Searching for Modulators of CD8+ T Cell Exhaustion

Manzhi Zhao

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Zoeken naar modulatoren van CD8+ T cel uitputting

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Manzhi Zhao

born in Beijing, China

Erasmus University Rotterdam

DOCTORAL COMMITTEE

Promotor
Prof.dr. P. Katsikis

Other members
Prof.dr. C.C. Baan
Dr. M. Turner
Dr. P.A. Boonstra

Copromotor
Dr. Y.M. Müller

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Chapter 1

General introduction

ABSTRACT

T cell exhaustion is found in various disorders including chronic infections and cancer. In these diseases, T cell exhaustion shares a core of common features, namely sustained high expression of multiple inhibitory receptors, deficiency of cytokine production and loss of memory potential, leading to impeded control of pathogens or cancerous cells. Reversing or ameliorating T cell exhaustion is the focus of many therapeutic interventions in cancer. Recent studies have implicated various roles for transcription factors and epigenetic imprinting on the development of T cell exhaustion. Here, we describe the current status on the molecular and cellular signatures of T cell exhaustion, especially novel developments in the understanding of the mechanisms that drive T cell exhaustion. We also highlight strategies of rejuvenating exhausted CD8+ T cell function and analyse their role in chronic viral infection and curing cancer.

INTRODUCTION

During viral and other intracellular infections, cytotoxic CD8+ T lymphocytes (CTL) play a crucial role in eliminating infected cells and thereby prevent pathogen dissemination. During initial recognition of viral antigens by the immune system, naïve CD8+ T cells differentiate into effector cells, which expand and exert effector functions. These effectors lyse the infected cells by production and release of, amongst other immune mediators, perforin and granzyme B (GzmB). Upon the clearance of infection, the effector cell population shrinks mostly due to apoptosis. However, a small subset of CD8+ T cells differentiate into long-lasting memory CD8+ T cells that are equipped with the capacity of self-renewal, allowing for rapid recall response upon re-infection (1).

In recent years, a different state of T cell differentiation, namely exhaustion, has been described. This state of T cells is still being characterized and intensely researched. In the case of chronic viral infection or cancer, CD8+ T cells are persistently stimulated due to elevated and unremitting antigen load (2). This continuous stimulation is demonstrated to be the main factor that drives the development of exhausted CD8+ T cells (3-5). T cell exhaustion is characterised by loss of pathogen control and memory potential, and is accompanied by altered metabolism and a unique transcriptional and epigenetic program (6, 7). The differentiation toward the exhausted state follows a distinct and progressive trajectory compared to memory and effector T cells.

Along with the in-depth understanding of the features of T cell exhaustion as well as the mechanisms leading to the related phenotypes, attempts have been made to restore the function or prevent the development of exhausted T cells. Understanding factors that contribute to T cell exhaustion is important for the development of interventions. Reversing the dysfunctional T cell state that arises in cancer and chronic viral infections is the focus of therapeutic interventions and key to restoring successful T cell immunity. This thesis has focused on understanding the factors that lead to T cell exhaustion and identifying the potential strategies that reverse/prevent CTL exhaustion to reinvigorate T cell immune responses in chronic infection and cancer.

In the introduction of this thesis, I will first review the functional, molecular and signalling changes that characterize exhausted T cells. I will then go on to present the factors and mechanisms that induce and regulate T cell exhaustion. Currently, there has been some progress in finding strategies to reverse or prevent T cell exhaustion with the objective of restoring effective CD8+ T cell responses. These potential novel approaches and the associated mechanisms, will be discussed. In the end, I will discuss the limitations in our knowledge about T cell exhaustion in the context of treating associated diseases.

Cellular, functional and molecular features of exhausted CD8+ T cells

Cellular features associated with T cell exhaustion

One of the most notable features of the CTL progression to exhaustion is a gradual and hierarchical loss of the capability for cytokine production, and this is accompanied by a decreased capacity to lyse target cells (8). As CTL become increasingly exhausted, they initially lose IL-2 production, then TNF-α and finally IFN-γ secretion. This loss of the ability to make more than one cytokine by the exhausted cells is known as loss of polyfunctionality. Exhausted CTL, despite having more granzyme B, present with impaired ability to degranulate, an essential requirement for target lysis. As CTL progress to exhaustion, they lose their proliferative potential. Concurrently to the loss of effector functions and ability to proliferate, multiple inhibitory receptors accumulate on the cell surface, which mediate inhibitory signaling that are induced by ligation of corresponding ligands presented in the environment (9, 10). The end result of the exhaustion process is apoptosis (2, 11). Exhaustion is believed to be a strategy employed by the host in an effort to prevent immune overreaction and the associated immunopathology during autoimmunity (12, 13).

CTL exhaustion and inhibitory receptors

A characteristic of exhausted CTL is the simultaneous expression of multiple inhibitory receptors. There are different types of inhibitor receptors that can be detected on exhausted T cells (Figure 1). In contrast to exhausted CTL, most of the inhibitory receptor expression is transient on effector cells during acute infection and diminishes on memory cells (14). Therefore, classifying T cells as exhausted by using single inhibitory receptor expression is unreliable, because this receptor expression could simply be a marker of recent T cell activation. The most well-studied inhibitory receptor in T cell exhaustion is PD-1, a CD28-family member. PD-1 was shown to be continuously overexpressed on virus-specific CD8+ T cells during chronic viral infection and in tumor infiltrating lymphocytes (TILs) during tumor progression (15, 16). An immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in the intracellular domain of PD-1 mediate its suppressive function. Upon binding to its ligands (PD-L1 or PD-L2), PD-1 forms clusters with the TCR (17). Subsequent phosphorylation of its intracellular tyrosine domain results in the recruitment of proteins that inhibits TCR signaling. It has been shown that the ITSM could recruit the tyrosine-protein phosphatase SHP1 (also known as PTPN6) and/or SHP2 (also known as PTPN11) (18, 19). However, this has not been validated in *in vivo* studies (20). It is also poorly understood what role the ITIM plays in PD-1's suppressive function. Downstream, activation of PD-1 was shown to lead to the upregulation of the basic leucine zipper transcription factor, activating transcription factor ATF-like protein (BATF) (21). Upregulation of BATF was shown to result in reduced proliferation and functionality of CD8+ T cells (2, 22).

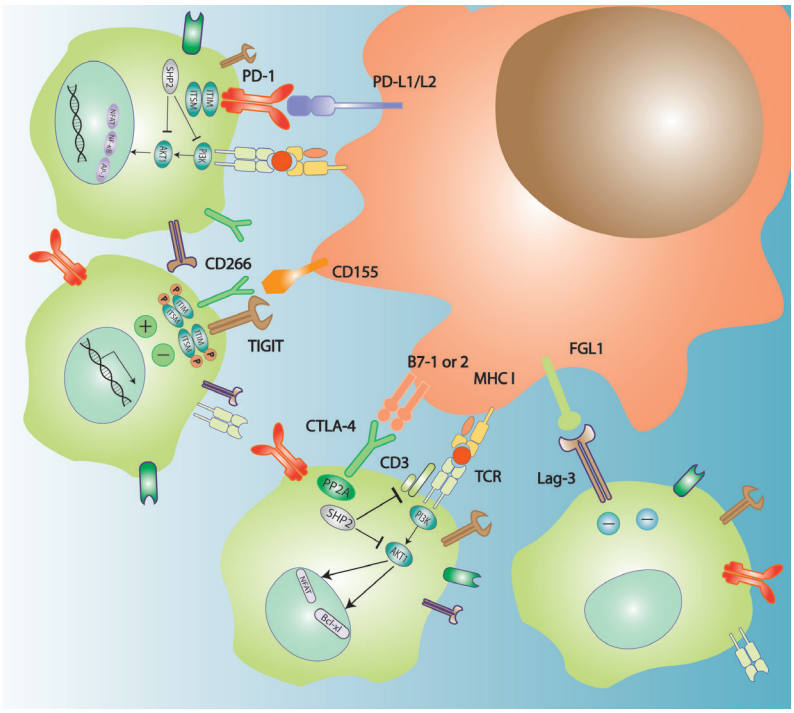

Figure 1. Inhibitory receptors and their ligands as well as the associated signalling cascades

There are multiple inhibitory receptors upregulated on the exhausted CTLs, such as PD-1, CTLA-4, et al. At the same time, there are correlated ligands expressed on tumor cell surface or antigen presenting cells. By binding to its ligand, inhibitory signaling would be transduced into the CD8+ T cells and regulating different pathways, finally inhibit the effector function of exhausted CTLs.

An important inhibitory receptor that is linked with PD-1 expression as a result of sustained CD8+ T cell activation is Lymphocyte Activation Gene-3 (LAG-3), an MHC class II ligand with structural similarities to CD4 (23). Downstream effects of LAG-3 signaling during T cell exhaustion, which are mediated through inhibiting TCR signaling, include reduction in cytokine production and proliferation. It has been shown that LAG-3 through binding to its ligand, Fibrinogen-like protein 1 (FGL1), inhibits the *in vitro* and *in vivo* CD8+ T cell response. Mechanistically, LAG-3 is associated with the inhibition of calcium flux, which compromises downstream TCR signaling (23).

Expression levels of T-cell immunoglobulin and mucin domain 3 (TIM-3) have also been shown to be correlated with the severity of exhaustion although the exact mechanism is not clear. It has been shown that as CD8+ T cells progress to the more terminal stage of exhaustion and lose their ability to produce cytokines, TIM-3 is concomitantly upregulated. TIM-3 is known to bind multiple ligands that lead to context-specific downstream effects (24, 25). In addition to the above mentioned inhibitory receptors, there are still more which are

associated with exhaustion such as T cell immunoglobulin and ITIM domain (TIGIT), CD160, CD244 (2B4) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (the related inhibitory signaling is schematically shown in the Figure 1).

Molecular and transcriptomic changes associated with T cell exhaustion

All published reports on gene expression profiling agree that exhausted T cells possess a distinct transcriptome compared to that of naïve, effector or memory cells (6, 26, 27). Although there is no true metric for accurately determining how different exhausted cells are from their naïve and memory counterparts, there are numerous differentially expressed genes identified as well as key transcription factors driving the observed transcriptomic changes that create the exhaustion profile.

A number of transcription factors play complex roles in driving T cell exhaustion. There are two T-box transcription factors, T-box transcription factor TBX21 (T-bet) and Eomesodermin (Eomes), which were first reported to play key roles in regulating both functional and dysfunctional CD8+ T cell responses. During chronic viral infections, downregulation of T-bet was associated with greater dysfunction of antigen-specific T cells. During acute infection, in the absence of T-bet, the development of T cells underwent central memory skewing (28, 29). T-bet directly regulates PD-1 expression on exhausted cells through repression of the PD-1 encoding gene (30). In HIV infected patients, CTL from elite controllers were observed to express higher levels of T-bet than the chronic progressors and its high expression was positively correlated with effector function (31). At the same time, increased expression of Eomes at the mRNA level was observed in acute viral infection compared to memory cells (8). Eomes is indispensable in memory development (32). While Eomes' contribution to T cell exhaustion remains controversial, it has been shown to correlate with the dysfunctional phenotype but also works as a maintainer of the effector function. Eomes was observed to be up-regulated in exhausted CD8+ T cells during chronic infection, but the $Eomes^{hi}$ population was still able to proliferate (33). Furthermore, in *Eomes*-deficient mice, CD8+ T cells were found to expand less during chronic infection. Due to the fact that transcription factors may affect multiple stages of T cell differentiation, the study of the function of transcription factors *in vivo* is very challenging.

Other transcription factors that have been found to correlate with the development of T cell exhaustion include transcription factors that belong to the nuclear factor of activated T-cells (NFAT)-family. These were one of the first transcription factors to be implicated in the development of T cell exhaustion, whereby increased expression was observed in T cells isolated from mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (8). Transcription factors belonging to the nuclear receptor 4A (NR4A)-family have been shown to play an important role in T cell exhaustion of both TILs and virus-specific CD8+ T cells (34, 35). Compared to fully functional effector and memory T cells, exhausted T cells expressed higher

levels of the transcription factors IRF4 and BATF, which are linked to TCR-responsiveness. NFAT1 and NFAT2 were shown to be required to induce IRF4 expression. IRF4, BATF and NFAT were found to bind to exhaustion-specific gene promoters genome-wide. Reduction in IRF4 expression was shown to favor the formation of TCF1-expressing memory-like antigen-specific T cells through restoration of anabolic metabolism (27). Enforced expression of BATF was sufficient to cause T cell proliferation and cytokine secretion impairment. Confirming this regulation of function, silencing BATF in T cells was shown to rescue HIV-specific T cell function (21).

Another transcription factor important for T cell exhaustion and T cell development is T cell factor 1 (TCF1; corresponding gene name *Tcf7*). TCF1, originally found to be crucial for memory T development, has recently been linked to progenitor exhausted T cells in both viral infections and tumors (26, 36, 37). The question about the existence of progenitor exhausted T cells or memory-like subpopulations within exhausted CD8+ T cells was raised because exhausted antigen-specific T cells undergo large expansions after transfer to naive mice (38). TCF1+ CD8+ T cells were subsequently identified as one subpopulation of memory-like exhausted antigen-specific CD8+ T cells in chronic infection. This subpopulation represents an earlier developmental state of exhaustion. Although TCF1-expressing CD8+ T cells sharing some characteristic of conventional memory cells, they also exhibit the hallmarks of exhaustion. After Tcf7-/- mice were infected with lymphocytic choriomeningitis virus clone 13 (LCMV Cl13), a strain of virus that induces a chronic viral infection, antigen-specific CD8+ T cells could expand as much as the wild-type cells on day 8, however, the expansion could not be maintained and viral control was lost by day 56 (26). Furthermore, exhausted CTL from chronically infected Tcf7-/- mice lost proliferation capacity when engrafted into acutely infected hosts. The reduction of TCF1+CD8+ T cells was accompanied by higher amounts of antigen and prolonged viral loads. Therefore, when antigen persists, TCF1+CD8+ T cells (the progenitor exhausted CTL) gradually differentiate into TCF1-CD8+ T cells (the terminally exhausted CTL) (39). The differential program of exhausted CTL has been illustrated in figure 2.

The tissue distribution of progenitor and terminally differentiated exhausted CTL indicated additional differences. The latest findings suggest that $TCF1^+$ and $CXCR5^+$ T cells can only be found in the lymphoid organs of chronically infected animals (40). Progenitor or "stem-like" exhausted CTL bear different epigenetic and transcriptomic signatures from that of central memory CTL in acute infection. These "stem-like" quiescent CTL reside in lymphoid tissues, which provide a protective niche and maintain the resource to generate "effector like" CTL during chronic infection (41). In contrast terminally exhausted CTL reside in blood and infected organs. In tumor patient studies, the presence of antigen presenting cell dense regions was shown to serve as an intratumoral niche for $PD\text{-}1^+$ $TCF1^+$ "stem-like" T cells, which contribute to sustaining the terminally differentiated T cell population as well

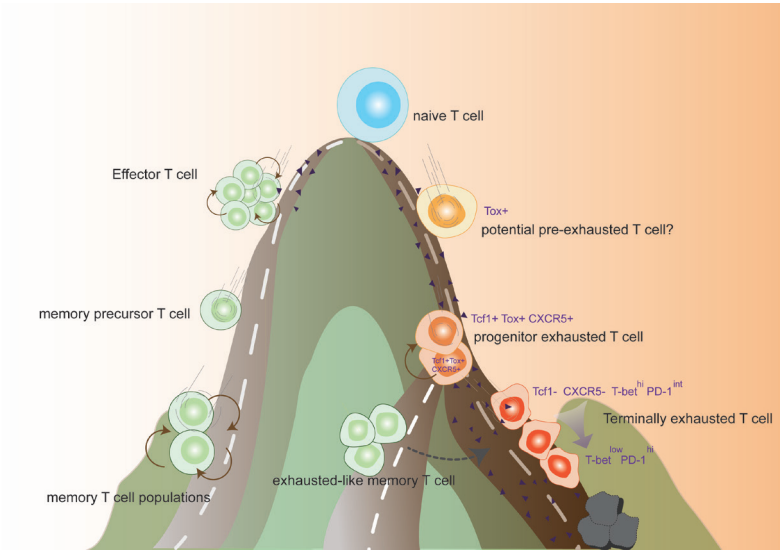

Figure 2. Different developmental stages of exhausted T cells as well as the key markers of identification

When there is an antigen presented, naïve CD8+ T cell could recognize the antigen, be primed and undergo differentiations. However, depending on the how long the antigen stimulation persists, their fates would be rather different. In the acute infectious diseases, the antigen could be cleared rapidly by the expanded effector T cells. After that, small pool of effector T cells further differentiated into memory precursor cells and finally become memory cells, which are polyfunctional and have the potential to quickly respond as the same antigen present again. On the other side, In the context of chronic viral infection or cancer, where the antigen persists. Naïve T cells would differentiated to the exhausted cells. It remains to be a question, if there is "potential pre-exhausted T cells" stage before they become exhausted. Before being terminally exhausted, at the early stage of the disease, the exhausted cells are progenitor exhausted T cells, which can be identified by the TCF1 and CXCR5 expression. The expression of TOX would drive the cells to be exhausted. There is an exhausted-like memory T cells would formed when the chronic antigen is eliminated. It is unclear, when the antigen reappears, whether exhausted-like memory T cells would function like real memory cells that responds rapidly and eliminate the infected cells or tumor cells. However, if the antigen maintained, the terminally exhausted T cell, marked by increasing PD-1 and downregulated T-bet expression will progressively formed, these cells will eventually end up in apoptosis.

as contributing to the anti-tumor immune response (42). It has been proposed that between "stem-like" progenitor exhausted CTL and terminally exhausted T cells, a transitory cell stage exists identified by $CD101^{-}$ $Tim\text{-}3^{+}$ (41). After check point blockade treatment, progenitor exhausted CTL give rise to transitory effector cells with cytotoxic and proliferative capacity, which can be detected in lymphoid and non-lymphoid organs. The transitory CTL can further differentiate into $CD101^{+}$ $Tim3^{+}$ terminally differentiated exhausted CTL. Importantly, the "stem-like" progenitor exhausted CTL in chronic infections or cancer are necessary for the animal or individual to respond to check point blockade (16, 43).

Recently, thymocyte selection associated high mobility group box protein (TOX) was found to play a critical role in programming and maintaining exhausted cells in chronic viral

infection and tumors (44-48). Mass cytometry studies of human CD8+ T cells found that TOX was expressed in the vast majority of exhausted T cells from patients with HIV infection and lung cancer (49). Interestingly, it was shown to be largely dispensable for the formation of effector and memory T cells (45). By using single cell RNA-seq, Yao C *et al* found that the progression to exhaustion was initiated before the peak of the CD8+ T cell response, where the progenitor-like CD8+ T cells in chronic infection distinguished themselves from memory precursor cells by their enrichment of the gene module containing TOX (47). TOX also promotes the long-term persistence of virus-specific CD8+ T cells during chronic LCMV infection. The expression of TOX is induced by high antigen stimulation of the TCR. In the absence of TOX, initially CD8+ T cells mediate increased effector function but cause more severe immunopathology (44). In chronic viral infection, in the absence of TOX, the number of antigen-specific T cells was decreased dramatically. Similarly, in the context of tumors, it was confirmed that expression of TOX was driven by chronic TCR stimulation and NFAT activation (45). Deletion of TOX in TILs abrogated the exhaustion program and T cells retained high expression of the transcription factor TCF1. Despite this however, they remained dysfunctional. Therefore it seems that sustained TCR activation causes the upregulation of TOX, whereby TOX works as a primary regulator to induce the exhaustion program which prevents the overstimulation of T cells and limits host immunopathology (50). These landmark findings dramatically influenced the understanding of the molecular mechanisms regulating exhaustion. However, given the multiple effects and roles transcription factors can have, one needs to be cautious in assigning exclusive roles in exhaustion. TOX is an example of this, as TOX was found to be expressed by most human blood effector memory CD8+ T cell subsets, including polyfunctional CTL, and was not exclusively linked to exhaustion (51). Despite these limitations, identifying critical transcription factors, associated with the origins and drivers of exhausted T cells provides further elucidation of exhaustion at the molecular level.

DNA methylation

The distinct gene expression profile of exhausted T cells, requires in addition to the interplay of multiple transcription factors, also the promotor accessibility of the gene loci. This accessibility is controlled by DNA methylation and chromatin accessibility. It has been shown that multiple forms of epigenetic modifications are present in exhausted cells. Due to the limitations of material and techniques, initial epigenetic modifications detected from exhausted cells were mainly focused upon single genes. Youngblood B *et al* first identified that the regulatory region of *Pdcd1* (corresponding gene of PD-1) was de-methylated in exhausted cells and the methylation status was unchanged even after viral clearance. This was in sharp contrast to acute viral infection, where the *Pdcd1* locus transiently lost DNA methylation in the effector stage but re-gained methylation in functional memory cells (52, 53). This finding was correlated at the protein level with the upregulation of PD-1 expression

on effector cells and diminished expression on memory cells. Unlike in acute resolving infection, exhausted T cells maintained high expression levels of PD-1. This transient DNA demethylation in effector cells, however, may reflect the replacement of these cells in the memory pool by memory precursor effector cells which may retain DNA methylation on the *Pcdc1* locus. Recently, global methylation sequencing was performed to compare the epigenetic landscape modifications between exhausted cells and functional counterparts (7, 54). Pauken *et al* revealed that exhausted T cells acquired a distinct epigenetic profile compared to effector and memory cells, which supported the idea that exhausted T cells are a distinct lineage (54). PD-1 blockade therapy was shown to be incapable of rewriting the epigenetic landscape of exhausted cells, which was suggested by the limited reengagement of effector circuitry in exhausted cells (54, 55). Related to DNA methylation, the enzyme, *de novo* methyltransferase 3a (DNMT3a) has been shown to contribute to *de novo* methylation during cell proliferation and development (56). DNMT3a plays a critical role in directing early CD8+ T cell effector and memory fate decisions (57). In the context of T cell exhaustion, the demethylation of the *Pdcd1* locus was due to the reduced expression of DNMT3a isoform 2.

Chromatin accessibility

Compared to DNA methylation sequencing, the results from chromatin accessibility assays are more directly correlative with transcriptional changes. When compared to memory cells, it has been shown that exhausted T cells in cancer and chronic viral infection present distinct accessible chromatin landscapes (7). The exhaustion-specific accessible regions in TILs were identified by filtering out activation-related accessible regions. Importantly, accessible regions were found to be shared by exhausted T cells from chronic viral infection and cancer (58). To confirm that epigenetic modifications play an important role in the establishment of T cell exhaustion, Zhang F *et al* showed that histone deacetylase inhibitors can restore de-acetylated histone H3 levels of exhausted CD8+ T cells *in vitro* and demonstrated functionally that their immune response to the antigen was improved (59). These effects were maintained even after adoptively transferring the inhibitor-treated cells into virus infected animals.

Using these methods, an enhancer site of the *Pdcd1* locus was discovered in exhausted T cells (60). Exhaustion-specific chromatin accessibility regions also aided in identifying strongly enriched Nr4a and NFAT binding motifs in exhausted T cells from both chronic infection and tumor settings (58). In the meanwhile, the chromatin accessibility landscape between HIV-specific and LCMV-specific CD8+ T cells show high degree of similarity based upon transcription factor binding motif comparisons (7). This finding suggested that a shared core of T cell exhaustion features in chronic viral infections and tumor is governed epigenetically.

What factor induces these epigenetic changes that impart T cells with an exhausted phenotype? Recent publications uncovered the critical function of TOX in LCMV, mouse

melanomas and human melanomas which shed light on this question (45, 48, 61). TOX drives exhaustion by recruiting the HBO1 complex that consists of multiple chromatin remodeling proteins, including proteins that are capable of acetylating histones H3 and H4 (45). The downstream effects of TOX resulted in both the shutting down of effector function associated genes and the opening of exhaustion associated genes. Overexpression of TOX was sufficient to induce a transcriptional program that leads to epigenetic changes in exhausted T cells.

All things considered though, not all the exhaustion-related molecular changes, such as the upregulation of inhibitory receptors, can be directly attributed to epigenetic modifications. This suggests that other complex regulatory mechanisms and circuitries contribute to the exhaustion-related changes and remain to be further identified.

Post-transcriptional regulation

There are variety of mechanisms by which gene expression is regulated at the post-transcriptional level. By binding to a specific sequence or secondary structure of RNA transcripts, microRNA (miRNA) and RNA binding proteins (RBP) can control gene expression in different tissues and various biological processes. MiRNAs are 18-22 nucleotide long non-coding RNA molecules. Typically, by complementary binding, often to particular sequences in the 3 'untranslated region (3'-UTR) of the target mRNA, miRNA can cause translational silencing or the degradation of mRNA (62-64). Initial experiments demonstrated the importance of miRNAs in T cell development when Dicer, an enzyme critical for the maturation of miRNAs (14), was deleted specifically in T cell compartments (65). By knocking out the enzyme, which results in the absence of mature miRNA in T cells, the function of the T cell compartment was heavily influenced. When Dicer was knocked out specifically in CD8+ T cells, it affected effector CD8+ T cells (66). Thus, it is reasonable to hypothesize that miRNAs could play a critical role in T cell exhaustion. Previous studies have reported that *ex vivo* subsets of human CD8+ T cells demonstrated unique expression patterns of miRNAs, notably the upregulation of miR-21, miR-155 and miR-146a and the downregulation of miR-19b, miR-20a, miR-92, and miR-26a in differentiated effector cells (67). However, as antigen stimulation and the local inflammatory environment during an active infection can heavily impact CTL differentiation (68), the miRNA expression profiles of exhausted CD8+ T cells are expected to differ from that observed for effector cells.

Although the changes in miRNA expression in relation to T cell exhaustion have yet to be delineated, experiments with individual miRNA have shown that these can regulate the induction of T cell exhaustion or alter the dysfunctional state of exhausted CTLs. One of the most prominent and widely studied miRNAs is miR-155. Previous studies have highlighted the contribution of miR-155 to CD8+ T cell responses in both virus and cancer models (69, 70). The expression of miR-155 is induced by TCR activation and increased expression was observed in terminally exhausted T cells during chronic LCMV infection (70, 71). Overexpression of

miR-155 leads to preservation of progenitor exhausted T cells and enhances their differentiation towards a terminally exhausted state while promoting the proliferation of terminally exhausted CTL (71). In cancer, Martinez-Usatorre *et al* found that the expression of miR-155 correlated with the responsiveness to antigen in the tumor microenvironment (TME) and thus increased tumor control (72). As an underlying mechanism, miR-155 enhances *Batf* and *Nfat* while repressing the transcription of *Fosl2*, a component of the AP-1 pathway, resulting in reduced signaling of inflammatory cytokines (69, 70, 72). Another miRNA that is sharply upregulated as a result of chronic TCR stimulation in mice and humans is miR-31 (73, 74). By using the LCMV model, Moffett *et al* showed that sustained stimulation of the TCR leads to the expression of miR-31 and attenuates the effect of IFN-β signaling in T cells. MiR-31 was demonstrated to suppress T cell effector function in chronic viral infection. The deficiency of miR-31 was largely beneficial to animals recovering from LCMV infection compared to wild-type mice (74). The above underscore the potential of post-transcriptional regulation in affecting T cell exhaustion.

Factors leading to the development of CTL exhaustion

Persistent antigen stimulation

It is well established that persistent infections both in humans and animal models induce T cell responses that display progressive dysfunction, which is thought to contribute to the further persistence of the pathogen in the host (Illustrated in Figure 3). T cell exhaustion was originally observed in human immunodeficiency virus-1 (HIV-1) infected patients in 2006, when it was shown that HIV-specific CD8+ T cells strongly upregulated PD-1 in untreated patients (15, 75, 76). Moreover, upregulation of PD-1 expression was found to be correlated with impaired functionality and disease progression, as measured by viral load and CD4+ T cell counts. In human chronic viral infection, it is not surprising that lower antigen burden correlates with less exhausted CTL. Indeed, patients that were treated with antiviral therapy showed reduced viral load which correlated with a decrease in expression of PD-1 on HIV-specific CD8+ T cells, indicative of less T cell exhaustion (77). Similar profiles of CD8+ T cell exhaustion have been shown in other chronic viral infections, namely, hepatitis B virus (HBV) and hepatitis C virus infections (HCV) (78-80). In these diseases, earlier intervention with antiviral therapy is recommended as it is linked to decrease antigen and preserves antigen-specific CD8+ T cell function (81-83).

In contrast to these chronic viral infections, other persistent viral infections, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) do not seems to cause CTL exhaustion, although the individuals do not completely eradicate the virus (2, 84). Despite the increased and sustained high levels of PD-1 expressed on CMV-specific CD8+ T cells, these levels are much lower when compared to HIV- or HBV-specific CD8+ T cells (85). EBV-specific CD8+ T

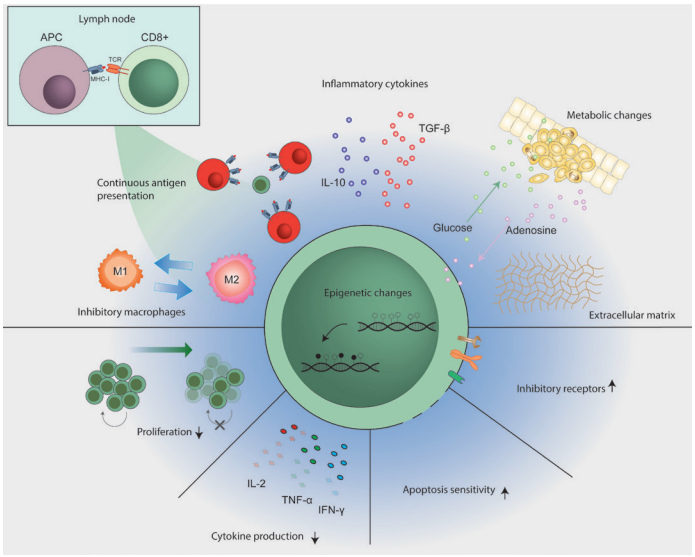

Figure 3. Multiple factors contribute to the development of CTL exhaustion

CTL exhaustion is mainly induced by persistent antigen presenting. Besides that, in the tumor microenvironment, there are different types of cell, like increased M2 cells and MDSCs that contribute to CTL exhaustion. The presence of inflammatory cytokines, such as IL-10 and TGF-β also play a role in CTL exhaustion. The other factors, like the accumulation of adenosine and glucose depriving, the existence of extracellular matrix could also play a role in CTL exhaustion development in the tumor. These factors lead to the epigenetic changes of CTL in chronic infection and cancer. The exhausted CTLs, which lost proliferation potential, hieratically reduced cytokine production, increase sensitivity to apoptosis and gradually upregulate inhibitory receptors, finally developed.

cells have been described as expressing an exhausted-like phenotype, yet still remain functional (86). These results can be explained by the dormant periods present during CMV and EBV infections in which antigen-specific CD8+ T cells are not persistently stimulated with cognate antigen.

LCMV infection is the most frequently used mouse model to study T cell exhaustion. When infected with the Armstrong strain (Arm) of LCMV, the animals develop an acute infection where the virus is cleared by day 8-10 post-infection. During this acute infection, functional effector and memory CD8+ T cells are generated. In contrast, although there are only two amino acid variations, the LCMV Cl13 strain causes chronic infection. Importantly, the immunodominant CTL epitopes are shared between these two strains, allowing researchers to investigate and compare the dynamics of the CD8+ T cell responses in acute and chronic infection (87).

The concept of persisting high levels of cognate antigen presentation contributing to CTL exhaustion was further supported in animal models. One such model, utilizes bone marrow (BM) chimeric mice, in which non-BM derived cells were MHC class I deficient. Infection of

these mice with LCMV Cl13 results in a chronic infection and CTL exhaustion. Mueller SN *et al*, reported more antigen specific CD8+ T cells in the mice lacking MHC I on non-BM derived cells, despite antigen load being less than in the class I-sufficient mice. These results provided direct evidence that persistence of antigen-MHC complexes drive functional exhaustion in T cells in the setting of an infection, leading to antigen-specific CD8+ T cells which were rapidly exhausted within 4-6 weeks (4).

Another interesting question, relevant to exhaustion, is what kind of antigens can cause the T cells to be exhausted? By using recombinant antigen variant-expressing LCMV strains, Utzschneider D T. *et al* revealed that low-level antigen exposure promotes the formation of T cells with an acute effector phenotype in chronic infection (5). Thus, whether one antigen can induce exhaustion or not depends critically on the frequency of T cell receptor (TCR) engagement and less upon the strength of TCR stimulation, i.e. the quantity of the antigen is more significant than the affinity to influence T cell exhaustion. However, LCMV infection models cannot completely exclude that the effect of chronic antigen stimulation on T cell exhaustion also requires the presence of inflammatory cytokines. This was addressed by repeatedly challenging animals with i.p. influenza virus *in vivo*, an approach that does not induce systemic inflammation (3). These studies demonstrated that chronic antigen stimulation alone is sufficient to drive CD8 T cell exhaustion (3). Thus it is not the nature of antigen but the dose of the antigen and the length of the antigen persistence that drives T cells to a dysfunctional and exhausted state.

Evidence that chronic antigen stimulation drives CTL exhaustion, also arose from HIV infection in patients. As mentioned above, combined antiretroviral therapy (cART), an antiviral therapy that strongly suppresses HIV replication and therefore viral loads, decreases the exhaustion phenotype of HIV-specific CD8+ T cells (77). As mentioned, in HCV infection, HCV-specific T cells are exhausted (78, 80) and checkpoint blockade therapy could benefit the HCV-specific CD8+ T cell antiviral response (88). In contrast to HIV suppression however, HCV clearance by direct-acting antivirals failed to restore the functionality of exhausted HCV-specific CD8+ T cells (89). These findings could be explained by the persistence of antigen months after the virus was cleared, however, this remains to be proven. Thus more evidence is needed to confirm the role of antigen in human chronic infections.

Similarly to chronic infection, the presence and persistence of antigen in the tumor environment, is what leads to tumor-specific T cells being dysfunctional at an early developmental stage in cancer (90). The difference therefore between T cells undergoing commitment to either an effector cell program or an exhausted fate is determined by this threshold of antigen exposure.

Cytokines and inhibitory immune cells

Besides sustained high levels of antigen, there are other factors that correlate with CTL exhaustion. Many inflammatory or immunosuppressive cytokines play distinct roles in both chronic viral infections and the tumor microenvironment and therefore can influence CD8+ T cell exhaustion.

IL-10 is an immunosuppressive cytokine secreted by amongst other cell types, regulatory CD4+ T cells (Tregs). When wild type mice are infected with LCMV Cl13, antigen-specific CTL are rapidly lost. However, in IL-10-deficient mice infected with LCMV Cl13 or after blockade of the IL-10 receptor (IL-10R) with a neutralizing antibody, the functional activity of CD8+ T cells is preserved (91, 92). Antigen-specific CD8+ T cells from *Il10r-/-* mice during chronic LCMV infection retained polyfunctionality and they expressed decreased levels of PD-1 expression (91). Concordantly, increased IL-10 levels have been observed during HIV infection. Multiple peripheral blood mononuclear cell subsets (PBMC) in HIV-infected subjects, particularly monocytes, T, B, and natural killer (NK) cells were confirmed to be major sources of IL-10. In line with the findings in animal models, blocking IL-10 has been shown to enhance T cell function in HIV-infected subjects (93). In addition to having a direct effect on CD8+ T cells, IL-10 can also affect exhaustion by inducing ligands to inhibitory receptors. Studies have shown this in cancer where IL-10 promotes tumor growth and induces PD-L1 expression in melanoma cells. Indeed, blocking IL-10 can increase tumor control by tumor infiltrating lymphocytes (TILs) (94).

Another immunosuppressive cytokine that was found to be highly expressed during chronic viral infection is tumor growth factor β (TGF-β). Mechanistically, TGF-β causes functional defects and promotes apoptosis in CD8+ T cells which ultimately compromises viral control (95). By selectively blocking TGF-β signalling in T cells, persistence-prone virus failed to establish a chronic infection. Blocking TGF-β signalling in T cells, increased the numbers of polyfunctional CD8+ T cells and enabled their development into memory cells while it eradicated the virus. In cancer models, it was shown that TGF-β induced a transcription factor known as Maf which induced functional defects (functionally by repressing *GzmB*) and some but not all features of exhaustion independent of antigenic stimulation (96). This effect may be mediated via IL-10 production (96). Additionally, other studies in cancer have shown that TGF-β enhances the induction of PD-1 expression under the influence of chronic antigen stimulation (97). Combination therapy of anti-TGF-β and tumor peptide vaccine has been shown to induce an increased number of tumor antigen-specific CTL with potent antitumor capacity, which indicated that TGF-β may contribute to CTL exhaustion in tumor models. However, systemic TGF-β blockade did not have any effect on T cell exhaustion in mice and therefore additional studies are required to determine the exact role of TGF-β in T cell exhaustion (98-101). The above indicate that IL-10 and TGF-β could contribute to some

of the functional T cell defects and the development of T cell exhaustion induced by chronic TCR signalling.

The type I interferon (IFN-I) response plays a prominent role during infections as it induces an antiviral state in cells (102) but also promotes the cytotoxic CD8+ T cell response (103-105). Type I interferon receptor (IFNR)-stimulated genes (ISG), signal transducer and activator of transcription (STAT) genes as well as the IFN-I regulatory factors (IRF) and viral clearance have similar kinetics in chronic infection (106, 107). Exogenous addition of IFN-α to HIV-infected samples *in vitro* has been shown to suppress viral replication (108). In both HIV-infected patients or simian immunodeficiency virus (SIV)-infected animals, IFN-α levels during the later stage of infection were found to be increased (109, 110). Peak plasma IFN-α levels and viral loads correlate negatively during acute SIV infection supporting a potential antiviral role for IFN-α, but during chronic SIV infection IFN-α levels and viral loads show a positive correlation, indicating that either the viral loads drive IFN-α production or chronic IFN-α contributes to the loss of viral control (111). This indicated that there was a link between prolonged IFN-I signalling and immunosuppression in chronic infection. Furthermore, type IFN-I renders T cells more sensitive to apoptosis, which was associated with abnormal expression of pro- and anti-apoptotic molecules in HIV-infected patients (111). In the LCMV model, genes downstream of the IFN receptor were shown to be upregulated in PD-L1^{hi} IL-10^{hi} expressing cells and blocking IFN-α resulted in an increased number of polyfunctional CD8+ T cells (TNF-α+IFN-γ+) and better control of viral infection (112-114). However, these effects of blocking type I IFN appear to be mediated by CD4+ T cells and their IFN-γ secretion (112-114). Moreover, the correlation between type I IFN and resistance to tumor treatment indicates a similar role for the type IFN-I response for TILs (94, 115). Despite the evidence, the exact role and direct contribution of Type IFN-I production to the development of CD8+ T cell exhaustion remains unclear (116).

Despite the accumulated evidence of the aforementioned immunosuppressive cytokines and their role in compromising CD8+ T cells in exerting their effector function in chronic infection, the regulator pathways that are related with these cytokines and how the immunosuppressive cytokines are regulated are far less clear. *In vivo* animal experiments showed that the bystander inflammatory environment could have an impact on the memory CD8+ T cell development, but would not induce CD8+ T cells to be exhausted (117) which might suggest the requirement of additional factors or that the inflammatory environment does not directly drive CD8+ T cells to become exhausted. In chapter 4 of this thesis, the data from our group illustrates that beyond immunosuppressive cytokines, the use of cognate antigen alone is sufficient to induce murine CD8+ T cell exhaustion *in vitro*.

The CD8+ T cell response in both chronic viral infection and tumors is dependent on CD4+ T helper cells. T helper cells are critical sources of IL-2 and IL-21, cytokines that both have been shown to enhance the CD8+ T cell response. Experimentally upon LCMV Cl13 infection,

wild type mice depleted of CD4+ T cells present with a more severe T cell exhaustion phenotype (118) and reduced T cell functionality. The indirect reason for this could be that CD4+ T cell depleted animals harbour increased viral load which induced more CTL exhaustion. Alternatively, CD4+ T cell depletion could promote CTL exhaustion because of reduced IL-2 and IL-21 production. During chronic viral infection in both mice and humans, IL-2 was shown to maintain the memory-like potential of CD8+ T cells as evidenced by decreased expression of inhibitory receptors and upregulation of CD127, which is the IL-7R and associated with greater memory potential (119, 120). Additionally, exhausted TILs from melanoma patients were capable of proliferating when supplied with exogenous IL-2 *in vitro* (121). IL-21 is another cytokine that has been associated with CD8+ T cell exhaustion. T follicular helper (THF) and Th17 CD4+ T cells are the most important sources of IL-21. Cell-autonomous IL-21 receptor (IL-21R)-dependent signaling plays an especially important role in chronic infection as it was shown to sustain the CD8+ T cell response (122, 123).There were fewer antigen-specific CD8+ T cells and higher viral titers in LCMV Cl13 infected IL-21R-/- mice on 30 days post-infection (122, 123). Furthermore, treatment with IL-21 enhanced CD8+ T cell function as well as viral clearance in CD4+ T cell depleted LCMV Cl13 infected mice (124). These findings highlight the importance of CD4+ T cell help during the CD8+ T cell response in chronic infection and tumors. Overall the cytokines IL-2 and IL-21 derived from CD4+ T cells suppress the development of T cell exhaustion in chronic viral infection and tumors by counteracting the induction of exhaustion features.

The role of immune regulatory cells in CTL exhaustion

Immune regulatory cells, normally assigned to dampen immunity and control autoreactivity have also been implicated in regulating CTL exhaustion. The two major cell types that have been investigated in the context of exhaustion are regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC). Below, I will discuss these in the context of chronic infection and will discuss them separately in cancer.

Beyond classical helper function, subsets of CD4+ T cells can affect CTL exhaustion by their immunoregulatory effects. Tregs were suggested to play an important role in driving T cell exhaustion as they secrete the immunosuppressive cytokines, IL-10 and TGF-β (125, 126). Depletion of Tregs during chronic LCMV infections resulted in an improved CD8+ T cell response (127), which was reflected by a much higher expansion of functional antigen-specific CD8+ T cells. However, the viral load remained unchanged despite the augmented virus-specific CD8+ T cell population. Strikingly, this was caused by an upregulation of PD-L1 in various cells, leading to inhibition of virus-specific CD8+ T cell effector function (127). Thus Tregs appear to have a dual role in exhaustion. While suppressing CTL numbers and promoting CTL exhaustion, they also repress immune activation and the expression of ligands

for inhibitory receptors on exhausted CTL. A similar role for Tregs has been described in the tumor microenvironment (128-130), which will be discussed later.

MDSC may regulate CD8+ T cell responses negatively in human chronic infectious disease (131). MDSC have been found to be enriched in cancer and chronic infection (132, 133). However, the role of MDSC in human chronic viral infectious disease remains controversial (134). Norris B et al studied MDSC effects on T cell immunity by using LCMV Cl13. Similarly, it was shown that the number of Ly6C^{hi} monocytic and Gr-1^{hi} neutrophilic cells, which resembled MDSC and play a suppressive role, was higher in chronic LCMV Cl13 than in acute infection. Further, in the animals when this population was depleted, enhanced T cell function was detected (135). Therefore, MDSC contributes to the dysfunction of CD8+ T cells in chronic infection and tumors (136, 137) .

Thus immune regulatory cells such as Treg and MDSC appear to play a conflicting role in cancer and chronic infections, on the one hand suppressing T cell immunity while also suppressing T cell exhaustion at the same time. Depending on the environment and context, the outcome of this conflicting effect can be either reduced or enhanced protection. In the overwhelming majority of tumor studies, depletion of Treg or MDSC resulted in better protection most likely due to prevention of their negative effects on effector T cell expansions which is critical for this protection.

Cancer-specific factors that promote T cell exhaustion

In cancer, CTL dysfunction may not only be the result of CTL exhaustion. CTL dysfunction in cancer has been attributed to factors and cells within established tumors, including the immunosuppressive microenvironment (e.g., MDSC) (137), tumor-associated macrophages (136), FOXP3+ Tregs (acting via IL-10, TGF-β, indoleamine-2,3 dioxygenase [IDO]) (138, 139), checkpoint inhibitory signaling pathways (e.g., PD1 and PD-L1) (140), and physiological changes (e.g., hypoxia and low nutrient levels) (Figure 3). Additionally, tumor neo-antigens recognized by CD8+ T cells are only weakly immunogenic and antigen recognition and co-stimulation is impaired in both the tumor microenvironment (TME) and draining lymph nodes. Thus, different to the T cell dysfunction developed in chronic infection, the dysfunctional status of CD8+ T cells in cancer could be induced by the complex immunosuppressive environment faced by TILs in the TME (138, 139).

Firstly, tumor cells express high levels of PD-L1 and B7 superfamily member 1 (B7S1), whose expression is mediated by IL-10 and IL-6. Both PD-L1 and B7S1 are capable of binding receptors on exhausted CD8+ T cells. Ligation of these receptors results in downstream reduced cytokine production and proliferation (138, 140, 141). In addition to the effects discussed before, TGF-β and IL-10 lead to increased expression of PD-L1 and promote the differentiation of naïve CD4+ T cells to Tregs in the TME (142). As mentioned before, these

cells in turn produce high levels of IL-10 and TGF-β, further increasing the immunosuppressive microenvironment and the accumulation of Tregs.

Beyond their production of immunosuppressive cytokines, Treg cells exert other functions that compromise CD8+ T cell effector functions. Expression of the ectoenzymes CD39 and CD73 on the Tregs surface, which can produce extracellular adenosine, further serves to inhibit CD8+ T cells in the TME (143, 144). The adenosine receptor (A2aR) has been detected on multiple immune cell types including CD8+ T cells and activation of this receptor results in impaired effector function (145). A2aR signaling has also been shown to induce CTL exhaustion (146). Thus, the infiltration of Tregs in the TME results in a high concentration of PD-L1, cytokines and adenosine, all of which contribute to T cell exhaustion of antigen-specific TILs within the TME.

Another immunosuppressive cell in the TME is the tumor associated macrophage (TAM), which is recruited through the secretion of, amongst other molecules, vascular endothelial growth factor (VEGF) produced by tumor cells. VEGF secretion recruits monocytes into the TME that can then differentiate into TAMs. In the tumor tissue, two classes of macrophages compose the TAM population, they are known as classically active macrophages (M1) and alternatively activated macrophages (M2). The M2 tend to be more abundant due to the environment established by the tumor (147, 148). Instead of playing a phagocytosis role, these cells contribute to the immunosuppressive environment and help tumor cells survive, by secreting IL-10 and TGF-β, further suppressing proliferation of CD8+ T cells (148) and promoting CTL exhaustion as mentioned above.

The fast and uncontrolled tumor growth rate also poses an additional feature that inhibits TIL function. CD8+ T cells in the TME must adjust to the unsupportive metabolic environment, which highly influences the effector function of CD8+ T cells (129, 140). As a result of gene defects, malignant cells use glycolysis as the main means to generate energy, a pathway that is similar to the one used by activated CD8+ T cells and therefore both cell populations compete for metabolites, namely glucose. Additionally, tumor cells excrete excessive amounts of by-products (e.g., Adenosine) in the TME, adding to the suppressive conditions CD8+ T cells must overcome and most likely contribute to the development of exhaustion (140). Taken together, tumor cells establish a stifling immunosuppressive network rich in factors that contribute to CD8+ T cell exhaustion on multiple levels.

Exhaustion however does not only affect the endogenous T cell response in cancer but also adoptively transferred T cells. Chimeric antigen receptor (CAR) T cell therapy in patients brought considerable success on curing hematological malignancies (149-151). However, clinical results with CAR T cell therapy for solid tumors have been less promising (152-154). It seems multiple factors contribute to this failure (155) and one of these is that the tumor antigen together with suppressive TME can drive the CAR T cells to become dysfunctional or exhausted (156). To overcome these obstacles, CAR T cell therapy has been proposed to be

combined with cytokine administration, checkpoint blockade (157) and/or tumor vaccines (158, 159).

Distinguishing T cell exhaustion from anergy

Although exhausted T cells were compared with other forms of T cell dysfunction (141), it is important to briefly compare T cell exhaustion to T cell anergy. Distinguishing CD8+ T cell exhaustion from CD8+ T cell anergy, however, suffers from the paucity of data on CD8+ T cell anergy. According to studies published thus far, many similarities in terms of phenotype can be ascribed to both exhausted and anergic cells (141). Both demonstrate poor responsiveness to antigen re-stimulation, namely, reduced expansion and cytokine production, especially, IL-2 production (160). However, there are defining differences between exhaustion and anergy. Anergy is caused by defective priming of cells. When naïve T cells are activated by antigen in the absence of co-stimulatory signaling, an anergic program ensues (161). Exhaustion, however, is driven by sustained antigen stimulation. Accompanying this repeated antigenic stimulation, exhausted cells accrue multiple inhibitory receptors on their cell surface. Anergic cells do not exhibit these features. Further, IL-2 not only prevents anergy, but also is used to reverse it (162). Similarly, other cytokines, IL-7 and IL-15, were shown to be capable of overriding the induction of anergy (161). In contrast, exhausted cells are no longer sensitive to IL-7 or IL-15 treatment. In previous research from our lab as well as others, the molecular features of exhaustion and anergy were compared and these were found to be different forms of T cell dysfunction (8) (this thesis Chapter 4). However, more systematic comparisons remain to be performed.

Therapeutic targeting of T cell exhaustion to treat chronic viral infection, cancer and autoimmunity

Check point blockade therapies can reinvigorate exhausted CTLs response

It has been shown that genetic deletion of PD-1 at the onset of chronic infection will not protect T cells from exhaustion, and contrary to what may be expected, some phenotypes of exhaustion were more severe. The absence of PD-1 led to increased cytotoxicity but forced cells toward further terminal differentiation (163). Thus, PD-1 plays a critical role in preserving the exhausted T cell population from overstimulation. Why then, does *in vivo* blockade of the PD-1 pathway mediate decrease viral load or tumor burden? Is it because this therapy can reinvigorate the exhausted T cell response, or maybe a subset of the population? Or are the effects attributable to another novel T cell population? When the effects of blocking PD-1 related pathways are evaluated in terms of efficacy, it is apparent that both tumor growth and viral load were better controlled (164-166). These effects made check point blockade become one of the most promising immunotherapies in cancer treatment. Despite

this immunotherapy's successes, however, the mechanisms behind the beneficial effects are only gradually being further elucidated.

Initial observations of the antigen-specific T cells from animals or non-human primates receiving anti-PD-1 or anti-PD-L1 therapy, demonstrated CD8+ T cells producing more IFN-γ and having greater polyfunctionality, which resulted in decreased viral load (166, 167). Furthermore, *ex vivo* experiments utilizing anti-PD-1 or anti-PD-L1 treatment enhanced the *in vitro* HIV-specific CD8+ T cell function and improved their capability to proliferate in response to cognate peptide stimulation. Clinical benefit, however, has yet to be demonstrated for anti-PD-L1/PD-1 antibody therapy (often referred to as checkpoint blockade) in HIV infection, despite HIV+ cancer patients being treated with checkpoint blockade (168, 169). In SIV-infected animals there remains a lack of clear evidence with contradicting results on whether checkpoint blockade can improve viral control (170, 171). Checkpoint blockade therapy, however, has been intensively utilized to treat cancer patients, but despite significant antitumor effects in some patients, outcomes are considerably variable (11, 172, 173). Simultaneously, the effects on different types of tumors are also variable and it remains difficult to predict which patients will benefit from these treatments (165, 174).

Early studies using the LCMV model found that there were different subpopulations of exhausted T cells. The terminally exhausted subset, identified by PD-1^{hi}CD44^{int} expression, did not respond to treatment with anti-PD-L1. However, the PD-1^{int}CD44^{hi} subset did respond to treatment, yielding an expansion of virus-specific CD8+ T cells and increased protective immunity (172). Indeed, later studies have shown that the epigenetic imprinting of the terminally exhausted T cell populations in both chronic viral infection and tumors was irreversible and anti-PD-L1 treatment does not result in epigenetic remodeling of genes involved in exhaustion. Thus, it seems anti-PD-L1 is not capable of reversing the exhausted status of T cells (54, 55, 58, 90).

More recent research has focused on further characterizing the exact subsets of exhausted T cells that respond to anti-PD-1/anti-PD-L1 treatment. There are several strategies to identify these populations by combining different markers. Im *et al* described that CXCR5^{+} progenitor exhausted T cells responded to anti-PD-L1 treatment which induced their proliferation to give rise to a new pool of CXCR5^{-} terminally exhausted T cells. These terminally exhausted T cells were highly cytolytic, which resulted in better control of the viral infection (43, 175). These findings were further confirmed by comparison of LCMV-specific CD8+ T cells and TILs from melanomas (16). Other studies also identified progenitor exhausted T cells based on high TCF1 expression and lower expression of inhibitory receptors (16, 26, 176). To circumvent technical problems, surrogate markers were identified to discriminate the progenitor and terminally differentiated populations of exhausted CTL. TIM-3^{+} cells normally lose TCF1 expression and are more terminally differentiated (43). Similar to chronic murine infection model systems, CD127^{+}PD-1^{int} HCV-specific CD8+ T cells exhibited similar features

to progenitor exhausted cells, which may serve to support the immune response following resolution of infection (37). TCF-1 and signaling lymphocyte activation molecule family 6 (Slamf6) were found to be highly co-expressed by progenitor exhausted cells. Therefore, the combination of Slamf6 and TIM-3 should identify progenitor and terminally differentiated exhausted cells (16). More recently, additional stages of exhausted CD8+ T cells are being distinguishing based on the expression of Slamf6 and CD69 (177).

At the same time, the markers to identify progenitor exhausted cells in chronic infections, were further validated by using tumor bearing animal models, where the progenitor exhausted population could reduce tumor growth and generate more TILs. These results indicated that the expansion capability of progenitor exhausted population was revived by check point blockade therapy (164). Besides examining anti-PD-L1 treatment in animal models, this treatment has also been intensively studied in cancer patients, though the exact mechanism that how anti-tumor immunity is enhanced is disputed (173).

The progenitor and terminally exhausted subsets of T cell exhaustion play an important role in checkpoint blockade therapy. In tumor experiments, it was found that the responses to check point blockade are heterogeneous and highly dependent on the type of tumor (173). For example, melanoma, which tends to have high mutational burden and strong immunogenicity, normally responds well to check point blockade. By sequencing the TCR before and after the check point blockade therapy, it was discovered that the expansion of T cell clones was not derived from pre-existing tumor-infiltrating T lymphocytes. Instead, the expanded clones were derived from novel clonotypes that had not previously been observed in the same tumor (178). *In vitro* results demonstrated that addition of anti-PD-L1 to the purified antigen-specific CD8+ T cells did not increase their cytotoxicity effects (9), which indicated that check point blockade might not affect the exhausted T cells per se. Taken together, check point blockade therapy induces a protective immune response when there are “progenitor” cells in lymphoid organs, which will further differentiate to cytolytic cells to kill the target cells. In the early stages of chronic viral infection or high mutation-bearing tumors, progenitor cells are better preserved. Because check point blocked therapy cannot remodel the pre-existing status of the exhausted cells, other approaches are necessitated to improve the T cell response.

One similar immunotherapy approach is targeting other inhibitory receptors or targeting combinations of more than one inhibitory receptors. TIM-3+ PD-1+ antigen-specific T cells in the tumor were found to be most dysfunctional, therefore TIM-3-TIM-3L blockade was tested as a checkpoint blockade strategy. By treating PBMCs from chronic HCV infected patients with TIM-3-TIM-3L blockade, enhanced cytokine production and proliferation were observed after stimulation with cognate peptide *ex vivo* (179). Furthermore, TIM-3-TIM-3L blockade synergized with PD-1-PD-L1 blockade (180). Blocking TIM-3 also resulted in increased *in vitro*

cytotoxicity and proliferation of virus-specific CD8+ T cells from HIV and HCV infections (88, 181).

Likewise, blocking LAG-3 alone had no result during chronic LCMV infections, though combinational therapy using PD-1 and LAG-3 blockade exerted a synergistic effect on reinvigorating CD8+ T cells (9). Importantly, improved pathogen control was observed in LCMV-infected mice and *Plasmodium falciparum* infected patients after blocking both PD-1 and LAG-3 (182, 183). CTLA-4 blockade therapy has been widely used in treating cancer patients (184-186). Whereas, its effects on chronic virally infected patients were not comparable to the effects observed in cancer patients (187). It could be surmised that the observed reinvigoration was CD4+ T cell-dependent, indirectly affecting exhausted CD8+ T cells. Thus, it is intriguing to understand why blockade of CTLA-4 *in vivo* failed to contain viral load or improve CD8+ T cell function in SIV infection (188). Other checkpoint blockade strategies target additional inhibitory receptors, such as TIGIT. TIGIT acts to transduce an inhibitory signaling cascade in CD8+ T cells by competing with CD266 and binding to CD155 on the tumor cell surface, thus anti-TIGIT or combining it with anti-PD-1/PD-L1 therapy could revive the anti-tumor immune responses in multiple cancers (189).

