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# THE ROLE OF MAP KINASES ON THE FUNCTIONAL HETEROGENEITY OF HUMAN CD8+ T CELL MATURATION SUBSETS

Tae Kon Kim

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**THE ROLE OF MAP KINASES ON THE FUNCTIONAL  
HETEROGENEITY OF HUMAN CD8+ T CELL MATURATION  
SUBSETS**

By

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Graduate School of Biomedical Sciences

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SUBSETS**

A

DISSERTATION

Presented to the Faculty of

The University of Texas  
Health Science Center at Houston

And

The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences

in Partial Fulfillment  
of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Tae Kon Kim, M.D.  
Houston, Texas

May 2010

## **DEDICATION**

To my parents (Kil Hyung Kim, Soon Ock Kim), my parents-in-law (Dong Suk Park, Eun Ok Lee), especially, my wife (Hye Jin Park) and daughter (Heesuh Kim), and other all my family members for their continual support and devotion.

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# THE ROLE OF MAP KINASES ON FUNCTIONAL HETEROGENEITY OF HUMAN CD8+ T CELL MATURATION SUBSETS

Publication No. \_\_\_\_\_

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While prior studies have focused on naïve (CD45RA+CD27+) and early stage memory (CD45RA-CD27+) CD8+ T cells, late memory CD8+ T cells (CD45RA+CD27) have received less interest because this subset of T cells is generally recognized as effectors, which produce IFN $\gamma$  (but no IL-2) and perforin. However, multiple studies suggest that late memory CD8+ T cells may provide inadequate protection in infectious diseases and cancer models.

To better understand the unique function of late memory CD8+ T cells, I optimized multi-color flow cytometry techniques to assess the cytokine production of each human CD8+ T cell maturation subset. I demonstrated that late memory CD8+ T cells are the predominant producer of CC chemokines (e.g. MIP-1 $\beta$ ), but rarely produce IL-2; therefore they do not co-produce IL-2/IFN $\gamma$  (polyfunctionality), which has been shown to be critical for protective immunity against chronic viral infection. These data suggest that late memory CD8+ T cells are not just cytotoxic effectors, but may have unique functional properties.

Determining the molecular signature of each CD8+ T cell maturation subset will help characterize the role of late memory CD8+ T cells. Prior studies suggest that ERK1 and ERK2 play a role in cytokine production including IL-2 in T cells. Therefore, I tested

whether differential expression of ERK1 and ERK2 in CD8<sup>+</sup> T cell maturation subsets contributes to their functional signature by a novel flow cytometry technique. I found that the expression of total ERK1, but not ERK2, is significantly diminished in late memory CD8<sup>+</sup> T cells and that ERK1 expression is strongly associated with IL-2 production and CD28 expression. I also found that IL-2 production is increased in late memory CD8<sup>+</sup> T cells by over-expressing ERK1. Collectively, these data suggest that ERK1 is required for IL-2 production in human CD8<sup>+</sup> T cells.

In summary, this dissertation demonstrated that ERK1 is down-regulated in human late memory CD8<sup>+</sup> T cells, leading to decreased production of IL-2. The data in this dissertation also suggested that the functional heterogeneity in human CD8<sup>+</sup> T cell maturation subsets results from their differential ERK1 expression.

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## **CHAPTER 1**

### **INTRODUCTION**



In murine models, many investigators have studied memory T cell generation, genetic and epigenetic regulation of T cell maturation, the relationship between memory T cell generation and protective immunity, and the differential function of T cell memory subsets. However, much less is known about how those findings apply to human T cells. Despite inherent limitations of human immunology studies, recent technical advances have better enabled us to investigate the function of memory T cells and the relationship between functional heterogeneity of T cell maturation subsets and control of pathogens (e.g. viruses) and cancers. To better understand human T cell memory, we and others are examining the intrinsic functions of T cell maturation subsets defined by surrogate surface markers, and the underlying molecular mechanisms generating this functional heterogeneity. A better understanding of how T cell maturation subsets confer protective immunity will allow us to improve immune modulations, including immunization and adoptive cellular therapies. I have focused on the functional and molecular signatures of CD8<sup>+</sup> T cell maturation subsets and their relationships in this dissertation, because (a) CD8<sup>+</sup> T cells are critical effectors in the setting of infections and cancer; (b) there are still controversial studies about function, terminologies and markers of CD4<sup>+</sup> T cell lineage (e.g. central memory vs. effector memory, Th1 vs Th2 etc.); (c) CD4<sup>+</sup> T cells are less optimal for studies of late memory T cells due to lower cell numbers in this stage relative to the CD8<sup>+</sup> compartment. To formulate the hypothesis, I described the current opinions about memory T cell generation in mouse models, cross-sectional assessment of human memory T cells, functional signature of human memory T cells and differential T cell receptor (TCR) signal transduction in memory T cells in Chapter 1. More specifically, the relationship between polyfunctionality of memory T cells and protective immunity, and the role of MAP kinase pathways in T cells were described in Chapter 1.

## **IMMUNOLOGICAL MEMORY**

Immunological memory is one essential characteristic of adaptive immunity. Once the initial response to a pathogen is imprinted in a subset of immune cells, subsequent exposure to the pathogen previously encountered causes faster and more robust immune responses to clear infection. With two key properties (longevity and proliferation potential), both humoral and cellular immunity are important players in immunological memory (1).

A key player in humoral immunity, a B cell recognizes an virus and phagocytoses the viral particle (2). The internalized virus particle is degraded into peptides and the peptides are presented on the cell surface by MHC class II molecules. Helper T cells (mostly, Th2 subset) activate B cells through co-stimulation (e.g. via CD40-CD40L interaction) and cytokines (e.g. IL-4) to make antibodies that recognize viral proteins. Humoral immunity to infections lasts for decades in humans and in mice. Humoral immunity via prolonged antibody generation provides the first line of defense against pathogens through neutralization, opsonization and complement activation. The maintenance of high titers of antibody in serum is critical to humoral immunity (3). For the maintenance of high serum antibody levels, long-lived plasma cells constitutively produce antibody, and memory B cells remains differentiated into plasma cells. Otherwise, naïve B cells must become activated and differentiated to replace dying plasma cells.

## **T CELL MEMORY**

In contrast to humoral immunity, naïve T cells are activated in secondary lymphoid organs by antigens presented on MHC class I/II molecules on antigen-presenting cells (APC) along with co-stimulation, and then proliferate into numerous daughter cells (more than 10,000 progenies) over the period of 5~8 days and differentiate into effector cells.

Effector cells produce cytotoxic molecules (e.g. perforin and granzymes) and effector cytokines (e.g. IL-2, IFN $\gamma$  and TNF $\alpha$ ) for effective pathogen clearance. The clearance of infection follows a contraction phase, leading to apoptosis of ~90% of primed T cells by activation-induced cell death, to allow for response to new pathogens and to minimize autoimmune diseases, which could occur in the host with persistence of activated T cells. Despite this extensive apoptosis during the contraction phase, ~10% of activated cells become memory T cells (4).

Since Ahmed and Gray proposed that multiple models may explain how memory and effector T cells are related and how naïve cells differentiate to memory or effector cells (1), memory T cell differentiation and maintenance have been extensively studied (2, 3, 5-8). One of the most conventional models, supporting linear differentiation, proposes that antigens drive activation and expansion of naïve cells, called the effector phase (9, 10). Effector T cells either become terminally differentiated T cells or mature into effector memory T cells that are characterized by low levels of CD62L, and CC-chemokine receptor 7 (CCR7) and are replication incompetent. Effector memory T cells can differentiate into central memory T cells that express CCR7 and CD62L, and they reside in lymphoid tissues (4, 11-15). More recently, Sarkar and Ahmed demonstrated that naïve CD8<sup>+</sup> T cells differentiate into effector cells and it is from these effector cells that memory cells are eventually generated after antigen clearance (4).

Although several lines of evidence described above support that memory T cells emerge from activated effector T cells, recent studies in both murine and human models suggest that memory cells may not pass through an effector phase (16-21). A bifurcation model proposed by Chang *et al.*, suggests that one T cell primed by an antigen divides into two daughter cells with distinct phenotypes (22). For instance, by differential polarization

of cellular components (e.g. co-receptors and signaling molecules) between the two daughter cells of primed naïve T cells, the distal daughter cell differentiates into central memory T cells, while the proximal daughter cell matures into effector memory/effector T phenotype (15, 22-24). Stemberger *et al.* reinforced the bifurcation model with their studies, suggesting from a single adoptively transferred naïve CD8<sup>+</sup> T cell, diverse maturation phenotypes of T cells can be generated (25).

As described above, acute antigen exposure induces the evolvement of phenotypes and function of memory T cell populations, and it has also been suggested that cytokine exposure contributes to it (5, 26-28). In addition, several studies suggest that cytokine-driven proliferation is critical to maintain the numbers of memory T cells after acute infection (29-32), while persistent antigen exposure is not necessarily required for the maintenance(33). Under circumstances where acute infection is cleared, memory T cells are maintained by the common  $\gamma$ -chain receptor cytokines (e.g. IL-7 and IL-15). IL-7 and IL-15 seem to provide survival and proliferation signals to memory CD8<sup>+</sup> T cells, respectively (29-32). It has been suggested that this basal level of proliferation driven by cytokine must be balanced by a similar rate of apoptosis (memory turnover), leading to maintaining the size of the memory T cell pool (34). Moreover, through cytokine-driven memory turnover, the phenotypes of CD8<sup>+</sup> memory T cell subsets are directed to central memory and effector memory, and the cells continue to maintain polyfunctionality (34).

In contrast to acute antigen exposure models (linear, bifurcation) and the memory maintenance model (cytokine-driven), chronic infections induce substantial alterations in the phenotype and function of memory CD8<sup>+</sup> T cells (35, 36). This could result in the exhaustion or impairment of their effector functions (37). Recently, several studies examined the underlying mechanisms of immune exhaustion during chronic infections.

Barber *et al.* and Freeman *et al.*, demonstrated that the expression of Programmed cell death 1 (PD-1) of memory CD8<sup>+</sup> T cells decreased their functionality in persistently infected mice (38, 39). Velu *et al.* also demonstrated that immune responses during a chronic simian immunodeficiency virus (SIV) infection, are restored by blocking the engagement between PD-1 and PD-1 ligand (40). In addition, Brooks *et al.* and Ejrnaes *et al.* respectively showed that IL-10 impairs T cell responses during chronic viral infection, while IL-10 blockades restore anti-viral immunity (41, 42). More importantly, maintenance of memory CD8<sup>+</sup> T cells during chronic infection in contrast to acute infection requires the interaction of T cells with viral peptides, not with inflammatory cytokines such as IL-7 and IL-15 (43-46). Thus, continuous exposure to antigen forces CD8<sup>+</sup> T cells to extensively proliferate, leading to a signature of exhaustion, such as decreasing cell numbers and gradual loss of their ability to produce IL-2.

Taken together, there are many unresolved questions related to T cell memory. Outstanding issues include: (a) which murine models best approximate human memory T cell generation, (b) whether cytokine-driven T cell maintenance in murine studies are similar in humans, (c) if the exhausted T cell population following persistent infection in mice is identical to one certain T cell subsets in humans (e.g. effector, CD45RA revertant, late memory, or terminally differentiated T cells).

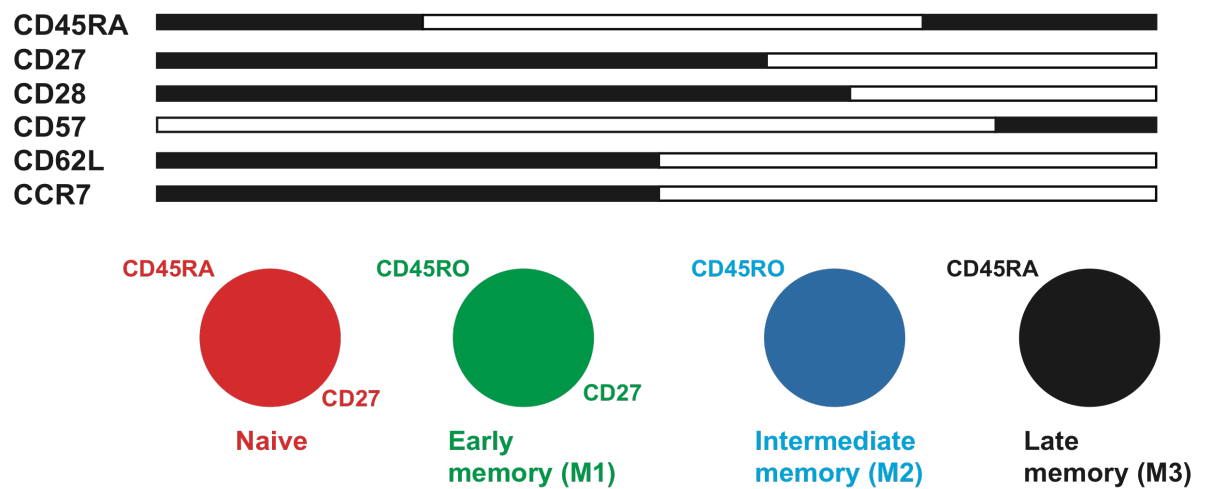
## **CROSS-SECTIONAL ASSESSMENT OF HUMAN MEMORY T CELLS**

We have been studying CD8<sup>+</sup> T cell responses to virus and T cell differentiation via a longitudinal analysis in murine models. To extend our understanding in murine models, Picher *et al.* demonstrated in nonhuman primates that Bromodeoxyuridine (BrdU)-labeled antigen-specific T cells in SIV-infected monkeys progressively differentiate from

CD28+CCR7+CCR5-, to CD28+CCR7-CCR5- or CD28+CCR7+CCR5+, and then to CD28+CCR7-CCR5+ (effector memory); CD28- T cells in this model seem to be terminally-differentiated or end-staged cells (47). A longitudinal analysis recently done by Miller *et al.*, suggests that human memory CD8+ T cell responses to smallpox and yellow fever vaccines are similar to a linear differentiation model in mice (48). However, in humans, we still have had to rely primarily on cross-sectional assessments of immune responses against chronic infections, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis C virus (HCV) and Epstein-Barr virus (EBV) to characterize T cell differentiation (49-57). One inherent problem is the need to use surrogate markers of naïve and memory T cell differentiation; variations in subsets defined by such surface markers have led to the lack of a consistent language used to define maturation stages. This has led to the use of semantic terms (e.g., central, effector and late memory cells) that are often inconsistent between studies, in part due to varying marker combinations or functional measures. Even basic terminologies have different definitions in the literature. In general, memory T cells are referred to as cells that have been primed with an antigen. Activated T cells are sometimes called memory T cells because they are no longer naïve. Effector T cells, more traditionally, are defined as cells which exist during the primary or secondary phases of an infection. But many researchers regard effectors as cells which contain effector functions (e.g. perforin production) *ex vivo* (58). Despite these limitations and the inconsistencies between studies due to inconsistent terminologies, we are beginning to better understand the consequences of T cell differentiation, especially as more advanced technological approaches to assess phenotypic and functional characteristics of fine T cell subsets have been developed.

The rapid advancement of flow cytometry has facilitated a better understanding of cell surface markers associated with human T cell maturation (**Figure 1**). Initially, CD45R isoforms were found to demarcate naïve (RA+/RO-) and memory (RA-/RO+) cells (59, 60). However, with the demonstration by Sprent and others that antigen-primed CD45RO+ cells could revert to CD45RA+ T cells (61-63), additional markers including CCR7 were suggested as a way to divide RA-/RO+ memory cells into earlier (“central” cells, capable of lymph node homing) and later “effector” memory cells (64). Additional studies demonstrated that down-regulation of CD27 and CD28 could also be used to define CD4+ and CD8+ T cell maturation stages (51, 65-68). In addition, it has been shown that CD57 expression defines a subset of terminal memory CD8+ T cells characterized by replicative senescence (69). Telomere length has also been shown to be different in T cell maturation subsets, suggesting that each maturation subsets have different cell division histories (70-72). Interestingly, the expression of CD57 revealed an inverse correlation with telomere length, suggesting that CD57+ T cells have divided more than other subsets (69, 71). Other markers, including CD62L, CD11a, HLA-DR and CD95 have been used to better distinguish naïve and memory subsets (73-77).

In contrast to CD8+ T cells, the phenotype and functionality of CD4+ T cell maturation subsets, remain relatively ambiguous (58). Earlier studies suggest that the expression of chemokine receptors in CD4+ T cells can delineate Th1 and Th2 responses (e.g. CCR5 and CXCR3: Th1 cytokine, CCR3, CCR4, and chemoattractant receptor-homologous molecule expressed on Th2 (CRTh2): Th2 cytokine) (78). Moreover, the markers used to define the CD8+ T cell populations (e.g. CD45RA, CCR7, CD27, CD28) have also been adapted to discriminate several CD4+ T cell subsets. Surprisingly, using



**Figure 1. More than two surface markers delineate human naïve and memory CD8+ T cell subsets.** CD45RA and CD27 can define naïve CD8+ T cells (CD45RA+CD27+) up to 90% and three other memory CD8+ T cell subsets (early memory (M1): CD45RA-CD27+, intermediate memory (M2): CD45RA-CD27-, late memory (M3): CD45RA+CD27-).

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these markers, functional and phenotypic similarities between T cell maturation subsets of CD4 and CD8 lineages have been found (68, 79, 80).

These studies support the view that the use of at least two surface phenotypic markers (e.g., CD45RA and CD27) is required to divide cells into earlier and later stages of the T cell maturation spectrum (**Figure 1**). However, results using all the surface markers together, suggest that while all the surface markers can precisely identify naïve T cells (CD45RA+CCR7+CD27+CD28+CD62L+), the combination of these markers gives rise to numerous subpopulations of T cell maturation subsets. This could be a reflection of the heterogeneity of CD4+ and CD8+ T cell maturation subsets. However, it is important to realize that the expression of numerous markers considerably overlap with each other (69, 81-86). Consistent with overlapping markers, general patterns of phenotype and functional attributes clearly exist within the heterogeneous T cell populations.

## **FUNCTIONAL SIGNATURE IN HUMAN MEMORY T CELL SUBSETS**

Far fewer studies have focused on the relationship of maturation stage with functional signature, as defined by the combination of cytokines produced by T cells. Hamann *et al.* demonstrated that CD8+CD45RA+CD27- cells do not secrete IL-2, but produce IFN $\gamma$  and TNF $\alpha$ , while CD45RA-CD45RO+ cells express higher levels of CD95 (Fas) and the integrin family members CD11a, CD18, CD29, CD49d, and CD49e, compared with naïve CD8+ T cells, and are able to produce multiple cytokines including IL-2 and IFN $\gamma$  (51). Sallusto and Lanzavecchia showed that CD4+ central memory T cells secrete more IL-2, but less IFN $\gamma$  than CD4+ effector memory T cells (64). Sandberg and colleagues found that IL-2 was not co-produced in perforin-positive T cells in response to staphylococcal enterotoxin B (SEB) and perforin-positive T cells were almost exclusively

CD57+ and CD45RA high; differential cytokine production occurred within surface marker-defined CD8+ T cell maturation stages (87). Tomiyama *et al.* also demonstrated that perforin expression, cytotoxic activity and CCR5 expression are different in the subpopulations (CD27+CD28+, CD27+CD28-, CD27-CD28-) of CD45RA-CD8+ T cells (85). In addition, Bratke *et al.* and Takata *et al.*, respectively showed the differential production of granzymes in CD8+ T cell maturation subsets. These data suggest that granzyme K production occurs in CCR7-CD27+CD28+ CD8+ T cells, whereas granzyme B is produced predominantly in CCR7-CD27-CD28- CD8+ T cells (88, 89). Furthermore, CD56, CD11a, CD11b, CCR5, KLGR1 and IL-7 receptor alpha have been used to define CD8+ T cell maturation subsets and demonstrate the relationship between their expression and function (90).

While many similarities have been observed between CD8+ and CD4+ T cell subpopulations defined by CD45RA, CCR7, CD28 and CD27, in terms of functional attributes and phenotypes, the expression of chemokine receptor has been used to define novel CD4+ T cell subsets. Recently, the expression of CCR6 and CCR4 has been demonstrated to identify a Th17 subset in humans (91). In contrast, the expression of CCR6, CXCR3, IL-23R and the transcription factor ROR gamma t, delineate populations consisting of Th1 cells and Th17/Th1 cells producing both IFN $\gamma$  and IL-17 (91, 92).

Despite extensive efforts to define the association between the phenotypes and the functional attributes of T cell subsets, the association is less obvious in virus-specific T cell responses because virus-specific T cells tend to be concentrated within certain T cell subsets. For instance, two independent studies in HIV-infected adults suggest that more than 50% of IL-2-producing CD4+ T cells specific for viruses associated with persistent infection (e.g. CMV, influenza virus, EBV, HSV) are in the effector memory subset (93,

94). Similarly, in mice infected with *Leishmania major* or in humans infected with vaccinia virus, IL-2 was produced predominantly in effector memory CD4<sup>+</sup> T cells (CCR7<sup>-</sup>) (95, 96). Duvall *et al.* demonstrated that in HIV-2 specific CD8<sup>+</sup> T cells, there was no significant difference in terms of functional profiles in T cell maturation subsets (97). These findings suggest that the type of infection or the duration of antigen exposure may also determine which T cell maturation subsets predominantly produce IL-2.

Despite all of the attempts to determine the relationship between T cell maturation subsets and function, few studies have analyzed T cells following activation with multiple stimuli and/or examined the production of multiple cytokines in a systematic fashion to determine the intrinsic capacity of each T cell maturation subset, as is now possible using modern cytometric approaches.

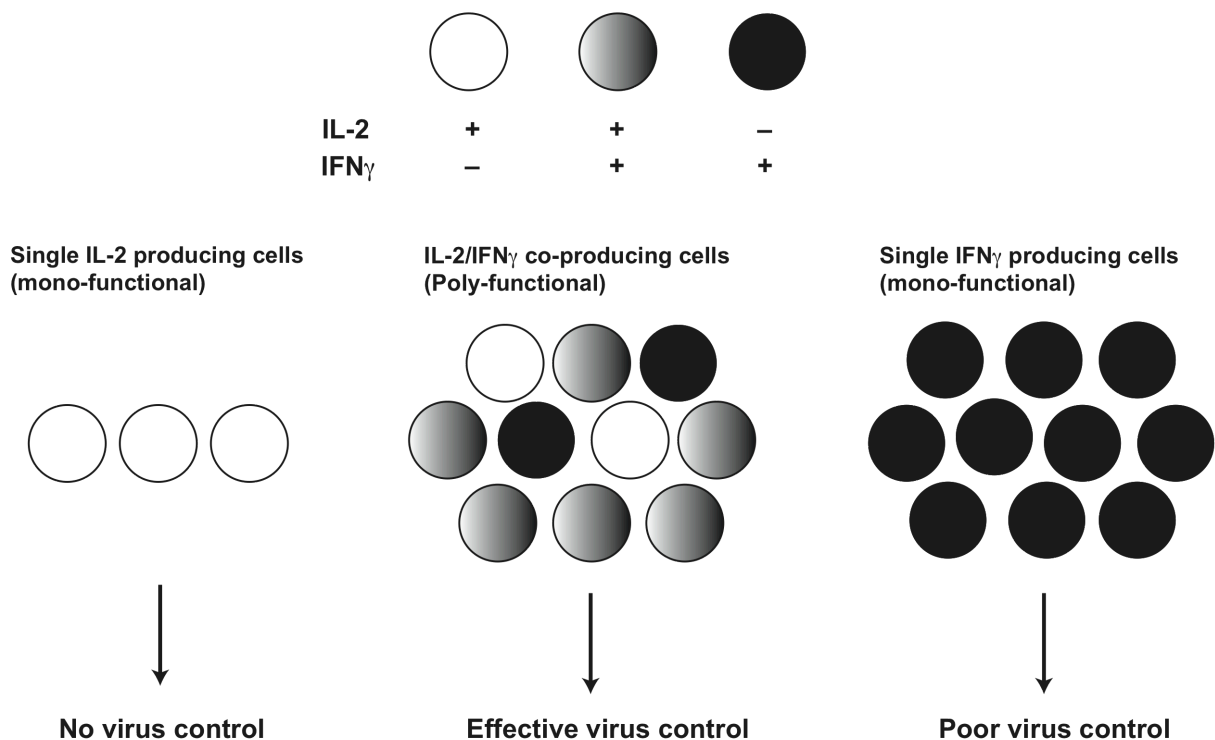
## **POLYFUNCTIONAL T CELLS AND PROTECTIVE IMMUNITY**

Although traditional studies focused on the magnitude of T cell responses, it is evident that T cell quality is also important in chronic viral infection and vaccination. The correlation between T cell quality (e.g. polyfunctionality) and clinical outcome has been studied in HIV models. Earlier studies analyzing dual cytokine production (IL-2 and IFN $\gamma$ ) by CD4<sup>+</sup> T cells from patients infected with HIV, showed that long-term non-progressors (LTNPs) or patients treated with anti-viral therapy, had increased frequencies of CD4<sup>+</sup> T cells producing IL-2 only or co-producing both IL-2 and IFN $\gamma$ , while CD4<sup>+</sup> T cells in progressors produced IFN $\gamma$  alone (98-101). Additional studies suggest that ~50% of the total HIV-specific T cell responses from LTNPs or patients on anti-retroviral treatment are polyfunctional (e.g. IL-2+IFN $\gamma$ +TNF $\alpha$ + or IL-2+IFN $\gamma$ +), while cytokine production pattern in patients with high viral loads was skewed to IFN $\gamma$  only (93, 102). Consistent results were

reported in the setting of infections with HCV (103), *Mycobacterium tuberculosis* (104) and CMV (105). These studies suggest that polyfunctional CD4<sup>+</sup> T cells that produce two or more cytokines are associated with viral clearance or control of viral reactivation (**Figure 2**).

The quality of CD8<sup>+</sup> T cell responses has been investigated in the context of protective immunity against viral infections and cancers. Migueles *et al.* demonstrated that HIV-specific CD8<sup>+</sup> T cells in HIV-1 infected LTNPs showed an early memory phenotype (CD45RO<sup>+</sup>CD28<sup>+</sup>CD27<sup>+</sup>), which maintained their proliferative potential following *in vitro* stimulation (106). Duvall, Koup and colleagues showed that cytokine signature of HIV-2 specific T cells is more polyfunctional than that of HIV-1 specific T cells, which can explain delayed disease progression in patients infected with HIV-2 (97). In a murine melanoma immunotherapy model, Gattinoni *et al.* demonstrated that fully differentiated melanoma-specific effector cells had impaired antitumor efficacy despite their ability to secrete abundant amounts of IFN $\gamma$  and perforin, while early memory cells with identical specificity effectively controlled tumor growth (107). Recently, Betts *et al.* found there was a significantly higher frequency of CD8<sup>+</sup> T cells that produce four or five effector molecules simultaneously (IFN $\gamma$ +TNF $\alpha$ +MIP1 $\beta$ +CD107+IL-2<sup>-</sup>, IFN $\gamma$ +TNF $\alpha$ +MIP1 $\beta$ +CD107+IL-2<sup>+</sup>) in LTNPs, compared with CD8<sup>+</sup> T cells from progressors (108). All these studies suggest that polyfunctional CD8<sup>+</sup> T cells or early memory CD8<sup>+</sup> T cells are strongly associated with protective immunity against viruses and cancer cells.

Several speculative explanations exist on why polyfunctional T cells are optimal for protective immunity (109). In mice vaccinated with *Leishmania major*, there was a progressive increase in the mean fluorescence intensity (MFI) of IL-2, IFN $\gamma$ , and TNF $\alpha$  in CD4<sup>+</sup> T cells that produce all three cytokines together, compared with dual- or single-cytokine-producing cells (95). For example, polyfunctional CD4<sup>+</sup> T cells made up to ten



**Figure 2. Polyfunctional T cells are associated with effective protective immunity.**

Polyfunctional T cells exhibit better T cell quality because polyfunctional CD4<sup>+</sup> T cells making more cytokine on a per-cell basis than monofunctional CD4<sup>+</sup> T cells.

Simultaneous secretion of both IFN $\gamma$  and TNF $\alpha$  from one polyfunctional T cell may mediate more efficient killing compared with either cytokine alone. Secretion of IL-2 from polyfunctional T cells promotes the expansion of T cells in an autocrine or paracrine manner, which could enhance CD8<sup>+</sup> T cells memory maintenance.

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times more IFN $\gamma$  on a per-cell basis than single-cytokine producing CD4 $^{+}$  T cells. These findings were also confirmed by ELISPOT (95). Similar results were reported in a study demonstrating the relationship between polyfunctionality of T cells and the effect of vaccination in humans immunized with the smallpox vaccine virus (102) or modified vaccinia virus Ankara (96). Secondly, the co-production of both IFN $\gamma$  and TNF $\alpha$  from one cell may mediate better cytotoxicity compared with either cytokine alone (110, 111). Finally, IL-2 from polyfunctional T cells induces the proliferation of T cells in an autocrine or paracrine fashion, which could help T cell memory maintenance (112).

Taken together, polyfunctional CD4 $^{+}$  and CD8 $^{+}$  T cells are associated with better responses to vaccination and protective immunity against chronic viral infections and cancers. Therefore, it is important to know which maturation stages of T cells are more polyfunctional, what mechanisms (e.g. signal transduction) lead to polyfunctionality in memory T cells and how to boost the polyfunctionality of memory T cells to better induce and maintain protective immunity.

