Virus-specific CD4+ T cells: ready for direct attack

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CD4+ T cells are classically thought to orchestrate adaptive immune responses. But recent studies demonstrate that they can also kill infected cells directly. A new paper shows that highly efficient processing of Epstein Barr virus (EBV) glycoproteins for presentation on MHC class II makes virus-transformed B cells susceptible to lysis by CD4+ T cells. Thus, antiviral vaccines should aim to stimulate both helper and cytolytic CD4+ T cells.

The immune system preserves the integrity of its host by recognizing and resisting invaders. Therefore, evolution has provided each species with the capacity to resist the pathogenic challenges in its ecological niche. The functions of different species' immune systems, tailored to meet these challenges, are therefore best explored by investigating host resistance to pathogens threatening the survival of that species. Examples of such pathogens are γ-herpesviruses, which include viruses that infect the majority of the human population and threaten host survival by their oncogenic capacity. In this commentary, we discuss recent evidence from studies of γ-herpesviruses that CD4+ T cells not only orchestrate adaptive immune responses in mice and man, but also have mechanisms to target infected cells directly.

CD4+ T cells are essential in the control of herpesvirus infections

Studies of mouse γ-herpesvirus 68 (MHV-68) have shown that CD4+ T cells can control a herpesvirus infection and its malignant consequences in vivo, independent of CD8+ T cells and B cells. MHV-68 establishes asymptomatic chronic infection in mice after respiratory challenge due to an immune response that contains but does not eliminate the virus. Immune control of MHV-68 infection can be established in the absence of CD8+ T cells, but depletion of both CD4+ and CD8+ T cells leads to a fatal primary infection (1). Depletion of CD8+ T cells resulted in higher virus loads in lung and spleen, suggesting that CD4+ T cells were more efficient at controlling MHV-68 infection in the presence of CD8+ T cells.

In addition to causing chronic infections, MHV-68 is oncogenic (although more weakly so than several of the human γ-herpesviruses). Virus-specific CD4+ T cells, but not CD8+ T cells, have been shown to eliminate tumors that were induced by injection of a MHV-68–infected B cell lymphoma cell line into T cell–deficient (*nude*) mice (2). After adoptive transfer of both CD4+ and CD8+ T cells, tumor-infiltrating lymphocytes were found to be primarily CD4 positive. More recently, CD4+ T cell–mediated immune control of MHV-68 has been shown to be independent of B cells (in addition to CD8+ T cells) (3).

There is also in vitro evidence that the human γ-herpesvirus Epstein Barr virus (EBV) and its malignant consequences are controlled by CD4+ T cells. EBV establishes persistent infection in more than 90% of the human adult population and is associated with tumors of epithelial and B cell origin, which fortunately develop in less than 10 per 100,000 individuals annually in most populations. Usually EBV-transformed B cells cannot grow out from peripheral blood mononuclear cells (PBMCs) of healthy EBV carriers unless the cultures are subject to immune suppression, for example, with cyclosporine A, which indicates that these individuals harbor immune cells that can target EBV-transformed B cells. Depletion of CD4+ T cells abolished this immune control (4), and addition of CD4+ T cells to T cell–depleted, EBV-infected PBMCs inhibited B cell transformation by EBV more efficiently than unsorted T cells. In agreement with this initial study, CD4+ T cell clones specific for nuclear antigen 1 or 2 of EBV (EBNA1 and EBNA2) were found to inhibit outgrowth of EBV-transformed B cells (5, 6). These studies suggest that memory CD4+ T cells specific for EBV antigens can control B cell transformation by EBV.

B cell transformation by EBV can also be inhibited by EBV-specific CD4+ T cells derived from the purified CD4+ T cells of EBV seronegative donors that were primed in vitro by dendritic cells (DCs) (7). Control over transformed latently infected B cells was evident in these cultures after 12 days, i.e., with the slow kinetics typical for primary immune responses.

Together these findings show that EBV infection in vitro can be controlled by virus-specific CD4+ T cells primed in vitro (i.e., a primary response) and by virus-specific CD4+ memory cells primed in vivo (i.e., a secondary response). It appears that this control can be exerted by CD4+ T cells directly, independent of CD8+ effector cells, which suggests that CD4+ T cells can mediate virus-specific immune control as effectors in their own right.

