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Cytolytic CD4⁺ T cells in viral immunity

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Abstract

It is generally believed that the role of CD4⁺ T cells is to coordinate the different arms of the adaptive immune system to shape an effective response against a pathogen and regulate nonessential or deleterious activities. However, a growing body of evidence suggests that effector CD4⁺ T cells can directly display potent antiviral activity themselves. The presence of cytolytic CD4⁺ T cells has been demonstrated in the immune response to numerous viral infections in both humans and in animal models and it is likely that they play a critical role in the control of viral replication *in vivo*. This article describes the current research on virus-specific cytolytic CD4⁺ T cells, with a focus on HIV-1 infection and the implications that this immune response has for vaccine design.

Keywords

cytolytic CD4⁺ T cells; HIV; vaccines; viral infection

The concept that T cells can play a helper role dates back to the 1960s, when it was established that thymocytes synergized with bone marrow cells to facilitate the production of antibodies [1,2]. The subsequent development of mouse monoclonal antibodies to lymphocyte antigens allowed for the further isolation of this helper activity to CD4⁺ T cells, which were also recognized to provide helper signals to cytolytic CD8⁺ T cells [3,4]. However, it was noticed by some groups that CD4⁺ T cells (first as class II reactive cells) could themselves exhibit cytotoxic activity [5]. Although this activity was recognized to only contribute a minor amount to the overall cytolytic potential of T cells in the blood, cytolytic CD4⁺ T-cell lines and clones could nonetheless readily be isolated [6,7]. At the time, it was suggested that this activity was a side effect of the conditions required to culture T cells *in vitro* [8]. For the most part, interest in cytolytic CD4⁺ T cells waned as immunologists focused on their helper functions. The Th1 and Th2 subsets were quickly established and became the paradigm for defining T-cell help in the ensuing years [9].

More recently, the CD4⁺ subset lineages have been redefined, not only on the basis of their effector functions, but also by the expression of characteristic transcription factors. By now, seven different CD4⁺ T-cell subsets have been defined in humans but it is likely that more will be distinguished in the future. Moreover, recent advances in CD4⁺ T-cell research have

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begun to revise the concept of immunomodulatory CD4⁺ T-cell help and have indicated that a direct antiviral activity by these cells may be critical for pathogen clearance. However, in the case of HIV-1 infection, where activated CD4⁺ T cells are the main targets, the importance of CD4⁺ T cells in the control of the virus is still controversial [10]. The induction of cytolytic CD4⁺ T-cell responses has therefore been seen with great skepticism. This article will focus on the current evidence and knowledge of the cytolytic properties of CD4⁺ T cells and will evaluate their potential role in vaccine design.

CD4⁺ T cells as direct effectors

The primary function of CD4⁺ helper T cells is to direct and focus immune responses to maximize antipathogenic processes, while suppressing nonessential immune responses. This modulatory capacity of CD4⁺ helper T cells is central to the proper functioning of the immune system. However, recent achievements in CD4⁺ T-cell research have changed our thinking regarding these cells dramatically. While it was commonly believed that Th1 cells provide help to CD8⁺ T cells and Th2 cells generally provide help to B cells, this dichotomy has been revised by the description of additional subsets (Th9, Th17, Th21, T follicular helper [TFH] cells and T regulatory cells [Tregs]), each of which plays a distinct role in the overall immune response.

