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CD8⁺ T-cell effector function and transcriptional regulation during HIV pathogenesis

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Summary

A detailed understanding of the immune response to human immunodeficiency virus (HIV) infection is needed to inform prevention and therapeutic strategies that aim to contain the acquired immunodeficiency syndrome (AIDS) pandemic. The cellular immune response plays a critical role in controlling viral replication during HIV infection and will likely need to be a part of any vaccine approach. The qualitative feature of the cellular response most closely associated with immunologic control of HIV infection is CD8⁺ T-cell cytotoxic potential, which is responsible for mediating the elimination of infected CD4⁺ T cells. Understanding the underlying mechanisms involved in regulating the elicitation and maintenance of this kind of effector response can provide guidance for vaccine design. In this review, we discuss the evidence for CD8⁺ T cells as correlates of protection, the means by which their antiviral capacity is evaluated, and transcription factors responsible for their function, or dysfunction, during HIV infection.

Keywords

T cells; cytotoxicity; cytokines; transcription factors; HIV

Introduction

A recent UNAIDS report estimates that there are currently 34 million people infected with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS)(1). With almost 2 million deaths each year from AIDS-related illnesses, HIV/AIDS remains one of the leading causes of death globally. While extensive prevention education initiatives and therapeutic intervention have contributed to reducing incidents of HIV infection and mortality over the last decade, the number of new infections remains high at more than 2 million new infections annually (1). These data indicate that there continues to be a pressing need for the development of an effective vaccine to control the HIV pandemic.

There are three potential strategies for a HIV vaccine design: a humoral approach, a cellular approach, or a combination of the two. While initial attempts to induce protection via humoral responses (AIDSVAX B/B and AIDSVAX B/E) or cellular responses (STEP trial) provided little to no protection (2–4), the Thai RV144 vaccine trial, which aimed to elicit both humoral and cellular immunity, met with partial success and provided the first evidence that it may be possible to protect against HIV acquisition (5). However, the RV144 vaccine

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strategy failed to elicit strong neutralizing antibody activity or CD8⁺ T-cell responses (6, 7). In addition, the protective effects were modest and of limited durability, and there is some question as to whether the results are generalizable to groups with greater risk of HIV acquisition than the cohort examined in the RV144 trial (8). It will take several years to determine if the RV144 ALVAC-HIV/AIDS VAX B/E approach is truly protective or can be improved upon, and it is important that alternate vaccine strategies be pursued to either complement the ALVAC-HIV/AIDS VAX B/E vaccine or replace it in the case of its failure.

A truly efficacious HIV vaccine will include the induction of antiviral cellular responses mediated by CD8⁺ T cells. As such, a detailed understanding of the properties of CD8⁺ T cells that correlate with virologic control is essential to focus vaccine development on strategies that will elicit appropriate cellular responses. The recent failure of the Merck STEP trial would appear to suggest that CD8⁺ T-cell responses do not prevent infection nor lower viral set-point following infection (9). However, there is substantial correlative evidence indicating CD8⁺ T-cell responses play a significant role in controlling HIV infection at some level, if not completely (10–16). Additionally, recent data from preclinical rhesus macaque studies suggests that CD8⁺ T cells induced by vaccination can indeed provide some degree of protection from SIV infection (17–20), supporting the idea that if strong responses in the right state of activation and anatomical location can be induced they could be effective. Together, these studies tell us we must extend our understanding of CD8⁺ T-cell responses beyond the examination of a single function, such as IFN- γ , to define the optimal measures on which to infer vaccine efficacy.

In this review, we discuss the various CD8⁺ T-cell functions elicited during infection, potential correlates of protection, and transcription factors involved in CD8⁺ T-cell differentiation and effector function. Understanding the responses that are associated with control of HIV disease progression as well as the complex network of transcription factors involved in CD8⁺ T-cell differentiation may identify useful targets for development of therapeutics as well as vaccines.

Cytotoxic CD8⁺ T-cell function

Antigen-specific CD8⁺ T cells are a heterogeneous population capable of performing multiple functions. Several studies have demonstrated this heterogeneity in the responses to HIV-1, cytomegalovirus (CMV), and Epstein-Barr virus (EBV) infections (21–25). These responses include production of cytokines and chemokines, cytolytic effector molecules, and antigen-specific lysis of major histocompatibility complex (MHC) class I matched target cells (Fig. 1). The majority of responding CD8⁺ T cells exert multiple functions following stimulation, but they can also respond with as little as one (depending on the number of parameters measured). Many of these functions are readily detectable by enzyme-linked immunospot (ELISpot) assay or flow cytometry and play specific, and potentially differential, roles in immunity against a variety of viruses. Several of the functions associated with CD8⁺ T cells that are frequently assessed in response to HIV infection are described in brief below.

Interferon- γ

Interferon- γ (IFN- γ) is the only member of the type II class of interferons, a family of cytokines originally discovered for their ability to interfere with influenza virus replication (26). IFN- γ is the single most commonly used function to assess CD8⁺ T cell responses to infection or vaccination. It promotes a general antiviral state by inducing the conversion of the constitutive proteasome to the immunoproteasome (27), upregulating expression of the TAP transporter proteins (28, 29), and increasing expression and stability of MHC class I molecules (30, 31). In some contexts, IFN- γ also increases the susceptibility of virally

infected cells to apoptosis by increasing the expression of the TNF- α receptors and Fas/FasL (32, 33). However, IFN- γ may also enhance HIV replication (34). Thus in the context of HIV infection, IFN- γ is potentially both beneficial and detrimental to inhibiting viral replication.

Interleukin-2

Interleukin-2 (IL-2) is the primary growth factor for T cells (35). Although typically considered a CD4⁺ T-cell cytokine, CD8⁺ cells are also quite capable of producing IL-2 (36). It has no direct antiviral effector function, but it does promote expansion of CD4⁺ and CD8⁺ cells, thereby amplifying the effector response to pathogens (37). IL-2 may also be important for programming CD8⁺ T cells for better memory generation and effector function (38). IL-2 production by CD8⁺ T cells is correlated with proliferation of CD8⁺ cells independent of CD4⁺ T-cell IL-2 production (36), and both IL-2 production and proliferation are preserved in nonprogressive HIV infection (39, 40). However, IL-2-induced activation and proliferation of CD4⁺ T cells may also increase the availability of target cells for infection as well as increase viral replication by infected cells (41).

