Cryopreservation Decreases Receptor PD-1 and Ligand PD-L1 Coinhibitory Expression on Peripheral Blood Mononuclear Cell-Derived T Cells and Monocytes


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The B7-CD28 immunoglobulin superfamily of costimulatory and coinhibitory ligands and their cell receptors play a critical role in modulating immune responses. Imbalances in these immune regulatory signals occur in pathological conditions characterized by chronic antigenic stimulation. Clinical studies often rely on the use of cryopreserved peripheral blood mononuclear cells (PBMC) to evaluate cellular immune responses. The impact of cryopreservation on these coinhibitory ligands and their cell receptors is unknown. In our studies, cryopreservation significantly reduced the expression of both PD-1 and PD-L1 on PBMC-derived CD3+/CD8+ T cells and CD45+/CD14+ monocytes obtained from adult control subjects. Blockade of PD-1, PD-L1, and PD-L2 using both freshly isolated and cryopreserved PBMC led to higher levels of phytohemagglutinin (PHA) and Candida-induced gamma interferon (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α) with no effect on IL-10 production. Coinhibitory signaling blockade of freshly isolated, PHA-stimulated PBMC from normal adult controls and human immunodeficiency virus (HIV)-infected subjects led to increased production of IL-4 and IL-5. Candida-stimulated PBMC preferentially induced IFN-γ and TNF-α production, with reduced production of IL-2 and IL-10. This is in contrast to high levels of IFN-γ, IL-2, and TNF-α production with PHA-stimulated cells. The effects of coinhibitory blockade on PHA and Candida-induced lymphoproliferation were varied, with freshly isolated PBMC from adult control subjects and HIV-infected patients yielding higher levels of lymphoproliferation in response to PD-1/PD-L1 blockade. Immune function studies employing cryopreserved cells may lead to increased T-cell effector cytolytic and regulatory immune responses.

PD-1 is a member of the CD28/CTLA-4 subfamily of the immunoglobulin (Ig) superfamily. Upon interaction with its natural ligands (PD-L1 and PD-L2, members of the B7 family) expressed on T cells, B cells, and macrophages/dendritic cells, as well as viral-infected target cells, PD-1 abrogates the effector function of both immunoregulatory CD4 T cells and TH-1 cytolytic CD8 T cells while promoting the induction of TH-2 cytokines, such as interleukin-10 (IL-10) (7). Blockade of these interactions with antibodies directed against PD-1 or its ligands restores TH-1 immunoregulatory and T-cell cytolytic functions (1, 5, 8, 16). These coinhibitory receptors and ligands, along with their costimulatory counterparts (CD28/CD80), have a regulatory role in immune responses (13). Pathological conditions characterized by chronic antigenic stimulation elevate coinhibitory signaling and antigen-specific anergy (4, 15). Clinical studies often rely on cryopreserved cells in order to monitor immune function. The possible alteration of coinhibitory receptor/ligand expression on cryopreserved T cells is suggested by recent studies that compared levels of PD-1 expression on T-cell subsets as improved markers of human immunodeficiency virus (HIV) disease progression using fresh versus cryopreserved samples (9). PD-1 expression on T-cell subsets using freshly prepared samples had a greater predictive value for HIV disease progression than CD38 expression on T cells or viral load, a correlation that was lost using cryopreserved samples. The downregulating effect of cryopreservation on other T-cell markers, such as CCR5 and CD62L, has also been described (6). This study describes the impact of cryopreservation on PD-1/PD-L1 expression on T-cell subsets and monocytes in a cohort of adult control donors. In addition, the influence of PD-1/PD-L1/PD-L2 blockade on mitogen- and antigen-induced TH-1/TH-2 cytokine production and lymphoproliferation is evaluated with adult control subjects and with a cohort of HIV-infected adult subjects on maintenance antiretroviral therapy.

MATERIALS AND METHODS

Preparation and cryopreservation of PBMC. Peripheral whole-blood samples collected in K2 EDTA were obtained from 10 consenting adult control subjects (three males and seven females, ages 19 to 49) and from 10 consenting HIV-infected adult subjects on maintenance antiretroviral therapy who are followed at the University of Pennsylvania HIV Clinic. Because blood samples from HIV-infected subjects arrived in our laboratory in the late afternoon, samples from both HIV-infected subjects and healthy adult controls were held overnight at room temperature for processing the next day. Peripheral blood mononuclear cells (PBMC) were isolated by routine Ficoll-Hypaque density gradient sedimentation. In addition, the evaluation of freshly prepared PBMC, cells were cryopreserved and later thawed for evaluation using methods previously described (18).