Virus-specific CD4+ T cells have cytolytic functions

Although it is widely accepted that CD4+ T cells provide helper functions for antigen-presenting cells and can restrict viral replication by secreting cytokines (1), there is also data that virus-specific CD4+ T cells can directly kill infected cells. EBV-specific cells were among the first CD4+ cytolytic T lymphocytes (CTLs) to be isolated, using EBV-transformed B cells, such as

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lymphoblastoid cell lines (LCLs), as targets. The initial EBV-specific CD4$^+$ CTLs to be studied were clones derived from healthy donors' PBMCs after prolonged in vitro expansion with LCLs (8), and therefore, there was the possibility that these had acquired cytolytic behavior in culture. However, subsequent studies described CD4$^+$ CTLs in PBMCs without the need for prolonged in vitro expansion. These CD4$^+$ CTLs recognized a variety of different EBV proteins expressed in both latent—EBNA1 (9), EBNA2 (10), EBNA3C (10), and latent membrane protein 2 (LMP2) (11)—and lytic—BHRF1 (12), BALF4, and BLLF1 (13)—stages of EBV infection. Apoptosis induced by the ligand of the death receptor Fas (FasL) (5, 9) and killing by the death effector molecule perforin (11) were both identified as cytotoxic mechanisms of EBV-specific CD4$^+$ CTLs. Interestingly, the EBNA1-specific CD4$^+$ CTLs and the BLLF1-and BALF4-specific CD4$^+$ CTLs described in this issue were found to target Burkitt's lymphoma, an EBV-associated B cell lymphoma which resists recognition by CD8$^+$ T cells due to down-regulation of the MHC class I antigen processing machinery. Therefore, particularly in humans where nearly all tissues can express MHC class II molecules after immune activation, virus-specific CD4$^+$ CTL could fill the gap left by viral immune escape from CD8$^+$ CTL recognition.

The presence of CD4$^+$ CTL is neither rare nor exclusive to EBV immunity and to in vitro–cultured T cell lines. For example, in mice infected with the murine lymphocytic choriomeningitis virus (LCMV), killing by CD4$^+$ CTLs was observed in vivo after injection of fluorescent-labeled target cells coated with viral peptides (14). This LCMV-specific CD4$^+$ T cell cytotoxicity was at least in part due to FasL-induced apoptosis. In addition, human peripheral blood contains CD4$^+$ T cells that are positive for the cytotoxic effector molecules perforin and granzyme (15). These CD4$^+$ CTL are fully differentiated effector cells and some are specific for the β-herpesvirus human cytomegalovirus (HCMV). Therefore, cytotoxic CD4$^+$ T cells have now been reported in vitro and in vivo.

Antigen processing pathways that lead to CD4$^+$ T cell recognition of virus-infected cells

The new concept of direct immune control by CD4$^+$ CTL raises questions on the pathways that lead to presentation of viral antigens on MHC class II molecules by target cells. Classically, MHC class II molecules display peptides processed from extracellular antigens after endocytosis. However, for CD4$^+$ T cell recognition of virus-infected cells, both exogenous and endogenous pathways for peptide loading of MHC class II molecules have been described (Fig. 1). Uptake of virus particles or fragments of infected cells has been found to mediate CD4$^+$ T cell recognition of EBV-associated tumor cell lines. The early lytic EBV antigen BHRF1 elicited CD4$^+$ T cell–mediated cytolysis of EBV-transformed B cells (16). MHC class II presentation of BHRF1 was most likely due to uptake of antigen-containing fragments (Fig. 1, pathway 1) from the few LCLs where EBV spontaneously entered lytic replication (typically less than 5%). However, the cytolysis was only moderately effective (40–80% of LCLs were killed), and 21 days were required for antigen transfer. CD4$^+$-mediated lysis that is much more efficient with respect to processing kinetics and required antigen amount is reported by Adhikary et al. in this issue (p. 995), who examined targeting of the late lytic EBV antigens and envelope proteins BLLF1 and BALF4 (13). Transfer of less than 1 EBV virion per target cell was sufficient to trigger IFNγ secretion by BLLF1-and BALF4-specific CD4$^+$ T cells as early as 12 hours after infection. EBV virion uptake for this MHC class II presentation was mediated by the interaction of BLLF1 with the B cell surface receptor CD21 (Fig. 1, pathway 4). Presentation of transferred BLLF1 and BALF4 even led to lysis of Burkitt's lymphoma cell lines, an EBV-associated malignancy that can support higher levels of lytic replication than LCLs in vitro and might, therefore, be especially well targeted via this pathway.

