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Flow Cytometric Enumeration of Antigen-Specific T Lymphocytes

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Until the second half of the 1990s, quantification of MHC-restricted, peptide-specific T lymphocytes required cell culture–based techniques of mononuclear cell suspensions that were often laborious, cumbersome, and prone to bias towards cellular subsets with growth advantage. Initially, limiting dilution assays were used. These assays include multiple days of culturing T lymphocytes in the presence of antigen and antigen-presenting cells, and the functional read-outs produced were T-cell proliferation (1) or cytolytic activity (2). However, such assays may not accurately reflect the function and frequency of T cells in vivo, due to selective proliferation and apoptosis that occur over time in culture. Alternatively, assays for T-cell activation have been developed based on the upregulation of the early activation marker CD69 (3) and/or the production of cytokines upon specific stimulation. The cytokines thus being secreted by the T cells can be measured in the culture supernatant by ELISA (4,5), or, after immobilization, on nitrocellulose plates coated with anti-cytokine mAbs (ELISPOT) (6,7). The advent of three-color flow cytometry enabled the direct visualization of cytokine production by individual cells (8). Visualization was achieved either intracellularly (8,9) or on the cell surface following retention of secreted cytokine in an affinity matrix allowing for subsequent staining with a fluorochrome-labeled mAb (10). This affinity matrix was later replaced with a bispecific mAb, specific for a cell surface marker (e.g., CD45) on the one hand, and for cytokine on the other (11). Alternatively, amplification of the fluorescence signal by fluorescent liposomes was shown to allow detection of surface-expressed IFN-γ on IFN-γ–producing cells (12). Among the flow cytometric techniques, the first approach, termed cytokine flow cytometry (CFC), is currently the most widely used. In all these functional assays, single peptides (13), mixtures of peptides (14), or more complex molecules (e.g., whole proteins or mixtures of proteins) (9), can be used as an antigen source.

An alternative approach for the direct visualization of antigen-specific T cells has been established by the construction of multimers consisting of recombinant MHC molecules folded with the appropriate peptides. The low affinity and fast off-rates of MHC-peptide ligands to the T-cell receptor (15) precluded the direct detection of MHC-restricted, antigen-specific T cells using recombinant MHC-peptide monomers. This problem was overcome by the construction of soluble MHC-peptide multimers that were able to bind specifically to the TCR with higher affinity than the sum of the single monomeric affinities. Initially, tetrameric complexes of Class I MHC-peptide monomers were constructed (16), followed by the development of Class II MHC-peptide tetramers (17). Similar approaches have been taken to detect other types of antigen-specific lymphocytes such as NK cell subsets (18), NK-T lymphocytes (19,20) and TCR-γδ$^+$ T-cell subsets (21). Alternatively, MHC dimers have been developed, which are MHC-immunoglobulin fusion proteins that bind to antigen-specific T cells via the bivalent nature of the two binding sites available to interact with the TCR (22).

Here, we discuss the principles and practice, and summarize clinical applications and relevance of antigen-specific T-cell enumeration using MHC-peptide tetramers, CFC, and a combination of both techniques. Extensive reviews of the methodological aspects of these assays and their performance in relation to other assays for detection of antigen-specific T lymphocytes have been published elsewhere (23,24).

MHC-Peptide Tetramers

For flow cytometric enumeration of antigen-specific T lymphocytes using tetramer technology, the HLA type of the test individual and the specific peptide epitope(s) to be studied must be known in advance.

Class I MHC tetramers. The Class I MHC transmembrane protein consists of a single heavy chain that contains the complete peptide-binding groove and is stable in soluble form when complexed with its essentially invariant light chain, β_2-microglobulin (16). The β_2-microglobulin and the recombinant Class I MHC heavy chain, on the COOH terminus of which a recognition tag for the BirA enzyme has been engineered, are synthesized using the *Escherichia coli* expression system. Following purification, the β_2-microglobulin and heavy chains are then refolded in molar excess of the appropriate 8- to 10-mer peptide. Subsequently, the peptide-loaded Class I MHC

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monomers are connected by the addition of fluorochrome-labeled streptavidin; its four biotin-binding sites result in the formation of tetrameric complexes. For the enumeration of MHC-restricted, antigen-specific T cells, PE is commonly preferred for its high signal to noise ratio; alternatively, FITC or APC are used.

