Published in final edited form as:

J Acquir Immune Defic Syndr. 2012 July 1; 60(3): 244–248. doi:10.1097/QAI.0b013e31825a313c.

Increased Program Cell Death - 1 (PD-1) Expression on T Lymphocytes of Patients with Progressive Multifocal Leukoencephalopathy (PML)

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Abstract

The cellular immune response is important in the containment of progressive multifocal leukoencephalopathy (PML). We examined program cell death-1 (PD-1) expression, a marker of cellular immune exhaustion, on T-lymphocytes in PML. PD-1 expression was elevated on total CD4⁺ and CD8⁺ T-cells (medians 36% and 24%) in PML patients compared to healthy control subjects (medians 14% and 18%; p=0.0015 and p=0.033). In PML patients, JCV-specific CD8⁺ cytotoxic T-lymphocytes expressed PD-1 more frequently than total CD8⁺ T-lymphocytes (means 39% and 78%, p=0.0004). Blocking the PD-1 receptor increased **JCV-specific T-cell immune response** in a subgroup of PML patients.

Introduction

Progressive multifocal leukoencephalopathy (PML) is an often fatal brain disease caused by the reactivation of the human polyomavirus JC (JCV)¹. While asymptomatic JCV primary infection occurs in up to 80% of the general population², reactivation resulting in PML is observed mainly in immunosuppressed individuals, including 3-7% of HIV-positive patients³⁻⁴. The events leading to JCV reactivation and disease induction are not completely understood. Although anti-JCV antibodies do not prevent viral reactivation, the presence of JCV-specific CD8⁺ cytotoxic T-lymphocytes (CTL) is associated with containment of PML progression and improved survival⁵. Furthermore, the detection of a JCV specific cellular immune response in healthy individuals suggests this response is likely instrumental in preventing PML⁶. While some patients survive PML, more than half die within weeks to

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months⁷. Thus, better understanding of pathogenesis of JCV cellular immune response is necessary in devising targeted therapy.

T-lymphocyte exhaustion, a form of T-cell dysfunction, is commonly found in chronic viral infections, autoimmunity, and in tumor lysis⁸. The inhibitory receptor, programmed cell death 1 (PD-1), is a member of the CD28 family and is expressed on activated T-cells, natural killer T cells (NKT), B-cells, and macrophages⁹. Exhausted CD8⁺ T-cells express high levels of PD-1 in many chronic human viral infections including HIV¹⁰. Binding of PD-1 to its' ligands, PD-L1 and PD-L2, renders T-lymphocytes anergic, preventing proliferation and the production of interleukin-2 (IL-2). Furthermore, blockade of the PD-1/PD-L1 pathway resulted in increased expansion of activated virus-specific T-cells, lower viral load, and extended life span in monkeys with chronic SIV infection¹¹⁻¹². Recently, increased PD-1 expression levels was associated with decreased HIV-specific effector memory CD8⁺ T-cells in HIV progressors, and blockade of this pathway restored the function and proliferation of these CD8⁺ T-cells¹³.

Little is known of effects of immune exhaustion on JCV-specific CTL in the context of PML. Specifically, it is important to elucidate whether JCV reactivation in HIV infected patients would further enhance PD-1 expression and increase immune exhaustion. Here we examined the contribution of PD-1 to host cellular immune dysfunction in PML patients with and without HIV.

Materials and Methods

This study was approved by the Beth Israel Deaconess Medical Center institutional review board and all subjects provided written consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. Direct staining was performed using fluorescently conjugated antibodies specific for CD8-FITC (clone SK1), CD4-PE (clone L200), CD3-PerCP Cy5.5, (clone SP34.2), and PD-1-APC (clone EH12.2H7) as previously described¹⁴. To control for inter experimental variations of the flow cytometry, an isotype control, IgG₁ (clone MOPC-21, Biolegend), was used at 10ug/ml as per manufacturer's recommendation. For the blocking experiments, we used unconjugated PD-1 antibody (clone EH12.2H7) per manufacturer's instructions and previously published methods¹⁵. Intracellular staining for IFN- γ and tetramer staining using previously mapped A*0201-restricted JCV VP1_{p36} and _{-p100} peptides were conducted as previously described.¹⁴ All antibodies were obtained from either BD Biosciences (San Jose, CA) or Biolegend (San Diego, CA). Data were acquired by flow cytometry on BD FACSCalibur (Becton Dickinson, New Jersey) and analyzed using FlowJo (Treestar, Oregon). Comparison of PD-1 expression levels between study groups were analyzed with Mann-Whitney U test. Statistical analysis was performed using Prism 5.

