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Assessment of Cellular Immunity to Human Cytomegalovirus in Recipients of Allogeneic Stem Cell Transplants

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ABSTRACT

Effective reconstitution of cellular immunity following hematopoietic stem cell transplantation (HCT) is thought to be important for protection from the morbidity caused by cytomegalovirus (CMV) reactivation and disease. This review critically discusses current methods for assessment of CMV-specific cellular immune responses, with emphasis on flow cytometry–based methodologies such as MHC-I and MHC-II tetramer staining and intracellular cytokine assays. The advantages and weaknesses of these assays are considered in comparison to traditional immunologic techniques. Application of these newer methodologies has provided insight into the dynamics of the levels of CMV-specific $CD4^+$ and $CD8^+$ T-lymphocytes following HCT, and into the sources and diversity of these cells. Data from preliminary clinical studies suggest that CMV-specific $CD8^+$ T-lymphocyte levels greater than 1×10^7/L of peripheral blood may correlate with protection from CMV disease. Studies on the functional phenotypes of CMV-specific $CD8^+$ T-lymphocytes such as cytokine production, degranulation, and cytotoxicity have indicated that these cells are heterogenous with regard to these properties. Future research will focus on establishing whether any of these immunologic assays will serve as a correlate of protection and inform as to which patients are at high risk for CMV reactivation and disease. Identification of an informative assay may allow its incorporation into standard clinical practice for monitoring HCT patients.

KEY WORDS

Human cytomegalovirus • Hematopoietic stem cell transplantation • Cytotoxic T-lymphocytes • Cellular immunity • Tetramers • Intracellular cytokine assay

INTRODUCTION

In the early days of allogeneic hematopoietic cell transplantation (HCT), cytomegalovirus (CMV) was observed to reactivate 30 to 60 days after transplantation, and disease occurred in approximately one half of patients [1]. Among the risk factors for disease, reconstitution of CMV-specific immunity was recognized to be important [2-4]. With the advent of preemptive antiviral therapy, the incidence of CMV infection remained unchanged, but the occurrence of CMV-associated disease dramatically decreased [5]. With this change, the time of onset of disease increased to approximately 6 months after HCT, and mortality due to late-onset CMV caused the preponderance of deaths at a rate approaching 10% for allogeneic HCT recipients [6]. Immunologic reconstitution remains quintessentially important for protection from late-onset CMV disease [5], as it did for early disease before the advent of ganciclovir (GCV) therapy. Thus, it is important to understand the assays that are in use to document and measure CMV-specific immunologic functions. The purpose of this review is to describe the relevance of these immunologic assays for predicting who is susceptible to disease, and to assess their potential utility in predicting clinical outcomes.

CMV IN HCT PATIENTS

Human CMV establishes persistent lifelong infections in most (50%-85%) individuals [7,8]. The virus

primarily infects endothelial cells in a range of tissues and, after a lytic cycle, establishes an asymptomatic latent infection [9]. The principal site of virus latency in the peripheral circulation is likely the monocyte. Although the factors that govern virus reactivation are not fully understood, it seems that periodic chronic low-grade reactivation occurs in healthy individuals. In the context of immunologic impairment due to conditioning for HCT, 60% to 70% of high-risk (CMV-seropositive) patients will experience CMV reactivation during the first 100 days after conventional or nonmyeloablative HCT, and approximately 20% will develop CMV disease during the first year. The lungs and digestive tract are the organs most often affected [10,11]. The effect of modern preemptive GCV treatment—based on early detection of CMV reactivation by methods [12] including polymerase chain reaction [13] or antigenemia assays [14,15]—has been to increase the time to onset of CMV disease, with the result that late disease is the current main CMV-related problem in HCT [16-19].

Several lines of study have presented persuasive evidence that $CD8^+$ cytotoxic T lymphocytes (CTLs) and CMV-specific helper $CD4^+$ lymphocytes play an important role in controlling CMV infection. First, Reddehase et al. [20-24] have used murine CMV models to demonstrate the importance of $CD8^+$ lymphocytes in this context. Second, experiments using in vitro culture methods to investigate CMV-specific cellular immune responses in immunocompromised transplant recipients indicated that CMV disease was associated with impairment of these responses [25-27]. Since then, rapid assays, often based on flow cytometry, have been used to examine the phenotype and functionality of the T cells involved in the immune responses to CMV and to address their clinical significance.

VIRAL ANTIGENS RECOGNIZED BY THE CELLULAR IMMUNE SYSTEM

The CMV genome contains approximately 200 open reading frames, which are expressed in a temporal sequence of immediate-early, early, and late proteins. Some of these, including the structural pp65 and pp150 proteins, induce vigorous immune responses. Because these are late gene products, this may seem counterintuitive, but when CMV infects the host cell, mature CMV pp65 protein is transferred with the virion [28,29] and is available to the immune system before viral protein synthesis. It is also the most abundantly produced tegument protein and is a major constituent of noninfectious viral "dense bodies" that accumulate in the cytoplasm of CMV-infected cells and are released by these cells [30,31]. Thus, given the reagents available in the past decade, pp65 has been the most commonly studied CMV protein, and, as such, it has been considered the predominant target of immune responses against the virus. Other proteins recognized to lesser degrees include the surface glycoprotein, which is more likely to be important in mediating virus neutralization by antibody; the immediate-early protein; the pp50 protein; and the tegument pp150 protein [32-37]. These polypeptides contain certain amino acid sequences that represent cytotoxic epitopes that are presented by the major histocompatibility complex (MHC) on the surface of the virus-infected antigen-presenting cell (APC) [38]. Some of these epitopes are immunodominant in that they are more likely to be presented as a result of the pathway that begins with generation of the peptide by cleavage of the viral protein by the endosome; continues with transport by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum, further processing of the peptides, and integration into the complex with the MHC polypeptides; and concludes with the appearance of the mature MHC-I complex on the surface of the cell (Figure 1). Each of these steps exerts selective pressure on the pools of peptides, but the most stringent step is probably the binding of the peptide to the MHC-I complex [39].

