Microenvironment and Immunology

PD-1/PD-L1 Interactions Contribute to Functional T-Cell Impairment in Patients Who Relapse with Cancer After Allogeneic Stem Cell Transplantation

Wieger J. Norde1, Frans Maas1, Willemijn Hobo1, Alan Korman6, Michael Quigley7, Michel G.D. Kester5, Konnie Hebeda4, J.H. Frederik Falkenburg5, Nicolaas Schaap2, Theo M. de Witte3, Robbert van der Voort1, and Harry Dolstra1

Abstract

Tumor relapses remain a serious problem after allogeneic stem cell transplantation (alloSCT), despite the long-term persistence of minor histocompatibility antigen (MiHA)-specific memory CD8+ T cells specific for the tumor. We hypothesized that these memory T cells may lose their function over time in transplanted patients. Here, we offer functional and mechanistic support for this hypothesis, based on immune inhibition by programmed death-1 (PD-1) expressed on MiHA-specific CD8+ T cells and the associated role of the PD-1 ligand PD-L1 on myeloid leukemia cells, especially under inflammatory conditions. PD-L1 was highly upregulated on immature human leukemia progenitor cells, whereas costimulatory molecules such as CD80 and CD86 were not expressed. Thus, immature leukemic progenitor cells seemed to evade the immune system by inhibiting T-cell function via the PD-1/PD-L1 pathway. Blocking PD-1 signaling using human antibodies led to elevated proliferation and IFN-γ production of MiHA-specific T cells cocultured with PD-L1–expressing leukemia cells. Moreover, patients with relapsed leukemia after initial MiHA-specific T-cell responses displayed high PD-L1 expression on CD34+ leukemia cells and increased PD-1 levels on MiHA-specific CD8+ T cells. Importantly, blocking PD-1/PD-L1 interactions augment proliferation of MiHA-specific CD8+ memory T cells from relapsed patients. Taken together, our findings indicate that the PD-1/PD-L-1 pathway can be hijacked as an immune escape mechanism in hematological malignancies. Furthermore, they suggest that blocking the PD-1 immune checkpoint offers an appealing immunotherapeutic strategy following alloSCT in patients with recurrent or relapsed disease. Cancer Res; 71(15); 1-12. ©2011 AACR.

Introduction

Alloreactive CD8+ T cells play a crucial role in the graft-versus-tumor (GVT) response following allogeneic stem cell transplantation (alloSCT) and donor lymphocyte infusion (DLI; ref. 1). In HLA-matched alloSCT, these alloreactive CD8+ T-cell responses are directed against minor histocompatibility antigens (MiHA; ref. 2). Previously, we have characterized CD8+ T-cell immunity toward a hematopoietic-restricted MiHA, designated LRH-1, which is presented by HLA-B7 and encoded by the P2×5 purinergic receptor gene (3). LRH-1–specific CD8+ T-cell responses can be frequently detected in myeloid leukemia patients following DLI, and has been associated with leukemic remission (3, 4). Moreover, we showed that CD34+ myeloid leukemia progenitor cells can be efficiently targeted in vitro by LRH-1–specific CD8+ CTLs, indicating that these CTLs play a significant role in graft-versus-leukemia (GVL)-specific immunity. However, we have observed that despite the presence of LRH-1–specific CD8+ memory T (Tmem) cells for many years, late relapses do occur in patients with advanced myeloid leukemia. Furthermore, we noted that LRH-1–specific CD8+ Tmem cells do not always efficiently expand with recurrence of leukemia cells, suggesting that these T cells become functionally impaired.

Mechanisms exploited by tumor cells to inhibit CD8+ T-cell–mediated immunity include disruption of antigen presentation, downregulation of HLA molecules, and induction of immunosuppressive components such as programmed death-1 (PD-1) signaling (5, 6). PD-1 plays a crucial role in T-cell regulation in various immune responses and is involved in peripheral tolerance, autoimmunity, infection, and antitumor immunity (7). Elevated PD-1 expression on antigen-specific
CD8+ T cells in chronic viral infection has been recognized as hallmark of T-cell exhaustion resulting in diminished cytokine production, proliferation, and cytolysis activity upon antigen restimulation (8). PD-L1 binds 2 ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273; ref. 7). Although PD-L2 expression is mainly restricted to antigen presenting cells (APC) like dendritic cells (DC) and macrophages, PD-L1 is also expressed by many nonhematological cell types (7). Furthermore, PD-L1 can be expressed on multiple tumor types and its expression is elevated following IFN-γ exposure (9). PD-L1 molecules on tumor cells can deliver negative signals through PD-L1 to tumor-reactive CTL, thereby inhibiting antitumor immunity (10). In agreement, PD-L1 expression has been associated with poor prognosis in various cancers including hepatocellular carcinoma and melanoma (9, 11). Notably, studies in models of murine myeloid leukemia have also shown that PD-1/PD-L1 interactions play an important role in immune evasion (12, 13). Interestingly, PD-L1 is also able to bind CD80, resulting in T-cell inhibition (14). Therefore, PD-L1 molecules on human leukemia cells may restrain CD8+ T-cell responses involved in GVL immunity after alloSCT.

