Increased Programmed Death-1 Expression on CD4⁺ T Cells in Cutaneous T-Cell Lymphoma

Implications for Immune Suppression

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Objectives: To investigate the expression profile of programmed death-1 (PD-1) on T cells derived from patients with cutaneous T-cell lymphoma (CTCL), analyze a potential mechanism responsible for upregulation of PD-1, and assess the correlation between blockade of its signaling pathway and improvement in immunological function.

Design: Translation research study.

Setting: University medical center.

Participants: Patients with Sézary syndrome, patients with mycosis fungoides, and healthy volunteers.

Main Outcome Measures: Programmed death-1 expression on T cells by flow cytometry and interferon-γ (IFN-γ) production by enzyme-linked immunosorbent assay.

Results: We report significantly increased PD-1 expression on CD4⁺ T cells from patients with Sézary syndrome compared with CD4⁺ T cells from patients with mycosis fungoides and healthy volunteers. Both CD26⁻ and CD26⁺ populations of CD4⁺ T cells demonstrated increased expression of PD-1, which was upregulated by the engagement of the T-cell receptor with anti-CD3/CD28 antibodies. In addition, blockade of the signaling pathway with blocking antibodies to PD-1 or its ligand PD-L1 led to an increase in the capacity to produce IFN-γ among some patients. Finally, longitudinal studies of 1 patient revealed a progressive decrease in PD-1 expression on CD4⁺ T cells with improvement of clinical disease.

Conclusion: Our data imply that increased PD-1 expression in Sézary syndrome may play a role in attenuating the immune response and provide further insight into the immunosuppressive nature of CD4⁺ T cells in Sézary syndrome and suggest another potential means of targeted therapy for these patients.

respectively.\textsuperscript{10} Programmed death-1, a member of the B7-C4D28 family, has 2 known ligands, PD-L1 and PD-L2; PD-L1 is expressed on many cell types, such as T cells, dendritic cells, and tumor cells, whereas PD-L2 expression is limited to antigen-presenting cells.\textsuperscript{11} Engagement of PD-1 by its ligands transduces a signal that leads to inhibition of T-cell function, including proliferation and cytokine production.\textsuperscript{12} Therefore, it is postulated that a major function of PD-1 is to attenuate the immune response.

Increased expression profiles of PD-1 have been identified in models of defective immune function, including chronic viral infection\textsuperscript{13-15} and adult T-cell leukemia/lymphoma,\textsuperscript{16} indicating a role in disease progression and immunosuppression. Murine models have demonstrated the crucial role that PD-1 plays in maintenance of self-tolerance and prevention of autoimmunity.\textsuperscript{17} In these models, alteration of the PD-1 gene and/or complete knockout of the gene has led to diabetes mellitus,\textsuperscript{18} lupus,\textsuperscript{19} and autoimmune cardiomyopathy.\textsuperscript{20} It has been proposed that the loss of PD-1 may lower the threshold for antigen recognition in peripheral tissues evidenced by increased numbers of antigen-specific cytotoxic cells and increased cytokine production when compared with wild-type mice.\textsuperscript{21} Furthermore, blockade of the PD-1/PD-L1 pathway results in the restored ability to proliferate and secrete cytokines.\textsuperscript{13,22-24}

In this study, we investigate the expression profile of PD-1 on T cells derived from patients with CTCL, and its functional significance for IFN-γ production. Our data imply a role for PD-1 in attenuating the immune response and antitumor immunity, providing further insight into the immunosuppressive nature of CD4+ T cells in CTCL and the potential for immunotherapy.

**METHODS**

**PATIENTS**

Blood samples were collected from healthy donors (HDs), and from patients with MF and SS who were seen and followed at the University of Pennsylvania Cutaneous Lymphoma Clinic, Philadelphia. Donation of blood by patients and HDs conformed to the protocols approved by the University of Pennsylvania institutional review board, and written informed consent was obtained. Patients were considered to have a low to medium tumor burden if their percentage of circulating malignant T cells with the flow cytometric phenotype of CD4+ CD26− ranged from 20% to less than 50% of total lymphocytes. Patients whose percentage of circulating phenotypically abnormal T cells was 50% or higher were considered to have a high tumor burden.\textsuperscript{25} Peripheral blood cells from a total of 21 patients with SS, 4 patients with MF (skin-only involvement without blood involvement with less than 5% CD4+ CD26− T cells), and 5 age-matched HDs were evaluated over the course of this study. The diagnoses of SS and MF were made clinically, histologically, and by flow cytometry of the blood and conformed to the criteria of the International Society for Cutaneous Lymphomas and the European Organization of Research and Treatment of Cancer.\textsuperscript{26}

**PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION AND CULTURE**

Venous blood was collected into heparinized syringes using uniform standards. The heparinized blood was diluted 1:2 with phosphate-buffered saline (PBS) and was layered on a Ficoll-Hypaque gradient (Amersham, Pittsburgh, Pennsylvania). The gradient was centrifuged at 2400 rpm for 30 minutes at room temperature. The peripheral blood mononuclear cell (PBMC) band was collected and washed twice in PBS. Cells were cultured in medium (RPMI 1640; Life Technologies, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (all from Gibco-BRL, Grand Island, New York).

**FLOW CYTOMETRY**

To detect PD-1 expression on T cells, approximately 1 million PBMCs per sample were stained with CD8 fluorescein isothiocyanate, CD26 phycoerythrin, CD4 peridinin chlorophyll protein complex, and PD-1 allophycocyanin (all from BD Pharmingen, San Jose, California) for 30 minutes on ice. Cells were analyzed with the FACS Calibur flow cytometer using CELLQuest software (BD Biosciences, San Jose) at the Flow Cytometry and Cell Sorting Core, Abramson Cancer Center, University of Pennsylvania. Approximately 50,000 cells were collected for each analysis.

**ELISA FOR CYTOKINE ANALYSIS**

Culture supernatants for cytokine analysis or assessment of PD-1 expression on cultured CD4+ T cells by flow cytometry.

**STIMULATION WITH ANTI-CD3/CD28 ANTIBODIES: ASSESSMENT OF PD-1 EXPRESSION AND IFN-γ LEVELS AFTER PD-1/PD-L1 BLOCKADE**

Freshly isolated PBMCs from patients and HDs were plated at a density of 1 × 10⁶/mL per well in the presence or absence of anti-CD3/CD28 antibodies (R&D Systems, Minneapolis, Minnesota) (0.5 µg each) with medium alone, isotype control antibody (ebioscience, San Diego, California) (17 µg/mL), purified anti PD-1 (R&D Systems) (17 µg/mL), or purified anti–PD-L1 (ebioscience) (17 µg/mL). Cells were incubated at 37°C in 5% carbon dioxide for 72 hours prior to the collection of supernatants for cytokine analysis or assessment of PD-1 expression on cultured CD4+ T cells by flow cytometry.

**STATISTICAL ANALYSIS**

Data are expressed as means (SDs) in Figure 1 for tested individuals. Statistical significance was determined using the t test when applicable.

**RESULTS**

**SIGNIFICANT INCREASE IN CIRCULATING PD-1+CD4+ T CELLS FROM PATIENTS WITH SS COMPARED WITH CD4+ T CELLS FROM THOSE WITH MF OR HDs**

Increased levels of PD-1 or its ligand PD-L1 have been observed on so-called exhausted T cells during chronic viral infections and on various tumor cells.\textsuperscript{13,15,16,23,27,28}
We postulated that a similar increase in PD-1/PD-L1 expression would be evident on T cells of patients with SS. Seven patients with SS (of a total of 14 patients tested), 4 patients with MF, and 5 age-matched HDs were tested for PD-1 expression in association with CD4, CD8, and CD26 by flow cytometry using antibody against PD-1. The PD-1+CD4+ T cells were significantly increased in patients with SS compared with CD4+ T cells from patients with MF or age-matched HDs (P < .01) (Figure 1). The percentage of PD-1+CD8+ T cells from patients with SS, although increased in comparison to those from HDs, was not statistically significant (P = .05) (data not shown). Expression of PD-L1 or PD-L2 was not detected on either population of T cells. However, variable and/or low-level expression of PD-L1 (range, 0.5%-7.4%) was observed on CD14+ monocytes from some, but not all, HDs and patients (data not shown). It is noteworthy that the CD4+CD26+ population from patients with SS (range, 20%-98% of CD4+ T cells) showed a small, but statistically insignificant (P = .42), upregulation of PD-1 expression compared with the CD4+CD26+ population (range, 2%-80% of CD4+ T cells) (Figure 1).