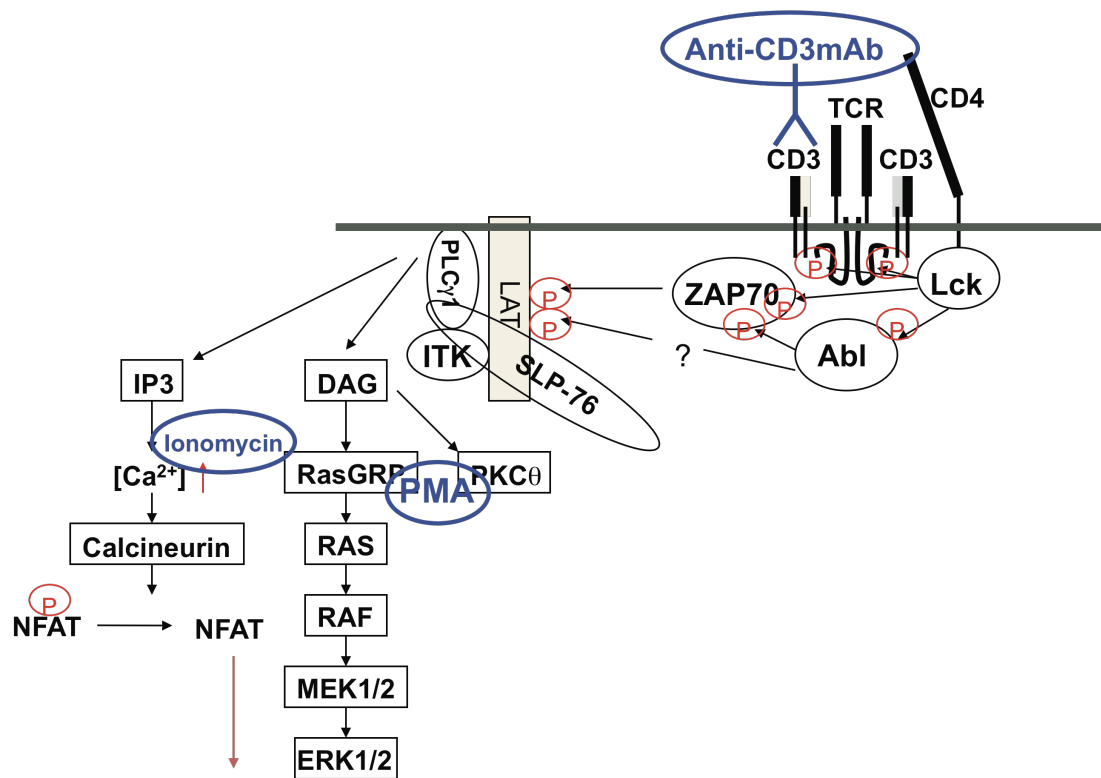
## **DIFFERENTIAL SIGNAL TRANSDUCTION IN T CELL MATURATION SUBSETS**

For T cells to be activated, the T cell receptor (TCR) binds with a ligand on MHC class molecule of an antigen presenting cell (APC). It leads to activation of Lck and Fyn, which in turn phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) within the cytoplasmic domain of CD3 $\zeta$  chain. The phosphorylated ITAMs provide docking sites for ZAP-70. ZAP-70 activates adaptor molecules such as LAT and SLP-76, which subsequently activate phospholipase C $\gamma$ 1 (PLC $\gamma$ 1). PLC $\gamma$ 1 generates inositol 1, 4, 5-triphosphate (IP $_3$ ) and diacylglycerol (DAG), second messengers involved in intracellular

Ca<sup>2+</sup> release and protein kinase C (PKC) activation, respectively (113) (**Figure 3**). These pathways are crucial for cytokine production, proliferation and differentiation in T cells through transcription factors (NFAT and AP-1).

Although recent studies have better characterized human T cell maturation, less is known about how signal transduction is associated with functional heterogeneity of T cell maturation subsets. Farber *et al.* demonstrated murine CD4<sup>+</sup> memory T cells produce fewer species of tyrosine-phosphorylated substrates than naïve T cells and fail to phosphorylate ZAP-70 (114). Hall and coworkers showed that human CD45RA<sup>+</sup> CD4<sup>+</sup> T cells were more active in TCR-dependent tyrosine phosphorylation, especially CD3 $\zeta$  and ZAP-70 than human CD45RO<sup>+</sup> CD4<sup>+</sup> T cells though the total expression levels were equivalent between the two subsets (115). Krishnan *et al.* demonstrated that human effector CD4<sup>+</sup> T cells generated *in vitro* by polyclonal activation exhibit elevated levels of intracellular tyrosine phosphorylation concomitant with reduced expression of CD3 $\zeta$  and CD3 $\epsilon$  (116). Hussain *et al.* explained the more rapid responses of CD4<sup>+</sup> effector T cells with higher SLP-76 expression, compared with CD4<sup>+</sup> memory T cells in a murine model (117). More recently, Kersh *et al.* showed that murine memory CD8<sup>+</sup> T cells do not increase TCR sensitivity nor initial CD3 signaling events, yet have more extensive lipid rafts to more efficiently induce phosphorylation of LAT and its downstream molecules than naïve and effector CD8<sup>+</sup> T cells (118).

Additionally, to define the distinct molecular signature of each T cell maturation subset, several investigators performed high-throughput genomic studies in mouse and human T cell maturation subsets. Holmes *et al.* showed that human naïve CD8<sup>+</sup> T cells (CD45RA<sup>+</sup>CD27<sup>+</sup>) have distinct gene expression patterns from the effector subset (CD45RA<sup>+</sup>CD27<sup>-</sup>) (119). Willinger and colleagues also demonstrated that human effector





memory CD8<sup>+</sup> T cells (CD45RA-CCR7<sup>-</sup>) express genes encoding lytic granule proteins (e.g. granzyme A, B, perforin etc.), genes involved in protein sorting to granules and granule transport/exocytosis and genes encoding IFN $\gamma$  and chemokines (e.g. CCL5 (RANTES)), whereas central memory CD8<sup>+</sup> T cells (CD45RA-CCR7<sup>+</sup>) are characterized by high basal and cytokine-induced STAT5 phosphorylation (120). Recently, Appay *et al.* revealed that CD4<sup>+</sup> and CD8<sup>+</sup> T cell gene expression is very similar depending on maturation stages (80). On the other hand, Kaech and Ahmed demonstrated in a murine model, that the mRNA level of genes associated with TCR signaling, intracellular Ca<sup>2+</sup> signaling, and cytokine signaling, are increased in effector CD8<sup>+</sup> T cells, leading to enhancement of signaling pathways and faster responses to antigens (121). They also found that other genes involved in cytokine production (p38, JNK etc.) and cell cycles (cyclin E1, E2 and B1) are up-regulated in memory CD8<sup>+</sup> T cells. Their data also demonstrated that mRNAs of effector molecules (e.g. granzyme B and IFN $\gamma$ ) are constitutively expressed, which facilitates rapid recall responses in memory CD8<sup>+</sup> T cells.

Despite all these studies, it remains to be elucidated (a) which genes or pathways dictate the production of effector molecules such as cytokines and chemokines, and cellular proliferation and migration in T cell maturation subsets; (b) which genes regulate T cell maturation.

## **THE ROLE OF MAPK PATHWAYS IN POST-THYMIC T CELLS**

Of the TCR signaling molecules, mitogen-activated protein (MAP) kinases have been extensively studied in T cells. Three key MAP kinase pathways have been investigated in mammalian cells. These are the extracellular signal-regulated kinase (ERK) (122, 123), the Jun NH2-terminal kinase (JNK) (124-126), and the p38 kinase (127-129). In addition to

the critical roles of these three MAP kinase pathways in early thymocyte development (130-136), positive/negative selection and lineage commitment of thymocytes (137-143), these pathways have been reported to play an important role in IL-2 production in peripheral T cells (144-147). ERK has been shown to phosphorylate and increase the activity of the protein Elk-1, which induces c-fos expression (148, 149). JNK has been reported to phosphorylate c-Jun for transcriptional activity (150). p38 also increases c-fos expression mediated by Elk-1 (151). C-fos and Jun dimerize into AP-1, which binds to the IL-2 promoter to induce IL-2 production along with other transcription factors (e.g. NFAT, NFκB etc.) (152-157).

It has been shown that the MAP kinase pathways regulate cellular proliferation. Several studies demonstrated that ERK1 and ERK2 are necessary for G1- to S- phase transition, induce positive regulators of the cell cycle (e.g. D-type cyclins, c-Myc) (158-161) and down-regulate genes inhibiting cell growth (Tob1, Ddit3, and JunD) (162). However, it is still under debate whether proliferation and cell cycle progression in T cells rely on TCR signaling induced ERK1/2 activation or results from IL-2 production, leading to cell cycle entry or both (157, 163, 164). P38 also has been shown to play a role in T cell proliferation, while JNK is not involved in T cell proliferation, but differentiation into Th1 (JNK2) or Th2 (JNK1) subsets (165-168).

MAP kinases are also involved in T cell survival. Several studies revealed that ERK1/2 activation promoted cell survival by suppressing the pro-apoptotic machinery in the Jurkat T cell line (169-171). It was also shown that ERK1/2 activation protects cells from glucocorticoid-induced apoptosis in T cells (172). In contrast, it has been demonstrated that ERK pathway is involved in activation-induced cell death (AICD) of T cells (173, 174). JNK and p38 have also been shown to play a role in AICD in T cells (175, 176).

## THE DIFFERENTIAL ROLE OF ERK1 AND ERK2

Of the key three MAP kinase pathways, ERK1 (MAPK3) and ERK2 (MAPK1) were the first mammalian MAPKs to be cloned, which are activated by mitogenic factors, differentiation stimuli and cytokines (122, 123, 177). This pathway consists of Ras-Raf-MEK-ERK. GTP-bound form of Ras activates Raf-1 (MAPKKK). Activated Raf-1, subsequently, phosphorylates MAP kinase kinase (MAPKK), MEK1 and MEK2 (178). MEK phosphorylates ERK1 and ERK2 on two critical residues, Tyr<sup>183</sup> and Thr<sup>185</sup>. Activated ERK1 and ERK2 then translocate into the nucleus and activate several transcription factors. The functional differences of ERK1 and ERK2, however, are not clearly demonstrated.

ERK1 and ERK2, which are 44kDa and 42 kDa in size, respectively, show 83% amino acid sequence homology and have been thought functionally redundant (140, 179, 180). Several lines of evidence in various species suggest that ERK1 and ERK2 have differential roles in development and differentiation (140, 181-187). Knockout mice have been used to study the distinct function of ERK1 and ERK2. ERK1<sup>-/-</sup> mice are viable, fertile, and of normal size. Additionally, Lefloch *et al.* demonstrated that there is no significant difference in cell proliferation, requirement of growth factor or cell cycle entry between ERK1<sup>-/-</sup> fibroblasts and wild-type cells (180). On the other hand, disruption of the ERK2 gene leads to early embryonic lethality (184, 185). ERK2<sup>-/-</sup> mice die *in utero* at day E7.5 due to impaired development of the trophoblast (185).

In T cells, however, there has been very limited evidence for differential ERK1 and ERK2 expression leading to divergent signaling and function in T cells (138, 140, 188). Initially, Pages *et al.* demonstrated that in ERK1 knockout mice, thymocyte maturation was impaired and thymocyte proliferation following polyclonal stimulation was severely reduced

(140). But subsequent studies in ERK1 knockout mice, demonstrated that ERK1<sup>-/-</sup> mice exhibit normal T cell effector function (e.g. cytokine production), but ERK1<sup>-/-</sup> T cells show a slightly reduced proliferation (189). On the other hand, an earlier study using an ERK2 conditional knockout approach to overcome embryonic lethality in ERK2 knockout mice, demonstrated that ERK2 is critical for checkpoints of thymocytes development especially in the DN3 and DN4 stages (188). Recent studies also suggest that ERK2 is required for murine CD8<sup>+</sup> T cell proliferation and survival, while ERK1 is dispensable for all aspects of CD8<sup>+</sup> T cell activation (179). More recently, Wille, Kemp and other colleagues demonstrated with short hairpin RNA, that both ERK1 and ERK2 equivalently contribute to IL-2 production in human and mouse T cell lines (190, 191). However, it has yet to be determined whether ERK1 and ERK2 play differential roles in primary human CD8<sup>+</sup> T cell maturation subsets.

## **SUMMARY OF PROBLEM AND HYPOTHESIS**

While many studies have examined CD8<sup>+</sup> memory T cell generation after either acute infection or persistent exposure to pathogens in mouse models, the precise mechanisms of CD8<sup>+</sup> memory T cell generation and maintenance have not been completely understood in humans. Since we still have to rely on cross-sectional assessment of memory T cell subsets by a multi-color flow cytometric approach, ***it is of significant importance to define the relationship between phenotype and function and the underlying molecular mechanisms that dictate the functional heterogeneity of maturation subsets.***

Moreover, few studies have focused on the role of late memory T cells, despite their known association with immunodeficiency states in humans. The frequency of late stage memory T cells has been reported to increase with aging, possibly leading to increased risk

for infection in the elderly (192-194). Our prior clinical studies have found that delayed immune reconstitution after cord blood transplantation is associated with skewing of the circulating T cell pool toward late memory cells, a likely result of a failure of thymopoiesis to renew the naïve and early memory T cell pool in this setting (195). Hamann *et al.* and Champagne *et al.* have suggested that late memory T cells show impaired proliferative capacity. In a murine model, Gattinoni *et al.* demonstrated that fully differentiated melanoma-specific effector cells had impaired antitumor efficacy, despite their ability to secrete abundant amounts of IFN $\gamma$  and perforin and induce cytotoxicity, while earlier memory cells with identical specificity effectively controlled tumor growth (107). These studies support the rationale that *late memory CD8<sup>+</sup> T cells may have a unique role, based on studies described above, suggesting that CD8<sup>+</sup> late memory T cells are poorly associated with protective immunity.* We and others have demonstrated that late memory CD8<sup>+</sup> T cells rarely produce IL-2. Therefore, *it is important to understand the molecular mechanism that determines the functional signatures of late memory CD8<sup>+</sup> T cells.*

Initially, I demonstrated that human late memory CD8<sup>+</sup> T cells have less ERK1/2 phosphorylation following polyclonal stimulation with PMA: Ionomycin, leading to my hypothesis that down-regulation of the MAP kinase pathway including ERK1/2, contributes to lack of IL-2 production in human late memory CD8<sup>+</sup> T cells. Further studies also demonstrated that total ERK1, but not total ERK2, is down-regulated in human CD8<sup>+</sup> late memory T cells. Given these preliminary data and prior studies suggesting that the MAPK pathway is critical for IL-2 production in T cells, the hypothesis tested in this dissertation is that *down-regulation of ERK1 leads to lack of IL-2 production in human late memory CD8<sup>+</sup> T cells.* To test this hypothesis, I first investigated the functional signature of human naïve and memory CD8<sup>+</sup> T cell subsets using multi-color cytokine flow cytometry.

Secondly, I determined the level of phospho-ERK1/2, and total ERK1 and ERK2 in activated and resting human naïve and memory CD8<sup>+</sup> T cell subsets by multi-color molecular flow cytometry, western blotting and real-time RT PCR. Finally, I determined whether ERK1 is sufficient or necessary for IL-2 production in human naïve and memory CD8<sup>+</sup> T cell subsets by (a) a novel flow cytometry technique to measure cytokine production simultaneously with ERK1, and (b) manipulating ERK1 expression using over-expression in late memory CD8<sup>+</sup> T cells.

Work in this dissertation has led to a understanding of the intrinsic functional signature of human CD8<sup>+</sup> T cell maturation subsets. This work enabled us to understand the molecular signature of human CD8<sup>+</sup> T cell maturation subsets, dictating the functional signature. This work also demonstrated the differential role of ERK1 and ERK2 isoforms in human CD8<sup>+</sup> T cell maturation subsets, and confirmed that late memory CD8<sup>+</sup> T cells have a unique functional and molecular signature.

**CHAPTER 2**  
**MATERIALS AND METHODS**

## **CELLS**

Peripheral blood mononuclear cells (PBMCs) were obtained by sedimentation using Histopaque 1077 (Sigma, St. Louis, MO) or Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) from CPD (Citrate-Phosphate-Dextrose) treated whole blood from healthy volunteers (obtained from Gulf Coast Regional Blood Bank, TX and from Continental Services Group, FL) (ranging in age from 32-60) and cryopreserved for further analysis. All cell preparations were more than 95% viable by trypan blue exclusion. For sorting CD8<sup>+</sup> T cell maturation subsets, three methods for enriching CD8<sup>+</sup> T cells (RosetteSep, EasySep (Stem Cell Technologies, Vancouver, BC, Canada), MACS CD8<sup>+</sup> T cell isolation kit (Miltenyi Biotec, Auburn, CA)), were used during or after the sedimentation process.

## **PREPARATION OF DENDRITIC CELLS**

Monocyte-derived dendritic cells (DCs) were generated *in vitro* from PBMCs by a previously described method (196). Briefly, PBMCs were plated in a tissue culture flask in AIM V media supplemented with 10% human AB serum, penicillin, and streptomycin (Sigma, St Louis, MO) for 3 hours at which point non-adherent cells were discarded. Adherent cells were cultured with 500 IU/ml of recombinant interleukin 4 (rIL-4, R&D Systems, Minneapolis, MN) and 1000 IU/ml of granulocyte-macrophage colony stimulating factor (GM-CSF, Berlex Pharmaceuticals) and incubated at 37° C with 5% CO<sub>2</sub>. After 5 days, media was supplemented with 10 ng/ml of tumor necrosis factor alpha (TNF- $\alpha$ ), 15 ng/ml of IL-6, 10 ng/ml IL-1 $\beta$ , and 1 ug/ml of PGE2 (R&D Systems, Minneapolis, MN). After an additional 48 hours, mature dendritic cells were harvested and subsequently irradiated (25 Gy) before co-culture with responder PBMCs.



## **CYTOKINE FLOW CYTOMETRY (CFC)**

$10^6$  thawed or fresh PBMCs were incubated with 10  $\mu$ g/ml Staphylococcal enterotoxin B (SEB) (Sigma, St. Louis, MO), or 1 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1  $\mu$ M ionomycin together (Sigma, St. Louis, MO) in 96-well V-bottom plates in 200  $\mu$ l of media (RPMI1640; GIBCO Life Technologies, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). For CMV-specific T cell stimulation, pools of pentadecapeptides spanning the CMV pp65 capsid antigen (Jerini Peptide Technologies, Berlin, Germany) were used to stimulate T cells in the presence of antibodies to CD28 and CD49d, as previously described (197, 198). For inhibition, 1~10  $\mu$ M U0126 or 1~10 nM tacrolimus were added. After 1 hr, brefeldin A (Sigma, St. Louis, MO) was added to enable accumulation of intracellular cytokines. Following an additional 5 hr incubation, cells were fixed and permeabilized with Fix & Perm A/B (Caltag, Burlingame, CA) and assessed the simultaneous expression of surface markers and intracellular effector proteins.

## **ASSESSMENT OF NAÏVE AND MEMORY T CELLS SEGREGATION BY FLOW CYTOMETRY**

FACS analyses were performed using monoclonal antibodies (mAbs) conjugated with the fluorochromes FITC, PerCP-Cy5.5, APC, PE-Cy7, PE, PE-Texas Red, APC-Alexa750 or pacific blue and specific for human CD4, CD8, IL-2, TNF $\alpha$ , and MIP-1 $\beta$  (BD Pharmingen, San Jose, CA), CD45RA (Beckman Coulter, Fullerton, CA), CD27, IFN $\gamma$  (eBiosciences, San Diego, CA) and CD57 mAb conjugated with biotin (BD Pharmingen, San Jose, CA) and furthermore Qdot705-streptavidin (Invitrogen, Camarillo, CA). After staining, cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by 8-color 10-parameter (or 9-color, for analyses including CD57) flow cytometry in a

Cyan cytometer (Dako, Carpinteria, CA) or an LSR-II cytometer (BD, San Jose, CA) using FlowJo software (Treestar, San Carlos, CA). For most analyses, at least  $3 \times 10^5$  total events ( $10^6$  for CMV studies) were analyzed, with sequential gating of PBMC in a lymphocyte region (by scatter), on T cells (by assessing CD4+ or CD8+ and CD45RA+/CD27+ to demarcate naïve and memory cells staining for intracellular IL-2, IFN $\gamma$ , TNF $\alpha$ , and MIP-1 $\beta$ ). Gates defining cytokine-positive populations were defined based on the upper limits of fluorescence of unstimulated cells stained with the same antibodies. The data were also confirmed by using dump channels (using monoclonal antibodies conjugated with FITC specific for CD14, CD19, CD16 and CD56) to exclude monocytes, B cells and NK cells, with equivalent results.

## **MOLECULAR FLOW CYTOMETRY**

$10^6$  thawed or fresh PBMCs were incubated with 1 ng/ml PMA and 1  $\mu$ M ionomycin together (Sigma, St. Louis, MO) in 96-well V-bottom plates in 200  $\mu$ l of media (RPMI1640; GIBCO Life Technologies, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) for 5 min. Following stimulation, cells were immediately fixed and permeabilized cells with Fix & Perm A/B (Caltag, Burlingame, CA) and assessed the simultaneous expression of surface markers and intracellular phospho-ERK1/2. For total ERK1 and total ERK2 measurement, resting cells were fixed and permeabilized cells with Fix & Perm A/B.

FACS analyses were performed using monoclonal antibodies (mAbs) conjugated with the fluorochromes FITC and specific for human CD4, CD14, CD19, CD56 (BD Pharmingen, San Jose, CA) (for the dump channel), and mAbs conjugated with the fluorochromes V450, PE, ECD and specific for human CD8, CD27 (BD Pharmingen, San

Jose, CA), and CD45RA (Beckman Coulter, Fullerton, CA). Alternatively, analyses were performed using mAbs conjugated with the fluorochromes PE-Cy7, V450, APC H-7, FITC and specific for human CD4, CD8, CD27, CD57 (BD Pharmingen, San Jose, CA), and mAbs conjugated with the fluorochromes PE, ECD and specific for human CD28 or CMV pp65 A2 Tetramer (HLA-A2-restricted (NLVPMVATV, amino acids 495-503; hereafter referred to as A2-pp65) and CD45RA (Beckman Coulter, Fullerton, CA). The expression of phospho-ERK1/2, total ERK1 and total ERK2 were assessed using rabbit anti-phospho-ERK1/2 (1:50 in Caltag Fix/Perm buffer B) (Cell Signaling Technology), mouse anti-ERK1 IgG2a (1:25 in Caltag Fix/Perm buffer B) (R&D Systems, Minneapolis, MN), and rabbit anti-ERK2 (1:25 in Caltag Fix/Perm buffer B) (Cell Signaling Technology, Danvers, MA), followed by secondary staining with donkey anti-rabbit IgG conjugated with Alexa 647 (1:1,000 in Caltag Fix/Perm buffer B) (Molecular Probes, Eugene, OR) and donkey anti-mouse IgG2a (1:500 in Caltag Fix/Perm buffer B) conjugated with APC (Jackson ImmunoResearch, West Grove, PA). The same staining protocol was applied for PKC $\theta$ , Ras, C-Raf, MEK1, MEK2, Elk-1, JNK, p38, pan-calcineurin (rabbit IgG) (Cell Signaling Technology, Danvers, MA). After staining, cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by 5-color 7-parameter flow cytometry (or 7-color, for analyses including CD28, CD57) in a Cyan cytometer (Dako, Carpinteria, CA) or an LSR-II cytometer (BD, San Jose, CA) using FlowJo software (Treestar, San Carlos, CA).

For the most analyses, at least  $3 \times 10^5$  total events were analyzed, with sequential gating of PBMC in a lymphocyte region (by scatter), on T cells (by assessing CD8<sup>+</sup> and CD45RA<sup>+</sup>/CD27<sup>+</sup> to demarcate naïve and memory cells staining for intracellular phospho-ERK1/2 and total ERK1 and ERK2). Gates defining phospho-ERK1/2 positive populations and total ERK1 and ERK2 were defined based on the upper limits of fluorescence of

unstimulated cells stained with the same antibodies (phospho-ERK1/2), and of isotype controls (total ERK1 and ERK2).

## **COMBINED FLOW CYTOMETRY**

Following the same stimulation protocol as described in CFC (6 hours with PMA: Ionomycin or with 10 µg/mL CMV pp65 A2 restricted peptide (NLVPMVATV, amino acids 495-503), FACS analyses were performed using mAbs conjugated with the fluorochromes Alexa 700, FITC, PE-Cy7, V450, PE, APC-H7, PE-Texas Red, and specific for human CD4, CD8, IL-2, IFN $\gamma$ , MIP-1 $\beta$ , CD27 (BD Pharmingen, San Jose, CA), CD45RA (Beckman Coulter, Fullerton, CA). The expression of total ERK1 was assessed using mouse anti-ERK1 IgG2a (1:25 in Caltag Fix/Perm buffer B) (R&D Systems, Minneapolis, MN), followed by secondary staining with donkey anti-mouse IgG2a (1:500 in Caltag Fix/Perm buffer B) conjugated with APC (Jackson ImmunoResearch, West Grove, PA). After staining, cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by 8-color 10-parameter flow cytometry in an LSR-II cytometer (BD, San Jose, CA) using FlowJo software (Treestar, San Carlos, CA). For most analyses, at least  $3 \times 10^5$  total events were analyzed, with sequential gating of PBMC in a lymphocyte region (by scatter), on T cells (by assessing CD4 $^+$  and CD8 $^+$  and CD45RA $^+$ /CD27 $^+$  to demarcate naïve and memory cells staining for intracellular IL-2, IFN $\gamma$  and MIP-1 $\beta$  and total ERK1).

## **MULTIPLEX BEAD IMMUNOASSAY**

CD8 $^+$  T cell subsets sorted by FACS (over 99% purity), following staining using mAbs specific for CD45RA, CD27, and CD8 (excluding cells staining in a “dump” channel positive for CD4, CD14, CD16, CD19, and/or CD56) were stimulated with PMA and

Ionomycin for 6 hr. Supernatants were stained using human chemokine 5-Plex reagents (Invitrogen, Camarillo, CA) following the manufacturer's instructions and analyzed with a Luminex 100<sup>TM</sup> instrument (Luminex, Austin, TX).

### ***IN VITRO* DIFFERENTIATION**

Naïve cells were sorted on the basis of CD45RO negativity and expression of CD27 by MACS microbeads (Miltenyi Biotec, Germany). To induce T cell differentiation, 5µg Phytohemagglutinin (PHA) (Roche, Mannheim, Germany) and 100 IU/ml of recombinant human interleukin-2 (rh IL-2) (Sigma, St. Louis, MO) were added on d 1 and d 3 respectively in RPMI media supplemented with 10% FBS. Cells were split at 1:5 every three days and supplemented with rh IL-2.

### **QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)**

CD8+ T cell subsets sorted by FACS (over 99% purity), following staining using mAbs specific for CD45RA, CD27, and CD8 (excluding cells staining in a “dump” channel positive for CD4, CD14, CD16, CD19, and/or CD56) were used for RNA extraction. Total RNA was extracted from cells by using RNeasy Mini Kit (Qiagen, Valencia, CA) as followed by the manufacturer protocol. RNA (100 ng) was reverse transcribed by using Oligo dT-primed cDNA reaction (Bio-Rad iScript Select cDNA synthesis kit). The resulting cDNA was employed for qRT-PCR reactions. These were performed in triplicate with 1 µl of cDNA from the reverse transcription mixture using Taqman Gene Expression Master Mix and Assays (corresponding to *ERK1* (*MAPK3*), *ERK2* (*MAPK1*) and *GAPDH*) (Applied Biosystems, NJ). The total reaction mix was preincubated at 50°C for 2 min. The protocol

for PCR consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 15 seconds denaturation at 95°C and 1 minute of annealing and elongation at 60°C.

## **IMMUNOBLOTTING**

Whole-cell lysates from CD8<sup>+</sup> T cell subsets were prepared in lysis buffer (Radioimmunoprecipitation assay buffer, (RIPA)) (50mM Tris-HCl, 150mM NaCl, 0.5% Na.Deoxycholate, 0.1% SDS, 1% NP-40) (Thermo Scientific, Waltham, MA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO). The lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were collected and quantified for protein concentration (by modified Bradford assay, Bio-Rad, Inc.). A total of 25µg of protein was resolved on a 10% SDS-PAGE gel and then transferred to a PVDF membrane using a semidry transfer cell apparatus (Bio-Rad, Hercules, CA). The membranes were treated with 5% nonfat milk for 1 hr to block nonspecific binding, rinsed, and incubated with mouse anti-human ERK1 (1:2,000) (R&D system), rabbit anti-human ERK2 (1:2000) (Cell Signaling Technology) and mouse anti-human β-actin (1:3,000) (Sigma) overnight. Signals were detected with HRP-conjugated anti-mouse IgG (1:3,000 for ERK1, 1:10,000 for β-actin) or anti-rabbit IgG (1:3,000) (Santa Cruz) using the ECL system (PerkinElmer, Waltham, MA).

The same protocol was applied for whole-cell lysates from regulatory T cells (CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>) or conventional CD4<sup>+</sup> T cells, sorted by FACS, following staining using mAbs specific for CD4, CD25, and CD127 (excluding cells staining in a “dump” channel positive for CD8, CD14, CD16, CD19, and/or CD56).

## **LENTIVIRAL TRANSDUCTION**

I sub-cloned human *ERK1* cDNA into a Lenti-MP2 plasmid. Briefly, transfection of lentiviral plasmid, pMDL (gag/pol), pREV, and pVSVG (vesicular stomatitis virus glycoprotein) into 293T cells were done with jetPEI solution (Polyplus transfection, NY). 72 hr after, viral particles were collected, cleared from cell debris by filtering and concentrated by spinning at 26,000 rpm for two hours. Viral titer was measured by the concentration of p24.

For over-expressing ERK1 in human late memory CD8<sup>+</sup> T cells sorted by FACS, 30 TU/cell of Lenti-MP2-*ERK1* cDNA virus were transduced into 200,000 cells/100  $\mu$ L media in 96-well flat bottom plate, with ViraDuctin Lentivirus Transduction kit (Cell Biolabs, Inc., San Diego, CA) as well as 2,500 rpm high-speed centrifugation for 1 hr. 5 ng/ml IL-7, and IL-15 and 25 IU/ml IL-2 were added for maintaining cell viability (199). At the 72 hr timepoint, cells were incubated with 1 ng/ml PMA and 1  $\mu$ M ionomycin together in 96-well V-bottom plates in 200  $\mu$ L of RPMI1640 supplemented with 10% FBS. After 1 hr, brefeldin A was added to enable accumulation of intracellular cytokines. Following an additional 5 hr incubation, I fixed and permeabilized cells with Fix & Perm A/B (Caltag, Burlingame, CA) and assessed the simultaneous expression of surface markers and intracellular ERK1 and effector proteins (IL-2 and IFN $\gamma$ ).

FACS analyses were performed using Aqua dye for dead cell exclusion (Molecular Probes, Eugene, OR) and mAbs conjugated with the fluorochromes, PE, V450, and specific for human IL-2, IFN $\gamma$  (BD Pharmingen, San Jose, CA). The expression of total ERK1 was assessed using mouse anti-ERK1 IgG2a (1:25 in Caltag Fix/Perm buffer B) (R&D Systems), followed by secondary staining with donkey anti-mouse IgG2a (1:500 in Caltag Fix/Perm buffer B) conjugated with APC (Jackson ImmunoResearch). After staining, cells were washed, resuspended in PBS with 1% paraformaldehyde, and analyzed by flow cytometry in

an LSR-II cytometer (BD, San Jose, CA) using FlowJo software (Treestar, San Carlos, CA). The production of IL-2 and IFN $\gamma$  and the expression of ERK1 were assessed within live cell population (Aqua negative population) and compared with empty Lenti-MP2 virus-transduced cells and mock-transduced (no virus) cells.

