



Cytotoxic CD4 T Cells: Differentiation, Function, and Application to Dengue Virus Infection

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Dengue virus (DENV) has spread through most tropical and subtropical areas of the world and represents a serious public health problem. The control of DENV infection has not yet been fully successful due to lack of effective therapeutics or vaccines. Nevertheless, a better understanding of the immune responses against DENV infection may reveal new strategies for eliciting and improving antiviral immunity. T cells provide protective immunity against various viral infections by generating effector cells that cooperate to eliminate antigens and memory cells that can survive for long periods with enhanced abilities to control recurring pathogens. Following activation, CD8 T cells can migrate to sites of infection and kill infected cells, whereas CD4 T cells contribute to the elimination of pathogens by trafficking to infected tissues and providing help to innate immune responses, B cells, as well as CD8 T cells. However, it is now evident that CD4 T cells can also perform cytotoxic functions and induce the apoptosis of target cells. Importantly, accumulating studies demonstrate that cytotoxic CD4 T cells develop following DENV infections and may play a crucial role in protecting the host from severe dengue disease. We review our current understanding of the differentiation and function of cytotoxic CD4 T cells, with a focus on DENV infection, and discuss the potential of harnessing these cells for the prevention and treatment of DENV infection and disease.

Keywords: CD4 T cells, cytotoxicity, dengue virus, protection, vaccines

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DENV INFECTION AND A PROTECTIVE ROLE FOR CYTOTOXIC CD4 T CELLS

Dengue virus (DENV) is a major public health problem in tropical and subtropical areas with 390 million estimated infections per year (1). DENV has four serotypes (DENV 1–4), and infection with one of the serotypes can be asymptomatic or result in a range of diseases spanning from dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The more severe forms of DHF and DSS are more likely to develop following secondary infections with a different serotype (2). Other than supportive care there is currently no specific therapy available for the treatment of dengue diseases. Tremendous efforts have been devoted to the development of DENV vaccines since World War II, and a tetravalent chimeric vaccine, Dengvaxia[®], has recently been licensed in several countries including Mexico, Brazil, and the Philippines (3). However, several clinical trials of Dengvaxia[®] raise concerns about the efficacy of the vaccine. A phase 2b study in Thailand showed an overall efficacy of 30.2% with only 9.2% protection against DENV 2 (4). Additionally, two large-scale phase 3 trials in Asia and Latin America reported that the average efficacies against the four serotypes

were 56.5 and 60.8%, respectively, and further confirmed the lowest level of protection against DENV 2 (5, 6). Furthermore, a long-term follow up of these trials reveals that Dengvaxia® was less effective in seronegative vaccinees and resulted in an increased incidence of hospitalization among vaccinated children who were under 9 years old (7). Given the importance of host immunity in the protection of DENV infection, it is crucial to gain a better understanding of anti-DENV immune responses and identify the correlates of protection or susceptibility in order to improve the development of immunotherapies and vaccines for dengue disease.

T cells play important roles in fighting infections with intracellular pathogens; however, the roles of T cells during DENV infection may be complex. Although some studies suggest that T cells may contribute to the pathogenesis of DENV infection *via* the production of inflammatory cytokines, and that the expansion of preexisting cross-reactive memory T cells may impair viral control upon secondary heterologous infections (original antigenic sin), others indicate that T cells may play important roles in the protection against severe dengue disease (8). Stronger T cell responses generated following natural infection or vaccination with DENV as measured by the production of effector cytokines such as interferon- γ (IFN- γ) have been associated with better protection against subsequent DENV infection (9, 10). Additionally, our laboratory has demonstrated that protective human leukocyte antigen (HLA) alleles against dengue disease are associated with robust and polyfunctional CD8 T cell responses (11). Furthermore, the observation that the frequency of T cells that express CD107a, a degranulation marker, correlates with less severe dengue disease, supporting the notion that the roles of T cells during DENV infection may depend upon their functionality and that T cells with cytotoxic potentials may be crucial for the control of DENV infection (12).

