

**CHARACTERIZATION OF VIRUS-SPECIFIC CD8<sup>+</sup> T CELL DIFFERENTIATION**

By

Aki Hoji

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This dissertation was presented

by

**Aki Hoji**

It was defended on

March 29, 2005

and approved by

Olivera J. Finn, Ph. D.  
Professor and Chair  
Department of Immunology  
School of Medicine  
University of Pittsburgh

Massimo M. Trucco, M.D.  
Professor and Director  
Department of Pediatrics  
Pittsburgh Diabetes Institutes  
School of Medicine  
University of Pittsburgh

Phalguni Gupta, Ph.D.  
Professor and Vice Chair  
Department of Infectious Diseases and Microbiology  
Graduate School of Public Health  
University of Pittsburgh

Simon M. Barratt-Boyes, D.V.M., Ph.D.  
Assistant Professor  
Department of Infectious Diseases and Microbiology  
Graduate School of Public Health  
University of Pittsburgh

Dissertation Director:  
Charles R. Rinaldo, Jr., Ph.D.  
Professor and Chair  
Department of Infectious Diseases and Microbiology  
Graduate School of Public Health  
University of Pittsburgh

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Virus-specific memory CD8<sup>+</sup> T cells play a prominent role in protection of a host from recurring and persistent virus infection. It is known that memory CD8<sup>+</sup> T cells undergo a series of differentiation stages to become fully matured effector cells. There are several important aspects of the current CD8<sup>+</sup>T cell memory phenotype model that need to be more thoroughly defined.

In specific aim 1, it was hypothesized that CD27<sup>+</sup>CD28<sup>+</sup> undifferentiated CD8<sup>+</sup> memory T cells specific for non-persistent virus influenza A (FluA) would have phenotypic markers associated with more differentiated (effector) phenotypes. Results showed that in spite of the phenotypic enrichment of FluA-specific memory CD8<sup>+</sup> T cells in the undifferentiated stage, they displayed effector markers indicative of late stage differentiated effector cells.

In specific aim 2, it was further hypothesized that the most undifferentiated CD62L<sup>+</sup> central memory CD8<sup>+</sup>T cells would have the effector function including immediate cytoplasmic production of IFN- $\gamma$  upon antigenic-stimulation.. Results showed that CD62L<sup>+</sup> CD8<sup>+</sup> T cells are capable of immediate IFN- $\gamma$  production after antigen-specific stimulation in the presence of the CD62 sheddase inhibitor, GM6001, highlighting the need to re-evaluate the defining markers of virus-specific central memory CD8<sup>+</sup> cells and/or their functions.

In specific aim 3, this dissertation tests the hypothesis that memory-effector differentiation of HIV-1-specific memory CD8<sup>+</sup> T cells is impaired during the course of

persistent HIV-1 infection. Detailed comparison of CD27 and CD57 co-expression on HIV-1-specific CD8<sup>+</sup> T cells showed that these cells had a significantly lower proportion of the CD27<sup>-</sup>CD57<sup>high</sup> effector subset. Moreover, these cells did not display progression from CD27<sup>+</sup>CD57<sup>-</sup> (immature memory), through CD27<sup>low</sup>CD57<sup>low</sup> (transitional memory-effector) to CD27<sup>-</sup>CD57<sup>high</sup> (effector subset) that was seen in well differentiated EBV-specific CD8<sup>+</sup> T cells and was common in CMV-specific CD8<sup>+</sup> T cells. These observations suggest that the normal course of HIV-1-specific CD8<sup>+</sup> T cell memory-effector differentiation is impaired during the course of persistent HIV-1 infection.

Elucidation of memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells has significant public health implications. Understanding the impairment of memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells, for instance, will greatly facilitate a design of effective vaccine against progressive HIV-1 infection.



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## I.

### INTRODUCTION

#### A. Virus-Specific CD8<sup>+</sup> T Cell Memory-Effector Differentiation

##### 1. MHC Class I Tetrameric Reagent

CD8<sup>+</sup> T cells play a major role in the cell mediated immunity against various types of pathogens. One of the most critical features that differentiates CD8<sup>+</sup> T cells from other immune cells is their antigen-specificity. Characterization of CD8<sup>+</sup> T cells specific for a particular antigen is therefore, essential for understanding the biology of CD8<sup>+</sup> T cells, and there has been great interest in accurately quantitating antigen-specific CD8<sup>+</sup> T cells.

Historically, the frequency of antigen-specific CD8<sup>+</sup> T cells was quantitated by direct cloning and limiting dilution assay (LDA). There are several caveats, however, of using the LDA for estimation of the frequency of antigen-specific CD8<sup>+</sup> T cells. The most significant caveat is a requirement of *in vitro* manipulation involving expansion of antigen-specific CD8<sup>+</sup> T cells over many weeks. The frequency of antigen-specific CD8<sup>+</sup> T cells derived from the LDA is markedly affected by the proliferative potential of antigen-specific CD8<sup>+</sup> T cells. In fact, the LDA greatly underestimates the *ex vivo* frequency of antigen-specific CD8 T cells, particularly if the antigen-specific CD8<sup>+</sup> T cells contain a large proportion of cells with less than adequate proliferative potential.

A replacement for LDA that could accurately quantitate antigen-specific CD8<sup>+</sup> T cells *ex vivo* appeared in 1996. Altman et. al. (2) reported on the new revolutionary methodology using



an MHC class I tetrameric reagent that allowed visualization and quantitation of the antigen-specific CD8<sup>+</sup> T cells *ex vivo* by flow cytometry. As its name suggests, the MHC Class I tetrameric reagent consists of four recombinant MHC class I monomers, each loaded with a peptide derived from an epitope of a particular antigen. Two components of the recombinant MHC class I monomer (heavy chain and  $\beta$ 2M) are produced in bacteria. After purification, they are allowed to recombine and form the mature MHC class I monomer. The recombinant MHC class I monomer has a biotinylation tag where biotin is covalently added by a biotinylation transferase (Figure 1). A tetrameric complex formed in the presence of streptavidin labeled with a fluorescent dye, allows detection of the antigen-specific CD8<sup>+</sup> T cells by the flow cytometer.

Another benefit of using the MHC class I tetrameric reagent is to conduct phenotypic and functional assessments of antigen-specific CD8<sup>+</sup> T cells. With advances in the modern flow cytometry, this reagent makes it possible to address many functional and phenotypic aspects of antigen-specific CD8<sup>+</sup> T cells. The phenotypic assessment of many antigen-specific CD8<sup>+</sup> T cells has helped establish a model of antigen-specific CD8<sup>+</sup> T cell development and differentiation. Also, combined with functional assays, it is possible to measure capacities of antigen-specific CD8<sup>+</sup> T cells for apoptosis, proliferation and effector functions both *in vitro* and *in vivo* (3, 4).

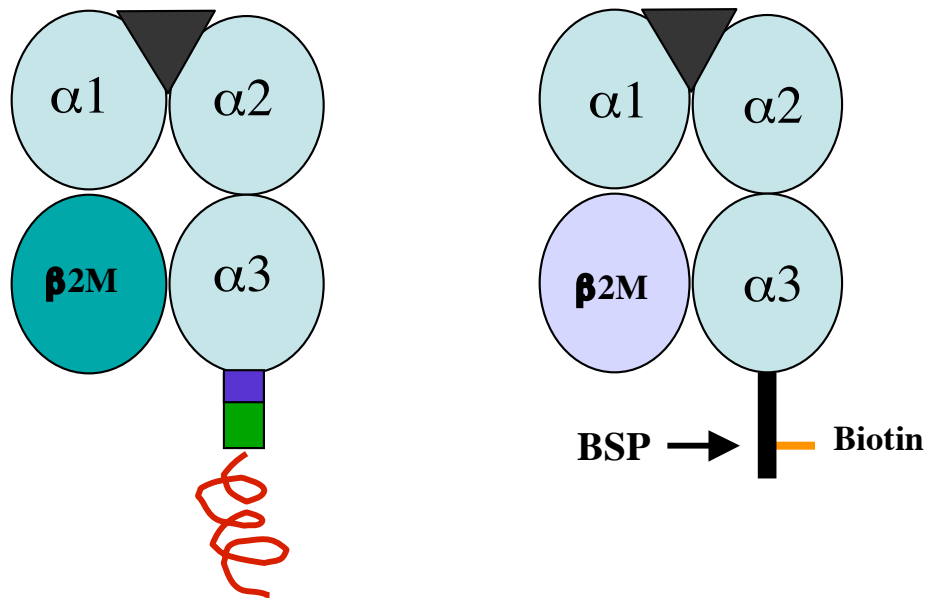


Figure 1. A theoretical structure of a MHC Class I tetrameric complex. (A) Four recombinant MHC Class I monomers (dark blue) are oligomerized with streptavidin (light blue) conjugated with phycoerythrin (red) Adapted from McMichael et. al. (5) (a copyright permission granted from Nature Publishing Group (<http://www.nature.com>)). (B) Schematic drawings of a wild type (left) and a recombinant (right) MHC class I monomers. A heavy chain (light blue) contains three globular domains associated non-covalently with  $\beta 2M$  (green and purple) and a peptide (black triangle). While the wild type monomer has transmembrane (blue and green) and cytoplasmic (red) domains, the recombinant monomer has a biotinylation sequence (BSP) where biotin is attached covalently.

Significantly, the recent development of MHC class II tetrameric reagents in addition to the MHC class I tetrameric reagent will offer nearly complete phenotypic and functional assessments of T cell immunity to various pathogens and tumors.

## 2. Differentiation Associated Phenotypic Markers

Prior to the development of the MHC class I tetrameric reagent, memory-effector differentiation of virus-specific  $CD8^+$  T cells was modeled by phenotypic and functional assessment of bulk  $CD8^+$  T cells. These early studies found several surface molecules that could be used as reliable phenotypic markers for distinguishing functional subsets such as naïve, memory and effector

subsets. More recent studies using MHC class I tetrameric reagents have added many more surface molecules to a growing list of differentiation-associated phenotypic markers. Importantly, the most frequently used phenotypic markers typically have clear cellular functions. CD62L (L-selectin) mediates adhesion of lymphocytes to high endothelial venules of secondary lymphoid tissues (6), and augments recruitment of activated effector CD8<sup>+</sup> T cells to inflammatory sites (7). CCR7 is a chemokine receptor for a class of CC chemokines, ELC (CCL19) and SLC (CCL21), and a critical molecule for T lymphocyte homing to secondary lymphoid organs (8). CD27 belongs to the TNFR superfamily. Its primary cellular function is to induce memory and effector differentiation through interaction with CD70 (9). CD28 belongs to an immunoglobulin superfamily, and its main function is to provide T cell co-stimulation to promote proliferation, survival, and effector differentiation (10). CD57, also known as HNK-1 antigen, is a modified carbohydrate (glucuronic acid 3-sulfate) (11). Its primary cellular function is to mediate cell-cell interaction, and is also a co-receptor for IL-6 (12). CD45R isoforms have a common cytoplasmic phosphatase domain, and appear to regulate T cell receptor (TCR) signals. Isoform switch occurs by alternative splicing, which generates the longer RA and shorter RO products. An exact triggering mechanism of isoform switch is not clearly understood (13).

Phenotypic subsets are usually defined by combination of at least two phenotypic markers. Modern flow cytometers allow simultaneous detection of 4 parameters (4 distinct surface markers). Usually, two parameters (a virus-specific MHC Class I tetrameric reagent and CD8) are reserved for defining virus-specific CD8<sup>+</sup> T cells, and the remaining two parameters are used to delineate phenotypic subsets. Notably, the most advanced cytometer has 11

parameters available for simultaneous determination of phenotypic marker expression on a single cell (14), permitting a more sophisticated phenotypic analysis.

### 3. Memory-Effector Differentiation Models

Early studies characterizing memory-effector CD8<sup>+</sup> T cell differentiation showed that antigen-specific CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) precursors were enriched in a CD45RA<sup>-</sup>CD45RO<sup>+</sup> subset (15). Later studies found that the CD45RA<sup>+</sup> subset contained CD8<sup>+</sup> T cells with low CD62L (16), low CD27 (17), and high CD11c/18 expression (18), which were phenotypic characteristics of effector cells. Following these studies, Hamann et al. (19) proposed a model of memory-effector CD8<sup>+</sup> T cell differentiation based on CD27 and CD45RA expression. By direct examination of functional properties (cytokine production and cytotoxic potential) of an enriched CD27<sup>+/-</sup>CD45RA<sup>+/-</sup> subset, they were able to show that naïve (CD27<sup>+</sup>CD45RA<sup>+</sup>), memory (CD27<sup>+</sup>CD45RA<sup>-</sup>) and effector (CD27<sup>-</sup>CD45RA<sup>+</sup>) subsets of CD8<sup>+</sup> T cells could be distinguished phenotypically.

More recent studies have added greater complexity to the model by incorporating migratory properties of T cells. These studies also provided evidence that the memory subset is highly heterogeneous phenotypically and functionally. The migratory property of CD8<sup>+</sup> (and CD4<sup>+</sup>) T cells is determined by a pattern of chemokine receptor expression. The most relevant chemokine receptor to CD8<sup>+</sup> T cell memory-effector differentiation is CCR7. It was shown that the memory CD8<sup>+</sup> T cells could be further divided into two subsets based on expression of CCR7 and CD45RA (20). CCR7<sup>+</sup>CD45RA<sup>-</sup>(CD62L<sup>+</sup>) CD8<sup>+</sup> T cell, a central memory cell (T<sub>CM</sub>), have the homing capacity to lymphoid organs, and lack cytoplasmic effector molecules (e.g. perforin and granzymes) and immediate effector function (e.g. cytoplasmic IFN- $\gamma$  production). In

contrast,  $CCR7^-CD45RA^-$  (CD62L<sup>low</sup>)  $CD8^+$  T cells are effector memory ( $T_{EM}$ ) cells that have homing capacity to non-lymphoid organs, and possess immediate effector functions. Based on these observations, Sallusto et. al. (20) proposed 4 stages of  $CD8^+$  T cell differentiation:  $CCR7^+CD45RA^+$  (naïve),  $CCR7^+CD45RA^-$  ( $T_{CM}$ ),  $CCR7^-CD45RA^-$  ( $T_{EM}$ ), and  $CCR7^-CD45RA^+$  (effector). Furthermore,  $T_{CM}$  appears to have two new subsets,  $CCR4^+$  type 2 polarized, and  $CCR4^-$  conventional type 1 polarized  $T_{CM}$  (21).

These studies have built a foundation of the memory-effector  $CD8^+$  differentiation model. Indeed, when the MHC class I tetrameric reagent became widely available, a similar combination of phenotypic markers was used to characterize virus-specific  $CD8^+$  T cells. Recent studies have shown that virus-specific  $CD8^+$  T cells undergo similar phenotypic stages of memory-effector differentiation defined by these earlier studies, though the earlier models of memory-effector  $CD8^+$  T cell differentiation have been greatly refined in recent years.

By utilizing a large number of MHC class I tetrameric reagents, Appay et. al. (22) determined and compared phenotypes of  $CD8^+$  T cells specific for human persistent virus during acute and chronic infection. They found that such  $CD8^+$  T cells regardless of virus-specificity expressed predominantly activated ( $CD38^+HLA-DR^+Ki-67^+$ ), immature/undifferentiated ( $CD27^+CD28^+$ ) phenotype, and cytotoxic effector molecules (perforin and granzymes). During chronic infection, however, these virus-specific  $CD8^+$  T cells were enriched in the particular stage of memory-effector differentiation according to their specific virus. This implies that classical models of  $CD8^+$  T cell differentiation may not accurately ascribe differentiation of virus-specific  $CD8^+$  T cells. Based on these results, four stages of virus-specific  $CD8^+$  T cell differentiation have been proposed based on expression of CD27 and CD28 (22, 23). The model shows virus-specific  $CD8^+$  T cell memory-effector differentiation progressing from

CD27<sup>+</sup>CD28<sup>+</sup> (early memory) through CD27<sup>+</sup>CD28<sup>-</sup> (intermediate memory) to CD27<sup>-</sup>CD28<sup>-</sup> (late memory effector).

Another equally important model (24) of virus-specific CD8<sup>+</sup> T cell memory-effector differentiation was proposed based on the earlier work of Hamman et. al. (19) and the phenotypic and functional characterization of CMV-specific CD8<sup>+</sup> T cells (25). Unlike the previous linear model, these authors proposed a branched model of virus-specific CD8<sup>+</sup> T cell memory-effector differentiation. Virus-specific CD8<sup>+</sup> T cells progress from CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup> (naïve precursor), through CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>-</sup> (acute effector stage) and CD27<sup>+</sup>CD28<sup>+/-</sup>CD45RA<sup>-</sup> (recovery stage), to either CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup> (effector 1) or CD27<sup>+</sup>CD28<sup>-</sup>CD45RA<sup>+</sup> (effector 2).

It is worth noting mouse models of CD8<sup>+</sup> T cell memory-effector differentiation because there are several important aspects that human models cannot provide. One of such important aspect is that the dynamics of CD8<sup>+</sup> T cell differentiation is fully known in the mouse model (26). During primary infection, naïve precursors of virus-specific CD8<sup>+</sup> T cells expand and become effectors; soon after primary infection (during a contraction/death period), a majority of these effectors undergo apoptotic death. A fraction of the virus-specific CD8<sup>+</sup> T cell survives during a contraction period forms the immunological memory.

Although a similar process occurs in the human (24), there are important differences between human and mouse virus-specific memory-effector CD8<sup>+</sup> T cells with respect to their phenotypic and functional characteristics. The most significant difference is that unlike virus-specific CD8<sup>+</sup> T cells in humans, the phenotype of virus-specific mouse CD8<sup>+</sup> T cells during the acute phase of infection is the effector type (CCR7<sup>low</sup>CD27<sup>low</sup>CD28<sup>low</sup>), and a majority of these cells differentiate to T<sub>EM</sub>, and then become T<sub>CM</sub> in a later phase of acute infection (1) (Figure 2).

In the mouse,  $T_{CM}$  cells are thought to be a source of long-term memory. Conceptually, this makes sense, since  $T_{CM}$  cells have a greater proliferative capacity and lack immediate effector functions, and their phenotype resembles naïve and immature memory subsets (27). Recently, mouse  $T_{CM}$  cells have been shown to be equally or more effective in protecting the host from systemic or peripheral viral challenge than  $T_{EM}$  cells (28). Also, in humans, a significantly higher proportion of  $T_{CM}$  cells have recently been shown to have immediate effector functions (29), challenging the previously proposed function of  $T_{CM}$  (20). However, unlike mouse  $T_{CM}$ , the actual protective immunity of  $T_{CM}$  in human has not been assessed *in vivo*.

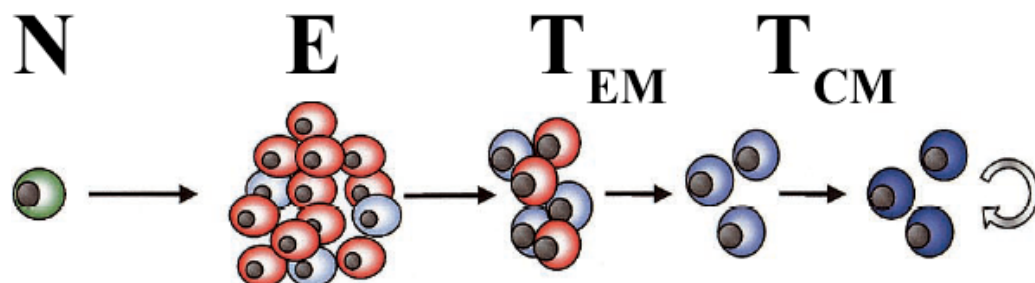


Figure 2. A mouse model of memory-effector virus-specific  $CD8^+$  T cells proposed by Wherry et. al. (1) (a copyright permission granted from Journal Department, American Society of Microbiology).

There are similarities between human and mouse models of virus-specific  $CD8^+$  T cell memory-effector differentiation. The most important similarity is significance of  $CD4^+$  T cells in modulating  $CD8^+$  T cell memory-effector differentiation through so-called “CD4 help.” In the mouse, the absence of “CD4 help” results in complete functional impairment of lymphocytic choriomeningitis virus (LCMV)-specific  $CD8^+$  T cells, resulting in failure to control persistent LCMV infection (30). During primary infection, primed virus-specific  $CD8^+$  T cell in the absence of “CD4 help” have substantially reduced potential to expand after secondary stimulation (31). Similarly, in humans, the absence of “CD4 help” due to progressive HIV-1

infection results in the impairment of EBV- and HIV-1-specific CD8<sup>+</sup> T cell differentiation (32). Also, survival of adoptively transferred HIV-1-specific CD8<sup>+</sup> T cells correlates with the presence of CD4<sup>+</sup> T cells (33). These observations imply that the function of CD4<sup>+</sup> T cells is evolutionarily conserved, and critically involved in the regulation of CD8<sup>+</sup> T cell memory-effector differentiation.

### **B. The CD8<sup>+</sup> T Cell Response to Persistent Virus Infection**

There are several families of viruses that can establish persistent infection in humans. Among these persistent viruses, CD8<sup>+</sup> T cell responses to EBV and CMV (herpesvirus) and HIV-1 (lentivirus) are exceptionally well characterized. This is due to the fact that these viruses infect a relatively large population of humans, and produce robust CD8<sup>+</sup> T cell responses. As mentioned in the previous section, the present methods commonly used to measure and characterize CD8<sup>+</sup> T cells responses to these viruses are the MHC class I tetrameric reagent, intracellular cytokine staining (ICS), and ELISPOT analysis. The MHC class I tetrameric reagent measures *ex vivo* frequency and also characterizes phenotypes (with a panel of monoclonal antibodies against surface markers) of virus-specific CD8<sup>+</sup> T cells. ICS measures *ex vivo* frequency of cytokine producing antigen-stimulated virus-specific CD8<sup>+</sup> T cells and also can characterize phenotypes of cytokine producing cells. Likewise, ELISPOT quantitates the number of cytokines, primarily IFN- $\gamma$ , secreting antigen-stimulated virus-specific CD8<sup>+</sup> T cells in a given number of PBMC.

There are several limitations to each of these methods. The MHC Class I tetrameric reagent is limited by the number of known immunodominant epitopes restricted by particular HLA types. It is, also severely limited by a number of recombinant MHC class I molecules available. Both functional assays, ICS and ELISPOT (measuring IFN- $\gamma$  production), are limited



by the fact that not all the virus-specific CD8<sup>+</sup> T cells are capable of IFN- $\gamma$  production, in part due to functional heterogeneity (34). Moreover, unlike ICS, ELISPOT does not allow phenotypic characterization of antigen-stimulated virus-specific CD8<sup>+</sup> T cells. Notably, to offset all the limitations, most recent studies use at least two of these assays, typically a combination of the MHC class I tetrameric reagent with either ELISPOT or ICS. It is also possible to combine the MHC class I tetrameric reagent and ICS in a single assay (35).

### **1. Responses to Epstein-Barr Virus Infection**

EBV is a member of a gamma-herpesvirus family, and establishes both lytic and persistent infections in human (36). There are two strains of EBV, EBV-1 and EBV-2, in the human population. In North America and Europe, seroprevalence of EBV-1 reaches over 90%, whereas less than 10% of the population is infected with EBV-2 (37). While EBV infection does not cause significant morbidity and mortality, primary infection with EBV especially in adolescents and adults can occasionally result in a relatively severe clinical condition known as infectious mononucleosis, and persistent infection is a suspected cause of several malignancies (38). In a majority of cases, primary EBV infection leads to establishment of asymptomatic latent infection in B cells with occasional virus reactivation and replication (39).