Tumor necrosis factor- α

Tumor necrosis factor- α (TNF- α) is a member of the TNF superfamily and was first identified by its ability to induce necrosis in solid tumors (42). It has subsequently been shown to be an important antiviral factor, due to its role as a mediator of apoptosis as well as inflammation and immunity (43–45). TNF- α is initially expressed as a biologically active homotrimer on the cell surface that the matrix metalloprotease TNF- α converting enzyme can subsequently cleave into its soluble form (46). Soluble TNF- α preferentially binds TNF-RI, which, upon being bound, initiates a signaling cascade that induces apoptosis of infected cells. Membrane-bound TNF- α binds TNF-RII and plays an important role in driving NF- κ B activation and inflammation (47, 48). TNF- α also promotes an antiviral state by enhancing expression of MHC class I and by inducing expression of IL-12 and IL-18, which are both important for upregulating production of IFN- γ by CD8⁺ T cells (31, 49, 50). However, similar to IFN- γ and IL-2, activation of cells induced by TNF- α can also result in increased production of virus (51–53).

Cytotoxic inhibitory mechanisms

Perhaps the most important function of CD8⁺ T cells is to recognize and kill infected cells. This function has been shown to be important for control of several infections, including EBV (54), CMV (22, 55), hepatitis B virus (HBV) (56), and hepatitis C virus (HCV) (57). CD8⁺ T cells predominantly mediate killing through the secretion of granzymes and perforin (58, 59). Granzymes are serine proteases that cleave caspases to induce apoptosis (60, 61), and perforin is a pore-forming protein that is required for delivery of granzymes into a target cell (62, 63). Both of these proteins are contained within lytic granules and are released early after CD8⁺ T-cell activation into the immunological synapse formed between the CD8⁺ T cell and a target cell. This process of degranulation is MHC class I restricted and antigen specific and likely plays an important role in control of viral infection *in vivo* (64).

CD8⁺ T cells can also mediate killing by the Fas-Fas ligand (FasL) pathway. FasL is upregulated by CD8⁺ T cells following activation by a target cell (65). Cross-linking of membrane bound FasL and the cell surface death receptor Fas expressed on targets cells induces assembly of an intracellular death-inducing signaling complex (DISC) (66). DISC formation causes activation of a caspase cascade that ultimately leads to apoptosis of the target cell. Individual CTL are thought to be capable of both FasL- and perforin-mediated killing (67); however, cytolysis of HIV-infected target cells appears to be largely perforin-mediated with no clear evidence of a contribution of FasL-mediated killing by HIV-specific

CD8⁺ T cells (59). In addition, reports that a soluble form of FasL can not only block apoptosis but also induce proliferation and NF- κ B activation of HIV target cells raises the possibility that its role in infection is not always directly antagonistic (68, 69).

Noncytotoxic inhibitory mechanisms

Both CD4⁺ and CD8⁺ T cells secrete a variety of chemotactic cytokines (chemokines) upon activation (70). Chief among them are the β -chemokines macrophage inflammatory protein-1 α (MIP-1 α) and MIP-1 β and regulated upon activation normal T-cell expressed and secreted (RANTES). MIP-1 α and MIP-1 β can be found in cytotoxic granules, while RANTES is stored in a separate secretory compartment called the RANTES secretory vesicle (RSV) (71, 72). Both types of granules are rapidly released following T-cell activation. New MIP-1 α and MIP-1 β synthesis occurs within a few hours of activation, while RANTES can take several days to be upregulated following its initial release. All three contribute to an inflammatory response primarily by recruiting leukocytes to the site of injury or infection.

β -chemokines were the first noncytotoxic factors secreted by CD8⁺ T cells to be identified that directly inhibit HIV replication (73). They inhibit replication *in vitro* by binding their cognate chemokine receptor, CCR5, which serves as a coreceptor for viral binding and entry into target cells. Binding of β -chemokines to CCR5 is thought to block access to and induce the internalization of the receptor (74). The exact role the β -chemokines play during HIV infection *in vivo* may still be a matter for debate. β -chemokines do not appear to prevent infection of monocytes and may actually enhance viral replication in these cells (75–77). RANTES (but not MIP-1 α or MIP-1 β) can increase attachment of HIV to cells in a manner independent of both CD4 and CCR5 and increase replication by activating signal transduction pathways (78). Serum β -chemokine concentrations do not correlate with HIV disease status, as patients with progressive infection tend to have higher levels than those with non-progressive infection (79). There is also the suggestion that physiologic levels of β -chemokines are not high enough to exert anti-HIV activity (80), although there is the possibility that concentrations are sufficient for inhibition in the microenvironment of the CD8⁺ T cell. Thus, while these molecules have been shown to have inhibitory effects *in vitro*, they may in fact fuel infection *in vivo* by not only recruiting uninfected target cells to sites of active viral replication but also by enhancing infection of those cells.

Another noncytotoxic function, CD8⁺ T-cell antiviral factor (CAF) was originally defined in the context of HIV infection, and the demonstration of its activity provided the first indication that CD8⁺ T cells possess the ability to inhibit HIV replication (81). CAF is a diffusible lymphokine that lacks identity with IFN- α , IFN- β , TNF- α , IL-4, IL-6, or the β -chemokines MIP-1 α , MIP-1 β and RANTES (82–85). Aside from this, there is little known and much debate about the exact nature of CAF (83, 86, 87). It may be the activity of one or more cytokines or chemokines acting together, or it could be an as yet unidentified molecule (88). In the case of HIV, CAF appears to function by suppressing HIV long terminal repeat (LTR)-mediated gene expression in CD4⁺ T cells (89). It does not block HIV entry (89), proviral integration (90), or reverse transcription (87), nor is it MHC class I restricted (86). Due to CAF activity being neither HIV-antigen specific nor produced only by CD8⁺ T cells, it has been hypothesized that it may in fact be part of an innate rather than an adaptive immune response (88, 91). Despite this, the suppressive capacity of CAF appears to be real, and further investigation is warranted to determine its identity.

Work from two separate groups provides evidence of a significant role for noncytolytic inhibitory mechanisms in viral control during chronic infection. Wong *et al.* (92) and Klatt *et al.* (93) both used a combination of experimental depletion of CD8⁺ T cells and ART treatment in SIV-infected rhesus macaques to examine the *in vivo* effect of CD8⁺ T cells on

lifespan of productively infected cells. They each found the estimated decay rate of infected cells is similar in the presence or absence of CD8⁺ T cells, suggesting clearance of infected cells by CD8⁺ T-cell cytotoxic mechanisms does not account for the entirety of CD8⁺ T-cell-mediated control during chronic infection. However, whether control is the result of CAF, β -chemokines, or some other noncytolytic function has not been determined. These studies also do not rule out the possibility of a greater role for cytotoxic effects in the context of acute or controlled infection.