Although cytotoxic functions are usually associated with CD8 T cells, accumulating evidence has demonstrated that a range of other cells can elicit cytotoxic effector functions. Dendritic cells (DCs) are the early, primary targets of DENV in natural infection, and the vigor of cell-mediated immunity is modulated by the relative presence or absence of IFN- γ in the microenvironment surrounding the virus-infected DCs (13). DCs including Langerhans cells (LCs) express CD1d, a molecule responsible not only for the presentation of lipopeptides but also conventional antigens that have a specific binding motif, i.e., hydrophobic amino acids in position 1, 4, and 7 (14). CD1d-restricted natural killer T (NKT) cells are a distinct subset of T cells that rapidly produce an array of cytokines upon activation and play a critical role in regulating various immune responses. NKT cells are classified into two groups based on differences in T-cell receptor usage. Type I NKT cells have an invariant T-cell receptor α -chain (iNKT), while Type II NKT cells have a more diverse T-cell receptor repertoire, and it has been shown that CD4 engagement by CD1d potentiates activation of CD4⁺ NKT cells (15, 16). Recent evidence suggests iNKT involvement in DENV pathogenesis, and the level of iNKT cell activation associates with the disease severity (17–19). Finally, another unconventional T cell subset, gamma delta ($\gamma\delta$) T cells, has been shown to be able to kill dengue-infected cells

and contribute to the immune response during DENV infection by providing an early source of IFN- γ (20).

This review focuses on CD4 T cells that can also acquire a cytotoxic phenotype, which has been investigated by numerous studies over the past three decades (21). The ability of CD4 T cells to acquire cytotoxic functions have been mostly attributed to T helper type 1 (Th1) cells after viral infections; however, it is now clear that other CD4 T cell subsets including regulatory T (Treg) cells can also secrete effector molecules and exert cytotoxic effects (22, 23). Moreover, recent studies further suggest that cytotoxic CD4 T cells may represent a separate lineage independent of other CD4 T cell subsets and are induced by distinct environmental cues and transcriptional regulators, highlighting the versatility of CD4 T cell responses (24–26). Notably, cytotoxic CD4 T cells are readily detectable following DENV infection and correlate with enhanced protection against dengue disease (12, 27). We discuss the differentiation and function of cytotoxic CD4 T cells, especially in the context of DENV infection, and anticipate future studies into the therapeutic potentials of these intriguing cells in the development of anti-DENV vaccines and immunotherapies.

