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Viral Inhibitory Activity of CD8+ T Cells in HIV Infection

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Abstract

Cytotoxic T lymphocytes, or CD8+ T cells, play an important role in the control of replication of HIV. Inducing effective and durable HIV-specific CD8+ T cell responses are, therefore, a major objective in prophylactic and curative strategies for HIV infection. To evaluate such strategies, reliable immunological assays are needed that measure the capacity of CD8+ T cells to exert their effector functions and control viremia. Classical immunological assays such as interferon-γ (IFN-γ) enzyme-linked immunospot or intracellular cytokine staining measure the production of one or several effector molecules but do not actually show suppression of viral replication. Perhaps unsurprisingly, these assays do not correlate with either prevention of infection or lower viral set-points after infection. Therefore, more relevant assays are needed which directly measure the viral inhibitory activity (VIA) of CD8+ T cells and are more likely to predict success or failure of different immune interventions. The present review discusses the methodology of the VIA in detail as well as the practical implications of the several variations that have been described. We then go onto discuss existent literature on the relationship between VIA and HIV control, give an overview of examples where VIA has been induced or boosted in vivo or in vitro, and finally discuss observed associations between VIA and other immunological parameters. We conclude that while VIA is complex and laborious, it provides functional information about CD8+ T cells that no other assay can deliver. **(AIDS Rev. 2019;21:115-125)**

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Key words

Viral inhibitory activity. Viral inhibition assay. Cytotoxic T cells. CD8+ T cells.

Introduction

A wide range of evidence supports the idea that CD8+ T cells play an important role in controlling viremia during HIV and simian immunodeficiency virus (SIV) infection. First, the emergence of HIV-specific CD8+ T cells during acute infection is quickly followed by a decline in viremia[1]. Second, the depletion of CD8+ T cells from SIV-infected rhesus macaques results in loss of viral control[2]. Finally, the emergence of mutations enabling viruses to escape CD8+ T cell responses indicates that selective pressure is exerted by the

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same[3]. Taken together, these observations clearly demonstrate the importance of CD8+ T cells in the control of HIV infection, whether CD8+ T cells exert this control through mainly cytolytic or non-cytotoxic activities remain a matter of debate (reviewed in detail by McBrien et al.[4]) but is beyond the scope of this review.

In the search for an HIV-1 vaccine able to induce protective CD8+ T cell responses, robust assays are crucial to evaluate vaccine candidates. In this regard, the failure of interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) to predict the failure of Merck's STEP trial showed the limits of these classical immunological assays[5,6]. These assays are conceptually limited in that they only measure the production of one or several cytokines in response to a peptide stimulation, often at (high) peptide concentrations which might not even be biologically relevant. Earlier work often used the chromium release assay (CRA) which shows the direct cytolytic activity of CD8+ T cells against HIV-infected CD4+ T cells[7]. However, besides being laborious, technically demanding and variable, the assay also gives little information about the CD8+ T cells themselves, other than that they can kill. Alternatively, peptide-major histocompatibility complex (MHC) staining (e.g., tetramer) is used to determine the number of HIV-specific cells but gives little functional information about these cells and can only be used with the limited number of known human leukocyte antigen (HLA) allele/peptide combinations[8]. Measuring proliferation in response to HIV peptides provides useful information but does not directly inform about the cytotoxic potential of proliferating CD8+ T cells. Taken together, it is probably more relevant to use assays which directly measure the actual inhibition of viral replication instead of using separate, indirect measures of CD8+ T cell functionality.

Such assays measuring the capacity to inhibit viral replication (named viral inhibitory activity [VIA]) generally consist of cocultures of superinfected CD4+ target T cells with CD8+ effector T cells and have been used since more than 30 years in HIV research[9]. Conceptually, this assay has the advantages of measuring inhibition of viral replication through both contact-dependent and independent mechanisms, and to measure exactly what an HIV vaccine aims to induce, i.e., suppression of viral replication.

In this review, we use studies dating from 1986 to 2019. We first summarize and discuss the different methods that have been described to measure *ex vivo* VIA. Next, we discuss how elite controllers (ECs) distinguish themselves from chronic progressors (CPs) in terms of VIA and discuss what other clinical parameters correlate with VIA. We give an overview of studies where VIA has been shown to be induced both *in vivo* and *in vitro* and finally, we discuss observed correlations with virological and immunological parameters.

Measuring VIA

As detailed in a number of papers[10-13], the main steps of a typical VIA assay are (a) activation of target CD4+ T cells, (b) preparation of effector CD8+ T cells, (c) superinfection of target CD4+ T cells, (d) coculture of target and effector cells, and (e) measurement of viral replication. Underneath, we discuss these different steps in detail. Figure 1 gives a flow chart of a typical VIA assay.

Target CD4+ T cell activation

Autologous CD4+ T cells are used as target cells when investigating contact-dependent inhibition. Alternatively, non-contact dependent inhibition of viral replication can be studied using autologous CD4+ T cells in trans-well experiments or heterologous CD4+ T cells in coculture[11,14]. Other cell types, such as macrophages, can be used as target cells as well[15].