Results

Study population

We enrolled 33 subjects, including 10 HIV-positive PML patients (8 male, 2 female), 10 HIV-negative PML patients (4 male, 6 female), 6 HIV-positive patients without PML (6 male), and 7 healthy controls (4 male, 3 female). The healthy control group had a median age of 25 (range 23-29) and were younger than the other groups. The median age of the HIV-positive group was 45 (range 38-73) and equal to the median age of the HIV-positive PML group (range 20-52). The HIV-negative PML group was older than the others with a median age of 63 (range 24-80). HIV-positive PML patients had lower median CD4 count (176/ μ l, range 12-800/ μ l) compared to the HIV-positive patients (741/ μ l, range 425-782/ μ l). While all patients in the HIV-positive group were on combined antiretroviral therapy,

nine out of ten of the HIV-positive PML patients were also on therapy. HIV-positive PML patients were farther from their PML diagnosis (median 257 days, range 4-2970 days) than HIV-negative PML patients at the time of testing (median 51 days, range 8-718 days). The immunosuppressive diagnosis of the HIV-negative PML patients were CLL(3 patients), sarcoidosis, multiple sclerosis with natalizumab treatment, dermatomyositis, lung transplantation, rheumatoid arthritis with methotrexate treatment, lymphomatoid granulomatosis, and NK cell leukemia. One subject in the HIV-positive PML group was also hepatitis C positive.

Increased PD-1 expressions in CD4⁺ and CD8⁺ T-cells of PML and HIV-infected patients

The mean percentages of CD4⁺ T-cells expressing PD-1 was elevated in both the HIV-positive and the HIV-negative PML groups(39% and 33%). The means of these two groups combined was elevated as compared to the healthy control group (36% vs 13%). The medians were significantly elevated as compared to the median in the healthy controls group (36% vs 14%, $p=0.0015$)(Figure 1A). However, the PML group did not have significantly different median PD-1 expression levels in CD4⁺ T-cells compared to HIV-positive control group (36% vs 42%, $p=0.65$). Similarly, both the HIV-positive and HIV-negative PML groups had elevated mean PD-1 expression levels in CD8⁺ T-cells (28% and 32%). The combined mean of these two groups were elevated as compared to the healthy control group (30% and 14%), and comparison of the medians were significantly different (24% vs 18%, $p=0.033$)(Figure 1B). The PML group did not have significantly different median PD-1 expression levels in CD8⁺ T-cells compared to the HIV-positive control group (24% vs 36%, $p=0.07$).

Increased PD-1 expression on JCV specific CD8⁺ cytotoxic T-lymphocytes

Reliable detection of JCV-specific CD8⁺ CTL in HLA A*0201⁺ patients requires stimulating PBMC with previously mapped A*0201-restricted JCV VP1_{p36} and _{p100} peptides in culture for 10 days in the presence of IL-2. Therefore, 8 paired PBMC samples, comparing fresh and cultured PD-1 expression on CD8⁺ T-cells, were used to assess possible augmentation of PD-1 expression caused by culturing condition. PD-1 staining of CD8⁺ T-cells was performed on PBMC from 8 (4 HIV-positive and 4 HIV-negative) subjects within 24 hours of blood collection and again after 10 days of culturing with JCV VP1 peptides. There was no significant difference of PD-1 expression between the fresh and the cultured cells (data not shown).

Next, PBMC from 4 HIV-positive and 6 PML (1 HIV-negative, 5 HIV-positive) HLA A*0201⁺ subjects were cultured with JCV VP1 peptides, VP1_{p36} and _{p100}, prior to staining with anti-PD-1 as well as anti-CD8 antibodies and JCV VP1_{p36} and _{p100} tetramers. Experiments above did not show significantly different PD-1 expression levels of CD8⁺ T-cells between the HIV-positive PML and HIV-negative PML groups. We therefore combined these two groups for further analysis. In PML patients, JCV specific CD8⁺ CTL had elevated levels of PD-1 expression compared to PD-1 expression levels on total CD8⁺ T-cells (Figure 1C). However, the HIV-positive control group had no detectable differences of PD-1 expression in JCV-specific CD8⁺ CTL and total CD8⁺ T-cells. These results suggest that immune exhaustion is associated with JCV-specific CD8⁺ T-cells in PML patients.

