HIV-1 Infection Abrogates CD8⁺ T Cell Mitogen-Activated Protein Kinase Signaling Responses

Timothy Q. Crawford,¹ Lishomwa C. Ndhlovu,¹ Alice Tan,² Alex Carvidi,² Frederick M. Hecht,³ Elizabeth Sinclair,² and Jason D. Barbour¹*¹

Hawaii Center for HIV/AIDS, John A. Burns School of Medicine, University of Hawaii Manoa, Honolulu, Hawaii;² Division of Experimental Medicine, Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California; and HIV/AIDS Division, Department of Medicine, San Francisco General Hospital, University of California, San Francisco, California

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Mitogen-activated protein kinase (MAPK) signaling pathways are dynamic and sensitive regulators of T cell function and differentiation. Altered MAPK signaling has been associated with the inflammatory and autoimmune diseases lupus and arthritis and with some pathogenic viral infections. HIV-1 infection is characterized by chronic immune inflammation, aberrantly heightened CD8⁺ T cell activation levels, and altered T cell function. The relationship between MAPK pathway function, HIV-1-induced activation (CD38 and HLA-DR), and exhaustion (Tim-3) markers in circulating CD8⁺ T cells remains unknown. Phosphorylation of the MAPK effector proteins ERK and p38 was examined by "phosflow" flow cytometry in 79 recently HIV-1-infected, antiretroviral-treatment-naïve adults and 21 risk-matched HIV-1-negative controls. We identified a subset of CD8⁺ T cells refractory to phorbol 12-myristate 13-acetate plus ionomycin-induced ERK1/2 phosphorylation (referred to as p-ERK1/2-refractory cells) that was greatly expanded in HIV-1-infected adults. The CD8⁺ p-ERK1/2-refractory cells were highly activated (CD38⁺ HLA-DR⁺) but not exhausted (Tim-3 negative), tended to have low CD8 expression, and were enriched in intermediate and late transitional memory states of differentiation (CD45RA⁻ CD28⁻ /CD27⁺⁻). Targeting MAPK pathways to restore ERK1/2 signaling may normalize immune inflammation levels and restore CD8⁺ T cell function during HIV-1 infection.

Activation of ERK, and p38 MAPK signaling molecules modulates T cell function, exerting differential effects on T cell development, cell cycle progression, and apoptosis (8, 14, 26). ERK signaling is critical for positive selection, promotes cell cycle progression, and inhibits apoptosis (13, 19, 20), while p38 signaling is necessary for negative selection, promotes cell cycle arrest, and induces apoptosis (1, 12). Alterations in ERK signaling have been associated with chronic inflammatory autoimmune conditions such as lupus and rheumatoid arthritis (15, 25) and with pathogenic viral infections (30). Several viral proteins are known to interact with MAPK signaling pathways (29). Attenuated ERK1/2 phosphorylation responses to T cell receptor stimulation have been observed in uninfected peripheral blood mononuclear cells (PBMCs) in HIV-1 infection (18).

HIV-1 disease is characterized by immune inflammation, with highly elevated CD8⁺ T cell-activation levels and lower levels of CD4⁺ T cell-activation, measured by joint surface expression of CD38 and HLA-DR markers. A set point CD8⁺ T cell-activation level is established in early untreated HIV-1 infection and predicts clinical outcome independently of plasma HIV-1 RNA levels (9). However, the functional significance of CD38 and HLA-DR coexpression on CD8⁺ T cells, a population that is not infected by HIV-1, has not been resolved. A detailed understanding of the functional changes to activated CD8⁺ T cells may aid in the development of therapeutic strategies to halt or reverse HIV immunopathogenesis.

HIV-1-associated CD8⁺ T cell activation has been linked to atypical T cell differentiation, (5) a process that involves MAPK signaling pathways (11). Previous studies of HIV-1-infected adults have reported altered CD8⁺ T cell differentiation profiles, specifically, a large expansion of transitional intermediate/late memory (CD45RA⁻ CD28⁻ CD27⁺⁻) subsets and a reduction in the proportion of naive (CD27⁺ CD28⁺ CD45RA⁺) subsets (2, 3, 22). An expansion of intermediate memory cells during HIV-1 infection may have negative functional consequences, such as increased CD8⁺ T cell replicative senescence or a failure to differentiate into functional effectors (28). In contrast, CD8⁺ T cells in the "terminally differentiated" CD45RA⁺ CD27⁺ pool, referred to as the effector/memory RA (EMRA) pool, exhibit enhanced effector activities (27). An expanded T_EMRA CD8⁺ T cell population has been associated with a lower viral load set point in early HIV-1 infection (21).