METHODS FOR ASSESSMENT OF CMV-SPECIFIC CELLULAR IMMUNE RESPONSES

Limiting Dilution Analysis, Cytotoxicity, and T-Cell Proliferation Assays

These methods were routinely used during the last 2 decades to enumerate T-cell responses to antigens. These responses, when absent after HCT, have been shown to be associated with the risk of CMV disease [3]. Essentially, populations of peripheral blood mononuclear cells (PBMCs) that potentially contain antigen-specific CTL precursors are serially diluted into multiwell culture plates in the presence of antigen and irradiated APCs. After multiple days of in vitro stimulation, the expanded effector populations are detected by proliferation assays [40] or by lysis of APCs loaded with antigen and radioisotope, usually chromium 51 [41,42]. Although such limiting dilution analysis assays are very sensitive, especially when multiple rounds of in vitro stimulation are performed, they have serious deficiencies. The CTL precursors undergo proliferation and apoptosis in culture; therefore, the phenotypes of the T-cell effectors that are obtained by these in vitro methods probably do not accurately reflect the cells present in vivo [43-45]. In addition, these assays are laborious and time consuming and require relatively large quantities of patient material. For these reasons, they are not well suited for examining the CMV-specific T-cell responses of HCT patients and will not be the focus of this review.

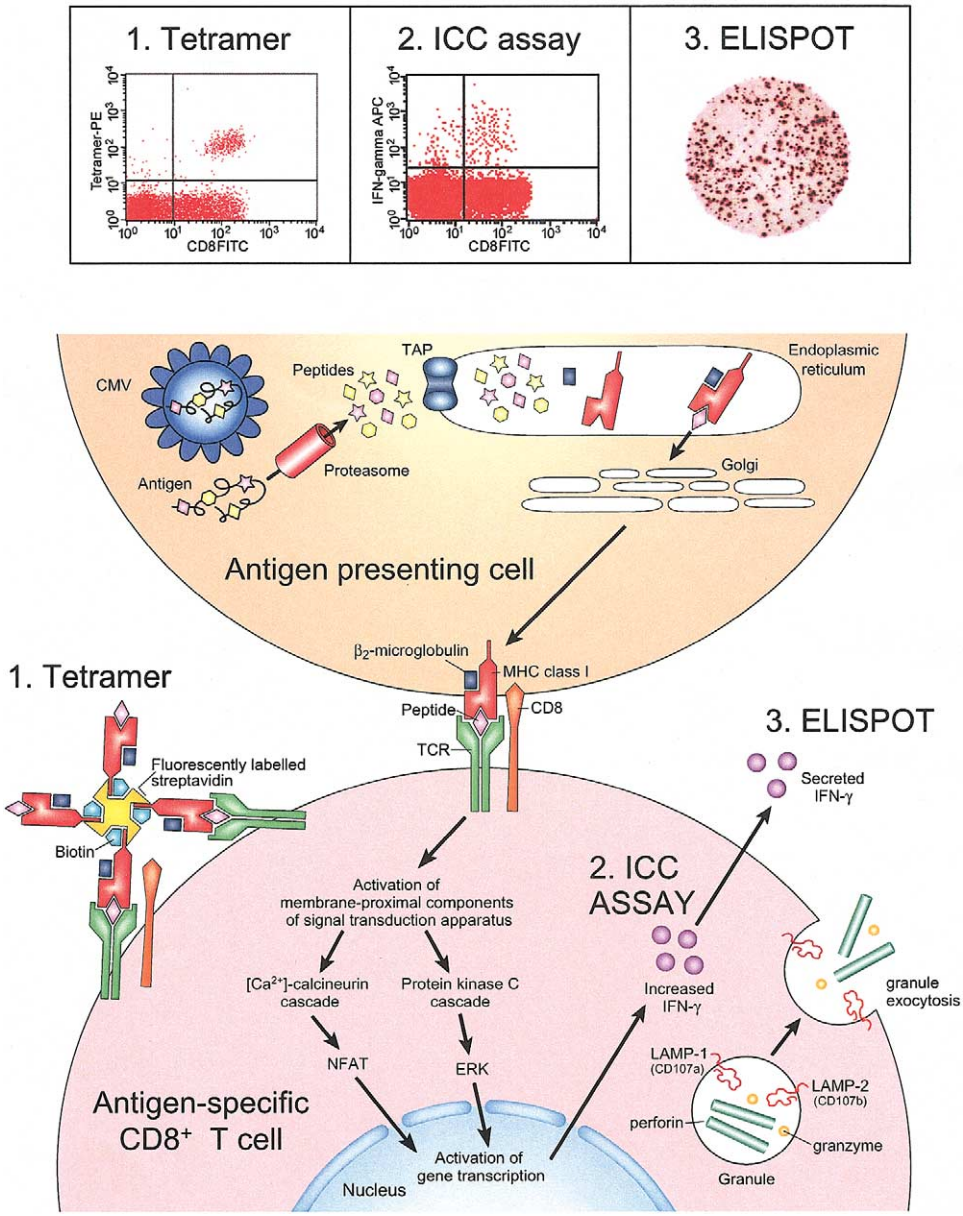

Figure 1. Simplified depictions of MHC-I antigen processing, T-cell receptor signaling, and detection of their consequences by 3 widely used assays of cellular immune responses. The main panel outlines in a schematic manner a simplified version of the mechanisms by which CMV antigens are processed to generate peptides and their presentation via the MHC-I pathway on the surface of antigen-presenting cells (APC). Interaction between peptide/MHC-I complexes on the APC cell surface and T-cell receptor (TCR) complexes on the surface of antigen-specific $CD8^+$ effector lymphocytes leads via complex signal transduction pathways (abbreviated here) to increased IFN-γ production by the effector cells. The flow cytometer–based tetramer-binding assay, an example of which is shown in the top inset panel (1), uses a synthetic fluorescently conjugated probe that specifically binds T-cell receptors that have affinity for the peptide/MHC-I complex. The ICC assay (2) also uses flow cytometry after cell permeabilization and staining with fluorescent antibodies to detect cytokines such as IFN-γ and TNF-α that are produced by $CD8^+$ effectors or $CD4^+$ helper T lymphocytes as the result of interaction with APCs. The ELISPOT assay (3) detects these cytokines after secretion from the cells by their capture on antibody-coated culture dishes and detection with enzyme-conjugated secondary antibodies. NFAT indicates nuclear factor of activated T cells; ERK, extracellular signal-regulated kinase.

MHC-I Tetramers