For flow cytometric enumeration of Class I MHC-restricted $CD8^+$ T cells, PBMC suspensions have been most commonly used, while bulk-lysed leukocyte suspensions can also be used (25), as well as the stain-lyse-wash method. Typically, tetramers are counterstained with CD8, after which lymphocytes are selected by gating on FSC and SSC, and the Class I MHC-restricted T cells are identified within the $CD8^{bright}$ subset of lymphocytes (16). As tetramers tend to bind nonspecifically to other cell types such as monocytes, we prefer to include CD3 in this assay in order to allow optimal identification of the T cells on the basis of SSC versus CD3 gating (26). Using this "T-gating" approach, $CD3^-,8^{dim}$ NK lymphocytes can be excluded from analysis, providing more reliable assessment of the percentage of Class I MHC-restricted T cells as a fraction of the $CD8^+$ T cells (25). Alternatively, cells other than $CD8^+$ T lymphocytes can be excluded, by staining them with a mAb cocktail containing, e.g., CD4 (T-helper cells), CD14 (monocytes), CD16 (NK cells), and CD19 mAb (B cells) labeled with the same fluorochrome (so-called "dump gating") (27). An example of the T-gating-based analysis is shown in Figure 1. Absolute numbers of Class I MHC-restricted, antigen-specific $CD8^+$ T cells are subsequently calculated from the percentage within the $CD8^+$ T cells and the simultaneously-obtained absolute $CD8^+$ T-cell count (28,29).

Depending on the pathogen or disease under study, the phase of the disease (e.g., acute versus chronic infection) or the timing relative to the clinical intervention (e.g., prior to or post vaccination) and the sampling site (e.g., peripheral blood versus biopsy), the proportions of antigen-specific $CD8^+$ T cells as measured using Class I MHC-peptide tetramers may range from undetectable to several tens of percentages of total $CD8^+$ T cells. The detection limit may vary between 0.01% and 0.1% of $CD8^+$ T cells, depending on the method for sample processing, the tetramer used, and the number of $CD8^+$ T cells acquired (23,25). The functional status of the tetramer-binding $CD8^+$ T cells can be assessed by stimulating the tetramer-labeled lymphocytes with the corresponding peptide and measuring intracellular cytokine production, as described below (30).

The procedure for staining T lymphocytes with MHC dimers is very similar to that for MHC tetramers, as summarized above (22).

Class II MHC tetramers. The construction of Class II MHC-peptide tetramers has been technically more demanding, and successful use of these reagents was not shown until late 1999 (16). Class II MHC molecules are heterodimers of α and β chains. For the production of recombinant α and β chains, insect cells such as Drosophila S2 are used (16,26). The α and β chains are cloned in separate expression vectors; both chains are extended