CD8⁺ T cells and correlates of protection

Evidence that cytotoxic CD8⁺ T lymphocytes (CTL) are important for control of HIV replication comes from both HIV infection in humans and simian immunodeficiency virus (SIV) infection in nonhuman primates (NHP). First, the resolution of peak viremia during acute HIV infection is temporally associated with the expansion of HIV-specific CD8⁺ T cells (10, 11). Second, immunologic pressure exerted by HIV- and SIV-specific CD8⁺ T cells is linked to the emergence of viral escape mutations during acute and chronic infection (12–14, 94, 95). Third, there is a strong correlation between specific MHC class I alleles and non-progressive infection in both humans and rhesus macaques (15, 16, 96–98). Finally, experimental depletion of CD8⁺ T cells in SIV-infected rhesus macaques results in a concomitant loss of control of viral replication (99, 100).

Despite a clear role for CD8⁺ T cells in the initial control of HIV replication, a correlate of protection has remained elusive. Much of the research in the field has focused on HIV seropositive individuals who maintain normal CD4⁺ T-cell counts and are clinically healthy for 10 or more years [long-term nonprogressors (LTNPs)] and those who control viral replication to below the limit of detection (elite controllers or ECs). Although the initial immune responses in these individuals fail to prevent infection, by determining the nature of the responses that subsequently control HIV replication we may better inform vaccine designs that are therapeutic if not prophylactic. Studies seeking to define the mechanism(s) of control have largely relied on the comparison of HIV-specific CD8⁺ T-cell responses from ECs to those from chronic progressors (CPs). Characterization of these responses has evolved over the past two decades and has provided some of the first clues to cellular mechanism(s) of control.

Early work in the field employed cytotoxicity assays, such as the chromium release assay (CRA), to measure HIV-specific CTL activity. The CRA was used to show that HIV-specific CD8⁺ T cells have direct cytotoxic effects against HIV-infected CD4⁺ T cells (101) and was important in establishing the link between emergence of HIV-specific CTL and resolution of peak viremia during primary HIV infection (10). However, as discussed later, questions were raised about the ability of the assay to link CTL activity and HIV viral load during chronic infection (102). The CRA is also laborious, relatively insensitive, highly variable, and, most importantly, provides little information about the cytolytic CD8⁺ T cells themselves other than that they can kill.

Direct detection and quantification of antigen-specific CD8⁺ T cells by MHC class I tetramer technology or IFN- γ production offered more rapid, more sensitive, and less variable assays than measurement of cytotoxicity by the CRA. While an inverse relationship between simple frequency of HIV-specific CD8⁺ T cells and plasma viral load was initially established on the basis of tetramer staining (102), this finding was not supported by subsequent studies that found no relationship between the frequency of IFN- γ -producing HIV-specific CD8⁺ T cells and HIV viral load (103–105). It was proposed that these disparate findings were the result of a significant portion of the circulating tetramer-staining CD8⁺ T-cell population being functionally impaired (106, 107). While early studies in this

area have shown that the majority of tetramer-positive HIV-specific CD8⁺ T cells can produce IFN- γ (23, 108), high expression of inhibitory markers including PD-1, CD160, 2B4, and Lag-3 on HIV-specific CD8⁺ T cells may indicate some degree of functional insufficiency (109–113).

IFN- γ was presumed to be an antiviral marker largely because CD8⁺ T-cell clones that produced it early after stimulation became CTLs after further culture (114, 115). This observation, combined with its ease of measurement by ELISpot or flow cytometry, made IFN- γ a popular choice for detecting HIV-specific cellular responses in both HIV-infected individuals and participants in HIV vaccine trials. However, several studies have demonstrated the inadequacy of IFN- γ as a surrogate marker for HIV control. One study found a positive correlation between frequency of IFN- γ -producing HIV-specific CD8⁺ T cells and HIV viral load (105), while two others showed that although HIV-specific CD8⁺ T cells from ECs and CPs can both produce IFN- γ , IFN- γ -producing CD8⁺ T cells from CPs are impaired in both cytotoxicity and proliferative capacity compared to cells from ECs (40, 116). The failure of the STEP trial further highlighted the misinterpretation of IFN- γ as a surrogate of protection: 77% of vaccinees had an IFN- γ response to one or more HIV antigens by ELISpot, yet there was no protection from infection or enhanced viral control following infection (9). While increased rates of escape mutations within vaccine-targeted CD8⁺ T-cell epitopes indicate that vaccine-induced immune pressure was exerted on the virus, the nature of the selective forces has not been defined (117). More recently, studies have shown more directly that the magnitude of IFN- γ responses does not correlate with CD8⁺ T-cell HIV inhibitory activity (116, 118). Thus, while IFN- γ may be a good indicator of the presence of a response, it cannot be used alone to infer the anti-HIV capacity of T cells.

Polyfunctional T cells

As discussed earlier, antiviral CD8⁺ T-cell responses have the capacity to produce several different functions, and measuring a single function such as IFN- γ likely does not describe the true extent of an antigen-specific immune response. This idea was confirmed by studies based on the murine lymphocytic choriomeningitis virus (LCMV) model of infection. The Armstrong strain of LCMV is cleared following acute infection, while the clone 13 strain results in chronic infection. Following resolution of acute infection by the Armstrong strain, a subset of LCMV-specific IFN- γ -producing CD8⁺ T cells emerges that also produces IL-2 (119). This same subset does not appear following chronic infection with LCMV clone 13, as LCMV-specific cells continue to produce only IFN- γ . Wherry *et al.* (120) extended these findings to show that CD8⁺ T cells are not only multifunctional but also that functional capacity of LCMV-specific CD8⁺ T cells is gradually lost in the context of chronic clone 13 infection. Cells producing IL-2 were the first functional subset lost, followed by those producing TNF α , while IFN- γ was the most resistant to this ‘functional exhaustion’. These studies demonstrate that individual CD8⁺ T cells are capable of responding with multiple functions simultaneously and that measuring IFN- γ alone fails to exclude T cells that are potentially impaired in their functional capacity. They also indicate that including more than one functional marker during the assessment of cellular responses to infection or vaccination will identify cells with greater antiviral potential.