The practical application of using soluble, tagged MHC-I complexes to bind antigen-specific T cells was first described by Altman et al. [46] but owed much to the earlier work by Garboczi et al. [47] and Silver et al. [48], who assembled and refolded these complexes in vitro as part of their biochemical and crystallographic studies. The key development was the multimerization of the complex into a tetrameric molecule capable of engaging more than 1 T-cell receptor (TCR) molecule and thus of overcoming the relatively low affinity [49] of the MHC-I complex for the TCR.

The MHC-I transmembrane protein consists of (1) a heavy chain that contains the complete peptide-binding groove and that exists in multiple allelic types and subtypes and (2) an invariant light chain termed β_2-microglobulin. The binding of peptides to the complex is restricted by the HLA subtype of the heavy-chain polypeptide [50]. In the original procedure for making tetramers [46], the heavy chain of choice was expressed in *Escherichia coli* as a truncated molecule that comprised just the extracellular domains but had a recognition tag for *BirA* biotin ligase engineered at the carboxy terminus. The heavy and light chains are separately expressed and purified under denaturing conditions from the bacteria and then combined with a molar excess of the MHC-I binding 8-11-mer peptide in a refolding buffer. After refolding, the complex is treated with *BirA* to biotinylate the heavy chain and is purified by high-performance liquid chromatography before the addition of fluorochrome-conjugated streptavidin, which has 4 binding sites for biotin. This forms a tetrameric complex that is normally stable for months at 4°C.

Refinements to these method have been described; for example, the introduction of a point mutation in the MHC-I heavy chain at a position known to interact with the $CD8^+$ co-receptor was made to reduce nonspecific interaction of the tetramer with irrelevant $CD8^+$ cells [51]. Chemical rather than enzymatic biotinylation of the MHC-I heavy chain [52] and the use of peptide-β_2-microglobulin fusion molecules rather than free peptide for complex formation [53] are other variations, but the basic procedure is straightforward and can be performed in any adequately prepared and equipped biochemistry laboratory.

Tetramer staining can be performed on freshly isolated or fixed PBMCs or on whole blood, and the results are quantified by fluorescence-activated cell sorter (FACS) analysis. Although less commonly described, in situ staining of tissue samples has also been performed, by, for example, Skinner et al. [54] and Skinner and Haase [55]. Counterstaining is normally performed with fluorescent antibodies to the CD8 cell-surface antigen. For FACS analysis, a primary gate is set on lymphocytes, either based on their forward and sideways light-scattering properties or, preferably, by co-staining with antibodies to the CD3 lymphocyte surface marker. Some anti-CD8 antibodies have been reported to interfere with tetramer binding of the TCR [54,56], so it may be necessary to select a different antibody clone or to label with tetramer before CD8 antibody staining. Tetramer-binding cells are identified as a subset of the $CD8^+$ T lymphocytes and may range from undetectable levels to as high as 15% of the $CD8^+$ T lymphocytes in some HCT recipients.

