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REVIEW

The CD8$^+$ T Cell Noncytotoxic Antiviral Responses

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SUMMARY The CD8$^+$ T cell noncytotoxic antiviral response (CNAR) was discovered during studies of asymptomatic HIV-infected subjects more than 30 years ago. In contrast to CD8$^+$ T cell cytotoxic lymphocyte (CTL) activity, CNAR suppresses HIV replication without target cell killing. This activity has characteristics of innate immunity: it acts on all retroviruses and thus is neither epitope specific nor HLA restricted. The HIV-associated CNAR does not affect other virus families. It is mediated, at least in part, by a CD8$^+$ T cell antiviral factor (CAF) that blocks HIV transcription. A variety of assays used to measure CNAR/CAF and the effects on other retrovirus infections are described. Notably, CD8$^+$ T cell noncytotoxic antiviral responses have now been observed with other virus families but are mediated by different cytokines. Characterizing the protein structure of CAF has been challenging despite many biologic, immunologic, and molecular studies. It represents a low-abundance protein that may be identified by future next-generation sequencing approaches. Since CNAR/CAF is a natural noncytotoxic activity, it could provide promising strategies for HIV/AIDS therapy, cure, and prevention.

KEYWORDS CD8$^+$ T cells, HIV transcription, elite controllers, human immunodeficiency virus, innate immunity, noncytotoxic antiviral activity, soluble antiviral factor

(I) INTRODUCTION

A major component of the immune system, CD8$^+$ T cells are often inaccurately known only as "killer T cells" or CTLs, for cytotoxic T lymphocytes. This terminology defines only one of the effector functions of CD8$^+$ T cells, which is their ability to recognize and kill pathogen-infected cells and cancer cells. In reality, CD8$^+$ T cells are polyfunctional (1); their various functions include not only target cell killing (cytotoxicity) but

TABLE 1 Characteristics of the innate and adaptive immune responses and association with CD8$^+$ T cell activity[a]

Characteristics	Characteristics of immune responses and antiviral CD8$^+$ T cell activity	
	Adaptive response	**Innate response**
Germline invariant receptor	−	+
Clonal receptor gene rearrangement	+	−
Specificity:		
Conserved motifs	−	+
Highly specific antigens	+	−
HLA restricted	+	−
Present without previous exposure to pathogens	−	+
Memory response	+	−[b]
Priming needed	+	−
Vaccine inducible	+	+
Speed of response	Slow	Fast
Cell-to-cell contact	+	+/−
Cytotoxicity	+	+/−
Soluble factor secretion	+	+
Cell population	B and T cells	ILCs, NK cells, $\gamma\delta$ T cells, phagocytes, mast cells
	CTL activity	**CNAR/CAF activity**
Germline invariant receptor	−	Potentially conserved viral motif recognition
Clonal receptor gene rearrangement	TcR	−
Specificity		
Conserved motifs	−	Affects several different retroviruses
Highly specific antigens	+	Not HIV specific
HLA restricted	+	Not restricted by HLA molecules, but histocompatibility improves the activity
Present without previous exposure to pathogens	−	Found in some uninfected individuals
Memory response	+	Found in exposed uninfected individuals and decreases after exposure; no recall response
Priming needed	+	−/+
Vaccine inducible	+	+
Speed of response	Days to wks	Rapid, early response to HIV infection (min to days)
Cell-to-cell contact	+	CNAR activity improved by cell contact
Cytotoxicity	+	−
Soluble factor secretion	TNF-α, IFN-γ, Gzm	Mediated by CAF, a secreted factor that inhibits viral transcription (Table 4)
Major CD8$^+$ T cell subset[c]	T_{EM}	T_{TM}, T_{RM}

[a]No shading represents adaptive immunity, dark gray shading represents innate immunity, and light gray shading represents both.
[b]Limited recall response for some innate cell subsets such as innate lymphoid cells and NK cells.
[c]T_{TM}, transitional memory CD8$^+$ T cells; T_{EM}, effector memory CD8$^+$ T cells.

also expansion via cell proliferation, the secretion of soluble proteins (cytokine production), and antiviral responses mediated by noncytotoxic mechanisms. The importance of the CD8$^+$ T cell noncytotoxic immune activity is of major interest to our laboratory and the topic of this review.

The initial observation of a CD8$^+$ T cell noncytotoxic antiviral response (CNAR) was made in the context of human immunodeficiency virus (HIV) infection many years ago (2). It was found in asymptomatic subjects infected by the virus. Indeed, CNAR was the first discovered immune response against HIV. This noncytotoxic CD8$^+$ T cell anti-HIV activity does not require major histocompatibility complex (MHC) recognition or cell-to-cell contact and is not the result of blocking virus entry, reverse transcription, or integration (section VI). Since then, this noncytotoxic antiviral activity has been shown to be active against all HIV isolates and other retroviruses, and similar CD8$^+$ T cell noncytotoxic antiviral activities have been observed with other viral infections (section VIII).

This review will provide background information on how characteristics of a noncytotoxic CD8$^+$ T cell-mediated antiviral response, or CNAR, differ from the antiviral cytotoxicity mediated by CD8$^+$ T cells (Table 1). The article will cover the discovery and clinical relevance of CNAR as well as the major secreted protein associated with its anti-HIV activity, CAF (the CD8$^+$ T cell antiviral factor). Furthermore, the assays and experimental approaches used to characterize this noncytotoxic antiviral response and

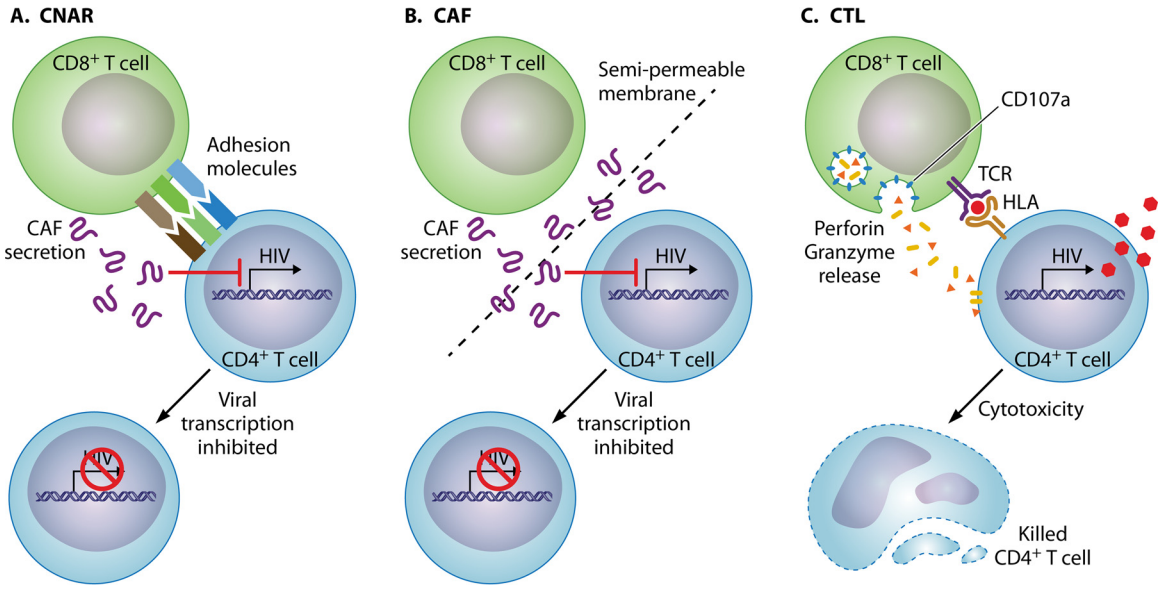


FIG 1 Anti-HIV activities of CD8$^+$ T cells. To control HIV replication, CD8$^+$ T cells can use the following activity: (A) the CD8$^+$ T cell noncytotoxic antiviral response (CNAR), which requires cell-cell contact, is improved by the interaction of adhesion molecules to their ligands, and is mediated by a soluble factor; (B) secretion of the CD8$^+$ T cell antiviral factor (CAF), which can pass through a semipermeable membrane (transwell) or is found in the culture supernatants of stimulated CD8$^+$ T cells; or (C) killing of the infected target cells by cytotoxic CD8$^+$ T lymphocytes (CTL) via perforin and granzyme release, which requires degranulation (cell-surface CD107a expression). As opposed to the latter case, CNAR/CAF does not result in target cell death, and instead, HIV transcription is blocked by the CD8$^+$ T cells.

its mode of action will be described. Because the search for CAF has uncovered the existence of several proteins that have anti-HIV activity but are not CAF, such as the β-chemokines, we will also review several of these soluble antiviral proteins (section IX).

The noncytotoxic role of CD8$^+$ T cells is important for the development of immune-based therapies for HIV, for understanding viral latency, and for approaches toward an HIV vaccine and a cure, as well as for an appreciation of this immune activity in other viral infections.

(II) THE CYTOTOXIC ANTIVIRAL ACTIVITY OF CD8$^+$ T CELLS

(A) Characteristics of CD8$^+$ T Cell-Mediated Cytotoxicity

CD8$^+$ T cell cytotoxicity is defined as cell death caused by the action of activated effector CD8$^+$ T cells on viable target cells recognized via a specific T cell receptor, or TcR, that binds to antigen-presenting HLA class I molecules (Fig. 1). The mechanism used by those CD8$^+$ CTLs to carry out this function involves the release of two types of soluble proteins, perforin and granzymes, from secretory vesicles, or granules. This cellular process is called degranulation and occurs at the effector/target cell interface, or immunological synapse. During this process, perforin creates pores in the target cell membrane, allowing granzymes to enter the cell and induce targeted programmed cell death (or apoptosis) (3, 4). When applied alone to target cells, perforin or granzymes do not cause cell death, but some granzymes have direct noncytotoxic antiviral activity (section IX).

CD8$^+$ T cells are protected from their own perforin/granzyme toxicity by CD107a, a protein coating the inside of the granules. CD107a is expressed at the CD8$^+$ T cell surface after vesicle/cell membrane fusion that occurs during degranulation (Fig. 1C). For CD8$^+$ T cell cytotoxicity to occur effectively, all 3 components, perforin, granzymes, and degranulation, need to be present. Most CD8$^+$ T cells are able to respond to TcR stimulation during the degranulating process, which can be measured by assessing CD107a expression by flow cytometry. However, the vast majority of resting CD8$^+$

T cells are not "ready to kill," in contrast to natural killer (NK) cells. To kill, the CD8$^+$ T cell must be primed for several days before expressing the cellular proteins needed for cytotoxicity: perforin and granzymes (5). For this reason, measuring only CD107a without confirming that either the target cells are killed or that both perforin and granzymes are present in effector cells is only a marker of degranulation and not CD8$^+$ T cell cytotoxicity.

The above-mentioned comment underlines the importance of determining specifically if target cell killing is the mechanism for virus control. Indeed, only a few complex *in vitro* procedures permit the accurate determination of target cell death, such as the chromium 51 (^{51}Cr) (6) or fluorophore release (7) assays and time-lapse microscopy imaging of real-time cytotoxicity (8). These labor-intensive assays are one of the reasons that most research in immunologic activity relies on the indirect and imperfect measure of degranulation to suggest cytotoxic function. These assays do not consider the possible presence of a noncytotoxic antiviral response (Fig. 1A and B).

(B) CD8$^+$ T Cell IFN-γ and TNF-α Production

Antigen-activated CD8$^+$ T cells can produce gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), other markers used to measure indirectly the potential for cytotoxicity. IFN-γ is a cytokine without cytotoxic capabilities that can directly inhibit the replication of some viruses, but not HIV (9). TNF-α is involved in death signaling, but its role in inducing cell death is minor given its weak signaling potential. Both of these cytokines can inhibit the replication of some viruses directly (section VIII) and play a role in immune responses by activating immune cells (10). Measuring their intracellular cytokine production, or release, only demonstrates that those CD8$^+$ T cells are responding to an antigen, not that they are cytotoxic. As noted above, CD8$^+$ T cells are mostly polyfunctional: they produce and secrete cytokines, as well as kill target cells or have a noncytotoxic function.

IFN-γ and TNF-α are also released from extracellular vesicles. As such, this response could lead to some level of CD107a expression at the cell surface; however, this process does not necessarily indicate that the activity is cytotoxic. Therefore, in the context of HIV and other infections, simply measuring CD107a, IFN-γ/TNF-α production, or granzyme release does not definitively indicate killing by the effector T cells. Confirmation of target cell death is needed. This review focuses on CNAR/CAF, the innate noncytotoxic antiviral mechanism that could be involved in controlling a virus infection, but is often underrecognized and underappreciated (11–15) (sections III and V).

(C) Activation-Induced Cell Death Activity

Another cytotoxicity mechanism through which immune cells can kill target cells is activation-induced apoptosis via the Fas/Fas-ligand (FasL) (16, 17) or the TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor (TRAIL-R) (18) pathways. However, evidence of Fas- or TRAIL-mediated cell killing of infected cells by CD8$^+$ T cells in the context of viruses remains limited in humans (19). In very rare cases, such as tumor elimination, viral defense in the nervous system (20), or in autoimmune diseases, CD8$^+$ T cells express FasL or TRAIL as well as other lymphocyte-activating receptors for this process. FasL and TRAIL have been reported, through an interaction with Fas or TRAIL-R expression on the target cell surface, to cause activation-induced cell death by apoptosis (21). However, whereas CD8$^+$ T cells can express FasL or TRAIL, there is very little evidence in humans that CD8$^+$ T cell cytotoxicity against infected target cells is mediated by the Fas/FasL or TRAIL/TRAIL-R (22) pathways. In this regard, a role for a Fas/FasL or TRAIL/TRAIL-R interaction has not been observed in HIV infection during CNAR (23) (C. E. Mackewicz, unpublished data). With CD8$^+$ T cells, these death ligands, when expressed, are mostly acting as costimulatory receptors to induce cell activation and proliferation (24).

(III) THE NONCYTOTOXIC ANTIVIRAL ACTIVITY OF CD8$^+$ T CELLS

(A) Discovery

During studies to determine why cultured peripheral blood mononuclear cells (PBMC) from asymptomatic HIV-seropositive individuals did not yield any infectious

viruses, Walker and colleagues observed that the removal of $CD8^+$ cells from the PBMC by immunologic panning with CD8 antibodies (25) led to HIV production (2). The subsequent reintroduction of the $CD8^+$ cells to the $CD8^+$ cell-depleted culture, even after 3 weeks, suppressed virus replication. This finding confirmed the role of $CD8^+$ cells in the inhibition of HIV replication in the infected cells. Most importantly, the studies indicated that the $CD8^+$ cells did not kill the infected $CD4^+$ cells: these target cells remained viable with HIV in a latent state. That observation uncovered a previously unknown antiviral activity of $CD8^+$ cells, the $CD8^+$ T cell noncytotoxic antiviral response (CNAR) (Fig. 1A).

In this seminal study, the $CD8^+$ cell suppression effect was shown to be dose dependent, detectable at low input ratios of $CD8^+$ cells to HIV-infected $CD4^+$ cells, more effective with autologous $CD8^+$ cells (although suppression was also observed with heterologous $CD8^+$ cells), and independent of NK cell activity (2). Most importantly, unlike CTL activity, the noncytotoxic antiviral response of the $CD8^+$ cells did not eliminate the infected $CD4^+$ cells from the cultures (2), an observation confirmed by other early studies (26, 27).

CNAR is not found with any other hematopoietic cells, including $CD4^+$ cells, NK cells, or macrophages (2, 28, 29). The antiviral response appears to be induced and maintained by viral antigen expression (30, 31). Therefore, in some HIV-infected asymptomatic individuals (i.e., elite controllers [ECs]) (section III.E.i), in whom viremia is not often seen, CNAR may not be observed. This CNAR activity is associated with production of an anti-HIV protein (32), the $CD8^+$ T cell antiviral factor (CAF) (section V). CNAR and CAF production are maintained by persistent exposure to viral motifs and seem to be linked by the same mode of action (section VI). Therefore, in this review, the two activities may sometimes be cited together as CNAR/CAF. It is also possible that these responses represent two different mechanisms for control of HIV. The $CD8^+$ T cell noncytotoxic anti-HIV activity was reviewed 2 decades ago (13). This current comprehensive review provides the most up-to-date information on this notable immunologic function of $CD8^+$ T cells.

(B) $CD8^+$ T Cell Noncytotoxic Antiviral Response (CNAR)

In early studies of CNAR, a dose response was readily shown: a threshold number of $CD8^+$ T cells was needed for the antiviral effect. When the total number of $CD8^+$ cells added to the HIV-infected $CD4^+$ cells exceeded 75% of the $CD8^+$ cell number in the original PBMC of the infected subjects, HIV replication was completely inhibited (2, 26). The antiviral effect was optimal when the $CD8^+$ T cells and the target cells were MHC-matched or semimatched (26, 33) (section IV.E). However, suppression of virus replication was also observed when HIV-infected target $CD4^+$ cells were cocultured with MHC-mismatched $CD8^+$ T cells (32, 34–38). This observation indicated a mechanism of virus suppression that acted independently of TcR-mediated, HLA-restricted cytotoxic activity. Importantly, HIV replication returned in 3 to 7 days after removal of $CD8^+$ T cells from the cultures (2, 26, 27). This anti-HIV activity confirmed the lack of target cell death and the maintenance of HIV in a silent state (section III.D). A similar noncytotoxic role for $CD8^+$ T cells was later described in HIV-infected newborn and young children (37).

Shortly after CNAR was described in HIV infection, the same $CD8^+$ cell antiviral immune activity was shown with simian immunodeficiency virus (SIV) infection (39). The removal of $CD8^+$ cells from the PBMC of SIV-infected rhesus monkeys led to increased virus replication *in vitro*. A similar observation was later made with the PBMC of HIV-infected chimpanzees (40). These studies provided early supporting evidence that noncytotoxic $CD8^+$ T cells play a critical role in controlling HIV/SIV replication.

This CNAR activity can be demonstrated *in vitro* with naturally infected $CD4^+$ cells from HIV-infected individuals (endogenous virus infection assay) or uninfected $CD4^+$ cells or macrophages (41) that are acutely infected with various strains of HIV before coculture with activated $CD8^+$ cells from an asymptomatic infected subject (acute virus

infection assay) (27, 33, 42–44) (section VII.A). Moreover, some studies have suggested that certain CD8$^+$ T cell clones can have both CTL and CNAR activity (36, 45).

The absence of CD8$^+$ T cell cytotoxicity as a major mechanism is also supported by an infectious center assay that quantifies the number of infected CD4$^+$ cells (44, 46, 47) following a CNAR assay (section VII.A). Briefly, CD4$^+$ cells from HIV-infected subjects or nonhuman primates (NHP) are first cultured alone or with CD8$^+$ cells from the same subject (autologous) or from different subjects or animals (heterologous). The CD8$^+$ T cells are removed from the cocultures 4 to 6 days later, and the CD4$^+$ cells are serially diluted (up to 10-fold) and added to replicate cultures of PBMC from uninfected subjects. Virus replication in the PBMC cultures is then measured. The assay provides a sensitive endpoint titration of infected CD4$^+$ cells cultured in the presence or absence of CD8$^+$ T cells (44, 46, 47).

In one infectious center assay involving CNAR with autologous human CD8$^+$ T cells (44), 10% or 35% of CD4$^+$ cells were found infected, as measured by immunofluorescent cell staining for HIV antigens or reverse transcriptase (RT) activity, respectively (48). This number was comparable to that of the CD4$^+$ cells cultured alone (44). In another study, the frequency of infected CD4$^+$ cells was the same when cultured alone or with CD8$^+$ T cells, whether autologous or heterologous (46, 47). Thus, both concordant and discordant CD8$^+$ T cell-mediated suppression of HIV replication was observed without killing the infected cells. Moreover, the infected CD4$^+$ cells continued to proliferate in the presence of the CD8$^+$ T cells. These results further confirmed that HIV replication was inhibited in these studies without elimination of the infected CD4$^+$ cells and without any substantial effect on their proliferation.

The CD8$^+$ T cell suppression of HIV replication in target cells was also observed when CD4$^+$ cells naturally infected with HIV-1 were superinfected with a different HIV isolate (e.g., HIV-2). The second virus infected and integrated into the genome of the target cells but was not produced unless the CD8$^+$ T cells were removed. After CD8$^+$ cell removal from the culture, both the endogenous (HIV-1) and the exogenous (HIV-2) viruses were produced (49). A similar observation has been made *in vivo* after homologous and heterologous HIV-2 superinfection of baboons previously infected with HIV-2 (50). Thus, CNAR activity against the initial virus infection also controls the superinfecting virus.

(C) CNAR in Nonhuman Primate Studies

(i) *In vivo* CD8$^+$ cell depletion studies. Several studies have demonstrated CNAR activity *in vivo* by CD8 antibody-mediated depletion of CD8$^+$ cells from HIV- or SIV-infected NHP. This antibody-mediated depletion of CD8$^+$ cells was first conducted with an AIDS-associated retrovirus 2 (ARV-2 [also known as HIV-1$_{SF2}$])-infected chimpanzee (51, 52), in which the virus could not be recovered from the PBMC 8 years after virus inoculation. Following *in vivo* removal of CD8$^+$ cells on two separate occasions, HIV became detectable (52). Subsequently, a similar observation was made in macaques infected with SIV (53, 54) as well as with a live attenuated SIV (SIVmac239Δnef) used to protect macaques against pathogenic SIVmac251 challenge (55). Some of these early experiments used different depleting antibodies that targeted the CD8α subunit of the CD8 molecule over the course of several years (56). Those studies demonstrated as well that when the CD8α^+ cell compartment was reduced, the viral load, previously under control in the NHP, rose from undetectable to up to 10 million RNA copies of SIV/ml of plasma. This response was not observed in the animal groups treated with control antibodies. Once the CD8$^+$ cells in the primates recovered from the cell depletion, the viral load was again controlled. Multiple CD8$^+$ cell depletions in the same animal led to the same observation of virus rebounds (52, 57). Again, these studies demonstrated that the infected cells were not eliminated by the CD8$^+$ cells and that the proviral DNA was stable and not transcribed.

This *in vivo* effect of CD8$^+$ cell depletion was also found in SIV-infected macaques on antiretroviral therapy (ART) (58). The removal of CD8$^+$ cells gave rise to viremia. The study indicated that CD8$^+$ cells were also involved in SIV control during ART. The

return of $CD8^+$ cells in all 13 rhesus macaques studied led to suppression of the virus. Notably, the earliest virus isolates that infected the animals were recovered. This finding presumably reflected those viruses that established latency (or a proviral state) due to the antiviral activity by the $CD8^+$ cells during the acute or early chronic phase of infection. In these studies, in comparison to cells grown alone, the frequency of SIV DNA-containing $CD4^+$ cells in the animals either expanded after $CD8^+$ cell depletion or remained the same for the duration of the experiment. These findings confirmed that the infected $CD4^+$ cells constituting the SIV reservoir were not eliminated.

$CD8^+$ cells also contribute to suppression of virus replication in SIV-infected rhesus macaques during short-term ART (58). Treatment with the depleting CD8 antibody during ART caused a rebound in SIV replication that did not subside until the $CD8^+$ cells returned. Moreover, SIV recovered after $CD8^+$ cell depletion had point mutations in the viral protein Nef, which is expressed during the early phase of infection, and not in the viral protein Gag, which is expressed later in infection (59). This observation is consistent with the early emergence of anti-HIV $CD8^+$ cells that provide rapid and prolonged noncytotoxic suppression of virus replication during the initial acute phase of infection (59–61).