EBV-specific CD8<sup>+</sup> T cells have been well characterized with MHC class I tetrameric reagents, ICS and ELISPOT, though a majority of studies has been limited to CD8<sup>+</sup> T cells specific for several known immunodominant EBV epitopes. CD8<sup>+</sup> T cell responses to immunodominant epitopes from EBV lytic proteins are more robust than those for latency proteins, hence, CD8<sup>+</sup> T cell responses to lytic proteins are better characterized (40).

Regarding the frequency of EBV-specific CD8<sup>+</sup> T cells, it was first reported that during primary infection, up to 12% of CD8<sup>+</sup> T cells (estimated by MHC class I tetrameric reagents and ICS) were specific for a HLA-A2 restricted epitope (GLCTLVAML) derived from a lytic protein, BMLF1, and surprisingly, up to 44% of CD8<sup>+</sup> T cells were specific for HLA-B8 restricted epitope (RAKFKQLL) derived from another lytic protein BZLF1 (41). The proportions of these EBV-specific CD8<sup>+</sup> T cells were significantly higher than values previously estimated by LDA (42). Interestingly, assessment of the usage of a variable region of TCR-β chain (TCR-Vβ) of EBV-specific CD8<sup>+</sup> T cells revealed a high degree of oligoclonal expansion (43), which was consistent with the earlier finding of a narrowly focused CD8<sup>+</sup> T cell response to EBV during primary infection (44, 45).

The average frequency of EBV-specific CD8<sup>+</sup> T cells (estimated by MHC class I tetrameric reagents) during the latency period in asymptomatic virus carriers is on the average 6% of total CD8<sup>+</sup> T cells (46). The decrease in frequency of EBV-specific CD8<sup>+</sup> T cells during latent infection is thought to be due to massive apoptosis of EBV-specific CD8<sup>+</sup> T cells upon resolution of primary viremia (43), reminiscent of the contraction period described in the mouse model of virus-specific CD8<sup>+</sup> T cells (1). Phenotypic analysis of CD8<sup>+</sup> T cells specific for lytic epitopes reveals that during primary infection, these cells uniformly display an activated phenotype, CCR7-CD27<sup>+</sup>CD28<sup>+/-</sup>CD45RO<sup>+</sup> (CD45RA<sup>-</sup>) CD38<sup>+</sup>HLA-DR<sup>+</sup>, while during latent infection, these cells displayed a resting memory phenotype, CD45RA<sup>+</sup>CD27<sup>+</sup>CD28<sup>+/-</sup> (40, 47). Additionally, a comprehensive phenotypic study showed that the majority of CD8<sup>+</sup> T cells specific for EBV lytic epitopes were CD27<sup>+</sup>CD28<sup>+/-</sup> Ki-67<sup>+</sup>CD38<sup>+</sup>, while during latent infection these cells were phenotypically enriched in a CD27<sup>+</sup>CD28<sup>+/-</sup> subset (22).

In contrast, phenotypic and functional characterization of CD8<sup>+</sup> T cells specific for EBV latent epitope-specific has not been as extensive. This is largely due to limited number of potential epitopes available for a study, and often the frequency of EBV latent epitope-specific CD8<sup>+</sup> T cells is quite low for an accurate analysis, even with a modern methodology available. During primary infection, the average *ex vivo* frequency of immunodominant EBV latent epitope (EBNA 3A; HLA-A2, CLGGLLTMV)-specific CD8<sup>+</sup> T cells (estimated by the MHC class I tetrameric reagent or ELISPOT) is typically less than 5%, and in many cases, frequency is well below the threshold of detection limit (40, 47, 48). During latent infection, the frequency of EBV latent epitope-specific CD8<sup>+</sup> T cells slightly raises but essentially remains below 3% (40, 47). As expected, EBV latent epitope-specific CD8<sup>+</sup> T cells during primary infection display an activated phenotype (CCR7<sup>+</sup>CD45RA<sup>-</sup>(CD45RO<sup>+</sup>) much like EBV lytic epitope-specific CD8<sup>+</sup> T cells, whereas during latent infection, unlike EBV lytic epitope-specific CD8<sup>+</sup> T cells, the phenotype of EBV latent epitope-specific CD8<sup>+</sup> T cells remains essentially identical (47).

## **2. Responses to Human Cytomegalovirus Infection**

CMV is a member of the beta-herpesvirus family, and establishes both primary and persistent-latent infection. As with EBV, primary infection of CMV is usually asymptomatic or associated with mild clinical symptoms, but can cause a severe mononucleosis in normal adults (49). Following primary infection, CMV causes asymptomatic persistent/latent infection in healthy individuals. However, in immunocompromised individuals such as bone marrow recipients and HIV-1 infected persons CMV infection can be associated with severe clinical manifestations (50, 51). The seroprevalence of CMV infection is about 50% in the North America Caucasian population, who remain latently infected (51); seroprevalence and latent infection can be as high

as 90-100% in African-Americans and Hispanic-Americans, and in resource poor countries. Cellular tropism of CMV is broader than EBV; CMV is found in leukocytes, epithelial cells (salivary glands and cervix) and PBMC (predominantly in PMNs and monocytes) (51).

Although recent studies have uncovered some novel epitopes (52) and found a broader HLA usage of an immunodominant pp65 antigen (53), most of the knowledge of CD8<sup>+</sup> T cell responses to CMV infection mainly comes from elucidation of CD8<sup>+</sup> T cell responses to HLA-A2 or HLA-B7 restricted immunodominant epitopes (A2;NLVPMNATV, B7;TPRVTGGGAM) derived from the pp65 matrix protein. During primary infection, these CMV-specific CD8<sup>+</sup> T cells expand to on the average 5% of total CD8<sup>+</sup> T cells in asymptomatic individuals, while there is delayed expansion of the CMV-specific CD8<sup>+</sup> T cells in symptomatic patients (54). Phenotypically, these cells are predominantly of the intermediate effector phenotype (CCR7<sup>-</sup>CD27<sup>+</sup>CD45RA<sup>-</sup>) and also express granzyme B (54); others have reported CMV-specific T cells to be CD27<sup>+</sup>CD28<sup>+</sup> (22).

During latent infection, the frequency of CMV-specific CD8<sup>+</sup> T cells is similar to the frequency found during primary infection (54), though these cells are phenotypically enriched in the effector stage (CD27<sup>-</sup>CD45RA<sup>+</sup>) (25) or the late memory stage (CD27<sup>-</sup>CD28<sup>-</sup>) (22). Down-regulation of CD27 is associated with the number of latent CMV reactivation events *in vivo*, and is largely due to expression of CD70 (a ligand of CD27) by proliferating CMV-specific CD8<sup>+</sup> T cells in the presence of IL-2 (9). Such CD27-CD70 interaction via CMV reactivation also regulates the size of the pool of CMV-specific CD8<sup>+</sup> T cells. This also explains the age-related increase in the frequency of CMV-specific CD8<sup>+</sup> T cells (55, 56). Moreover, as with EBV-specific CD8<sup>+</sup> T cells, oligoclonal expansion of HLA-A2 restricted pp65 epitope-specific CD8<sup>+</sup> T cells has been documented (57, 58).

### 3. Responses to Human Immunodeficiency Virus Infection

HIV-1 is a member of the lentivirus subfamily of *Retroviridae*, and is the etiological agent responsible for AIDS. HIV-1 establishes both primary and persistent infection predominantly in CD4<sup>+</sup> T cells and in other immune cells (i.e., macrophages/monocytes and dendritic cells) (59). Primary infection lasts typically for 12 weeks, and is characterized by an initial burst of viremia with a rapid but transient decrease in the CD4<sup>+</sup> T cell counts (60). Following primary infection, HIV-1 establishes an asymptomatic persistent infection; however, unlike herpesviruses, persistent HIV-1 infection is not latent but progressive; viral replication continues predominantly in lymphoid organs during this asymptomatic period (61, 62). Clinical manifestations of HIV-1 infection appear on average 10 years after primary infection without antiviral drug intervention, although there are substantial differences in rate of disease progression among HIV-1 infected individuals (63).

After 20 years of intense research, the immune correlates of long-term protection of the host from progressive HIV-1 infection still remain elusive. There are, however, observations implicating an antiviral role of HIV-1-specific CD8<sup>+</sup> T cells. A few earlier studies showed a temporal correlation between increase in frequency of HIV-1-specific CD8<sup>+</sup> T cells and decline in acute viremia (64, 65). TCR-V $\beta$  analysis of HIV-1-specific CD8<sup>+</sup> T cells during primary infection revealed marked oligoclonal expansion of some TCR-V $\beta$  populations associated with a decline in viremia (66). High levels of HIV-1-specific CD8<sup>+</sup> T cell responses, slow decline in CD4<sup>+</sup> T cell counts, and low viral load correlated in a longitudinal study (67). Also, robust HIV-1-specific CD8<sup>+</sup> T cell responses were found among HIV-1 exposed yet uninfected prostitutes in Gambia (68, 69). Moreover, viral escape from the CTL epitope has been implicated in accelerated progression to AIDS (70, 71). More recent studies, however, showed that the

breadth and magnitude of the HIV-1-specific CD8<sup>+</sup> T cell response does not correlate with long-term non-progression (72, 73).

Direct evidence supporting the critical role of HIV-1-specific CD8<sup>+</sup> T cells in controlling HIV-1 progressive infection comes from a nonhuman primate model of lentiviral infection. *In vivo* depletion of CD8<sup>+</sup> T cells and a subsequent simian immunodeficiency virus (SIV) challenge in rhesus macaques resulted in uncontrolled viral replication and rapid development of AIDS (74, 75). The temporal association between emergence of the SIV-specific CD8<sup>+</sup> T cell and clearance of SIV was clearly evident (76-78). There are also several cases of vaccinated monkeys with stronger anti-viral CD8<sup>+</sup> T cell responses that correlate with better clinical outcomes (79-82).

The mechanisms underlying CD8<sup>+</sup> T cell mediated immunologic control of progressive HIV-1 infection are still incompletely understood. The majority of studies have focused on immunological determinants for protection by contrasting function and phenotype of HIV-1-specific CD8<sup>+</sup> T cells from long-term nonprogressors and progressors. These studies showed that a wider breadth and greater magnitude of HIV-1-specific CD8<sup>+</sup> T cell responses were the best correlates of long-term survival (72, 73). However, the minimal quantitative and qualitative requirements for HIV-1-specific CD8<sup>+</sup> T cell responses for long term survival have yet to be determined (83).

Recent phenotypic assessment of HIV-1-specific CD8<sup>+</sup> T cells by tetramer staining techniques has addressed quantitative and qualitative aspects of HIV-1-specific CD8<sup>+</sup> T cells. At present, phenotypic correlates of long-term survival are not yet clear. After primary infection, HIV-1-specific CD8<sup>+</sup> T cells appear to be phenotypically enriched at an intermediate maturation stage (CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>-</sup>) (22, 84, 85). This suggests that fully matured (effector) HIV-1-

specific CD8<sup>+</sup> T cells may not be necessary for long-term survival. In contrast, a relatively higher proportion of late stage CD27<sup>-</sup>CD45RA<sup>+</sup> (CD27<sup>-</sup>CD28<sup>-</sup>) HIV-1-specific CD8<sup>+</sup> T cells appears to correlate with long term survival (86), suggesting that further maturation to the terminally differentiated effector CD8<sup>+</sup> T cell offers better protection.

Functional assessment of HIV-1-specific CD8<sup>+</sup> T cells has documented evidence for functional impairment. This ranges from increased sensitivity to apoptosis (87, 88), increased TGF- $\beta$  production (89), diminished IFN- $\gamma$  production (90), decreased costimulatory molecule expression (91) and diminished granzyme and perforin production (92, 93). It has been reported that IFN- $\gamma$  production is not defective in HIV-1-specific CD8<sup>+</sup> T cells from progressors, while perforin production is defective in HIV-1-specific CD8<sup>+</sup> T cells from progressors (35, 93). Lack of perforin production is primarily due to diminished proliferative capacity of the HIV-1-specific CD8<sup>+</sup> T cells (93). Loss of proliferative potential of HIV-1-specific CD8<sup>+</sup> T cells from progressors appears to occur soon after primary infection is resolved; however, proliferative potential can be restored by addition of autologous HIV-1-specific CD4<sup>+</sup> T cell isolated during primary infection *in vitro* (94). This exemplifies a recurring theme of HIV-1 pathogenesis, that functional impairment of HIV-1-specific CD8<sup>+</sup> T cells is related to lack of CD4<sup>+</sup> T cell help (95).

In contrast, activation and expansion of some types of CD4<sup>+</sup> T cells by progressive HIV-1 infection can be a cause of functional impairment of HIV-1-specific CD8<sup>+</sup> T cells. For instance, regulatory CD4<sup>+</sup> T cells (Tregs) recently found in the Foxp3 expressing CD25<sup>+</sup> compartment of mice and humans constitutes a central part of a complex immunoregulatory network (96, 97). Such an immunoregulatory network can be disrupted by progressive HIV-1-infection, leading to excessive suppression of the antiviral CD8<sup>+</sup> T cell response. In fact, enriched human CD25<sup>+</sup>CD4<sup>+</sup> T cells suppress IFN- $\gamma$  production by antigen-stimulated HIV-1-

and CMV-specific CD8<sup>+</sup> T cells in a dose dependent manner (98). Intriguingly, a later study found restimulable memory (CD45RO<sup>+</sup>) HIV-1-specific CD25<sup>+</sup>CD4<sup>+</sup> cells (99).

Another recent study (100) showed contradictory results. A higher frequency of the CD45RO<sup>+</sup>CD25<sup>high</sup>CD4<sup>+</sup> Tregs was strongly correlated with immunological markers (e.g., CD4<sup>+</sup> T cell counts, viral load, and CD8<sup>+</sup> T cell function) indicative of a positive clinical outcome. Oddly, this study suggests a clinical benefit of suppressing HIV-1-specific CD8<sup>+</sup> (and CD4<sup>+</sup>) T cells as opposed to stimulating HIV-1-specific CD8<sup>+</sup> (and CD4<sup>+</sup>) T cells in controlling progressive HIV-1 infection. Interestingly, such clinical benefit by suppressing CD8<sup>+</sup> T cell responses to HIV-1 infection has already been suggested. Prolonged excessive stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells during progressive HIV-1 infection has been implicated as a primary cause of gradual dysregulation and eventual destruction of the host immune system (23, 101). Historically, because of a resemblance between HIV-1 and LCMV pathogenesis, it was originally argued by Zinkernagel that immune unresponsiveness to HIV-1 infection would be beneficial to the host (102). Whether long-term survival from progressive HIV-1 infection requires immune unresponsiveness remains to be seen, however, it is known that healthy African green monkeys (a natural host of SIV) harbor high levels of SIV plasma viremia (greater than 6x10<sup>6</sup> plasma RNA copies/ml), yet their immune systems remain unresponsive to SIV (103).

Additionally, immune control of CTL escape mutants is an equally important mechanism of immune protection from progressive HIV-1 infection. CTL escape mutants emerge as a result of failed recognition of HIV-1-specific CD8<sup>+</sup> T cell epitopes by either TCR or failed loading of such epitopes to cognate MHC class I molecules. This is largely due to accumulation (due to selection) of HIV-1 pseudostrains carrying amino acid variations within the epitope created by an error-prone HIV-1 reverse transcriptase and continuous high virus turnover (104). Immune



escape has been seen in certain epitopes during primary infection (105) and chronic infection (70), and clearly undermines development of a vaccine to control HIV-1 infection.

Containment of progressive HIV-1 infection by CD8<sup>+</sup> T cells does not necessarily require cytolytic activity. A novel non-cytolytic HIV-1 suppression by CD8<sup>+</sup> T cells was first reported in 1986, and later shown to be a soluble factor(s), known as CAF, secreted from activated CD8<sup>+</sup> T cells (106). The suppression activity by activated CD8<sup>+</sup> T cells has been demonstrated in rhesus monkeys infected by SIV (107), and more importantly, greater suppression is correlated with better clinical status (108). Although there are numerous recent reports demonstrating CAF, the exact molecular nature of CAF has remained elusive.

## II.

### STATEMENT OF PROBLEM/SPECIFIC AIMS

Understanding memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells is an essential component of modern immunology. Recent technological advances in characterizing virus-specific CD8<sup>+</sup> T cells have generated phenotypic and functional details of memory-effector differentiation of these cells. According to the current model of memory-effector differentiation, virus-specific CD8<sup>+</sup> T cells undergo a series of phenotypically defined differentiation stages, and display phenotypic enrichment according to the virus specificity. This model has been supported by results from many earlier studies. However, more recent studies have pointed out several limitations in the current model. Thus, the goal of the present Ph.D. project was to improve and expand on the current model of virus-specific CD8<sup>+</sup> T cells by focusing on three significant issues based on the hypothesis that the most critical determinant of memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells is an interplay between the etiology of the virus infection and the host responses to such virus infection.

#### **Specific Aim 1: Assess memory-effector differentiation of Influenza A M1-specific CD8<sup>+</sup> T cells based on a broad phenotypic characterization.**

The specific aim 1 is based on the hypothesis that CD27<sup>+</sup>CD28<sup>+</sup> undifferentiated CD8<sup>+</sup> memory T cells specific for non-persistent virus influenza A (Flu A) would have phenotypic markers associated with more differentiated (effector) phenotypes. A primary goal of this aim is the

phenotypic assessment of CD8<sup>+</sup> memory T cells specific for Influenza A virus (as a model of the nonpersistent virus) with an array of phenotypic markers reveals details of memory-effector differentiation that were not described by the current model of CD8<sup>+</sup> T cell differentiation. Memory and effector cell subsets of Flu A-specific CD8<sup>+</sup> T cells in healthy virus carriers will be determined by expression of memory–effector phenotypic markers (CD27, CD28, CD45RA, CD62L, CD94 and granzyme A) and chemokine receptors (CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5), with tetramer staining reagents specific for Flu A matrix protein 1.

**Specific Aim 2: Determine the functional capacity of CD62L<sup>+</sup> central memory EBV-specific CD8<sup>+</sup> T cells as a model for the most undifferentiated virus-specific CD8<sup>+</sup> T cells.**

This specific aim is based on the hypothesis that a CD62L<sup>+</sup> subset of EBV-specific CD8<sup>+</sup> T cells includes the more mature effector-like subset that is capable of immediate cytoplasmic IFN- $\gamma$  production upon peptide-specific stimulation *in vitro*. The circulating memory CD8<sup>+</sup> T cell can be divided into two functional subsets, T<sub>CM</sub> and T<sub>EM</sub>, based on their migratory, functional, and phenotypic characteristics. T<sub>CM</sub> is the immature memory subset characterized by expression of CD62L and CCR7 and lack of immediate cytoplasmic IFN- $\gamma$  production. Since a majority of virus-specific CD8<sup>+</sup> T cells stained by MHC class I tetramers lack expression of CCR7, a CD62L<sup>+</sup> subset is thought to represent T<sub>CM</sub> with respect to the virus-specificity. Supporting this notion, the CD62L<sup>+</sup> subset of EBV-specific CD8<sup>+</sup> T cells is not able to immediately produce cytoplasmic IFN- $\gamma$ . However, this view has been challenged in a mouse model which has shown immediate IFN- $\gamma$  production from CD62L<sup>+</sup> virus-specific mouse CD8<sup>+</sup> T cells. A goal of the specific aim is to further investigate the functionality of virus-specific CD62L<sup>+</sup> T<sub>CM</sub>.

**Specific Aim 3: Investigate limited differentiation of HIV-1-specific CD8<sup>+</sup> T cells from the intermediate stage to the late stage.**

This specific aim is based on the hypothesis that enrichment of HIV-1-specific T cell in the intermediate stage (CD27<sup>+</sup>CD28<sup>-</sup>) is due to impaired differentiation to the late/effector stage (CD27<sup>-</sup>CD28<sup>-</sup>). Recent phenotypic and functions studies have shown that HIV-1-specific CD8<sup>+</sup> T cells are enriched in an intermediate stage (CD27<sup>+</sup>CD28<sup>-</sup>) of memory-effector CD8<sup>+</sup> T cell differentiation, in contrast to EBV-specific (early) and CMV-specific (late)-CD8<sup>+</sup> T cells. However, unlike EBV and CMV, HIV-1 infection establishes chronic progressive infection which provides continuous presence of HIV-1 antigens. The continuous supply of antigens should drive HIV-1-specific CD8<sup>+</sup> T cells toward effector cells (resembling CMV-specific CD8<sup>+</sup> T cells) unless memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells (in the absence of CD4 help) is impaired. The experiments described in this aim address whether HIV-1-specific CD8<sup>+</sup> T cells endure impaired memory-effector differentiation.

### **III.**

## **ASSESS MEMORY-EFFECTOR DIFFERENTIATION OF INFLUENZA A M1-SPECIFIC CD8<sup>+</sup> T CELLS BASED ON A BROAD PHENOTYPIC CHARACTERIZATION.**

**Human CD8<sup>+</sup> T cells specific for influenza A virus M1 display broad expression of maturation-associated phenotypic markers and chemokine receptors**

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Aki Hoji<sup>1</sup> and Charles R. Rinaldo, Jr.<sup>1,2</sup>

Department of Infectious Diseases and Microbiology<sup>1</sup> and Pathology<sup>2</sup>, Graduate School of Public Health and School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261

Short title: Immunophenotyping of antigen-specific CD8<sup>+</sup> T cells

Key words: tetramer, EBV, influenza, T cells, flow cytometry, phenotyping

Abbreviations: EBV: Epstein-Barr Virus, TCR $\alpha\beta$ : T cell receptor  $\alpha\beta$  chain

## **A. ABSTRACT**

To define the role of memory T cells in a nonpersistent viral infection, we have delineated the phenotype of memory CD8<sup>+</sup> T cells specific for influenza A virus (FluA; epitope matrix protein M1<sub>58-66</sub>) based on expression of several memory/effector lineage markers and relevant chemokine receptors. We found a majority of FluA-specific CD8<sup>+</sup> T cells expressed CD27 and CD28, and variably expressed CD45RA, CD62L, CD94, and granzyme A. A majority of FluA-specific CD8<sup>+</sup> T cells expressed high levels of CXCR3, and moderate levels of CCR5 and CXCR4, whereas a limited proportion expressed CCR7, CCR6 and CXCR5. A phenotypic profile based on these observations showed that there are both immature and mature memory CD8<sup>+</sup> T cells specific for FluA.

## B. INTRODUCTION

Generation of virus-specific memory CD8<sup>+</sup> T cells appears to be a multi-stage developmental process, characterized by phenotypic alterations associated with changes in T cell functional and migratory capacity (109). Most of our knowledge of memory CD8<sup>+</sup> T cell differentiation is based on phenotypic analyses of CD8<sup>+</sup> T cells specific for persistent viruses including herpesviruses and lentiviruses. The predominant phenotype of CD8<sup>+</sup> T cells specific for non-persisting viruses, such as influenza A virus (FluA), has not been well characterized.

FluA infection causes severe acute airway infection associated with substantial mortality (110). Although CD8<sup>+</sup> T cells specific for the immunodominant FluA matrix epitope (FluM1<sub>58-66</sub>) persist after acute infection, their frequency in the circulation remains barely detectable by MHC class I tetrameric analysis (34, 111). This low frequency makes it difficult to perform accurate multi-parametric flow cytometric analysis with FluA-specific MHC class I tetramers. Hence, there is a little known about the range of the phenotypic diversity of circulating FluA-specific CD8<sup>+</sup> T cells.

We have therefore determined the phenotype of FluA-specific CD8<sup>+</sup> T cells by using 4-color flow cytometric analysis employing HLA-A\*0201 FluM1 tetramers, together with an extensive set of lineage/maturation markers including several chemokine receptors, from asymptomatic individuals. We found that FluA-specific CD8<sup>+</sup> T cells largely consisted of a phenotype of immature memory cells based on expression CD27 and CD28. However, a significant proportion of FluA-specific CD8<sup>+</sup> T cells also expressed CXCR3, CCR5, CD94 and granzyme A, suggesting a heterogeneous population of more mature, effector memory cells.