Tetramer-based assays have proven to be a versatile technology and have been used in a wide range of applications, including detection of virus-specific T cells in human immunodeficiency virus [46,57,58], Epstein-Barr virus [58-61], influenza [62], hepatitis C virus [58], and CMV [63-67] infections; detection of tumor-specific CTLs [68-70]; isolation of antigen-specific cells for characterization; and even expansion for immunotherapeutic use by flow sorting [71,72] or by antibody bead capture. The disadvantage of MHC-I tetramers lies in their epitope specificity and the HLA restriction of the peptides; a given tetramer in samples from individuals with the corresponding HLA phenotype will detect only that subset of virus-specific CTL that recognizes the peptide with which the tetramer is refolded. Thus, for a complete survey of cellular immune responses to a given viral polypeptide, it is necessary to have previously identified the immunodominant cellular epitopes, such as the CMV $pp65_{495-503}$ sequence in the case of HLA-A*0201, and to have prepared the appropriate tetramer refolded with the appropriate HLA heavy-chain subtype. In addition, even when the tetramer reagent is available, it could represent an epitope that is not immunodominant in all individuals because of certain combinations of HLA subtypes [73-75]. As more immunologic information on CMV cellular immune responses emerges, it may be necessary to assemble panels of tetramers to cover multiple epitopes restricted by different HLA types and represented at reasonable frequencies in the patient population under study. An analysis of the coverage of different ethnic populations with CTL epitopes combined theoretical and empirical studies to conclude that 90% coverage was attainable with a panel of 11 to 15 epitopes [76].

It is also possible to combine tetramer staining with other flow-based assays to gain more phenotypic information on the nature of antigen-specific cells. Intracellular cytokine (ICC) assays (see below) can provide insight into the functionality of CTLs in terms of their ability to secrete immunologically relevant molecules such as interferon (IFN)-γ or tumor necrosis factor (TNF)-α. Phenotyping with fluorescent antibodies to surface markers can determine activation status (CD28 and CD69) or information on the lineage and differentiation status (CDRA, CDRO, and CD62L) of the cells. Clonotyping of the tetramer-binding cells with antibodies to the Vβ region of the TCR will provide information on the clonality of the antigen-specific immune response [77-79]. Tetramer-binding cells can also be counterstained with antibodies to detect the presence of cytolytic effector molecules such as perforin [80] and the granzymes [81,82] that are stored within cytotoxic granules of effector CTLs and released during the fusion of these vesicles with the target cell membrane (degranulation). Rubio et al. [83] recently described, in the context of tumor immunity, a novel assay based on detection of mobilization of a vesicle membrane protein,

CD107a (lysosomal-associated membrane protein-1), to the cell surface during this degranulation process. Lymphocytes from vaccinated melanoma patients were incubated with target cells expressing melanoma antigen and were then stained with tetramer and antibodies to CD107a before flow analysis. These investigators reported that only effector cell clones that mobilized CD107a exhibited high recognition efficiency for target cells that presented the relevant tumor antigenic peptide. Mobilization of CD107a may be required as a means of breaking tolerance. Limited data from Betts et al. [84] suggest that degranulation, as measured by CD107a expression, correlates with the cytotoxic activity of $CD8^+$ T lymphocytes specific for the CMV $pp65_{495–503}$ epitope.

In response to the research community's need for tetramers, 4 years ago the National Institute of Allergy and Infectious Diseases instituted a central Tetramer Facility [85,86]. Located at Emory University, this resource provides custom tetramers to investigators around the world. A wide range of human, mouse, macaque, and chimpanzee MHC-I alleles are available for refolding with peptides, and the facility now offers a limited range of MHC-II tetramers.

MHC-I Dimers

MHC-I dimers can be regarded as a technical variation on the concept of MHC-I tetramers. For procedures, see Schneck et al. [87]. This approach, developed by Schneck et al. [88,89], was used to examine human T lymphotropic virus type 1 (HTLV-I)–specific cells [89,90]. The key molecule used by these investigators was an HLA-A2/immunoglobulin chimera engineered in a eukaryotic expression plasmid by fusing the α_1 to α_3 domains of HLA-A2 to murine immunoglobulin [91]. J558L cells in culture were transformed with this plasmid and with another plasmid expressing human β_2-microglobulin. A cell line secreting high levels of the chimeric protein was selected, and the dimer was purified from culture supernatant. This dimer was then loaded with the HLA-A2–binding peptide at a very high molar excess (660-fold) for 2 weeks before use in FACS. It is necessary to use a fluorescent antibody to mouse immunoglobulin to detect the dimer-labeled cells. One technical advantage of dimers compared with tetramers is that the HLA-A2/immunoglobulin complexes can be prepared in bulk, and then aliquots can be loaded with different peptides. Our laboratory used this approach to evaluate CMV pp65 epitope peptides that emerged from experiments with scanning synthetic combinatorial libraries [92]. Dimers have been less widely used than MHC-I tetramers, possibly because of the effort required to establish the required secretory cell lines. A recent report described their use as artificial APCs when coupled with CD28-specific antibody to beads [93]. These artificial APCs were used to expand CMV-specific CTLs and may have utility in adoptive immunotherapy.

MHC-II Tetramers

The first report of the synthesis and use of MHC-II tetramers to identify antigen-specific $CD4^+$ cells was in 1999 [94]. However, for technical reasons, their preparation and use is more difficult [95], and they have not become widely available, although recently they have become available commercially or via the National Institute of Allergy and Infectious Diseases tetramer facility. Furthermore, because of the very low levels of circulating $CD4^+$ cells specific for a given antigen—below the detection limit of flow cytometry, which is approximately 0.01% of $CD4^+$ or $CD8^+$ lymphocytes—it has generally been necessary to use an in vitro amplification step, combined with labeling of the cells with a fluorescent dye, to estimate the number of divisions undergone by the stimulated cells and, hence, the original frequencies of the antigen-specific precursor cells. Nonetheless, several reports have described the use of class II tetramers to investigate immunity to influenza [94], herpes simplex [96], and autoimmune antigens [97].