To evaluate MAPK signaling in activated CD8⁺ T cells during early untreated HIV-1 infection, we implemented a flow cytometry-based signaling assay termed "phosflow" (7, 24). Phosflow combines multiparameter phenotyping of surface antigen expression with simultaneous detection of phosphorylated forms of intracellular signaling protein intermediates. We examined ERK (ERK1/2) and p38 phosphorylation responses to phorbol 12-myristate 13-acetate and ionomycin

* Corresponding author. Mailing address: Hawaii Center for HIV/AIDS, Department of Tropical Medicine, John A. Burns School of Medicine, 651 Halo Street, BSQ 325B, University of Hawaii Manoa, Honolulu, HI 96813. Phone: (415) 287-0959. Fax: (510) 355-1082. E-mail: jbarbour@hawaii.edu.
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(PMA+I) stimulation at the single-cell level in T cell subsets defined by expression of CD38, HLA-DR, and Tim-3. PMA is an analog of diacylglycerol, a key mediator of MAPK signaling through protein kinase C (PKC) (4). Ionomycin stimulates Ca2+ release from the endoplasmic reticulum, activating Ca2+-sensitive enzymes and synergizing with PMA (6). PMA+I is a potent stimulator of MAPK signaling cascades, resulting in the accumulation of phosphorylated, kinase-active ERK1/2 and p38 signaling intermediates (10). We hypothesized that activated CD38+ HLA-DR+ CD8+ T cells would display intact but attenuated MAPK signaling responses in HIV-1-infected adults compared to HIV-1-negative controls. Our findings did not confirm our hypothesis but instead revealed a novel, large population of highly activated CD8+ T cells not merely lacking robust MAPK pathway signaling responses but also displaying a complete abrogation of signaling through the ERK1/2 MAPK module.

MATERIALS AND METHODS

Study subjects. Cryopreserved PBMCs were selected from the OPTIONS cohort study of early HIV-1 infection in San Francisco. The first clinical visit was always prior to administration of antiretroviral treatment. Early HIV-1 infection was identified as previously described (17). HIV-1-negative risk-matched controls were identified through OPTIONS project screening of adults with suspected HIV-1 sexual exposure who subsequently were found to be HIV-1 negative. All persons gave informed consent to participate, and the UCSF Committee on Human Research approved this study.

Cell culture, staining, stimulation, and flow-cytometric analysis. Cryopreserved PBMCs stored by the UCSF/AIDS Research Institute (ARI) AIDS Specimen Bank were thawed, and 1 × 10⁶ cells were immediately stained for T cell immunophenotypic markers. The remaining cells were allowed to rest for 18 h in 5% rPMI (RPMI 1640 medium; UCSF Cell Culture Facility [CCF]) supplemented with 5% fetal bovine serum (FBS) (JR Scientific). CD27 from eBiosciences, and Tim-3 from R&D Systems.

RESULTS

Clinical cohort description. Frozen peripheral blood mononuclear cells (PBMCs) were obtained from the OPTIONS Project cohort study of early HIV-1 infection (San Francisco General Hospital, University of California, San Francisco). The study included 79 recently HIV-1-infected adults and 21 HIV-1-negative adult risk-matched controls (Table 1). PBMC samples were obtained during early infection prior to initiation of an antiretroviral treatment (HIV-1-infected patients) or during screening for HIV-1 infection that subsequently proved negative (controls). HIV-1-infected adults had lower CD4+ counts and higher CD8+ T cell activation than controls and had been infected an estimated median 98 days at the time of the study (Table 1).

A subset of CD8+ T cells refractory to ERK1/2 phosphorylation is expanded in early untreated HIV-1 infection. To examine MAPK pathway intracellular signaling events in T cell subsets, phosflow analysis was performed on PBMCs to detect phosphorylation of ERK1 and -2 (p-ERK1/2) and p38 (p-p38). Figure 1 illustrates a typical example of the CD8+ T cell p-ERK1/2 and p-p38 response to PMA+I stimulation and shows an increase in median fluorescence intensity (MFI) of p-ERK1/2 and p-p38 expression relative to unstimulated controls. Strikingly, in HIV-1-infected subjects, a large population of CD8+ T cells do not respond to PMA+I stimulation and have a p-ERK1/2 MFI similar to that of the unstimulated control (Fig. 1A, panel i). The resulting bimodal p-ERK1/2 histogram profile was gated into two distinct CD8+ subsets based on p-ERK1/2 responsiveness. The histogram peak containing cells shifted positively along the p-ERK1/2 axis with a labeled p-ERK1/2, and this subset is referred to as p-ERK1/2 responsive. The term "refractory" was chosen to reflect the fact that while this CD8+ T cell subset exhibits an impaired p-ERK1/2 response after 15 min of PMA+I stimulation, the cells might still retain the capacity to respond through ERK1/2 with different signaling kinetics. As illustrated in Fig. 1A and in Table S1 in the supplemental material, the frequency of CD8+ p-ERK1/2-refractory T cells was significantly expanded in HIV-1-infected individuals relative to uninfected controls (median frequencies [IQRs], 12.1 [9.0, 12.1, 28.7] for the infected group and 7.1 [3.4, 10.4] for the uninfected group; P < 0.0001). The fraction of CD8+ T cells