Thus, FluA-specific CD8<sup>+</sup> T cells display degree of phenotypic profile indicative of a highly heterogeneous population of phenotypically mature memory and primed effector CD8<sup>+</sup> T cells.

## **C. MATERIALS AND METHODS**

### **Study subjects and isolation of PBMC**

Nine HLA-A\*0201 positive healthy volunteers (median age 52 yr, range 26-54 yr, all male Caucasians) were divided into two groups based on FluM1 tetramer or Epstein-Barr virus (EBV) BMLF1 tetramer reactivity: a FluM1 tetramer positive group (donor 01 through 06), and an EBV BMLF1 tetramer positive group (donor 01, and 07 through 09). PBMC were isolated from heparinized blood samples by centrifugation on Ficoll-Hypaque (Sigma, St. Louis, MO) density gradients. Except for CD62L staining, the PBMC were immediately frozen in 10% DMSO and 90% FCS by a controlled rate cryogenic freezer (Gordinier Electronics, Roseville, MI) and stored at -135 °C for later use.

### **Antibodies and HLA-A\*0201 tetramers**

The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD27, CD28, CD62L, CD94 HLA-DR, CCR5, granzyme A, and perforin monoclonal antibodies (mAbs), unlabeled mouse anti-human CCR6, CXCR4, CXCR5, CXCR6 mAbs (Becton Dickinson, San Jose, CA), FITC-conjugated goat anti-mouse polyclonal F(ab')<sub>2</sub> antibodies (DAKO, Carpinteria, CA), SPRD- conjugated goat F(ab')<sub>2</sub> anti-mouse mAb (Southern Biotechnology Birmingham, AL), FITC-conjugated mouse anti-rat polyclonal antibody (Jacksonimmuno, West Grove, PA), and FITC-conjugated mouse anti-human CD45RA, PE-Cy5 conjugated mouse anti-human TCR $\alpha\beta$ , and ECD-conjugated CD8 mAbs (Coulter-Immunotech, Miami, FL). Rat anti-human CCR7 mAb (3D12) was kindly provided by Dr. Reinhold Forster (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). Appropriate isotype matched

mAbs (BD and Coulter-Immunotech, Miami, FL) in the fluorescent minus one (FMO) control (112) were used throughout the course of the study.

HLA-A\*0201 EBV BMLF1<sub>280-288</sub> (GLCTLVAML) and Flu A M1<sub>58-66</sub> (GILGFVFTL) tetrameric agents were obtained from NIH Tetramer Synthesis Facility. These tetramers were labeled with PE, and used at a 1/50 dilution for staining  $2-5 \times 10^6$  cells.

### **Preparation of stained PBMC**

HLA-A\*0201 restricted EBV BMLF1<sub>280-288</sub> and Flu A M1<sub>58-66</sub> specific CD8<sup>+</sup> T cells were stained as described in a protocol obtained from NIH Tetramer Synthesis Facility with a few modifications. Briefly, for staining CD27, CD28, CD45RA, CD62L, HLA-DR, CD94 and CCR5,  $2-5 \times 10^6$  fresh (for staining CD62L) or thawed frozen PBMC were resuspended in 50ul of PBS with 4% heat-inactivated fetal calf serum (FCS) and 0.1% azide, and were first stained with 1ul of the appropriate tetramer at 4°C for 30 min. Then, the cells were washed and incubated with PE-Cy5 mouse anti-human TCR $\alpha\beta$  and ECD anti-human CD8 mAbs for 30 min at 4°C. Cells were fixed with 1% paraformaldehyde (PFA) and analyzed with an Elite XL flow cytometer (Beckman-Coulter) immediately after staining.

For detection of CCR6, CXCR3, CXCR4 and CXCR5, PBMC were first stained with the primary mAbs, washed, and incubated with goat anti-mouse antibody for 30 min 4°C. Cells were then washed and blocked with 10% mouse serum (Sigma, St. Louis, MO) for 15 min at room temperature. The tetramer, PE-Cy5 anti-human TCR $\alpha\beta$ , and ECD anti-human CD8 mAbs were added, and subsequently the cells were fixed with 1% PFA. For CCR7 staining, PBMC were first treated with 5% mouse serum for 15 min at room temperature, and then stained with

rat-anti CCR7 mAb for 30 min at 4°C. The cells were then stained with FITC conjugated mouse anti-rat polyclonal antibody, and the rest of the procedure was done as described.

For intracellular staining of granzyme A and perforin,  $2-5 \times 10^6$  fresh or frozen-thawed PBMC were first stained with the tetramer panel as described. After the initial wash, cells were resuspended in 300 ul of OrthoPermFix (OrthoDiagnostics, Raritan, NJ) or PermiFlow (Invirion, Frankfort, MI) for 60 min at room temperature. Subsequently, the cells were washed and stained with anti-human granzyme A or perforin mAbs for 40 min at 4°C, fixed with 1% PFA and immediately read on the flow cytometer.

### **Flow cytometric and statistical analysis**

The flow cytometer was calibrated daily for color compensation and laser fluctuation. A 4 color compensation matrix was created for acquisition of tetramer stained samples and subsequent software compensation by singly and FMO staining PBMC from donors. We followed a flow cytometric analysis for rare eventing described by Hoffman et. al. (111), and spectral compensation by Roderer (112). Approximately  $2 \times 10^5$  to  $1 \times 10^6$  total events were collected for the fully stained sample and FMO isotype control (the same number of events were collected for both samples). This resulted in more than 200 tetramer, CD8<sup>high</sup> and TCR $\alpha\beta$ <sup>+</sup> events based on a compounded gating scheme of Hoffman et.al. (111). The frequencies of FluM1- and BMLF1-specific CD8 T cells are shown in Table 1. Data analysis and graphic representations were done by using FlowJo (TreeStar, Cupertino, CA).

The phenotypic profile was constructed as follows. The percent marker expression of the tetramer positive and negative CD8<sup>+</sup> T cells was derived by the number of events in the upper right (tetramer-marker double positives) or bottom right (tetramer negative (bulk CD8<sup>+</sup> T cells)-

marker positives) quadrant of plots divided by number of events in the bottom right quadrant or the bottom left quadrant of the same plots respectively. The Student's T test for paired samples was used to show significant difference ( $P < 0.05$ ) in percent marker expression between tetramer positive and tetramer negative CD8<sup>+</sup> T cells. Statistical analysis and graphical representation used JMP v5.0 software (JMP Sales, Cary, NC).

## D. RESULTS

### Surface and intracellular expression of conventional functional and CD antigen markers on FluM1-specific CD8<sup>+</sup> T cells

To phenotypically characterize the memory-effector stage of FluM1-specific CD8<sup>+</sup> T cells, we first examined expression of CD27, CD28, CD45RA, CD62L, CD94, HLA-DR and granzyme A (Figure. 3). Immature and mature phenotypes were verified by the presence or absence of co-stimulatory molecules CD27 and CD28 (14, 22), naïve (6) and central memory marker (1) CD62L, and effector cell markers, granzyme A (113) and perforin (114). We found that a majority of FluM1-specific CD8<sup>+</sup> T cells expressed CD27 (median 98%, range 58-100%) and CD28 (median 90%, range 58-100%) (Table 2), though a moderate proportion of FluM1-specific CD8<sup>+</sup> T cells expressed the naïve cell/central memory marker, CD62L (median 40%, range 28-69%) (Table 2). Granzyme A expression was found in a relatively larger proportion of FluM1-specific CD8<sup>+</sup> T cells (median 63%, range 20-98%), although limited expression was seen in most of a lower FluM1-specific CD8<sup>+</sup> T cells (median 16 %, 4-68%) (Table 2).

CD45RA expression has been found on both naïve and effector, bulk CD8<sup>+</sup> T cells (113), and CD27<sup>-</sup>CD45RA<sup>+</sup> has been shown to be an effector phenotype (19). We observed that a small proportion of FluM1-specific CD8<sup>+</sup> T cells (except subject 02) expressed CD45RA (median 7%, 1-14%) (Table 2). Thus, a majority of FluM1-specific CD8<sup>+</sup> T cells appear to be CD27<sup>+</sup> CD45RA<sup>-</sup>, indicating that FluM1-specific CD8<sup>+</sup> T cells do not display an effector phenotype.

In addition, we assessed CD94 expression, because of its known association with terminal differentiation (115, 116), and HLA-DR, a late activation marker (117). A relatively

higher proportion of FluM1-specific CD8<sup>+</sup> T cells expressed CD94 (median 53%, range 11-82%) whereas a consistently lower proportion of FluM1-specific CD8<sup>+</sup> T cells expressed HLA-DR (median 14%, range 1-20%) (Table 2).

In summary, the phenotype of the majority of FluM1-specific CD8<sup>+</sup> T cells circulating in asymptomatic donors appears to be CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>-</sup>HLA-DR<sup>-</sup>, and a moderate proportion of FluM1-specific CD8<sup>+</sup> T cells express, CD62L, CD94 and granzyme A.

### **Surface expression of chemokine receptors on FluM1-specific CD8<sup>+</sup> T cells**

To further delineate memory and effector phenotypes among FluM1-specific CD8<sup>+</sup> T cells, we determined surface expression of 6 chemokine receptors: CXCR3, CXCR4, CXCR5, CCR5, CCR6 and CCR7 (Figure 4). CXCR4 appears to be expressed preferentially on naïve CD8<sup>+</sup> T cells (118, 119); in contrast, expression of CCR5 or CXCR3 is known to be associated with activated memory cells (118). CCR5 also appears to be expressed primarily on the late memory to effector stage of viral-specific CD8<sup>+</sup> T cells (120). Thus, these latter two chemokine receptors serve as potential mature memory/primed\_effector markers. We found that moderate proportion of FluM1-specific CD8<sup>+</sup> T cells expressed CCR5 (median 43%, range 20-92%), and higher proportion expressed CXCR3 (median 84%, range 32-99%), whereas there was a variable proportion of CXCR4 expression (median 45%, range 19-97%) (Table 2).

A previous report showed that expression of CCR6 was restricted to the memory subset (CD45RO<sup>+</sup>) of CD8<sup>+</sup> T cells (121). In contrast, we found that CCR6 was minimally expressed on FluM1-specific CD8<sup>+</sup> T cells (median, 5%, range 2-18%) (Table 2).

The expression of CCR7 differentiates CCR7<sup>+</sup> central memory T cells and CCR7<sup>-</sup> effector/memory T cells (20). We found a lower proportion of CCR7 expression on FluM1-

specific T cells (median 20%, range 2-33%) (Table 2). A similar, low level of CCR7 expression has been observed in HIV-specific and CMV-specific CD8<sup>+</sup> T cells (25, 122, 123).

Thus, our data show that a large proportion of FluM1-specific, memory CD8<sup>+</sup> T cells express moderate levels of chemokine receptors CCR5, CXCR3 and CXCR4 while lacking major expression of CXCR5, CCR6 and CCR7 receptors. These T cells fit the conventional definition of memory/effector cells based on chemokine receptor expression.

### **Comparison of the phenotypic profile between FluM1- and BMLF1-specific CD8<sup>+</sup> T cell**

We next determined the extent of FluM1-specific CD8<sup>+</sup> T cell maturation in relation to bulk CD8<sup>+</sup> T cells. For this, we constructed the phenotypic profile by comparing differences in the percent marker expression on tetramer positive CD8<sup>+</sup> T cells and corresponding tetramer negative CD8<sup>+</sup> T cells (bulk CD8<sup>+</sup> T cells). Then, we compared the phenotypic profiles of FluM1-specific CD8<sup>+</sup> T cells to BMLF1-specific CD8<sup>+</sup> T cells (Table 3) as an example of more mature CD8<sup>+</sup> T cells. As expected, expression of early stage memory markers (CD27, CD28, CD62L, CCR7 and CXCR4) on FluM1-specific CD8<sup>+</sup> T cells was favored in the bulk CD8<sup>+</sup> T cells more than in BMLF1-specific CD8<sup>+</sup> T cells, whereas expression of some more mature memory markers (HLA-DR, granzyme A, and CCR5) was slightly more extensive in BMLF1-specific CD8<sup>+</sup> T cells (Figure. 5). Furthermore, FluM1-specific CD8<sup>+</sup> T cells displayed a higher degree of a skewed CD45RA<sup>-</sup> phenotype (from the bulk CD8<sup>+</sup> T cells) than BMLF1-specific CD8<sup>+</sup> T cells (Figure. 5). In contrast, except for CD45RA, CD94 and CXCR3, there were no statistically significant differences in percent marker expression between FluM1 specific CD8<sup>+</sup> T cells and bulk CD8<sup>+</sup> T cells. These data suggest that BMLF1-specific CD8<sup>+</sup> T cells are skewed toward a more mature phenotype than FluM1-specific CD8<sup>+</sup> T cells.



## E. DISCUSSION

In the present study, we characterized phenotypes of FluM1-specific CD8<sup>+</sup> T cells for a large number of phenotypic markers and chemokine receptors. Our data show that FluM1-specific CD8<sup>+</sup> T cells appear to be CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>-</sup>. This phenotypic enrichment is suggestive of a less mature or early stage of memory-effector cells. Consistent with our findings, the only other phenotypic study on the FluM1-specific CD8<sup>+</sup> T cell has recently shown that circulating FluM1-specific CD8<sup>+</sup> T cells (CD28<sup>+</sup>CCR7<sup>-</sup>CD45RA<sup>-/low</sup>) are less mature than that of the CD8<sup>+</sup> T cell specific for persistent virus infection (115).

Our analysis shows only slight differences in marker expression between FluM1-specific and BMLF1-specific CD8<sup>+</sup> T cells despite well known, distinct differences in underlying viral infection and persistent antigen burden. This is not surprising since EBV-specific CD8<sup>+</sup> T cells have been shown to be phenotypically enriched in the early stage of CD8<sup>+</sup> T cells maturation (22). However, persistent EBV infection causes repeated stimulation of BMLF1-specific CD8<sup>+</sup> T cells, and this undoubtedly drives BMLF1-specific CD8 T cells to skew towards more mature phenotype than FluM1-specific CD8<sup>+</sup> T cells. This may reflect our observation that although BMLF1-specific CD8<sup>+</sup> T cells show a greater degree of down-modulation of early stage memory markers (CCR7, CD28, CD62L and CXCR4), FluM1-specific CD8<sup>+</sup> T cells appear to show the similar degree of differences in the percent of some effector markers (perforin, CCR5 and CXCR3) from the bulk CD8<sup>+</sup> T cells. Thus, it is possible that down-modulation of some phenotypic markers such as CD27 and CD28 may not be strongly correlated with development of effector cell phenotype.

Expression of effector and activation markers could also be modulated by the nature of viral infection rather than memory CD8<sup>+</sup> T cell maturation. For instance, preferential CD94 expression on FluM1 specific CD8<sup>+</sup> T cells may have little to do with terminal differentiation of such cells. Possibly, expression of the CD94-NKG2A heterodimer averts over-activation of FluM1-specific CD8<sup>+</sup> T cells to prevent a fatal pulmonary edema due to acute infection. Such regulation is presumably not necessary for persistent EBV infection. Similarly, persistent EBV infection appears to cause constitutive activation of a small number of antigen specific CD8<sup>+</sup> T cells, indicated by a higher proportion of HLA-DR<sup>+</sup> BMLF1-specific CD8<sup>+</sup> T cells.

In conclusion, the present study shows that circulating FluM1-specific CD8<sup>+</sup> T cells are composed of multiple subsets, each displaying a distinct set of naïve, memory and effector markers. Conceivably, such multiple subsets could be generated as a result of the antigen-specific CD8<sup>+</sup> T cell maturation (5), or they could simply signify subsets with distinct migratory and functional capacities. These results suggest that phenotypic maturation may not always accurately ascribe functional maturation.

## **F. ACKNOWLEDGEMENTS**

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Table 1. Frequency of BMLF1 and FluM1 tetramer positive cells detected *ex vivo* in peripheral blood.

Antigen	Donors	Tetramer Frequency * (%TCR $\alpha\beta^+$ CD8 $^+$ )
FluM1	01	0.16
	02	0.13
	03	0.23
	04	0.56
	05	0.11
	06	0.14
BMLF1	01	4.91
	07	0.40
	08	0.32
	09	1.08

\* Average frequency of FMO isotype control; 01 BMLF1(n=7, stdev=0.36), 01 FluM1(n=7, stdev=0.03), 02 BMLF1(n=7, stdev=0.09), 03 BMLF1(n=6, stdev=0.05), 04 BMLF1 (n=7, stdev=0.06) 05 FluM1 (n=8, stdev=0.02), 06 FluM1 (n=10, stdev=0.02), 07 FluM1 (n=7, stdev=0.05), 08 FluM1 (n=7 stdev=0.01), and 09 FluM1 (N=5, stdev=0.05).

Table 2. Percent TCR $\alpha\beta^+$  CD8 $^+$  tetramer and percent bulk TCR $\alpha\beta^+$  CD8 $^+$  (tetramer minus) in parentheses expressing each marker among six volunteers from the FluM1 group<sup>1,2</sup>.

Marker	Subject ID					
	01	02	03	04	05	06
CD27	58 (71)	91 (92)	97 (85)	99 (81)	99 (57)	100 (72)
CD28	24 (48)	74 (82)	93 (73)	86 (76)	96 (58)	98 (67)
<b>CD45RA</b>	<b>6 (25)</b>	<b>7 (54)</b>	<b>16 (38)</b>	<b>1 (28)</b>	<b>7 (32)</b>	<b>3 (41)</b>
CD62L	35 (45)	38 (47)	40 (46)	41 (63)	28 (48)	70 (60)
<u>CD94</u>	<u>63 (14)</u>	<u>60 (2)</u>	<u>32 (9)</u>	<u>82 (15)</u>	<u>45 (7)</u>	<u>11 (17)</u>
HLA-DR	20 (24)	19 (12)	12 (26)	15 (28)	6 (34)	1 (14)
Granzyme A	79 (70)	73 (33)	52 (55)	98 (56)	20 (65)	53 (38)
Perforin	68 (27)	27 (5)	5 (14)	27 (20)	5 (29)	4 (33)
<u>CXCR3</u>	<u>32 (24)</u>	<u>62 (36)</u>	<u>83 (53)</u>	<u>99 (85)</u>	<u>85 (74)</u>	<u>90 (57)</u>
CXCR4	19 (18)	39 (64)	23 (32)	52 (41)	97 (88)	59 (52)
CXCR5	6 (4)	6 (2)	9 (4)	1 (2)	12 (1)	7 (1)
CCR5	57 (34)	20 (13)	42 (30)	91 (41)	29 (37)	44 (37)
CCR6	18 (7)	6 (13)	4 (6)	2 (10)	15 (10)	3 (6)
CCR7	12 (22)	27 (64)	16 (35)	2 (40)	21 (17)	33 (38)

<sup>1</sup>Percent marker expression is derived from the number of marker positive tetramer $^+$  events (upper right quadrant of plots in Fig. 1 and 2) or tetramer $^-$  (bulk CD8 $^+$  T cells) events (bottom right quadrant) divided by number of total tetramer $^+$  or tetramer $^-$  (bulk CD8 $^+$  T cells) events (Upper or bottom right and left quadrants).

<sup>2</sup>Bolded or underlined markers across the donor indicate that the mean percent marker expression of the tetramer $^+$  CD8 $^+$  T cell is significantly lower (bold) or higher (underline) than that of the tetramer $^-$  CD8 $^+$  T cell (bulk CD8 $^+$  T cells) with  $p < 0.05$ , determined by the paired Student's t test. See the graphical representation of these values in Fig. 3.

Table 3. Percent TCR $\alpha\beta^+$  CD8 $^+$  tetramer and percent bulk TCR $\alpha\beta^+$  CD8 $^+$  (tetramer minus) in parentheses expressing each marker among five volunteers from the BMLF1 groups<sup>1,2</sup>.

Marker	Subject ID			
	01	07	08	09
CD27	81 (73)	83 (88)	64 (93)	95 (78)
CD28	26 (49)	24 (81)	35 (77)	92 (70)
CD45RA	13 (28)	20 (63)	90 (84)	2 (38)
<b>CD62L</b>	33 (47)	16 (59)	7 (44)	41 (67)
<u>CD94</u>	26 (19)	26 (6)	25 (3)	44 (11)
<u>HLA-DR</u>	34 (18)	27 (7)	32 (9)	19 (8)
<u>Granzyme A</u>	93 (74)	95 (51)	91 (31)	91 (48)
Perforin	42 (33)	26 (8)	16 (8)	24 (23)
CXCR3	25 (32)	71 (59)	55 (63)	81 (61)
<b>CXCR4</b>	6 (20)	13 (56)	35 (67)	2 (16)
CXCR5	2 (13)	3 (0)	8 (3)	1 (3)
CCR5	40 (33)	43 (22)	29 (7)	89 (27)
CCR6	2 (8)	6 (15)	1 (6)	4(5)
<b>CCR7</b>	2 (18)	5 (68)	7 (59)	18 (53)

<sup>1</sup>Percent marker expression is derived from the number of marker positive tetramer $^+$  events (upper right quadrant of plots in Fig. 1 and 2) or tetramer $^-$  (bulk CD8 $^+$  T cells) events (bottom right quadrant) divided by number of total tetramer $^+$  or tetramer $^-$  (bulk CD8 $^+$  T cells) events (Upper or bottom right and left quadrants).

<sup>2</sup>Bolded or underlined markers across the donor indicate that the mean percent marker expression of the tetramer $^+$  CD8 $^+$  T cell is significantly lower (bold) or higher (underline) than that of the tetramer $^-$  CD8 $^+$  T cell (bulk CD8 $^+$  T cells) with  $p < 0.05$ , determined by the paired Student's t test. See the graphical representation of these values in Fig. 3.