"Shock and kill" strategy to cure HIV requires a functional CTL response

As one of the most widespread chronic viral infectious diseases, HIV infection, is also the most well-studied in the field of human CD8+ T cell exhaustion. Since the inception of cART, the mortality of HIV-infected individuals has been significantly reduced. However, little progress has been made to develop a protective vaccine or find a drug to cure HIV infection. Exhaustion of HIV-specific CD8+ T cells, and thus incomplete clearance of the virus necessitates reinvigoration of the CD8+ T cell response against HIV-1 and remains extremely important for curing of the disease.

Latent infection is the biggest hurdle to overcome in the curing of HIV. This latent infection gives rise to the latent HIV reservoir that can harbour virus for many years. The proviral DNA is transcriptionally silent, which results in latently infected cells being virtually undetectable by the immune system. Persistence of the virus results from the latent reservoir being mainly found in quiescent cells (190, 191). A number of factors have been proposed to reactivate latent HIV, these have been termed latency reversing agents (LRAs), and they include Histone Deacetylase inhibitors (HDACi), protein kinase C (PKC) agonists, bromodomain and extraterminal domain protein (BET) inhibitors and BRG-Brahma associated factor (BAF) inhibitors. Targeting latent infections is critical for curing HIV infection, and thus the "shock and kill" approach has been proposed. In this therapy, simultaneous application of LRAs to reactivate HIV-1 provirus transcription is employed alongside cART to prevent the infection of new cells. This reactivation of infected cells makes latent cells immunologically visible and thus targets to be lysed by cytotoxic cells (192, 193). In order to kill the infected cells, apparently,

it is essential that an effective immune response can be mounted, especially functional HIV-specific CD8+ T cells capable of eliminating target cells. In theory, the combination of LRAs, cART, and sufficient immune responses could achieve HIV latent reservoir elimination and thereby cure HIV infection. However, thus far, attempted functional cures of HIV infection have failed (194, 195). This could be due to lack of an effective way to eliminate infected cells (196).

Although several reagents have demonstrated the capacity to reactivate latently HIV infected cells, there are several issues to be solved before a successful intervention can be reached. Firstly, the LRAs should not adversely affect the functions of other cells, especially CD8+ T cells. We and others have found that different classes of LRAs greatly vary in their cytotoxic effects on immune cells (196) (this thesis chapter 2). Given the above described epigenetic changes in exhausted CD8+ T cells, LRAs that are epigenetic modifiers could directly affect exhausted CTL. In this thesis chapter 2, we present data on different LRAs toxicity effects on immune cells from healthy donor and HIV+ patients, the potential of these reagents to reverse or rejuvenate CD8+ T cell function is also discussed. Identifying LRAs that improve exhausted CTL would be most valuable as preserved or rejuvenated cytotoxic T cells are likely necessary to guarantee an effective cure for HIV.

Induction of exhaustion to benefit autoimmune disease patients

Whereas T cell exhaustion in tumors and chronic infections favors disease progression, the opposite might be true for T cell exhaustion in autoimmune disorders. Indeed, expression of T cell exhaustion associated markers in multiple autoimmune disorders has been associated with a better prognosis during the course of disease (12). Furthermore, checkpoint blockade therapy to treat cancer induced in some cases as a side effect autoimmunity (197, 198) and has even led to fatal cases where patients developed lethal immune-mediated diseases as a result of autoreactive T cells (13). This provides strong evidence that inhibitory receptors are important component of peripheral tolerance. Thus, inducing T cell exhaustion in T cells of patients with autoimmune disorders might be an interesting therapeutic approach.

A study by McKinney *et al* has found that *in vitro* incubation with Fc-chimeric PD-L1 induced exhaustion features in CD8+ T cells (13). Future studies should clarify whether inducing $CD8^+$ T cell exhaustion incurs adverse effects like viral relapse or the development of malignancies. A study by Kulshrestha *et al* proposed that dysregulation of T cell exhaustion might be at the very foundation of autoimmune disorders (199). Mice that expressed transgenic autoimmune regulator (Aire), a transcription factor that promiscuously transcribes sets of tissue related antigens, under the control of a DC-specific promoter were not observed to develop immune-mediated diabetes. Notably, this was attributed to increased exhausted CD4+ and CD8+ T cell populations. These cells possessed a poor expression profile of IFN-γ and TNF-α, and high expression of PD-1 (199). Thus, the effect of Aire could rely on T cell

exhaustion and autoimmune disorders might represent a failure of the induction of the T cell exhaustion program. However, the role of T cell exhaustion in autoimmune diseases, remains far less conclusive than the much more studied relationship between T cell exhaustion and chronic infection or cancer.

CONCLUDING REMARKS

The foundations of T cell exhaustion are primarily based on results from the LCMV Cl13 mouse model. The knowledge gained from this model has become the stepping stones for therapies that are currently being employed to treat patients with various types of cancer. Despite its success, immune checkpoint blockade with antiPD-1/antiPD-L1 and antiCTLA-4 has demonstrated inconsistent results and variations in outcome have been found depending on the cancer type. These variations have been related to the mutation load of tumors (150, 200-202), but could also be a result of context-specific heterogeneity within the T cell exhaustion subpopulations, which is highly dependent on the microenvironment in which CD8+ T cells encounter their cognate antigen in chronic infection or tumors.

In order to search for therapeutic interventions or novel compounds that work as immune regulators to reverse CD8+ T cells exhaustion, a systematic analysis is necessary. On one side genome-wide screens revealed critical factors influencing T cell development and effector function (203, 204). On the other side, there has been attempts to screen candidate drugs to enhance exhausted T cell function (205). Major hurdles still exist for the assaying of these compounds, for instance the lack of an *in vitro* model to assess candidate efficacy prior to assessment in clinical trials. Discover the strategies of reversing or preventing CTL exhaustion is depending on a deeper understanding of T cell exhaustion biology and the collaboration of different fields of researchers. In this thesis, I examine the effect of LRAs on T cell exhaustion and survival (chapter 2), and then describe the establishment of an *in vitro* model of CD8+ T cell exhaustion that has the potential to be used for drug screens (chapter 3). In chapter 4, we studied the effects of miR-139 on regulating effector, memory and exhausted CTL differentiation. In chapter 5, I examine the effect of interleukin-2-inducible T-cell kinase (ITK) inhibition and the amelioration of continuous TCR activation on T cell exhaustion. The discussion in chapter 6 puts these findings in context and discusses future studies.

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Chapter 2

T cell toxicity of HIV latency reversing agents

Manzhi Zhao[a], Elisa De Crignis[b], Casper Rokx[c], Annelies Verbon[c], Teun van Gelder[d], Tokameh Mahmoudi[b], Peter D. Katsikis[a], Yvonne M. Mueller[a,*]

[a]Department of Immunology, Erasmus MC University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
[b]Department of Biochemistry, Erasmus MC University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
[c]Department of Internal Medicine, Section of Infectious Diseases, Erasmus MC University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
[d]Department of Hospital Pharmacy, Erasmus MC University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.

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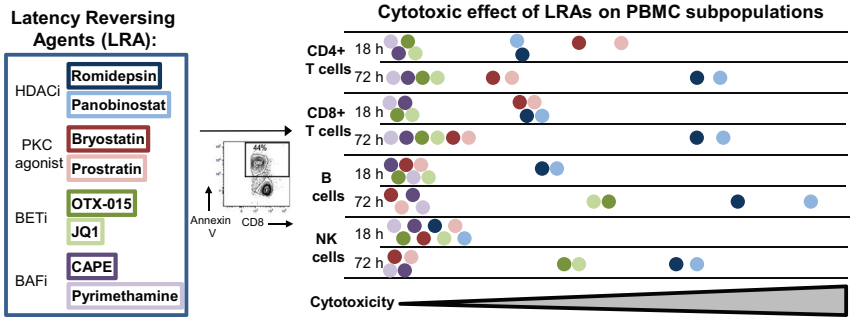
ABSTRACT

Combination antiretroviral therapy reduces morbidity and mortality in HIV infected patients. However, the cure of HIV infection is hindered by the persistence of the latent HIV reservoir. Latency reversing agents (LRAs) are developed to target the HIV latently infected cells for HIV reactivation. In addition to reversal of HIV latency, the eradication of HIV latently infected cells will require effector HIV-specific CD8+ T cells. Therefore it is imperative to understand how LRAs affect immune cells. We have performed a comparative in depth analysis of the cytotoxicity of several compounds belonging to four LRA classes on T cells, B cells, and NK cells. In addition, the effect of these LRAs on activation and inhibitory receptor expression of CD8+ T cells was examined. We show that the HDAC inhibitors romidepsin and panobinostat are highly cytotoxic for CD4+ and CD8+ T cells, whereas the PKC agonists bryostatin and prostratin and BET inhibitors JQ1 and OXT-015 were less cytotoxic. The BAF inhibitors CAPE and pyrimethamine exhibit no cytotoxicity. Drug-specific cytotoxicity on CD8+ T cells was comparable between healthy controls and cART-treated HIV-infected patients. Bryostatin and both BET inhibitors downregulated the expression of CD279 on CD8+ T cells without affecting their activation. Our comparison of LRAs identified differences in cytotoxicity between LRA classes and members within a class and suggests that some LRAs such as bryostatin and BET inhibitors may also downregulate inhibitory receptors on activated HIV-specific CD8+ T cells. These findings may guide the use of LRAs that have the capacity to preserve or restore CD8+ T cell immunity.

Keywords: Cytotoxicity; T cells; HIV infection; latency reversing agents.



INTRODUCTION

The morbidity and mortality of HIV-infected individuals have been significantly reduced mainly due to the effectiveness of combination anti-retroviral therapy (cART). However, the functional cure of HIV infection has proven elusive (reviewed in [1]). During infection HIV integrates into the DNA of infected cells as proviral HIV DNA and establishes a reservoir of latently infected cells. This provirus is transcriptionally silent, making latently infected cells undetectable by the immune system. The latent reservoir is mostly found in resting memory CD4+ T cells [2,3]. A promising approach to reactivate HIV and purge the latent reservoir is the "shock and kill" strategy [4,5]. In this strategy, reactivation of HIV is accomplished with latency reversing agents (LRAs) which will induce the transcription of the provirus in latently infected cells while concurrent treatment with ART prevents the infection of new cells. However, because the cytopathic effect of the virus is not sufficient to kill all HIV infected cells [6], it is essential that the immune system and specifically HIV-specific CD8+ T cells eliminate HIV infected cells. If successful, the combined effects of LRAs, cART and immune system could lead to the elimination of the HIV latent reservoir and therefore may cure HIV infection.

In recent years it was proposed that one underlying reason for the failure of HIV-specific CD8+ T cells to control HIV infection is the exhausted state of these cells [7-12]. Because such CD8+ T cell exhaustion associates with inhibitory receptors expression [10-14] and this has been linked to epigenetic changes [15-18], LRAs may also affect the expression of such inhibitory receptors and the functionality of these cells. Determination of the therapeutic potential of LRAs should therefore include not only the compound's cytotoxicity and capacity to induce HIV re-activation in CD4+ T cells but importantly also its potential effects on CD8+ T cells exhaustion and function.

Over the last years, different classes of LRAs were developed to overcome one or more of the many different mechanisms which control the latency of integrated HIV provirus. Major LRA classes include histone deacetylase inhibitors (HDACi), protein kinase C (PKC) agonists, bromodomain and extraterminal domain protein inhibitors (BETi), and BRG-Brahma Associated Factor inhibitors (BAFi). Several of these LRAs are already being used in clinical trials [19]. Histone deacetylases (HDACs) deacetylate lysine residues on histones, resulting in a chromatin structure that is more compact and less accessible to activating transcriptional regulators but renders the recruitment of repressive factors that together inhibit transcription of the HIV provirus [20]. HDACi such as romidepsin, panobinostat and vorinostat/SAHA have been shown ex vivo and in vitro to induce reactivation of the HIV latent reservoir in CD4+ T cells from HIV infected patients [21-24]. Since HDACs deacetylate not only lysine residues on histones but also on non-histone proteins including transcription factors and signalling molecules, HDACi have effects beyond altering the structure and function of chromatin

(reviewed in [25,26]). The PKC agonists prostratin and bryostatin activate classical and novel PKC [27,28]. One of the downstream effects of PKC activation is the nuclear translocation of NF-κB and the recruitment of activating histone acetyltransferase complexes and the positive transcription elongation factor b (P-TEFb), leading to the activation of the HIV long terminal repeats (LTR) and HIV transcription [28-31]. Although neither the phorbol ester prostratin nor non-phorbol ester bryostatin by itself induce proliferation of T cells, they can enhance T cell proliferation in the presence of IL-2, CD28 costimulation or CD3 stimulation [32,33]. Induction of the elongation phase of HIV gene transcription is another mechanism to reverse latency. In actively HIV infected cells, HIV Tat binds P-TEFb and the complex is recruited to the HIV LTR leading to transcription elongation [34]. However, in HIV latently infected cells, the bromodomain-containing protein 4 (BRD4) competes with Tat for the binding of P-TEFb and therefore prevents transcriptional elongation. BETi like JQ1 and OXT-015 are small molecules which bind BRD4, therefore inhibiting P-TEFb binding to BRD4, resulting in the release of P-TEFb. This allows the recruitment of P-TEFb by Tat to the HIV-1 promoter [35,36], leading to HIV reactivation [36,37]. The BAF chromatin remodeling complex is another cellular factor contributing to latency by positioning the transcriptionally repressive nucleosome nuc-1 in the HIV LTR and altering DNA accessibility [38,39]. Indeed BAFi like caffeic acid phenethyl ester (CAPE) and pyrimethamine were shown to act as LRAs in human HIV-infected PBMC [38,40].

Since the shock and kill strategy depends on an effective immune system to kill reactivated latent HIV-infected cells, the question arises what effect these LRAs have on immune cells. In recent years, cytotoxicity of LRA has been studied mostly in latently infected cell lines and primary CD4+ T cells [6,31,36,38,41-44]. Some studies have examined the effect of LRA on viability of natural killer (NK) cells, since these cells also play a role in killing HIV-infected cells [45]. The HDACi SAHA (vorinostat) at concentrations up to 500 nM had no cytotoxic effect on NK cells after 24 hours and 4 days incubation [46,47], whereas higher concentrations of SAHA [47] and the HDACi panobinostat and romidepsin [46] reduced viability. Within the LRA class of PKC agonist, no reduction of NK cell viability was observed when prostratin and ingenol were used [46]. Several studies have investigated the effect of LRAs on viability of PBMC and cytotoxic CD8+ T cells (CTL) [6,48-53], the main immune cell population eliminating HIV-infected cells in an antigen-specific manner. However, a direct comparison of the cytotoxicity of different LRA classes and also between members of a LRA class based on these studies is challenging since studies differ both in experimental set up and LRA exposure time. SAHA was shown to be non-toxic for CD8+ T cells when exposed for 7 days [6], nor was proliferation or killing function of CD8+ T cells impaired when these cells were pre-incubated with SAHA for 24 hours [51]. Although resting CD8+ T cells from one healthy donor did not show any cytotoxicity after incubation of PBMC for 4 and 21 hours with romidepsin, panobinostat and SAHA [50] when either HIV-specific CTL clones or activated CD8+ T cells were analyzed,

significant cytotoxicity was observed for the HDACi romidepsin and panobinostat but not for SAHA [50]. In contrast, romidepsin-mediated cytotoxicity was reported for CD4+ and CD8+ T cells, and NK cells when PBMC were exposed for 24 hours [49]. When cells were exposed to romidepsin for 6 hours, washed and analyzed after additional 42 hours of cultivation in the absence of romidepsin, reduced viability was observed for CD8+ T cells from HIV-negative controls [53] as well as for PBMC from cART-treated HIV+ patients [48]. However, the cytotoxic effect of the PKC agonist bryostatin and prostratin differed between these two studies, with one showing no cytotoxic effect on day 2 [53], whereas the second reported significant reduction in viability for both PKC agonists [48].

To allow for a direct comparison of the cytotoxic effect of LRAs on immune cell subpopulations, we included two members of 4 different LRA classes at concentrations shown to reverse HIV latency. The cytotoxicity of these LRA using PBMC from healthy individuals and HIV-infected patients were analyzed. We chose two time points, a short term (18 hours) and a long-term (72 hours) continuous exposure to the compound. Since LRAs have to enter the cells and act intracellularly either in the cytoplasm or the nucleus to induce epigenetic modifications, the LRAs accumulate in the cells, leading to a higher concentration within a cell compared to plasma [54]. This may also suggest that intracellular LRA will not follow the pharmacokinetics indicated for plasma concentrations of LRAs, and that it may take more time to reach a steady-state intracellular concentration. We furthermore compared the effect of the LRAs on viability of unactivated and activated T cells. For LRAs with low cytotoxicity we additionally investigated the effect these LRAs have on the expression of the chronic activation marker CD38 and three different inhibitory receptors (CD279, CD160, CD244) on CD8+ T cells. Our findings demonstrate that LRAs greatly vary in their cytotoxicity on immune cells with the HDACi romidepsin and panobinostat showing the highest cytotoxicity. Importantly, T cell activation did not affect the cytotoxicity of most of the LRA analyzed. LRA cytotoxicity on CD8+ T cells did not differ between HIV-uninfected controls and cART-treated HIV-infected patients. Finally, some LRAs such as PKC agonists and BETi could modulate inhibitory receptors expression on CD8+ T cells, suggesting that these LRAs may also modulate the function of these cells.

MATERIALS AND METHODS

Cell samples and ethical approval

Buffy coats from healthy donors were purchased from Sanquin Amsterdam. Leukapheresis from three HIV-infected patients were included in this study after receiving approval from the Erasmus MC Medical Ethics Committee and receiving written informed consent. All three

patients initiated cART during chronic HIV infection and were on stable cART for at least three years with effective viral suppression below 50 copies/ml for at least 12 months.

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Hypaque, GE Healthcare life sciences). Cells were frozen in freezing media (90% Fetal bovine serum (FBS) supplemented with 10% DMSO) and stored at -80°C or in liquid nitrogen.

Cell culture

After thawing, cells were cultured in culture media RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100g/ml streptomycin-sulfate at a concentration of $1x10^6$cells/ml in 24-well plates (Thermo Scientific) that were either uncoated (unactivated cells) or coated with anti-human CD3 (1µg/ml, clone UCHT1, no azide/low endotoxin, BD Bioscience) and anti-CD28 (10µg/ml, clone CD28.2, no azide/low endotoxin, BD Bioscience) monoclonal antibodies (activated cells). LRAs at indicated concentrations were added 60 min after cells were added to the plate. Since LRAs were dissolved in dimethyl sulfoxide (DMSO), DMSO was added to the DMSO control culture at 0.1%, the DMSO concentration of the highest compound concentration to control for the effect DMSO may have on cell viability. Cells were cultured at 37°C in 5% CO_2 incubator for either 18 or 72 hours.

Reagents

The following LRAs were used in this study: HDAC inhibitors romidepsin (Sigma, cat number SML1175), panobinostat (Cayman Chemical, 13280-50) and SAHA/vorinostat (Selleck Chemicals, S1047), BAF inhibitors pyrimethamine (Sigma, 46706) and CAPE (MP Biomedicals, 0219586005), BET inhibitors OTX-015 (ApexBio, A3692) and JQ1 (Cayman Chemical, 11187-1), and PKC agonists bryostatin (Santa Cruz, sc-201407) and prostratin (Sigma, P0077). All drugs were reconstituted in DMSO. Concentrations of LRAs included in this study were based on previously published studies: HDACi romidepsin 1 nM – 100 nM [22,44], panobinostat 1 nM – 50 nM [22,43,44,55], and SAHA 350 nM [46,52]; BAFi pyrimethamine 1 µM – 10 µM [38], and CAPE 0.1 µM – 1 µM [38]; BETi OTX-015 0.1 µM – 5 µM [37], and JQ1 0.01 µM – 10 µM [31,37,43,53]; PKC agonists bryostatin 0.1 nM – 50 nM [43,44,53], and prostratin 0.1 µM – 2.5 µM [28,31,37].

Flow cytometry and viability measurement

For staining of surface antigens, $0.6\text{-}1.0x10^6$ cells were washed with FACS wash (FW; Hanks buffered saline solution (HBSS, Life Technologies), 3% fetal bovine serum (FBS, Life Technologies), 0.02% NaN_3) and incubated with a 20 µl mix of pre-determined optimal

concentrations of monoclonal antibodies for 30 min at 4° C, washed two times with FW and fixed with 1% paraformaldehyde in PBS.

The following directly conjugated monoclonal anti-human antibodies were used to analyze CD8+ T cells (CD3+CD8+), CD4+ T cells (CD3+CD4+), B cells (CD3-CD19+), and NK cells (CD3-CD56+): CD3-BV421 (UCHT1), CD4-BV650 (SK3), CD8-BV786 (RPA-T8), CD14-PE-Cy7 (61D3, eBioscience), CD19-PerCP-Cy5.5 (HIB19, eBioscience), CD56-PE-Cy5.5 (CMSSB, eBioscience), Annexin V-PerCP-Cy5.5 or AnnexinV-Cy5.5, CD279-BV711 (CD279, EH12.1), CD160-Alexa Fluor 488 (clone BY55), CD38-PE-Cy7 (HIT2), CD244-PE (eBioC1.7, eBioscience). All antibodies were purchased from BD Biosciences unless otherwise indicated. When Annexin V was used to measure cell death and exclude dead cells, 2.5 mM $CaCl_2$ was added to all solutions. Between 1-4x10^5 events were collected per sample on a LSRFortessa (BD Biosciences, 4 lasers, 18 parameters) and analyzed using FlowJo software (version 9.7.4, Tree Star). For cell death analysis, doublets were excluded and percentage of apoptotic (Annexin V+) cells determined of CD3+CD8+ T cells, CD3+CD4+ T cells, CD19+ B cells and CD3-CD56+ NK cells (Supplementary Figure 1A). To analyze expression of the activation marker CD38 and inhibitory receptors CD160, CD244, and CD279, first doubles were excluded and then within live (Annexin V-) cells, the expression of these antigens were determined using Fluorescence Minus One samples (FMO) for gate definition (Supplementary Figure 1B).

Data are represented as frequency within a defined population. Drug-specific cell death was calculated using the following formula [56]:

[(% Drug-induced cell death – % cell death in DMSO only)/(100 - % cell death in DMSO only)]*100

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad Prism5 for Windows, Version 5.04). For statistical analysis of the effects LRAs have on immune cells, repeated Measures ANOVA with Tukey's Multiple Comparison Test was performed (paired analysis). To compare the cytotoxic effect these LRAs have on CD8+ T cells from healthy controls and HIV-infected individuals, one-way analysis of variances with Tukey's Multiple Comparison Test (unpaired analysis) was performed. P values equal or lower than 0.05 were considered statistically significant with the numbers of stars in the figures indicating the p value: * = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$. If no star is indicated in figures, then the comparison was not statistically significant.

RESULTS

HDACi exhibit cytotoxicity for T cells at low concentrations

We tested the HDACi romidepsin and panobinostat at concentrations of 1 nM – 100 nM and 1 nM – 50 nM, respectively. These concentrations are in the range of those used previously for ex vivo and in vitro HIV reactivation and observed in plasma of treated patients [22,43,44,53,55]. We found significant differences in HDACi cytotoxicity depending on the length of drug exposure of PBMC from healthy donors. HDACi induced low yet significantly increased cytotoxicity when CD4+ and CD8+ T cells were incubated for short term (18h) even with the highest concentration of 100 nM for romidepsin and 50 nM for panobinostat (Supplementary Table 1). B cells showed slightly lower survival rates than T cells with up to 22±6.3% (mean±SE) cell death for romidepsin and 27±7.3% for panobinostat (Supplementary Table 1). Both HDACi exhibited low cytotoxicity for NK cells (Supplementary Table 1).

When cells were continuously exposed to these two LRAs for 72h, both HDACi at 10 nM concentration induced significantly increased cell death in unactivated and activated CD8+ T cells (Figure 1A, B, C). Romidepsin and panobinostat showed a similar cytotoxic effect on CD4+ T cells with 10 nM induced significantly reduced survival (Figure 1B, C). B cells were very sensitive to HDACi in long-term cultures as concentrations of 10 nM and higher of either romidepsin or panobinostat decreased the survival of these cells significantly (Table 1). NK cells also showed high sensitivity to the cytotoxic effect of panobinostat after 72 hours culture, as already the lowest concentration induced significantly increased cell death (Table 1). NK cells were less sensitive to the cytotoxic effect of the lowest concentration of romidepsin, however higher concentrations also induced significant cell death (Table 1).

We also tested the T cell-specific cytotoxicity of a third HDACi, SAHA (vorinostat) in the long-term cultures at 350 nM, the concentration most frequently used in cell culture experiments [46,52]. As shown before [6,50,51], SAHA does not statistically significantly increase cell death of unactivated (2.0±3.0% and 2.2±1.5% specific cell death for CD4+ and CD8+ T cells, respectively, n=4) and activated CD8+ T cells (1.6±0.6% specific cell death, n=4). Activated CD4+ T cells however showed a small but statistically significant increase in cytotoxicity after long term culture with SAHA (11±2.1% specific cell death, n=4, $p \leq 0.001$). The above suggest that not all HDACi exhibit T cell toxicity and suggests careful consideration of this property when selecting these for HIV latency reversal.

Cytotoxic effect of PKC agonists is drug dependent

The PKC agonists bryostatin and prostratin were used at concentrations that were reported to reactivate HIV ex vivo and in vitro [28,31,37,43,44,53]. In short-term cultures, bryostatin did not increase significantly cell death of unactivated and activated CD8+ T cells independent of concentrations used (Supplementary Table 1). Prostratin however showed

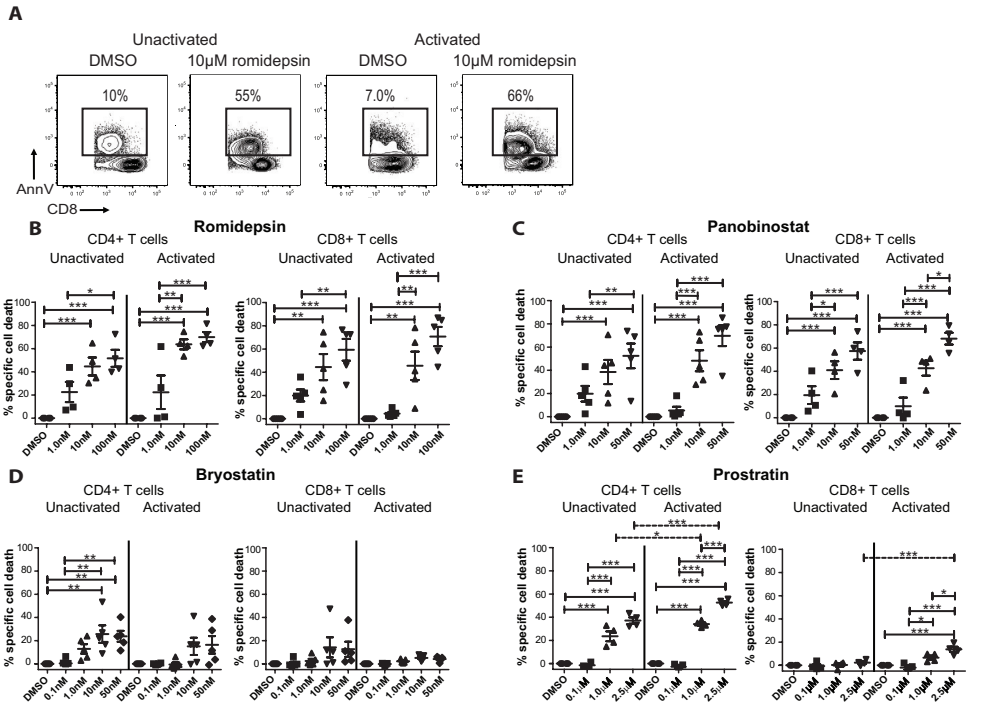


Figure 1. HDACi are cytotoxic for unactivated and activated CD4+ and CD8+ T cells.

PBMC from healthy donors were incubated with the indicated LRAs for 72 hours either under unactivated conditions or activated with anti-CD3/anti-CD28 antibodies. Cell death was analyzed using flow cytometry and Annexin V staining. (**A**) Representative Facs plots depicting AnnexinV+ CD8+ T cells. Numbers in plot depict frequency of gated cells. Pooled data of drug-specific cell death is shown for CD4+ and CD8+ T cells for the HDACi romidepsin (**B**) and panobinostat (**C**) and for the PKC agonists Bryostatin (**D**) and Prostratin (**E**). Each symbol represents one healthy donor (n = 4-5 from 4 independent experiments). Statistical significance is indicated by stars (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, no star = not significant = P > 0.05), solid line depicts statistical comparison within unactivated and activated cultures and dashed line depicts comparison between unactivated and activated cultures treated with the same concentration of LRA.

concentration dependent significant cytotoxicity on unactivated and activated CD8+ T cells (Supplementary Table 1). Unactivated and activated CD4+ T cells were more sensitive to PKC agonists as bryostatin and prostratin induced significant cell death with several of the concentrations tested (Supplementary Table 1). We observed a low non-significant drug-specific cytotoxicity when B cells and NK cells were analyzed (Supplementary Table 1).

In long-term cultures, the PKC agonist bryostatin did not induce significant drug-specific cell death in unactivated and activated CD8+ T cells and activated CD4+ T cells (Figure 1D) whereas unactivated CD4+ T cells showed reduced survival with bryostatin concentrations of 10 nM and above (Figure 1D). Prostratin, the second PKC agonist tested, elicited cytotoxic

Table 1. Drug-specific cell death in long-term cultures

		B cells	NK cells
HDAC inhibitor	**Romidepsin**		
	1nM	47±25%^	12±3.3%
	10nM	79±19%*	53±12%***
	100nM	79±20%*	69±8.1%***
	Panobinostat		
	1nM	84.5±5.8%***	32±9.7%*
	10nM	97.8±0.8%***	80±2.7%***
	50nM	97.6±1.0%***	77±3.4%***
PKC agonist	**Bryostatin**		
	0.1nM	-14±8.1%	8.4±3.0%
	1nM	-17±9.3%	-4.1±0.5%
	10nM	-17±8.9%	-4.2±1.3%
	50nM	-16±8.7%	-4.1±1.3%
	Prostratin		
	0.1µM	-13±7.5%	-3.8±0.9%
	1µM	-3.4±7.5%	6.5 ±4.2%
	2.5µM	-2.0±7.6%	12±8.8%
BET inhibitor	**JQ1**		
	0.01µM	0.01±2.8%	3.5±3.2%
	0.15µM	25±12%	12±3.0%
	1µM	56±12%***	39±13%***
	10µM	76±12%***	61±8.7%***
	OTX-015		
	0.1µM	18±8.3%**	5.9±4.0%
	0.5µM	52±14%***	16±7.3%
	1µM	62±17%***	20±5.8%*
	5µM	72±19%***	38±8.1%***
BAF inhibitor	**Pyrimethamine**		
	1µM	1.8±2.3%	2.0±2.2%
	5µM	2.8±2.0%	1.4±1.6%
	10µM	4.8±1.9%	3.9±2.8%
	CAPE		
	0.1µM	2.7±1.9%	0.0±1.0%
	0.5µM	0.9±1.7%	-0.3±0.5%
	1µM	4.1±2.0%	-0.8±0.4%

^Mean±SE

N = 3 - 5 from 3 - 5 independent experiments

Statistical analysis: * = P ≤ 0.05, ** = P ≤ 0.01, and *** = P ≤ 0.001 compared to DMSO control; values without a star are not statistically significantly different from DMSO control.

effects on CD4+ T cells with a concentration of 1µM and higher (Figure 1E). No significant increase of cell death was observed for unactivated CD8+ T cells when treated for 72 hours with bryostatin (Figure 1E). Although prostratin induced significant cytotoxicity in activated CD8+ T cells, this was still below 20% drug-specific cell death (Figure 1E). No significant drug induced cytotoxicity was observed for B cells and NK cells when PKC agonists were tested in long-term cultures (Table 1).

BETi at low concentrations show little cytotoxic effect

We investigated the effect of two BETi, JQ1 and OXT-15, on viability of immune cells at concentrations known to reactivate HIV in vitro [31,37,43,53]. When incubated short term, both drugs have a low effect, although partially statistically significant, on survival of unactivated and activated CD8+ and CD4+ T cells (Supplementary Table 1). The survival of B cells and NK cells was also only slightly reduced when PBMC are treated for 18 hours with the BETi (Supplementary Table 1).

The BETi JQ1 induced in long term cultures significant drug-specific cell death in T cells when concentrations above 0.15 µM were used (Figure 2A). OTX-015 was non-cytotoxic for unactivated CD4+ T cells and unactivated and activated CD8+ T cells with the exemption of the highest concentration (5 µM) which showed a small but statistical significant increase in drug-specific cell death (Figure 2B). Activated CD4+ T cells were more sensitive to the cytotoxic effect of OTX-015 with already a significant small increase in drug-specific cell death with 0.5 µM of OTX-015 (Figure 2B). However, even the highest BETi concentration did not induce more than 20% drug-specific cell death in CD4+ and CD8+ T cells. In contrast, B cells and NK cells showed a concentration dependent significant sensitivity to the death-inducing effect of BETi with >70% drug-specific cell death for B cells and >40% for NK cells at the highest concentrations (Table 1). These findings demonstrate that BETi are safe for T cells even at the highest concentrations tested.

BAFi CAPE is non-cytotoxic for immune cells

BAFi are a new class of LRAs which showed promising results in HIV reactivation [38]. When PBMC were cultivated in the presence of two representatives of BAFi at concentrations previously shown to induce HIV latency reversal [38] for 18 hours, neither CAPE nor pyrimethamine induced significant cytotoxicity in the immune subpopulations examined (Supplementary Table 1). The survival of B cells and NK cells was also not significantly compromised after long-term culture in the presence of both BAFi (Table 1). When unactivated or activated T cells were analyzed after long-term exposure to pyrimethamine (Figure 2C), no significantly increased cytotoxicity was observed for unactivated CD4+ and CD8+ T cells (Figure 2C). A small but statistically significant increase of drug-specific cytotoxicity was observed however for activated CD4+ T cells with pyrimethamine concentrations of 5µM and above and for activated CD8+ T cells for the highest pyrimethamine concentration used (Figure 2C). CAPE, the second BAFi tested, did not show any cytotoxic effect on unactivated or activated CD4+ and CD8+ T cells (Figure 2D). These findings indicate that the BAFi show very low cytotoxic potential for immune cells even after longer exposure periods.

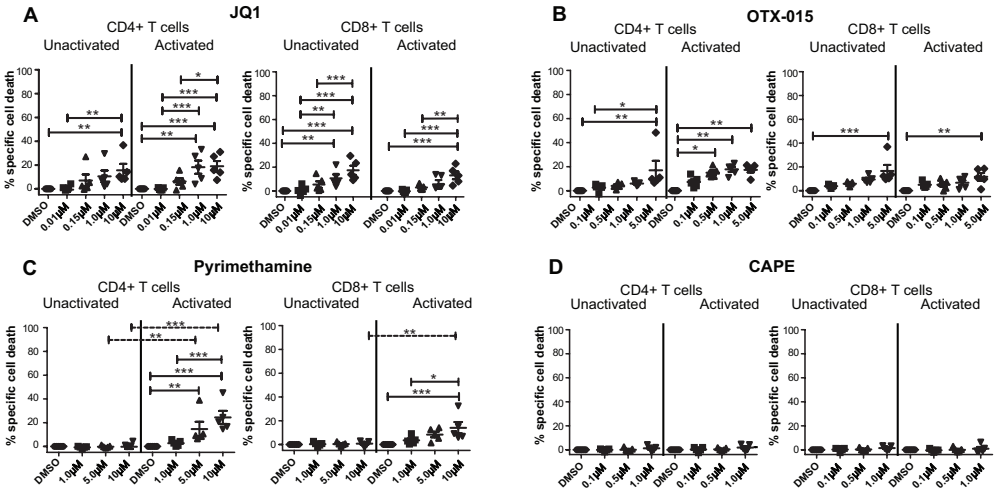


Figure 2. Cytotoxicity of BET and BAF inhibitors on unactivated and activated CD4+ and CD8+ T cells.

Either unactivated or anti-CD3/anti-CD28 activated PBMC from HIV-uninfected donors were cultured in the presence of the BETi and BAFi for 72 hours. Pooled data of drug-specific cell death is shown for CD4+ and CD8+ T cells for the BET inhibitors JQ1 (**A**) and OTX-015 (**B**) and for the BAF inhibitors Pyrimethamine (**C**) and CAPE (**D**). Each symbol represents one healthy donor (n = 5 from 4 independent experiments). Statistical significance is indicated by stars (* = $P \leq 0.05$, ** = $P \leq 0.01$, no star = not significant = $P > 0.05$), solid line depicts statistical comparison within unactivated and activated cultures and dashed line depicts comparison between unactivated and activated cultures treated with the same concentration of LRA.

The toxicity of LRAs for CD8+ T cells does not differ significantly between PBMC from HIV-infected individuals and healthy donors

The targeted patient group for LRA-induced purging of the latent HIV reservoir are HIV-infected patients with undetectable plasma viral loads due to cART. Given the chronic activation, exhaustion and propensity of CD4+ and CD8+ T cells to undergo apoptosis in HIV infection [56-60], we also evaluated the effect of these LRAs on CD8+ T cells from three cART-treated HIV-infected patients with undetectable plasma viral loads. HDACi in short term cultures resulted in significantly increased drug-specific cell death in unactivated and activated CD8+ T cells when higher concentrations of LRAs were used (Supplementary Figure 2A). No significant induction of cell death was observed when PBMC from HIV-infected patients were incubated for 18 hours (Supplementary Figure 2B). The BETi JQ1 did not show any cytotoxicity whereas OTX-015 induced significant but low level cell death in unactivated CD8+ T cells with only the highest concentration included and on activated CD8+ T cells with all concentrations used with cell death not above 6.0±0.9% (Supplementary Figure 2C). None

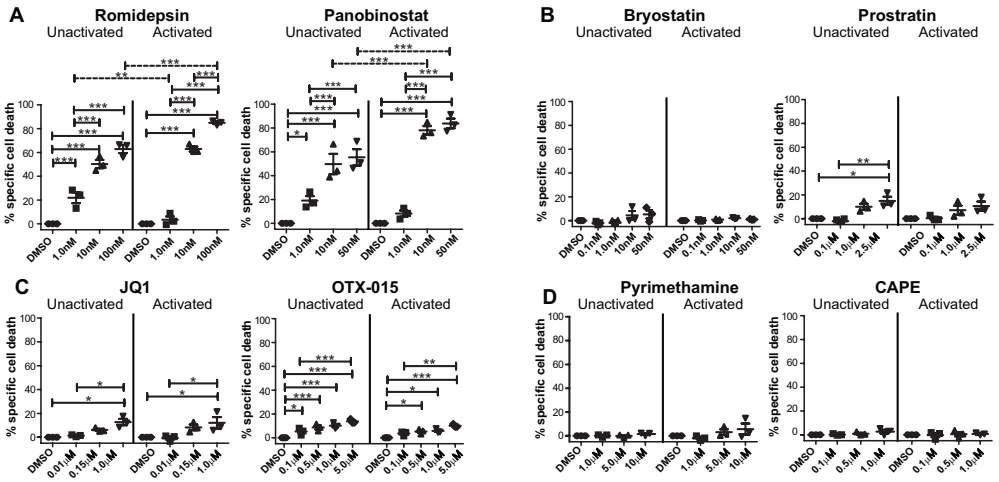


Figure 3. Cytotoxicity of LRAs in long term cultures of CD8+ T cells from HIV+ patients.

PBMC from HIV-infected patients were either activated with anti-CD3/anti-CD28 antibodies or left untreated (unactivated) and cultured in the presence of the indicated LRAs for 72 hours. The frequency of drug-specific cell death is shown for the HDACi romidepsin and panobinostat (**A**), PKC agonists bryostatin and prostratin (**B**), BETi JQ1 and OTX-015 (**C**) and BAFi pyrimethamine and CAPE (**D**). Each symbol represents one HIV-infected donor (n= 3 from 3 independent experiments); Statistical significance is indicated by stars (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, no star = not significant = P > 0.05), solid line depicts statistical comparison within unactivated and activated cultures and dashed line depicts comparison between unactivated and activated cultures treated with the same concentration of LRA.

of the two BAFi included in this study reduced significantly the viability of unactivated or activated CD8+ T cells from HIV+ patients (Supplementary Figure 2D).

In long term cultures of PBMC from HIV+ patients, the HDACi romidepsin and panobinostat induced significant drug-specific cell death in unactivated CD8+ T cells already with the lowest concentration and this increased up to 62±4.4% and 55±7.1%, respectively with the highest concentration used (Figure 3A). Activated CD8+ T cells were less sensitive to the lowest concentrations of both HDACi but showed significant cytotoxicity to higher concentrations of these LRAs (Figure 3A). The PKC agonists bryostatin did not induce significant drug-specific cell death in unactivated and activated CD8+ T cells (Figure 3B) while prostratin, at the highest concentration (2.5 µM), induced only a slight but significant increase of cell death in unactivated (15±3.5%) and activated (11±3.8%) CD8+ T cells (Figure 3B). As observed for CD8+ T cells from healthy donors, CD8+ T cells from HIV-infected patients showed only low cytotoxicity when cultivated in the presence of the BETi JQ1 and OXT-015 (Figure 3C), with JQ1 drug-induced cell death reaching significance for only the highest concentration, whereas all concentration of OTX-015 induced low but significant cell death. The BAFi pyrimethamine and CAPE did not significantly compromise the cell survival of CD8+ T

cells from HIV-infected patients when cultivated for 72 hours under unactivated or activating conditions.

To investigate whether the LRAs included in this study exhibit a similar toxicity on CD8+ T cells from healthy controls and HIV-infected individuals, the drug-specific cell death after short-term and long-term culture was compared between these two groups. No significant difference of the cell death frequency of unactivated and activated CD8+ T cells was found for romidepsin, CAPE, pyrimethamine, JQ1, OTX-015 and bryostatin (data not shown). Statistical significant differences in drug-specific cell death was only observed for unactivated CD8+ T cells from HIV-infected individuals after long-term culture in the presence of the highest concentration of prostratin (2.5μM) ($p \leq 0.05$) and for activated CD8+ T cells in the presence of 10 nM panobinostat ($p \leq 0.05$) (data not shown).

These findings indicate that the LRAs investigated in this study have a comparable effect on CD8+ T cell viability when CD8+ T cells from healthy donors and HIV-infected patients are compared.

BETi reduce frequency of CD279-expressing CD8+ T cells

One of the mechanisms leading to the failure of the immune system to control HIV infection is exhaustion of HIV-specific CD8+ T cells. The inhibitory receptor CD279 (Programmed cell death protein 1, PD-1) is upregulated on exhausted CD8+ T cells [11,12,14] which is due to epigenetic modification of the CD279 promotor region [15,17]. Since LRAs may reactivate HIV by directly or indirectly altering the epigenetic silencing of the HIV LTR, we reasoned that LRAs-induced epigenetic changes may affect CD279 expression. Because CD279 and other inhibitory receptors are not only upregulated during chronic antigen stimulation but also during acute activation and some LRAs may affect the activation status of cells, we analyzed the expression of the activation marker CD38 on CD8+ T cells from HIV infected patients. We did not include HDACi in this analysis due to their high cytotoxic effect on T cells.

PKC agonists at concentrations > 1nM for bryostatin and > 0.1μM for prostratin induced a significant upregulation of CD38 on unactivated CD8+ T cells from HIV+ individuals (Figure 4B). In activated CD8+ T cells, neither the PKC agonist bryostatin nor prostratin altered significantly the expression of CD38 (Figure 4B). BETi did not change significantly the frequency of CD38-expressing unactivated CD8+ T cells (figure 4A, C). However, both BETi at the highest concentration significantly reduced the frequency of CD38+ cells within activated CD8+ T cells compared to cells cultured in the presence of DMSO only (Figure 4A, C). We did not observe a significant change in the frequency of CD38+ CD8+ T cells in the presence of the BAFi pyrimethamine and CAPE in long term cultures (Figure 4D).

We next examined whether LRAs alter the frequency of CD279-expresssing CD8+ T cells. We observed a small, significant increase of CD279+CD8+ T cells when unactivated CD8+ T cells were treated with the PKC agonist prostratin (Figure 5B). The frequency of CD279+CD8+

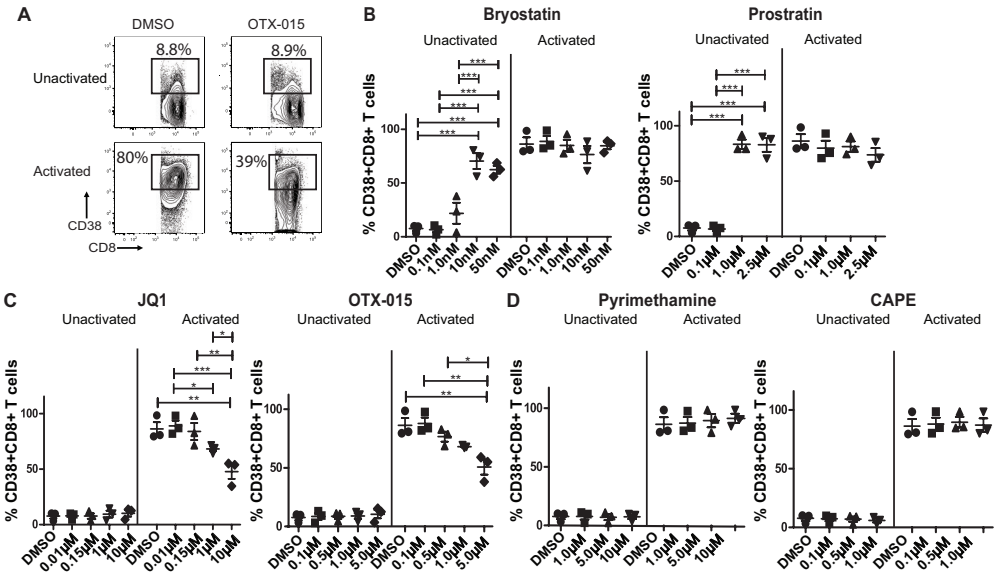


Figure 4. PKC agonists activate CD8+ T cells from HIV+ patients.

PBMC from HIV+ patients were cultured with indicated concentrations of LRAs either under unactivated or anti-CD3/anti-CD28 antibody activating conditions for 72 hours. Expression of CD38 was analyzed by flow cytometry. (**A**) Representative facs plots depicting the frequency of CD38+CD8+ T cells of unactivated and anti-CD3/anti-CD28 antibody activated PBMC either cultured in the presence of DMSO or OTX-015 (5μM) for 72h. Pooled date showing the frequency of CD38+CD8+ T cells in cultures with PKC agonists bryostatin and prostratin (**B**); BETi JQ1 and OTX-015 (**C**); and BAFi pyrimethamine and CAPE (**D**). Each symbol represents one HIV-infected donor (n= 3 from 3 independent experiments). Statistical significance is indicated by stars (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, no star = not significant = P > 0.05);

T cells was however significantly reduced when activated PBMC were treated with 10 and 50 nM bryostatin and 1 μM and 2.5 μM prostratin, respectively (Figure 5B). The expression of CD279 on unactivated CD8+ T cells was not significantly altered by the BETi JQ1 and OTX-015 (Figure 5C). Both BETi induced significant downregulation of CD279 on activated CD8+ T cells at higher concentrations (Figure 5A, C). The BAFi pyrimethamine and CAPE had no significant effect on CD279 expression on either unactivated or activated CD8+ T cells (Figure 5D). We also analyzed the frequency of inhibitory receptors CD160+ and CD244+ CD8+ T cells in the above cultures in the presence and absence of LRAs, however, none of the LRAs significantly affected the expression of these inhibitory receptors (data not shown).

Since CD279 expression is also modulated by activation with activated cells upregulating CD279 expression and inhibition of activation would prevent CD279 upregulation, we examined whether the reduction in CD279-expression on activated CD8+ T cells is independent on the activation status of the cells. We therefore calculated from activated CD8+ T cells the ratio of LRA-altered CD38 expression to DMSO-only CD38 expression and a comparable

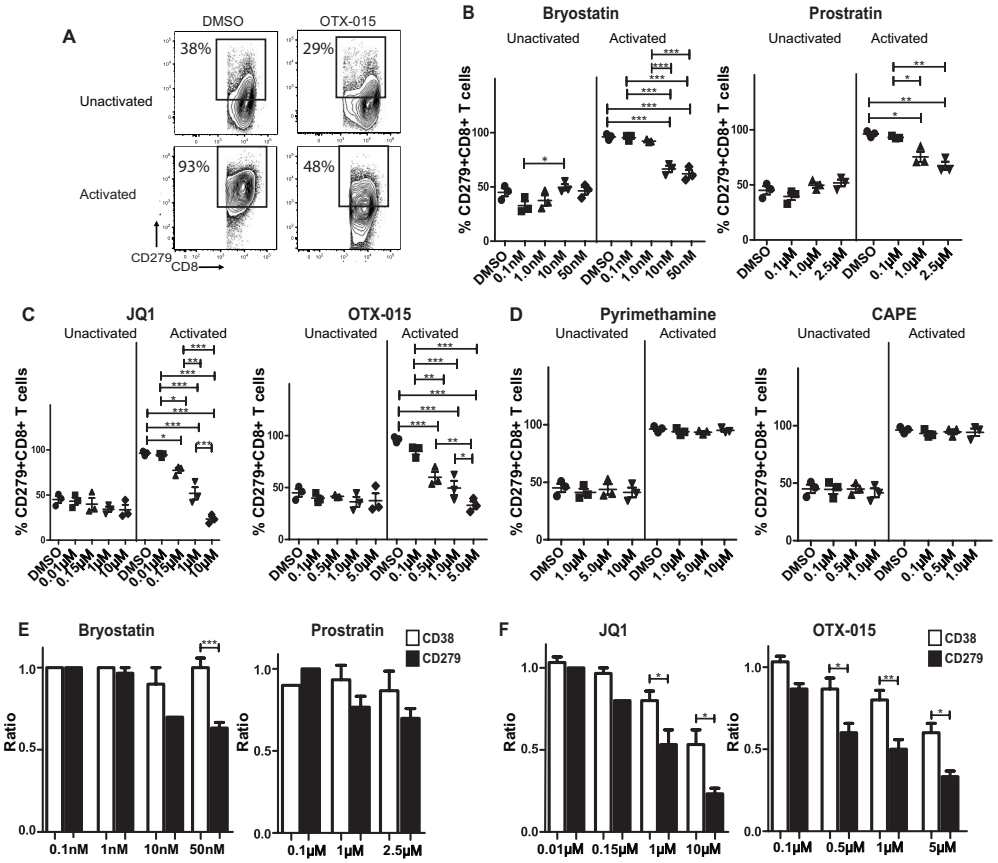


Figure 5. BETi reduce CD279 expression on CD8+ T cells from HIV+ patients.

PBMC from HIV-infected patients were either left untreated (unactivated) or activated with anti-CD3/anti-CD28 antibody and cultured with indicated concentrations of LRAs for 72 hours. (**A**) Representative facs plots depicts the frequency of CD279-expressing CD8+ T cells either in unactivated or activating cultures in the presence of DMSO or OTX-015 (5μM). Pooled data showing the frequency of CD279+ CD8+ T cells in cultures with PKC agonists bryostatin and prostratin (**B**); BETi JQ1 and OTX-015 (**C**); and BAFi pyrimethamine and CAPE (**D**). Each symbol represents one HIV-infected donor (n= 3 from 3 independent experiments). (**E**)**.** Ratio of CD38 expression on activated CD8+ T cells of PKC agonist treated cultures to CD38 expression on activated CD8+ T cells of DMSO-only cultures (white column) and Ratio of CD279 expression on activated CD8+ T cells of PKC agonist treated cultures to CD279 expression on activated CD8+ T cells of DMSO-only cultures (black column) shown. (**F**)**.** Ratio of CD38 expression on activated CD8+ T cells of BETi treated cultures to CD38 expression on activated CD8+ T cells of DMSO-only cultures (white column) and Ratio of CD279 expression on activated CD8+ T cells of BETi treated cultures to CD279 expression on activated CD8+ T cells of DMSO-only cultures (black column) shown. Column depicts mean ± SE with n=3 from 3 independent experiments. Statistical significance is indicated by stars (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, no star = not significant = P > 0.05).

ratio of LRA-altered CD279 expression to DMSO-only CD279 expression for the PKC agonists (Figure 5E) and the BETi (Figure 5F). A ratio of 1 indicates that LRAs did not alter the expression of CD38 or CD279 whereas a ratio below 1 suggests that the expression of the antigen is reduced by the LRA compared to DMSO only.

In activated CD8+ T cells, 50 nM bryostatin treatment resulted in the downregulation of CD279-expressing cells which was not accompanied by a reduction in CD38-expresssing CD8+ T cells, and the difference between CD38 expression and CD279 expression was highly significant (Figure 5E), suggesting that the effect of bryostatin was not due to an overall reduction of activation by bryostatin. We also observed a higher reduction in the frequency of CD279+CD8+ T cells than of CD38+CD8+ T cells with 1 µM and 2.5 µM prostratin, however, this difference did not reach significance (Figure 5E). Although both BETi reduced the frequency of CD38-expression cells, the reduction of CD279-expression CD8+ T cells was significantly higher (Figure 5F). The above findings indicate that the PKC agonist bryostatin and the BETi JQ1 and OTX-015 can decrease specifically the frequency of CD279 expressing CD8+ T cells indicating that this CD279 reduction may be independent of effects on T cell activation.

DISCUSSION

The “shock and kill” approach has been proposed as a strategy to reactivate the latent HIV reservoir and successfully eliminate previously quiescent HIV infected cells [4,5] thereby curing HIV infection. However, the cytotoxic impact that different classes of LRAs have on immune cells is not fully understood. Several immune subpopulations are involved in controlling HIV infection including NK cells, B cells and specifically cytotoxic CD8+ T cells (CTL). Since LRAs reverse HIV latency, the latently infected cells re-express HIV peptides which enables CTL recognition. The importance of the CTL response when using LRAs is highlighted by the fact that the viral cytopathic effects of reactivated virus are not generally sufficient to eliminate HIV-infected cells [6]. Thus it is imperative to understand the effects LRAs have on CTL survival and function.

In the present study we performed a comparative analysis of the cytotoxic effect several different LRAs have on CTL but also CD4+ T cells, NK cell, and B cells. Overall, our data show that LRAs differ widely in their cytotoxic effects with different members within a class having very heterogeneous effects on cell viability. The sensitivity to drug-specific cell death differs also when immune cell subpopulations are compared.

We show here that the HDACi romidepsin and panobinostat have little but significant cytotoxic effect on T cells in short term cultures. This is in accordance with several studies analyzing cytotoxicity of these LRA on purified CD4+ T cells [43] and PBMC from HIV-infected and uninfected donors [50]. However, when cytotoxicity was analyzed after long-term 3

day exposure, both HDACi reduced significantly viability of CD4+ and CD8+ T cells. Reduced viability was previously reported when cells were incubated for only 6 hours and viability was tested on day 2 for romidepsin [48,53] and panobinostat [48]. Concentration dependent cytotoxicity of romidepsin was also observed for CD4+T cells, CD8+ T cells and NK cells after 48 hour exposure time [49]. Thus HDACi may induce a slow death of T cells which is underestimated in short term cultures. A small three week study in HIV+ patients found that romidepsin increase plasma HIV RNA load, however, no reduction of the latent reservoir was detected [61]. Although the authors conclude that romidepsin does not affect the T cell compartment, increased frequencies of peripheral naïve CD8+ T cells together with a small, non-significant decline in total CD8+ T cells could indeed indicate that effector memory cells are deleted by the romidepsin treatment as indicated by our study.

To further examine whether high T cell cytotoxicity is an overall characteristic of HDACi, we included SAHA (vorinostat) in our assay since SAHA showed no cytotoxic effects on T cells in previous studies when concentrations below 500 nM were used [6,46,47,50-52]. We indeed observed that SAHA did not compromise the viability of T cells after 3 days of continued exposure. This data indicate that even within one LRA class, cytotoxic effects differ significantly between inhibitors which also offers the opportunity to choose a LRA candidate with lowest cytotoxic potential.

Previous studies suggested that the effect of HDACi on NK cells is drug dependent with lower cytotoxicity induced by romidepsin than panobinostat [46]. We also observed low cytotoxicity for the HDACi at short-term cultures, however, long-term exposure with both drugs were cytotoxic for the majority of NK cells. Detrimental effect on NK cells was also reported for HDACi treatment in advanced cutaneous T cell lymphoma patients where romidepsin treatment resulted in decreased NK cell function [49] while in HIV infected patients treated with panobinostat and romidepsin a decrease in NK cell viability, antiviral activity, and cytotoxicity was observed [46]. Thus both NK cells viability and function can be affected by HDACi.