A major critique of these $CD8^+$ cell depletion studies was that the CD8α antibodies can deplete other cell types besides $CD8^+$ T cells, since the CD8α subunit is also expressed on NK cells, NKT cells, and $\gamma\delta$ T cells (but not $CD4^+$ cells) in NHP (62). That issue was addressed when those findings were confirmed in subsequent depletion experiments using an antibody specific for the CD8β subunit (57, 63). Because this molecule is only expressed on $CD8^+$ T cells in NHP, these studies indicated that only $CD8^+$ T cells were responsible for viral control.

(ii) Mathematical modeling of the $CD8^+$ T cell antiviral response. In addition to the *in vivo* studies described above, mathematical modeling has permitted further evaluation of the potential impact of different $CD8^+$ cell functions (e.g., cytotoxic and noncytotoxic) on viral dynamics under various conditions, including the use of antiviral drugs (56). For example, in studies of a successful SIV/HIV (SHIV) vaccination approach in NHP, the peak viral loads and the resulting decay rates of virus observed were compared to the dynamics expected from mathematical models of cytotoxic cell clearance versus noncytotoxic cell control. The modeling suggested that the experimental data were consistent only with a noncytotoxic antiviral response induced by the SHIV vaccine (64).

Other mathematical modeling of SIV infection also strongly supported the role of a $CD8^+$ cell noncytotoxic antiviral response. For example, an approach evaluating the $CD8^+$ T cell-mediated antiviral response in rhesus monkeys during the acute phase of SHIV infection demonstrated that the increase in viral loads following $CD8^+$ cell depletion and the similar life span of productively infected cells in the presence and absence of $CD8^+$ cells could not be explained solely by $CD8^+$ cell-mediated killing. The studies concluded that the contribution of a noncytotoxic antiviral activity was central to control of viremia and disease progression (60, 65).

Nevertheless, other groups proposed another model: the early eclipse phase observed during SHIV infection could be compatible only with viral clearance mediated by cytotoxic $CD8^+$ cells (66). Subsequently, further modeling studies unified these contradictory findings by explaining that the role of $CD8^+$ T cells in SHIV infection was much more dynamic and included both infected target cell killing and noncytotoxic antiviral activity (67, 68). Notably, cytotoxic $CD8^+$ T cells can exert immune selection pressure on HIV, resulting in the emergence of variants that acquired escape mutations in HLA class I epitopes (59). By mathematical modeling, the noncytotoxic $CD8^+$ T cell antiviral response appeared to be especially critical in suppressing replication of the class I HLA-driven escape mutants (69). Thus, CNAR that does not depend on a TcR-HLA/peptide interaction may contribute to suppression of these CTL escape variants soon after they emerge (70, 71).

The clinical relevance of the above-described findings is not completely clear. Escape mutants are also found in ECs (those HIV-infected individuals who have low viremia

without treatment) and not just in disease progressors (72) (section III.E). Yet, because the ECs with their ongoing CNAR activity can control replication of the escape mutant viruses sufficiently, the viral load can remain low for several years without ART.

Not only is there no difference between how wild-type and escape mutant viruses are controlled by CD8$^+$ T cells, more escape mutant viruses are also generated during the first steps in HIV replication (section VI.B). This finding further implies that, in addition to a potential cytotoxic response by CD8$^+$ T cells, a CD8$^+$ T cell-mediated noncytotoxic mechanism is critical to suppress not only the initial virus but also the *de novo* production of mutant viruses. Indeed, cytotoxic immune pressure will result in escape mutants, which need to be immediately kept under control. The latter response can only be accomplished via some kind of rapid, non-antigen- non-HIV-specific immune response with innate features such as CNAR. Finally, cell-cell interaction using three-dimensional (3D) modeling has also suggested that while the CD8$^+$ T cell noncytotoxic antiviral response might also lead to the emergence of HIV mutants, those viruses would be kept under better, more durable, immune control by CNAR than by CTL activity (70) (Table 1).

(D) CNAR in Latent Infection

The effect of this CD8$^+$ T cell noncytotoxic antiviral response on HIV latency has also been studied in cell culture. During HIV infection, some cells have latent or silent HIV infection, in part because of virus suppression by CNAR. In one study, resting unstimulated CD4$^+$ cells from uninfected subjects were infected with HIV, cultured to induce a latent state, and then treated for 3 days with an antiretroviral drug to prevent viral spread (73). At the same time, autologous CD8$^+$ T cells activated by CD3/CD28 antibody beads were added to the culture. Viral reactivation by latency reversal agents (LRA) was substantially inhibited after 3 days by a noncytotoxic antiviral CD8$^+$ T cell response (74).

Other *in vivo* studies with NHP have further supported a role of CNAR in maintaining latency. Latently infected cells in the animals were activated *in vivo* with LRA to induce virus production. The primates that had a higher number of CD8$^+$ T cells showed better control of the reactivated SIV (74). Then, when CD8$^+$ cells were depleted from the animals, virus replication was detected (57, 74).

Recently, an *in vitro* noncytotoxic antiviral immune mechanism of HIV inhibition by CD8$^+$ T cells in the context of virus reservoir formation was reported using RNA sequencing (RNA-Seq) of CD8$^+$ and CD4$^+$ cell cocultures (75). This process that used CD4$^+$ and CD8$^+$ T cells from uninfected individuals had many characteristics of CNAR: virus suppression mediated by CD8$^+$ T cells only, suppression of HIV replication at the transcription level, and the activity is HLA class I independent as shown with an HLA blocking antibody. The researchers suggested that soluble factors secreted by CD8$^+$ T cells (e.g., interleukin-4 [IL-4], IL-5, and IL-13) could be mediating the viral suppression, but this possibility was not tested directly. The CD8$^+$ T cell anti-HIV activity was attributed to a decrease in target cell proliferation and activation, along with increased infected CD4$^+$ cell survival. However, previous findings by others showed that these cytokines were not antiviral and that the frequency of infected CD4$^+$ cells is not affected by CNAR or CAF (44, 46, 47) (see above and sections III.B and III.C).

(E) Clinical Relevance

(i) Phenotype of HIV-infected subjects. HIV-infected individuals, before receiving ART, are classified according to their clinical status after infection (76). In the first category are HIV-infected individuals who are able to keep the virus under control, with HIV RNA levels lower than 50 RNA copies/ml (on average, 40 to 500 copies/ml) during several years of follow-up. As noted above, these individuals are called ECs and represent 1% or less of HIV-infected subjects. They share characteristics with uninfected individuals and long-term survivors (LTS) (see below) (Appendix 1). Those who maintain their viral load lower than 1,000 to 2,000 RNA copies/ml of blood are considered HIV controllers (HIC) or viremic controllers (VC). In contrast, HIV-infected individuals who

cannot control the virus, with a viral load higher than 10,000 copies/ml of HIV RNA, are categorized as noncontrollers (NC) or viremic individuals (VI). Another category is HIV-infected individuals for whom the disease progresses slowly over several years (from 10 to 25 years and more) without clinical symptoms of AIDS. Individuals in this category have $CD4^+$ cell counts on average of $>$500 cells/μl (with a range of 300 to 1,000 cells/μl), a stable $CD4^+$ cell slope overtime, and a low viral load (usually $<$10,000 RNA copies/ml). These are slow progressors (SP) or long-term nonprogressors (LTNP). We also call them long-term survivors (LTS) (77) (Appendix 1), some of whom we have been following for more than 35 years. The average time for development of symptoms of HIV infection is 10 years (76, 78, 79). At that later stage, they are called progressors. Finally, rapid progressors (RP) are HIV-infected individuals with a fast disease course, with $CD4^+$ cell counts of $<$300 cells/μl and clinical manifestations of AIDS within 3 years of the initial infection (80).

(ii) Viral phenotype. The clinical course of HIV infection can depend on the biological characteristics of the infecting virus. When HIV infects target cells, it begins to express two viral envelope glycoproteins that can be found at the cell surface, gp41 and gp120. This Env multimer is used by HIV particles to bind to the major HIV cell receptor CD4 and its coreceptors, CCR5 and CXCR4. The viruses with gp120 that binds to the CCR5 coreceptor are called R5-tropic; the gp120 of X4-tropic viruses binds to CXCR4. Some viruses are dual-tropic: their envelope protein can bind to either or both coreceptors.

When gp41-gp120 multimers on the infected cell surface bind with CXCR4 on other cells in the vicinity, the strong interaction results in a fusion of cellular membranes. A large multinucleated cell forms called a syncytium. Therefore, the X4-tropic viruses can be associated with cytopathic effects *in vitro* and have also been called syncytium-inducing (SI) viruses. The viruses using the CCR5 receptor are less capable of forming syncytia *in vitro* and are known as non-syncytium-inducing (NSI) viruses (81). The R5-tropic/NSI viruses are less cytopathic, associated with a slow disease progression, and are able to infect macrophages. The X4-tropic/SI viruses infect established T cell lines and are associated with a more rapid disease course (82, 83). The latter viruses generally appear at a later stage in HIV infection (84, 85).

(iii) Association of CNAR/CAF with an HIV-infected asymptomatic clinical state. Multiple studies have confirmed that CNAR/CAF is found in clinically asymptomatic HIV-infected individuals (11, 43, 77, 86–91). $CD8^+$ cells from these asymptomatic individuals suppress virus replication with $CD8^+/CD4^+$ cell ratios as low as 0.05:1. In contrast, $CD8^+/CD4^+$ cell ratios as high as 4:1 are needed to suppress 90% of the HIV replication in $CD4^+$ cells from AIDS patients with $<$200 $CD4^+$ cells/μl (43, 46). Also, $CD8^+$ T cells from AIDS patients exhibit a 4- to 20-fold lower antiviral activity when cocultured with autologous, naturally infected $CD4^+$ cells or with acutely infected $CD4^+$ cells (43). Thus, substantial differences in the $CD8^+$ T cell response between progressors and LTS are observed. With the EC and LTS, this CNAR activity can remain stable for up to 20 years or more in some subjects not receiving ART.

Notably as well, the levels of integrated HIV-1 proviral DNA are lower in the PBMC from clinically asymptomatic HIV-1-seropositive individuals than in progressors (88, 92–94). This integrated proviral HIV DNA increases when the $CD8^+$ T cells are removed from their cultured PBMC. Therefore, $CD8^+$ T cells can block the virus spread by suppressing the levels of viral mRNA as well as progeny virus: this action also reduces the total number of infected $CD4^+$ cells (section VI). Similar observations have been made with asymptomatic HIV-infected infants in whom autologous $CD8^+$ T cells showed suppression of HIV replication during an endogenous virus CNAR assay that was detected at as early as 3 weeks of age. In contrast, HIV-infected infants with $CD8^+$ T cells that did not suppress virus replication had rapidly progressing disease and died within 3 years of life (37).

In acute or primary HIV-1 infection, a high level of plasma viremia is associated with a more rapid disease course (95). In the context of the HIV-1 infection, CNAR appears

before the production of neutralizing antibodies and is associated with a lower viral load (87). Developing CNAR early is therefore critical for effective prevention of HIV disease progression and for a relatively asymptomatic long-term clinical course. The extent of the CD8$^+$ cell immune response soon after infection can also determine the prognosis for infected individuals. In asymptomatic individuals with low viral load, substantial levels of CNAR are observed in cocultures of CD8$^+$ T cells with endogenously and acutely infected CD4$^+$ cells (30). The noncytotoxic antiviral activity is particularly strong in HIV-infected LTNP (or LTS) (77), and maintenance of the LTNP status involves robust CNAR/CAF activity (11).

(F) CNAR Shares Characteristics of an Innate Immune Response

Innate immunity is a combination of evolutionarily conserved defense mechanisms (Table 1) found in many different species, including bacteria, plants, invertebrates, and vertebrates. Adaptive (or acquired) components of the immune system later emerged from the innate immune response in an antigen-specific manner (96, 97). Innate immunity is characterized by a very rapid response (minutes to days) against foreign pathogens or substances and by its lack of specificity for a defined foreign antigenic epitope; the latter could be a viral peptide that has been processed and presented by antigen-presenting cells to T and B cells. These latter cells, part of adaptive immunity, recognize defined epitopes by using a receptor (e.g., TcR and BcR) encoded by rearranged genes (section II.A). In the case of innate immunity, conserved viral or bacterial pathogen-associated motifs or stress molecules induced by infection are recognized by germ line-encoded, genetically invariant, and conserved receptors expressed by innate immune cells (98). This interaction triggers, among other functions, the secretion of soluble defense proteins, such as interferons, cytokines, or chemokines, by the innate immune cells. These soluble proteins then attract or stimulate other cell populations of the immune system to fight off the pathogens synergistically.

CNAR shares several characteristics of an innate immune response (Table 1). It occurs very early after HIV infection (87), is independent of the TcR, and has broad antiretroviral inhibitory activity. It suppresses multiple HIV-1 and HIV-2 strains, including all biotypes of HIV-1 and HIV-2 (R5- and X4-tropic) as well as drug-resistant isolates, escape mutant viruses, and other unrelated retroviruses (11, 12, 14) (sections VIII.A and VIII.B). Therefore, it does not require TcR-mediated recognition of specific viral epitopes presented by HLA molecules. HIV isolates resistant to CNAR have not been recovered or identified after multiple passages in the presence of CNAR (K. R. Bonneau and J. A. Levy, unpublished data). Nevertheless, the activity of CNAR/CAF associated with CD8$^+$ T cells from HIV-infected subjects is restricted to retroviruses; it has no effect on other virus families (section VIII.C). This observation suggests that it can be elicited only by the recognition of certain conserved retrovirus motifs (99) by germ line-encoded, invariant pattern receptors. Both of these remain to be elucidated.

Also, as another characteristic of innate immunity, CNAR does not have a strong recall response characteristic of adaptive immunity. Aside from NK cells that are considered innate lymphocytes and have such "memory-like" recall responses (100), innate immune cells previously presented with viral antigens are not typically characterized by an increased antiviral response (101). They can then quickly react to any incoming pathogen without the need for previous exposure. Notably, CNAR is also found in individuals exposed to HIV who remain uninfected (see below). The CD8$^+$ T cell antiviral response decreases over time if exposure to HIV ceases. For example, this observation has been made with spouses of hemophiliacs (102) (see below). Reexposure to HIV is needed to observe the antiviral activity, but the subsequent CNAR activity is not stronger. Finally, as with some other innate immune responses, CNAR is linked to the production of a soluble antiviral protein (i.e., CAF) (32) (section V).

(G) CNAR in HIV-Exposed Seronegative Individuals

CNAR is also observed in frequently exposed seronegative individuals who have no detectable HIV infection (Table 2). This finding was particularly evident in four studies

TABLE 2 Presence of CNAR in exposed seronegative individuals[a]

Descriptor	Frequency of CNAR$^+$ individuals (no/total [%])
Heterosexual partners	15/18 (83)[b]
MSM partners[c]	18/25 (72)[b]
Newborns of HIV-infected mothers	16/31 (52)
Intravenous drug users	7/30 (23)

[a]These data were obtained by studies conducted in the laboratory of Jay Levy (section III.G).
[b]If exposed within the past 6 months. CNAR is not present after 1 year of no exposure.
[c]MSM, men having sex with men.

of subjects who had unprotected sexual activity with HIV-infected partners (102). Two cohorts comprised heterosexual women and men with unprotected receptive sexual activity. The other two cohorts both had a mix of male and female subjects with unprotected sexual activity. Subjects were stratified according to the time since the last unprotected exposure to an HIV-infected partner. In these studies, CD8$^+$ T cells from all 60 subjects were evaluated for CD8$^+$ T cell suppression of one or more strains of HIV-1, including those sensitive and not sensitive to β-chemokines. Strong inhibition of both primary and laboratory-derived HIV isolates was observed in all four cohorts. Thirty-five randomly selected HIV-exposed seronegative (HESN) individuals from each of the four cohorts had levels of suppression greater than the maximum percentage observed in control populations ($<$45%). At the same time, there was no HIV-1-specific CTL activity detected in this population. Very-high-risk behavior was defined as multiple exposures in the last 6 months; moderate-risk behavior was defined as multiple exposures in the last 6 to 12 months before the study was conducted. The lower-risk group had multiple exposures more than 1 year ago. The data indicated that the low-risk and moderate-risk populations showed fewer subjects with CNAR than the very-high-risk population (0 to 25% versus 50 to 100%) depending on the virus isolate used in the assay. Among heterosexual women, CNAR was observed as long as the women were exposed to HIV via their infected partner. Once unprotected sexual contact ceased, the CD8$^+$ T cell antiviral activity was no longer detected (102). Moreover, HESN individuals with CNAR were observed in the population of men having sex with men (MSM) (102) (Table 2).

CNAR has also been seen in uninfected children born to HIV-infected mothers. In one study, 54% of the 16 uninfected children showed CNAR that suppressed HIV replication $>$90% in the heterologous acute infection assay (103). In another study, 81% (29/36) of HIV-infected mothers who did not transmit HIV to their children exhibited $>$50% CNAR at a 1:1 (CD8$^+$ T cell/infected CD4$^+$ cell) ratio compared to 44% (4/9) of HIV-infected mothers who transmitted the virus (104).

In certain cases, evidence of low-level CTL and HIV-specific proliferative responses to HIV antigens has been noted in some HESN individuals (105, 106). However, those activities could be explained in part by CD4$^+$ T cells with effector functions (102). All these studies indicate that CNAR can be induced following an exposure to HIV but without necessarily an established and productive HIV infection (Table 2). Thus, CNAR shares the innate immune response characteristic of not having an antigen-specific recall response: the anti-HIV activity is not retained once HIV exposure is no longer present (see above) (Table 1).

(H) CNAR in Uninfected Subjects

Anti-HIV activity by CNAR/CAF can also be seen with CD8$^+$ T cells from uninfected individuals but usually at lower and limited activity; this finding depends on the amount of infectious virus used in the cell culture assay (107). In these studies, both R5- and X4-tropic viruses gave similar results. Notably, CNAR is best observed with the autologous acute virus infection assay (2, 75) (F. C. Teque and M. G. Morvan, unpublished data) (section VII.A.ii). In several studies, phytohemagglutinin (PHA)- or CD3-stimulated CD8$^+$ T cells from uninfected subjects were found to suppress HIV replication when cocultured with acutely virus-infected heterologous or autologous CD4$^+$

cells (33) (F. C. Teque, unpublished data). Levels of CD8$^+$ T cell suppression are generally lower than those observed in HIV-infected subjects (28, 102, 103) (F. C. Teque, unpublished data). Moreover, the stimulated CD8$^+$ T cells from some uninfected subjects with CNAR activity retain usual memory phenotypic markers when stimulated via CD3 and are able to produce soluble factors such as CAF that control HIV in acutely infected CD4$^+$ cells (108) (M. G. Morvan, unpublished data). Antiretroviral suppressing activity can also be observed in clonal CD8$^+$ cell lines derived from HIV-seronegative individuals (38, 109, 110) (see above). However, CNAR has not been observed with resting CD8$^+$ T cells from uninfected subjects (2, 29, 33, 111).

Finally, PHA-stimulated CD8$^+$ cells from uninfected sooty mangabeys (SM) suppressed SIV replication in the CD4$^+$ cells from rhesus macaques (112). Conceivably, the CD8$^+$ cells play a central role in the prevention of immunodeficiency disease in the SM primates (61). Taken together, these observations underscore the relative anti-HIV potential of the CD8$^+$ cells from uninfected as well as HIV-infected subjects. The findings also are not unexpected since CNAR, as noted above, is not a TcR-antigen-dependent, acquired immune activity against specific antigenic peptides but is, presumably, an innate immune response to conserved retroviral motifs (section III.F).

(IV) FURTHER CHARACTERISTICS OF CNAR

(A) Memory Phenotype of CD8$^+$ T Cells with CNAR Activity

During virus infection, antigen-primed CD8$^+$ T cells undergo a differentiation process leading to the establishment of memory cell populations with rapid recall potential. Memory cells fall within the following successive states, which can be defined by expression of cell-surface markers (Fig. 2): central (T_{CM}), transitional (T_{TM}), and effector (T_{EM}). Late-stage effector memory cells with CD45RA reexpression, designated T_{EMRA}, have also been defined. They have mostly a secretory function and a senescent phenotype as defined by the absence of cell division, shorter telomeres, and the expression of CD57 (113–115) and programmed cell death protein 1 (PD-1) (see below). The HIV-specific cytotoxic CD8$^+$ T cells (i.e., CTLs) are primarily found in the T_{EM} compartment but not within the T_{EMRA} compartment (116, 117).

The CD8$^+$ T cells mediating CNAR express CD28, the costimulatory cell membrane receptor for B7.1 (CD80) and B7.2 (CD86), and CD3 but not CD11b (Fig. 2) (86). CNAR is also associated with an activated phenotype (for example, HLA-DR expression) (86). Moreover, some of these cells express PD-1, an activation marker (section IV.D) (29). In HIV-infected individuals, a decline in CD8$^+$ CD28$^+$ cell number is associated with loss of CNAR activity and disease progression (86). This phenotype is characteristic of recently activated, transient-stage memory CD8$^+$ T cells, called transitional memory (T_{TM}) cells (Fig. 2).

In this regard, our studies have shown that T_{TM} is a major CD8$^+$ T cell subset mediating CNAR (29). In contrast, CD8$^+$ T cells with cytotoxic function lack CD28 expression and are CD11b$^+$. Thus, phenotypically distinct CD8$^+$ T cell subpopulations mediate CNAR versus CTL activities (Table 1). Notably, in the case of HIV infection, "memory" can define a CD8$^+$ T cell subset with the innate function of mediating CNAR. However, as noted above (section III.F), the absence of a classic T cell memory response is characteristic of this noncytotoxic response. Therefore, these CD8$^+$ T cells have a memory cell phenotype but also an innate antiviral function that does not involve a greater recall response specific to HIV antigens.

Engagement of CD28 on the CD8$^+$ T cells of HIV-seropositive subjects results in increased CD8$^+$ T cell activation and robust anti-HIV activity (118–120). In addition, CD8$^+$ T cell costimulation with CD3 and CD28 antibodies, compared to that with CD3 antibody alone, enhances the CD8$^+$ T cell anti-HIV response as measured by CNAR (121). Similarly, the interaction of the CD28 molecule on CD8$^+$ T cells with the natural ligands CD80/CD86 on autologous antigen-presenting cells (APCs), particularly macrophages, results in increased antiviral suppression by CD8$^+$ T cells when cocultured with acutely HIV-infected CD4$^+$ cells (122) (Fig. 3). Finally, blocking the CD80/CD86

CD8$^+$ T cell:	Naïve	T_{CM}	T_{TM}	T_{EM}	T_{EMRA}	T_{RM}
Maturation						
CD45	RA	RO	RO	RO	RA	RA, RO
CD27/CD28	High/+	High/+	+/+	Low/–	–/–	+,–/+,–
CCR7	+	+	–	–	–	Low
PD–1	–	+	High	–/+	+	+
CD57	–	–	Low	+	High	?
CD69	–	–	–	–	–	+
HLA–DR	–	–/+	–/+	+	–	?
CD38/CD11a (LFA–1)	Low/Low	–/High	?	–/High	–/High	?
CD11b	–	–	–	+	+	?
CD62L	+	+	–	–	–	Low
CD103	–	–	–	+	–	+
KLRG1	–	Low	+	+	High	Low
VCAM–1						+
Turnover	Very low	Low	?	High	Low	?
Granzymes	–	Low	+	High	High	+/–
CNAR/CTL	–	–	CNAR	CTL	?	CNAR

FIG 2 Memory phenotype and maturation stages of CD8$^+$ T cell subsets. The successive differentiation stages of peripheral CD8$^+$ T cells from T_N (naive) are T_{CM} (central memory), T_{TM} (transitional memory), T_{EM} (effector memory), and T_{EMRA} (effector memory reexpressing CD45RA). In addition, T_{RM} (resident memory) cells are CD8$^+$ T cells found in some tissues. These various CD8$^+$ T cell subsets have different expression profiles for the cell surface and intracellular markers listed. In addition, the major CD8$^+$ T cell subsets mediating CNAR or CTL are specified.

ligands on macrophages with the CTLA4-Ig fusion protein prior to exposure to CD8$^+$ T cells prevents the interaction of the CD80/CD86 and CD28 molecules and abrogates the CD8$^+$ T cell suppression of virus replication in CD4$^+$ cells (123).