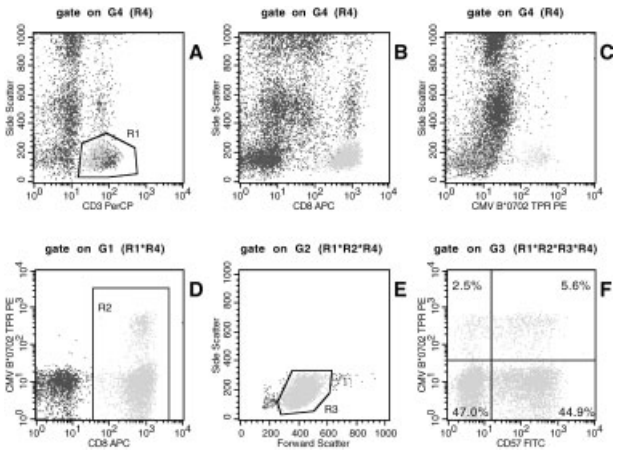

FIG. 1. Enumeration of $CD8^+$ T lymphocytes specific for the CMV pp65-encoded peptide TPRVTGGGAM presented by HLA-B*0702. The subset of $CD3^+,8^+$ T cells are depicted in green, other $CD3^+$ T cells are in red, and cells other than $CD3^+$ T cells are in gray. Inadvertently acquired air bubbles are excluded by selecting only list mode data with a stable rate of events per second (time gate G4, not shown). T lymphocytes are identified by region R1 as $CD3^+$ events with low sideward light scatter (A). Plots B and C serve to verify the positions of the total $CD8^+$ T-cell population and the tetrameter-binding $CD8^+$ cells, respectively. Among the $CD3^+$ T lymphocytes, the $CD8^+$ T cells are selected by region R2 (D). Events with low and high forward light scatter signals relative to the major $CD8^+$ T-cell cluster, representing dead cells and cell aggregates, respectively, are excluded by placing region R3 as shown in (E). Within the viable single $CD8^+$ T cells (gate G3), the percentage of tetramer-binding cells is assessed, while quadrant statistics allow the analysis of coexpression of a fourth marker by tetramer-binding $CD8^+$ T cells (F). In this sample, the $CD8^+$ subset constituted 68% of the $CD3^+$ T cells; 8.1% of the $CD8^+$ T cells were specific for TPRVTGGGAM presented by B*0702; and 69% of the latter cells coexpressed CD57. By adapting this technique for intracellular staining, coexpression of intracellularly expressed markers such as granzyme A or perforin by the tetramer-binding $CD8^+$ T cells can be assessed (30).

with leucine zipper motifs and flexible linkers. Furthermore, a biotinylation site is added to the COOH terminus of the β chain. Following purification, biotinylation is performed. The leucine zippers allow stable pairing of the soluble α and β chains, allowing loading with the appropriate peptide. The flexible linkers prevent steric hindrance when the αβ heterodimers are complexed to the fluorochrome-linked streptavidin molecule.

The use of Class II MHC-peptide tetramers to detect and monitor antigen-specific $CD4^+$ T cells is less straightforward than the use of Class I tetramers for $CD8^+$ T cells (27). $CD4^+$ T cells specific for particular antigens, as detected by Class II tetramers, typically circulate at very low frequencies (i.e., $<0.01\%$ of $CD4^+$ T cells). Such low frequencies are below the detection limit of flow cytometry (see above). In order to allow the detection of these low frequency $CD4^+$ T cells, these T cells are stimulated with the study antigen so that following their proliferation, their frequencies are higher and their detection is facilitated (17). The incorporation of carboxyfluorescein diacetate succinimidyl ester, a fluorescent dye, into the cell surface membrane prior to culture allows the quantitation of the number of cell divisions (i.e., up to 10) during these expansion cultures. In this way, the original fre-

quency of antigen-specific $CD4^+$ T cells—which are capable of dividing—in the original sample can be extrapolated (17).

Cytokine Flow Cytometry

CFC is based on the detection of intracellular cytokines with fluorochrome-conjugated mAb that occurs as a consequence of short-term stimulation of leukocytes with recall antigens (e.g., virus- or tumor-derived peptides or proteins) and polyclonal activators such as mitogens or inductors of intracellular signal cascades (i.e., phorbol 12-myristate 13-acetate [PMA] plus ionomycin). Normally, unstimulated leukocytes produce little or no cytokines. PBMC suspensions or whole blood are typically used for this assay (31–33). An incubation time of 6 h is sufficient for inducing cytokine production by T cells using polyclonal activators and peptides. The peptides must have the appropriate format for direct loading of Class I and II MHC molecules in order to achieve efficient activation of $CD8^+$ and $CD4^+$ T cells, respectively. In contrast, whole proteins need to be taken up by antigen-processing cells for endosomal processing (34); the resulting peptides are mainly presented by Class II MHC molecules to $CD4^+$ T cells. However, in a minority of individuals some proteins may enter the endogenous processing pathway so that the derived peptides are presented by Class I MHC molecules to $CD8^+$ T cells (35,36). However, T cells are much less efficiently stimulated via this route of presentation than via exogenously loaded peptides (36). Consequently, the use of complete proteins is recommended for stimulating $CD4^+$ T cells, but not $CD8^+$ T cells.