The high cytotoxic effects of HDACi on B cells may impact the clearance of virally infected cells since HIV-specific antibodies could support elimination of HIV-infected cells through mechanisms like antibody-dependent cellular cytotoxicity. In a clinical study, no increase was found in HIV-specific antibodies in patients treated with panobinostat despite an increase in HIV RNA and presumably HIV antigens [62]. The authors concluded that the panobinostat-induced HIV reactivation is not sufficient to induce an increase in antibody level, however, our study suggests that the treatment with panobinostat may have affected the viability of B cells and this may contribute to the failure of panobinostat to boost antibody levels. Although no decrease in peripheral blood lymphocyte numbers were reported in patients treated with panobinostat [62], the effects of the HDACi on plasma B cells/memory B cell numbers in organs like bone marrow cannot be excluded.

If LRA induce cytotoxicity in in vitro settings, as indicated in our study, then this leads to the question whether cytotoxic effects of LRA have been observed in vivo in clinical trials for HIV latent reversal [19]. Although no serious side effects on the immune system was reported in clinical studies treating HIV+ patients, transient decline in immune cells was observed in several studies such as romidepsin-induced significant reduced WBC [61], and thrombocytopenia after SAHA treatment [64,66]. A reduction in the frequency of peripheral blood CD4+ and CD8+ T cells was observed in cutaneous T cell lymphoma patients when treated with higher concentrations of romidepsin than in HIV latency reversal clinical trials [49]. Other adverse events reported from studies with romidepsin-treated T cell lymphoma patients included transient thrombocytopenia, leukopenia and granulocytopenia in the majority of patients [72]. Importantly, only peripheral blood was analyzed in these studies, an effect on immune cells in organs like lymph nodes where HIV reactivation will occur was not included. For LRA classes others than HDACi few clinical studies were performed so far. Using bryostatin in a phase Ib trial for refractory malignancies, anemia was found in around half of the patients whereas leukopenia and thrombocytopenia was rarely observed [73].

The question arises how HDACi like romidepsin induce immune cell death and could this affect HIV-specific CTL preferentially. Indeed, an impairment of anti-viral functions of HIV-specific CD8+ T cells partially due to increased death of activated CD8+ T cells was linked to HDACi, specifically romidepsin [50]. Findings from HDACi studies in cancer point out that HDACi upregulate several pathways which increase apoptosis sensitivity of transformed cells including the death receptor CD95 and CD95-L [81]. Whilst non-transformed cells are resistant to this apoptosis [81], HIV-specific CD8+ T cells are sensitive to CD95-induced apoptosis [56,60], suggesting that in patients, HIV-specific CD8+ T cells may be preferentially killed, which further cautions the use of HDACi in a shock and kill strategy.

We examined a number of other potential LRAs for their toxicity. The PKC agonist bryostatin did not induce significant cytotoxicity in any of the immune subpopulations examined besides unactivated CD4+ T cells. The second PKC agonist, prostratin, significantly increased cell death in CD4+ T cells and CD8+ T cells but not in B cells and NK cells. This is in agreement with a previous study indicating that prostratin is non-toxic for NK cells [46] but induced death in CD4+ T cells [42]. Our findings indicate that BETi have only moderate cytotoxicity in T cells even at the highest concentration tested which is in line with studies analyzing the effect of OTX-015 [37] and JQ1 [36,42,43] on primary CD4+ T cells. Whether BETi will succeed in clinical trials to reactive latent HIV reservoir remains to be seen. A promising new class of LRAs are the BAFi for which in vitro data indicate a potent latency reversing effect [38], however, in vivo data are lacking so far. Our findings indicate that these small molecules induce no significant cytotoxicity in T cells with exemption of pyrimethamine at higher concentrations. Finally, the cytotoxicity of LRA we tested was comparable when PBMC from uninfected controls and HIV+ patients were tested. This suggests that T cells from HIV+ patients are

not more susceptible to the cytotoxic effects of these LRAs, which is an important point to consider when treating HIV-infected patients.

The cytotoxicity of LRAs we observed was limited to in vitro studies, and has some constraints including the question of appropriate exposure time. The half-life of these LRAs are very different when in vitro and in vivo conditions are compared. Specifically, the absence of liver, kidney and other means of metabolism in vitro will influence the availability of drugs and metabolites. Clinical studies using LRAs on latent HIV reservoir are being conducted for several weeks and are aimed at achieving therapeutic concentrations in serum of patients [67,69,70,82]. Furthermore, LRA exert their function inside the cells in the cytoplasm and/or nucleus and accumulate intracellularly [54]. Therefore, half-life of drugs measured in peripheral blood will not necessarily reflect the exposure time of cells either due to differences in intracellular levels or due to differences in drug concentrations in tissues like lymph nodes compared to peripheral blood. We analyzed the effect of these drugs after short term (18 hours) and long-term (72 hours) exposure which is within the exposure range previously published for in vitro studies [6,46,47,49-51].

It is important to keep in mind that LRAs may have additional effects beyond HIV reactivation. Bryostatin acts as a Toll-like receptor 4 ligand [83] and therefore may directly activate TLR4-expressing CD8+ T cells [85], thereby enhance the effector function of CTL. We have indeed observed an upregulation of CD38 on unactivated CD8+ T cells through bryostatin, whether this however is due to a TLR4-mediated signalling or due to the PKC agonistic effect of bryostatin remains unclear. We also observed an upregulation of CD38 on CD8+ T cells with prostratin, the second PKC agonists tested which was also reported for CD4+ T cells [43]. The BETi JQ1 and OTX-15 did not induce CD38 on unactivated cells, and this is in agreement with a previous study showing that OTX-015 does not induce CD69, CD25 or HLADR on primary CD4+ T cells after 48 hour incubation [37]. Both BETi reduced significantly the CD38 expression on activated cells, indicating that these inhibitors may inhibit activation of CD8+ T cells at higher concentrations. The BAFi pyrimethamine and CAPE did not affect CD8+ T cell activation which is in agreement with the previously reported absence of the activation marker CD25 and proliferation marker Ki-67 on purified CD4+ T cells from healthy donors after 72 hours of incubation [38].

CTL from HIV infected patients are exhausted [7-12] leading to reduced effector functions and proliferative capacity of these cells. Epigenetic changes [15-18] in HIV-specific CD8+ T cells may lead to the increased expression of inhibitory receptors [10-14] . We have analyzed the effect of LRAs on the expression of the inhibitory receptors CD160, CD244 and CD279 on unactivated and activated CD8+ T cells from HIV infected patients. No changes in expression of CD160 and CD244 on CD8+ T cells in the presence of the LRAs were detected. However, CD279 expression on CD8+ T cells was significantly decreased by bryostatin, JQ1 and OTX-015 and this was also significantly different from the effect these LRAs have on CD38 expression.

This indicates that the reduced frequency of CD279 is not due to reduced activation of T cells but to a drug-specific downregulation of the inhibitory receptor. These preliminary results indicate that LRA can affect inhibitory receptor expression on CD8+ T cells and this effect of LRA merits further investigation.

CONCLUSIONS

We executed a comparative study of the cytotoxic effect that different LRA classes have on immune cells. Although LRA-class-dependent effects on the survival of immune subpopulations including CD4+ and CD8+ T cells were observed, there were differences between LRA even from the same class. HDACi romidepsin and panobinostat convey high T cell cytotoxicity while SAHA, the PKC agonist bryostatin, BETi and BAFi exhibit the lowest cytotoxicity on CD8+ T cells. The impact of LRAs on immune subpopulations is comparable between HIV-uninfected and HIV-infected individuals. Finally, PKC agonist bryostatin, and BETi reduce the frequency of CD279 expressing CD8+ T cells without affecting activation suggesting potential ability to restore CTL immunity. Our studies suggest that different classes of LRAs have diverse impact on CTL viability with some LRAs having additionally the potential to reduce exhaustion of HIV-specific CD8+ T cells, thereby improving the elimination of HIV-infected cells in a “shock and kill” approach.

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AUTHORS CONTRIBUTIONS

M.Z., E.D.C. and Y.M.M. performed experiments and analyzed data. M.Z., T.M., A.V., C.R., T.v.G., P.D.K. and Y.M.M. contributed to study design. Manuscript was written by M.Z., P.D.K., and Y.M.M. All authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest or financial interests.

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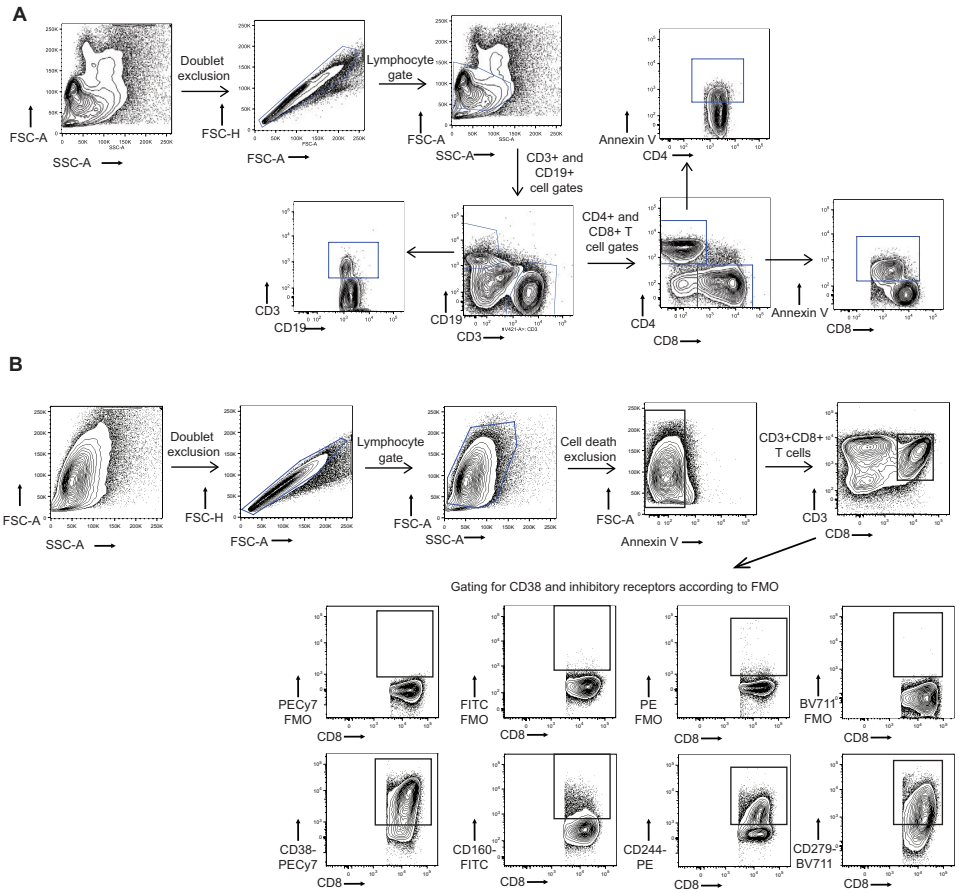
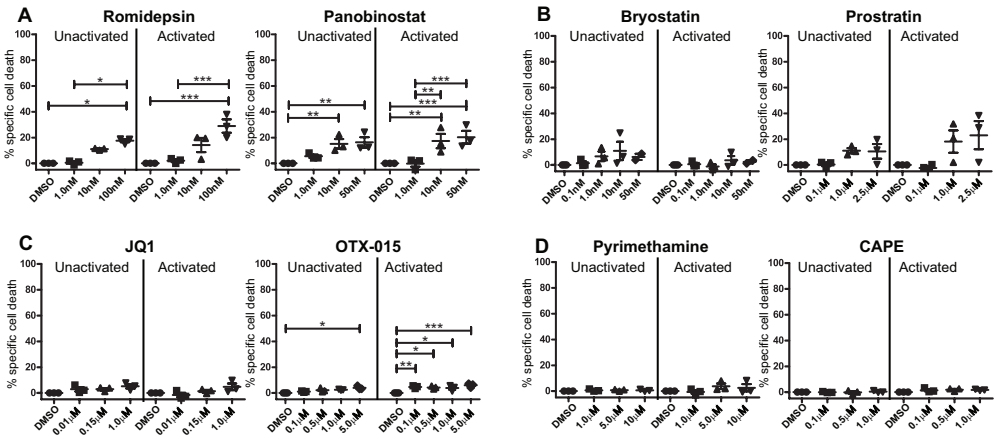


Supplementary Figure 1. Gating strategy for A. cytotoxicity and B. expression of activation marker and inhibitory receptors.

A. Doubles were excluded using FSC height (H) and area (A), followed by a lymphocyte gate. Using immune cell subset defining markers, Annexin V+ cells were defined for CD3+CD4+ T cells, CD3+CD8+ T cells and CD19+ B cells. **B**. For defining CD38+ and inhibitory receptor+ populations, samples were run with Minus One Fluorescence (MFO) for each of these markers (CD38, CD160, CD244, CD279). Analysis was performed by first excluding doubles and dead cells (Annexin V+) and then gating for CD3+CD8+ T cells. The gates for CD38 and inhibitory receptors were set on the MFO samples.



Supplementary Figure 2. LRA-specific cell death of unactivated and activated CD8+ T cells from HIV-infected patients after short-term culture.

Unactivated or anti-CD3/anti-CD28 antibody activated PBMC from HIV+ patients were cultured in the presence of the indicated concentrations of LRAs for 18 hours. Pooled data indicating the drug-specific cell death of CD8+ T cells are shown for HDACi romidepsin and panobinostat (**A**), PKC agonists bryostatin and prostratin (**B**), BETi JQ1 and OTX-015 (**C**), and BAFi pyrimethamine and CAPE (**D**). Each symbol represents CD8+ T cells from one HIV-infected patient (n= 3 from 3 independent experiments), horizontal line depicts mean ± SE. Statistical significance is indicated by stars (* = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, no star = not significant = P > 0.05).

Supplementary Table 1. Drug-specific cell death of immune populations in short-term cultures.

		CD4		CD8		B cells	NK cells
		Unactivated	Activated	Unactivated	Activated		
HDAC inhibitor	**Romidepsin**						
	1nM	6.8±5.0%^	7.2±4.8%	7.9±5.2%	7.1±4.5%	8.6±6.7%	1.3±0.3%
	10nM	12±4.3%*	16±2.3%**	14±4.8%*	17±2.3%**	14±6.5%	0.8±1.1%
	100nM	14±4.6%*	16±3.2%**	17±4.7%**	21±2.7%***	22±6.3%**	5.2±1.9%*
	Panobinostat						
	1nM	8.5±3.3%	5.0±3.5%	9.7±3.8%	6.3±2.7%	13±8.1%	3.0±2.5%
	10nM	13±3.0%**	10±4.0%*	16±2.9%**	16±5.9%**	19±7.8%*	5.9±2.5%
	50nM	17±3.4%***	16±3.4%***	21±2.2%***	21±4.9%***	27±7.3%**	5.0±3.2%
PKC agonist	**Bryostatin**						
	0.1nM	1.1±0.9%	2.5±1.7%	1.4±1.2%	0.0±0.3%	0.8±7.0%	2.8±0.9%
	1nM	9.3±4.3%	36±8.6%***	5.3±2.1%	9.3±4.0%	0.3±6.7%	1.9±2.3%
	10nM	41±11%***	61±7.5%***	23±11%	24±12%	3.3±5.0%	5.5±4.4%
	50nM	29±6.0%**	44±3.3%***	17±5.9%	19±7.8%	0.6±5.1%	4.2±2.4%
	Prostratin						
	0.1µM	1.1±2.0%	6.4±0.9%	1.1±1.9%	2.0±0.6%	-11±2.9%	0.4±0.2%
	1µM	56±12%***	72±5.6%***	26±11%*	26±9.7%*	6.4±5.4%	9.0±4.3%
	2.5µM	76±6.4%***	77±4.8%***	27±5.6%*	27±8.1%*	12±7.0%	5.2±2.9%
BET inhibitor	**JQ1**						
	0.01µM	0.4±0.3%	0.7±0.3%	0.6±0.4%	0.5±0.9%	6.2±6.2%	2.0±1.2%
	0.15µM	1.5±0.5%	4.2±1.3%*	1.5±0.5%	6.9±3.1%*	5.7±7.1%	2.4±1.0%
	1µM	2.8±0.7%	7.1±1.8%***	3.8±1.8%	9.4±3.3%**	8.3±8.8%	3.6±1.8%
	10µM	5.5±1.1%***	7.1±1.0%***	7.2±2.0%*	9.6±2.6%**	16±9.5%	5.0±1.7%*
	OTX-015						
	0.1µM	0.4±0.2%	1.3±2.0%	0.3±1.0%	0.7±1.8%	-1.3±1.6%	1.3±0.4%
	0.5µM	-0.9±1.0%	3.2±1.6%	2.5±1.2%	3.9±2.3%	-0.8±3.1%	3.0±0.9%
	1µM	0.7±0.7%	3.5±1.0%	2.6±1.0%	3.2±1.5%	0.2±3.6%	3.6±1.6%
	5µM	1.7±0.8%	4.1±0.9%*	3.5±1.3%	5.0±1.8%**	4.9±6.3%	4.9±2.5%*
BAF inhibitor	**Pyrimethamine**						
	1µM	0.9±0.5%	1.0±0.6%	0.6±0.4%	1.5±1.0%	2.8±4.4%	0.9±0.9%
	5µM	1.4±0.5%	0.0±0.7%	1.0±0.4%	0.5±0.6%	6.7±5.9%	0.5±1.2%
	10µM	1.6±0.9%	2.5±3.0%	1.5±0.7%	3.8±2.6%	6.6±7.3%	2.7±1.3%
	CAPE						
	0.1µM	0.6±0.2%	1.2±1.1%	0.9±0.2%	0.6±1.3%	0.2±1.4%	1.2±0.9%
	0.5µM	0.8±0.7%	1.2±1.4%	2.1±0.7%	0.8±1.5%	3.0±2.5%	1.7±1.3%
	1µM	1.6±0.9%	2.1±1.8%	1.9±0.7%	1.1±1.8%	1.5±1.9%	2.0±2.3%

^ Mean±SE

Statistical analysis: * = P ≤ 0.05, ** = P ≤ 0.01 and *** = P ≤ 0.001 compared to DMSO control; values without a star are not statistically significantly different from DMSO control;

N = 3 - 5 from 3 - 5 independent experiments

Chapter 3

Rapid *in vitro* generation of bona fide exhausted CD8+ T cells is accompanied by *Tcf7* promotor methylation

Manzhi Zhao[1], Caoimhe H. Kiernan[1], Christopher J. Stairiker[1], Jennifer L. Hope[6], Leticia G. Leon[1], Marjan van Meurs[1], Inge Brouwers-Haspels[1], Ruben Boers[2,4], Joachim Boers[2,4], Joost Gribnau[2,4], Wilfred F.J. van IJcken[5], Eric M. Bindels[3], Remco M. Hoogenboezem[3], Stefan J. Erkeland[1], Yvonne M. Mueller[1], Peter D. Katsikis[1]

[1]Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands.
[2]Department of Developmental Biology, Erasmus University Medical Center, Rotterdam, The Netherlands.
[3]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands.
[4]Oncode Institute, Erasmus University Medical Center, Rotterdam, The Netherlands.
[5]Center for Biomics, Erasmus University Medical Center, Rotterdam, The Netherlands.
[6]Cancer Immunology and Tumor Microenvironment Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, United States.

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ABSTRACT

Exhaustion is a dysfunctional state of cytotoxic CD8+ T cells (CTL) observed in chronic infection and cancer. Current *in vivo* models of CTL exhaustion using chronic viral infections or cancer yield very few exhausted CTL, limiting the analysis that can be done on these cells. Establishing an *in vitro* system that rapidly induces CTL exhaustion would therefore greatly facilitate the study of this phenotype, identify the truly exhaustion-associated changes and allow the testing of novel approaches to reverse or prevent exhaustion. Here we show that repeat stimulation of purified TCR transgenic OT-I CTL with their specific peptide induces all the functional (reduced cytokine production and polyfunctionality, decreased *in vivo* expansion capacity) and phenotypic (increased inhibitory receptors expression and transcription factor changes) characteristics of exhaustion. Importantly, *in vitro* exhausted cells shared the transcriptomic characteristics of the gold standard of exhaustion, CTL from LCMV cl13 infections. Gene expression of both *in vitro* and *in vivo* exhausted CTL was distinct from T cells anergy. Using this system, we show that *Tcf7* promoter DNA methylation contributes to TCF1 downregulation in exhausted CTL. Thus this novel *in vitro* system can be used to identify genes and signaling pathways involved in exhaustion and will facilitate the screening of reagents that prevent/reverse CTL exhaustion.

AUTHOR SUMMARY

In this manuscript, we describe an *in vitro* method that rapidly establishes large numbers of exhausted CD8+ T cells. The exhaustion of CTL induced by this method has been fully validated by multiple approaches (cytokine production, polyfunctionality, cytotoxicity, *in vivo* proliferation, inhibitory receptors, transcription factors, RNAseq and DNA methylation). This method will facilitate not only the study of T cell exhaustion but also the screening of drugs. As proof of point, we use this method to show that TCF1 downregulation in terminally exhausted T cells is accompanied by *Tcf7* DNA promoter methylation and show that a transmethylase inhibitor can prevent TCF1 downregulation. Our method presents a critical resource for the study of CTL exhaustion and the screening of drugs and interventions.

INTRODUCTION

Cytotoxic CD8+ T cells (CTL) play a critical role in eliminating viral infection and controlling cancer development. During chronic viral infection and cancer, CTL acquire a state of dysfunction that is often referred as CTL exhaustion which was originally described in chronic Lymphocytic Choriomeningitis Virus (LCMV) infection of mice (1). CTL exhaustion has been documented in humans in chronic viral infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections and in most human cancers (2-9) and is thought to be a central mechanism behind the failure of CTL to eliminate chronically infected and cancerous cells. Preventing and/or reverting exhaustion therefore constitutes a promising approach to restore the function of these CD8+ T cells. This requires however an in depth understanding of the mechanisms that lead to exhaustion and the stimuli that affect exhausted CD8+ T cells.

In recent years, the characteristics of exhausted CTL have been intensively researched by comparing antigen-specific CTL in chronic viral infection or cancer with effector and memory cells in acute virus infection (10-12). Exhaustion is characterized by loss of cytokine production, such as interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), decreased cytokine polyfunctionality, diminished expansion potential (13) and sustained high expression of multiple inhibitory receptors such as PD-1, Tim3, Lag3, TIGIT, CD160 and CD244 (14-17).

The phenotypic and functional changes of exhausted CTL arise from an altered transcriptional profile and modified epigenetic landscape (12, 18-20). Altered expression of transcription factors and repressors such as T cell factor-1 (TCF1) (21, 22), Thymocyte selection-associated HMG box protein (TOX) (23-27), T-box transcription factor 21 (T-bet) (12), Eomesodermin (EOMES) (1), IRF4 (28), NR4a (29) and BAFT (30) are indicative for the exhaustion phenotype.

For example, transcription factor T-bet and TCF1 commonly expressed by functional effector and memory CTL, are also expressed by exhausted CTL, but are associated with distinct gene expression (12, 31, 32). TOX expression has been associated with molecular and epigenetic programs of CTL exhaustion (23, 24, 26, 27). In addition, in comparison to effector and memory CTL, exhausted CTL also have a distinct epigenetic landscape that contributes to their phenotypic changes and gene expression (19, 20).

To study T cell exhaustion, *in vivo* mouse models are commonly used where T cell exhaustion is either induced through chronic viral infection or cancer. LCMV infection is a well-characterized mouse model for CD8+ T cell exhaustion (10, 33). The exhausted phenotype is also detected among tumor infiltrating lymphocytes (TIL) in tumor mouse models (34, 35). The persistence of the antigen has been shown to be the crucial element (36) and chronic antigen stimulation alone is sufficient to induce CTL exhaustion *in vivo* (37, 38). Previous *in vitro* exhaustion protocols have failed to validate T cell exhaustion, assuming inhibitory receptor expression and reduction of IL-2 production as surrogates for dysfunction (39). Some of these changes, however, such as expression of PD-1 and CD160 also accompanies T cell activation while loss of IL-2 production is a feature of effector differentiation. Thus a fully validated *in vitro* exhaustion system has yet to be established.

The importance of CTL exhaustion in chronic infections and cancer demands rapid *in vitro* methods to screen new approaches that can prevent or revert CTL exhaustion. Inducing CTL exhaustion *in vivo* is time-consuming, requiring more than 30 days and yields limited numbers of exhausted cells as experimental material. Most importantly the *in vivo* milieu in these models is characterized by inflammation, high viral loads, suppressive cytokines or cells; all of which can obscure the phenotype of exhausted CTL and therefore make it hard to dissect true exhaustion-associated changes. Our newly developed *in vitro* method circumvents all of the above mentioned issues and allows gene manipulation and screening of small molecules or antibodies that could restore the function of exhausted CTL and/or prevent the induction of exhaustion. Using this system, we can show that the *Tcf7* promotor is methylated in exhausted CTL and using DNA transmethylase inhibitors one can prevent the downregulation of TCF1. Therefore, this 5-day *in vitro* exhaustion system enables the rapid testing of new approaches to prevent and/or revert exhaustion in T cells and therefore may facilitate the development of new therapies for chronic infections and cancer.

RESULTS

Repeat stimulation with cognate peptide to induce CTL exhaustion

Because of the critical role sustained antigen stimulation plays in driving CD8+ T cells to exhaustion (36), we utilized repeat peptide stimulations of $OVA_{(257-264)}$ –specific TCR

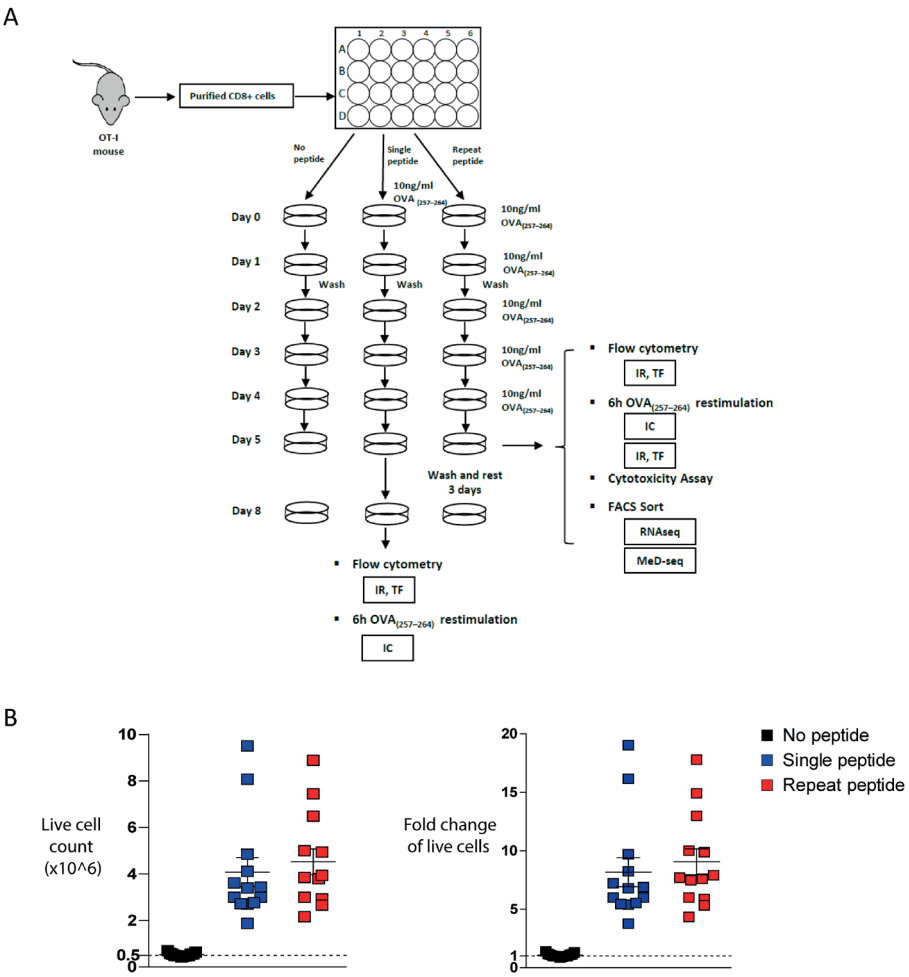

Figure 1. Scheme of the exprimental protocol and numbers of live exhausted CTL generated by in vitro exhaustion. (**A**) Scheme of the experimental set up. (**B**) Purified CD8+ T cells ($0.5x10^6$ cells per well) were cultured either unstimulated, stimulated once with peptide or repeat peptide stimulated. Live cells were counted on day 5. Pooled data showing absolute cell numbers (left) and fold expansion (right). Data are from n=13 and 10 independent experiments.

transgenic OT-I cells to induce CTL exhaustion *in vitro.* To induce exhaustion, CD8+ T cells purified from OT-I mice were stimulated daily for five days with 10ng/ml $OVA_{(257-264)}$ peptide in the presence of IL-15 and IL-7 (Repeat peptide stimulation, Fig 1A). As controls, cells were either left unstimulated or stimulated only once with $OVA_{(257-264)}$ peptide for 48 hours and then washed and cultured without peptide for an additional 3 days. All cells were cultured with IL-7 and IL-15. On day 5, cells were either harvested for analysis or in some instances

cells were cultured an additional 3 days in the presence of IL-7 and IL-15 without any additional peptide stimulation (Fig 1A).

An important feature of this *in vitro* exhaustion system is that it rapidly yields large numbers of exhausted cells within 5 days (Fig 1B). Single peptide stimulations yielded comparable numbers while unstimulated culture numbers remained largely unchanged. The expansion in the numbers of exhausted T cells represents a ~9-fold increase from the T cell numbers seeded. Thus this *in vitro* exhaustion system can yield large numbers of exhausted CTL that can be used to further study and manipulate the pathways that drive exhaustion.

Repeat peptide stimulation reduces cytokine production and leads to loss of polyfunctionality

Since hierarchical loss in the capacity to produce cytokines is one of the most critical characteristics of exhausted CD8+ T cells (10), we first examined the capacity for cytokine release upon peptide re-stimulation. After five days of culture, the cells were harvested and stimulated with $OVA_{(257–264)}$ peptide in the presence of Golgi plug for 6 hours. More than 40 percent of the unstimulated cells could produce IFN-γ upon re-stimulation (46±4%, mean ± standard error Fig 2A and 2B). Single peptide stimulated cells were also able to produce IFN-γ (58±4%). In contrast, repeat peptide stimulated cells had impaired IFN-γ production with only 27±3% of cells able to produce IFN-γ after re-stimulation (Fig 2A and 2B). In addition, very few repeat peptide stimulated cells (4.8±0.4%) could produce TNF-α compared to single peptide stimulated cells (44±4%) and unstimulated cells (71±4%) (Fig 2A and 2B). IL-2-producing cells could barely be detected in the repeat peptide stimulated cells after re-stimulation, while IL-2 was readily produced by single peptide stimulated cells (37±3%) and unstimulated cells (60±4%) (Fig 2A and 2B). Ultimately, these results demonstrate that repeat peptide stimulation of CD8+ T cells *in vitro* leads to impaired cytokine production in a manner that has been previously reported in exhausted CD8+ T cells, first IL-2 production is lost followed by TNF-α and finally IFN-γ.

Exhausted CD8+ T cells, unlike memory cells, cannot produce multiple cytokines at the same time, and we therefore assessed the polyfunctionality of our *in vitro* generated cells. For this, the percentage of single, double and triple cytokine producing cells were determined and visualized using the SPICE software. In unstimulated cultured cells, 33% produce all three cytokines simultaneously upon peptide stimulation while significant percentages of these cells were double producer (Fig 2C). Only 15% of the cells did not produce any of these three cytokines. Similarly, in the single peptide stimulated cells, 18% were triple positive for all three cytokines, large numbers were double cytokine producers, while 26% were incapable of releasing any of these cytokines (Fig 2C). In contrast, 70% of the repeat peptide stimulated cells could not produce any cytokines after re-stimulation. IFN-γ single producers were 25% of the cells, while very few cells produced other cytokines (Fig 2C). To demonstrate the

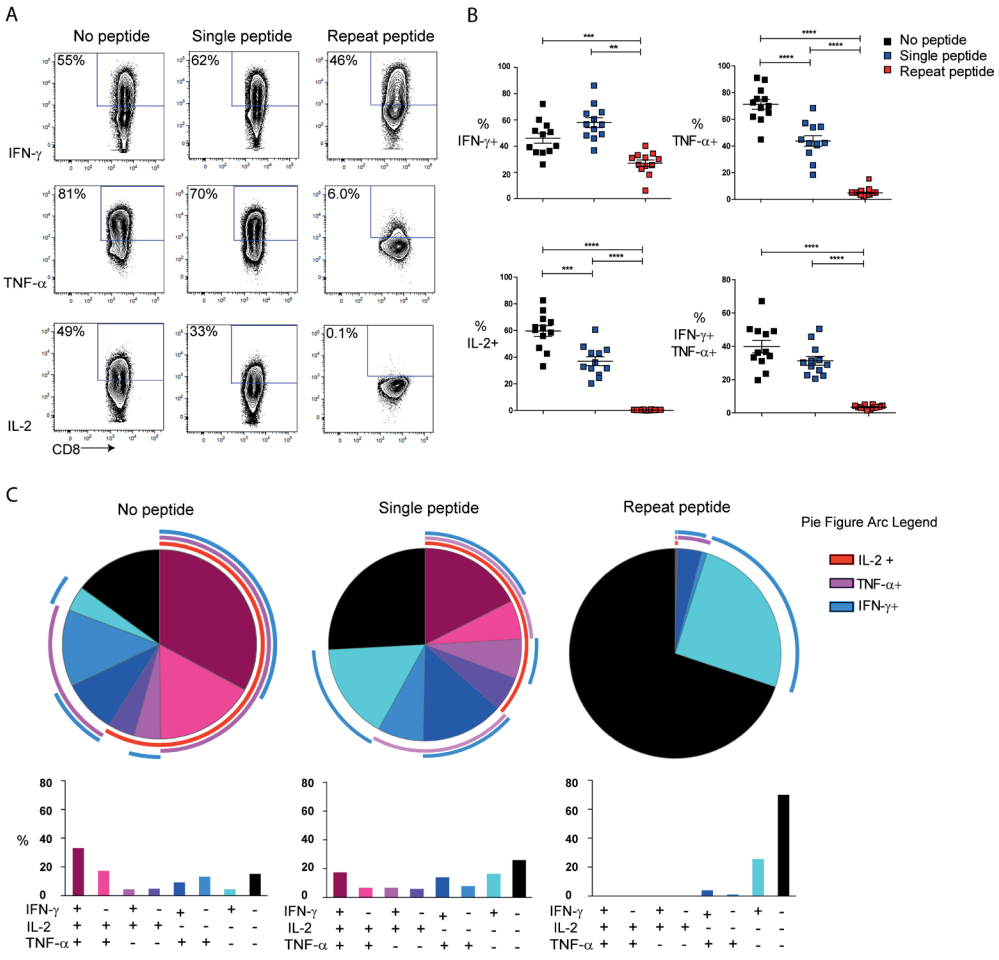

Figure 2. Repeat peptide stimulated CD8+ T cells show reduced cytokine production capacity and lose polyfunctionality.

Purified OT-I CD8+ T cells were cultured either without peptide (no peptide), stimulated one time for 2 days with OVA peptide (single peptide) or stimulated with OVA peptide daily (repeat peptide). On day 5 cells were harvested and re-stimulated with OVA peptide. (**A**) Representative flow cytometry plots showing frequency of cytokine producing live CD8+ T cells after re-stimulation. (**B**) Pooled data showing the frequency of cytokine producing cells after re-stimulating with OVA peptide. (**C**) SPICE figures depicting the frequency of cells producing one, two or three cytokines in different combinations. Each symbol represents one animal (n=11-12), 9 independent experiments performed. Line depicts mean ± SE. Between the groups, Student's t test with Welch's correction was performed except for % IFN-γ+ (ANOVA with Tukey's post hoc test). *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

persistence of the cytokine profile of repeat peptide stimulated cells, we rested cells without peptide for another 3 days before examining their cytokine production. We found that the

reduced cytokine production of repeat peptide stimulated cells was maintained after resting (Supplementary Fig 1A).

Degranulation is an important step for the CD8+ T cell cytotoxicity. We therefore analyzed the degranulation marker CD107a and Granzyme B (GzmB) expression in our cells. When cells were analyzed without peptide re-stimulation, cells either unstimulated or single peptide stimulated were negative for CD107a and GzmB (Fig 3A and 3B), while repeat peptide stimulated cells showed a significantly higher level of both markers (Fig 3A and 3B). When cells were re-stimulated with $OVA_{(257-264)}$ peptide, cells cultivated in the presence of no peptide and single peptide stimulated cells upregulated CD107a (Fig 3A). In contrast, repeat peptide stimulated cells failed to increase CD107a expression after re-stimulation, indicating that these cells are not able to degranulate further upon re-stimulation. Granzyme B was increased in repeat peptide stimulated cells in agreement with the terminal differentiation of exhausted cells (Fig 3B). No increase of GzmB was detected after 6 hours of re-stimulation for any of the conditions analyzed (Fig 3B). Repeat peptide stimulated cells exhibited reduced cytotoxic capacity against $OVA_{(257–264)}$ peptide-loaded tumor cells (Fig 3C). These findings clearly show that after repeat stimulation *in vitro*, CTL lose their ability to make cytokines, have reduced polyfunctionality, cannot degranulate further, have increased GzmB

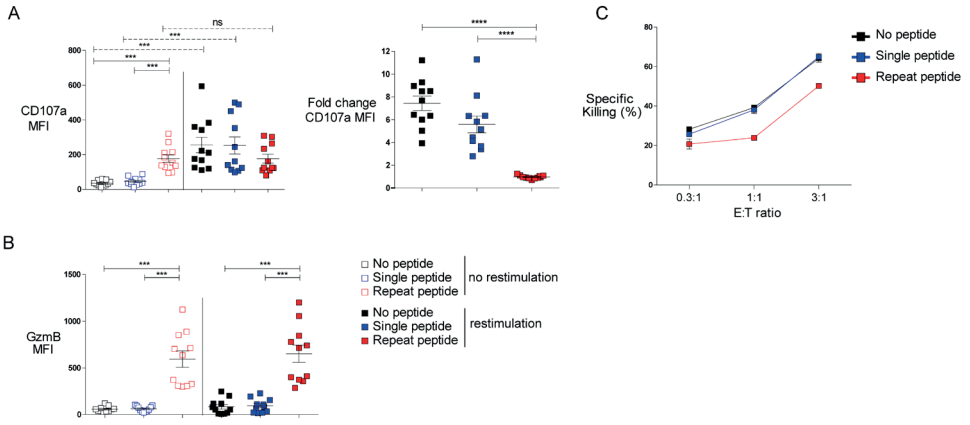

Figure 3. Repeat peptide stimulated CD8+ T cells have decreased cytotoxic function.

On day 5, cells were harvested and re-stimulated with OVA peptide. (**A**) Median fluorescence intensity (MFI) of the degranulation marker, CD107a, shown (left panel). Fold change of CD107a MFI induced by peptide re-stimulation depicted on the right panel. (**B**) MFI of Granzyme B (GzmB) depicted for the different culture conditions. Each symbol represents one animal (n=11-12), 9 independent experiments performed. (**C**) No peptide, single peptide and repeat peptide stimulated cells were co-culture with target cells (OVA-pulsed AE-17 cells) at different ratios. Percentage of specific killing is depicted. One of five independent experiments shown. Line depicts mean ± SE. Between the groups, Student's t test with Welch's correction was performed except for CD107a MFI (Wilcoxon signed rank test). *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

and reduced cytotoxicity. All of these features are characteristics of the dysfunctional state of *in vivo* exhausted CTL.

Multiple inhibitory receptors were upregulated following repeat peptide stimulation

An increase in inhibitory receptor expression corresponds to a more exhausted state (17). After harvesting on day five, the cells were stained immediately for the surface expression of the inhibitory receptors PD-1 (CD279), CD244, CD160, Lag3 (CD223), Tim-3 (CD366) and TIGIT. As expected, inhibitory receptors were barely expressed on the cells without peptide stimulation and single peptide stimulated cells (Fig 4A and 4B), except for PD-1, where 20±4% of the single peptide stimulated cells expressed this inhibitory receptor. In contrast, there was a significant upregulation of multiple inhibitory receptors on repeat peptide stimulated cells. Almost all of these cells became PD-1 positive (98±0.3%, Fig 4A and 4B). Furthermore, 74±5% and 76±4% of the cells, respectively, express TIGIT and Lag3. The expression of Tim3 (64±2%), CD160 (58±4%) and CD244 (14±2%) was also significantly increased on the repeat peptide stimulated cells.

We next examined the simultaneous co-expression of multiple inhibitory receptors (PD-1, Lag3, CD160 and CD244) using SPICE (Fig 4C). Repeat peptide stimulated cells were 39% double positive for the inhibitory receptors and another 39% of the cells co-expressed three of these inhibitory receptors simultaneously. Furthermore, 11% of the cells expressed all 4 inhibitory receptors (Fig 4C). In contrast, very few of the no peptide and single peptide stimulated cells expressed two or more inhibitory receptors (Fig 4C). The differences in inhibitory receptor expression remained even after resting repeat peptide stimulated cells for 3 days (Supplementary Fig 1B).

To exclude that differences in inhibitory receptor expression were due to a different activation status, all the cells were re-stimulated for 6 hours after harvesting them on day 5. Although reactivation induces a slight upregulation of some inhibitory receptors on the no peptide and single peptide cultures, they still remain much lower than the repeat peptide stimulated cultures (Supplementary Fig 2A). The exception was CD160 which was upregulated to similar levels in all cells. Overall, these findings confirm that multiple inhibitory receptors are expressed on the repeat peptide stimulated cells.

Expression of transcription factors is altered in repeat peptide stimulated cells

Previous studies have reported that exhausted CTL express and utilize transcription factors (TF) differently compared to effector and memory cell (11, 12, 40). To characterize the expression pattern of TF in the *in vitro* exhausted cells, four of the core TFs (TCF1, TOX, T-bet, and EOMES) were measured. TCF1 has been reported to play a critical role in identifying

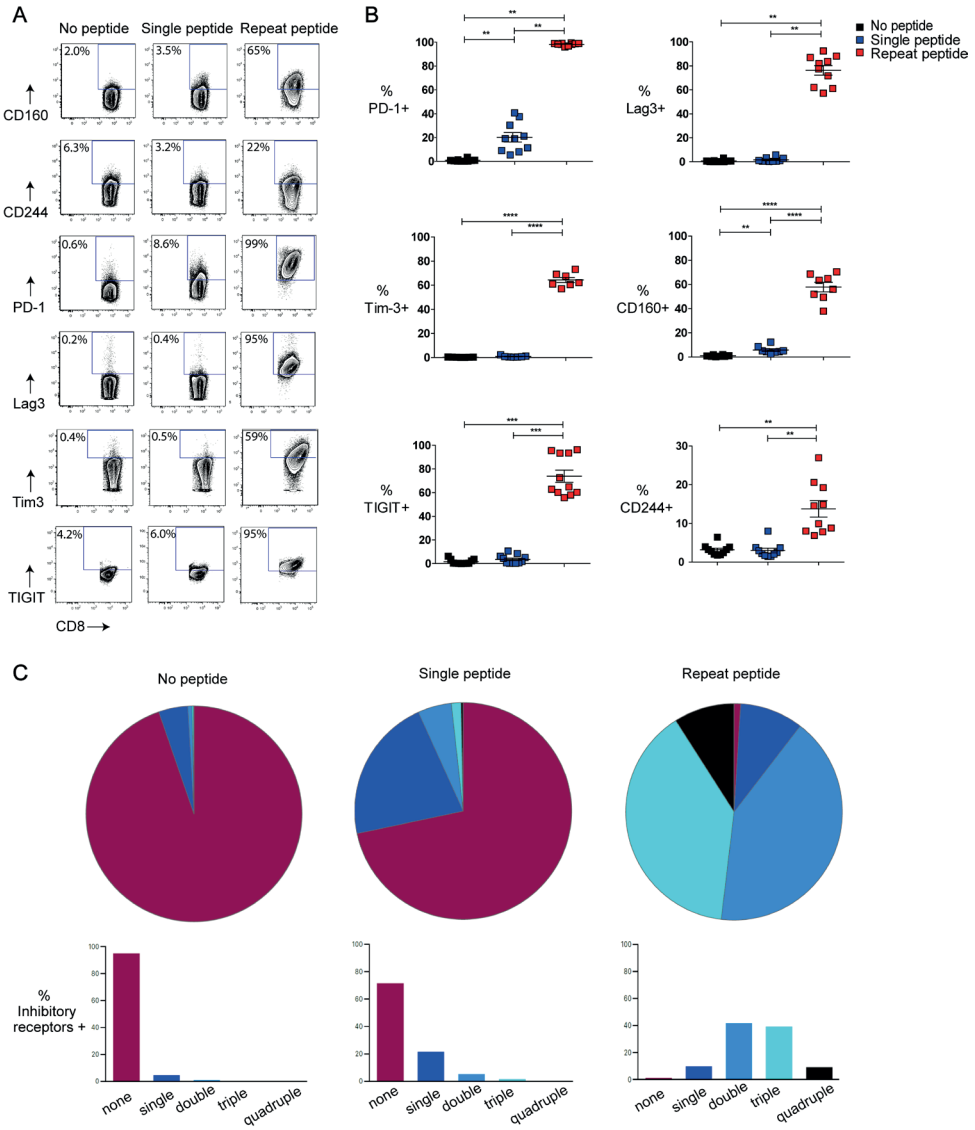

Figure 4. Repeat peptide stimulated cells express multiple inhibitory receptors.

The expression of the inhibitor receptors PD-1, CD244, CD160, Lag3 were determined by flow cytometry on day 5 of *in vitro* culture of OT-I CD8+ T cells. Cells were cultured either without peptide (no peptide), one time OVA peptide stimulation (single) or daily peptide stimulations (repeat peptide). (**A**) Representative flow cytometry plots depicting the frequency of live CD8+ T cells expressing inhibitory receptors. (**B**) Pooled data of frequency of CTL expressing individual inhibitory receptors within the different culture conditions. (**C**) SPICE figures depicting the frequency of CD8+ T cells expressing one, two, three or four inhibitor receptors (PD-1, CD244, CD160, Lag3) simultaneously. Each symbol representative one animal (n=7-11), with 7-9 independent experiments performed. Line depicts mean ± SE. Between the groups, Wilcoxon signed rank test was performed with exception of % Tim-3+ and % CD160+ for which a Student's t test with Welch's correction was used. *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

subsets of exhausted T cells (22, 41). Early exhausted or the progenitor exhausted cells maintained TCF1 expression, while terminally exhausted T cells downregulate its expression. In comparison to single peptide stimulation, repeat peptide stimulation induced down-regulation of TCF1 expression and upregulation of GzmB (Fig 5A and 5B). TOX expression was reported to be increased on exhausted cells (2, 23-25, 40), and indeed we also found TOX to be significantly upregulated in the repeat peptide stimulated cells in comparison to unstimulated and single peptide stimulated cells (Fig 5A and 5B). These differences in TOX and TCF1

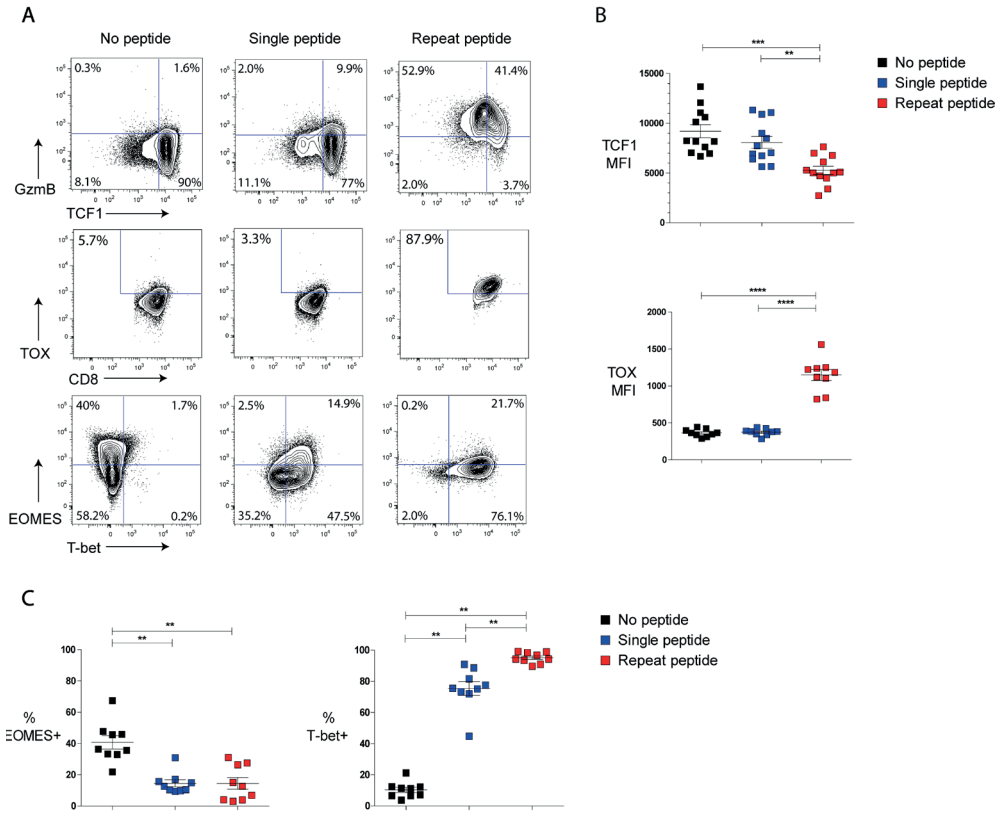

Figure 5. Repeat peptide stimulation induces a distinct pattern of transcription factor expression.

Transcription factors (TF) were analyzed in OT-I CD8+ T cells on day 5 of culture. Cells either unstimulated (no peptide), stimulated one time (single peptide) or daily (repeat peptide) shown. (**A**) Representative flow cytometry plots of the frequency of TF-expressing live CD8+ T cells shown for the differently treated cultures. (**B**) Pooled data depicting the MFI of TCF1 and TOX in live CD8+ T cells. (**C**) Pooled data showing the frequency of EOMES and T-bet expressing live CD8+ T cells. Each symbol represents one animal (n=9-12), with n=7-9 independent experiments performed. Line depicts mean ± SE. ANOVA with Tukey's post hoc test was performed on TCF1 MFI, Student's t test with Welch's correction was used for TOX MFI and Wilcoxon signed rank test for data in **C**. *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

expression persisted even after resting 5 day cultures for another 3 days (Supplementary Fig 1C and D) but also after reactivation of cells (Supplementary Fig 2B and C). T-bet and EOMES expression in exhausted CD8+ T cells were dependent on the stage of exhaustion (1, 42). Although *in vitro* single peptide stimulation already increased the expression of T-bet, repeat peptide stimulation further significantly upregulated T-bet (Fig 5A and 5C). Around 90% of the repeat peptide stimulated cells were T-bet+/PD-1+ double positive whereas in single peptide simulated cells T-bet+ cells were mostly PD-1 negative (Supplementary Fig 2D and E). *In vitro* peptide stimulation downregulated EOMES, with no detectable difference of EOMES expression comparing repeat with the single peptide stimulated cells (Fig 5A and 5C). The increased TOX and T-bet accompanied by decreased TCF1 expression in repeat peptide stimulated CTL is in agreement with the TF profile of *in vivo* exhausted CTL (25, 41, 42).

Repeat peptide stimulated cells have decreased *in vivo* expansion capacity

To evaluate the *in vivo* expansion capacity of repeat peptide stimulated cells, we sorted live CD8+ T cells from *in vitro* cultures (no peptide, single peptide and repeat peptide stimulated cells) and adoptively transferred 10^4 live CD45.1+ CD8+ T cells into wild type CD45.2+ mice that were infected 3h later with WSN-OVA influenza virus. Freshly isolated naïve CD8+ T cells from an OT-I spleen were also transferred as controls. At day 10 post infection, mice were harvested and the number of donor CD45.1+ cells in the lung were measured. We found a significant reduction in the frequency (23% versus 68% and 66%, for repeat peptide, single peptide and no peptide, respectively, Fig 6A) as well as absolute cell numbers ($3.2x10^6$, $11.8x10^6$ and $12.6x10^6$, for repeat peptide, single peptide and no peptide respectively, Fig 6B) for the repeat peptide stimulated cells compared to all controls. Freshly isolated naïve cells did not differ from cells of single and no peptide cultures. The numbers of repeat peptide stimulated donor cells were also reduced in mediastinal lymph nodes (MLN) and spleens (Supplementary Fig 3). This indicated that repeat peptide stimulated cells are less capable of expanding after being exposed to their specific antigen. Meanwhile, the mice that received the repeat peptide stimulated cells possessed a larger frequency and number of endogenous CD45.2+ $OVA_{(257–264)}$-specific CTL in their lungs than mice that received unstimulated or single peptide stimulated cells (frequency: 4.5% versus 0.8% and 1.1%, Fig 6C; cell numbers: $0.6x10^6$ versus $0.1x10^6$ and $0.2x10^6$, for repeat peptide, single peptide and no peptide respectively, Fig 6D). This indicates that the adoptively transferred repeat peptide stimulated cells were less efficient than the other donor cells to compete with the endogenous antigen-specific CTL response. These results indicated that *in vitro* repeat peptide stimulation resulted in a reduced capacity of CTL to expand when re-exposed to their cognate antigen. This further supports our notion that *in vitro* repeated antigen stimulation results in exhaustion of these cells.

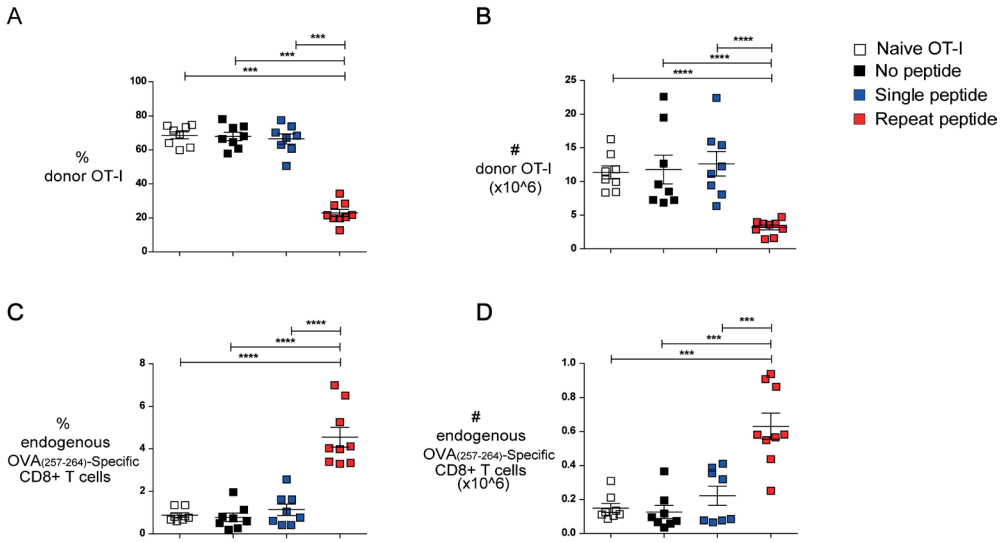

Figure 6. Repeat peptide stimulation decreases *in vivo* CTL expansion capacity.

OT-I CD8+ T cells were cultured without peptide stimulation (no peptide), one-time stimulation (single peptide) or daily stimulation (repeat peptide) and sorted on day 5. Live CD8+ T cells were adoptively transferred into wild type mice which were then infected with the OVA(257–264)-expressing influenza virus WSN-OVA. Freshly isolated OT-I CD8+ T cells from a naïve mouse were also transferred (naïve OT-I). Lungs were harvested on day 10 post infection. (**A**) Frequency of donor CD45.1+ OT-1 CD8+ T cells within total lung CD8+ T cells shown. (**B**) Absolute number of lung donor CD45.1+ OT-1 CD8+ T cells depicted. (**C**) Frequency of endogenous CD45.2+ OVA-specific CD8+ T cells within the total lung CD8+ T cells shown. (**D**) Absolute number of endogenous CD45.2+ OVA-specific CD8+ T cells shown. Each symbol represents one animal (n=8-9) from n=3 independent experiments. Line depicts mean ± SE. To determine significant differences between the different animal groups, a Mann–Whitney U test was used except for data in **a** (ANOVA with Tukey's post hoc test). ***P<0.001, ****P<0.0001.