CD4+ target T cells are activated to render them more susceptible to infection. The most common agents used for stimulation include phytohemagglutinin (PHA) in combination with interleukin 2 (IL-2), beads coupled to anti-CD3 and anti-CD28 antibodies or bispecific monoclonal antibodies targeting CD3 and CD8. Depending on the agent and its concentration, target cell stimulation takes 3-7 days. When using PHA+IL-2 or anti-CD3/CD28 beads, CD4+ T cells are enriched before stimulation. With bispecific monoclonal anti-CD3/CD8 antibodies, CD4+ T cells are enriched after stimulation, as the bispecific antibodies need to crosslink CD4+ and CD8+ T cells, thereby causing preferential proliferation of the CD4+ T cells as well as killing of CD8+ T cells[16]. Enrichment before stimulation is more reliable and typically results in higher yields and purities.

Effector CD8+ T cell preparation

As effector cells, three types of cells are commonly used, namely, (i) CD8+ T cell clones or cell lines, (ii) non-stimulated, resting CD8+ T cells, and (iii) stimulated, expanded CD8+ T cells.

Much of the research on VIA has been done using CD8+ T cell lines or clones. These are prepared by repeated HIV peptide stimulations in combination with

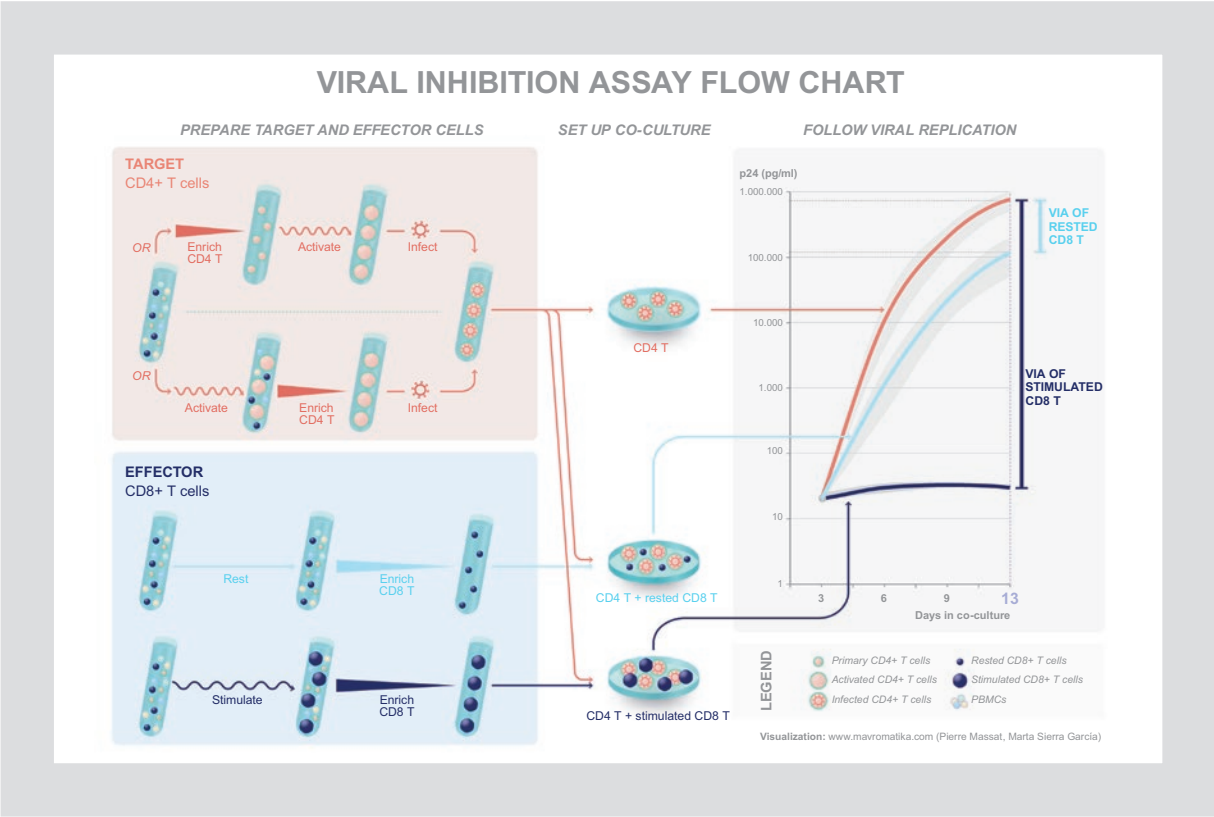

Figure 1. *Flow chart of a typical viral inhibition assay. Target CD4+ T cells are enriched, activated, and infected while CD8+ T cells are either rested or stimulated. Target cells alone and cocultures of target and effector cells are set up after which viral production is monitored over time by measuring p24 concentrations in the supernatant. The difference in viral production is a measure of the viral inhibitory activity of the effector cells.*

a polyclonal stimulus and/or γ-chain cytokines (IL-2, IL-7, and IL-15) to induce proliferation. Cell lines/clones are useful tools to perform in-depth studies on epitope specificity, functional avidity, transcription profiles or the influence of HLA restriction but are less suited to study differences between different patients and the relation of VIA activity with clinical parameters. In addition, epitope-specific clones, which are kept in long-term *in vitro* cultures, use a single T cell receptor and might have less cross-reactivity than primary epitope-specific CD8+ T cell populations that are polyclonal[17].