In order to let the cytokines accumulate in the stimulated cells, protein transport inhibitors such as brefeldin A or monensin are added after 2 h of incubation. Following stimulation, the leukocytes are collected, washed, and fixed in paraformaldehyde. When whole blood has been used, simultaneous red cell lysis and fixation can be achieved by the use of FACS Lysing Solution (BD Biosciences, San Jose, CA). Subsequently, the cell membranes are permeabilized using mild nonionic detergents to allow intracellular staining. Staining is then performed using conjugated mAbs that have been selected and formulated to react with antigens in fixed and permeabilized cells. As with the tetramers, we recommend the use of CD3 to allow thresholding on total T cells during data acquisition, combined with the use of CD4 and CD8 to identify the helper and cytotoxic T cells, respectively. Alternatively, dump gating may be used to exclude unwanted cell populations from analysis (33). The third or fourth color is then used for cytokine detection. The analytical procedure of a sample stained with CD3, CD4, CD8 and anti-IFN-γ or mouse IgG_1 isotype control mAb is shown in Figure 2. Cytokines are commonly counterstained with CD69 mAb in order to visualize the cytokine-producing cells as a 2D cluster (31–33). However, as we have rarely, if at all, observed $CD69^-$,IFN-γ^+ T cells after peptide or protein stimulations, we consider the use of CD69 staining redundant. Similarly, the use of isotype control mAb

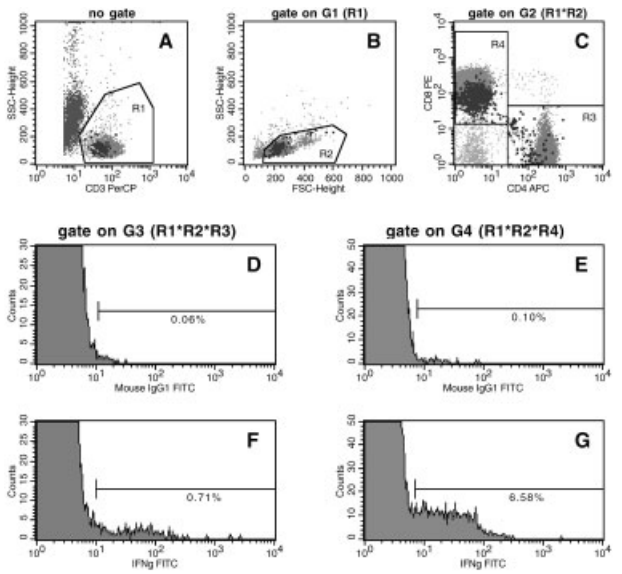

FIG. 2. Enumeration of $CD4^+$ and $CD8^+$ T lymphocytes producing IFN-γ after 6 h stimulation with a pool of overlapping 15-mer peptides spanning the CMV pp65 protein. In (A–C), $CD4^+$ T cells are depicted in blue; among them, IFN-γ^+ cells are highlighted in dark blue. $CD8^+$ T cells are depicted in red; among them, IFN-γ^+ cells are highlighted in violet. Other $CD3^+$ T cells are shown in green; other cells than $CD3^+$ T lymphocytes are in gray. During data acquisition, a generous threshold is set on CD3 PerCP in order to exclude most $CD3^-$ events; this threshold should not be set too tight on the $CD3^+$ population to avoid exclusion of $CD3^{dim}$ T cells that have downregulated CD3 as a consequence of their activation. During list mode data analysis, viable T cells are identified by region R1 as $CD3^+$ events with low sideward light scatter (A) combined with exclusion of dead cells with low forward light scatter (region R2) (B). The gated viable T cells are shown in (C); region R3 selects the $CD4^+$ T cells and region R4 selects the $CD8^+$ T cells. The reactivities of $CD4^+$ and $CD8^+$ T cells with the IgG_1 isotype control mAb are shown in (D) and (E), and the reactivities of these subsets with the anti-IFN-γ mAb are shown in (F) and (G). The markers discriminating positive from negative events are placed on the isotype control histograms, and the proportions of cells exceeding these thresholds are subtracted from the respective percentages of IFN-γ^+ cells. In this example, 0.65% of $CD4^+$ T cells and 6.48% of $CD8^+$ T cells are IFN-γ^+. Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinyl chlorophyllin.

is controversial (37,38), in particular when a negative procedure control (see below) is included that frequently yields similar information (T Bunde and F Kern, unpublished results). If an isotype control mAb staining is used, it must be appropriately matched and formulated, i.e., it should approximate the anti-cytokine mAb as much as possible (i.e., same concentration and fluorochrome-to-protein ratio), except for the very antigen binding portion (i.e., the paratope).