Flow cytometry immunophenotyping. Three-color flow cytometry was used to evaluate PD-1 and PD-L1 expression on CD3+/CD8+ and CD3+/CD14+ T lymphocytes and CD45+/CD14+ monocytes using freshly prepared and cryopreserved PBMC from adult control subjects. The directly conjugated murine monoclonal antibodies used included allophycocyanin (APC)-conjugated anti-CD3,
were accumulated for CD3
strategies as illustrated in Fig. 1. A total of 2,500 PE–PD-1 and PE–PD-L1 events
resuspended in 0.3 ml 2% (vol/vol) formaldehyde prepared in PBS. The samples
of calcium- and magnesium-free, phosphate-buffered saline (PBS), pH 7.3, and
lysing buffer (BD Biosciences, San Diego, CA) was added. After incubation for
measurement of PD-1 and PD-L1 expression on T-cell subsets and monocytes.

PD-L1 on CD3
(20
freshly prepared PBMC stimulated for 3 days with phytohemagglutinin (PHA)
tive coinhibitory blockade experiments was determined by flow cytometry using
control antibody.
cursors set to yield less than 2% positive signals using the PE-conjugated isotype
cells. Positive and negative signals for PD-1 and PD-L1 expression were based on
added 20
/H9262 cryopreserved PBMC were resuspended in 50
l of the cell suspension (2
lo l of PE-conjugated anti-PD-1, APC-con-
gated anti-CD3, and PerCP-conjugated mu-
peridinin chlorophyll protein (PerCP)-conjugated anti-CD8, PerCP-conjugated anti-
CD45, APC-conjugated anti-CD14, and phycoerythrin (PE)-conjugated mu-
rine isotype control antibody (BD PharMingen, San Diego, CA). PE-conjugated
anti-CD1 and anti-PD-L1 antibodies were obtained from eBiosciences, San
Diego, CA. Sterile and azide-free antibodies prepared against PD-1, PD-L1, and
anti-PD-1 and anti-PD-L1 antibodies were obtained from eBiosciences, San
Diego, CA. A BD fluorescence-activated cell sorter Calibur flow cytometer was used for all immunophenotyping
studies using a lyse-wash procedure. Briefly, 1 x 10^6 freshly prepared or thawed,
cryopreserved PBMC were resuspended in 50 l of fetal calf serum to which was added 20 l each of directly conjugated monoclonal antibodies that allow for the measurement of PD-1 and PD-L1 expression on T-cell subsets and monocytes. After incubation for 30 min at 4°C, 2 ml of fluorescence-activated cell sorter lysing buffer (BD Biosciences, San Diego, CA) was added. After incubation for 10 min at room temperature, the fixed and stained cells were washed with 2 ml of calcium- and magnesium-free, phosphate-buffered saline (PBS), pH 7.3, and resuspended in 0.5 ml 2% (vol/vol) formaldehyde prepared in PBS. The samples were stored in the dark at 4°C until fluorescence intensity was evaluated by flow

cytometry.

Fluorescence measurements were performed using flow cytometry gating strategies as illustrated in Fig. 1. A total of 2,500 PE–PD-1 and PE–PD-L1 events were accumulated for CD3
/CD8
, CD3
/CD8
, and CD45
/CD14
 gated cells. Positive and negative signals for PD-1 and PD-L1 expression were based on
cursors set to yield less than 2% positive signals using the PE-conjugated isotype
control antibody.