CELLULAR AND ENVIRONMENTAL FACTORS THAT MEDIATE THE GENERATION OF CYTOTOXIC CD4 T CELLS

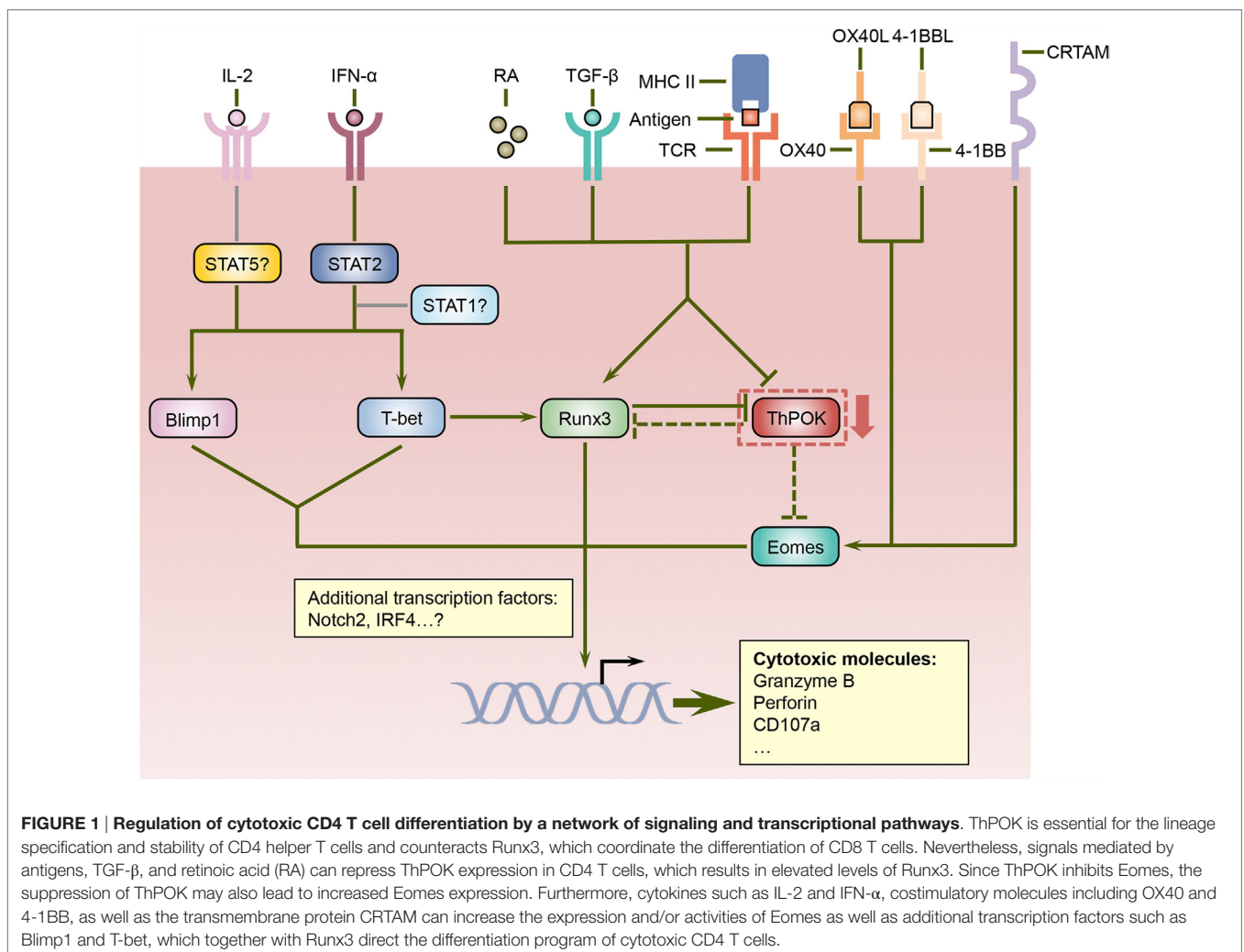
The differentiation of diverse CD4 T cell subsets is induced and guided by antigens, costimulation, and distinct sets of cytokines, which are integrated to regulate the expression of transcription factors that are crucial for CD4 T cell lineage specification (28). In line with this notion, costimulatory signals mediated by OX40–OX40L and 4-1BB (29–31), as well as cytokines such as transforming growth factor- β (TGF- β), type I interferons and IL-2 (25, 32–34), have been suggested to promote the differentiation of cytotoxic CD4 T cells. Although cytotoxic CD4 T cells are often observed during chronic infections such as HIV, Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), and mouse CMV (MCMV) infections (35–39), they are also readily detectable following acute lymphocytic choriomeningitis virus (LCMV), influenza virus, and ectromelia virus infections (40–44). Therefore, persistent antigenic stimulation may not be absolutely required for cytotoxic CD4 T cell differentiation. Furthermore, Brown et al. reported that IL-2 substantially enhances the cytotoxic functions of CD4 T cells that are activated with low antigen dose *in vitro*, suggesting that inflammatory cytokines may amplify T cell receptor (TCR) signals to promote the differentiation of cytotoxic CD4 T cells (32). Two additional common cytokine receptor γ -chain (γ_c) family cytokines, IL-7 and IL-15, however, are dispensable for the formation of cytotoxic CD4 T cells, although IL-15 may promote their effector functions upon reactivation with TCR stimulus (24). Interestingly, IL-21, which is another member of the γ_c family, has been shown to increase the production of the cytotoxic molecule granzyme B in CD8 T cells both *in vivo* and *in vitro* (45, 46). Thus, it would be interesting to investigate

whether IL-21 also plays a role in the generation and/or functional maturation of cytotoxic CD4 T cells.

MOLECULAR REGULATION OF CYTOTOXIC CD4 T CELL DIFFERENTIATION

The integration and interpretation of numerous cellular and environmental parameters are mediated by transcriptional factors, and a number of transcriptional regulators have been implicated in the differentiation program of cytotoxic CD4 T cells (**Figure 1**). T-helper-inducing POZ/Kruppel-like factor (ThPOK) and Runt-related transcription factor 3 (Runx3), which suppress each other's expression, control the development of CD4 and CD8 T cells in the thymus, respectively (47). After exiting the thymus, mature CD4 T cells continue to express ThPOK, which suppresses Runx3 and maintains the lineage stability of CD4 T cells (48–50). Ectopic expression of ThPOK in CD8 T cells results in reduced expression of CD8, the T-box transcription factor eomesodermin (Eomes), as well as effector molecules such as