Non-stimulated primary CD8+ T cells are either rested for the duration of the CD4+ T cell activation or kept frozen until the start of coculture and are used to measure the "*ex vivo*" VIA of a patient's CD8+ T cells. To measure VIA after *in vitro* stimulation/expansion of the effector cells, CD8+ T cells can be stimulated in a non-specific, polyclonal way (e.g., bispecific monoclonal anti-CD3/CD4 antibodies in combination with IL-2[11]) or in more specific ways, using HIV peptides or monocyte-derived dendritic cells (Mo-DCs) transfected with HIV mRNA or pulsed with peptides. As non-specific stimulation methods are usually strong and induce proliferation, few cells are needed, and viral suppression is easily detectable. An important disadvantage, however, is higher "background inhibition" by T cells from non-infected subjects and a questionable *in vivo* relevance of the results. Relatively few studies have used the more specific stimulation methods, using pulsed Mo-DCs[18] and HIV peptides in combination with IL-2[19-23]. From our own experiments, we have observed that the use of Mo-DCs and peptides in combination with IL-2 also suffers from considerable background viral inhibition (unpublished data), likely due to the strong activation of CD8+ T cells by the Mo-DCs and IL-2. We, therefore, recommend to stimulate effector cells with peptides and as little IL-2 as possible (maximum 10 U/mL), if feasible without IL-2 altogether.

It is important that effector cells are pure CD8+ T cells before being put in coculture to avoid interference from other cell fractions (e.g., natural killer cells). Therefore, negative selection methods need to be used to remove CD8+ non-T cells. In addition, negative selection ensures that effector cells are not influenced in non-specific ways by binding to beads coupled to cognate antibodies.

Superinfection of CD4+ target T cells

After stimulation, activation, and enrichment, CD4+ target cells are superinfected. While most studies have used lab-adapted viral strains, a few have used autologous viruses previously obtained from primary culture of CD4+ T cells[19,24,25]. Although VIA against autologous virus was observed to be slightly stronger for some patients, no consistent discrepancies have been reported with lab-adapted viral strains[24]. The choice between will depend on the feasibility to obtain autologous virus and ultimately the research question to be answered. Using autologous virus is warranted for in-depth studies investigating, for example, escape mutations or epitope-specific CD8+ T cells. The choice of lab-adapted viral strain depends on the subtype prevalence within the study population and the epitopes targeted by a vaccine. While some groups routinely use a C-C chemokine receptor type 5 (CCR5) tropic HIV-1 (such as BaL) in parallel with a C-X-C chemokine receptor type 4 (CXCR4) tropic HIV-1 strain (such as III_B)[26], this might not be necessary as an influence of HIV tropism on CD8+ T cell activity has not been reported yet. Besides, it has been shown multiple times that effective and clinically relevant cytotoxic T lymphocytes (CTL) responses do not target the variable Env epitopes but rather the more conserved Gag and Pol epitopes[27,28].

The infection dose or multiplicity of infection should be such that a sufficiently large amount of virus is produced at the peak of viral replication to allow discrimination between weak and strong CD8+ T cell responses. In his Nature Protocol paper Sáez-Cirión described that when using p24 enzyme-linked immunosorbent assay (ELISA) for the evaluation of suppression, between 100 and 1000 ng/mL of p24 should be produced at the peak of viremia. Lower amounts do not allow discrimination between weak and strong responses, whereas higher viral replication is probably too strong even for the most potent CD8+ T cells. Similarly, when using intracellular *gag* staining, the ideal level of infection should be around 10-30%[12].

Infecting versus superinfecting virus

Since autologous CD4+ T cells are used from HIV+ patients, it is possible that autologous virus will replicate as well during the VIA assay beside the virus used for superinfection. However, in HIV+ patients on combination antiretroviral therapy (cART) with suppressed viremia, given the relatively short duration of culture and small amounts of (infected) CD4+ T cells, the probability of autologous virus emerging during the assay is rather small, and amounts of autologous viral replication are usually much smaller than that of the superinfecting virus. Nevertheless, it is good practice to include non-superinfected CD4+ T cells as controls to be able to correctly interpret the VIA data. Alternatively, a superinfecting virus which is resistant to an antiretroviral drug can be used, allowing the addition of this antiretroviral in the culture medium and blocking replication of any autologous virus[26,29].

Coculture of target and effector cells

Superinfected CD4+ target T cells are then put in coculture with CD8+ effector T cells. This is usually done in medium containing relatively high amounts of IL-2 to keep the cell-cultures alive[11,12,23]. From our experience, high amounts of IL-2 in the coculture (as opposed to during the prior stimulation of effector cells) do not influence VIA as it does not increase background inhibition by effector CD8+ T cells from non-infected subjects (unpublished data).

The choice of effector to target ratio (E: T) will depend on the expected VIA. The literature describes the suppression of viral replication at E: T ratios ranging from 5:1 to as low as 1:10[13,14]. Since it is hard to predict VIA beforehand, it is again good practice to test several E: T ratios in parallel.

Measurement of viral replication

Finally, VIA is calculated as the difference in viral replication between target cells alone and target cells in coculture with effector cells. The percentage suppression is calculated as:

$$\%\,\text{Suppression} = \frac{(\text{Viral replication in target cells only}) - (\text{Viral replication in coculture})}{(\text{Viral replication in target cells only})}$$

Viral replication can be measured by quantifying p24 in the supernatant with ELISA[10,11,30-32], by determining intracellular *gag* with flow cytometry[12,33] or alternatively by infecting reporter TZM-Bl cells with VIA assay supernatants[34,35]. Measuring p24 levels in the supernatant by ELISA are more sensitive and quantitative than measuring the number of infected cells with intracellular *gag* staining but is also more expensive and requires more cells[12].