An estimate of effective concentrations of unlabeled functional anti-PD-1 antibodies in mitogen- and antigen-induced TH-1/TH-2 lymphoproliferative
coinhibitory blockade experiments was determined by flow cytometry using freshly prepared PBMC stimulated for 3 days with phytohemagglutinin (PHA)
(20 l/ml). These culture conditions result in maximum expression of PD-1 and
PD-L1 on CD3
/CD8
 and CD3
/CD8
 T cells (Fig. 1). Briefly, PHA-stimu-
lated PBMC were harvested at day 3 and resuspended in fetal calf serum at a cell
density of 4 x 10^6/ml. A total of 50 l of the cell suspension (2 x 10^5 cells) was
distributed to 12- by 75-mm tubes in duplicate, to which was added 20 l of PBS or
seriously diluted (0.6 l/ml to 20 l/ml in PBS) unlabeled functional anti-PD-1 antibody (final antibody concentrations 0.02 l/ml to 2.7 l/ml). After a 10-min
incubation at room temperature, 20 l of PE-conjugated anti-PD-1, APC-con-
jugated anti-CD3, and PerCP-conjugated anti-CD8 were added and incubated for
30 min at 4°C in the dark. The stained cells were then processed as described
above and stored at 4°C in the dark until evaluated for fluorescence intensity by
flow cytometry. PE signals were accumulated based on both a physical gate of the
entire PBMC population and CD3
/CD8
 fluorescence signals. The unblocked
samples were evaluated first with the photomultiplier tube (FL2), used for the
expression of PE signals, and adjusted to allow signals to fall within the second
decade of the fluorescence histogram. A total of 5,000 events were accumulated
each for each sample. The remaining antibody-blocked samples were then evaluated
with the geometric mean value for the fluorescence distribution recorded. The

average of fluorescence geometric mean values of duplicate samples was com-
puted with the percent inhibition of fluorescence intensity for each antibody-
blocked sample calculated, based on the average geometric mean value of the
unblocked samples.

PHA and Candida-induced PBMC TH-1/TH2 cytokine production by CBA
assay. A flow cytometry-based cytometric bead array (CBA; BD Biosciences, San
Diego, CA) assay was used to measure TH-1/TH2 cytokines in culture fluids of
PHA and Candida-stimulated PBMC at multiple time points (24, 48, and 72 h
poststimulation). Fresh and cryopreserved whole-blood-derived PBMC obtained from
10 consenting adult control subjects and 10 consenting HIV-infected sub-
jects (obtained through the University of Pennsylvania HIV Clinic) were cultured in the presence and absence of combinations of monoclonal antibodies
eBioscience, Inc., San Diego, CA) directed against the coinhibitory receptor (PD-1)
and its ligands (PD-L1 and PD-L2), as well as costimulatory receptors (CD28
and CD40). PBMC were cultured at a cell density of 1 x 10^6/ml in RPMI 1640
media containing 10% pooled normal human AB sera at 37°C under 5% CO2.
A flow cytometry-based cytometric bead array (CBA; BD Biosciences, San

Diego, CA) was used to measure TH-1/TH2 cytokines in culture fluids of
PHA and Candida-stimulated PBMC obtained from 10 consenting healthy adult control subjects and 10 consenting HIV-infected sub-
jects (obtained through the University of Pennsylvania HIV Clinic) at day 3 poststimulation). Fresh and cryopreserved whole-blood-derived PBMC obtained from
10 consenting adult control subjects and 10 consenting HIV-infected sub-
jects (obtained through the University of Pennsylvania HIV Clinic) were cultured in the presence and absence of combinations of monoclonal antibodies
eBioscience, Inc., San Diego, CA) directed against the coinhibitory receptor (PD-1)
and its ligands (PD-L1 and PD-L2), as well as costimulatory receptors (CD28
and CD40). PBMC were cultured at a cell density of 1 x 10^6/ml in RPMI 1640
media containing 10% pooled normal human AB sera at 37°C under 5% CO2.

Lymphoproliferative responses to the presence and absence of coinhibitory antibodies. Lymphoproliferative responses to PHA and Candida were evalu-
ated using freshly isolated and cryopreserved whole-blood-derived PBMC obtained from consenting healthy adult control subjects and HIV-infected adults followed at the University of Pennsylvania HIV Clinic. Cells were
maintained in culture for 6 days (using suboptimal PHA concentrations) in the presence and absence of coinhibitory antibodies directed against PD-1, PD-L1, and PD-L2 (eBiosciences, San Diego, CA). Cells (1 x 10^6 cells in 0.2
ml RPMI containing 10% pooled normal human AB sera) were cultured in triplicate at 37°C under 5% CO2 for 6 days in the presence and absence of all
three antibodies, each having a final concentration of 1 l/ml. The cultures

![FIG. 1. Three-color flow cytometric evaluation of PD-1 and PD-L1 expression on unstimulated and PHA-stimulated PBMC-derived CD3
/CD8
 and CD3
/CD8
 T cells. Freshly prepared PBMC from an adult control were cultured for 3 days with and without PHA (20 l/ml final concentration).](http://cvl.asm.org/)

![TABLE 1. Influence of cryopreservation on PD-1/PD-L1 expression on PBMC-derived T-cell subsets and monocytes obtained from healthy adult controls.](http://cvl.asm.org/)