As with any functional assay, negative and positive procedure controls are recommended. The negative control consists of the same blood sample stimulated with "irrelevant" antigen, e.g., a lysate from uninfected fibroblasts as control for CMV-infected fibroblasts. Background cytokine production in healthy donors is typically low (i.e., ≤0.02% of $CD4^+$ or $CD8^+$ T cells) (39,40), but it may be higher in patients with constitutively activated T cells. Nevertheless, very low frequencies of antigen-specific T cells can

be detected if a sufficiently large list mode data set is collected (23,39).

Clinical Applications and Relevance

Viral infections. The main application of the combination of Class I MHC-peptide tetramers, CFC, and these techniques, has been, and still is, the analysis of antiviral $CD8^+$ T cells. In patients infected with HIV, a dynamic equilibrium between the levels of HIV-specific $CD4^+$ and $CD8^+$ T cells and viral load was observed. In patients starting on (highly active) antiretroviral therapy, HIV-specific $CD8^+$ T-cell counts increased sharply in the face of decreasing viral load (41–44), but gradually decreased when the viral load was efficiently controlled by the therapy, indicating that the continuous presence of HIV antigens is necessary to drive the proliferation of HIV-specific $CD8^+$ T cells (41,45–48). However, high levels of HIV-specific $CD8^+$ T cells were found to persist in patients progressing to AIDS and in long-term nonprogressing HIV carriers (49). Importantly, the combined use of tetramer staining and CFC suggested that significant proportions of HIV-specific $CD8^+$ T cells were nonfunctional, as evidenced by the failure of (part of) these cells to accumulate IFN-γ after stimulation with the cognate peptide (50,51). An earlier CFC-based study had already indicated the important role of HIV-specific $CD4^+$ T cells in this respect, being more prominently present in nonprogressing patients than in those progressing to AIDS (52). Specifically, the loss of HIV-specific $CD4^+$ T cells was associated with a decline in functional (i.e., IFN-γ–producing) HIV-specific $CD8^+$ T cells, increments of HIV load, and progression to AIDS (48,51,53).

With respect to the herpesviruses, Class I MHC tetramers have been used to detect and monitor virus-specific $CD8^+$ T cells in primary and latent infections with CMV (54–56) and EBV (57–59). In patients with AIDS and in iatrogenically immunosuppressed patients such as organ and blood stem cell transplant (SCT) recipients, CMV and EBV infections may reactivate and if not controlled, progress to CMV disease and EBV^+ B-lymphoproliferative disease. The use of CFC allowed the detection of CMV-specific $CD4^+$ T cells in the blood of CMV carriers; their frequency was higher in HIV-infected than in noninfected individuals (9). Even in healthy virus carriers, CMV-specific T cells showed significant fluctuations over time (60), and circulated in significantly higher numbers than T cells specific for herpes simplex or varicella zoster virus (61). The combined results of these three studies indicate that CMV presents a considerable and recurrent challenge to the immune systems of its carriers. Tetramer-based studies of CMV- and EBV-specific $CD8^+$ T cells have confirmed their crucial role in keeping viral infection under control; for both viruses, a combination of quantitative monitoring of viral load and virus-specific $CD8^+$ T-cell reconstitution allowed the identification of SCT recipients at high risk for progressive viral infection and disease (62,63). The combination of tetramer staining with CFC revealed nonfunctional CMV-specific $CD8^+$ T cells (i.e., $TNF\text{-}\alpha^-$ upon stimulation with the cognate peptide), particularly in heavily immunosuppressed SCT recipients (i.e., those receiving corticosteroid therapy for acute graft-versus-host disease); the occurrence of these nonfunctional $CD8^+$ T cells was associated with recurrent CMV antigenemia (64) By the same token, increased EBV loads progressing to EBV^+ lymphoma were observed in HIV-infected patients with declining $CD4^+$ T cell counts and nonfunctional (i.e., $IFN\text{-}\gamma^-$) EBV-specific $CD8^+$ T cells (65). The important role of CMV-specific $CD4^+$ T cells was illustrated in HIV-infected patients and SCT recipients. In the first group of patients, CMV disease developed in patients without detectable CMV-specific $CD4^+$ T cells, but not in those with CMV-specific $CD4^+$ T cells, while these cells increased in number after highly active antiretroviral therapy (66). Furthermore, significant numbers of CMV-specific $CD8^+$ T cells only appeared in the circulation of HIV-infected patients with detectable CMV-specific $CD4^+$ T cells (67). Finally, the reconstitution of CMV-specific $CD4^+$ and $CD8^+$ T cells was required for the resolution of active CMV infection in allogeneic SCT recipients, while the lack of these cells in CMV-infected patients at three months post-SCT was associated with the development of late CMV disease (68).