Upon presentation of viral peptides by antigen-presenting cells, CD4⁺ T helper cells become activated, secrete cytokines and clonally expand. The differentiation of naive CD4⁺ T helper cells into distinct subsets occurs during the progression of an infection and depends fundamentally on the effect that the infection has on the antigen-presenting cell. The affinity and strength of the T-cell receptor–major histocompatibility complex (MHC) interaction, as well as the cytokine/chemokine milieu present during initial CD4⁺ T-cell activation, greatly influence subsequent T-cell differentiation [11]. The CD4⁺ subsets that result from this differentiation are defined primarily on the basis of their ability to secrete different cytokines and the expression of specific transcriptional factors. Th1 cells are characterized by the expression of the transcription factor Tbet and the production of their cardinal cytokine/chemokine, IFN- γ , along with TNF- α and IL-2 [12]. Th2 cells, on the other hand, produce IL-4, IL-5 and IL-10 under the control of the transcription factor GATA3 and preferentially act on the humoral arm of the adaptive immune system. These cells have been shown to be important for the induction of antibody class-switching to the IgE and certain IgG isotypes [13]. TFH cells have been recently described to be central for B-cell proliferation, maturation and the induction of somatic hypermutation within the germinal center of B-cell follicles. TFH cells are defined by the transcription factor B-cell lymphoma 6 (BCL-6) and surface expression of CXCR5 and inducible costimulator (ICOS) [14]. However, both surface antigens are also present on non-TFH cells, demonstrating the overlap of effector potential with other CD4⁺ subsets. The functional properties of TFH cells in humans are currently not well understood; TFH cells in general produce the cytokine IL-21, which functions to stimulate B cells but may also act in an autocrine fashion to amplify TFH activity [15]. The production of IL-21 is especially interesting as IL-21 has been linked to important antiviral helper functions for CD8⁺ T cells [16–18]. It has been suggested that, in addition to TFH cells, a separate CD4⁺ T-cell subset primarily secreting IL-21 may exist to mediate these antiviral activities [19]. Th17 CD4⁺ T cells, which produce IL-17, have also been suggested to secrete IL-21. Th17 cells have been defined on the basis of the transcription factor RAR-related orphan receptor γ t (ROR- γ t) and are induced in the presence of IL-6, TGF- β and IL-1 β [20–22]. IL-17 attracts and activates neutrophils and facilitates proinflammatory responses from various other cell types (e.g., endothelial cells). By contrast, CD4⁺ Tregs have been described as a CD4⁺ subset with suppressive activity on innate and adaptive immunity and have been shown to be enriched in chronic viral infections. Tregs are defined by the coexpression of the zinc-finger transcription factor

forkhead box P3 (FoxP3) and the IL-2 receptor (CD25). Although it is known that Tregs can be induced through the presence of TGF- β (iTregs), they also can be selected in the thymus in the early stages of differentiation (natural Tregs). They are believed to act by a cell–cell contact-mediated mechanism that does not depend on soluble factors such as TGF- β or IL-10, although these factors might have an impact *in vivo* [23,24]. Interestingly, it is also notable that CD4⁺ CD25⁺ T cells have been linked to a cytolytic phenotype and it has been speculated that, despite their suppressive nature, Tregs have a second, cytolytic function. However, cytolytic CD4⁺ T cells might also represent their own, less well described, CD4⁺ T-cell subset.

Cytolytic CD4⁺ T cells

Very little is known about the phenotype, function and transcriptional profile of cytolytic CD4⁺ T cells. Although cytotoxic activity is traditionally thought to be the role of natural killer (NK) cells or CD8⁺ cytotoxic T lymphocytes (CTLs), there is no evidence to suggest that these cells are linked to the differentiation of cytolytic CD4⁺ T cells. Although controversy persists about their relevance, cytolytic CD4⁺ T cells have been implicated in the control of a variety of persistent viral infections, such as Epstein–Barr virus (EBV), hepatitis C virus (HCV) and HIV-1 [25–27]. Moreover, cytolytic killing of virally infected cells by CD4⁺ T cells has been also recently observed *in vivo* in the lymphochoriomeningitis virus (LCMV) mouse model [28]. It is therefore possible that these cells comprise a novel CD4⁺ subset with a unique lineage and functionality. This underestimated direct effector activity of CD4⁺ T cells raises questions about their nature, role and the mode of induction of cytotoxicity.

Phenotype

Early studies describing the presence of cytolytic CD4⁺ T cells in the context of influenza and poliovirus categorized these cells broadly as belonging to the Th1 subset. This conclusion was based on the cytokine secretion profiles of cytolytic CD4⁺ clones as well as the observation that cells polarized *in vitro* to the Th1 phenotype could exert cytolytic activity in killing assays, in contrast to Th2 cells [29,30]. These findings have been extended to other viruses, such as West Nile Virus and EBV [31–33], as well as to TB infection [34] and cancer [35]. However, the functional profile of cytolytic CD4⁺ T cells appears to be distinct from CD4⁺ T cells with a Th1 phenotype. Moreover, while classical Th1 cells show the ability to secrete IL-2, the majority of cytolytic CD4⁺ T cells lose this effector function [36]. By contrast, Th1 cells show higher levels of the costimulatory molecules CD28 and CD27 after initial antigenic stimulation, while cytolytic CD4⁺ T cells lack expression of these markers [26,36]. Similar differences have been observed for the expression of the integrin α chains CD11a and CD11b, which are upregulated on CD4⁺ T cells with cytolytic effector potential [36]. This marker profile is suggestive of a terminally differentiated effector cell phenotype; indeed, the expression patterns of other markers associated with such a phenotype – such as CD57^{high} and CCR7^{low} – are found on perforin or granzyme-positive CD4⁺ T cells [36,37]. Interestingly, perforin or granzyme-expressing CD4⁺ T cells with this terminally differentiated phenotype are present even in healthy individuals and are markedly expanded in those with chronic viral infections [36].

