

# Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection

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## Introduction

More than 25 million people have died of AIDS as a result of infection with the human immunodeficiency virus (HIV), and between 36 and 45 million individuals are living with the virus. While HIV is rampant in sub-Saharan Africa and the Caribbean, there are growing epidemics in Eastern Europe and Central Asia. Despite significant advances in our understanding of the virus, increased public awareness and intervention, and the development of effective treatment regimens, the annual rate of new HIV infections threatens to *increase* around the world without some kind of novel intervention, according to recent projections by UNAIDS. There is, thus, a desperate need for the development of a preventative vaccine against HIV infection.

HIV vaccine development has been hindered primarily by the difficulty of inducing antibodies capable of neutralizing the virus. Antibody-producing B cells recognize the variable loops of gp120, but the high error rate of viral reverse transcriptase (RT), along with the rapid turnover of plasma virions, provides a broad base of variants that escape detection by humoral immunity [1, 2]. The infidelity of RT during viral replication also promotes changes in envelope glycosylation patterns that render neutralization-sensitive domains inaccessible to HIV-specific antibodies [3–6]. In the simian immunodeficiency virus (SIV)/non-human primate model of HIV infection, no vaccine candidate to date has been shown to stimulate

effective neutralizing antibodies capable of protecting its host against a heterologous virus challenge. Furthermore, a study in which neutralizing antibodies were passively transferred into rhesus macaques necessitated unreasonably high titers of antibody to achieve protection from infection [7]. Similar studies in HIV-infected humans have not only yielded very limited evidence of protection but have even suggested the promotion of viral escape [8].

Although efforts continue in the hope of developing an immunogen that will induce neutralizing antibodies, researchers have more recently focused on vaccine candidates that will primarily stimulate cellular immune responses against HIV. The cellular immune responses are known to provide effective control of several chronic human pathogens, including Epstein–Barr virus (EBV) [9, 10], cytomegalovirus (CMV) [11–13], and hepatitis viruses B [14–16] and C [17]. The cytotoxic CD8<sup>+</sup> T lymphocytes (CTL) are thought to be the primary mediators of control of viral replication due to their ability to recognize and eliminate infected autologous cells. CD4<sup>+</sup> T helper (Th) cells also play a critical role in protection from viral and bacterial pathogens, as these cells provide support for both cellular and humoral immune responses. The precise mechanism(s) of control in different pathogenic settings, however, remains elusive.

The SIV/rhesus macaque experimental system has provided compelling evidence in support of a role for T cells in the control of HIV/SIV replication. The depletion of CD8<sup>+</sup> T cells during either primary or chronic SIV infection in Mamu A\*01-positive rhesus macaques is directly correlated to an increase in SIV viral load [18, 19]. In addition, the presence of vaccine-induced SIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells leads to a reduction in viral load during primary SIV infection [20–22]. A similar *in vivo* evidence in the setting of HIV infection has been difficult to

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obtain, but several lines of evidence suggest that CD8<sup>+</sup> T cells play a critical role in controlling HIV infection:

1. HIV-specific CD8<sup>+</sup> T cells are capable of directly killing HIV-infected CD4<sup>+</sup> T cells [23–25].
2. The control of initial viremia during primary HIV infection occurs concomitant to the appearance of HIV-specific CD8<sup>+</sup> T cells in the blood [26, 27].
3. Several major histocompatibility complex (MHC) class I haplotypes have been associated with non-progressive HIV infection [28–31].
4. Immunologic pressure mediated by CD8<sup>+</sup> T cell recognition promotes the outgrowth of virologic escape mutants [28, 32–35].

While it remains clear that CD8<sup>+</sup> T cells are a critical component of effective anti-HIV immunity, the precise correlates of control remain a mystery. In this review, we will discuss the general aspects of CD8<sup>+</sup> T-cell function that may be relevant to the control of viral replication in the context of evaluating the efficacy of a candidate HIV vaccine.

### Cytotoxic (CD8<sup>+</sup>) T-cell function

CD8<sup>+</sup> T cells recognize peptides derived from endogenously produced antigens, typically 8–11 amino acids in length, presented by MHC class I molecules. Upon engagement of the T-cell receptor (TCR) with its cognate peptide–MHCI (pMHCI) complex, a cascade of signaling events leads to the activation of the cell. An activated CD8<sup>+</sup> T cell is capable of a plethora of functions, some of which are listed in Table 1.

The functions can be grouped into two general categories: those involving immediate release of pre-formed factors (degranulation) and those requiring de novo protein synthesis. Many of these functions can be directly assessed by short-term in vitro stimulation with specific peptides or polyclonal stimulation. As shown in Fig. 1, CD8<sup>+</sup> T cells respond in a variety of ways when stimulated with *Staphylococcus* enterotoxin B (SEB) (top row), CEF (a mixture of peptides derived from CMV, EBV, and influenza, middle row), and HIV-Gag peptides (bottom

row). Although some cells react to stimulation with only a single function, the vast majority of responding cells exert multiple mechanisms. This phenomenon can only be appreciated by assessing the various functions simultaneously. Each of these functions plays a specific role in antiviral immunity and T-cell maintenance, as discussed below.