ICC Assays

These assays represent the current major alternative to the tetramer-based approach. The principle is that antigen is added to PBMC preparations in culture or to samples of whole blood to induce the production of cytokines such as IFN-γ or TNF-α. The antigen may be a single peptide or pools of peptides [80,98,99], purified viral antigenic polypeptides [100], or even virus-infected cell lysates [80,101] containing complex mixtures of viral antigenic polypeptides and peptides. If $CD8^+$ immune responses are being studied, it is generally necessary to use peptides or cell lysates containing peptides, because the cross-presentation by the MHC-I pathway of endocytosed antigens [102] can be inefficient except in the $CD8^+$ dendritic cell subpopulation [103]. Alternatively, APCs can be infected with recombinant vaccinia or modified vaccinia Ankara virus expressing the antigen of interest. Ubiquitination of this antigenic protein can improve the processing and, thus, presentation of peptides [104,105]. For $CD4^+$ analysis involving the MHC-II complex, purified or recombinant virus protein may be used [100]. Interaction between the antigenic peptide displayed on the MHC-I or MHC-II complex on the surface of APCs in the culture and the TCR of virus-specific T cells induces cytokine production in the latter. The addition of an inhibitor of transport, such as brefeldin A, prevents secretion and so causes accumulation of the cytokine in the cell [106]. After the induction period, the cells are fixed

and permeabilized before staining with fluorescent antibodies to the cytokine and analysis by flow cytometry. Counterstaining with antibodies to CD4 and CD8 permits discrimination between these 2 lymphocyte subsets. This method lends itself well to combination with MHC-I tetramers, because it is thus possible to determine the proportion of tetramer-binding $CD8^+$ lymphocytes that are capable of producing cytokine on virus-specific antigenic stimulation [66,78]. Because engagement of peptide with the TCR tends to lead to internalization and, hence, reduced tetramer binding [107], it is often helpful to stain the cells with tetramer before the antigen-stimulation step. As with tetramer assays, it is also possible to combine ICC assays with flow-based clonotyping [79].

A great strength of the ICC assay when used with peptide libraries or whole antigen is that the assay can be used to analyze samples from patients who possess any HLA phenotype. A discussion of the issues involved in the design of the pools of overlapping peptides in such libraries may be found in Roederer and Koup [108].

In an extracellular variation of the ICC assay, the PBMCs or blood samples from patients are incubated with viral antigens, and the secreted cytokines are detected in the medium by using enzyme-linked immunosorbent assay [74,109] or cytokine bead array [110]. If enzyme-linked immunosorbent assay is used, this method has the advantage of not requiring a flow cytometer. Although the cytokine concentrations in the medium or plasma can be quantitated by reference to standards, this assay has the weakness that it does not identify the cell subpopulations that produce the detected cytokines.

Enzyme-Linked Immunospot

Enzyme-linked immunospot (ELISPOT) permits detection of antigen-induced cytokine secretion at the single-cell level [111,112] without a flow cytometer. This assay is based on the principle of the enzyme-linked immunosorbent assay. A 96-well tissue culture plate is coated with an antibody that binds the marker cytokine of choice (usually TNF-α or IFN-γ, but ELISPOT assays have been described for a range of molecules, including interleukin [IL]-1, IL-2, IL-4, IL-5, IL-6, IL-10, and granzyme B). By using cells (which may be unseparated PBMCs or a mixture of effector cells and APCs such as peptide-loaded TAP-deficient T2 cells [113] or autologous mature dendritic cells [114] and which are then incubated in the antibody-coated wells in culture medium containing antigen), cytokines are produced in response to specific stimulation. The immobilized antibody on the surface of the plate binds the cytokine, and the culture wells are then washed, thus removing the cells. The bound cytokine is detected by using an enzyme-labeled detection antibody and a chromogenic substrate; this results in colored spots, each of which represents the position of a single cytokine-secreting cell. Evaluation of the results can be assisted by computer image-analysis systems, increasing the speed and, possibly, the objectivity [115]. The ELISPOT assay has the advantage of being highly sensitive, easy to perform, and rapid, and it requires no expensive equipment. However, it yields much less information than the flow-based ICC or tetramer assays on the phenotypes of the antigen-specific cells. The sensitivity of ELISPOT seems to be comparable to that of the ICC assays: some researchers report lower limits of detection in the former [116] and others in the latter [117,118]. Background may be a problem because of constitutive secretion by PBMCs of some cytokines, such as TNF-α [119], or because of the choice of culture vessels [120]. A recent technical development termed Lysispot detects lysis in a single cell assay. A marker protein (β-galactosidase) is introduced into antigenic peptide-pulsed APCs by infection with a herpes simplex amplicon that is capable of infecting a wide range of human and mouse cells. Lysis of these cells by effector cells is perforin dependent and produces blue spots in the culture wells. Furthermore, this Lysispot assay can be combined with ELISPOT for a 2-color ELISPOT that is capable of detecting both the cytolysis and cytokine-secretion functions of effector cells. For this, the plates are coated with a mixture of antibodies to IFN-γ and β-galactosidase and with 2 different conjugated secondary antibodies. A mixture of red and blue spots results [121]. ELISPOT remains a viable alternative to flow-based assays because of its sensitivity and simplicity (Table 1).