Further studies have suggested that cytolytic CD4⁺ T cells may also express molecules that are normally found on NK cells such as NKG2D, KIR2DS2 and KARAP/DAP12 [38–40]. The role of these receptors on cytolytic CD4⁺ T cells, however, is unknown. In CD8⁺ cells, NKG2D, for example, can act as a costimulatory receptor; signaling through NKG2D on these cells can fine-tune CTL activity in the absence of other costimulatory molecules like CD28 [41–43]. In cytolytic CD4⁺ T cells lacking CD28, it has been hypothesized that NKG2D may serve a similar role [44].

Similarly puzzling is the close phenotypic relationship of cytolytic CD4⁺ T cells with Tregs. Tregs have been defined on the basis of the expression of the transcription factor FoxP3 and the expression of CD25. While the inhibitory action of Tregs is unknown, studies of both mice and human cells suggest that a subset of CD25⁺CD4⁺ T cells can exert cytolytic activity. CD25⁺CD4⁺ T cells isolated from human subjects have been shown to express high levels of perforin and granzyme [45]. Both iTregs, as well as stimulated CD25⁺ natural Tregs, were confirmed to perform MHC-unrestricted killing of autologous targets [46]. In mice, however, Tregs were observed to lyse target cells in an antigen-dependent fashion [47]. Although further studies must be performed to understand the species and context-specific differences behind these activities, work so far has underscored the possibility that Treg-mediated cytotoxicity may play an important role in immune regulation. Most recently, it was demonstrated that a tumor antigen-specific CD25⁺CD4⁺ cell line was able to kill dendritic cells when infused into tumor-bearing mice, suggesting that cytolytic Tregs may represent a mechanism to blunt T-cell priming by antigen-presenting cells [48].

Cytolytic effector function

Cytolytic CD4⁺ T cells share common cytolytic pathways with CD8⁺ CTLs and NK cells. These cell types can kill target cells by one of two primary effector mechanisms. In the first, ligation of the Fas ligand (FasL; CD95L/CD178) on effector cells with its cognate receptor, Fas (CD95), on target cells results in the activation of caspase-mediated apoptosis programs in the target [49]. In the second, cytotoxic granules that contain toxic effector molecules like perforin and granzymes, are released during exocytotic degranulation, triggering target cell death both directly and indirectly, by activating downstream apoptosis pathways [50].

The earliest mechanistic studies of cytolytic CD4⁺ T cells implicated a Fas/FasL-based pathway. Analysis of cytolytic CD4⁺ T-cell lines revealed that they killed in a manner that was dependent on the level of Fas in the target; indeed, Fas-deficient targets from *lpr* mice could not be lysed [51]. A similar dependence on the Fas killing pathway was found in an early *in vivo* study of CD8⁺ T-cell-deficient mice infected with LCMV [52]. However, more recent reports have also suggested a second, cytotoxic granule-based mechanism. CD4⁺ T cells expressing high levels of perforin and granzyme or granulysin have been observed in the context of several viral infections as well as in healthy subjects. These cells have been shown to degranulate upon antigenic stimulation directly *ex vivo* [27,53–55]. A distinct subset of CD4⁺ T cells expressing both perforin and granzymes has been demonstrated in cytomegalovirus (CMV)-infected patients; these cells can release their cytotoxic granules in an antigen-specific manner [54]. Likewise, in HIV-1-infected patients, up to half of HIV-1 Gag-specific CD4⁺ T cells were observed to be able to degranulate, measured by the expression of CD107a/b (LAMP) on the cell surface [27].