IFN- γ , granzyme B, and perforin, further supporting the notion that ThPOK restricts the initiation of cytotoxic T lymphocyte (CTL) differentiation program in CD4 T cells (51). In contrast, Runx3 promotes CD8 expression by binding its enhancer regions (52, 53) and also cooperates with Eomes and another T-box transcription factor, T-bet, to induce the manufacture of IFN- γ , perforin, and granzyme B (54, 55). Intriguingly, a portion of CD4 T cells downregulates their expression of ThPOK in the intestine, especially in the intraepithelial lymphocyte (IEL) compartment, under unimmunized conditions or following activation with their cognate antigen (24, 25). Conversely, these ThPOK^{low} CD4 T cells upregulate the expression of Runx3, thus resembling their CD8 T cell counterparts (24, 25). Consequently, these cells showed enhanced expression of cytotoxic effector lymphocytes-associated molecules, including 2B4, granzyme B, and CD107a, and demonstrated cytotoxicity *in vitro* (24, 25). It is further proposed that sustained antigenic stimulation and/or local environmental cues such as TGF- β and retinoic acid (RA) induce the downregulation of ThPOK and the upregulation of Runx3, although the underlying signaling and transcriptional mechanisms are less well defined (24, 25). Thus, the antagonistic



expression of ThPOK and Runx3 not only dictate the lineage stability of CD4 and CD8 T cells but also direct the establishment of cytotoxic CD4 T cells. Further studies are needed to determine whether and how the expression of ThPOK and Runx3 dictate the development of cytotoxic CD4 T cells following viral infections as well as the cellular and environmental factors that modulate their expression.

In addition to ThPOK and Runx3, several other transcription regulators have been suggested to regulate the differentiation of cytotoxic CD4 T cells. T-bet promotes the differentiation of effector CD8 T cells (56) and also induces the expression of Runx3 in CD4 T cells (54), suggesting that it may play a role in the formation of cytotoxic CD4 T cells. Indeed, T-bet-deficient CD4 T cells demonstrated substantially reduced production of granzyme B following influenza virus infection (33). Furthermore, the acquisition of cytotoxic functions by CD4 T cells is also dependent upon B lymphocyte-induced maturation protein 1 (Blimp1), which facilitates the binding of T-bet to the granzyme B and perforin promoters (33). The upstream signals that drive the expression of T-bet and Blimp1 in CD4 T cells include IL-2 and IFN- α , and IFN- α may also exert its effects partially *via* signal transducer and activator of transcription 2 (STAT2) as evidenced by decreased T-bet and granzyme B expression in the absence of STAT2 (33). Since heterodimerization with activated STAT1 is required for the nuclear accumulation of phosphorylated STAT2 (57), whether STAT1 promotes cytotoxic CD4 T cell development in conjunction with STAT2 warrants further investigation. Surprisingly, STAT4, which has been shown to promote T-bet expression and Th1 cell differentiation in response to IL-12 signals (58–60), is dispensable for the upregulation of T-bet and granzyme B in CD4 T cells following influenza virus infection (33), indicating that cytotoxic CD4 T cells and Th1 cells may rely on distinct pathways for their differentiation. As discussed above, ThPOK suppresses the expression of Eomes, which cooperates with T-bet and Runx3 to promote the effector functions of CD8 T cells (55, 61–63), suggesting that Eomes may also participate in programming cytotoxic CD4 T cells when its expression is increased. Indeed, the engagement of costimulatory molecules OX40 and/or 4-1BB induces the expression of Eomes, which then upregulates the production of granzyme B by CD4 T cells and enhances their antitumor activities (29–31). In addition, a recent report shows that class I-restricted T cell-associated molecule (CRTAM) can promote the expression of Eomes and cytotoxic proteins including granzyme B and perforin in CD4 T cells (64). Therefore, Eomes may coordinate multiple signaling pathways to direct the development of cytotoxic CD4 T cells. Additional transcriptional regulators such as Notch2 (65), STAT5 (66), and interferon regulatory factor 4 (IRF4) (67) have been implicated in the manufacture of cytotoxic weaponry in CD8 T cells, and it would be interesting to investigate whether these transcriptional regulators also modulate the cytotoxic potential of CD4 T cells. In sum, the signals mediated by TCR, cytokines, costimulatory molecules, and other cell surface receptors are integrated and interpreted by a network of transcriptional regulators, which collectively orchestrate the differentiation of cytotoxic CD4 T cells.