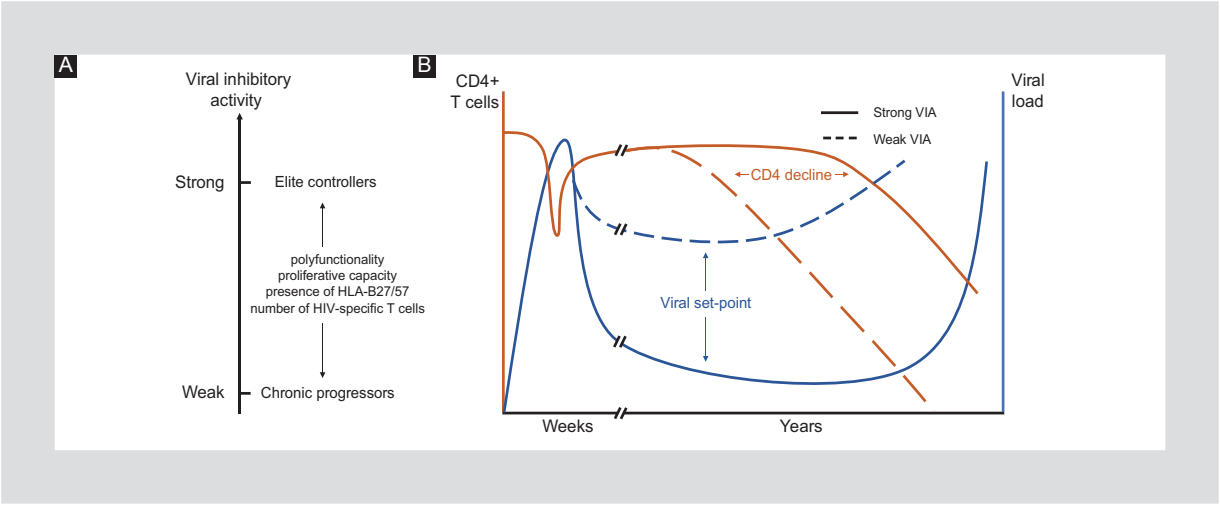

Figure 2. A: *Elite controllers (ECs) show much stronger viral inhibitory activity (VIA) than chronic progressors (CPs). At the same time, ECs are characterized by the presence of protective human leukocyte antigen alleles, higher numbers of circulating human immunodeficiency virus-specific T cells, which also frequently show polyfunctionality and higher proliferative capacity as compared to CPs, possibly explaining the difference in VIA.* **B:** *Stronger VIA in viremic patients off combination antiretroviral therapy has been associated with slower CD4+ T cell decline and lower viral set-point.*

VIA in ECs versus CPs

All studies comparing VIA in EC or viremic controllers (VC) versus CPs have observed much stronger VIA in controllers, be it in patients infected with HIV-1 or HIV-2[36], who are treatment-naive or on cART[14] and who started treatment early or late[14,22,24,34-37]. This difference is consistent whether or not effector cells are stimulated and regardless of the type of autologous target cells: resting or activated CD4+ T cells or monocyte-derived macrophages[15,20]. Besides this remarkably stronger VIA, EC CD8+ T cells are characterized by more frequent polyfunctionality[38], sustained proliferative capacity,[39] and frequent presence of the HLA-B*27 and B*57 alleles[40,41] as compared to CPs.

Interestingly, the total number of HIV-specific CD8+ T cells is comparable between ECs and CPs off cART but is much higher than in suppressed CPs on cART[24]. Thus, CPs off treatment fail to control viremia despite their high numbers of CD8+ HIV specific T cells while CPs on treatment fail to sustain high numbers of HIV specific cells despite their similarly low viral loads (VLs) and therefore antigenic exposure as ECs. Clearly, the ability to maintain high levels of circulating HIV specific CD8+ T cells in the face of very low peripheral VL seems to be an intrinsic characteristic of ECs.

As a possible explanation, Migueles et al. established that ECs have a stronger per cell capacity to lyse target cells and respond better to peptide stimulation than CPs, as evidenced by higher proliferation and granzyme B (GrB) and perforin production[37]. Akinsiku et al. also found a correlation between polyfunctionality, including IL-2 production and VIA in ECs[34] (Fig. 2A).

Evidence of induction of VIA

Induction by in vivo vaccination

In the field of SIV infection, several groups have reported increased *ex vivo* VIA after *in vivo* prime/boost vaccination with or without subsequent viral challenge. VIA correlated with higher numbers of virus-specific CD8+ T cells[30] and inversely correlated with VL peak and set-point[32]. Stephenson et al. confirmed this inverse correlation between VL set-point and VIA after vaccination, and in addition demonstrated that VIA correlated with *gag*-specific and not *pol/env*-specific responses[42]. Thus, VIA can be induced by *in vivo* vaccination, is correlated with *gag*-specific cellular immune responses, and is clinically relevant as it correlates with viral control after break-through infection. In the field of HIV-1, a number of Phase 1 vaccination trials, all using DNA prime/viral vector boost strategies have similarly shown clear induction of VIA after vaccination[11,21,25,43,44]. As will be discussed in the following chapter, the same *gag*-specificity and correlation with viral set-point mentioned above for SIV has been reported for VIA in HIV as well[16,24,45].