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median value (IQR) for group</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>38.1 (32.4, 40.9) NA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HIV-1 RNA level</td>
<td>4.7 (4.2, 5.1)</td>
</tr>
<tr>
<td>CD4+ T cell count (cells/µl)</td>
<td>1,038 (816, 1,133) 593 (466, 750)</td>
</tr>
<tr>
<td>% Male (no.)</td>
<td>45.3 (35.9, 90.5) 27.5 (19, 96.2)</td>
</tr>
<tr>
<td>% p-ERK1/2 refractory at time of study</td>
<td>0 (0, 0) 76 (72, 130)</td>
</tr>
<tr>
<td>Time since infection (days)</td>
<td>NA 98 (72, 130)</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not applicable. <sup>b</sup> ART, antiretroviral treatment.
falling within the p-ERK1/2-refractory gate was also significantly increased in HIV-1-infected individuals relative to uninfected controls (medians [IQRs], 3.1 [2.3, 5.1] for the infected group and 1.8 [1.4, 2.8] for the uninfected group; \( P < 0.0007 \)) (Fig. 1B and data not shown), although the effect was more modest than for CD8\(^+\) T cells. The frequency of CD8\(^+\) p-ERK1/2-refractory T cells exhibited a significant correlation with viral load (Spearman \( r = 0.39, P < 0.0007 \)) but not with CD4\(^+\) T cell count (Spearman \( r = -0.02, P = 0.8 \)).

To examine the relationship between activation and the p-ERK1/2-refractory population, CD8\(^+\) T cells were divided into subsets with the activation markers CD38 and HLA-DR. We found that p-ERK1/2-refractory cells were primarily contained within the highly activated CD38\(^{+}\)HLA-DR\(^{+}\) population (Fig. 1A, panel iv). In HIV-1-infected patients, the frequency of CD8\(^+\) p-ERK1/2-refractory cells strongly correlated with CD38\(^{+}\)HLA-DR\(^{+}\) frequency (Spearman \( r = 0.71, P < 0.0001 \)).

To define the p-ERK1/2-refractory phenotype, CD8\(^+\) T cells were gated for the presence or absence of CD38, HLA-DR, and Tim-3 markers individually, and histogram profiles of p-ERK1/2 MFI in the single-marker subsets were examined (Fig. 2). Of the p-ERK1/2-refractory CD8\(^+\)T cells in HIV-1-infected subjects, 69.9% (IQR, 53.1, 84.1) were CD38\(^{+}\) and 50.7% (IQR, 39.0, 70.4) were HLA-DR\(^{+}\), but only 11.7% (IQR, 8.6, 16.6) were Tim-3\(^{+}\). We then examined the MFIs of CD38, HLA-DR, and Tim-3 in p-ERK1/2-refractory and -responsive populations. The CD38 MFI of p-ERK1/2-refractory cells was significantly higher than that of the p-ERK1/2-responsive population (medians [IQRs], 705 [397, 1,221] for p-ERK1/2-refractory cells and 262 [224, 341] for p-ERK1/2-responsive cells; \( P < 0.0001 \)). We observed a similar difference for HLA-DR (medians [IQRs] 835 [545, 1,548] for p-ERK1/2-refractory cells and 289 [225, 415] for p-ERK1/2-responsive cells; \( P < 0.0001 \)), while Tim-3 expression showed the opposite pattern (medians [IQRs] 74.6 [67.0, 88.3] for p-ERK1/2-refractory cells and 129 [110, 149] for p-ERK1/2-responsive cells; \( P < 0.0001 \)). Together, these data indicate that...
To explore the p-ERK1/2-refractory phenotype, individually gated CD38, HLA-DR, and Tim-3 subsets (Fig. 2) were used to generate eight Boolean gate populations in both HIV-1-infected and uninfected populations (Fig. 3). As expected, we observed significant expansions of activated (CD38⁺ HLA-DR⁺ Tim-3⁺) subsets, and a reduction in the nonactivated (CD38⁻ HLA-DR⁻ Tim-3⁻) subset, in recently HIV-1-infected patients versus controls (Fig. 3A). The frequency of p-ERK1/2-refractory cells was significantly increased in 7 of 8 Boolean subsets in HIV-1-infected patients versus uninfected controls (Fig. 3B). Notably, the highly activated CD38⁺ HLA-DR⁺ Tim-3⁻ subset contained the largest proportion of p-ERK1/2-refractory cells in both HIV-1-infected and control subjects (medians [IQRs], 47.6% [32.1, 66.8] for the infected group and 15.4% [9.7, 36.7] for the uninfected group) (Fig. 3B, columns 3 and 4), and the level was significantly higher in HIV-1-positive patients (P > 0.0001).