#### Cytotoxicity

CD8<sup>+</sup> T cells release perforin and granzymes A and B, the mediators of target cell killing, via *degranulation*, the polarized, microtubule-mediated transport of granules to the immunological synapse formed between the CD8<sup>+</sup> T cell and its target cell [36, 37]. Evidence also suggests that *Fas*-mediated cytotoxicity requires degranulation [38]. Although it has not been formally demonstrated, it is likely that the same cytotoxic granules responsible for *Fas* killing also contain perforin and granzymes.

CD8<sup>+</sup> T-cell killing activity can be readily detected by <sup>51</sup>Cr release and flow-based killing assays; however, these techniques are limited by the fact that neither technique examines the CD8<sup>+</sup> T cell itself but rather the effect of the CD8<sup>+</sup> T cell on the target. The presence of perforin within CD8<sup>+</sup> T cells stained with MHC class I tetramer or peptide-stimulated CD8<sup>+</sup> T cells can be alternatively examined [39]. This technique, however, suffers from a substantial flaw, namely, that perforin is *released* from activated cells; even MHC class I tetramer labeling of CD8<sup>+</sup> T cells can provide sufficient activation to induce the release of perforin.

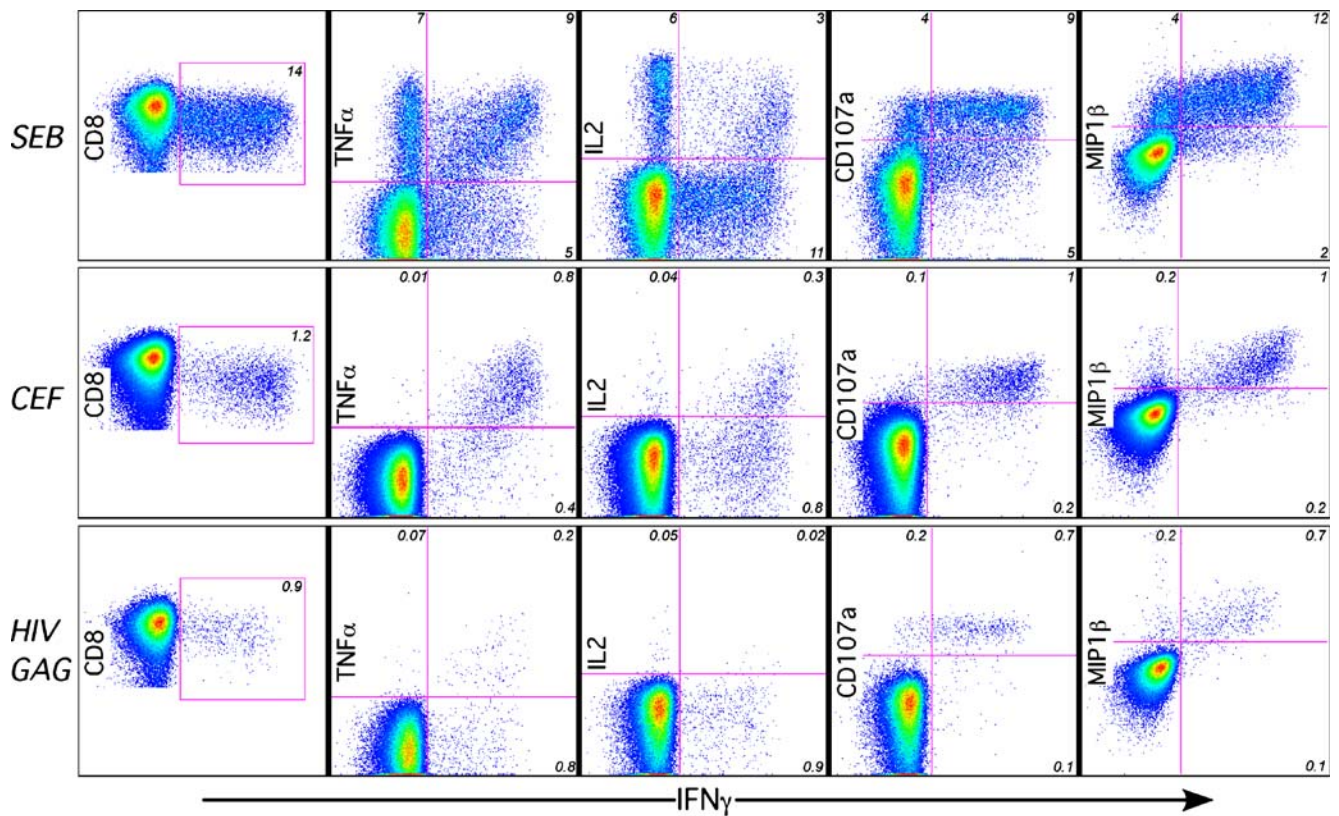
As a result, an alternate method was developed, in which the ability of CD8<sup>+</sup> T cells to degranulate is assessed by detecting the exposure of CD107a and b on the surface of CD8<sup>+</sup> T cells [40]. These molecules are not normally found on the surface of CD8<sup>+</sup> T cells but are found in the cytotoxic granule membrane [41]. Thus, when CD8<sup>+</sup> T cells degranulate, CD107a and b are made accessible on the cell surface for direct labeling by specific antibody. This assay, when used in concert with intracellular cytokine staining, allows the simultaneous measurement of both arms of the CD8<sup>+</sup> T-cell response to viral peptides. It has been shown in both CD8<sup>+</sup> T cells ex vivo as well as in CD8<sup>+</sup> T-cell clones that the ability to degranulate is directly linked to the ability to induce cytotoxicity [40, 42].