T-CELL RECEPTOR ACTIVATION AND UPREGULATION OF PD-1 EXPRESSION ON CD4+ T CELLS FROM PATIENTS WITH SS

To further investigate the underlying mechanisms responsible for upregulated PD-1 expression on patients’ CD4+ T cells, we examined the effect of anti-CD3/CD28, IFN-α, or IFN-γ on the expression of PD-1. The engagement of the T-cell receptor by anti-CD3/CD28 led to enhanced expression of PD-1 on CD4+ T cells from 12 patients with SS. Stimulation of PBMCs from patients with a low to medium tumor burden (and from HDs; data not shown) with anti-CD3/28 resulted in an increase in the percentage of PD-1 expression on both CD4+CD26− and CD4+CD26+ T cells, and an increase in density of PD-1 molecules on the surface of those cells (mean fluorescent intensity [MFI], 1.2- to 1.3-fold increase, respectively) (Figure 2A). In contrast, patients with a high tumor burden did not demonstrate an increased percentage of PD-1+ within CD4+CD26+ T cells. However, an increase in MFI (1.0-fold) suggests an increase in density of PD-1 molecules on some CD4+CD26+ T cells on TCR stimulation. The CD4+CD26+ population maintained the ability to upregulate expression of PD-1 on anti-CD3/CD28 engagement, resulting in an increased percentage of CD4+PD-1+ T cells and intensity of PD-1 expression on cell surfaces (1.2-fold increase in MFI) (Figure 2B).

Both IFN-α and IFN-γ have been used as therapeutic modalities for CTCL, and consequently we theorized that they might play a role in the expression of PD-1. However, findings from cultures of PBMCs from patients and HDs for 3 days with either interferon (at 10 ng/mL) did not alter the expression profile of PD-1 on CD4+ T cells. Both interferons also failed to upregulate PD-L1 on T cells, but in concordance with previously published data, IFN-γ, and, to a lesser extent, IFN-α were able to upregulate expression of PD-L1 on monocytes (data not shown).²⁹

**PD-1/PD-L1 BLOCKADE AND ENHANCED IFN-γ PRODUCTION**

The expression of PD-L1 on monocytes of some patients (4 patients; range, 0.7%-7.4% of CD14+ T cells), although low, suggested the potential for the engagement of the PD-1/PD-L1 pathway to affect the end production of cytokines, such as IFN-γ. To analyze the effect of blocking PD-1 and PD-L1 on cytokine production, we performed coculture assays using PBMCs from 6 patients with SS and 2 HDs in the presence of blocking antibody to PD-1 or PD-L1. We incubated the cells in medium alone or in the presence of CD3/CD28 to determine a change in IFN-γ production. After the cells had been in the culture for 72 hours, we noted enhanced production of IFN-γ in 3 of 6 patients and 1 HD when cells were activated with anti-CD3/CD28 in the presence of blocking antibodies (Figure 3). It is worth noting that 2 of 3 patients demonstrated increased IFN-γ production resulting from the blockade of both PD-1 and PD-L1, while 1 patient demonstrated increased IFN-γ production in the presence of PD-1 blocking antibody alone.

**CORRELATION OF DECREASE IN PD-1 EXPRESSION WITH CLINICAL IMPROVEMENT**

To further our understanding of the biological significance of PD-1 expression on patients’ CD4+ T cells, we examined PBMCs from patients collected at different times and different stages of disease. In 1 patient with SS, clinical remission in association with clearing of circulating malignant T cells correlated with progressive changes of PD-1 expression on CD4+ T cells from initial high...
Figure 2. Activation of CD4⁺CD26⁻ T cells by anti-CD3/CD28 antibodies varies between patients with a low and high tumor burden in circulation. The peripheral blood mononuclear cells were isolated from either a patient with a low tumor burden (A) or a high tumor burden (B) and were cultured with medium alone or with anti-CD3/CD28. After 48 hours of stimulation, cells were harvested, stained with antibodies, and analyzed by flow cytometry. In both A and B, the top panels demonstrate cells cultured with medium alone, and the lower panels demonstrate cells cultured with anti-CD3/CD28 antibodies. Panels on the left reveal the expression of CD26 on CD4⁺ T cells, the center panels reveal the expression of programmed death-1 (PD-1) on the CD4⁺CD26⁻ population, and panels on the right reveal the expression of PD-1 on the CD4⁺CD26⁺ population. The numbers in the quadrants represent the percentages of cells that are double positive for each condition, and the numbers in parentheses represent the mean fluorescent intensity for PD-1 expression. Results shown are representative of a total of 12 patients tested. APC indicates allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; and PerCP, peridinin chlorophyll protein complex (all from BD Pharmingen, San Jose, California).
cells. However, in patients with SS, PD-1 is not limited to the malignant population of cells but is also found on CD4+ T cells, currently defined as the malignant population of cells but is also found on CD4+ T cells, considered normal and immuno-competent. Additional studies on the mechanisms responsible for the upregulation of PD-1 on patients’ CD4+ T cells demonstrated upregulated PD-1 expression on both CD4+CD26+ and CD4+CD26- T cells on T-cell receptor activation. Interestingly, an increase in the percentage of PD-1+ CD4+CD26- T cells was apparent only in patients with a low to medium tumor burden and not in those with a high tumor burden, despite the presence of CD3 on their surface. Similarly, as we have previously reported, these patients also fail to upmodulate CD40 ligand on their CD4+ T cells in response to anti-CD3 ligation.30