(B) CNAR/CAF Antiviral Activity in Lymphatic Tissues

(i) Overview. HIV replication remains active in lymphatic tissues during the asymptomatic phase of infection (124). Therefore, the control of virus replication in these tissues is also important for maintaining a clinically asymptomatic state (section III.E). Most studies of CNAR in HIV-infected individuals have used CD8$^+$ and CD4$^+$ cells isolated from peripheral blood. However, notably, noncytotoxic CD8$^+$ T cell activity is also present in lymph node mononuclear cells (LNMC), although requiring, in cell culture, 2 to 10 times more CD8$^+$ T cells than found in PBMC (46). As in the peripheral blood, a direct beneficial clinical correlation is observed with LNMC CNAR activity. Low CD8$^+$/CD4$^+$ cell ratios (0.05:1 to 0.25:1) are required to suppress HIV replication in target CD4$^+$ cells with lymphoid CD8$^+$ T cells from asymptomatic HIV-infected individuals (46). In contrast, higher numbers of CD8$^+$ T cells (up to 20 times compared to that in LTS) are needed for CNAR measured with the lymphatic cells from AIDS patients and in association with the higher viral loads found in progressors (46). In addition, the retention of a normal lymph node architecture, as seen by histological examination, correlates with the suppression of virus replication by CD8$^+$ T cells in cocultured autologous CD4$^+$ cells (46). Taken together, these findings indicate that CD8$^+$ T cells with CNAR can play a major role in controlling virus production at the site of virus replication in the lymphatic tissues.

(ii) CD8$^+$ T cell subset and phenotype. Recently, a study that included RNA sequencing conducted on CD8$^+$ T cells from ECs confirmed the noncytotoxic antiviral function of lymphoid tissue CD8$^+$ T cells. The responsible cells had a resident memory

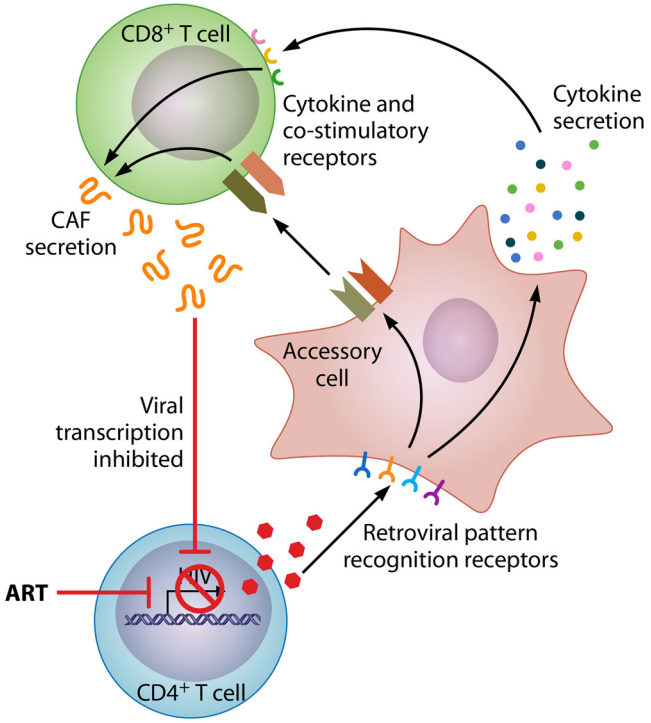


FIG 3 Tripartite immune cell interactions can induce CNAR/CAF activity. Infected $CD4^+$ cells (below) release HIV particles containing conserved retroviral motifs that can be sensed by germ line-encoded invariant pathogen pattern receptors (e.g., TLRs) on accessory cells (e.g., DCs and macrophages). This recognition can (i) upregulate the expression of costimulatory ligands at the surface of the accessory cells (e.g., CD40, and CD80/CD86), and/or (ii) stimulate cytokine secretion (e.g., IL-2, IL-15, and IL-21). These cell surface or secreted molecules can, in turn, activate the subset of $CD8^+$ T cells that can mediate CNAR activity by binding to costimulatory receptors (e.g., CD154 and CD28) or cytokine receptors (e.g., CD25, CD215, and CD360). This action would induce or enhance CAF production. CNAR/CAF activity then inhibits HIV transcription, resulting in fewer virus particles and lowered accessory cell (and $CD8^+$ T cell) activation. Similar to the effect of ART treatment (31, 162), this action decreases CNAR/CAF activity over time, for as long as HIV is kept in a reduced and/or latent state.

(T_{RM}) phenotype (see below) (Fig. 2) and a distinct transcriptional signature of tissue resident $CD8^+$ T cells (125). The gene candidates for CAF could potentially be found among those differentially expressed genes (125, 126) (section X.C).

Moreover, in lymphoid tissues, T resident memory (T_{RM}) cells are found that do not express CD27/CD28 and have a more mature phenotype that closely resembles the peripheral $CD8^+$ T_{EM} cells (127) (Fig. 2). However, these cells, for reasons including low expression of perforin, granzyme, and transcription factors regulating cytotoxic function, are not equipped to mediate cytotoxicity in contrast to activated T_{EM} cells (128). Therefore, the T_{RM} cells represent another notable $CD8^+$ T cell subset found in tissues that demonstrates a strong CNAR (125–128) (see below).

(iii) $CXCR5^+$ $CD8^+$ T cells. The chemokine receptor CXCR5 is another $CD8^+$ T cell marker that is expressed on cells with CNAR but not CTLs (127). Originally found in the context of chronic infections in animal models (e.g., lymphocytic choriomeningitis mammarenavirus [LCMV], human T cell lymphotropic virus [HTLV], or SIV) (129, 130), the $CD8^+$ T cells expressing CXCR5 are particularly present in lymphoid follicles (131, 132) where cells express its ligand, CXCL13. These $CXCR5^+$ $CD8^+$ T_{RM} cells have a memory phenotype and express lower levels of transcription factors associated with cytotoxicity such as EOMES and T-bet (131). Nevertheless, these differentially regulated $CD8^+$ T cells do not release large amounts of perforin and granzymes. However, they are still able to degranulate and express CD107a at the cell surface (section II.A). Importantly, as noted above, the $CD8^+$ T_{RM} cells can suppress virus replication without

TABLE 3 Adhesion molecules in HIV infection[a]

Name	Other name	Ligand	Role in HIV	Reference(s)
CD11a/CD18	LFA-1 (Itgα_L/Itgβ_2)	CD50, CD54, CD102, CD242, ICAM-5, CD321	Associated with CD8$^+$ T cell trafficking to tissues; may induce CAF	137, 180
CD11b/CD18	CR3, Mac-1 (Itgα_M/β_2)	iC3b, CD54 (ICAM-1)	Absent from CD8$^+$ T cells mediating CNAR	86
CD62L	L-selectin	GlyCAM-1, CD34, MadCAM-1, PSGL-1	Expression inversely correlated with viral loads	134
CD103	IntegrinαE (Itgα_E)	CD324	Found on (gut) CD8$^+$ T_{RM} cells of HIV$^+$ subjects	136
CD106	VCAM-1	VLA-4 (CD49d/CD29)	Marker of CD8$^+$ T cells mediating CNAR/CAF	133, 330
CD324	E-cadherin	CD103, KLRG1	Expression correlates with higher viral loads, inhibits CNAR *in vitro*	135

[a]See section IV.C.

killing the infected cells (127). Thus, lymphoid tissue CD8$^+$ T_{RM} cells have CNAR activity. Nevertheless, it has been suggested that when these CD8$^+$ T_{RM} cells leave the lymph node, they can then become antigen-specific CTLs (125, 127).

(C) Adhesion Molecules

Vascular cell adhesion molecule 1 (VCAM-1) is an adhesion molecule whose expression was detected unexpectedly on CD8$^+$ T cells with a noncytotoxic anti-HIV response (133). Up to 12% of CD8$^+$ T cells from HIV-infected subjects with CNAR express VCAM-1 at the cell surface compared to 0.8% in uninfected individuals. In cell culture studies, fewer sorted VCAM-1$^+$ CD8$^+$ T cells than bulk CD8$^+$ T cells were needed to inhibit HIV replication in infected CD4$^+$ cells. Moreover, this cell type was also associated with a greater production of CAF as demonstrated by a transwell assay (133). Therefore, this endothelial cell surface adhesion molecule, which favors cell-to-cell contact, is another marker of a CD8$^+$ T cell subset with CNAR activity.

Other adhesion molecules (Table 3), such as L-selectin (CD62L, a protein found on the surface of naive CD8$^+$ T cells and central memory CD8$^+$ T cells), are expressed at lower levels on the CD8$^+$ T cells of HIV-infected subjects who are not on ART; their expression is inversely correlated with viral loads (134). Compared to those from HIV-seronegative controls and regardless of clinical status, the CD8$^+$ T cells of HIV-infected subjects coexpress lower levels of CD62L. In contrast, the expression of adhesion molecule E-cadherin (CD324) is found at higher levels on CD8$^+$ T cells of HIV-infected subjects; it correlates with higher viral loads and impaired CD8$^+$ T cell functions, including noncytotoxic activity (135). Moreover, integrinαE (CD103) is expressed at high levels on the noncytotoxic tissue-resident memory CD8$^+$ T cells of HIV-infected subjects (136). Finally, lymphocyte function-associated antigen (LFA-1; CD11a/CD18) is associated with anti-HIV CD8$^+$ T cell trafficking to tissues (137). Like VCAM-1, all these adhesion molecules could have a beneficial effect on CNAR by stabilizing the cell-to-cell interaction required for improving CD8$^+$ T cell-mediated HIV suppression.

(D) Activation

(i) Overview. Stimulation of CD8$^+$ T cells via the TcR, together with the costimulation receptor CD28, "activates" the cells. This activation leads to antigen-specific cell proliferation, differentiation into an effector phenotype (Fig. 2), and a reduction in overall cell numbers by programmed cell death (apoptosis) (138). In terms of the latter state, the activated CD8$^+$ T cells can survive if they also express cell surface inhibitory receptors (see below) that counter overactivation and are often used as well as phenotypic markers of CD8$^+$ T cell activation.

Two of the main inhibitory receptors used as cell activation markers are CD69, which is expressed early after cell stimulation, and HLA-DR, which is detected later (139) and is one of the markers of CD8$^+$ T cells mediating CNAR. Other cell surface molecules induced after cell stimulation that can also be used as activation markers are CD25, CD38, and CD154 (Fig. 2). Additional markers, such as CD57, CD95 (Fas), or CD279 (PD-1), have been commonly cited as activation markers (140–142), but they are now primarily used to characterize T cell "exhaustion" (see below). The latter term

refers to CD8$^+$ T cells chronically exposed to their cognate antigens, which have diminished cytotoxic function (143–145) but not necessarily decreased CNAR; PD-1$^+$ CD8$^+$ T cells have this noncytotoxic anti-HIV activity (29). Finally, CD27 and CD28 expression also increases after CD8$^+$ T cell stimulation. However, when these cells transition to an effector cell function, the CD27/CD28 expression is downregulated and their absence at the cell surface can identify them as effector memory CD8$^+$ T cells (146). The latter cells do not mediate CNAR but now have CTL function (Fig. 2).

(ii) Clinical relevance of CD8$^+$ T cell activation. An asymptomatic clinical state in HIV-infected individuals is associated with higher levels of CD28 and HLA-DR expression on CD8$^+$ T cells that reflects cell activation. Moreover, low levels of CD11b as well as the activation marker CD38 and the senescence marker CD57 are often identified on CD8$^+$ T cells in asymptomatic infected individuals and not in AIDS patients (86).

In cell culture, CD8$^+$ T cells that are HLA-DR$^+$ CD28$^+$ CD11b$^-$ found in asymptomatic HIV-infected individuals, as well as *in vitro* PHA-stimulated CD8$^+$ T cells, exhibit the strongest CNAR activity against HIV replication (86). This early finding was later confirmed when the CD27/CD28$^+$ transitional memory CD8$^+$ T cells and PD-1$^+$ CD8$^+$ cells were identified as major subsets mediating CNAR (29) (see below and section IV.A). Notably, high PD-1 expression during chronic HIV infection has been associated with "exhaustion" via continuous TcR stimulation of HLA-restricted CD8$^+$ T cells (see above). The susceptibility of noncytotoxic CD8$^+$ T cells to exhaustion during chronic infection is unknown, but perhaps unexpected given TcR engagement is not a feature of CNAR activity. CD8$^+$ T cells that mediate CNAR do express PD-1 (29), but in this circumstance, it is more likely a marker of activation than exhaustion. Notably, PHA, as a potent mitogen, can elicit broad cell activation that can lead to the general skewed expression of some phenotypical memory markers (e.g., CCR7 and CD45RA) on CD8$^+$ T cells. This finding is not the case with CD3 stimulation (147). Therefore, CD8$^+$ T_{TM} cells, a CD45RA$^-$ CCR7$^+$ subset of cells mediating CNAR, may not be correctly identified following PHA stimulation.

Moreover, some activated CD8$^+$ T cell clones from asymptomatic HIV-infected subjects (section III.E.iii) have suppressed viral replication in heterologous CD4$^+$ cells, including in a transwell assay, and were noncytotoxic in response to HIV antigens (148). This noncytotoxic suppressing activity was TcR independent, since different clones with the same TcR exhibited variable degrees of suppression of HIV replication, including no response (148). Moreover, the noncytotoxic clones produced antiviral soluble factors without any correlation of this activity with their activation phenotype. Importantly, the expression of activation markers such as CD28, HLA-DR, or CD11b/CD18 was highly variable among the clones. These findings suggest that CD8$^+$ T cells can suppress virus replication at various stages of the activation cycle after antigen or mitogen stimulation.

(E) Role of HLA Class I Compatibility in CNAR

In HIV infection, CNAR differs from the mechanism(s) involved in the activity of HIV antigen-specific cytotoxic CD8$^+$ CTLs. While CTL activity is dependent on the histocompatibility of HLA class I from cocultured CD8$^+$ effector T cells and HIV-infected CD4$^+$ target cells (149) (section II), CNAR is observed when there is minimal or no HLA matching between the two cell populations (2, 33). In the case of heterologous target cells, HIV suppression requires 2- to 5-fold more MHC-discordant CD8$^+$ T cells (26). Notably, 20-fold fewer autologous CD8$^+$ T cells are required to suppress HIV replication than CD8$^+$ T cells with different HLA molecules (33). Moreover, HLA blocking antibodies do not affect CNAR (75) (C. E. Mackewicz, unpublished data). Similar findings on the lack of histocompatibility dependence, even across species barriers, were made in NHP. Cultured CD8$^+$ cells from SIV-infected sooty mangabeys suppressed viral transcription in rhesus macaque CD4$^+$ cells transiently transfected with a long terminal repeat (LTR)-driven CAT reporter gene, without killing the target cells (112).

Studies directed at demonstrating the requirements of HLA compatibility for HIV control by CNAR focused on a cohort of uninfected and HIV-infected subjects,

including two pairs of HIV-discordant identical twins (33). Optimal HIV suppression without cytotoxicity was observed when effector $CD8^+$ T cells and infected $CD4^+$ cells from the same subject were used. This noncytotoxic viral suppression was also observed, but at a reduced level, when $CD8^+$ T cells and infected target $CD4^+$ T cells from different subjects were cocultured. Importantly, compared to autologous $CD8^+$ T cells, 20-fold more heterologous $CD8^+$ T cells were needed to suppress virus replication in the infected $CD4^+$ cells from HIV-seropositive subjects. This difference in $CD8^+$ T cell suppression was due neither to the HIV replication capacity nor to the sensitivity to CNAR of the $CD4^+$ cells (33).

Furthermore, in a reciprocal coculture of $CD8^+$ T cells with acutely HIV-infected $CD4^+$ cells from an HLA-mismatched subject, differing levels of HIV suppression were observed (33). For instance, when the $CD8^+$ T cells and infected $CD4^+$ cells from the same subject were cocultured (having an identical HLA genotype), HIV suppression was very strong (97% at the 0.25:1 $CD8^+/CD4^+$ cell ratio). However, when culturing the $CD8^+$ T cells with the infected $CD4^+$ cells from another subject, HIV suppression was noted but at lower levels (48% at the same 0.25:1 cell ratio) (33). This observation indicated that, in a heterologous coculture, $CD8^+$ T cell-mediated suppression of HIV can occur at a reduced level but is not dependent on matched HLA genotypes. Overall, all these observations demonstrate that CNAR activity suppresses HIV replication independent of genetic histocompatibility.

Another evaluation of $CD8^+$ T cell anti-HIV responses indicated, via cell clonal lines, that both cytotoxic and noncytotoxic activities can be involved depending on the HLA concordance. Acutely HIV-1-infected HLA-matched $CD4^+$ cell lines and primary $CD4^+$ cells cocultured with HIV-specific $CD8^+$ T cell clones isolated from infected individuals suppressed virus replication at lower $CD8^+/CD4^+$ cell ratios (0.25:1) than HLA-unmatched target and effector cells cultured at a 1:1 ratio (45). This low number of $CD8^+$ T cells in the coculture assays also supports the lack of CTL activity, where ratios of 10 to 25 $CD8^+$ T cells to one target cell may be required for antigen-specific lytic responses (3, 5, 150, 151). Moreover, these $CD8^+$ T cell clones were found to suppress virus replication by up to 60% via the production of soluble anti-HIV factors, as demonstrated by coculture with HLA-mismatched HIV-infected $CD4^+$ cells in a transwell assay (45) (section V).

Other HLA studies used $CD8^+$ T cell clones generated from two infants with $CD8^+$ T cell anti-HIV activity. When cocultured with autologous $CD4^+$ cells, these clones did not show CTL activity by using a ^{51}Cr release assay, but suppressed virus replication by more than 50% (37). The results confirmed CNAR in the absence of killing even in the presence of HLA-matched effector and HIV-infected target cells. In summary, CNAR can be improved by HLA class I compatibility, but the activity is not HLA restricted.

(F) Factors that Induce or Enhance CNAR/CAF

The definitive mechanism of how conserved retroviral motifs (section III.F) trigger CNAR and CAF production remains to be elucidated. The induction of CNAR in NHP with an SIV vaccine (64) may uncover the process involved. A variety of factors or processes can induce or increase the innate activity of CNAR/CAF (Appendix 2). Notably, the loss of the $CD8^+$ T cell-mediated anti-HIV activity in symptomatic patients has been attributed to a transition from a decreased expression of cytokines (e.g., IL-2) that typically promote cell-mediated immunity (the T helper 1 [T_h1] response) to an increased production of cytokines (e.g., IL-4 and IL-10) that downregulate this response (the T helper 2 [T_h2] response) (35, 152).

In this regard, when PHA-stimulated $CD8^+$ T cells from LTS or progressors were treated in culture with IL-2, a substantial increase in suppression of HIV replication was observed compared to that for $CD8^+$ cells grown in medium alone (35, 153). Conversely, when these $CD8^+$ T cells were treated with either IL-4 or IL-10, a decrease in HIV suppression was noted (Fig. 4). Furthermore, IL-2 treatment of $CD8^+$ T cells from LTS prevented and reversed the inhibitory effects of IL-4 and IL-10 on CNAR. Notably

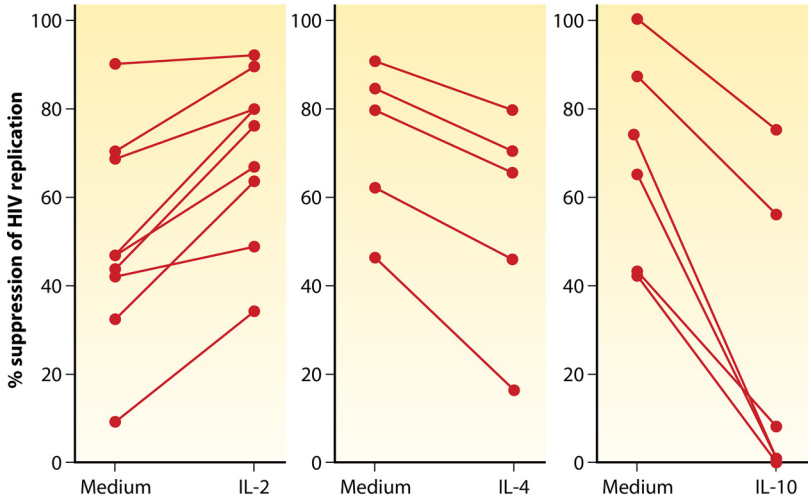


FIG 4 Effect of different (T_h1 and T_h2) cytokines on HIV replication. To determine the effect of cytokines in the CD8$^+$ T cell noncytotoxic anti-HIV response (CNAR), CD8$^+$ T cells were stimulated in the presence of type 1 (IL-2) or type 2 (IL-4, IL-10) cytokines for 3 days. After being washed, these cells were tested for their ability to suppress HIV replication in acutely infected CD4$^+$ lymphocytes. (Modified from references 153 and 417 with permission.)

as well, prolonged exposure of CD8$^+$ T cells from progressors to IL-2 (3 to 6 days) improved the HIV suppressing activity (i.e., CNAR) of these cells (153).

As noted above, CD8$^+$ T cells that mediate CNAR express CD28 (86, 121), a costimulation receptor that enhances IL-2 production when engaged with its ligands or antibodies (154, 155). Some studies have revealed that, whereas culture fluids from CD8$^+$ T cells treated only with a CD3 antibody contained minimal levels of IL-2, these same cells costimulated with both CD3 and CD28 antibodies had detectable IL-2 levels (121). IL-2 production by the CD8$^+$ T cells also resulted in increased expression of the IL-2 receptor (CD25) on the stimulated CD8$^+$ T cells. Furthermore, preventing the IL-2/CD25 interaction with neutralizing or blocking antibodies abrogated the CNAR of these CD8$^+$ T cells without affecting their viability (121). Overall, CD3/CD28 stimulation of CD8$^+$ T cells from asymptomatic HIV-seropositive subjects resulted in IL-2 production and CD25 expression associated with virus suppression.