Repeat peptide stimulated cells have the transcriptional profile of exhausted CTLs

To determine whether repeat peptide stimulated cells also have a distinct transcriptional profile, we performed RNAseq on sorted live CD8+ T cells from the different culture conditions. As shown in the principle component analysis (PCA) (Fig 7A), the samples clustered together and where distinctly separated depending upon their *in vitro* treatment. We identified 1196 genes with more than 2-fold increased expression and 1218 genes with more than 2-fold downregulation in the repeat peptide stimulated cells relative to that in single peptide stimulated cells. The transcriptional prolife of the repeat peptide stimulated cells is clearly more distinct than those of the single and non-stimulated cells (Fig 7B and Table S1). Among the upregulated genes, multiple inhibitory receptors encoding genes, including PD-1 (*Pdcd1*), *Lag3*, Tim3 (*Havcr2*), *CD160*, *Tigit* and *CTLA4* were presented on the top

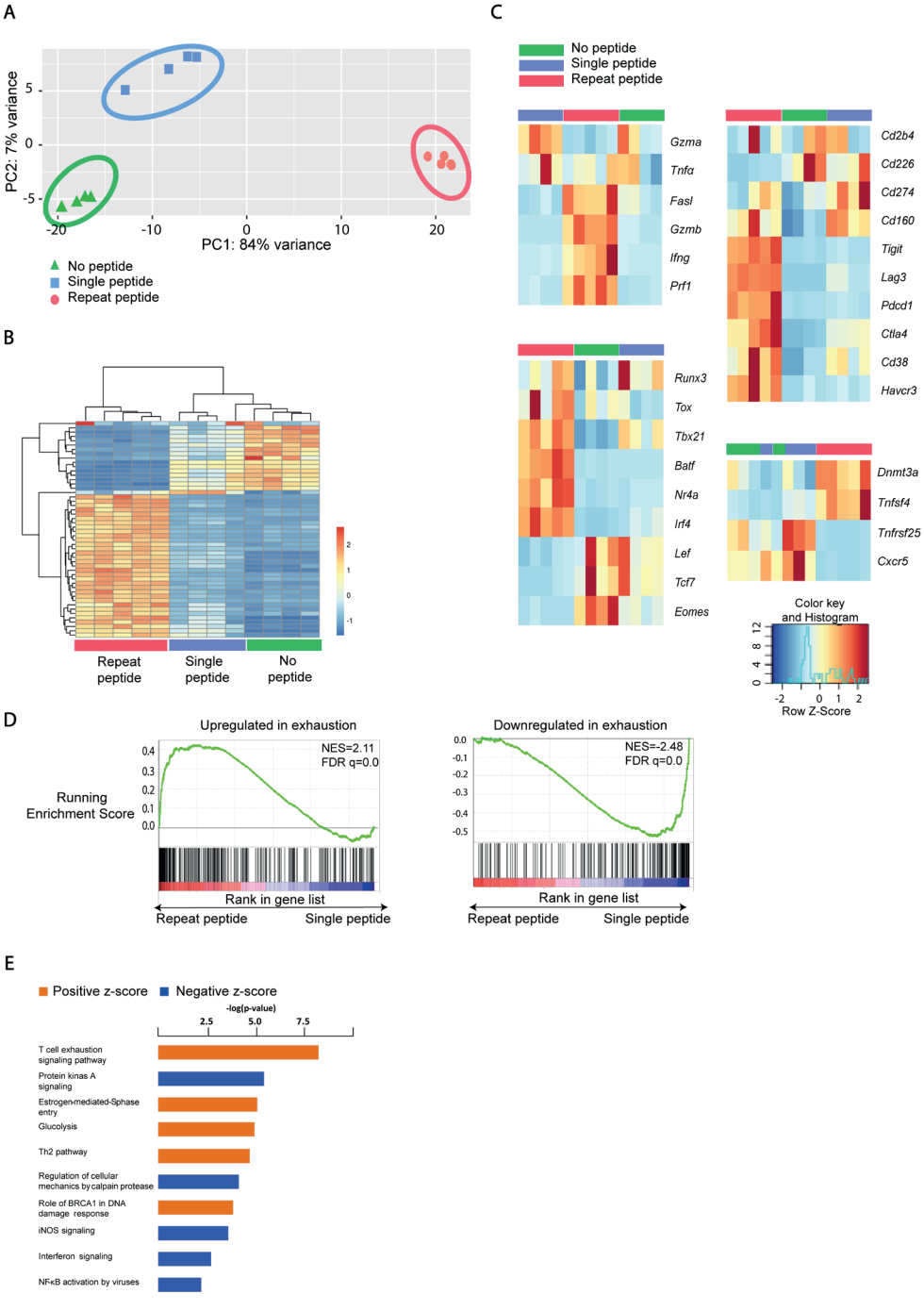
A
PC2: 7% variance
PC1: 84% variance
No peptide
Single peptide
Repeat peptide
B
Repeat peptide
Single peptide
No peptide
C
No peptide
Single peptide
Repeat peptide
Gzma
Tnfa
Fasl
Gzmb
Ifng
Prf1
Runx3
Tox
Tbx21
Batf
Nr4a
Irf4
Lef
Tcf7
Eomes
Cd2b4
Cd226
Cd274
Cd160
Tigit
Lag3
Pdcd1
Ctla4
Cd38
Havcr3
Dnmt3a
Tnfsf4
Tnfrsf25
Cxcr5
Color key and Histogram
Row Z-Score
D
Upregulated in exhaustion
NES=2.11
FDR q=0.0
Running Enrichment Score
Rank in gene list
Repeat peptide
Single peptide
Downregulated in exhaustion
NES=-2.48
FDR q=0.0
Rank in gene list
Repeat peptide
Single peptide
E
Positive z-score
Negative z-score
-log(p-value)
T cell exhaustion signaling pathway
Protein kinas A signaling
Estrogen-mediated-Sphase entry
Glucolysis
Th2 pathway
Regulation of cellular mechanics by calpain protease
Role of BRCA1 in DNA damage response
iNOS signaling
Interferon signaling
NFκB activation by viruses

Figure 7 (see previous page). Repeat peptide stimulated cells have distinct transcriptional profile.
RNAseq analysis was performed on live CD8+ T cells sorted from 5 day culture of OT-I CD8+ T cells. Cells were either without peptide (no peptide), one-time stimulation (single peptide) or daily peptide stimulations (repeat peptide). (**A**) PCA plot of RNA-seq data show that cells from identical culture conditions cluster together and away from each other. (**B**) Heatmap shown for the top 50 differentially expressed genes within CTL from the different culture conditions. (**C**) Heatmaps for the individual genes clustered based on function presented. (**D**) Repeat peptide stimulated CTL transcriptomes were enriched in gene signatures of LCMV-Cl13 exhausted CTL. Differentially expressed genes in repeat peptide stimulated CTL were analyzed by GSEA for their enrichment in gene sets found in exhausted CTL from LCMV-Cl13 infected mice. Enrichment in upregulated (left) and downregulated (right) genes shown. (**E**) Ingenuity pathway analysis was performed on the differentially expressed genes in repeat peptide stimulated cells (compared to single peptide). Significantly upregulated pathways (orange) and downregulated pathways (blue) in repeat peptide stimulated cells are depicted. P values (hypergeometric test) are presented as –log10.

of this list. As expected, the genes for the markers of the terminal differentiated effector cells, like *GzmB*, *GzmC* and *Prf1* were also significantly upregulated on the repeat peptide stimulated cells (Fig 7C). The transcription factors, *Eomes* and *Tcf7* were downregulated by 4.0 and 6.6 fold, respectively, while *Tox* was found to be 2.9 fold increased in the repeat peptide stimulated cells compared to single peptide stimulated cells (Fig 7C), confirming our flow cytometry findings of these TFs (Fig 5B and 5C). Furthermore, TFs, which are associated with CTL exhaustion such as *IRF4* (28), *NR4a* (29) and *Batf* (30) were also upregulated in the repeat peptide stimulated cells in comparison to single peptide and no peptide stimulated cells (Fig 7C).

To assess whether differentially expressed genes in repeat peptide stimulated cells were enriched for genes that characterize *in vivo* exhausted CTL from LCMV cl13 infections, we performed gene set enrichment analysis (GSEA). Differentially expressed genes between repeat peptide stimulated cell and single peptide stimulated cells were compared to public gene sets of upregulated or downregulated in *in vivo* exhausted CTLs (gene set GSE87646). Gene sets upregulated in exhaustion were found to be significantly more enriched in the repeat peptide stimulated upregulated genes compared to single peptide stimulated cells (Fig 7D). Conversely, the gene sets that were reported to be downregulated in exhausted cells were more enriched in the genes that were downregulated in repeat peptide stimulated cells versus the single peptide stimulated cells (Fig 7D). Thus, GSEA demonstrated significant transcriptional similarity between *in vitro* repeat peptide stimulated cells and *in vivo* exhausted cells. To further investigate signaling pathways that distinguish repeat peptide stimulated cells from single peptide stimulated cells, we performed IPA analysis. The most significant pathway with upregulated activity in repeat peptide stimulated cells was the T cell exhaustion signaling pathway (Fig 7E). The above findings clearly show that *in vitro* repeat peptide stimulation results in the transcriptional changes of *in vivo* exhausted CD8+ T cells from LCMV cl 13 infections which serve as the benchmark of CTL exhaustion.

Repeat peptide stimulation results in hyper-methylation of the Tcf7 transcriptional regulatory region

Changes in methylation of transcription regulatory region have been described in exhausted CTLs (18, 43). In order to identify whether *in vitro* repeat peptide stimulated cells also possess similar epigenetic characteristics of exhausted CTLs, whole genome methylated DNA sequencing (MeD-seq) (44) was performed on the sorted live CD8+ T cells. Stimulated T cells undergo distinct genome wide DNA methylation changes depending on the type of treatment (Fig 8A and Table S2). In comparison to unstimulated and single peptide stimulated cells, the transcriptional regulatory region of *Pdcd1* had significantly less DNA methylation in the repeat peptide stimulated cells (Fig 8B, left). When comparing the promotor methylation status (2kb region surrounding the TSS) of *Tcf7*, there was more DNA methylation detected in repeat peptide stimulated than in the single peptide stimulated cells or unstimulated cells (Fig 8B, center). Meanwhile, in the promotor region of the *GzmB*, significantly less DNA methylation was found in the repeat peptide stimulated cells than in the other cells (Fig 8B, right), which was in line with the higher expression of the protein upregulated in the repeat peptide stimulated cells (Fig 3B). Besides *Pdcd1*, none of the other inhibitory receptor genes' regulatory or promotor regions were found to possess significant differences in methylation status, although some of them showed expected trends in DNA methylation changes at their TSS. Interestingly, the DNA methylation states of the cytokine genes *IL-2, IFN-γ* and *TNF-α* were not significantly different. This indicated that other gene expression control mechanisms, like histone modifications or transcription factor abundance, might regulate the differential expression of these cytokines. Overall, these findings indicate that the repeat peptide stimulated cells have distinct DNA methylation patterns and reveal that the downregulation of TCF1 expression is accompanied by increased promotor methylation of *Tcf7*.

To further confirm that DNA promotor methylation contributes to the downregulation of TCF1 expression in exhausted cells we treated cells with a DNMT inhibitor for the last 3 days of culture and examined whether TCF1 expression was modified in the repeat peptide stimulation cultures. Indeed, treatment of these cells with DNMT inhibitor resulted in an increase in TCF1 levels in exhausted CTL (Fig 8C). This further supports that DNA methylation plays a role in silencing TCF1 as exhausted cells progress from "progenitor exhausted" to the "terminally exhausted" subpopulation.

DNMT inhibitor also reduced the expression of PD-1 and Tim3 in repeat peptide stimulated cells (Supplementary Fig 4). Because PD-1 and Tim3 expression are controlled by promoter region DNA methylation (43, 45), our findings suggest that DNMT inhibitor prevented exhaustion rather than reverted the exhaustion of T cells. These experiments suggest that the *in vitro* exhaustion system we describe can be used to test reagents that modulate T cell exhaustion.

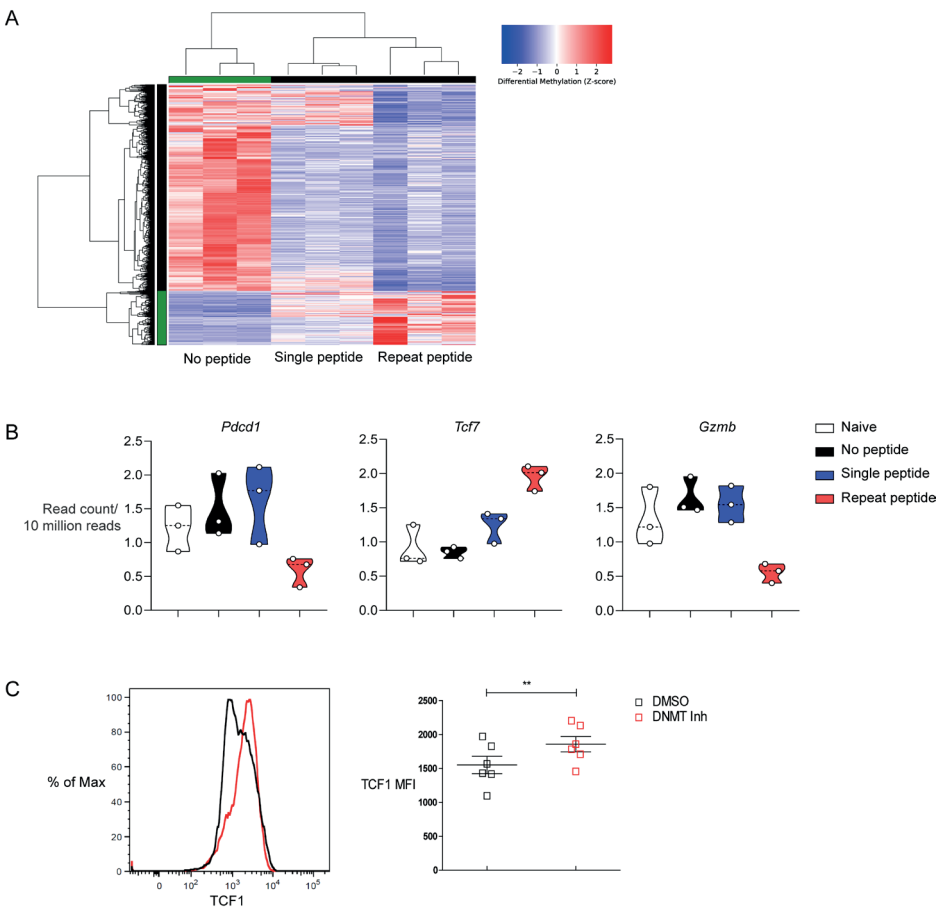

Figure 8. Genome wide DNA methylation changes during T cell stimulation reveal *Tcf1* promotor methylation. Sorted live CD8+ T cells were processed and whole genome methylated DNA sequencing (MeD-seq) was performed. (**A**) Hierarchical clustering on DMRs (differentially methylated regions) found between the three different peptide exposure conditions are shown. (**B**) Boxplot of DNA methylation read count data in a 2kb window surrounding the TSS of mentioned genes are shown. The samples were collected from three independent experiments. (**C**) Representative histogram of TCF1 expression (left) and pooled data showing TCF1 MFI on live CD8 cells (right) in the presence or absence of 20µM DNMT inhibitor during the last 3 days of repeat peptide stimulated cells shown. Data are from n=6 performed in 3 independent experiments, paired Student's t test performed. **P<0.01.

Anergy is not a feature of *in vitro* and *in vivo* exhausted CTL

The high purity of T cells in our culture system raises the question whether the absence of CD28 costimulation induces anergy (46-48) and this explains some of the phenotypes we observe. However, this is unlikely as our cells are cultured in the presence of IL-7 that has been suggested to prevent anergy (49) and furthermore we still see exhaustion when cells

are cultured with IL-2 (Supplementary Fig 5A and B), another anergy preventing cytokine (50, 51). Some CD28 costimulation, however, may be present in our culture system, as repeat peptide stimulated cells express CD80 mRNA levels 74-fold and 13-fold higher compared to unstimulated and single peptide stimulated cultures, respectively. Flow cytometry confirmed the high surface expression of CD80 on repeat peptide stimulated cells (Supplementary Fig. 5C).

To further exclude that our repeat peptide stimulated cells have features of anergy, we performed GSEA for sets of genes identified by *in vitro* anergy induction (gene set GSE5960) (46). We find that both genes that are upregulated and downregulated in anergic T cells, are enriched in our repeat peptide stimulated cells (Supplementary Fig 6A and B). Thus there is no evidence for the presence of anergy. We found a similar enrichment for genes both upregulated and downregulated in anergic T cells in *in vivo* exhausted gp33-specific CTL from LCMV clone 13 infected mice (gene set GSE41867) (12) (Supplementary Fig 6C and D).

Finally, we performed pathway enrichment analysis by IPA on the genes differentially expressed between repeat peptide stimulated cells and the unstimulated cells. We found that anergy related signaling pathway was neither up- or down-regulated in the repeat peptide stimulated cells (Supplementary Fig 6E). In this comparison, the T cell exhaustion signaling pathway was again the most significant upregulated pathway. Taken together, these findings argue that anergy is not a major feature of our *in vitro* exhausted CTL.

DISCUSSION

Persistent antigen stimulation is a key driver of T cell exhaustion (37, 38). In this study, we show that repeat stimulation with peptide is sufficient to induce T cell exhaustion *in vitro.* Repeat peptide stimulation rapidly, within 5 days, induced all characteristics of CTL exhaustion including loss of cytokine production and polyfunctionality, the expression of multiple inhibitory receptors, reduced expansion capacity and competitive fitness, transcription factor and gene expression patterns all compatible with *in vivo* generated exhausted CTL (10, 11). This phenotype was largely preserved when cells were rested for several days. Our *in vitro* repeat peptide stimulation culture conditions include cytokines IL-7 and IL-15 which are known to have anti-apoptotic potential and ensure T cells do not undergo activation induced cell death (AICD) (52). In particular, IL-15 has been shown to promote the survival of exhausted HIV-specific CD8+ T cells by upregulating Bcl-2 and Bcl-xL (7, 53). Its inclusion in the repeat peptide stimulations, we argue, promotes the survival of these cells as they become gradually more exhausted while avoiding the increased apoptosis mediated by IL-2, a known promotor of T cell AICD (54).

The *in vitro* exhausted CTL we generate have many of the molecular features of exhaustion. In particular transcription factors that associate and orchestrate T cell exhaustion are clearly modulated in the same manner as seen with *in vivo* induced CTL exhaustion of chronic infection models like LCMV clone 13 infections and cancer (11, 31). The transcription factor TOX is known to be increased in *in vivo* exhausted CTL (23, 24, 26), while TCF1 is downregulated in exhausted cells and its presence is associated with precursor exhausted CTL that retain some proliferative capacity (11, 40). All *in vitro* exhausted CTL we generate have high protein expression for TOX and *Tox* mRNA is upregulated by 2.6-fold. Furthermore, *in vitro* exhausted CTL have downregulated TCF1 and at the mRNA level its gene *Tcf7* is decreased by 6.6-fold. A recent report indicated that *Prdm1* and *c-maf* transcription factors control a co-inhibitory module in T cells (55). Although we found that *Prdm1* expression was upregulated by 4.5-fold in our *in vitro* exhausted CTL, we did not find any changes in *c-maf* transcription factor. Activated protein C receptor (PROCR) and podoplanin (PDPN), recently reported to be co-inhibitory receptors (55), were not upregulated in our *in vitro* exhausted cells. This may not be surprising as these markers were only found in tumor infiltrating T cells and IL-27 may be mediating this profile (55). Exhausted T cells from chronic infection however are not affected by IL-27 signaling (56) and thus PROCR and PDPN expression may depend on the tumor microenvironment and not characterize all exhausted CTL. Our *in vitro* exhaustion method generates exhausted T cells which may more closely reflect the core features of exhaustion. Our method is independent of environmental factors that may occur in the *in vivo* setting of chronic infection or tumor microenvironment. Such factors can obscure direct exhaustion related transcriptional changes from those induced by bystander effects such as high viral loads.

Our *in vitro* exhaustion method utilizes peptide-stimulated purified CD8+ T cells and therefore one could question whether TCR stimulation in the absence of costimulation induces anergy in our T cells. Although, T cell anergy and exhaustion may share some traits and potential signaling pathways of unresponsiveness to restimulation, the mechanisms of their induction is very different. Analyzing gene expression and signaling pathways we found no evidence for anergy in our *in vitro* exhausted CTL. Similar data were obtained with *in vivo* exhausted CTL. The reason we do not induce anergy in our cultures is because they are carried out in the presence of IL-7 and IL-15. It is well established that γc chain cytokines such as IL-2 and possibly IL-7 and IL-15 can override the induction of anergy when CD8+ T cells are stimulated in the absence of CD28 costimulation (47, 49-51). Cells were exhausted even when cultured with IL-2, albeit IL-2 in these cultures increases cell death significantly. Interestingly, our *in vitro* exhausted CTL express high levels of mRNA and surface CD80 and this may also contribute to preventing anergy. This is not surprising as activated mouse T cells can express CD80 both *in vitro* but also *in vivo* in autoimmune mice (57, 58). Thus *in*

vitro exhausted CTL do not present key anergy traits although one cannot exclude that some downstream signaling or inhibitory pathways are shared between exhaustion and anergy.

Previous studies have reported that there are subsets of exhausted CTLs, one known as "progenitor exhausted" cells which express TCF1 and TOX, corresponding to cells that retain the capacity to expand with checkpoint blockade therapy and the other subpopulation known as the "terminally exhausted" cells which are TCF1 low and cannot respond to checkpoint blockade (26, 40, 41, 59). In our *in vitro* exhausted cell cultures, we observed both subsets; a subset of cells that express low levels of TCF1 and high levels of TOX and GzmB, which is in line with the characteristics of "terminally exhausted" T cells and a subset that is TCF1+TOX+ corresponding to the "progenitor exhausted" cells. All cells are T-bet^{hi} PD1+, thus our TOX+ cells may retain some "progenitor exhausted" characteristics as T-bet^{hi} cells are reported to retain proliferative capacity in the exhaustion setting (42). Thus, these two subsets exist in our exhausted cultures and this allows us to perform future in depth characterization of these cells and establish their relationship and the factors that control them. CXCR5+ T cells were described as "less exhausted" and responders to check-point blockade in chronic viral infection (21, 59). Although "progenitor exhausted" TCF1+TOX+ are present in our repeat peptide stimulated T cells, we could not detect any CXCR5 expression. CXCR5+ exhausted CD8+ T cells are found in lymphoid organs but not in peripheral blood or other infected organs of LCMV cl13 infected animals (21), and therefore our CXCR5- *in vitro* exhausted cells may resemble more the later than those found in lymphoid organs.

TCF1 downregulation in exhausted cells was reported to be accompanied by reduced chromatin accessibility (24, 25). Our findings suggest that DNA methylation also contributes to reduced TCF1 expression as both the *Tcf7* promotor was found to be hyper-methylated and the inhibition of DNA methyltransferases during the last 3 days of culture results in the retention of TCF1 expression in our exhausted CTL. Whether these TCF1 high cells generated by DNMT inhibitor treatment retain all the characteristics of the "progenitor exhausted" cell subset remains to be determined. If true it may indicate that such inhibitors can be combined with checkpoint inhibition therapy as such TCF1+ cells are the major responders to therapy.

By studying these *in vitro* exhausted CTLs, the accumulation of inhibitory receptor expression on exhausted cells can be better understood. PD-1, Tim-3 and TIGIT are rapidly expressed on the early exhausted cells, while the other receptors, like CD160 and CD244, were induced more slowly by repeat antigen stimulation. Because of the promising effects of the checkpoint blockade therapy, such as anti-PD1/PD-L1, on the treatment of human cancer(60), there is considerable interest to revive the function of exhausted T cells by co-blockade of multiple inhibitory receptors simultaneously. Our *in vitro* method, which induces multiple inhibitory receptors in just a few days, can serve as a feasible tool to test blocking approaches before performing *in vivo* animal experiments. For example, TIGIT blockade can be tested, because these cells not only upregulated TIGIT, but also downregulated its competitor of the

same ligand, CD266, on their surface. Moreover, the underlying mechanisms of checkpoint blockade on reversal of T cell exhaustion need to be further investigated as do the pathways that restrain these cells from developing into terminally exhausted cells. Clearly the balance of these exhausted cells can be modulated with large effects on the numbers and potentially the function of these cells. As we recently have demonstrated, by overexpressing a single miRNA, namely miR-155, we can ameliorate the attrition of virus-specific CTL during chronic infection and *in vivo* increase their numbers by 2 logs (61). Our *in vitro* system of T cell exhaustion can be used to screen the overexpression of genes with retroviruses or their inhibition or deletion using CRISPR-Cas9 to discover new targets which modulate T cell exhaustion.

The method we describe allows for the rapid, within 5 days, generation of large numbers of fully exhausted CTL and can therefore be used for medium to high throughput screening of compounds, reagents or gene modifications that can prevent, ameliorate, reverse or accelerate T cell exhaustion. In particular, the extreme loss of IL-2 and TNFα production in our repeat peptide stimulated cells allows for the first time a truly medium to high throughput approach for drug screening since changes in IL-2 or TNFα production can be easily determined in the supernatants.

In conclusion, we have established a rapid *in vitro* system of CD8+ T cell exhaustion. These exhausted CTL exhibit all the known molecular and functional characteristics of exhaustion yet can be induced in large numbers within 5 days as opposed to the small numbers generated after 30 days of chronic infection of *in vivo* mouse models of exhaustion. This *in vitro* method can not only be used as a screening system to prevent/revert CD8+ T cell exhaustion, thereby identifying new therapies, but also for research aiming at revealing mechanisms of CD8+ T cell exhaustion. Using this *in vitro* method we show that TCF1 silencing during exhaustion is in part controlled by DNA methylation. Overall, this *in vitro* method makes the future study of CTL exhaustion more feasible and reduces the need for *in vivo* studies.

MATERIALS AND METHODS

Mice

OT-I CD45.1+ mice on the C57BL6/J background were generated by backcrossing C57BL/6 Tg (TcraTcrb)1100Mjb/J (OT-I) with B6.SJL-Ptprca Pepcb/BoyJ (CD45.1+) mice (both from Charles River France, a registered vendor of The Jackson Laboratories C57BL/6 mice). C57BL/6J mice and OT-I mice were housed in a certified barrier facility at Erasmus University Medical Center.

Ethics statement

Animal work was performed under Project Proposal (AVD101002015179) by the animal welfare body (AWB) of the Instantie voor Dierenwelzijn (IvD). All animal experiments were conducted in compliance with the Netherlands' government laws of the Centrale Commissie Dierproeven (CCD).

Repeated antigen stimulation *in vitro*

CD8+ T cells were purified from spleens of OT-I mice by negative selection with magnetic beads (EasySep, Stemcell Technologies). After purification, cells were 97.7±0.5% CD8+ T cell and contained 0.11±0.04% CD11b+ CD11c- monocytes and 0.09±0.05% CD11b+ CD11c+ dendritic cells. In each well of a 24-well plate, $5x10^5$ of the purified CD8+ T cells/ml were cultured in complete media (RPMI 1640, 10% FBS (Gibco), 1% 2mM L-glutamine (Life Technologies), 1% HEPES (Life Technologies), 1% 100nM Sodium Pyruvate (Life Technologies), 1% non-essential amino acides (Life Technologies), 100U/ml penicillin (Gibco) and 100µg/ml Streptomycin-sulfate (Gibco), 0.05mM Betamercaptoethanol (Sigma)) with IL-15 (5ng/ml, Peprotech , Cat 210-15) and IL-7 (5ng/ml, Peprotech, Cat 210-07) with or without 10ng/ml $OVA_{(257-264)}$ peptide (Anaspec Cat AS-60193).

For single peptide stimulation, cells were cultured in the presence of $OVA_{(257-264)}$ peptide for 48 hours. The peptide was then removed by washing the cells two times with complete media. For the remaining 3 days, the cells were cultured in the complete media with cytokines. For repeat peptide stimulation, 10ng/ml $OVA_{(257-264)}$ peptide was added daily for five days. The cells were washed also on day 2 to allow for comparable culture conditions. Unstimulated control cells were cultured in media with cytokines but without adding peptide. Cells from all three conditions were checked daily, and when the cells were confluent, they were split and cultured with fresh complete media containing cytokines. After day 5, some of the cell were washed two times with complete media and maintained in the media only with cytokines for another three days. In some experiments cell cultures were treated on day 2 with 20µM DNA methyltransferase (DNMT) inhibitor SGI-1027 (Tocris, Bio-techne) that targets DNA methyltransferases DNMT3B, DNMT3A and DNMT1.

On day five, cells were harvested and counted using an automated counting system (Countess, Life Technologies). Cells were stained with DAPI Viability dye (Beckman Coulter, Cat B30437) and Acridine Orange (Biotium, Cat 40039) to distinguish live and dead cells.

In vitro killing assay

AE17 cells were maintained in RPMI 1640 supplemented with 10% FBS (Gibco), 100 units/mL Penicillin/Streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), 0.05 mM 2-mercaptoethanol (Sigma), and were cultured at 37°C in 5% CO2. AE17 cells were pulsed with 1 µg/ml $OVA_{(257-264)}$ Peptide (Anaspec Cat AS-60193) for 1 hour and then the cells

were washed thoroughly before they were labelled with the CellTrace™ Far Red fluorescent dye (ThermoFisher Scientific Cat C34564/15396613). Un-pulsed cells were not labeled. A 1:1 mix of peptide pulsed and un-pulsed AE17 cells (10^5 each) were mixed and different amounts of T cells (Effector: Target ratios: 3:1, 1:1, 0.3:1) were added. The cells were harvested after 16 hours, the ratio of labeled and unlabeled tumor cell were detected by flow cytometry.

Flow cytometry

To investigate phenotypic and functional changes, cells were stained with monoclonal antibodies and analyzed using flow cytometry. The following fluorochrome-conjugated monoclonal antibody combinations against surface and intracellular antigens were used; Inhibitory receptors and ligands: anti-CD8-eFluor 450 (53-6.7, eBioscience), anti-Lag3-APC (C9B7W, BD Biosciences), anti-PD-1-APC-Cy7 (19F.1A12, Biolegend), anti-CD244-PE (2B4, BD Biosciences; eBio244F4, eBioscience), anti-Tim3-PE-Cy7 (RMT3-23, Invitrogen), anti-CD160-PE-CF594 (CNX46-3, BD Biosciences), anti-TIGIT-FITC (GIGD7, eBioscience); Activation and differentiation: anti-CD44-BV785 (IM7, BD Biosciences) , anti-CD25-APC-Cy7 (PC61, BD Biosciences); Cytokines and effector molecules: anti-IFN-γ-APC (XMG1.2, eBioscience), anti-TNF-α-AF488 (MP6-XT22, eBioscience), anti-IL-2-PE (JES6-5H4, eBioscience), anti-GranzymB-PE-Cy7 (NGZB, eBioscience); Intracellular expression of transcription factors anti-Tbet-PE-Cy7 (4B10, Biolegend), anti-TCF1-APC (C63D9, Cell Signaling), anti-EOMES-PE- eFluor F610 (Dan11mag, eBioscience), anti-Tox-PE (TXRX10, eBioscience). To exclude apoptotic and dead cells, Annexin V conjugated with APC, Cy5.5 or PerCP-Cy5.5 (BD Biosciences) was included in all the stains and 2.5 mM $CaCl_2$ was added to all solutions.

On day 5, cells were harvested and immediately stained for surface and intracellular antigens. For surface staining, cells were washed with FACS wash (HBSS containing 3% FBS and 0.02% sodium azide) and incubated with 20µL mix of the pre-determined optimal concentrations of the fluorochrome-conjugated monoclonal antibodies at 4°C in the dark for 20 minutes. The cells were then washed once with FACS wash and fixed with 1% PFA. For the transcription factor staining, cells were first stained for surface antigens as described above. Following the washing step, cells were fixed with FoxP3 Fixation Buffer (005523, eBioscience) for 60 minutes in the dark at 4°C and then washed with Perm/Wash buffer (008333, eBioscience) and stained with a mix of antibodies against transcription factors for 1 hour at 4°C in the dark. Cells were then washed twice with Perm/Wash buffer and fixed with 1% PFA. Appropriate isotype controls were included for staining of transcription factors.

To analyze cytokine production, cells were re-stimulated with the 10µg/ml $OVA_{(257-264)}$ SIINFEKL peptide for 6 hours at 37°C, 5% CO_2 in the presence of GolgiPlug (BD Biosciences) and anti-CD107a-APC-Cy7 antibodies (ID4B, Biolegend). Cells were then stained with surface marker antibodies as described above. After washing with FACS wash, cells were fixed with IC Fixation Buffer (88-8824, eBioscience) at 4°C overnight, washed with Perm/Wash buffer

and stained for intracellular cytokines for 45 min in the dark at 4°C. After staining, cells were washed twice with Perm/Wash buffer and fixed with 1% PFA.

Cells were measured on a LSRFortessa (BD Biosciences) using application settings and at least 200,000 events were collected. Data was then analyzed with FlowJo software (Version 9.9.4, Treestar, Ashland, OR, USA).

In vivo influenza model

For influenza virus infection studies, live CD45.1+ OT-I cells were sorted from *in vitro* cultures on a FACSAria III (BD Biosciences) using fluorochrome-conjugated Annexin V and anti-CD8 antibodies and 10,000 cells were intravenously transferred into 8–12 weeks old CD45.2+ C57BL/6J wild-type recipient mice. Naïve CTL were also freshly isolated from spleen of an OT-I mouse and adoptively transferred. Three hours later, mice were anesthetized with 2.5% isoflurane gas and infected intranasally with influenza virus strain A/WSN/33-expressing OVA (WSN-OVA, a gift from Dr. D. Topham, University of Rochester Medical Center). Body weight was measured daily to track the influenza infection.

Ten days post infection, the lung, spleen and mediastinal lymph nodes were harvested and single cell suspensions were obtained after processing the tissues. As described previously(62), lungs were digested for 2 h at 37°C with 3.0 mg/ml collagenase A and 0.15 µg/ml DNase I (Roche) in RPMI 1640 containing 5% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin. Digested lungs were then filtered through a 40-µm cell strainer (Falcon) and washed in the same media as above. PE-conjugated tetramers of H-2K^{b} major histocompatibility complex class I loaded with $OVA_{(257–264)}$ were used to identified the antigen-specific CTLs in the lungs(63).

RNA sequencing

To compare the gene expression between the different culture conditions, RNA sequencing was performed. On day 5, 1x10^{6} live CD8+ T cells were sorted from the three different culture conditions and immediately lysed with TRIzol LS reagent (Life Technologies) and stored at -80°C. RNA was extracted according to manufacturer's instructions and a bioanalyzer (Agilent) was used to determine the integrity of the extracted RNA. Barcoded sequencing libraries were generated using the KAPA RNA HyperPrep kit (Roche Diagnostics) with RiboErase (HMR) rRNA depletion. Library quality was assessed with the bioanalyzer and KAPA qPCR was performed for quantification before cluster generation and 100-bp paired-end sequencing on a Hiseq2500 machine (Illumina). Data sets are deposited in the Gene Expression Omnibus (GEO) database repository under accession numbers GSE 150120.

Analysis of differential transcript abundance, normalization of read counts by gene size, and downstream analyses

Four independent biological replicates were analyzed for each condition. The quality of the sequencing was verified using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The demultiplexed fastq files were aligned using STAR software (v.2.5.3e) (64) with default settings and *mus musculus* GRCm38 as alignment reference. Then bam files, generated during alignment, were annotated using FeatureCount software (v1.6.1)(65) to obtain the annotated files (count files). The annotation reference was gencode.vM15.gtf.

Count data was preprocessed to remove low expressed genes. Then, rlog transformation was applied to the clean counts for visualization and comparison purposes which include correlation and clustering analysis generating the heatmaps and PCA plots during the process. For differential expression analysis, DESeq2 (DESeq2 R package, v1.22.2)(66) was used directly in the clean count data.

The list of differentially expressed genes was used to perform Ingenuity Pathway Analysis (IPA, Qiagen, USA version 01-12) to further discern which pathways are involved in the CD8+ T cell exhaustion process. Pathway enrichment P-values (Fisher's exact test) and activation Z-scores were calculated by IPA and used to rank the significant pathways.

The same list of significantly differentiated genes was used for enrichment analysis (GSEA Desktop Application, v2.2.1). The CD8+ T cell exhaustion gene-sets were downloaded from GMO datasets (gene set GSE87646) based on the publication of Bengsch B, et al (2). Enrichment analysis in CTL exhaustion genes was determined for the upregulated and downregulated genes separately. Normalized Enrichment Scores (NES) values were used to determine whether an expression gene-set was enriched or not in CTL exhaustion genes. For anergy GSEA analysis, gene set GSE5960 (46) and gene set GSE41867 (12) were used.

DNA methylation profiling detection

DNA methylation profiling was done as previously described by the MeD-seq method(44). For MeD-seq sample preparation LpnPI (New England Biolabs) digestions were carried out on DNA samples according to manufacturer's protocol. Reactions contained 50 ng and digestion took place overnight in the absence of enzyme activators. Digests of genomic DNA with LpnPI resulted in snippets of 32 bp around the fully-methylated recognition site that contains CpG. The DNA concentration was determined by the Quant-iT™ High-Sensitivity assay (Life Technologies) and 50 ng dsDNA was prepared using the ThruPlex DNA-seq 96D kit (Takara). Twenty microliters of amplified end product were purified on a Pippin HT system with 3% agarose gel cassettes (Sage Science). Stem-loop adapters were blunt end ligated to repaired input DNA and amplified (4 +10 cycles) to include dual indexed barcodes using a high fidelity polymerase to yield an indexed Illumina NGS library. Multiplexed samples were sequenced

on Illumina HiSeq2500 systems for single read of 50 base pairs according to manufacturer's instructions. Dual indexed samples were demultiplexed using bcl2fastq software (Illumina).

MeD-seq data processing was carried out using specifically created scripts in Python version 2.7.5. Raw fastq files were subjected to Illumina adaptor trimming and reads were filtered based on LpnPI restriction site occurrence between 13-17 bp from either 5' or 3' end of the read. Reads that passed the filter were mapped to mm10 using bowtie2.1.0. Multiple and unique mapped reads were used to assign read count scores to each individual LpnPI site in the mm10 genome. BAM files were generated using SAMtools for visualization. Gene and CpG island annotations were downloaded from UCSC (MM10). Genome wide individual LpnPI site scores were used to generate read count scores for the following annotated regions: transcription start site (TSS) (1 kb before and 1 kb after), CpG islands and genebody (1 kb after TSS till TES).

MeD-seq data analysis was carried out in Python 2.7.5. DMR detection was performed between two datasets containing the regions of interest (TSS, genebody or CpG islands) using the Chi-Squared test on read counts. Significance was called by either Bonferroni or FDR using the Benjamini-Hochberg procedure. Differently methylated regions were used for unsupervised hierarchical clustering, the Z-score of the read counts was used for normalization and is also shown in the heatmaps. In addition, a genome wide sliding window was used to detect sequentially differentially methylated LpnPI sites. Statistical significance was called between LpnPI sites in predetermined groups using the Chi-squared test. Neighbouring significantly called LpnPI sites were binned and reported, DMR threshold was set at a minimum of ten LpnPI sites, a minimum size of 100 bp and either a twofold or fivefold change in read counts. Overlap of genome wide detected DMRs was reported for TSS, CpG island and gene body regions.

Statistical analysis

Statistical analyses were performed using Prism software (GraphPad Prism5 for Windows, Version 5.04). The normality of data distribution was assessed using the Shapiro-Wilk normality test. Homogeneity of variance was tested with Bartlett's test. When data were normally distributed and group variances were equal, an ANOVA with Tukey's Multiple Comparison Test was performed. When data were normally distributed but group variances were unequal, a Student's t test with Welch's correction was performed. If data were not normally distributed a Wilcoxon signed rank test or a Mann–Whitney U test was performed. P values equal or lower than 0.05 were considered statistically significant with the numbers of stars in the figures indicating the p value: * = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$.

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AUTHOR CONTRIBUTIONS

M.Z., S.E., Y.M.M., J.G., P.D.K. contributed to study design. M.Z., R.B., C.H.K., M.V.M., I.B.H., E.M.B., R.M.H. and Y.M.M. performed experiments, J.L.H., L.G.L., R.B., J.B., W.V.I. and J.G. generated and analyzed the sequencing data and DNA methylation sequence data. M.Z., C.H.K., R.G.B., Y.M.M., C.J.S., and P.D.K. contributed on writing the manuscript.

COMPETING INTERESTS

The authors declare no conflict of interest or financial interests except for R.B., J.B., W.V.I. and J.G., who report being shareholder in Methylomics B.V., a commercial company that applies MeD-seq to develop methylation markers for cancer staging.

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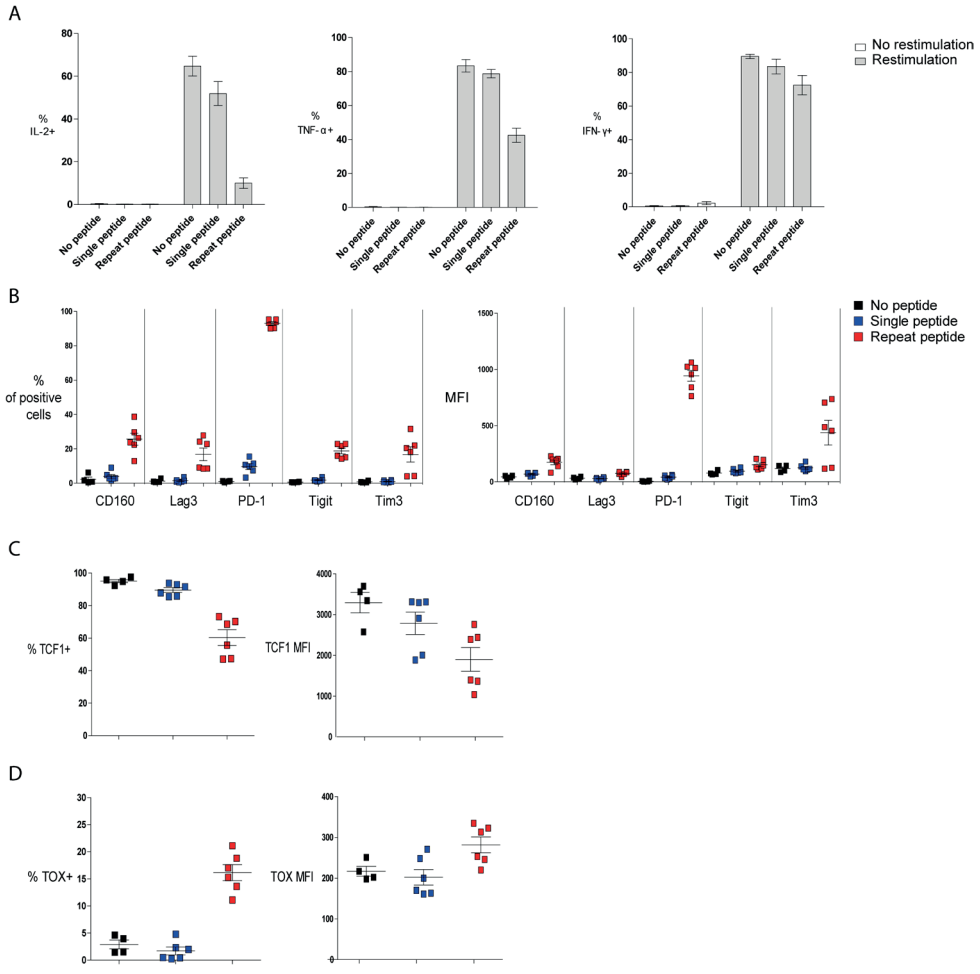
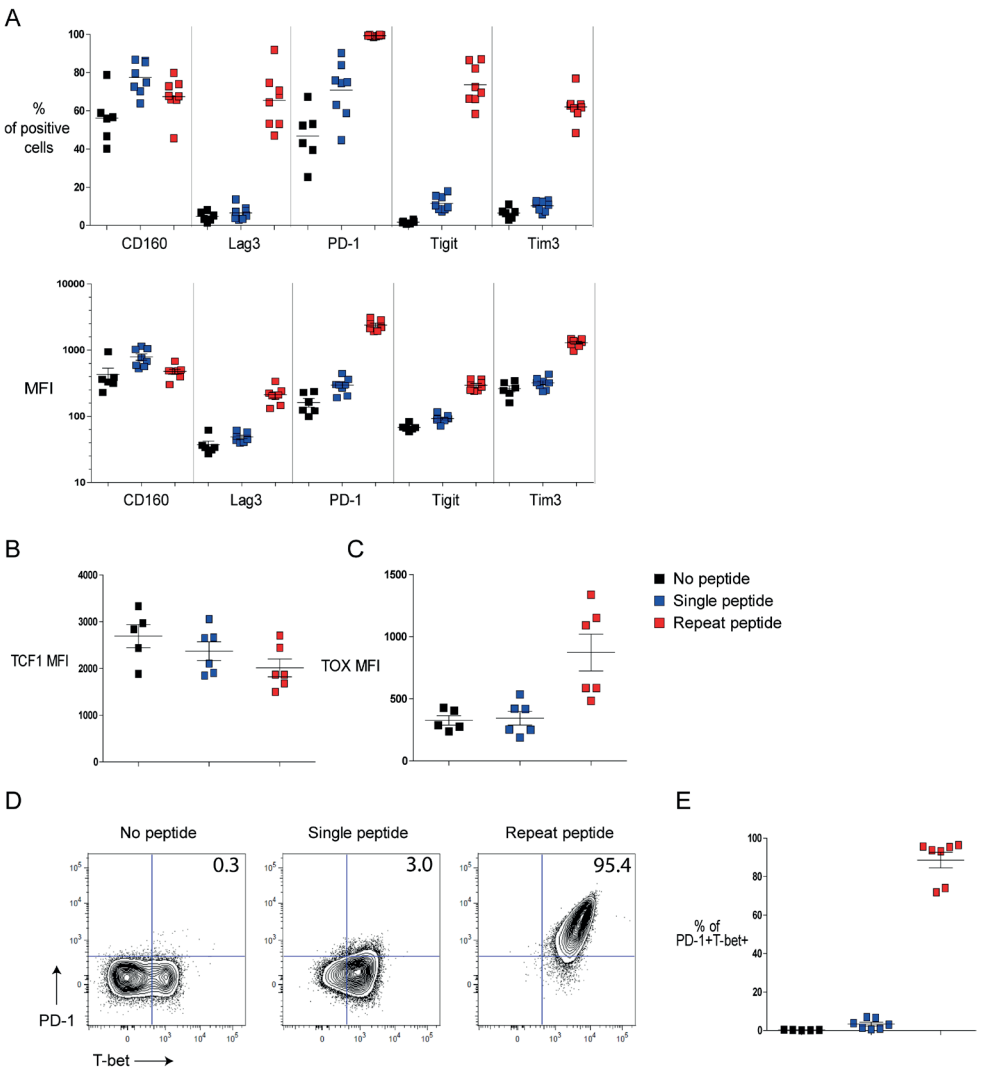
SUPPLEMENTAL DATA



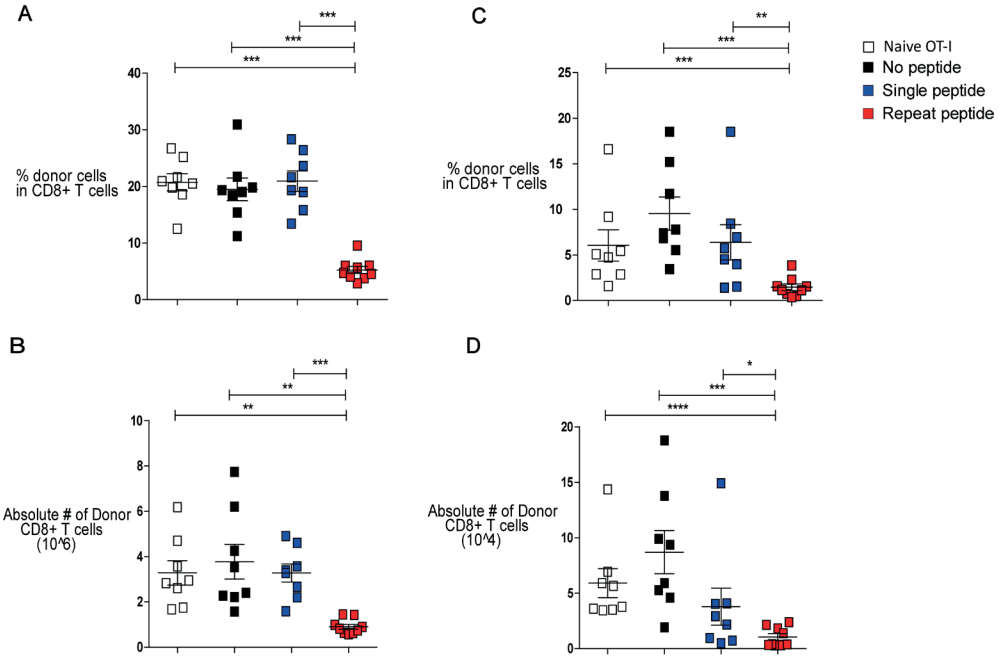
Supplementary Figure 1. Exhaustion related features are retained after 3 days resting.

Cells were harvested on day 5, peptide was washed away and the cells were rested for 3 days without peptide. On day 8, cytokine production after restimulation is shown (**A**). The expression of inhibitor receptors (**B**) and transcription factors (**C** and **D**) are also shown on day 8. Each symbol represents one animal. Line depicts mean ± SE.



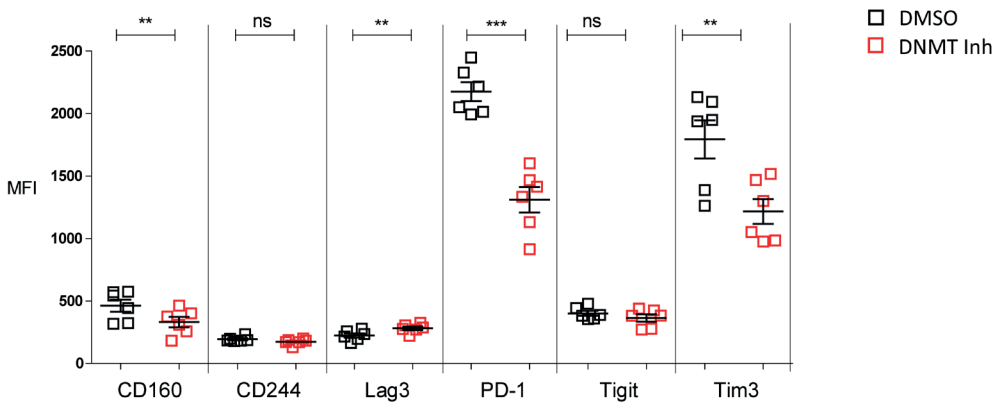
Supplementary Figure 2. Repeat-peptide stimulated cells maintain inhibitory receptor and transcription factor differences after 6 hours of restimulation.

After extra 6 hours of stimulation with OVA peptide (10µg/ml) on day 5, the percentage and MFI of inhibitory receptors (**A**) and the MFI of TCF1 (**B**) and TOX (**C**) are depicted. Representative FACS plots (**D**) and pooled data (**E**) of the frequency of PD-1 and T-bet co-expression on day 5 shown. Line depicts mean ± SE. Each symbol represents one animal. Data from 5-6 experiments.



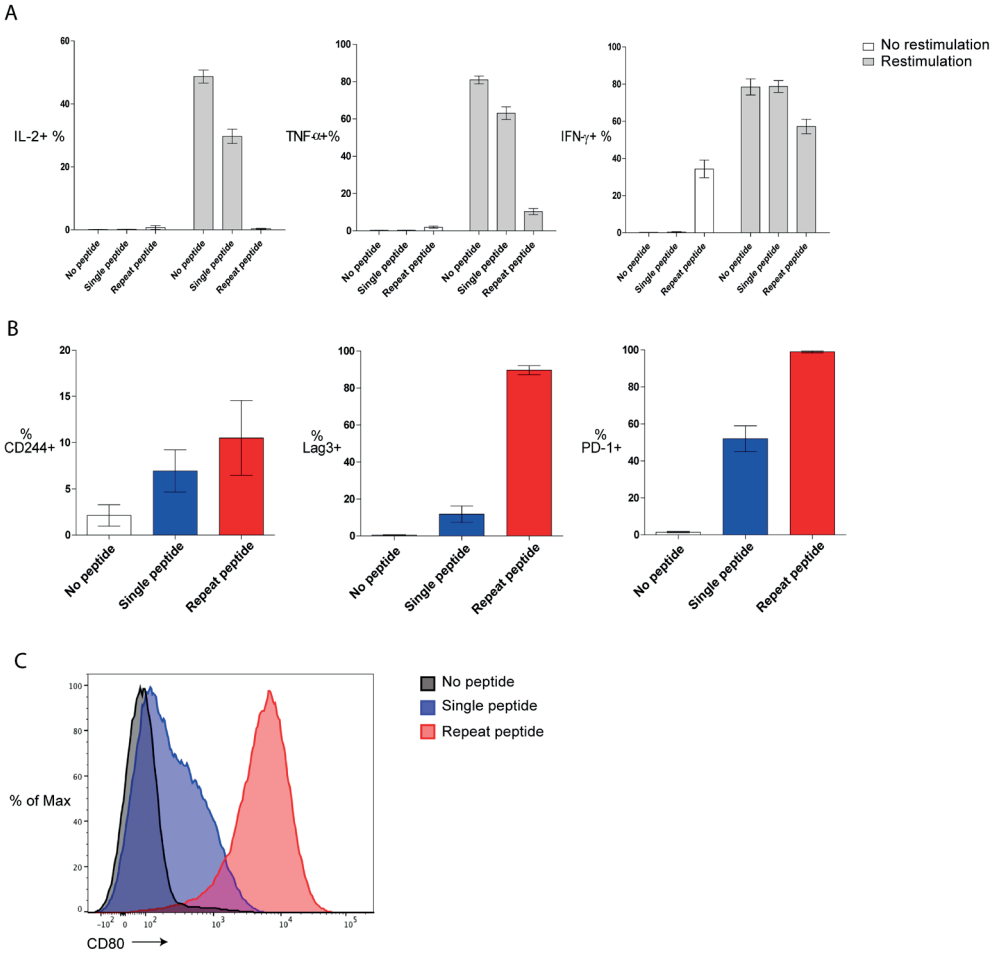
Supplementary Figure 3. Repeat peptide stimulated cells expanded less in mediastinal lymph nodes and spleens *in vivo.*

OT-I CD8+ T cells were cultured without peptide stimulation (no peptide), one-time stimulation (single peptide) or daily stimulation (repeat peptide) and sorted on day 5. Live CD8+ T cells were adoptively transferred into wild type mice which were then infected with the $Ova_{(257-264)}$-expressing influenza virus WSN-OVA. Freshly isolated OT-I CD8+ T cells from a naïve mouse were also transferred (naïve OT-I). Mediastinal lymph nodes (MLN) and spleens were harvested on day 10 post infection. Frequency of donor OT-I CD8+ T cells within spleen (**A**) and MLN (**C**) in total CD8+ T cells and the absolute number of donor cells in spleen (**B**) and MLN (**D**) are presented. Each symbol represents one animal (n=8-9) from n=3 independent experiments. Line depicts mean ± SE. To determine significant differences between the different animal groups, Mann-Whitney U test was used except for data in (**A**) (ANOVA with Tukey's post hoc test). *P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.



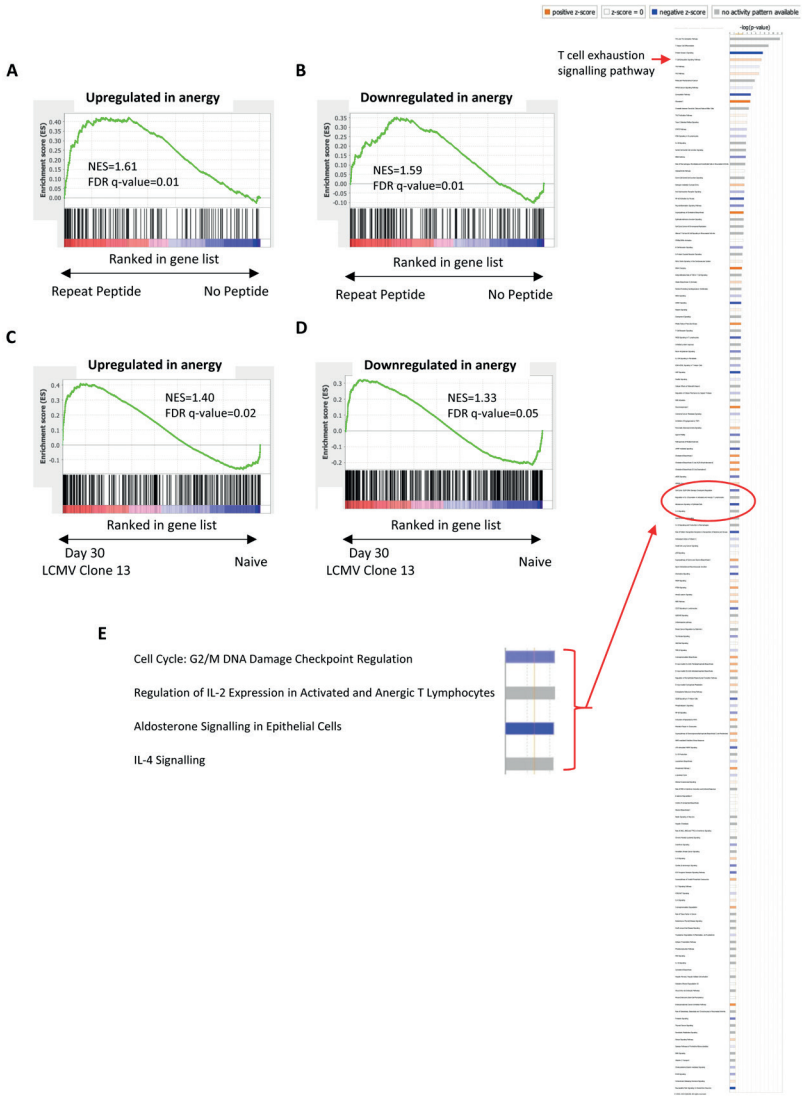
Supplementary Figure 4. DNMT inhibitor decreases expression of inhibitor receptors on the repeat peptide stimulated cells.