### **ALLOGENEIC DENDRITIC CELL INDUCED T CELL PROLIFERATION**

To assess T cell proliferation in the presence or absence of U0126 (Cell Signaling Technology) or tacrolimus (FK506, Sigma), PBMCs were labeled them with 2carboxyfluorescein diacetate (CFSE) (Molecular Probes, Eugene, OR), a highly fluorescent dye that is transferred to daughter cells, resulting in a linear decrease in fluorescence. Briefly, I labeled PBMCs with 2 $\mu$ M CFSE for 10 min at room temperature, quenched the reaction with FBS, and washed them twice with RPMI 1640 containing 10% FBS. The frequency of proliferating CD4 $^{+}$  and CD8 $^{+}$  T cells was estimated after 7 d of mixed lymphocyte reaction (MLR) culture with allogeneic dendritic cells.

### **STATISTICAL ANALYSES**

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Intergroup comparisons were performed using Wilcoxon matched pair analysis and paired t-test. All P values were two-tailed and considered significant if less than 0.05. Results were presented using Prism (GraphPad, San Diego, CA) and Illustrator (Adobe, Seattle, WA) using Macintosh computers (Apple, Cupertino, CA).



## **CHAPTER 3**

**FUNCTIONAL SIGNATURE OF HUMAN CD8+ T CELL**

**MATURATION SUBSETS: THE UNIQUE FUNCTION OF HUMAN**

**LATE MEMORY CD8+ T CELL**

While most studies have focused on naïve and early stage memory CD8<sup>+</sup> T cells, late memory CD8<sup>+</sup> T cells have been less well studied with many assuming that late memory CD8<sup>+</sup> T cells are simply exhausted cells. Therefore, the extensive assessment of surface markers in combination with the production of effector cytokines in human CD8<sup>+</sup> T cell maturation subsets will enhance our understanding of the role of late memory CD8<sup>+</sup> T cells in protective immunity and determine whether they are identical to the exhausted T cells in mice or whether they have unique function besides cytotoxicity. In this chapter, the functional signature of human CD8<sup>+</sup> T cell maturation subsets by multi-color cytokine flow cytometry is presented to better define the unique function of human late memory CD8<sup>+</sup> T cells.

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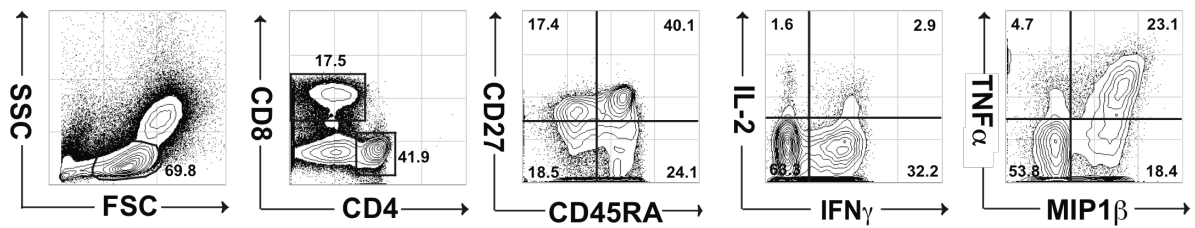
Dr. Krishna V. Komanduri and the other co-authors (Lisa St. John, Eric D. Wieder, Jahan Khalili, Qing Ma) certify that Tae Kon Kim was the primary author of the text.

## **RESULTS**

### **Higher order cytokine flow cytometry of human T cells.**

Cytokine flow cytometry was initially developed by Picker, *et al.* (200) to characterize both cell surface markers of human T cells and the production of effector cytokines at a single-cell level. Following earlier methods published by de Rosa, *et al.* (201) and Roederer and colleagues (202) as well as other studies that have established the importance of polyfunctionality (previously defined by co-production of IL-2, IFN $\gamma$ , TNF $\alpha$ , and MIP-1 $\beta$  (CCL4) (95, 96, 105, 109, 203)), I optimized an 8-color, 10-parameter flow cytometric approach that allowed us to simultaneously assess CD8 $^{+}$  lineage, maturation stages (defined by CD45RA and CD27) and the production of three cytokines (IL-2, IFN $\gamma$  and TNF $\alpha$ ) and one chemokine (MIP-1 $\beta$ ).

Analyses for subsequent studies were based on sequential gating of lymphocytes by forward/side scatter, CD8 $^{+}$  staining, and then on naïve and memory T cell subsets defined by the four quadrants demarcated by CD45RA and CD27 staining (**Figure 4**). Although additional markers (e.g., CD62L, CCR7) may assist in the discrimination of naïve T cells, CD27 and CD45RA co-expression defines naïve human CD8 $^{+}$  T cells with up to 93% specificity, while the remaining three quadrants defined by these markers demarcate memory cells (201). Individual studies have used inconsistent terms to describe memory subsets defined by CD27, CCR7 and CD45RA expression, including “central memory”, “effector memory” and “late memory” subsets. However, it is widely accepted that the loss of CD27 expression is associated with maturation and that CD45RA re-expression characterizes late memory cells (61-63, 201, 204-206). Thus, for the purpose of clarity I



**Figure 4. Optimization of 8-color 10-parameter cytokine flow cytometry.** For the most analyses, at least 300,000 total events were acquired, with sequential gating of PBMC in a lymphocyte region by forward and side scatter, on CD8+ T cells and assessment of intracellular IL-2, IFN $\gamma$ , TNF $\alpha$ , and MIP-1 $\beta$  within naïve and three CD8+ T cell maturation subsets demarcated by CD45RA and CD27 expression.

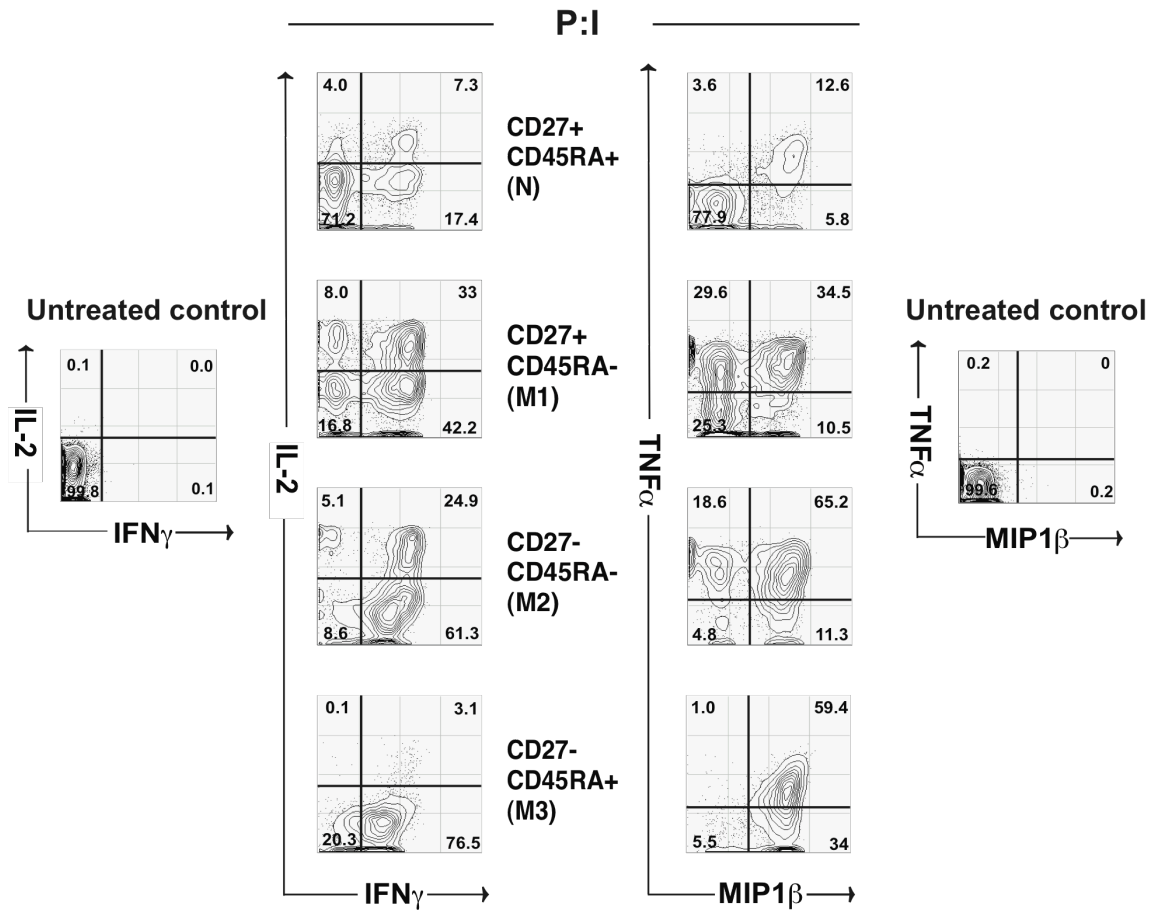
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chose to classify cells as naïve (CD27+CD45RA+) or as progressively mature M1, M2 and M3 memory cells (M1: CD27+CD45RA–, M2: CD27–CD45RA, M3: CD27–CD45RA+).

Within CD8+ T cell subsets divided by maturation stage, I analyzed the simultaneous production of IL-2, IFN $\gamma$ , TNF $\alpha$  and MIP-1 $\beta$ . In most cases, for clarity, we displayed effector cytokine/chemokine production using 2-D plots (e.g., IL-2 vs. IFN $\gamma$  staining or TNF $\alpha$  vs. MIP-1 $\beta$  staining) although all 15 possible combinations of one or more cytokines were always assessed.

### **Maturation-dependent cytokine production signatures are independent of TCR stimulation.**

PBMCs were stimulated with PMA and ionomycin (P:I) to induce T cell activation. PMA and ionomycin directly activate protein kinase C-theta (PKC $\theta$ ) while mobilizing intracellular calcium stores (207); this activation does not require TCR engagement or its most immediate downstream signaling events. Consequently, P:I stimulation reflects the intrinsic ability of a cell to become activated irrespective of the expression of a given surface TCR and/or cell surface proteins (including CD27 and CD45RA) that might themselves modulate the activation threshold of a T cell. I consistently observed a strong association between maturation stage and functional signature, as determined by cytokine production. The cytokine signatures by maturation stage induced by P:I stimulation in a representative subject are shown in **Figure 5**. These results demonstrated significant polarization in naïve cells relative to M3 cells. In contrast to the polarized production of individual cytokines noted in naïve and M3 cells, M1 and M2 cells produced heterogeneous combinations of cytokines (e.g. IL-2+IFN $\gamma$ + and TNF $\alpha$ +MIP-1 $\beta$ + cells). In addition, MIP-1 $\beta$  production



**Figure 5. CD8+ T cell maturation is associated with stage-specific cytokine signature.** The relationship between maturation stage and cytokine production pattern in PMA: Ionomycin-stimulated healthy donor PBMC was assessed as shown for a representative subject. Two-dimensional contour plots depict IL-2 vs IFN $\gamma$  staining and TNF $\alpha$  vs MIP-1 $\beta$  staining within four CD8+ T cell maturation subsets; naïve (N: CD45RA+CD27+), early memory (M1: CD45RA-CD27+), intermediate memory (M2: CD45RA-CD27-), late memory (M3: CD45RA+CD27-).

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increases with progressive maturation of memory cells and is most prominent in M3 CD8<sup>+</sup> T cells.

**Superantigen stimulation via the TCR induces characteristic cytokine signatures defined by maturation stage.**

Superantigens, including SEB, activate significant fractions of the T cell repertoire (~30%) (208, 209) by bridging MHC class II on APC and cognate TCR V $\beta$  subsets unique to each superantigen (210). Similar to the native activation of T cells by their cognate MHC-peptide ligands, superantigens activate the TCR signaling pathway through Lck-ZAP70-PLC $\gamma$  and other downstream intermediates (211, 212). For this reason, SEB stimulation allows us to model the activation of antigen-specific T cells via the TCR, while affording the advantage of activating sufficient proportions of rare T cell subsets to yield informative results. Based on this rationale, I stimulated peripheral PBMC derived from healthy subjects with SEB and examined the production of IL-2, IFN $\gamma$ , TNF $\alpha$  and MIP-1 $\beta$  within naïve and memory (M1, M2 and M3) subsets. While a lower proportion of CD8<sup>+</sup> T cells are activated by SEB relative to P:I, I observed a similar polarization in cytokine signature from naïve to progressively mature memory CD8<sup>+</sup> T cells (not shown).

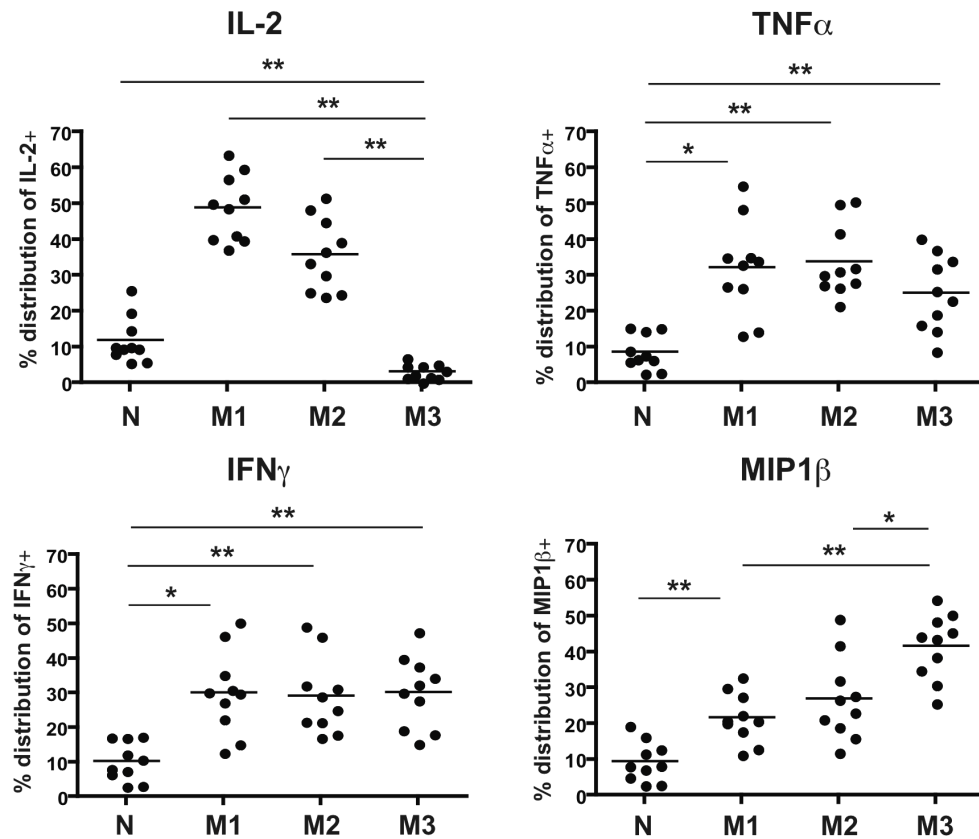
**Late memory CD8<sup>+</sup> T cells have a unique cytokine signature characterized by MIP-1 $\beta$  production without IL-2**

While this approach allowed me to individually analyze all 15 possible combinations of the four cytokines/chemokines, I first examined intracellular production of individual T cell effector proteins. Aggregate results from ten subjects, consistent across the group, are shown in **Figure 5**. For this analysis, the total production of each cytokine/chemokine was

summed, irrespective of whether production originated in a cell producing only one or multiple cytokines. As shown in **Figure 6**, the bulk of IL-2 production in P:I-stimulated CD8<sup>+</sup> T cells emanated from stimulated M1 cells. While M2 cells did produce substantial amounts of IL-2, M3 cells produced little IL-2. Similar results were seen with SEB stimulation (data not shown), suggesting that cytokine signature is an intrinsic property of cells at a given differentiation stage and independent of whether T cell activation occurs via the TCR (as for SEB) or by post-TCR activation (for P:I). Production of IFN $\gamma$  and TNF $\alpha$  was relatively consistent across M1, M2, and M3 subsets, and were increased significantly relative to the naïve subset (**Figure 6**,  $P < 0.01$ ). In contrast, MIP-1 $\beta$  production progressively increased with maturation.

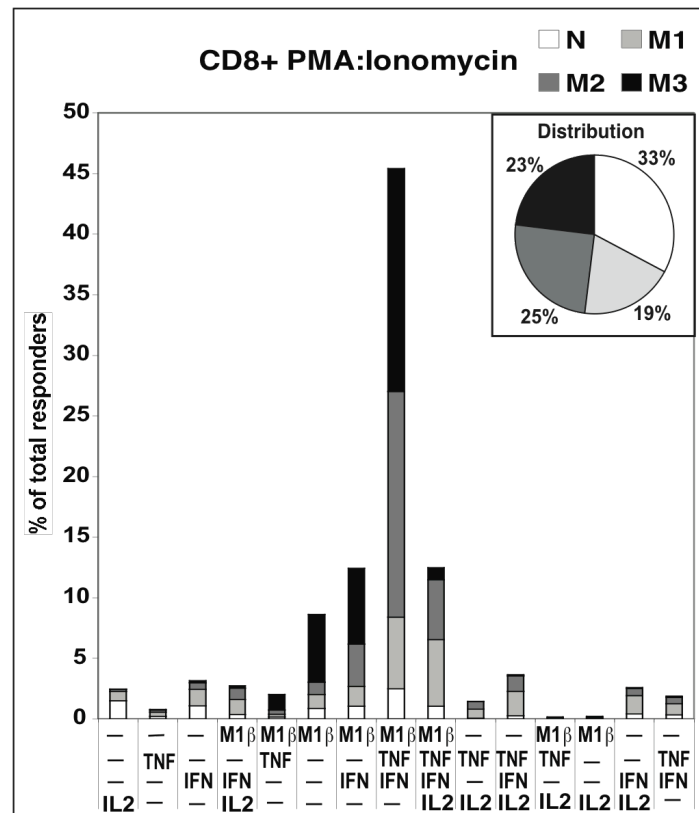
To better define how individual combinations contribute to the overall production of effector cytokines/chemokines within CD8<sup>+</sup> T cell subsets, I assessed all of the 15 possible cytokine/chemokine combinations based on IL-2, IFN $\gamma$ , TNF $\alpha$  and MIP1 $\beta$  production. The production of each individual cytokine (or combination) was then represented as a percentage of the overall cytokine/chemokine production. Data from ten healthy donors were analyzed in this fashion, and presented in the form of stacked bar graphs in **Figure 7**. These analyses allow me to clearly see which combination of cytokines/chemokines is predominant within the overall CD8<sup>+</sup> population. The overall composition of the CD8<sup>+</sup> repertoire by maturation stage in these subjects is also shown (Fig. 2B, inset pie chart, mean  $\pm$  SD N: 33.5 $\pm$ 12.4%, M1: 18.6 $\pm$ 7.0%, M2: 24.6 $\pm$ 8.1% M3: 23.3 $\pm$ 7.2%). This analysis demonstrates that the dominant cytokine-producing combination is one characterized by MIP1 $\beta$ /TNF $\alpha$ /IFN $\gamma$  co-production without IL-2; this functional subset primarily consists of M2 and M3 cells. The next most common signatures consist of MIP1 $\beta$ /IFN $\gamma$  co-producing cells (also mostly M2 and M3 cells) and the most polyfunctional (MIP1 $\beta$ /TNF $\alpha$ /IFN $\gamma$ /IL-2)





**Figure 6. CD8<sup>+</sup> T cell maturation is associated with decreasing IL-2 and increasing MIP-1 $\beta$  production.** Production of individual cytokines/chemokines by maturation stage. Total production of each cytokine/chemokine following PMA:Ionomycin (P:I) stimulation was summed, irrespective of whether such production originated in a cell producing only one or multiple cytokines, and then analyzed by production within N, M1, M2, and M3 stages within the CD8<sup>+</sup> T cell population. Results depict aggregate results from 10 healthy donors. \*\*  $P < 0.01$ , \*  $P < 0.05$

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**Figure 7. MIP-1 $\beta$ -producing polyfunctional cells are dominant within the CD8+ T cell population.** All 15 possible combinations of cytokines were analyzed with each combination assessed as a fraction of all cells producing one or more cytokines/chemokines in combination. Each stacked bar reflects the overall proportion of cells producing a given combination of cytokines/chemokines; each bar is further divided by its production across the memory spectrum (white: N, light gray: M1, dark gray: M2, black: M3). Results from 10 subjects were combined and presented in aggregate, with the inset pie graph revealing the distribution of naïve and memory cells across the population.

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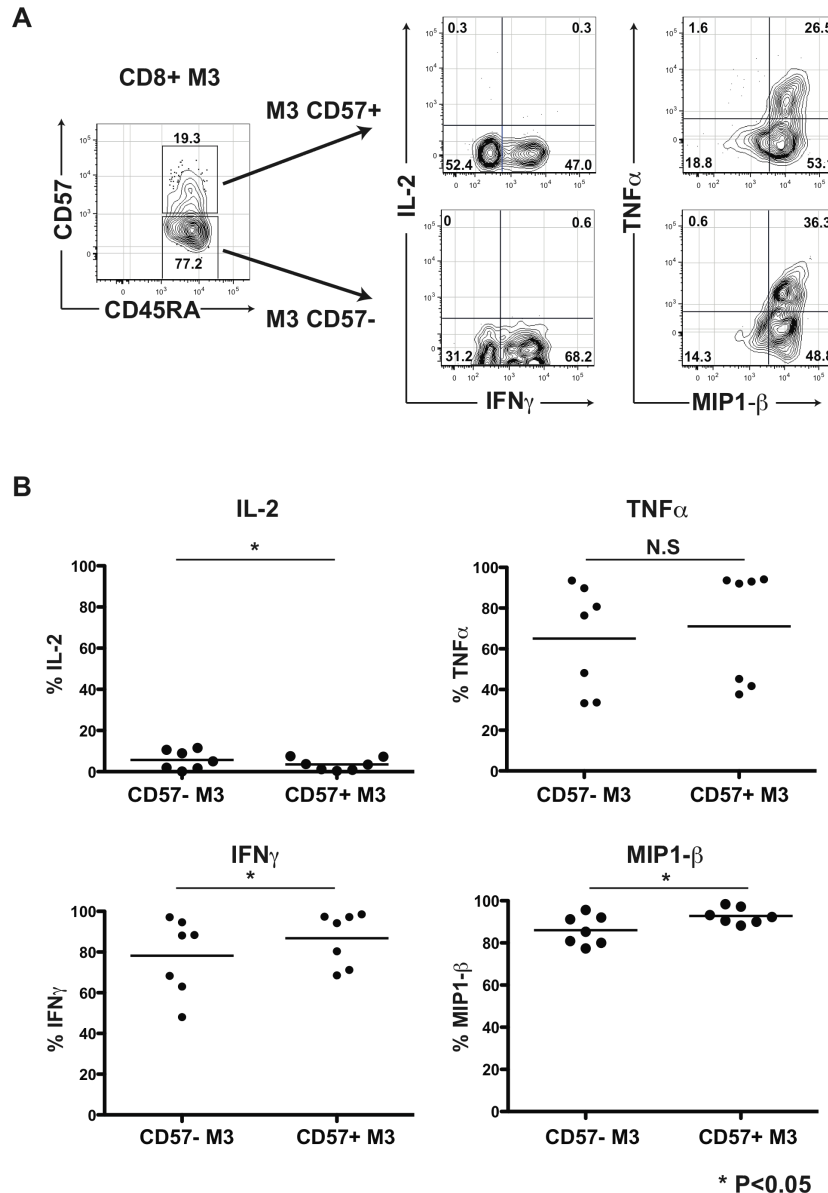
subset. Notably, all of the four most dominant signatures of cytokine/chemokine production include MIP1 $\beta$  production, while only one contains IL-2 producing cells.

**CD8+ CD57+ terminal memory T cells have further skewing to MIP-1 $\beta$  production.**

I further determined whether CD57 within the M3 population could define distinct subsets of these cells. Using a 9-color flow cytometric approach similar to that used above but also incorporating staining for CD57, I assessed an additional seven donors to determine whether CD57-positive and -negative M3 cells exhibited unique patterns of cytokine/chemokine production (**Figure 8**). Consistent with our overall conclusion that memory maturation is associated with skewing in MIP1 $\beta$ :IL-2 production, I found relatively subtle, yet statistically significant, increases in MIP1 $\beta$  production and decreases in IL-2 production in CD57-expressing M3 cells, consistent with their more differentiated phenotype.

**The CC-chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES are abundantly produced by late memory CD8+ T cells.**

To confirm that late memory maturation is characterized by increasing CC-chemokine production and to determine whether the increasing MIP-1 $\beta$  production observed in late memory cells by cytokine flow cytometry reflects its actual secretion into supernatants, I performed a Multiplex Bead Immunoassay to measure the production of five chemokines (MIP-1 $\alpha$ /CCL3, MIP-1 $\beta$ /CCL4, RANTES/CCL5, MCP-1/CCL2, and eotaxin/CCL11) simultaneously in supernatants produced by CD8+ T cell subsets (e.g. N, M1, M2, and M3) from five healthy donors, following stimulation with P:I for 6 hours. Consistent with the observation by intracellular staining that M3 CD8+ T cells produce



**Figure 8. CD57+ M3 CD8+ T cells produce more MIP1-β and less IL-2.** A. The relationship between CD57 expression and cytokine production in M3 CD8+ T cells. We assessed the relationship between CD57 expression and functional signature in P:I-stimulated healthy donor PBMC, as shown for a representative subject. 2-D dot plots depict IL-2 vs. IFN $\gamma$  staining and TNF $\alpha$  vs. MIP1-β staining within CD57+/- M3 CD8+ T cell maturation subsets. B. The difference of production of individual cytokines/chemokines by CD57. Percentage of each cytokine/chemokine following P:I stimulation was calculated within CD57- M3 and CD57+ M3 stages in the CD8+ T cell population, irrespective of whether such production originated in a cell producing only one or multiple cytokines. Results depict aggregate results from 7 healthy donors. \*P<0.05

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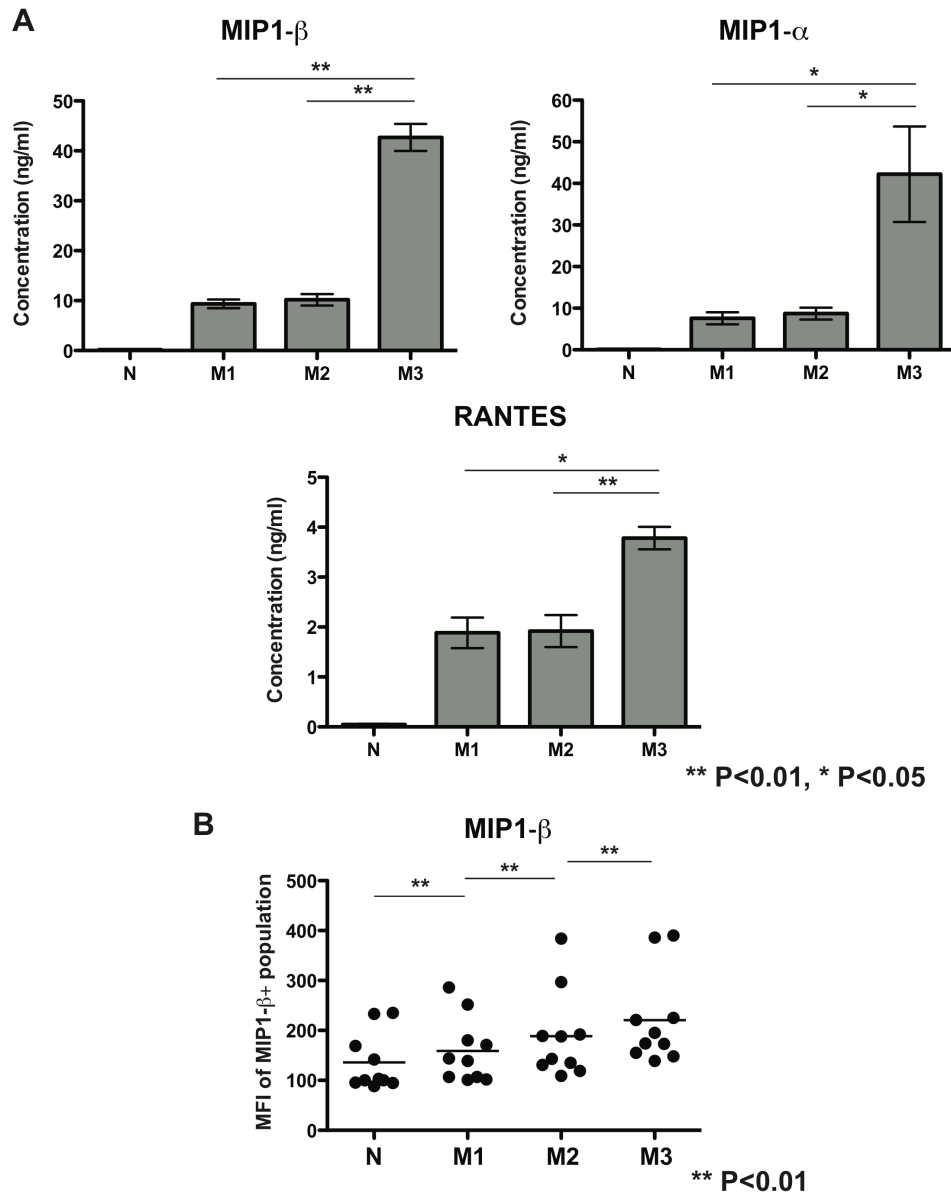
abundant MIP-1 $\beta$  (**Figure 6**), the concentration of secreted MIP-1 $\beta$  was highest in M3 CD8+ T cells than other CD8+ T cell subsets (N, M1, M2,  $P<0.01$ ) (**Figure 9A**).

Furthermore, I also observed the per cell MIP-1 $\beta$  production, as assessed by measurement of the mean fluorescence intensity (MFI) of MIP-1 $\beta$  within chemokine-positive cells, was increased significantly in a stepwise fashion from the naïve to M3 phenotypic stages (**Figure 9B**). I also found that the CCR5-binding CC-chemokines MIP-1 $\alpha$  and RANTES were also primarily produced in M3 cells ( $P<0.05$ ) (**Figure 9A**). MCP-1 and eotaxin levels were too low to be determined (data not shown). Taken together, late memory M3 CD8+ T cells are dominant producers of MIP-1 $\beta$ , MIP-1 $\alpha$ , and RANTES, supporting our intracellular cytokine flow cytometry data and confirming that CC-chemokine production is strongly associated with late memory CD8+ T cell maturation.