Appay *et al.* (23) and Sandberg *et al.* (121) were the first to demonstrate the functional complexity of human CD8⁺ T-cell responses. They examined cellular responses to either HIV and CMV (23) or CMV alone (121) and showed that different cells specific for the same antigen were capable of producing TNF- α , IFN- γ , and MIP-1 β or IL-2, TNF- α and IFN- γ , respectively. Technical limitations prevented either of these studies from examining the capacity of individual antigen-specific cells to co-produce cytokines or chemokines; however, advances in flow cytometry technology allowing the concurrent measure of up to

18 functional and phenotypic markers soon provided a new tool for assessment of responses (122). The first true demonstration of multifunctional T cells in humans came when De Rosa *et al.* (123) examined responses of antigen-specific T cells elicited by HBV- and HIV- vaccination or natural infection, measuring five different functions (IL-2, TNF- α , IFN- γ , MIP-1 β , and IL-4) simultaneously. Both CD4⁺ and CD8⁺ T cells displayed a surprising breadth and complexity of responses that could not have been captured by the measurement of any single function alone. Antigen-specific CD8⁺ T cells were capable of producing IL-2, TNF- α , IFN- γ , and MIP-1 β alone or in combination. Importantly, there were several functional subsets that did not include IFN- γ production and thus would have been missed had IFN- γ been measured alone. This study established that measurement of multiple functions provides a more sensitive and complete evaluation of T-cell responses elicited by vaccination or natural infection. It also presented the possibility that distinct functional expression patterns might provide correlates of protection or disease progression.

Historically, neither the quantity nor the breadth of the HIV-specific CD8⁺ T-cell response has correlated with protection. However this is based almost entirely on analysis of IFN- γ responses alone, which is likely not representative of the true response to infection. Given the capacity of CD8⁺ T cells to produce multiple functions, it is possible that the key to protection is a matter of quality of the response rather than simply quantity. Zimmerli *et al.* (36) were the first to show that higher quality HIV-specific CD8⁺ T-cell responses consisting of simultaneous production of IL-2 and IFN- γ , compared to IFN- γ alone, differentiate nonprogressive and progressive HIV infection, respectively. The ability of nonprogressor CD8⁺ T cells to produce IL-2 was consistent with the maintenance of greater proliferative capacity than for cells from progressors (36, 40). These data suggested that quality of the response was indeed important. To investigate this association further, our laboratory measured five T-cell functions (IL-2, IFN- γ , TNF- α , MIP-1 β , and CD107a) simultaneously to characterize HIV-specific CD4⁺ and CD8⁺ T-cell responses in a cohort of progressors and nonprogressors (39). This study demonstrated that while the absolute frequency of HIV-specific CD8⁺ T cells does not correlate with viral control, the frequency of specific functional subsets does correlate with control. Comparing the functional profiles of the HIV-specific CD8⁺ T-cell responses between progressors and nonprogressors, the two groups were differentiated by a higher degree of functionality in the nonprogressors than in the progressors. A population of CD8⁺ T cells capable of producing all five functions was observed almost exclusively in the nonprogressors, and a subset of CD8⁺ T cells producing IFN- γ , TNF- α , MIP-1 β , and CD107a was also more prevalent in this group than in progressors. These responses were consistent across multiple HIV proteins (Gag, Pol, Env and Tat/Rev/Vif/Vpr/Vpu). Furthermore, within the progressor group, the magnitude and proportion of the HIV-specific CD8⁺ T-cell responses positive for these same five- and four-function subsets inversely correlated with viral load. This demonstrates that by measuring multiple functions simultaneously it is possible to discern a difference between progressive and nonprogressive HIV infection based on the functional capacity of HIV-specific CD8⁺ T-cell responses.

In support of an association between polyfunctional cells and protection during infection, Darrah *et al.* (124, 125) demonstrated that vaccine-elicited polyfunctional CD4⁺ T cells are far better at providing protection to mice challenged with *Leishmania major* than dualfunctional or monofunctional cells. However, a similar phenomenon for CD8⁺ T cells has not been established in this or other models of infection, and the role of polyfunctional CD8⁺ T cells in the context of HIV infection is surrounded by questions. First, how can polyfunctionality be a significant correlate of protection, when only a small proportion of nonprogressor HIV-specific CD8⁺ T cells actually produce five functions and some nonprogressors do not have this or any other measurable functional subset (39, 126)? Second, why do polyfunctional HIV-specific CD8⁺ T cells induced in progressors during

antiviral therapy generally fail to impart virologic control upon cessation of therapy (127–129)? Finally, how do IL-2, TNF- α , IFN- γ , and MIP-1 β become protective in combination when they are not individually associated with protection and they all have potentially pleiotropic effects in the context of HIV infection (as discussed earlier)? Is it a synergistic effect of multiple functions acting in concert, or the ability of polyfunctional antigen-specific CD8⁺ T cells to produce more cytokine on a per cell basis (130, 131)? Is it simply that these cells are functional in a way not yet measured? All of these issues come down to a single question: is polyfunctionality a cause or consequence of control? Polyfunctionality, as defined by the above measures, is not indicative of the protective capacity of a cell, and their use as a surrogate for a protective response in vaccine studies is not yet founded on direct evidence that they themselves mediate control.

Cytotoxicity and perforin

Cytotoxicity is one function of CD8⁺ T cells that is unequivocally antiviral, as direct killing of virally infected cells will certainly impact viral replication. Cytotoxic capacity can be assessed directly or indirectly by several different methods, each with its own advantages and disadvantages. As discussed earlier, direct cytotoxic capacity of CTLs can be assessed *in vitro* using the chromium release assay or by a similar fluorescence-based assay, and these assays were important for establishing the role of CTL in controlling HIV infection (10, 11, 101). Although several studies have since used the CRA to show HIV-specific CD8⁺ T cells from ECs maintain greater cytotoxicity than those from CPs (23, 132, 133), the connection between the results of these assays and the *in vivo* state has been questioned (102). Critics of this interpretation cite the prolonged culture and expansion of cells *in vitro* required to generate sufficient numbers of CTLs for quantitative CRA (134), which likely introduces bias in CTL analysis as it only measures the subset of memory and effector cells capable of proliferating and remaining functional under artificial conditions. Additionally, these assays only measure the effects of CTL on the target cells, without providing any phenotypic or functional characteristics about the CTL itself. This represents a critical loss of information when trying to determine the exact nature of these cells.