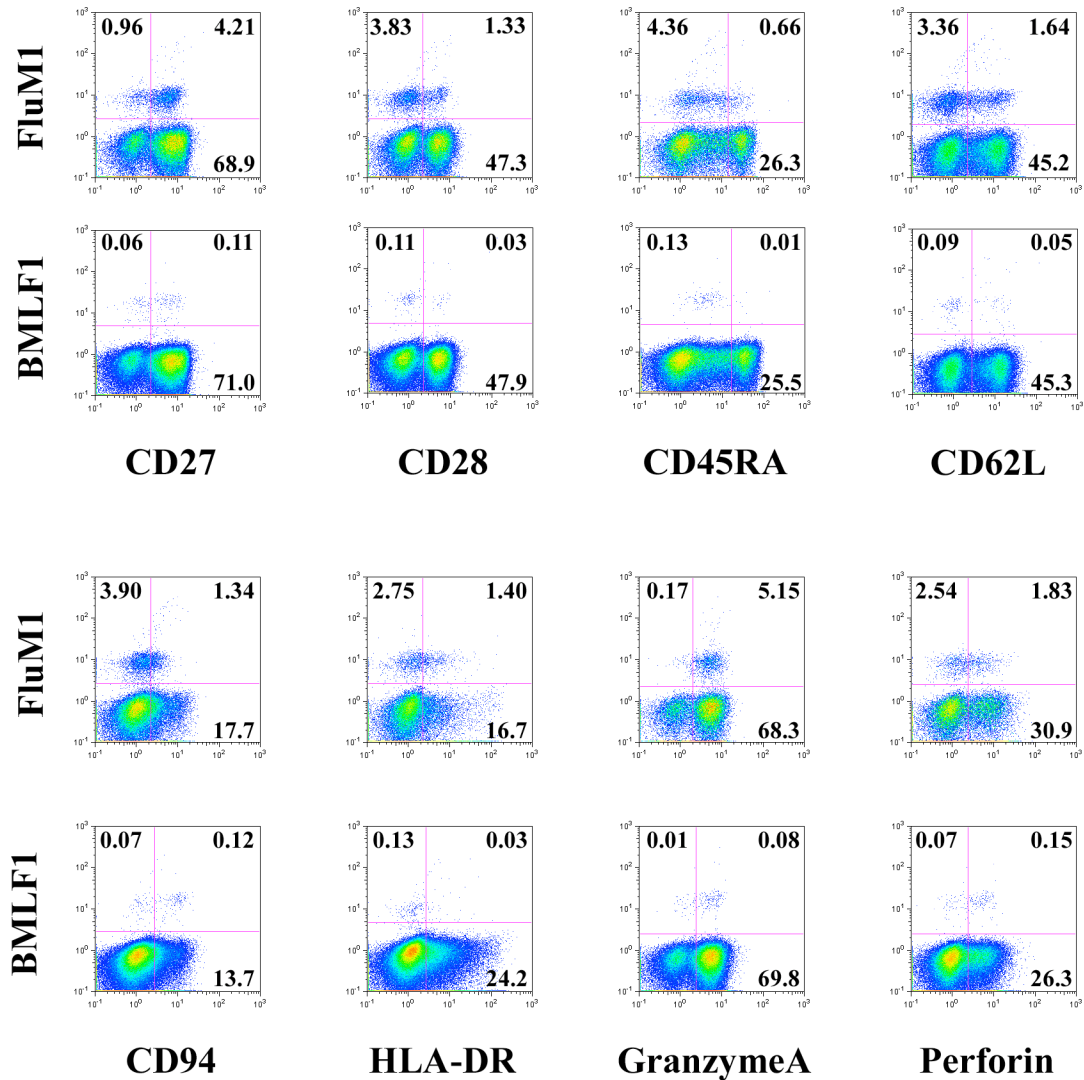


Figure 3. Surface and intracellular expression of memory/effector markers on BMLF1- or FluM1-specific TCR $\alpha\beta$  CD8<sup>+</sup> cells

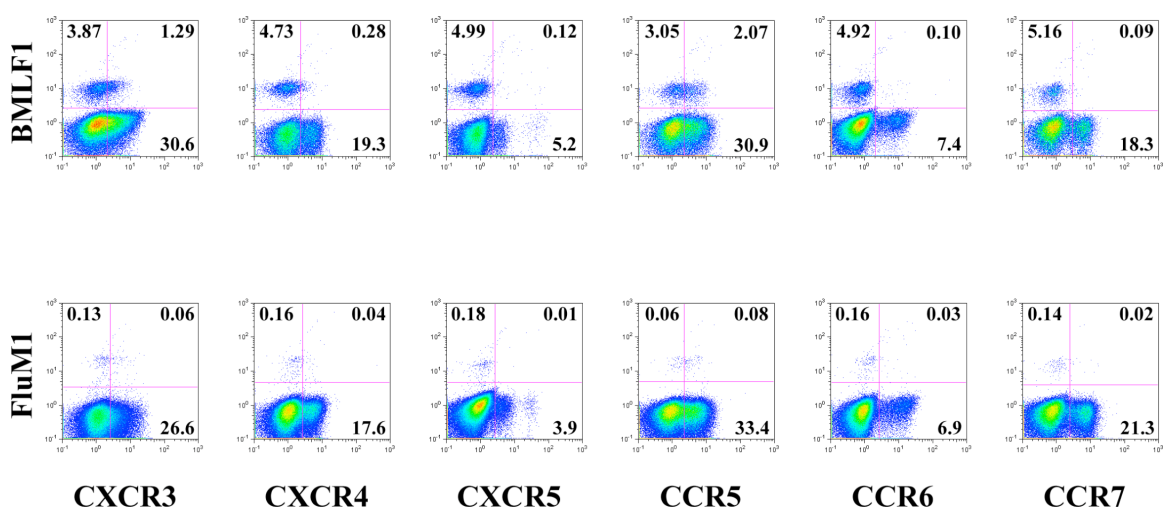


Figure 4. Surface expression of chemokine receptors on BMLF1- or FluM1-specific TCR $\alpha\beta$  CD8<sup>+</sup> cells.



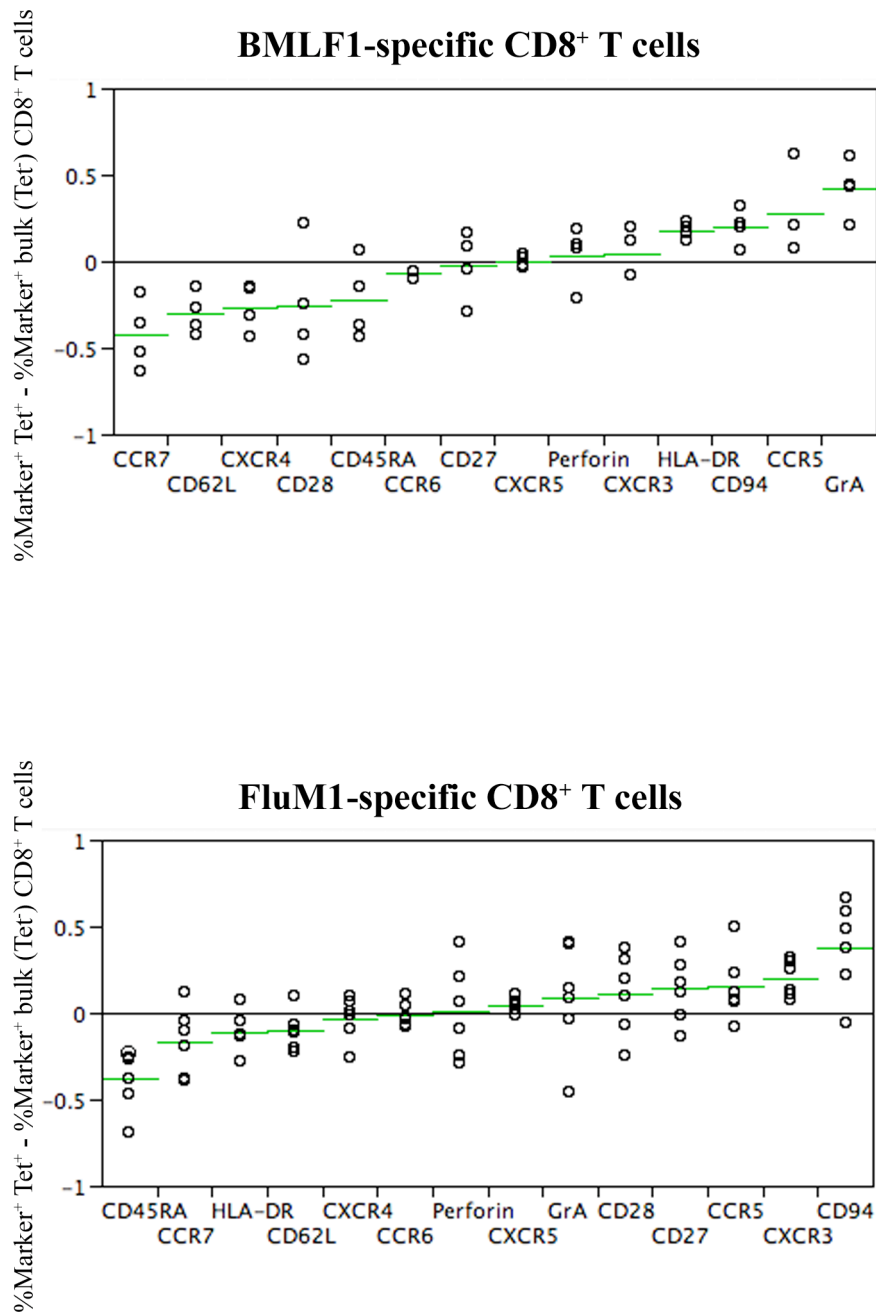


Figure 5. Comparison of the proportion of marker expression between tetramer<sup>+</sup> and tetramer<sup>-</sup> (bulk) CD8<sup>+</sup> T cells

## H. Addendum

### ***In vitro* stimulation and intracellular IFN- $\gamma$ staining**

*In vitro* peptide-specific stimulation and detection of intracellular IFN- $\gamma$  production by the antigen specific CD8<sup>+</sup> T cells from the thawed frozen specimen was based on a protocol obtained from L. Gamadia (Academic Medical Center, Amsterdam; personal communication). Briefly, frozen PBMC were thawed carefully and placed in a 37°C incubator for 20min. After washing, 2-3 x 10<sup>6</sup> cells were resuspended in 200ul fresh RPMI medium (GIBCO/Invitrogen, Grand Island, NY) containing 10% FCS, and immediately treated with 10uM of appropriated peptide, 50ng/ml PMA and 1ug/ml ionomycin, or DMSO alone. After an hour of incubation, 5ug/ml of brefeldin A was added and the cells were further incubated for 5 hours. The cells were then washed once with medium, and stained with anti-human TCR $\alpha\beta$ , and anti-human CD8 mAbs as described. The cells were washed again and incubated in 300ul of PermiFlow (Invirion, Frankfort, MI) overnight at room temperature. After incubation, the cells were washed and incubated with FITC-conjugated anti-human IFN- $\gamma$  and PE conjugated anti-human CD69 mAbs for 40 min at 4°C, washed, and fixed in 1% PFA.

To determine proportions of functional cells, we assessed antigen-specific intracellular IFN- $\gamma$  production *in vitro* (Figure. 6A). The frequency of IFN- $\gamma$  producing, CD69<sup>+</sup>CD8<sup>+</sup> T cells in the BMLF1 peptide-stimulated group was 53% to 74% of the BMLF1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells (Figure. 6B). There was a greater frequency of IFN- $\gamma$  producing, CD8<sup>+</sup> T cells (82% to 100%) per FluM1 tetramer<sup>+</sup>CD8<sup>+</sup> T cells. A similar discrepancy between the frequency of the tetramer and intracellular IFN- $\gamma$  positive cells has been reported for BMLF1-specific CD8<sup>+</sup> T cells (124, 125). Development of effector functions and phenotype by antigen driven differentiation may,

however, be independent of the cellular aging process. As indicated by the similar proportion of cytoplasmic IFN- $\gamma$  producing cells in both BMLF1- and FluM1-specific CD8<sup>+</sup> T cells, it is possible that FluM1-specific CD8<sup>+</sup> T cells are functionally as mature as BMLF1-specific CD8<sup>+</sup> T cells.

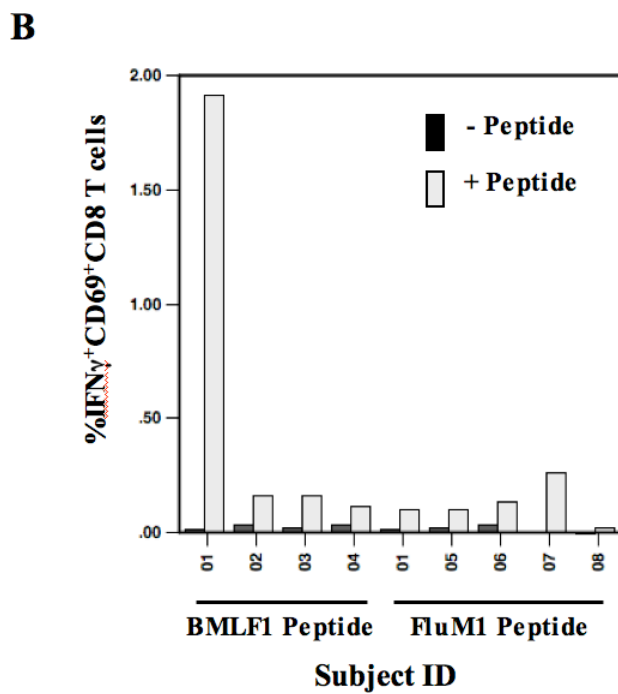
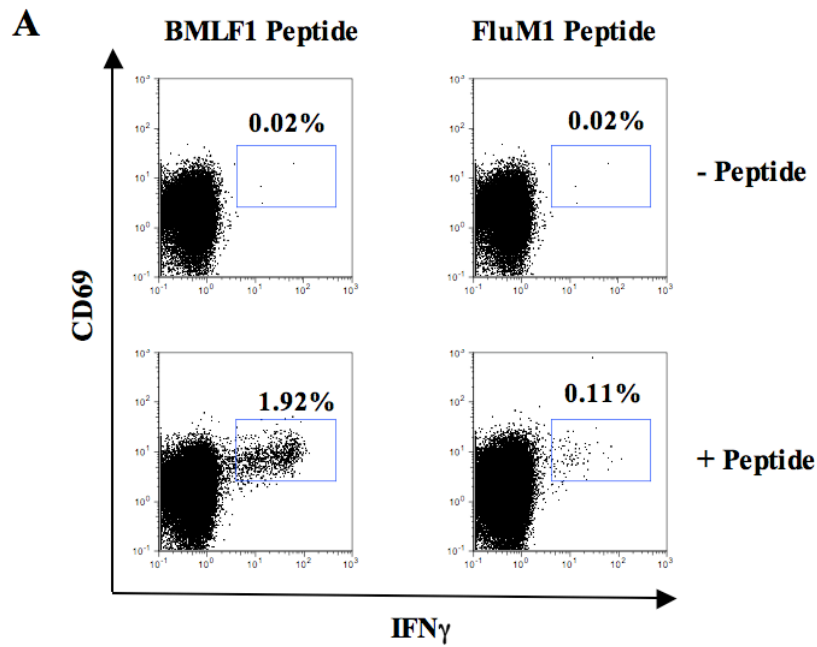


Figure 6. (A) Representative IFN- $\gamma$  production by of CD69<sup>+</sup>CD8<sup>+</sup> T cells stimulated by either BMLF1 or FluM1 peptide *in vitro*. (B) Comparison of percent IFN- $\gamma$ <sup>+</sup>CD69<sup>+</sup>CD8<sup>+</sup> T cells of study subjects from BMLF and FluM1 groups.

**IV.**

**DETERMINE THE FUNCTIONAL CAPACITY OF CD62L<sup>+</sup> CENTRAL MEMORY  
EBV-SPECIFIC CD8<sup>+</sup> T CELLS AS A MODEL FOR THE UNDIFFERENTIATED  
VIRUS-SPECIFIC CD8<sup>+</sup> T CELLS.**

**Immediate IFN- $\gamma$  Production by Epstein Barr virus-specific CD62L<sup>+</sup> Central  
Memory CD8<sup>+</sup> T cells**

(Manuscript in preparation, 2005)

Aki Hoji<sup>1</sup>, and Charles R. Rinaldo, Jr.<sup>1,2</sup>

Department of Infectious Diseases and Microbiology, Graduate School of Public Health<sup>1</sup>, and  
Department of Pathology, School of Medicine<sup>2</sup>, University of Pittsburgh, Pittsburgh, PA 15261.

## A. ABSTRACT

Central memory T cells ( $T_{CM}$ ) have been distinguished from effector memory T cells ( $T_{EM}$ ) by the presence of surface CD62L (L-selectin), and the absence of immediate effector functions (e.g. IFN- $\gamma$  production). However, surface CD62L is known to be proteolytically cleaved and rapidly shed following T cell activation, which could alter phenotypic characteristics during T cell stimulation. In the present study, we show that EBV-specific CD62L<sup>+</sup> CD8<sup>+</sup> T cells express intracellular IFN- $\gamma$  upon antigen-specific stimulation if activation-induced shedding of CD62L is circumvented. These results suggest that CD62L<sup>+</sup>  $T_{CM}$  have  $T_{EM}$  capacity, and that the current definition of antigen-specific CD8<sup>+</sup>  $T_{CM}$  using lineage markers needs to be re-evaluated.

## B. INTRODUCTION

Central memory T cells ( $T_{CM}$ ) are a subset of memory T cells that lack characteristics of effector cells. Two definable characteristics of  $T_{CM}$  are co-expression of homing receptors for lymphoid organs, CCR7 and CD62L, and lack of immediate cytoplasmic IFN- $\gamma$  production after antigen stimulation(20). In particular, two recent studies have shown that Epstein-Barr virus (EBV) specific CD62L<sup>+</sup>CD8<sup>+</sup> T cells stimulated with EBV peptides in short-term culture do not produce cytoplasmic IFN- $\gamma$  (124, 125). This supports the notion that antigen-specific CD62L<sup>+</sup>  $T_{CM}$  lack immediate effector, cytokine-producing capacity.

Surface expression of CD62L molecules can be altered by proteolytic cleavage and shedding of the extra-cellular portion of CD62L after cell activation(126-129). In the present study, we demonstrate that pre-enriched CD62L<sup>+</sup>CD8<sup>+</sup> T cells produced IFN- $\gamma$  after short-term peptide-stimulation. Moreover, in the presence of the CD62L sheddase inhibitor, GM6001(130), antigen-stimulated EBV-specific CD8<sup>+</sup> T cells contain CD62L<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells, indicating that CD62L<sup>+</sup>CD8<sup>+</sup> T cells can express intracellular IFN- $\gamma$  during short-term stimulation. These results suggest that use of CD62L expression and lack of immediate IFN- $\gamma$  production for defining antigen-specific  $T_{CM}$  and its lineage relationship to  $T_{EM}$  need to be re-evaluated.



## **B. MATERIALS AND METHODS**

### **Peptides, antibodies and HLA-A\*0201 tetramers**

Two peptides GLCTLVAML (HLA-A\*0201 EBV BMLF1<sub>280-288</sub>; GLC) and RAKFKQLL (HLA-B\*0801 EBV BZLF1<sub>190-197</sub>; RAK) were produced by the University of Illinois at Chicago Protein Research Laboratory. Monoclonal antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-CD62L and FITC or phycoerythrin (PE) conjugated anti-IFN- $\gamma$  (Becton Dickinson, San Jose, CA), and PE-Cy5 anti-CD62L and PE-Cy7 anti-CD8 (Beckman-Coulter-ImmuneTech, Miami, FL). PE conjugated HLA-A\*0201 EBV BMLF1<sub>280-288</sub> tetramer was purchased from Immunomics (Beckman-Coulter, San Diego, CA), and HLA-B\*0801 EBV BZLF1<sub>190-197</sub> tetrameric agents was obtained from the NIH Tetramer Synthesis Facility. Tetramer staining was done at 4C° for 30 min or 37C° for 25 min as indicated in the figure legend.

*Isolation of peripheral blood mononuclear cells (PBMC), and enrichment of CD62L<sup>+</sup> PBMC by magnetic beads.*

PBMC were isolated on Ficoll-Hypaque (Sigma, St. Louis, MO) density gradients.

Approximately 20 x 10<sup>6</sup> cells were stained with FITC mouse anti-human CD62L antibody for 30 min at 4°C. CD62L<sup>+</sup> cells (greater than 96% purity) were positively enriched by two rounds of magnetic bead separation (MACS, Miltenyl, Bergisch-Galdbach, Germany).

### *In vitro stimulation and intracellular IFN- $\gamma$ staining*

Freshly donated  $2-3 \times 10^6$  PBMC, and CD62L<sup>+</sup> or CD62L<sup>-</sup> cell populations, were resuspended in 200  $\mu$ l of RPMI 1640 with 10% FCS in the presence of 10 $\mu$ M GLC or RAK peptide. PBMC were also stimulated in the presence of 200  $\mu$ M GM6001 (Calbiochem, San Diego, CA) and pre-stained with GLC or RAK tetramer (37°C for 25 min) where appropriate. After an hour of incubation, 5 $\mu$ g/ml of brefeldin A was added and the cells were further incubated for 5 hours. The cells were then washed once with medium and stained with PE-Cy5 anti-CD62L, and PE-Cy7 anti-CD8 mAbs. The cells were washed and incubated in 300  $\mu$ l of PermiFlow (Invirion, Kalamazoo, MI) overnight at room temperature. Following overnight incubation, cells were washed and incubated with FITC or PE anti-human IFN- $\gamma$  for 40 min at 4°C, and washed.

### **Flow cytometric analysis**

Stained samples were run on a Coulter XL flow cytometer (Beckman-Coulter, Miami, FL). Data analysis and graphical representations were done using FlowJo (TreeStar, Cupertino, CA). A gating strategy based on the compounding gating method (111) was used throughout the study.

## D. RESULTS AND DISCUSSION

Two recent reports have provided evidence that the CD62L<sup>+</sup> subset of EBV-specific CD8<sup>+</sup> T cells does not produce cytoplasmic IFN- $\gamma$  upon short-term stimulation(124, 125). Other studies, however, have shown that PMA and ionomycin-stimulated, bulk CD62L<sup>+</sup>CD8<sup>+</sup> T cells(130), and antigen-stimulated CCR7<sup>+</sup> virus-specific CD8<sup>+</sup> T cells produce IFN- $\gamma$  (29). To address this discrepancy, PBMC were stimulated with GLC or RAK peptides for 6 hours, followed by intracellular staining for IFN- $\gamma$ . Although nearly 50% of GLC- and 37% of RAK-specific CD8<sup>+</sup> T cells expressed surface CD62L (Figure 7A), IFN- $\gamma$  production was not associated with CD62L expression (Figure 7B). This is in accordance with the observations made by Tussey et.al (125), and Hislop et.al (124).

Surface CD62L molecules can be rapidly cleaved and shed following cell activation(126-129). Enrichment of CD62L<sup>+</sup>CD8<sup>+</sup> T cells prior to peptide-specific stimulation will circumvent CD62L shedding, and demonstrate immediate IFN- $\gamma$  production by CD62L<sup>+</sup> GLC or RAK-specific CD8<sup>+</sup> T cells. As shown in Figure 7C, peptide stimulated, CD62L pre-enriched, GLC- and RAK-specific CD8<sup>+</sup> T cells produced IFN- $\gamma$  during short-term culture.

To further circumvent activation-induced CD62L shedding, bulk CD8<sup>+</sup> T cells were stimulated with specific peptides in the presence or absence of GM6001, a CD62L sheddase inhibitor (130). Treatment of the cells with 200uM of GM6001 significantly inhibited shedding of surface CD62L without affecting IFN- $\gamma$  production, clearly demonstrating immediate cytoplasmic IFN- $\gamma$  production in over 50 % of GLC- and RAK-specific CD62L<sup>+</sup>CD8<sup>+</sup> T cells (Figure 8A and B). Notably, there were also GLC- and RAK-specific CD62L<sup>+</sup>CD8<sup>+</sup> T cells that did not express IFN- $\gamma$  in response to antigen-specific stimulation.

Recently, CD62L<sup>+</sup>CD8<sup>+</sup> T cells stimulated with PMA and ionomycin were shown to produce several cytokines including IFN- $\gamma$  (130). Moreover, antigen-stimulated CCR7<sup>+</sup> viral-specific CD8<sup>+</sup> T cells were able to produce immediate IFN- $\gamma$  and TNF- $\alpha$  (29), and antigen-stimulated EBV-specific T<sub>CM</sub> (CD45RA<sup>low</sup>CCR7<sup>dull</sup>) produced IFN- $\gamma$  and IL-2 (131). Even naïve CD8<sup>+</sup> T cells have the capacity to produce IFN- $\gamma$  although such cells require longer stimulation (132). These observations suggest that T<sub>CM</sub> have the capacity for immediate IFN- $\gamma$  production.

Our data demonstrated that antigen-specific CD62L<sup>+</sup> T<sub>CM</sub> have immediate effector function (Figure 8C). Apparently, CD62L<sup>-</sup>CD8<sup>+</sup> T<sub>EM</sub> are able to re-express surface CD62L as long as CD62L transcription is not permanently silenced (109). Indeed, murine T<sub>CM</sub> (CD62L<sup>+</sup>CCR7<sup>+</sup>) can evolve directly from a surviving fraction of CD62L<sup>low</sup> T<sub>E/EM</sub> (1). Presumably, such CD62L<sup>+</sup> T<sub>CM</sub> retain the capacity for immediate effector function including immediate IFN- $\gamma$  production following antigenic stimulation. This explains the capacity of CD62L<sup>+</sup> EBV-specific CD8<sup>+</sup> T cells to produce immediate IFN- $\gamma$  upon antigenic stimulation. However, the presence of IFN- $\gamma$  negative CD62L<sup>+</sup> EBV-specific CD8<sup>+</sup> T cells in our study suggests that CD62L<sup>+</sup> T<sub>CM</sub> have more than one functional subset. Potentially, these cells may represent CD62L<sup>+</sup> T<sub>CM</sub> that have remained undifferentiated, or become dysfunctional (Figure 8C).