Pooled data showing day 5 inhibitor receptor MFI on repeat stimulated cells in the presence or absence of 20μM DNMT inhibitor. Inhibitor was added during the last 3 days of culture. Data are from n=6 animals, performed in 3 independent experiments. Line depicts mean ± SE. To determine significant differences between the different treatment, paired t-test was used *P<0.05, ** P<0.01, ***P<0.001.



Supplementary Figure 5. Exposure to IL-2 does not alter the exhaustion phenotypes in repeat peptide stimulated cells and CD80 is upregulated on repeat stimulated cells.

CD8+ T cell exhaustion was induced in the presence of IL-2 (20U/ml) and IL-7/IL-15 (5ng/ml each). Cytokine production after OVA peptide restimulation for 6 hours (**A**) and inhibitory receptor expression on day 5 (**B**) are shown. n=8 animals from 5-6 independent experiments depicted. Representative histogram of CD80 expression on the cells is shown in (**C**). One of two independent experiments shown (n=3).



Supplementary Figure 6. Anergy gene sets and pathways do not characterize repeat peptide exhausted T cells.

Both upregulated and downregulated genes in anergic T cells are enriched in exhausted T cells. **A.** GSEA of genes upregulated and **B.** downregulated in anergic T cells (gene set GSE 5960) are both enriched in differentially expressed repeat peptide stimulated cells (repeat peptide versus no peptide cell). **C.** GSEA of genes upregulated and **D.** downregulated in anergic T cells (gene set GSE 5960) are both enriched in differentially expressed Day 30 gp33-specific CTL from LCMV clone 13 infected animals (Day 30 versus gp33-specific CTL naïve CD8+ T cells; data from GSE41867). **E.** In repeat peptide stimulated cells the anergy related pathway in IPA analysis was neither upregulated not downregulated. IPA analysis was performed on the differentially expressed genes in repeat stimulated cells (compared to no peptide). The "Regulation of IL-2 expression in activated and anergic T lymphocytes" pathway is showed enlarged and the overall positioning in the global IPA pathway analysis is indicated by the arrow and red circle. Significantly upregulated pathways (orange) and downregulated pathways (blue), no activity pattern available (grey) are depicted. Bar graphs depict q values (hypergeometric test) presented as –Log10.

Chapter 4

MicroRNA-139 expression is dispensable for the generation of CD8+ T cell responses

Jennifer L. Hope[†‡$], Manzhi Zhao[†$], Christopher J. Stairiker[†‡], Alison J. Carey[‡§], Yvonne M. Mueller[†‡], Marjan van Meurs[†], Inge Brouwers-Haspels[†], Alex Maas[¶], Hans de Looper[| |], Paolo M. Fortina[††], Isidore Rigoutsos[‡‡], Stefan J. Erkeland[†], and Peter D. Katsikis[†‡]

† Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands
‡ Department of Microbiology & Immunology, Drexel University College of Medicine, Philadelphia, PA, USA
§ Department of Pediatrics, Drexel University College of Medicine, Philadelphia, PA, USA
¶ Department of Cell Biology, Erasmus University Medical Center, Rotterdam, The Netherlands
| |Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands
†† Department of Cancer Biology, Thomas Jefferson University, Philadelphia, PA, USA
‡‡ Computational Medicine Center, Sidney Kimmel Medical Center, Thomas Jefferson University, Philadelphia, PA, USA

$ Shared authors

Under revision

ABSTRACT

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs that are important post-transcriptional regulators with clear roles in the development of the immune system and immune responses. Using miRNA microarray profiling, we characterized the expression profile of naïve and *in vivo*-generated murine effector anti-viral $CD8^+$ T cells. We observed that out of 362 measurable mature miRNA, 120 were differentially expressed by at least 2-fold in influenza-specific effector cytotoxic $CD8^+$ T lymphocytes (CTL) compared to naïve $CD8^+$ T cells. One miRNA found to be highly downregulated on both strands in effector CTL was microRNA-139 (miR-139). As previous studies have indicated a role for miR-139-mediated regulation of CTL effector responses, we hypothesized that deletion of miR-139 would enhance anti-viral CTL responses during influenza virus infection. We generated miR-$139^{-/-}$ mice or overexpressed miR-139 in T cells in order to assess the functional contribution of miR-139 expression in $CD8^+$ T cell responses. Our study demonstrates that the development of naïve T cells and generation or differentiation of effector or memory $CD8^+$ T cell responses to influenza virus infection are not impacted by miR-139 deficiency or overexpression. Using an *in vitro* model of T cell exhaustion, we confirmed that miR-139 expression similarly does not impact the development of T cell exhaustion. We conclude that despite significant downregulation of miR-139 following *in vivo* and *in vitro* activation, miR-139 expression is dispensable for CTL responses.

INTRODUCTION

MicroRNAs (miRNAs) are the most well-studied group of non-coding RNAs (1). MiRNAs are small (19-22 nucleotides) single-stranded non-coding RNAs that modulate gene expression by directly binding to target mRNA in a sequence specific manner (2), resulting in the inhibition of mRNA translation or RNA instability and thereby promoting the degradation of target mRNAs. We and others have previously demonstrated that modulating the expression of a single microRNA in immune cells can have a profound impact on the immune response to infection and tumors (3-7). It has also become clear that miRNA-directed gene regulation is heavily influenced by cell-extrinsic and -intrinsic factors such as the local inflammatory environment or the activation state of a given cell type (8, 9). It is therefore of great interest to explore miRNA expression and regulation of their specific targets in the context of different infection and tumor systems as the miRNAs involved and their effects may differ in these conditions.

A crucial component of the immune response to infection is the development of effector cytotoxic $CD8^+$ T cells (CTL), as these cells are ultimately responsible for the clearance of intracellularly infections and the control of tumors (10, 11). Influenza virus infection of an immunocompetent host, both in humans and mice, results in an acute viral infection localized in the lungs that is efficiently cleared by $CD8^+$ T cells followed by the establishment of long-lived influenza-specific memory $CD8^+$ T cells (12). Over the course of this response, $CD8^+$ T cells undergo significant changes in gene expression (13) that are, in part, mediated by miRNAs. MiRNAs are of critical importance to overall T cell development as well as the development of effective responses to pathogens. The importance of miRNAs in T cell development was first shown when Dicer, an enzyme critical for the maturation of miRNAs (14), was deleted in T cells (15). While the role of specific miRNAs has been studied previously including by our own group (3, 5), large scale changes in miRNA expression during the development of CTL responses to influenza virus infection remains unknown. Deletion of Dicer in CTL has demonstrated that aspects of the CTL response are affected by the absence of miRNAs (15). We hypothesized that *in vivo*-differentiated influenza virus-specific $CD8^+$ T cells would demonstrate miRNA expression patterns unique to the activation state of the cell. To address this, we performed miRNA microarray analysis on naïve or *in vivo*-generated effector influenza virus-specific $CD8^+$ T cells to determine the relative miRNA expression profiles during acute viral infection in a murine model. We determined the miRNA profile of an *in vivo* CTL response and identified 120 mature miRNAs with significant changes in expression that accompany effector CTL differentiation.

One miRNA that was greatly downregulated in effector CTL was microRNA-139 (miR-139). MiR-139 has previously been identified as a tumor suppressor such as in myeloid progenitors where miR-139 regulates the expression of EIF4G2 and TSPAN3, and during interstrand

cross-links (ICL-)-induced stress where miR-139-3p was suggested to repress the RNA binding protein HuR (16-19). In addition, miR-139 is downregulated in chronic myeloid leukemia (CML) where miR-139-5p serves as a regulator of proliferation and Brg1, an ATP-dependent helicase with a known role in BCR-ABL transformation in CML (20). Decreased expression of miR-139-5p has also been associated with several other types of cancers, including breast cancer (21) and non-small cell lung cancer (22). However, a role for miR-139 in immune cell responses remains poorly understood. One study by Trifari *et al*, was the first to identify a role for miR-139 (particularly, miR-139-3p) in $CD8^+$ T cell differentiation and function by assessing the impact of miR-139-overexpression in wild type or Dicer-deficient (and therefore lacking all mature miRNAs) $CD8^+$ T cells(23). Trifari *et al* specifically noted the regulation of the transcription factor Eomes by miR-139. Our miRNA profiling identified that miR-139 was significantly downregulated in effector CTL and upon *in vitro* activation. We therefore sought to test the direct contribution of miR-139 in effector and memory anti-influenza CTL responses.

Our study reports the miRNA changes that CTL undergo as they differentiate from naïve $CD8^+$ T cells into effector CTL in an *in vivo* viral infection. IPA analysis of our miRNA profiling suggests that effector CTL are characterized by a unique miRNA profile that regulates cytokine signaling and cellular migration. We specifically examine miR-139, which previously has been reported to affect effector CTL differentiation and using miR-$139^{-/-}$ mice demonstrate that *in vivo* miR-139 expression does not affect effector or memory CTL development during influenza virus infection. Using two models of influenza virus infection (PR8 and WSN-OVA), we evaluated the ability of miR-139 to regulate Eomes by both knocking out miR-139 and overexpressing it in $CD8^+$ T cells. Our studies conclude that *in vivo* miR-139 is not a major regulator of Eomes levels in $CD8^+$ T cells which is upregulated in exhausted $CD8^+$ T cells (24, 25); therefore, we sought to evaluate if miR-139 deficiency promoted the development of exhausted $CD8^+$ T cells with an *in vitro* model system of T cell exhaustion. By every parameter evaluated, we found no evidence for miR-139 deficiency affecting the development of T cell exhaustion. We therefore conclude that, despite significant downregulation upon activation of $CD8^+$ T cells, miR-139 expression is dispensable for the *in vivo* generation of effector and memory antigen-specific $CD8^+$ T cell responses.

MATERIALS AND METHODS

Animals and infections

C57BL/6 Tg(TcraTcrb)1100Mjb/J (OT-I) were backcrossed with B6.SJL-Ptprca Pepcb/BoyJ ($CD45.1^+$) mice (both from the Jackson Laboratory) to generate OT-I $CD45.1^+$ mice on the C57BL6/J background. MiR-139-deficient (miR-$139^{-/-}$) mice were generated on the

C57BL/6 background using CRISPR/Cas9 nickase (Cas9n) mRNA, and four sgRNAs containing sgRNA 5' plus strand: 5'–GTCAGTACAGTGGGAGTGCC–3', sgRNA 5' minus strand: 5'–GGTGATAGAGAGTGGGAAGG–3', sgRNA 3' plus strand: 5'–GTGAGTGAAAGGCACGTCTC–3', sgRNA 3' minus strand: 5'–GGTGTAAAGACCGGGATGTA– 3', to introduce four DNA nicks in the specific regions of the gene encoding miR-139 in C57BL/6J zygotes prior to transplantation into a BCBA (F1: C57BL/6J x CBA) female (Harlan) as published (Supplementary Fig. 3A) (26). F1 heterozygote offspring were crossed until complete homozygote knockouts were confirmed using PCR and sequence analysis of multiple tissues. MiR-139$^{-/-}$ mice were backcrossed with OT-I CD45.1 mice until complete miR-139-deficiency was confirmed using PCR analysis and OT-I CD45.1 expression was confirmed using PCR and flow cytometry analysis. C57BL/6J mice, OT-I CD45.1 mice, miR-139$^{-/-}$, and miR-139$^{-/-}$ OT-I mice (both on the C57BL/6J background) were kept in a barrier facility (certified by the Association for the Assessment and Accreditation of Laboratory Animal Care) at Drexel University College of Medicine, or in a barrier facility at Erasmus University Medical Center. This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee (IACUC) or the Instantie voor Dierenwelzijn (IvD). The protocols were approved by the IACUC or IvD. Mice 8-12 weeks old were anesthetized with 2.5% isoflurane gas and were infected intranasally with influenza virus strain A/Wilson Smith Neurotropic/33 expressing OVA$_{(257\text{-}264)}$ (WSN-OVA; a gift from D. Topham, University of Rochester Medical Center) or influenza virus strain A/Puerto Rico/8/1934 (PR8; H1N1; a kind gift from W. Gerhard, Wistar Institute).

MicroRNA microarray

Naïve (CD44^{-}CD62L^{+}) and day 9 post-influenza virus infection anti-viral donor (CD45.1^{+}) OT-I CD8^{+} T cells were sorted from the spleens or lungs of naïve or infected mice, respectively, and frozen at -80^{o}C in TRIReagent (Molecular Research Center, Cincinnati, OH). Total RNA was isolated from sorted cells and miRNA gene expression was assessed using an Affymetrix murine miRNA 4.0 gene chip array by the Thomas Jefferson University Cancer Genomics Laboratory (Thomas Jefferson University, Philadelphia, PA). Microarray probe intensity data (CEL) files were normalized and analyzed using Affymetrix Expression Console (ThermoFisher Scientific); miRNA annotation is from miRBASE version 21. Expression data were exported to GraphPad Prism 7 (Graph Pad, La Jolla, CA, USA). To assess miRNA expression in naïve and effector cells, expression was counted if the average detection p-value was $p \leq 0.05$. To calculate fold-change of miRNA expression in effector cells, naïve expression values were averaged, and individual effector expression value fold change was calculated against the naïve average; overall effector fold-change was calculated by averaging the individual effector fold-change. Fold-change p-value was calculated using GraphPad Prism 7's "multiple T tests" feature analyzing each miRNA independently and using the recommended two-stage step-up method of Benjamini, Krieger, and Yekutieli false discovery rate (FDR) method with

an FDR of 5%. MiRNAs were considered significantly upregulated or downregulated if effector cells had a fold-change ≥ 2 or ≤ -2 compared to naïve cells, a p-value ≤ 0.05, and a q-value ≤ 0.05.

Pathway analysis

Significantly upregulated and downregulated miRNAs in effector $CD8^+$ T cells and their fold-change values, miRNAs detected in naïve $CD8^+$ T cells only, and effector $CD8^+$ T cells only were uploaded into Ingenuity Pathway Analysis (IPA) (Qiagen). IPA miRNA Target Filter analysis paired with mRNA expression data from day 10 influenza virus infection effector OT-I $CD8^+$ T cells (27) identified canonical pathways predicted to be affected by the upregulated and downregulated miRNAs. Targets were limited to those expressed in day 10 CTL and targets predicted by pairing miRNA upregulation with mRNA downregulation or conversely miRNA downregulation with mRNA upregulation. In the figures, blue indicates downregulated genes; red indicates upregulated genes in effector versus naïve OT-I $CD8^+$ T cells.

Adoptive transfer experiments

Spleens from uninfected female $CD45.1^+$ OT-I or $CD45.1^+$ miR-$139^{-/-}$ OT-I transgenic mice were processed into a single-cell suspension by passing cells through a 40 µM cell strainer (BD Falcon, BD Biosciences) into RPMI 1640 containing 5% FBS + 1% penicillin/streptomycin and 1% L-glutamine. Red blood cells were lysed using ammonium-chloride-potassium (ACK) solution. Cells were counted using trypan blue staining and a hemocytometer and the frequency of $CD8^+$ T cells were determined by flow cytometry. OT-I splenocytes were resuspended at a concentration of 10^5 $CD8^+$ T cells/mL in sterile 0.9% NaCl. Equal numbers (10^4) of $CD45.1^+$ OT-I $CD8^+$ T cells were transferred intravenously (I.V.) into female $CD45.2^+$ wild-type C57BL/6J recipient mice through tail vein injection of 100 µL. At the time of transfer, all wild-type recipient host mice were between 8-12 weeks of age. All donor OT-I mice were between 8-12 weeks of age. Three hours later, the recipient mice were anesthetized using 2.5% isoflurane gas and infected intranasally with influenza virus WSN-OVA. Only female donor and recipient mice were used for these experiments.

Retroviral transductions

The murine miR-139-encoding region was cloned into the MSCV-IRES-Thy1.1 vector (provided by P. Marrack, University of Colorado). A scrambled control insert producing no functional microRNA was similarly cloned into the MSCV-IRES-Thy1.1 vector. Retroviruses were produced in the Platinum-E cell line (Cell Biolabs, San Diego, CA). Retroviral transduction of primary OT-I $CD8^+$ T cells was completed as previously described (3). Splenic $CD8^+$ T cells were isolated by negative selection with magnetic beads (EasySep; Stemcell Technologies) from uninfected OT-I $CD45.1^+$ female mice 8-10 weeks of age. The purity of $CD8^+$ T cells was

>90% as determined by flow cytometry. Isolated $CD8^+$ T cells were activated for 48 hours using solid-phase α-CD3 (0.25 µg/mL, clone: 17A2; eBioscience, San Diego, CA) and α-CD28 antibodies (5 µg/mL, clone: 37.51; eBioscience) in 10% RPMI medium with 20 U/mL recombinant human IL-2 (Roche, Switzerland), 5 ng/mL of recombinant murine IL-7 and 5 ng/mL recombinant murine IL-15 (both from PeproTech, Rocky Hill, NJ). Cells were collected and plated at a density of 3 x 10^6 cells per 2 mL in poly-D-lysine plates (ThermoFisher, Waltham, MA) coated with 20 µg/mL of Retronectin (Takara, Japan) and pre-loaded with retroviral supernatants. Cells were incubated for an additional 48 hours. Transduction efficiency was determined by expression of Thy1.1 (CD90.1). Transduced cells were sorted with a FACS Aria III sorter (BD Biosciences, San Jose, CA).

Activation of $CD8^+$ T cells

Splenic OT-I $CD45.1^+$ and OT-I miR-$139^{-/-}$ $CD45.1^+$ $CD8^+$ T cells were isolated by negative selection with magnetic beads (EasySep; Stemcell Technologies) from uninfected female mice 8-10 weeks of age. The purity of $CD8^+$ T cells was >90% as determined by flow cytometry. Isolated $CD8^+$ T cells were activated for 24 or 72 hours using solid-phase α-CD3 (0.25 µg/mL, clone: 17A2; eBioscience, San Diego, CA) and α-CD28 antibodies (5 µg/mL, clone: 37.51; eBioscience) in 10% RPMI medium with 20 U/mL recombinant human IL-2 (Roche, Switzerland), 5 ng/mL of recombinant murine IL-7 and 5 ng/mL recombinant murine IL-15 (both from PeproTech, Rocky Hill, NJ).

In vitro T cell exhaustion assay

Splenic OT-I $CD45.1^+$ and OT-I miR-$139^{-/-}$ $CD45.1^+$ $CD8^+$ T cells were isolated by negative selection with magnetic beads (EasySep) from uninfected female mice 8-10 weeks of age. Cells were cultured at 5 x 10^5 $CD8^+$ T cells either without cognate antigen stimulation (no stim), one time stimulation with 10 ng/mL of $OVA_{(257-264)}$ peptide for 48 hours (single stim), or with daily peptide stimulation with 10 ng/mL of $OVA_{(257-264)}$ peptide for 5 days (repeated stim). Cultures contained 5 ng/mL of recombinant murine IL-7 and 5 ng/mL recombinant murine IL-15 throughout culture. For all conditions, after 48 hours of culture, cells were washed twice (RPMI + 10% FBS medium containing 1% Pen/Strep, 1% L-glutamine, 1% Non-essential amino acids, 1% sodium pyruvate, and BME) and re-seeded. On day five, all conditions were harvested and cells were counted. $CD8^+$ T cells were stained as described below to measure inhibitory receptor and transcription factor expression, or stimulated with $OVA_{(257-264)}$ peptide in the presence of GolgiPlug for 6 hours followed by intracellular cytokine staining as described below to measure cytokine production.

Quantitative real-time PCR

For *ex vivo* measurement of miR-139-3p and miR-139-5p expression, total RNA, including miRNA, was extracted using the miRNeasy mini kit (Qiagen, Germantown, MD) as per the manufacturer's instructions. cDNA was synthesized from 100 ng of total RNA with the High Capacity cDNA Reverse Transcription Assay (ThermoFisher). The expression of miR-139 was measured by quantitative real-time PCR with the TaqMan mmu-miR-139-5p and mmu-miR-139-3p MicroRNA Assays (ThermoFisher). The expression of snoRNA-429 served as endogenous control. All assays were run using a 7900 HT Real-Time PCR System. Expression was evaluated by the comparative cycling threshold ($\Delta\Delta C_T$) method.

Flow cytometry

Flow cytometry staining was completed as previously described (5). In all stains, cells were pre-treated with anti-CD16/32 (Fc Block; 2.4G2; BioLegend, San Diego, CA) for 15 minutes before continuing with surface staining. For surface stains, cells were stained for 20 min on ice. Cells were stained with the following fluorochrome conjugated monoclonal antibodies: CD8a (clone 53-6.7), CD45.1 (clone A20), CD45.2 (clone 104), Thy1.1 (clone HIS51) (all from eBioscience), CD25 (clone PC61), CD69 (clone H1.2F3), CD44 (clone 1M7), CD62L (clone MEL-14) (all from BD Bioscience, San Jose, CA), KLRG1 (clone 2F1/KLRG1), IL-7R/CD127 (clone A7R34), PD-1 (clone 29F-IAI2) (all from BioLegend). Cells were also stained with Cy5.5-labeled Annexin V (BD Biosciences) and APC labeled-tetramers of H-2K^b major histocompatibility complex class I loaded with $OVA_{(257-264)}$. After staining, cells were washed 2 times with HBSS containing 3% FBS and 0.02% sodium azide and fixed with 1% paraformaldehyde solution (PFA). For Annexin V staining, all buffers contained 2.5 mM $CaCl_2$. For staining of intracellular cytokines, cells were stimulated with the indicated peptides for 6 hours at 37°C, 5% CO_2 in the presence of GolgiPlug (BD Biosciences) and monoclonal antibody against CD107a (clone ID4B) or isotype control. Cells were surface stained as above including fluorochrome-conjugated monoclonal antibody against CD107a (clone ID4B) or the appropriate isotype control (both Biolegend), then fixed overnight at 4°C with IC Fixation Buffer (eBioscience), washed using Perm/Wash buffer (eBioscience) and stained for intracellular cytokines for 45 minutes at 4°C. Fluorochrome-conjugated anti-IFNγ monoclonal antibody (clone XMG1.2), anti-TNFα monoclonal antibody (clone MP6-XT22) or the appropriate isotype controls (all from eBioscience) were used for intracellular stains. After staining, cells were washed twice with Perm/Wash buffer (eBioscience) and fixed with 1% PFA. For staining of transcription factors, cells were surface stained as above then fixed for 1 hour at 4°C with FoxP3 Fixation Buffer, washed using Perm/Wash buffer (eBioscience) and stained for transcription factors for 1 hour at 4°C. The following antibodies were used in combination with intracellular flow cytometry: anti-T-bet antibody (clone 4B10, BioLegend), anti-Eomes antibody (clone DAN11MAG, eBioscience), anti-TCF1 antibody (clone C63D9, Cell Signaling),

anti-TOX antibody (clone TXRX10, eBioscience), or the appropriate isotype controls. After staining, cells were washed twice with Perm/Wash buffer (eBioscience) and fixed with 1% PFA. Anti-T-bet antibody staining was titrated using wild type (T-bet^{+}/$^{+}$) and T-bet$^{+/-}$ splenocytes to achieve a clear distinction between heterozygote and homozygote T-bet expression. All samples were collected with an LSR-Fortessa (BD Biosciences) and analyzed with FlowJo v10 software (Treestar, Ashland, OR).

Statistics

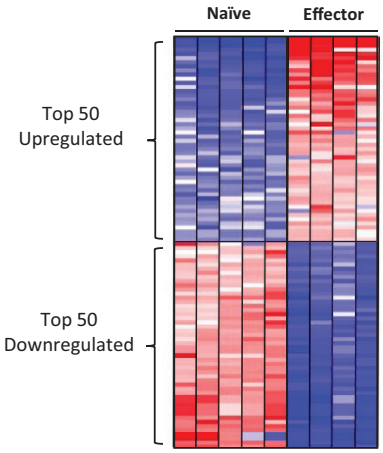
For flow cytometry and qRT-PCR data analysis, the normality of the population distribution was assessed using the Shapiro-Wilk normality test by GraphPad Prism 8. Significant differences between normally distributed populations were assessed using a two-tailed, unpaired *t*-test; significant differences between non-normally distributed populations were assessed using a two-tailed Mann Whitney exact test. The tests performed are denoted in each figure legend and subsequent p-values are annotated in the associated figure.

RESULTS

MiRNA are differentially expressed in anti-viral CTL of acute influenza virus infection

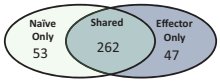
Effector CTL are phenotypically and functionally different from naïve CD8^{+} T cells (28, 29). We and others have demonstrated that specific miRNAs are upregulated or downregulated upon activation of CD8^{+} T cells (3, 30, 31), such as the increased expression of miR-155 upon *in vitro* activation or *in vivo* in anti-viral CTL (3). We hypothesized that miRNA expression within the naïve and effector CD8^{+} T cell compartment would reflect the phenotypic and functional differences of naïve versus *in vivo*-generated effector CD8^{+} T cells. To test this, we performed miRNA microarray expression profiling miRNA in naïve and effector OT-I CD8^{+} T cells (CD8^{+} T cells expressing a transgenic T cell receptor (TCR) recognizing the $OVA_{(257-264)}$ epitope). Our use of transgenic OT-I cells for these analyses ensured that any differences in miRNA expression were not a result of differences in T cell receptor affinity or avidity. To generate effector CTL *in vivo*, we performed adoptive transfers of FACS sorted naïve (CD44^{-}CD62L^{+}) CD45.1^{+} OT-I CD8^{+} T cells into CD45.2^{+} wild-type recipient mice which were then infected with WSN-OVA influenza virus (an $OVA_{(257-264)}$-expressing strain of A/WSN/33 influenza virus); a subset of sorted naïve OT-I CD8^{+} T cells were kept for naïve cell miRNA profiling analysis. On day 9 post-infection, during the peak of the effector CD8^{+} T cell response to influenza virus infection, donor CD45.1^{+} OT-I CD8^{+} T cells were sorted from the lungs of CD45.2^{+} wild-type recipient mice. MiRNA profiling of these naïve and effector OT-I CD8^{+} T cells was performed.

A



B

Number of mature miRNAs Detected



C

Top 30 mature miRNAs upregulated in effector CTL

Rank	Mature miR	Fold Change	p-value	q-value
1	miR-126a-3p	103.90	0.0130	0.0025
2	miR-182-5p	46.43	0.0001	0.0001
3	miR-183-5p	23.24	0.0001	0.0001
4	miR-195a-3p	14.08	0.0470	0.0077
5	miR-31-5p	12.38	0.0001	0.0001
6	miR-23a-5p	10.32	0.0001	0.0001
7	miR-143-3p	10.20	0.0098	0.0020
8	miR-145a-5p	10.09	0.0165	0.0030
9	miR-125b-5p	9.62	0.0001	0.0001
10	miR-27a-5p	9.24	0.0002	0.0001
11	miR-421-3p	7.59	0.0001	0.0001
12	miR-27b-5p	7.44	0.0001	0.0001
13	miR-146a-5p	6.92	0.0001	0.0001
14	miR-210-3p	5.98	0.0013	0.0004
15	miR-449a-5p	5.85	0.0013	0.0004
16	miR-7030-5p	5.80	0.0360	0.0062
17	miR-7666-3p	4.90	0.0002	0.0001
18	miR-183-3p	4.51	0.0077	0.0017
19	miR-301a-3p	4.43	0.0165	0.0030
20	miR-132-3p	3.74	0.0001	0.0001
21	miR-147-3p	3.65	0.0001	0.0001
22	miR-7648-3p	3.44	0.0017	0.0004
23	miR-6937-5p	3.30	0.0001	0.0001
24	miR-155-5p	3.20	0.0003	0.0001
25	miR-18a-5p	3.03	0.0001	0.0001
26	miR-6968-5p	3.02	0.0009	0.0003
27	miR-1934-3p	2.97	0.0038	0.0009
28	miR-21a-5p	2.90	0.0013	0.0004
29	miR-22-3p	2.88	0.0006	0.0002
30	miR-298-5p	2.84	0.0008	0.0003

D

Top 30 mature miRNAs downregulated in effector CTL

Rank	Mature miR	Fold Change	p-value	q-value
1	miR-669c-5p	-11.09	0.0001	0.0001
2	miR-181a-5p	-10.86	0.0001	0.0001
3	miR-192-5p	-10.77	0.0001	0.0001
4	miR-466a-3p	-9.71	0.0004	0.0002
5	miR-466e-3p	-9.71	0.0004	0.0002
6	miR-466b-3p	-9.12	0.0020	0.0010
7	miR-466c-3p	-9.12	0.0020	0.0010
8	miR-466p-3p	-9.12	0.0020	0.0010
9	miR-467c-5p	-7.85	0.0001	0.0001
10	miR-466d-3p	-7.34	0.0004	0.0002
11	miR-194-5p	-7.05	0.0005	0.0003
12	**miR-139-5p**	**-6.89**	**0.0001**	**0.0001**
13	miR-26b-5p	-6.83	0.0242	0.0086
14	miR-28a-3p	-6.64	0.0001	0.0001
15	miR-30e-5p	-6.53	0.0002	0.0002
16	miR-669a-3p	-6.48	0.0001	0.0001
17	miR-669o-3p	-6.48	0.0001	0.0001
18	miR-378a-5p	-6.41	0.0006	0.0003
19	miR-339-5p	-6.21	0.0001	0.0001
20	miR-466c-5p	-6.21	0.0087	0.0033
21	miR-342-5p	-6.17	0.0001	0.0001
22	miR-466h-5p	-5.92	0.0067	0.0026
23	miR-342-3p	-5.88	0.0001	0.0001
24	miR-181c-5p	-5.45	0.0001	0.0001
25	miR-378a-3p	-5.43	0.0001	0.0001
26	miR-467a-5p	-5.06	0.0005	0.0003
27	miR-128-3p	-4.67	0.0026	0.0012
28	miR-181b-5p	-4.64	0.0001	0.0001
29	miR-3068-3p	-4.58	0.0019	0.0010
30	miR-30b-5p	-4.12	0.0002	0.0002

E

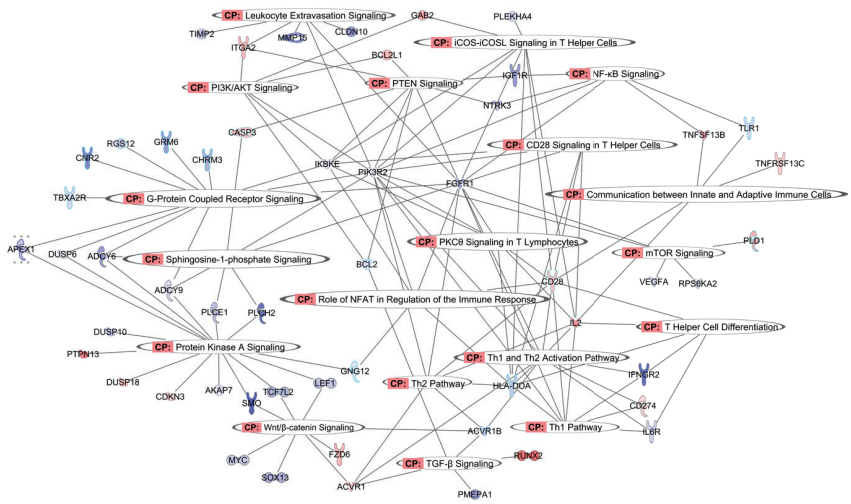
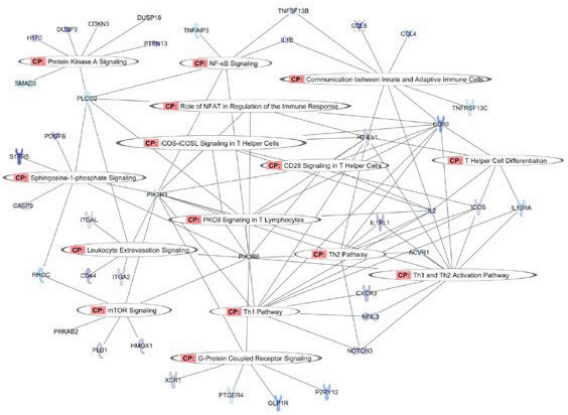


Figure 1 (previous page). Anti-viral effector CTL demonstrate unique miRNA profiles after *in vivo* differentiation. **(A)** Heatmap of miRNA expression levels as assessed by miRNA microarray in sorted CD44-CD62L^{+} naïve and day 9 acute influenza virus infection effector OT-I T cells shown. The 50 most upregulated and 50 most downregulated miRNA are represented relative to the total average expression for the given miRNA. **(B)** Venn diagram of mature miRNAs present exclusively or shared between naïve and effector OT-I CD8^{+} T cells. **(C)** Table of the 30 most upregulated and **(D)** 30 most downregulated miRNA relative to naïve expression levels in effector CTL. (**E**) IPA pathway analysis showing predicted canonical pathway genes regulated by miRNAs. MiRNA with >5-fold up-or downregulated in effector anti-viral OT-I cells and genes with >1.5-fold differential and reciprocal expression in effector CTL compared to naïve cells were used for analysis. Pathways with at least 5 predicted regulated genes shown. Blue genes are downregulated and red genes are upregulated in effector OT-I cells.

MiRNA expression analysis revealed that naïve and effector CTL had significantly different miRNA profiles (Fig. 1A, Fig. S1). We observed that 315 mature miRNA were expressed above background level (miRNA with an average naïve or effector sample detection p-value ≤ 0.05) in naïve CD8^{+} T cells and 308 miRNA were expressed above background level in effector CTL. Of these, 53 miRNA were expressed only in naïve, and 47 miRNA were expressed only in effectors (Fig. 1B). We found that 51 miRNA in effector CTL demonstrated ≥ 2-fold upregulation compared to naïve expression levels (FDR = 0.05, $p \leq 0.05$), and 69 miRNA demonstrated ≥ 2-fold downregulation compared to naïve expression levels (FDR = 0.05, $p \leq 0.05$)(Fig. S1A-B). Of these, miR-126a demonstrated the highest upregulation (103-fold), followed by miR-182 (46-fold) and miR-183 (23-fold); miR-669c, miR-181a, miR-192, and miR-466 family members were the most downregulated and all had >9-fold lower compared to naïve expression levels (Fig. 1C-D). Strikingly, the transition from a naïve cell to an effector CTL was accompanied by a change of only 11 miRNA exhibiting more than 10-fold changes (up or down) in expression levels. MiR-155 expression levels in effector CTL were 3.3-fold greater than in naïve CD8^{+} T cells, confirming our previous findings by qRT-PCR (3).

Ingenuity Pathway Analysis (IPA) of effector CTL miRNAs (>5-fold up- or downregulated) paired to genes that reciprocally changed expression in effector CTL (27) identified multiple canonical pathways including leukocyte extravasation signaling, JAK/Stat signaling, Wnt/β-catenin signaling, PI3K/Akt signaling, and cell cycle regulation (Fig. 1E or Fig. S2B), underscoring the important role miRNAs play in the activation, cytokine signaling and proliferation of CTL responses. The sphingosine-1-phosphate (S1P) signaling and G-protein coupled receptor (GPCR) signaling pathways were the most enriched in predicted hits (26% and 21% of pathway molecules, respectively Fig. S2A-B). IPA of miRNA uniquely expressed in naïve and effector CTL, paired to genes reciprocally expressed in these populations (27) again revealed a preferential targeting of S1P and GPCR signaling pathways (Fig. 2A-D).

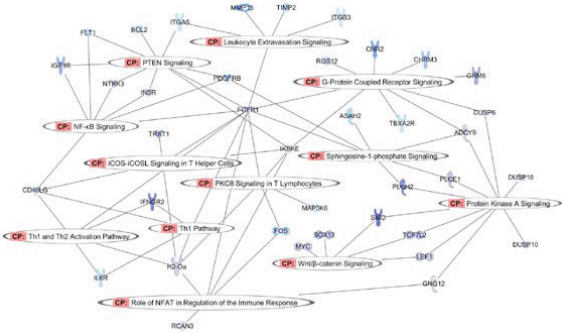
A



B

Pathways in naïve CD8+ T cells with predicted downregulated target genes by miRNAs expressed only in naïve CD8+ T cells.		
Rank	**Pathway**	**% Molecules Regulated**
1	Sphingosine-1-phosphate Signaling	26.9
2	Communication between Innate and Adaptive Immune Cells	20.0
3	Th1 Pathway	14.1
4	PKCθ Signaling in T Lymphocytes	12.5
5	iCOS-iCOSL Signaling in T Helper Cells	12.0
6	Th2 Pathway	11.5
7	Th1 and Th2 Activation Pathway	10.9
8	T Helper Cell Differentiation	10.6
9	mTOR Signaling	10.3
10	CD28 Signaling in T Helper Cells	8.9
11	Role of NFAT in Regulation of the Immune Response	7.7
12	NF-κB Signaling	7.0
13	Leukocyte Extravasation Signaling	7.0
14	Protein Kinase A Signaling	6.6

C



D

Pathways in effector CD8+ T cells with predicted downregulated target genes by miRNAs expressed only in effector CD8+ T cells.		
Rank	**Pathway**	**% Molecules Regulated**
1	Sphingosine-1-phosphate Signaling	23.1
2	PTEN Signaling	18.4
3	G-Protein Coupled Receptor Signaling	16.4
4	PKCθ Signaling in T Lymphocytes	10.4
5	iCOS-iCOSL Signaling in T Helper Cells	10.0
6	Protein Kinase A Signaling	9.4
7	Role of NFAT in the Immune Response	9.2
8	Wnt/B-catenin Signaling	8.2
9	NF-κB Signaling	8.1
10	Th1 Pathway	7.0
11	Th1 and Th2 Activation Pathway	4.5

Figure 2. Reciprocal regulation of canonical pathways in naïve and effector CD8⁺ T cells.

(A) IPA diagram showing predicted canonical pathway genes downregulated by miRNAs expressed exclusively in naïve OT-I cells. For IPA, miRNA were paired to genes downregulated >1.5-fold in naïve OT-I cells. **(B)** Ranked list of canonical pathways with at least 5 predicted regulated molecules shown. Percent of total genes in pathway targeted are indicated. **(C)** IPA pathway analysis depicting predicted canonical pathway genes downregulated by miRNAs expressed exclusively in day 10 effector CTL. IPA was done on miRNA paired to genes downregulated >1.5-fold in effector CTL. **(D)** Ranked list of canonical pathways with at least 5 predicted regulated molecules and the percent of total genes in pathway targeted are indicated.

MicroRNA-139 expression is downregulated in effector CTL *in vivo* and *in vitro*

MicroRNA-139 (miR-139) was one miRNA identified by microarray analysis to be expressed at higher levels in naïve CD8⁺ T cells and downregulated in effector CTL. Our findings were consistent with a previous study which identified the *in vivo* downregulation of miR-139 in KLRG1⁺CD127⁻ effector cells compared to KLRG1⁻CD127⁺ memory precursor cells from LCMV

Armstrong infected mice 8 days post-infection (23). Trifari *et al.* had also shown that overexpression of the full-length pri-miR-139 gene in P14 $CD8^+$ T cells, transgenic $CD8^+$ T cells that recognize the LCMV glycoprotein residues 33-41 (GP33), resulted in the decreased killing ability of activated CTL in response to GP33-expressing *Listeria monocytogenes* infection. Taken together with our miRNA profiling data, we hypothesized that miR-139 expression was essential in regulating mRNAs that are critical in maintaining a naïve or memory $CD8^+$ T cell state. We therefore chose to assess the role of miR-139 in CTL differentiation in response to influenza virus infection.

MiRNA profiling of influenza-specific effector CTL identified that miR-139-5p was significantly downregulated (-6.89 fold, p = 0.001)(Fig. 1D). To assess how quickly miR-139 was downregulated upon $CD8^+$ T cell activation and to exclude that this downregulation was a result of the OT-I transgenic TCR or cytokine driven, qRT-PCR analysis of both miR-139-3p (Fig. 3A) and miR-139-5p (Fig. 3B) was performed on splenic $CD8^+$ T cells purified from C57BL/6 mice that were either activated using solid phase-bound α-CD3/α-CD28 antibodies or treated with IFNγ, TNFα, IFNB1, or IL-1B for either 24 or 72 hours. For both miR-139-3p and -5p, only the addition of TNFα was sufficient to drive the downregulation of miR-139 in non-activated $CD8^+$ T cells at the 24 hour timepoint. While T cell receptor activation was sufficient to drive a high level of downregulation of miR-139-3p by 24 hours, miR-139-5p was slower to decrease. Regardless of the addition of cytokines, however, both miR-139-3p and miR-139-5p expression levels were undetectable or barely detectable by qRT-PCR after 72 hours of activation (Fig. 3A-B). Taken together, these data confirmed that both forms of miR-139 are highly downregulated upon activation of $CD8^+$ T cells.

Expression of miR-139 is dispensable for the normal development of CD4 and CD8 T cells

As we hypothesized that miR-139 expression was critical for maintaining the naïve state of $CD8^+$ T cells, we questioned if miR-139 played a functional role in the development of $CD8^+$ T cells. Using flow cytometry, we assessed if miR-139-deficiency impacted T cell phenotype in the absence of infection. The spleens of 8-week-old uninfected miR-$139^{+/+}$, miR-$139^{+/-}$, and miR-$139^{-/-}$ female mice (Supplementary Fig. 3A) were processed into single-cell suspensions and stained to assess the frequency and phenotype of $CD4^+$ and $CD8^+$ T cells. Total cell counts from the spleens were comparable across all conditions. Flow cytometry identified that there were no substantial differences in the frequencies of $CD4^+$ or $CD8^+$ T cells in the periphery when miR-139 was absent (Fig. 3C). Further, miR-139-deficiency did not affect the frequency of naïve $CD4^+$ or $CD8^+$ T cells (Fig. 3C). Thus, we conclude that miR-139 expression is not required for the normal development of $CD4^+$ and $CD8^+$ T cells and its deficiency did not result in the skewing of the steady-state naïve and memory phenotype of $CD4^+$ or $CD8^+$ T cells.

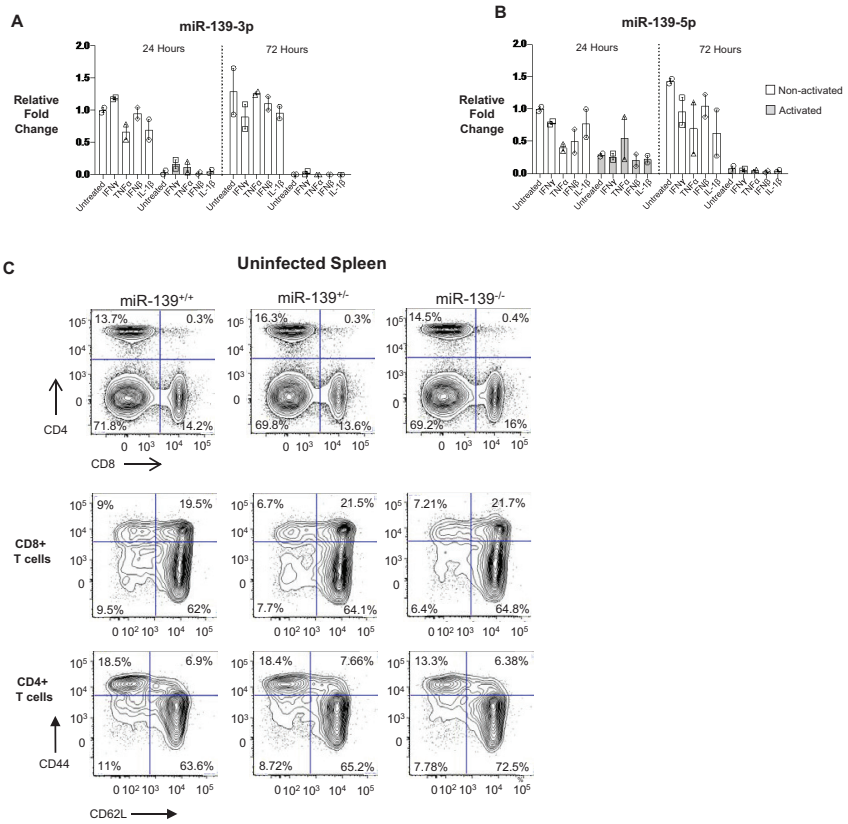


Figure 3. MiR-139 expression is downregulated after CD8⁺ T cell activation, but is not required for the development of CD4 and CD8 T cells.

MiR-139-3p **(A)** and miR-139-5p **(B)** expression levels (relative to the respective untreated 24 hour non-activated control) as determined by qRT-PCR at 24 and 72 hours after activation compared to non-activated OT-I CD8⁺ T cells in the presence and absence of immune activating (IFNγ, TNFα; both at 10 ng/mL) and inhibiting (IFN-β1, 1000 U/mL; IL-1β, 10 ng/mL) cytokines. Each dot represents a pooling of 6 C57BL/6 mice from two independent experiments (3 per experiment). **(C)** Representative FACS plots showing the frequency of CD4⁺ and CD8⁺ T cells from the splenocytes from miR-139$^{+/+}$, miR-139$^{+/-}$, and miR-139$^{-/-}$ uninfected female mice 8 weeks of age. The frequency of naïve (CD44^{-} CD62L^{+}) and virtual memory (CD44^{+}CD62L$^{+/-}$) CD8⁺ and CD4⁺ T cells are shown. Representative of 5-6 mice evaluated from 3 different litters.

MiR-139 deficiency does not affect effector or memory anti-viral CTL responses to influenza virus infection

To directly test if miR-139 impacts the generation of effector CTL responses to an active infection, we assessed CTL responses to an acute influenza virus infection using two model systems: 1) infection of total miR-139$^{-/-}$ mice with PR8 influenza virus infection, and 2) the

adoptive transfer of miR-139$^{-/-}$ OT-I CD8^{+} T cells into wild-type recipient host mice with WSN-OVA influenza virus infection, therefore limiting any effects of miR-139$^{-/-}$ to a CD8^{+} T cell-intrinsic mechanism. Regardless of the model, absence of miR-139 globally or exclusively in CD8^{+} T cells had no impact on the CTL response to influenza virus infection. When infected with PR8 influenza virus, miR139$^{-/-}$ mice lost on average the same percent body weight as wild-type control mice (Fig. 4A). At 10 days post-infection with influenza virus, the frequency of $NP_{(366-374)}$–specific CD8^{+} T cells in the lungs did not differ between wild-type and miR-139$^{-/-}$ (Fig. 4B-C). In addition, no difference was found in either spleens or mediastinal lymph nodes (MLN) as well (Fig. 4C). We also did not observe any differences in the frequency of proliferating virus-specific CD8^{+} T cells as assessed by Ki-67 expression (Fig. 4D). The failure of miR-139 to affect the development of influenza-specific CTL responses was also confirmed in the adoptive transfer experiments, where donor OT-I cells and miR-139$^{-/-}$ OT-I cells both generated similar CTL responses in terms of frequencies and numbers of cells in lungs, spleens, and MLN (Fig. 4E-G). MiR-139 deficiency also did not affect CTL expansion and there was also no evidence of impaired or enhanced trafficking, as the numbers of virus-specific CTL were similar in the lungs, spleens and MLN of infected animals (Fig. 4G). Similar to PR8 infection, there were no differences in donor miR-139$^{-/-}$ OT-I proliferation in either the lungs or spleens of WSN-OVA influenza virus infected mice (Fig. 4H).

We next assessed if lack of miR-139 altered the activation state of effector CD8^{+} T cells in the lungs following infection with either PR8 or WSN-OVA influenza virus. Both wild-type and miR-139-deficient CTL expressed similar levels of CD25 and CD69, markers of T cell activation, *ex vivo* from the lungs (Fig. 5A-B). Finally, to assess the functional impact of miR-139 expression on CTL responses, we performed intracellular cytokine staining to determine IFNγ and TNFα production. Again, no differences were observed between wild type and miR-139$^{-/-}$ CTL in terms of their ability to produce effector cytokines after the adoptive transfer of miR-139$^{-/-}$ OT-I or in WT OT-I mice (Fig. 5C-D). Therefore, regardless of the model used, absence of miR-139 in CD8^{+} T cells had no impact on the generation of the CTL effector response.

In previous studies assessing the role of miR-155 in the regulation of CD8^{+} T cells, we have observed that deletion versus overexpression of a miRNA can result in alteration of miRNA target selection (3, 5). To confirm if this was the case with miR-139-targeted regulation of CTL, we used retroviral transduction to overexpress miR-139 in OT-I CD8^{+} T cells (miR-139 OE). Control transduced or miR-139 OE OT-I CD8^{+} T cells were FACS sorted based on the expression of the selectable marker CD90.1/Thy1.1, and 10^{4} OT-I cells were adoptively transferred into wild-type recipient mice (CD90.2/Thy1.2) followed by infection with WSN-OVA as in previous experiments. At day 9 post-infection, we evaluated the donor OT-I cells in the lungs of influenza virus infected mice. Similar to our results from miR-139$^{-/-}$ OT-I donor transfer experiments, we did not observe any change in the frequency of donor OT-I cells

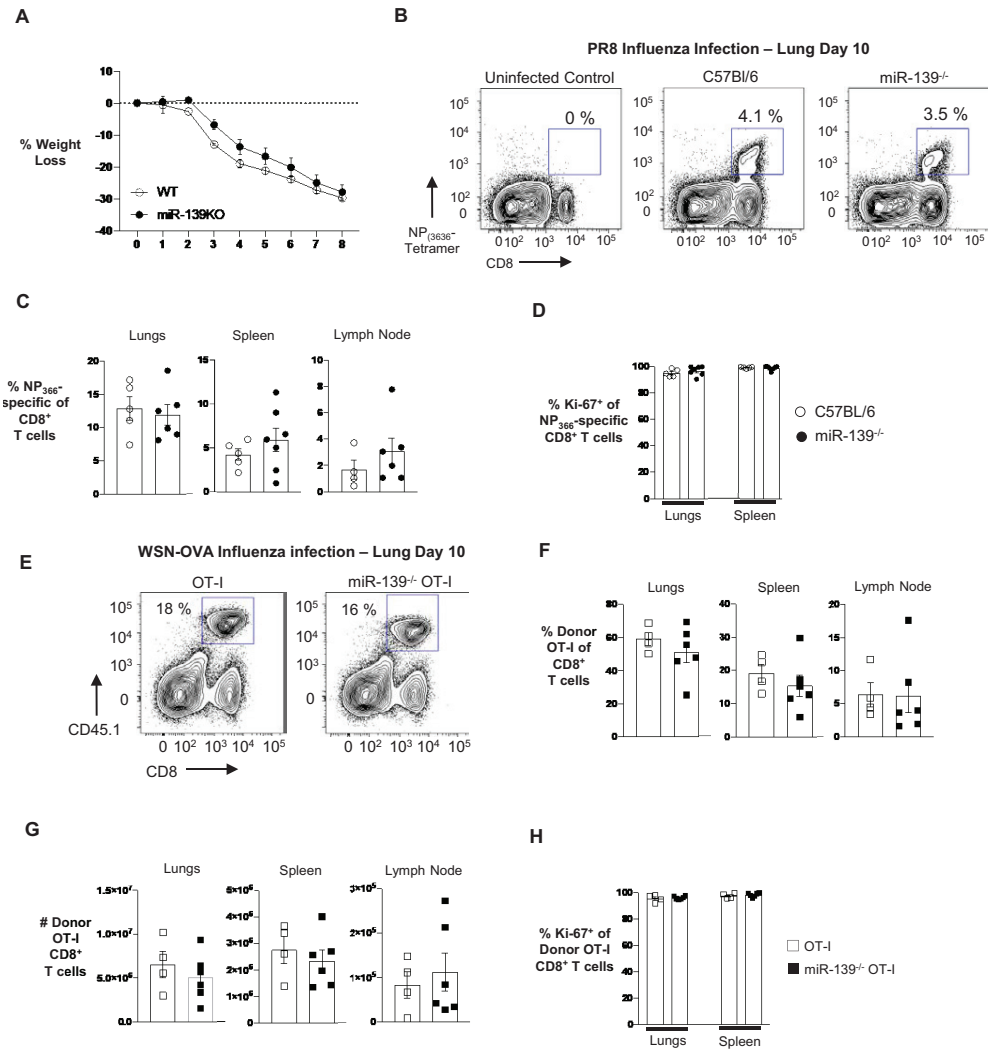


Figure 4. MiR-139$^{-/-}$ CD8^{+} T cells successfully protect mice from influenza virus infection challenge.

Complete miR-139$^{-/-}$ and control C57Bl/6J female mice infected with PR8 influenza virus respond equally to infection as shown by average % weight loss **(A)**. **(B)** Representative FACS plots of NP$_{(366)}$-specific CD8^{+} T cells in the lungs of wild-type and miR-139$^{-/-}$ mice infected with PR8 influenza virus. **(C)** Frequency of influenza-specific CD8^{+} T cells in the lungs, spleens, and mediastinal lymph nodes of wild-type and miR-139$^{-/-}$ mice at day 10 post-infection. **(D)** Frequency of Ki-67^{+} NP$_{(366)}$-specific CD8^{+} T cells in the lungs and spleens of wild-type and miR-139$^{-/-}$ mice. Adoptive transfer of miR-139$^{-/-}$ OT-I or wild-type OT-I CD8^{+} T cells is sufficient to control infection with WSN-OVA influenza virus. **(E)** Representative FACS plots of CD45.1^{+} donor wild-type or miR-139$^{-/-}$ OT-I in the lungs of mice 10 days after WSN-OVA influenza virus infection. **(F)** Frequency and **(G)** number of donor CD8^{+} T cells in the lungs, spleens, and mediastinal lymph nodes of host mice 10 days post-infection. **(H)** Frequency of Ki-67^{+} donor OT-I cells in the lungs and spleens of mice. All figures representative of 4-7 mice per group from two independent experiments.

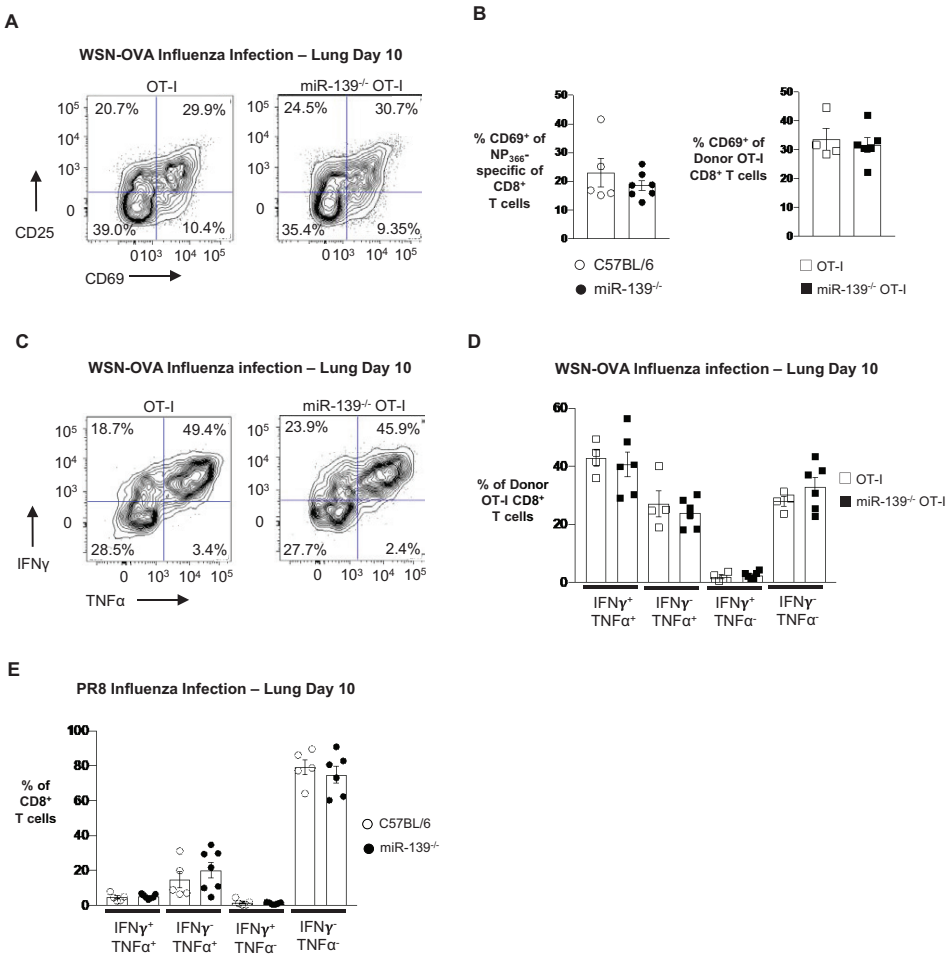


Figure 5. MiR-139-deficiency does not alter effector CD8⁺ T cell differentiation.