THE DEVELOPMENT OF CYTOTOXIC CD4 T CELLS FOLLOWING DENV INFECTIONS

DENV-specific CD4 T cells with cytotoxic potential were initially observed with T cell clones isolated from a DENV-infected donor (68). These CD4 T cell clones demonstrate *in vitro* killing of target cells that display DENV antigens as assessed by chromium release assays, which are restricted by HLA class II molecules (68). Subsequent studies discovered that DENV non-structural (NS) proteins especially NS3 are the major targets of cytotoxic CD4 T cell clones and that many of these cell clones exhibit cross-reactivity against several DENV serotypes (69–72). Thus, cytotoxic CD4 T cell may preferentially recognize conserved DENV antigens, and repeated antigenic stimulation may favor their formation. In addition to antigen-specific killing, anti-DENV cytotoxic CD4 T cell clones generated from DENV-immune donor can also mediate the lysis of non-antigen-presenting bystander target cells (73). While cytotoxic CD4 T cells lyse target cells pulsed with DENV antigens primarily *via* perforin-dependent mechanisms, the lysis of bystander target cells mainly relies on the Fas/Fas ligand (FasL) pathway (73). Additionally, a cytotoxic CD4 T cell clone has also been isolated from DENV-infected mice and is able to kill DENV antigen-pulsed target cells *in vitro* (74). Furthermore, Yauch et al. showed, using a mouse model of DENV infection, that CD4 T cells can mediate DENV-specific killing of target cells *in vivo*, although the production of cytotoxic molecules by CD4 T cells was not assessed (75). Immunization with CD4 T cell epitopes derived from DENV NS proteins NS2B and NS3 can accelerate viral clearance following DENV challenge, suggesting that the induction of cytotoxic CD4 T cells by vaccination may be beneficial for the control of secondary infections with DENV (75).

Previous studies have demonstrated that CD4 T cells with cytotoxic potential as assessed by the expression of CD107a are present in patients associated with both primary and secondary DENV infections, although the frequencies of these cells vary according to infection history and disease severity (12). Interestingly, the frequency of DENV-specific CD107a⁺ CD4 T cells is higher in DF patients compared with those who had a more severe form of the disease, DHF, implicating a protective role for cytotoxic CD4 T cells in DENV-infected patients (12). Our laboratory has recently discovered that a subset of CD4 T cells expand as a function of DENV infection history and is most prominently represented in donors associated with multiple DENV infections (27). These CD4 T cells display a CD45RA^{high}CCR7^{low} phenotype, which is distinct from their CD45RA^{low}CCR7^{high} central memory T (T_{cm}) and CD45RA^{low}CCR7^{low} effector memory T (T_{em}) counterparts, and thus are designated effector memory RA T (T_{emra}) cells. Compared with CD4 T_{cm} or T_{em} cells, a higher proportion of CD4 T_{emra} cells express CD8 α , the degranulation marker CD107a, as well as other cytotoxic effector molecules such as granzyme B and perforin, suggesting that CD4 T_{emra} population contains anti-DENV cytotoxic CD4 T cells (27). Additionally, CD4 T_{emra} cells also had increased expression of CD226 (27), which is a costimulatory molecule that has been shown to enhance the effector and cytotoxic functions of CD8 T cells (76, 77). Conversely, these cells downregulate CD28

expression (19), which is consistent with previous observations that CD4⁺CD28⁻ T cells are associated with enhanced cytotoxic functions following infections with CMV and hepatitis B virus (HBV) (78, 79). As discussed earlier, the T-box transcription factors T-bet and Eomes coordinate the development of cytotoxic CD4 T cells. Notably, the vast majority of CD4 Temra cells express high levels of T-bet and Eomes, further supporting the notion that CD4 Temra cells encompass cytotoxic CD4 T cell subset in terms of their phenotypic and functional attributes, as well as their transcriptional signatures. Since cytotoxic CD4 Temra cells are generally detected following secondary DENV infections, these findings further support that DENV-specific cytotoxic CD4 T cells are induced by repeated TCR stimulation from conserved DENV antigens, which is consistent with *in vitro* studies using cytotoxic CD4 T cell clones. Intriguingly, CD4 Temra cells with cytotoxic potential observed in secondary DENV-infected donors phenotypically and functionally resemble the live attenuated yellow fever vaccine 17D (YF-17D)-elicited CD8 Temra cells, which are highly proliferative and polyfunctional as evidenced by their ability to produce various cytokines and cytotoxic molecules including CD107a (80). Since vaccination with YF-17D has been tremendously successful in controlling yellow fever virus (81), Temra phenotype cytotoxic CD4 T cells may be highly relevant in vaccine-elicited protection against DENV infection.