In conclusion, we show that over 50% of CD62L<sup>+</sup>CD8<sup>+</sup> T cells specific for EBV antigen can produce cytoplasmic IFN- $\gamma$ . Our results suggest that CD62L and immediate IFN- $\gamma$  expression is not confined to either T<sub>CM</sub> or T<sub>EM</sub> but is broadly expressed among various memory CD8<sup>+</sup> T cell subsets. Thus, a further refinement of functional and phenotypic definitions and lineage relationships between T<sub>CM</sub> and T<sub>EM</sub> is needed.

## **E. ACKNOWLEDGEMENT**

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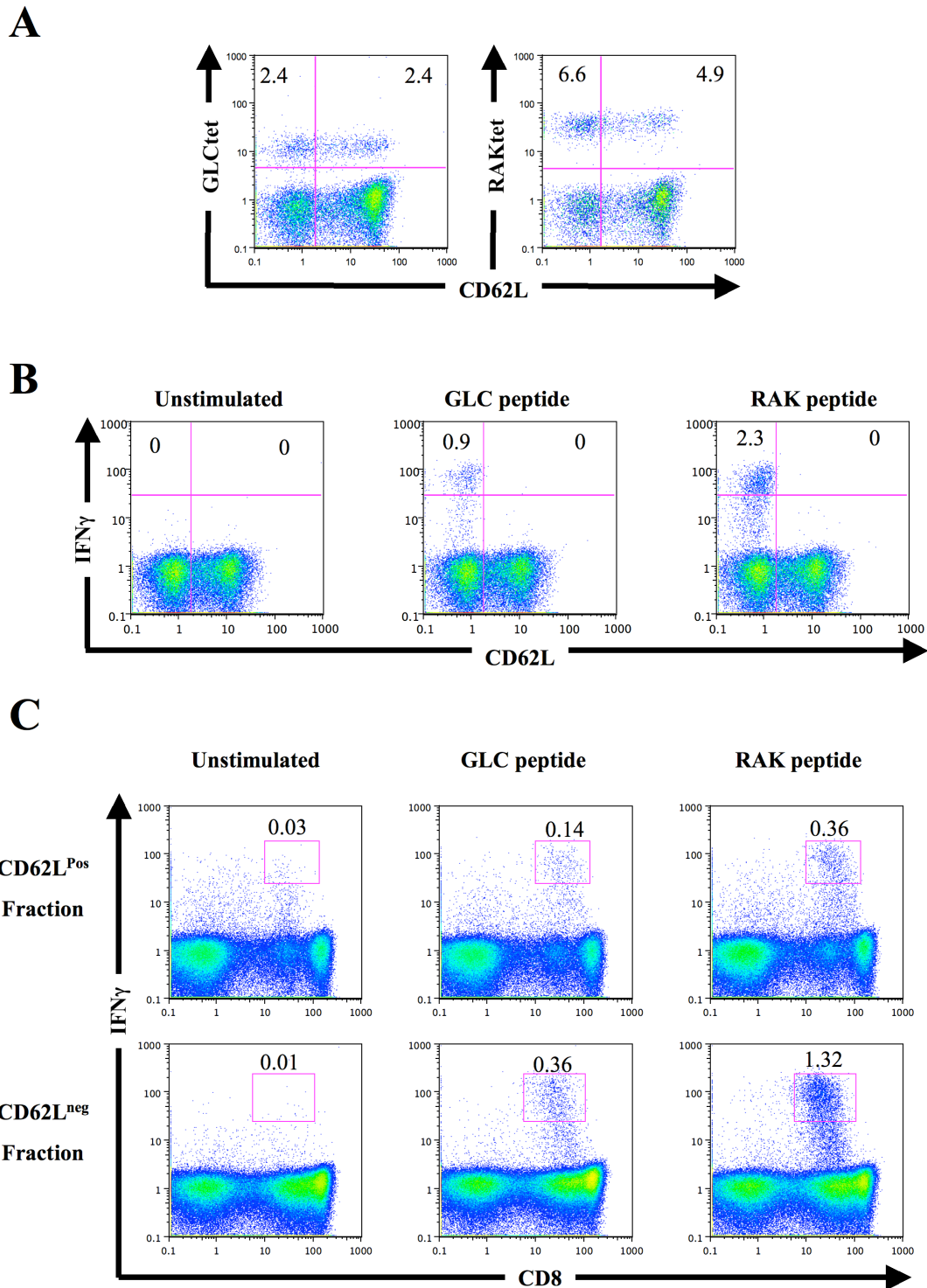


Figure 7. (A) Visualization of CD62L<sup>+</sup> T<sub>CM</sub> subset of GLC (A2 BMLF1)- and RAK(B8 BZLF1)specific CD8<sup>+</sup> T cells. (B) GLC and RAK-specific CD62L<sup>+</sup> cells in bulk CD8<sup>+</sup> T cells did not produce cytoplasmic IFN- $\gamma$  following peptide stimulation. (C) GLC- and RAK-specific CD62L<sup>+</sup> cells in enriched produced cytoplasmic IFN- $\gamma$  following peptide stimulation

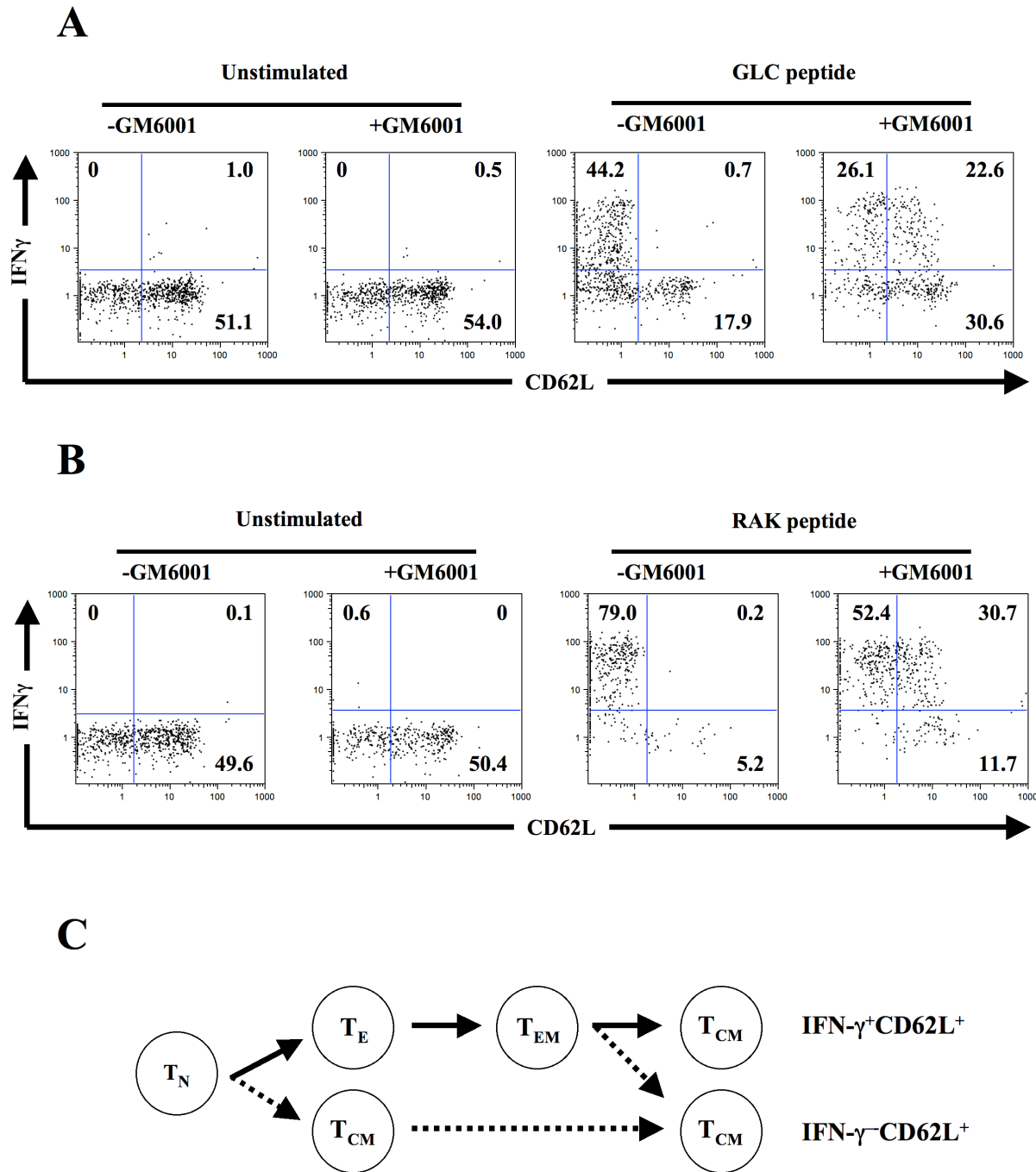


Figure 8. (A) GLC-stimulated CD62L<sup>+</sup> GLC tetramer-specific CD8<sup>+</sup> T cells produce cytoplasmic IFN- $\gamma$  in the presence of GM6001. (B) RAK-stimulated CD62L<sup>+</sup> RAK tetramer-specific CD8<sup>+</sup> T cells produce cytoplasmic IFN- $\gamma$  in the presence of GM6001. (C) A schematic diagram depicting two distinct lineages of T<sub>CM</sub>.

V.

**INVESTIGATE LIMITED DIFFERENTIATION OF HIV-1-SPECIFIC CD8<sup>+</sup> T CELLS  
FROM THE INTERMEDIATE STAGE TO THE LATE STAGE.**



**Limited memory to effector differentiation of  
HIV-1-specific CD8<sup>+</sup> T cells**

(Manuscript in preparation, 2005)

Aki Hoji<sup>1</sup> Nancy C. Connolly, William G. Buchanan, and Charles R. Rinaldo, Jr.<sup>1,2\*</sup>

Department of Infectious Diseases and Microbiology<sup>1</sup> and Pathology<sup>2</sup>, Graduate School of Public Health and School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261

## A. ABSTRACT

HIV-1-specific CD8<sup>+</sup> T cells play a major role in controlling acute HIV-1 viremia, however, these cells appear to eventually lose control of chronic infection. Failed control of chronic HIV-1 infection by HIV-1-specific CD8<sup>+</sup> T cells could be due to their inability to undergo memory-effector differentiation. In the present study, we used 6-color flow cytometric analysis to establish a detailed phenotypic assessment of HIV-1-specific CD8<sup>+</sup> T cell memory-effector differentiation by analyzing expression of CD27, CD28, CD57, and CD62L and by comparing phenotypes of EBV- and CMV-specific CD8<sup>+</sup> T cells as models of an immature memory and a mature effector CD8<sup>+</sup> T cells respectively. We found that HIV-1-specific CD8<sup>+</sup> T cells were phenotypically enriched in a late intermediate stage of CD8<sup>+</sup> T cell differentiation (CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>-/low</sup>CD62L<sup>-</sup>). Moreover, unlike EBV- and CMV-specific CD8<sup>+</sup> T cells, HIV-1-specific CD8<sup>+</sup> T cells did not display memory-effector differentiation associated down-regulation of CD27 and CD28 and up-regulation of CD57. Furthermore, these cells showed a comparably smaller proportion of a CD27<sup>-</sup>CD57<sup>high</sup> terminally differentiated effector subset. These results suggest that despite the continuous presence of viral antigen, HIV-1-specific CD8<sup>+</sup> T cells do not complete normal CD8<sup>+</sup> T cell memory-effector differentiation.

## B. INTRODUCTION

Assessing memory-effector differentiation of virus-specific memory CD8<sup>+</sup> T cells is essential to understand how virus-specific memory CD8<sup>+</sup> T cells are able to contain viral infection. Distinct stages of CD8<sup>+</sup> T cell differentiation can be assessed phenotypically based on expression of several phenotypic markers that are known to be up- or down-regulated during the natural course of CD8<sup>+</sup> T cell memory-effector differentiation. Currently, virus-specific memory CD8<sup>+</sup> T cells are shown to progress from the least mature stage of CD27<sup>+</sup>CD28<sup>+</sup>CD45RO<sup>+</sup>, to the more mature (effector) stage of CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup> (23, 32). The most mature or terminally differentiated effector cells can be further defined by expression of CD57 (88).

Recent phenotypic assessment based on these phenotypic markers has shown that virus-specific memory CD8<sup>+</sup> T cells display phenotypes that are associated with particular stages of T cell memory-effector differentiation depending on the nature of viral infection. Memory CD8<sup>+</sup> T cells specific for non-persistent virus such as influenza A virus show less differentiated memory phenotype (CD27<sup>+</sup>CD28<sup>+</sup>CD45RO<sup>+</sup>) (115, 133). In contrast, CD8<sup>+</sup> T cells specific for persistent viruses such as EBV and CMV show a further differentiated phenotype, characteristic of mature memory (CD27<sup>+</sup>CD28<sup>+/</sup>CD45RO<sup>+</sup>) and effector (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>) subsets, respectively (23, 32).

One of the critical factors that drives differentiation of CD8<sup>+</sup> T cells appears to be repeated antigen exposure. Unlike herpes viruses, HIV-1 replicates continuously providing a constant supply of HIV-1 antigens, hence, long-term HIV-1 infection theoretically drives HIV-1-specific CD8<sup>+</sup> T cells to terminal differentiation. While the dominant phenotype of HIV-1-specific CD8<sup>+</sup> T cells for long-term non-progressors has been shown to be the effector subset

(CD27<sup>-</sup>CD45RA<sup>+</sup>) (86), others have reported that the phenotype of the HIV-1-specific CD8<sup>+</sup> T cell remains essentially the same (CD27<sup>+</sup>CD28<sup>-</sup>) regardless of clinical status (85) and duration of infection (84). Thus, HIV-1-specific CD8<sup>+</sup> T cell memory-effector differentiation during the natural course of CD8<sup>+</sup> T cell maturation remains to be clarified.

In the present study, we assessed memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells by a refined phenotypic analysis based on 6-color flow cytometry. We found that unlike EBV- and CMV-specific CD8<sup>+</sup> T cells, HIV-1-specific CD8<sup>+</sup> T cells do not exhibit maturation associated regulation of CD27, CD28 and CD57 expression. We also found that in contrast to EBV and CMV-specific CD8<sup>+</sup> T cells, the majority of HIV-1-specific CD8<sup>+</sup> T cells are phenotypically enriched with a CD27<sup>+(low)</sup>CD57<sup>low</sup> (intermediary) subset and showed an irregular pattern of progression from a CD27<sup>+(high)</sup>57<sup>-</sup> (memory) to CD27<sup>-</sup>CD57<sup>high</sup> (effector) subset. These results suggest that the phenotypic enrichment of HIV-1-specific CD8<sup>+</sup> T cells (CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>-/low</sup>CD62L<sup>-</sup>) that persists in spite of the continuous presence of HIV-1 antigen is due to impaired effector differentiation.

## **C. MATERIALS AND METHODS**

### **Study Population**

The study population consisted of 10 HIV-1 uninfected volunteers (median age 52 yr, range 43-69yr, 1 female and 12 male Caucasians) and 13 HIV-1 infected subjects (median age 48 yr, range 25-70 yr, all male Caucasians) from the Pittsburgh portion of the national Multicenter AIDS Cohort Study (MACS). Their HLA types are listed in Table 1. CD4<sup>+</sup> T cell counts and viral load in the HIV-1 infected subjects and status of antiretroviral therapy at the time of blood draw are summarized in Table 2. HIV-1 serum viral load was assayed by a quantitative RT-PCR assay (Amplicor; Roche Diagnostics, Alameda, CA). HLA-typing was done at the Laboratory of Genomic Diversity, National Cancer Institute and the laboratory of Dr. M. Trucco, University of Pittsburgh.

### **Antibodies and Class I tetramers**

The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD57 and (phycoerythrin) PE-Cy5 conjugated mouse anti-human CD27 (BD Biosciences, San Jose, CA); ECD (PE-Texas Red)-conjugated mouse anti human CD28 and PE-Cy7 conjugated mouse anti-human CD8 (Coulter-Immunotech, Miami, FL); (allophycocyanine) APC-Cy7 conjugated mouse anti human CD62L. (Caltag, Burlingame, CA). Appropriate isotype-matched mAbs were obtained from the same companies, and used throughout the course of the study.

class I tetrameric reagents used in the study were listed in Table 2. All the tetrameric agents except A2pp65 CMV and A2BMLF1 EBV were obtained from NIH Tetramer Synthesis

Facility. A2pp65 CMV and A2BMLF1 EBV tetramers were obtained from Coulter Immunomics (San Diego, CA). All of the tetramers were labeled with PE, and used at a 1/50-1/200 dilution for staining  $2-5 \times 10^6$  PBMCs.

### **Isolation and preparation of stained PBMC**

Blood specimen were collected in BD Vacutainer Cell Preparation Tubes (CPT) with sodium heparin (BD Biosciences, San Jose, CA), and PBMC were isolated according to the manufacturer's protocol. PBMC were stained as described previously (133). Briefly,  $2-5 \times 10^6$  fresh PBMC were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) with 10% heat inactivated fetal calf serum (FCS) (Hyclone, Logan, UT) and were first stained with 1/50-1/200 dilution of the appropriate tetramer at 37°C for 25 min. Then, cells were washed and resuspended in PBS with 4% FCS and 0.1% sodium azide, and incubated with a cocktail of mAbs for 30 min at 4°C. After the final wash, cells were fixed with 1% paraformaldehyde (PFA) and analyzed by an MoFlo flow cytometer (DakoCytomation, Boulder, CO).

### **Flow Cytometry**

The MoFlo flow cytometer was calibrated daily for color compensation and laser fluctuation. A 6 color compensation matrix was created by FlowJo (TreeStar, Cupertino, CA) software compensation based on six singly stained PBMC from actual donors as recommended by manufacturer (TreeStar). A PE-conjugated mouse anti-human CD8 monoclonal antibody was used for PE compensation in a place for the MHC class I tetramer.

We used a flow cytometric analysis described previously (133) with a few modifications. A minimum of 500 total tetramer<sup>+</sup>CD8<sup>high</sup> events were collected for the fully stained sample and

isotype control (the same number of events were collected for both samples), and this resulted in collection of approximately  $1-3 \times 10^6$  total events. We used a compounded gating scheme previously described (111) (133) with necessary modifications. Briefly, cells were first gated on the CD8<sup>high</sup> population on a CD8 and SS-Log plot, followed by a lymphocyte gate on an FS and SS-Log plot. Potential doublets were excluded on FS and FS-integrated (FS-int), and the resulting tetramer<sup>+</sup> population was visualized on a tetramer-PE vs SSLog plot for determining phenotypic analysis and frequency of tetramer<sup>+</sup>CD8<sup>+</sup> T cells. Data analysis and graphic representations were done with FlowJo software (TreeStar, Cupertino, CA).

### **Statistical analysis**

We used Whitney-Mann U, Wilcoxon Rank-Sum and Tukey-Kramer HSD tests for determining statistical significance between and among group means. We used the Spearman correlation test and a standard least square method for determining r correlation coefficients and P values; P<0.05 was considered statistically significant. Statistical analysis and graphical representation were done using JMP IN v5.1 (JMP Sales, Cary, NC) and Aabel software (Gigawiz LTD Co, Tulsa, OK).

## D. RESULTS

### **EBV-, CMV, and HIV-1-specific CD8<sup>+</sup> T cells show distinct levels of CD27, CD28, CD57, and CD62L expression.**

We first used a battery of phenotypic markers and MHC class I tetramers to assess virus-specific T cell phenotypes comparable to those previously related to a unique virus-specific memory CD8<sup>+</sup> T cell during persistent viral infections (22). Virus-specific CD8<sup>+</sup> T cells were visualized by a host of MHC class I tetramers (Figure. 9A and Table 5), and phenotype of virus-specific CD8<sup>+</sup> T cells were assessed by co-staining with antibodies against CD27, CD28, CD57, and CD62L (Figure. 9B).

Comparison of percent marker expression revealed a phenotypic subset unique to each virus-specific CD8<sup>+</sup> T cells (Figure. 10A). The major phenotypic subsets of EBV-, HIV-1-, and CMV-specific CD8<sup>+</sup> T cells were CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>-</sup>CD62L<sup>+/-</sup>, CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>+</sup>CD62L<sup>-</sup>, and CD27<sup>-</sup>CD28<sup>-</sup>CD57<sup>+</sup>CD62L<sup>-</sup> respectively. These data suggest that EBV-specific CD8<sup>+</sup> T cells display the least differentiated phenotype (central memory), while CMV-specific CD8<sup>+</sup> T cells display the most differentiated phenotype (effector). HIV-1-specific CD8<sup>+</sup> T cells appear to be phenotypically in an intermediate stage of CD8<sup>+</sup> T cell differentiation. This is in agreement with observations made by Appay, et al. (22).

A few HIV-1 infected subjects (Table 4) had either or both EBV- and/or CMV-specific CD8<sup>+</sup> T cells, making it possible to compare phenotypes of three virus-specific CD8<sup>+</sup> T cells from the same immunological background. EBV- and CMV-specific CD8<sup>+</sup> T cells showed a similar pattern of percent CD27, CD28, CD57, and CD62L expression as compared to HIV-specific CD8<sup>+</sup> T cells from the same HIV infected subjects (Figure. 10B). Moreover, we found



no statistical differences ( $p>0.05$ ) in percent marker expression of EBV- and CMV-specific CD8<sup>+</sup> T cells between HIV-1 uninfected and infected subjects as predicted (Figure. 11A and B).

These differences can be attributed to equally skewed proportions of bulk CD8<sup>+</sup> T cells (tetramer<sup>-</sup> CD8<sup>+</sup> T cells) expressing these markers from HIV-1 infected subjects. Although bulk CD8<sup>+</sup> T cells from HIV-1 infected subjects show a relatively lower proportion of CD27 and CD62L and higher proportion of CD57 expressing T cells, only the proportion of CD28<sup>+</sup> T cells was significantly different from that of HIV-1 uninfected subjects (Figure. 11C).

These data suggest that even though HIV-1 infection affects expression of at least one marker (CD28) in bulk CD8<sup>+</sup> T cells, such impairment does not appear to significantly skew memory-effector differentiation of EBV- and CMV-specific CD8<sup>+</sup> T cells.

**HIV-1-specific CD8<sup>+</sup> T cells do now show correlations among percent marker expression, and correlations between percent marker expressions and frequency.**

We next examined correlations between percent marker expression of the virus-specific CD8<sup>+</sup> T cells to determine whether there is a memory-effector differentiation-associated modulation of these phenotypic markers. If the virus-specific CD8<sup>+</sup> T cell undergoes uninterrupted memory-effector differentiation, such a correlation would be expected especially between CD27 and CD28 expression. This is based on the known dichotomy of their differential expression during memory-effector CD8<sup>+</sup> T cell differentiation (22). Both EBV- and CMV-specific CD8<sup>+</sup> T cells showed a significant positive correlation between percent CD27 and CD28 expression (EBV:R=0.81,  $p<0.05$ , CMV:R=0.87,  $p<0.0001$ ), whereas there was not correlation for any combination of phenotypic markers on HIV-1-specific CD8<sup>+</sup> T cells (Figure. 12). In addition, there were significant correlations for EBV-specific CD8<sup>+</sup> T cells between CD27 and CD57

( $R=0.81$ ,  $p<0.05$ ), and CD28 and CD57 ( $R=0.97$ ,  $p<0.001$ ). Similarly, CMV-specific CD8<sup>+</sup> T cells show significant correlations between CD27 and CD62L ( $R=0.58$ ,  $p<0.05$ ), and CD28 and CD62L ( $R=0.62$ ,  $p<0.01$ ).