<table>
<thead>
<tr>
<th>T cell or monocyte</th>
<th>Expression</th>
<th>Fresh</th>
<th>Cryo</th>
<th>P value</th>
</tr>
</thead>
</table>
| CD3
/CD4
/CD8
/PD-1 | 4.2 ± 1.8 | 3.6 ± 1.0 | NS |
| CD3
/CD4
/CD8
/PD-1 | 10.1 ± 3.9 | 9.0 ± 3.3 | NS |
| CD3
/CD8
/CD8
/PD-1 | 7.4 ± 4.1 | 2.6 ± 1.5 | 0.001 |
| CD3
/CD8
/CD8
/PD-1 | 5.6 ± 3.6 | 1.3 ± 0.7 | 0.006 |
| CD45
/CD4
/CD8
/PD-1 | 3.0 ± 1.5 | 0.5 ± 0.3 | 0.003 |
| CD45
/CD4
/CD8
/PD-1 | 7.0 ± 4.4 | 2.2 ± 1.3 | 0.007 |

All data, with the exception of the P values, represent mean ± 1 standard deviation of the percent positive cells. n = 10 for all analyses. Cryo, cryopre-
served; NS, not significant.

![FIG. 2. Flow cytometric evaluation of competitive binding of various concentrations of unlabeled functional anti-PD-1 antibodies with a constant concentration of PE-conjugated anti-PD-1 antibodies for PD-1 receptors on 2 x 10^6 PHA-stimulated PBMC.](http://cvl.asm.org/)

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were pulsed with 1 μCi of [3H]TdR during the last 6 hours of culture. The cells were harvested onto a 96-well plate and dried overnight. Radiolabeling was measured with a TopCount scintillation counter (PerkinElmer, Inc., Shelton, CT). Mean counts per minute (cpm) were recorded for resting, PHA-stimulated, and Candida-stimulated cultures, with data expressed as net cpm (mean stimulated cpm – mean resting cpm).

Statistical analyses. The statistical analyses of differences in the levels of PD-1/PD-L1 expression on T-cell subsets and monocytes, lymphoproliferative responses, and cytokine levels between the various treatment groups were performed by the Student two-tailed t-test for paired observations using GraphPad Prism version 5.01 software (GraphPad Software, Inc., La Jolla, CA). The Student two-tailed t-test for unpaired observations was used in the analyses of cytokine levels of control and HIV-infected subjects.

RESULTS

Effect of cryopreservation on T-cell and monocyte PD-1 and PD-L1 expression. Table 1 summarizes PD-1 and PD-L1 expression on fresh versus cryopreserved, whole-derived PBMC obtained from healthy adult controls. Cryopreservation had little effect on both PD-1 and PD-L1 cell surface expression on CD3+/CD4+ T helper cells. A significant reduction in the detection of both molecules, however, was observed with CD3+/CD8+ T cells and CD45+/CD14+ monocytes.

Titration of functional anti-PD-1 antibody. In order to assess the blocking capacity of various concentrations of unlabeled functional anti-PD-1 antibody to be used in the cytokine induction and lymphoproliferation assays, PBMCs prepared from an adult control subject were placed in culture for 3 days in the presence of optimal PHA concentrations. Under these conditions, the vast majority of T cells express PD-1 and PD-L1 as assessed by flow cytometry using PE-conjugated anti-PD-1 and anti-PD-L1 antibody (Fig. 1). Fig. 2 illustrates an inverse linear relationship between increasing concentrations of unlabeled functional anti-PD-1 antibody and cell binding of a constant amount of PE-labeled antibody. The maximum amount of unlabeled antibody tested (20 μg/ml and a 5.2-μg/ml final concentration) provided 72% inhibition of PE-labeled antibody binding. The concentration selected for use in the cytokine induction and lymphoproliferation assays was a 1-μg/ml final concentration, which provided approximately 60% inhibition of the probe antibody cell binding.

Effect of coinhibitory blockade on PHA and Candida-induced lymphoproliferation. The influence of antibody blockade of coinhibitory receptor/ligand interactions on PHA and

![Figure 3](http://cvi.asm.org/) TH-1/TH-2 cytokine levels in culture fluids of PHA-stimulated adult control PBMC with and without coinhibitory blockade. Freshly isolated (A) and cryopreserved (B) PBMC were cultured for 24 h with and without monoclonal antibodies specific for the coinhibitory receptor/ligands PD-1, PD-L1, and PD-L2.
Candida-induced lymphoproliferation, as assessed by [3H]TdR uptake, is summarized in Table 2. Blockade of coinhibitory interactions resulted in significantly higher proliferative responses to PHA using freshly isolated PBMC from adult control subjects and HIV-infected patients.