In this study, we examined the role of PD-1/PD-L1 interactions in functional impairment of MiHA-specific CD8+ T cells. We observed that PD-L1 on myeloid leukemia cells is induced following IFN-γ and TNF-α exposure. Furthermore, we observed that activated LRH-1-specific CD8+ T cells express PD-1 during the course of the immune response. Importantly, we showed that blockade of PD-1/PD-L1 interactions using clinical grade human antibodies increases the proliferation and IFN-γ production of MiHA-specific CD8+ T cells when stimulated with PD-L1–expressing acute myelogenous leukemia (AML) cells and DC. Together, these findings indicate that the PD-1 signaling pathway suppresses MiHA-specific CD8+ T-cell responses and PD-L1 blockade may be an attractive approach to boost GVL immunity in patients with recurrent or relapsed disease.

Materials and Methods

Patient and donor material

Peripheral blood (PB) and bone marrow (BM) samples of leukemia patients have been collected after written informed consent in ongoing clinical SCT protocols approved by the RUNMC Institutional Review Board. We used PB mononuclear cells (PBMC) obtained from transplanted patients who developed MiHA-specific CD8+ T-cell responses. Patient 1 (Pt 1) suffered from accelerated phase (AP) chronic myeloid leukemia (CML) and was successfully treated with therapeutic DLI after alloSCT (3). However, the patient relapsed 4 years after DLI. Patient 2 (Pt 2) suffered from AML and developed an LRH-1–specific CD8+ T-cell response upon preemptive DLI, but developed extramedullary relapses without leukemic involvement in BM (4). Characteristics of these and other transplanted patients are included in Table 2.

DCs were generated from monocytes isolated from PBMC of healthy donors by plastic adherence. Immature DCs (iDC) were generated by culturing adherent monocytes in X-VIVO 15 medium (Lonza) supplemented with 2% HS, 500 U/mL interleukin (IL)-4, and 800 U/mL Granulocyte macrophage colony-stimulating factor (GM-CSF) (both Immunotools). After 3 days, cells were harvested, used for T-cell stimulations experiments or further cultured as described before (15). At day 8, mature DCs (mDC) were harvested and used in T-cell stimulation experiments. LRH-1–specific CD8+ CTL culture RP1 was isolated from CML-AP Pt 1 and was cultured as described previously (4). Before use in T-cell stimulation experiments, leukemia samples and DCs were cultured overnight with or without 100 U/mL IFN-γ and 1.25 ng/mL TNF-α (both Immunotools).

Mixed lymphocyte AML reactions and CTL stimulation assays

After culturing and preincubation, AML cells and DCs were washed, counted, and seeded in 96-well round bottom plates (Corning Costar). In lymphocyte AML reaction assays, allogeneic CD3+ T cells were isolated by direct magnetic labeling with the appropriate MicroBeads (Miltenyi Biotec) following the manufacturer’s instructions. Subsequently, 10⁴ CD3+ T cells were added to AML cells and mDCs at different E:T ratios. In MiHA-specific T-cell expansion assays, PD-L1+iDCs were loaded with 10 μM/L LRH-1 peptide TPNQQRVNC for 30 minutes at room temperature and cocultured with LRH-1–specific CTL RP1 at a stimulation ratio of 1:1.

Blocking antibodies were added at a final concentration of 10 μg/mL. Antibodies to PD-1 (BMS-936558; MDX-1106; and ONO-4538) and PD-L1 (BMS-936559 and MDX-1105) and a matching immunoglobulin (Ig) G4 isotype control were kindly provided by Dr. A. Korman (Bristol-Myers Squibb, Biologics Discovery). BMS-936558 and BMS-936559 are genetically engineered, fully human IgG4 antibodies currently evaluated in clinical trials for selected tumor treatments (16). All cocultures were performed in a total volume of 200 μL Iscove’s modified Dulbecco’s medium (IMDM)/10% HS. After 5 days of coculture, supernatant was harvested for cytokine analysis. At day 5, 0.5 μCi [³H]-thymidine (Perkin Elmer) was added to each well. After overnight incubation, [³H]-thymidine incorporation was measured using a 1205 Wallac Betaplate counter (Perkin Elmer).