As for hepatitis, $CD8^+$ T cells specific for hepatitis B (69–71) or C (72,73) virus were found to increase rapidly during the acute phase of infection, whereas after its resolution, virus-specific $CD8^+$ T cells persisted at higher levels in patients who had cleared viremia than in those who had not.

The studies summarized above have shown a dynamic interaction between persisting viruses and the immune systems of their carriers: functional virus-specific T cells of both $CD4^+$ and $CD8^+$ subsets are required to keep these viruses under control.

Cancer. Most studies using Class I MHC tetramers and CFC to detect and monitor tumor-specific $CD8^+$ T cells have been performed in patients with malignant melanoma. Tetramer technology allowed the detection and isolation of HLA-A*0201–restricted, Melan-A/MART-1–specific $CD8^+$ T cells from lymph nodes containing melanoma metastases, but not from lymph nodes without such metastases (74–76). In addition, these $CD8^+$ T cells were not only detectable in the circulation of patients with melanoma, but also in patients with vitiligo, an autoimmune disease characterized by the loss of epidermal melanocytes, and in healthy individuals (77–79). Other melanoma-associated proteins eliciting specific $CD8^+$ T-cell responses in vivo are tyrosinase, gp100, and the MAGE proteins (79–82). Class I MHC tetramers are also excellent tools to monitor specific $CD8^+$ T-cell responses after vaccination (83–85). Although melanoma-specific $CD8^+$ T cells, upon isolation and culture, can express high levels of specific cytolytic activity against melanoma target cells in vitro, their in vivo presence in melanoma lesions is not clearly associated with regression of these lesions (76,82). Detailed phenotypic and functional analyses of the melanoma-specific $CD8^+$ T cells revealed their heterogeneity within and between patients. Some exhibited a "naive" $CD45RA^{++}$,$45R0^-$,$CCR7^+$ phenotype lacking melanoma-

specific reactivity; this phenotype was predominant in healthy individuals (86,87). Others expressed an antigen-experienced a $CD45^-$,$45R0^+$,$CCR7^+$ or terminally differentiated $CD45RA^{(+)/++}$,$45R0^-$,$CCR7^-$ phenotype and were able to lyse appropriately matched melanoma cells or to secrete IFN-γ in response to these cells (86–88). The relative proportions of these subsets among melanoma-specific $CD8^+$ T cells in patients may vary over time (78,86). Thus, one explanation for the paradoxical presence of melanoma-specific $CD8^+$ T cells in the absence of regression of melanoma lesions could be the anergization of these T cells, e.g., due to the immunosuppressive environment of the tumor tissue or lack of appropriate costimulatory molecules on the tumor cells. However, in this context, little if anything is known about the role of tumor-specific $CD4^+$ T cells to initiate and maintain effective antitumor $CD8^+$ T-cell responses.

CFC has also been used to assess antitumor responses in other settings. Spontaneous $CD8^+$ T-cell reactivity against HLA-A*0201–restricted peptides derived from WT1 or proteinase 3, both overexpressed by AML blasts, was observed in 8 of 15 patients with AML (89). After vaccination of patients with adenocarcinoma using the MUC-1 peptide, clear peptide-specific $CD8^+$ T cell responses were seen (90). Specific $CD4^+$ T-cell responses were observed in patients with multiple myeloma after vaccination using autologous dendritic cells pulsed with autologous tumor immunoglobulin idiotype (91).