CD8⁺ T-cell cytotoxic potential can also be assessed directly by flow cytometry. Unlike the cytotoxicity assays, this method does not measure killing but rather determines killing potential based on the perforin and granzyme content of the CD8⁺ T cell, with the concept that CD8⁺ T cells that do not express these markers would be incapable of killing targets. The advantage to this method is that flow cytometry permits measurement of additional parameters, thus providing a more complete picture of the cells with killing potential. Perforin content of antigen-specific CD8⁺ T cells can be measured by staining resting cells with MHC class I tetramers or by peptide-stimulation. When Appay *et al.* (23) compared perforin content of CP antigen-specific CD8⁺ T cells identified directly *ex vivo* by tetramer staining, they found that HIV-specific CD8⁺ T cells were deficient for perforin compared to CMV-specific CD8⁺ T cells. However, MHC class I tetramer staining may provide sufficient stimulation to induce perforin release, resulting in an underestimation of perforin content. Also, by measuring perforin content of resting cells, only immediate killing potential is measured, and the sustainability of the cytotoxic response cannot be determined. To assess serial killing potential, CTLs had to be stimulated and perforin content measured after the cells were allowed to progress through multiple rounds of proliferation. Several studies measuring cytotoxic potential in this way suggested that HIV-specific CD8⁺ T cells from ECs maintain the ability to upregulate perforin, a property that was deficient in CPs (23, 40, 133). While these results were in line with the results from the cytotoxicity assays, they also shared the same potential for memory subset bias introduced by the need for prolonged culture.

As a result of the difficulty in measuring cytotoxic factors in activated cells directly *ex vivo*, we developed an assay that utilized CD107a expression on CD8⁺ T cells as a surrogate marker of killing capacity (135). CD107a lines the membranes of cytotoxic granules and is exposed on the cell surface following activation and degranulation of CD8⁺ T cells. This technique was used recently to suggest cytotoxicity is one of the primary mechanisms responsible for both the enhanced HIV inhibitory potential of CD8⁺ T cells from ECs as well as for the emergence of HIV escape mutants (136–138). It is important to note, however, that although expression of CD107a correlates well with the exocytosis of perforin and cytotoxicity (135, 139), it does not directly equate to actual killing, as not all granules contain perforin or granzymes (140).

Through further testing, we soon discovered that the most commonly used perforin antibody (clone δ G9) was sensitive to the degranulation assay, which required the use of monensin to neutralize intracellular pH (139). By using a different monensin-insensitive perforin antibody (clone D48), we found that CD8⁺ T cells are capable of rapidly (within 4–6 h) upregulating perforin following antigen-specific stimulation without the requirement for proliferation (139, 141). We also found that newly synthesized perforin can traffic directly to the immunological synapse in a process that largely bypasses cytotoxic granules (141). This new perforin antibody thus allowed a direct assessment of antigen-specific CD8⁺ T-cell cytotoxic potential directly *ex vivo*. Used in conjunction with standard intracellular cytokine staining (ICS), we could now assess cytokine production and cytotoxic potential simultaneously.

Perforin upregulation and polyfunctionality

In an attempt to better understand the HIV-specific CD8⁺ T-cell mechanisms of viral control, we re-evaluated HIV-specific CD8⁺ T-cell polyfunctional responses, including the ability to rapidly upregulate perforin. Comparing the functional profiles of HIV-specific CD8⁺ T-cell responses from a cohort of elite controllers and chronic progressors, we confirmed that elite controllers have a higher frequency of polyfunctional cells compared to chronic progressors based on the expression of IL-2, IFN- γ , TNF- α , MIP-1 β , and CD107a (142). HIV-specific CD8⁺ T cells from elite controllers also have a greater ability to rapidly upregulate perforin directly *ex vivo* compared to progressors. This identifies a function of the elite controller HIV-specific CD8⁺ T-cell response that confers an enhanced ability to directly eliminate HIV-infected cells. Interestingly, however, there was no link between perforin upregulation and polyfunctionality. Rather, perforin expression was associated with cells of limited functional capacity, most frequently seen in combination with production of MIP-1 α or degranulation (CD107a). Thus, while highly polyfunctional cells are a correlate of control, so too are oligofunctional cells, depending on the functions produced. The question then became which of these populations, if either, is involved in control of viremia. Freel *et al.* (138) recently demonstrated that HIV-specific CD8⁺ T-cell capacity to inhibit HIV replication *in vitro* is correlated with the expression of MIP-1 β and CD107a and not linked to upregulation of IL-2, TNF- α , or IFN- γ . While noncytolytic mechanisms likely play some role in inhibition (136, 143), given our findings it is not unreasonable to speculate that many of the cells expressing MIP-1 β and CD107a are also upregulating perforin and inhibiting mainly through cytotoxicity, although this hypothesis has not been confirmed. Still, these studies only establish that HIV-specific CD8⁺ T cells found during controlled HIV infection have greater functional capacity and inhibitory potential, they do not tell us if these responses are actively involved in virus control or they are a result of control by some other mechanism.

CD8⁺ T-cell responses in acute HIV infection

HIV-specific CD8⁺ T cells are elicited early following HIV infection and are associated with control of viremia as well as selection of escape mutants (13, 144, 145). As the long-term control of HIV is likely determined by CTL responses during the resolution of acute viremia, we decided to characterize the functionality of HIV-specific CD8⁺ T cells during this phase of infection. It stands to reason that if polyfunctionality or rapid perforin upregulation are responsible for control, they should be present during this critical time period. Similar to other reports (137, 146, 147), when we examined expression of IL-2, IFN- γ , TNF- α , MIP-1 α , and CD107a, we found that HIV-specific CD8⁺ T cells are rarely polyfunctional during acute infection with the highest frequencies of responding cells represented by the expression of MIP-1 α and CD107a singly or in combination (Makedonas G, Betts M, *et al.*, manuscript in preparation). In contrast to previous reports indicating perforin expression during acute infection is low (148, 149), we found that rapid perforin upregulation dominates the earliest HIV-specific CD8⁺ T-cell responses we could detect in nearly every acute subject (Makedonas G, Demers K, Betts M, *et al.*, manuscript in preparation). This finding suggests that the appearance of highly polyfunctional HIV-specific CD8⁺ T cells observed in ECs is likely a result of viremic control, while a robust perforin-mediated response may be responsible for clearance of acute viremia. This idea is supported by a recent study comparing the HIV-specific CD8⁺ T-cell responses of ECs with individuals who initiate antiretroviral therapy immediately following infection (150). In this study, early, long-term therapy resulting in control of viremia led to the establishment of polyfunctional HIV-specific CD8⁺ T-cell responses that were identical to controller responses. These data suggest that polyfunctional HIV-specific CD8⁺ T cells emerge as a result of effective control of HIV replication while CD8⁺ T-cell cytotoxicity mediated by perforin expression is likely responsible for viral control.