In clinical studies, the administration of IL-2 in combination with ART increased CD4$^+$ cell numbers in the peripheral blood and the lymph nodes while maintaining an undetectable viral load (156–159). Subsequent studies of primary HIV infection showed that highly active antiretroviral therapy (HAART) combined with intermittent subcutaneous doses of IL-2 led to an approximately 3-fold increase in CD4$^+$ cell number over the 48-week study (160, 161). No difference was observed with viral loads and CD8$^+$ T cell numbers. Notably, at baseline, the HAART-treated and untreated subjects had substantial levels of CNAR activity that declined over the 48-week period (162). The latter could reflect the lack of expression of viral motifs (section III.F), since stopping ART quickly resulted in CNAR (163). Seventy-five percent of the subjects treated with the IL-2/ART combination showed increased levels of CNAR activity into week 24 of the study when HIV replication was controlled (160).

To better understand the association between the IL-2 production and CNAR activity, some studies focused on cell-to-cell interactions to identify a potential mechanism for the T cell-mediated immunity. Previous work has noted that dendritic cells (DCs) are involved in inducing CD8$^+$ T cell responses (164, 165), particularly IL-2 production (166). As disease progresses, HIV-seropositive subjects undergo a decrease in DC numbers circulating in the periphery and reduced APC function (167). This action can lead to dysfunction of the DC/CD8$^+$ T cell interaction and could be responsible for a decrease in CNAR activity (Fig. 3).

During HIV infection, the loss of activated $CD4^+$ cells may also result in insufficient levels of CD40 ligand (CD40L) expression that helps with the maturation of DCs via CD40. In turn, DCs would be unable to sufficiently activate $CD8^+$ T cells to maintain CNAR activity (Fig. 3). When sufficiently activated with CD40L, DCs secrete both IL-12 and IL-15 (168–170). These two cytokines also expand and differentiate naive $CD8^+$ T cells into cells with a memory phenotype (171, 172). Furthermore, IL-12 promotes $CD8^+$ T cell responses, including CNAR activity (153).

Importantly, $CD8^+$ T cells from HIV-infected progressors (section III.E.i), cocultured with CD40L-matured DCs, had higher CNAR activity than those costimulated with only CD3/CD28 antibodies (173). IL-15 treatment of the $CD8^+$ T cells from these subjects resulted in increased $CD8^+$ T cell proliferation and CNAR activity compared to that with IL-12 treatment (153, 173). Since both the IL-15 receptor and the IL-2 receptor share the same β and γ subunits, each with a unique α chain, IL-15 and IL-2 may co-promote $CD8^+$ T cell-mediated antiviral immunity, such as CNAR, through similar downstream pathways.

Other mechanisms leading to increased anti-HIV activity via the secretion of soluble factors have been identified. For instance, when $CD8^+$ T cell clones from uninfected individuals were CD3/CD28 stimulated in the presence of IL-21, they exhibited higher antiviral activity *in vitro* than with the use of other cytokines (e.g., IL-2, IL-7, and IL-15). They also upregulated CD28 (110), a marker of $CD8^+$ T cells mediating CNAR. Despite the known increased cytotoxic potential of $CD8^+$ T cells treated with IL-21 (174), it is unlikely that these $CD8^+$ T cells killed the HIV-infected $CD4^+$ target cells, since the PBMC were from HIV-uninfected individuals (section III.H). Instead, CNAR could have been involved (110). In addition to the benefit of the cytokines, ART will sometimes result in an increase in CNAR activity at least for an initial period of 2 to 3 months. However, with reduction in HIV levels, CNAR decreases (31, 162, 175). The findings probably reflect the continued need for viral motif recognition (section III.F).

Allogeneic stimulation of polyclonal $CD8^+$ T cells from HIV-seronegative individuals as well as PHA stimulation can also produce an antiviral soluble factor, such as CAF (176–179). This finding supports observations that CAF is an innate immune factor that responds to cell activating signals (section III.F).

Another way of enhancing CNAR is the treatment of PBMC with an LFA-1 antibody (section IV.C). It binds to $CD8^+$ T cells and induces the production of a soluble factor with antiviral activity that is not any of the known anti-HIV cytokines or chemokines (180). In addition, thymosin α1 is a polypeptide derived from prothymosin-α (section IX.F) that enhances the secretion of antiviral soluble factors by lipopolysaccharide (LPS)-stimulated $CD8^+$ T cells (181). The supernatants from these treated $CD8^+$ T cells (probably containing CAF) are able to inhibit the replication of both HIV and HTLV-1 *in vitro* better than those stimulated in the absence of thymosin α1.

Finally, the use of Toll-like receptor (TLR) agonists with the cultured PBMC of uninfected individuals decreased HIV replication in autologous $CD4^+$ T cells. In particular, an agonist to TLR7/8, but not TLR4 or TLR1/2, induced a state of $CD8^+$ T cell activation that resulted in the secretion of multiple soluble factors, including presumably CAF, which suppressed HIV replication in $CD4^+$ T cells (182). Similarly, a successful vaccine should induce $CD8^+$ T cell responses, such as CNAR, which can decrease virus replication and protect $CD4^+$ cell numbers. To this point, a study evaluating the effect of a vaccine on SHIV infection in NHP indicated that a noncytotoxic $CD8^+$ cell function was primarily involved in viral control (64) (section III.C.ii).

(G) Summary: Major CNAR Characteristics

CNAR is distinct in function, regulation, and phenotype from CTL activity, an effector function essential to adaptive antiviral immunity (Table 1). Most importantly, CNAR does not involve target cell killing. The CTL activity is triggered by the interaction between a T cell receptor and an HLA molecule presenting a specific peptide from a foreign pathogen (e.g., HIV antigen) or compound (e.g., haptens). With CNAR, (i) the $CD8^+$ cells involved are innate immune cells that are responsible for inhibiting HIV

replication without cell killing, and the suppression is dependent on the number of CD8$^+$ T cells present; (ii) HIV suppression can be shown with autologous CD4$^+$ cell infection or acutely HIV-infected heterologous CD4$^+$ cells; (iii) MHC compatibility provides optimal virus inhibition in CNAR but is not required for suppression of HIV production; (iv) the activity is associated with a clinically asymptomatic state, and CNAR is substantially reduced in HIV-infected individuals with symptoms and AIDS; (v) the major phenotype of CD8$^+$ T cells mediating CNAR is CD28$^+$ CD11b$^-$ activated (HLA-DR$^+$) T_{TM} cells; (vi) CNAR is most effective when CD8$^+$ T cells are activated by various stimuli such as an interaction with DCs and CD3/CD28 cross-linking, and (vii) exposure to certain T_h1 cytokines (e.g., IL-2, IL-12, IL-15, and IL-21) (Fig. 3). (viii) Moreover, CNAR is an early response to HIV infection (44). Finally, (ix) CNAR is associated with the production of a soluble CD8$^+$ T cell antiviral factor (CAF).

(V) THE CD8$^+$ T CELL ANTIVIRAL FACTOR (CAF)

(A) Discovery

As reviewed above, the CD8$^+$ T cell noncytotoxic antiviral response was first demonstrated by coculturing CD8$^+$ cells with HIV-infected CD4$^+$ cells. While cell-cell contact gives optimal viral control, this approach is not necessary. Experiments performed using transwell devices, in which infected target CD4$^+$ cells and effector CD8$^+$ T cells are separated by a semipermeable membrane, showed effective virus suppression. The studies indicated that the CD8$^+$ T cells secrete a soluble factor that can pass through a filter and block virus replication in the target CD4$^+$ cells (32, 36, 183) (section III.B).

Similarly, supernatants taken from cultures of activated CD8$^+$ cells from HIV-infected asymptomatic individuals can suppress HIV replication in acutely infected CD4$^+$ cells or naturally infected CD4$^+$ cells stimulated to produce the virus. Likewise, cell culture fluids from the CD8$^+$ cells of baboons infected with HIV-2 have suppressed HIV-1 replication in acutely infected human CD4$^+$ cells and inhibited HIV LTR transcription in 1G5 cell lines (184). These fluids contain an unknown CD8$^+$ T cell antiviral factor (i.e., CAF) that can show antiviral activity in acute HIV infection assays (section VII.B). It is noteworthy that the presence in these fluids of other cytokines (e.g., TNF-α) that enhance HIV replication (185) may counter the antiviral activity observed with CD8$^+$ T cell culture fluids (186, 187). This control of HIV infection by soluble secreted factors was reviewed more than a decade ago (15). It is evident that CAF is a major anti-HIV protein produced by the CD8$^+$ T cells that was detected early in studies of immunologic control of HIV infection by asymptomatic HIV-infected individuals (32).

In the initial studies of the noncytotoxic CD8$^+$ T cell antiviral activity, the amount of CAF produced by CD8$^+$ T cells was found to be limited. The highest dilution of CD8$^+$ T cell culture showing a 50% suppression of HIV replication in CD4$^+$ cells was 1:4 (Fig. 5) (186). Thus, undiluted supernatants could have 4 units of anti-HIV activity, in which one unit of activity gives a reduction of HIV replication by 50%. Importantly, initially in measuring CAF activity, the biological assays often used HIV-1_{SF2} or the more cytopathic X4-tropic SI virus, HIV-1_{SF33} (83). Neither viral isolate is sensitive to the antiviral activity of the β-chemokines (188, 189).

(B) Characterization of CAF

The antiviral effect of CAF is not related to a direct inactivation of the RT enzyme nor the integrity of the virus particles. Cocultivation of virus with CAF does not affect virus infectivity (190). Moreover, mitogen-induced proliferation and expression of activation markers such as CD25, CD38, CD69, and HLA-DR by target CD4$^+$ cells are not affected by exposure to CNAR or to CAF (11, 191, 192).

Among the first experiments conducted to identify the nature of CAF were those directed at determining whether the factor is a protein, a lipid, or another biological compound. Using the serine protease trypsin in CAF assays eliminated the antiviral activity; thus, CAF was assumed to be a protein. Mixing CAF-containing fluids with polar solvents such as ether or acetone supported this conclusion. The HIV activity remained in the aqueous portion, not in the polar fraction. Thus, CAF was not a lipid but likely a

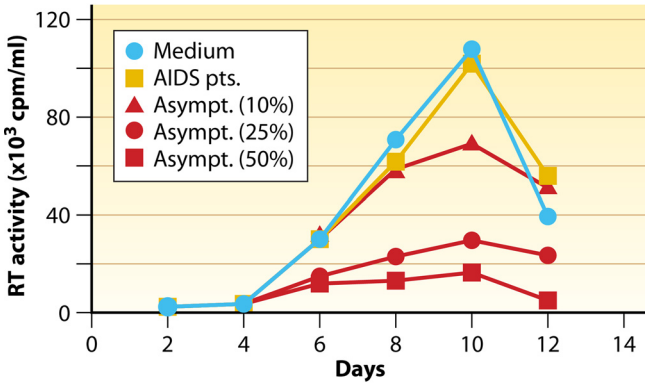


FIG 5 Level of anti-HIV activity of CAF. To measure the anti-HIV activity of CAF, $CD4^+$ cells acutely infected with $HIV\text{-}1_{SF2}$ were cultured in the presence of various dilutions of $CD8^+$ T cell culture supernatants (changed every 2 days) and monitored for viral reverse transcriptase (RT) activity. Examples from one of several experiments are shown. Medium, control; AIDS patients (pts), $CD8^+$ T cell culture fluid prepared from an AIDS patient diluted 50%; Asympt, $CD8^+$ T cell culture fluid prepared from an asymptomatic HIV seropositive subject diluted 10%, 25%, and 50%. Modified from reference 186 with permission from Elsevier.

protein (192). In other studies, CAF-containing fluids were passed through a 30-kDa filter and maintained their anti-HIV activity. This CAF activity was not removed by 10-kDa filters, but was after 3-kDa filtration. Therefore, its size is estimated between 10 kDa and 30 kDa (J. A. Levy, unpublished data). CAF is stable to lyophilization and pHs from 2 to 8. The CAF fluids yield fractions of anti-HIV activity after ammonium sulfate precipitation and withstand heating up to 10 min at 86°C with the antiviral activity intact (J. A. Levy, unpublished data) (184, 192) (Table 4).

In the above-described studies, a variety of protease inhibitors (leupeptin, antipain, and Pefabloc) were used to counter the potential proteolytic activity in cell culture of the proteases selected to determine if CAF was a protein. CAF was found to be particularly sensitive to Staph V8 protease and other serine proteases. While most of these protease inhibitors reduced the effect of proteases on CAF activity, surprisingly, only

TABLE 4 Characteristics of the $CD8^+$ T cell antiviral factor[a]

Property	CAF characteristics
Production	Only by activated $CD8^+$ T cells
	Not found in cellular granules
	Lacks identity with other known cytokines
	Precursor protein may be activated by proteolytic cleavage
Activity	Inhibits all HIV-1, HIV-2, and SIV isolates
	Blocks HIV replication in naturally and acutely infected cells
	Blocks HIV transcription
	Does not substantially affect cell activation or proliferation
Stability	Lyophilization and ether
	Dialysis
	56°C for 30 min
	86°C for 10 min
	Up to pH 8 and as low as pH 2
	$(NH_4)_2SO_4$ precipitation
	In polar phase separation, found in the aqueous fraction
	Protease sensitive (e.g., *Staphylococcus* V8 protease)
Mol wt	Between 10 and 30 kDa
Stable to protein purification methods	Mono Q anion exchange chromatography column
	Capto adhere multimodal chromatography resin
	HiTrap Q HP anion exchange chromatography column
	Hydrophobic interaction chromatography
	BioLC HPLC columns
	Stable-isotope labeling by amino acids in cell culture (SILAC)

[a]See sections V and X for references. Modified from reference 417 with permission.

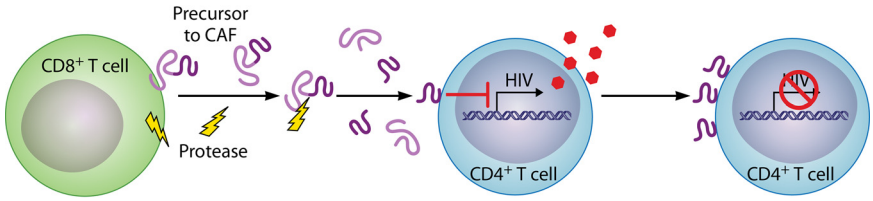


FIG 6 Cleavage of a CAF precursor by a protease may be needed for CAF activity. During production of the CD8+ T cell antiviral factor (CAF), it is proposed that noncytotoxic CD8+ T cells produce a serine protease that cleaves a precursor to CAF into an active moiety. This activated antiviral protein interacts with the HIV-infected CD4+ cell surface to induce an anti-HIV state; or, it could enter the cell to produce this response. If the CNAR/CAF-like activity is not blocked by a protease inhibitor (193), the CAF precursor could have already been cleaved into an active anti-HIV protein. (Modified from reference 192 with permission from Elsevier.)

the serine protease inhibitors, when added to infected CD4+ cells, directly blocked HIV replication (193). Several studies also indicated that when the serine protease inhibitors, such as leupeptin, were placed in a CNAR coculture, they also prevented this CD8+ T cell anti-HIV activity. However, in approximately 30% of cases, leupeptin showed no effect on CNAR/CAF. We suggest that the latter results could be explained by the fact that a cleavage of a CAF precursor secreted by CD8+ T cells is needed for its anti-HIV activity (Fig. 6). This observation therefore suggests that the active protein could have already been clipped by a CD8+ T cell serine protease during its production or soon after its release from the CD8+ cells. Then, neither CNAR nor CAF would be sensitive to the serine protease inhibitors. The identity of the specific CD8+ T cell protease has not been determined (192, 193), but it might be a granzyme (section IX.D).

In other strategies to identify CAF, many experiments were conducted with selected purified cytokines and antibodies to cytokines to evaluate anti-HIV activity (Table 5). Known amounts of purified cytokines with antiviral activity were used, and enzyme-linked immunosorbent assays (ELISAs) were performed to detect the level of potential anti-HIV cytokines in CAF-containing fluids. None of the cytokines had any identity with CAF (Table 5) (186). It is also conceivable that multiple factors may work together to inhibit HIV replication. This possibility was evaluated in cell culture with up to 16 different cytokines used in combination at clinical concentrations (Table 5). These experiments also gave negative results (L. Liu and J. A. Levy, unpublished data).

In the case of the β-chemokines that have been reported to show anti-HIV activity (194), the clinical importance of this effect is not conclusive (section IX.A). Many studies have shown that CAF activity observed with CD4+ lymphocytes and monocytes/macrophages is not related to β-chemokines (189, 195–197) and do not support the β-chemokines as major determinants of the clinical course in HIV infection. These cytokines may prove to be more involved with promoting viral spread, since they attract CD4+ cells to the site of HIV replication (198). Indeed, several studies confirm that β-chemokines can enhance HIV infection by X4-tropic isolates (199–201). This effect can be caused by the cross-linking of virus to the cell surface via CCL5 oligomers (200) and increased signal transduction (199). Continued efforts to identify CAF have included several biological, physiological, immunological, and chemical approaches. Thus far, no definitive answer is yet available (section IX).

(C) Summary

Whether CAF is one protein or a mixture of proteins is not clear. Its biologic and chemical properties suggest that it is one protein, unless multiple proteins with similar physical and chemical properties are involved. We have evaluated mixtures of several cytokines with potential anti-HIV activity and they do not show anti-HIV activity (Table 5). Moreover, the evaluation of CAF-containing fluids in comparison to fluids with no CAF activity has not indicated any levels of known CD8+ T cell secreted cytokines that could be CAF. For this reason, the research in our laboratory has been directed at

TABLE 5 Soluble factors lacking identity to the $CD8^+$ T cell antiviral factor

Protein name	Other name(s)	Receptor(s)	Anti-HIV	Reason for not being CAF	Reference(s)
IL-1[a]		IL-1R	−	No effect on HIV replication	186
IL-3[a]		CD123	−	No effect on HIV replication	186
IL-6[a]		CD126/CD130	−	No effect on HIV replication	186
IL-12[a]		CD212	−	No effect on HIV replication	186
CXCL10[a]	IP-10	CXCR3	−	No effect on HIV replication	431
Granzymes	Gzm	CD222, F2R, heparan sulfate	−	No effect on HIV replication	116, 327
IL-2, IL-15		CD25/CD122/CD132	−	Can enhance HIV replication	186
IL-4		CD124/CD132	−	Can enhance HIV replication	186
IL-5		IL-5R	−	Can enhance HIV replication	186
IL-7[a]		CD127/CD132	−	Can enhance HIV replication	186
IL-9		CD129/CD132	−	Can enhance HIV replication	186
G-CSF[b]	CSF3	CD114	−	Can enhance HIV replication	186
GM-CSF[a]	CSF2	CD116	−	Can enhance HIV replication	186
TNF-β	TNFSF1, LT-α	CD18	−	Can enhance HIV replication	186
IFN-γ		CD119/IFNGR2	−	Can enhance HIV replication	186
TNF-α[a]		CD120α or CD120β	+/−	Can enhance HIV replication at some concentrations	186
TGF-β[c]		TGFBR1, TGFBR2, or TGFBR3	+/−	Can enhance HIV replication at some concentrations	186
IL-8[a]	CXCL8	CD181/CD182	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	186
IL-10[a]	CSIF	CD210a/CD210b	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	186
IFN-α, IFN-β	Type 1 interferons	IFNAR1/IFNAR2	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity; no expression by $CD8^+$ T cells	186
CCL3	MIP-1α	CD191, CD195	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	41, 188, 195, 196, 284, 291, 293
CCL4	MIP-1β	CD195, CD198	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	41, 188, 195, 196, 284, 291, 293
CCL5	RANTES	CD191, CD193, CD195	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	41, 188, 195, 196, 284, 291, 293
IL-16	LCF	CD4	+	Can inhibit HIV replication, but blocking antibodies do not affect CAF activity	324
CCL22	MDC	CD194	+/−	Only truncated form has anti-HIV activity	198
CXCL12	SDF-1	CD184, ACKR3	+	Low/no expression by $CD8^+$ T cells	307
α-Defensins	DEFA	NA	+	Low/no expression by $CD8^+$ T cells	357
TOE1	FLJ13949	p53	+	Expressed by $CD8^+$ T cells and other cells	333
XCL1	Lymphotactin	XCR1	+	Expressed by $CD8^+$ T cells and other cells	198
IL-32	TAIF	?	+	Expressed by $CD8^+$ T cells and other cells	350
Peroxiredoxin	PRDX	NA[d]	+	Expressed by $CD8^+$ T cells and other cells	352
IL-13		CD124/CD213a	+	Effect on other cells (e.g., macrophages) than $CD4^+$ cells	432
Prothymosin-α	PTMA	?	+	Effect on other cells (e.g., macrophages) than $CD4^+$ cells	337, 340
IL-18	IGIF	CD218a/CD218b	+	Different mode of action than CAF	433, 434
Ribonucleases	RNases	NA	+	Different mode of action than CAF	11, 192
Antithrombin	AT III, Serpin C1	NA	+	Different mode of action than CAF	192

[a]These cytokines, as well as CCL2, CCL7, CCL8, CXCL5, IL-28, and sCD40L, were tested in combination; no effect on HIV-1_{SF33} replication was observed (L. Liu and J. A. Levy, unpublished data). GM-CSF, granulocyte-macrophage colony-stimulating factor.
[b]G-CSF, granulocyte colony-stimulating factor.
[c]TGF-β, transforming growth factor beta.
[d]NA, not applicable.

identifying CAF through proteomics and, more recently, molecular studies. As we note, CAF presents a challenge of a low-abundance protein, which joins the ongoing research by a large number of companies directed at identifying cancer biomarkers (section X).

Other soluble factors produced by $CD8^+$ T cells might have some anti-HIV activity (section IX) but they have specific characteristics that need to be appreciated. For example, if the β-chemokines are involved, their activity acts only on blocking entry of cells by R5-tropic viruses, which are generally present during an asymptomatic course

of infection and not during disease progression. Moreover, in cell culture, high levels of β-chemokines and a combination of the three chemokines are typically needed to suppress R5-tropic virus infection compared to what has been detected in culture fluids of CD8$^+$ T cells (or CD4$^+$ cells). However, if these chemokines (e.g., CCL5) are associated with proteoglycans, the amount needed may be less (202). This finding needs to be further evaluated.

Importantly, CAF activity is associated with a protein that blocks HIV transcription and affects all HIV isolates. Therefore, in the studies cited in this review, we expect the major mechanism for the noncytotoxic, cytokine-mediated anti-HIV response to involve the secretion of CAF. In our experience, CAF, although its identity is still unknown, has a much broader anti-HIV activity than any other potential cytokine that could be involved.

(VI) CNAR/CAF MODE OF ACTION

(A) Overview

The CD8$^+$ T cell noncytotoxic antiviral response (CNAR) and the CD8$^+$ T cell antiviral factor (CAF) suppress HIV replication and can inhibit the replication of other retroviruses, but not viruses from other families (section VIII). While the exact mechanism of action for CNAR or CAF remains to be defined, several studies have shown it is retrovirus restricted and involves a block of HIV transcription (sections II and V). Unlike β-chemokines that prevent HIV entry, the noncytotoxic CD8$^+$ T cell response inhibits HIV replication after the virus has infected the host target cells. Decreases in HIV replication and mRNA production are observed (44, 94, 191). The activity of CAF does not impact the processes involved in virus reverse transcription or integration of viral cDNA into the genome (44) and is not attributed to direct inactivation of the RT enzyme (190). The virus envelope is not affected and the infectivity of the HIV particles is also not reduced when exposed to CAF or CD8$^+$ T cells (190, 203).