### **IL-2/IFN $\gamma$ co-producing cells rarely exist beyond the M2 stage.**

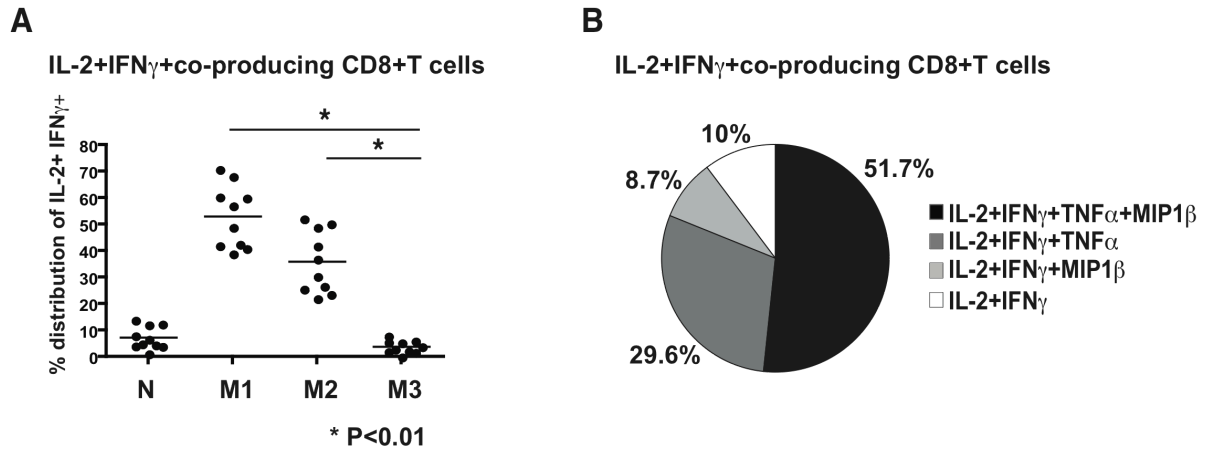
Recent studies have strongly suggested that the expression of individual effector cytokines (especially IFN $\gamma$ ) may be insufficient to identify human T cells most important for protective immunity to persistent viral pathogens (98, 108, 213-215). Pantaleo, *et al.* (98) demonstrated that polyfunctional T cells, defined by IL-2 and IFN $\gamma$  co-production, were critical for protective immune responses in studies of chronic and cleared infections. Based on prior studies, I hypothesized that IL-2/IFN $\gamma$  co-producing CD8+ T cells would be concentrated at certain stages of the maturation spectrum.

Confirming this view, I found that the naïve and M3 CD8+ T cell compartments rarely contained cells capable of IL-2/IFN $\gamma$  co-production, following either P:I or SEB stimulation (with results from ten subjects shown in **Figure 10A**). For example, after P:I stimulation, the proportion of CD8+ T cells (mean $\pm$ SD) with the IL-2+IFN $\gamma$ + phenotype by maturation



**Figure 9. Late memory (M3) CD8+ T cells are the predominant producer of CC chemokines.** A. CD8+ T cells were FACS-sorted from PBMC from five healthy donors and stimulated with PMA: Ionomycin 6 hr and secreted chemokines (MIP-1β/MIP-1α/RANTES) were measured using a multiplex bead immunoassay. (\*  $P<0.05$ , \*\*  $P<0.01$ ), B. Increasing MIP1β production per cell is a characteristic of memory maturation. We calculated the mean fluorescence intensity (MFI) of the MIP1-β+ population within CD8+ T cell maturation subsets (Naïve, N: CD27+CD45RA+, Early memory, M1: CD27+CD45RA-, Intermediate memory, M2: CD27-CD45RA-, Late memory, M3: CD27-CD45RA+). Results depict aggregate results from 10 healthy donors. \*\*  $P<0.01$

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**Figure 10. IL-2/IFN $\gamma$  co-producing cells exist primarily at M1 and M2 stages.** A. IL-2/IFN $\gamma$  co-producing cells, known to be critical for protective immunity, were assessed for their production across the CD8+ T cell naïve/memory spectrum within 10 healthy donors. The vast majority of these cells were present at the M1 and M2 stages (mean $\pm$ SD for N: 8.4 $\pm$ 3.7%, M1: 47.9 $\pm$ 9.8%, M2: 37.7 $\pm$ 11.7%, M3: 5.8 $\pm$ 1.4% \*P<0.01), B. Most IL-2/IFN $\gamma$  co-producing cells also produce IL-2 and/or MIP-1 $\beta$ . We assessed the polyfunctionality of IL-2/IFN $\gamma$  co-producing CD8+ T cells stimulated with P:I. (white: IL-2+IFN $\gamma$ +TNF $\alpha$ -MIP1 $\beta$ -, light gray: IL-2+IFN $\gamma$ +MIP1 $\beta$ +TNF $\alpha$ -, dark gray: IL-2+IFN $\gamma$ +TNF $\alpha$ +MIP1 $\beta$ -, black: IL-2+IFN $\gamma$ +TNF $\alpha$ +MIP1 $\beta$ +) )

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stage was as follows: N, 7.2%±4.0%; M1, 53.0%±11.2%; M2, 35.8%±11.1%; M3, 3.8%±2.3%. Similar results were seen in CD8<sup>+</sup> T cells following SEB stimulation (data not shown). These results demonstrate that differentiation to an M3 CD8<sup>+</sup> T cell is associated with the loss of the IL-2/IFN $\gamma$  co-production. Furthermore, I examined the production of TNF $\alpha$  and MIP-1 $\beta$  in IL-2/IFN $\gamma$  co-producing cells and found that the vast majority of such cells also produced either TNF $\alpha$  and/or MIP-1 $\beta$  (**Figure 10B**). Taken together, IL-2/IFN $\gamma$  co-producing cells are confined to the M1 and M2 subsets, and rarely exist at the M3 subset.

Moreover, these cells are functionally heterogeneous and usually produce other cytokines in addition to IL-2 and IFN $\gamma$ . These findings were also confirmed by an analysis of the maturation subsets producing each individual cytokine/chemokine or possible combinations. In **Table 1**, the location within the CD8<sup>+</sup> T cell maturation spectrum of the primary and secondary producer of each possible cytokine combination, in data derived from ten healthy subjects, is shown; the subset producing the greatest amount of each cytokine is highlighted in bold, while the standard typeface indicates the location of the second-most prolific producer. Cytokine signatures of CD8<sup>+</sup> T cells stimulated by P:I (or SEB) are shown in Table 1. It is evident from this analysis that the production of individual cytokines/chemokines (e.g., IL-2 without others) occurs primarily at the naïve or M1 stages; polyfunctional cells are increasingly produced at M1 and M2 stages, while progression to the M3 stage is again associated with monofunctionality, with prominent MIP-1 $\beta$  production. This analysis also reaffirms that the subset defined by Pantaleo, *et al.* as polyfunctional on the basis of IL-2 and IFN $\gamma$  co-production is actually quite heterogeneous, with IL-2/IFN $\gamma$  co-producing cells rarely producing these two cytokines without additionally



<b>N</b> <b>(CD27+CD45RA+)</b>	<b>M1</b> <b>(CD27+CD45RA-)</b>	<b>M2</b> <b>(CD27-CD45RA-)</b>	<b>M3</b> <b>(CD27-CD45RA+)</b>
<b>IL-2</b>	<b>TNF<math>\alpha</math></b>	IFN $\gamma$ /TNF $\alpha$ /MIP1 $\beta$	<b>TNF<math>\alpha</math>/MIP1<math>\beta</math></b>
<b>TNF<math>\alpha</math></b>	<b>IFN<math>\gamma</math></b>	IL-2/IFN $\gamma$ /TNF $\alpha$ /MIP1 $\beta$	<b>MIP1<math>\beta</math></b>
IFN $\gamma$	<b>IL-2/TNF<math>\alpha</math></b>	IL-2/IFN $\gamma$ /TNF $\alpha$	<b>IFN<math>\gamma</math>/TNF<math>\alpha</math></b>
	<b>IL-2/ IFN<math>\gamma</math>/TNF<math>\alpha</math></b>	IL-2/TNF $\alpha$	<b>IFN<math>\gamma</math>/TNF<math>\alpha</math>/MIP1<math>\beta</math></b>
	<b>IL-2/ IFN<math>\gamma</math>/TNF<math>\alpha</math>/MIP1<math>\beta</math></b>		
	<b>IL-2/IFN<math>\gamma</math></b>		
	<b>IFN<math>\gamma</math>/TNF<math>\alpha</math></b>		

**Table 1. Dominant cytokine signatures by CD8+ T cell maturation stage.** For each possible cytokine/chemokine permutation the bold listing highlights the maturation stage that is the dominant producer of the given combination; when appropriate the standard typeface indicates the stage producing the second highest amount of the combination. For example, cells producing IFN $\gamma$  alone are found most commonly in the M1 compartment, followed by the N compartment.

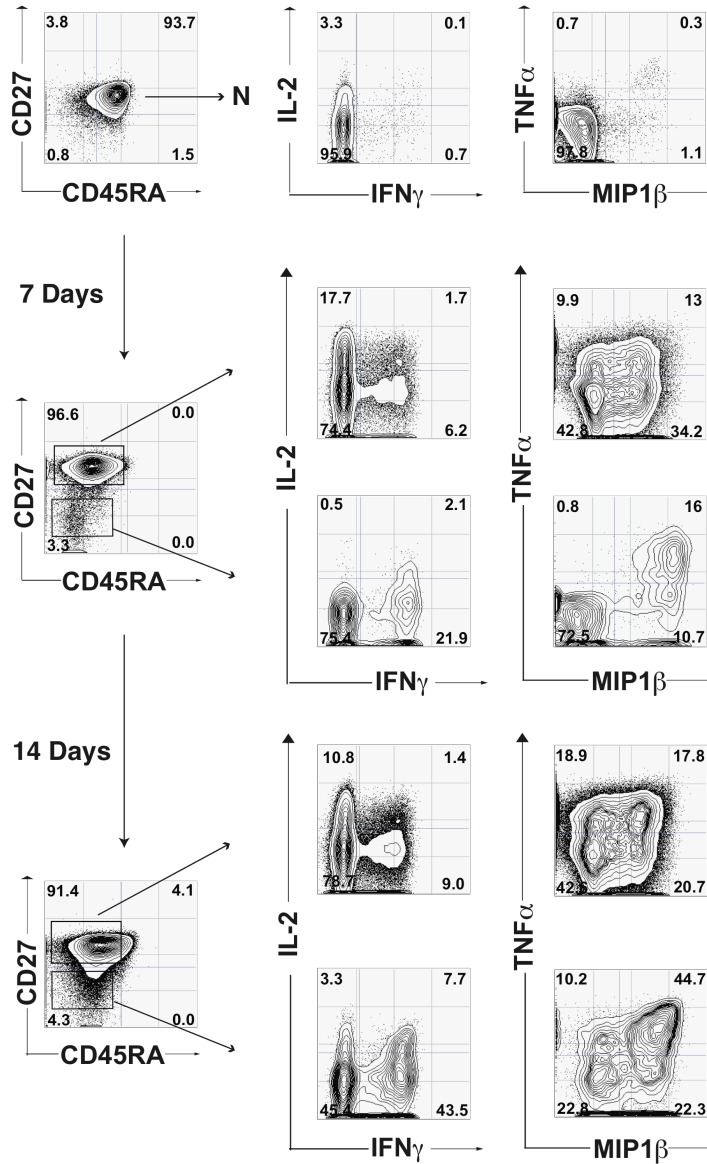
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producing TNF $\alpha$  and/or MIP-1 $\beta$ . Finally, while M3 cells are the dominant source of MIP-1 $\beta$  production, they rarely produce IL-2.

### **Cytokine signatures of T cell maturation subsets can be induced by *in vitro* differentiation**

To formally demonstrate that maturation of peripheral T cells leads to alterations in functional cytokine production, I induced T cell differentiation from sorted naïve human T cells *in vitro*. Previously, Brenchley *et al.* showed the relationship between cell division and functional or phenotypic changes following stimulation with PHA and SEB (216). I previously demonstrated that prolonged stimulation of naïve CD45RA+CD62L+ human cord blood cells with PHA and IL-2 induced down-regulation of CD45RA and CD62L (217). Based on these studies, I sorted naïve CD27+CD45RA+(CD45RO-) human peripheral T cells to at least 99% purity and stimulated them with P:I at baseline, and following seven and 14 days of culture in the presence of PHA and IL-2.

I then analyzed the functional profiles of CD8+ T cells (**Figure 11**) within naïve, M1 and M2 compartments. Under these strong stimulation conditions, I was easily able to induce differentiation of naïve cells to the M1 subset, while fewer T cells were present at the M2 stage. Similar to my prior experiments with peripheral CD8+ T cells, I found that naïve cells primarily produced IL-2; similarly, I saw the same transition toward polyfunctional T cells and toward MIP-1 $\beta$ -producing cells during differentiation through the M1 and M2 stages. These data provided direct confirmation that differentiation stage determines intrinsic functional status, as defined by cytokine production, in CD8+ T cells.



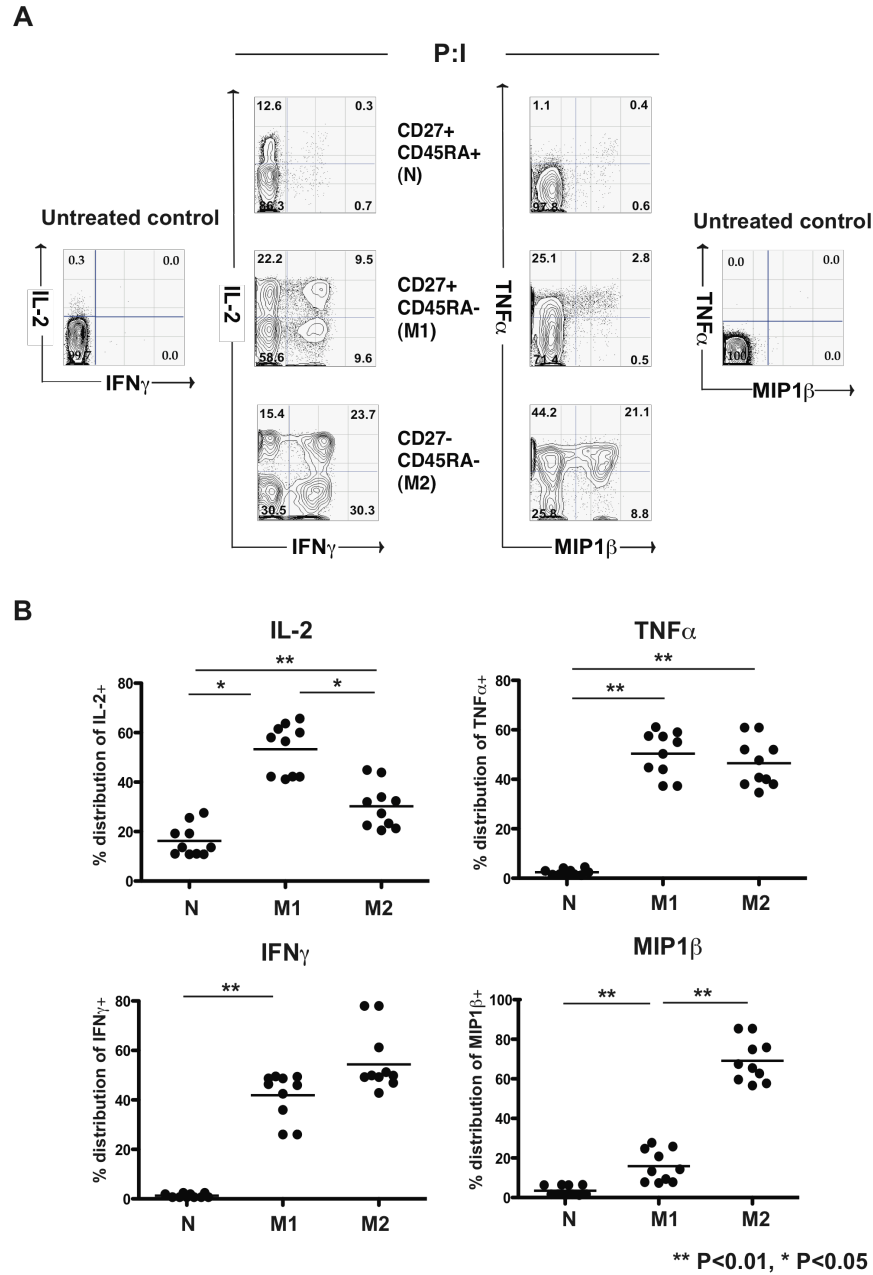
**Figure 11. Cytokine signatures of CD8+ T cell maturation subsets can be induced by *in vitro* differentiation.** CD27+CD45RO- CD8+ naïve cells were exposed to PHA and IL-2 for 14 days in liquid culture. Cells were harvested on day 0, 7 and 14 and re-stimulated with P:I for 6 hours to assess cytokine/chemokine productions in CD8+ T cell maturation subsets by multi-color CFC as described above. (Day 0, TNF $\alpha$ : 0.7%, IFN $\gamma$ : 0.8%, MIP1 $\beta$ : 1.4% of total CD8+; Day 7, M1, TNF $\alpha$ : 22.9%, IFN $\gamma$ : 7.9%, MIP1 $\beta$ : 47.2% of CD8+M1, M2, TNF $\alpha$ : 16.8%, IFN $\gamma$ : 24%, MIP1 $\beta$ : 26.7% of CD8+M2; Day 14, M1, TNF $\alpha$ : 36.7%, IFN $\gamma$ : 10.4%, MIP1 $\beta$ : 38.5% of CD8+M1, M2, TNF $\alpha$ : 54.9%, IFN $\gamma$ : 51.2%, MIP1 $\beta$ : 67.0% of CD8+M2), (IL-2+IFN $\gamma$ +, Day 0: 0.1% of CD8+, Day 7: M1, 1.7% of CD8+M1, M2, 2.1% of CD8+M2, Day 14: M1, 1.4% of CD8+M1, M2, 7.7% of CD8+M2).

**The cytokine production pattern of CD4<sup>+</sup> T cell maturation subsets is similar to that of CD8<sup>+</sup> T cell maturation subsets.**

Recently, the surface markers used to discriminate the CD8<sup>+</sup> T cell maturation subsets (e.g. CD45RA, CCR7, CD27, CD28) have been adapted for CD4<sup>+</sup> T cell lineage. Surprisingly, many phenotypic and functional similarities have been found between CD8<sup>+</sup> and CD4<sup>+</sup> T cell maturation subsets demarcated by same surface markers (68, 79, 80). My data suggest that (a) as human CD4<sup>+</sup> T cells progressively mature, MIP-1 $\beta$  production is increased, but IL-2 production is decreased, (b) IL-2/IFN $\gamma$  co-producing cells are confined to the M1 and M2 subsets, and rarely exist at the M3 subset (**Figure 12**). Consistent with others' studies, my data also suggest that each CD4<sup>+</sup> T cell maturation subset (N, M1, M2) has identical cytokine production pattern to corresponding CD8<sup>+</sup> T cell subset (N, M1, M2). The percentage of late memory (M3) CD4<sup>+</sup> T cell population is significantly low, so that it is impossible to consistently analyze the functional signature, although those small number of cells predominantly produce MIP-1 $\beta$ .

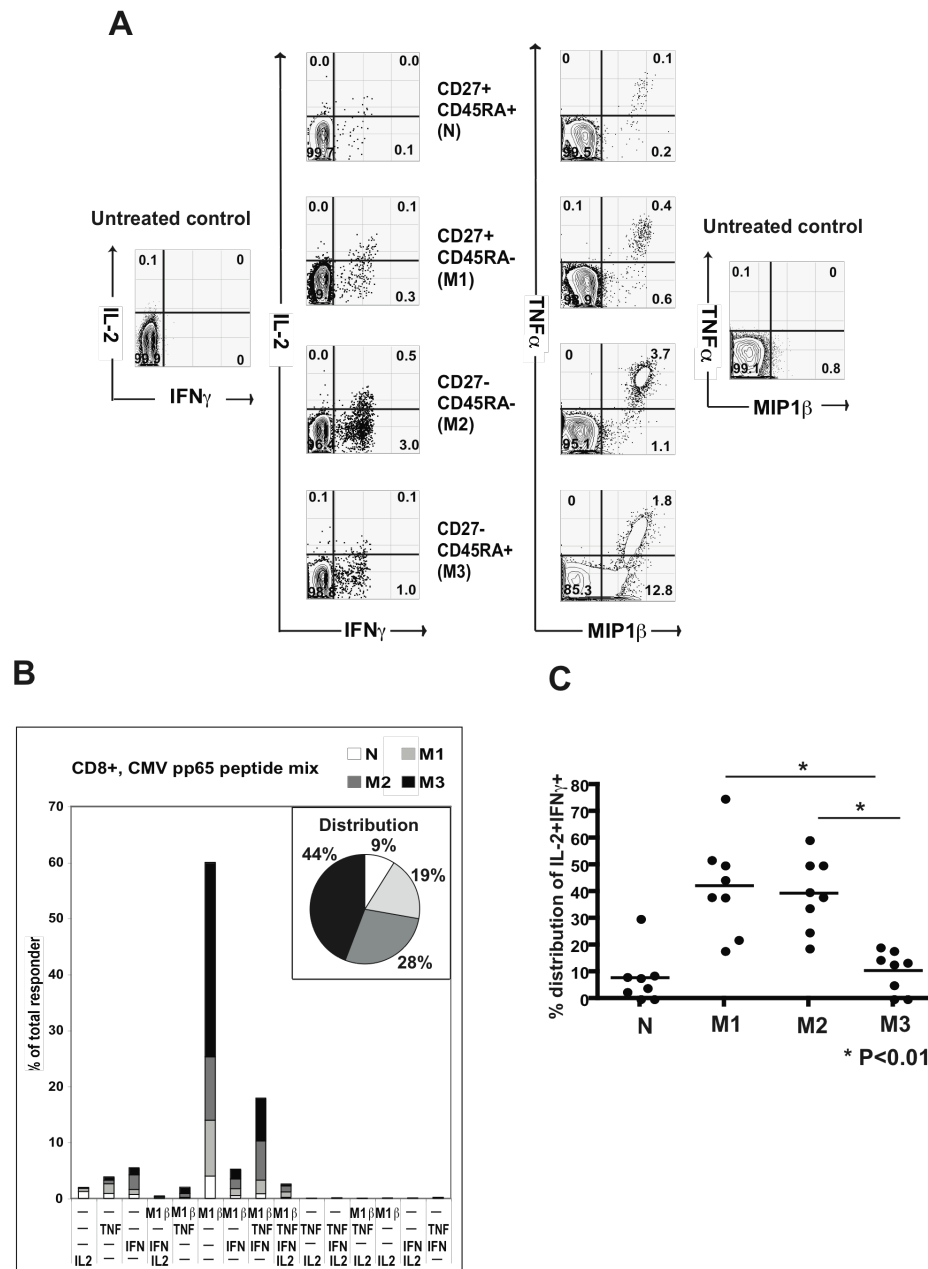
**The production of MIP-1 $\beta$ , and not IFN $\gamma$ , by late-stage memory cells is the hallmark of the CMV-specific CD8<sup>+</sup> T cell response.**

It has recently been suggested that mature CMV-specific CD4<sup>+</sup> T cells acquire antiviral effector function and that their predominant combination of cytokine production is IFN $\gamma$ /TNF $\alpha$ /MIP-1 $\beta$  (105). I sought to more carefully examine the cytokine signature of CD8<sup>+</sup> CMV-specific T cells, and the relationship of cytokine/chemokine production to maturation stage. Therefore, I stimulated PBMC from eight healthy CMV-seropositive donors with a CMV pp65 peptide mixture (197, 198), and examined maturation and function.



**Figure 12. CD4<sup>+</sup> T cell maturation is associated with decreasing IL-2 and increasing MIP-1 $\beta$  production.** A. The relationship between maturation stage and cytokine production pattern in PMA: Ionomycin (P:I)-stimulated healthy donor PBMC was assessed as shown for a representative subject. Two-dimensional contour plots depict IL-2 vs IFN $\gamma$  staining and TNF $\alpha$  vs MIP-1 $\beta$  staining within four CD4<sup>+</sup> T cell maturation subsets; naïve (N: CD45RA<sup>+</sup>CD27<sup>+</sup>), early memory (M1: CD45RA<sup>-</sup>CD27<sup>+</sup>), intermediate memory (M2: CD45RA<sup>-</sup>CD27<sup>-</sup>). B. Production of individual cytokines/chemokines by maturation stage. Total production of each cytokine/chemokine following P:I stimulation was summed, irrespective of whether such production originated in a cell producing only one or multiple cytokines, and then analyzed by production within N, M1, M2, and M3 stages within the CD8<sup>+</sup> T cell population. Results depict aggregate results from 10 healthy donors. \*\*  $P < 0.01$ , \*  $P < 0.05$ .

Consistent with prior observations suggesting the relative maturity of peripheral CMV-specific T cells (67), I found that most functional CMV-specific T cells were in the M2 and M3 stages, as shown for a representative subject (**Figure 13A**). I then analyzed the relative contribution of individual cytokines/chemokines (and combinations) to overall cytokine production in eight separate donors (**Figure 13B**). The most striking finding was the most abundant functional subsets consisted of cells producing either MIP-1 $\beta$  alone (>60% of the total cytokine/chemokine response) or MIP-1 $\beta$  and other cytokines (especially IFN $\gamma$ +TNF $\alpha$ +MIP-1 $\beta$ ). Consistent with my earlier observations that memory differentiation is associated with increasing production of MIP-1 $\beta$ , M2 and M3 cells were the primary source of this chemokine in CMV-specific CD8 $^{+}$  T cells. Finally, I analyzed the production of IL-2/IFN $\gamma$  co-producing cells. These data (in **Figure 13C**) demonstrate that the distribution of these cells was consistent with that observed following SEB and P:I stimulation, reinforcing the notion that differentiation stage, rather than the nature of the activating stimulus, determines the functional signature. Furthermore, these data suggest that skewing of the memory response to M3 results in functional T cells that produce abundant MIP-1 $\beta$ , but lack the ability to produce IL-2, a characteristic associated with pathogen clearance.



**Figure 13. Cytokine production signatures of CMV specific CD8<sup>+</sup> T cells. A.** Functional assessment of CMV-specific CD8<sup>+</sup> T cells across the maturation spectrum in a representative donor. **B.** MIP-1 $\beta$  is the dominant cytokine/chemokine produced by CMV-specific CD8<sup>+</sup> T cells. Cytokine production profiles across the CD8<sup>+</sup> T cell maturation spectrum in CMV pp65-stimulated PBMC from eight healthy CMV-seropositive donors. Inset pie charts depict the naïve/memory distribution of the functional CMV-specific CD8<sup>+</sup> T cell population (white: N, light gray: M1, dark gray: M2, black: M3) **C.** As for P:I-stimulated cells, IL-2/IFN $\gamma$  co-producing CD8<sup>+</sup> T cells are primarily present within M1 and M2 stages (mean $\pm$ SD, M1: 44.3 $\pm$ 10.3% M2: 35.0 $\pm$ 14.5% M3: 14.2 $\pm$ 5.4% \* P < 0.01, negligible cytokine production in CMV-stimulated naïve cells excluded)

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## **CHAPTER 4**

# **THE DIFFERENTIAL EXPRESSION OF ERK1 AND ERK2, AND FUNCTIONAL CONSEQUENCE IN HUMAN CD8+ T CELL MATURATION SUBSETS**



Studies in Chapter 3 demonstrated functional differences in human CD8<sup>+</sup> T cell maturation subsets with a cytokine signature that shifts from IL-2 production in early memory T cells to a pro-inflammatory chemokine production (CC-chemokine) in late memory T cells. Late memory CD8<sup>+</sup> T cells are characterized by the loss of IL-2 production, an increase in MIP1- $\beta$  production as well as an absence of co-production of IL-2 and interferon- $\gamma$ , along with the loss of proliferative capacity. My preliminary data suggest that late memory CD8<sup>+</sup> T cells have less ERK1/2 phosphorylation following polyclonal stimulation with PMA: Ionomycin. Given these preliminary data and prior studies suggesting that the MAP kinase pathway is critical for IL-2 production in T cells (144-147, 152-157), I hypothesized that down-regulation of MAP kinase pathway including ERK1/2 in human CD8<sup>+</sup> T cell maturation subsets alters their functional signature.

### **Multi-color phospho/molecular flow cytometry**

Flow cytometry has been a critical method of immunological research to characterize immune cells in terms of function, phenotype and other cellular events. Recently, flow cytometry started being employed as a tool of molecular biology for cell cycle analysis, fluorescent protein detection or apoptosis measurement. Furthermore, flow cytometry has been applied to single cell analyses of phosphorylation. Initially, Rossi and other colleagues measured the phosphorylation events in peripheral blood using anti-phospho tyrosine antibody by flow cytometry (218). Subsequently, overall phosphorylation status in CD4<sup>+</sup> and CD8<sup>+</sup> T cells following stimulation was measured, and employed for comparing HIV seropositive patients with non-infected individuals (219-221). With the advance of generating phospho-specific antibodies against individual proteins, and of optimal permeabilization protocols (222), the measurement of phosphorylated molecules has been

performed in several studies (223-226). These studies suggested that with an antibody specifically recognizing one phospho-epitope and optimal fixation/permeabilization methods, intracellular phospho proteins could be detected with flow cytometry.

Several studies using murine models, extended these technical advantages into measuring antigen-specific T cell responses. Rosette *et al.* (227) and Zell *et al.* (223) respectively, showed the change of phosphorylation status in antigen-specific T cell response by flow cytometry. In addition, Kaech *et al.* employed phospho-ERK1/2 as a read-out to determine the differential signaling properties of lymphocytic choriomeningitis virus (LCMV)-specific CD8<sup>+</sup> T cell memory subsets both *in vitro* and *in vivo* (121). More efforts have been devoted to develop multi-color flow cytometric analysis for activation of multiple proteins along with surface markers in mice and human immune cells (228-232). However, this approach requires careful optimization to consistently facilitate staining of multiple surface markers, reduce background, and to demonstrate consistency with other methodologies.