In addition to these exogenous pathways for MHC class II presentation of viral antigens from infected cells, efficient sensitization of virally infected cells for CD4$^+$ T cell recognition and killing has also been demonstrated after intracellular transport of viral antigens. Endogenous pathways for presentation of viral antigens include co-migration of viral membrane proteins with MHC class II molecules to MHC class II loading compartments (MIICs) via the secretory pathway (Fig. 1, pathway 2). Two prominent examples of viral proteins following the secretory pathway to intersect with MIICs are the HCMV envelope protein gB (17) and the influenza envelope protein hemagglutinin (HA) (18). Glial, epithelial, and endothelial cells were shown to present low levels of HCMV gB to cytolytic CD4$^+$ T cells (17). Similarly, mutated influenza HA, which was retained in the Golgi apparatus, sensitized human lymphoblastoid cells to killing by CD4$^+$ T cells after processing of HA through the secretory pathway (18). Later, it was proposed that association of HA with MHC class II molecules directs HA from the Golgi apparatus to the MIIC for efficient MHC class II loading. However, it remains unclear how efficient this pathway is since most cells block MHC class II loading in the endoplasmic reticulum by the invariant chain (19).

The second endogenous MHC class II antigen processing pathway that has been shown to elicit CD4$^+$ T cell recognition of virus-infected cells involves macroautophagy. During this degradation pathway, the cell engulfs its own cytoplasm by forming autophagosomes, which are then transported to lysosomes. The nuclear EBV antigen EBNA1 was found to accumulate in autophagosomes after inhibition of lysosomal degradation, and EBNA1-specific CD4$^+$ T cell recognition of EBV-transformed B cells could be down-regulated by pharmacological or siRNA-mediated inhibition of macroautophagy (20).

Together, these experiments show that cytosolic, nuclear, and membrane-bound viral antigens can sensitize cells