Studies using perforin inhibitors or cells from perforin-deficient mice have verified the important role that an exocytotic granule mechanism plays in CD4⁺ cytotoxicity. Treatment of CD4⁺ T-cell clones as well as CD4⁺ T cells directly *ex vivo* with concanamycin A, a vacuolar type H⁺-ATPase inhibitor that inhibits exocytotic granule activity, results in a greatly diminished capacity to lyse autologous targets [36,45]. Although this pathway occurs by a single general mechanism, it is likely that it can be differentially regulated within CD4⁺ T cells at the molecular level. For example, mycobacterium- and EBV-specific cytolytic CD4⁺ T cells in particular have been shown to express high levels of granulysin [56,57]. In studies of cytolytic Tregs, it was found that iTregs expressed high levels of granzyme B, while natural Tregs express granzyme A [46]. The exact conditions that lead to these differences remain unknown but will probably be important for vaccine design.

Additional cytotoxic mechanisms have been described, but their role in mediating cell death is less well studied. In CD8⁺ T cells, it has been demonstrated that the TNF-related

apoptosis-inducing ligand (TRAIL) plays a significant role in lysing virally infected target cells, including in HIV-1 and CMV infection [58,59]. The function of TRAIL in killing by cytolytic CD4⁺ T cells has been evaluated in a limited number of studies, which found that CD4⁺ T-cell clones expressed TRAIL and could induce bystander apoptosis in antigen-presenting cells [60] as well as in TRAIL-sensitive tumor cell lines [61]. However, the role of the TRAIL pathway in the control of viral infection by CD4⁺ T cells has not been evaluated. Target cells can be sensitized for TRAIL-mediated apoptosis by the presence of inflammatory cytokines such as TNF- α and IFN- γ [59]. In addition to their ability to potentiate apoptosis by other means, both may also exert a direct, contact-independent cytotoxic mechanism [62,63]. It has been shown that both cytokines can lead to cell death by inducing the production of nitric oxide and other free radicals or by activating death pathways within the target cell [64,65]. While cytolytic CD4⁺ T cells have been found to secrete these cytokines, their direct impact on CD4-mediated cytotoxicity has not been investigated.

Early efforts to quantify the contributions of the different killing pathways to the overall cytotoxic activity of CD4⁺ T cells led to the estimate that Fas/FasL mechanisms accounted for 30% of killing and the perforin/granzyme pathway played only a negligible role. Cytotoxic cytokines were hypothesized to account for the remainder of the observed activity [66,67]. However, this work was performed in a mouse model of graft-versus-host disease and is probably specific for that context. It has also been proposed that a perforin-based pathway is dominant in humans and that Fas-mediated killing is the primary cytotoxic pathway for CD4⁺ T cells in mice [68]. Evidence in support of this idea, however, is limited to studies of FasL-deficient mice or patients showing a hereditary perforin or Fas deficiency. Indeed, more recent studies in mice have shown that cytotoxic CD4⁺ T cells preferentially use a perforin pathway depending on the availability of IL-2 [69,70]. Although the impact of killing by CD4⁺ T cells through cytotoxic cytokine secretion or a TRAIL-based mechanism has not been evaluated, it is likely that multiple pathways coexist and can be differentially induced depending on the immunological context.

Differentiation

No specific transcription factor or underlying gene pathway has been implicated in mediating cytotoxicity of CD4⁺ T cells (Figure 1). Since cytotoxic CD4⁺ T cells have been suggested to resemble Th1 cells, a role for the transcription factor Tbet can be considered. Interestingly, Tbet has also recently been found to regulate the induction of cytotoxic programming in CD8⁺ T cells in combination with another T-box transcription factor, eomesodermin (Eomes) [71,72], potentially indicating an overlap in the molecular pathways responsible for cytotoxic function in both cell types. Indeed, transcriptional profiling studies have revealed remarkable similarities between the transcriptional profiles of CD4⁺ and CD8⁺ T cells. Both express high levels of perforin, granzyme and granulysin, among other proteins [73]. Although the role of Eomes in CD4⁺ T cells is not well characterized, it has been implicated in helping to drive the production of IFN- γ in Th1 cells [11,74] and has been shown to be able to induce the expression of cytotoxic genes when overexpressed in Th2 cells [75]. Nonetheless, CD4⁺ effectors have been found to express Eomes at a considerably lower level than CD8⁺ CTLs [76]. A related transcription factor, Runt-related transcription factor 3 (Runx3), has been shown to be important for the induction of Eomes and the expression of perforin and granzyme B in CD8⁺ T cells [77]. In CD4⁺ T cells, Runx3 is known to play a role in Th1 polarization and IFN- γ production [78,79]; however, whether it is also involved in the induction of cytotoxic activity in CD4⁺ T cells has not yet been established. Further transcriptional studies of specific CD4⁺ subsets are needed to more rigorously dissect the differences that lead to cytotoxic effector functions in certain CD4⁺ T cells.