#### Chemokine production

The chemokines MIP-1 $\alpha$  and MIP-1 $\beta$  can also be found in cytotoxic granules, the latter type of which is upregulated rapidly upon activation [43]. RANTES is stored in a separate granule and is released immediately upon activation of the T cell, independently of the cytotoxic granule [44]. The primary function of these chemokines is the

**Table 1** Antiviral CD8<sup>+</sup> T-cell functions

Degranulation		De novo synthesis and release	
Cytotoxicity	Chemokines	Cytokines	Chemokines
Perforin	MIP-1 $\alpha$	IFN- $\gamma$	MIP-1 $\alpha$ (late ?)
Fas	MIP-1 $\beta$	TNF- $\alpha$	MIP-1 $\beta$
Granulysin	RANTES	IL2	RANTES (late ?)
		IL-4 (?)	
		TGF- $\beta$	



**Fig. 1** Various patterns of CD8<sup>+</sup> T-cell responses after stimulation. Peripheral blood lymphocytes were stimulated for 6 h with *Staphylococcus* enterotoxin B (top row), peptides derived from cytomegalovirus, Epstein–Barr virus, or influenza virus (CEF, middle row), or

overlapping peptides derived from HIV-gag (bottom row). The values represent the percentage of total CD8<sup>+</sup> T cells positive for the specific function in each plot. All functions were measured simultaneously by a 12-parameter flow cytometry

recruitment of additional cells to the site of the inflammatory response. As shown in Fig. 1, chemokine production can dominate the antigen-specific response, with nearly every responding cell producing MIP-1 $\beta$ . As discussed later, these chemokines may have additional positive and negative effects upon HIV itself.

#### Interferon- $\gamma$

In addition to chemokines, CD8<sup>+</sup> T cells also upregulate various cytokines and cell surface markers that can be detected as early as 2–4 h after stimulation [45]. The production of interferon (IFN)- $\gamma$  is routinely used as a marker for antigen-specific T-cell activity and is readily detectable by standard flow cytometric assays (Fig. 1). IFN- $\gamma$  is the only member of the type II class of interferons, a family of proteins that was originally discovered to interfere with viral replication [46]. IFN- $\gamma$  is structurally unrelated to type I interferons (multiple  $\alpha$  subtypes,  $\beta$ ,  $\omega$ , and  $\tau$ ), and is bound by a receptor that is distinct from that used by the type I IFNs [47]. Mice with mutations in either the gene that encodes IFN- $\gamma$  or the receptor through which it signals demonstrate deficien-

cies in natural resistance to bacterial, parasitic, and viral infections such as vaccinia virus, Theiler's murine encephalomyelitis virus, *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, and several poorly virulent mycobacteria species [48–53].

IFN- $\gamma$ -induced signalling regulates the expression of transcription factors that, in turn, regulate the expression of key antiviral enzymes, such as protein kinase R (PKR) [54–57]. In addition, IFN- $\gamma$  signalling increases the sensitivity of virally infected cells to apoptotic mechanisms, by promoting the expression of the TNF- $\alpha$  receptor on the cell surface [58] and/or by inducing the cellular expression of Fas and Fas ligand [59, 60]. As a result, several viruses encode proteins designed to specifically interfere with IFN- $\gamma$  receptor signalling [61, 62]. In addition to promoting an antiviral environment in an infected cell, IFN- $\gamma$  induces many functions that collectively endorse the generation of an adaptive immune response against pathogens:

- IFN- $\gamma$  activates the immunoproteasome [63, 64], the TAP transporter proteins [65–67], and the synthesis of MHC class I molecules [68, 69], all of which favor efficient processing and presentation of viral antigens.

- IFN- $\gamma$  favors Th1 cell lineage commitment and inhibits Th2 cell differentiation [6, 70].
- A gene chip analysis of antigen-specific CD8<sup>+</sup> T cells from the acute phase of an antiviral response reveals the activation of multiple genes by IFN- $\gamma$  [71].

IFN- $\gamma$  secretion constitutes a first line of antiviral defence, as T cells release IFN- $\gamma$  immediately after stimulation with antigen [72]. The pleiotropic effects of IFN- $\gamma$  play a critical role in many aspects of the innate and adaptive immune response to many viral and bacterial pathogens. As discussed later, however, the role of IFN- $\gamma$  in HIV-specific immunity is somewhat questionable, as it could also play a role in disease exacerbation through upregulation of HIV replication.