(A) Representative FACS plot of CD25 and CD69 expression in wild-type or miR-139$^{-/-}$ OT-I CD8⁺ T cells in the lungs of mice 10 days post-infection. **(B)** Frequency of CD69⁺ virus-specific CD8⁺ T cells from mice 10 days post-infection with PR8 (left) or WSN-OVA (right) influenza virus infection. **(C)** Representative FACS plot of IFNγ and TNFα production by OT-I cells after 6 hour re-stimulation with OVA peptide followed by intracellular staining. Dot plots of the frequency of cytokine-producing donor OT-I **(D)** or total CD8⁺ T cells **(E)** in the lungs after WSN-OVA or PR8 influenza virus infection, respectively. All figures representative of 4-7 mice per group from two independent experiments.

between the control and miR-139 OE groups, nor in cytokines production or CD69 expression (Supplemental Fig. 3D-F).

We next examined if miR-139 deficiency impacts the development of memory CTL in response to influenza virus infection. Similar to our previous findings in uninfected mice, we observed no differences in the memory CTL frequency or skewing of memory CD8⁺ T cells

towards the central memory ($CD44^+CD62L^+$) or effector memory ($CD44^+CD62L^-$) phenotype as assessed by CD44 and CD62L expression (Fig. 6A-D). Taken together, our data demonstrates that the expression of miR-139 is dispensable for the development of both effector and memory $CD8^+$ T cell responses to acute infection with influenza virus.

Eomes is not a target of miR-139 in influenza virus-specific $CD8^+$ T cells

The transcription factor Eomesodermin (Eomes) is important to the development of memory CTL (32) and T cell exhaustion (24). Previous studies into the role of miR-139 and T cells have reported that overexpression of miR-139 resulted in decreased Eomes expression (23). We therefore reasoned that the absence of miR-139 should promote Eomes expression. We evaluated the frequency and level (MFI, median fluorescent intensity) of Eomes during the effector phase of the CTL response; we simultaneously assessed T-bet expression levels as the balance of expression between the two transcription factors is important in the development of $CD8^+$ T cell responses to acute viral infection (33). In both the spleen and lung, we did not observe any differences in the frequency or expression levels of Eomes and T-bet in influenza virus-specific $CD8^+$ T cells upon either PR8 infection of miR-$139^{-/-}$ mice or WSN-OVA infection following miR-$139^{-/-}$ OT-I adoptive transfer (Fig. 7A-G). This may explain the lack of any impact on effector or memory CTL development as we originally hypothesized. Unlike what was previously noted in T cell responses to *Listeria monocytogenes* (23), we observed no change in the expression levels of either Eomes or T-bet in miR-$139^{-/-}$ OE $CD8^+$ T cells (Fig. 7H-I).

Absence of miR-139 does not promote T cell exhaustion

During acute viral infection, $CD8^+$ T cells clear virally infected cells and can protect the host from re-infection through the generation of functional memory $CD8^+$ T cells. However, in cases of chronic infection such as murine infection with LCMV Clone 13 and HIV infection in humans, $CD8^+$ T cells enter a different differentiation pathway and become exhausted (34). T cell exhaustion is characterized by the increased co-expression of inhibitor receptors (including PD-1, LAG3, TIM-3, CD160 and TIGIT), decreased proliferative capacity, and loss of cytokine production (35). T cell exhaustion is also accompanied by significant changes in the transcriptome, including changes in the expression levels of T-bet and Eomes (36). High levels of Eomes expression have previously been implicated in the development or promotion of T cell exhaustion in response to chronic virus infection and cancer (24, 37). Despite the lack of miR-139-mediated regulation of Eomes in influenza virus infection, we reasoned that lack of miR-139 may enhance Eomes expression and therefore promote T cell exhaustion under conditions of repeated TCR stimulation. Using a novel method to develop T cell exhaustion *in vitro* (chapter 3), we assessed the development of T cell exhaustion in miR-$139^{-/-}$ $CD8^+$ T cells. As expected, repeated stimulation of wild-type $CD8^+$ T cells with peptide resulted

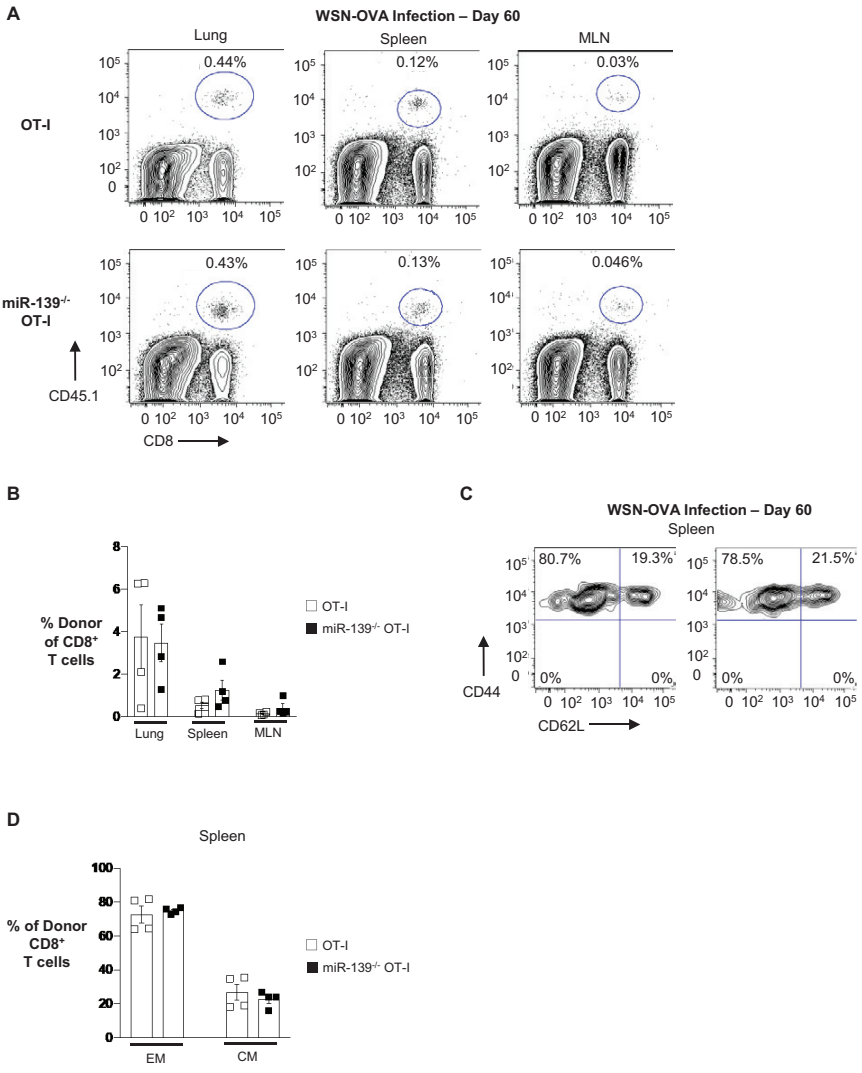


Figure 6. Memory CD8⁺ T cell development is not regulated by miR-139.

(A) Representative FACS plots of CD45.1⁺ donor OT-I and miR-139$^{-/-}$ OT-I cells in the lungs, spleen, and mediastinal lymph nodes of C57Bl/6 recipient host mice 60 days post-infection with WSN-OVA influenza virus. **(B)** Frequency of CD45.1⁺ donor T cells within the CD8⁺ T cell compartment of the respective tissues. **(C)** Representative FACS plots of effector memory (EM)(CD44⁺CD62L⁻) and central memory (CM)(CD44⁺CD62L⁺) frequency within the donor OT-I CD8⁺ T cells in the spleen of mice 60 days post-infection with WSN-OVA influenza virus. **(D)** Frequency of EM and CM donor OT-I CD8⁺ T cells in the spleen. All figures representative of 4 mice per group from two independent experiments.

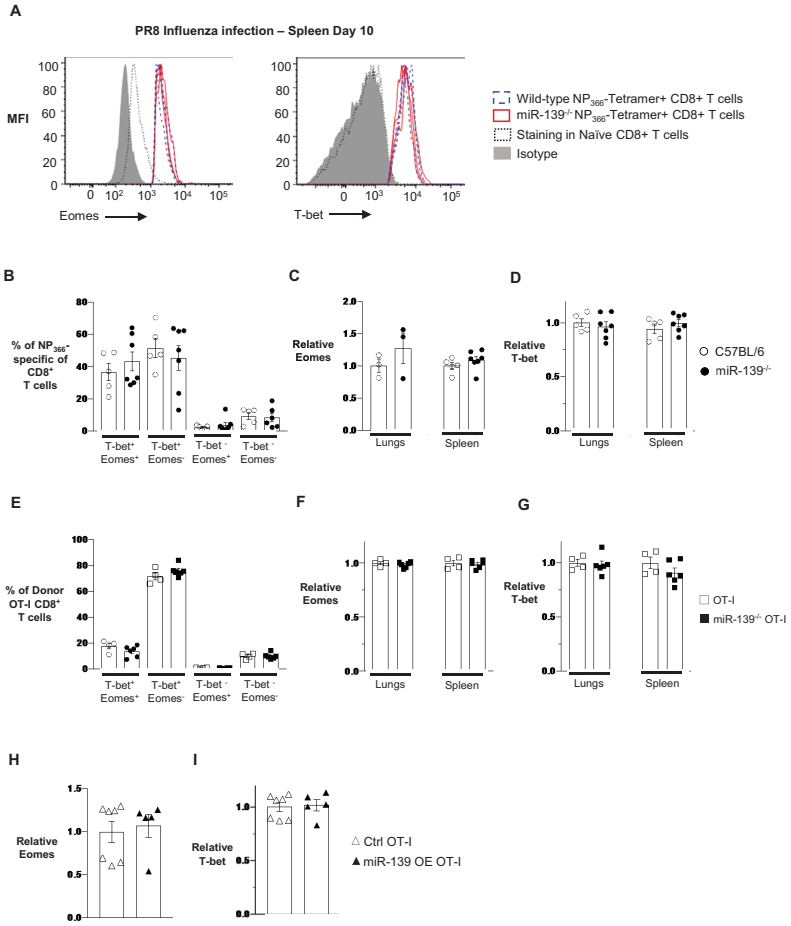


Figure 7. Eomes expression is not mediated by miR-139 in influenza virus-specific CD8⁺ T cells.

(A) Representative histogram of Eomes (left) or T-bet (right) expression in wild type (blue dotted line) or miR-139$^{-/-}$ (red) NP$_{(366)}$-specific CD8⁺ T cells from 3 mice each compared to isotype (gray) and naïve CD8⁺T cells (black dotted line). **(B)** Dot plot of the frequency of T-bet and Eomes expression in NP$_{(366)}$-specific CD8⁺ T cells in wild type (open circle) and miR-139$^{-/-}$ (filled circle) mice. Dot plots of the relative expression levels (MFI) of Eomes **(C)** and T-bet **(D)** in NP$_{(366)}$-specific CD8⁺ T cells in the lungs and spleens of wild type and miR-139$^{-/-}$ mice after PR8 infection. Data is shown relative to the expression in wild type cells of the indicated tissue. **(E)** Dot plot of the frequency of T-bet and Eomes expression in wild type (open square) and miR-139$^{-/-}$ (filled square) donor OT-I CD8⁺ T cells from WSN-OVA influenza virus infected mice. Dot plots of the relative expression levels (MFI) of Eomes **(F)** and T-bet **(G)** in donor OT-I CD8⁺ T cells in the lungs and spleens of mice after WSN-OVA infection. Data is shown relative to the expression in wild type cells of the indicated tissue. Activated wild type OT-I CD8⁺T cells were transduced with either a scrambled control retrovirus (open triangle) or a retrovirus inducing the overexpression of miR-139 (filled triangle) and adoptively transferred into wild type recipient mice; mice were then infected with WSN-OVA. Dot plots of the relative expression levels (MFI) of Eomes **(H)** and T-bet **(I)** in donor OT-I CD8⁺ T cells in the lungs of mice 9 days after WSN-OVA infection. All figures representative of 4-7 mice per group from two independent experiments.

in the loss of IL-2 and TNFα production (Fig. 8A); in agreement with our *in vivo* studies, there were no differences in cytokine production by "effector" miR-139$^{-/-}$ CD8^{+} T cells compared to wild-type cells after a single round of peptide stimulation (Fig. 8A). Importantly, we observed a similar loss of polyfunctionality by miR-139$^{-/-}$ CD8^{+} T cells compared to wild-type cells upon repeated peptide stimulation (Fig. 8A, B). Repeated stimulation of both wild-type and miR-139$^{-/-}$ CD8^{+} T cells resulted in equivalently high frequencies and expression levels of PD-1, LAG3, TIM3, CD160, and TIGIT (Fig. 8C). Similarly, neither single nor repeated peptide stimulation resulted in increases or decreases of the expression levels of Eomes or T-bet in miR-139$^{-/-}$ CD8^{+} T cells (Fig. 9A-B).

To evaluate if the absence of miR-139 increased or decreased the prevalence of TOX^{+}TCF-1^{-} terminally exhausted and TOX^{-}TCF1^{+} progenitor exhausted CTL, we evaluated the frequency of these after repeat stimulation. Repeated stimulation of wild-type and miR-139$^{-/-}$ CD8^{+} T cells resulted in an equivalent downregulation of TCF-1 expression (Fig. 9C, D) and upregulation of TOX (Fig. 9C, E), while single peptide stimulation failed to promote TOX expression in either wild-type or miR-139$^{-/-}$ CD8^{+} T cells, in agreement with their characterization as effector cells. Taken together, these data in combination with the *in vivo* influenza virus studies demonstrate that miR-139 expression is dispensable for the generation of effector CD8^{+} T cell responses.

DISCUSSION

Previously, microarray profiling of *in vitro* activated CD8^{+} T cells was performed by Wu, *et al* (38). However, as antigen presentation and the local inflammatory environment during an active infection can have a major impact on CTL differentiation (39), we direct assessed the miRNA expression in *in vivo* generated CTL. We analyzed the miRNA expression profiles of naïve CD8^{+} T cells and compared them to *in vivo*-generated effector anti-viral CTL taken directly from the site of infection, the lungs, in order to establish the relative dynamics of the CTL miRNome. Of the 362 mature miRNA detected by microarray analysis, large-scale changes in effector CTL are rather modest with only 11 miRNA of the miRNome changing more than 10-fold, while 120 miRNA change by >2-fold. This suggests that the overall miRNome is relatively stable in CD8^{+} T cells with large-scale changes potentially associated with cellular identity and lineage. The relatively modest changes in the majority of miRNAs meanwhile appears sufficient to impart effector CTL differentiation, function and expansion.

We found that the most upregulated miRNAs in effector CTL were miR-126a, miR-182, miR-183, miR-185 and miR-31 while miR-669c, miR-181, miR-378b and miR-192 were the most downregulated. *In vitro* activation of mouse CD4^{+} T cells showed the upregulation of miR-155 concurrent with the downregulation of miR-150, miR-146, miR-142, and miR-16

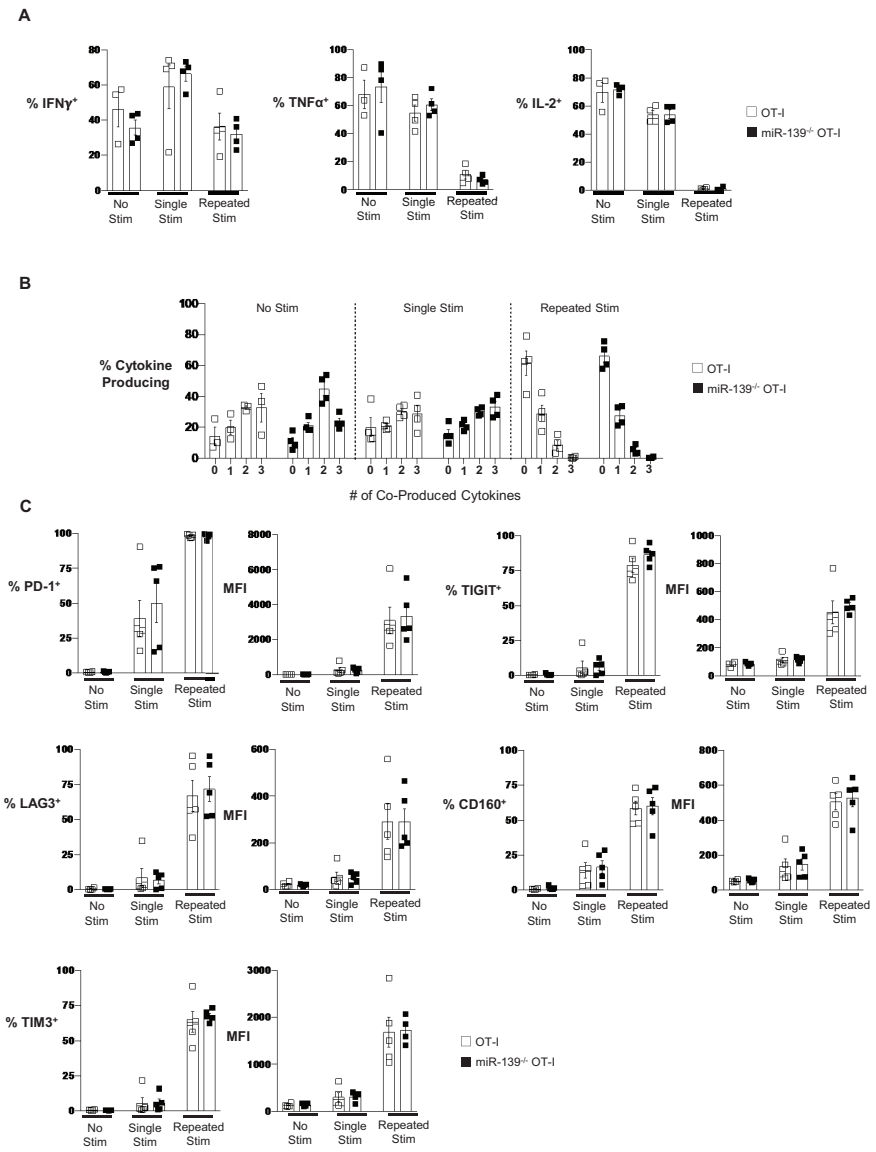


Figure 8. Lack of miR-139 expression does not alter the development of T cell exhaustion.

"Effector" and "exhausted" CD8$^+$ T cells were generated *in vitro* from OT-I or miR-139$^{-/-}$ OT-I mice through either single (single stim) or repeated (repeated stim) OVA-peptide stimulations and compared to non-stimulated (no stim) controls. **(A)** Dot plots of cytokine-producing OT-I cells and **(B)** dot plot of the frequency of polyfunctional CD8$^+$ T cells 5 days after initial activation *in vitro* (**C**). Dot plots of the frequency or expression level (median fluorescent intensity) of PD-1, LAG3, TIM3, TIGIT, and CD160 in *in vitro* exhausted wild-type or miR-139$^{-/-}$ OT-I CD8$^+$ T cells. Representative of 4-5 independent experiments.

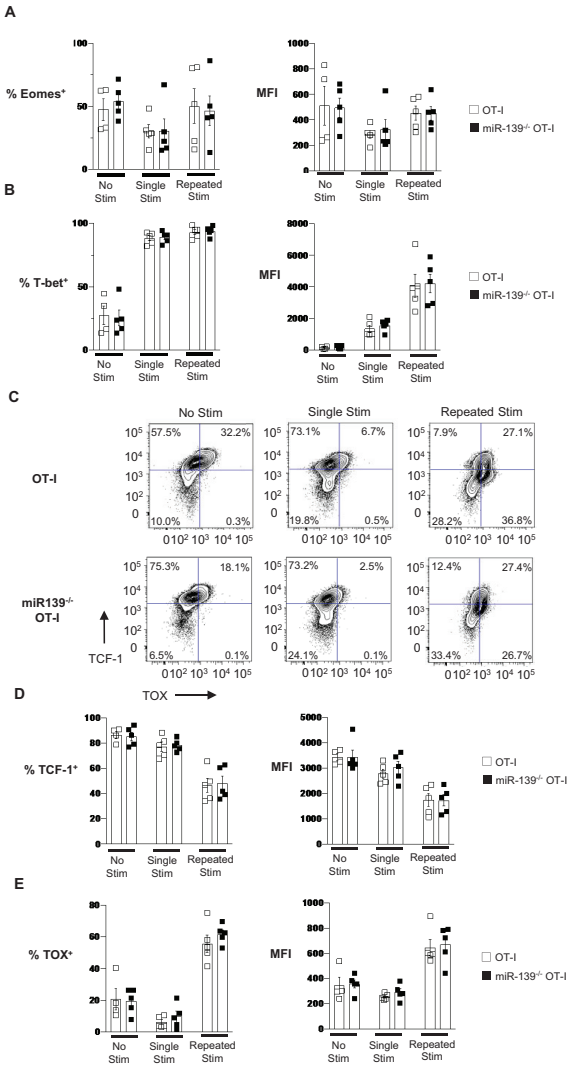


Figure 9. Mir-139 deficiency does not alter the expression of key transcription factors associated with T cell exhaustion.

(A) Dot plots of the frequency (left) and MFI (right) of Eomes expression in *in vitro*-generated effector and exhausted OT-I or miR-139$^{-/-}$ OT-I CD8^{+} T cells. **(B)** Dot plots of the frequency (left) and MFI (right) of T-bet expression in *in vitro*-generated effector and exhausted OT-I or miR-139$^{-/-}$ OT-I CD8^{+} T cells. **(C)** Representative FACS plots of TCF-1 and TOX expression in *in vitro*-generated effector and exhausted OT-I or miR-139$^{-/-}$ OT-I CD8^{+} T cells. Plots representative of 5 independent experiments. **(D)** Dot plots of the frequency (left) and MFI (right) of TCF-1 expression in *in vitro*-generated effector and exhausted OT-I or miR-139$^{-/-}$ OT-I CD8^{+} T cells. **(E)** Dot plots of the frequency (left) and MFI (right) of TOX expression in *in vitro*-generated effector and exhausted OT-I or miR-139$^{-/-}$ OT-I CD8^{+} T cells. Data represents 4-5 independent experiments.

(30), expression patterns that are distinct from our influenza virus-specific CD8$^+$ effector cells. Previous studies have reported that *ex vivo* subsets of human CD8$^+$ T cells demonstrated unique expression patterns of miRNAs, noting in particular the upregulation of miR-21, miR-155 and miR-146a and the downregulation of miR-19b, miR-20a, miR-92, and miR-26a in differentiated effector cells (40). Our profiling of mouse anti-viral effector CTL shared the upregulation of miR-21, miR-155, and miR-146a and downregulation of miR-92 and miR-26a. Surprisingly, we found that the second most downregulated miRNA in effector CTL was miR-181a (down by >10-fold), which is known to regulate TCR signaling by targeting phosphatases (41). At first glance, this seems counterintuitive as decreased miR-181a would result in reduced TCR activation which would likely impact CTL killing ability. However, miR-181a is downregulated after TCR signaling in thymocytes (41) and its downregulation in *in vivo* effector CTL could result from TCR engagement. In effector CTL, this downregulation may signify an increasing threshold of TCR activation at the peak of the CTL response and may relate to the forthcoming CTL contraction phase. Kinetic studies of miR-181a expression at early stages of effector CTL generation could address this and reveal if this downregulation in CTL occurs at the later stage of the response or that miR-181a may have some unanticipated targets and effects in the CTL response. Interestingly, miR-150 which has been shown to be required for optimal effector CTL responses to *Listeria monocytogenes* (42), was downregulated in effector CTL in our profiling, something also reported with *in vitro* activation of CTL (23). These studies highlight the contextual expression of miRNA; understanding the dynamic nature of the miRNA expression landscape during CTL differentiation and immune responses in acute or chronic infections and cancer and identifying the unique miRNAs associated with the different phases of CTL immunity may prove important for the enhancement of cellular immunotherapy approaches such as chimeric antigen receptor T cells or tumor-infiltrating lymphocyte cancer therapies.

IPA miRNA target analysis comparing our miRNA expression profiles to mRNA expression profiles of naïve and day 10 effector OT-I CD8$^+$ T cells from influenza infection predicted the regulation of multiple canonical pathways involved in T cell regulation by miRNAs. The two most-regulated pathways are S1P and GPCR signaling, both with more than 20% of the pathways' molecules predicted to be regulated by differentially expressed miRNA in effector CTL. IPA analysis also showed miRNA-regulation of the leukocyte extravasation signaling pathway in effector CTL. The S1P signaling pathway was the most targeted pathway of unique to naïve- or effector CTL expressed miRNA, and GPCR signaling was also targeted by miRNA uniquely expressed in effector CTL. As S1P and GPCR pathways are known to regulate T cell migration (43, 44), the above suggest that miRNA may preferentially control or direct CTL trafficking. This is in agreement with Dicer$^{-/-}$ CTL studies that revealed trafficking as a major defect of CTL lacking mature miRNA (45). Our analyses identified the regulation of multiple signaling pathways known to be important in T cell responses in both naïve and effector anti-viral CD8$^+$

T cells including PTEN, PI3K/Akt, NF-κB, and JAK/Stat signaling. We (46) and others (47, 48) have previously demonstrated the importance of PI3K and the p110δ PI3K isoform for CTL responses, while PTEN is a negative regulator of PI3K signaling and T cell activation (49, 50). Finally, JAK/Stat and NF-κB signaling pathways are critical for CTL proliferation, survival, and differentiation (51). Taken together, the potential regulatory role of miRNAs in controlling these key pathways in CTL emphasizes the need for greater understanding of specific miRNA contribution to effective CTL responses during infection and cancer.

Our study also calls into question the functional contribution of individual miRNAs over the course of the CTL response to infection despite evidence of the modulation of a particular miRNA's expression. Microarray analysis identified miR-139 as one microRNA highly downregulated in effector anti-viral CTL, downregulation which was confirmed by qRT-PCR of *in vitro* activated or cytokine treated $CD8^+$ T cells. In our study, we have directly tested the functional contribution of miR-139 expression in the development of T cells and maintenance of their naïve state in the periphery, as well as in the development of effector CTL and memory $CD8^+$ T cells following influenza virus infection. In two model systems (global deficiency and $CD8^+$ T cell intrinsic deficiency), the absence of miR-139 did not affect the frequency, numbers, or phenotype of virus-specific CTL at either acute primary infection or a memory time point of 60 days post-infection. We also did not observe any differences in weight-loss in these animals, indicating that miR-139 does not significantly contribute to influenza-induced morbidity. This is in contrast to a previous study by Trifari, *et al*., who observed a decrease in both Eomes and perforin expression in $CD8^+$ T cells in response to *Listeria* infection when miR-139 was overexpressed selectively in $CD8^+$ T cells by retroviral transduction (23). In our study, we also forced the overexpression of miR-139 by retroviral transduction. However, in response to influenza virus infection, miR-139 overexpression failed to significantly influence Eomes expression in $CD8^+$ T cells. This discrepancy may be reflective of *Listeria* resulting in a systemic infection whereas influenza infection is localized to the lung, but would also reaffirm that miRNA-mediated regulation of mRNA in T cells is context dependent (9).

Our previous findings that knocking out a single miRNA that is highly upregulated upon activation of $CD8^+$ T cells, miR-155, has profound effects on the ability of $CD8^+$ T cells to effectively control influenza virus infection (3). For this study, we used the readily-available technique of microarray profiling to evaluate changes in miRNA expression between the naïve and influenza virus-specific $CD8^+$ T cells. A major limitation of this method however is that we can only directly compare expression levels of the same miRNA between two conditions, and we are unable to draw any conclusions based on the relative abundance of different miRNAs. In future studies, the use of miRNA-deficient mice and RNA-seq quantification may provide key insight into whether a threshold of miRNA expression exists for which expression levels below this threshold hold negligible biological consequences for miRNA-deficiency. Whether

universally applicable thresholds can be determined, or whether target abundance dictates a wide range of such thresholds remains to be seen.

Taken together, our study provides greater insight into the dynamics of miRNA expression in $CD8^+$ T cells by providing us with a snapshot of the expression profiles of naïve and *in vivo*-generated influenza virus-specific $CD8^+$ T cells at the peak of the adaptive immune response to infection. Importantly, our findings identify a miRNA, miR-139, that while significantly downregulated in both *in vivo* and *in vitro* activated CTL, is dispensable for the development of functional CTL responses in response to acute influenza virus infection. We have also demonstrated through the use of an *in vitro* exhaustion model system that eliminating miR-139 neither enhances nor limits the development of T cell exhaustion. Our study therefore highlights the discrepancies that can be observed in miRNA targets between overexpression versus knockout studies and reinforces the importance in considering the impact of contextual microRNA-mediated regulation.

AUTHOR CONTRIBUTIONS

JH performed infections, *in vitro* assays, adoptive transfers, and flow cytometry; MZ developed and performed *in vitro* T cell exhaustion assays and flow cytometry; CS performed retroviral transductions, infections, and flow cytometry; AC, MM, AB, and RK performed infections, flow cytometry, RT-PCR, and mouse breeding; YM performed adoptive transfers and cell sorting; AM, HL, and SE generated miR-139 knockout mice; SE and CS generated the miR-139 OE plasmid; PF and IR provided expert guidance for the design and completion of the microarray profiling; JH and PK were responsible for study design; JH, MZ, and PK were responsible for data analysis and manuscript authorship; all authors discussed the results and commented on the manuscript.

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SUPPLEMENTAL DATA

A

Mature miRNAs ≥ 2 fold change upregulated in effector CTL.				
Rank	Mature miR	Fold Change	p-value	q-value
1	miR-126a-3p	103.90	0.0130	0.0025
2	miR-182-5p	46.43	0.0001	0.0001
3	miR-183-5p	23.24	0.0001	0.0001
4	miR-195a-3p	14.08	0.0470	0.0077
5	miR-31-5p	12.38	0.0001	0.0001
6	miR-23a-5p	10.32	0.0001	0.0001
7	miR-143-3p	10.20	0.0098	0.0020
8	miR-145a-5p	10.09	0.0165	0.0030
9	miR-125b-5p	9.62	0.0001	0.0001
10	miR-27a-5p	9.24	0.0002	0.0001
11	miR-421-3p	7.59	0.0001	0.0001
12	miR-27b-5p	7.44	0.0001	0.0001
13	miR-146a-5p	6.92	0.0001	0.0001
14	miR-210-3p	5.98	0.0013	0.0004
15	miR-449a-5p	5.85	0.0013	0.0004
16	miR-7030-5p	5.80	0.0360	0.0062
17	miR-7666-3p	4.90	0.0002	0.0001
18	miR-183-3p	4.51	0.0077	0.0017
19	miR-301a-3p	4.43	0.0165	0.0030
20	miR-132-3p	3.74	0.0001	0.0001
21	miR-147-3p	3.65	0.0001	0.0001
22	miR-7648-3p	3.44	0.0017	0.0004
23	miR-6937-5p	3.30	0.0001	0.0001
24	miR-155-5p	3.20	0.0003	0.0001
25	miR-18a-5p	3.03	0.0001	0.0001
26	miR-6968-5p	3.02	0.0009	0.0003
27	miR-1934-3p	2.97	0.0038	0.0009
28	miR-21a-5p	2.90	0.0013	0.0004
29	miR-22-3p	2.88	0.0006	0.0002
30	miR-298-5p	2.84	0.0008	0.0003
31	miR-7221-3p	2.82	0.0001	0.0001
32	miR-6970-5p	2.82	0.0001	0.0001
33	miR-199a-3p	2.69	0.0009	0.0003
34	miR-199b-3p	2.69	0.0009	0.0003
35	miR-346-3p	2.66	0.0001	0.0001
36	miR-7081-5p	2.58	0.0017	0.0004
37	miR-3082-5p	2.58	0.0098	0.0020
38	miR-18b-5p	2.52	0.0001	0.0001
39	miR-7658-5p	2.49	0.0015	0.0004
40	miR-149-3p	2.46	0.0009	0.0003
41	miR-222-3p	2.46	0.0006	0.0002
42	miR-193a-5p	2.45	0.0002	0.0001
43	miR-330-3p	2.30	0.0023	0.0006
44	miR-671-5p	2.19	0.0139	0.0027
45	miR-3077-5p	2.16	0.0004	0.0002
46	miR-3110-3p	2.16	0.0059	0.0013
47	miR-212-3p	2.09	0.0374	0.0063
48	miR-501-3p	2.09	0.0046	0.0010
49	miR-6909-5p	2.03	0.0350	0.0061
50	miR-1224-5p	2.03	0.0098	0.0020
51	miR-6914-5p	2.02	0.0143	0.0027

B

Mature miRNAs ≥ 2 fold change downregulated in effector CTL.				
Rank	Mature miR	Fold Change	p-value	q-value
1	miR-669c-5p	-11.09	0.0001	0.0001
2	miR-181a-5p	-10.86	0.0001	0.0001
3	miR-192-5p	-10.77	0.0001	0.0001
4	miR-466a-3p	-9.71	0.0004	0.0002
5	miR-466e-3p	-9.71	0.0004	0.0002
6	miR-466b-3p	-9.12	0.0020	0.0010
7	miR-466c-3p	-9.12	0.0020	0.0010
8	miR-466p-3p	-9.12	0.0020	0.0010
9	miR-467c-5p	-7.85	0.0001	0.0001
10	miR-466d-3p	-7.34	0.0004	0.0002
11	miR-194-5p	-7.05	0.0005	0.0003
12	**miR-139-5p**	**-6.89**	**0.0001**	**0.0001**
13	miR-26b-5p	-6.83	0.0242	0.0086
14	miR-28a-3p	-6.64	0.0001	0.0001
15	miR-30e-5p	-6.53	0.0002	0.0002
16	miR-669a-3p	-6.48	0.0001	0.0001
17	miR-669o-3p	-6.48	0.0001	0.0001
18	miR-378a-5p	-6.41	0.0006	0.0003
19	miR-339-5p	-6.21	0.0001	0.0001
20	miR-466c-5p	-6.21	0.0087	0.0033
21	miR-342-5p	-6.17	0.0001	0.0001
22	miR-466h-5p	-5.92	0.0067	0.0026
23	miR-342-3p	-5.88	0.0001	0.0001
24	miR-181c-5p	-5.45	0.0001	0.0001
25	miR-378a-3p	-5.43	0.0001	0.0001
26	miR-467a-5p	-5.06	0.0005	0.0003
27	miR-128-3p	-4.67	0.0026	0.0012
28	miR-181b-5p	-4.64	0.0001	0.0001
29	miR-3068-3p	-4.58	0.0019	0.0010
30	miR-30b-5p	-4.12	0.0002	0.0002
31	miR-1843a-5p	-4.03	0.0407	0.0131
32	miR-467d-3p	-3.99	0.0033	0.0015
33	let-7b-5p	-3.93	0.0001	0.000
34	miR-7a-1-3p	-3.72	0.0062	0.0025
35	miR-7033-5p	-3.55	0.0310	0.0107
36	miR-669a-5p	-3.52	0.0025	0.0011
37	miR-669p-5p	-3.52	0.0025	0.0011
38	miR-1839-5p	-3.50	0.0007	0.0004
39	miR-26a-5p	-3.46	0.0001	0.000
40	miR-151-5p	-3.31	0.0003	0.0002
41	miR-30d-5p	-3.28	0.0001	0.000
42	miR-200b-3p	-3.23	0.0124	0.0046
43	let-7f-5p	-3.20	0.0426	0.0136
44	miR-150-5p	-3.20	0.0001	0.0000
45	miR-1843b-5p	-3.03	0.0001	0.0001
46	let-7d-3p	-2.98	0.0081	0.0031
47	miR-320-3p	-2.94	0.0001	0.0001
48	miR-29a-3p	-2.87	0.0008	0.0005
49	miR-28a-5p	-2.87	0.0009	0.0005
50	miR-151-3p	-2.86	0.0036	0.0015
51	miR-140-5p	-2.74	0.0286	0.0100
52	miR-674-5p	-2.70	0.0001	0.0001
53	miR-29b-3p	-2.61	0.0046	0.0019
54	miR-328-3p	-2.58	0.0445	0.0137
55	miR-30e-3p	-2.44	0.0436	0.0137
56	miR-361-5p	-2.38	0.0001	0.0001
57	miR-30c-5p	-2.31	0.0004	0.0002
58	let-7a-5p	-2.29	0.0047	0.0019
59	miR-423-5p	-2.28	0.0190	0.0069
60	miR-17-3p	-2.28	0.0001	0.0001
61	miR-200c-3p	-2.27	0.0015	0.0008
62	miR-150-3p	-2.27	0.0001	0.0001
63	miR-6988-3p	-2.24	0.0350	0.0115
64	miR-466m-5p	-2.22	0.0334	0.0111
65	miR-669m-5p	-2.22	0.0334	0.0111
66	miR-19b-3p	-2.10	0.0015	0.0008
67	miR-30c-1-3p	-2.08	0.0455	0.0138
68	miR-92a-3p	-2.07	0.0001	0.0001
69	miR-467d-5p	-2.05	0.0058	0.0024

Supplementary Figure 1. MiRNA expression profiling of effector CTL.

All miRNAs upregulated **(A)** or downregulated **(B)** greater than 2-fold relative to naïve $CD8^+$ T cells; FDR = 0.05, $p \leq 0.05$ and $q \leq 0.05$.

A

Mature miRNAs predicted to regulate genes in canonical pathways in effector CTL as shown in Figure 1.						
Th1 and Th2 Activation Pathway	**Th1 Pathway**	**Th2 Pathway**	**T Helper Cell Differentiation**	**CD28 Signaling in T Helper Cells**	**Leukocyte Extravasation Signaling**	**Communication between Innate and Adaptive Immune Cells**
miR-28a-3p	miR-28a-3p	miR-30e-5p	miR-125b-5p	miR-126a-3p	miR-30e-5p	miR-30e-5p
miR-30e-5p	miR-125b-5p	miR-126a-3p	miR-143-3p	miR-143-3p	miR-126a-3p	miR-146a-5p
miR-125b-5p	miR-126a-3p	miR-143-3p	miR-145a-5p	miR-181a-5p	miR-421-3p	miR-181a-5p
miR-126a-3p	miR-143-3p	miR-181a-5p	miR-181a-5p	miR-378a-3p	miR-7030-5p	miR-339-5p
miR-143-3p	miR-145a-5p	miR-210-3p	miR-378a-3p	miR-449a-5p		miR-342-5p
miR-145a-5p	miR-181a-5p	miR-378a-3p		miR-7030-5p		miR-378a-3p
miR-181a-5p	miR-339-5p	miR-7030-5p				
miR-210-3p	miR-378a-5p					
miR-339-5p	miR-7030-5p					
miR-378a-3p						
miR-7030-5p						

Protein Kinase A Signaling	**iCOS-iCOSL Signaling in T Helper Cells**	**Role of NFAT in Regulation of the Immune Response**	**mTOR Signaling**	**PTEN Signaling**	**Sphingosine-1-phosphate Signaling**	**G-Protein Coupled Receptor Signaling**
miR-26b-5p	miR-126a-3p	miR-126a-3p	miR-126a-3p	miR-30e-5p	miR-30e-5p	miR-27a-5p
miR-27a-5p	miR-143-3p	miR-143-3p	miR-339-5p	miR-126a-3p	miR-145a-5p	miR-31-5p
miR-30e-5p	miR-146a-5p	miR-183-5p	miR-449a-5p	miR-139-5p	miR-182-5p	miR-125b-5p
miR-31-5p	miR-181a-5p	miR-449a-5p	miR-7030-5p	miR-143-3p	miR-7030-5p	miR-126a-3p
miR-125b-5p	miR-342-5p	miR-378a-3p		miR-145a-5p		miR-145a-5p
miR-145a-5p	miR-378a-3p	miR-7030-5p		miR-182-5p		miR-182-5p
miR-181a-5p	miR-449a-5p			miR-342-5p		miR-210-3p
miR-182-5p	miR-7030-5p			miR-449a-5p		miR-449a-5p
miR-183-5p				miR-7030-5p		miR-7030-5p
miR-210-3p						
miR-449a-5p						
miR-7030-5p						

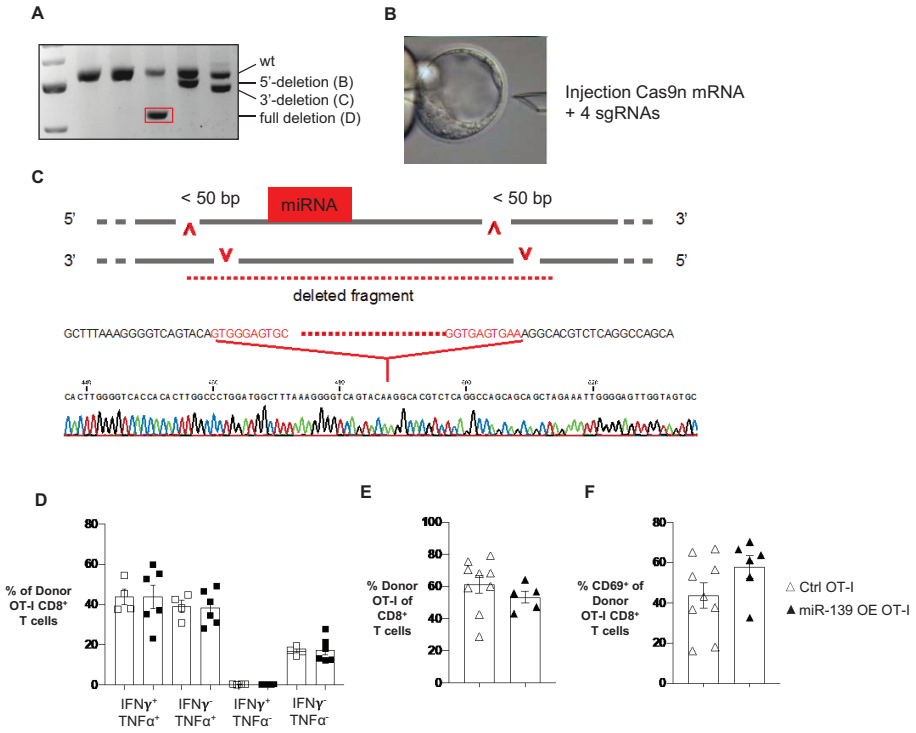
Wnt/β-catenin Signaling	**TGF-β Signaling**	**NF-κB Signaling**	**PKCθ Signaling in T Lymphocytes**	**PI3K/AKT Signaling**
miR-30e-5p	miR-30e-5p	miR-30e-5p	miR-126a-3p	miR-30e-5p
miR-125b-5p	miR-143-3p	miR-126a	miR-143-3p	miR-126a-3p
miR-145a-5p	miR-210-3p	miR-145a-5p	miR-181a-5p	miR-143-3p
miR-183-5p	miR-449a-5p	miR-146a-5p	miR-378a-3p	miR-342-5p
miR-194-5p	miR-7030-5p	miR-182-5p	miR-449a-5p	miR-449a-5p
miR-210-3p		miR-449a-5p	miR-7030-5p	miR-7030-5p
miR-449a-5p		miR-7030-5p		
miR-7030-5p				

B

Canonical pathways in effector CD8+ T cells with predicted miRNA-targeted genes.		
Rank	**Pathway**	**% Molecules Regulated**
1	Sphingosine-1-phosphate Signaling	26.9
2	G-Protein Coupled Receptor Signaling	21.8
3	PTEN Signaling	18.4
4	iCOS-iCOSL Signaling in T Helper Cells	16.0
5	Protein Kinase A Signaling	14.2
6	Wnt/β-catenin Signaling	13.1
7	Communication between Innate and Adaptive Immune Cells	12.5
8	PKCθ Signaling in T Lymphocytes	12.5
9	Th1 Pathway	11.3
10	CD28 Signaling in T Helper Cells	10.7
11	T Helper Cell Differentiation	10.6
12	Role of NFAT in Regulation of the Immune Response	9.2
13	Th1 and Th2 Activation Pathway	9.1
14	PI3K/AKT Signaling	9.1
15	mTOR Signaling	8.6
16	TGF-β Signaling	8.3
17	Th2 Pathway	8.0
18	Leukocyte Extravasation Signaling	7.0
19	NF-κB Signaling	7.0

Supplementary Figure 2. Regulation of canonical pathways by miRNA.

(A) Table of the canonical pathways predicted to be regulated by miRNAs expressed more than 5-fold upregulated or downregulated in effector anti-viral CD8$^+$ T cells, and the miRNAs predicted to be involved in regulating the molecules within each pathway. **(B)** Ranking of the canonical pathways based on the percentage of molecules predicted to be regulated in each respective pathway.



Supplementary Figure 3.

(A) Representative DNA gel electrophoresis showing full deletion of miR-139 in miR-139$^{-/-}$ mice. **(B)** Image of the insertion of Cas9n mRNA and 4 sgRNAs into a C57Bl/6 embryo. **(C)** Schematic for the deletion of miR-139. **(D)** Dot plot of the frequency of cytokine producing scrambled control or miR-139 OE donor OT-I cells from the lungs of WSN-OVA influenza virus infected mice 10 days post-infection. **(E)** Dot plot of the frequency of scrambled control or miR-139 OE donor OT-I cells within the CD8$^+$ T cell compartment in the lungs of WSN-OVA influenza virus infected mice 9 days post-infection. **(F)** Dot plot of the frequency of CD69$^+$ scrambled control or miR-139 OE donor OT-I cells in the lungs of WSN-OVA influenza virus infected mice 9 days post-infection. Representative of 4-9 mice per group from two independent experiments.

Chapter 5

Inhibition of ITK signaling with ibrutinib can directly reverse T cells exhaustion and enhances checkpoint blockade in solid tumors

Manzhi Zhao[1], Ling Li[1], Caoimhe H. Kiernan[1], Melisa D. Castro Eiro[1], Marjan van Meurs[1], Inge Brouwers-Haspels[1], Rik Ruijten[1], Merel Wilmsen[1], Tessa Alofs[1], Tamara van Wees[1], Dwin G.B. Grashof[1], Harmen J.G. van de Werken[1,2], Rudi W. Hendriks[3], Joachim G. Aerts[3], Yvonne M. Mueller[1], Peter D. Katsikis[1*]

[1] Department of Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands

[2] Cancer Computational Biology Center, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, the Netherlands

[3] Department of Pulmonary Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands

In preparation

ABSTRACT

CD8+ T cell (CTL) exhaustion is driven by chronic antigen stimulation and is characterized by specific molecular, phenotypic and functional changes. Reversing CTLs exhaustion with immune checkpoint blockade (ICB) has provided clinical benefits in different types of cancer. In some lymphoid malignancies, the antitumor effect of ICB is enhanced by ibrutinib, an inhibitor of both Bruton's tyrosine kinase (BTK) and IL2-inducible T-cell kinase (ITK). We therefore investigated whether modulating chronic antigen stimulation and TCR signaling by ibrutinib could directly reinvigorate exhausted CTL and enhance ICB therapy. Treatment of *in vitro* exhausted T cells with ibrutinib and ITK-specific inhibitor enhanced cytokine production, decreased inhibitory receptor expression and downregulated the transcription factor TOX while upregulating TCF1. RNAseq revealed that ibrutinib reduced the exhaustion related transcriptional profile of these cells. Ibrutinib mediated reversal of CTL exhaustion was BTK independent, indicating ITK targeting. Finally, *in vivo* treatment of tumor bearing mice with ITK inhibitors improved ICB in terms of solid tumor growth and CTL exhaustion. Our study demonstrates that ibrutinib and ITK inhibition can be used to directly ameliorate CTL exhaustion and enhance ICB immunotherapies in cancer.

INTRODUCTION

The functional impairment that accompanies CTL exhaustion represents a significant barrier for efficient immunity in chronic infections and cancer (1-4). Immunotherapies that target the reinvigoration of exhausted CTL can contribute to tumor control, especially in melanoma and other cancers (5-10). Up till now, the most successful immunotherapy has been the blocking of the PD-1/PD-L1 inhibitory receptor pathway. Critical for this ICB efficacy are progenitor exhausted CTL, which are defined as transcription factor T cell factor 1 (TCF1) positive (11-14). Despite ICB's success, there still remain large proportions of patients that do not benefit from ICB therapy. Therefore, developing novel treatments that could improve the efficiency of ICB is greatly needed (15).

Ibrutinib is a potent inhibitor of BTK, which is a critical kinase for normal B cell development and function but also malignant B cell survival (16, 17). Therefore, ibrutinib is an efficient therapy in CLL and other B-cell malignancies (18-20). This anti-tumor activity of ibrutinib has been attributed to its direct effect on malignant B cells, but there is accumulating evidence showing that it also affects T cell immunity. Ibrutinib treatment reduces chronic activation markers, like CD39 and HLA-DR on T cells in leukemia patients (21, 22). Ibrutinib improves CD19 chimeric antigen receptor T-cell (CAR T cell) expansion and decreases PD-1 expression on CAR T cells, and this was accompanied by better clinical responses (23, 24). T cells in ibrutinib treated CLL patients have reduced PD-1 expression and improved cytokine production, indicating possible effects on T cell exhaustion (25). Finally, in mouse lymphoma models, ibrutinib can synergize with anti-PD-L1 treatment (26). However, the mechanism by which ibrutinib affects T cells in hematopoietic tumors remains obscure. This effect could be either direct or indirect. Chronic antigen stimulation is a driving force of T cells exhaustion (27, 28), therefore by acting on malignant B cells and reducing tumor load and antigen stimulation, ibrutinib could be reducing chronic antigen stimulation and indirectly affect T cell exhaustion. On the other hand, ibrutinib can also indirectly affect T cell exhaustion by reducing PD-L1 expression in CLL (29).

In addition to indirect effects on T cells, ibrutinib however could deliver direct effects. Besides BTK, ibrutinib also targets other TEC-family tyrosine kinases, such as the bone marrow-expressed kinase (BMX), redundant resting lymphocyte kinase (RLK) and IL-2 inducible T-Cell kinase (ITK) (30-32). By acting on ITK, a critical kinase for TCR signaling (33), ibrutinib could be tempering TCR signaling in the face of chronic antigen stimulation, thus mitigating T cell exhaustion. Therefore, ibrutinib has the potential to directly affect T cell exhaustion by inhibiting ITK.

In this study, we investigated whether ibrutinib and ITK inhibitors can directly affect T cell exhaustion. We evaluated the functional and transcriptional effects of ibrutinib and ITK inhibitor in an *in vitro* CTL exhaustion model (34). We next examined ITK inhibitor antitumor

effects in combination with anti-PD-1 therapy. We report that inhibiting ITK can improve *in vitro* key aspects of T cell exhaustion while *in vivo* intermittent inhibitor treatment synergizes with immune checkpoint blockade in different solid tumors. Intermittent inhibition of ITK with ibrutinib or ITK inhibitors could be a strategy to reinvigorate exhausted CTLs and improve ICB immunotherapy.

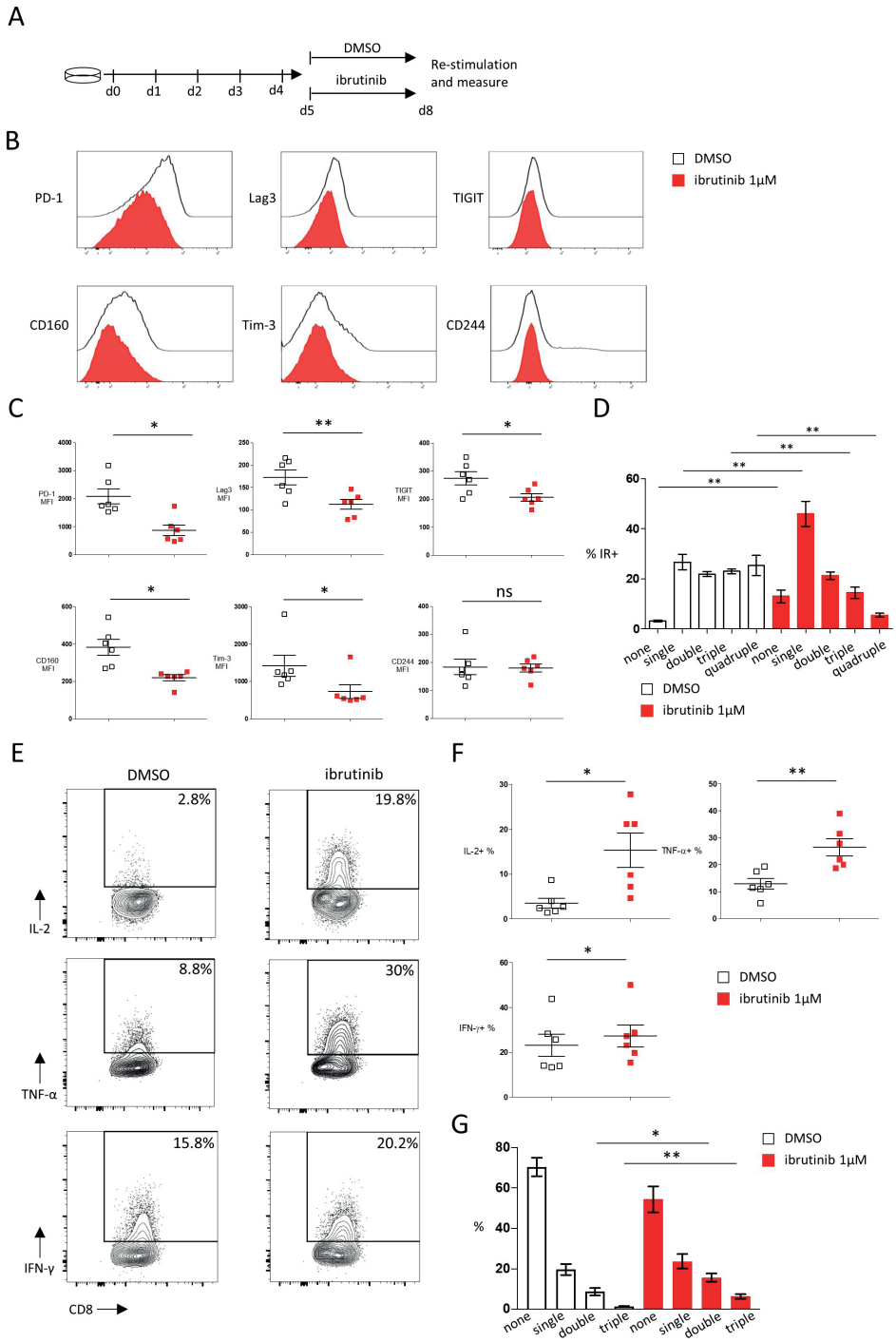
RESULTS

Ibrutinib and ITK inhibitors reverse the exhaustion-related phenotype of *in vitro* exhausted CTLs

CD8+ T cell function is improved in CLL patients who receive long term ibrutinib treatment (25), however, it is unclear whether ibrutinib is directly affecting CTL exhaustion. We therefore examined if ibrutinib would directly affect exhausted CTL in an *in vitro* exhaustion model we established (34). This model uses purified OT-I CD8+ T cells that are driven to be exhausted by repeated stimulation with $OVA_{(257-264)}$ peptide for 5 days. Using this model, *in vitro* exhausted cells were treated with 1μM ibrutinib for an additional 3 days, and then cells were analyzed for exhaustion characteristics (Fig 1A). We found that ibrutinib treatment lowered the expression of inhibitory receptors, including PD-1, Lag3, TIGIT, Tim3 and CD160 (Fig 1B and 1C). Cells expressing multiple inhibitory receptors were also decreased with ibrutinib treatment (Fig 1D and 2A). Cytokine production after peptide re-stimulation improved with ibrutinib treatment, and there were more cells producing IL-2 cells and TNF-α (Fig 1E

Figure 1 (see next page). Ibrutinib rejuvinates *in vitro* exhausted CD8+ T cell functions.