MiHA-specific Tmem-cell proliferation assays

MiHA-specific CD8+ T cells present in PBMC from patients Pt 1, 2, and 15 to 21 (Table 2) were stimulated for 1 to 3 consecutive weeks ex vivo with either MiHA peptide alone or with MiHA peptide-loaded PD-L1+ iDC or PD-L1+ PD-L2+ mDC as described previously (15). PD-1 and PD-L1 blocking antibodies were added at a final concentration of 10 μg/mL. After 5 days, 500 μL supernatant was removed and fresh IMDM/10% HS containing 50 μM/L IL-2 and 5 ng/mL IL-15 (Immunotools) was added. At day 7, cells were harvested, counted, and the percentage of MiHA-tetramer+ CD8+ T cells was determined.

Flowcytometry

Expression of co-signaling ligands on myeloid leukemia cells and DCs was analyzed by staining with the following fluorochrome-conjugated antibodies: CD14 (Dako), CD3,
CD34, CD117, CD54, CD80, CD83, CD86 (all from Beckman Coulter), anti-PD-L1, anti-PD-L2 (both from Becton Dickinson), and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2b PE (Beckman Coulter). PD-1 expression on and the percentage of MiHA-specific CD8\(^+\) T cells were determined as described previously (15) using anti-PD-1 (Becton Dickinson). Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

**Immunohistochemistry staining**

Paraffin-embedded chloroma tissues were stained as previously described (17). Briefly, antigen retrieval was done using 10 minutes of boiling in 0.01 mol/L of sodium citrate pH 6.0 followed by incubation with primary antibodies anti-PD-L1 (eBioscience), anti-PD-L2 (eBioscience), anti-CD8 (DAKO), anti-CD34 (Klinipath), and anti-FoxP3 (ITK Diagnostics). Staining was visualized either by 3,3-diaminobenzidine (DAB) or aminoethyl carbazole (AEC) staining.

**IFN-\(\gamma\) and granzyme B ELISA**

Production of IFN-\(\gamma\) and granzyme B by stimulated T cells was determined by enzyme-linked immunosorbent assay (ELISA; IFN-\(\gamma\): Pierce Endogen; granzyme B: Mabtech) according to manufacturer’s protocol.

**Real-time quantitative reverse transcription PCR and microarray analysis**

Total RNA was isolated from cell samples using Trizol (Invitrogen). cDNA synthesis and PCR amplification were carried out as described (18). The *hydroxymethylbilane synthase* (*HMBS*) housekeeping gene was used to normalize PD-L1 and PD-L2 expression. PD-L1 and PD-L2 mRNA expression is shown in \(\Delta\Delta C_t\) values and was quantified relative to cell line U266, which was set at 1 \(\Delta\Delta C_t\) value. \(\Delta C_t\) was calculated as follows: \(2^{\Delta\Delta C_t} = 2^{\frac{C_{t_{gene}}}{C_{t_{HMBS}}}}\), in which \(\Delta C_t\) was normalized for *HMBS* by calculating \(\Delta C_t = C_t_{gene} - C_t_{HMBS}\) per sample.

For microarray analysis, LRH-1-tetramer\(^{-}\) human CD8\(^+\) T cells were sorted (median sorted cell number = 1,300; range 200–15,000 cells) on an Epics Elite sorter (Beckman Coulter), resulting in more than 96% pure product, and resuspended in Trizol. RNA extraction, amplification, cDNA generation, and microarray analysis were done as described previously (19).

**Statistical analysis**

Paired 1-tailed student \(t\) test or 1-way ANOVA followed by a Bonferroni post hoc test was used when appropriate.

**Results**

**Myeloid leukemia progenitor cells differentially express PD-L1 compared with CD80 and CD86**

To investigate whether myeloid leukemia cells in relapsed patients after alloSCT express PD-L1 under inflammatory conditions, we analyzed leukemia samples from 2 patients who relapsed despite circulating LRH-1\(^{-}\)specific CD8\(^+\) T cells (Table 1). We found that CD34\(^+\) CML-AP cells from a relapsed patient (Pt 1) at 4 years after DLI express PD-L1 upon