#### Interleukin-2

IL-2 is a protein growth factor that is secreted by T cells to promote the proliferation and differentiation of antigen-specific T cells in attempt to respond to pathogenic infections swiftly and efficiently. Although typically considered a CD4<sup>+</sup> T-cell cytokine, CD8<sup>+</sup> T cells are also quite capable of producing IL-2. Unlike CD4<sup>+</sup> T cells though, CD8<sup>+</sup> T-cell production of IL-2 is typically much more restricted, as shown in Fig. 1. The selective impairment of IL-2 secretion following TCR stimulation is a critical determinant of immune dysfunction during HIV infection, resulting in the inability of HIV-specific CD4<sup>+</sup> T cells to mediate protective immunity upon restimulation [73, 74]. IL-2 secretion thus serves as a reliable marker of functional HIV-specific CD4<sup>+</sup> T cells.

#### Tumor necrosis factor (TNF)- $\alpha$

This member of the TNF superfamily of proteins was first identified as a macrophage and lymphocyte factor that induced hemorrhagic necrosis of solid tumors [75, 76]. The activities of TNF- $\alpha$  are broad and encompass both beneficial effects in inflammation, protective immunity, and immune system development, as well as detrimental effects during sepsis and various autoimmune disorders [77]. TNF- $\alpha$  is first displayed on the plasma membrane as a 26-kDa pro-protein [78], which is subsequently cleaved by the matrix metalloprotease TNF- $\alpha$  converting enzyme to yield the mature soluble monomer [79]. The biologically active form of the soluble protein is a homotrimer that binds to both the TNF-RI and TNF-RII receptors [80].

TNF- $\alpha$  is mainly produced by monocytes and macrophages but can also be secreted by T cells, natural killer cells, basophils, eosinophils, dendritic cells, neutrophils, and mast cells [81]. TNF- $\alpha$  synthesis can be stimulated by viral

infections, bacterial and parasitic products, complement, and cytokines [81]. In the case of T cells, engagement of the TCR triggers TNF- $\alpha$  release, which amplifies the Th1 response by inducing the synthesis of IL-12 and IL-18 [82]. These factors are important in upregulating IFN- $\gamma$  production. TNF- $\alpha$  can alternatively kill virally infected target cells by binding its cognate receptor on their cell surface. The TNF receptor contains an intracellular ‘death domain’ which triggers an apoptosis signalling cascade [77].

#### CD8<sup>+</sup> T cell function and control of HIV

Despite the potential functional breadth of the antiviral T-cell response, the immunogenicity of HIV-specific T-cell responses and candidate T-cell-based vaccine constructs is largely gauged by the production of IFN- $\gamma$ . However, other functional activities of CD8<sup>+</sup> T cells could play an important role in controlling or exacerbating HIV replication.

Although IFN- $\gamma$  is the most widely assessed function of CD8<sup>+</sup> T cells, there is no direct evidence that this cytokine itself has direct effects against HIV. An inverse correlation between the absolute frequency of HIV-specific CD8<sup>+</sup> T cells and HIV RNA copies in the plasma was initially established based on MHC class I tetramer staining [83]. Numerous subsequent studies examining the frequency of HIV-specific CD8<sup>+</sup> T cells based on IFN- $\gamma$  production have demonstrated, to the contrary, that the frequency of IFN- $\gamma$ -producing cells during primary HIV infection is directly proportional to increases in plasma viral loads [84–86]. This may result from the fact that IFN- $\gamma$  signalling upregulates NF $\kappa$ -B activity [87], which could increase the activation state of the cell, thereby making it a prime target for HIV infection. Enhanced NF $\kappa$ -B activity could similarly potentially promote viral replication in cells already infected with the virus. Furthermore, it has been demonstrated that IFN- $\gamma$  secretion may identify a population of cells that will clearly not develop into long-term protective memory cells [88]. Helper CD4<sup>+</sup> T-cell responses (Th1) defined by the secretion of IFN- $\gamma$  were shown to be short-lived, whereas a population of activated Th1-lineage cells that did not secrete IFN- $\gamma$  after primary antigenic stimulation persisted for several months *in vivo* and developed the capacity to secrete IFN- $\gamma$  upon subsequent stimulation [88]. Thus, the current literature on HIV infection suggests that IFN- $\gamma$  secretion alone is a poor correlate of *in vivo* protection against HIV. Yet despite these findings, the measurement of vaccine-induced IFN- $\gamma$  secretion remains the primary readout of HIV vaccine immunogenicity.

Chemokines are optimistically thought to serve as antagonists to HIV infection. The natural ligands of CCR5

(RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) and CXCR4 (SDF-1) were shown to be able to block viral entry in vitro, presumably by competing with HIV gp120 for binding sites on the receptors [89, 90]. Receptor downregulation as a result of cognate chemokine binding could, alternatively, potentially retard HIV infection. However, the regions in CCR5 required for ligand binding and HIV co-receptor activity only partially overlap, and not all primary isolates use the same structural elements of CCR5 to gain entry [91]. Upregulated chemokine production by HIV-1-specific CD8<sup>+</sup> T cells may also recruit uninfected, immune-competent T cells to sites of active viral replication to serve as new targets of infection. Finally, there is the possibility that chemokine release by CD8<sup>+</sup> T cells could play a role in driving the switch in HIV-1 coreceptor usage (CCR5  $\rightarrow$  CXCR4) that typically occurs before progression to AIDS.