Induction by in vitro stimulation

Robust *in vitro* models able to test the potential of vaccine candidates to induce *in vivo* VIA are needed,

but only limited data have been published so far. In 2001 Lu and Andrieu described a model where Mo-DCs pulsed with inactivated virus and used to stimulate peripheral blood mononuclear cells (PBMCs) led to the expansion of HIV-specific cells able to kill HIV-infected cells[18]. Two more recent publications used HIV-1 *gag* peptide pools in combination with IL-2 to directly stimulate PBMCs, increasing the capacity of CD8+ T cells to kill infected CD4+ T cells[19,20]. In recently published work from our own group, PBMCs specifically stimulated with an HIV-1 *gag* peptide pool but without the presence of any IL-2 caused an important upregulation of VIA in HIV+ patients on cART[23]. Finally, as it is known that latency-reversing agents (LRA) can inhibit CD8+ T cell function, one group investigated and indeed confirmed the inhibition of VIA by LRAs such as bryostatin-1, prostratin, and JQ1[46].

Clinical correlates with VIA

As early as the year 2000, it was reported that weaker VIA predicted faster CD4+ T cell decline in therapy naive HIV+ patients[47]. More than 10 years later, this was confirmed by a study which also observed an inverse correlation with the VL set-point[45], a finding which, in turn, was backed up by the results of an already mentioned study in vaccinated rhesus monkeys[42] (Fig. 2B).

These data indicate that strong CD8+ T cell VIA activity *in vitro* is associated with lowered *in vivo* viral replication, resulting in a low VL and a delayed decline of CD4+ T cell count. However, it can be hypothesized that sustained antigenic exposure is required for the maintenance of virus-specific CD8+ T cell responses and strong VIA activity. This hypothesis is supported by a publication from Freel et al. showing that VIA declined over time with resolving VLs[35]. Interestingly, the study even showed comparable VIA activity in ECs and CPs early in infection, while it is known that in chronic infection VIA is much stronger in ECs than in CPs, as discussed previously. Spentzou et al. showed that VC have stronger VIA than patients on cART[11]. Noel et al. further showed that VIA is significantly stronger in ECs whose CD4+ T cells produce more virus after stimulation with LRA than ECs with weaker VIA responses[48]. Patients with more easily inducible proviruses are more likely to have occasional, residual viremia, and antigenic exposure, presumably leading to continued boosting of the immune system and maintenance of potent CD8+ T cell responses, possibly explaining the above observations.

Certain protective HLA types (e.g., HLA-B*27, HLA-B*57) are enriched in ECs. Interestingly, ECs with such HLA-types also have the strongest VIA[14,49]. In CPs, the image is less clear, as the presence of protective HLA types does not always lead to stronger VIA[16,31]. In other words, there is no direct and straightforward relationship between HLA restriction and VIA.

Besides CD8+ T cell effector potency, the susceptibility of infected CD4+ T cell targets could also play a role. Buzon et al. observed that CD4+ T cells of ECs are more susceptible to cytotoxic T cell (CTL) killing than CD4+ T cells of CPs on cART[50]. Remarkably, this susceptibility was consistently higher in HLA-B*57 positive patients. Patients with higher CD4+ T cell susceptibility also had the smallest viral reservoir sizes.

In this regard, a very intriguing observation was made by Huang et al. on an apparently inherent resistance to CTL killing of CD4+ T cells containing intact pro-viruses[51]. In this study, resting CD4+ T cells were treated with a combination of LRA and autologous CD8+ T cells. While the total amount of proviral DNA was reduced, the replication competent fraction was not, indicating that only CD4+ T cells containing incomplete pro-viruses were being eliminated. Further research is needed to understand whether this preferential elimination is due to *nef* mediated downregulation of MHC I or due to another as yet unidentified inherent resistance mechanism of CD4+ T cells containing intact pro-viruses.

Correlations with cytokine production

The most studied cytokine in relation to cell cytotoxicity is IFN-γ. It is routinely measured in ELISPOT assays to determine the frequency of HIV-specific cells, the antigen specificity, as well as the breadth and avidity of the immune response. A study from Saez-Cirion et al. observed a strong correlation between the frequency of IFN-γ producing T cells and VIA in ECs[24]. Another study illustrated that the variability within epitope specificities showing a correlation between VIA and numbers of IFN-γ producing cells after *env* but not after *gag* stimulation[31]. Yet another study nicely showed that the breadth, and not the magnitude, of *gag* responses is a determining factor for VIA[16].

Interestingly, a study using CD8+ T cell lines specific for various gag epitopes observed 1000-fold differences in VIA between cell lines despite having comparable activity in IFN-γ ELISPOT[31]. This suggests that correlations with IFN-γ are possibly confounded by the production of other bio-molecules responsible for viral suppression. In this regard, Freel et al. delivered the

most convincing data. In their DNA prime/boost vaccination study, VIA was associated with cells coexpressing CD107a, macrophage inflammatory proteins (MIP)-1α, and IFN-γ[25]. Nevertheless, VIA was only associated independently with CD107a and MIP-1α expression and not with IFN-γ, strongly suggesting that associations of IFN-γ with VIA are a consequence of the frequent coexpression of this and other cytokines by cells with real suppressive activity.