CD8⁺ T-cell responses in gut mucosa and lymph nodes

The vast majority of HIV replication takes place in mucosal and lymphoid tissues, and it is not clear that HIV-specific CD8⁺ T-cell responses found in the blood are representative of responses found at these sites. As such, we need to broaden our understanding of CD8⁺ T-cell function within relevant tissues to pinpoint correlates of protection. Critchfield *et al.* (151) and Ferre *et al.* (131) both found associations between polyfunctional responses and disease status investigating production of IL-2, IFN- γ , TNF- α , MIP-1 β , and CD107a by HIV-specific CD8⁺ T cells from human gut mucosa. Although there was a paucity of cells producing all five functions, an IFN- γ /TNF- α /MIP-1 β /CD107a population clearly segregated controllers from noncontrollers, a finding largely in agreement with our observations from peripheral blood cells. However, Shacklett *et al.* (152) found little to no resting perforin expression by CD8⁺ T cells from rectal mucosa of both chronically infected HIV-positive and healthy individuals. Whether this finding is the same when looking at upregulated perforin or extends to the gut mucosa of ECs as well will need to be determined by more comprehensive studies comparing ECs and CPs.

Data from the lymph nodes are less consistent. While Altfeld *et al.* (153) found a greater magnitude of HIV-specific CD8⁺ T cells in the lymph nodes than in peripheral blood, Connick *et al.* (154) demonstrated that CD8⁺ T cells have limited access to lymphoid follicles, the primary site of HIV replication within these tissues. Further, an early study by Sunila *et al.* showed activated perforin-expressing CD8⁺ T cells localized within follicles, whereas subsequent studies have found no perforin expression by lymph node-resident CD8⁺ T cells (133, 148, 155), particularly by those that do gain access to the follicle (155). The discrepancies between peripheral blood HIV-specific CD8⁺ T-cell responses and those from the gut or lymph nodes may be related to phase of infection during which samples

were acquired, the progression status of the subjects studied, or simply a matter of differences in reagents used for analysis. It will be important to determine the true contribution of antiviral CD8⁺ T-cell responses at these sites given their role in HIV replication and dissemination. This is of particular importance given recent papers identifying lymph node CD4⁺ T-follicular helper cells as a new major reservoir of HIV infection (156–158).

Transcriptional regulation of CD8⁺ T-cell function and differentiation status

Accumulating evidence from our laboratory and others (40, 142, 159, 160) implicates the cytotoxic capacity of CD8⁺ T cells as a primary factor in the cellular control of HIV infection. Cytolytic potential is lost rapidly in most HIV-infected individuals, such that during chronic progressive infection only ~15% of HIV-specific CD8⁺ T cells express perforin, compared with ~40% in elite controllers (142). Understanding the underlying mechanisms involved in CD8⁺ T-cell differentiation and function may provide insight into the factors responsible for the dysfunction observed during chronic infection. To this end, several transcription factors have been identified that regulate the transition of CD8⁺ T cells into effector cells, including T-box expressed in T cells (T-bet), Eomesodermin (eomes), Runx3, and Blimp-1 (Table 1). While the majority of our current knowledge about the transcriptional control of CD8⁺ T-cell function comes from mouse models, we have recently begun assessing the role these transcription factors play in human T-cell effector function. Below, we discuss what we know from mouse models as well as our recent findings regarding CD8⁺ T-cell dysfunction in the context of HIV infection.

T-bet and Eomesodermin

T-bet and Eomes are both T-box binding transcription factors that play important roles in promoting CD8⁺ T-cell effector and memory differentiation and function. The T-box is a 180–190 amino acid sequence highly conserved across T-box family members that acts as a DNA-binding domain. T-bet and Eomes share 74% sequence identity in their T-box domains but lack sequence similarity outside this region. Like other T-box-binding transcription factors, they mediate developmental transitions through epigenetic modifications (161).

T-bet was originally identified as a determinant of T-helper 1 (Th1) cell lineage commitment (162), but subsequent work demonstrated its importance also as a regulator of CD8⁺ T-cell effector differentiation and function (163–165). T-bet positively regulates several genes associated with effector function, including perforin, granzyme B, β -chemokines, and IFN- γ (166), and negatively regulates genes that oppose effector function such as IL-2 and PD-1 (162, 166, 167). Although T-bet activity was originally described as being required for CD4⁺ and CD8⁺ T-cell IFN- γ expression and cytotoxicity, these functions were not completely ablated in T-bet knockout mice, suggesting the existence of a T-bet-independent regulatory mechanism (165, 168). This observation led to the discovery that CD8⁺ T cells also express Eomes, which shares both redundant and reciprocal functions with T-bet (169). The genes under the control of Eomes are not as well defined as for T-bet, but knockdown of Eomes in activated CD8⁺ T cells causes decreased expression of IFN- γ , perforin, and granzyme B, indicating there is at least some overlap with T-bet in promoting effector function. A combined T-bet and Eomes deficiency results in the loss of CTL identity and anomalous production of IL-17 by CD8⁺ T cells (170), suggesting that T-bet and Eomes coordinately regulate CTL differentiation.

