingly, the level of chronicity of the infection is directly correlating with TOX expression, confirming its role only in dysfunctional T-cells not only in mice, but as well in humans. Moreover, Tcf-1^+ HCV-specific $CD8^+$ T-cells, which were previously shown to have the characteristics of memory-like T-cells, express high levels of TOX. The discovery of this memory-like subset of HCV specific $CD8^+$ T-cells that is maintained even after cessation of chronic antigen stimulation have great implication for re-infection and therapeutic vaccination [121]. A further characterization of the role of TOX in regulating the Tcf-1^+ subset will give great chances of immune therapeutic interventions in addition to the anti-viral treatments. In addition, TOX is upregulated in T-cells infiltrating melanoma tumor (TILs) [181], indicating a role of TOX in regulating exhaustion also in human chronically stimulated T-cells. Further studies will be of great importance to reveal if TOX might be new key candidate in immunotherapeutic approaches striving chronic infections and cancer.

The present study gives some important points of discussion for future immunotherapeutic applications. Adoptive cell transfer (ACT) is a promising field of research in cancer immunotherapy [182]. ACT therapy uses cancer antigen-specific T-cells including TILs,

peptide-induced T-cells and engineered T-cells (TCR and CAR) to kill cancer cells by recognizing antigen targets expressed on cancer cells. However, there are functional challenges of engineered T-cell therapy concerning T-cell exhaustion. The exposure of T-cells from patients to the tumor microenvironment leads to a progressive loss of functionality of the transferred T-cells. Immuno-checkpoint blockade can reinvigorate antitumor activity of $CD8^+$ T-cells, but recent studies have shown that the reinvigoration is not sustained and that the PD-1 blockade therapy can not erase the epigenetic exhaustion program [113, 114]. Thus, novel immunotherapeutic strategy has to take into account the stability of the exhaustion differentiation program [117, 120, 124, 183]. The possibility to manipulate a key regulator of chronic phenotype features and stability represents a promising approach to increase success of adoptive cell therapy. However, the manipulation of the chronic differentiation program can induce a severe survival defect of antigen specific T-cells, compromising the success of the therapy. A better understanding of how TOX controls survival of dysfunctional T-cells and the maintenance of memory-like cells is essential for future clinical applications.

7. Conclusions and outlook

The interpretation of $CD8^+$ T-cell exhaustion as a terminal state has been progressively modified. Many recent studies at transcriptional and epigenetic level have shown how T-cells during chronic infections acquire a differentiation program that it is distinct form the effector, memory or anergic one. $CD8^+$ T-cells have to be finely tuned under persistent stimulation to maintain a certain level of protection, without causing overwhelming immunopathology. With our work, we have identified TOX as a key regulator of the

exhaustion program, but further studies are needed to place TOX in the network of factors that orchestrate $CD8^+$ T-cell dysfunction during chronic infection. Would be of highest importance to identify the downstream targets of TOX. The lack of a ChIP sequencing validated antibody and is an obstacle in obtaining confident results. Moreover, TOX binds to the DNA in a conformation and not sequence dependent manner, making the analysis even more complicated. Nevertheless, changes in transcriptional programs are controlled not only through the action of TFs near TSSs, but also through epigenetic changes in a variety of DNA and histone modifications at regulatory elements throughout the genome [184]. In our work have shown that TOX regulates the methylation and chromatin accessibility of dysfunctional T-cells, highlighting a major role in epigenetic modifications. TOX could act as a direct factor or by recruiting other factors, and further study will reveal this dynamics.

With this work, we were able to characterize TOX as a key regulator in the program that drives dysfunction in T chronically stimulated T-cells. The importance of TOX is highlighted by the fact that other two groups are focusing their research on this master factor.

The results we found provide new and important insights on the regulation and functional meaning of T-cell exhaustion, substantiating the possibility to restore a potent and efficient T-cell response for future therapeutic applications.

8. Abbreviations

ACT	Adoptive Cell Transfer
APL	Altered peptide ligand
BACH2	BTB Domain And CNC Homolog 2
BATF	Basic Leucine Zipper ATF-Like Transcription Factor
BLIMP-1	PR domain zinc finger protein 1
CAR-T	Chimeric antigen receptor-expressing T
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
Eomes	Eomesodermin
FACS	Fluorescence-Activated Cell Sorter

FOXO1	Forkhead Box O1
gp33	LCMV-glycoprotein33-41 derived epitope
gp276	LCMV-glycoprotein276-284 derived epitope
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFN-γ	Interferon- gamma
IL	Interleukin
i.p.	intraperitonealy
i.v.	intravenously
IRF-4	Interferon Regulatory Factor 4
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
KO	Knock-Out
Lag-3	Lymphocyte-activation gene 3
LCMV	Lymphocytic Choriomeningitis Virus
MFI	Mean fluorescent intensity
NFAT	Nuclear Factor Of Activated T Cells
np396	LCMV-nucleoprotein396-404 derived epitope
PD-1	Programmed cell death protein-1
PD-L1	PD-1 ligand-1
p.i.	post infection
PFU	Plaque-Forming Units
s.e.m	Standard error of the mean
SIV	Simian Immunodeficiency Virus
TCR	T-cell receptor
TNF	Tumor necrosis factor
Tcf-1	Transcription factor-1
TIL	Tumor-infiltrating lymphocytes
TIM3	(HAVCR2) Hepatitis A Virus Cellular Receptor 2
TOX	Thymocyte selection-associated high mobility group box factor
TNF	Tumor necrosis factor
Wt	Wild-type

9. References

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