| Table 1. Myeloid leukemia patient characteristics and PD-L1 expression |
|----------------|----------------|----------------|----------------|----------------|
| **Patient** | **Disease FAB classification** | **Sample type** | **WBC count at sample date** | **PD-L1 mRNA expression** | **PD-L1 surface expression (MFI)** |
| | | | | **Unstim IFN-\(\gamma\)** | **TNF-\(\alpha\)** | **Unstim IFN-\(\gamma\)** | **TNF-\(\alpha\)** |
| 1 | CML-CP | PB | 11 | ND | ND | 0.4 | 0.6 |
| 1-relapse | CML-AP | PB | 105 | ND | ND | 1.0 | 2.0 |
| 2-relapse | AML-M0 | Chloroma | 6 | ND | ND | \(+^a\) | ND |
| 3 | AML-M0 | BM | 14 | 0.4 | 140.1 | 1.2 | 7.7 |
| 4 | AML-M0 | BM | 54 | 1.1 | 73.5 | 1.2 | 5.2 |
| 5 | AML-M2 | BM | 47 | 0.2 | 122.8 | 2.0 | 26.5 |
| 6 | AML-M4 | BM | 114 | 0.3 | 64.8 | 2.0 | 15.6 |
| 7 | AML-M4 | BM | 87 | 0.3 | 483.7 | 1.8 | 14.7 |
| 8 | AML-M4 | BM | 89 | 2.1 | 237.9 | 1.3 | 7.9 |
| 9 | AML-M4 | BM | 20 | 0.9 | 2,156.4 | 1.8 | 39.2 |
| 10 | AML-M5 | BM | 108 | 0.4 | 2,674.4 | 1.5 | 20.0 |
| 11 | AML-M5 | BM | 259 | ND | ND | 5.9 | 15.4 |
| 12 | AML-M2 | BM | 6 | 4.8 | 254.2 | 1.8 | 6.0 |
| 13 | AML-M4 | BM | 17 | 4.9 | 236.9 | 1.6 | 10.3 |
| 14 | AML-M5 | BM | ND | 28.3 | 800.2 | 3.5 | 29.5 |

**NOTE:** Characteristics of myeloid leukemia patients who have been analyzed for PD-L1 expression. Patient samples at diagnosis, except indicated by "relapse." CML-CP, chronic myeloid leukemia chronic phase; CML-AP, chronic myeloid leukemia accelerated phase; unstim, unstimulated; MFI, mean fluorescence intensity; ND, not determined; WBC, white blood cell count in \(10^9/L\) PB; and \(^a\), determined by immunohistochemistry.
stimulation with IFN-γ (Fig. 1A), whereas expression of costimulatory molecules CD80 and CD86 on these CD34+ cells was low. Furthermore, we observed high PD-L1 expression on CD34+ leukemia cells in a chorioma biopsy of an AML patient (Pt 2) who relapsed 3 years after DLI (Fig. 1B). Again, we found that these extramedullary AML cells expressed low levels of CD80 and CD86 (data not shown). Staining of specific T-cell markers revealed that CD8- T cells extensively infiltrated the chorioma, whereas FoxP3+ regulatory T cells were hardly detectable (Supplementary Fig. S1). These data suggest that selective expression of PD-L1 on these relapsed leukemia cells could have been involved in evading LRH-1-specific CD8- T-cell immunity.

To determine whether selective induction of PD-L1 expression under inflammatory conditions is a general phenomenon in leukemia, we analyzed a panel of 12 primary AML samples for expression of co-signaling ligands following treatment with IFN-γ and TNF-α (Table 1). Indeed, these cytokines induced a 137-fold and 31-fold upregulation of PD-L1 and PD-L2 mRNA, respectively (Fig. 1C). Furthermore, consistent with the findings in the 2 relapsed leukemia patients, PD-L1 cell surface expression was significantly upregulated (>20% PD-L1+ cells) on AML cells of 7 out of 10 newly diagnosed patients, whereas expression of PD-L2 was only slightly induced (Fig. 1D).

Note: Characteristics of patients with hematological malignancies displaying MiHA-specific T-cell responses. Pt, patient; CML-AP, chronic myeloid leukemia accelerated phase; CML-BC, chronic myeloid leukemia blast crisis; MM, multiple myeloma; T-ALL, T-cell acute lymphoid leukemia; NHL, non-Hodgkin lymphoma; pDLI, preemptive DLI; tDLI, therapeutic DLI; MiHA-specific response: MiHA for which a response was observed; Effect PD-L1 blockade: ratio absolute number of tet+ cells DC+ peptide + aPD-1/absolute number of tet+ cells DC+ peptide + isotype control. Mo, months; GVHD, graft-versus-host disease.