Recent work by Brown *et al.* has provided insight into some of the signals involved in the induction and regulation of cytolytic CD4⁺ T cells in the mouse model [69,80]. Unpolarized CD4⁺ T cells as well as *in vitro*-generated Th1, Th2 and Th17 cells all demonstrated a cytolytic phenotype, although at significantly varying levels. While all groups exhibited some degree of cytotoxicity, unpolarized effectors generated in the presence of IL-2 alone showed the greatest cytolytic ability, followed (in order) by Th1, Th2 and Th17 cells [69,80]. The blockade of IL-6, IL-10, IL-12, TNF- α or TGF- β did not have an effect on the polarization of cells to a cytolytic phenotype. However, IL-2 was necessary for the granule-mediated killing of peptide-pulsed B-cell targets, while Fas/FasL killing did not show IL-2 dependency [69]. The authors speculated that this was due to an induction of the perforin/granzyme pathway by IL-2. Indeed, IL-2 has a regulatory effect on perforin and granzyme expression in CD8⁺ CTLs and the case may be similar for CD4⁺ T cells [81,82]. A role in the induction of cytolytic activity in CD4⁺ T cells has also been proposed for IL-15 [36]. Similarly to IL-2, other γ -chain receptor cytokines such as IL-15 and IL-21 can induce expression of perforin and other cytolytic effector molecules [83–85]. IL-15 signaling additionally elicits the preferential production of CD4⁺ effector memory cells [86]. Therefore, while a cytolytic CD4⁺ T-cell phenotype can be induced in the presence of high levels of IL-2, it is currently unknown whether the corresponding transcriptional profile is significantly different from the Th1 CD4⁺ T-cell subset.

Cytolytic CD4⁺ T cells in HIV-1 infection

HIV-1 infection is characterized by progressive infection and depletion of CD4⁺ T cells. This is especially the case in acute HIV-1 infection, where up to 60% of all activated memory CD4⁺ T cells are infected and subsequently depleted from all tissue compartments [87]. Initial HIV-1 vaccine efforts aimed to avoid any CD4⁺ T-cell activation to avoid further generation of viral target cells. However, recent evidence has suggested that the induction of CD4⁺ T-cell responses might be particularly important for successful antiviral vaccines [88]. Most HIV-1-specific CD4⁺ T-helper responses are detectable during acute HIV-1 infection, but appear to gradually decline over the course of viremia [89]. The majority of HIV-1-specific CD4⁺ T-cell responses are directed against Gag and the persistence of these responses into the chronic phase of the infection has been repeatedly associated with better control of viral replication [89,90]. In addition to IFN- γ secretion, HIV-1-specific CD4⁺ T-cell responses also produce TNF- α and IL-2, suggesting a Th1-dominant antiviral CD4⁺ T-cell response in the absence of detectable IL-4 or IL-13 secretion [89].

Some of the first HIV-specific CD4⁺ T cell clones isolated from infected individuals were able to kill HIV envelope peptide-pulsed B cells [91], suggesting a direct cytolytic potential on the part of HIV-1-specific CD4⁺ T cells. Similar reports described cytolytic CD4⁺ T cells obtained from healthy individuals who received a recombinant gp160 subunit vaccine during an early HIV-1 vaccine trial [92–95]. However, these and most other studies of HIV-specific cytolytic CD4⁺ T cells relied on cell lines or clones that were expanded under long-term culture conditions *in vitro*; it is therefore difficult to draw conclusions about the relevance of cytolytic CD4⁺ T cells for the control of HIV-1 replication *in vivo*.