T-bet and Eomes appear to play opposing roles in the generation of memory CD8⁺ T cells. Both transcription factors cause the upregulation of IL-2R β (CD122), which is required for long-term memory CD8⁺ T-cell survival and homeostatic proliferation in response to IL-15

signals (171). However, whereas high expression of Eomes correlates with central memory T (T_{CM}) cells, high expression of T-bet represses IL-7R α expression to drive formation of effector T and effector memory T (T_{EM}) cell subsets at the expense of T_{CM} cell generation (172, 173). T-bet is highest in early effector CD8 $^{+}$ T cells but progressively declines as memory cells form, while Eomes is initially upregulated in early effector CD8 $^{+}$ T cells but increases during the effector to memory transition (174). These divergent expression patterns may be partly explained by the way these transcription factors are induced. T-bet is rapidly induced in activated CD8 $^{+}$ T cells downstream of TCR signaling and augmented by inflammatory signals such as IL-12 (163, 164). Expression of T-bet initiates a positive feedback loop by upregulating IL-12R β , thereby increasing sensitivity to additional IL-12. Eomes induction is less well defined. However, studies indicate it is induced subsequent to T-bet, amplified by IL-2, and repressed by IL-12 (164). This suggests the differential expression patterns of T-bet and Eomes in CD8 $^{+}$ T cells, and in turn the differentiation state, depends on the degree of inflammation in the immediate environment of the cell.

Runx3

The runt domain-containing protein Runx3 is important for establishing the CD8 $^{+}$ T-cell lineage in the thymus, but its role in mature CD8 $^{+}$ T cells and mechanism of regulation are less clear. Previously, Runx3 knockout models showed reduced CD8 $^{+}$ T-cell cytolytic activity. This defect was later attributed to the role of Runx3 in driving CD8 $^{+}$ T-cell proliferation rather than a defect in cytolytic activity (175). More recently Cruz-Guilloty *et al.* (176) described a direct role for Runx3 in programming effector function, in cooperation with T-bet and Eomes, using *in vitro* differentiated CTLs. Similar to the findings described above, in this system, T-bet was induced early during differentiation, whereas Eomes was expressed at later stages. Interestingly, they showed only Eomes, not T-bet, could upregulate perforin, suggesting Eomes may be more important for CTL effector function. Importantly, Runx3-deficient CD8 $^{+}$ T cells failed to upregulate *Eomes*, *Ifng*, *Gzmb* (granzyme B), and *Prfl* (perforin). However, while Runx3 was found to bind to the promoters for *Ifng*, *Gzmb*, and *Prfl* in CTLs, it remains unclear if Runx3 upregulates these effector molecules directly or via its induction of Eomes, which binds to the same promoters.

Blimp-1 and Bcl-6

The B-cell transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) was first described for its ability to promote B-cell terminal differentiation into plasma cells. Three recent studies demonstrate that Blimp-1 plays a similar role in driving the terminal differentiation of effector CD8 $^{+}$ T cells over memory cells (177–179). These studies show that in antigen-specific CD8 $^{+}$ T cells, Blimp-1 expression increases during the acute response, has its highest expression in terminally differentiated CD8 $^{+}$ T cells, and decreases in memory cells following the resolution of infection, with the lowest expression levels observed in T_{CM} cells. Blimp-1 is critical for normal perforin and granzyme B expression and promotes trafficking to sites of infection by upregulating the chemokine receptor CCR5 (178, 179). Like T-bet, Blimp-1 is induced by IL-12, but it is also induced by IL-2 and IL-21. Bcl-6 is crucial for the formation of T_{CM} cells and is induced by IL-10 and IL-21 (180, 181). It is an antagonist of Blimp-1 activity, and its expression correlates inversely with Blimp-1 in effector and memory CD8 $^{+}$ T cells (182). Bcl-6 has also been shown to bind the T-bet promoter and T-bet itself in CD4 $^{+}$ T cells (183–185). While a similar interaction has not yet been demonstrated in CD8 $^{+}$ T cells, if confirmed, it would suggest Bcl-6 drives memory differentiation by repressing both Blimp-1 and T-bet directly, as well as the targets of these transcription factors.

Transcription factors in murine models of chronic infection

As discussed earlier, normal differentiation and function of virus-specific CD8⁺ T cells altered during chronic infection and exposure to persistent antigen. Effector cells fail to form functional memory populations and instead gradually become more exhausted. A few transcription factors have been shown to play a role in this process, including Blimp-1, T-bet, and Eomes (167, 177, 186, 187). Blimp-1 drives effector differentiation during acute infection; however, in the context of chronic infection, Blimp-1 also promotes the expression of the inhibitory receptors PD-1, CD160, 2B4, and Lag3, thereby inducing an exhausted state (177). T-bet sustains virus-specific CD8⁺ T-cell responses during chronic infection and suppresses the expression of PD-1. As T-bet expression decreases over time during chronic infection, PD-1 levels and CD8⁺ T-cell dysfunction both increase (167). Eomes also sustains virus-specific CD8⁺ T-cell responses during chronic infection, but its expression is also associated with a more terminally differentiated state, with high PD-1 expression levels, and Blimp-1 expression, which combined, indicate Eomes also plays a role in promoting exhaustion (187). Thus, the same transcription factors responsible for driving effector CD8⁺ T-cell differentiation and memory formation during acute infection are also responsible for limiting responses and preventing immunopathology in the context of chronic infection. Any attempt to manipulate these factors for purposes of immunotherapy will have to take these dual roles into account so that potential functional gains are not negated by simultaneously increasing terminal differentiation and exhaustion.

Transcriptional regulation of human CD8⁺ T cells

Although the molecules described above have been studied extensively in mouse models, much less is known about them in the context of human immunology. Investigation of transcription factors in human lymphocytes at the single cell level is largely limited to T-bet and Eomes as poor reagent availability precludes the assessment of Runx3 or Blimp-1. In the first report to fully characterize the expression of T-bet and Eomes in healthy human T cells, we found that Eomes is typically expressed with T-bet in CD8⁺ T cells and, in most cases, is bimodally distributed (188). T-bet, on the other hand, has a distinct trimodal expression pattern with a clear intermediate (or T-bet^{low}) population in the majority of individuals. We also found that differential T-bet expression levels are associated with specific functional characteristics: T-bet^{low} and negative cells are more likely to express IL-2 compared to T-bet^{high} cells, which tend to express perforin and granzyme B (25). These data are in agreement with data from mouse models in which T-bet promotes perforin and granzyme B expression, while repressing IL-2 production. T-bet and Eomes expression patterns also closely associate with memory phenotype: naive cells express little to no T-bet; T_{CM} cells express a little of both; there are more T_{EM} cells expressing T-bet overall, but with low MFI; and effector T cells express high levels of T-bet, with a large population of cells having high T-bet MFI. Eomes association with memory phenotype in healthy humans diverges somewhat from the mouse model, although there may be instances in human infections where these may be more similar (187). We found that significantly more cells in the effector T and T_{EM} subsets express Eomes than in the T_{CM} subset and, in the case of T_{EM} cells, Eomes is also expressed at a higher MFI. Polychromatic imaging cytometry (Amnis Imagestream^X) analysis of the T-bet^{high} and T-bet^{low} cells revealed an important difference in the localization of T-bet in human T cells; nuclear localization was generally observed in T-bet^{high} cells cytoplasmic localization was more common in T-bet^{low} cells (188). Looking at T-bet expression levels and localization in memory populations, T_{CM} cells generally express low levels of cytoplasmic T-bet, whereas effector T cells tend to express higher levels of nuclear T-bet. Thus while a cell may contain T-bet, if it is not expressed at high enough levels it remains sequestered in the cytoplasm of the cell and is transcriptionally inactive.