The majority of DENV-specific CD4 Temra cells are not associated with the phenotypes of Th1, Th2, or Th17 cells as evidenced by their lack of expression of the chemokine receptors CXCR3, CCR4, and CCR6, which have been used to distinguish between these distinct CD4 T cell subsets (27, 82). Since CD4 Temra cells encompass cytotoxic populations, these findings further support the notion that cytotoxic CD4 T cells may represent an

independent CD4 T cell lineage. Notably, Temra cells upregulate the expression of the chemokine receptor CX3CR1, which binds to CX3CL1 (fractalkine) and has been implicated in promoting the adhesion and migration of CD8 T cells (83, 84). Thus, CX3CR1 may facilitate the trafficking of DENV-specific CD4 T cells, particularly cytotoxic CD4 T cells, to infected tissues. Intriguingly, accumulating studies have demonstrated that CD4 tissue-resident memory T (Trm) cells reside in sites of pathogen entry and are crucial for the control of viral pathogens such as influenza virus and herpes simplex virus by providing immediate effector functions (85, 86). Therefore, it would be interesting to investigate whether DENV-specific CD4 Trm cells develop following infections and whether CX3CR1 plays a role in their establishment and maintenance in non-lymphoid tissues such as the skin. Importantly, the expression of CX3CR1 is also associated with the cytotoxic functions of CD4 T cells as isolated CX3CR1⁺ CD4 T cells demonstrated specific killing of DENV epitopes-pulsed target cells *ex vivo* (27). This is consistent with a recent report showing that CX3CR1⁺ CD8 T cells have potent cytotoxic functions and express elevated levels of granzyme B and perforin (87). Thus, CX3CR1⁺ cytotoxic CD4 T cells may play an important role in viral control by directly targeting DENV-infected cells in tissues.

CYTOTOXIC CD4 T CELLS CORRELATE WITH HLA-ASSOCIATED PROTECTION AGAINST DENV INFECTION

Additional insights into the question of whether cytotoxic CD4 T cells are beneficial in the context of DENV infections come

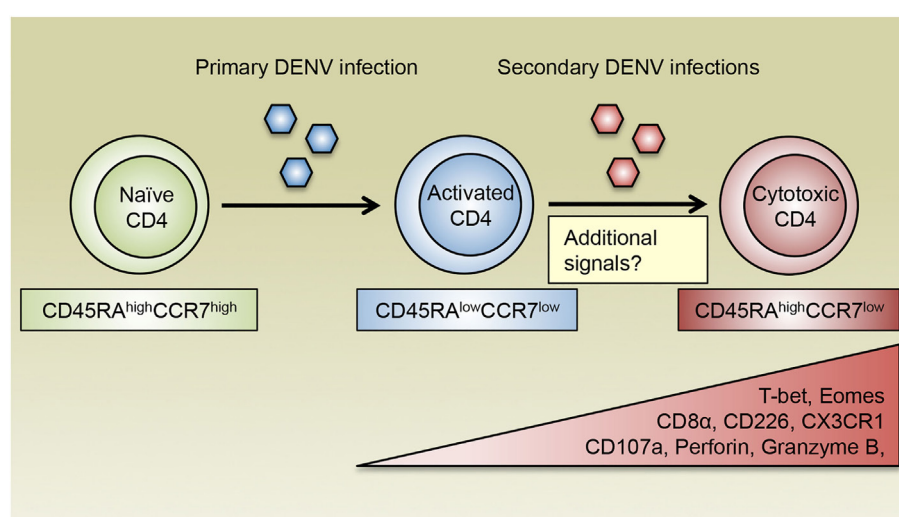


FIGURE 2 | The generation of cytotoxic CD4 T cells following DENV infections. DENV-specific naïve CD4 T cells are activated during primary DENV infection and acquire an effector phenotype and the ability to produce inflammatory cytokines such as IFN- γ . Following reexposure to secondary heterologous infections, DENV-specific CD4 T cells receive repeated antigenic signals and differentiate into cytotoxic CD4 T cells, which display a CD45RA^{high}CCR7^{low} Temra phenotype and are characterized by their expression of the chemokine receptor CX3CR1. Cytotoxic CD4 T cells also upregulate the expression of CD8 α and CD226, as well as the transcription factors T-bet and Eomes, which may cooperate with additional transcription regulators to induce the production of cytotoxic molecules such as CD107a, perforin, and granzyme B. In addition to recurring antigens, costimulatory molecules, cytokines, and other environmental cues are all likely to modulate the differentiation of cytotoxic CD4 T cells.