In terms of the infected target cells, mitogen-induced proliferation of the CD4$^+$ cells is not substantially affected by exposure to CAF-containing supernatants or coculture with CD8$^+$ T cells (C. E. Mackewicz, unpublished data; M. G. Morvan, unpublished data) (section V.B). Furthermore, CD4$^+$ cell activation remains unchanged after an interaction with CNAR or CAF, as demonstrated by similar levels of mitogen-induced activation markers at the cell surface, such as CD25, CD38, CD69, and HLA-DR (C. E. Mackewicz and J. A. Levy, unpublished data). We have also found that antibodies to CD3, CD8, CD11a (LFA-1), CD15 (LFA-3), CD18, CD44, or DRGG2 (homing receptor) do not inhibit CNAR activity when added to cocultures of the CD8$^+$ T cells (J. A. Levy, unpublished data). These findings confirmed that this antiviral activity is not dependent on the engagement of any of those cell-surface receptors individually (section IV.F).

(B) No Effect on Early Steps in HIV Infection

As noted above, CNAR/CAF activity is not affected during the early steps in the HIV replication cycle, including virus attachment and entry, viral uncoating, reverse transcription, the formation and transport of the preintegration complex to the nucleus, and integration into the host genome. Moreover, coculturing infected CD4$^+$ cells with CD8$^+$ T cells does not substantially reduce the amounts of (i) early HIV cDNA, as detected by LTR U3-R, (ii) HIV *gag* cDNA in various fractions of cellular DNA, or (iii) HIV *gag* cDNA in the nucleus (190) (Fig. 7). Quantification of HIV-integrated proviral cDNA by a sensitive PCR assay has confirmed that coculture with CD8$^+$ T cells has no effect on the amount of virus cDNA reaching the integration stage, with no major difference observed compared to that in CD4$^+$ cells cultured alone. Thus, viral replication proceeds unaffected through proviral integration but is then interrupted by the CD8$^+$ T cell-mediated antiviral response. Only viral transcription and virus particle production are specifically reduced (44, 190, 203, 204).

(C) Major Effect on HIV Transcription

As demonstrated by Northern blot analysis of total RNA extracted from infected CD4$^+$ cells cocultured with CD8$^+$ T cells, the transcription of all subtypes of HIV RNA

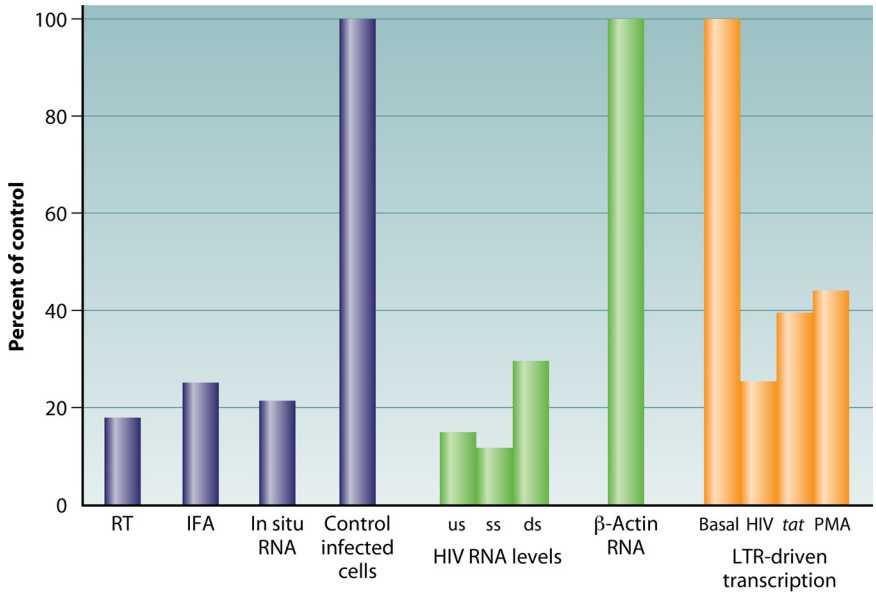


FIG 7 Effect of CNAR and CAF on parameters of HIV replication. The $CD8^+$ T cell noncytotoxic antiviral response blocks viral replication, as indicated by decreased RT activity, viral protein expression measured by immunofluorescent antibody (IFA) techniques, and *in situ* RNA production. This activity has no effect on the number of infected cells in the culture. The antiviral effect is observed as well by a reduction in unspliced (us), single-spliced (ss), and double-spliced (ds) HIV RNA levels compared to a normal expression of β-actin RNA. Finally, the suppressing effect of $CD8^+$ T cells or CAF does not affect the basal-level expression of HIV LTR-driven transcription but blocks induction of this transcription by HIV, simian virus 40 *tat* expression, or phorbol myristate acetate (PMA) using cells in which the HIV LTR has been linked to a reporter gene (section VI). (Modified from reference 417 with permission.)

(unspliced, single spliced, and double spliced) is found to be downregulated by CNAR compared to that in infected $CD4^+$ cells cultured alone (44) (Fig. 7). Since the transcription of housekeeping genes such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-2, or IL-2 receptor (IL-2R) was only slightly reduced in the presence of $CD8^+$ T cells (44), the CNAR/CAF antiviral activity appears to inhibit specifically HIV transcription (Appendix 3). These results were confirmed using quantitative reverse transcription-PCR (RT-PCR), a highly sensitive method to assess the effect of $CD8^+$ T cells on the kinetics of viral mRNA transcription in HIV-1-infected $CD4^+$ cells (190). The addition of $CD8^+$ T cells to infected $CD4^+$ cells drastically reduced HIV *tat* and *gag* mRNA levels as early as 3 h after infection, by 7- to 30-fold for *tat* and by 10- to 100-fold for *gag* (190). A recent study has further confirmed that $CD8^+$ T cells cocultured with infected $CD4^+$ cells suppress HIV replication at the transcriptional level (75). Taken together, these findings show that the $CD8^+$ T cell-mediated antiviral activity specifically inhibits HIV transcription, not global transcription, in infected target cells.

(D) Effect on Viral LTR

In further support of a specific and inhibitory effect of CNAR/CAF on HIV transcription, we and others have demonstrated that this antiviral activity occurring after HIV integration can block the LTR-dependent transcription of a reporter gene in primary $CD4^+$ cells (44, 204). In initial studies with SIV, both the Tat-mediated enhancement of LTR-dependent transcription and basal transcription via the LTR were suppressed by CNAR (204). Notably, the inhibition of transcription by coculture with $CD8^+$ T cells is specific to the HIV promoter, since transcription of a reporter gene via a cytomegalovirus (CMV) promoter is not reduced (204). Moreover, when studying the effect of $CD8^+$ T cell supernatants alone on the 1G5 T cell line, a stable Jurkat cell line containing the luciferase reporter gene under the control of the HIV-1_{SF2} LTR (205), CAF only affected Tat-mediated HIV transcription, not the basal LTR-dependent transcription (44).

CD8$^+$ T cells and their supernatants from SIV-infected NHP also inhibited SIV replication and LTR-dependent transcription, which was demonstrated in assays using either primary CD4$^+$ cells (204) or a B cell line (112) transfected with an LTR-chloramphenicol acetyltransferase (CAT) reporter construct. However, in the case of the B cell line, in contrast to observations with the 1G5 cell line (44), only basal LTR-dependent transcription was affected, not Tat-mediated enhancement of LTR-dependent transcription (112). In this study, the CD8$^+$ T cells from two different species of NHP, sooty mangabey and rhesus macaque, inhibited the CAT activity (112). However, Tat, introduced via a different LTR vector, reduced this effect. The studies also showed that NF-κB in the LTR was necessary for CAT expression and thus appeared to be affected by CNAR/CAF. However, whether CNAR/CAF suppression could have a direct effect on Tat or indirectly affect the transcription factors interacting with Tat (206) was not determined. Notably, an effect on Tat/transactivation-responsive region (TAR) was not supported by later studies using a Tat/TAR-deleted mutant that was susceptible to CNAR/CAF (207).

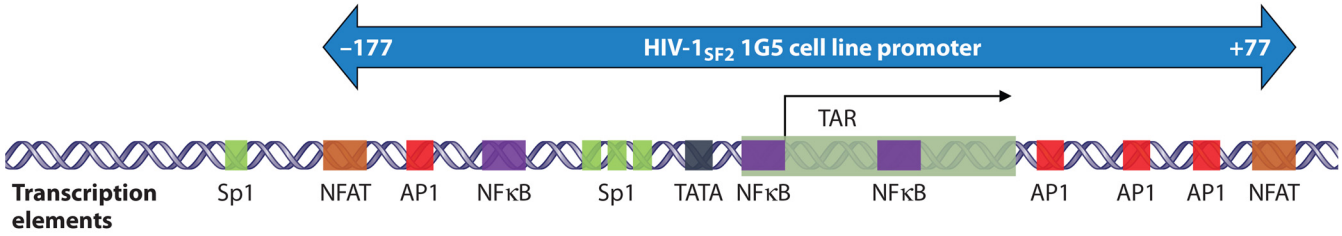
In further experiments, the expression of a CAT reporter gene under the control of the HIV LTR was reduced by 50% to 70% in Jurkat cells when supernatants from stimulated primary CD8$^+$ T cells or an immortalized CD8$^+$ T cell clone taken from HIV-infected individuals were used in the assays (208). This reduction was independent of LTR transcription being induced either by Tat or mitogenic activation via phorbol myristate acetate (PMA) and ionomycin. In addition, CD8$^+$ T cell supernatants also decreased transcription of the CAT reporter gene when it was under the control of a minimal promoter only containing two NF-κB elements (112). Moreover, the CD8$^+$ T cell supernatants were able to suppress transcription via the retrovirus promoters of HTLV-1 and Rous sarcoma virus (RSV), which also contain two NF-κB elements (208). Thus, all these findings suggested that NF-κB-related events can play an important role in CD8$^+$ T cell-mediated suppression. However, these LTR elements may not be necessary, since an SIV mutant virus lacking NF-κB and the Spl binding domain was also sensitive to CAF inhibition (209).

The activity of CD8$^+$ T cell line supernatants on transcription has also been studied using LTR promoters with various mutations, including the NF-κB, Sp-1, and nuclear factor of activated T cells (NFAT) elements (207, 210) (see below). The results varied depending on the construct used. Some studies, as noted above, showed that the NF-κB elements were necessary for induction of LTR-dependent transcription by mitogenic activation of the infected cells (112). Cultured CD8$^+$ T cell supernatants blocked this activity. Yet, as noted above with SIV (209), CD8$^+$ T cell-mediated inhibition was observed even in the absence of NF-κB elements (207). Notably, in these studies, Tat-mediated enhancement of LTR-dependent transcription was suppressed by CD8$^+$ T cell supernatants (210). Moreover, this inhibition was not observed when the NFAT element was mutated. Thus, the reduction in Tat-mediated transcription appeared to be dependent on NFAT activity. Yet, when NFAT binds to the IL-2 promoter and IL-2 is added to cultures of PBMC, the ability of CD8$^+$ T cells to suppress HIV replication increases (35, 153) (section IV.F).

In other studies using the 1G5 cell line, Tat-mediated transcription was still suppressed by CNAR and by supernatants from primary CD8$^+$ T cells with antiviral activity (44). The 1G5 cell line is derived from Jurkat cells transfected with the luciferase reporter gene under the control of an HIV promoter cloned from the −177 to +77 sequence of the HIV-1_{SF2} LTR (205). As such, the HIV-LTR in 1G5 lacks all the NFAT binding elements (as well as the 5′ AP-1, USF, and COUP elements). Therefore, in these studies, the suppression of Tat-induced transcription was not dependent on a functional NFAT site in the promoter.

(E) Studies with LTR Mutants

Importantly, in assessing the mode of action of CNAR/CAF, Bonneau et al. generated multiple infectious HIV mutants with defective major elements of the LTR (207). In some studies, a Tat/TAR-deleted virus was used. All those mutants were able to infect



Element	Sp1	NFAT		Sp1	TATA and downstream	TAR		NFκB		AP1
Role	Regulatory			Promoter		Tat binding		Enhancer		Regulatory
Needed for CAF effect	?	+	−	−	+?	+	−	+	−	−
References		Copeland, 1996	Mackewicz, 1995 Bonneau, 2008	Copeland, 1996 Locher, 2001 Bonneau, 2008	Shridhar, 2014	Chen, 1993 Mackewicz, 1995	Powell, 1993 Copeland, 1995 Bonneau, 2008	Chen, 1993 Copeland, 1995 Powell, 1993	Copeland, 1996 Locher, 2001 Bonneau, 2008	Mackewicz, 1995 Bonneau, 2008

FIG 8 Effect of CNAR/CAF on transcription elements of the HIV LTR. This schematic representation of the main transcription elements of the HIV LTR shows (i) the relative position of these elements in the HIV promoter; (ii) which transcription elements are present in the HIV-1_{SF2} promoter cloned and transduced into the 1G5 cell line used in many studies discussed in this review; and (iii) the potential role of these various transcription elements in CNAR/CAF activity (section VI). References are as follows: Copeland, 1996 (210); Mackewicz, 1995 (44); Bonneau, 2008 (207); Copeland, 1995 (208); Locher, 2001 (209); Shridhar, 2014 (211); Chen, 1993 (204); Powell, 1993 (112).

PHA-stimulated primary $CD4^+$ cells and were still susceptible to the $CD8^+$ T cell-mediated suppression by CNAR and CAF (207). These findings indicated that the following elements of the LTR do not appear involved in the transcriptional block (Fig. 8): NFAT, AP-1, IL-2 homology region, interferon-sensitive response element (ISRE), NF-κB, Sp-1, Tat, or TAR (207).

Other studies have suggested that the effects on the HIV LTR involved in virus suppression may depend on elements downstream of the CATA box (211). In this regard, we have shown that noncytotoxic $CD8^+$ T cells can reduce the amount of RNA polymerase II (RNAPII) on the HIV LTR in infected $CD4^+$ cells by 3-fold compared to that in infected $CD4^+$ cells cultured alone. Furthermore, the recruitment of RNAPII on the viral *gag* and *tat* sequences was reduced 4-fold (212). These observations support the other studies showing that CNAR/CAF blocks HIV replication at transcription. Moreover, CNAR/CAF may affect preinitiation complex assembly (212). These findings suggested an important role for $CD8^+$ T cell activity on the positive transcription elongation factor (P-TEFb) complex (cyclin T1, Cdk9, and hexim1) involved in cellular transcription. The apparent sensitivity of HIV-Tat to P-TEFb (213, 214) could explain the lack of effect of the noncytotoxic $CD8^+$ T cell response on other activities of normal cells, such as IL-2R expression and IL-2 production (190).

(F) Summary

The mechanism by which CNAR and CAF block retroviral replication through transcriptional inhibition of the LTR domain is complex and multifactorial (Fig. 8). The hijacking of an array of cellular transcription factors by HIV-1 may reflect the exquisite adaptability of retroviruses to replicate in diverse types of cells and, in the case of HIV-1, infect subpopulations of $CD4^+$ lymphocytes, macrophages, and monocytes. The different cell lines used to measure CNAR and CAF activity may also explain the diversity of the LTR responses described above. Cell lines and subpopulations of $CD4^+$ T lymphocytes have various degrees of cell surface receptor expression and kinase regulatory patterns that modulate transcription factors in a differential manner. Therefore, $CD4^+$ lymphocytes are guided by IL-2 toward the memory phenotype for adaptive immune responses. Also, CAF production appears to be IL-2 dependent (35) (section

TABLE 6 Comparison of various assays measuring anti-HIV CD8+ T cell activity[a]

Assay	HIV-infected CD4+ cells	CD8+/CD4+ cell ratio	CD8+ T cell stimulation	Assay duration (days)	Target cell killing?	CD8+ T cell function
Endogenous	Autologous	0.05:1 to 2:1		6	No	CNAR
Acute	Heterologous	0.05:1 to 2:1	3 days, PHA or resting	6	No	CNAR
Transwell	Autologous/ heterologous	1:1 to 4:1	3 days, PHA	6	No	CNAR
sCNAR	Autologous	Physiological	3 days, PHA	7	No	CNAR
VIA	Autologous	5:1 to 25:1	Various stimuli or resting	Up to 14	Possible	CNAR and CTL
CAF	Heterologous	NA[b]	3 days, anti-CD3 beads; for production up to 2 wks	4–6	No	CAF secretion

[a]See section VII for details.
[b]NA, not applicable.

IV.F). Thus, CNAR activity may help retain CD4+ cell immunological memory function by blocking HIV-1 transcription without killing the cells.

In addition, regulation of HIV-1 in the context of the architecture of the lymphatic tissues must be considered. The close proximity of the CD8+ and CD4+ cells surrounding the germinal center may provide greater control of HIV-1 transcription than found in the *in vitro* assays used to measure CNAR and CAF activity. Furthermore, the potent innate immune responses of CNAR and CAF activity observed in immunized (215) and HIV-2-infected NHP such as baboons (47, 50) suggest that this animal model provides an excellent resource for *in vivo* study of noncytotoxic mechanisms of CD8+ cell control. Further studies in well-controlled and well-characterized cell lines and animal models are required to better define the activity of specific transcription factors and their effect on the LTR by CNAR and CAF.

(VII) CNAR/CAF ASSAYS

(A) CNAR Assay

CNAR against HIV was initially observed using an endogenous virus assay. At the time, it was known that HIV could be readily isolated from the PBMC of individuals with symptoms of AIDS. In contrast, when the PBMC came from individuals who were seropositive but asymptomatic, the virus was difficult to isolate. Then, as noted above, when CD8+ cells were removed from the PBMC cultures, HIV replication was detected (2). For CNAR, the CD8+ T cell antiviral activity is typically observed by the cocultivation of CD8+ cells at various ratios with HIV-infected CD4+ cells. Initially, the CD8+ cells were isolated by immunologic panning in a petri dish (2, 25). Presently, CD8 antibodies bound to immunomagnetic beads are used.

Three distinct assays were developed by our group to measure CNAR activity (Table 6): (i) the endogenous virus infection assay (2); (ii) the acute virus infection assay (26); and (iii) the screening assay for detection of CNAR activity (sCNAR or Rapid CNAR) (28). Each of these assays contributes in different ways to the understanding of how CNAR controls HIV replication and spread. In addition, others have adapted CNAR assays into protocols called the virus inhibition assay (VIA) (151, 216–222) or HIV suppression assay (223).

(i) Endogenous virus infection assay. In this assay, endogenously infected CD4+ cells isolated from the PBMC of an HIV-seropositive subject are cocultured for up to 6 days with autologous CD8+ T cells at CD8+/CD4+ cell ratios ranging from 0.05:1 to 2:1. The CD4+ cells are cultured alone as a control, and virus replication is measured via the RT assay (48) or a p24 ELISA of the culture supernatants. CNAR activity is determined by how much endogenous HIV replication is suppressed in the CD8+/CD4+ cell cocultures compared to virus replication in the infected CD4+ cells cultured alone (2). This assay was established early in the HIV epidemic when numerous HIV-infected subjects progressed to AIDS. Notably, these subjects often had low CD4+ cell counts, making it difficult to isolate enough cells to assess CNAR activity through an endogenous assay.

(ii) Acute virus infection assay. To circumvent the limitation above, the acute virus infection assay was developed in which CD4+ cells are isolated from the PBMC of

uninfected subjects, PHA stimulated for 3 days, and infected with an X4-tropic HIV-1 isolate (e.g., SF33) (83). It is optimal to use a β-chemokine-resistant virus to avoid any possible virus suppression mediated by CCL3, CCL4, and CCL5 secreted by $CD8^+$ T cells (see below; section IX.A). Concurrently, $CD8^+$ T cells are isolated from the PBMC of an HIV-infected individual that have been stimulated with PHA for 3 days. The acutely HIV-infected $CD4^+$ cells are cocultured with these $CD8^+$ cells at $CD8^+/CD4^+$ cell ratios ranging from 0.05:1 to 2:1. Virus replication is measured 4 to 6 days postinfection as noted above, and CNAR activity is determined by the extent of virus suppression in the $CD4^+/CD8^+$ cell cocultures compared to that in the HIV-infected $CD4^+$ cells cultured alone.

The acute virus infection assay is more efficient in time and breadth than the endogenous infection assay to study CNAR. Uninfected donor $CD4^+$ cells, as well as a variety of viral isolates with various pathogenic and biological properties, are more readily available. Early studies using the acute infection assay showed that $CD8^+$ T cells from HIV-infected subjects were able to suppress the replication of all HIV-1, HIV-2, and SIV isolates tested (section III).

(iii) sCNAR/rapid CNAR assay. A screening CNAR (sCNAR), or rapid CNAR assay, was developed in order to have a rapid assay to study CNAR activity in HIV-infected subjects, particularly from underserved communities (28). In this assay, PBMC from an HIV-infected subject are PHA stimulated for 3 days and acutely infected with a known amount of a β-chemokine-resistant HIV isolate. Concurrently, $CD8^+$ cell-depleted PBMC (mostly $CD4^+$ cells) from the same subject are PHA stimulated, infected with the same virus, and cultured as a control. After 7 days, virus replication is measured in culture supernatants as noted above. The extent of HIV suppression by $CD8^+$ T cells is determined by comparing HIV replication in the total PBMC to that in the $CD8^+$ cell-depleted PBMC. The rapid CNAR assay is a time- and cost-effective alternative to the acute virus infection assay.

(iv) Virus inhibition assay. The VIA and HIV suppression assays were designed by other groups to measure $CD8^+$ T cell-mediated anti-HIV responses *in vitro*. They resemble the rapid CNAR assay described above (28), in which the objective is to have an effective screening assay. These tests generally use PBMC to isolate and coculture acutely HIV-infected $CD4^+$ cells, usually with autologous $CD8^+$ T cells. The $CD8^+$ T cells can be stimulated (151, 217, 221) or resting (216, 218–220, 222, 223). As with the other assays described above, anti-HIV activity is measured in the culture supernatants by RT or p24 antigen assays, and the extent of HIV replication is compared in the exogenously infected PBMC or the $CD4^+/CD8^+$ cocultures to that found in the infected $CD4^+$ cells cultured alone.

In contrast to the VIA, the acute virus infection assay, described above, is usually conducted with $CD8^+$ T cells and $CD4^+$ cells from different individuals. A heterologous setting excludes any possible HLA-restricted $CD8^+$ T cell-mediated cytotoxicity that could be observed with autologous, acutely HIV-infected target $CD4^+$ cells. As such, under certain conditions, the VIA can be used to quantify noncytotoxic $CD8^+$ T cell-mediated viral suppression. Importantly, in some VIA protocols, more $CD8^+$ T cells are cocultured with the infected $CD4^+$ cells, with $CD8^+/CD4^+$ cell ratios ranging from 5:1 (221, 222) to 25:1 (151). $CD8^+$ T cells may also be cocultured for a longer period of time (13 to 14 days) (216, 218, 221, 223), during which they could acquire the cellular machinery required to kill target cells (151).