Effect of coinhibitory blockade on PHA and Candida-induced TH-1/TH-2 cytokine production. Fig. 3 to 6 summarize results obtained in the study of coinhibitory blockade on TH-1/TH-2 cytokine production by freshly isolated and cryopreserved PBMC from normal adult controls and HIV-infected patients. Coinhibitory blockade of freshly isolated and cryopreserved PBMC from normal adult controls and HIV-infected patients resulted in significant increases in both PHA and Candida-induced IFN-γ, IL-2, and TNF-α production. No increase in IL-10 production was observed. PHA-stimulated, freshly isolated PBMC from normal adult controls and HIV-infected patients yielded significant increases in IL-4 and IL-5 with coinhibitory blockade, in contrast to the effect on IL-10 production. Further, PHA-induced IL-5 production by PBMC from HIV-infected patients was significantly higher than that observed with normal adult controls with or without coinhibitory blockade. Candida stimulation of both freshly isolated and cryopreserved PBMC resulted in levels of IFN-γ and TNF-α production similar to that achieved with PHA. IL-2 and IL-10 production with Candida stimulation was significantly reduced compared to PHA.

DISCUSSION

The delineation of hematopoietic cell surface molecules has led to further investigations of the effects of in vitro manipulation of PBMC on regulatory and effector immune function (3, 9). The Ig superfamily of coinhibitory/costimulatory molecules, which are expressed on both lymphoid and antigen-
presenting cells, have been investigated in studies of pathological conditions characterized by chronic antigenic stimulation and elevated expression of coinhibitory receptor/ligand molecules on immune cells (1, 7). Coinhibitory signaling in HIV infection and potential regulatory pathways is the subject of a recent review by Kaufmann and Walker (10). The level of infection and potential regulatory pathways is the subject of a recent review by Kaufmann and Walker (10). The level of expression of these molecules on freshly isolated CD8+ T cells has a high predictive value of CD4+ T-cell loss, an indicator of HIV disease progression. This correlation was lost when using cryopreserved cells (9). Other molecules important in the pathogenesis of HIV disease, such as the HIV coreceptor CCR5, have also been found to be sensitive to various in vitro manipulations (3). The mechanism(s) leading to the loss of these molecules is unknown, and the influence of various manipulations on in vitro correlates of immune function has not been investigated systematically. In our study, cryopreservation of PBMC led to a marked reduction of PD-1 and PD-L1 expression on CD3+CD8+ T cells and CD45+CD14+ monocytes, with no significant effect on CD3+CD4+ T cells. These findings offer an explanation for the observations of Holm et al. (9), who demonstrated the loss of correlation of PD-1 expression of these molecules on freshly isolated CD8+ T cells with CD4 T-cell loss using cryopreserved PBMC. Our study indicates a pattern of enhanced lymphoproliferative responses to PHA and Candida upon antibody blockade of PD-1, PD-L1, and PD-L2 ligation in a cohort of HIV-infected adults. The enhanced lymphoproliferation is in keeping with our observations of enhanced IL-2 production following co-inhibitory receptor/ligand blockade of PHA and Candida-stimulated PBMC. PD-1/PD-L1 blockade of PHA and Candida-stimulated PBMC consistently resulted in significant increases in the TH1 cytokines IFN-γ, TNF-α, and IL-2, with no impact on IL-10 production. The observed insensitivity of IL-10 production to PD-1/PD-L1 blockade parallels previous observations of increased IL-10 production and elevated co-inhibitory receptor/ligand expression with disease progression in HIV infection (14, 17). Our findings of levels of PHA-induced, PBMC-derived IL-4 and IL-5 production in HIV-infected subjects that are significantly higher than those in healthy adult controls (with or without PD-1/PD-L1 blockade; Fig. 6) support previous observations of elevated Ig E levels and eosinophilia in HIV-infected subjects (2, 11, 12). A comparison of mitogen- and antigen-induced lymphoprolifera-

tive responses and TH-1/TH-2 cytokine production in the presence and absence of coinhibitory signaling blockade may lead to an improved functional immune measure for monitoring HIV disease progression or response to antiviral therapy and is the subject of further study.

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