Cytolytic CD4⁺ T cells have also been proposed to be a byproduct of chronic viral infection, such as CMV or HIV, where persistent antigenic stimulation could result in the terminally differentiated phenotype that corresponds to cytolytic activity [96]. In the case of HIV infection, a population of perforin-positive CD4⁺ T cells can be found in all stages of disease progression; this subset is largest in patients with chronic, untreated disease and can represent up to half of the CD4⁺ T cells in the blood, although this percentage is highly patient dependent [36]. Additional analysis of lymphocytes from HIV-1-infected patients

stimulated with Gag peptide bolstered these findings, showing that approximately 50% of Gag-specific CD4⁺ T cells are able to degranulate, as measured by the presence of the surrogate degranulation marker CD107a [27]. Several studies have demonstrated cytolytic activity of CD4⁺ T cells from HIV-1 patients directly *ex vivo* in the killing of peptide-pulsed B cell target cells in chromium release assays [97–100]. In addition, in a recent Simian immunodeficiency virus (SIV) study, *ex vivo* peptide-pulsed and fluorescently-labeled target cells disappeared after reinfusion, even when test animals were depleted of CD8⁺ T cells [101]. This suggested a non-CD8⁺ T-cell-dependent mechanism of target cell lysis and led the authors to speculate that cytolytic CD4⁺ T cells might be involved. However, a direct contribution of these cells to the control of viral replication has not been shown. Thus, it is unknown whether cytolytic CD4⁺ T cells are also able to inhibit HIV-1 replication.

Two recent publications have begun to address the question of whether cytolytic CD4⁺ T cells may play a direct role in the control of virus in HIV-1-infected macrophages or CD4⁺ T cells. Sacha *et al.* demonstrated that macaques able to control SIV infection display strong Gag- and Nef-specific CD4⁺ T cell responses [102]. After long-term culture, these CD4⁺ T-cell responses were able to inhibit viral replication in SIV-infected macrophages, but were unable to recognize and kill infected CD4⁺ T cells in an *in vitro* viral replication assay [102]. By contrast, Zheng *et al.* demonstrated that Nef-specific CD4⁺ T-cell clones derived from human elite controllers were able to suppress viral infection in both macrophages and CD4⁺ T cells [103]. Both studies suggested a direct contribution of cytolytic CD4⁺ T-cell responses to the control of viral replication. The concept that cytolytic CD4⁺ T cells might recognize virally infected macrophages is interesting and potentially important for HIV-1 vaccine design. Although it has been shown that HIV-1-specific CD8⁺ T cells are strong contributors to the control of viral replication, escape mutations in the targeted epitopes can substantially impair their efficiency to recognize and kill HIV-1-infected cells [104]. Cytolytic CD4⁺ T cells, however, recognize targets via a class II pathway; infected MHC class II-expressing cells, such as other CD4⁺ T cells or macrophages, could therefore be recognized by a second mechanism. It has also been shown that macrophages play a role in the pathogenesis of HIV-1 by acting as long-lived reservoirs for viral persistence [105]. The combined recognition of virally infected antigen-presenting cells and CD4⁺ T cells by both cytolytic CD4⁺ and CD8⁺ cells may therefore play an important role in HIV-1 pathogenesis. However, it remains to be seen whether cytolytic HIV-1-specific CD4⁺ T-cell responses have any effect on viral inhibition *in vivo*.

Expert commentary

Compelling evidence suggests an unprecedented and critical role for cytolytic CD4⁺ effector responses in the control and clearance of pathogens in mice and humans. Although early studies have already reported on the presence of cytolytic CD4⁺ T cells, their existence has only recently been widely acknowledged and investigated in greater detail. Especially in viral infections – where pathogens can replicate unhindered within the confines of a cell – recognition through the antigen presentation machinery is pivotal for viral containment. Historically, the effector CD8⁺ T-cell response has been recognized to be the major contributor to the control of chronic viral infection. Virus-specific CD8⁺ T cells recognize target cells after viral antigen presentation through the MHC class I complex, and significant associations exist between the expression of certain HLA class I alleles and the ability to control the replication of viruses like HCV and HIV-1. Thus, there is strong evidence that CD8⁺ T cells play a major role in the lysis of infected target cells and therefore contribute to the control of viral replication. However, CD8⁺ T cells are also often unable to control viral replication alone, emphasizing the need for potential alternative pathways that can be induced by vaccines.