We next examined correlations between the frequency of the virus-specific CD8<sup>+</sup> T cells and percent marker expression (Figure. 13). EBV- and CMV-specific CD8<sup>+</sup> T cells displayed a significant correlation between percent CD27 expression and frequency (EBV:  $R=0.86$ ,  $p<0.02$ , CMV:  $R=0.55$ ,  $p<0.05$ ). In addition, CMV-specific CD8<sup>+</sup> T cells showed significant correlations between percent and frequency of CD28 and CD62L expression (CD28:  $R=0.48$ ,  $p<0.05$ , CD62L:  $R=0.55$ ,  $p<0.05$ ). In contrast, HIV-1-specific CD8<sup>+</sup> T cells did not show any significant correlations for expression or frequency of any of the markers.

These results show that coordinated down-modulation of CD27 and CD28 expression is the most consistent phenotypic marker alteration associated with memory-effector differentiation, based on EBV- and CMV-specific CD8<sup>+</sup> T cells from HIV-1 uninfected subjects. Also, memory-effector CD8 T cell differentiation of HIV-1-specific CD8<sup>+</sup> T cells appears to be arrested at a stage of CD27 down-modulate. Alternatively, these results indicate a possible deletion of a CD27<sup>-</sup> subset of HIV-1-specific CD8<sup>+</sup> T cell.

**HIV-1-specific CD8<sup>+</sup> T cells are enriched in the CD27<sup>-</sup>CD57<sup>+(high)</sup> subset and do not appear to display transition from CD27<sup>+(high)</sup>CD57<sup>-</sup> to CD27<sup>-</sup>CD57<sup>+(high)</sup>.**

It is possible that majority of HIV-1-specific CD8<sup>+</sup> T cells differentiated into effector cells without down-regulating CD27 expression. To address this, we examined CD57 expression as an additional marker of the effector phenotype. In agreement with results from previous sections, relative phenotypic enrichment based on CD27 and CD57 expression of virus-specific

CD8<sup>+</sup> T cells indicates that HIV-1-specific CD8<sup>+</sup> T cells are accumulated in intermediate stage (CD27<sup>+</sup>CD57<sup>+</sup>) of memory-effector differentiation. Likewise, EBV-specific CD8<sup>+</sup> T cells are enriched in undifferentiated memory stage (CD27<sup>+</sup>CD57<sup>-</sup>), and CMV-specific CD8<sup>+</sup> T cells are enriched in terminal effector stage (CD27<sup>-</sup>CD57<sup>+</sup>) (Figure. 15).

EBV- and CMV-specific CD8<sup>+</sup> T cells appear to uninterruptedly progress from CD27<sup>high</sup>CD57<sup>-</sup> (undifferentiated memory) to CD27<sup>low</sup>CD57<sup>low</sup> (transitional stage), and finally to CD27<sup>-</sup>CD57<sup>high</sup> (effector) (Figure. 14B). This pattern of progression from immature to mature states becomes more apparent in the relatively more differentiated EBV-specific CD8<sup>+</sup> T cells (Figure. 14B 1) and generally in most of the CMV-specific CD8<sup>+</sup> T cell (Figure. 14B 2). Even EBV- and CMV-specific CD8<sup>+</sup> T cells from the same HIV-1 infected individuals show a similar pattern (Figure. 14B 4). However, a majority of HIV-1-specific CD8<sup>+</sup> T cells (Figure. 14B 3 and 4) seldom display such pattern, even for some HIV-1-specific CD8<sup>+</sup> T cells with relatively well developed CD27<sup>-</sup>CD57<sup>+</sup> subset (Figure. 14B 5). Based on these results, HIV-1-specific CD8<sup>+</sup> T cells appear to be enriched in the transitional stage (CD27<sup>high</sup>CD57<sup>low</sup>), and do not appear to differentiate to the effector subset (CD27<sup>-</sup>CD57<sup>-</sup>) even during chronic, untreated HIV-1 infection.

## E. DISCUSSION

Recent 4-color phenotypic studies characterizing HIV-1-specific CD8<sup>+</sup> T cells have shown that these cells are phenotypically enriched in the intermediate stage of memory-effector CD8 T cell differentiation, relatively more differentiated than immature EBV-specific CD8<sup>+</sup>T cells and less differentiated than effector CMV-specific CD8<sup>+</sup> T cells (22, 84-86). In agreement with these studies, our 6-color study showed that the majority of HIV-1-specific CD8<sup>+</sup> T cells, regardless of clinical status of the HIV-1 infected study subjects, displayed the phenotype (CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>-/low</sup>CD62L<sup>-</sup>). This is indicative of the advanced intermediate stage of memory-effector differentiation as compared to the average phenotype of EBV-specific CD8<sup>+</sup>T cells (CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>-</sup>CD62L<sup>+/-</sup>) and of CMV-specific CD8<sup>+</sup> T cell (CD27<sup>-</sup>CD28<sup>-</sup>CD57<sup>low/high</sup>CD62L<sup>-</sup>) (Figure. 15).

If memory-effector differentiation of the antigen-specific CD8<sup>+</sup> T cell depends primarily on the antigen load, progressive HIV-1 infection, unlike predominately latent EBV and CMV infection, would provide continuous presence of HIV-1 antigens that in theory should drive HIV-1-specific CD8<sup>+</sup> T cells to be more differentiated effectors. However, HIV-1-specific CD8<sup>+</sup> T cells remain largely less differentiated than CMV-specific CD8<sup>+</sup> T cells. Our results favor the possibility that an impaired immune system caused by progressive HIV-1 infection has a negative impact on memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells. One could argue that such immune impairment would affect differentiation of any antigen-specific CD8<sup>+</sup> T cells. However, our data show that EBV- and CMV-specific CD8<sup>+</sup> T cells from HIV-1 infected individuals display similar phenotypic enrichment to T cells from HIV-1-uninfected individuals.

This strongly suggests that immune impairment posed by progressive HIV-1 infection appears to predominately affect differentiation of HIV-1-specific CD8<sup>+</sup> T cells.

Evidence supporting impaired differentiation of HIV-1-specific CD8<sup>+</sup> T cells has been documented (32), though the proposed underlying mechanism of such impairment remains largely speculative. A possible mechanism provided by our data is that HIV-1-specific CD8<sup>+</sup> T cells lose the capacity to undergo differentiation from the advanced intermediate stage (CD27<sup>high</sup>CD57<sup>low</sup>) to the effector stage (CD27<sup>-</sup>CD57<sup>high</sup>), potentially due to inability to down-regulate CD27. It has been shown that CD27 down-regulation on CMV-specific CD8<sup>+</sup> T cells requires CD70 expression induced by antigen-specific activation (or activation through the T cell receptor-CD3 complex) and the presence of IL-2 (9). Thus, long-term continuous activation of HIV-1-specific CD8<sup>+</sup> T cells in the absence of IL-2 due to CD4<sup>+</sup> T cell loss possibly causes HIV-1-specific CD8<sup>+</sup> T cells to fail to down-regulate CD27.

Alternatively, the effector subset (CD27<sup>-</sup>CD57<sup>high</sup>) of HIV-1-specific CD8<sup>+</sup> T cells may have developed but was depleted during chronic HIV-1 infection. This is possible considering the fact that CD57<sup>+</sup>CD8<sup>+</sup> T cells are more prone to activation-induced apoptosis (88). Also, chronic antigen stimulation could prolong CD27-CD70 interaction due to over expression of CD70 on HIV-1-specific CD8<sup>+</sup> T cells (134). However, in the absence of CD28, co-stimulation through CD27 is sufficient for survival of effector T cells in mice (135). Similarly, antigen-specific stimulation of CD27<sup>+</sup> HIV-1-specific CD8<sup>+</sup> T cells with CD27 co-stimulation results in prolonged survival of proliferating cells (136). Thus, it appears that excessive CD27-CD70 interaction may lead to generation and subsequent deletion of CD27<sup>-</sup>CD57<sup>high</sup> HIV-1-specific CD8<sup>+</sup> T cells. This may explain the relatively lower range of frequency of HIV-1-specific CD8<sup>+</sup> T cells observed in our study. Also, the average frequency of HIV-1-specific CD8<sup>+</sup> T cells (in

total CD8<sup>+</sup> T cells) is estimated to be around 0.1%-1% (137), although higher frequencies (over 5%) of HIV-1-specific CD8<sup>+</sup> T cells have been found occasionally by tetramer staining (85) and ELISPOT and intracellular IFN- $\gamma$  staining (138).

It is not entirely clear whether a particular phenotypic enrichment or differentiation stage of the virus-specific CD8<sup>+</sup> T cells can provide necessary and sufficient protection from the virus infection. Intuitively, having a large proportion of effector virus-specific CD8<sup>+</sup> T cells seems to be advantageous because of their capacities to mount immediate protection with direct cytotoxic functions. However, having a large proportion of effector cells may not be suitable for long-term protection if effectors readily undergo activation-induced apoptosis. This issue has remained controversial in the context of assessing phenotypic correlates of long-term survival in HIV-1 infection (23, 32). The mode of long-term nonprogression can be multifaceted, with HIV-1 infected individuals including long-term survivors being heterogeneous in their current and previous clinical conditions (e.g., viral load, CD4<sup>+</sup> T cell counts, duration of infection, types of medications) and basis of immune control of HIV-1 infection.

Our study shows that HIV-1-specific CD8<sup>+</sup> T cells from a cross section of HIV-1 infected individuals show remarkably consistent phenotypes, despite of a range of clinical status. Our observations are in agreement with those of Papagno et. al. (85) that long-term progressive HIV-1 infection, regardless of clinical conditions, does not considerably skew phenotypic enrichment of HIV-1-specific CD8<sup>+</sup> T cells.

In conclusion, we assessed the state of memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cell by characterizing and comparing phenotypes of HIV-1-, EBV-, and CMV-specific CD8<sup>+</sup> T cells based on 6-color, 8-parameter flow cytometry. We found that HIV-1-specific CD8<sup>+</sup> T cells predominantly displayed the phenotype (CD27<sup>+</sup>CD28<sup>-</sup>CD57<sup>low</sup>CD62L<sup>-</sup>)

characteristic of the advanced intermediate stage of CD8<sup>+</sup> T cell memory-effector differentiation. Moreover, we noted that some EBV- and majority of CMV-specific CD8<sup>+</sup> T cells displayed coordinated modulation of CD27, CD28, and CD57 expression during memory-effector differentiation, while a majority of HIV-1-specific CD8<sup>+</sup> T cells did not. Particularly, HIV-1-specific CD8<sup>+</sup> T cells did not demonstrate differentiation to the effector subset (CD27<sup>-</sup>CD57<sup>high</sup>). These results indicate HIV-1-specific CD8<sup>+</sup> T cells sustain impaired effector differentiation, or alternatively this effector subset could be deleted during the course of progressive HIV-1 infection.

## **F. ACKNOWLEDGEMENTS**

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Table 4. HLA types, tetramer reactivity, and clinical data of study subjects

Subjects	HLA type	Tetramer reactivity <sup>a</sup>	Clinical status <sup>b</sup>	CD4 counts <sup>c</sup>	Viral load <sup>d</sup>
1.	A2, A3, B27, B37	A2p17, A2pol, A2CMV A2EBV	HIV-1 <sup>+</sup> , no HAART	459	939
2.	A3, A24, B27, B15	A3p17, A3pol	HIV-1 <sup>+</sup> , noHAART	385	24968
3.	A1, A32, B40, B8	B8p24, B8Nef	HIV-1 <sup>+</sup> , no HAART	270	18646
4.	A2, A26, B44, B15	A2p17, A2CMV	HIV-1 <sup>+</sup> , HAART	457	50
5.	A1, A32, B8, B40	B8p24	HIV-1 <sup>+</sup> , HAART	483	50
6.	A2, A33, B15, B42	A2p17	HIV-1 <sup>+</sup> , no HAART	1029	101
7.	A2, A2, B27, B13	A2p17, A2pol, A2EBV, A2CMV	HIV-1 <sup>+</sup> , no HAART	990	23737
8.	A3, A31, B13, B51	A3p17	HIV-1 <sup>+</sup> , no HARRT	453	22580
9.	A2, A3, B57, B40	A2p17, A2pol, A3p17, A3pol, A2EBV, A2CMV	HIV-1 <sup>+</sup> , no HARRT	129	66752
10.	A2,	A2CMV	HIV-1 <sup>-</sup>		
11.	A2, A2, B8	A2EBV, B8EBV	HIV-1 <sup>-</sup>		
12.	A2, A31, B15, B44	A2CMV	HIV-1 <sup>-</sup>		
13.	A2, A24, B44, B7	A2EBV, A2CMV	HIV-1 <sup>-</sup>		
14.	A2,	A2EBV, A2CMV	HIV-1 <sup>-</sup>		
15.	A1, A2, B8, B56	A2CMV	HIV-1 <sup>-</sup>		
16.	A1, A11, B8, B15	B8EBV	HIV-1 <sup>-</sup>		
17.	A1, A2, B18, B51	A2EBV, A2CMV	HIV-1 <sup>-</sup>		
18.	A2, A23, B13, B44	A2CMV	HIV-1 <sup>-</sup>		
19.	A2, A23, A39, B41	A2CMV	HIV-1 <sup>-</sup>		

<sup>a</sup> Each tetramer is designated by the HLA-type and the antigen. See table 2. for more detail on tetramer designation.

<sup>b</sup> Clinical status of study subjects at the time of their blood draw. HIV-1<sup>+</sup> indicates HIV-1 infected individuals. HAART indicates a recipient of highly active anti-retroviral therapy.

<sup>c</sup> CD4 count indicates number of CD4<sup>+</sup> T cells per ml of blood.

<sup>d</sup> Viral load indicates number of HIV-1 viral genome per ml of serum sample.

Table 5. List of epitopes used for MHC class I tetramer production

HLA alleles	Epitope amino acid sequence	Protein	Epitope location <sup>a</sup>
A2	SLYNTVATL	HIV-1 p17Gag	77-85
A2	ILKEPVHGV	HIV-1 Pol	309-317
A2	GLCTLVAML	EBV BMLF1	280-288
A2	NLVPMVATV	CMV pp65	493-503
A3	RLRPGGKKK	HIV-1 p17Gag	20-28
A3	AIFQSSMTK	HIV-1 Pol	158-166
A3	QVPLRPMTYL	HIV-1 Nef	73-82
B8	EIYKRWII	HIV-1 p24Gag	128-135
B8	FLKEKGGL	HIV-1 Nef	90-97
B8	RAKFQLL	EBV BZLF1	190-197

<sup>a</sup> Epitope location is indicated by number of amino acids spanning the epitope from N-terminus of the corresponding protein.

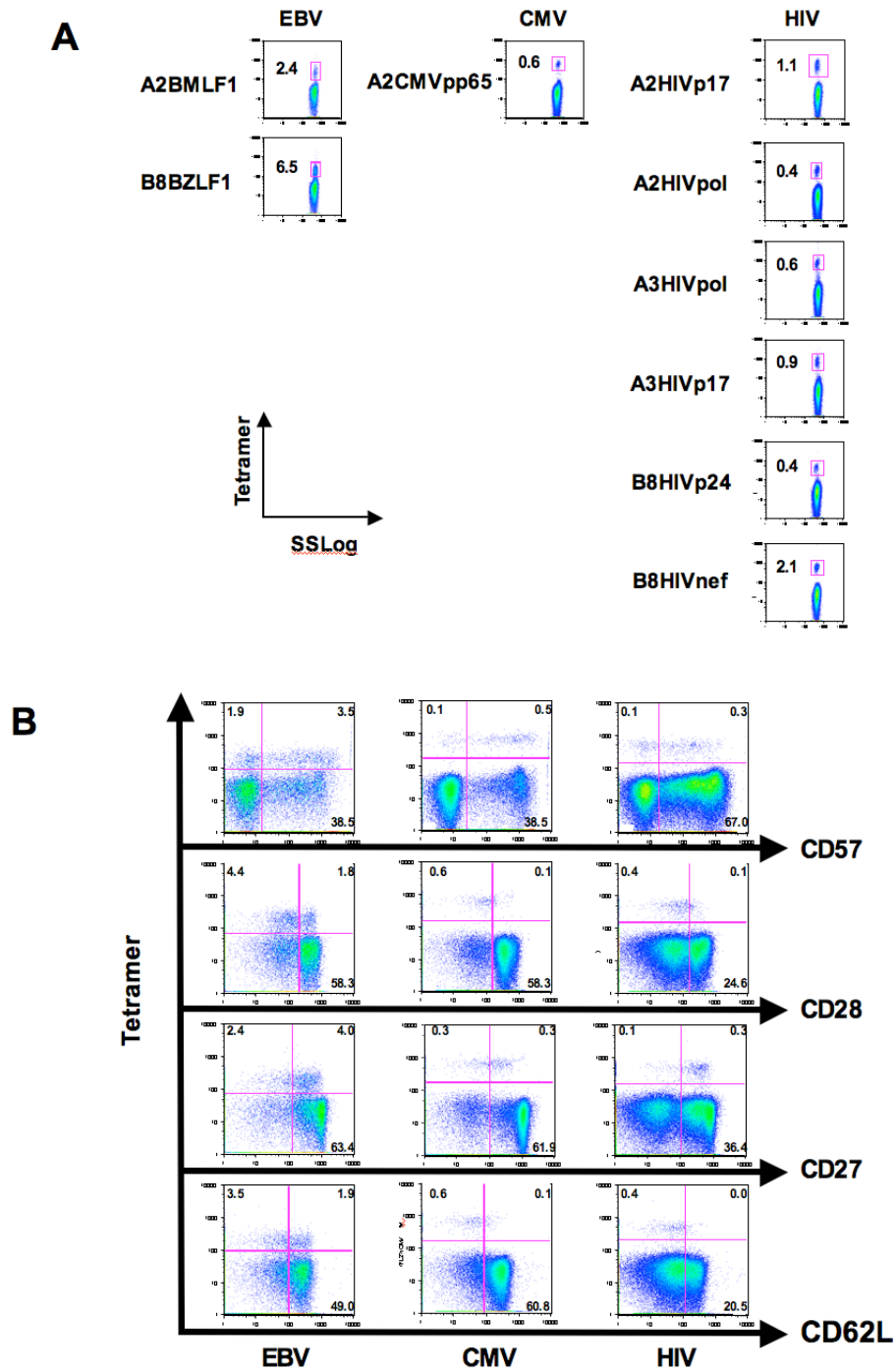


Figure 9. (A) Visualization of virus-specific CD8<sup>+</sup> T cells by staining *ex vivo* with MHC class I tetramers and surface expression of memory/effector markers. (B) Surface expression of memory/effector phenotypic markers on virus-specific CD8<sup>+</sup> T cells

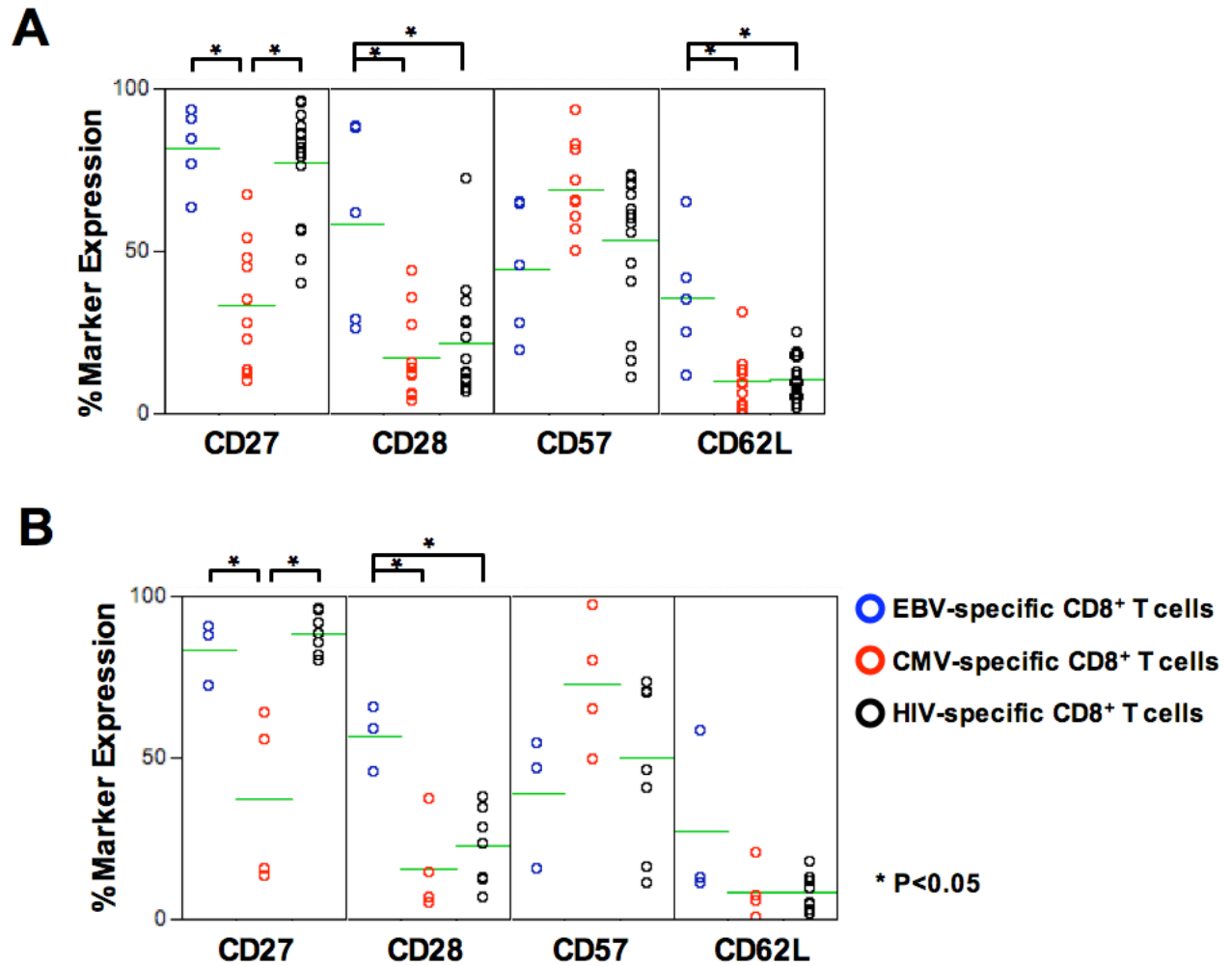


Figure 10. (A) Comparison of percent CD27, CD28, CD57, and CD62L expressing EBV-, CMV-, and HIV-1-specific CD8<sup>+</sup> T cells. (B) EBV- and CMV-specific CD8<sup>+</sup> T cells from HIV-1 infected subjects displaying the pattern of marker expression similar to those CD8<sup>+</sup> T cells from HIV-1 uninfected subjects.