Because of some differences between the VIA and the CNAR assays, especially the high number of $CD8^+$ cells used and the extended length of each assay (e.g., up to 2 weeks), both CTL and CNAR activity could be involved in some VIAs measuring $CD8^+$ T cell suppression of HIV replication (Table 6) (5). This possibility is particularly important to consider since the VIA has been used by many researchers to evaluate $CD8^+$ T cell function in the context of vaccine studies, both in humans and in NHP. Because the VIA cannot discriminate CTL from CNAR activity, the possible contribution from each mechanism to viral suppression for various vaccine approaches must be

TABLE 7 CD8+ T cell noncytotoxic antiviral response in virus infections besides HIV[a]

Lentiviruses	Other retroviruses	Herpesviruses	Other families
Simian immunodeficiency (SIV)	Human T lymphotropic	Cytomegalovirus	Hepatitis B
Feline immunodeficiency (FIV)	Rous sarcoma	Epstein-Barr	Hepatitis C
		Herpes simplex	Influenza

[a]See section VIII for details.

considered. Also, since the acute virus infection assay uses stimulated CD8+ T cells, the full HIV-suppressing activity of these cells can be measured, which is often not achieved with resting CD8+ cells. Finally, acute infection of CD4+ cells from an uninfected individual circumvents the variability caused by superinfection of autologous CD4+ cells from HIV-infected subjects. The CNAR assays also facilitate the consistent use of various HIV isolates and lab-adapted viral strains as well as CD4+ cell sources.

(B) CAF Assays

This procedure measures the antiviral activity of the soluble anti-HIV factor produced by CD8+ cells (i.e., CAF) and can be utilized to evaluate other potential anti-HIV proteins (186). The assay is more quantitative and convenient than the initial transwell culture technique (32). It involves magnetic bead isolation and 3-day anti-CD3 bead stimulation of CD8+ cells obtained from the PBMC of HIV-infected subjects. These cells are then cultured for 2 weeks, with culture medium changes, filtering, and storage (at −80°C) of the spent culture supernatants every 2 to 3 days. Subsequently, these fluids (usually at a 1:2 dilution) are added to HIV-1 acutely infected CD4+ cells (section VII.A.ii). The extent of HIV replication in the fluid-treated CD4+ cells is measured by the RT or p24 antigen assay. The extent of HIV production is determined by comparing the HIV levels in the fluid-treated CD4+ cells with that in control infected CD4+ cells grown alone. Generally, stimulation of CD8+ T cells (preferably with anti-CD3 beads) is needed for CAF production. Unstimulated CD8+ T cells do not usually show an antiviral effect when cultured in a transwell assay (C. E. Mackewicz C, unpublished data; M. G. Morvan, unpublished data).

(VIII) CD8+ T CELL NONCYTOTOXIC CONTROL OF OTHER VIRUSES

As reviewed in this article, CNAR with CAF activity was first described as an immune response in HIV infection. Then this activity was observed with other retroviruses in humans (e.g., HTLV), NHP (e.g., SIV), and cats (e.g., feline immunodeficiency virus [FIV]). Notably, a CD8+ T cell noncytotoxic antiviral immune response, differing from the CNAR with HIV, has also been recognized in infections caused by other virus families (Table 7). In these cases, CAF is most likely not the soluble secreted antiviral protein involved.

(A) Lentiviruses

(i) SIV. The NHP AIDS virus, SIV, is most similar genetically to HIV-1 and HIV-2. Its infection has been identified in at least 45 species of NHP, causing nonpathogenic infection in natural host species and eliciting pathogenic infection (i.e., simian AIDS) in some NHP, such as rhesus macaques (224). Also, SIV infection in NHP exhibits pathology comparable to that from HIV infection in humans, including CD4+ T cell depletion, immunodeficiency onset, and a beneficial response to ART (225).

Several *in vivo* SIV studies in NHP detail the role for CD8+ T cells in a noncytotoxic antiviral response (i.e., CNAR). In these studies, previously identified soluble anti-HIV factors such as IL-16 (226) and β-chemokines (227, 228) produced by the immune system were considered involved but did not always correlate with SIV controls (226). In longitudinal studies of SIV-infected cynomolgus macaques, a soluble CD8+ cell antiviral factor was detected within the first week after viral infection, and this increase in the factor activity correlated with low plasma viral load over 15 months. The antiviral effect was neither cytotoxic nor MHC restricted (226).

Other studies, involving SIV-challenged macaques followed by $CD8^+$ cell depletion, further supported the noncytotoxic antiviral function of $CD8^+$ T cells. When the $CD8^+$ cells were depleted, both at early (day 57 post-SIV infection) and late (day 177 post-SIV infection) times, the infected $CD4^+$ cells survived and released virus from a latent state. This finding indicated the presence of $CD8^+$ cells that did not affect the life span of SIV-infected $CD4^+$ cells (section III.C). Thus, virus control was not due to CTL activity but, rather, to CNAR (60). Furthermore, *in vivo* studies of SIV-challenged NHP, such as African green monkeys, sooty mangabeys, baboons, and HIV-challenged chimpanzees, can involve soluble $CD8^+$ cell antiviral factors such as IL-16 (229, 230), the β-chemokines (231), and a CAF-like cytokine (52). SIV is sensitive to CAF (209).

(ii) FIV. Although FIV is genetically and antigenically distinct from HIV, these cat viruses possess structural, pathological, immunologic, and physiologic similarities to HIV (232, 233). In particular, during the acute and asymptomatic stage of FIV infection, noncytotoxic $CD8^+$ cells produce a soluble antiviral factor that suppresses virus replication independent of MHC restriction (234–238). Moreover, when either autologous or heterologous T cells or feline skin fibroblasts were infected with FIV isolates and cocultured with autologous or heterologous $CD8^+$ cells, suppression of FIV replication by the $CD8^+$ cells was observed (239).

The activity of a soluble anti-FIV factor was also detected when FIV-infected target cells were cultured with the supernatants of $CD8^+$ T cells from HIV-infected individuals (235, 237). Moreover, *ex vivo* feline studies, similar to HIV studies, demonstrated that a $CD8^+$ cell anti-FIV soluble factor suppressed FIV in a dose-dependent manner (238). The soluble anti-FIV factor(s) was distinct from IFNs and chemokines (235, 240). During FIV pathogenesis and progression, the production of this feline $CD8^+$ cell antiviral factor was observed as early as 1 week postinfection in blood, spleen, and lymph nodes. This response was sustained for several months to years in peripheral and mesenteric lymph nodes in animals that remained clinically asymptomatic (237). Finally, the immunization of cats with a whole inactivated FIV vaccine led to strong noncytotoxic anti-FIV activity of $CD8^+$ cells from the blood and lymph nodes. This response was mediated by a soluble factor, as demonstrated with a transwell device (241). Therefore, the cat lentivirus FIV that causes immunodeficiency in its host also induces a $CD8^+$ cell noncytotoxic response involving a soluble anti-FIV factor similar to CAF. Moreover, FIV is sensitive to human CAF in our CAF assay (J. K. Yamamoto and J. A. Levy, unpublished data).

(iii) Other lentiviruses. Other less-studied lentiviruses that induce immunodeficiency have been described in cattle (e.g., bovine immunodeficiency virus [BIV]) and ungulates, such as sheep (e.g., visna-maedi virus [VMV]), goats (e.g., caprine arthritis encephalitis [CAEV]), and horses (e.g., equine infectious anemia [EIA]). Early *in vivo* $CD8^+$ cell depletion studies in sheep demonstrated that over the course of 10 days, VMV replication was comparable in $CD8^+$ cell-depleted sheep and non-$CD8^+$ cell-depleted sheep (242). Thus, in this animal model, $CD8^+$ cell control of VMV was not observed. Limited studies have been conducted to find a CNAR-like response in other lentivirus infections.

(B) Other Retrovirus Infections

Similar to HIV, HTLV-1 is a retrovirus in the subfamily of *Orthoretrovirinae*. It infects mainly $CD4^+$ cells through a receptor complex consisting of glucose transporter 1, neuropilin-1, and heparan sulfate proteoglycans (243). Once inside the host cell, the virus encodes structural proteins, such as Gag and Env, viral enzymes, and the regulatory proteins Tax and Rex (244).

An association of virus-specific $CD8^+$ T cell cytotoxic responses with a decreased HTLV proviral load and an asymptomatic infection has been observed (245–247). However, in some patients, the $CD8^+$ CTLs are linked with the development of neurologic diseases, particularly myelopathy or tropical spastic paraparesis (248). A $CD8^+$ T cell noncytotoxic response controlling HTLV infection has also been reported (249). Using $CD8^+$ cell depletion studies with cultured PBMC from HTLV-infected individuals,

an increased level of HTLV-I p19 antigen expression in peripheral white cells was noted. Reconstitution of the autologous CD8$^+$ cells or heterologous CD8$^+$ cells resulted in dose-dependent viral suppression without target cell killing (249). Furthermore, HTLV inhibition by a soluble factor was observed when autologous CD8$^+$ cells and HTLV-infected CD8$^+$ cell-depleted PBMC were separated by a semipermeable membrane (249). Similar to that in HIV studies, the viral suppression by a soluble factor was less efficient than that seen with cell-to-cell contact cultures (249).

In an attempt to identify the CD8$^+$ cell anti-HTLV soluble factor(s), cultures of CD8$^+$ cells from uninfected human donors stimulated with LPS and thymosin α1 showed production of a 10-kDa to 30-kDa-sized protein(s) that suppressed HTLV-1 replication in uninfected human donor PBMC (181). To further identify the anti-HTLV-1 protein(s), the CD8$^+$ cells were evaluated by DNA microarray for 440 genes involved in autoimmunity and inflammatory immune responses. In comparison to untreated CD8$^+$ cells, the LPS- and thymosin α1-treated CD8$^+$ T cells differentially upregulated certain chemokines (CCL22 [MDC], CCL3 [MIP1α], CCL4 [MIP1β], and interleukins [IL-1β and IL-8]) (181). The specific role of these proteins in HTLV inhibition has not been reported. The action of the soluble factor on HTLV transcription has not been described, but it seems to resemble CAF (181, 249).

As discussed in this review (section VI), CNAR can be studied using LTR constructs from certain retroviruses, such as HIV-1, HTLV-1, and the RSV of chickens. Copeland et al. (208) developed gene constructs of the HIV-1 and HTLV-1 LTRs fused to the CAT reporter gene. To enhance the efficiency of viral transcription, the constructs were each cotransfected with their respective regulatory proteins (e.g., Tat and Tax) into human Jurkat T cell lines. Subsequently, the Jurkat T cell lines were stimulated with PMA and calcium ionophore and treated with the supernatants obtained from the primary CD8$^+$ cells or CD8$^+$ cell clones of asymptomatic HIV-infected subjects. Treatment of these Jurkat cells with the culture fluids resulted in substantial reduction in HIV-1 LTR, HTLV-1 LTR, and RSV LTR activity (208). The activity against the avian retrovirus, RSV, indicated the broad antiviral effect of the CD8$^+$ T cell fluids on retroviruses. Similar observations were made with LTR constructs from HIV-1, HIV-2, and SIV (112) (section VI). A CAF-like protein seems to be involved.

(C) CNAR with Other Virus Families

Whereas CNAR was first described in studies of HIV, a similar noncytotoxic response is present in other virus infections, although CAF is not known to be involved (Table 7).

(i) Hepatitis B virus. Extensive studies have identified the role of IFN-γ in its control of viral infections, such as hepatitis B virus (HBV) and hepatitis C virus (HCV). Early studies of acute HBV infection in transgenic mice and chimpanzees suggested that antigen-specific CD8$^+$ T cells suppress virus replication in infected hepatocytes via a noncytotoxic mechanism (250–252). Subsequently, *in vitro* studies using matched HLA-A2$^+$ human CD8$^+$ T cell lines or clones, and an HBV-infected human hepatoma cell line, demonstrated similar antiviral activity with minimal cell killing via cell-to-cell contact or when the cells were separated by a semipermeable membrane (253). These effector CD8$^+$ T cells produced IFN-γ and TNF-α that suppressed HBV replication in the HBV-infected cell line without cytotoxicity. Moreover, IFN-γ and TNF-α treatment of the HBV-producing cell line resulted in a noncytotoxic CD8$^+$ cell antiviral response. Blockade with IFN-γ and TNF-α antibodies abrogated this antiviral activity (253). Thus, both of these CD8$^+$ cell cytokines help to control HBV infection.

(ii) Hepatitis C virus. Similar to HBV, HCV infects hepatocytes to induce a noncytolytic immune antiviral response that is both cell and cytokine mediated. Researchers developed a coculture assay that assessed CD8$^+$ T cell antiviral responses via cell-to-cell contact and in a transwell assay with a semipermeable membrane (254). Importantly, 20% of the anti-HCV activity observed was linked to a CD8$^+$ cell cytokine-mediated noncytotoxic response. Moreover, supernatants from CD8$^+$ T cell cultures containing IFN-γ inhibited HCV replication in the replicon system (255). This finding, supported with antibody blocking studies, showed that a CD8$^+$ T cell noncytotoxic

antiviral response was partially due to IFN-γ in addition to FasL-mediated cytotoxicity (254, 256). Subsequent *in vitro* studies utilizing a matched HLA-A2 epitope of the replicon system and an HCV-specific CD8$^+$ T cell clone demonstrated both cytotoxic and noncytotoxic anti-HCV responses that reflected high and low CD8$^+$ cell inputs, respectively (256). Concurrent *ex vivo* studies showed that CD8$^+$ cell depletion of HCV-infected PBMC resulted in increased HCV RNA replication. Adding CD8$^+$ T cells back to the depleted PBMC again inhibited HCV replication (256). Therefore, CD8$^+$ T cell control of HCV replication in these cell culture models is due to both cytotoxic and noncytotoxic mechanisms.

(iii) Hepatitis E virus. Hepatitis E virus (HEV) typically causes a self-limiting asymptomatic infection (257). However, in some immunocompromised individuals (e.g., with HIV infection), a chronic HEV infection can develop, with neurologic disorders and hepatic failure (258). Whereas HEV-specific T cells have been noted (259), cytokines produced by CD8$^+$ T cells, particularly IFN-γ and TNF-α, can also play a role in controlling HEV replication via a noncytotoxic CD8$^+$ T cell antiviral response (260, 261).

(iv) Epstein-Barr virus. Other studies have identified a noncytotoxic function of Epstein-Barr virus (EBV)-specific CD8$^+$ cells when cocultured with autologous EBV-transformed B cells. Notably, the former cells produce IFN-γ at concentrations that inhibit EBV-transformed B cell outgrowth without cytotoxicity. This inhibitory activity can be neutralized with IFN-γ monoclonal antibodies (262).

(v) Herpes simplex virus. CD8$^+$ T cells mediate an immune response to maintain herpes simplex virus 1 (HSV-1) in latency and control HSV-1 reactivation (263, 264). Specifically, *ex vivo* mouse studies of dissociated, latently HSV-1-infected trigeminal ganglions (TG) have demonstrated that depletion of CD8$^+$ T cells or IFN-γ neutralization results in enhanced HSV-1 reactivation in TG cultures without neuronal damage and death (265). Further studies have revealed that in mice and humans, the lytic granules of CD8$^+$ T cells can reduce latent HSV-1 reactivation through a nonlytic mechanism involving granzyme B (GzmB) (266). The GzmB from CD8$^+$ T cells directly cleaves IPC4, a viral protein essential for early and late stage viral transcription (267). Therefore, HSV infection can also be controlled by two different mechanisms involving IFN-γ and granzymes, without cytotoxicity.

(vi) Cytomegalovirus. Human CMV (HCMV) is another herpesvirus that is inhibited by a CD8$^+$ cell noncytotoxic response. The CD8$^+$ T cells exhibit cytotoxic or noncytotoxic functions that eliminate virus-infected cells or maintain viral latency, respectively. In terms of CNAR activity, CD8$^+$ cells, during mouse CMV infection, migrate to the lungs and produce IFN-γ that blocks CMV replication without killing the infected cells (268). Other studies have demonstrated that granzyme M (GzmM) inhibits HCMV replication in infected human cells via an NK or CD8$^+$ cell noncytotoxic mechanism (269). In particular, GzmM interacts with the HCMV proteome and efficiently cleaves the viral tegument protein phosphoprotein 71 (pp71) through a CD8$^+$ cell noncytotoxic antiviral response that does not kill the infected cells.

(vii) Influenza A virus. Influenza A virus (IAV) infects airway and alveolar epithelial cells of several animal species (270, 271). During virus replication in humans, the viral nucleoprotein (NP) associates with viral RNAs (vRNA) to form a viral nucleoprotein (vRNP) complex and combines with the viral RNA polymerase, resulting in virus production (272).

Studies have demonstrated that the host cell factors importin α/β dimer and the importin β homolog receptor, by binding to the subunits of the RNA polymerase or NP, initiate virus replication (273–275). The proteolytic activity of granzyme K (GzmK) dissociates these two transporter proteins, disrupting the transport of viral NP to the nucleus. The result is suppression of IAV replication via GzmK without cell death (276).

(IX) ANTIVIRAL PROTEINS THAT ARE NOT CAF

CAF is a soluble factor secreted by CD8$^+$ T cells that suppresses HIV transcription, but its protein structure remains to be identified. Several other cytokines that have

been found to inhibit HIV replication include IFN-α and -β (277) but not -γ (9, 278) and not IL-4, IL-6, TNF-α, or -β (279, 280). However, of those cytokines, only IFN-γ and TNF-α are produced by CD8$^+$ T cells. None are associated with the noncytotoxic anti-HIV activity mediated by CD8$^+$ T cells from HIV-infected individuals (186), including some of these cytokines in combination (Table 5). Notably, not observed with CAF, the interferons induce the 2′,5′-oligoadenylate synthetase/RNase L pathway to inhibit virus replication in CD4$^+$ cells (186). In the efforts to define the nature of CAF, several additional CD8$^+$ cell soluble factors with anti-HIV activity were discovered. These proteins that lack an identity to CAF are described below and summarized in Table 5 (section V.C).

(A) β-Chemokines

Chemokines (chemotactic cytokines), are small secreted proteins that bind to their cell surface chemokine receptor, which induces cell chemotaxis. This response defines the movement of cells toward chemical gradients of soluble molecules. The β-chemokines, characterized by two adjacent cysteines on their N terminus (CC chemokine ligands [CCL]), have been shown to have an anti-HIV effect. They were identified in the search for CAF by high-performance liquid chromatography (HPLC) using supernatants from cultured HTLV-transformed CD8$^+$ T cell lines (194). The β-chemokines, found in secretory granules, block viral attachment and entry by binding to the chemokine receptor CCR5 (281, 282), which is also an HIV coreceptor. This activity was primarily linked to the NSI viruses that use CCR5 for entry, called R5-tropic viruses (section III.E.ii). These cellular proteins do not inhibit the replication of HIV isolates that enter the cell via another HIV coreceptor, CXCR4 (X4-tropic viruses) (189, 196, 283). However, in some cases, these cytokines can enhance X4-tropic virus replication (199–201). The virus inhibition is mediated by the synergistic effect of the three β-chemokines: MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) (at 500-pg to 500-ng concentrations) (194). Indeed, only when a combination of antibodies to the three β-chemokines is used is the previously observed viral inhibition reversed (283, 284). Notably as well, CCL5 activity in monocytes/macrophages is optimal when the chemokine is in complex with proteoglycans (202).

Several studies have confirmed and expanded on the role of β-chemokines and their chemokine receptors in HIV infection (285). They have also characterized additional possible virus entry coreceptors that bind other chemokines such as stromal cell-derived factor 1 (SDF-1) (see below). In this regard, a genetic mutation corresponding to a 32-bp deletion in CCR5 (CCR5Δ32) protects individuals who carry it from R5-tropic virus infection. Cells expressing CCR5 receptors with this mutation are resistant to infection by R5-tropic viruses (286, 287). This genetic advantage is found in approximately 1% of the Caucasian population (288). In some cases, this CCR5 mutation has also been associated with an inhibitory effect on X4-tropic virus replication (289, 290).

The antiviral effect of the β-chemokines is unrelated to the noncytotoxic anti-HIV activity mediated by CD8$^+$ T cells via CAF. When using a combination of CCL3-, CCL4-, and CCL5-blocking antibodies with culture supernatants from HIV LTS CD8$^+$ T cells, the CAF-mediated virus suppression was not eliminated (284, 291). Also, separate studies have confirmed that antibodies blocking these β-chemokines do not neutralize viral suppression mediated by the noncytotoxic CD8$^+$ T cell anti-HIV activity either in HIV-infected CD4$^+$ cells (188, 196, 291–293) or in macrophages (41, 195). In addition, the usual chemokine levels in the CD8$^+$ T cell CAF-containing culture supernatants (0.1 to 4 ng/ml) do not correlate with the levels needed for virus suppression (see above) (284). Importantly, suppression by CAF is also observed with viruses not susceptible to β-chemokine inhibition, such as the X4-tropic (SI) and dual-tropic HIV isolates (188, 294) (section III.E.ii).

The clinical relevance of β-chemokines in long-term nonprogression remains unclear. Similar serum levels of β-chemokines are observed in HIV-infected progressors and NP (295). Moreover, high levels of β-chemokines in supernatants from CD8$^+$ T cells from HIV-infected subjects sometimes do not correlate with the asymptomatic clinical state in these subjects (296, 297), nor do plasma β-chemokine levels always

correlate with lower viral loads (298). To the contrary, high expression of β-chemokines by $CD8^+$ T cells has been associated, at times, with disease progression and higher viral loads (299), perhaps linked with chronic T cell activation. Furthermore, $CD8^+$ T cell clones obtained from long-term-asymptomatic HIV-infected subjects produce slightly higher levels of β-chemokines than those from uninfected subjects and progressors, but the amount does not correlate with strong viral suppression (197). Moreover, in contrast to CAF, the β-chemokines affect only HIV entry into cells; they do not affect HIV LTR-driven transcription (284) (section VI). This observation has been further confirmed by other studies in which suppression of HIV-1 LTR transcription by $CD8^+$ T cell culture supernatants was not dependent on β-chemokines levels (300), and the addition of recombinant β-chemokines did not prevent the transcription of HIV-1 LTR genes (301).

Very puzzling is the fact that β-chemokines are induced when $CD4^+$ cells are infected with R5-tropic viruses, and yet virus replication is not prevented in these cells. Neutralizing antibodies to β-chemokines cause low-level virus production to occur in the cultured $CD4^+$ cells, indicating that endogenous β-chemokines can affect HIV replication *in vitro* (189, 302). In contrast, infection with X4-tropic viruses decreases production of β-chemokines by $CD4^+$ cells (189, 294, 302). There is also no consistent correlation between β-chemokine production by $CD4^+$ cells and the resulting levels of virus replication in these cells (303). In fact, the β-chemokines could play a more important role in recruiting $CD4^+$ cells to the site of HIV infection (303), thereby enhancing HIV spread on transmission.

Macrophage-derived chemokine (MDC or CCL22) is another β-chemokine identified in culture fluids from an HTLV-transformed $CD8^+$ T cell line. It was reported to have anti-HIV activity for both R5-tropic and X4-tropic viruses (304). CCL22 is only known to bind to the chemokine CCR4, not any of the HIV coreceptors. Therefore, its antiviral activity could not involve a virus entry blockade, and it was hypothesized that the activity could be mediated by downstream CCR4 signaling (305). In early studies, we were not able to show this anti-HIV activity with CCL22 (198). The reason for that finding was explained by a subsequent study showing that a truncated form of CCL22 produced by the cell line, but not the full natural sequence, was responsible for anti-HIV activity (306). CCL22 is also not found routinely in amounts high enough in $CD8^+$ T cell fluids to inhibit HIV replication (294) (C. E. Mackewicz, unpublished data).