Our data suggest that increased expression of PD-1 on CD4+ T cells from patients with SS results mainly from activation of the TCR on patients’ CD4+ T cells with a putative tumor antigen. Furthermore, both CD4+CD26+ and CD4+CD26- populations, comprising normal immunocompetent cells and malignant cells, respectively, are activated, particularly in patients with low tumor burden, suggesting a less clearly defined population of potential tumor cells than previously assumed. The “normal” CD4+CD26- T cells may in fact contain a subset of cells that have not yet lost CD26 on their cell surface and represent an activated phenotype resulting from ongoing stimulation with tumor antigen. In addition, the CD4+CD26- T cells may contain a subset of cells that are still sensitive to TCR stimulation implicated by an increase in intensity of PD-1 expression (MFI) on CD4+CD26- T cells in patients with a high tumor burden. As tumorigenesis progresses, there seems to be a diminished capacity of peripheral blood T cells to respond to TCR stimulation. These malignant cells may represent an “exhaustion” phenomenon, suggesting that the level of PD-1 expression likely correlates with an impaired T-cell response.14 At present, more detailed studies are needed to fully understand the mechanism responsible for the impairment of TCR signaling in patients with a high tumor burden.

In the case of 1 patient followed longitudinally, we noted a clear correlation between a decrease in PD-1 expression with improvement in clinical disease. The change in percentage of CD4+PD-1+ T cells paralleled a decrease in the CD4:CD8 ratio and a decrease in the absolute numbers of the CD4+CD26- population in the circulation. Similar findings are noted in human immunodeficiency virus, in which PD-1 expression is shown to correlate positively with plasma viral load and inversely with a CD47 T-cell count.14 As a result, decreased expression of PD-1 that correlates with improvement of disease status may result from either a diminished number of tumor cells expressing PD-1 owing to therapy, a loss of PD-1 on exhausted immune-compatible cells and recovery of immune function owing to therapy, or a combination of both. Of note, our previous study3 of patients with SS revealed normalization of dysregulated immune functions during remissions induced by immune modulatory therapy.

Blockade of the PD-1/PD-L1 pathway revealed a relative increase in IFN-γ production by PBMCs of patients with CTCL. Patients with active CTCL demonstrate a skewed cytokine profile, resulting in a deficiency of IFN-γ production. Interferon γ is critical for the generation of

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**COMMENT**

Our study demonstrates increased PD-1 expression on circulating CD4+ T cells of patients with SS compared with CD4+ T cells of patients with MF and HDs. To our knowledge, this has not been previously described in patients with SS. The presence of PD-1 on CD4+CD26- T cells, currently defined as the malignant population of cells in CTCL, implies a role for an increase in PD-1 expression in tumor progression. This is demonstrated in the case of adult T-cell leukemia lymphoma, in which the neoplastic T cell, characterized by a CD4+CD25+ phenotype, demonstrates increased PD-1 expression with a relative increase in the absolute number of tumor cells.10 However, in patients with SS, PD-1 is not limited to the malignant population of cells but is also found on CD4+CD26+ T cells, considered normal and immuno-

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**Figure 3.** Blocking the pathway of programmed death-1 (PD-1) and its ligand PD-L1 results in increased interferon γ (IFN-γ) production by patients’ peripheral blood mononuclear cells (PBMCs) stimulated with anti-CD3/CD28. The PBMCs of patients with Sézary syndrome were cultured for 72 hours with either medium or anti-CD3/CD28 alone, and with the following: murine IgG (mIgG), anti–PD-1, or anti–PD-L1. Subsequently, culture supernatants were collected and tested for the presence of IFN-γ. With Clinical Improvement of Diseasea