Although the overall role of cytolytic CD4⁺ T cells in the containment of infections *in vivo* remains to be determined, the presence of these responses raises important considerations for vaccine design. While MHC class I is ubiquitously expressed, MHC class II expression is limited to professional antigen-presenting cells such as B cells, monocytes/macrophages, dendritic cells and some epithelial cells [106]. The induction of cytolytic CD4⁺ T-cell responses by a vaccine might be especially important in the case of HIV-1 infection, where error-prone reverse transcription during viral replication results in a vast diversity of circulating strains and the rapid generation of escape mutations in CD8⁺ T-cell-targeted epitopes. These escape mutations often impair CD8⁺ T-cell recognition and contribute to an accelerated disease progression [104,107]. Viral immune escape has also been observed at CD4⁺ T-cell-targeted epitopes in several infections, including HCV and the LCMV mouse model [108,109]. While this phenomenon has not been widely studied to date, CD4⁺ T-cell-driven escape mutations have also been suggested to occur in the case of HIV infection, indicating the potential importance of CD4⁺ T-cell-mediated immune selection pressure in the control of viral replication [110]. Moreover, the HIV-1 accessory protein Nef has the ability to downmodulate the expression of MHC class I and therefore facilitates the evasion of CD8⁺ T-cell recognition directly [111]. Thus, it is possible that the simultaneous induction of cytolytic CD4⁺ T-cell responses as well as CD8⁺ T-cell responses bears several advantages in the context of viral infection: first, dual recognition through MHC class I and II increases the chance of killing virally infected target cells (Figure 2). Second, the nature of the MHC class II structure allows for a greater diversity in the type and number of viral epitopes that can be presented in comparison to MHC class I. Thus, the potential ability of a vaccine to induce virus-specific cytolytic CD4⁺ T cells to recognize and kill virally infected targets in concert with cytolytic CD8⁺ T cells represents a unique opportunity to contain viral replication early after HIV-1 acquisition. Infected antigen-presenting cells like macrophages may be particularly important targets of such a strategy, as these cells are believed to act as long-lived viral reservoirs [105].

Eliciting cytolytic CD4⁺ responses *in vivo* will require the development of innovative vaccines consisting of intelligently designed antigens and immunogens. Vaccines designed to specifically induce cytolytic CD4⁺ T-cell responses have not been studied, primarily due to the lack of understanding regarding their induction. However, efforts to broadly stimulate CD4⁺ T-cell responses in general have been more thoroughly investigated. Adenovirus vectors, for example, have been shown to induce potent memory CD4⁺ T-cell responses [112]. Likewise, vaccination of rhesus macaques with a DNA-prime, adenovirus-boost SIV vaccine regimen resulted in the generation of durable tissue-wide virus-specific CD4⁺ T-cell responses [113]. In the context of EBV, it was found that dendritic cells vaccinated with a modified vaccinia virus Ankara vector could effectively induce both CD4⁺ and CD8⁺ T-cell responses to antigen *in vitro* [114]. DNA vaccination efforts have also been successful at stimulating CD4⁺ T-cell responses. A test of an experimental anti-HIV-1 DNA vaccine in mice revealed that the promiscuous class II-targeting epitopes encoded by the vaccine could induce broad CD4⁺ T-cell responses in the context of several different MHC class II molecules [115]. Although the molecular mechanisms of antigen processing that lead to efficient presentation of peptides on MHC class II molecules are still unclear, it has also been found that CD4⁺ responses to specific DNA vaccine epitopes can be strengthened through the addition of lysosomal targeting sequences [116]. Lastly, rationally designed adjuvants are likely to be important for the efficient targeting of cytolytic CD4⁺ T-cell responses. Adjuvants like Toll-like receptor ligands, cytokines and costimulatory molecules can provide the proinflammatory and conditioning signals necessary to drive T-cell differentiation towards a particular lineage [117]. As new immunogens are designed and as the pathways that lead to the generation of cytolytic CD4⁺ T cells are uncovered, the specific stimulation of these responses *in vivo* is likely to move closer to reality.