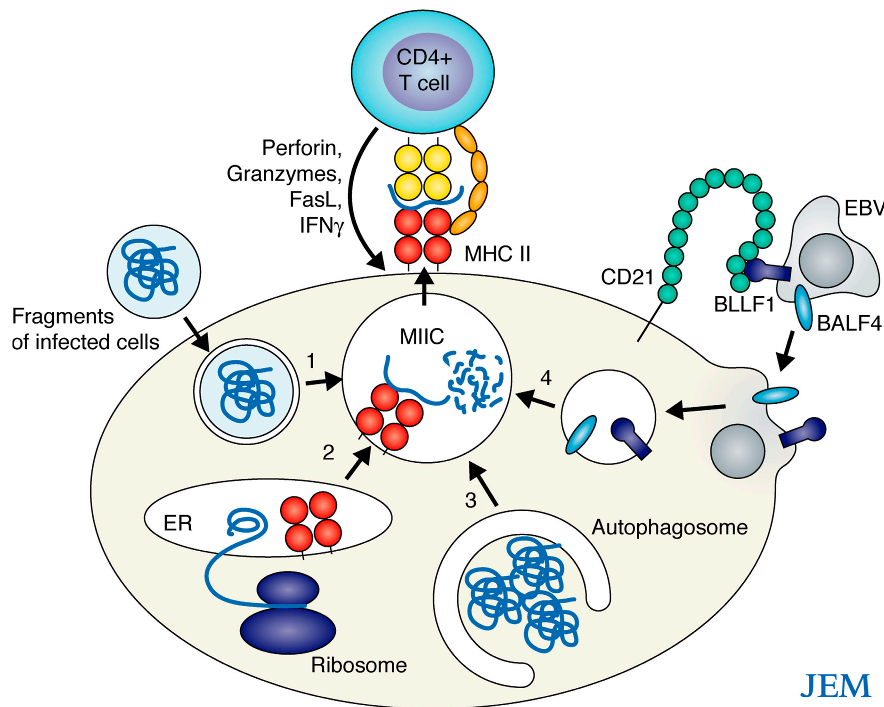

Figure 1. Exogenous and endogenous MHC class II antigen processing pathways lead to direct recognition of virus-infected cells by CD4+ T cells. Uptake of infected cell fragments (1) or virions (4) leads to MHC class II presentation of viral antigens. In addition, viral envelope as well as cytosolic or nuclear antigens reach the MIIC via the secretory pathway from the endoplasmic reticulum (ER) (2) or after macroautophagy of cytoplasm (3), respectively. After transport to the cell surface, the complexes of MHC class II molecules and viral antigen fragments are recognized by CD4+ T cells, which then either secrete IFNγ to restrict viral replication or kill the infected cell via FasL and perforin-granzyme–dependent mechanisms.

for CD4+ T cell–mediated cytolysis in vitro. Whether these endogenous and exogenous antigen presentation pathways contribute to the immune control of infections and tumors in vivo remains to be established.

Vaccines should induce both helper and cytolytic virus-specific CD4+ T cells

The discovery of a cytolytic effector function of virus-specific CD4+ T cells indicates that CD4+ T cells can do more than simply help other lymphocytes. Cytolytic CD4+ T cells have been targeted in recent approaches to rational vaccine design, a good example being the development of vaccines specific for EBV. Earlier immunization attempts focused on inducing CD8+ CTLs against the viral antigens present in EBV-associated malignancies, for example LMP2, which is expressed in nasopharyngeal carcinomas (NPCs). DCs pulsed with CD8+ T cell epitopes of LMP2 were injected into inguinal lymph nodes of NPC patients, where they induced detectable but transient LMP2-specific CD8+ CTLs. Unfortunately, as with other antitumor vaccines that have focused solely on the induction of CD8+ T cells, the epitope-specific responses were either too weak or too transient to achieve clinical responses (21). Since CD4+ T cells are necessary to maintain protective CD8+ T cell immunity and can target infected cells directly, more recent vaccine attempts against EBV-associated tumors have focused on inducing both CD4+ and CD8+ T cells. NPC cells also express EBNA1, which is a strong immunogen for CD4+ T cells in healthy EBV carriers. To achieve simultaneous stimulation of CD4+ and CD8+ T cells against NPC, a modified vaccinia virus vector encoding a fusion between the CD4+ T cell epitope-rich C-terminal domain of EBNA1 and the full-length LMP2 protein was constructed. DCs infected with this vector were able to reactivate both LMP2-specific CD8+ and EBNA1-specific CD4+ T cell responses from blood of healthy virus carriers in vitro (22). Furthermore, in order to directly target viral epitopes to the MIIC, a polyepitope protein incorporating multiple class II–restricted viral epitopes of EBV, including EBNA1 peptides, was fused to the lysosomal-targeting sequence of the lysosome-associated membrane protein, LAMP1. When the gene encoding this fusion protein was delivered to PBMCs with recombinant vaccinia viruses, activation of virus-specific CD4+ memory CTLs was observed (23). Stimulation of CD4+ CTLs against EBV-infected tumors is crucial especially for Burkitt's lymphoma, where EBNA1 is the only viral protein expressed and MHC class I antigen presentation for CD8+ T cell recognition is severely impaired. However, the work presented by Adhikary et al. (13) shows that vaccines against Burkitt's lymphoma and other EBV-associated malignancies should stimulate not only EBNA1-specific CD4+ CTLs, but also BLLF1-and BALF4-glycoprotein–specific CD4+ CTLs. Indeed, vaccination with BLLF1 encoding recombinant vaccinia viruses protected cottontop tamarins from lethal EBV challenge, and part of this protection might have been due to BLLF1-specific CD4+ CTL (24).

Whether any of the therapeutic immunization approaches that have stimulated CD4+ CTLs in vitro will work in vivo remains to be seen. One caveat of the current vaccine approaches is that, although many of them use DCs to stimulate T cells in vitro, they do not target DCs in vivo. Novel immunization approaches (25) that target antigens directly to endocytic receptors of DCs in vivo promise to achieve a more robust stimulation of CD4+ CTLs, which in turn might lead to a direct attack on infected cells as well as efficient establishment of T cell memory.

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