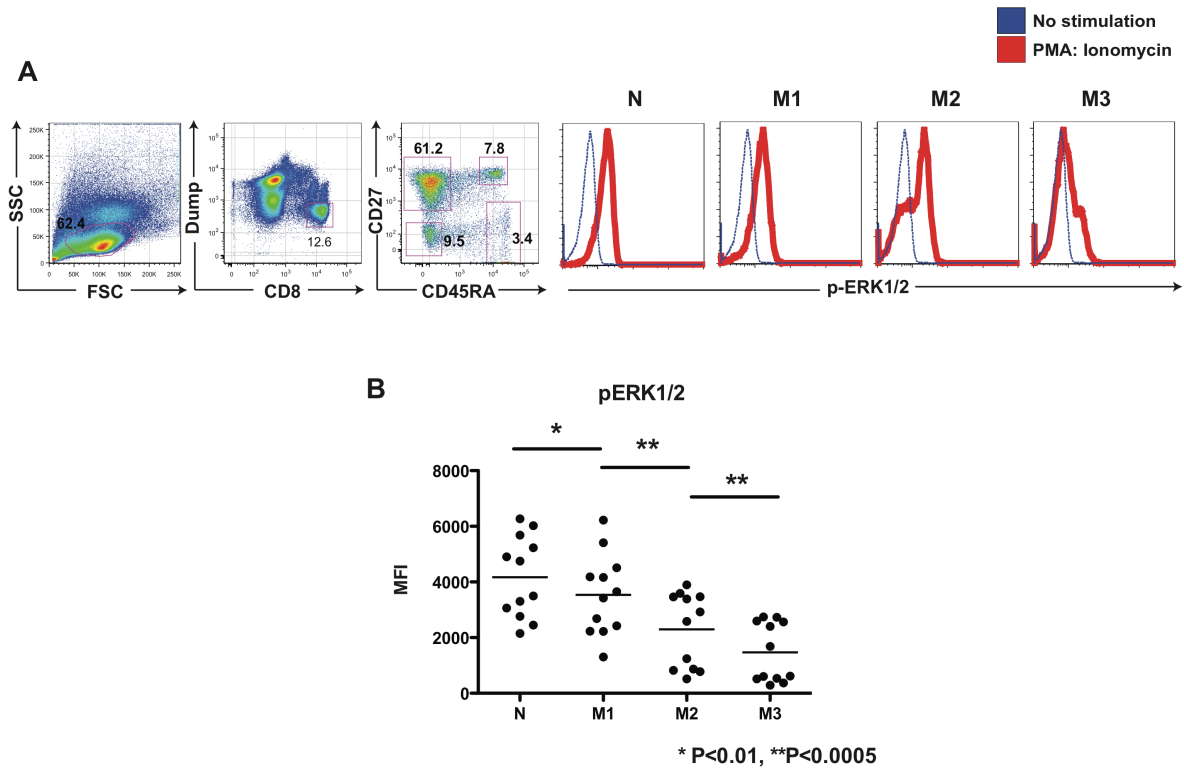
Multi-color flow cytometry has several advantages as an approach to measure intracellular phospho-proteins (233). The first advantage is the nature of objectively quantitative analysis, while immunohistochemistry relies on more subjective quantification or a semiquantitative manner of analysis. Secondly, multi-color flow cytometry enables us to simultaneously measure multiple parameters (e.g. intracellular staining for effectors or molecules with surface markers or with apoptosis/proliferation/cell cycle indicators) at single cell level. Most importantly, it is possible to assess the activation state of kinases in important signaling pathways within rare populations. Currently, one of the major obstacles to studying signal transduction in rare populations such as human regulatory T cells or human late memory CD8<sup>+</sup> T cells, is the lack of attaining suitable numbers of cells within

each subset to perform immunoblotting or standard kinase assays. Immunoblotting also has a limited ability to define T cell heterogeneity, which can be more easily assessed using flow cytometry. In this dissertation, I took advantage of multi-color flow cytometry to detect phosph-ERK1/2 and total ERK1 and ERK2 along with four surface markers as well as two effector cytokines to define the relationship between the molecular and functional signatures of CD8<sup>+</sup> T cell maturation subsets.

## **RESULTS**

### **Optimization of multi-color phospho/molecular flow cytometry**

Given prior studies suggesting that the MAP kinase pathways, including ERK1/2 are critical for IL-2 production in T cells (144-147, 152-157) and my data (described in Chapter 3) suggesting that late memory CD8<sup>+</sup> T cells rarely produce IL-2, I sought to determine whether ERK1/2 phosphorylation is down-regulated in late memory CD8<sup>+</sup> T cells. I optimized a 5-color, 7-parameter flow cytometric approach that allowed me to simultaneously assess CD8<sup>+</sup> lineage, maturation stages (defined by CD45RA and CD27) and the phosphorylation of ERK1/2, following polyclonal stimulation (**Figure 14A**) (PMA: Ionomycin (P:I), or anti-CD3 mAb). Analyses for subsequent studies were based on sequential gating of lymphocytes by forward/side scatter, CD8<sup>+</sup> gating following excluding unnecessary populations by dump channel (CD14, CD19, CD56, CD4), and then on naïve and memory T cell subsets demarcated by CD45RA and CD27 staining (naïve: CD27<sup>+</sup>CD45RA<sup>+</sup>, Early memory (M1): CD27<sup>+</sup>CD45RA<sup>-</sup>, Intermediate memory (M2): CD27<sup>-</sup>CD45RA<sup>-</sup>, Late memory (M3): CD27<sup>-</sup>CD45RA<sup>+</sup>). Within CD8<sup>+</sup> T cell subsets divided by maturation stage, I analyzed the phosphorylation of ERK1/2 (pERK1/2



**Figure 14. Late memory CD8<sup>+</sup> T cells have impaired ERK1/2 phosphorylation.** A. Optimization of 5-color 7-parameter phospho flow cytometry. For the most analyses, at least 300,000 total events were acquired, with sequential gating of PBMC in a lymphocyte region by forward and side scatter, on CD8<sup>+</sup> T cells (excluding unnecessary populations (CD4, CD14, CD19, CD56) and assessment of intracellular phospho-ERK1/2 within naïve and three CD8<sup>+</sup> T cell maturation subsets demarcated by CD45RA and CD27 expression. B. Mean Fluorescence Intensity (MFI) of pERK1/2 following PMA:Ionomycin (P:I) stimulation was measured within the CD8<sup>+</sup> T cell population. Results depict aggregate results from 12 healthy donors. \*\*  $P < 0.01$ , \*  $P < 0.05$

hereafter). In most cases, I displayed histogram of pERK1/2 within CD8<sup>+</sup> T cell subpopulations in PBMCs stimulated with P:I and anti-CD3 mAb (not shown), overlaid with one in corresponding unstimulated subpopulations (**Figure 14A, not shown for anti-CD3 mAb stimulation**).

### **ERK1/2 phosphorylation is diminished in human late memory CD8<sup>+</sup> T cells.**

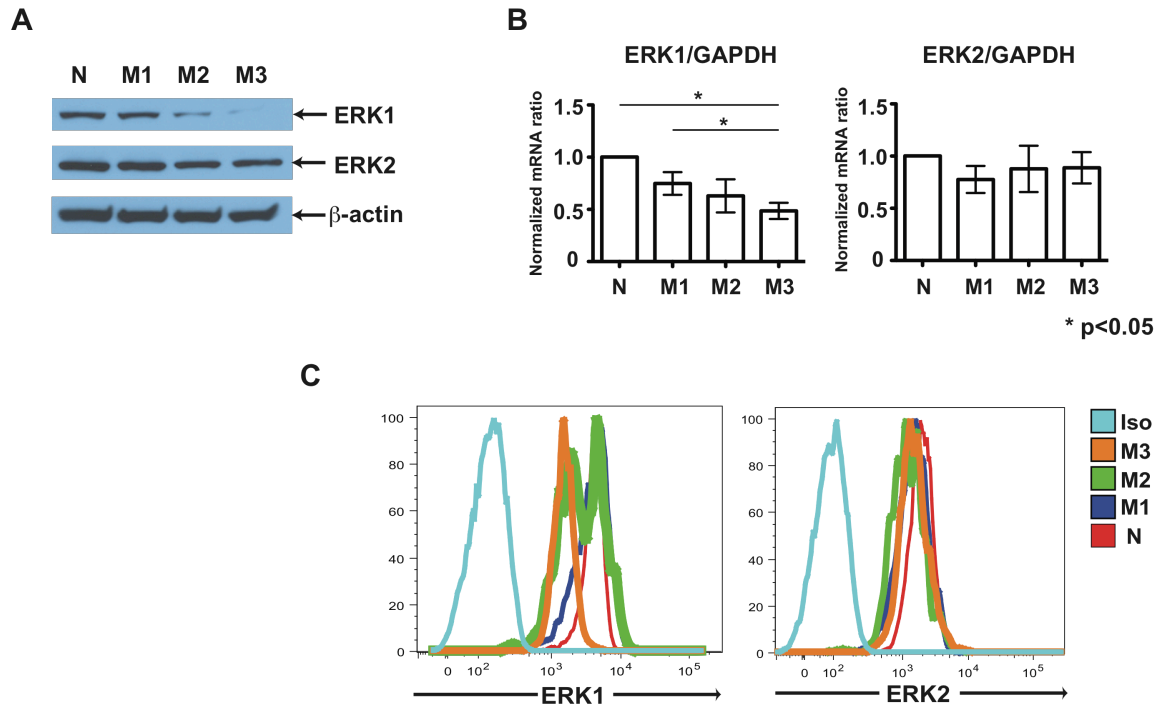
Initially, I assessed pERK1/2 (and phopho-CD3 $\zeta$ ) within CD8<sup>+</sup> T cell subpopulations in PBMCs stimulated with anti-CD3 mAb. I observed that the levels of pERK1/2 and phopho-CD3 $\zeta$  were decreased in CD8<sup>+</sup> M2, and M3 T cells (**Figure 21 in Chapter 5**)

In order to determine whether a lower level of pERK1/2 results from the activity of TCR proximal signaling molecules (e.g. CD3 $\zeta$ , Zap-70) or from the activity or expression level of molecules distal to PKC $\theta$  (e.g. MEK1/2) or both, I stimulated PBMC with PMA and ionomycin (P:I) to induce T cell activation. As mentioned in Chapter 3, P:I stimulation does not require TCR engagement or its most immediate downstream signaling events. Consequently, P:I stimulation reflects the signaling property of molecules downstream to PKC $\theta$  to become activated irrespective of the expression of a given surface TCR and/or intracellular molecules proximal to PKC $\theta$ . I consistently observed a strong association between maturation stage and phosphorylation of ERK1/2, as determined by cytokine production. Following 5 min P:I stimulation, ERK1/2 phosphorylation was displayed as bimodal distribution (lower peak was superimposed by unstimulated control) in M2 cells. In M3 CD8<sup>+</sup> T cells, ERK1/2 phosphorylation was almost superimposed with the unstimulated control, although there was a small shoulder of a positive peak (**Figure 14A**). I, then, measured the mean fluorescence intensity (MFI) of pERK1/2 in CD8<sup>+</sup> T cell maturation

subsets from 12 healthy donor PBMC (**Figure 14B**). Interestingly, the MFI of pERK1/2 in M3 CD8<sup>+</sup> T cells is significantly lower than that of N, M1 and M2 CD8<sup>+</sup> T cells ( $P<0.0005$ ), while the MFI of pERK1/2 in M1 CD8<sup>+</sup> T cells is slightly lower than that naive CD8<sup>+</sup> T cells ( $P<0.01$ ). These data suggest that in addition to down-regulated TCR-proximal signaling activity, the activity of signaling molecules downstream to PKC $\theta$  is diminished in M2 and M3 CD8<sup>+</sup> T cells.

### **ERK1 expression is diminished in human late memory CD8<sup>+</sup> T cells**

To demonstrate whether lower phosphorylation of ERK1/2 in late memory CD8<sup>+</sup> T cells results from down-regulation of total ERK1 or total ERK2 expression, I measured ERK1 and ERK2 protein expression respectively in FACS-sorted CD8 maturation subsets by immunoblotting. I found that ERK1 expression is diminished by ~90% (by measuring densitometry) in M3 CD8<sup>+</sup> T cells compared with M1 CD8<sup>+</sup> T cells, while ERK2 expression is equivalent among maturation subsets (**Figure 15A**). I also demonstrated MEK1 expression was decreased, but not as much as ERK1 (Figure 22 in Chapter 5). To further demonstrate whether *ERK1* mRNA is also decreased in late memory CD8<sup>+</sup> T cells, I performed quantitative RT-PCR with RNA extracted from FACS-sorted CD8<sup>+</sup> T cell maturation subsets. I showed that *ERK1* mRNA level is also lower in M3 CD8<sup>+</sup> T cells than in M1 CD8<sup>+</sup> T cells (**Figure 15B**). Since flow cytometry enables us to determine phenotype and function (e.g. cytokine production, cell proliferation, apoptosis) in a population containing very small number of cells, I optimized multi-color flow cytometry to measure total ERK1 and total ERK2 expression individually along with other surface markers. Using this approach, I confirmed diminished total ERK1 expression in M3 CD8<sup>+</sup> T cells compared with the M1 population by single cell level analysis (**Figure 15C**). More interestingly, I



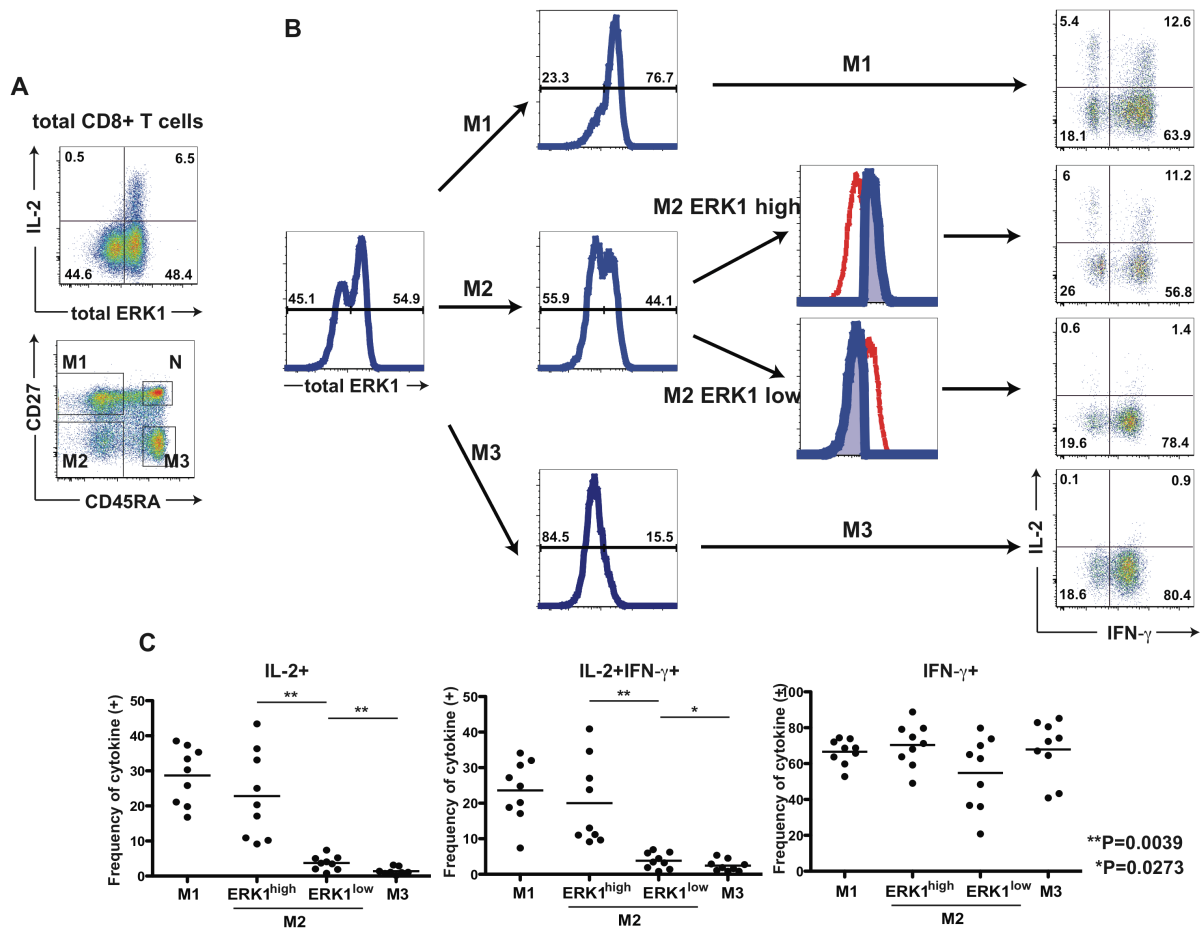
**Figure 15. ERK1 expression is diminished in late memory CD8<sup>+</sup> T cells.** A. ERK1 and ERK2 protein expression in FACS-sorted CD8 maturation subsets respectively were assessed by immunoblotting. By densitometry, ERK1 expression is diminished at ~90% in M3 CD8<sup>+</sup> T cells (75% in M2) compared with M1 CD8<sup>+</sup> T cells, while ERK2 expression is equivalent among maturation subsets. B. Quantitative RT-PCR with RNA extracted from FACS-sorted CD8<sup>+</sup> T cell maturation subsets was performed to determine the mRNA level of ERK1 and ERK2. ERK1 and ERK2 mRNA level were normalized by the level of GAPDH. Relative ratio of ERK1/GAPDH or ERK2/GAPDH of each subset to one of naïve CD8<sup>+</sup> T cells was compared. C. Multi-color molecular flow cytometry was used to assess ERK1 expression of CD8<sup>+</sup> T cell maturation subsets in PBMC. Overlaid histograms indicate differential expression of ERK1 in CD8<sup>+</sup> T cell maturation subsets. (Red: naïve (CD45RA<sup>+</sup>CD27<sup>+</sup>, N), Blue: early memory (CD45RA<sup>+</sup>CD27<sup>+</sup>, M1), Green: intermediate memory (CD45RA<sup>+</sup>CD27<sup>-</sup>, M2), Orange: late memory (CD45RA<sup>+</sup>CD27<sup>-</sup>, M3), Light blue: Isotype control)

found that M2 CD8<sup>+</sup> T cells have bimodal expression of ERK1 (ERK1<sup>high</sup> and ERK1<sup>low</sup>). Taken together, ERK1 expression is decreased in M3 CD8<sup>+</sup> T cells and a subpopulation of M2 CD8<sup>+</sup> T cells, leading to a lower level of pERK1/2, while ERK2 expression is equivalent among CD8<sup>+</sup> T cell maturation subsets.

### **ERK1 expression is associated with IL-2 production in human CD8<sup>+</sup> maturation subsets**

Based on prior studies suggesting that ERK1/2 is important for IL-2 production in T cells (144-147, 152-157), and my data suggesting that late memory (M3) CD8<sup>+</sup> T cells rarely produce IL-2 and have down-regulated expression of ERK1, I hypothesized that down-regulation of ERK1 in human late memory CD8<sup>+</sup> T cells leads to decreased production of IL-2. To better demonstrate whether ERK1 expression is tightly associated with cytokine production in human CD8<sup>+</sup> maturation subsets, I further optimized flow cytometry to simultaneously measure cytokine production (IL-2 and IFN $\gamma$ ) and ERK1 expression in human CD8<sup>+</sup> T cell subsets (CD45RA, CD27) in PBMC stimulated with P:I for 6 hours. As shown in **Figure 16A**, most of IL-2 production emanated from the ERK1<sup>high</sup> population in CD8<sup>+</sup> T cells (11.8% in ERK1<sup>high</sup>, vs 1.1% in ERK1<sup>low</sup>). Accordingly, M3 CD8<sup>+</sup> T cells, which are mostly ERK1<sup>low</sup>, produced IL-2 at a significantly lower level, compared to M1 CD8<sup>+</sup> T cells (**Figure 16B**). As I showed in Chapter 3, the cytokine production pattern in M1 CD8<sup>+</sup> T cells is heterogeneous, including IL-2<sup>+</sup> and IL-2/IFN $\gamma$  co-production. Therefore, M1 CD8<sup>+</sup> T cells, which are a mostly ERK1<sup>high</sup> population, produced IL-2 in a higher level than any other populations. Since I observed two subpopulations of M2 CD8<sup>+</sup> T cells in terms of ERK1 expression, I further analyzed cytokine production pattern in ERK1<sup>high</sup> and ERK1<sup>low</sup> M2 subpopulations by sequential



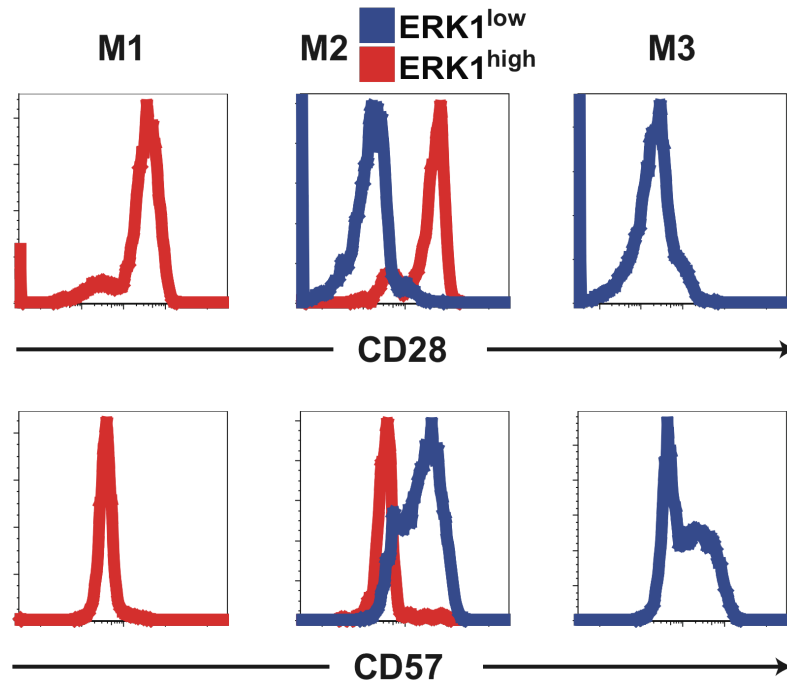


**Figure 16. IL-2 is produced by the ERK1<sup>high</sup> population of CD8+ T cells.** A. Multi-color flow cytometry was optimized to simultaneously measure cytokine production (IL-2 and IFN $\gamma$ ) and ERK1 expression in human CD8+ T cell subsets (CD45RA, CD27) in PBMC stimulated with P:I for 6 hours. In total CD8+ T cells, IL-2 was mostly generated from ERK1<sup>high</sup> population. B. By sequential gating, strong association between ERK1 and IL-2 was shown in each maturation subsets. C. Frequency of IL-2+, IL-2+IFN $\gamma$ + or IFN $\gamma$ + following PMA:Ionomycin (P:I) stimulation was measured within CD8+ T cell maturation subsets (M1, M2, and M3) and ERK1<sup>high</sup> and ERK1<sup>low</sup> subpopulation of M2. Results depict aggregate results from 9 healthy donors. \*\*  $P=0.0039$ , \*  $P=0.0273$

gating. The ERK1<sup>low</sup> subpopulation of M2 CD8+ T cells has a similar cytokine production signature to M3 CD8+ T cells (e.g. relatively low IL-2 production), while the ERK1<sup>high</sup> subpopulation in the same subset produces abundant IL-2, similar to what I observed in M1 CD8+ T cells. Furthermore, my observations are very consistent among human healthy donors, suggesting ERK1 is a more critical marker to predict IL-2 production in human CD8+ T cell maturation subsets than any surface markers (**Figure 16C**). Notably, the level of IFN $\gamma$  production is identical regardless of CD8+ T cell maturation subsets and ERK1 expression, as I found in Chapter 3. These data suggest that ERK1 expression is strongly associated with IL-2 production in human CD8+ T cell maturation subsets.

### **ERK1 expression is associated with CD28 expression**

It has been demonstrated that CD28-mediated signals potently enhance IL-2 production, and IL-2R expression (234-237) and CD28 co-engagement induces ERK1/2 phosphorylation only with engagement of the TCR-CD3 complex (238-240). To determine whether the diminished expression of ERK1 and poor production of IL-2 in later stage CD8+ memory T cells (M3, and a subpopulation of M2), I assessed the expression of CD28 with ERK1 expression in CD8+ T cell maturation subsets by multi-color flow cytometry. I found that M3 CD8+ T cells expressed low levels of CD28, while CD28 expression in M1 CD8+ T cells was uniformly high. Interestingly, CD28 expression was substantially decreased in the ERK1<sup>low</sup> population of M2 (equivalent to M3), compared with the ERK1<sup>high</sup> M2 population (**Figure 17**). In addition, to examine whether a terminal stage marker, CD57 has any association with ERK1 expression in CD8+ T cell subsets, I further assessed CD57 expression in association with ERK1 expression. I found that CD57 expression was higher in the ERK1<sup>low</sup> population of M2 CD8+ T cells than in the ERK1<sup>high</sup> population. Many M3



**Figure 17. ERK1 expression is strongly associated with CD28 expression, and inversely correlated with CD57 expression.** Multi-color flow cytometry was optimized to simultaneously measure CD28, CD57 and ERK1 expression in human CD8<sup>+</sup> T cell subsets (CD45RA, CD27) in PBMC. In M2 CD8<sup>+</sup> T cells, ERK1<sup>high</sup> subpopulation highly expresses CD28, but rarely expresses CD57. ERK1<sup>low</sup> subpopulation of M2 CD8<sup>+</sup> T cells does not express CD28, but does CD57. Notably, most M1 cells express CD28, but not CD57, while M3 has opposite pattern of expression (CD28<sup>-</sup>, CD57<sup>high</sup>)

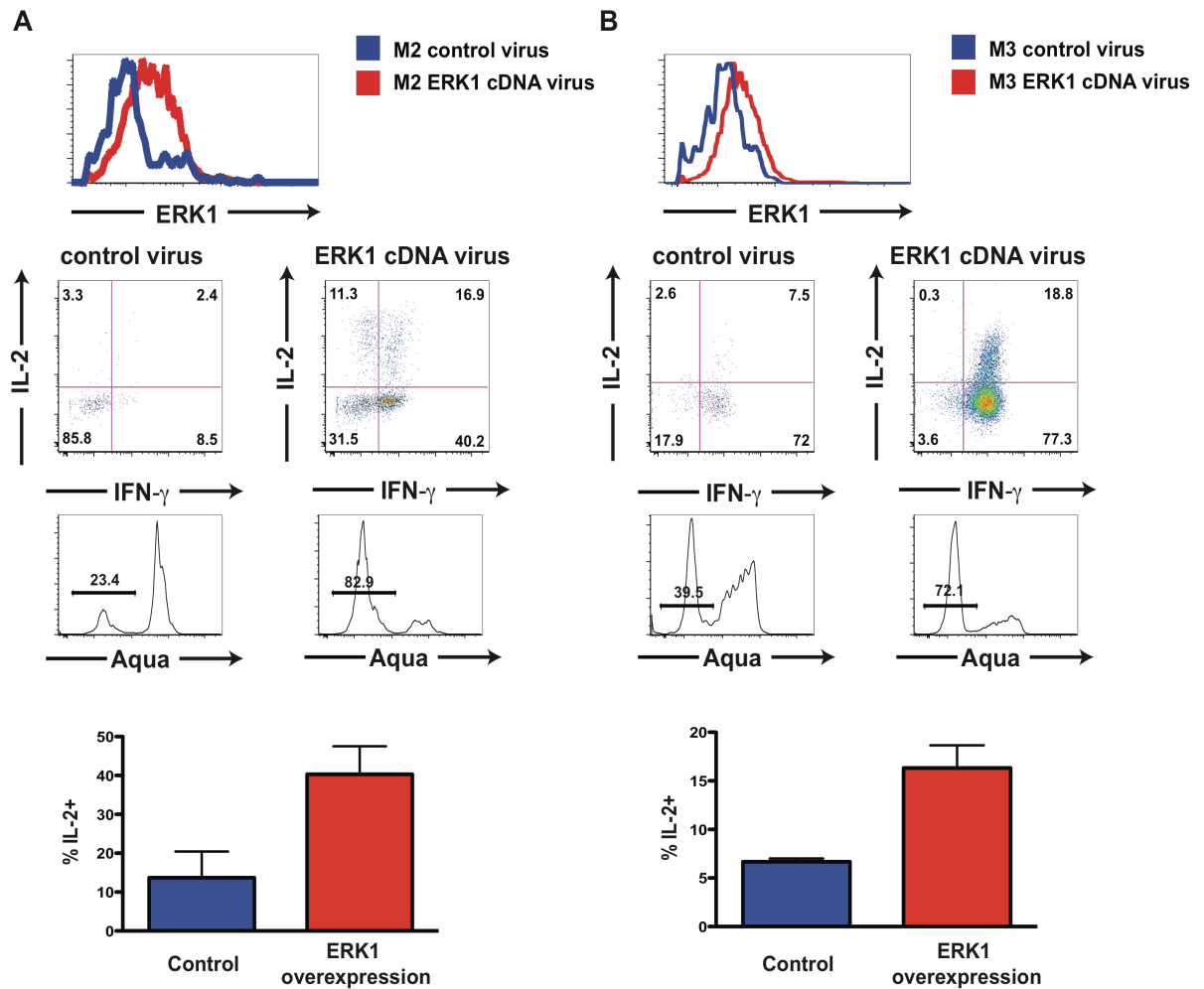
CD8+ T cells had high CD57 expression, while most of M1 CD8+ T cells expressed a low amount of CD57 (**Figure 17**). Taken together, ERK1 expression is strongly associated with CD28 expression, and inversely correlated with CD57 expression.

### **ERK1 expression is required for IL-2 production**

To determine whether ERK1 down-regulation is responsible for decreased production of IL-2, I delivered lentivirus-expressing human *ERK1* cDNA into purified intermediate and late memory (M2, M3) CD8+ T cells. Following P:I stimulation, I examined whether IL-2 production is increased in ERK1 over-expressed M2 and M3 CD8+ T cell populations. M2 CD8+ T cells transduced with lentivirus expressing *ERK1* cDNA, produced ~3 times more IL-2 than control virus transduced M2 CD8+ T cells (n=3) (**Figure 18A**). Additionally, M3 CD8+ T cells transduced with lentivirus expressing *ERK1* cDNA, produced ~2.5 times more IL-2 than control M3 CD8+ T cells (n=3) (**Figure 18B**). Notably, I also found by using the Aqua, live-dead exclusion dye that ERK1 over-expression increased cell viability 2~4 times in M2 and M3 T cells (**Figure 18A & B**). These data suggest that ERK1 expression is required for IL-2 production and cell survival in human CD8+ T cell maturation subsets.