**Table 2.** Characteristics of transplanted patients and effect of PD-1 blockade on MiHA-specific T-cell proliferation

<table>
<thead>
<tr>
<th>Pt</th>
<th>Disease</th>
<th>MiHA T-cell response</th>
<th>Sample date (months post-SCT or post-DLI)</th>
<th>Effect PD-1 blockade</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CML-AP</td>
<td>LRH-1</td>
<td>52 post-tDLI</td>
<td>19.8</td>
<td>Hematologic relapse at 47 mo post-tDLI</td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>LRH-1</td>
<td>7 post-pDLI</td>
<td>15.4</td>
<td>Chloroma relapses at 33, 70, and 78 post-pDLI</td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>LRH-1</td>
<td>36 post-pDLI</td>
<td>2.8</td>
<td>Chloroma relapses at 33, 70, and 78 post-pDLI</td>
</tr>
<tr>
<td>15</td>
<td>CML-BC</td>
<td>HA-1</td>
<td>9 post-pDLI</td>
<td>1.5</td>
<td>Remission; death due to GVHD at 11 mo post-pDLI</td>
</tr>
<tr>
<td>16</td>
<td>MM</td>
<td>HA-1</td>
<td>6 post-SCT</td>
<td>5.5</td>
<td>Active disease at 6 mo post-SCT. Plasma cells 5%; M-protein 7 g/L</td>
</tr>
<tr>
<td>17</td>
<td>Pre-T ALL</td>
<td>HA-2</td>
<td>12 post-SCT</td>
<td>2.5</td>
<td>Remission; alive at 174 mo post-SCT</td>
</tr>
<tr>
<td>18</td>
<td>CML</td>
<td>SP110</td>
<td>20 post-SCT</td>
<td>8.7</td>
<td>Cytogenetic relapse at 14 mo post-SCT</td>
</tr>
<tr>
<td>19</td>
<td>AML</td>
<td>HA-8</td>
<td>12 post-SCT</td>
<td>0.7</td>
<td>Remission; alive at 213 mo post-SCT</td>
</tr>
<tr>
<td>20</td>
<td>NHL</td>
<td>HA-1</td>
<td>12 post-SCT</td>
<td>5.9</td>
<td>Remission; alive at 165 mo post-SCT</td>
</tr>
<tr>
<td>21</td>
<td>MM</td>
<td>HA-1</td>
<td>6 post SCT</td>
<td>3.3</td>
<td>Remission; alive at 31 mo post-SCT</td>
</tr>
</tbody>
</table>

Effect of PD-L1–expressing AML cells on allogeneic T cells

To investigate whether PD-L1 expression on AML cells can dampen allogeneic T-cell responses, we performed mixed lymphocyte reactions between PD-L1–expressing AML cells and allogeneic CD3+ T cells in the absence or presence of anti-PD-1/BMS-936558 and anti-PD-L1/BMS-936559 blocking antibodies. Blocking with either anti-PD-1 or anti-PD-L1 antibody significantly increased the proliferation of CD3+ T cells upon stimulation with allogeneic PD-L1+ AML cells from AML-M4 Pt 9 (Fig. 2A), whereas allogeneic T-cell proliferation stimulated with PD-L1+ and PD-L2+ mDCs could only be...
Figure 1. Myeloid leukemia cells express PD-L1 under inflammatory conditions. Expression of co-signaling ligands on leukemia cells was determined by FCM, immunohistochemistry, and RT-PCR. A, FCM analysis of PD-L1, PD-L2, CD80, and CD86 expression on CD34^+ progenitor cells of CML-AP Pt 1 at time of relapse, 5 years after DLI. Leukemia cells were exposed to 100 IU/mL IFN-γ and 1.25 ng/mL TNF-α 16 hour before analysis. B, expression of PD-L1 and PD-L2 by CD34^+ AML cells in a chloroma biopsy of Pt 2 at time of relapse, 3 years after DLI, was determined by immunohistochemistry. Staining was visualized using DAB (CD34) or AEC (PD-L1, PD-L2, and isotype control). Magnification 1,000x. C, CD33^+ AML cells from 9 to 12 different patients were incubated with 100 IU/mL IFN-γ and 1.25 ng/mL TNF-α for 16 hours, after which PD-L1 and PD-L2 mRNA expression with (+) and without (−) IFN-γ and TNF-α was measured. D, expression of costimulatory ligands on CD33^+ AML cells, in the absence (−) or presence (+) of IFN-γ and TNF-α, was determined by FCM. E, expression of PD-L1, PD-L2, CD80, CD86, and CD54 on CD33^+CD117^+CD14^+ progenitor AML cells from 8 patients. Expression is depicted as mean ± SD. Paired 1-tailed student t test was performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 2. PD-1 blockade enhances T-cell responses to stimulation by primary AML cells and DCs. Allogeneic CD3\(^+\) T cells were cocultured with PD-L1\(^-\) expressing leukemia cells of AML Pt 9 (A) and (C) or PD-L1\(^+\)PD-L2\(^+\) mDC (B) and (D) with addition of blocking anti-PD-1 or anti-PD-L1 antibodies. Proliferation was measured on day 5 (A) and (B) and IFN-\(\gamma\) production was evaluated (C) and (D). LRH-1-specific CTL RP1 was cocultured with peptide-loaded primary leukemic cells of AML Pt 11 (E), (G), and (I) or peptide-loaded PD-L1\(^+\) iDC (F), (H), and (J), combined with blockade of PD-1 or PD-L1. Subsequently, proliferation was measured (E) and (F) in addition to IFN-\(\gamma\) production (G) and (H). Cytotoxicity was measured by granzyme B secretion (I) and (J). One representative experiment of 3. One-way ANOVA was performed compared with isotype antibody as control. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
inhibited with anti-PD-1 (Fig. 2B). This difference can be explained by the high PD-L2 expression on mDC resulting in insufficient interference of PD-1 signaling by the PD-L1 antibody. In agreement with the T-cell proliferation data, IFN-γ production was also increased by blocking of PD-1 interactions between T cells and PD-L1⁺ AML cells (Fig. 2C) or PD-L1⁺/L2⁺ mDC (Fig. 2D). These results show that PD-L1 expression on AML cells decreases T-cell proliferation and cytokine production.