RECONSTITUTION OF CELLULAR IMMUNITY AFTER HCT

After HCT, the recipients are immunocompromised for at least a year and have defects in both cellular and humoral immunity. During this period, in addition to the danger of relapse or development of a secondary malignancy [122], there is an increased risk of opportunistic bacterial, viral, or fungal infections [123]. These risks continue even after successful engraftment, which takes place by day 30 after transplantation in both bone marrow and stem cell recipients, because a high proportion of allograft recipients have at least 1 postengraftment infection [124,125], the occurrence of which is the predominant independent factor associated with nonrelapse mortality [125]. The nature of the deficiency after engraftment probably lies in the mononuclear cell subsets, because low numbers of these cells, especially $CD4^+$ lymphocytes, have been associated with increased infections [126-128]. Storek et al. [129] performed a detailed comparison of immune reconstitution after transplantation of bone

Table 1. *Summary of Assays Available for Assessment of Cellular Immune Responses to Pathogens Such as CMV*

Variable	Cells Detected	Advantages	Disadvantages
MHC-I tetramer	$CD8^+$ T lymphocytes	Specific, rapid, sensitive. Identifies single cells; allows further cell characterization by co-staining with Abs or combination with ICC assay	Restricted by HLA type. Nonfunctional assay. Knowledge of immunodominant epitopes required. Costly to prepare large panels of tetramers
MHC-II tetramer	$CD4^+$ T lymphocytes	Specific, rapid, sensitive. Identifies single cells; allows further cell characterization by co-staining with Abs or combination with ICC assay	MHC-II tetramers difficult to prepare. Low levels of specific CD4 cells may require in vitro amplification step
ELISPOT	$CD8^+$ and $CD4^+$ T lymphocytes	Very sensitive. Detection of single cells. Does not require a flow cytometer. Not restricted by HLA type	Limited information on phenotypes of cells secreting cytokine
Intracellular cytokine assay (ICC)	$CD8^+$ and $CD4^+$ T lymphocytes	Specific, rapid, sensitive. Identifies single cells; allows further cell characterization by co-staining with Abs. Panels of peptides (CD8), virus-infected APC, or use of whole antigens (CD4) permits coverage of many epitopes. Not restricted by HLA type	Background levels and nonspecific detection. Requires metabolically active cells
In vitro stimulation combined with cytotoxicity assay	$CD8^+$ T lymphocytes	Sensitive. Assesses cytotoxic function	Labor intensive and time consuming. Culture and in vitro stimulation may alter cell phenotype
Proliferation assays	$CD8^+$ and $CD4^+$ T lymphocytes	Simple assay. Does not require knowledge of cellular epitopes	Provides only general information on immune responses

Abs indicates antibodies.

marrow or mobilized stem cells. Using flow cytometry to examine cell-surface markers, they found that patients who received stem cells had significantly higher mononuclear cell subset counts, particularly $CD4^+$ lymphocytes. The functionality of the cells was comparable as measured by lymphoproliferation, but the HCT recipients had a 2.4-fold higher rate of documented severe infections.

One of the more interesting debates in the field of immune reconstitution after allogeneic HCT concerns the contribution of the thymus to the T-lymphocyte repopulation of the recipient. In the fetus and the neonate, the thymus is the primary site of T lymphopoiesis. In young children, recovery of naive CD4 cell populations (bearing the CD45RA marker) is rapid after chemotherapy, a phenomenon termed *thymic rebound* [130]. However, in adults, the thymus is involuted, and functional thymic tissue is replaced by adipose tissue [131]. This seems to exert a constraint on CD4 recovery after chemotherapy without transplantation [132]: recovery of $CD4^+$ $CD45RA^+$ cells is limited in many patients even after 2 years. Even after allogeneic HCT, adults were found by flow cytometry analyses to have a limited ability to generate naive $CD4^+$ cells [133]. This view seemed to be challenged by findings with a new assay [134] that measures the number of TCR-rearrangement excision circles (TREC) in peripheral blood T cells. These DNA products, which arise during thymocyte development, are stable and T-cell specific and are diluted during each cellular division. They therefore seemed to present an elegant tool for measuring thymic output. Douek et al. [135] reported that, as quantified by this assay, substantial numbers of naive T cells produced by the thymus appeared in the peripheral circulation as little as 100 days after transplantation and accumulated to supranormal levels. This interpretation has since been challenged as misleading because of failure to consider the complexities of T-cell dynamics [136-138]. First, naive T cells are very long-lived, with a life span of 1000 to 10000 days [139]; thus, the presence of TREC-containing naive T cells is not evidence of ongoing thymic production. In addition, after transplantation, there are so few T cells in the peripheral blood compartment that the arrival of a small number of naive $TREC^+$ cells can have a large effect on the TREC levels, which are normally measured as TREC content per microgram of cell DNA. In summary, this approach to evaluation of thymic function is indirect and should be used with caution.

RECONSTITUTION OF CMV-SPECIFIC IMMUNITY AFTER ALLOGENEIC HCT

A number of studies have been published that used 1 or more of the available immunologic techniques to investigate the reconstitution of CMV-specific cellular immunity in HCT transplant recipients and to correlate them with CMV-related clinical events. The overall message from these studies confirmed the central importance of T lymphocytes for protection

against CMV disease. Notably, CMV disease is more common in recipients who receive a graft from a CMV-seronegative donor [140-142].