Cytotoxic activity by HIV-specific CD8<sup>+</sup> T cells is generally considered a necessary requirement for effective control of viral replication; however, it has been suggested that this activity could also be detrimental. Though not considered to be a major contributor, bystander-killing by CD8<sup>+</sup> T cells has been proposed to play a role in CD4<sup>+</sup> T cell depletion [92, 93]. Furthermore, the direct killing of HIV-infected CD4<sup>+</sup> T cells may curtail any residual beneficial effects the CD4<sup>+</sup> T cells may have for providing help. The elimination of infected HIV-specific CD4<sup>+</sup> T cells by CD8<sup>+</sup> T cells may indeed in part result in the profound defects observed later in disease in the HIV-specific CD8<sup>+</sup> T cell pool.

### The importance of CD4<sup>+</sup> T cells

The CD4<sup>+</sup> T-helper lymphocytes are critical in the maintenance of effective immunity against several viral infections. Virus-specific CD4<sup>+</sup> T cells proliferate and secrete cytokines, for example IL-2, that promote antiviral functions from other arms of the immune system, especially CTL [94]. In mice infected with lymphocytic choriomeningitis virus (LCMV), the depletion of CD4<sup>+</sup> T cells by the administration of a CD4<sup>+</sup> T-cell-specific monoclonal antibody results in the abrogation of CTL control of viral replication during the chronic stage of infection [95, 96]. CD4<sup>+</sup> T-cell-depleted mice experience persistent high viral loads and are unable to sustain effective CTL responses to the virus [95, 96].

In the setting of HIV infection, CD4<sup>+</sup> T-cell proliferation in response to stimulation with p24 antigen is inversely related to the plasma viral load in patients with chronic infection [97]. Individuals who display long-term control of viremia in the absence of antiviral therapy maintain

polyclonal HIV-1-specific CD4<sup>+</sup> T-cell proliferative responses throughout the course of HIV infection, whereas this effector function is absent in individuals with persistent viral loads [97]. The selective depletion of activated CD4<sup>+</sup> T helper cells during early HIV infection is considered to be responsible for the impairment of CTL function during chronic infection. It has been shown that aberrant HIV-specific CD8<sup>+</sup> T-cell proliferation can be restored, in vitro and in vivo, by the addition of fully competent autologous HIV-specific CD4<sup>+</sup> T cells that secrete IL-2 [98]. Thus, CD4<sup>+</sup> T lymphocytes appear to be an important correlate of immunity against HIV infection.

It is not surprising that chronically infected HIV patients exhibit impaired CD8<sup>+</sup> memory T-cell function, as the IL-2-producing HIV-specific CD4<sup>+</sup> T cells that are capable of rapid proliferation during recall stimulation are functionally impaired in these individuals [74]. The HIV-specific CD8<sup>+</sup> T cells from HIV infected patients with persistent viremia are able to secrete IFN- $\gamma$ , but they are low in perforin expression, poorly cytotoxic, and incapable of antigen-specific proliferation [99]. In mice chronically infected with the lymphocytic choriomeningitis virus, virus-specific CD8<sup>+</sup> memory T cells progressively lose their effector capabilities [100]. IL-2 secretion appears to be the most sensitive to persistently high viral loads, followed by TNF- $\alpha$  production, and finally IFN- $\gamma$  secretion [100]. Functional exhaustion and deletion of antigen-specific CD8<sup>+</sup> memory T cells have also been observed in primates infected with SIV [101, 102], in humans infected with hepatitis B virus (HBV) [103] and hepatitis C virus [104], and during malignant melanomas [105]. Thus, the measurement of IFN- $\gamma$  secretion alone may focus on T cells that are actually impaired in their ability to control viral replication.

### The problem with phenotyping

Considerable efforts have been made to delineate and characterize the subsets of antigen-specific memory T cells. Cell surface markers, including CD45RA/RO, CD27, CD28, CD57, CD62L, CD127, and CCR7 have all been used in various combinations to define memory cell populations that are responsible for effective antiviral immunity. For example, the expression of CCR7 on memory T cells was postulated to separate central memory T cells from effector memory T cells [106]. In this model, the former cells, by virtue of CCR7 expression, efficiently home to lymph nodes but are incapable of exerting antiviral effector functions. In contrast, antiviral immunity, such as cytokine secretion and cytotoxicity, is mediated by the CCR7<sup>-</sup> effector memory T cell subgroup. Recent studies using both murine [107] and human systems [108, 109],

however, have questioned this hypothesis, as antigen-specific CCR7-expressing cells have been shown to express similar levels of perforin as CCR7<sup>-</sup> cells and to be potent secretors of TNF- $\alpha$  and IFN- $\gamma$  also.