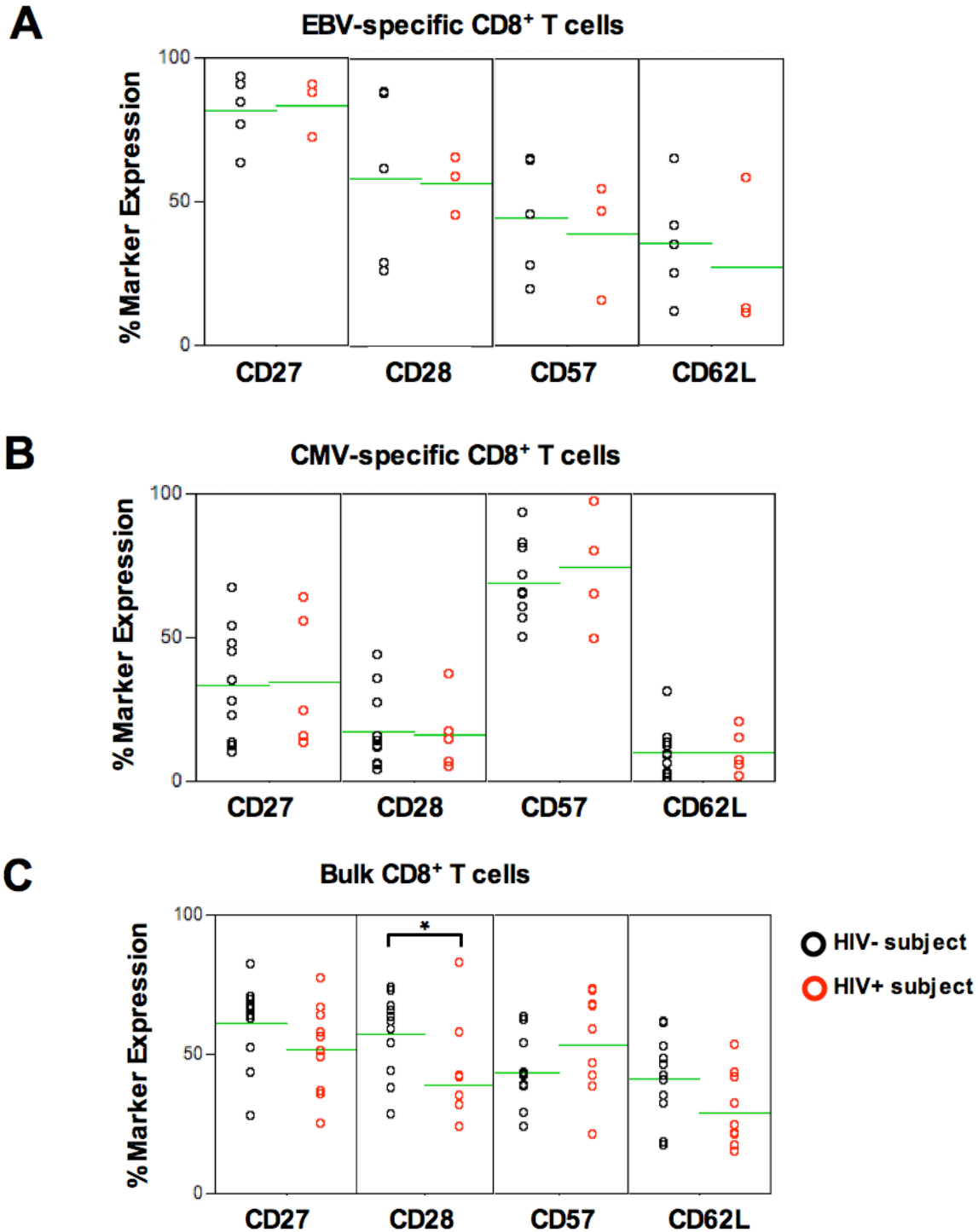


Figure 11. (A) EBV (A)-specific CD8<sup>+</sup> T cells from HIV-1 infected and uninfected subjects show similar proportions of CD27, CD28, CD57 and CD62L subsets. (B) CMV (B) -specific CD8<sup>+</sup> T cells from HIV-1 infected and uninfected subjects show similar proportions of CD27, CD28, CD57 and CD62L subsets. (C) Comparison of proportions of CD27, CD28, CD57 and CD62L subsets of bulk CD8<sup>+</sup> T cells expression (C) from HIV-1 infected (HIV-1<sup>+</sup>) and uninfected (HIV-1<sup>-</sup>) subjects

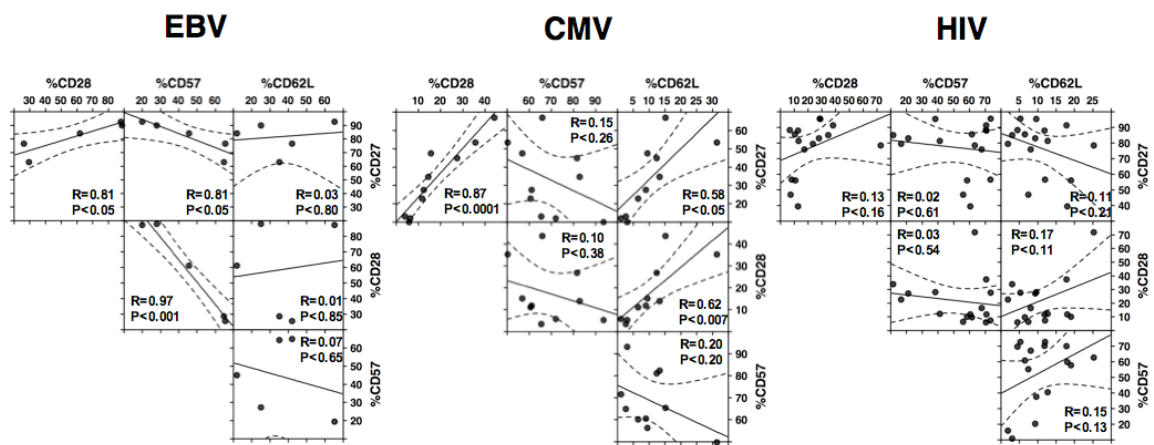


Figure 12. A scatter matrix plot of each pairs of percent marker expression of EBV-, CMV-, and HIV-1-specific CD8<sup>+</sup> T cells.

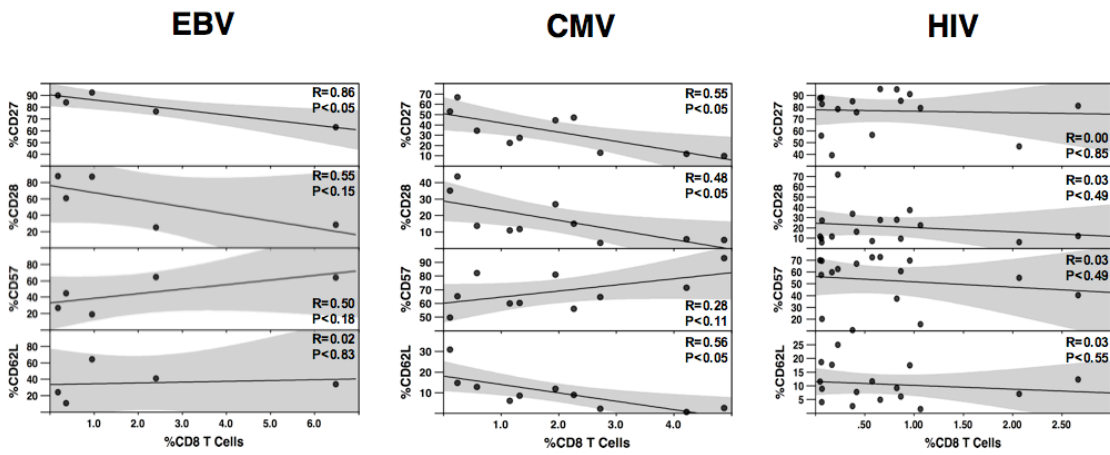
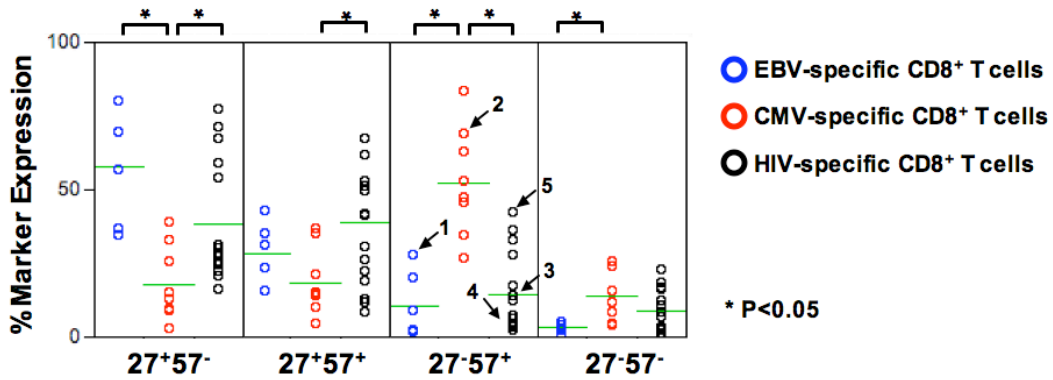


Figure 13. Correlation between the marker expression and frequency of EBV-, CMV-, HIV-specific CD8<sup>+</sup> T cells

**A**



**B**

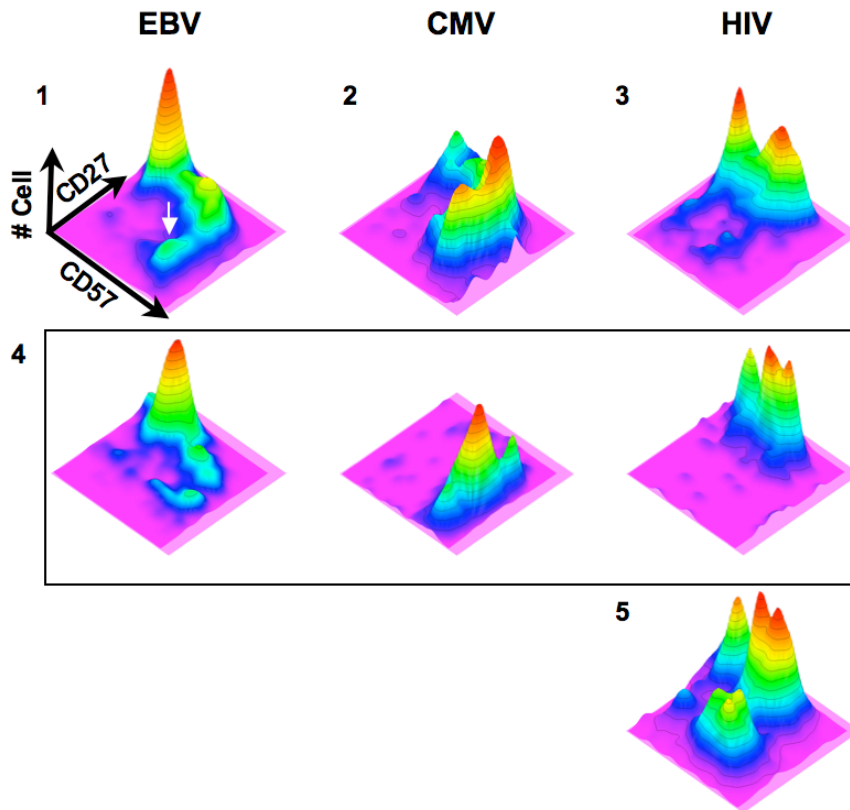


Figure 14. (A) Comparison of percent CD27<sup>+</sup>CD57<sup>+</sup>, CD27<sup>+</sup>CD57<sup>-</sup>, CD27<sup>-</sup>CD57<sup>+</sup>, CD27<sup>-</sup>CD57<sup>-</sup> of EBV-, CMV-, and HIV-1-specific CD8<sup>+</sup> T cells. (B) Representative three dimensional topographic plots of EBV-, CMV-, and HIV-1-specific CD8<sup>+</sup> T cells. Color variation indicates a level of cell number from low (purple) to high (red). A white arrow indicates a CD27<sup>-</sup>CD57<sup>high</sup> cluster. Representative subjects 1 and 2 are HIV-1 uninfected, while subjects 3, 4, and 5 are HIV-1 infected subjects. Representative subject 4 is HIV infected, and has EBV (BMLF1)-, CMV-, and HIV-1-specific CD8<sup>+</sup> T cells.



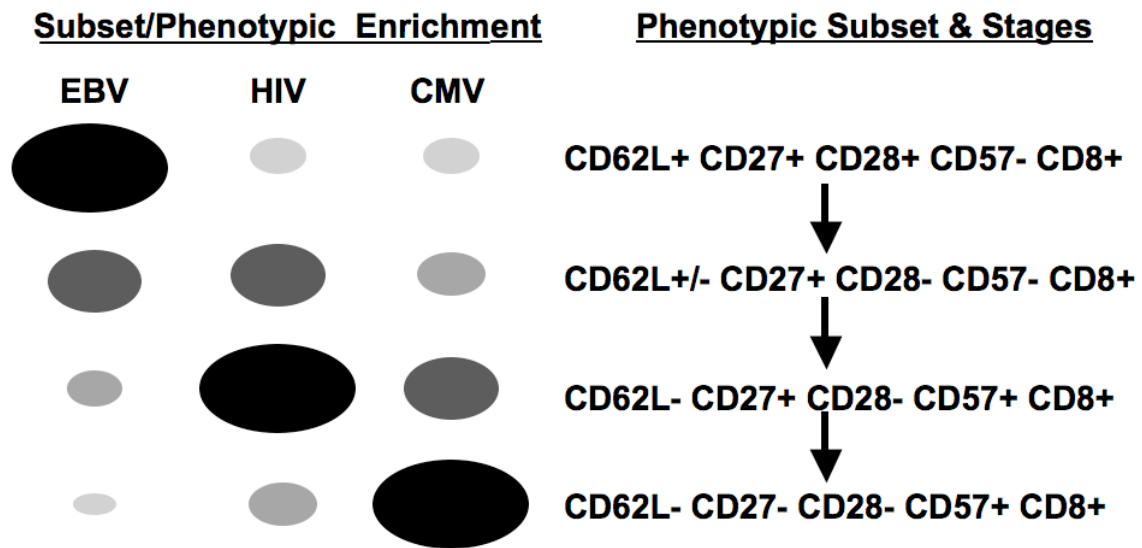


Figure 15. A schematic diagram showing proposed CD8<sup>+</sup> T cell functional subset enrichment of antigen-specific CD8<sup>+</sup> T cells based on our 6-color study.

## VI. DISCUSSION

Phenotypic characterization of memory CD8<sup>+</sup> T cells specific for FluA, EBV, CMV and HIV-1 in post-primary infection revealed phenotypic enrichment of virus-specific CD8<sup>+</sup> T cells according to each virus specificity. The collective phenotypic enrichment of FluA-, EBV-, CMV- and HIV-1-specific CD8<sup>+</sup> T cells were CD27<sup>+</sup>CD28<sup>+</sup>, CD27<sup>+</sup>CD28<sup>+</sup>, CD27<sup>-</sup>CD28<sup>-</sup>, CD27<sup>-</sup>CD28<sup>+</sup> respectively (Figure. 5 and 15, and Table 2 and 3). This is in agreement with the current model of virus-specific memory-effector CD8<sup>+</sup> T cell differentiation originally proposed by Appay et. al. (22). Thus, FluA- and EBV-specific CD8<sup>+</sup> T cells are in the early/immature memory stage (CD27<sup>+</sup>CD28<sup>+</sup>), HIV-1-specific CD8<sup>+</sup> T cells are in the intermediate memory stage (CD27<sup>+</sup>CD28<sup>-</sup>), and CMV-specific CD8<sup>+</sup> T cells are in the effector/mature stage (CD27<sup>-</sup>CD28<sup>-</sup>) of memory-effector differentiation.

In the first part of this dissertation, it was hypothesized that relatively unknown FluA-specific CD8<sup>+</sup> T cells considered to be the undifferentiated by the conventional definition (CD27<sup>+</sup>CD28<sup>+</sup>) (115) display the effector phenotype if characterized by a large number of phenotypic markers associated with the effector subset. Although, results from the present study (based on analysis of 14 distinct phenotypic markers) clearly showed that FluA-specific CD8<sup>+</sup> T cells were in a less differentiated memory stage than EBV-specific CD8<sup>+</sup> T cells (Figure. 5), FluA-specific CD8<sup>+</sup> T cells had similar degree of skewness toward effector cells as EBV-specific CD8<sup>+</sup> T cells by their expression of effector markers (e.g. CCR5, CXCR3, granzyme A, CD94)

(Figure. 5). Also, a similar proportion of FluA- and EBV-specific CD8<sup>+</sup> T cells had immediate cytoplasmic IFN- $\gamma$  production (Figure. 6B). These results suggest that FluA-specific CD8<sup>+</sup> T cells are as mature or differentiated memory cells as EBV-specific CD8<sup>+</sup> T cells. Thus, it appears that phenotypic assessment based on a lesser number of conventional maturation-associated phenotypic markers (e.g. CD27 and CD28) does not accurately ascribe the memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells.

Another important observation is that the proportion of CCR7-expressing FluA-specific CD8<sup>+</sup> T cells was consistently lower than expected (Figure. 4 and 5, Table 2). This observation was rather unusual, considering that the FluA-specific CD8<sup>+</sup> T cell represents the immature/undifferentiated memory cell (24). Accordingly, immature/undifferentiated memory cells should consist of a large number of T<sub>CM</sub> (CCR7<sup>+</sup>CD62L<sup>+</sup>). There are two possible explanations for this contradictory observation: T<sub>CM</sub> cells lack virus-specific memory CD8<sup>+</sup> T cells, or alternatively, in the absence of CCR7 expression, a CD62L<sup>+</sup>CCR7<sup>-</sup> subset becomes T<sub>CM</sub>. The latter appears to be the more likely, since the CD62L<sup>+</sup> subset retains a capacity for lymph node homing, which is the most critical characteristic of T<sub>CM</sub> cells (139) by CXCR4 surface up-regulation (induced by cross-linking of CD62L) (140).

Work done in the second part of this dissertation tested the hypothesis that CD62L<sup>+</sup> virus-specific central memory CD8<sup>+</sup> T cells had a capacity of the immediate effector function. Specifically, experiments in this section addressed whether a CD62L<sup>+</sup> subset of EBV-specific CD8<sup>+</sup> T cells had potential for immediate cytoplasmic IFN- $\gamma$  production, an one of critical immediate effector functions. Results showed that CD62L<sup>+</sup> CD8<sup>+</sup> T cells specific for EBV antigen can produce cytoplasmic IFN- $\gamma$  (Figure. 8A and B). This suggests that CD62L and IFN- $\gamma$  are not confined to either T<sub>CM</sub> or T<sub>EM</sub>' but broadly expressed among various memory CD8<sup>+</sup> T

cell subsets. These observations are in accord with recent data that, in contrast to their CD4<sup>+</sup> T cell counterparts, even naïve, cord blood-isolated CD8<sup>+</sup> T cells are efficient producers of IFN- $\gamma$  following short-term stimulation (132). Markedly, there is a fraction of IFN- $\gamma$  negative CD62L<sup>+</sup> EBV-specific CD8<sup>+</sup> T cells (Figure. 8A and B). These cells may represent the classical T<sub>CM</sub>, if antigen-specific stimulation induces IL-2 production in these cells (131). Further experiments are needed to confirm this.

A similar study showed that virus-specific CCR7<sup>+</sup>CD8<sup>+</sup> T cells possess granzyme B, and produce IFN- $\gamma$  upon peptide-specific stimulation (29). The same authors concluded that CCR7 expression on antigen-specific CD8<sup>+</sup> T cells may not agree with the functional definition of T<sub>CM</sub>. Thus, antigen-specific IFN- $\gamma$  response *ex vivo* does not appear to distinguish effector memory (CCR7<sup>-</sup> and/or CD62L<sup>-</sup>) from central memory cells (CCR7<sup>+/-</sup>CD62L<sup>+</sup>). It is increasingly clear that memory with respect to antigen-specificity is far more heterogeneous, and clear functional and phenotypic correlation will likely be difficult to establish. Nonetheless, these considerations provide the rationale for further refinement of the functional and phenotypic definitions of virus-specific CD8<sup>+</sup> T<sub>CM</sub> and T<sub>EM</sub>.

In the previous parts of this work, a high degree of heterogeneity was noted within virus-specific CD8<sup>+</sup> T cell memory population. This illustrates a markedly more complex stage of memory-effector differentiation of virus-specific memory CD8<sup>+</sup> T cells than those defined to date using primarily phenotypic markers CD27 and CD28. A larger combination of phenotypic markers is therefore necessary to ascertain more elaborate stages of memory-effector differentiation of virus CD8<sup>+</sup> T cells. Thus, experiments in specific aim 3 used 6-color flow cytometric analysis allowing the simultaneous detection of 4 distinct phenotypic markers (CD27,

CD28, CD57 and CD62L) on single virus-specific CD8<sup>+</sup> T cells (Figure. 9B). This permitted more accurate phenotypic assessment of virus-specific CD8<sup>+</sup> T cells.

Accurate phenotypic assessment of virus-specific CD8<sup>+</sup> T cells is critical for understanding significance of virus-specific CD8<sup>+</sup> T cell memory-effector differentiation in controlling viral pathogenesis. Of the several virus pathogenesis models in humans, HIV-1 infection provides the best model system to study an interaction between virus-specific CD8<sup>+</sup> T cells and virus pathogenesis. Unlike EBV and CMV infection in asymptomatic virus carriers, HIV-1 infection is progressive, and onset and duration of infection and longitudinal clinical data (e.g. viral load) are typically available. The caveat appears to be the significant clinical heterogeneity found among HIV-1 infected individuals. This could potentially hinder the collective analysis of memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells.

In the third part of this work, it was hypothesized that differentiation of HIV-1-specific CD8<sup>+</sup> T cells from the intermediate to late/effector stage was impaired even though there is a continuous presence of HIV-1 antigens, which in theory should drive HIV-1-specific CD8<sup>+</sup> T cells toward effector stage. Previous reports showed phenotypic enrichment of HIV-1-specific CD8<sup>+</sup> T cells in the intermediate stage (CD27<sup>+</sup>CD28<sup>-</sup>) of CD8<sup>+</sup> T cells differentiation regardless of clinical stages and duration of persistent HIV-1 infection (22, 84, 85, 141). In agreement with these reports, 6-color phenotypic assessment in specific aim 3 showed the same overall phenotypic enrichment of HIV-1-specific CD8<sup>+</sup> T cells based on the phenotypic markers used in this study (Figure. 15).

Notably, CD27 and CD57 expression of some EBV- and CMV-specific CD8<sup>+</sup> T cells showed the peculiar pattern of progression from the most undifferentiated (CD27<sup>high</sup>CD57<sup>-</sup>) to the most differentiated stage (CD27<sup>-</sup>CD57<sup>high</sup>) (Figure. 14B). This strongly suggested that

memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells might be impaired. Although neither the conventional flow cytometric analysis (Figure. 14A) nor the conventional model (Figure. 15) did not show the exact nature of the differentiation impairment, the three dimensional topographical model (Figure. 14B) revealed that HIV-1-specific CD8<sup>+</sup> T cells did not complete effector differentiation associated down-regulation of CD27 while these cells can nearly complete up-regulation of CD57. Moreover, Global immune dysfunction due to progressive HIV-1 infection did not appear to be a cause of this since phenotypic enrichment of CMV-specific CD8<sup>+</sup> T cells from HIV-1 infected subjects were not affected (Figure. 11B).

Alternatively, the differentiation impairment might be a result of deletion of the effector subset (CD27<sup>-</sup>CD57<sup>high</sup>) of the HIV-1-specific CD8<sup>+</sup> T cell over the course of progressive HIV infection. Deletion of the effector subset can be caused by apoptosis, since CD57<sup>+</sup>CD8<sup>+</sup> T cells are particularly prone to activation induced apoptosis (88), and Fas-mediated apoptosis(87). Interestingly, Fas-mediated apoptosis of the effector subset can be minimized by addition of IL-15 (142), indicating that cytokine environment clearly influences a fate of the effector subset. Also, it is not clear as to how deletion of the effector subset occurs specifically for HIV-1-specific CD8<sup>+</sup> T cells (and not for CMV-specific CD8<sup>+</sup> T cells). These issues must be addressed further to determine how the effector subset of HIV-1-specific CD8<sup>+</sup> T cells might be selectively deleted by activation-induced apoptosis.