Therefore, the evidence is not conclusive as to whether β-chemokines influence disease progression. At best, the β-chemokines secreted by $CD8^+$ T cells only show antiviral activity against HIV isolates with a CCR5 tropism. In contrast, CAF explains the noncytotoxic anti-HIV activity of $CD8^+$ T cell culture supernatants from LTS against all HIV biotypes (R5- or X4-tropic) or dual-tropic viruses.

(B) α-Chemokines

Another group of chemokines is characterized by the presence of a C-X-C motif. In this group, SDF-1, or CXCL12, is an α-chemokine whose mRNA transcripts were found to be expressed, although at very low levels, in $CD8^+$ T cells with anti-HIV activity (307). CXCL12 has two receptors, CXCR4 (308) and the atypical chemokine receptor 3 (ACKR3; formerly CXCR7) (309). Like the β-chemokines with CCR5, CXCL12 can block the entry of some X4-tropic HIV isolates into the cell by interfering with CXCR4. This finding was demonstrated using a cell line transfected with CD4 and CXCR4 (310). However, some X4-tropic HIV isolates are not sensitive to CXCL12, even when used in combination with the β-chemokine CCL5, particularly in the case of dual-tropic isolates (311). For example, the novel recombinant SDF-1/54, a mutant form of CXCL12, blocks virus entry via CXCR4 despite reduced chemotactic function (312). In addition, a recessive mutation, SDF-1-3′A, located in an untranslated region of the gene coding for CXCL12, can confer resistance to HIV infection as shown by genetic studies (313), although the mechanism remains to be defined. The role of CXCL12 in suppressing HIV replication is independent from the $CD8^+$ T cell CAF-mediated suppression of $HIV\text{-}1_{LAI}$ (314).

The clinical benefits of CXCL12 in HIV infection is unclear. Even if it blocks the entry of some X4-tropic viruses, it has also been shown to increase the infectivity of R5-tropic viruses by stimulating proviral gene expression (315). Moreover, while more CXCL12 is detected in the PBMC of HIV-infected individuals, particularly asymptomatic carriers, higher levels of CXCL12 transcripts after PHA stimulation do not correlate with a better CD8$^+$ T cell antiviral activity *in vitro* (314). Finally, as mentioned above, CD8$^+$ T cells express very little CXCL12, and there is no correlation between CXCL12 expression by CD8$^+$ T cells and the ability of these cells to suppress virus replication (307). Thus, CXCL12 is not responsible for the CAF-mediated activity.

(C) Interleukin-16

CD8$^+$ T cell clones from LTNP that produce β-chemokines also secrete high levels of IL-16, a monomeric 13.5-kDa polypeptide previously known as lymphocyte chemoattractant factor (LCF) (197). The antiviral role of this soluble factor was initially suggested in studies examining the lack of pathogenicity in natural SIV infection in NHP. Their CD8$^+$ cells were found to suppress virus replication in the CD4$^+$ cells by secreting this cytokine (229). Like CAF, IL-16 is not antigen specific and can suppress replication of virus isolates with various tropisms (316). Similar to the β-chemokines, IL-16 blocks virus entry by binding to an HIV receptor, the major cell surface virus-binding protein CD4 (317). It is a lymphocyte chemoattractant factor (318) that brings immune cells to the site of infection and is expressed by CD8$^+$ T cells after serotonin and mitogen stimulation (319). Because IL-16, like the β-chemokines, recruits activated CD4$^+$ cells to the site of infection, this effect could also lead to an increase in infected cells; its clinical relevance remains unclear.

Supernatants from Jurkat cells transfected to express IL-16 can also suppress virus replication up to 99% by blocking viral mRNA production (320). This activity reflects a reduction in the HIV LTR promoter activity (321). Therefore, it is hypothesized that IL-16 may inhibit CD4$^+$ T cell activation resulting from HIV infection by binding to the CD4 receptor (316). This interaction can trigger downstream signaling that negatively regulates the HIV promoter in the LTR region (321, 322). The mechanism is potentially mediated by the tyrosine kinase p56-Lck (323).

Importantly, treatment of infected cells with recombinant IL-16 suppresses HIV replication only by 45% at the very high concentrations of >5 μg/ml. In addition, IL-16 was not detected in most of the CD8$^+$ T cell culture supernatants with antiviral activity produced in our laboratory, and when found, it was only in small amounts (0.3 to 1.1 μg/ml). Moreover, IL-16-neutralizing antibodies do not block the viral suppression mediated by CAF-containing CD8$^+$ T cell culture supernatants (324). Thus, the antiviral activity of the CD8$^+$ T cell culture supernatants from HIV-1-infected LTS is not likely to be due to the effect of IL-16. Furthermore, the clinical importance of IL-16 remains to be determined, since elevated IL-16 levels have been associated with poor health status (325).

(D) Granzymes

Granzymes, serine proteases, have been shown to be antiviral without killing target cells, by cleaving proteins required for virus replication (e.g., HSV, CMV) (section VIII.C). These include proteolytic processes affecting entry receptors and viral and host proteins involved in viral metabolism or in viral defense mechanisms that block antiviral responses (326). However, incubating HIV-infected cells with granzymes A and B did not indicate that these serine proteases were involved in CNAR (327). Granulysin, another cell membrane-perforating protein found in the cytolytic granules of NK and CD8$^+$ T cells, was also excluded from being involved directly in CNAR/CAF activity (328). Moreover, extracts of CD8$^+$ T cell granules did not show the presence of CAF-like activity (116).

(E) Target of Egr1

Target of Egr1 (TOE1) is a nuclear protein that has been reported to be expressed by activated CD8$^+$ T cells. This protein appears to have an anti-HIV activity similar to

CAF (329). It is found as two active isoforms, including one resulting from cleavage by the serine protease GzmB. Both isoforms are secreted by $CD8^+$ T cells and can enter HIV-infected target cells. TOE1 has been found to act at the HIV transcription level by interfering with Tat and directly affecting the viral LTR (329). Moreover, TOE1 (described initially as hypothetical protein FLJ13949) transcripts were found in one of our studies using representative differential gene analysis (RDA) (suppressive subtractive hybridization PCR [SSH-PCR]) (section X.B). It was expressed at higher levels by $CD8^+$ T cells with noncytotoxic anti-HIV activity (330). However, the latter finding has not been confirmed in more recent gene expression studies using $CD8^+$ T cells from NP (125, 331, 332). Moreover, TOE1 is also expressed by $CD4^+$ cells, whereas CAF is not. Since studies correlating TOE1 levels with better clinical status in HIV-infected subjects have not been reported, its clinical relevance remains to be determined (333).

(F) Prothymosin-α

Prothymosin-α (ProTα) is an acidic polypeptide with anti-HIV activity that was originally found in the culture supernatant of $CD8^+$ T cell lines established from HIV-infected children (334), LTNP, and uninfected subjects (335). These immortalized cell lines were derived by transformation using herpesvirus saimiri (HVS) in order to study the antiviral activity of soluble factors produced by $CD8^+$ T cells. Culture supernatants from those $CD8^+$ T cell lines showed antiviral activity on macrophages infected with primary HIV isolates resistant to chemokines as well as on virus reactivation in U1, a chronically infected promonocytic cell line (334). Protein purification by ion-exchange chromatography and reverse-phase HPLC revealed a novel antiviral polypeptide with a size between 3 and 10 kDa, later identified as ProTα by *de novo* protein sequencing (336). ProTα induces cytokine production by activating the STAT1 pathway (335) via TLR4 (337), although some ProTα isoforms interact with other receptors (338).

There are indeed several variants and isoforms of ProTα, which can be expressed by $CD8^+$ T cells and have various levels of anti-HIV activity (339). ProTα isoforms inhibit HIV replication very early in the virus replication cycle, without affecting cellular proliferation or virus transcription. This activity results from its interaction with TLR4 that induces the secretion of antiviral cytokines, such as type 1 interferons and CCL5 (337), from infected macrophages and monocyte-derived DCs but not $CD4^+$ T cells (337, 340). Thus, while ProTα was found during a search for CAF and could have value as a novel anti-HIV protein, it is not CAF.

(G) Ribonucleases

Ribonucleases are enzymes that catalyze the cleavage of mRNA. The initial observation that some RNases had anti-HIV activity was made when two members of the RNase A superfamily from other species, ranpirnase (northern leopard frog) and the bovine seminal RNase (BS-RNase), were found to inhibit HIV replication substantially in a leukemia cell line (341). Later, the supernatants of mixed lymphocyte reactions (MLR) using HLA-mismatched PBMC from uninfected subjects were also found to reduce HIV replication (342). Using blocking antibodies and an RNase inhibitor, the anti-HIV activity of these supernatants was attributed to another member of the RNase A superfamily, the eosinophil-derived neurotoxin (EDN; encoded by the *RNASE2* gene in humans). However, because of the nature of MLR, during which both $CD4^+$ T cells and $CD8^+$ T cells expand (343), which cell populations secreted the RNase could not be ascertained.

RNase 4 and angiogenin (RNase 5) are other members of the RNase A superfamily found in humans. Their antiviral activity was demonstrated after these two enzymes were identified by HPLC in the supernatant of $CD8^+$ T cell lines derived from LTNP (344). RNase 4 is only secreted by cell lines derived from $CD8^+$ T cells (344). Angiogenin, or RNase 5, has been found in the supernatants of both $CD4^+$ and $CD8^+$ cell lines from LTNP. X4-tropic strains of HIV can be inhibited by a mixture of these two RNases and β-chemokines (344). Recombinant EDN, RNase A, and angiogenin ribonucleases can suppress HIV replication *in vitro* when added before, during, and up to 4 h after infection (345).

While the exact mechanism of action of RNases on HIV infection is still to be determined, these enzymes appear to act primarily during early phases of the virus replication

cycle, such as destroying viral RNA before virus entry, during internalization in the endosomes, or in the nucleus before reverse transcription (346). Moreover, extracellular RNases may be able to enter the virus particles and cleave the viral RNA outside the cell before virus infection. That activity has been shown with other RNA virus infections where exposure of the virus to RNases has decreased infectivity (347, 348).

RNases do not have characteristics of CAF. For instance, RNases block HIV replication before integration, not transcription (179), and antibodies to RNase did not block CAF/CNAR activity (192). Also, CAF does not directly inactivate HIV particles (11). Finally, RNases are not often found in CAF-containing fluids (C. E. Mackewicz, unpublished data).

(H) Interleukin-32

IL-32 is a cytokine that has 9 known isoforms (IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, IL-32ζ, IL-32η, IL-32θ, and IL-32sm [for small]) resulting from alternate splicing of the IL-32 gene transcripts (349, 350). IL-32 is expressed by CD8$^+$ T cells, CD4$^+$ T cells, and NK cells after stimulation by mitogen agents such as PHA. While this protein is commonly referred to as a proinflammatory cytokine, its isoforms can have very different, even opposite, activities. For example, IL-32α is a nonproinflammatory isoform that could explain the (seemingly contradictory) immunomodulatory role of IL-32 observed in the lymphoid tissues of HIV-infected patients (351). Indeed, CD8$^+$ T_{RM} cells from those lymphoid tissues inhibit HIV replication in a noncytotoxic fashion (127) but express higher levels of IL-32 transcripts (as detected by single-cell RNA-Seq analysis), but not at the isoform level (125) (section X.C). Since this cytokine is expressed by several hematopoietic cells and induces IFN, it does not have characteristics of CAF.

(I) Peroxiredoxin

Using a cDNA expression array with activated CD8$^+$ T cells, two NK cell-enhancing factors, NKEF-A and NKEF-B, were found upregulated in CD8$^+$ T cells from HIV-infected individuals compared to those from uninfected individuals (352). The recombinant proteins in microgram per milliliter concentrations inhibited HIV replication by 50% (352). Elevated levels were found in the plasma of HIV-infected individuals. The results suggested that these antioxidant proteins could be involved in CNAR/CAF. Using a gene expression microarray, these peroxiredoxin proteins were found to be upregulated with anti-HIV activity (352). The proteins are primarily associated with enhancing NK cell antiviral activity. The researchers proposed that these proteins affect the NF-κB pathway and inhibit HIV transcription. Since these proteins are also made by NK cells, are not present in large amounts in CD8$^+$ T cell fluids, and their clinical relevance is not evident, they would not be candidates for CAF.

(J) Antithrombin III

Some investigators have found that a heparin-binding protein in cell culture fluids modifies antithrombin III in fetal bovine serum to become antiviral. They propose that CD8$^+$ T cells from HIV-infected subjects produce anti-HIV activity by activating antithrombin III in the culture medium (353). As a member of the serine protease inhibitor protein family (serpins), the modified antithrombin III has antiviral and anti-inflammatory properties. In HIV infection, these factors can affect virus replication prior to reverse transcription (354). We have also observed that antithrombin III has antiviral activity in microgram amounts, but this effect is not consistently observed nor clinically relevant (192). Furthermore, we can demonstrate CNAR/CAF activity in cultures lacking serum proteins (192).

(K) Interferons

IFNs are stimulatory cytokines released by infected cells and immune cells in response to viral infections. They can be classified into three families: type 1 IFNs that include IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω in humans, type 2 IFN that includes only IFN-γ in humans, and type 3 IFNs that are IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4. While type 1 IFNs have strong anti-HIV activity, these cytokines have

never been found to be produced by CD8$^+$ T cells. To the contrary, CD8$^+$ T cells only express IFN-γ. The type 2 IFNs do not inhibit viral replication (9). Thus, CNAR cannot be attributed to any type of IFN.

(L) Defensins

Another group of antiviral factors that were considered CAF was the defensins. Defensins are small proteins with broad antiviral, anti-fungal, and antimicrobial activities that are conserved among various species. The main mechanism of action of defensins is preventing viral entry into target cells, but some defensins can also induce infected cell death. Defensins are mainly expressed by innate immune cells such as neutrophils, monocytes, macrophages, NK cells, and $\gamma\delta$ T cells. One study reported that a mixture of α-defensins was expressed by CD8$^+$ T cells from HIV-infected LTNP, and the authors noted that they had anti-HIV activity comparable to CAF (355). This study, however, was quickly retracted after the authors found that the α-defensins were likely produced by the irradiated feeder cells used to grow the CD8$^+$ T cells (356). Our laboratory also determined that while α-defensins indeed have anti-HIV activity, they lack identity to CAF. CD8$^+$ T cells do not express these proteins, which has been confirmed at both the transcript and protein levels (357).

(M) Wingless-Type Integration Sites

Wingless-type integration sites (Wnts) are a network of secreted glycoproteins that are found in many different organisms (358). In humans, there are 19 Wnts that primarily act as regulators of transcription (359, 360). The canonical Wnt signaling pathway (or Wnt/β-catenin pathway) is involved in the inhibition of HIV replication in astrocytes and various other cells, such as monocytes, macrophages, and CD4$^+$ lymphocytes (361, 362).

Past studies have specifically underscored the role of the Wnt/β-catenin pathway in CD8$^+$ T cells and its effect on the control of HIV replication in human astrocytes transplanted into humanized mice (363). Recent findings maintained that primary human CD8$^+$ T cells express all 19 Wnt genes upon CD3/CD28 stimulation (364). In cell culture, these CD8$^+$ T cells induce the canonical Wnt/β-catenin pathway in infected cells, resulting in the inhibition of HIV transcription (364). In particular, the latter study noted the structural, biological, and functional similarities of Wnts to CAF (Table 4), such as (i) the size ($<$50 kDa) and heat stability, (ii) that these proteins are secreted by CD8$^+$ T cells in a noncytotoxic non-MHC-restricted manner, and (iii) that both the Wnt and CNAR/CAF signaling pathways result in transcriptional inhibition in HIV-infected CD4$^+$ cells.

Nevertheless, there are substantial distinctions between Wnts and CNAR/CAF. The Wnts have a broad effect on various cellular mechanisms in many organisms; in contrast, CNAR/CAF is a response limited to retroviral infections (sections VIII.A and VIII.B), with no effect on other biological processes (190). Also, while Wnts have been described in the signaling pathways of different cell types, such as lymphocytes and astrocytes, CNAR/CAF is a response restricted to CD8$^+$ T cells.

The initial observations of the CNAR/CAF response in cell culture correlated with an asymptomatic clinical status (section III.E.iii). In comparison, while CD8$^+$ T cells expressed comparable levels of all 19 Wnts, only Wnt2b, -3a, -9b, and -10b expression levels have been studied with HIV status. Notably, only antibody depletion of Wnt2b and Wnt9b in CD8$^+$ T cell culture fluids was shown to result in abrogation of HIV replication (364). While these results indicate a potential role for CD8$^+$ T cells expressing Wnts in HIV pathogenesis, the function of most Wnts in controlling virus replication remains to be determined. To this point, there is a potential for other Wnts to have redundant or modulatory functions to those of Wnt2b and Wnt9b in HIV replication. Overall, CNAR/CAF-mediated HIV suppression is distinct from the broad biological activities of Wnt proteins.

(X) APPROACHES TO IDENTIFY CAF

CAF is produced at low levels by cultured CD8$^+$ T cells (we estimate at $<$5 pg/ml of culture supernatant) and therefore difficult to purify for structural studies. However,

specific physical and chemical characteristics of this novel antiviral cytokine can be appreciated (e.g., heat stability, molecular weight, and pH stability) (section V) (Table 4). Notably, how CAF differs from the other proteins that have been identified as candidates for CAF is of considerable interest (section IX). Thus, biochemical, molecular, immunological, and protein chemistry approaches to determine the nature of CAF are currently being used. However, detection of a low-abundance protein such as CAF even with sensitive mass spectrometry has not yet revealed the identity nor structure of this naturally occurring antiretroviral protein. This challenge is similar to that of finding other low-abundance proteins such as cancer biomarkers (365).

(A) Mass Spectrometry

Because of the high sensitivity of mass spectrometry in identifying proteins, our laboratory devoted several years toward evaluating the identification of CAF by using this procedure, following successive steps of size exclusion, NH_3SO_4 precipitation, ion-exchange chromatography, and stable-isotope labeling by amino acids in cell culture (SILAC) (Table 4). We soon realized that mass spectrometry can detect femtomoles (10^{-15}) of a protein that could, on average, represent 50 pg of a low-abundance protein (366). Most cytokines have been detected by immunologic approaches and are usually produced at 5 pg/ml of plasma (367). We have therefore realized the important challenge of identifying CAF by mass spectrometry unless very large quantities of CAF starting material are available for purification.

(B) Microarrays and Gene Expression Methods

To determine if CAF could be identified genetically from CD8$^+$ T cells isolated from HIV-infected and uninfected individuals, identical twins were compared using representative differential gene analysis (RDA) or suppressive subtractive hybridization PCR (SSH-PCR) (330). The SSH-PCR approach (368) and the RDA approach were described around the same time, but the latter is more complicated and thus less popular (369, 370). The SSH-PCR limits the biological variations of an assay due to genetic differences. Nevertheless, this procedure did allow the comparison of a differential gene expression profile of the CD8$^+$ T cells from an HIV-infected twin that suppressed HIV replication with CD8$^+$ T cells from the uninfected twin that demonstrated no suppression of HIV replication.

Genes associated with the immune response, apoptosis, and cell maintenance, as well as unknown genes, were found to be differentially expressed in the CD8$^+$ T cells from the twins (330). Some of those unknown genes have since been identified: FLJ13949 (*TOE1*), c3orf4 (*CLDND1*), FLJ13046 (*XPO4* [exportin-4]), and KIAA0212 (*EDEM1*). When the expression of the most upregulated genes found in the anti-HIV CD8$^+$ T cells was assessed by quantitative PCR in the CD8$^+$ T cells of other HIV-infected subjects, only three genes coding for soluble proteins were consistently found to be higher: *ICT1* (encoding a peptidyl tRNA hydrolase of the mitochondrial ribosome), *NKG7* (encoding a protein with undefined function found in the granules of NK and T cells), and *TOE1*. The TOE1 protein was later evaluated by others and showed anti-HIV activity but is not CAF (329) (section IX.E). Notably, our differential gene expression analysis studies also led to the discovery of VCAM-1 as a potential marker of CD8$^+$ T cells mediating CNAR (133, 330) (section IV.C).

In subsequent studies, the genes differentially expressed between the CD8$^+$ T cells of HIV-infected subjects with strong antiviral activity in CNAR assays and the CD8$^+$ T cells of uninfected individuals with no suppressing activity were determined via cDNA microarrays (332). This assay, using the Affymetrix GeneChip Human Genome technology, showed that 18% of the genes were differentially expressed between the two groups, half of the 568 genes were higher in the CD8$^+$ T cells with strong antiviral activity (332). Using stringent selection criteria to select only transcripts with high confidence, this list of upregulated genes was narrowed down to 52 by RNA PCR analysis. As expected, 13 of those genes were associated with the immune response, including the β-chemokines, granzymes H and K, and IFN-γ, but 36 other genes were also part of various known and unknown cellular processes (for example, cell cycle and proliferation).

Three unknown genes were also found to be upregulated in the CD8$^+$ T cells with HIV-suppressing activity. They were FLJ35091 (a gene encoding a protein similar to ANKRD18A), FLJ39873 (*TIGIT*), and TncRNA (*NEAT1*, a long noncoding RNA derived from the trophoblast involved in transcriptional regulation). Notably, the increased expression of TIGIT, an inhibitory checkpoint receptor like PD-1, on CD8$^+$ T cells of HIV-infected subjects has since been confirmed by many groups (371–373). TIGIT negatively regulates the cytotoxic function of CD8$^+$ T cells when it interacts with its ligand CD155 (poliovirus receptor), which is expressed at the surface of HIV-infected target cells (374). Whether it plays a role in promoting CNAR merits further evaluation. None of the genes that were highly expressed in the CNAR$^+$ CD8$^+$ T cells encoded proteins with known anti-HIV activity (332).

(C) Next-Generation Sequencing Approaches

Next-generation sequencing (NGS) is a set of cutting-edge molecular biology technologies that use high-throughput DNA sequencing methods. One application for NGS is RNA sequencing (RNA-Seq), which utilizes cDNA libraries generated by reverse transcription of the RNA extracted from cell samples. By sequencing those libraries using NGS platforms, it is now possible to analyze the full transcriptome of RNA samples generated from large-scale experiments (375). This approach could prove very useful in identifying genes encoding proteins such as CAF, which is expressed at low levels yet is differentially expressed in CD8$^+$ T cells from HIV-infected subjects. This powerful new approach could also help determine known and novel pathways involved in both the production of CAF in CD8$^+$ T cells and its downstream effects in infected CD4$^+$ cells.

In one study (125), the transcriptional signature of blood and lymph node HIV-specific CD8$^+$ T cells from HIV-infected subjects was analyzed by bulk RNA-Seq and single-cell RNA-Seq (scRNA-Seq). CD8$^+$ T cells from the blood, as well as activated lymph node CD69$^+$ CD8$^+$ T cells, had a transcriptional profile corresponding to a cytotoxic phenotype, which was not the case for resting lymph node CD69$^-$ CD8$^+$ T cells. Some differentially expressed genes upregulated in the CD69$^+$ CD8$^+$ T cells were of particular interest, such as the gene for VCAM-1 (section IV.C), as well as those for some secreted proteins such as IFN-γ, lymphotactin (both were previously excluded from being CAF) (Table 5), and platelet-derived growth factor D (PDGFD). Notably, one gene that was strongly upregulated in the CD69$^-$ CD8$^+$ T cells is *SVIL1*, which encodes supervillin, a small soluble protein usually localized in the nucleus that warrants further evaluation. This study showed strong differences between distinct subsets of CD8$^+$ T cells in HIV-infected subjects. However, it did not address what genes in the transcriptional profile of these CD8$^+$ T cells, all from ECs (section III.E), could code for proteins that suppress HIV replication.