Dynamics of Levels of CMV-Specific CD8$^+$ Cells

Quinnan et al. [27] used conventional assays as early as 1982 to detect CMV-specific CTLs in HCT recipients and to show that these correlated with protection from CMV infection. Similarly, later investigators who used the same cytotoxicity and lymphoproliferation assay techniques also reported that detection of CMV-specific CD8$^+$ CTLs was associated with protection from CMV disease [25,143]. With the advent of MHC-I tetramer technology in the late 1990s, several groups applied this assay either alone [142,144,145] or in combination with ICC assays [78,146-149] to enumerate CMV pp65-specific CD8$^+$ lymphocytes. With these flow-based methods, CMV-specific lymphocytes can be detected as early as 21 days after transplantation [144,145], although there is considerable variation in the time of detection. For example, Hebart et al. [114] reported a range of 35 to >180 days after transplantation, with a median of 90 days. The peak observed proportions of CMV-specific CD8$^+$ T lymphocytes are quite extraordinary, often exceeding 10% and occasionally 20% of the CD8$^+$ lymphocytes.

Cwynarski et al. [144] studied a cohort of 13 patients with allogeneic transplants from siblings and 11 with transplants from matched unrelated donors and reported that no patient with levels of CMV-specific CD8$^+$ lymphocytes $>1 \times 10^7$ per liter of peripheral blood developed CMV disease. In another small study, Gratama et al. [142] found that in a cohort of 21 donor/recipient pairs in which either or both were CMV seropositive, no recipient with CMV-specific CD8$^+$ T lymphocyte levels $>0.2 \times 10^7$/L developed disease. Despite the absence of large definitive studies, the consensus of these smaller reports suggests that enumeration of CMV-specific lymphocytes by tetramer/ICC assays has potential value for identifying individuals at increased risk of CMV disease.

Source and Diversity of CMV-Specific Cells

Studies in our laboratory using tetramer staining combined with fluorescent antibodies to the variable region (Vβ) of the TCR have suggested that CMV-specific CD8$^+$ T lymphocytes detected in CMV-seropositive recipients of allogeneic stem cell transplants from CMV-seropositive donors are likely of donor origin and expand in the recipient after antigenic stimulus due to CMV reactivation [78]. Similarly, Peggs et al. [150] reported data suggesting antigen-driven expansion of CMV-specific CD8$^+$ cells after HCT. This issue was addressed in more detail by Weekes et al. [77], who generated cell clones from HCT donors and recipients. In concordance with the previous report from our group [78], all CMV-specific CD8$^+$ cell clones in recipients were found by single-nucleotide DNA polymorphism analysis to be of donor origin. Sequencing of the hypervariable region of the TCR β chain indicated expansion and diversification of these clones within the recipient. In addition, their data suggested, but did not prove, the existence of de novo generation of CMV-specific clones from donor-derived progenitor cells in seropositive recipients of stem cell transplants from seronegative donors [151].

Functionality of CMV-Specific CD8$^+$ Cells

The combination of tetramer staining and ICC assays permits assessment of the functionality (in terms of cytokine production) of CMV-specific CD8$^+$ T lymphocytes at the single-cell level [57,78,147,152]. Ozdemir et al. [147] used this combined technique to examine samples from 87 HCT recipients and found that in individuals who experienced CMV reactivation, as measured by CMV antigenemia, the proportion of CMV-specific tetramer-binding CD8$^+$ T cells that produced TNF-α was lower than in patients who did not. They also reported that patients with higher immune suppression, such as those receiving steroids for treatment of acute graft-versus-host disease, had lower levels of these cytokine-producing cells. They suggested that failure to control CMV reactivation might be due to impaired function of these CMV-specific cells.

Characterization of the cytolytic function of CMV-specific CD8$^+$ T lymphocytes is more technically challenging because of the limited numbers of these cells in clinical samples. Expansion of virus-specific T lymphocytes by using in vitro stimulation to obtain the numbers required for conventional cytotoxicity assays will inevitably affect their activation status and phenotype. Lacey et al. [78] used ex vivo cytotoxicity assays to evaluate the CMV-specific cytolytic function of unsorted and unstimulated PBMCs from HCT donors and recipients. They observed striking differences between the CMV-specific ex vivo cytolytic activity associated with PBMCs from HCT donors compared with HCT recipients. They detected little or no CMV-specific cytolytic activity in donor PBMC samples, even when significant levels of CMV tetramer-binding CD8$^+$ lymphocytes capable of producing IFN-γ were present. By contrast, samples from several HCT recipients possessed significant levels of ex vivo CMV-specific cytotoxicity that correlated with the frequency of tetramer-binding cells [78]. This was consistent with earlier data that reported the absence of ex vivo CMV-specific cytotoxicity in samples from healthy donors and the presence of such cytotoxicity in samples from human immunodeficiency virus–infected individuals [153]. It may be

that reactivation of CMV in the immunocompromised HCT recipients drives a change in phenotype of the tetramer-binding cells to a cytotoxic one. A critical question that remains unanswered is whether the adaptive immune response controls CMV reactivation after HCT. When CMV seropositive recipients of HCT from seropositive donors (D^+R) are compared with seropositive recipients of HCT from seronegative donors (D^-R^+) recipients for CMV reactivation, as measured by antigenemia rate, there are no differences. However, the probability of having a high level of antigenemia (>10 cells per slide) is significantly higher in the D^-R^+ patient [154]. Thus, it is likely that the adaptive immune system contributed by the donor influences the frequency or magnitude of CMV reactivation.