Next, we examined the magnitude of the PMA+I-induced p-ERK1/2 response in CD38, HLA-DR, and Tim-3 Boolean gated subsets, recorded as fold change (FC) in p-ERK1/2 MFI upon stimulation. The p-ERK1/2 FC response tended to be lower for the HIV-1-infected group than for uninfected controls in all subsets. Significant differences between infected and uninfected groups were observed in highly activated CD38⁺ HLA-DR⁺ Tim-3⁻ and CD38⁺ HLA-DR⁻ Tim-3⁻ populations (Fig. 4A). There was no significant difference in p-ERK1/2 FC between groups in any of the CD8⁺ T cell populations that did not express CD38. In HIV-1-infected patients, the activated CD38⁺ HLA-DR⁺ Tim-3⁻ subset exhibited the lowest p-ERK1/2 response (FC = 2.47 [IQR, 1.28, 3.93]). The reduced magnitude of p-ERK1/2 FC response in highly activated subsets was not attributable to differences in unstimulated (basal) p-ERK1/2 levels, as there were no significant differences in p-ERK1/2 MFIs between CD38⁺ HLA-DR⁺ double-positive subsets and subsets not expressing HLA-DR (Fig. 4B, compare column 1 with column 5 and column 3 with column 7). Taken together with the finding that almost 50% of CD8⁺ T cells in the CD38⁺ HLA-DR⁻ Tim-3⁻ compartment were p-ERK1/2 refractory (Fig. 3B), these results indicate that the reduced p-ERK1/2 responsiveness (p-ERK1/2 FC) observed in the highly activated CD38⁺ HLA-DR⁺ Tim-3⁻ subset in HIV-1-infected individuals is primarily due to the large percentage of p-ERK1/2-refractory cells present in this compartment, not simply an attenuated but intact per-cell responsiveness.

Similar to the p-ERK1/2 response, the PMA+I-induced p38 phosphorylation response (fold change in p-p38 MFI) was significantly reduced in highly activated CD38⁺ HLA-DR⁺ subsets compared to populations not coexpressing these two markers (left four columns of Fig. S2A in the supplemental material). However, unlike with p-ERK1/2, significantly higher unstimulated basal p-p38 levels were observed in CD38⁺ HLA-DR⁺ subsets (Fig. S2B). These data indicate that, in contrast to p-ERK1/2, reduced p-p38 fold changes in activated CD8⁺ T cell compartments during early untreated HIV-1 infection are at least partially driven by higher basal levels of p38 phosphorylation.

Impairment of p-ERK1/2 responses, which participate in cell cycle and cell differentiation processes in CD8⁺ T cells (13),...
might associate with the shifts in T cell maturation profiles observed in HIV-1 infection. We therefore examined the co-expression of CD45RA, CD28, and CD27 in PMA+i-stimulated samples stained for p-ERK1/2 expression. Five maturation stages were defined and are described in Materials and Methods. As previously described, recently HIV-1-infected, treatment-naïve patients exhibited significantly elevated frequencies of intermediate (CD45RA-CD28+CD27-) and late (CD45RA-CD28-CD27-) memory maturation stages relative to HIV-1-negative controls (Fig. 5A; see also Table S1 in the supplemental material).