**Table. Decrease in PD-1 Expression Correlating With Clinical Improvement of Diseasea**

<table>
<thead>
<tr>
<th>Date</th>
<th>CD4·PD-1+ T Cells, %</th>
<th>CD4·CD26+ T Cells, %</th>
<th>CD4·CD26- T Cells, %</th>
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<td>August 2008</td>
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</table>

Abbreviations: LCP, large-cell population; PD-1, programmed death-1; asterisk, the evidence of a malignant LCP, which correlates with a peak in the percentage of PD-1 expression.

aPeripheral blood mononuclear cells from 1 patient were collected over 1 year and examined for PD-1 and CD26 expression on CD4+ T cells and for the ratio of CD4:CD28.

levels at the time of diagnosis to normal levels as the patient responded to immunotherapy (Table).

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antitumor immunity and plays an important role in stabilizing a T<sub>p</sub> phenotype, particularly when used as a therapeutic agent. In studies using human T cells, activation of those cells using anti-CD3 in the presence of PD-L1 demonstrates decreased IFN-γ secretion. Recovery of IFN-γ production in some patients with CTCL during PD-L1/PD-L1 blockade implies an operational PD-L1 or cytokine profile. In the case of LCMV and human immunodeficiency virus infections, blockade of PD-L1 or PD-L2 results in enhanced T-cell proliferation, cytokine production, and restored function of exhausted CD8<sup>+</sup> T cells. 

Unlike PD-1, PD-L1 and PD-L2 are not frequently or highly expressed on monocytes and dendritic cells of patients with CTCL without stimulation by IFN-γ. The engagement of the TCR, which provides a strong signal for upregulation of PD-1 in patients with a low to medium tumor burden, results in a rather weak upregulation of PD-L1 or PD-L2 (data not shown). It is therefore possible that blockade of this pathway, as our results demonstrate, would not consistently result in upregulation of T<sub>p</sub> cytokine production and improvement of T-cell responses in patients with CTCL.

One of the most important implications of fully understanding the role of both PD-1 and PD-L1 on malignant T cells in CTCL relates to the application of novel therapies. Blockade of PD-1 can increase immune activating cytokine production and may enhance functioning of cytotoxic T cells, which would lead to enhanced disease eradication. Injection of tumors into PD-1 double negative mice has revealed increased effector cytokine production, greater recruitment of T cells, and reduced spread of tumor cells. To further develop PD-1-targeted therapy, it will be necessary to acknowledge the complexity of its role in T-cell homeostasis by considering treatment that spares normal, immunocompetent T cells, induces a vigorous antitumor response, and avoids the consequence of inducing autoimmune disease.

While PD-1 seems to act as a negative regulator of the immune response, its function in T cells, specifically in patients with SS, largely remains unclear. It is unknown whether an increase in PD-1 expression serves to protect tumor cells from elimination, particularly if PD-1 ligands are not in abundance, or if it represents an “exhausted phenotype” of immunocompetent cells chronically stimulated with tumor antigen. More detailed investigations are needed to fully elucidate its role in this specific patient population.

Accepted for Publication: May 6, 2010. Published Online: August 16, 2010. doi:10.1001/archdermatol.2010.200

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Author Contributions: Drs Samimi, Wysocka, and Rook had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Samimi, Evans, Wysocka, and Rook. Acquisition of data: Samimi, Benoit, Evans, and Wysocka.

Analysis and interpretation of data: Samimi, Wherry, Showe, Wysocka, and Rook. Drafting of the manuscript: Samimi, Wysocka, and Rook. Critical revision of the manuscript for important intellectual content: Samimi, Benoit, Evans, Wherry, Showe, Wysocka, and Rook. Obtained funding: Rook. Administrative, technical, and material support: Samimi, Benoit, Evans, Wherry, Showe, and Rook. Study supervision: Evans, Wysocka, and Rook.

Financial Disclosure: Dr Wherry has a licensing agreement on a PD-1 pathway blockade patent with Genentech.

Funding/Sponsor: This work was supported in part by grant CA122569 from the National Institutes of Health, by grant NCI ROI CA132098, and by a Translational Research Grant from the Leukemia and Lymphoma Society.

Role of the Sponsors: The sponsors had no role in the design and conduct of the study; in the collection, analysis, and interpretation of data; or in the preparation, review, or approval of the manuscript.

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