Class II MHC-peptide tetramers. The use of Class II MHC-peptide tetramers has been reported to detect and monitor Class II-restricted, antigen-specific $CD4^+$ T cells in viral (17,92) and bacterial infections (93), autoimmune diseases (94–96), and before and after dendritic cell-based vaccination (97). The restricted range of Class II MHC alleles and the complicated assay formats required to detect Class II-restricted, antigen-specific $CD4^+$ T cells (see above) has thus far prevented their widespread application.

CONCLUSIONS AND PERSPECTIVE

Flow cytometric detection of individual antigen-specific T lymphocytes has significantly advanced, and will continue to do so. It has much increased our insight into the interactions between viruses and tumors, and their carriers. Two major approaches exist, i.e., MHC multimers (i.e., currently mainly Class I HLA tetramers) and cytokine flow cytometry. Both techniques can be used to monitor and isolate antigen-specific T cells in a clinical setting. They can even be combined to increase information.

For detection and monitoring of antigen-specific T cells in a clinical setting, Class I HLA tetramers have advantages and limitations. Importantly, the combination of Class I HLA tetramer staining and stimulation with the cognate peptide in a single assay yields information on the functional status of the $CD8^+$ T cells, which is important in the immunodeficiency setting. The advantages of Class I HLA tetramer staining are rapidity (the assay can be completed in 2 h) and excellent reproducibility (intra- and interassay CVs of $<10\%$ are realistic) (98). A significant limitation in clinical routine is that the patient's HLA type must be known, as well as a T-cell epitope presented in the respective context. Some applications, e.g., general surveys of T-cell reactivity against a particular virus or protein, are limited by the fact that the currently available synthetic HLA molecules cover only a minority of all Class I HLA alleles, and that cognate peptides are not known for each HLA allele. Thus, it is practically impossible to fully comprehensively cover the $CD8^+$ T-cell response against a protein by Class I HLA tetramers only. Rather, these reagents offer "snapshot views" of the $CD8^+$ T-cell response, which have nevertheless thus far yielded very useful information, as reviewed above. Unfortunately, the number of commercial suppliers of Class I HLA tetramers is very limited and reagent costs are high (typically US$ 30–40 per test).

Cytokine flow cytometry is less rapid than Class I HLA tetramer staining: the test takes approximately 8 h if cells are stimulated with antigen for 6 h. The reproducibility is good (intraassay CVs $<10\%$ and interassay CVs $<25\%$ are realistic) (33). When protein-spanning peptide pools are used as source of antigen, the patient's HLA type only needs to be known in advance when fine specificities of the T-cell responses have to be unraveled (14,99). When using pools of 15-mer peptides that overlap each other by 11 amino acids, both $CD4^+$ and $CD8^+$ T-cell responses can be analyzed in a single assay. Therefore, this approach offers a much more comprehensive view of the T-cell response against specific proteins than Class I HLA tetramers. Practical points of attention are: 1) the amino acid sequence of the studied protein must be known; and 2) in many cases, protein-spanning peptide pools must be manufactured to order. This can be very costly depending on the size of the protein and the synthesized amount per peptide. Once the peptides are synthesized, protein-spanning peptide pools can be aliquotted and frozen at –80°C, yielding a price per test in the range of US$ 5–10.

Both Class I HLA tetramers and cytokine flow cytometry, in combination with magnetic particle technology for cell enrichment, have considerable potential to isolate T lymphocytes for adoptive immunotherapy (100,101). $CD8^+$ T cells have been isolated using Class I HLA tetramers from the peripheral blood of CMV-seropositive SCT donors and administered to SCT recipients with CMV reactivation in order to protect them against further progression of this complication (M. Cobbold, personal communication). CMV-specific T cells derived from SCT donors have also been adoptively transferred to recipients with CMV reactivations not responding to antiviral therapy using a scaled-up cytokine secretion assay (102). Thus, flow cytometric detection of individual antigen-specific T cells has great potential not only for diagnostic, but also for therapeutic purposes.

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