(A) Scheme of testing ibrutinib effects on *in vitro* CTL exhaustion. By repeatedly stimulating the purified OT-I T cells with $OVA_{(257-264)}$ peptide for 5 days, *in vitro* exhausted CTLs were induced. From day 5, the cells were treated with DMSO or 1μM ibrutinib. On day 8, function and phenotype of the cells were determined. **(B)** Representative histograms depicting the expression of inhibitory receptors on DMSO or ibrutinib treated exhausted cells on day 8 of the *in vitro* exhaustion. **(C)** Pooled data showing the median fluorescence intensity (MFI) of the inhibitory receptors expressed on DMSO or ibrutinib treated cells. **(D)** Bar chart depicting frequency of cells expressing either one, two, three or four of the inhibitory receptors PD-1, Lag3, Tim-3 and TIGIT. **(E)** Representative FACS plots illustrating percentage of cytokine producing CD8+ T cells upon $OVA_{(257-264)}$ peptide re-stimulation. Exhausted OT-I CD8+ T cells were re-stimulated on day 8 for 6 hours with $OVA_{(257-264)}$ peptide and intracellular cytokines were measured by flow cytometry. **(F)** Pooled data showing the frequency of cytokine producing CD8+ T cells. **(G)** Bar graph depicting the frequency of cells producing either one, two, or three of the cytokines (IL-2, TNF-α and IFN-γ) simultaneously. Each symbol represents one animal (n=6), 4 independent experiments performed. Lines depict mean ± SE. Between the groups, paired-t test was performed, except for MFI of PD-1, CD160 and Tim3, where the Wilcoxon matched-pairs signed rank test was used. *<0.05, **P<0.01.


A
DMSO
ibrutinib
Re-stimulation and measure
d0
d1
d2
d3
d4
d5
d8
B
PD-1
Lag3
TIGIT
CD160
Tim-3
CD244
DMSO
ibrutinib 1μM
C
PD-1 MFI
Lag3 MFI
TIGIT MFI
CD160 MFI
Tim-3 MFI
CD244 MFI
ns
D
% IR+
none
single
double
triple
quadruple
DMSO
ibrutinib 1μM
E
DMSO
ibrutinib
2.8%
19.8%
8.8%
30%
15.8%
20.2%
IL-2
TNF-α
IFN-γ
CD8
F
IL-2+ %
TNF-α+ %
IFN-γ+ %
DMSO
ibrutinib 1μM
G
%
none
single
double
triple
DMSO
ibrutinib 1μM

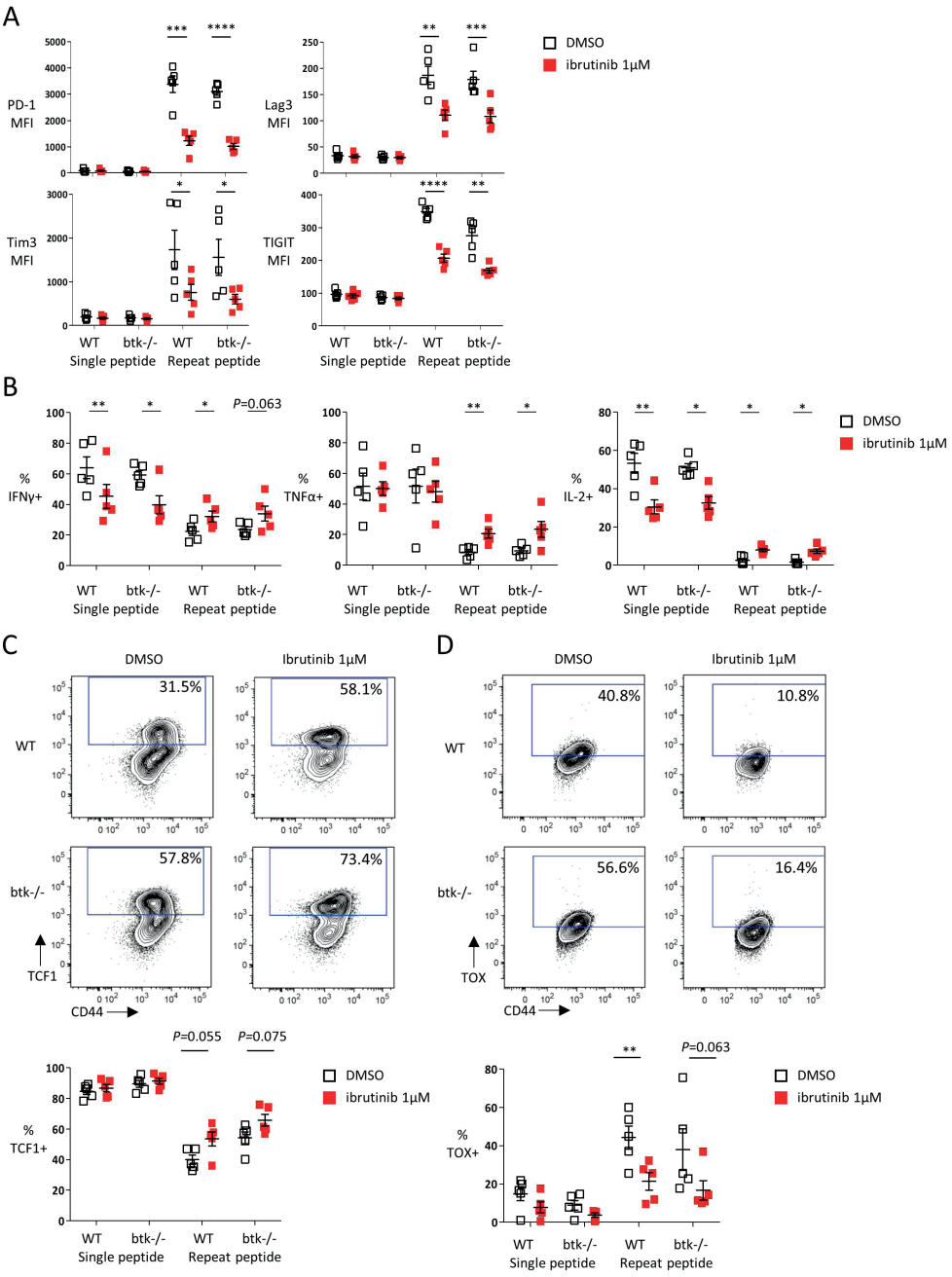
and 1F, 2B). When the simultaneous production of these three cytokines was analyzed, the frequency of double and triple cytokine producing cells were significant increased upon ibrutinib treatment (Fig 1G), which indicates that ibrutinib treatment partially rescues the polyfunctionality of exhausted T cells. Ibrutinib treatment also improved the exhaustion-related transcription factor profile of the exhausted cells as it decreased TOX expression while increasing TCF1 expression in wild type cells (Fig 2C and 2D). Overall, these results indicated that ibrutinib could directly ameliorate key features of CTL exhaustion by downregulating inhibitory receptors, improving cytokine production and polyfunctionality while downregulating TOX and increasing TCF1 transcription factors.

Ibrutinib improves CD8+T cell exhaustion independent of BTK

In order to understand how ibrutinib is acting to directly reverse exhaustion, we first performed Western blot to exclude that BTK is expressed in *in vitro* exhausted T cells (Fig S1A). To further exclude a role for BTK in ibrutinib's effect, we submitted *btk*-/- OT-I cells to our *in vitro* exhaustion protocol and found that *btk*-/- T cells, similar to wild type OT-I were readily exhausted *in vitro.* When *btk*-/- OT-I cells were *in vitro* exhausted and treated with ibrutinib, ibrutinib retained its ability to ameliorate exhaustion-related features of these cells. Ibrutinib could decrease inhibitory receptor expression (Fig 2A) as well as improve cytokine production in *btk*-/- OT-I cells (Fig 2B). Ibrutinib also prevented the upregulation of TOX in *in vitro* exhausted *btk*-/- T cells (Fig 2D). Additionally, a higher frequency of repeat peptide stimulated *btk*-/- T cells maintained TCF1 expression after ibrutinib treatment (Fig 2C).

Since *btk*-/- OT-I cells could still be driven to exhaustion and ibrutinib could reverse this, we examined whether ITK may play this role in exhaustion. Ibrutinib is known to inhibit ITK and downstream PLC-γ1 phosphorylation in T cells (35), and ITK is an important kinase that regulates TCR signaling in CD4+ T cells and CD8+ T cells (33). In addition, chronic TCR signaling can lead to exhaustion (27, 28). Therefore, we first tested if ITK activity was increased

Figure 2 (see next page).The T cell exhaustion decreasing effect of Ibrutinib is independent of Btk expression.
Purified OT-I cells from wild type (WT) or *btk* deficient (*btk-/-*) mice were stimulated once with $OVA_{(257-264)}$ peptide or repeatedly stimulated for 5 days, and then treated with DMSO or 1μM ibrutinib till day 8, when phenotype and function of cells was determined. **(A)** Pooled data depicting the expression of inhibitory receptors. **(B)** Pooled data showing the frequency of cytokine producing cells re-stimulated on day 8 with $OVA_{(257-264)}$ peptide for 6h. **(C)** Representative flow cytometry plots (top) and pooled data (bottom) illustrating the frequency of TCF1+ CD44+ CD8+ T cells from either WT or *btk-/-* mice after treatment with DMSO or 1μM ibrutinib. **(D)** Representative flow cytometry plots (top) and pooled data (bottom) showing the frequency of TOX+ CD44+ CD8+ T cells from either WT or *btk-/-* mice after DMSO or 1μM ibrutinib treatment. Each symbol represents one animal (n=5), 5 independent experiments performed. Lines depict mean ± SE. To compare different groups, paired-t test was performed with exemption of (B) % of IL-2+ cells and **(D)** % of TOX+ cells, where Wilcoxon matched-pairs signed rank test was used . *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.


A
PD-1 MFI
Lag3 MFI
Tim3 MFI
TIGIT MFI
DMSO
ibrutinib 1μM
WT
btk-/-
Single peptide
Repeat peptide
B
% IFNγ+
% TNFα+
% IL-2+
P=0.063
C
DMSO
Ibrutinib 1μM
31.5%
58.1%
57.8%
73.4%
WT
btk-/-
TCF1
CD44
% TCF1+
P=0.055
P=0.075
D
40.8%
10.8%
56.6%
16.4%
TOX
% TOX+

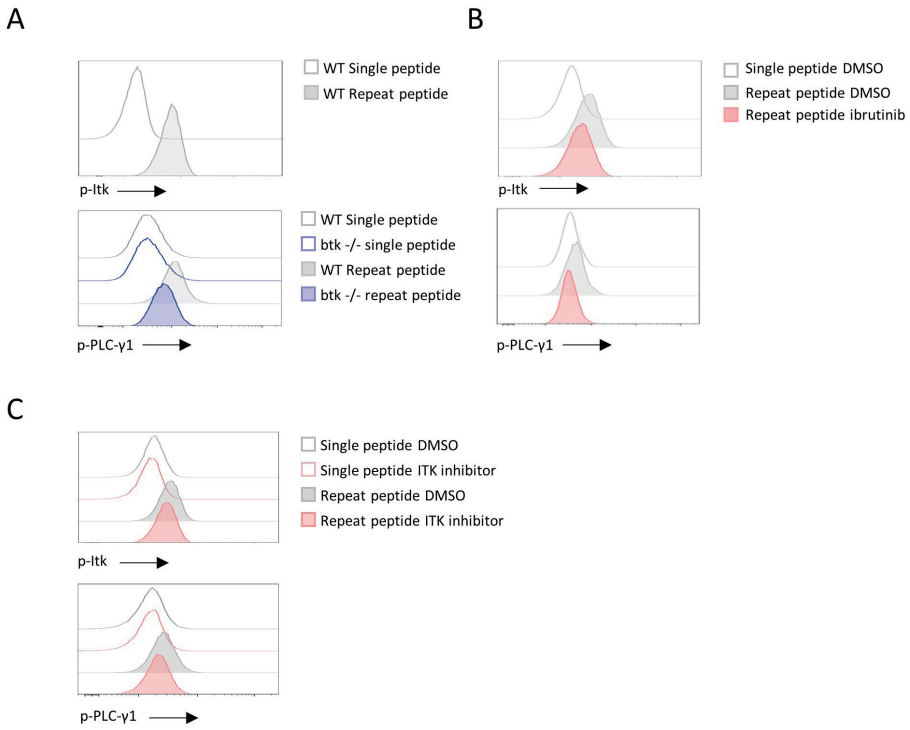


Figure 3. ***In vitro*** **exhausted CTL upregulated ITK activity.**
Purified OT-I cells from wild type (WT) or btk deficient (btk-/-) mice were stimulated one time with $OVA_{(257-264)}$ peptide or repeatedly stimulated with the peptide for 5 days. ITK (Tyr180) and PLC-γ1 (Tyr783) phosphorylation was determined by flow cytometry on day 5. **(A)** Representative histograms showing ITK and PLC-γ1 phosphorylation in single or repeat peptide simulated WT or btk-/- cells. **(B)** Representative histograms depicting the ITK and PLC-γ1 phosphorylation in single or repeat peptide stimulated cells that were treated on day 5 with ibrutinib or DMSO and harvested on day 8. **(C)** Representative histograms depicting the ITK and PLC-γ1 phosphorylation in single or repeat peptide simulated WT cells that were treated on day 5 with ITK inhibitor (BMS-509744) and stained on day 8.

in *in vitro* exhausted CTL. We find that both ITK (Tyr180) and its downstream target, PLC-γ1 (Tyr783), were highly phosphorylated in exhausted OT-I cells (Fig 3A). Treatment with ibrutinib or ITK inhibitor BMS-509744 decreased phosphorylated ITK and PLC-γ1 in these exhausted T cells (Fig 3B and 3C).

We next examined whether the ITK inhibitor BMS-509744 could also affect exhaustion in the *in vitro* exhaustion model. ITK inhibition, we found, downregulated multiple inhibitory receptors (Fig 4A). Furthermore, the frequency of IL-2 and TNF-α producing cells increased after ITK inhibitor treatment (Fig 4B). Finally, ITK inhibition downregulated TOX expression (Fig 4C left) although the expression of TCF1 remained unchanged.

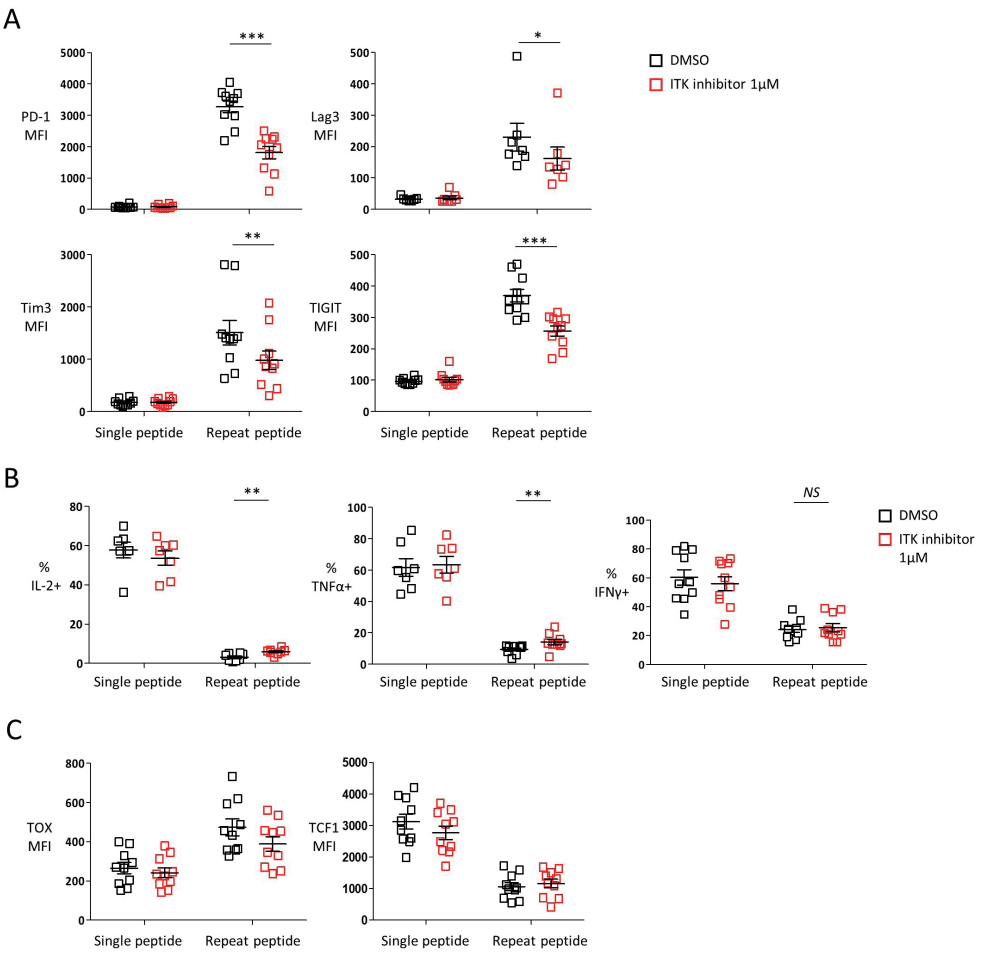


Figure 4. Inhibiting ITK reverses CTL exhaustion.

OT-I T cells were stimulated with $OVA_{(257-264)}$ peptide once (single peptide), or repeatedly for 5 days (repeat peptide) and treated either with ITK inhibitor (BMS-509744) or DMSO for another three days. On day 8 cells were harvested and phenotype and cytokine production were analyzed. **(A)** Pooled data showing the MFI of inhibitory receptors on cells. **(B)** Pooled data showing the frequency of cytokine producing cells. Cells were harvested on day 8 and re-stimulated for 6 hours with $OVA_{(257-264)}$ peptide and intracellular cytokines were measured by flow cytometry. **(C)** Pooled data depicting the MFI of transcription factors. Each symbol represents one animal (n=7-10), 7-9 independent experiments performed. Lines depict mean ± SE. Between the groups, paired-t test was performed to test for statistical significance, except for A) Lag3 MFI, Tim3 MFI, B) frequency of TNF-α+ and IFN-γ+ where Wilcoxon matched-pairs signed rank test was used. *<0.05, **P<0.01, ***P<0.001.

From the above, it is clear that dampening ITK signaling activity with pharmacological inhibitors, such as ibrutinib and BMS-509744, can directly reverse key features of CTL exhaustion *in vitro*.

Ibrutinib improves the transcriptional profile of *in vitro ex*hausted CD8+ T cells

To further confirm that ibrutinib treatment of *in vitro* exhausted CD8+ T cells, could directly change the transcriptional profile of these cells, we performed RNAseq. For this we first exhausted OT-I cells for 5 days and then treated cells with ibrutinib or left without treatment till day 8. Samples then were collected for RNAseq. By visualizing the RNAseq results with Principle Component Analysis (PCA), we found that single peptide stimulated cells and repeat peptide stimulated cells were distinctly separated. However, ibrutinib treatment drove the repeat peptide stimulated cells to clustere more closely with single peptide stimulated cells (Fig 5A). There were 1746 significant differentially expressed genes between the exhausted cells with or without ibrutinib treatment (Fig 5B). Specifically, ibrutinib downregulated the expression of genes encoding inhibitory receptors *Pcdc1* (1.8 fold), *Lag3* (1.9 fold) and *Tigit* (2.1 fold), but did not affect *Havcr2*/*Tim3a*, *Cd160* and *Cd244a* (Fig 4C). Ibrutinib also corrected some of the transcription factor changes that are induced by T cell exhaustion. *Tox* and *Irf4* were downregulated by 1.6 and 2 fold, respectively. *Tcf7* and *Eomes* were upregulated (1.8 fold and 2.2 fold, respectively) while *Tbx21*, Batf and Nfatc1 were not affected (Fig 4C). We next used gene set enrichment analysis (GSEA) to confirm whether ibrutinib reduced the exhausted related transcriptional profile of *in vitro* exhausted CD8+ T cells. We found that ibrutinib treated cells downregulated genes that are upregulated by T cell exhaustion while upregulating genes that are downregulated by exhaustion (Fig 5D) thus mitigating the gene expression changes induced by exhaustion. Overall, these results indicated that ibrutinib acts directly on exhausted CD8+ T cells and decreases their exhaustion-associated gene transcriptional signature.

ITK inhibition enhances the anti-tumor effect of checkpoint blockade therapy

Ibrutinib has been shown to surprisingly enhance the effect of checkpoint blockade in breast and colon cancer solid tumor models in Balb/c mice (26). To validate our hypothesis that inhibiting ITK activity would reverse CTL exhaustion and enhance anti-tumor effects of checkpoint blockade, we tested the effect of ITK inhibitor BMS-509744 in C57BL/6 mouse tumor models. To avoid, continuous ITK inhibition blocking T cell activation, something that would impair the T cells response, we administrated the inhibitor intermittently using a 3-day cycle. We first tested animals injected with the B16-OVA melanoma tumor that expresses ovalbumin, and 7 days later we performed adoptive transfers of OT-I T cells that are TCR

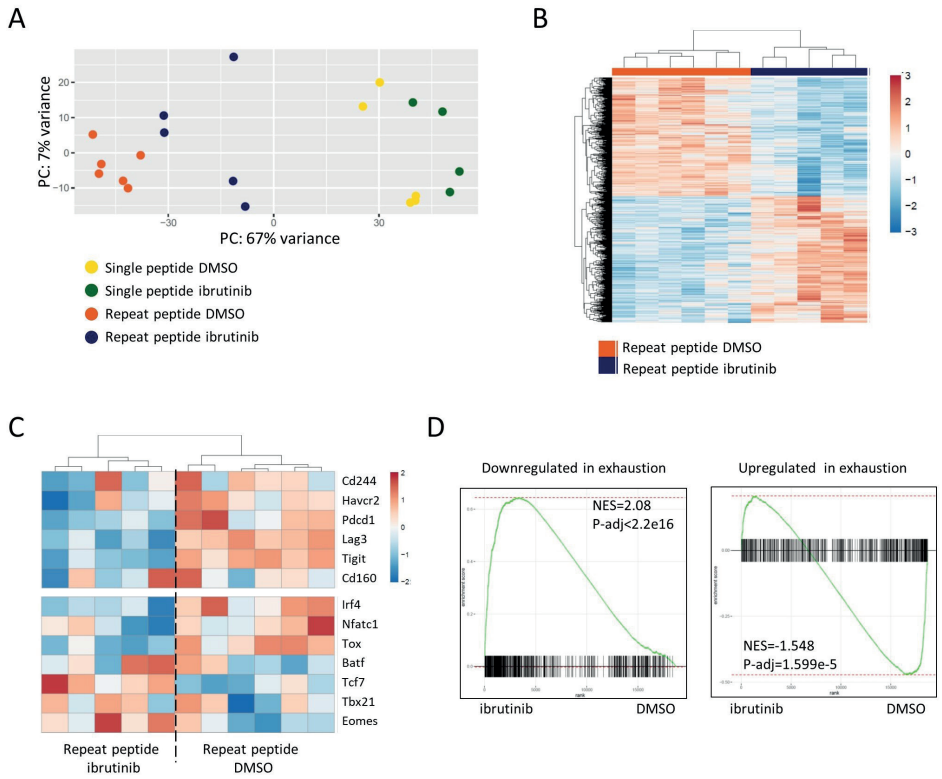


Figure 5. Ibrutinib reduces the exhaustion-related transcriptional signature of *in vitro* exhausted cells.
RNAseq was performed with the single peptide stimulated and repeat peptide stimulated cells after treatment of cells with ibrutinib or DMSO from day 5 to day 8. **(A)** Principle Component Analysis (PCA) plot of RNAseq results. **(B)** Heatmap depicting the significantly differentially expressed genes between ibrutinib-treated cells and DMSO-treated exhausted T cells. **(C)** Heatmap showing a specific subset of exhaustion-related genes (inhibitory receptors and Transcription factors). **(D)** Differentially expressed genes expression changes induced by ibrutinib in exhausted T cells were analyzed using Gene set enrichment analysis (GSEA). The gene set downregulated in exhausted T cell is enriched in genes upregulated by ibrutinib (left) while the gene set upregulated in exhausted T cell is enriched in genes downregulated by ibrutinib (right).

transgenic cells that recognize the SIINFEKL peptide of ovalbumin (Fig 6A). We treated with BMS-509744 animals with established tumors after day 12 of tumor injection to avoid inhibition of naïve T cell priming. As expected, B16-OVA melanoma was sensitive to anti-PD-1 therapy, however, addition of ITK inhibitor treatment further improved tumor growth inhibition and survival (Fig 6B). The mice that received isotype control treatment had a median survival time of 24 days and ITK inhibitor treatment alone resulted in a similar median survival of 27 days. However, combining ITK inhibitor with anti-PD-1 therapy could further extend the

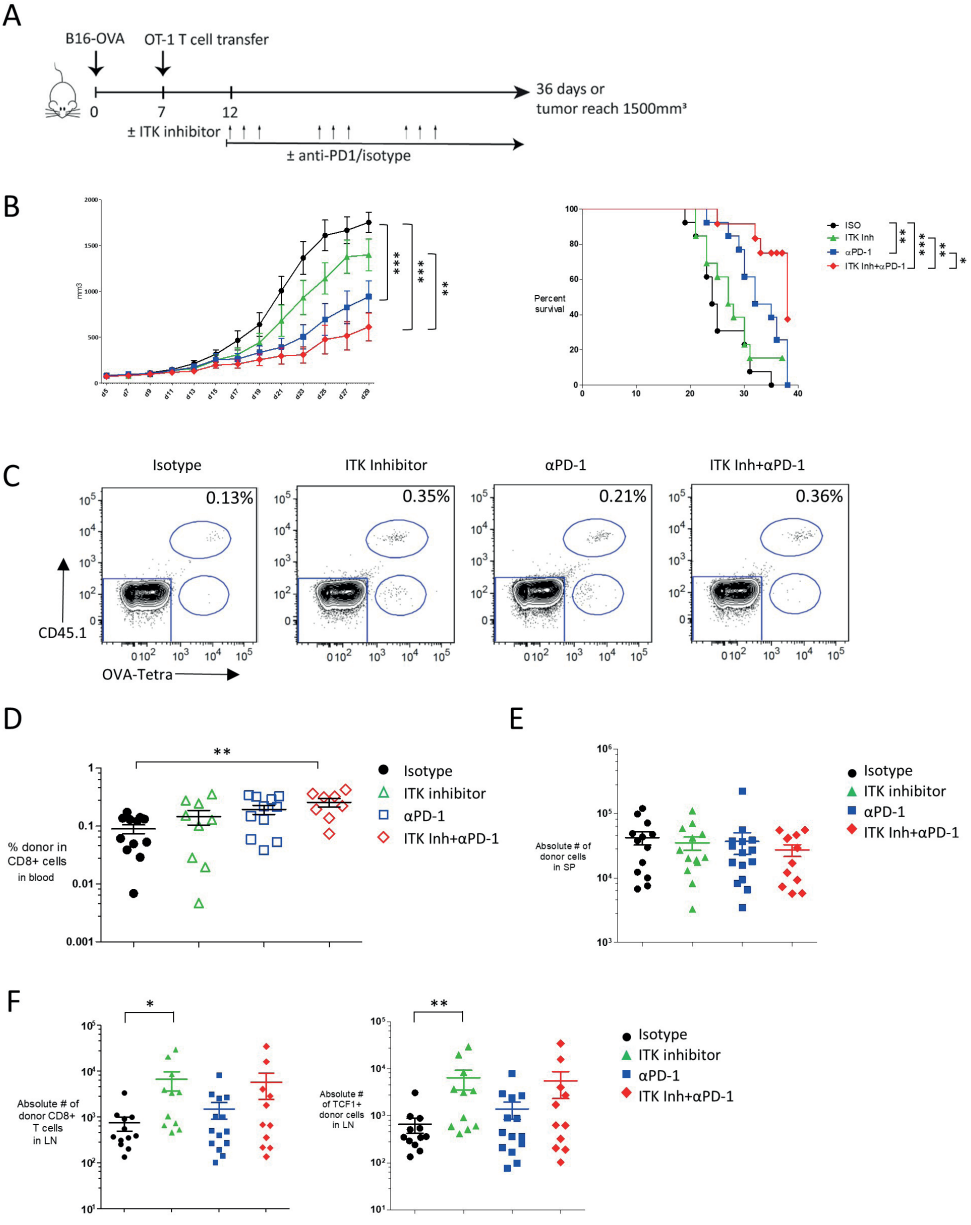

A
B16-OVA
OT-1 T cell transfer
0
7
12
± ITK inhibitor
± anti-PD1/isotype
36 days or
tumor reach 1500mm³
B
mm3
Percent survival
ISO
ITK Inh
αPD-1
ITK Inh+αPD-1
C
Isotype
ITK Inhibitor
αPD-1
ITK Inh+αPD-1
0.13%
0.35%
0.21%
0.36%
CD45.1
OVA-Tetra
D
% donor in CD8+ cells in blood
Isotype
ITK inhibitor
αPD-1
ITK Inh+αPD-1
E
Absolute # of donor cells in SP
F
Absolute # of donor CD8+ T cells in LN
Absolute # of TCF1+ donor cells in LN

Figure 6 (see previous page). ITK inhibitor enhances the anti-tumor effects of anti-PD-1 in the murine B16-OVA model.

(A) Scheme of *in vivo* experimental set up shown. **(B)** Group based tumor volume curve (left) and Kaplan-Meier survival curve (right) are depicted. **(C)** Representative flow cytometry plots showing the percentage of donor CD45.1+ OVA-specific CD8+ T cells in the peripheral blood of tumor-bearing mice. **(D)** Pooled data showing frequency of donor cells within total CD8+ T cells in peripheral blood from indicated groups 20 days post tumor injection. One way ANOVA with Tukey's post test was used to compare frequency of donor CD8+ T cells from the different treatment groups. **(E)** Pooled data depicting the absolute numbers of donor CD8+ T cells (CD45.1+CD8+) in spleen of mice shown. **(F)** Absolute numbers of donor CD8+ T cells and absolute numbers of TCF1+ donor CD8+ T cells in the draining lymph nodes. Mann Whitney test was used to test significant differences between treatment groups. Each symbol represents one animal (n=9-15), 3-4 independent experiments performed. Lines depict mean ± SE. *<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

median survival time (from 32 days to 38 days) (Fig 6B right). Anti-PD-1 treatment alone or in combination with ITK inhibitor increased the frequency of donor CD8+ T cells in peripheral blood 20 days post-tumor injection (Fig 6C and 6D). CD8+ T cells in lymphoid organs were assessed when animals reached the endpoint of 1500mm^3 tumor size or at the end of the experiment. Although there was no difference in the donor cell number in spleens (Fig 6E), more donor cells could be found in the tumor draining lymph node of animals treated with ITK inhibitor (Fig 6F) and nearly all of these cells maintained TCF1 expression. Thus TCF1+ donor cells that respond to ICB were increased in draining lymph nodes with ITK inhibition.

We next examined the tumor infiltrating CD8+ T cells (TILs). Anti-PD-1 treatment alone or combined with ITK inhibitor induced higher absolute numbers of donor OT-I cells and endogenous OVA-specific TILs in tumors (Fig 7A and 7B). Addition of ITK inhibitor to anti-PD-1 treatment did not change TCF1 or TOX expression in intratumor donor OT-I cells but did improve these for the endogenous CD8+ T cell response (Fig 7C and 7D). The addition of ITK inhibitor to anti-PD-1 reduced the MFI of PD-1 and CD160 on donor OT-I cells but did not affect Lag3 or Tim3 expression (Fig S2A). ITK inhibitor added to anti-PD-1 also reduced the frequency of PD-1+, CD160+ and PD-1+Tim3+ cells on the endogenous TILs in tumors (Fig S2B). When the co-expression of multiple inhibitory receptors was analyzed, it was found that ITK inhibitor added to anti-PD-1 treatment decreased the frequency of the endogenous TILs expressing all four inhibitory receptor at the same time (Fig 7E) and increased cells with no inhibitory receptor. Together, these data illustrated that ITK inhibitor improves antitumor effects of ICB, by accumulating pre-terminally exhausted TCF1+ tumor-specific donor cells in the draining lymph nodes but also reducing the exhaustion state of TIL even at the tumor endpoint.

ITK inhibition enhances anti-tumor effects of ICB in resistant tumors

We next evaluated the anti-tumor effects of ITK inhibitor in combination with ICB, in two tumors less sensitive to ICB therapy, the AE17 mesothelioma and 4662 pancreatic tumor cell lines (36, 37). With the AE17 tumor, the treatment started 20 days after the inoculation of

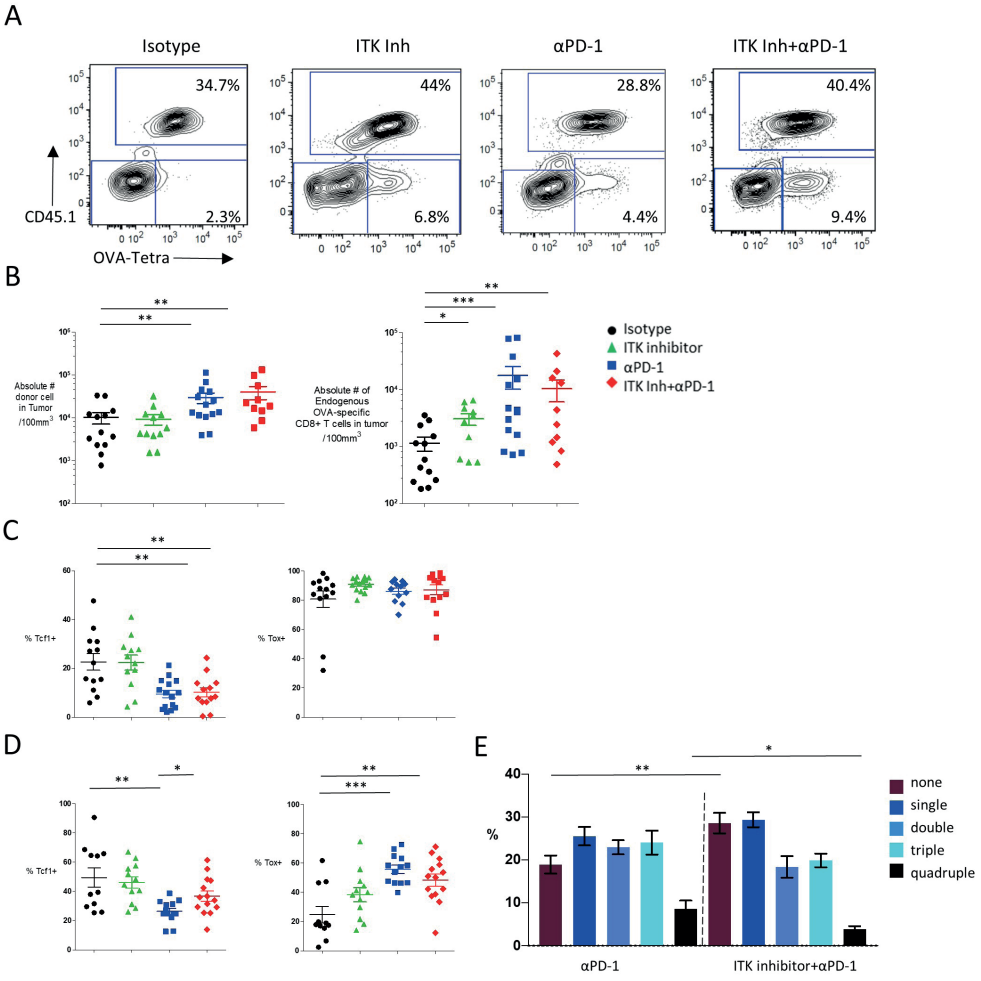


Figure 7. Reduced exhaustion of endogenous anti-tumor T cells with ITK inhibition.

(A) Representative flow cytometry plots showing the frequency of donor OT-I cells and endogenous OVA-specific CD8+ T cells in tumor infiltrating lymphocytes (TIL). **(B)** Absolute number of donor CD8+ T cells and endogenous OVA-specific CD8+ T cells per 100mm^3 of tumor are shown. **(C)** Pooled data depicting the percentage of TCF1- and TOX-expressing donor cells in tumor. **(D)** Pooled data showing the frequency of TCF1- and TOX-expressing endogenous tumor infiltrating CD8+ T cells. **(E)** The frequency of endogenous tumor infiltrating CD8+ T cells expressing one, two, three or four inhibitory receptors at the same time are shown. Mann Whitney test was performed between the groups, except for C were an unpaired t test with Welch's correction was performed. Each symbol represents one animal (n=10-14), 4 independent experiments performed. Lines depict mean ± SE. *<0.05, **P<0.01, ***P<0.001.

tumor (Fig 8A) and only ITK inhibitor combined with anti-PD-1 had an effect on tumor growth (Fig 8B). The beneficial effects of the combined therapy was also demonstrable in terms of survival of these tumor bearing animals (Fig 8C).

In the 4662 pancreatic tumor model, treatments were started when the tumor was established (Day 5) (Fig 8D). Similar to the AE17 tumor model, combined ITK inhibitor and anti-PD-1 treatment could decreased tumor growth, and improved ICB alone therapy (Fig 8E). Overall, the results from ICB insensitive tumor models indicated that tumor growth could be suppressed by the combined therapy with ITK inhibitor.

DISCUSSION

Ibrutinib's effects on T cell immunity in B cell malignancies has been described in recent years but it remains unclear if this is the result of reduced antigenic load as a consequence of BTK inhibition of malignant B cells or a potential indirect effect on other immune cell populations because of off-target inhibitory effects. In a previous clinical study, it was found that the CLL patients who received ibrutinib treatment, maintained increased CD4+ and CD8+ T cells counts while it markedly diminish PD-1 and CTLA-4 expression on T cells (38). Since chronic antigen stimulation plays a critical role in T cell exhaustion (27, 28), reducing B cell tumor load with ibrutinib could indirectly affect T cell exhaustion. Indeed, these T cell effects were suggested to be due to a BTK independent mechanism, because the highly specific BTK inhibitor, acalabrutinib could not induce these T cell changes (39). When CD19-targeted CAR T cell therapy was combined with ibrutinib, it was found that ibrutinib enhanced the function and engraftment of CAR T cells (23). Although the effects of enhancing CAR T cells expansion could also be caused by the reduced B cell tumor burden, better CAR T cells expansion was also observed in the patients who had failed ibrutinib treatment alone (40). Therefore, the greater expansion of polyfunctional CAR T cells could be due to ibrutinib acting on CAR T cells or other immune cells populations. In our study, we demonstrate that ibrutinib can directly act on T cells and mitigate T cell exhaustion. We show this by using an *in vitro* CTL exhaustion induction method, where only CD8+ T cells are present (34). In this system, ibrutinib ameliorates many of the functional, transcription factor and transcriptional changes of T cell exhaustion. Ibrutinib not only downregulated inhibitory receptors, but also improved these cells functionally as it increased cytokine production. Ibrutinib resulted in a less exhausted transcriptional signature with changes in key exhaustion-related transcription factors. Thus ibrutinib can act directly on T cells and reverse or prevent T cells exhaustion.

By far, the most promising tumor immunotherapy to date, ICB, exhibits only limited efficacy in many solid tumors. Therefore, identifying treatments that enhance ICB effects are highly desirable. Ibrutinib has been shown to augment ICB in tumor models in Balb/c mice

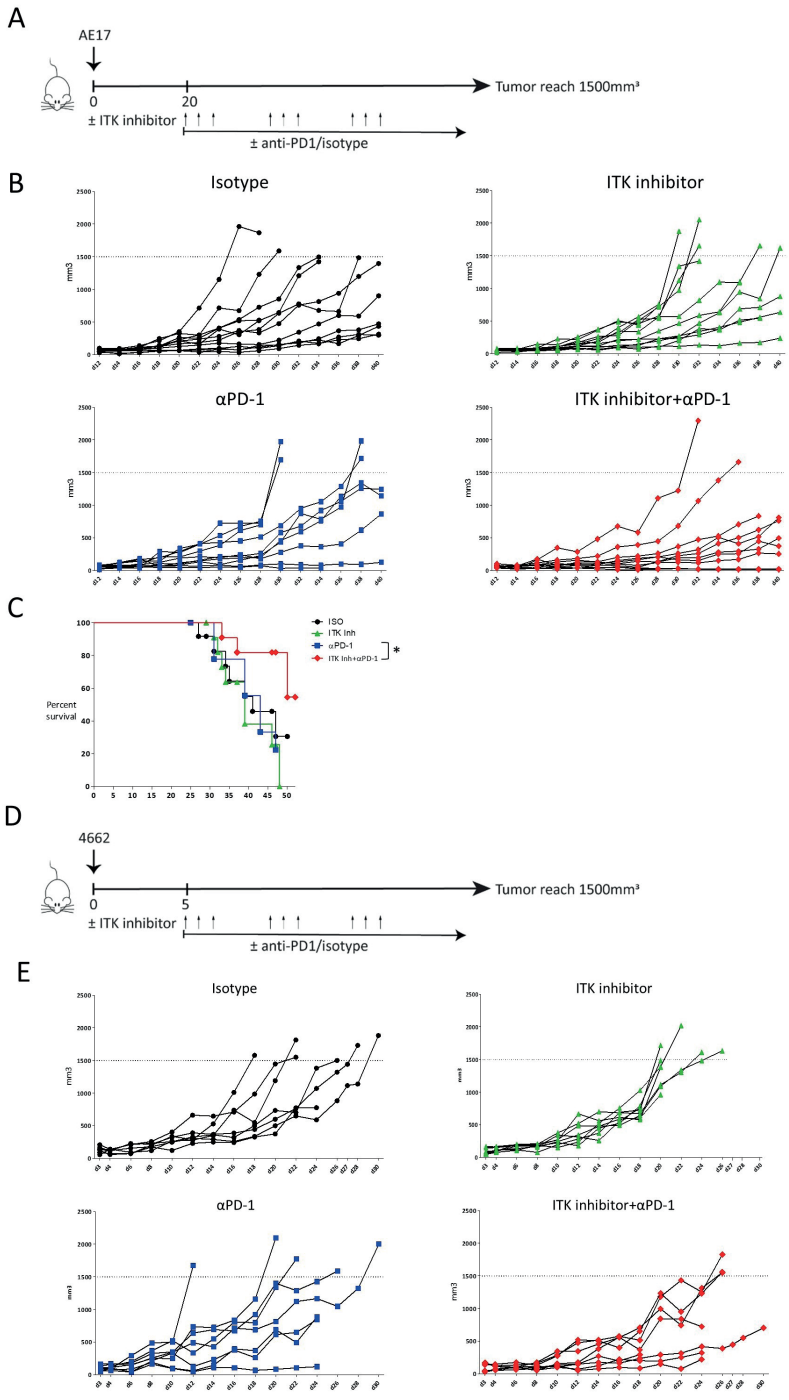

A
AE17
0
20
± ITK inhibitor
± anti-PD1/isotype
Tumor reach 1500mm³
B
Isotype
ITK inhibitor
αPD-1
ITK inhibitor+αPD-1
mm3
C
ISO
ITK Inh
αPD-1
ITK Inh+αPD-1
*
Percent survival
D
4662
0
5
± ITK inhibitor
± anti-PD1/isotype
Tumor reach 1500mm³
E
Isotype
ITK inhibitor
αPD-1
ITK inhibitor+αPD-1

Figure 8 (see previous page). Combination treatment of ITK inhibitor and anti-PD-1 augments the antitumor effect of ICB in ICB unresponsive tumors.

(A) Scheme of *in vivo* experimental set up of AE17 tumor experiments is shown. Tumor growth curve **(B)** and Kaplan-Meier survival curve **(C)** is depicted. n=9-11, 3 independent experiments performed. **(D)** Scheme of *in vivo* experimental set up of 4662 tumor experiments. **(E)** Individual tumor growth curve is shown for n=7, 2 independent experiments performed. Kaplan-Meier survival analysis was performed. *<0.05.

(26). Ibrutinib could serve as an ICB enhancer based on its known ITK inhibitory capacity and our findings that inhibiting ITK can improve the anti-tumor effect of ICB. We used BMS-509744 in three solid tumor models to demonstrate ITK inhibition can improve ICB. BMS-509744 is a selective and potent ITK kinase inhibitor that can block ITK phosphorylation and downstream PLC-γ phosphorylation in both human and mouse cells (41, 42). Ibrutinib is actually a more potent inhibitor of ITK compared to BMS-509744 (42) and this may explain why *in vitro* ibrutinib had a stronger effect restoring *in vitro* exhausted CTL. However, the higher potency of ibrutinib may also be due to its ability to inhibit additional targets, such as MEK1/2 (42), that could also contribute to the T cell exhaustion improvement seen in the *in vitro* experiments. Indeed, an inhibitor of MEK, which suppresses MAPK-PI3K-mTOR pathways activity, has been shown to improve the anti-tumor effects of CD8+ T cells and ICB (43-46). Thus using ibrutinib to enhance ICB may have additional benefits compared to exclusively targeting ITK.

The question arises how is ITK inhibition improving ICB in solid tumor models. Our data suggest that the prevention of CTL exhaustion may be a major factor in this ICB treatment improvement. We find that ITK inhibition *in vivo* results in more TCF1+ cells in the draining lymph nodes of tumor bearing mice. ITK addition to anti-PD-1 treatment also improved the TCF1 positivity of intra-tumor exhaustion profile of the endogenous CD8+ T cell response. The efficacy of ICB therapy depends on stem cell-like TCF1+ T cells (13, 47), therefore the accumulation of such cells when ITK was inhibited could explain the increased efficacy of ICB in combination treatment. Our studies were performed using animals with established tumors. Because ITK plays a critical role in CD8+ T cells activation (33, 48), treatments were started a few days after tumor growth and, when applicable, adoptive T cell transfer, in order to avoid effects on antigen-specific T cells priming. We applied intermittently ITK inhibitor *in vivo*, namely, in series for three day treatments and then resting for another three days, to avoid a continuous blocking of T cell activation which would be detrimental to the anti-tumor response. Further optimization of this ITK inhibition scheme potentially could improve the effect of ICB therapy combination. As mentioned above, ibrutinib enhances CAR-T cell therapy in B cell malignancies. Our data suggest that ICB combined with ibrutinib could further improve CAR-T cell therapy not only for B cell malignancies but also solid tumors.

Our study demonstrates the direct effect of ibrutinib on exhausted CTLs, and provides insight into the extra benefits brought by ibrutinib treatment to CLL patients and CAR T cells

therapy. Importantly, our study shows that ibrutinib and ITK inhibition, by dampening TCR signaling, can be used to mitigate T cell exhaustion and augment ICB therapy in cancer. In conclusion, we provide evidence and a rationale for ibrutinib and ITK inhibitors to be used together with ICB for the treatment of solid tumors.

MATERIAL AND METHODS

In vitro CTL exhaustion induction and inhibitor treatment

We have previously described in detail the induction of *in vitro* exhausted murine CD8+ T cells (34). In brief, CD8+ T cells were purified from splenocytes of an OT-I mouse based on negative selection (EasySep, Stemcell Technologies). Repeat peptide stimulated cells were induced by adding OVA peptide $_{(257\text{-}264)}$ daily for five days. For single peptide stimulated cells, OVA peptide $_{(257\text{-}264)}$ was added once to the cells for 48 hours and washed away, followed by resting for 3 days. After a total of 5 days, cells were harvested and phenotypically and functionally characterized to analyze their exhaustion status. From day 5, single peptide and repeat peptide stimulated cells were treated with different concentrations, as indicated in the figures, of ibrutinib (MedChemExpress, No. HY10997) or BMS509744 (ITK inhibitor) (MedChemExpress, No. HY11092). On day 8, cells were harvested and either stained or reactivated with peptide. Concentrations of ibrutinib used in this study were based on results from previous clinical studies, which indicate a peak plasma concentration of ibrutinib between 340 nmol/L and 450 nmol/L after oral administration (18, 20). In addition, our preliminary experiments indicate that 1μM of ibrutinib does not induce cell death.

Animals

In-house-bred OT-I CD45.1+ mice on the C57BL6/J background were generated by backcrossing C57BL/6 Tg (TcraTcrb)1100Mjb/J (OT-I) with B6.SJL-Ptprca Pepcb/BoyJ (CD45.1+) mice (both from Charles River France). Btk deficient (*btk-/-*) OT-I mice were generated by cross breeding OT-I CD45.1+ mice with *btk* deficient C57BL/6J (kindly provided by Prof. Rudi W. Hendriks). *Btk-/-* OT-I mice were housed in a certified barrier facility at Erasmus University Medical Center. OT-I CD45.1 expression was confirmed using PCR and flow cytometry analysis. *Btk-/-* deficiency was confirmed by PCR. All the experiments in this study were carried out under ethical approval by the Instantie voor Dierenwelzijn (IvD). The Project Proposal (AVD101002015179) was approved by CCD (Centrale Commissie Dierproeven).

Tumor cell culture and tumor establishment

The B16-OVA melanoma cell line (kindly provided by M. Wolkers, Sanquin, Amsterdam) was cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco), 2 mM glutamine (Life Technologies), 100U/ml penicillin (Gibco),100µg/ml Streptomycin-sulfate (Gibco), and 50 µM β-mercaptoethanol (Sigma). In the T cell adoptive transfer experiment, 6-weeks-old C57BL/6J mice received 0.5*10^6 B16-OVA melanoma cells per mouse subcutaneously in the shaved right flank. Adoptive transfer of 0.5*10^6 CD8+ T cells from naïve OT-I CD45.1+ mice was performed on day 7 when the tumors reached an average size of 60-80mm3. All of the treatments started from day 12 post tumor injection.

AE-17 cells (kind gift from Dr. Delia Nelson, Curtin University, Perth, Australia) were maintained in RPMI 1640 supplemented with 10% FBS, 48 mg/L Gentamicin, 60 mg/L benzylpenicillin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol (Sigma). Murine Pancreatic tumor cell line 4662 (Pancreatic Ductal Adenocarcinoma, PDA) (kind gift from Prof. Robert H. Vonderheide , University of Pennsylvania, PA, USA) was cultured in DMEM supplemented with 10% FBS, 100 units/mL Penicillin/Streptomycin, 2 mM Glutamine, and 100 mg/L Gentamicin (Sigma). Six-week-old C57BL/6 mice received 0.5*10^6 of either AE-17 or PDA 4662 cells subcutaneously in the flank. Due to the variability in tumor growth between cell lines, treatment was started for the two different tumor cell lines at different time points when the tumor reached an average size of 60-100mm^3. The 4662 cell line grew faster *in vivo*, and therefore the treatment was started on day 5 post tumor injection. For the AE-17 cell line, the treatment for the mice started from day 20 post tumor cell injection. Size of the tumors were measured every other day using a digital vernier caliper. The volume of tumor was calculated using the formula: $V = L*W*H$, where V is tumor volume, L is the length of the tumor (longer diameter), W is the width of the tumor (shorter diameter) and H is the height of the tumor. Mice were monitored for tumor growth and survival. Mice were euthanized and organs were harvested when the tumor volume reached 1500mm^3, or when they met the humane endpoint criteria defined in the project proposal such as necrosis.

In vivo treatment of mice

The ITK inhibitor BMS509744 (MedChemExpress, cat. No. HY11092) was injected intraperitoneally (i.p.) at a dose of 5mg/kg per day. Each treatment interval consisted of 3 days continuous treatment followed by 3 days off treatment, with this interval repeated 3 times over a period of 18 days. Anti-PD-1 antibodies (RMP1-14, cat: 114119, Biolegend) or isotype control antibodies (RTK2758, cat: 400565, Biolegend) were i.p. injected at a dose of 100µg/mouse, twice per week. Treatment of mice was started when tumors reached an average size of 60-100mm^3.

Tissue collection and sample preparation

When the tumor volumes reached 1500mm^3, tumor, spleen and draining lymph nodes were harvested. Single cell suspensions were obtained after processing these tissues. As described previously (49), spleen and lymph nodes were mechanically dissociated and filtered through a 40-µm cell strainer (Falcon). After washing two times with medium (RPMI medium containing 5% heat-inactivated FBS, and 2 mM L-glutamine), single cell suspensions were obtained. Tumors were first cut into small pieces and then digested for 30 min at 37°C with tumor dissociation reagent (cat: 661563, BD Biosciences). Digested tumor were filtered through a 40 µm cell strainer (Falcon) and washed in RPMI 1640 medium to acquire single cell suspensions. Cells were counted using Trypan blue on an automated cell counter (Countess, Life Technologies).

Antibodies and reagents

To exclude apoptotic and dead cells, Annexin V conjugated with either APC, Cy5.5 or PerCP-Cy5.5 (BD Biosciences) was included in all stains and 2.5 mM calcium chloride ($CaCl_2$) was added to all solutions and washes. The following fluorochrome-conjugated monoclonal antibody combinations against surface and intracellular antigens were used to stain the *in vitro* or *ex vivo* harvested cells: anti-CD8a-eFluor 450 (53-6.7, eBioscience), anti-CD4-BV510 (RM4-5, Biolegend), anti-CD160-PE-CF594 (CNX46-3, BD Biosciences), anti-Lag3-APC (C9B7W, BD Biosciences), anti-CD244-PE (2B4, BD Biosciences; eBio244F4, eBioscience), anti-PD-1-APC-Cy7 (19F.1A12, Biolegend), anti-Tim3-PE-Cy7 (RMT3-23, Invitrogen), anti-TIGIT-FITC (GIGD7, eBioscience); anti-CD44-BV786 (IM7, BD Biosciences), anti-CD45.1-FITC (A20, BD Biosciences), anti-CD45.1-PE-CF594 (A20, BD Biosciences), anti-IFN-γ-APC (XMG1.2, eBioscience), anti-TNF-α-AF488 (MP6-XT22, eBioscience), anti-IL-2-PE (JES6-5H4, eBioscience), anti-Tox-PE (TXRX10, eBioscience), anti-Ki67-AF700 (16A8, Biolegend), anti-TCF1-AF647 (C63D9, Cell Signaling).

For surface staining, cells were washed with FACS wash (HBSS containing 3% FBS and 0.02% sodium azide) and incubated with Fc receptor blocking antibody (2.4G2, BD Biosciences) for 10 minutes on ice, followed by pre-determined optimal concentrations of the fluorochrome-conjugated monoclonal antibodies at 4°C in the dark for 20 minutes. The cells were then washed one time with FACS wash and fixed with 1% PFA. For the intranuclear staining of Ki-67 and transcription factors, cells were first stained for surface antigens as described above. After washing, cells were fixed with FoxP3 Fixation Buffer (005523, eBioscience) for 60 minutes in the dark at 4°C, washed with Perm/Wash buffer (008333, eBioscience) and stained with a mix of antibodies against transcription factors and Ki-67 for 45 minutes at 4°C in the dark. Cells were then washed twice with Perm/Wash buffer and fixed with 1% PFA. Appropriate isotype controls were included for staining of transcription factors.

To analyze phosphorylation at day 5 and 8 of the *in vitro* cell cultures, cells were first surface stained (see above) followed by fixation with IC Fixation Buffer (88-8824, eBioscience) for 30 minutes at 4°C to enable cytoplasmic staining of phosphorylated-protein specific antibodies . Anti-BTK Phospho (Tyr223)/ITK Phospho (Tyr180) antibody (A16128C, Biolegend) and anti-PLCγ1 Phospho (Tyr783) antibody (A17025A, Biolegend) were added and incubate for 45 minutes in the dark at 4°C, cells were washed twice with Perm/Wash buffer and fixed with 1% PFA.

In order to detect cytokine production, *in vitro* cultured cells or the *ex vivo* samples were re-stimulated with 10µg/ml $OVA_{(257-264)}$ SIINFEKL peptide for 6 hours at 37°C, 5% CO2 in the presence of GolgiPlug (BD Biosciences). Cells were then stained with surface antibodies as described above. After washing with FACS wash, cells were fixed with IC Fixation Buffer (88-8824, eBioscience) at 4°C overnight, washed with Perm/Wash buffer and stained for intracellular cytokines for 45 min in the dark at 4°C. After staining, cells were washed twice with Perm/Wash buffer and fixed with 1% PFA. All the samples were measured within 48 hours after fixation.

Samples were measured on a LSRFortessa (BD Biosciences) using application settings and at least 200,000 targeted events for *in vitro* samples and 500,000 targeted events from *ex vivo* samples were collected. Data was then analyzed with FlowJo software (Version 9.9.4, Treestar, Ashland, OR, USA).

RNA sequencing

Raw fastq files were analyzed with the nf-core/rnaseq pipeline (v3.0) using Nextflow (v20.11.0-edge) (50, 51). This workflow reported the quality of the sequencing with FastQC (v11.9). Subsequently, read sequences with low Phred scores (<= 30) were either trimmed or removed using Trim Galore! (v6.6) with default nf-core/rnaseq parameters. Trimmed fastq files were mapped to the mouse reference genome version GRCm38 with Ensembl GRCm38.81.gtf file for gene annotations, using RSEM (v1.3.1) which umbrellas STAR (v2.7.6a) for read alignments, Samtools (v 1.10) processed the alignment files and extracted mapping statistics of the post-alignment (52-54). Subsequently, RSEM estimated the number of transcripts using an expectation-maximization technique. Quality of each sample alignment was visually inspected using reports derived from RSeQC (v3.0.1), Qualimap (v2.2.2-dev) and Preseq (v2.0.3), including read inner distance plots, splice junction annotations, the genomic origin of the mapped reads, and the estimated complexity of the sequencing library (55-57). RSEM estimated counts were imported into R (v4.0.3) using the packages tximport (v1.18) and analyzed with DESeq2 (v1.30.0) (58, 59). Prior to calculating gene expression differences between samples, genes were filtered on biotype using biomaRt (v2.46.0), only taking genes with a biotype of “protein_coding” or “lincRNA” (60). Counts were transformed with respect to library size using the “rlog” function of DESeq2 for visual inspection. The Principal

Component Analysis (PCA) plots were generated utilizing the "plotPCA" function of DESeq2 with rlog transformed data.