from the association of HLA allelic variants with relative susceptibility or resistance to severe DENV-associated diseases. For instance, HLA class II molecules DRB1*04:01 and DRB1*08:02 are associated with resistance and susceptibility to severe DENV diseases, respectively (88–90). Intriguingly, the frequency of DENV-specific CD4 Temra cells is higher in donors expressing the protective allele DRB1*04:01 compared with those expressing the susceptible allele DRB1*08:02. Moreover, CD4 Temra cells restricted by DRB1*04:01 express substantially higher levels of cytotoxic proteins including CD107a, perforin, and granzyme B compared with their DRB1*08:02-restricted counterparts. Thus, increased abundance and functionality of cytotoxic CD4 Temra cells may be associated with enhanced protection against severe dengue disease. Furthermore, the capacity of CX3CR1⁺ CD4 T cells to kill target cells varies according to HLA restrictions with the protective allele DRB1*04:01-restricted cells showing higher cytotoxic activities than their susceptible allele DRB1*08:02-restricted counterparts, which again indicates that cytotoxic CD4 T cell responses correlate with protection from severe dengue disease. Since DENV primarily infects major histocompatibility complex (MHC) class II-expressing antigen-presenting cells such as monocytes, macrophages, and DCs (91, 92), which could be exacerbated by antibody-dependent enhancement (ADE) mechanism (93), cytotoxic CD4 T cells may play important roles in controlling the spread of DENV infection by directly eliminating these cells. Other cell types such as epithelial cells can be induced to upregulate the expression of MHC class II molecules following viral infection and potentially become additional targets for cytotoxic CD4 T cells (43). Therefore, cytotoxic CD4 T cells may contribute to HLA-associated protection against DENV infection by targeting DENV-infected cells of various types. Taken together, we propose a model where repeated antigenic signals as well as other potential cellular and environmental stimuli facilitate the formation of DENV-specific cytotoxic CD4 T cells, which exhibit a Temra phenotype with upregulated CX3CR1 expression and mediate protective responses against DENV infection (Figure 2).

CONCLUSION AND PERSPECTIVE

Despite the recent approval of Dengvaxia[®] in several countries where DENV is epidemic, our need for an efficacious DENV vaccine is still unsatisfied. Accumulating studies strongly indicate that, in addition to CD8 T cells, cytotoxic CD4 T cells may

play an important role in eliminating DENV-infected cells and protecting the hosts from severe dengue disease. Since the generation of cytotoxic CD4 T cells is concurrent with multiple DENV infections, it would be interesting to identify the TCR-specificity of cytotoxic CD4 T cells and confirm whether they respond to conserved epitopes shared by different DENV serotypes. This would allow for the design of vaccines that include such epitopes and preferentially induce the formation of cytotoxic CD4 T cells. In addition to vaccines, adoptive transfer of engineered CD4 T cells that are specific for such antigens may accelerate viral clearance and benefit the treatment of DENV infection, as clinical trials of T cells that are engineered to express transgenic TCRs or chimeric antigen receptors (CARs) have generated promising results in treating cancers (94). Furthermore, costimulatory molecules and cytokines that are involved in the differentiation and function of cytotoxic CD4 T cells may potentially be used as adjuvants to enhance the cytotoxic effects of DENV-specific CD4 T cells. Additionally, elucidating the factors that control the development of cytotoxic CD4 T cells may allow one to manipulate their expression, availability, and activity in order to reshape CD4 T cell responses in patients expressing susceptible alleles and redirect antiviral CD4 T cells to differentiate into potent cytotoxic cells. Finally, T cell differentiation and function is greatly influenced by nutrients and metabolism (95). For instance, glucose and glycolysis promote the expression of cytotoxic molecules in CD8 T cells (96). Thus, modulation of the metabolic program may provide additional opportunities to enhance cytotoxic CD4 T cell response. In summary, future investigations into the antigenic, environmental, and cellular parameters that configure the formation, migration, and maintenance of cytotoxic CD4 T cells may reveal novel strategies for developing and improving vaccines and therapies that fight DENV as well as other emerging pathogens.

AUTHOR CONTRIBUTIONS

YT wrote the manuscript; YT and DW designed the figures; AS and DW critically edited the manuscript.

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