In a follow-up analysis, the same group compared differences in the transcriptomes of lymph node CD8$^+$ T cells between ECs and progressors by single-cell RNA-Seq (scRNA-Seq). In addition to the difference in cytotoxic potential of CD8$^+$ T cells and their ability to suppress HIV in the absence of a cytotoxicity-mediated mechanism described above (section IV.B), the EC lymph node CD8$^+$ T cells had a very distinct transcriptional profile. For example, the lymph node CD8$^+$ T cells from ECs expressed less perforin and GzmB at the transcript and protein levels than those from progressors, confirming their noncytotoxic phenotype (126). Moreover, the expression of various genes involved in CD8$^+$ T cell cytotoxicity, such as those encoding other granzymes and the death receptors Fas and TRAIL, were similarly lower in the lymph node CD8$^+$ T cells from ECs. Recently, HIV-specific CD8$^+$ T cells with a noncytotoxic T_{TM} phenotype were detected more frequently in the thoracic ducts than in the blood (435).

In contrast, CD8$^+$ T cells from progressors expressed higher levels of the transcription factor EOMES and other inhibitory molecules, including checkpoint blockade receptors such as TIGIT, LAG3, and 2B4. This finding indicated that CD8$^+$ T cells from progressors not only have an effector phenotype but also present many inhibitory receptors that are markers of what has been called T cell "exhaustion;" however, these

inhibitory receptors are possibly associated with other functions such as CNAR (sections IV.B and IV.D). Importantly, lymph node CD8$^+$ T cells from ECs showed substantially upregulated expression of many genes encoding secreted proteins with known or potential antiviral activity, including CCL5 (section IX.A), IL-32 (section IX.H), IL-1β, TNF, lymphotoxin β (also named TNFSF3), and RNase1 (section IX.G) (Table 5). The possible anti-HIV activity of the other secreted proteins found by scRNA-Seq, including the phospholipase DDHD1, the metalloprotease ADAMTS4, and the chemokines CXCL2, CXCL3, and CCL2, remain to be elucidated.

The molecular pathways that are enriched in the CD8$^+$ T cells of ECs are noteworthy. Some linked to transcription (metabolism of RNA, viral transcription, and activation of mRNA) are also of interest given our knowledge of how CNAR suppresses HIV replication at the transcriptional level (section VI). In particular, many of the pathways that are enriched in the CD8$^+$ T cells of progressors, which means they are downregulated in the CD8$^+$ T cells of ECs, confirm the absence of a role played by some known antiviral pathways such as signaling. This finding has been made with those induced by type 1 interferons, IL-2, PD-1, Ras, or extracellular signal-regulated kinases (i.e., ERKs). Taken together, these molecular studies confirmed the finding that HIV suppression mediated by CD8$^+$ T cells in ECs is not the result of direct killing of the infected target cells; these CD8$^+$ T cells lack the cellular ability to mediate cytotoxicity. Moreover, the reports provide new insights into potential gene and pathway candidates in the search for CAF.

In addition to CD8$^+$ T cells mediating antiviral activity, the NGS approaches could also be relevant to elucidate the intracellular pathway(s) creating the antiviral state elicited by CAF in CD4$^+$ cells. In one study, RNA-Seq analysis was performed on the CD4$^+$ cells of ECs (376). Although this particular study focused on subjects who were resistant to R5-tropic virus infection, this NGS approach may help to determine how the transcriptome of target cells is affected by CNAR/CAF in future experiments. Another study using the PBMC from a cohort of ECs and progressors found several genes coding for intracellular proteins for which the transcripts were lower in the EC group (377). These included the enzymes GTPase of the immunity-associated protein 4 (GIMAP4) and phospholipid scramblase 1 (PLSCR1) involved in known antiviral and cell apoptosis pathways (378, 379), as well as N-Myc and STATs interactor (NMI), which regulates transcription factors (380). However, because this experiment was conducted with bulk PBMC, any pathway associated with these genes will need to be confirmed in infected target cells such as isolated CD4$^+$ cells.

Finally, computational analysis of the transcriptome of *in vitro* HIV-exposed DCs obtained by scRNA-Seq from ECs helped find a novel, previously unknown, functional antiviral pathway that involves the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) and TLR3 sensing pathways and could be associated with an asymptomatic clinical state in these subjects (381). This type of analysis could also be used in future studies with acutely infected CD4$^+$ cells in which HIV replication is suppressed by CNAR/CAF *in vitro*. We are currently evaluating the effects of CNAR/CAF on particular pathways in infected CD4$^+$ cells using these approaches.

This pursuit of the antiviral pathways induced by noncytotoxic CD8$^+$ T cells in infected CD4$^+$ cells was recently attempted using RNA-Seq (75). Some notable cellular pathways in the CD4$^+$ cells were found to be downregulated, including pathways related to cell death, polarization, and proliferation. However, because the experimental conditions tested were just CD4$^+$ cells cultured alone or CD4$^+$ cells cocultured with CD8$^+$ T cells, those differences could be mostly caused by the alloantigen-stimulated HIV-inhibiting factors found in the supernatants of a mixed lymphocyte reaction (section IX.G) (342), not the CD8$^+$ T cell response to HIV infection itself.

(XI) PERSPECTIVES AND CONCLUSIONS

This review was written to bring further attention to the novel immunological activity first discovered in HIV infection. While the finding of a CD8$^+$ T cell noncytotoxic

anti-HIV response (CNAR) was surprising more than 3 decades ago, this antiviral activity of the immune system may, in fact, be much more common than was initially expected (section VIII). Traditionally, most immunologists were trained to consider that $CD8^+$ T cell antimicrobial control occurs by target cell killing via $CD8^+$ CTL. However, without the proper *in vivo* or even *in vitro* assays (section VII), the breadth of the true functions of $CD8^+$ T cells may be overlooked (Table 1; Fig. 1). Simply measuring $CD8^+$ T cell secretion of IFN-γ or CD107a expression does not conclusively identify the type of antiviral response mediated by these cells (section II.B). Particular assays are needed (Table 6), and these, as well as considering the major effect of CNAR/CAF on viral transcription (section VI) (Fig. 6 and 7), can help to determine the best approaches at promoting or inhibiting this activity.

If one focuses solely on cytotoxic activity, which can eliminate many infected target cells, this strategy would not be necessarily in the best interests of the infected host. CTL killing of the virus-infected cells could damage several organs of the body, particularly the brain and, conceivably, the heart and kidneys (Appendix 5). Instead, holding the virus in check or in a "locked" state by CNAR/CAF could maintain the function of the infected cells and thus accomplish the same objective as CTLs with minimal deleterious side effects (382). Unfortunately, one finds too often that any activity of $CD8^+$ T cells is considered to be cytotoxic (section II). This conclusion can be misleading and influence the anti-HIV approaches taken. Certainly, CNAR offers an advantage to the infected host that is not yet fully appreciated (Appendix 3). Hopefully, this review will encourage attention to this important issue.

We have considered that CNAR/CAF is an innate mechanism for preserving the viability of the infected target cells (Appendix 4) so they can return to relatively normal function. At first glance, the activity of CNAR/CAF appeared to share properties of an innate immune response (section III.F) (Table 1) (14). Notably, unlike adaptive epitope-specific CTLs, the $CD8^+$ T cells that mediate CNAR show broad responses against all retroviruses. Thus, even mutant viruses resulting from the selection pressure of cellular immunity or drug treatment can be controlled (section III.B).

However, CNAR/CAF may share characteristics of both the innate and adaptive arms of the immune system (Table 1). For example, similar to NK cells, which are considered innate lymphocytes yet possess adaptive characteristics such as a "memory-like" recall response (383), $CD8^+$ T cells in HIV infection could also bridge these two types of immune responses. They have the innate feature of mediating CNAR/CAF with its broad antiretroviral activity. Yet, they are classified as adaptive lymphocytes, since they have various antigen-specific receptors encoded by rearranged genes and a memory cell phenotype (Appendix 3; Table 1).

Also, because CD3/CD28 stimulation associated with CTLs increases CNAR activity, conceivably, an HIV-specific TcR engagement activating the same intracellular pathways and leading to CNAR could occur as part of an adaptive immune response (section II.A). Similarly, the direct or indirect exposure to conserved retroviral motifs recognized by germ line-encoded, invariant pattern receptors can promote CNAR, leading to the secretion of the antiviral cytokine, CAF. The latter reflects an innate immune response. Moreover, the $CD8^+$ T cells mediating CNAR have a memory cell phenotype (Fig. 2) similar to CTLs, but memory, defined as a better antigen-specific recall response, is not one of their functional characteristics (section IV.A). Their main activity, the secretion of antiviral soluble factors in response to conserved retroviral motifs, again is part of an innate immune activity (Table 1).

At the same time, it is tempting to suggest that CNAR/CAF, being a rapid early innate immune activity, can suppress the initial expression of HIV so that the later adaptive immune response to the virus (e.g., CTL) does not have time to occur. In this case, cooperation between the two arms of this novel function would not be evident. This possibility could explain why only CNAR is found in some individuals who have been exposed to HIV yet remain uninfected (section III.G) (Table 2). The transient, low

antigen expression that occurs is not sufficient to elicit an adaptive immune response, such as CTL activity and antibody production.

Obviously, with HIV infections, both innate and adaptive immune responses should be present and contribute to the control of the virus. As noted, both seem involved with CNAR activity, lending attention to both arms of the immune system. Information gained from studying the CNAR/CAF response is also important for our general knowledge of innate immunity as the first line of defense against retroviruses. This type of innate immune response with uninterrupted expression of CAF could eventually lead to the elimination of replicating virus reservoirs and lock HIV into a persistent latent state—essentially a functional cure (section III.D).

Indeed, the objective of knowing the true function of CD8$^+$ T cells is especially important when trying to induce a long-term HIV cure. This virus integrates into the genome of the infected cells and captures the genetic machinery for progeny production. Fortunately, this action can be suppressed by CD8$^+$ T cells via CNAR/CAF for extended periods of time (section VI).

Nevertheless, because of the recent objective by some researchers to achieve a sterilizing cure in HIV infection, this noncytotoxic response seems to be opposing that aim (75). Yet, for others, elimination of every infected cell in the body for a cure does not seem feasible (382, 384). Thus, whereas some researchers would choose to eliminate CNAR, others, in contrast, would prefer to induce and maintain this noncytotoxic control (382, 384). CNAR can eventually narrow down the number of infected cells and reduce the need for potentially toxic antiretroviral drugs.

In any case, full identification of the CD8$^+$ T cell antiviral factor (CAF) will be a benefit to any group of investigators (section V) (Table 4). This protein is stable and fits the definition of a low-abundance protein, similar to cancer biomarkers (365). Thus, proteomics approaches with better resolution are needed to identify such rare proteins. Those of us who observe the beneficial role of CAF would like to find the gene encoding it in order to develop an immune-based therapy that would be naturally occurring and nontoxic. Those wishing to accomplish eradication of HIV reservoirs would like, presumably, to find neutralizing antibodies or drugs against CAF and its production.

How sustained the CNAR/CAF activity can be over time raises the important question of the impact of aging on this CD8$^+$ T cell function. Since most immune cells become senescent, with decreased functional capacity as individuals age (section IV.D), future research should focus on developing a multidisciplinary plan for the treatment of HIV. Such an approach could include an early promotion of innate antiviral responses such as CNAR/CAF, with subsequent ART treatment to maintain HIV control if needed. This strategy could lead to the induction of viral latency, and further therapy would not be needed. Such viral control has been reported in recent studies on individuals after long-term ART (385).

As for CAF, there are today very few immune factors secreted by CD8$^+$ cells that have anti-HIV activity (section IX). Those known include the chemokines, IFNs, and a few cytokines such as TNF-α/β (Table 5). A major discovery would be the identification of this CD8$^+$ T cell antiviral factor. This objective has been our challenge for several years (section X). Once identified, CAF could be found to have other beneficial functions in the host besides its anti-HIV activity. Just as IFNs were initially discovered as an anti-influenza response (386), these cytokines and their related proteins have been useful in other diseases such as cancer (387). Likewise, CAF could also be playing an important immunologic role in autoimmune diseases and cancer. Indeed, a noncytotoxic CD8$^+$ T cell response has been reported in non-virus-induced tumors (388–390).

With CAF identified, its general purpose in physiological contexts can be better explored. In this regard, there are reports of antiviral factors within exosomes (391–393) or immune cell secretomes that do not yet have an identity or function. One of these may indeed be CAF. The CD8$^+$ T cell exosomes contain a factor with the

characteristics of CAF (391). Also, because CNAR/CAF can inhibit all HIV subtypes, including drug-resistant viruses, escape mutant viruses, and other retroviruses, it can provide broad protection from all these viruses, including HTLV, that cause leukemia and neurological diseases (sections III.B, III.E, and VIII.B). Moreover, the full identity of CAF would enable the development of products or processes that could enhance or maintain its production by $CD8^+$ T cells (section IV.F) (Appendix 2; Table 3). The resulting natural, nontoxic antiviral therapy would provide a promising approach. Furthermore, an ELISA measuring this protein would be helpful in screening potential CAF-enhancing agents and determining if enough CAF is being produced to suppress HIV replication.

Finally, the development of a vaccine that induces CNAR/CAF (64, 241) has the additional benefit of promoting an early innate immune response that can provide protection against any retrovirus. As seen in HESN individuals (section III.G), this innate activity appears to be the major immune response that is associated with the lack of infection in these individuals.

This discovery of a $CD8^+$ T cell noncytotoxic antiviral activity was made in the context of HIV infection (section III.A). While the effect of CAF is restricted to retroviruses (sections VIII.A and VIII.B), a similar immune activity may be found with many if not all viruses. Some examples are presented in this review (section VIII.C). Currently, we are faced with another pandemic: COVID-19. Most evidence fortunately suggests that coronavirus infection does not substantially influence HIV transmission, infection, or pathogenesis (394). Certainly, the early studies of HIV have helped infectious disease experts appreciate the challenges presented by COVID-19. Perhaps a noncytotoxic $CD8^+$ T cell response active against this virus will be found. We encourage virologists and microbiologists to investigate this possibility further. CAF-like products may be made very early in these infections and hold the promise of better antimicrobial drugs and immune factors to combat drug resistance.

This background on CNAR, its characteristics, and potential presence in many viral infections provides a foundation to encourage researchers and new students in the field to take on the challenges of answering key questions surrounding this immune activity.

What is the nature of the $CD8^+$ T cell antiviral factor? How is this T cell antiviral response elicited in comparison to CTL activity? How commonly is CNAR-like activity found in viral infections, especially in uninfected individuals? For example, this type of response could be involved in controlling other common viruses that may be kept in a latent state and not be evident until long after the infection has taken place. Could this be the mechanism that silences viruses such as chickenpox and measles for many years until they reemerge as causes of other diseases (e.g., shingles and encephalitis) (395, 396)?

The mediators of CNAR-like activity in other infections would be, most likely, different for each virus family. However, the overall effect would be shared with the $CD8^+$ T cell noncytotoxic responses to HIV infection described in this review (Appendix 3). We look forward to seeing further innovative findings in this important immunological field.

APPENDIX 1

Characteristics of long-term survivors (LTS) of HIV infection include the following:

- Clinically asymptomatic for $>$10 years*
- Not on ART*
- Normal $CD4^+$ cell number* (77, 216, 397)
- Large number of $CD4^+$ cells in the gastrointestinal (GI) tract* (398)
- Low virus load (measured by plasma viremia; infected PBMC)* (77, 216, 397)
- Low immune activation* (398)
- CCR2-V641 polymorphism as well as HLA-B54 allele (399)

- Presence of the HLA-B57 class I allele (400)
- Viral envelope V2 region is increased in length (401)
- Virus with deletion in Nef region (402, 407)
- Presence of a predominantly nonvirulent HIV isolate (e.g., R5 virus)* (403)
- Virus with Vpr mutation (404) or deletion in SP-1 site of LTR (405)
- Strong cellular CD8$^+$ cell cytotoxic antiviral response* (89, 406, 407)
- HIV-specific CD4$^+$ T cell and CD8$^+$ T cell responses* (152, 397, 408)
- Presence of CNAR/CAF
- Presence of anti-HIV neutralizing antibodies (216, 409, 410)
- Presence of anti-Tat antibodies (411, 412)
- No enhancing antibodies (409)
- IgG2 antibodies that react with gp41 together with CD4$^+$ cell anti-p24 responses (413, 414)
- Type 1 cytokine production by PBMC* (407)
- Strong NK cell activity (415)
- Active antibody-dependent cellular cytotoxic (ADCC) (416)
- Lymph node structure is normal* (46, 397)

LTS are also called LTNP (section III.E.i). Some of these characteristics have been observed in only a few cases and not fully confirmed. Major factors are noted by an asterisk (*). Modified from reference 417 with permission.

APPENDIX 2

Factors that induce and enhance CNAR/CAF include the following:

- HIV replication (2)
- HLA concordance (33)
- Conserved retroviral motifs
- Activation: CD3 or PHA stimulation of CD8$^+$ T cells
- IL-2 (121, 160, 161)
- CD40/CD40L-stimulated DCs (168, 169)
- CD28 costimulation (121)
- Early ART treatment (31, 162, 175)
- IL-15 (153, 173)
- IL-21 (110)
- Allogeneic mixed lymphocyte reaction (176–179, 342)
- LFA-1 agonist antibody (180)
- Thymosin α1 (181)
- TLR7/8 agonists (182)
- Potential vaccine (64)

APPENDIX 3

Characteristics of the CD8$^+$ T cell noncytotoxic anti-HIV response (CNAR) include the following:

- Does not involve cell killing
- Mediated by CD8$^+$ T cells only, not CD4$^+$ cells, NK cells, or macrophages
- Mediated by heterogenous, polyclonal CD8$^+$ T cell populations
- Exhibited predominantly by the HLA-DR$^+$ CD28$^+$ CD11b$^-$ CD8$^+$ T cell subset
- Correlates directly with asymptomatic clinical status and normal to high CD4$^+$ cell counts
- Early response to HIV infection that occurs before seroconversion
- Active against all isolates of HIV-1, HIV-2, and SIV tested

- Can block HIV replication at low $CD8^+/CD4^+$ cell ratios ($<0.05:1$)
- Not MHC restricted
- Blocks HIV replication in naturally or acutely infected $CD4^+$ cells
- Has characteristic of innate immune response (Table 1)
- Dose dependent
- Associated with VCAM-1 expression on $CD8^+$ cells (133, 330)
- Blocks HIV at the transcriptional level; does not affect earlier steps in virus replication (section VI)
- Optimal activity with cell-cell contact, potentially due to integrins and adhesion molecules (section IV.C)
- Mediated (at least in part) by a novel soluble anti-HIV factor, CAF (Table 4)
- No substantial effect on activation or proliferation of $CD4^+$ cells
- Observed with $CD8^+$ cells from infected nonhuman primates
- Can be observed with stimulated $CD8^+$ T cells from uninfected individuals

Measured by *in vitro* assays. Modified from reference 417 with permission. See also references 11, 12, and 192.

APPENDIX 4

Potential clinical value of CNAR/CAF IN HIV infection includes the following:

- Could ensure long-term survival
- Not affected by viral heterogeneity (HIV-1 and HIV-2); cytopathic and noncytopathic strains are sensitive; can control drug-resistant and mutant viruses
- Can prevent several log units of virus replication *in vitro* (and possibly 3 to 5 log units *in vivo*)
- Can prevent emergence of drug-resistant strains and immune "escape" mutants by blocking virus replication at transcription
- Can inhibit replication of a superinfecting virus
- Does not substantially affect viability, activation, or proliferation of $CD4^+$ cells
- Prevents effects of CTL activities that could be destructive to the host (e.g., in brain, lung, and kidney) (see list below)

This list was modified from reference 417 with permission.

APPENDIX 5

Possible detrimental characteristics of CTL activity in HIV infection include the following:

- Lysis of uninfected $CD4^+$ cells (418, 419) or APCs (420, 421)
- CTL response is absent in asymptomatic NHP naturally infected with simian lentiviruses (11)
- CTL activity can be harmful in viral infections (HTLV, LCMV, and Sin Nombre virus [SNV]) (248, 422–424)
- Involved in lung lymphocytic alveolitis (425)
- CTL infiltration of lymph nodes (426, 427)
- Found in the cerebrospinal fluid of symptomatic disease patients (428)

This list was adapted from reference 417 with permission. See also references 11, 429, and 430.

APPENDIX 6

Abbreviations used in this review include the following:

APC, antigen-presenting cell
ART, antiretroviral therapy
BcR, B-cell receptor
CAF, $CD8^+$ T cell antiviral factor

CAT, chloramphenicol acetyltransferase
CCL, chemokine (C-C motif) ligand
CCR, C-C chemokine receptor
CMV, cytomegalovirus
CNAR, CD8$^+$ T cell noncytotoxic antiviral response
CTL, cytotoxic T lymphocyte
DC, dendritic cell
EBV, Epstein-Barr virus
EC, elite controller
ELISA, enzyme-linked immunosorbent assay
FAS (Fas receptor), apoptosis antigen 1 (APO-1), TNF receptor superfamily member 6 (CD95)
FIV, feline immunodeficiency virus
Gzm, granzyme
HBV, hepatitis B virus
HCV, hepatitis C virus
HESN, highly exposed seronegative
HEV, hepatitis E virus
HIC, HIV-infected controller
HLA, human leukocyte antigen
HSV, herpes simplex virus
HTLV, human T cell lymphotropic virus
HVS, herpesvirus saimiri
IAV, influenza A virus
IFA, immunofluorescent antibody
IFN, interferon
IL, interleukin
Itg, integrin
LCMV, lymphocytic choriomeningitis mammarenavirus
LFA, lymphocyte function-associated antigen
LNMC, lymph node mononuclear cells
LPS, lipopolysaccharides (endotoxins)
LRA, latency-reversing agent
LTS, long-term survivor
LTNP, long-term nonprogressor
MHC, major histocompatibility complex
NC, noncontrollers
NGS, next-generation sequencing
NHP, nonhuman primates
NK cell, natural killer cell
NSI, non-syncytium-inducing
PBMC, peripheral blood mononuclear cells
PD-1, programmed cell death protein 1 (CD279)
PHA, phytohemagglutinin
PMA, phorbol myristate acetate
P-TEFb, positive transcription elongation factor b
R5-tropic, using CCR5
RP, rapid progressor
RSV, Rous sarcoma virus
RT, reverse transcriptase
SHIV, simian human immunodeficiency virus
SI, syncytium inducing
SILAC, stable-isotope labeling by amino acids in cell culture
SIV, simian immunodeficiency virus
SM, sooty mangabey
T_{CM}, central memory T cell
TcR, T cell receptor

T_{EM}, effector memory T cell
TLR, Toll-like receptor
TNF, tumor necrosis factor
TRAIL, TNF-related apoptosis-inducing ligand (CD253)
T_{RM}, tissue-resident memory T cell
T_{TM}, transitional memory T cell
VCAM-1, vascular cell adhesion molecule 1 (CD106)
VI, viremic individual
VIA, virus inhibition assay
X4-tropic, using CXCR4

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