In parallel, CD28 was hypothesized to distinguish functional effector cell populations, but data derived from studies using human [110] and rhesus macaque [111] peripheral blood mononuclear cells (PBMC) clearly show that both CD28<sup>+</sup> and CD28<sup>-</sup> cells are adept at antiviral cytokine production. Moreover, recent evidence suggests that cytokine expression patterns between HIV-specific CD8<sup>+</sup> T cells of various memory phenotypes can be shared [112]. Thus, it is entirely unclear what relevance memory phenotype may have if the functionality of the cells does not differ substantially between the various potential phenotypes.

### Polyfunctional T cell analysis

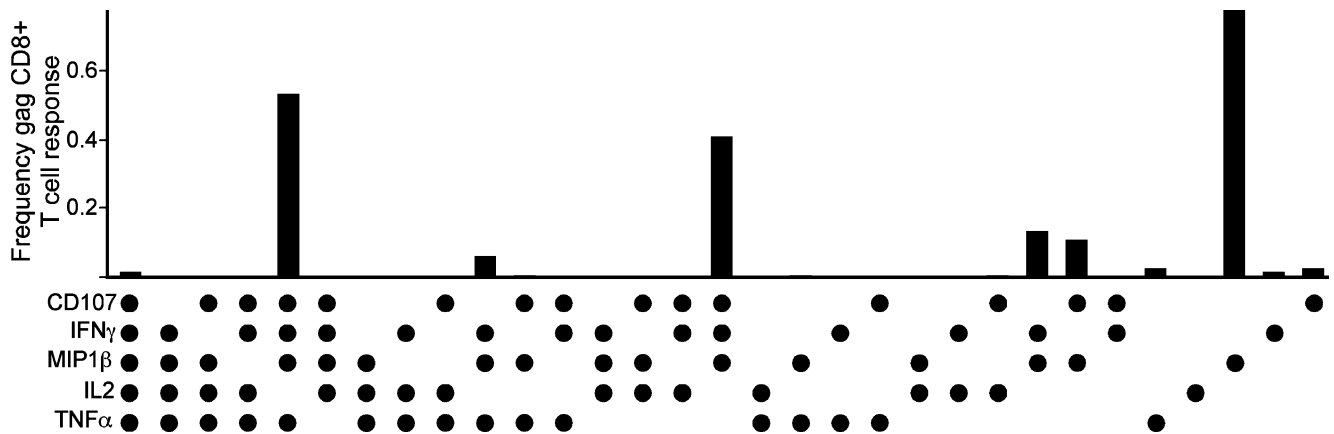
It is clear that there is no discernible relationship between phenotypic and functional heterogeneity among human memory T-cell populations. Functional heterogeneity has been demonstrated in the course of nearly every viral infection. As such, antigen-specific T cells must be characterized functionally, and then the functional profile must be correlated to protective T-cell immunity. Only recently have researchers begun to explore the breadth of the functional T-cell repertoire.

Antigen-specific IL-2 production has been demonstrated in several experimental systems to be a more accurate predictor of the protective capacity of responder T-cell populations than IFN- $\gamma$  secretion alone. The observation that IL-2 knockout (-/-) mice are severely impaired in their ability to control viral infections is likely explained by drastically reduced CD8<sup>+</sup> T-cell responses [113]. It has been demonstrated that antigen-specific CD8<sup>+</sup> T cells are capable of secreting IL-2 [114, 115]. In some systems, they can even outnumber the quantity of CD4<sup>+</sup> IL-2-producing cells during acute infection [116]. In a murine model of influenza-specific memory T-cell development, antigen-specific CD8<sup>+</sup> T cells in the resolution phase of infection after both primary and secondary challenges were shown to exhibit a functional hierarchy in their cytokine response: IL-2<sup>+</sup> CD8<sup>+</sup> T cells are a subset of TNF- $\alpha$ <sup>+</sup> cells, which are a subset of IFN- $\gamma$ <sup>+</sup> cells [117]. During HIV infection, it has been shown that patients who maintain CD8<sup>+</sup>, as well as CD4<sup>+</sup>, T cells with the capacity to produce both IFN- $\gamma$  and IL-2 typically experience a milder HIV disease course than those who have a greater proportion of single cytokine-positive T-cell responses [74, 118]. Thus, the capacity to produce IL-2 may reflect a stage of superior functional differentiation than the secretion of IFN- $\gamma$ .

TNF- $\alpha$  has been shown, in combination with IFN- $\gamma$ , to clear HBV from hepatocytes and LCMV from acutely infected mice [16, 119, 120]. In murine studies of memory T cells, TNF- $\alpha$  production represents an earlier stage of functional development compared to IFN- $\gamma$  secretion, as the loss of TNF- $\alpha$  production precedes the loss of IFN- $\gamma$  production [100]. By incorporating the detection of TNF- $\alpha$  secretion into studies of CD8<sup>+</sup> T-cell functional diversity, it is clear that the functional heterogeneity of responding antigen-specific CD8<sup>+</sup> T-cell populations is more complex than what was thought based on studies that measured fewer parameters. In a study by Sandberg et al., stimulating PBMC from normal human donors with CMV pp65 revealed that IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells were the most prevalent in the responding CD8<sup>+</sup> T-cell population [121]. In response to SEB stimulation, it was determined that approximately 30% of the cytokine-secreting CD8<sup>+</sup> T cells were TNF- $\alpha$ <sup>+</sup>, IL-2<sup>+</sup>, and IFN- $\gamma$ <sup>+</sup> [121]. A kinetic analysis of the cytokine secretion profile of the SEB-stimulated cells revealed that TNF- $\alpha$  is produced first, followed by IFN- $\gamma$  and then IL-2 [121]. Despite the fact that all three cytokines were not stained for simultaneously, the results clearly indicated a novel level of functional complexity.