While the causal relationship between IFN-γ production and suppressive activity is dubious, the evidence for CD107a, a marker of degranulation on CD8+ T and NK cells, is more convincing. Several independent studies, both cross-sectional and prospective vaccination trials, in HIV as well as SIV models, have linked CD107a expression to *in vitro* VIA[25,32,52]. Thus, the capacity to deliver lytic granules seems to be predictive of *in vitro* VIA. Migueles et al. previously showed that lytic granule content is strongly associated with killing capacity, observing increases in perforin and GrB expression in response to peptide stimulation in HIV controllers and not in progressors[37]. The importance of perforin was later confirmed[32]. Besides these classical cytotoxicity markers, a number of other cytokines have been linked to viral suppression, such as IL-2, MIP-1α, MIP-1β, and tumor necrosis factor-α[52].

Taken together, the evidence suggests that VIA cannot be predicted by the expression of one marker only. Rather, the ability to produce several cytokines simultaneously and to degranulate with high perforin and grB content in response to cognate antigen stimulation seems necessary to achieve potent suppression of viral replication.

Epitope specificity and avidity

Specificity

T cell responses to different parts of the HIV proteome have been associated with variable levels of protection against disease progression. Responses to *gag* peptides have most often been correlated to lower VLs[27,28], but so have responses to *pol* and *vif*[21,26,53] and even to some *env* peptides[26,54]. These differences are also reflected in associations between T cell specificity and VIA. In general, CD8+ T cells or clones targeting *gag* epitopes display the strongest VIA, but responses specific for *pol, tat,* and *nef* have also been reported to be suppressive[16,24,26,31,33,53,55]. More precisely, while there is no correlation with the breadth or magnitude of bulk CD8+ T cells[11], the breadth of *gag* specific responses is associated with VIA, polyfunctionality, and even reduced *in vivo* VLs[16,24,49,56]. As with *in vivo* control, *gag* responses thus seem to be crucial for *in vitro* viral suppression, while (most) *env* responses are not. Presumably, *gag* peptides from incoming virions can be presented on HLA molecules within hours after infection, while *env* peptides first need to go through synthesis and processing and are only presented 24 h after entry at the earliest[57-59]. Besides this rapid antigen presentation of *gag* peptides after infection, the role of other determining factors such as protein expression levels, amino acid composition, processability, immunogenicity, and escape potential need further investigation.

Concerning the importance of antigen specificity for VIA, Pohlmeyer et al. made two interesting observations. They observed that ECs were able to suppress viral replication of both wild type NL4.3 virus and mutants containing escape mutations in HLA-B*57 restricted *gag* epitopes[60]. No CPs were included in these experiments, so it is unknown whether the recognition of escape mutants distinguishes ECs from CPs. Nevertheless, another study showed that a single amino acid difference can significantly change epitope binding avidity and strongly affect VIA in CPs[61]. It is, therefore, unlikely that VIA in CPs are very forgiving for escape mutations, while this might be the case in ECs.

Interestingly, Pohlmeyer et al. also demonstrated that microbial peptides can cross-react with HIV-specific T cells and induce VIA against HIV-1[62]. The T cell receptor diversity was shaped differently in different patients in response to the same microbial peptide pool, indicating that anti-HIV immunity can be modulated by non-HIV, microbial peptides.

Avidity

Avidity is commonly defined as the peptide concentration, which elicits half-maximal response rates in assays such as IFN-γ ELISPOT or ^{51}Cr release, i.e., the lower this concentration, the higher the avidity. T cell responses directed at protective epitopes have higher avidity in ECs than in CPs[29]. In the same way, avidity of T cell responses has been positively correlated with VIA[26,31,61,63-66]. As an example, Bennett et al. nicely illustrated how avidity could explain the discrepancy between cross-reactivity in ^{51}Cr release assays and VIA[61]. While responses against several peptides were detected in the former assay (which utilizes supra physiological peptide concentrations), not all of these responses proved to cause suppression in the latter