### **ERK1 expression can predict IL-2 production in CMV-specific CD8+ T cells.**

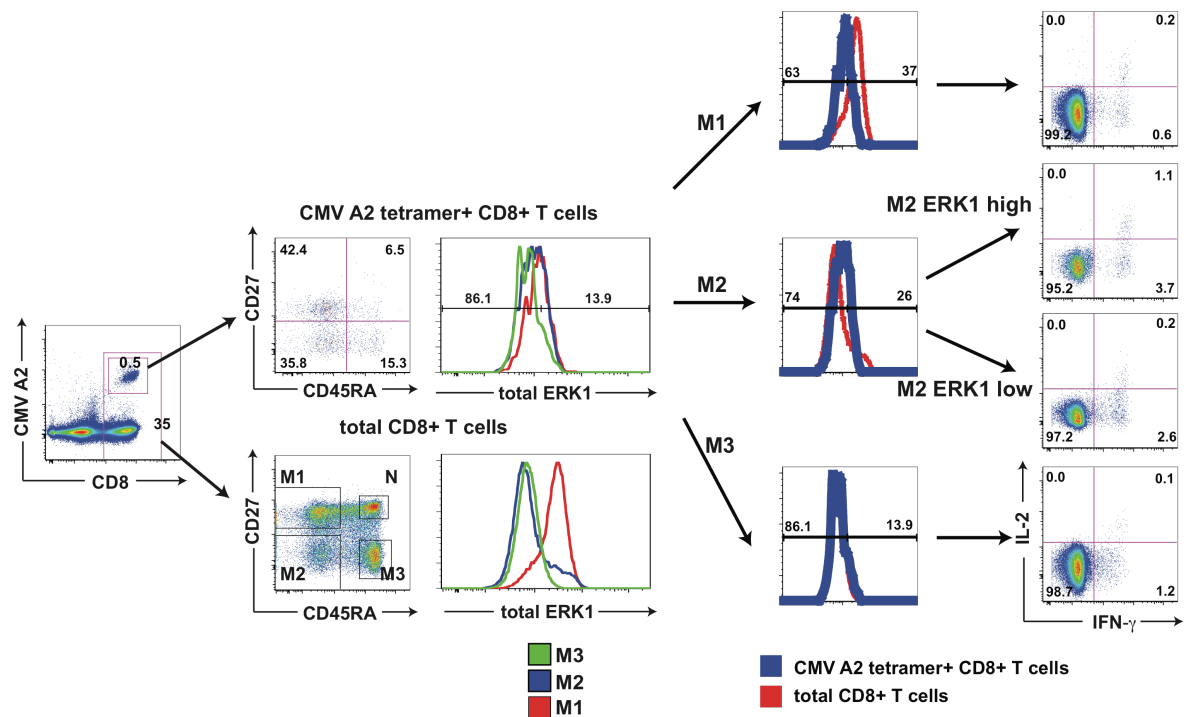
To demonstrate whether ERK1 expression dictates IL-2 production in antigen-specific CD8+ T cell response, I measured ERK1 expression in CMV A2-tetramer+ CD8+ T cells (hereafter, CMV A2+ cells) along with surface markers. As I expected from my prior data and others' studies, CMV A2+ T cells were mostly in M2 and M3 CD8+ T cell subsets. I, then, assessed ERK1 expression in each subset and gated ERK1<sup>high</sup> and ERK1<sup>low</sup>



**Figure 18. ERK1 restoration reconstitutes IL-2 production, and cell survival.**

Following transducing lentivirus expressing human ERK1 cDNA into purified intermediate (A) and late memory (B) CD8<sup>+</sup> T cells, IL-2 production was measured by multi-color flow cytometry. At the same time, cell viability was assessed by Aqua live-dead cell exclusion dye. Experiments were done with cells from three healthy donors (n=3).

subpopulations based on the separation of ERK1 expression in total CD8<sup>+</sup> T cells (**Figure 19**). Compared with ERK1 expression in an M2 subset in total CD8<sup>+</sup> T cells, CMV A2<sup>+</sup> M2 T cells had relatively higher percentage of ERK1<sup>high</sup> population (19% vs 26%). Consistent with my prior observation suggesting that ERK1 expression is associated with IL-2 production, CMV A2<sup>+</sup> ERK1<sup>high</sup> M2 cells produced higher level of IL-2 than CMV A2<sup>+</sup> ERK1<sup>low</sup> M2 cells (1.1% vs 0.2%). Of the responders (IL-2<sup>+</sup> or IFN $\gamma$ <sup>+</sup>), the percentage of IL-2 production was 23% in CMV A2<sup>+</sup> ERK1<sup>high</sup> M2 cells, and 7% in CMV A2<sup>+</sup> ERK1<sup>low</sup> M2 cells. As expected, the ERK1 expression in M3 CMV A2<sup>+</sup> cells was as low as that in M3 CD8<sup>+</sup> T cells. I, also, found that M3 CMV A2<sup>+</sup> cells rarely produce IL-2 (0.1%, 7% of total responder). Although the total response of CMV A2<sup>+</sup> M1 cells is relatively low (0.8%), the percentage of IL-2 production was 25% of the responders. These data suggest that ERK1 expression predicts IL-2 production in CMV-specific T cells, and that CMV A2<sup>+</sup> ERK1<sup>high</sup> M2 cells are similar to M1 cells and CMV A2<sup>+</sup> ERK1<sup>low</sup> M2 cells are functionally similar to M3 cells.



**Figure 19. ERK1 expression predicts IL-2 production in CMV-specific CD8+ T cells.** Using multi-color flow cytometry, ERK1 expression was assessed in CMA A2-tetramer+ CD8+ T cells along with surface markers. Separately, following stimulation with CMV A2 restricted peptide, IL-2/IFN $\gamma$  production with ERK1 expression was determined in CD8+ T cell maturation subsets from the same CMV seropositive healthy donor. Overlaid histograms indicate differential expression of ERK1 in CMV A2+ CD8+ T cell maturation subsets (upper), and total CD8+ T cells (lower). (Red: early memory (CD45RA-CD27+, M1), Blue: intermediate memory (CD45RA-CD27-, M2), Green: late memory (CD45RA+CD27-, M3)). In maturation subsets of CMV A2+ CD8+ T cells, ERK1 expression was determined. For comparison, ERK1 expression in corresponding CD8+ T cell maturation subsets was overlaid (Blue: CMV A2 tetramer+ CD8+ T cells, Red: total CD8+ T cells).

## **CHAPTER 5**

### **OTHER CHARACTERISTICS OF HUMAN LATE MEMORY CD8+ T CELLS**



In Chapter 3, I described the functional uniqueness of human late memory (M3) CD8<sup>+</sup> T cells. In Chapter 4, I determined the differential expression of ERK1/2 in human CD8<sup>+</sup> T cell maturation subsets to explain their functional heterogeneity and better understand the underlying mechanisms dictating the unique function of M3 CD8<sup>+</sup> T cells. Although I proved that ERK1 expression is critical for IL-2 production in human CD8<sup>+</sup> T cells and further showed that ERK1 down-regulation decreased IL-2 production in M3 CD8<sup>+</sup> T cells, it is evident that other signaling molecules and surface co-receptors can directly or indirectly control CD8<sup>+</sup> T cell activation, and also IL-2 production. Therefore, I sought to determine which TCR signaling molecules are key to the activation of human M3 CD8<sup>+</sup> T cells by molecular flow cytometry. These findings, along with data showing the differential expression of  $\alpha 4\beta 1$  integrin in human T cell maturation subsets, leading to differential co-stimulatory effects of  $\alpha 4\beta 1$  integrin on each subset, are presented in this chapter.

## **RESULTS**

### **Human late memory CD8<sup>+</sup> T cells have impaired proximal TCR signaling cascades**

CD3 $\zeta$  and ZAP70 are among the most proximal TCR signaling molecules, and are immediately phosphorylated by stimulation through the TCR/CD3 complex. Therefore, it is important to determine the expression and phosphorylation of these two molecules in T cell maturation subsets to explain their functional heterogeneity, such as differential proliferation and cytokine production. As I explained in Chapter 4, it has been difficult to study signal transduction in rare populations such as human Tregs or M3 CD8<sup>+</sup> T cells, because of the difficulty obtaining suitable numbers of cells within each subset to perform immunoblotting

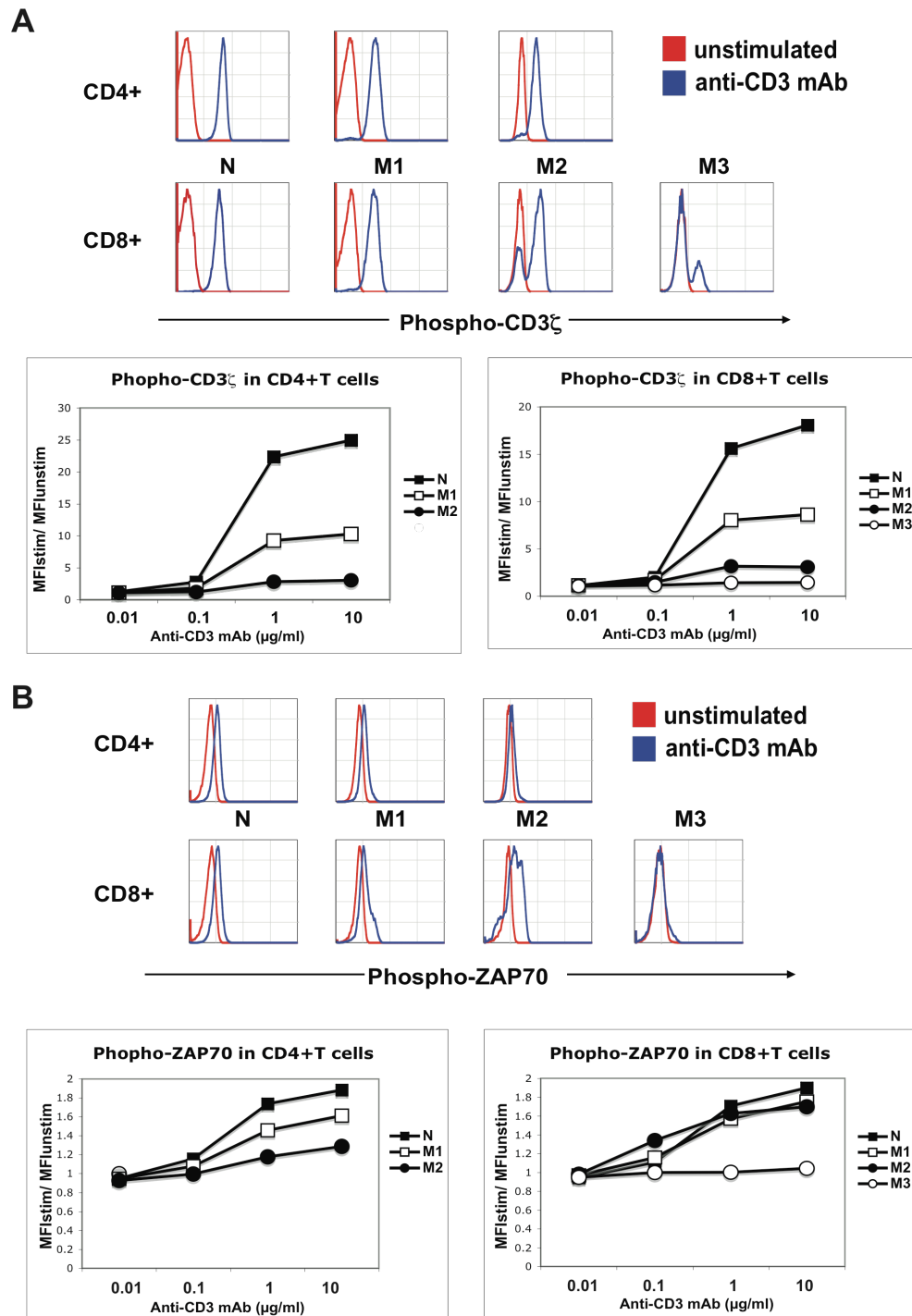
or kinase assays. Again, I took advantage of multi-color flow cytometry to examine whether there are differential proximal signaling events (e.g. via phospho-CD3 $\zeta$  and phospho-ZAP70) in CD8<sup>+</sup> T cell maturation subsets.

Following anti-CD3 mAb engagement for 5 min (with cross-linking with secondary antibody), I assessed phosphorylation of CD3 $\zeta$  and ZAP70 within human CD8<sup>+</sup> T cell maturation subsets present in purified bulk T cells. I found that the phosphorylation of CD3 $\zeta$  and ZAP70 was much lower in M2 and M3 CD8<sup>+</sup> T cells than in Naïve and M1 subsets (**Figure 20**). I also observed similar findings in CD4<sup>+</sup> T cells (lower in M2). Interestingly, there was no increase in phosphorylation of CD3 $\zeta$  and ZAP70 in M3 CD8<sup>+</sup> T cells regardless of anti-CD3 mAb dose, suggesting that the expression of total CD3 $\zeta$  or ZAP70 is lower or the initiation of stimulation is impaired in M3 CD8<sup>+</sup> T cells.

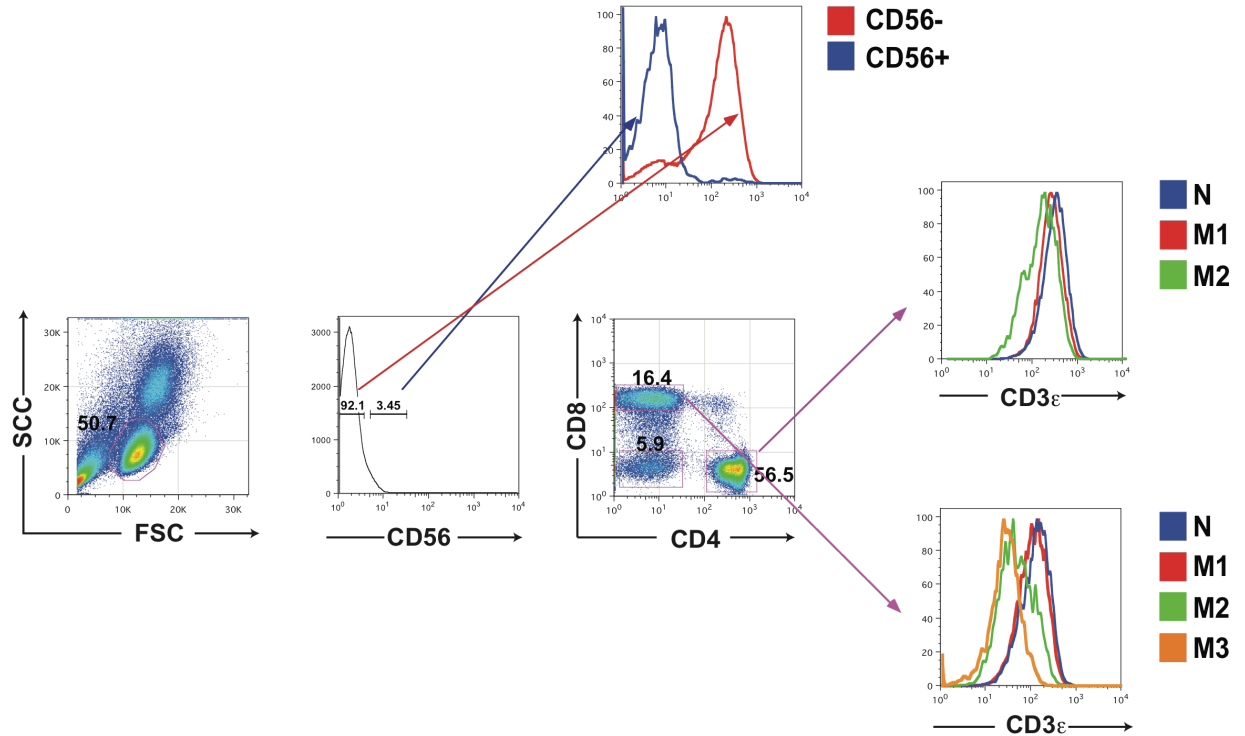
To determine whether the initiation of stimulation is impaired in M3 CD8<sup>+</sup> T cells, I assessed the expression of CD3 $\epsilon$ , a cognate target for anti-CD3 mAb (clone: OKT3) in CD8<sup>+</sup> T cell maturation subsets by multi-color flow cytometry. To my surprise, the expression of CD3 $\epsilon$  was substantially down-regulated in M3 population and partially in M2 cells (**Figure 21**). M2 cells in the CD4<sup>+</sup> lineage also demonstrated down-regulation of CD3 $\epsilon$ , but not to the same extent as M2 or M3 CD8<sup>+</sup> T cells. Given the observation from my preliminary studies, suggesting the expression of CD3 $\zeta$  (not shown) is similar among CD8<sup>+</sup> T cell maturation subsets, these data suggest that TCR-proximal signaling is impaired in late memory CD8<sup>+</sup> T cells, due to down-regulation of CD3 $\epsilon$ .

### **JNK is down-regulated in late memory CD8<sup>+</sup> T cells**

While I demonstrated the functional consequence of ERK1 down-regulation in human late memory (M3) CD8<sup>+</sup> T cells, I sought to determine whether other TCR signaling



**Figure 20. T cell receptor proximal signaling is impaired in late memory CD8+ T cells.** Using multi-color phospho flow cytometry, phospho-CD3 $\zeta$  (A) and phospho-ZAP-70 (B) were assessed in CD4+ and CD8+ T cell maturation subsets following anti-CD3 mAb stimulation (Blue: stimulation with anti-CD3 mAb, Red: unstimulated). It is noted that even 10 $\mu$ g/ml anti-CD3 mAb rarely induce phosphorylation of CD3 $\zeta$  and ZAP-70 in late memory (M3) CD8+ T cells, compared with other subsets.



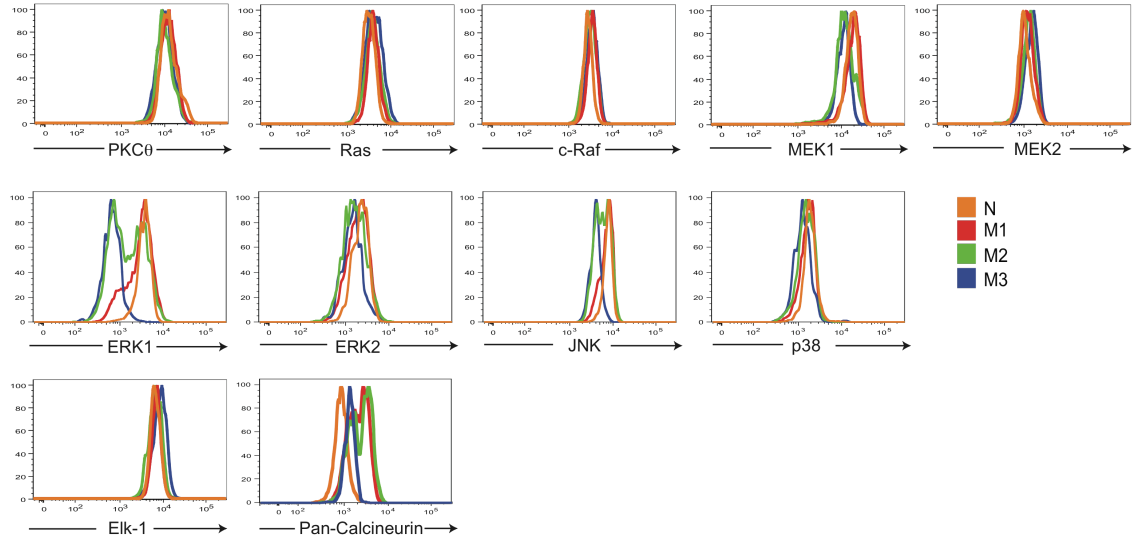
**Figure 21. The expression of CD3ε is down-regulated in late memory CD8<sup>+</sup> T cells.**

Using multi-color flow cytometry, CD3ε expression was assessed in CD4<sup>+</sup> and CD8<sup>+</sup> T cell maturation subsets. CD56<sup>+</sup> population (NK cells) (blue on far upper histogram) was used as a negative control for specific CD3ε staining. CD56<sup>+</sup> population was also removed to rule out possible contamination into M3 cells. Overlaid histograms indicate differential expression of CD3ε in CD8<sup>+</sup> T cell maturation subsets. (Red: naïve (CD45RA+CD27<sup>+</sup>, N), Blue: early memory (CD45RA-CD27<sup>+</sup>, M1), Green: intermediate memory (CD45RA-CD27<sup>-</sup>, M2), Orange: late memory (CD45RA+CD27<sup>-</sup>, M3))

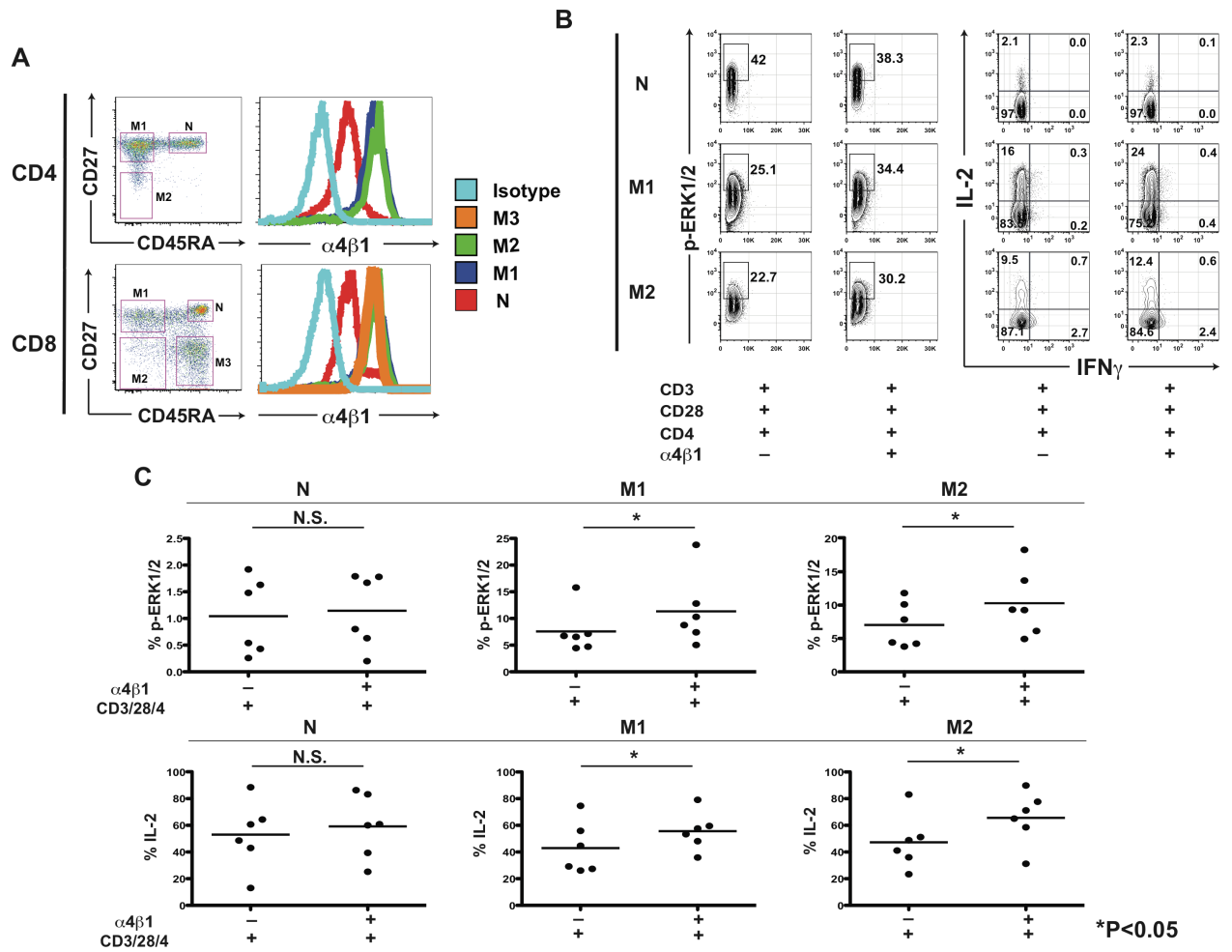
molecules are down-regulated in M3 cells, and if I can further explain the functional characteristics of M3 cells with other molecular changes. So, I characterized the expression of other signaling molecules in CD8<sup>+</sup> T cell maturation subsets by flow cytometry. While I consistently observed ERK1 down-regulation in M3 population, I also found that MEK1 and JNK were similarly down-regulated in M3 cells (**Figure 22**). However, by comparing the relative expression of each molecule in M3 cells to N, it was noted that the extent of down-regulation of MEK1 and JNK was not nearly as much as that of ERK1 in M3 cells (ERK1<sub>MFI</sub> of N/ ERK1<sub>MFI</sub> of M3=5.5; JNK<sub>MFI</sub> of N/ JNK<sub>MFI</sub> of M3=1.9; MEK1<sub>MFI</sub> of N/ MEK1<sub>MFI</sub> of M3=1.5). Interestingly, the expression of calcineurin in M3 cells was higher than in naïve CD8<sup>+</sup> T cells, but equivalent to those of M1 and M2 CD8<sup>+</sup> T cells (**Figure 22**, immunoblotting not shown). These data suggest that alterations in other downstream TCR signaling in M3 CD8<sup>+</sup> T cells, besides ERK1, may affect functional signature such as decreased production of IL-2 and prolific production of MIP-1 $\beta$ .

#### **VLA-4 ( $\alpha 4\beta 1$ ) integrin is highly expressed in late memory CD8<sup>+</sup> T cells**

Several studies demonstrated that integrin  $\alpha$  or  $\beta$  chain is highly expressed in effector T cells (51, 74, 201). However, few studies demonstrated the expression of integrin by an antibody recognizing both the  $\alpha$  and  $\beta$  chain (241). To determine whether the augmentation of T cell activation by VLA-4 ligation differs in T cell maturation subsets, I evaluated the expression of VLA-4 using an antibody that recognizes both the  $\alpha$  and  $\beta$  chain (241), while simultaneously measuring the phosphorylation of ERK1/2 and the production of IL-2 in maturation CD4<sup>+</sup> T cell lineages, demarcated by CD45RA and CD27 staining. Although naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressed VLA-4, its costimulatory effect on phosphorylation of ERK1/2 and IL-2 production was not significant (**Figure 23**). In



**Figure 22. TCR molecular signature of CD8+ T cell maturation subsets.** Multi-color molecular flow cytometry was used to assess ERK1 expression of CD8+ T cell maturation subsets in PBMC. Overlaid histograms indicate differential expression of TCR signaling molecules in CD8+ T cell maturation subsets. (Orange: naïve (CD45RA+CD27+, N), Red: early memory (CD45RA-CD27+, M1), Green: intermediate memory (CD45RA-CD27-, M2), Blue: late memory (CD45RA+CD27-, M3))



contrast, M1, M2 and M3 T cells expressed higher levels of VLA-4 and demonstrated significantly augmented phosphorylation of ERK1/2 and IL-2 production in both CD4 and CD8 lineages (**Figure 23**, data not shown for CD8+ T cell compartment). These data suggest that VLA-4 binding augmented ERK1/2 phosphorylation and the IL-2 production in memory, but not in naïve subsets.

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Dr. Krishna V. Komanduri and the other co-authors (Matthew Billard, Bradley W. McIntyre) certify that Tae Kon Kim was the primary author of the text.



## **CHAPTER 6**

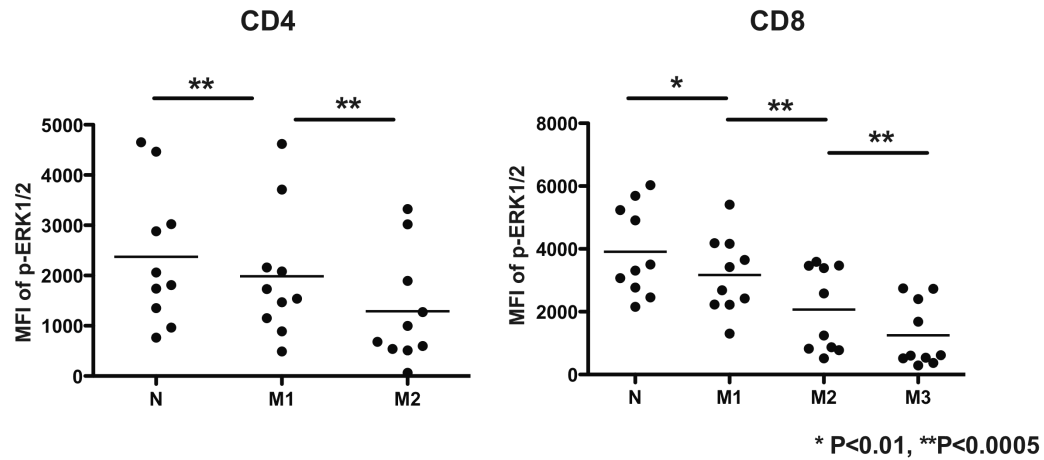
### **NOVEL APPROACH TO TREAT GRAFT-VERSUS-HOST DISEASE WITH RELATIVE SPARING OF CYTOMEGALOVIRUS (CMV)- SPECIFIC IMMUNITY: SELECTIVE INHIBITION OF T CELL MATURATION SUBSETS**

Allogeneic stem cell transplantation (SCT) has been widely used for patients with hematologic malignancies. One of the limitations for successful allogeneic SCT is graft-versus-host disease (GVHD) (242-245). GVHD is an immune response mounted against the recipient of an allograft by mature donor T cells. To reduce the risk of GVHD, most patients receive immunosuppressive medications after SCT or they receive manipulated allografts (e.g. depleted of T cells). However, these strategies delay immune reconstitution, which increases the risk of infections, leading to the high mortality in allogeneic SCT setting. Especially, current immunosuppressive drugs, such as steroids (246) and calcineurin inhibitors (247) are not selective enough to spare pathogen-specific T cells leading to an increased risk of life-threatening infections (e.g., CMV reactivation). Current immunosuppressive drugs also abrogate T cell mediated graft-versus-tumor effects (GVT) (e.g. graft-versus-leukemia) (248). Therefore, the development of novel approaches to promote the GVT effect of donor T cells and spare protective immunity against pathogens without inducing GVHD is critical for better therapeutic outcome of allogeneic SCT.

Recent studies demonstrated the differential roles of T cell subsets on inducing GVHD. Initially, Anderson, Shlomchik and colleagues demonstrated that memory CD4<sup>+</sup> T cells induce neither clinical nor histological GVHD, while naïve CD4<sup>+</sup> T cells do (249). Chen *et al.* also showed that adoptive transfer of allogeneic CD62L<sup>-</sup> memory T cells does not induce GVHD in a murine model (250). Zheng *et al.* found that central memory T cells, but not effector memory T cells, induce GVDH (251, 252). These studies suggest that naïve and central memory (or early memory) CD4<sup>+</sup> and CD8<sup>+</sup> T cells may be relatively more potent at inducing GVHD than effector memory (or intermediate) or late memory T cell subsets (249-254).