Figure 5B illustrates the proportion of p-ERK1/2-refractory cells that were identified within each of the five maturation stages (see Materials and Methods for descriptions of the stages). We observed p-ERK1/2-refractory cells in all stages in both HIV-1-infected and uninfected subjects, but they differed substantially in distribution by HIV infection status and differentiation state. The frequency of p-ERK1/2-refractory cells was lowest in the naïve (CD45RA+CD28+CD27+) compartment and increased in proportion through to the late memory stage (CD45RA-CD28-CD27-) in both HIV-1-infected and HIV-1-negative individuals (Fig. 5B). With the exception of naïve-phenotype CD8+ T cells, the p-ERK1/2-refractory population was significantly expanded or trended toward significant expansion in every maturation stage in recently HIV-1-infected, treatment-naïve adults relative to HIV-1-negative adults. These expansions of the p-ERK1/2-refractory population were most pronounced in the intermediate (CD45RA-CD28+CD27+) and late (CD45RA-CD28-CD27-) memory populations. These are the same two memory differentiation stages that are themselves expanded in frequency in HIV-1 infection (2, 3, 22).
DISCUSSION

We observed a highly expanded p-ERK1/2-refractory CD8+ T cell population residing within the activated (CD38+ HLA-DR+) CD8+ T cell compartment in HIV-1-infected adults. In contrast, we did not observe a large expansion of a p-ERK1/2-refractory population in CD4+ T cells in HIV-1 infection. In HIV-1-negative risk-matched controls, we observed a smaller, lower-frequency population of p-ERK1/2-refractory CD8+ T cells, suggesting that a functional blockade in the ERK1/2 MAPK pathway is a normal, if far less common, process in CD8+ T cells in healthy adults. CD8+ T cells refractory to phosphorylation of ERK1/2 not only were highly activated but also were enriched in intermediate (CD45RA- CD28+ CD27+) and late memory (CD45RA- CD28- CD27+) CD8+ T cell populations, which are greatly expanded in HIV-1-infected persons (3, 22). However, we found the ERK1/2-refractory phenotype to be present in both CD28+ and CD28- CD8+ T cells, suggesting that the loss of PMA+I-induced ERK1/2 responsiveness was not due exclusively to the loss of CD28 expression, which signals through ERK1/2.

In contrast, CD8+ T cells bearing the immunomodulatory receptor Tim-3 did not display this refractory signaling phenotype, suggesting that a proportion of Tim-3-bearing cells retain the ability to respond to certain stimuli during HIV-1 infection (16) and may be readily converted to effectors through blockade of Tim-3 ligands. Tim-3, commonly referred to as an exhaustion marker, may mark a population of CD8+ T cells that, while strongly repressed in exhibiting effector activities, retain functional signaling pathways and perhaps full effector activities, given the appropriate stimuli. Additionally, we observed the appear-
ance of a bilobed CD8+ population among HIV-1-infected individuals not apparent in HIV-1-negative risk-matched controls (Fig. 2; also Fig. S1 in the supplemental material and data not shown) (23). We observed that the majority of activated (CD38+/HLA-DR+) CD8+ “low” cells exhibited the p-ERK1/2-refractory phenotype (data not shown).

In this study, we found evidence for and against the previously reported higher basal MAPK pathway phosphorylation in HIV-1-infected persons (24). Specifically, we found that p38 basal phosphorylation levels were higher in activated (CD38+/HLA-DR+) CD8+ T cell subsets, which likely contributed to the lower fold changes in p-p38 MFI observed in activated compartments (see Fig. S2 in the supplemental material). In contrast, reduced fold changes in p-ERK1/2 MFI observed in activated CD8+ T cells was not due to higher basal phosphorylation levels (Fig. 4B) but instead was driven primarily by the presence of cells refractory to potent PMA+I stimuli (Fig. 3B). These data suggest that HIV-1 infection, and associated activation levels, may differentially impact ERK1/2 and p38 MAPK modules.

An abrogated ERK1/2 signaling response indicates the presence of a block in the MAPK pathway. PMA induces ERK1/2 phosphorylation through activation of PKC, which in turn positively modulates activity of the Raf scaffolding protein upstream of ERK1/2 (4). This suggests a functional impediment either at the level of PKC or in one of the downstream components in the pathway, such as Raf, MEK, or ERK1/2 itself. Alternatively, low ERK1/2 expression levels or the induction of phosphatases with specificity for ERK1/2 might confer the observed phenotype. Abrogation of ERK1/2 signaling in a large fraction of CD8+ T cells could have multiple deleterious functional consequences. These include reduced T cell prolif-
eration, altered differentiation profiles, changes to apoptotic programs, and altered effector functions, all of which are observed in CD8+ T cells in HIV-1 infection. Targeting MAPK pathways to restore ERK1/2 signaling may normalize immune inflammation levels and restore CD8+ T cell function during HIV-1 infection.

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REFERENCES