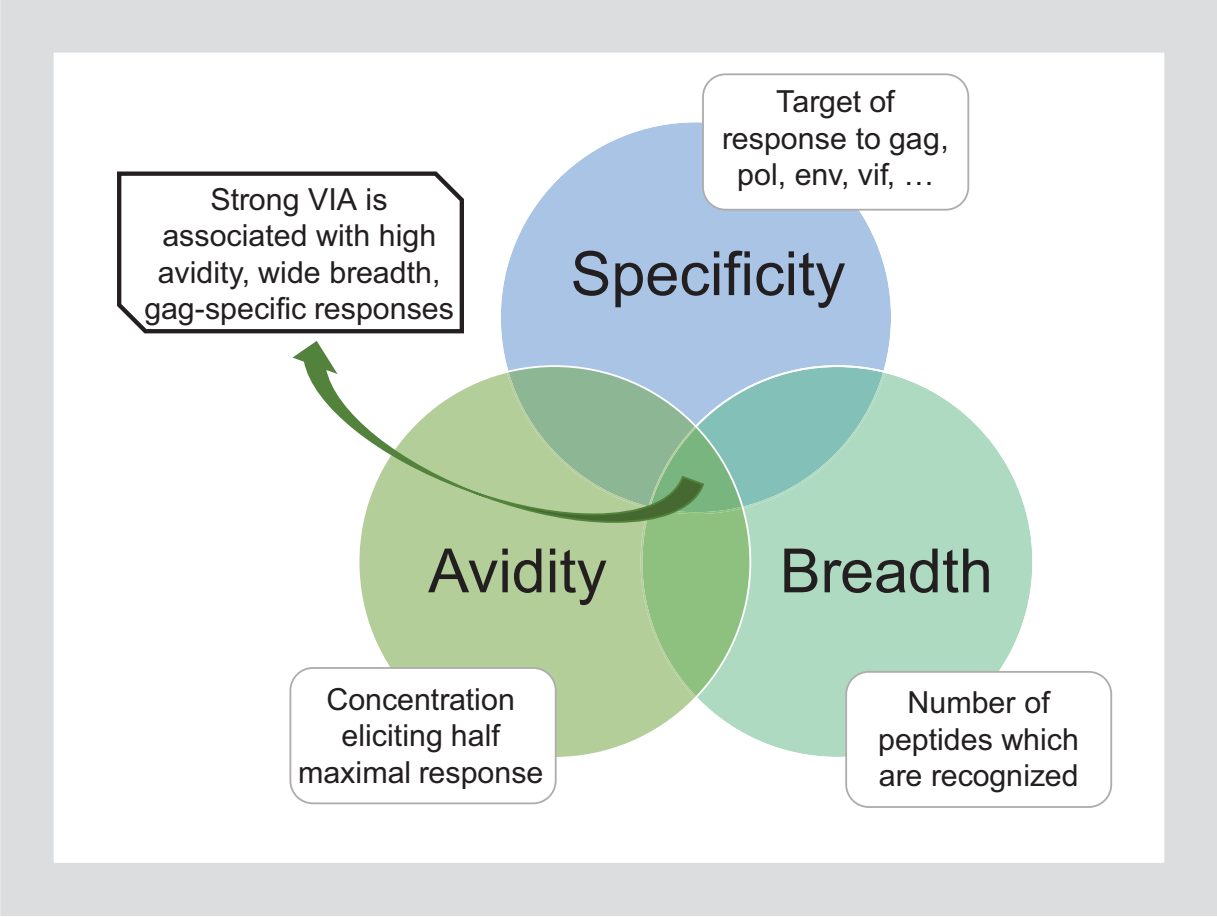

Figure 3. *In terms of T-cell responses, strong viral inhibitory activity (VIA) has been separately associated with the capacity to recognize a wide range of epitopes (breadth), a response mainly targeting gag-epitopes (specificity) with a strong functional affinity of the T-cell receptor for the peptide (avidity). This suggests that T-cell responses corresponding to these three criteria will show strongest VIA.*

assay (where peptides are presented by target cells at lower concentrations). Only responses with high avidity were able to suppress viral replication. Furthermore, they determined that the relationship between avidity and VIA follows a sigmoidal curve, where suppressive activity quickly drops below a certain avidity threshold (Fig. 3).

Despite the clear association between avidity and viral suppressive activity, the following nuance must be made. Chen et al. and Lissina et al. both found that (1) T cell responses against *gag* resulted in stronger VIA than responses against *env* and (2) responses against *gag* had higher avidity than responses against *env*[31,65]. Nevertheless, when *gag* and *env* responses were analyzed separately, no correlation was observed anymore between avidity and VIA. At the same time, other studies have established a correlation between VIA and avidity within the same epitope specificity, and as a result, it remains under debate whether epitope specificity is more important than avidity in determining VIA.

Remarkably, high avidity responses have been linked both to higher[63] and lower polyfunctionality[67]. On the one hand, strong binding of the cognate antigen to the T cell receptor might more easily induce activation cascades, leading to polyfunctional cells. On the other hand, high avidity will also lead to stronger expansion and higher turnover of the stimulated cells, which might negatively affect their lifespan and lead to irreversible exhaustion[68].

Differentiation stage and phenotype

Terminally differentiated CD8+ T cells directed against HIV-1 are more frequent in ECs than in CPs[69]. In addition, two independent papers on HIV and SIV have reported that VIA can be measured in all effector and memory T cell subsets, except for naïve T cells. In a study by Julg et al., where broad *gag* responses were correlated with stronger VIA, these same broad *gag* responses were also correlated with