Importance of CMV-Specific CD4 Cells

It is clear from several lines of evidence that CMV-specific helper $CD4^+$ lymphocytes are important for reconstitution of $CD8^+$ CMV-specific cell responses and for protection from disease. First, in HCT recipients, low levels of CMV-specific $CD4^+$ T lymphocytes were associated with reduced reconstitution or functionality of CMV-specific CD8 cells [142,147,155]. In fact, for HCT recipients, the absence of lymphocyte proliferation to CMV antigen in vitro has been linked to risk for disease [2-5]. Second, Gamadia et al. [156] reported that in renal transplant recipients with symptomatic CMV disease, CMV-specific $CD4^+$ T-cell responses were delayed and could be detected only after antiviral therapy. Third, in Riddell's classic trials of adoptive immunotherapy against CMV by infusion of expanded CMV-specific T-cell clones from the donor, it was found that in patients deficient in $CD4^+$ CMV-specific T-helper cells, the infused clones decreased, suggesting that these cells are necessary for the persistence of the transferred $CD8^+$ cells [157]. A second adoptive transfer study, in which CMV antigen-stimulated $CD4^+$ T cells were infused into patients with chemotherapy-resistant CMV viremia and who lacked a CMV-specific $CD4^+$ helper response, led to a decrease in CMV load in all 7 patients, an increase in CMV-specific T-cell proliferation, and reconstitution of $CD4^+$ and $CD8^+$ T cells in most patients. From the studies described previously, it is clear that assessment of cellular immunity to CMV should include evaluation of both CMV-specific $CD4^+$ T-helper cells and CMV-specific $CD8^+$ effector T cells.

CMV DISEASE IN HCT AND SOLID ORGAN TRANSPLANTATION

Ideally, after HCT, the reconstitution of immunity occurs in a timely fashion such that the patient is gradually protected from infection. Because many infections will occur before immune reconstitution, the selective use of antibiotics and antivirals is necessary to protect the patient during the early months after HCT. At some point, of course, depending on the particular clinical regimen, the prophylactic therapy is usually stopped, and then, if specific immunity has not been restored, infection and disease can still occur. This is especially true for patients at risk for CMV, for which preemptive use of GCV was developed and used during the first 2 to 3 months after HCT. The widespread use of prophylactic therapy has had the effect of increased late-onset CMV disease, defined as CMV-associated disease occurring >100 days after HCT [158]. The risk factors for this late CMV morbidity have been determined to be CMV infection, lymphopenia, failure to reconstitute a lymphoproliferative response to CMV, and graft-versus-host disease before day 90 after HCT [5].

CMV infections are a serious clinical problem in the context of transplantation of solid organs, including liver, lung, and kidneys. The CMV reactivation and disease incidence in these immunosuppressed patients are often higher than in patients who receive stem cell transplants [159-161]. Tetramers, ICC, and lymphoproliferation assays have been used to examine CMV-specific $CD8^+$ and $CD4^+$ T lymphocytes in liver [64,162] and kidney [26,156,163] transplant recipients. Taken together, these studies confirm the importance of both helper T cells and CTLs in protection from CMV reactivation and disease in solid organ transplant recipients.

A criterion for clinical utility of 1 or more of the newer assays of CMV immunity discussed in this review is the ability to aid in the prediction of which patients might safely stop CMV preemptive therapy. Data from ongoing multicenter trials, such as the Blood and Marrow Clinical Trials Network Protocol 0201 (which is testing the immunophenotypic characterization of stem cell grafts and measuring posttransplantation immune reconstruction and T-cell responses from approximately 500 HCT recipients), may address this question. Beckman Coulter is also sponsoring an evaluation of the tetramer-binding assay for detection of CMV-specific $CD8^+$ T lymphocytes in HCT recipients. If such tests, when used to monitor immune reconstitution, can identify a patient population no longer at risk for CMV complications on the basis of immune status, then they could be important in patient management. However, assays for specific immune function, such as ICC or cytotoxicity assays, will probably remain research tests, and future clinical tests of CMV immunity will most likely serve as surrogate markers for these more complex assays, as

do the current CMV polymerase chain reaction and antigenemia measurements.

CONCLUSION

Several assays are now available to characterize CMV-specific $CD8^+$ and $CD4^+$ immune responses at the cellular level. The MHC-I tetramer assay is a powerful and convenient method for measurement of CMV-specific $CD8^+$ cells, but it requires the availability of a panel of tetramers of the appropriate HLA type corresponding to several immunodominant epitopes. It, however, lends itself to further characterization or selection of CMV-specific cells. The ICC assay and its technical variants, when performed with pools of peptides, permit the identification of lymphocytes that recognize a wide range of epitopes within different viral antigens and restricted by different HLA types. The ICC assay, because it measures cytokine secretion by CMV-specific $CD8^+$ and $CD4^+$ lymphocytes, is also a functional assay, although it does not assess the primary cytotoxic function of $CD8^+$ lymphocytes. Both the tetramer and ICC assays have a role in the assessment of CMV-specific cellular immune responses in immunocompromised individuals. The data that have emerged from studies to date suggest that immunologic assays will be of value in identifying individuals at risk for reactivation and disease and will be helpful in guiding future immunotherapeutic interventions.

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