Another important T cell subset in GVHD is regulatory T cells (Tregs). Preclinical transplantation murine models demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> Tregs reduce acute GVHD while sparing the GVT effect (255). Additional studies suggest that higher donor graft CD4<sup>+</sup> FoxP3<sup>+</sup> T cell doses are associated with less acute GVHD in allogeneic peripheral blood SCT (256). Currently, several groups are performing clinical trials on the adoptive transfer of *ex vivo* expanded Tregs to prevent GVHD (unpublished). While the clinical utility of Tregs remains to be determined, Tregs are thought to be a critical player to suppress GVHD in allogeneic SCT.

In addition, Appay *et al.* and I have shown that most CMV-specific T cells exist as relatively differentiated cells, and only a small number of these cells are found at early stages of the T cell maturation spectrum (66, 257). Therefore, I reasoned that if there are signaling differences in T cell maturation subsets, they could be used to selectively inhibit naïve and early memory T cells, which have been reported to preferentially induce GVHD while sparing CMV-specific immune responses. I found in Chapter 4, that naïve and early memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells express two-fold higher expression of p-ERK1/2 than intermediate and late memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells ( $p < 0.0005$ ) (**Figure 24**). Given this rationale combined with my data on differential phosphorylation of ERK1/2 in human T cell maturation subsets, I hypothesized that the effects of MEK inhibition on T cell activation depend on levels of ERK1/2 phosphorylation. To test this hypothesis, I measured cytokine production in activated T cell maturation subsets in the presence of MEK inhibitor (U0126) and the results are presented in this Chapter.



**Figure 24. CD4<sup>+</sup> and CD8<sup>+</sup> T cell maturation subsets reveal differential phosphorylation of ERK1/2.** Optimization of 5-color 7-parameter phospho flow cytometry. Using multi-color phospho flow cytometry, assessment of intracellular phospho-ERK1/2 was assessed within naïve and two CD4<sup>+</sup> and three CD8<sup>+</sup> T cell maturation subsets demarcated by CD45RA and CD27 expression, following PMA:Ionomycin (P:I) stimulation. Mean Fluorescence Intensity (MFI) of pERK1/2 was measured within CD4<sup>+</sup> and CD8<sup>+</sup> T cell maturation subsets. Results depict aggregate results from 12 healthy donors. \*\*  $P < 0.01$ , \*  $P < 0.05$

## **RESULTS**

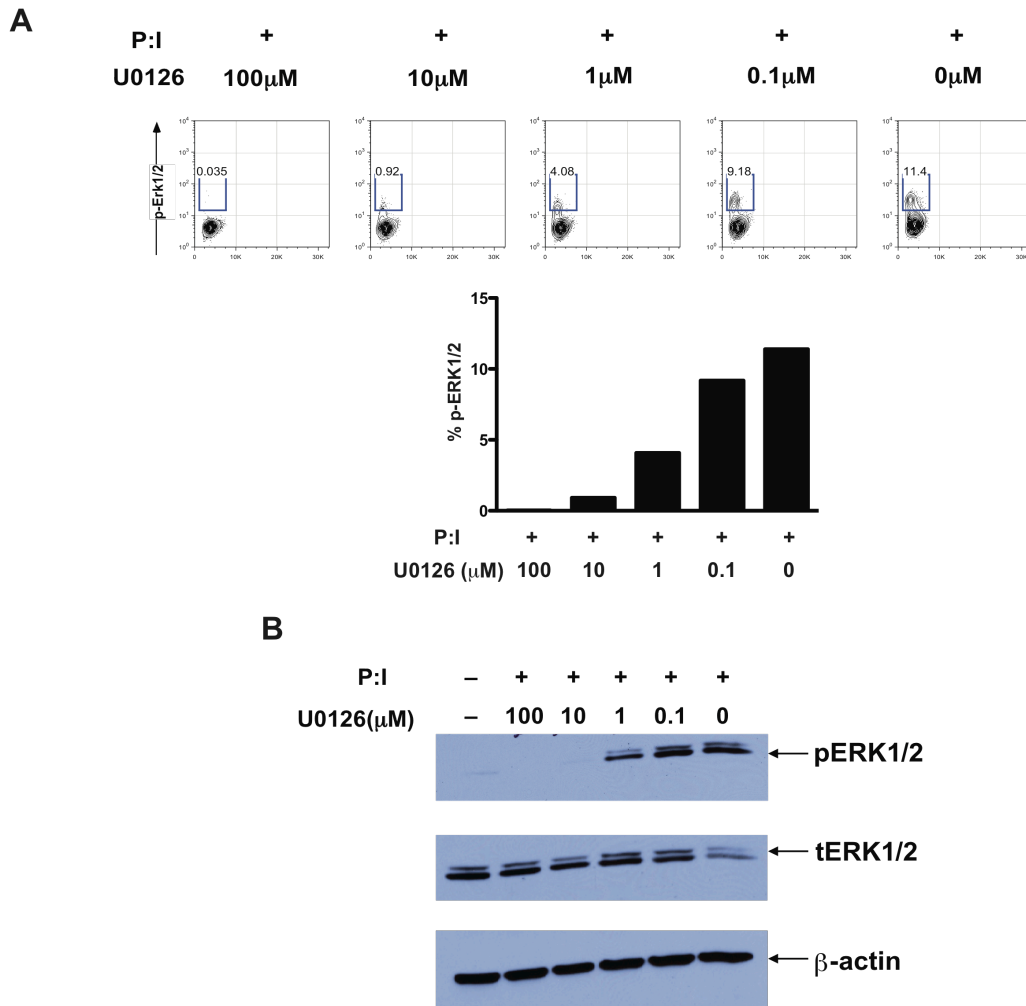
### **U0126 inhibits cytokine production in T cells in a stage-dependent manner**

I first determined the optimal dose of U0126 to suppress ERK1/2 phosphorylation. By both immunoblotting and phospho flow cytometry, I found that 1~10 $\mu$ M U0126 efficiently suppressed ERK1/2 phosphorylation in human T cells *in vitro* (**Figure 25**).

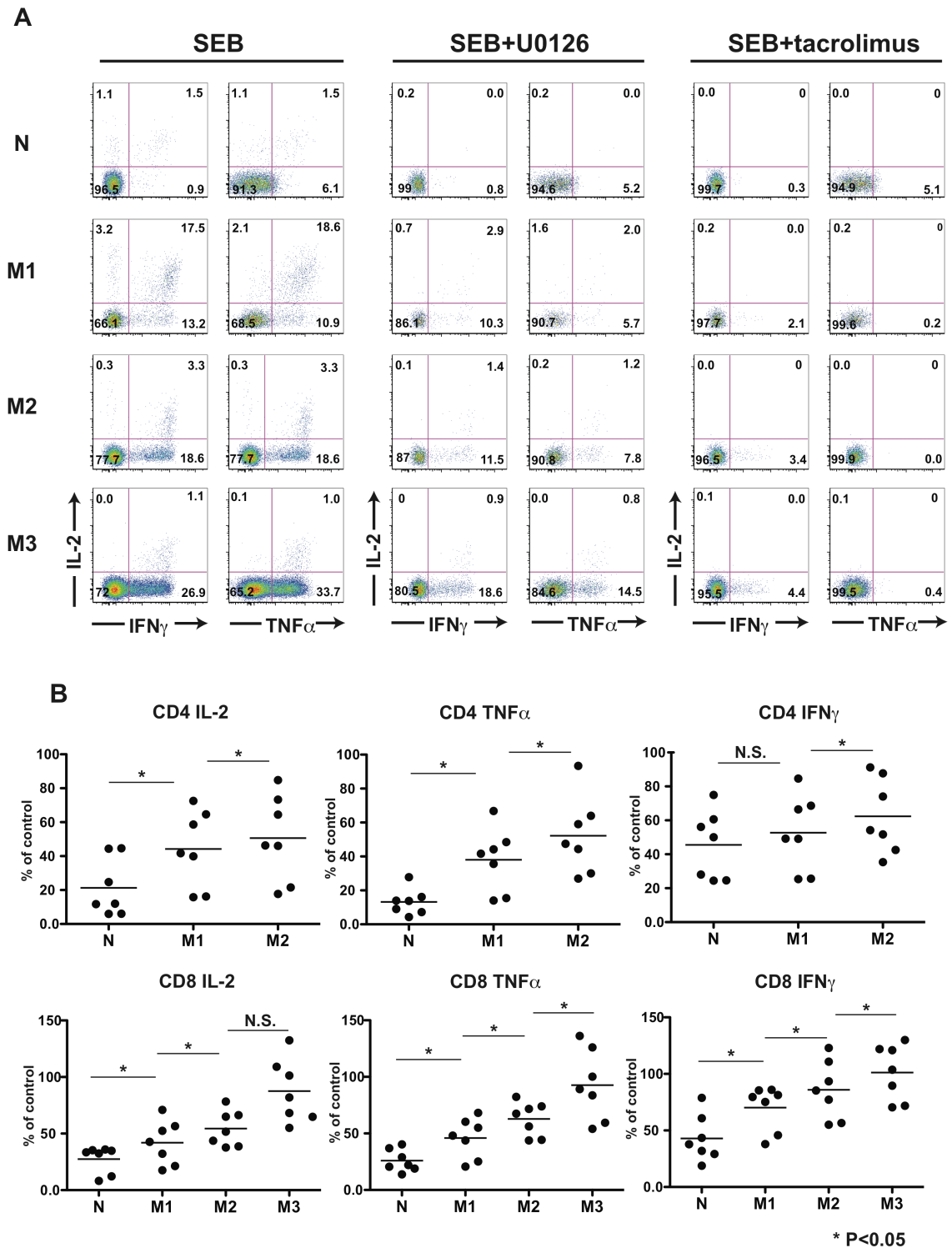
Next, I determined whether U0126 selectively suppresses cytokine production in a stage-dependent manner. Using multi-color cytokine flow cytometry, I stimulated T cells in PBMC obtained from seven healthy donors by SEB in the presence of U0126 and measured IL-2, IFN $\gamma$  and TNF $\alpha$  production in T cell maturation subsets. In CD4<sup>+</sup> T cells, both IL-2 and TNF $\alpha$  production were significantly more suppressed in naïve and early memory subset (Mean reduction of IL-2 production, N: 79%, M1: 56%, M2: 50%, Mean reduction of TNF $\alpha$ , N: 87%, M1: 62%, M2: 48%). In CD8<sup>+</sup> cells, I observed similar pattern of reduction in IFN $\gamma$  and TNF $\alpha$  production following MEK inhibition (Mean reduction of IFN $\gamma$  production, N: 58%, M1: 30%, M2: 15%, M3: 0%, Mean reduction of TNF $\alpha$ , N: 70%, M1: 55%, M2: 38%, M3: 5%) (P<0.05) (**Figure 26**). Notably, a calcineurin inhibitor, tacrolimus, which is widely used in the clinical setting, suppressed the production of all three cytokines regardless of maturation stages at the dose equivalent to the usual therapeutic plasma concentration.

### **U0126 suppresses allogeneic dendritic cell (allo-DC)-induced T cell proliferation with sparing of CMV-specific T cell function**

To determine whether U0126 suppresses allo-DC-induced T cell activation in a degree equivalent to tacrolimus, I set up mixed lymphocyte reactions with mature DC from



**Figure 25. MEK inhibitor, U0126 efficiently suppresses ERK1/2 phosphorylation in T cells.** Using phospho flow cytometry (A), and immunoblotting (B), pERK1/2 was assessed in human T cells stimulated with P:I, in the presence of U0126. For immunoblotting, total ERK1/2 and  $\beta$ -actin were measured for loading control.



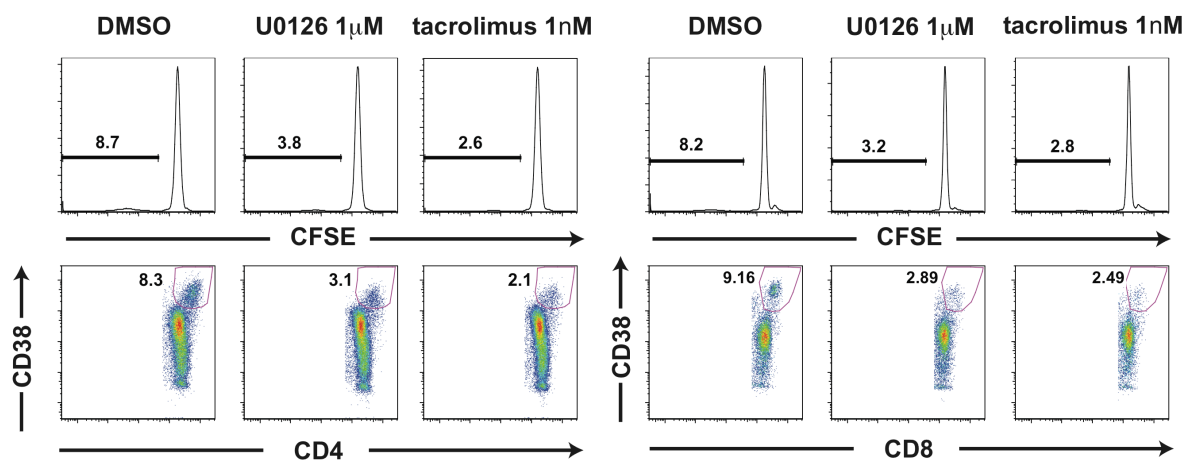
**Figure 26. Naïve and early memory T cells are preferentially inhibited by U0126.** A. Using multi-color cytokine flow cytometry, I stimulated T cells in PBMC by SEB in the presence of 1 $\mu$ M U0126 and 1nM tacrolimus for 6 hours and measured IL-2, IFN $\gamma$  and TNF $\alpha$  production in T cell maturation subsets. B. The ratio of frequency of cytokine positive events treated with U0126 to frequency of cytokine positive events without U0126 treatment (% of control) were measured within CD8+ T cell maturation subsets. Results depict aggregate results from 7 healthy donors. \*  $P<0.05$

human monocytes in the presence of U0126 and tacrolimus. U0126-treated CD4<sup>+</sup> and CD8<sup>+</sup> T cells were ~70% less activated than DMSO-treated cells, as assessed both by up-regulation of the late activation marker, CD38 and by the CFSE proliferation assay. Tacrolimus-treated T cells were suppressed equivalently to U0126 (**Figure 27**). Therefore, U0126 has the potency to suppress *in vitro* allo-DC-induced T cell proliferation at a similar efficiency to tacrolimus.

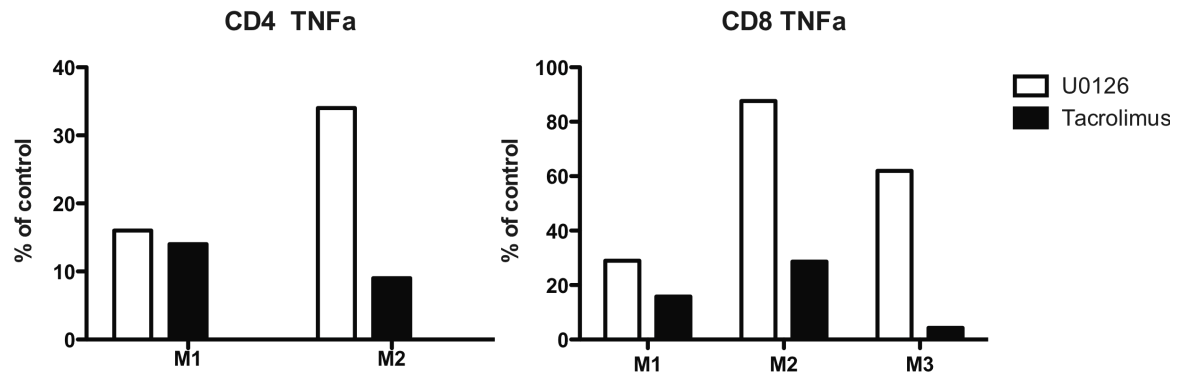
In Chapter 3, I demonstrated that CMV-specific CD8<sup>+</sup> T cells are mostly in M2 and M3 stages (**Figure 13B**). Based on prior studies suggesting that most of allo-specific T cells are derived from the N and M1 (249-254) and my data suggesting MEK inhibition decreases cytokine production in SEB-stimulated T cells in a stage-dependent manner, I tested whether MEK inhibition spares cytokine production in CMV-specific T cells. PBMC from a CMV seropositive donor were exposed to CMV pp65 peptide mix in the presence or absence of U0126 and tacrolimus. 6 hours after, TNF $\alpha$  production were assessed in T cell maturation subsets. Tacrolimus substantially suppressed TNF $\alpha$  production irrespective of maturation stages (CD4<sup>+</sup> M1: 85%, M2: 90%; CD8<sup>+</sup> M1: 80%, M2: 70%, M3: 90%) of both CD4<sup>+</sup> and CD8<sup>+</sup> lineages. However, U0126 did not suppress TNF $\alpha$  production in M2 and M3 stages as much as tacrolimus, although suppression was similar in the M1 stage (CD4<sup>+</sup> M1: 87%, M2: 65%; CD8<sup>+</sup> M1: 75%, M2: 15%, M3: 30%) (**Figure 28**).

These data suggest that MEK inhibition results in potent suppression of allogeneic DC-induced T cell activation. Moreover, MEK inhibition is maturation stage-dependent, and preferentially inhibits naïve and early memory cells thought to be critical for GVHD initiation, while relatively sparing late memory subsets, where CMV-specific immunity is concentrated.





**Figure 27. U0126 suppresses T cell activation induced by allogeneic dendritic cells equivalently to tacrolimus.** For mixed lymphocyte reaction (MLR), CFSE-labeled PBMCs were cultured with allogeneic mature dendritic cells in the presence of U0126 and tacrolimus for 7 days. CFSE dilution and CD38 were measured by flow cytometry in CD4<sup>+</sup> and CD8<sup>+</sup> T cells by sequential gating.

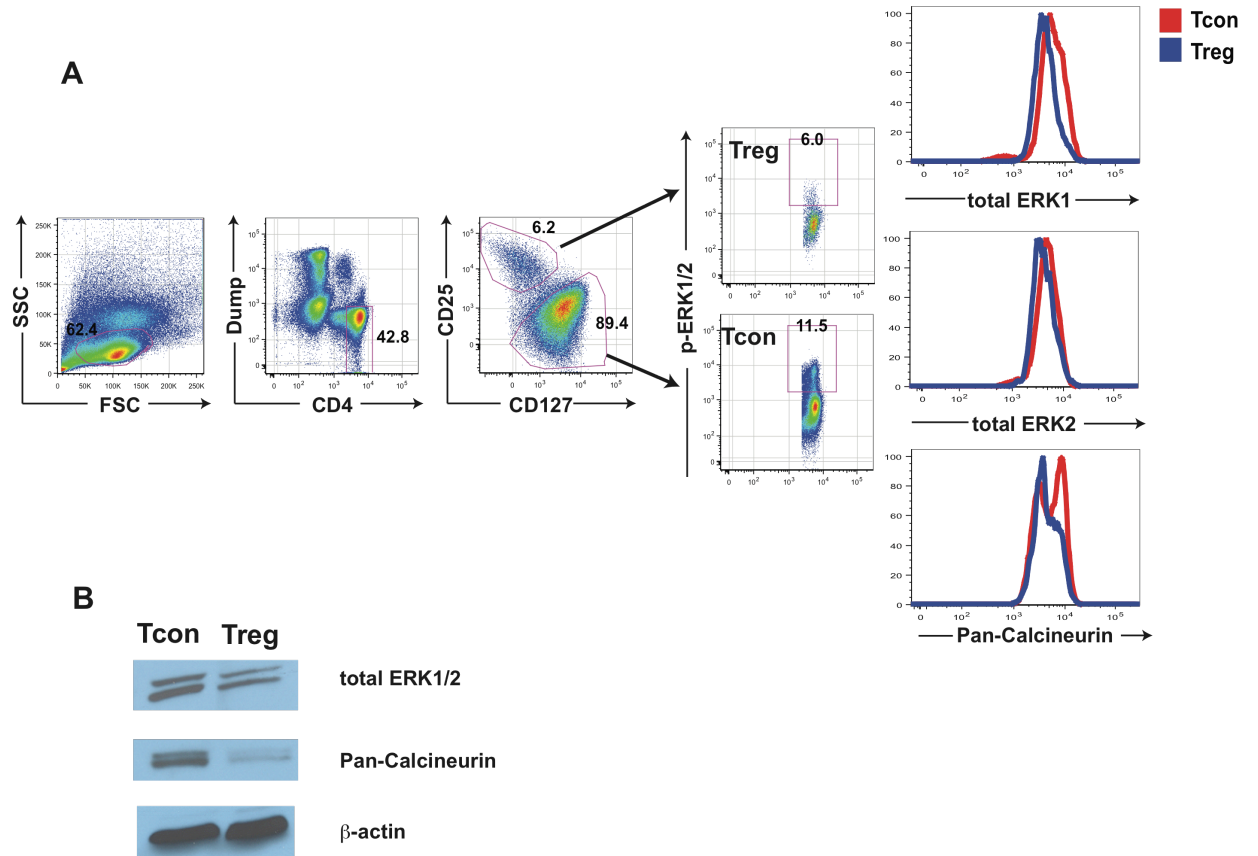


**Figure 28. U0126 selectively spares cytokine production in CMV-specific T cells compared with tacrolimus-treated T cells.** Using multi-color cytokine flow cytometry, I stimulated T cells in PBMC from a CMV seropositive donor by CMV pp65 peptide mix in the presence of 1 $\mu$ M U0126 and 1nM tacrolimus for 6 hours and measured TNF $\alpha$  production in T cell maturation subsets. The ratio of frequency of cytokine positive events treated with U0126 to frequency of cytokine positive events without U0126 treatment (% of control) were measured within CD8<sup>+</sup> T cell maturation subsets. Results depict aggregate results from a representative donor.

## **MEK inhibition does not suppress TGF- $\beta$ secretion in human regulatory T cells (Tregs)**

As explained above, Tregs are a critical player to suppress GVHD in allogeneic stem cell transplantation. Recently, Zeiser *et al.* demonstrated that the function of Tregs is suppressed *in vivo* by the calcineurin inhibitor, Cyclosporine A (CsA) (258). These data suggest that the inhibition of IL-2 production from other T cells by CsA suppresses Treg function. In contrast, Kogina *et al.* showed that tacrolimus, another form of calcineurin-inhibitor promoted proliferation of Tregs, while it did not induce cell conversion to Tregs (259). These contradictory studies suggest that the effect of calcineurin inhibitors on Tregs *in vitro* and *in vivo* needs to be further investigated, since these drugs are widely used for reducing GVHD in the transplantation setting. On the other hand, Luo *et al.* demonstrated that MEK inhibition induced FoxP3 expression in naïve CD4+CD25- T cells and MEK inhibitor-induced CD4+CD25+FoxP3+ T cells had suppressive function *in vitro* (260). More recently, Chen and other colleagues showed that blockade of IL-6 signaling augments Treg reconstitution *in vivo* and reduce GVHD (261). Taken together, these studies suggest that small molecular inhibitors and other types of blockade can manipulate the generation and function of Tregs to suppress GVHD, as an alternative therapeutic approach to *ex vivo* expansion and subsequent infusion of Tregs.

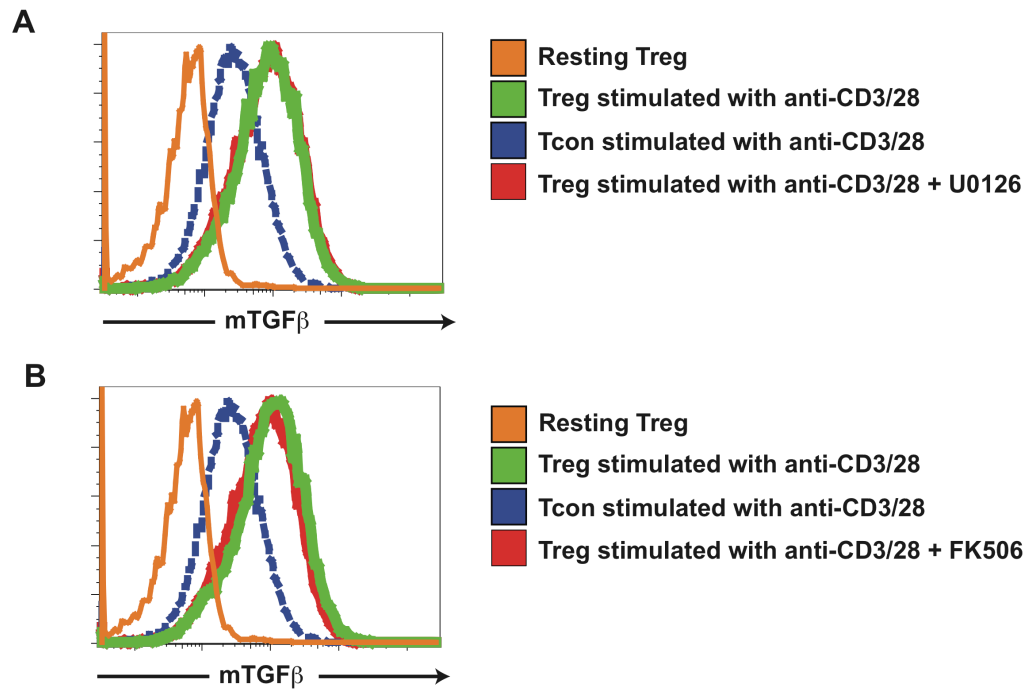
It has been reported that TCR signaling is down-regulated in Tregs (262, 263). For initial characterization, I examined the expression or phosphorylation of three key molecules in the TCR pathway, ERK1, ERK2 and pan-calcineurin in FACS-sorted Tregs defined by CD4+CD25<sup>high</sup>CD127<sup>low</sup> and in conventional CD4+ T cells (Tcons) by molecular flow cytometry and immunoblotting (**Figure 29**). Consistent with prior data (262, 263), I found that following anti-CD3/28 or SEB stimulation, the level of ERK1/2 phosphorylation in



**Figure 29. Regulatory T cells (Tregs) have distinct TCR signaling cascades.** A. Using multi-color phospho/molecular flow cytometry, phospho-ERK1/2, total ERK1, total ERK2 and Pan-calceinurin were assessed in Tregs and conventional CD4<sup>+</sup> T cells (Tcons) in PBMC. A least 300,000 total events were acquired, with sequential gating of PBMC in a lymphocyte region by forward and side scatter, on CD4<sup>+</sup> T cells (excluding unnecessary populations (CD8, CD14, CD19, CD56) and assessment of intracellular phospho-ERK1/2 within CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs, and remaining Tcons following P:I stimulation. Similarly, total ERK1, total ERK2 and pan-calceinurin were assessed in Tregs and Tcons by overlaid histogram (Red: Tcon, Blue: Treg). B. The expression of total ERK1/2 and pan-calceinurin were determined in FACS-sorted Tregs and Tcons by immunoblotting.  $\beta$ -actin indicates loading control.

Tregs was lower than Tcons. The expression of total ERK1/2 was also slightly, but not significantly, down-regulated, suggesting that upstream molecules of ERK1/2 from TCR can be impaired. I also found that pan-calcineurin expression was lower in Tregs than in Tcons. These data suggest that two important arms in TCR signaling to activate T cells are impaired in Tregs.

In order to determine whether MEK inhibition directly affect the function of Tregs, I measured membrane TGF- $\beta$  (mTGF- $\beta$ ) in FACS-sorted Tregs, stimulated by anti-CD3/28 in the presence or absence of U0126 for 6 hours (264). Interestingly, U0126 did not alter the expression of mTGF- $\beta$  in activated Tregs (**Figure 30**). Additionally, tacrolimus did not change the expression of mTGF- $\beta$  in activated Tregs. These data suggest that neither MEK inhibition nor calcineurin inhibition directly affect TGF- $\beta$  secretion in Tregs.



**Figure 30. MEK inhibition does not suppress TGF- $\beta$  secretion in human Tregs.** Membrane TGF- $\beta$  (mTGF- $\beta$ ) in FACS-sorted Tregs, was measured by flow cytometry following stimulation with anti-CD3/28 in the presence or absence of U0126 for 6 hours. Overlaid histogram was shown for comparison (Orange: resting Treg, Green: Treg stimulated with anti-CD3/28, Blue: Tcon stimulated with anti-CD3/28, Red: Treg stimulated with anti-CD3/28 in the presence of U0126 (A) or tacrolimus (B). Tcons stimulated with anti-CD3/28 in the presence of U0126 or tacrolimus have similar intensity as Tcon stimulated with anti-CD3/28 (not shown)

## **CHAPTER 7**

### **DISCUSSION**

## **The unique function of human late memory CD8<sup>+</sup> T cells**

Recent technological advances, including development of improved instrumentation and reagents to facilitate “polychromatic” flow cytometry using monoclonal antibodies recognizing surface and intracellular proteins, have led to the recognition that there is dramatic phenotypic and functional heterogeneity of the peripheral T cell repertoire. To better characterize the relationship between CD8<sup>+</sup> T cell maturation stages and their cytokine signatures, we examined peripheral human CD8<sup>+</sup> T cells in PBMC, stimulated with polyclonal activators and viral antigens, and systematically characterized the production of IL-2, IFN $\gamma$ , TNF $\alpha$  and MIP-1 $\beta$ . My results confirm the following: 1) Late memory (M3) CD8<sup>+</sup> T cells produce little IL-2 (including IL-2/IFN $\gamma$  co-production), but are prolific producers of MIP-1 $\beta$ , MIP-1 $\alpha$  and RANTES; 2) MIP-1 $\beta$  is the most abundant cytokine produced by CMV-specific CD8<sup>+</sup> T cells; 3) Nearly all IL-2 production occurs in N, M1, and M2 CD8<sup>+</sup> T cells, while nearly all IL-2/IFN $\gamma$  co-production occurs at the M1 and M2 stage; and 4) Functional cytokine signature is strongly associated with T cell maturation stage in CD8<sup>+</sup> T lineage and is largely independent of the type of stimulus initiating activation.

Using both TCR-dependent stimulation (e.g., CMV antigens) and stimuli that bypass the TCR, requirements for co-stimulation and proximal signaling events (P:I), I found that maturation stage was closely tied to cytokine signature. While I observed complexity in the various combinations of cytokines/chemokines produced by early and late memory CD8<sup>+</sup> T cells, relatively simple rules could be used to model the effects of maturation on cytokine signature: 1) Early stage CD8<sup>+</sup> T cells, including naïve cells, were more likely to be monofunctional with respect to cytokine/chemokine production, with IL-2 and TNF $\alpha$  production defining their cytokine signature; 2) Differentiation from naïve to the M1/M2



stages of CD8<sup>+</sup> T cells is associated with increasing polyfunctionality, including co-production of IL-2 and IFN $\gamma$ , while progression to the M3 stage is again associated with monofunctionality; and 3) M3 CD8<sup>+</sup> T cells are characterized by the production of abundant amounts of MIP-1 $\beta$  (often in concert with other cytokines) and other CC-chemokines (MIP-1 $\alpha$  and RANTES) and little, if any, IL-2. These results are consistent with the results of Hamann *et al.* (51) and Sandberg *et al.* (87), who also found that CD27-CD8<sup>+</sup> T cells rarely produce IL-2, although they produce perforin following polyclonal stimulation. They also confirm the findings of Duvall *et al.*, who demonstrated that subsets of HIV-2-specific CD8<sup>+</sup> T cells making MIP-1 $\beta$  were more likely to express markers associated with more differentiated maturation states (97).