**MiHA-specific CTL expansion and function is enhanced by PD-1 blockade**

To elucidate the role of PD-1 on AML cells in inhibiting the recognition by MiHA-specific CD8⁺ T cells, we performed antigen restimulation experiments using CTL clone RP1 that recognizes the hematopoietic-restricted MiHA LRH-1 on AML progenitor cells (4). RP1, as well as CTLs against other MiHA, upregulates the expression of PD-1 upon coculture with MiHA⁺ AML (Supplementary Fig. S3 and data not shown). Antibody blockade of PD-1 signaling using human antibodies resulted in improved proliferation and IFN-γ production by CTL. RP1 upon engagement of PD-1–expressing primary AML cells from Pt 11 loaded with MiHA peptide (Fig. 2E and G). As hypothesized, we found that PD-1 blockade strongly elevated the proliferation and IFN-γ production by CTL, but as expected, we saw no cytotoxicity in AML cells was also enhanced after PD-1 and PD-L1 blockade (Fig. 2I), whereas no cytotoxicity was observed versus iDC (Fig. 2J). These data indicate that MiHA-specific CD8⁺ effector T cells can be inhibited via the PD-1/PD-L1 pathway either by AML or resident APC populations that selectively express PD-L1 in the leukemia microenvironment.

**PD-1 is highly expressed by circulating MiHA-specific CD8⁺ T cells in vivo**

Next, we investigated whether PD-1 is expressed by LRH-1–specific T cells in CML-AP Pt 1 and AML-M0 Pt 2 who relapsed 3 and 4 years, respectively, after the initial DLIIs that induced long-lasting LRH-1–specific CD8⁺ T-cell responses in these patients. PD-1 expression could be detected on LRH-1–specific CD8⁺ T cells during the complete course of the immune response after DLI which peaked at week 28 for CML-AP Pt 1 and at week 10 for AML-M0 Pt 2 (Fig. 3A). We observed relatively elevated expression of PD-1 on LRH-1–tetramer-positive T cells compared with tetramer-negative CD8⁺ T cells in the same patient (Fig. 3A and B). After DLI, PD-1 levels at the cell surface of LRH-1–specific CD8⁺ T cells gradually declined, but more than 95% of the tetramer-positive T cells remained PD-1 positive during the contraction phase. To determine PD-1 expression of the apparently impaired LRH-1–specific CD8⁺ Tmem cells in the relapsed patients several years after DLI, we sorted LRH-1–specific CD8⁺ Teff cells (Pt 1: 1.8% at week 28; Pt 2: 2.9% at week 15) and low frequencies of LRH-1–specific CD8⁺ Tmem cells (Pt 1: 0.08% at week 225; Pt 2: 0.05% at week 151) and performed microarray analysis using amplified cDNA. PD-1 mRNA levels of LRH-1–specific CD8⁺ Tmem cells at the time of relapse were elevated or similar compared with LRH-1–specific CD8⁺ Teff cells at the peak of the response for Pt 1 and Pt 2, respectively (Fig. 3C). These data indicate that LRH-1–specific CD8⁺ T cells express elevated levels of PD-1 on the cell surface, which remain present during the contraction and late memory phase of the immune response following DLI.

**PD-1 blockade augments proliferation of MiHA-specific CD8⁺ T cells**

To further elucidate the role of PD-1 in impairment of LRH-1–specific CD8⁺ Tmem cells, we performed functional assays using PBMCs from CML-AP Pt 1 containing 0.05% to 0.10% LRH-1–specific CD8⁺ Tmem cells several years after the initial response. Stimulation of PBMC of Pt 1 with peptide alone in the presence of IL-2 and IL-15 did not result in an increase of LRH-1–specific T cells (data not shown). Notably, PD-1 or PD-L1 blockade resulted in a 2- to 4-fold increase in the number of LRH-1–specific T cells (Fig. 4A). However, peptide stimulation in the presence of PD-1 blockade, but in the absence of professional APCs, resulted in insufficient T-cell outgrowth. Therefore, we stimulated PBMCs containing LRH-1–specific Tmem cells with peptide-loaded PD-L1–expressing iDC in the presence of PD-1 blockade. Blockade with anti-PD-1 antibody resulted in a 20 times higher number of LRH-1–specific CD8⁺ T cells after 3 stimulations with peptide-loaded PD-L1⁺ iDCs (Fig. 4B). Consistently, we observed a specific increase to 4.4% LRH-1–specific CD8⁺ T cells compared with 0.6% with the isotype control after repeated DC stimulations using blockade with anti-PD-1 (Fig. 4C).