Blocking PD-1 may augment JCV-specific immune response in PML patients

We next explored whether *in vitro* blockade of PD-1 expression would augment the function of JCV-specific CD8⁺ T-cells in 11 experiments performed in 7 subjects (4 healthy controls and in 3 HIV-positive PML patients). In one of the healthy control subjects with absent JCV-specific CD8⁺ T-cells for either epitope peptide before blockade, blocking PD-1 did

not result in detection of these cells. Likewise, the IFN- γ expression of CD8⁺ or CD4⁺ T-cells did not change after PD-1 blockade. In three other healthy controls with absent JCV-specific CD8⁺ T-cell for one of two epitope peptides at baseline, blocking PD-1 did not result in detection of these cells either. However, two of these three individuals had detectable JCV-specific CD8⁺ T-cells for the other epitope peptide at baseline, and PD-1 blockade resulted in the expansion of these cell populations in three of four distinct experiments. For example, in a healthy control subject with detectable JCV tetramer positive cells prior to blockade, blocking PD-1 augmented JCV tetramer positive cells from 1.78% to 7.45% (data not shown). Similarly, the IFN- γ expression level of total CD8⁺ T-cells increased from 0.38% to 4.62% after PD-1 blockade. However, the IFN- γ expression level of CD4⁺ T-cells did not change (0.1% before to 0.29% after blocking).

We performed 5 PD-1 blocking experiments in 3 HIV-positive PML patients. Two patients were long-term PML survivors (HIV+/PML S, determined by >1 year survival from PML diagnosis) and had no detectable changes in the number of JCV epitope-specific **T-cell responses** after PD-1 blockade. In one of these two PML survivors, there were increased expression of IFN- γ in both the JCV_{p36} and _{-p100} stimulated CD4⁺ T-cells in one experiment and increased expression of IFN- γ in JCV_{p100} stimulated CD8⁺ T-cells in two separate experiments. For example, results of one PML survivor are shown in Figure 2. After blocking PD-1, the percentage of CD8⁺ T-cells expressing PD-1 decreased from 17.1% to 0.6% (Figure 2A) and JCV tetramer positive cells changed from 0.3% before to 0.1% after blockade (Figure 2B). The IFN- γ expression level in CD8⁺ T-cells remained at 0.1% while the IFN- γ expression in CD4⁺ T-cells changed from 0.08% to 0.04% (Figure 2B). Interestingly, in an HIV-positive early PML patient (HIV+/PML E, still alive within 6 months of PML diagnosis), blocking PD-1 augmented JCV-specific **T-cell immune response in one of two experiments**. Indeed, while PD-1 blocking decreased PD-1 expression on CD8⁺ T-cells from 38.6% to 3% (Figure 2A), the JCV tetramer positive cells increased from 8.3% to 15% (Figure 2B). The IFN- γ expression of total CD8⁺ T-cells increased from 3.6% to 8.6%, and the IFN- γ expression of total CD4⁺ T-cells increased from 0.01% to 0.09% (Figure 2B). These results suggest that *in vitro* blockade of PD-1 receptors on CD8⁺ T-cells may increase JCV-specific T-cell function in a subset of PML patients.

Discussion

We have shown that both HIV-positive and HIV-negative PML patients harbor lymphocytes with significant PD-1 expression, suggesting that this cellular marker might play a role in the pathogenesis of PML. Better understanding of factors associated with immune dysregulation leading to uncontrolled JCV proliferation may aid in the development of therapies for this deadly disease.

This study is the first to demonstrate a potential role that immune exhaustion may play in loss of JCV-specific cellular immune response. We have shown that blocking the PD-1 receptors leads to an increase of JCV-specific CD8⁺ CTL in a subgroup of patients. Furthermore, concurrent increase of IFN- γ expression indicates a possible increase in lymphocyte functions. These increases are detected in both healthy and HIV-positive PML subjects with detectable JCV-specific CTL prior to blockade. While blocking PD-1 cannot generate *de novo* JCV-specific cellular immune response **in either healthy controls or PML patients**, blocking PD-1 may increase JCV-specific CD8⁺ T-cell response in a patient with early PML and increase IFN- γ expression in T-lymphocytes in some PML survivors. We postulate that PD-1 blocking may better augment existing JCV-specific CD8⁺ CTL response and improve cellular immune control of JCV in subjects with active JCV infection. It is possible that further increase in JCV-specific CTL may be obtained by interference with additional receptors, including the PD-L1 and PD-L2 ligands.