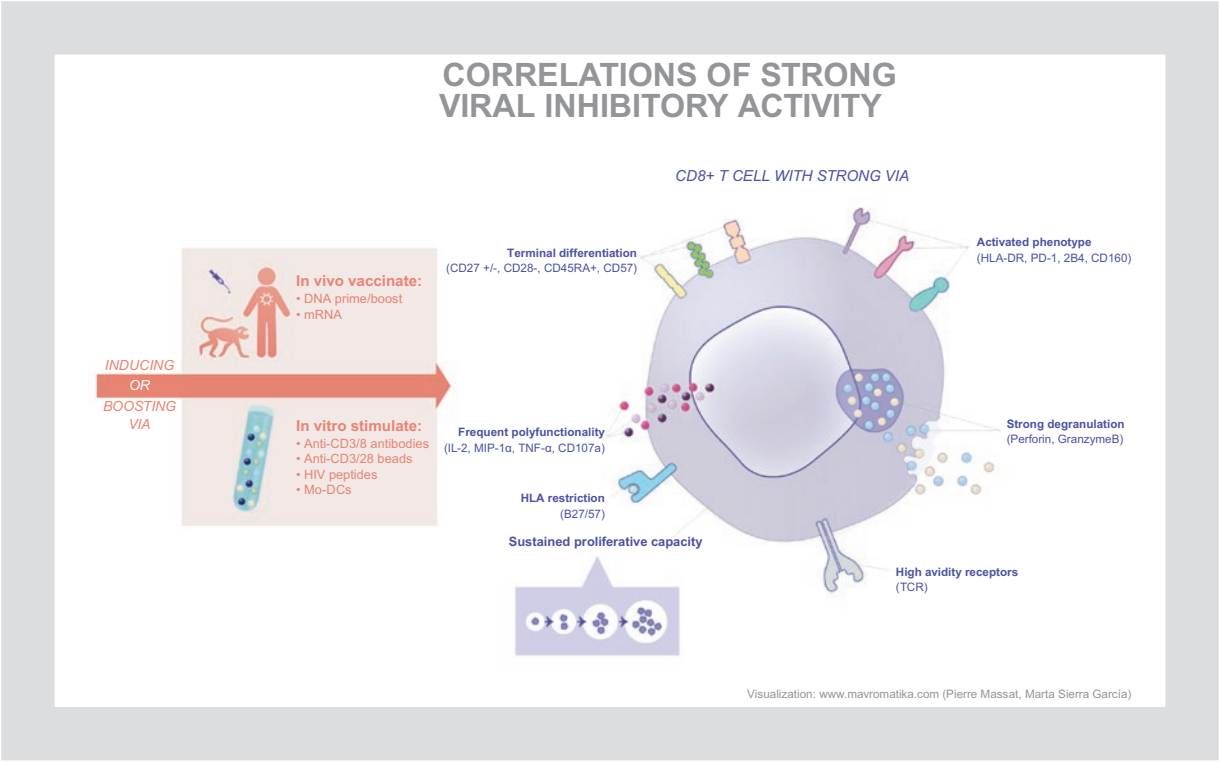

Figure 4. *Schematic summary of the most important characteristics of CD8+ T cells that have been associated to viral inhibitory activity (VIA) as well as a number of evidenced ways to induce or boost VIA in vivo and in vitro.*

higher numbers of terminally differentiated RA$^+$ effector memory T cells (T_{EMRA}) as a percentage of bulk CD8+ T cells. Interestingly, Jensen et al. found that VIA was correlated with higher CD57 expression on effector T cells and higher PD1/2B4/CD160 coexpression on effector memory T cells (T_{EM}). CD57 is a marker of terminal differentiation and senescence, while PD1/2B4/CD160 are all activation/exhaustion markers. In our own research, we have observed similar findings, with increased CD57 expression in sub-clusters of T_{EM} and T_{EMRA} cells as well as increased coexpression of PD1/CD160 in sub-clusters of T_{EMRA} cells in patients with high VIA[23]. Even though the evidence base is still small, taken together, it seems that cells responsible for suppressing viral replication tend toward more activated and (terminally) differentiated phenotypes. Such cells may be more likely to quickly mediate cytolytic activities than earlier memory phenotypes, although they also might be shorter lived.

Finally, ECs also have higher HLA-DR expression on CD8+ T cells than patients on cART[14]. This HLA-DR^{high} profile has been associated with stronger VIA[24]. In our own work, we have observed a similar trend, with higher HLA-DR expression in CPs with higher VIA[23]. HLA-DR is a typical cell activation marker but is also associated with proliferation[70]. Consistent with reports on higher proliferative capacity of HIV specific T cells in ECs, this higher HLA-DR expression on CD8+ T cells showing strong VIA might reflect the proliferative potential of these cells.

Conclusions

It is becoming increasingly clear that a functional cure for HIV-1 cannot be achieved by merely reducing the size of the viral reservoir. A series of treatment interruption studies including patients with extremely small viral reservoirs have had disappointing outcomes, with hardly any delayed viral rebound[71-74]. Immune interventions targeting cytotoxic CD8+ T cells will most likely be necessary to achieve sustained suppression of viremia in the absence of cART. However, a number of therapeutic vaccination trials have been equally unsuccessful in achieving *in vivo* control of viremia, even though classic immune parameters such as IFN-γ ELISPOT and ICS provided evidence of vaccine immunogenicity. Nevertheless, it is believed that therapeutic vaccination still has a lot of potentials, attributing the failure of previous trials to flawed immunogen design, rather than a flawed strategy altogether. Clearly, an assay able to predict *in vivo* viral

control is needed to evaluate therapeutic vaccination candidates before going into clinical trials.

In vitro VIA assays have been convincingly associated with CD4+ T cell count maintenance, *in vivo* viral suppression, polyfunctionality, and the cytotoxic potential of CD8+ T cells (Fig. 4). There is ample evidence that VIA can be induced *in vivo* and we even know which antigenic targets have the most potential in terms of protection. Today, research is needed to determine whether *in vitro* VIA can actually predict *in vivo* viral remission or not.

VIA assays are challenging to set-up, are labor intensive and take several weeks to finish. In addition, due to their complexity, a lot of different set-ups have been published, making comparisons between studies less straight-forward as compared to, for example, IFN-γ ELISPOT results. In short, VIA assays are not the easiest, cheapest or fastest, but they should be studied further in future prospective treatment interruption trials.

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