Technological advancements in flow cytometry now permit the staining and detection of up to 18 different markers on human T cells. Staining panels and procedures have been developed to permit the examination of five T-cell functions simultaneously: CD107a, IFN- $\gamma$ , MIP-1 $\beta$ , TNF- $\alpha$ , and IL-2. As shown in Fig. 1, each of these functions can be assessed individually or in combination with each other. This flow panel yields a phenomenal amount of data; by measuring five different T-cell functions concurrently, it becomes possible to subdivide the functional profile of the response into 32 (2<sup>5</sup>) different ‘flavors’. The advantage of this type of analysis over traditional techniques is that response quality, rather than simple magnitude, can be assessed.

This type of analysis was recently used to examine HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in a large group of HIV-infected subjects with varying disease progression rates [112]. The goal of these studies was to assess the *quality* of the HIV specific response in these individuals, in an attempt to identify a correlate of immune protection mediated by CD8<sup>+</sup> T cells. Figure 1, bottom row, shows the five different T-cell functions measured in response to HIV Gag in one of the subjects. It is noteworthy that nearly every responding cell population (to any HIV antigen) produced MIP-1 $\beta$ , to the point where production of this chemokine dominated the HIV-specific T-cell response. Furthermore, as shown in Fig. 2, some of the major responding populations did not even produce IFN- $\gamma$ . In the subject shown in Fig. 2, a substantial proportion of the HIV-Gag-specific CD8<sup>+</sup> T cells produced only MIP-1 $\beta$ . As



**Fig. 2** Functional profile of HIV-Gag-specific CD8<sup>+</sup> T cells in a HIV-infected progressor. Polychromatic flow cytometry was performed measuring five different CD8<sup>+</sup> T-cell functions simultaneously, shown

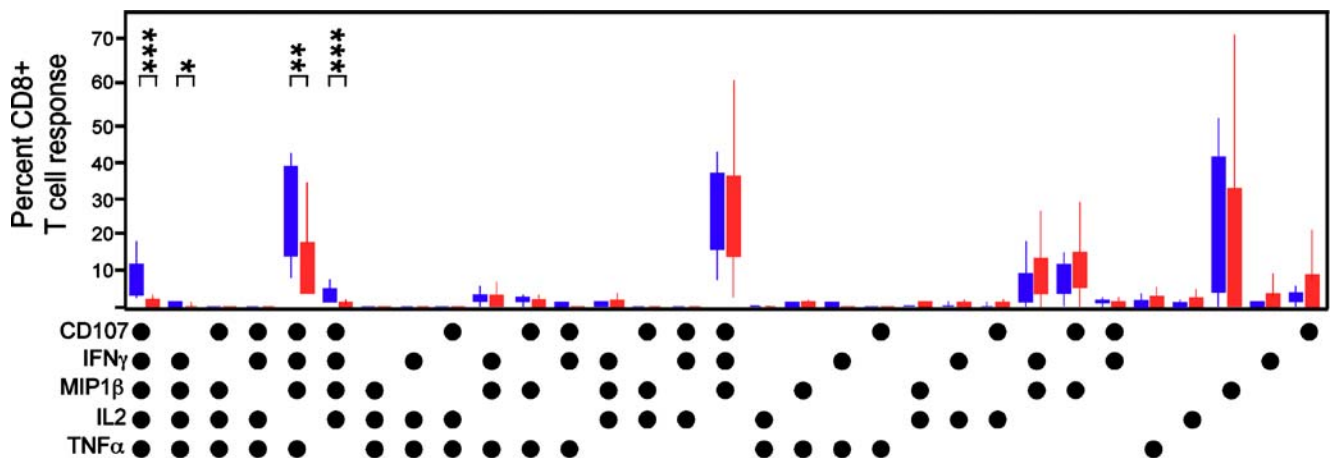
in the figure key on the *left*. *Solid circles* denote positive responses for the particular function. The data are background corrected

a consequence, the measurement of multiple functions simultaneously allowed the detection of virus-specific responses that would have otherwise been overlooked.