Similar assays were performed with PBMC obtained 7 and 36 months post-DLI containing low numbers of LRH-1–specific Tmem cells from AML-M0 Pt 2. In these assays, we used mDC to prevent repetitive T-cell stimulation. At 7 months post-DLI, blocking with anti-PD-1 and anti-PD-L1 antibody resulted in increased outgrowth of LRH-1–specific CD8⁺ T cells up to 8.1% and 6.4%, respectively, compared with 2.0% in the presence of an isotype control (Fig. 4D). In addition, during relapse at 36 months post-DLI, upon PD-1 and PD-L1 blockade LRH-1–specific CD8⁺ T cells increased to 1.16% and 0.86%, respectively, compared with 0.59% for isotype control (Table 2). To confirm effect of PD-1/PD-L1 blockade on the proliferative capacity of other MiHA-specific T cells, we stimulated PBMC from a relapsed multiple myeloma (MM) patient (Pt 16) containing HA-1–specific T cells. In concordance with results obtained with LRH-1–specific CD8⁺ T cells, blockade of PD-1/PD-L1 interactions leads to enhanced mDC-stimulated proliferation of HA-1–specific CD8⁺ T cells (Fig. 4E). In addition, we investigated whether PD-1 blockade increased the absolute amount of MiHA-specific T cells. For AML Pt 2 and MM Pt 16, we observed a robust absolute increase of MiHA-specific T cells upon PD-1 and PD-L1 blockade (Fig. 5A).

Next, we investigated whether the effect of PD-1/PD-L1 is exclusive for dysfunctional MiHA-specific T cells in relapsed patients, or that it also affects potential nonimpaired T cells in patients with remission after alloSCT. Therefore, we investigated the effect of PD-1/PD-L1 blocking in CML-BC Pt 15 and pre-T ALL Pt 17 (Table 2). PD-1 and PD-L1 blockade does enhance the absolute number of MiHA-specific T cells, but the
effect is moderate (Fig. 5B). Finally, we compared the effect of PD-1 blockade on MiHA-specific T cells from relapsed patients to those from patients in remission. Importantly, we showed that PD-1 blockade has a significantly superior effect on dysfunctional MiHA-specific T cells from relapsed patients (Fig. 5C).

Collectively, these results show that PD-1 signaling impairs the proliferative capacity of MiHA-specific CD8<sup>+</sup> T cells upon antigen stimulation prior to or during relapse, and this functional impairment can be abrogated by PD-1/PD-L1 immune checkpoint blockade.

Discussion

AlloSCT is a potentially curative treatment for advanced myeloid leukemia (1). The effect largely depends on alloreactive CD8<sup>+</sup> T cells targeting MiHA on leukemic blasts and progenitor cells (20). However, MiHA-specific CD8<sup>+</sup> T-cell responses induced after transplantation are in many patients not sufficient to sustain complete remission. Distinct mechanisms are involved in reducing antitumor T-cell responses, allowing malignant cells to escape immune destruction. Among these mechanisms, T-cell inhibition or even exhaustion due to signaling of the PD-1/PD-L pathway may diminish immune responses by limiting the expansion and functionality of CD8<sup>+</sup> T cells (12, 21). Recently, we showed that LRH-1 leukemia can relapse without inducing secondary LRH-1–specific T<sub>mem</sub> cell expansion, suggesting that these T<sub>mem</sub> cells are either suppressed or not activated (3, 4). In this study, we examined the role of PD-1/PD-L1 interactions in functional impairment of LRH-1–specific CD8<sup>+</sup> T cells reactive to myeloid leukemia. Interestingly, we showed that PD-L1