Differential expression analysis

Differentially expressed genes were calculated with the functions "DESeq" and "results" from DESeq2 consecutively, where the FDR was set at 0.1, p-values were calculated using Wald statistical test, and r_DMSO was defined as the reference in both analyses. Benjamini-Hochberg multiple hypothesis testing method was used to calculate adjusted p values. Fold Changes were shrunk with the DESeq2 function "lfcshrink" using method "apeglm" (61). Genes were indicated as differential expressed with an adjusted p-value <= 0.05. Heatmaps were made using the R package pheatmap (v1.0.12), center scaling on gene rlog counts and performing complete clustering on the Euclidean distances for both the samples and individual genes.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed with the complete gene-set after shrinkage of the fold changes (62). Genes were ranked on the FC and compared with CD8+ T cell specific exhaustion gene-sets. The CD8+ T cell exhaustion gene-sets were based on the publication of Bengsch B, et al. and downloaded from PubMed Central (63). Enrichment plots were made using R package fgsea (v1.16.0) with 100.000 permutations. The GSEA was calculated separately for the upregulated and downregulated genes from Bengsche B et al.

Data accessibility

The RNA-seq data is accessible at Gene Expression Omnibus (GEO) platform (ref https://academic.oup.com/nar/article/41/D1/D991/1067995), accession number: to be completed.

Statistics

Statistics analysis was performed using Prism software (GraphPad Prism 5, Version 5.04). Normal distribution of the data was evaluated by Shapiro-Wilk normality test. For the paired samples, two-tailed, paired-t test was used for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. As the *in vivo* data was normally distributed, one way ANOVA with Tukey's post-test was used, for the non-normally distributed data, two-tailed, Mann Whitney test was utilized as mentioned in the figure legends. Tumor growth curves were compared using two-way RM ANOVA. Kaplan-Meier survival analysis was used to determine the survival of treated tumor bearing mice. P values lower than 0.05 were considered statistically significant with the numbers of stars in the figures indicating the p value: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

AUTHORS CONTRIBUTIONS

MZ, LL, RR, TA, MvM and IB performed tumor injections, adoptive transfers and tumor measuring. MZ, CHK, MDCE, MW, TW contributed to the *in vitro* experiments, ex vivo measurement. MZ, LL, YM and PK contributed to data analysis. DG, HW performed the RNA sequence analysis. Study was designed by MZ, RH, JA, YM and PK. Study was conceived by MZ, YM and PK. Manuscript was written by MZ, LL,YM and PK. All authors have read, discussed the results and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest or financial interests.

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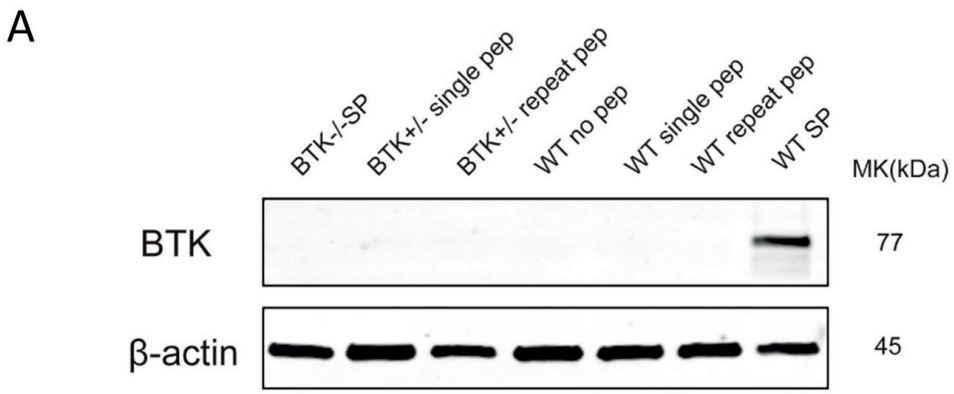
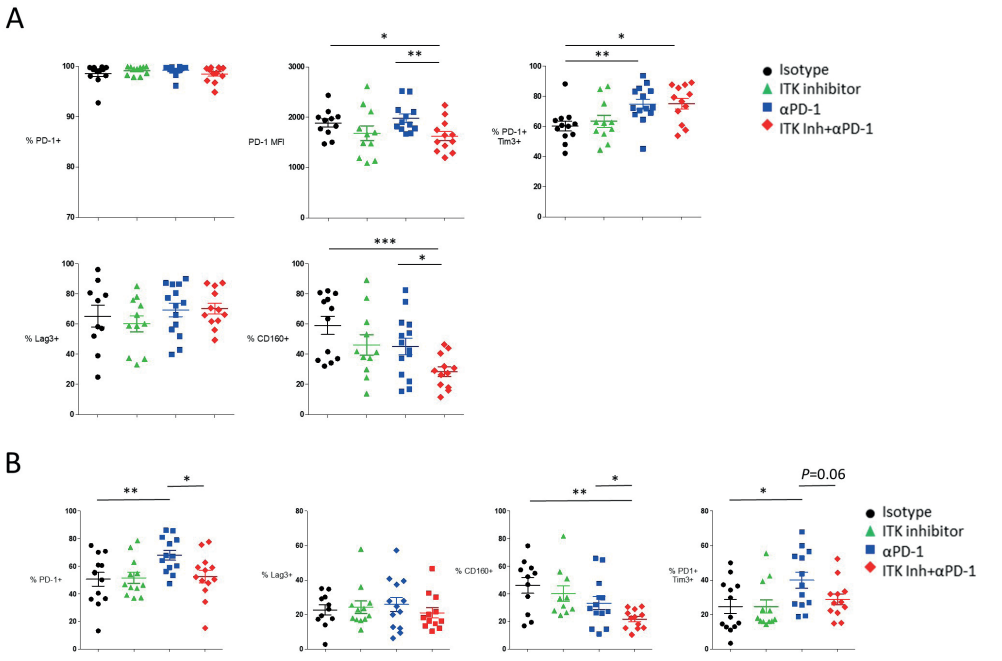
SUPPLEMENTAL DATA



Supplementary Figure 1.

(A) BTK is not expressed in exhausted T cells. Western blot examining the expression of BTK in *in vitro* exhausted OT-I cells (day 5 of repeat peptide stimulation) or OT-I cells stimulated once with peptide. WT and *btk+/-* OT-I cells shown. WT and *btk-/-* splenocytes shown as positive and negative controls.



Supplementary Figure 2.

Tumor tissues were harvested from B16-OVA bearing animals, Flow cytometry measurements were performed with the samples. **(A**) Pooled data showing the frequency of inhibitory receptor expression on donor (CD45.1+) tumor infiltrating CD8+ T cells. Mann Whitney test performed between the groups**. (B)** Pooled data showing the percentage of inhibitory receptors (indicated in the graph) expressing endogenous tumor infiltrating CD8+ T cells are shown. Each symbol represents one animal (n=7-9), 3 independent experiments performed. Line depicts mean ± SE. Mann Whitney test performed between the groups. *<0.05, **P<0.01.

Chapter 6

General discussion

GENERAL DISCUSSION

T cell exhaustion is a term used to define the differentiation state of dysfunctional T cells that develop under chronic antigen stimulation (1-5). This differentiation state was originally identified in the setting of chronic viral infection. However, now T cell exhaustion has also been widely described in cancer (6-9). The exhausted T cells from both settings share the similar characteristics. These include broadly expressing different inhibitory receptors, hierarchical loss of cytokine production and polyfunctionality, decreased proliferation potential and a propensity to undergo apoptosis (1). The failure to control pathogen replication and tumor growth has been attributed to the exhaustion of responding T cells. This has resulted in intensive research efforts to understand the exhaustion of CD8+ T cells amongst antiviral and antitumor immunology studies. Especially intensive challenging has been the quest to improve the function of exhausted T cells with the aim to facilitate pathogen clearance in the chronic infectious disease and tumor control in cancer (10-13). These studies have led to novel tumor immunotherapies that have brought a true revolution in cancer therapy (6, 14-16). Here in this thesis, we embarked on a series of studies to examine T cell exhaustion and explore potential strategies of reversing and/or preventing the development of T cell exhaustion.

In **chapter 2** of this thesis, some of the effects of epigenetic modifiers on CD8+ T cells viability and phenotype were systematically studied. The study was prompted by HIV functional cure strategies, such as “shock and kill” strategy, where HIV latency reversing agents (LRAs) are administrated to reactivate HIV transcription in the quiescent HIV latent reservoir (17). A large number of LRAs are currently in development, although a majority of current LRAs are epigenetic modifiers, like histone deacetylase inhibitors (HDACi). It is well established that HIV-specific CD8+ T cells are exhausted (18, 19) and exhaustion is characterized by epigenetic changes (20-22). The success of the “shock and kill” HIV cure strategy greatly depends on the ability of potent CD8+ T cells being able to kill CD4+ T cells of the reservoir that are expressing HIV antigens (23). We therefore examined, whether 4 major classes of LRA also affected T cell function and viability. We observed cytotoxicity of the four categories of LRAs on different immune cells and the potential of these LRAs to modulate the function of CD8+ T cells was also illustrated. These findings not only facilitate the study of function curing HIV by establishing a the immune toxicity of classes of LRA, but also provide preliminary data that highlight the ability of LRA to influence the function of CD8+ T cells. Understanding how LRAs affect exhausted T cells, however, was hampered by the very low number of HIV-specific CD8+ T cells in the blood of HIV+ patients or tumor antigen specific CD8+ T cell in tumor bearing animals. Therefore, how these LRAs could modify exhausted CTL by acting at the epigenetic level was not feasible with such samples in **chapter 2**.

The requirement of abundant amounts of exhausted T cells needed to perform different functional and molecular assays, in our quest to understand T cell exhaustion and the low numbers of exhausted T cells isolated from patients or animals, triggered us to establish a method that yields large number of exhausted T cells. We therefore initiated the studies to establish an *in vitro* CTL exhaustion model, which was presented in **chapter 3**. Standing on the widely accepted knowledge that exhaustion is caused by chronic antigen stimulation (24-26), especially on the absence of CD4+ T cells help, we set up the experiment by using purified CD8+ T cells from transgenic OT-I mice and repeatedly stimulated them with cognate peptide. Strikingly, we found that after repeatedly stimulating CD8+ T cells for 5 days, the characteristic spectrum of features associated with exhausted CTL were present in the cells. These included upregulation of multiple inhibitory receptors, loss of polyfunctionality, reduced cytotoxicity as well as the exhaustion associated transcriptional changes. Furthermore, exhaustion related DNA methylation changes were also detected in these repeated stimulated cells. Beyond using this exhaustion model to understand CTL exhaustion, this model can be used to test or screen drugs that may affect exhaustion. Indeed, we presented that DNMT inhibitor, a DNA methylate inhibitor, could retain the expression of TCF1 expression, a critical transcription factor that confers proliferative capacity in progenitor exhausted cells. This not only confirmed the mechanisms of TCF1 decrease but also highlighted the potential application of this *in vitro* model to drug testing. Using this model, we hope that we can further compare TCF1+ cells and TCF1- exhausted cell which both exist in these cultures. In future studies, using reporter mice we plan to use this *in vitro* model to study the effect of individual miRNA and small molecules on development or reversion of CTL exhaustion.

MicroRNAs (miRNAs) being important factors that regulate gene expression at the post-transcriptional level (27-29), play a critical role in CD8+ T cell differentiation and development (30, 31). Some miRNAs have been shown to contribute to CTL exhaustion development and modify their exhausted CTL function (32-34). In **chapter 4**, we first identified that there were 120 significantly differentially expressed miRNAs when comparing effector CD8+ T cells to naïve CD8+ T cells in the influenza infection model. miR-139 was found to be significantly downregulated in the effector CD8+ T cells. In addition to our finding, other studies also indicated that miR-139 played an important role in regulating CD8+ T cells responses at the effector phase (35). Therefore, we hypothesised that further downregulating miR-139 may enhance effector CD8+ T cell function or expansion. However, when we overexpressed miR-139 by using retroviral transduction or when we used the cells from miR-139 deficient mice in the context of influenza virus infections, we found that neither the development of naïve cells nor the differentiation of effector cells depended on the expression of miR-139. In the influenza virus infection experiments, miR-139 was also dispensable for the memory CD8+ T cells development. Specifically, the expression of the transcription factor, EOMES, was not

different in the miR-139 overexpressing cells, which was in conflict with other studies that examined CTL responses in *Listeria* infection (35). Additionally, the results showed in **chapter 4** was also an application example of our *in vitro* CTL exhaustion model. We performed miRNA sequencing of *in vitro* exhausted cells (data not presented in this thesis). When we analysed the differential expressed miRNA that are shared by the *in vitro* exhausted cells and exhausted tumor infiltrating CD8+ T cells, miR-139 was significantly downregulated (data not presented). Therefore, we evaluated the function of miR-139 in T cell exhaustion by using the *in vitro* exhaustion model. Surprisingly, we found that the development of CTL exhaustion did not depend on miR-139 expression. From the results we presented in **chapter 4**, we concluded that miR-139 was significantly differential expressed by effector CD8+ T cells comparing to naïve cells, however it is dispensable for the development of functional effector and memory CD8+ T cells in murine influenza infection. The function and development of CTL exhaustion was not influenced in the absence of miR-139. Due to constraints in the number of miR-139 overexpressing cells obtained after transduction, we could not address whether overexpressing miR-139 would affect the development of CTL exhaustion. Beyond this miR-139 study, our group is also trying to investigate other miRNAs, whose overexpression or knockdown could reverse CTL exhaustion. Our studies point out that the differential expression levels of certain miRNA between different CD8+ T cells should not be the only criteria to select the target miRNAs to study. The abundancy the miRNA, the abundancy of potential targets of the miRNA, the redundancy between different miRNA that share targets are all expected to influence the results and suggests that such complex interactions explain why miRNA expression levels alone do not always predict the outcome of miRNA manipulation.

Although the effects of Ibrutinib on enhancing antitumor immune responses has been implicated in different tumor models (36), whether it is mediated by any direct effects on CD8+ T cells, the specific target and the associated mechanism are not known (37, 38). In **chapter 5** of this thesis, we took advantage of our *in vitro* CTL exhaustion model, where CD8+ T cells are the only population present, to study the effect of Ibrutinb on exhausted CTLs. We discovered that one of Ibrutinib's targets, ITK, is a key kinase in CTL exhaustion. Inhibiting ITK with a pharmacological small molecule inhibitor, decreased the expression of TOX, a crutial transcription factor of CTL exhaustion development (39-42). We also demonstrated that intermittent use of ITK inhibitor in combination with anti-PD-1, enhanced antitumor efficiency. These results indicated that the immune regulatory effects of Ibrutinib observed in other studies (37, 43, 44), may be mediated by its inhibitory function on ITK and that regulating the activity of ITK in exhausted CD8+ T cells could improve their function. The work included in **chapter 5**, is a further example of the applicability of the *in vitro* CTL exhaustion model for drug testing.

In line with what we found in **chapter 5**, other investigators also found that the factors of TCR activation signalling pathway play a critical role in modifying CTL exhaustion. It was

reported that FOXO3, member of phosphatidylinositol 3-kinase (PI3K)-Akt signalling cascade, was hyperphosphorylated in T cells on day 8 post infection with LCMV-Cl13, the strain that results in chronic infection (45). In FOXO3 deficient animals, not only were there more antigen specific CD8+ T cells in the chronically infected animals, but they also preserved polyfunctionallity and more efficiently controlled virus (45). Driving the CD8+ T cells to memory instead of exhaustion, was another strategy of enhancing CD8+ T cells function in chronic antigen stimulation settings. It has been found that PI3K-Akt-mTOR signalling pathway plays a critical role in regulating memory CD8+ T cell formation (46). By inhibiting Akt pharmacologically, tumor infiltrating T cells isolated from human tissue develop features of immunologic memory (47), which improves the function of donor cells in the adoptive T cell therapy. Related to this signalling, PI3Kδ inhibitor leads to less differentiation of donor T cells, which increase CD62L and CD127 expression without influencing their expansion capacity. Donor CTL cells pre-treated with PI3Kδ inhibitor show more potent anti-tumor activity (48). There are additional reports about the effects of inhibiting MAPK/ERK kinase (MEK; also known as mitogen-activated protein kinase kinase) in CD8+ T cells (49, 50). Although the priming of the naïve CD8+ T cells could be dampened, inhibiting the downstream of TCR signalling by MEK selective inhibitors, reverses exhaustion related phenotypes and potentiates effector functions of CD8+ T cells. Combining MEK inhibitor and anti-PD-1 checkpoint blockade also synergized and prolonged anti-tumor effects (49). Thus, the observations that chronic TCR signalling is the cause of CTL exhaustion, has led to targeting and inhibiting the kinase in the TCR signalling pathway and this could reverse the development of CTL exhaustion.

Beside the applications of the *in vitro* exhaustion model we demonstrated in this thesis, there are other potential ways that this *in vitro* model could facilitate the study of discovering novel strategies to reverse CTL exhaustion. Regarding the large yield of exhausted cells from this *in vitro* model, it has the potential to be used in high throughput screening of molecules and reagents to reverse or prevent CTL exhaustion. This high throughput screening is especially applicable if critical cytokines, such as IL-2, or crucial transcription factors, such as TCF1 or TOX are tagged with reporter genes in CD8+ T cells (Figure 1A) (51). Utilizing techniques to manipulate the expression of genes or using pools of sgRNAs with CRISPR/Cas approaches to screen the genes that contribute to exhaustion development and further identify the novel interventions of regulating CTL exhaustion are also possible applications of this model (Figure 1B) (52). Modulating such promising genes or targeting their pathways with inhibitors could be used in adoptive T cell therapy. In this way either the dysfunctional tumor infiltrating CD8+ T cells can be rejuvenated before transferring or antigen-specific CD8+ T cells from different tissues can be treated to prevent them to be exhausted.

Although immune checkpoint blockade (ICB) for the treatment of cancer patients has shown great promise (53-57), there are still concerns whether blocking a single inhibitory receptor would broadly benefit all kinds of cancer patients (58). Indeed, only a subset cancer

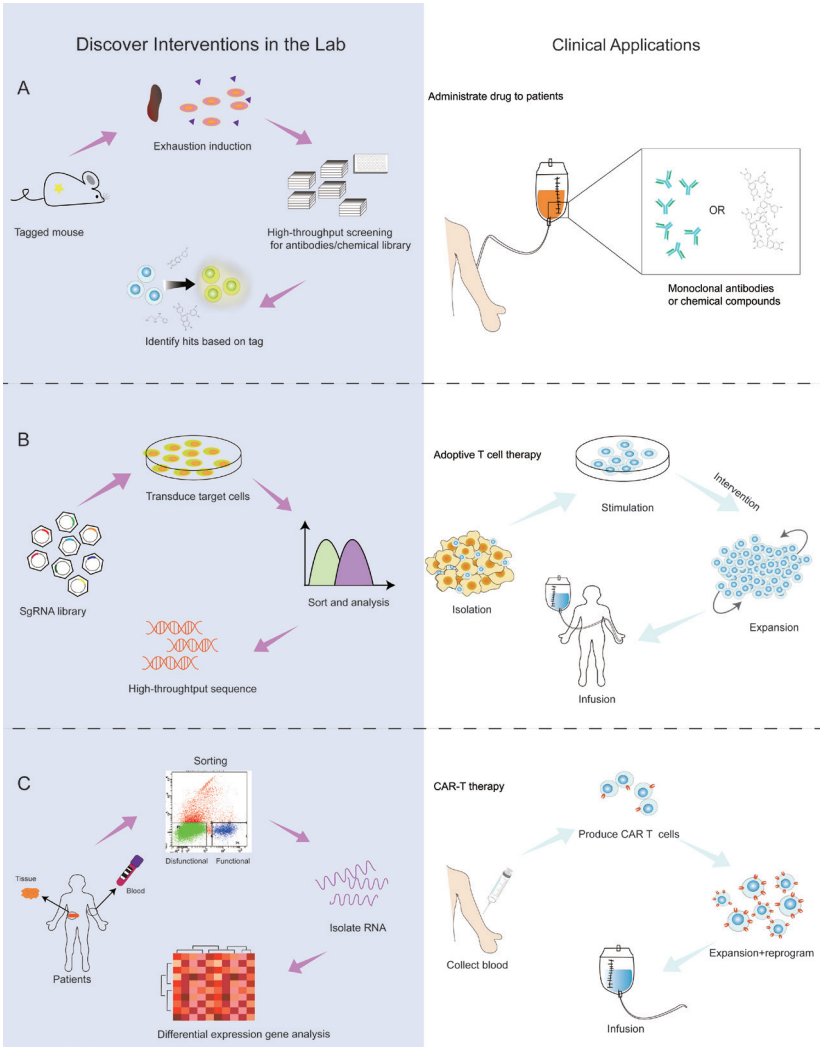


Figure 1. Discovering novel interventions with fundamental research to improve the treatment of chronic infectious disease and cancer.

(A) Transgenic T cells could be used to induce CTL exhaustion *in vitro*, when the critical exhaustion genes or genes related to function are tagged, the *in vitro* exhaustion model could be utilized to screen antibodies or chemical libraries that prevent, reverse or accelerate T cell exhaustion. **(B)** CRISPR/Cas approaches using sgRNA pools could be used to screen exhausted T cells associated genes. By sorting exhausted versus non-exhausted cells and sequencing these, novel exhaustion related genes could be identified. **(C)** Sort the functional and dysfunctional cells from patients, then compare their transcriptome characteristics. By analysing differentially expressed genes, crucial factors could be identified. The above approaches could benefit immunotherapies and adoptive T cell therapies. Small molecules that prevent or promote exhaustion could be employed in chronic infection, cancer or autoimmunity patients. For adoptive therapies T cells are isolated from the patients and modified with identified genes or treatments.

patients respond to ICB therapy. Therefore, treatment combination approaches have been proposed to improve the ICB efficiency. This has already been shown to improve ICB effectiveness when anti-PD-1 and anti-CTLA-4 treatments have been combined (59, 60). Additional combinations of different ICB therapies, such as anti-PD-1 and Tim3, were also found to be a promising strategy (61). Because the efficacy of ICB relies on the existence of progenitor exhausted CD8+ T cells (62-65), expanding or preserving these would augment antitumor effects of ICB. Strategies that expand such cells, such as the one we employed in **chapter 5,** as we show, can enhance ICB anti-tumor activity. Recruiting additional antigen-specific T cells to anti-tumor responses is also expected to enhance the effect of ICB. It has been shown that tumor vaccines can induce de novo antigen-specific T cells responses to tumors (66, 67) and ICB efficacy correlates with the mutational load of tumors and neoantigens (68-72). Therefore the combination of tumor vaccines and check point blockade is another promising strategy to enhance the efficacy of ICB therapies (73, 74). Further, therapeutic combination strategies are being tested in order to enhance ICB. Such strategies include combining ICB with radiotherapy or antibodies that target phosphatidylserine (75-77).

FUTURE PERSPECTIVE

Despite the great progress in our understanding of the molecular and cellular mechanisms involved in T cell exhaustion and the development of therapies such as ICB that target exhausted cells, there still remain major questions on the application of ICB in different disease. Below I will discuss some of those main questions.

Does ICB work the same in chronic viral infections and in cancer?

Although exhausted CD8+ T cells from chronic viral infection and cancer shared comparable phenotypic and transcriptome characteristics, their response to ICB is not identical. When anti-PD-1/PD-L1 therapy was prescribed to chronic HCV infected patients, there were synergetic effects and improvement the antiviral efficiency (78). These effects could be attributed to the improved function of CD8+ T cells and CD4+ T cells (79, 80). In another clinical study, it was demonstrated that blocking PD-1/PD-L1 could not completely restore the function of HCV-specific CD8+ T cells (81). Administrating anti-PD-L1 to the chronic HBV patients could increase the proliferation capacity and cytokine release of intrahepatic and peripheral HBV-specific T cells (82). Although *in vitro* studies have indicated that ICB may be beneficial in HIV+ patients (18, 83-85), this has not been directly tested. ICB has been tested in SIV infected non-human primates with conflicting findings regarding the therapeutic benefit of ICB (86-89). Thus the benefit of ICB in chronic infections has not been proven and this is reflected by the fact that none of the ICB approaches have been approved to be used

clinically in chronic infections. Why ICB in chronic infections has failed to show clinical benefit whereas in the setting of cancer it has shown great promise, remains poorly understood and should be a subject for investigation.

In contrast to chronic infections, ICB was approved to be used in the treatment of different cancers, such as melanoma and non-small-cell lung cancer (54, 55, 57, 73, 90). However, despite the tremendous progress that ICB has brought to the field of tumor immunotherapy, the fundamental understanding of how these therapies work to delay or halt the progression or induce the regression of cancer is still missing (91-93). So far, it was found that the mutation burden of tumors positively correlates with treatment efficiency (94) and this is attributed to increased neoantigens and responding T cells. In addition, the maintenance of progenitor exhausted cells are also necessary for the tumor bearing animal or patients to respond to ICB (62, 63, 95-98). Further efforts have focused on identifying the characteristics of the population that responds to the checkpoint blockade therapy, and the more precise surrogate markers are required to define the responsible populations (63, 99, 100). Therefore, combining strategies that boost tumor neoantigen-specific T cells, such as tumor vaccines, or facilitate the preservation or increase of the less terminally differentiated progenitor exhausted T cell population would hopefully further enhance the effects of ICB therapy.

From the above it is clear ICB in cancer and chronic infections has shown very different efficacy. The reasons why this is so remain unknown and although exhaustion is shared in both settings, subtle differences in the molecular characteristics of exhaustion, the cells involved and their location may underlie these differences and beg for further investigation.

Could other immune cells develop the exhausted state?

There is no clear answer if exhausted CD4+ T cells exist and how it should be defined, however, similar phenotype and state of dysfunction as CD8+ T cells exhaustion could be detected on CD4+ T cells after chronic antigen stimulation (101-104). Lately, by using antigen-specific CD4+ T cells from TCR transgenic mice, Fu J et al showed that after their adoptive transfer into tumor bearing mice, there were multiple inhibitory receptors, such as PD-1, Tim3, Lag3, expressed on the surface of these cells. CD4+ T cells from tumor tissue also lost TCF1 expression, which was similar to exhausted CD8+ T cells. Surprisingly, it was found that anti-PD-L1 treatment could largely prevent the exhaustion of CD4+ T cells in this study (105). How checkpoint blockade influence the function of CD4+ T cells function and what is the contribution of these exhausted CD4+ T cells in the disease development are to be determined.

Could innate immunity members, such as natural killer cell (NK cells), be exhausted? It has been demonstrated that NK cell will decrease effector function, such as degranulation, after over-stimulation. The hypo-functional state of NK cells was also correlated with poor pregnosis of different tumors (106, 107). Characteristics and mechanism of NK cell

exhaustion have been reviewed by J. Bi et al (108). However, we are still limited by the lack of deep understanding of NK cell exhaustion. It is expected that further studies into NK cell exhaustion may bring novel immunotherapies targeting on NK cells. Finally, currently there is a paucity of evidence relating to the exhaustion state of other immune cells.

Inducing T cell exhaustion-a therapeutic strategy to overcome autoimmune disease?

The development of T cell exhaustion cannot be clearly classified as a positive or negative outcome. Some researchers suggest that exhaustion is an adaptive change under the persistence of antigen stimulation in order to restrain immunopathology and host damage (5, 109). Sandu. I et al reported that TCR signalling of antigen-specific CD8+ T cell from *in vivo* chronic LCMV infection was low, although there was antigen presented on MHC-class I molecule (110). This inhibitory state of TCR signalling was reversed by prescribing anti-PD-1/PD-L1 treatment to the animal. Such a depression of TCR signalling can occur in autoreactive T cells as demonstrated in the patients that receive ICB therapy as these are at risk to develop autoimmune diseases because of breaking of self-tolerance (111, 112).

Based on the above, inducing T cell exhaustion was proposed to be a potential therapeutic strategy in autoimmune disease (109). There is now further proof that inducing T cells exhaustion would slow down the progression of autoimmune disease, such as type 1 diabetes (113). From clinical trial, it was found that Alefacept, a LFA-3-Ig fusion protein that binds CD2, could induce the CTL exhaustion related transcriptome profile, and this resulted in highly preserved endogenous insulin production (114). In contrast to the settings of chronic viral infection and cancer, enhancing inhibitory receptor signalling is proposed to treat autoimmune disease, where inducing peripheral tolerance is needed (114). Whereas, it is far less clear how to induce CD4+ and CD8+ T cells exhaustion in the setting of autoimmunity and the benefit of such exhaustion induction or inhibitory receptor stimulation remains to be determined. Despite this, there is hope that such approaches may yield novel treatments for autoimmune diseases.

Overcoming T cell exhaustion to facilitate the application of CAR T cells to solid tumors

CAR T cells therapy has demonstrated great success by improving the outcome of patients with B cell malignancies (115, 116). In patients who achieved complete remissions, a higher proportion of CAR T cells maintained a memory signature than the CAR T cells from partial remission or progressive patients (117). This indicated that infusion of less differentiated or memory-like autologous CAR T cells further improves the efficiency of this therapy. Despite its success in blood malignancies, it has been proven more difficult to design highly efficient CAR T cells to treat solid tumors (118, 119). Besides optimizing antigen targeting, the chimeric

receptor selection and vector designing, identifying strategies that can expand the CAR T cells while preventing terminal differentiation and facilitate the CAR T cell migration into lymphoid organs, are expected to improve CAR T cell therapy. It has been shown that expanding CAR T cells with IL-7/IL-15 instead of IL-2 could achieve highly functional antitumor T cells (120). Therefore, the strategies, such as culture conditions, pharmacological interventions and genetic modifications, have the potential to promote CAR T cells persistence or memory formation. Having *in vitro* exhaustion models available as the one we have developed could greatly facilitate the discovery of such factors.

In summary, our studies on CTL exhaustion that attempt to further define the features of exhaustion through different approaches in this thesis, have implications for chronic infections, tumor immunotherapy and autoimmunity. In this chapter, we further discussed the potential contributions of our data to understand how CTL exhaustion develops. By repeat stimulation of murine CD8+ T cells with cognate peptide *in vitro*, we successfully generate abundant bona fide exhausted CD8+ T cells. Subsequently, this *in vitro* CTL exhaustion model was utilized to screen the candidate pharmacological interventions and molecules to reverse and/or prevent CTL exhaustion. Overall, these findings deepened our understanding about T cell exhaustion biology and demonstrate the applicability of this platform to drug discovery. This in turn might contribute to the discovery of novel immune therapies for chronic infectious diseases, cancer and autoimmunity.

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Addendum

Abbreviations

Summary

Samenvatting

Acknowledgements

Curriculum Vitae

PhD Portfolio Summary

Publications

ABBREVIATIONS

ACT	Adoptive T cell therapy
Aire	Autoimmune regulator
Arm	Armstrong strain
BAFi	BRG-Brahma associated factor inhibitor
BATF	Basic leucine zipper transcription factor, activating transcription factor ATF-like protein
BETi	Bromodomain, and extra-terminal domain inhibitor
BRD4	Bromodomain-containing protein 4
BTK	Bruton's tyrosine kinase
CAPE	Caffeic acid phenethyl ester
CAR	Chimeric antigen receptor
cART	Combination anti-retroviral treatment
CMV	Cytomegalovirus
CTL	Cytotoxic CD8+ T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMSO	Dimethyl sulfoxide
DNMT3a	DNA methyltransferase 3a
EBV	Epstein-Barr virus
FGL1	Fibrinogen-like protein 1
GSEA	Gene set enrichment analysis
GzmB	Granzyme B
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDACi	Histone deacetylase inhibitor
HIV-1	Human immunodeficiency virus-1
IDO	Indoleamine-2,3 dioxygenase
IFN	Interferon
IFNR	Interferon Receptor
IL-10R	IL-10 receptor
ITK	IL-2-inducible T-cell kinase
ITSM	Immunoreceptor tyrosine-based switch motif
LAG-3	Lymphocyte activation gene-3
LCMV Cl-13	Lymphocytic choriomeningitis virus clone-13
LRAs	Latency reversing agents
LTR	Long terminal repeats
MDSC	Myeloid-derived suppressor cells

MEK1/2 (MAPKK1/2)	Mitogen-activated protein kinase kinase
miRNA	MicroRNA
NFAT	Nuclear factor of activated T-cells
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PCA	Principle component analysis
PD-1	Programmed cell death protein 1
PKC	Protein kinase C
PLC-γ1	Phospholipase C-γ1
P-TEFb	Positive transcription elongation factor b
RBP	RNA binding proteins
SIV	Simian immunodeficiency virus
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophage
TCR	T cell receptor
TGF-β	Tumor growth factor B
TIGIT	T cell Immunoglobulin and ITIM domain
TILs	Tumor infiltrating lymphocytes
TIM-3	T cell immunoglobulin and mucin domain 3
TME	Tumor microenvironment
TOX	Thymocyte selection associated high mobility group box protein
Tregs	Regulatory CD4+ T cells
VEGF	Vascular endothelial growth factor

SUMMARY

Chronic antigen stimulation drives CD8+ T cells into a dysfunctional state known as exhaustion, which has been documented in a variety of chronic infectious diseases and cancer. Under chronic antigenic stimulation, CD8+ T cells progressively develop exhaustion-related characteristics such as upregulating multiple inhibitory receptors, an ordered loss of the capacity to produce cytokines, a diminishing potential to proliferate and finally an increasing propensity to apoptosis. Epigenetic modifications and transcriptional changes underlie these phenotypic and functional changes. The deepening of our knowledge on CD8+ T cells exhaustion, has revealed different differentiation stages of exhausted T cells, namely, progenitor exhausted T cells and terminally exhausted T cells. Progenitor exhausted T cells still maintain a stem-cell like potential to proliferate, and can further differentiated into terminally exhausted T cells, while terminally exhausted T cells only preserve some cytokine production and cytotoxic capacity. Due to the critical function of CTL in eliminating viral infections and lysing tumors, preventing or reversing T cells exhaustion is becoming a major research goal in the field of infectious diseases and tumor immunology, especially after immune check point blockade therapies have brought enormous benefits to cancer treatment. However, to date, the factors that can reverse or prevent T cells from undergoing exhaustion remain largely unknown. The quest to identify such factors motivated us to embark on a series of studies to search for modulators of T cell exhaustion.

In this thesis we first introduce our current knowledge about T cell exhaustion and describe the characteristics, the factors that induce T cells exhaustion, and the T cell exhaustion-related potential clinical applications for treating different types of diseases (**Chapter 1**). Starting off from the premise that epigenetic modifications accompany the development of T cells exhaustion, we began to evaluate the cytotoxicity effects of eight HIV latency reverse agents (LRA), which are mainly epigenetic modifiers (**Chapter 2**). Our reasoning was that LRA used to reactivate HIV need foremost to have little T cell cytotoxicity, otherwise the so called “shock and kill” strategy would fail, but that also some of these LRA epigenetic modifiers may modulate T cells exhaustion which is mediated by epigenetic changes. We found that different LRAs have distinct cytotoxic effects on diverse types of immune cells in human PBMC. Furthermore, BET inhibitors and PKC agonists could downregulate inhibitory receptor expression, indicating a potential role in modifying the function and exhaustion of CD8+ T cells (**Chapter 2**).

A major bottleneck in the study of T cell exhaustion is the quantity of exhausted T cells that can be isolated from either animal models or human samples to perform different experiments. We, therefore, initiated a series of experiments to establish an *in vitro* T cell exhaustion model (**Chapter 3**). We found that repeatedly stimulating CD8+ T cells with the cognate peptide for five days could induce the development of bona fide exhausted CD8+ T

cells, which not only showed the whole spectrum of molecular and functional characteristics of exhausted cells, but also displayed the expected epigenetic modifications found in the exhausted cells. In these studies, we also discovered that the well-known downregulation of TCF1 in exhausted T cells results from hyper methylation of the promotor region of *Tcf7*, the gene encoding TCF1 (**Chapter 3**). By using this model, we next tested if microRNA miR-139 played a role in T cell exhaustion development (**Chapter 4**). However, we found that miR-139 deficient CD8+ T cells could still undergo exhaustion. Furthermore, miR-139 deficiency or overexpression could not influence the development of functional effector or memory CD8+ T cells in a murine influenza infection model, although miR-139 was found to be significantly differentially expressed between naïve and effector CD8+ T cells (**Chapter 4**). While searching for potential modulators that reverse T cell exhaustion, we identified that one small molecular inhibitor of BTK and ITK, ibrutinib, could improve the cytokine producing capacity, reduce inhibitory receptor expression and reverse the transcriptional features of *in vitro* exhausted T cells (**Chapter 5**). Since BTK is not expressed in T cells, we confirmed that ibrutinib was not acting via BTK by showing that BTK deficient T cells could still be driven to exhaustion and that they respond the same as wild type cells after ibrutinib treatment. These observations indicated that the effects of ibrutinib on exhausted T cells could due to its targeting of ITK, which we find is highly activated in exhausted cells. This was confirmed using a specific ITK inhibitor that could decrease inhibitory receptor expression and improve cytokine production of exhausted cells. To examine the translational potential of these findings, we tested and found that combining ITK inhibitor with check point blockade enhanced anti-tumor effects even in check point blockade resistant solid tumors (**Chapter 5**). In summary, the *in vitro* T cell exhaustion model we established, enables us to deepen our understanding of T cell exhaustion and facilitates the studies to discover novel strategies of rejuvenating CD8+ T cell exhaustion and these future directions are discussed in the final chapter (**Chapter 6**). The T cell exhaustion method we developed and the modulators we identified in our research could potentially contribute to new treatments for chronic infectious diseases and cancer.

SAMENVATTING

Chronische antigeen stimulatie drijft CD8+ T-cellen naar een disfunctionele staat van uitputting, wat gedocumenteerd is in een scala van chronische infectieziekten en kanker. Gedurende chronische antigeen stimulatie ontwikkelen CD8+ T-cellen geleidelijk uitputtings-gerelateerde kenmerken zoals de verhoogde expressie van diverse inhiberende receptoren, een geordend verlies van het vermogen om cytokines te produceren, een afnemende mogelijkheid om te vermeerderen en tot slot een toenemende neiging tot apoptose. Epigenetische modificaties en transcriptionele veranderingen liggen ten grondslag aan deze fenotypische en functionele veranderingen. Het vergroten van onze kennis over CD8+ T-cel uitputting heeft verschillende differentiatie stadia van uitgeputte T-cellen onthuld, zoals een voorstadium van uitgeputte T-cellen en een staat van terminale uitgeputte T-cellen. Uitgeputte T-cellen in het voorstadium hebben nog steeds een stamcel-achtige mogelijkheid om te vermeerderen, en kunnen verder differentiëren in terminaal uitgeputte T-cellen, terwijl terminaal uitgeputte T-cellen slechts een gelimiteerde cytokineproductie en cytotoxische capaciteit behouden. Vanwege de kritische functie van cytotoxische T-cellen in het elimineren van virale infecties en tumoren is het voorkomen van of het omkeren van T-cel uitputting een groot onderzoeksdoel geworden in het onderzoeksveld van infectieziekten en tumorimmunologie, met name nadat immuun checkpoint blokkade therapieën enorme voordelen hebben opgeleverd voor de behandeling van kanker. Echter, tot op heden zijn de factoren die T-cel uitputting kunnen omkeren of voorkomen grotendeels onbekend. De zoektocht om deze factoren te identificeren heeft ons gemotiveerd om een reeks studies te beginnen om modulatoren van T-cel uitputting te vinden.

In dit proefschrift introduceren we eerst de huidige kennis over T-cel uitputting en beschrijven we de kenmerken en de factoren die T-cel uitputting veroorzaken, alsmede de potentiële klinische toepassingen die verband houden met uitputting van T-cellen voor de behandeling van verschillende soorten ziekten (**Hoofdstuk 1**). Uitgaande van het standpunt dat epigenetische modificaties hand in hand gaan met de ontwikkeling van T-cel uitputting, zijn we de cytotoxische effecten van acht HIV latency reverse agentia (LRA), die met name epigenetische modificatoren zijn, gaan evalueren (**Hoofdstuk 2)**. Onze redenering was dat LRA, dat gebruikt wordt om HIV te reactiveren, in de eerste plaats geen toxisch effect moet hebben op de T-cellen, anders zou de zogenoemde “shock and kill” strategie mislukken. In plaats daarvan zou de LRA T-cel uitputting kunnen beïnvloeden door middel van epigenetische veranderingen. We ontdekten dat diverse LRAs verschillende cytotoxische effecten hebben op diverse soorten immuun cellen in humane PBMC. Bovendien konden BET remmers en PKC agonisten de expressie van inhiberende receptoren verminderen, wat wijst op een mogelijke rol in het veranderen van de functie en uitputting van CD8+ T-cellen (**Hoofdstuk 2**).

Een groot knelpunt in het onderzoek naar T-cel uitputting is de hoeveelheid uitgeputte T cellen, nodig voor diverse experimenten, die geïsoleerd kunnen worden uit diermodellen of humane monsters. We hebben daarom een reeks experimenten opgezet om een *in vitro* T-cel uitputtingsmodel te ontwikkelen (**Hoofdstuk 3**). We ontdekten dat herhaald stimuleren van CD8+ T-cellen met hun bijbehorend peptide voor een periode van 5 dagen de ontwikkeling van bonafide uitgeputte CD8+ T-cellen kan induceren. Deze cellen vertoonden niet alleen het hele spectrum van moleculaire en functionele kenmerken van uitgeputte cellen, maar lieten ook de verwachte epigenetische modificaties van uitgeputte T cellen zien. In deze studies hebben we ook ontdekt dat de welbekende verminderde expressie van TCF1 in uitgeputte T-cellen het gevolg is van hyper-methylering van de promotor regio van *Tcf7*, het gen dat codeert voor TCF1 (**Hoofdstuk 3**). Met dit uitputtingsmodel hebben we vervolgens getest of microRNA miR-139 een rol speelde in de ontwikkeling van T-cel uitputting (**Hoofdstuk 4**). Echter, miR-139 deficiënte CD8+ T-cellen raakten nog steeds uitgeput. Bovendien had miR-139 deficiëntie of over-expressie geen effect op de ontwikkeling van functionele effector of geheugen CD8+ T-cellen in een muis influenza infectie model, hoewel eerder ontdekt was dat miR-139 significant verschillend tot expressie kwam bij naïeve en effector CD8+ T-cellen (**Hoofdstuk 4**). Zoekend naar mogelijke modulatoren die T-cel uitputting kunnen omkeren, ontdekten we dat ibrutinib, een kleine moleculaire remmer van BTK en ITK, de cytokine productie capaciteit kon verbeteren, de expressie van inhiberende receptoren kon verminderen en de transcriptionele eigenschappen van *in vitro* uitgeputte T-cellen kon omkeren (**Hoofdstuk 5**). Omdat BTK niet tot expressie komt in T-cellen, probeerden we te bevestigen dat ibrutinib niet werkt via BTK. BTK deficiënte T-cellen raakten nog steeds uitgeput en, na behandeling met ibrutinib reageerden deze cellen hetzelfde als wild type cellen. Deze observaties tonen aan dat de effecten van ibrutinib op uitgeputte T-cellen mogelijk het gevolg waren van de inhibitie van ITK. Tevens was ITK inderdaad sterk geactiveerd in uitgeputte cellen. Het effect van ibrutinib op ITK werd bevestigd door gebruik te maken van een specifieke ITK remmer die de expressie van inhiberende receptoren kon verlagen en de cytokine expressie van uitgeputte cellen kon verbeteren. Om de translationele mogelijkheden van deze bevindingen te onderzoeken hebben we een combinatie van een ITK remmer en check point blokkade getest in tumoren. Uit deze experimenten bleek dat het anti-tumor effect sterk was verbeterd, zelfs in check point blokkade resistente, vaste tumoren (**Hoofdstuk 5**). Samenvattend stelt het *in vitro* T-cel uitputtingsmodel dat we opgezet hebben ons in staat om ons begrip van T-cel uitputting te vergroten en vergemakkelijkt dit model het onderzoek naar nieuwe strategieën om T-cel uitputting om te keren en te voorkomen. Deze mogelijkheden worden verder besproken in het laatste hoofdstuk (**Hoofdstuk 6**). Het T-cel uitputtingsmodel dat we hebben ontwikkeld en de modulatoren die we hebben geïdentificeerd in ons onderzoek kunnen mogelijk bijdragen aan nieuwe behandelingen voor chronische infectieziekten en kanker.

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Thank you Rik Ruijten for choosing to work in this lab after you finished your internship here. I had a really good time with you when you were a student in the lab and I knew you would be a good technician or a good researcher in the future. When you were back in the lab and worked on the ConCovid project, I saw the professionalism and the sense of responsibility you showed in your daily work. Thank you so much for your help with my experiments. I am truly grateful because it is not required of you to help me. Thank you for listening to my complaints and always telling me to look at things in an optimistic way.

Merel Wilmsen, I am glad we have you in this group, you are one of the people that would spend lots of time thinking about what you need to do before doing it. I learned a lot from you. Thank you for always saying yes to me when I asked for help, even if you already have a very packed schedule every day.

Thank you Tamara van Wees , Danique Laport and Tessa Alofs, thank you for your understanding and the help you offered in the lab. Tamara, as I told you, you are a warrior. I believe nothing can stop you from pursuing the life you want. You can always tell right from wrong. Thank you for always being there for me when it is needed. Dear Danique and Tessa, although you are always calm and quiet, your talents will never be buried. Thank you for always asking "what can I do for you" when you have time and never saying "I could not help".

Dear Dr. Christopher Schliehe, thank you for the time you spent talking with me. You are the PI outside of our lab who spent the most of time discussing scientific questions with me. I learned a lot from you. I wish to have as many good scientific ideas as you have in the future. I am sure the others will discover these and your genius ideas will be funded by lots of grants, as long as you keeping on submitting. You will be famous!

Thank you both Prof. dr. Anton W. Langerak and Dr. Marjan A. Versnel, it was you that presented in my PhD committee meeting every year to evaluate and inquiring what happened, how is my PhD study going from time to time. I am glad we have this confidential policy in this department to help all the PhD students finish their study.

Dear Anne Bras, you are the most talented person I have ever known. I am not the kind of person who easily admits that there is something I can't do, but I would immediately say "I can't do what Anne does". Your level of talent is somewhere I don't think I can reach. I shouldn't say I like you a lot, but worship you a lot. I appreciate that I got to know you and talk to you in these years. Your attitude towards work and life also opened another world for me, I learned a lot from you. I hope you can do what you want to do like always and that there is no limitation on your way to success.

Thank you my dear Chinese colleagues, Ziye and Verna, because of you both, I was not the only Chinese in this department. I wish I could give you more guidance for your life in the Netherlands. Thank you for the time we spent together and the encouraging words you said to me. I believe you will have a good study experience in the Netherlands and harvest fruitful PhD.

Thank you to my office mates, Emma Tondeur and Olivia Manusama, I clearly feel we are totally different and facing different problems during our PhD, but to be real is what we all persue. Thank you for listening to my complaints and helping me to clear my mind again and again. Your friendly support made me feel our office was more like a family instead of a simple office.

I would like to thank you Christiaan J. Stavast, we used to be the only two PhD students in the lab before Ling came and after Chris left. Your hard working attitude also guided me

when I went through the most difficult period during my PhD, and your sense of humour facilitate the hardest time. Your optimistic and unwavering attitude will bring you to many future successes.

Dear Fabian Kaiser, thank you for your company every day in these passing years, we complained to each other and shared good news from time to time. Your braveness and hard-working indeed impressed me. I believe you will complete your story about Pax5 finally!

There are lot of other PhD students I would like to thank, Jorn Assmann, Martijn Kolijn, Benjamin Schrijver, Paul Hengeveld, your greet and chat every day made my daily life in the Netherlands. You always share your ideas and opinions with me ,and made me feel I am not fighting alone.

The PhD students sitting in the 11th floor, the MS group members Steven Koetzier and Jamie van Langelaar, I believe the MS group is the most productive group in this department. Your presentations in all the conferences always impressed me. Thank you for your help and sharing your experiences with me. Javad M. Wahadat you are the person who has the best presentation skills in my opinion. I wish I could be as confident as you. I think because we have a similar educational background it was easier for us to talk. Your natural leadership is also what I wish to have. Dear Astrid van Stigt and Sander van Tilburg, although you started your PhD just recently, you all already spent some time in the department, which made us easily know each other.

Thank you the members of “scientist girls” group, Panagiota and Laurine. I really enjoyed the time of talking to you. It is hard to believe that we are separated, because we live in three different countries. However, I believe that the similarities among us will keep us together.

Dear Dr. Leticia G. Leon, thank you so much for the help you gave me with analyzing those RNA-seq results for me. It was your effort that made those data finally being published. I enjoyed the time when you were around, just a few meters away from my office.

It was a great opportunity for me to work with the great bio-informaticians Dr. Harmen van de Werken and Dwin Grashof, thanks a lot for your help, It was your help that makes my publication possible, I also learnt a lot during the process.

Dear Maaike, my old office mate and friend, you always offered to help when I had problems at the beginning of my PhD, and always listened to my difficulties. Your calm and professional working attitude were the best which I can learn from.

The “real boss” of this department, Gellof, you were the first person that helped me in this department. I replied to your email extremely fast which surprised you, but that was only because I was so sure that I would like to leave the old department. In these four or five years, you did not change at all, and continuously helped me when I was facing troubles. Your help made my life and study in the Netherlands much easier. Thanks for your enthusiastic help. Hearing your hearty laughter around in the department every day made me feel confident to solve all the problems.

Marie-Joan Heijne den Bak-Glashouwer, Harm de Wit, Peter van Geel, Sascha van Vuuren-Ligthelm, Bibi van Bodegom and Daniëlle Korpershoek, thanks to all of you supporting me in the lab and my study in this department.

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I would like to thank the EDC isolation colleagues, Ingeborg, Vincent, Simone, thank you so much for your tireless help at the last part of my PhD. This made it possible for me to finish my PhD. Thank you for caring for those mice together with us.

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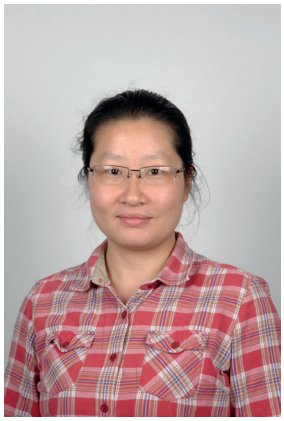
Thank you to my aunt, Yang Shuping, my mother in law and sister in law, thank you for your caring and support. Without your help, it is impossible for me to finish my PhD. My dear brother, Zhao Mancang, thank you for your quiet support and listening. Although we are always separated, I know your love is always around me.

Last but not least, my dearest husband, Dr. Maoxing Tang, thank you for your company in the last 13 years. You always support my decisions. The difficulties we went through and experienced in learning abroad solidified the love between us and deepened our affections. Thank you for establishing a good model to do a successful PhD and decisive to overcome all the difficulties during the process. I believe after these, there is a successful career in front of us and we are going to enjoy the family life together. My dearest son, Muyao Tang, I wish I could have spent more time with you, but I hope you can understand that it was because of COVID-19 pandemic that stopped you coming back to the Netherlands. Thank you for coming to our life, bringing surprise and happiness to us. Your existence inspires me to do my best always.

CURRICULUM VITAE



Manzhi Zhao was born in Beijing, China on May 7th, 1988. In 2007, after graduating from Beijing Pinggu high school, she started the clinical medicine (5-year program) study at Huazhong University of Science and Technology, Tongji Medical College. During her bachelor study, Prof. Ming Tang inspired her into the research field and offered her an opportunity to study in her physiology lab. In 2012, she finished the fundamental medical training and clinical internship rotation in Tongji Hospital as an outstanding graduate. Then she was offered exemption from master program entrance examination to study as a master student in Huazhong University of Science and Technology, Tongji Medical College, Tongji hospital, where she started her first stage of professional training for residents.

At the same time, she began her clinical research study to establish a diagnostic formula of diagnosing adult FUO patients under the supervision of Prof. Jianxin Song. In the summer of 2014, Manzhi joined an international summer school in molecule medicine hosted by the university of Ulm: Cancer: from Molecules to Disease. This experience motivated her to become a researcher and studying abroad. In 2015, she finished her resident training and master study.

Since November 2015, Manzhi Zhao started her PhD program supported by China Scholarship Council (CSC) at Erasmus Medical Center, Rotterdam, the Netherlands. She became a group member in the lab of Prof. dr. Peter D. Katsikis, Department of Immunology. Since August 2016, under the supervision of Prof. dr. Peter D. Katsikis and Dr. Yvonne M. Müller, Manzhi started the project of discovering novel strategies to reinvigorate CTL exhaustion. In the process, she first evaluated the toxicity effects of LRAs on the cells from HIV patients, providing evidence that LRAs have the potential capacity to preserve or restore CD8+ T cell immunity. She established an *in vitro* CTL exhaustion model which could rapidly generate bona fide exhausted T cells, which could facilitate the screening of reagents that prevent/reverse CTL exhaustion. By using this model, she identified that ITK inhibition could be a potential therapy to reverse CTL exhaustion. These findings may shed light on new therapeutic immunological approaches for the treatment of chronic infections and cancer diseases. From the spring of 2021, Manzhi will return to China and become a physician specialized in infectious diseases.

PHD PORTFOLIO

Name:	Manzhi Zhao
Department:	Immunology
Research school:	Molecular Medicine
PhD Period:	2016-2021
Promotor:	Prof. Dr. Peter D. Katsikis
Copromotor:	Dr. Yvonne M. Müller

Courses and workshops

2016	Workshop Microsoft Excel 2010: Basic
2016	Workshop Microsoft Excel 2010: Advanced
2016	MolMed course
2016	Galaxy for NGS
2016	Course on R
2016	Survival analysis course
2016	Virology 2016
2016	Gene expression data analysis using R
2016	Article 9
2016	Biomedical Research Techniques XV
2018	Advanced immunology
2018	Advanced course on Applications in flow cytometry
2019	Data analysis with Python
2019	Scientific English writing
2019	Scientific Integrity
2020	The FlowJo: High dimensional analysis of flow cytometry data

(Inter)national Scientific meetings and presentations

2018	European Congress Immunology (ECI), Amsterdam, the Netherlands (Poster)
2019	Annual MolMed meeting (Oral presentation)
2019	2nd Human and Translational Immunology Conference, Kos, Greece. (Oral presentation)
2019	NVVI Annual Meeting, Noordwijkerhout, The Netherlands (Oral presentation)
2020	AAI Annual Meeting, Honolulu, USA. (Poster) (Cancelled)

Seminars, Symposiums

2019	2nd Erasmus MC Lymphocyte Consortium meeting
2020	Cancer Research Institution Day
2021	2nd annual symposium ACE TI-IT

Teaching

2016-2019	Histology teaching (1st year medical students or 2nd year medical students)
2017-2019	Supervising master students (research internships in the lab and research review writing)

Other Activities

2016-2020	Journal club at the Department of Immunology
2016-2021	Seminars and mini symposia at the Department of Immunology

LIST OF PUBLICATIONS

Manzhi Zhao, Ling Li, Caoimhe H Kiernan, et al, Inhibition of ITK signaling with ibrutinib can directly reverse T cells exhaustion and enhances checkpoint blockade in solid tumor. In preparation.

Jennifer L Hope, **Manzhi Zhao**, Christopher J Stairiker et al. MicroRNA-139 expression is dispensable for generation of CD8+ T cells responses. Journal of Immunology. Under revision.

Manzhi Zhao, Caoimhe H Kiernan, Christopher J Stairiker et al. Rapid *in vitro* generation of bona fide exhausted CD8+ T cells is accompanied by *Tcf7* promotor methylation. PLoS Pathog. 2020; 16(6): e1008555. (IF: 6.21)

Manzhi Zhao, Elisa De Crignis, Casper Rokx et al. T cell toxicity of HIV latency reversing agents. Pharmacol Res. 2019; 139: 524-534. (IF: 5.89)

Manzhi Zhao, Qiurong Ruan, Mingyou Xing et al. A diagnostic tool for identification of etiologies of fever of unknown origin in adult patients. Curr Med Sci. 2019; 39(4): 589–596. (IF: 1.27)

Dong Xu, **Manzhi Zhao**, Yuhu Song et al. Novel insights in preventing Gram-negative bacterial infection in cirrhotic patients: review on the effects of GM-CSF in maintaining homeostasis of the immune system. Hepatol Int. 2015; 9(1): 28-34. (IF: 5.10)

Chunhui Zhu, **Manzhi Zhao**, Guang Chen et al. Baseline HBV load increases the risk of anti-tuberculous drug-induced hepatitis flares in patients with tuberculosis. J Huazhong Univ Sci Technolog Med Sci. 2017; 37(1): 105-109.

Underlined: these authors contribute equally as first authors