A precise correlate of immune protection in HIV-infected long-term nonprogressors (LTNP) has remained elusive, as the simple frequency of HIV-specific CD8<sup>+</sup> T-cell responses in these subjects is not dramatically different than in progressor subjects [122], aside from some differences in proliferative potential and perforin upregulation after extended culture *in vitro* [99]. However, when we compared the functional profile of the HIV-specific CD8<sup>+</sup> T-cell responses between the progressors and nonprogressors (Fig. 3), the nonprogressors (blue box plots) had a higher degree of functionality (four or five

different functions simultaneously) than the progressors (red box plots). The presence of HIV-Gag-specific CD8<sup>+</sup> T cells that were positive for all five measured functions was a discriminating factor between nonprogressors and progressors (on Fig. 3; \*\*\*= $P < 0.001$ ; \*\*= $P < 0.01$ ; \*= $P < 0.05$ ). This population was largely absent in most progressors. In addition, Gag-specific CD8<sup>+</sup> T cells expressing various combinations of the four functions were more prevalent in the nonprogressors. In a similar fashion, HIV-specific CD8<sup>+</sup> T-cell responses to Pol, Env, and Tat/Rev/Vif/Vpr/Vpu were also more functional in nonprogressors (data not shown).

Thus, the measurement of several functions concurrently enabled the discernment of a fundamental difference



**Fig. 3** The HIV-specific CD8<sup>+</sup> T-cells from nonprogressors (*blue boxes*) have a qualitatively different functional profile compared to progressors (*red boxes*). The *box plots* on the figure represent the 10th, 25th, 75th, and 90th percentiles of the proportion of the respective functional response towards the total CD8<sup>+</sup> T-cell response against HIV Gag. The responses from the cohorts were standardized so that

the profiles could be compared irrespective of any frequency differences. *Asterisks* are placed above response pairs that are significantly different: \*\*\*= $P \leq 0.001$ ; \*\*= $P \leq 0.01$ . Marginal differences (\*= $P \leq 0.05$ ) are designated as a *single asterisk*. Each *dot* in the legend denotes a positive response for the function indicated at the *bottom left*

between HIV-specific CD8<sup>+</sup> T-cell responses in LTNP compared to progressor subjects.

### Polyfunctional analysis of vaccine-induced responses

Historically, most immunological assessments of candidate HIV vaccines have been performed using lymphoproliferation and chromium release assays. The IFN- $\gamma$  ELISPOT and intracellular cytokine staining/ MHC-class I tetramer binding have more recently been the assays of choice. It is clear that these relatively simple assessments may drastically underestimate the potential breadth of vaccine-induced responses. A few recent studies highlight the polyfunctional nature of vaccine-induced responses in the setting of hepatitis B virus and HIV vaccines.

The first such study, by De Rosa et al., examined the functional nature of HBV- and HIV-vaccine-induced responses in human volunteers, using a functional panel that examined IFN- $\gamma$ , IL-2, TNF- $\alpha$ , MIP-1 $\beta$ , and IL-4 [123]. It was surprising that complex functional profiles were observed for CD4<sup>+</sup> T-cell responses against the vaccines. Rather than being limited to only IFN- $\gamma$ , each vaccine also stimulated IL-2, TNF- $\alpha$ , and MIP-1 $\beta$  production in various combinations from vaccine-induced CD4<sup>+</sup> T cells. A similar complexity was observed in the functional response against the HIV vaccine from CD8<sup>+</sup> T cells. It is important to note that in no subject was IFN- $\gamma$  the best indicator of a vaccine-induced response. This study provided the first indication of the importance of measuring multiple functional responses of T cells in a vaccine setting.

A subsequent study focused on the HIV vaccine-induced response in a single individual immunized with a canary pox-HIV vector [124]. This individual mounted a potent response against HIV Gag (compared to the majority of canary pox vaccinees) from both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These responses also displayed considerable functional complexity; however, substantial responses were detected that were positive for only a single function: for CD4<sup>+</sup> T cells, a substantial IL-2 only response was found, and for CD8<sup>+</sup> T cells a CD107a only response was detected. For both T-cell subsets, the frequency of cells producing IFN- $\gamma$  was quite low. Thus, without measuring additional functions beyond IFN- $\gamma$ , a substantial proportion of the vaccine-induced response would have gone undetected.

An important caveat to these studies is that all of the various vaccine vectors utilized stimulated qualitatively *different* functional profiles in responding T cells. This is further accentuated by differential functionality observed in individuals vaccinated with vaccinia (NYVAC and modified vaccinia Ankara) and HIV-DNA and Ad5 vectors (R. Koup, personal communication). Hence, it is becoming

clear that polyfunctional analysis is critical to the accurate assessment of vaccine-induced T-cell responses.

### Concluding remarks

Over the past 10 years, major improvements in our understanding of memory T-cell generation and differentiation has prompted the design of sophisticated vaccine constructs aimed at inducing potent and long-term antigen-specific cellular immunity. In addition to the vaccine vector and the composition of the construct, the route of administration, the dose, the timing of the prime/boost regimens, and the processing and presentation of the immunogens all influence the type and quality of immunity induced by the candidate vaccine. These factors are difficult to modulate in humans, thus making the assessment of promising vaccine strategies extremely challenging. The delineation of an effective HIV-vaccine-induced response capable of preventing infection or controlling viral replication will require the simultaneous analysis of specific functions elicited from responding T cells. It is important to note, however, that we must also understand the functional nature and protective aspects of T cells induced by natural infection. Only by drawing such a comparison can we begin to assess whether a candidate vaccine will induce a potentially protective or an irrelevant immune response.

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