My analysis of CMV-specific T cells confirmed the results of other studies demonstrating the abundance of later memory cells (i.e., M2 and M3 cells) within this antigen-specific population. In contrast to my expectation that the most abundant product of CMV-specific T cells would be either IFN $\gamma$  or TNF $\alpha$ , which have been most commonly utilized as functional markers of antigen-specific T cells (98, 105, 197, 198, 200, 265, 266), I found that MIP-1 $\beta$  was by far the most abundant cytokine produced by CD8<sup>+</sup> CMV-specific T cells stimulated *ex vivo*. Besides cells producing MIP-1 $\beta$  alone, I found fairly robust numbers of cells producing MIP-1 $\beta$  in conjunction with effector cytokines, including IFN $\gamma$ /TNF $\alpha$ /MIP-1 $\beta$ , IL-2/IFN $\gamma$ /TNF $\alpha$ /MIP-1 $\beta$ , and IFN $\gamma$ /MIP-1 $\beta$  were observed. These data suggest that MIP-1 $\beta$  may actually be a more sensitive marker than IFN $\gamma$  for the detection of CMV-specific T cells, and that conventional approaches (e.g., CFC or ELISPOT assays based on IFN $\gamma$  detection alone) may significantly underestimate the frequency of CMV-specific T cells. In addition, late memory (M3) CMV-specific T cells are also characterized by the lack of IL-2 production.

Taken together, my results establish an unequivocal link between maturation stage and functional cytokine/chemokine signature in CD8<sup>+</sup> T cells, and demonstrate that late memory maturation results in polarization toward CC-chemokine production without IL-2 production. I also found that CMV-specific CD8<sup>+</sup> T cells in healthy donors were heavily skewed toward late memory subsets, and that MIP-1 $\beta$  production dominates the functional signature of the human CMV-specific T cell response. Further studies in model systems and in the context of clinical trials, will be required to determine how the accumulation of late memory cells that are polarized toward CC-chemokine production influences outcomes in the setting of human disease states.

### **The functional role of ERK1 in human CD8<sup>+</sup> T cell maturation subsets**

I demonstrated that late memory CD8<sup>+</sup> T cells are characterized by the loss of IL-2 production, an increase in MIP1-  $\beta$  production and limited co-production of IL-2 and IFN- $\gamma$ . Given prior studies suggesting that the MAP kinase pathway is critical for IL-2 production in T cells (144-147, 152-157), I tested the hypothesis that down-regulation of MAP kinase pathway including ERK1/2 in human CD8<sup>+</sup> T cell maturation subsets contributes to their functional signature. My results confirm the following: 1) ERK1/2 phosphorylation is lower in late memory (M3) CD8<sup>+</sup> T cells than other subsets; 2) the expression of total ERK1, not ERK2, is down-regulated in M3 cells and in a subpopulation of M2 cells; 3) ERK1 expression is strongly associated with IL-2 production and CD28 expression in CD8<sup>+</sup> T cell maturation subsets; and 4) ERK1 is required for IL-2 production in CD8<sup>+</sup> T cell maturation subsets (**Table 2**).

I further investigated the differential expression of TCR signaling molecules in CD8<sup>+</sup> T cell maturation subsets. Phospho/molecular flow cytometry enabled me to

	IL-2	Erk1	Erk2	CD28	MIP1- $\beta$	IFN $\gamma$	IL-2+IFN $\gamma$ +
<b>M1</b>	+++	+++	+++	++	+	++	+++
<b>M3</b>	+/-	+	+++	+/-	+++	++	+/-

**Table 2. Summary of functional and molecular signatures of human late memory (M3) CD8+ T cells.** The uniqueness of M3 CD8+ T cells were described, compared with M1 (early memory) CD8+ T cells.

determine the expression of TCR signaling molecules even in M2 and M3 subsets, where the cell number is less than 15% of CD8<sup>+</sup> T cells. I found that TCR proximal signaling events (phospho-CD3 $\zeta$ , phospho-ZAP-70) are down-regulated due to impaired expression of CD3 $\epsilon$  in M3 cells (**Figure 21 in Chapter 5**). This finding can explain the poor proliferation potential of M3 cells (204). However, since the more distal signaling molecules, ultimately regulating transcription factors may more directly determine functions including cytokine production, I focused on the MAP kinase pathway distal to PKC $\theta$ . PMA and ionomycin directly activate PKC $\theta$  while mobilizing intracellular calcium stores (207); this activation does not require TCR engagement or its most immediate downstream signaling events. I consistently observed M2 and M3 cells express lower amounts of phosphorylated ERK1/2 following P:I stimulation. Additionally, this observation was reproduced in a time-course experiment, suggesting impaired ERK1/2 phosphorylation in M3 cells is due to lower activity of upstream molecules or lower expression of total ERK1/2. In contrast to relatively equivalent expression of upstream molecules (PKC $\theta$ , Ras, c-Raf, MEK1/2) (**Figure 22 in Chapter 5**), ERK1 expression, but not ERK2, was much more down-regulated in M3 CD8<sup>+</sup> T cells. These data suggest that the differential phosphorylation of ERK1/2 depends on the level of ERK1 expression in CD8<sup>+</sup> T cell maturation subsets. More interestingly, I found that M2 CD8<sup>+</sup> cells had a bimodal distribution of ERK1 (ERK1<sup>high</sup> and ERK1<sup>low</sup>), which would have been impossible to discover with traditional techniques such as immunoblotting.

To better understand the relationship between the functional profile of M3 CD8<sup>+</sup> T cells and the down-regulation of ERK1, I developed a multi-color flow cytometry approach to simultaneously detect ERK1, IL-2 and IFN $\gamma$  along with surface markers of differentiation (CD8, CD45RA, C27, CD28 and CD57). I found that 1) most IL-2 is produced by ERK1<sup>high</sup> CD8<sup>+</sup> T cells; 2) CD28 expression is associated with ERK1 expression; 3) the cytokine

production pattern (high production of IL-2 and IL-2+IFN $\gamma$  co-production) and higher CD28 expression in the ERK1<sup>high</sup> subpopulation of M2 cells is close to M1 cells, whereas the features of the ERK1<sup>low</sup> subpopulation of M2 cells (poor production of IL-2 and lack of IL-2+IFN $\gamma$  co-production, no CD28 expression) is similar to M3 cells; and 4) ERK1 expression can predict IL-2 production in CMV-specific CD8<sup>+</sup> T cells. These data suggest that ERK1 expression is strongly associated with IL-2 production and CD28 expression. These data also suggest that ERK1 expression is a more accurate marker of functional memory CD8<sup>+</sup> T cell maturation than the surface markers that are classically used to define maturation.

Knockout models have been utilized to determine the function of one gene in mice and zebrafish. In humans, the manipulation of gene expression has been performed by delivering dominant-negative constructs or gene-expression vectors into cell lines *in vitro* to elucidate the relationship between gene expression and its functional consequence. The recent advancement of delivery systems of gene-expression vector using lentivirus enabled us to examine the function of ERK1 in primary human CD8<sup>+</sup> T cells. I showed that over-expression of ERK1 increased IL-2 production in M2 and M3 cells. These data suggest that IL-2 production is dependent on ERK1 expression in human CD8<sup>+</sup> T cell maturation subsets.

I also determined the association between ERK1 and IL-2 production in CMV pp65 A2<sup>+</sup> T cells. Along with tetramer staining, I found that CMV A2<sup>+</sup> ERK1<sup>high</sup> M2 cells produced higher levels of IL-2 than CMV A2<sup>+</sup> ERK1<sup>low</sup> M2 cells. These observations are consistent with my prior observation suggesting that ERK1 expression is associated with IL-2 production in human CD8<sup>+</sup> T cells. As expected, M3 CMV A2<sup>+</sup> cells rarely produce IL-2. However, it remains to be elucidated why ERK1 expression in CMV A2<sup>+</sup> cells is skewed into the center rather than the bimodal distribution in overall CD8<sup>+</sup> compartment, leading to

relatively ambiguous separation of ERK1<sup>high</sup> and ERK1<sup>low</sup> subpopulations, and why the response of CMV A2+ M1 cells is low.

In conclusion, I demonstrated a strong association between ERK1 expression and IL-2 production in human CD8+ T cells. I also demonstrated that the functional consequence of ERK1 down-regulation in human late memory CD8+ T cells is poor production of IL-2. My studies are one of the first demonstrations that genetic modulation may skew the functional signature of primary human CD8+ T cells. In addition, my studies demonstrated loss of ERK1 expression may explain prior studies demonstrating why late memory CD8+ T cells are functionally impaired with a poor ability to produce IL-2, to respond polyfunctionally and to proliferate in response to stimulation.

### **MEK inhibition decreases cytokine production in human T cells in a stage-dependent manner**

Recent studies suggest that most allo-reactive T cells, which induce GVHD, exist in naïve and early memory stages (249-254), while most CMV-specific T cells are in intermediate and late memory stages (66, 257). Therefore, it would be ideal to selectively inhibit naïve and early memory T cells, and spare later stage memory T cells following allogeneic stem cell transplantation (SCT). In other words, GVHD could be reduced without impairing immunity to CMV reactivation. Therefore, I sought to determine signaling difference between T cell maturation subsets that could be explored to develop more rational immunosuppressive strategies. Given this rationale and my data about differential phosphorylation of ERK1/2 in human T cell maturation subsets (pERK1/2 in N & M1 is greater than pERK1/2 in M2 or M3), I tested the hypothesis that the effects of MEK inhibition on T cell activation depend on levels of ERK1/2 phosphorylation. I found that: 1)

MEK inhibitor (U0126) suppresses cytokine production in T cells in a stage-dependent manner, while a calcineurin inhibitor, tacrolimus at a therapeutic plasma concentration suppressed the production of all three cytokines regardless of maturation stage; 2) MEK inhibitor (U0126) suppresses allogeneic dendritic cell (DC)-induced T cell proliferation while relatively sparing CMV-specific T cell responses; and 3) MEK inhibition does not suppress TGF- $\beta$  secretion in human Tregs.

Very recently, ERK1/2 have been reported to be phosphorylated in T cells in a mouse GVHD model and that MEK inhibition reduces GVHD (267). My results showed that the expression of calcineurin in memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells (not shown for CD4<sup>+</sup> T cells, **Figure 22 in Chapter 5** for CD8<sup>+</sup> T cells) was higher than in the naïve population. If the expression levels of calcineurin reflect the functional significance of the pathway, one could expect that a calcineurin inhibitor, tacrolimus would suppress memory subsets preferentially. In fact, I found that tacrolimus suppressed SEB- induced cytokine production non-selectively in T cell maturation subsets. In contrast to tacrolimus, U0126 suppressed cytokine production preferentially in naïve and early memory stages of T cells. These data suggest that the phosphorylation level of ERK1/2 more accurately reflects the functional significance of the pathway at each maturation stage of T cells, and that MEK inhibition induces T cell memory stage-specific immunosuppression following polyclonal stimulation.

To mimic GVHD initiation with an experimental model, I performed an MLR with mature allogeneic DC and measured CMV-specific T cell responses in the presence of U0126 or tacrolimus. I demonstrated that U0126 suppressed potent allo-DC induced T cell proliferation equivalently to tacrolimus, but spared CMV-specific T cell responses. Tacrolimus, however, suppressed CMV-specific T cell responses in all memory T cell stages. These data suggest that the MEK inhibitor suppressed activation of allo-reactive T

cells, which are mostly in naïve and M1, but did not suppress activation of CMV-specific T cells, which are mostly in M2 and M3.

Tregs also play a role in reducing GVHD, and are potentially targets of immunosuppression, which may undermine the potential benefit MEK inhibition could provide in sparing CMV immunity. Therefore, to determine whether MEK inhibition suppresses Treg function, I measured mTGF $\beta$  in Tregs stimulated with anti-CD3/28 in the presence or absence of U0126. I found that U0126 did not suppress the expression of mTGF $\beta$  in Tregs, suggesting that the MAP kinase pathway is not important in releasing TGF $\beta$ . However, it has to be determined whether the MAP kinase pathway is important in the synthesis of TGF $\beta$ . Interestingly, tacrolimus also did not suppress the expression of mTGF $\beta$  in Tregs. Recent studies suggest that cyclosporine A (CsA) suppresses Treg function, because IL-2 production from other T cells, which is also important for the function of Treg, was suppressed by CsA (258). In contrast, other studies suggested that U0126 increased FoxP3 expression, the number of inducible Tregs, and their suppressive function (260). Taken together, neither MEK inhibitors nor calcineurin inhibitors directly affect the expression of mTGF $\beta$ , but others' data suggest that MEK inhibitors could positively affect the Treg number or function, while calcineurin inhibitors may negatively affect them.

In conclusion, MEK inhibition suppresses T cell activation in a stage-dependent manner, leading to selective immunosuppression of allo-reactive T cells with sparing of the CMV-specific T cell response and the function of Tregs. These experimental models also suggest that MEK inhibition might have therapeutic potential to treat GVHD instead of or in addition to calcineurin inhibitors, although further studies in murine models and humans are necessary.



## **FUTURE PERSPECTIVES**

Collectively, this dissertation demonstrates the functional heterogeneity of human CD8<sup>+</sup> T cell maturation subsets and the role of ERK1 on CD8<sup>+</sup> T cell maturation subsets. More specifically, this dissertation shows that ERK1 is down-regulated in human late memory CD8<sup>+</sup> T cells, leading to poor production of IL-2.

However, there remain potential problems and several important questions regarding the functional signature of late memory CD8<sup>+</sup> T cells. A major limitation of my study was the use of only two markers to define naïve and memory subsets. I acknowledge that the use of additional markers to define naïve and memory subsets, while adding complexity to my experimental approach, would likely have defined both naïve and memory subsets with greater specificity. For example, de Rosa *et al.* (201) found that the addition of a third memory/naïve marker increased the specificity of definition of naïve CD8<sup>+</sup> T cells from up to 87-93% to approximately 96%. A second limitation of this work is that I only studied antigen-specific immunity to only one virus. My studies of CMV-specific CD8<sup>+</sup> T cells should be cautiously interpreted with respect to their relevance to T cell responses to other pathogens. It is very likely that individual viruses differ in their susceptibility to individual mechanisms of CD8<sup>+</sup> T cell control; the caveat that “individual results may vary” should be considered when considering the unique functional profiles that constitute protective immune responses to specific pathogens. Third, the balance between the functions of M3 cells (e.g. chemotaxis) and those of earlier memory cells that appear to be more important for viral clearance (e.g., polyfunctional M1, M2 cells) deserves further investigation. It will also be important to elucidate the mechanisms that govern maturation and migration through activated functional stages and to determine how the production of CC-chemokines is associated with CCR5 expression on T cells and myeloid subpopulations *in vivo*.

Additional questions regarding the molecular signature of late memory (M3) CD8<sup>+</sup> T cells to dictate their function also need to be answered. First, I acknowledge that there must be additional genetic changes that contribute to the function of CD8<sup>+</sup> T cell maturation subsets. I already demonstrated that JNK was also down-regulated in M3 cells, suggesting that the down-regulation of JNK can contribute to poor production of IL-2 in M3 CD8<sup>+</sup> T cells and that JNK-Jun pathway can be also important for maximal IL-2 production in human CD8<sup>+</sup> T cells. Recent studies in mouse models suggest that ERK2 is critical for CD8<sup>+</sup> T cell proliferation and survival (179). The function of ERK2 also needs to be investigated in human CD8<sup>+</sup> T cell maturation subsets. Secondly, it will be critical to better define the role of epigenetic changes associated with phenotypic and functional differentiation. It has been shown that CpG methylation of cytokine promoter regions modify functional cytokine profiles of both CD4 and CD8 memory T cells (268, 269). I observed that naïve CD8<sup>+</sup> T cells do not produce as much IL-2 as M1 cells, although those cells have considerable expression of ERK1. These data raise a possibility that the IL-2 promoter could be hyper-methylated in the naïve population compared to M1 cells. Additionally, the mechanism of ERK1 down-regulation could be direct or indirect epigenetic changes, via methylation or microRNA expression. Finally, it is important to investigate what other pathways are involved in cytokine and chemokine secretion in M3 CD8<sup>+</sup> T cells. Recently, Krishnan *et al.* demonstrated that FcR $\gamma$  and Syk kinase replace CD3 $\zeta$ -chain and ZAP-70 in human effector CD4<sup>+</sup> T cells (270). These studies suggest that there could be other pathways, possibly FcR $\gamma$  and Syk kinase to induce cytokine and chemokine secretion in M3 CD8<sup>+</sup> T cells.

Chapter 6 in this dissertation demonstrates that MEK inhibition suppresses cytokine production in T cells in a maturation stage-dependent manner. It was based on the

hypothesis that the differential activation and expression of ERK1/2 reflect their unique utility in T cell subsets. These data suggest a novel potential therapeutic approach to reduce GVHD while sparing protective immunity against CMV and other critical pathogens in the allogeneic SCT setting. Several questions regarding subset or stage-specific immunosuppression also need to be answered. First, I acknowledge that MEK1-ERK1/2 pathway is not an ideal target for selective inhibition because ERK1/2 expression shows a spectrum through naïve and later stage memory T cells. Accordingly, I observed that cytokine suppression by MEK inhibitor was revealed as a spectrum, not as ‘all or none’. Therefore, it is important to find other targets, which are more exclusively expressed or activated at certain stages of T cell maturation by novel high throughput methods, such as by reverse phase protein array. Our results suggest a proof of concept that stage-specific inhibition is achievable. Secondly, it needs to be determined whether MEK inhibitor reduces T cell survival in a stage-dependent manner. Finally, the effect of MEK inhibitor on reducing GVHD with sparing of virus-specific T cell responses needs to be confirmed in murine models to understand if this strategy might be translated *in vivo* for therapeutic benefit.

## **BIOLOGICAL/CLINICAL SIGNIFICANCE AND CONCLUSIONS**

This dissertation highlighted the functional significance of human late memory CD8<sup>+</sup> T cells. It provided new insight to understand the role of late memory CD8<sup>+</sup> T cells in the physiologic (desired) (e.g. chemotaxis besides cytotoxicity) and pathologic (undesired) immunological processes (e.g. GVHD) in the human body. More importantly, results from these studies will provide a better understanding of the relationship between maturation stage and functional heterogeneity and facilitate ***better immune monitoring strategies*** and ***immune modulation***.

Our group's prior studies of late memory CMV-specific T cells (e.g., those expressing CD57, which defines cells that would most likely have been defined as M3 in this study) suggests that greater frequencies of cells residing within this population are associated with poor control of CMV reactivation after stem cell transplantation, supporting the view that polyfunctional cells producing IL-2 may be better at controlling pathogenic viral infections. Studies by Restifo and colleagues (107) in a cancer immunotherapy model also suggest that late effector T cells, characterized by strong cytotoxic function and IFN $\gamma$  production, may be paradoxically inferior in their ability to control cancer growth. While higher-order cytometric analyses were not reported, this report showed that late effector cells, while producing IFN $\gamma$  and having greater cytolytic capacity, were deficient in IL-2 production. Taken together with prior translational studies in the setting of human infections, my present results suggest that ***human clinical studies in the setting of cancer and infectious disease should consider not only the overall precursor frequency of responsive T cells, but also differentiation stage, polyfunctional capacity (especially that of IFN $\gamma$ /IL-2 co-production) or both.*** Additionally, my results confirm that assays that measure IFN $\gamma$  alone (including ELISPOT assays) will measure cells across the memory spectrum and may significantly underestimate the precursor frequencies of antigen-specific CD8 $^{+}$  T cells, especially those at later stages of the maturation spectrum.

While studies like this one cannot directly confirm mechanisms operative *in vivo*, my results suggest that late memory CD8 $^{+}$  T cells that are abundant producers of MIP-1 $\beta$ , MIP-1 $\alpha$  and RANTES may have a unique functional role in human immune responses. It is known that CCR5 up-regulation occurs with T cell activation (271), raising the possibility that late memory (M2 and M3) cells may have an autocrine or paracrine role in regulating immune responses. Indeed, Wherry, Ahmed and colleagues (272) demonstrated

upregulation of MIP-1 $\beta$  and MIP-1 $\alpha$  mRNA in a murine model, and suggested that chemotactic properties of murine CD8<sup>+</sup> exhausted cells might “sound the alarm”.

Consistent with this hypothesis, MIP-1 $\beta$ , MIP-1 $\alpha$  and RANTES are potent chemotactic factors, inducing migration of monocytes and macrophages to the site of local elaboration.

***This raises the possibility that the activation of late memory CD8<sup>+</sup> T cells (especially those specific for CMV and other herpes viruses that reside mostly in late memory stages) may serve as important attractors of cells capable of clearing debris and magnifying antigen presentation locally.***

My analysis of CMV-specific T cells confirmed the results of other studies demonstrating the abundance of later memory cells (i.e., M2 and M3 cells) within this antigen-specific population. In contrast to my expectation that the most abundant product of CMV-specific T cells would be either IFN $\gamma$  or TNF $\alpha$ , which have been most commonly utilized as markers of antigen-specific T cells, I found that MIP-1 $\beta$  was by far the most abundant cytokine produced by CD8<sup>+</sup> CMV-specific T cells stimulated *ex vivo*. Besides cells producing MIP-1 $\beta$  alone, I found fairly robust numbers of cells producing MIP-1 $\beta$  in conjunction with effector cytokines, including IFN $\gamma$ /TNF $\alpha$ /MIP-1 $\beta$ , IL-2/IFN $\gamma$ /TNF $\alpha$ /MIP-1 $\beta$ , and IFN $\gamma$ /MIP-1 $\beta$  were observed. These data suggest that ***MIP-1 $\beta$  may actually be a more sensitive marker than IFN $\gamma$  for the detection of CMV-specific T cells, and that conventional approaches (e.g., CFC or ELISPOT assays based on IFN $\gamma$  detection alone) may significantly underestimate the frequency of CMV-specific T cells.*** This is relevant for clinical studies of CMV immunity in humans, and similar rules might apply to other disease settings and pathogens.

This dissertation also demonstrated the differential expression of ERK1/2 and its functional consequence in human CD8<sup>+</sup> T cell maturation subsets. Although there have

been several studies examining the molecular signature of T cell maturation subsets and how genetic modulation might dictate T cell memory maturation (80, 119-121), few studies demonstrated a direct link to individual proteins determining functional heterogeneity including IL-2 production in human CD8<sup>+</sup> T cell maturation subsets. Moreover, none of studies highlighted human late memory CD8<sup>+</sup> T cells in humans, in part due to technical limitations such as (a) limited cell number (b) low viability in liquid culture (c) difficulty manipulating gene expression. By overcoming these limitations, I found that ERK1 is highly down-regulated in human late memory CD8<sup>+</sup> T cells and that ERK1 down-regulation leads to poor production of IL-2 in human late memory CD8<sup>+</sup> T cells. These findings suggest that ***loss of ERK1 expression is a more accurate marker to delineate human late memory CD8<sup>+</sup> T cells than classical surface markers*** and that ***ERK1 expression dictates functional heterogeneity in human CD8<sup>+</sup> T cell maturation subsets.***

Adoptive transfer of virus-specific T cells isolated and expanded from a healthy donor, has been shown to be one of the potential therapeutic modalities against human viral infections in allogeneic SCT recipients (273-275). Based on the relationship between polyfunctionality in T cells and virus control, as described in Chapter 1, adoptive transfer of polyfunctional T cells may be critical for a successful immunity against pathogens. One recent study demonstrated that the reintroduction of CD28 in CD28<sup>-</sup> human CD8<sup>+</sup> T cells increased IL-2 production (276). My data suggest that ***polyfunctionality could be reconstituted in late memory CD8<sup>+</sup> T cells by augmenting ERK1 expression by introduction of the gene with a constitutive promoter, leading to a strategy for optimal adoptive transfer to control viral reactivation in the allogeneic SCT setting.***

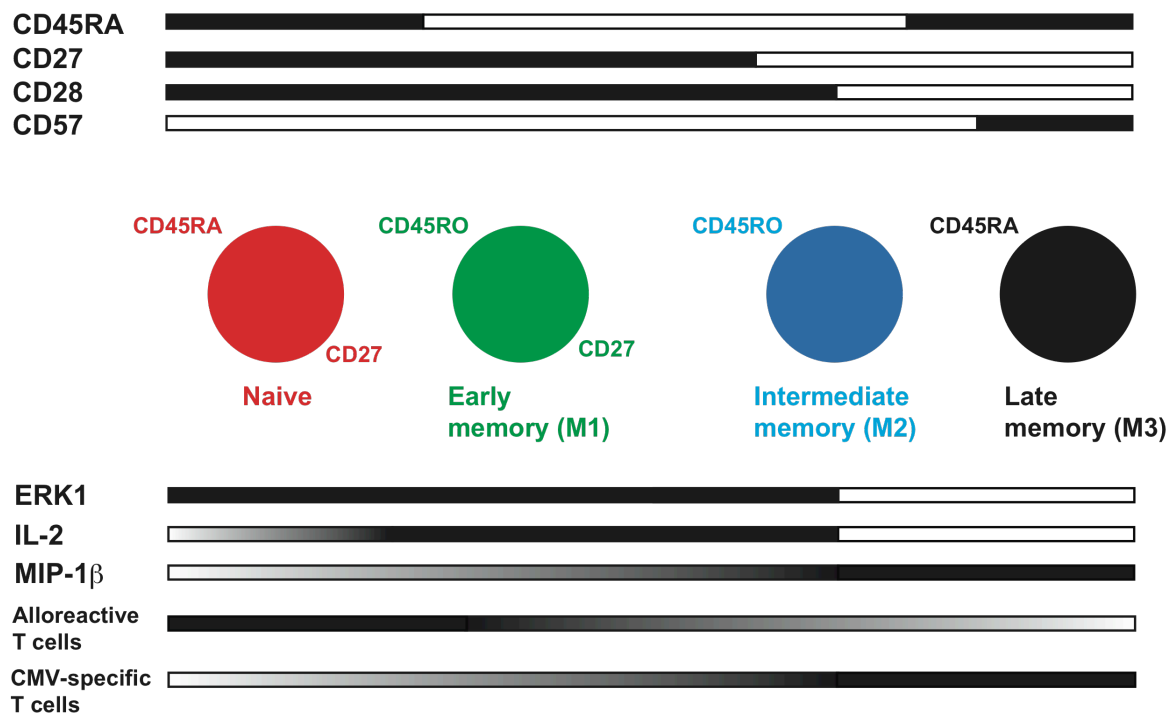
At the end of this dissertation, a novel therapeutic approach to reduce GVHD was introduced based on the observation suggesting that ERK1/2 phosphorylation differs in T

cell maturation subsets. For the last years, small molecular inhibitors or monoclonal antibodies have been used to modulate immune function in humans. Of many available drugs (Cyclosporin A, tacrolimus, steroids etc.), Sirolimus (Rapamycin) has recently been used as an immunosuppressive drug in SCT. Recent studies suggest that Sirolimus has potentiating effects on CD4+CD25+ Tregs (277-279), and that activated conventional CD4+CD25- T cells become highly sensitive to Sirolimus, while Sirolimus rarely induces apoptosis in CD4+CD25+Tregs (277). I hypothesized that differential phosphorylation of ERK1/2 reflects its functional utility in human T cell maturation subsets. I demonstrated that the MEK inhibitor, U0126 suppressed cytokine production in T cells in a stage-dependent manner, and that U0126 efficiently suppressed alloreactive T cell proliferation while sparing CMV-specific T cell responses and preserving TGF $\beta$  production in Tregs. This novel concept provides *a better rationale to selectively inhibit undesired inflammation (GVHD) induced by early stage T cells (naïve and M1) and spare desired immune function by later stage memory T cells (M2, M3) (protective immunity against viral reactivation) in allogeneic SCT*. These results suggest that it may be more practical to find more selective targets and manipulate T cell subsets *in vivo*, to reduce GVHD and restore protective immunity, than to adoptively transfer *ex vivo* expanded T cell subsets.

In conclusion, my studies establish the relationship between functional heterogeneity and molecular signature in human CD8+ T cell maturation subsets (**Figure 31 and Table 2**). I demonstrated in this dissertation that a) human late memory CD8+ T cells have a distinct cytokine production signature characterized by prolific production of MIP-1 $\beta$  (and other CC chemokines) and poor production of IL-2; b) These functional characteristics result from ERK1 down-regulation; c) ERK1 expression is a superior marker to define polyfunctional memory CD8+ T cells. Moreover, these findings can be translated into important clinical

settings a) by reintroducing polyfunctionality in late memory CD8<sup>+</sup> T cells by reexpressing ERK1 via genetic or pharmacological approaches, and b) by suggesting more rational and selective strategies to inhibit GVHD while relatively sparing pathogen-specific memory cells in allogeneic SCT recipients.





**Figure 31. Functional (lack of IL-2 production) and molecular (loss of ERK1) markers are best to define late memory CD8<sup>+</sup> T cells.** Summary of the relationship between functional heterogeneity and molecular signature in human CD8<sup>+</sup> T cell maturation subsets and the rationale for selective inhibition based on differential expression of MAP kinases.

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## **VITA**

Tae Kon Kim was born on January 21, 1976 in the Republic of Korea, the Son of Soon Ock Kim and Kil Hyung Kim. After completing his work at Banpo High School, Seoul, Korea in 1994, he entered Seoul National University in Seoul, Korea in 1994. He completed pre-medicine course in 1996, then received the degree of Doctor of Medicine in 2001, both from Seoul National University, Seoul, Korea. For the next year, he worked as an Intern (Postgraduate Year-1) in Seoul National University Hospital (SNUH), Seoul, Korea. He also achieved the Intern of the Year, 2001 from the SNUH. He then spent two and half years working as a visiting scholar in the University of Pennsylvania from 2002 to 2005. In August of 2005, he entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston, TX. He immediately joined the Immunology program of the University of Texas, M.D. Anderson Cancer Center. He moved to University of Miami with his thesis advisor, Dr. Krishna V. Komanduri in 2008 to complete the Ph.D. work.

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