Nonetheless, the induction of cytolytic CD4⁺ T-cell responses may be a double-edged sword. Research in LCMV-infected mice has shown that an induction of CD4⁺ effector responses can result in an increase in inflammation and a corresponding enhancement of viral pathogenesis [118]. Furthermore, while cytolytic and other desirable CD4⁺ effectors may be stimulated, unwanted regulatory CD4⁺ responses can also be amplified, potentially dampening any therapeutic response. Possible virus-specific pitfalls must also be considered. Activation and generation of virus-specific cytolytic CD4⁺ T-cell responses by a preventative vaccine may put patients at greater risk for HIV-1 infection, for example, as activated memory CD4⁺ T cells have been shown to be preferentially targeted by the virus [10]. In EBV infection, where cytolytic CD4⁺ T cells are recognized to play an important role in viral suppression, it has also been found that EBV-specific CD4⁺ T-cell responses can reactivate latently infected cells, possibly leading to the proliferation of cancerous transformed B cells [119]. More broadly, the function and role of cytolytic CD4⁺ T cells *in vivo* must be evaluated to determine their impact on the modulation of other antigen-specific B and CD8⁺ T-cell responses.

Five-year view

Within the next 5 years, considerable advances in our understanding of cytolytic CD4⁺ T cells are expected. One specific unanswered question is precisely when CD4⁺ T cells commit to the cytolytic CD4⁺ T-cell program during their lineage development. It is currently unknown if cytolytic activity is simply a function acquired by terminally differentiated CD4⁺ Th1 cells or Tregs, or if it represents a characteristic of a unique CD4⁺ T-cell subset (Figure 1). We expect that careful multiparametric analysis of the various CD4⁺ T-cell phenotypes that have shown cytolytic potential, in combination with detailed microarray profiling, will shed further light on this question. The identification of specific markers or transcriptional profiles will lead to a better *ex vivo* assessment of cytolytic CD4⁺ T-cell responses and therefore help to evaluate their role in different disease settings. A closely related question – and one especially relevant in the context of vaccine design – is how can cytolytic CD4⁺ responses be induced and/or maintained *in vivo*? Here it will be important to understand which antigens – but also whether specific adjuvants – play a role in the directed stimulation of cytolytic CD4⁺ T-cell responses. Vaccine trials in animals will start to elucidate if the induction of such responses can bring about better results than the primary induction of antigen-specific cytolytic CD8⁺ T cells. In terms of HIV-1 vaccine design, we will obtain a better understanding of whether the induction of cytolytic CD4⁺ T cells is beneficial for viral control or rather leads to the generation of activated target cells and accelerates disease progression. Finally, in the next few years, we expect to develop a better understanding of the differences between cytolytic CD4⁺ and CD8⁺ T-cell responses and under which circumstances one or the other pathway is preferentially elicited. This knowledge will be pivotal in guiding rational, reverse vaccine design, especially against viral infections that have so far eluded efforts to create effective vaccines.

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Figure 1. Cytolytic CD4⁺ T-cell lineage

Cytolytic CD4⁺ T cells can resemble both Th1 cells and regulatory T cells. However, it is unknown whether a cytolytic phenotype represents a highly differentiated effector state that can be assumed by different CD4⁺ subsets, or if cytolytic CD4⁺ T cells represent an entirely unique CD4⁺ T-cell lineage instead (dotted line). Although acquisition of cytolytic potential seems to be at least partially dependent on IL-2, the underlying transcriptional pathways have not been determined. Based on the similarities of cytolytic CD4⁺ T cells to regulatory T cells, Th1 and CD8⁺ T cells, it is possible that FoxP3, Tbet, Eomes and Runx3 may play important roles in inducing this activity. Similarly, it is possible that γ -chain cytokines such as IL-15 and IL-21 may participate in the generation of cytolytic CD4⁺ T-cell responses.

Figure 2. Hypothetical model for dual-pathway killing by cytolytic CD4⁺ and CD8⁺ T cells
Certain viruses like HIV-1 are able to establish infection within cells that express both MHC class I and class II molecules, such as antigen-presenting cells. Antigen presentation by these molecules is critical for recognition by circulating cytolytic CD4⁺ and CD8⁺ T cells. Even if the virus were to inhibit recognition through one pathway – for instance by downregulating MHC class I molecules or via the acquisition of escape mutations – effective recognition and lysis of the infected cell could still be achieved through the other pathway.