T-bet expression by HIV-specific CD8⁺ T cells

Having previously shown that HIV-specific CD8⁺ T cells from chronic progressors have a reduced ability to upregulate perforin compared to ECs, we examined the potential role of T-bet in driving effector function in each of these two groups (189). Similar to the expression pattern observed in normal healthy donors, perforin and granzyme B were closely associated with T-bet^{high} CD8⁺ T cells in the context of HIV infection. More importantly, we demonstrated that T-bet is significantly lower in HIV-specific CD8⁺ T cells from CPs compared to ECs. A recent report by Ribeiro-Dos-Santos *et al.* (190) confirmed our results through T-bet (and eomes) mRNA expression, finding reduced T-bet mRNA production during chronic HIV infection.

Given our recent findings regarding T-bet localization in human CD8⁺ T cells, the differential T-bet expression levels observed in the context of HIV control suggests that T-bet in elite controller HIV-specific CD8⁺ T cells may be predominately nuclear and therefore able to drive the effector gene cassette, while T-bet in chronic progressor HIV-specific CD8⁺ T cells is cytoplasmic and cannot upregulate effector function. If the association between localization and T-bet expression levels is confirmed in HIV-specific CD8⁺ T cells, these observations will provide a tangible mechanism to explain defects in the antiviral activity of HIV-specific CD8⁺ T cells in chronic progressors. Additionally, it will be important to determine when during the infection course this defective expression pattern is established. Two potential models are (i) HIV-specific CD8⁺ T cells from both ECs and CPs express T-bet at high levels during acute infection but failure to control viral replication results in loss of the T-bet^{high} population in CP, or (ii) EC HIV-specific CD8⁺ T cells are better able to express high levels of T-bet from the outset thereby providing a more robust antiviral response and better control.

It will also be important to look at the role of other transcription factors in the context of acute and chronic HIV infection, as T-bet does not act alone to regulate CD8⁺ T-cell differentiation and function (Table 1). For instance, Paley *et al.* (187) recently examined the expression of both T-bet and Eomes in HCV-specific CD8⁺ T cells from individuals who were either chronically infected with HCV or who had spontaneously cleared the infection. HCV-specific CD8⁺ T cells during chronic infection had higher levels of Eomes and relatively low levels of T-bet compared to HCV-specific CD8⁺ T cells from cleared HCV infection. This result mirrored their findings in a murine model of chronic LCMV infection. In addition, their murine model showed high Eomes expression was associated with high levels of Blimp-1 and the inhibitory receptors PD-1, Lag3, and Tim3, corresponding to a more exhausted phenotype. These data suggest that not only are low levels of T-bet unable to drive effector function in these cells, but Eomes is simultaneously promoting terminal differentiation and exhaustion which likely contributes to lack of control. Understanding the connection between CD8⁺ T-cell differentiation and function, and the complex network of transcription factors that regulates them, may provide a target for prophylactic or therapeutic strategies that lead to enhanced antiviral capacity in the face of chronic infection.

Concluding remarks

The identification of immune correlate(s) of protection will help form the basis on which to engineer and assess a prophylactic HIV vaccine. Although multiple studies have established the importance of measuring several T-cell functions simultaneously, the recent results of the Merck STEP trial emphasized the need to identify a true correlate of protection rather than relying on surrogate markers such as coproduction of IL-2, IFN- γ , and TNF- α . The ability of HIV-specific CD8⁺ T cells to clear infected cells through cytotoxic mechanisms represents the strongest indication of protective potential to date and should serve as a benchmark for cellular vaccine strategies. However, evaluation of additional functions

remains important as noncytolytic mechanisms may also play a role in protection, and a multi-faceted assessment of responses offers the greatest odds of predicting vaccine efficacy. Additionally, understanding how novel factors, such as transcription factors, are involved in the regulation of CD8⁺ T-cell differentiation and effector function during acute and chronic infection may provide new targets for prophylactic or immunotherapeutic strategies that elicit protective cellular responses to HIV.

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Fig. 1. Multifunctional nature of the CD8⁺ T cell-mediated HIV antiviral response

Schematic of CD8⁺ T cell (blue) interacting with HIV-infected or uninfected CD4⁺ T cells (red). CD8⁺ T cells directly inhibit replication by perforin and granzyme-mediated lysis of infected cells. They also promote a generalized antiviral state through secretion of cytokines (IFN-γ and TNF-α), prevent HIV transcription in infected cells by as yet unidentified molecules (CAF), and block HIV infection of target cells through β-chemokines (MIP-1α, MIP-1β, and RANTES).

Table 1Transcriptional regulators of CD8⁺ T cell differentiation and function

Transcription factor	Effect	References
T-bet	Cytotoxicity generation, granzyme and perforin production, terminal differentiation, represses PD-1 expression	25, 165, 166, 167, 172, 174, 189
Eomes	Cytotoxicity generation, granzyme and perforin production, expression of IL-15 receptor (CD122), upregulation of exhaustion markers	169, 171, 172, 174, 181, 187
Runx3	CTL proliferation, Eomes production	175, 176
Blimp-1	Cytotoxicity generation, granzyme and perforin production, terminal differentiation, represses Bcl-6 and Id3, upregulation of exhaustion markers	177, 178, 179, 187
Bcl-6	Inhibits Blimp-1 and T-bet directly as well as their target genes	180, 183, 184, 185
Id2	CTL expansion, terminal differentiation	191, 192
Id3	Promotes survival during memory transition	192, 193
Notch1	<i>Eomes</i> and perforin production	194
Notch2	Granzyme B production	195
STAT3	Sustains Bcl-6 and Eomes expression	182
STAT4	T-bet expression and terminal differentiation	196, 197
STAT5	Perforin production	181, 198, 199