There are several limitations to our study. PML is a rare disease and the number of patients available is limited; thus increasing variability of the clinical characteristics in our study groups. For instance, the HIV-positive PML group had lower CD4⁺ T-cell counts as compared to the HIV-negative group. The reduction of CD4⁺ T-cells may contribute further to T-cell exhaustion and enhanced PD-1 expression in the remaining cells. However, our data showed similar percentages of CD4⁺ T-cells and CD8⁺ T-cells with PD-1 expression in the two groups. Although the underlying causes of immunosuppression leading to PML may also contribute to immune exhaustion, there was no significant difference in PD-1 expression in T-lymphocytes of HIV-positive and HIV-negative PML patients. Therefore, we postulate that active JCV-infection and resulting immune response was responsible for the increase in PD-1 expression. We initially hypothesized that co-infection of JCV and HIV would result in greater immune exhaustion as compared to infection with HIV alone. Our data demonstrate that presence of both viruses does not appear to have an additive effect on immune exhaustion. The relative contributions of HIV and JCV in enhancement of PD-1 expression need further investigation. Additional PD-1 blockade experiments are needed to explore the full potential of repairing immune function in PML patients through the PD-1 pathway. In order to measure JCV-specific tetramer positive cells, our blocking experiments were limited to HLA_A*0201 positive PML patients. Future studies performed on a more diverse group of PML patients will help better define genetic and other factors that may influence PD-1 blockade. Finally, the effects of other immune exhaustion receptors should also be explored, including the membrane glycoprotein T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3), which was detected in terminally differentiated Th1 cells¹⁶. Lastly, while it is outside the scope of this study, PD-1 expression on lymphocytes specific for other viral infections in HIV patients needs to be investigated.

PML patients harbor an exhausted immune phenotype with elevated expression of PD-1 on their T-cells. Reversing immune exhaustion by blocking the PD-1 receptor, in conjunction with other immunotherapies¹⁷ may prove valuable in the containment of PML.

Acknowledgments

This work was conducted with support from Harvard Catalyst | The Harvard Clinical and Translational Science Center (NIH Award #UL1 RR 025758 and financial contributions from Harvard University and its affiliated academic health care centers). In addition, NIH grants R01 NS 047029, NS 041198, and K24 NS 060950 supported IJK, and NIH grant K08 NS 064215-01 supported CST. SG is funded by NIH grant T32 AI07387-21 and is a fellow of the Clinical Investigator Training Program: Beth Israel Deaconess Medical Center – Harvard/MIT Health Sciences and Technology, in collaboration with Pfizer Inc. and Merck & Co. IJK consults for Geneuro, Cytheris, Perseid, Vertex, Johnson and Johnson.

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Figure 1. Percentage of PD-1⁺ CD4⁺ and CD8⁺ T-cells in PML patients and controls

Blocking PD-1 receptors increased JCV-specific CTL response to JCV VP1_{p36} restricted by HLA A*0201 in a PML early patient but not in a PML survivor. Seven million PBMC were incubated with blocking antibody, anti-human CD279 (PD-1) (clone EH12.2H7) at 10ug/ml half-hour prior to addition of HLA-A*0201-restricted nonamer epitope peptides of the JCV VP1 protein, VP1_{p36}-SITEVECF or VP1_{p100}-ILMWEAVTL, at final concentrations of 2.5 μg/ml and 1 μg/ml, respectively. IL-2 was added 72 hours later; and every 2 days thereafter, half the medium was replaced. PD-1 blocking antibody was replenished on day 7. Intracellular staining assay was performed after 11 days in culture. HIV+/ PML E: HIV-positive PML early patient who was within 6 months of PML diagnosis; HIV+/PML S: HIV-positive PML survivor who was at least 1 year after PML diagnosis. Shaded histograms indicate IgG isotype control. Numbers on upper right quadrant indicate percentage of cells.

Figure 2. JCV-specific T-cell response can be augmented by blocking PD-1 in some patients

While PML patients with and without HIV have significantly elevated median PD-1 expression on CD4⁺ T-cells (A) and CD8⁺ T-cells (B) compared to healthy controls, their PD-1 expression levels on T-cells are similar to HIV-positive patients without PML. PD-1 staining was performed with anti-human CD279 (PD-1) (clone EH12.2H7, Biolegend) at 25 μ g/ml, and IgG₁ (clone MOPC-21, Biolegend) was used as isotype control at 10 μ g/ml as per manufacturer's recommendation. JCV-specific CD8⁺ T-cells had significant elevated levels of PD-1 expression when compared to total CD8⁺ T-cells in PML patients with and without HIV (C). This difference did not reach significance in the HIV-positive study group. Seven million PBMC of three HIV-positive patients and six patients with PML were stimulated with HLA-A*0201-restricted nonamer epitope peptides of the JCV VP1 protein, VP1_{p36}-SITEVECF or VP1_{p100}-ILMWEAVTL, at final concentrations of 2.5 μ g/ml and 1 μ g/ml, respectively. Cells were cultured in RPMI 1640-12% FCS in a well of a 12-well plate. After 72 h, an equal volume of medium containing 50U of recombinant interleukin-2 (IL-2) was added to each well, and every 2 days thereafter, half of the medium was replaced. Cells were then stained with JCV VP1_{p36} and -p100 tetramers on days 10-11 of culture. Horizontal line illustrates median of each group and Mann Whitney U test was used to compare groups in A) and B). Bar graphs express means and standard deviations of means and paired t-test was used to compare groups in C).