In conclusion, the present study characterized phenotype and function of virus-specific CD8<sup>+</sup> T cells in humans with three specific aims that focused on memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells. The present study found that memory-effector differentiation of virus-specific CD8<sup>+</sup> T cell deviated from the currently accepted model when a larger number of phenotypic markers were examined. To refine the memory-effector CD8<sup>+</sup> T cell differentiation,

a 6-color flow cytometric analysis was employed, and revealed finer stages of memory-effector differentiation (Figure 17). Furthermore, the 6-color flow cytometric analysis was used for phenotypic assessment of HIV-1-specific CD8<sup>+</sup> T cells to ascertain viral modulation of memory-effector CD8<sup>+</sup> T cell differentiation. Refined analysis revealed an impaired memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cell based on CD27 and CD57 expression. Further study is needed to delineate the molecular defects causing the impairment in memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells.

## VII.

### FUTURE DIRECTIONS

Phenotypic assessment of virus-specific CD8<sup>+</sup> T cells will undoubtedly continue to progress with even more able technologies. The most advanced flow cytometer currently available allows simultaneous analysis of up to 15 distinct markers on single cells (14). When this type of flow cytometry becomes widely available, it will be possible to assess exceedingly detailed memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells, given that a larger number of MHC class I tetrameric reagents become available. A comprehensive list of virus-specific CD8<sup>+</sup> T cell phenotypes clearly helps refine the model of memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells.

Equally important is the functional assessment of virus-specific CD8<sup>+</sup> T cells. The current functional assessment is far more limited than phenotypic assessment in defining stages of memory-effector virus-specific CD8<sup>+</sup> T cells. This will soon change since modern functional assays combined with the MHC class I tetrameric reagents have been developed to characterize various functions of virus-specific CD8<sup>+</sup> T cells by flow cytometry (3).

Elucidation of memory-effector differentiation of virus-specific CD8<sup>+</sup> T cells not only has enormous impact on advancement of modern immunology but also has significant public health implications. Understanding the impairment of memory-effector differentiation of HIV-1-specific CD8<sup>+</sup> T cells, for instance, will greatly facilitate a design of effective vaccine against progressive HIV-1 infection. Beyond HIV-1 vaccine development, knowledge from this type of



research is applicable for designing effective vaccines against practically any infectious diseases and also for effective treatment of neoplastic diseases.

## **APPENDIX A.**

### **Modified MHC Class Monomer and Oligomer Synthesis Protocol**

## INTRODUCTION

A conventional method for producing MHC class I tetramers requires a large scale prokaryotic protein synthesis and complicated procedures that typically span over a week. Before MHC class tetramers became freely available to NIH grant recipients about 5 years ago, this laboratory had to develop a more simplified “in house” procedure for obtaining recombinant MHC-peptide monomers and oligomers for completion of this Ph.D. dissertation project. The modified method consisted of incorporation of an unpaired cysteine residue at the C-terminus of the HLA-A\*2011 heavy chain, allowing site-specific biotinylation by a SH-specific biotinylating reagent. This significantly shortens the production time of MHC class I monomers and oligomers (including tetramers) .

Another advantage is that the modified method generates highly flexible MHC class I oligomers for a variety of different applications. The recombinant MHC-peptide containing free sulfhydryls can be coupled to any solid surface (e.g. tissue culture plates, BIACORE chip™, glass/plastic array slides, microspheres, and Qdots™) that contains avidin or sulfhydryl reactive reagents. The sulfhydryl-specific coupling reagents allow conjugation of free sulfhydryl containing molecules to almost any solid surface. These oligomers can be used to generate a superior antigen-specific staining reagent, an artificial antigen presenting particle and a T cell microarray for a variety of CD8<sup>+</sup> T cells studies and therapy. Thus, the modified method provides a simple, efficient and inexpensive procedure for making recombinant MHC class I - peptide oligomers, a highly specific and very useful reagent with a number of important applications in basic and clinical T cell research.

## INCLUSION BODY PREPARATION AND PURIFICATION

### Materials

- 1M MES (0.195g in 1ml dH<sub>2</sub>O)
- 2M DTT (0.3085g in 1ml dH<sub>2</sub>O)
- Cell Lytic BII (Sigma) +10mM DTT
  - Prepare 100ml of 5ml CLBII + 95ml of dH<sub>2</sub>O+ 50ul of 2M DTT.
- Washing Buffer – 500ml
  - 50mM Tris (pH 8.0) – 25ml 1M Tris (pH 8.0)
  - 100mM NaCl — 50ml 1M NaCl
  - 1mM NaEDTA — 1ml 0.5M NaEDTA
  - 1mM DTT — 250ul 2M DTT
- Washing Buffer with 0.5% Trition
  - 350ml of Washing Buffer + 1.75ml of Trition
- Lysozyme (10mg/ml) 1ul of Ready-Lyse Lysozyme (Epicenter)
- SS-34 rotor
- Teflon coated Spatula (autoclaved)
- Sonicator
- Duncce Homegenizer (7ml or bigger) Autoclaved
- LB Medium (prepare and autoclaved a day before)
- IPTG (a stock is 0.5M)
- Tubes
  - 15 and 50 mL Falcon tubes
  - 50ml Oakridge tubes (autoclaved)

- Antibiotics
  - Kanamycin —30ug/ml (stock 10mg/ml)
  - Carbenicillin – 100ug/ml (50-100mg/ml)
  - Chroloamphinicol – 34ug/ml (34mg/ml)

## Method

- Prepare and autoclave 1L LB medium in a 4L flask and store in a cold room.
- Inoculate a colony in 3ml of LB (for A2CP Kanamycin (30ug/ml final) / for b2M Carbenicillin (100ug/ml final) + Chroloamphinicol (34ug/ml final).
- Incubate overnight.
- Warm 1L LB to room temp., and add 1-1.5ml of overnight culture.
- Incubate 1L culture with rigorous shaking.
- When O.D600 reaches 0.6-0.8 (it usually takes 2-2.5hrs), add 2ml of 0.5M IPTG (1mM final).
- Continue incubate for another 4hrs.
- Take a 1ml sample and run SDS-PAGE gen to see levels of induction (Figure 16).
- Transfer 500ml of the culture to 4x 750ml centrifuge tube, and spin down bacteria at 4Co 3000rpm (don't want a hard pellet !!) for 30min.
- Discard LB and drain a last bit of LB by placing the tube upside down a paper towel (do not disturb the pellet).
- Store the pellet in –80Co freezer for overnight or longer if you wish.
- **Prepare all the buffers, and place them on ice.**
- Brake the pellet by tapping bottom of the tube (careful not to smash the tube. The whole pellet should come off the bottom.), and catch pellet in a 50ml Falcon tube.

- Wash our remaining pellet with 5ml of Cell Lytic 10mM DTT.
- Start sonication, when pellet is somewhat dissolve or settle at bottom.
- Place the tube on an ice
- Place a tip of a probe near bottom.
- 20-pules at output 4, followed by 20-pulses at output 5 (take 10-30 sec break and mix in between each pulses). Repeat this 10 times.
- At the end of sonication, the solution should pore like water. If not, continue to sonicate more.
- Add 1ul of Ready-Lyse Lysozyme, and incubate for 10min at room temp.
- Add 1/20 diluted Cell Lytic with 10mM DTT to 50ml, and transfer to 50ml Oakridge tube.
- Spin the inclusion body down for 15min at 15000 rpm.
- **(From now on, everything must be done on ice !!)**
- Discard the liquid, and take pellet out of the tube with a spatula and into a homogenizer.
- Add about 7ml or less of the Washing Buffer with 0.5% triton to the homogenizer.
- Insert a piston and start homogenizing.
- Transfer the liquid to an new 50ml Oakridge tube, and fill the tube with the same buffer.
- Spin 15000rpm for 15min.
- Repeat this washing step 3 times, and a last wash is done in the buffer w/o Triton.

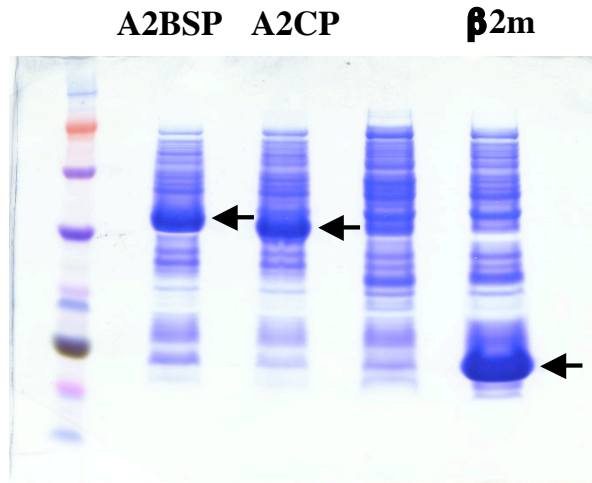


Figure 16. SDS-PAGE of three proteins after 4hr induction in 1L culture. Typically, BL21(DE3) bacteria produce up to 60% of total protein in the inclusion body when induced. Smaller protein such as b2m is produced more than bigger proteins.

### Inclusion Body Denaturation

#### Materials

- Denaturant Solution (make 20ml)
  - 8M Urea (9.608g)
  - 25mM MES (250ul of 1M MES)
  - 10mM NaEDTA (400ul of 0.5M NaEDTA (8.0))
  - 0.1mM (1ul of 2M DTT)

#### Method

- After the last wash w/o Triton, the inclusion body should look pale white pellet.
- Transfer the inclusion body pellet to 15ml Falcon tube.
- Add a denaturant solution (about 5ml for A2CP or 10ml for b2M) and incubate in the cold room for overnight on a rotator.
- Spin junks down at 45000rpm for 20min 20Co

- Carefully take the denaturant out and make 0.5 ml aliquots in 1.5ml Eppendorf tubes.
- Take small sample and measure protein concentration by BCA (use 1ul), and also run SDS-PAGE (need only 1-5ul). Expected protein concentration is around 15-20mg/ml for A2Cp and twice as much for b2M (Figure 17).
- Store them in -80 freezer.

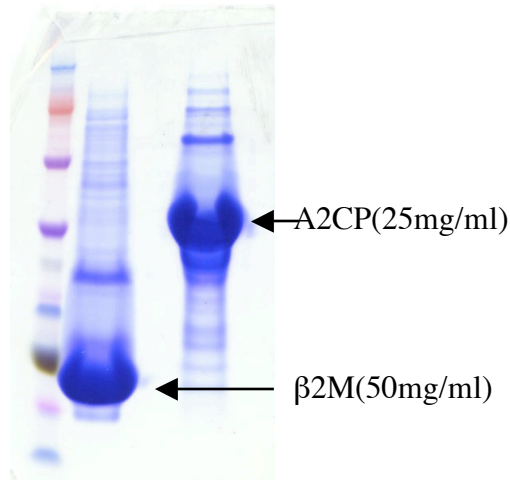


Figure 17. SDS-PAGE analysis of inclusion bodies dissolved in 8M Urea solution for overnight. 5ul of dissolved inclusion bodies are loaded. Typically, over 98% of inclusion bodies contain expressed A2CP or b2m protein.

## HLA REFOLDING

### Materials

- Refolding Buffer (250ml) (pH 8.0-8.3) : RB
- **w/o Glutathione** can be stored at 4C<sup>0</sup>
  - 100mM Tris-HCl (25ml of 1M Tris-HCl (pH8.0))
  - 400mM L-Arginine HCl (21g)
  - 2mM NaEDTA (1ml of 0.5M stock)
  - 0.5mM Oxid. Glutathione (76.5mg)
  - 5mM Redu. Glutathione (3.7g)



- 3M Guanidine Solution pH 4.2 (100ml) Store at 4C<sup>0</sup>
  - 3M Guanidine-HCl (28.7g)
  - 10mM NaOAC (82mg)
  - 10mM NaEDTA (2ml of 0.5M stock)
- Protease Inhibitor Cocktail (Calbiochem PI set III)
  - 1000 fold dilution (200uL)
- Heavy Chain – 18.6mg total (3X6.2mg) 1uM final conc.
- $\beta$ 2m – 13.2mg total (3x4.4mg) 2uM final conc.
- Peptide – 12mg dissolve in 100 uL of DMSO 30uM final conc. (upto 100uM best for some peptides)
- Two 27 gauge needles and 1ml syringes

## Methods

(Preparation of Refolding Buffer in the morning or a day before to accommodate the first 8hr incubation.)

- To 100ml of dH<sub>2</sub>O in a 250ml bottle, add Tris-HCl, L-arginine, and EDTA, bring a volume to 240ml.
- Add oxidated and reduced glutathione (do not add glutathione if leaving RB overnight in the cold room).
- Add a protease inhibitor cocktail
- Check pH, and if necessary, adjust pH to 8.0.
- Bring the volume to 250ml.
- Cool RB to 10C<sup>0</sup> in ice.
- Dissolve the peptide in 100ul DMSO

- Inject peptide with vigorous stirring.

#### Preparation of Guanidine Solution.

- In 250ml beaker, add 28.7 g of Guanidine and add dH<sub>2</sub>O to 50ml.
- After dissolving Guanidine, add NaOAC and NaEDTA.
- Bring the volume to 95ml, and adjust pH to 4.2, and then adjust the volume to 100ml
- Store GS at 4C<sup>0</sup> but keep it at RT before the injection.

#### Heavy chain and $\beta$ 2m injection.

- Aliquot appropriate amount of HC and b2m in three 1.5ml eppendorf tubes. Store them at -20C<sup>0</sup> till use.
- Add 500ul of GS into thawed HC and b2m in the eppendorf tubes, vortex and quick spin.
- Stir vigorously.
- Using 27G 1ml syringe, forcefully inject b2m-GS right into the vortex. Slow injection will result in the precipitation. One should be able to see a huge plume coming out a tip of the needle.
- Wait 1-5 min, and proceed with the second injection with HC-GS
- Reduce stirring velocity, and incubate for 8hrs till the second injection (Do the first injection around noon, and second injection at 8pm).
- Third injection will be 6-14hrs after the second injection (around 10am next morning).
- Incubate additional 24-48 hrs. (constantly monitoring the temp. )

### **CONCENTRATION OF THE REFOLDED HLA COMPLEX**

#### **Materials**

- Amicon Stirred Concentrator (a model 8200, max capacity 200ml)
- YM10 membrane

- 15ml Ultrafree Concentrator (Biomax 10)
- 500ml beaker
- 10ml pipet and removable pipeter

### **Methods (in the cold room)**

- Cool Sorval RC-5C to 4C°.
- Spin down RB in 4x 50ml polypropene tubes (for a SS-34 rotor) at 15000rpm for 10min.
- Carefully collect RB into a 250ml glass flask.
- Assemble the stirred cell , and make sure everything is tight and secure.
- Before applying the pressure, maker sure a regulator is set to less than 55psi.
- Add 20ml of dH<sub>2</sub>O, assemble the rest of the stirred cell, and set the stirred cell on the top of the stirrers. Start stirring.
- Open a main valve of compressed N<sub>2</sub> tank, and increase pressure to 55psi with a regulator.
- Wash the membrane for 5min (for the brand new membrane) MilliQ water, or longer for the stored membrane (do not go down 5ml).
- Close the valve, and discard the remaining water with a 10ml pipet.
- Spin RB 15000rpm for 15min if there is a precipitate, otherwise it will clog membrane.
- Place RB in the stirred cell. Put a cap on (make sure tight and secure), and start stirring.
- Open the valve, and make sure pressure stays 55psi during incubation.
- Concentrate 200ml RB to around 10ml. Normally, it would take 2hrs with 55psi.
- Option: Exchange buffer with PBS by adding 10ml of PBS at the time. Don't forget to add protease inhibitor cocktail !!
- Cool Centrifuge to 4C°.
- Load 4ml of RB into 4ml Ultrafree in 15 ml polypropene Faclon tubes.

- Spin at 6000g (roughly 6000rpm on Sorval RT) at 4C°. (can go up to 7500g).
- Further concentrate RB to 2ml. It would roughly take 1-2hrs.

## **PURIFICATION OF HLA MONOMERS BY FPLC**

### **Materials**

- FPLC Buffer (for BSP) pH 8.0
  - 20mM Tris-HCl
  - 50mM NaCl
- FPLC Buffer (for A2CP) PBS pH 7.0

### **Methods**

- Filter RB with 0.2um filter before loading.
- A sample loop is 1ml so you need to run twice.
- Collect fractions that correspond to A2 monomer. Usually 3-5ml (Figure 18)
- Concentrate A2 monomers to around 0.5-1ml
- Measure concentration by BCA, and adjust it to 1-2mg/ml
- Add protease inhibitor cocktail (1000x).

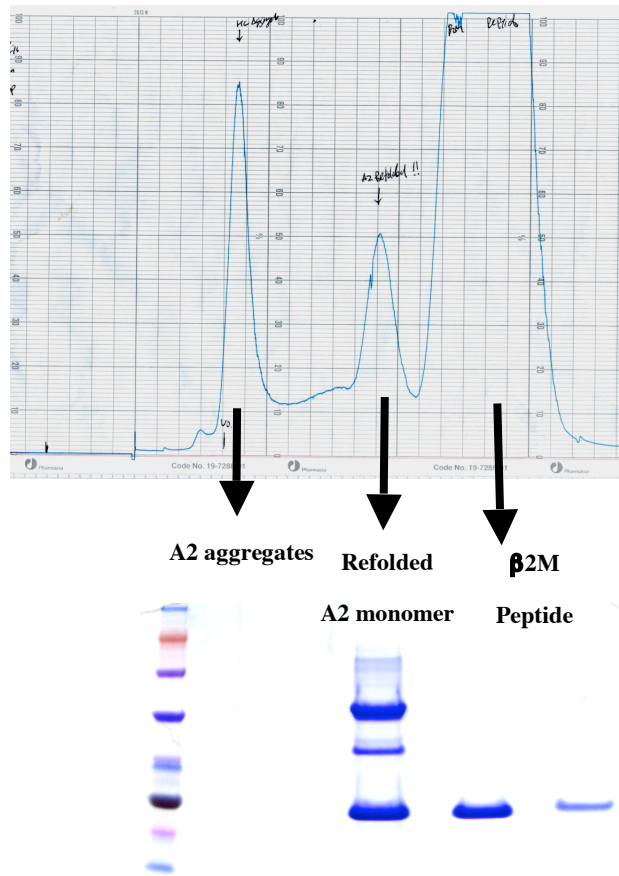


Figure 18. FPLC gel filtration profile shows multiple peaks of the concentrated refolding mixture. A middle peak corresponds to the refolded recombinant HLA-A2 molecule with C-terminus sulfhydryl group (SH). Coomassie stain of a SDS-PAGE gel from the fraction taken from each peaks shows that only the middle peak contains both recombinant HLA-A2 heavy chain and b2m.

## BIOTINYLATION OF HLA MONOMERS

### Materials

- DTT – 1M Stock (154mg in 1ml MQ) – final conc. is 5mM
- Class I monomer
- PEO-Maleimide activated Biotin
- PBST—PBS (7.0) + 5mM EDTA
- 0.5M NaEDTA

- Protease Inhibitor Cocktail

## Methods

- Add 5ul of 0.5M NaEDTA to 500ul RB (roughly contains 0.5-1mg of the monomer.)
- Add 2.5ul of 1MDTT (final is 5mM) to 500ul of the monomer solution
- Incubate 90min room temp (use a heat block).
- Bring a volume to 2.5ml with PBST.
- Get rid of DTT by running PD-10 desalting column.
- Concentrate the monomer by 4ml Ultrafree down to 500ul (takes 5min).
- Add PEO-maleimide-biotin (5 fold molar excess, and make fresh)
- Incubate overnight.
- Get rid of free biotin by FPLC.
- Perform biotinylation test.
  - Add an 10fold excess FITC-SA, and incubate about an hour.
  - Add an 10fold excess biotinylated A2 monomer to FITC-SA and incubate an hour.
  - Run SDS-PAGE. (Figure 19)
- Concentrate the monomer to 2mg/ml, and add the protease inhibitor cocktail.
- Aliquot the monomer into 200ug/ml , snap freeze, and store them at -80.

## General Comment

Unlike the BSP construct, A2CP can be chemically conjugated with variety of sulfhydryl (SH) reactive molecules (e.g. maleimide) that are currently available. At certain pH, these thiol reactive molecules are very specific to thiol group, and reaction is completed in a matter of

hours. Cochran et.al. took this advantage to produce a class II multimers. This is a cheaper and easier alternative to biotinylation to BSP.

Cochran et.al. reported that a SH group at a carboxyl terminus is capped with glutathione during refolding. This is why 5mM DTT is used to liberate -SH group. Apparently, Class II proteins can tolerate 5mM DTT though Class I may not. Perhaps, smaller amount may be sufficient since 1mM DTT (60min 37°C) can break SS bond between two heavy chains of IgG.

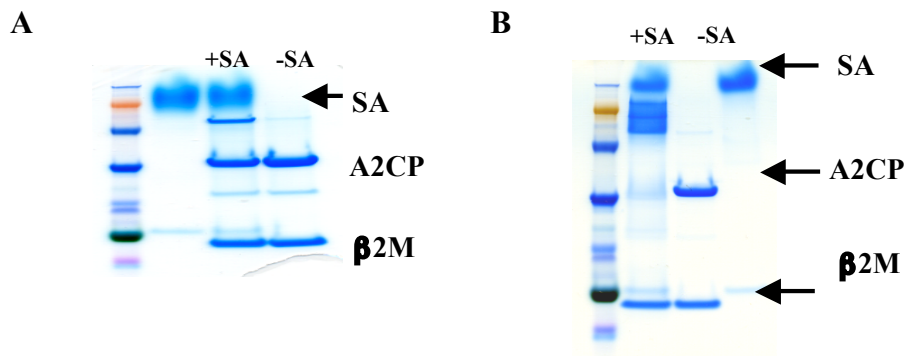


Figure 19. (A) The recombinant A2CP without a biotin-tag does not bind streptavidin. If binding occurs, A2CP band in the presence of SA (+SA) will shift to the arrow indicated by SA. (B) The recombinant A2CP (but not b2m) with a biotin-tag bind streptavidin. A2CP band only in the presence of SA (+SA) lane shifted to the arrow indicated by SA, whereas B2m band remained at the same size. Notice that A2CP band in SA+ lane is almost completely gone, suggesting, biotinylation proceeded nearly 100% efficiency and specificity.

## OLIGOMERIZATION OF HLA MONOMERS

### Materials

- Streptavidin (non-Carbohydrate versions can also be used but staining may be less intense)
- Conjugated with any fluorochromes (**unlike an NIAID's BSP based monomer**), and also HRP or AP for a tissue staining and signal amplification (i.e. by TSA).

## Methods

- Calculate molar concentration of SA conjugated with a particular flouoroforme (see NIAID's protocol)
- Add 1/10 of SA-flourchrome at time to the 200ug/ml monomer aliquot, and incubate 30min.
- Do this until all the SA is added.

## General Comment

This procedure produces mixture of monomer-, dimmer-, trimer-, and tetramer-SA. SA-PE is known to form oligomers which give stronger signal than other SA-florescent dye conjugates. If the tetrameric form is needed, it is necessary to purify it only from a tetrameric peak by FPLC. Technically, this procedure requires a bigger column (i.e. 2x HR10/30 with superdex 200, or HR16/30 HighLoad™ column), and a downside of using the bigger columns is that it takes a long time, and may lose substantial amount of tetramers. One could also run a small sample on SDS-PAGE or FPLC (use a smaller sample loop (e.g. 50-100 ul)) to determine a degree of tetramerization. For flow cytometry purpose, these steps are not necessary, and in fact, NIAID Tetramer Synthesis Facility does not further purify the tetrameric form, however other groups do purify the tetrameric form for flow cytometry.

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