Figure 3. MiHA-specific T cells express elevated levels of PD-1 in vivo. A, PD-1 expression of LRH-1–specific T cells in CML-AP Pt 1 and AML-M0 Pt 2 was determined by FCM. Time post DLI is indicated in weeks, and percentage of LRH-1–specific T cells of total CD8<sup>+</sup> T cells in brackets and MFI of LRH-1–specific T cells is displayed. B, PD-1 expression of LRH-1–specific T cells compared with total CD8<sup>+</sup> T cells in the same patient. C, LRH-1–specific T cells were isolated by FCM-assisted sorting. Subsequently, RNA was isolated and PD-1 mRNA levels were determined at the peak of the response, and in LRH-1–specific T<sub>mem</sub> cells 4.5 or 3 years after initial response. Raw intensity values measured from microarray analysis are depicted.
Figure 4. PD-1 blockade increases ex vivo proliferation of MiHA-specific CD8$^+$ Tmem cells. A, PBMC of CML-AP Pt 1 containing low levels of LRH-1–specific Tmem cells years after initial responses were stimulated by addition of LRH-1 peptide in the presence of blocking antibodies against PD-1 or PD-L1. Numbers of LRH-1–specific cells were enumerated by FCM, and numbers at isotype control were set to 1. Results are from 3 independent experiments from a sample 225 weeks after DLI (▲, ▼, and ★) and 1 sample taken 275 weeks after DLI (★). B, iDCs were loaded with LRH-1 peptide and added weekly to PBMC containing LRH-1–specific Tmem cells for 3 weeks, combined with blocking antibodies. C, LRH-1–specific T-cell percentages of total CD8$^+$ were identified by FCM. iDC stimulation assays with Pt 1 are representative of 3 separate experiments. D, mDCs loaded with LRH-1 peptide were used to stimulate LRH-1–specific Tmem cells of AML-M0 Pt 2. Subsequently, the percentage of LRH-1–specific T cells after 1 week was determined by FCM. E, mDCs loaded with HA-1 peptide were used to stimulate HA-1–specific Tmem cells of relapsed MM Pt 16. Subsequently, the percentage of HA-1–specific T cells after 1 week was determined by FCM.
and to some extent, PD-L2 was expressed by CD34+ progenitor myeloid leukemia cells of 2 patients with relapses after initial efficient T-cell responses. Furthermore, we confirmed expression of PD-L1 on a broader panel of AML samples at diagnosis. Previously, it has been shown that PD-L1 expression is elevated on relapse AML compared with diagnosis material (22). We investigated this in 1 CML patient, and indeed, PD-L1 expression was higher on relapse tumor cells than cells at diagnosis (Table 1). Especially, CD117+CD14−/CD10 early progenitor myeloid leukemia cells, which contain the leukemic stem cells, highly expressed PD-L1. PD-L1 expression increased upon exposure to inflammatory cytokines, whereas expression of CD80 and CD86 remained low. Consequently, prolonged PD-1/PD-L1 interactions may lead to functional exhaustion of LRH-1–specific Tmem cells, and relapse of the leukemia may occur without induction of a secondary immune response.

To investigate whether LRH-1–specific T cells display an impaired phenotype, we analyzed T cells of 2 patients with LRH-1–specific responses. It is known that PD-1 is elevated on T cells specific for viral epitopes in chronic viral infections (23). Also, during CML disease, PD-1 levels of the total population of CD8+ T cells are elevated (12). Here, we showed for the first time that MiHA-specific Tmem cells can have an elevated level of PD-1. Both patients with the nonresponding Tmem cells had leukemia relapses following a robust initial LRH-1–specific T-cell response. Whether or not elevated PD-1 expression on MiHA-specific T cells correlates with immune escape and subsequent relapse of myeloid leukemia needs to be investigated.
be determined in a larger cohort of patients. However, we found that the PD-1/PD-L1 pathway negatively influences the function of PD-1-expressing LHB-1–specific CTL. Most importantly, we show that blocking PD-1/PD-L1 interactions with human blocking antibodies resulted in increased outgrowth of MiHA-specific Tₘₑₙₖ cells. We also observed a stimulatory effect of PD-1 blockade on MiHA-specific T cells from patients in remission, which is not unexpected due to the role of PD-1 in regulation of T-cell activation. However, the abrogation of PD-1 signaling had a significant stronger effect on the proliferation of MiHA-specific T cells in relapsed patients than those in patients in remission.

Besides PD-1, several other inhibitory receptors play a role in functional T-cell exhaustion, such as CTLA-4, LAG-3, BTLA, TIM-3, CD160, and CD244 (24). In future years, the influence of this array of coinhibitory receptors will be further elucidated. Perhaps, combinations of blocking antibodies to PD-1 and LAG-3 will result in highly reactivated MiHA-specific T-cell responses (25). But, as PD-1 is involved in peripheral tolerance, autoimmune events following PD-1 blockade therapy may occur (26). In a recent phase I study, the clinical grade anti-PD-1 antibody BMS-936558, also used in our study, was administered to patients with solid tumors. Anti-PD-1 was well tolerated and only 1 serious adverse event, inflammatory colitis, was observed in a melanoma patient. Remarkably, 1 durable complete response and 2 partial responses were observed (16).

Our current in vitro data illustrate that PD-1 blockade is an attractive approach to reinvigorate impaired MiHA-specific T cells in patients with persisting or relapsed leukemia. However, in the setting of alloSCT, PD-1 blockade could aggravate GVHD. For optimal boosting selective GVT immunity in the post-SCT setting, we would like to combine active immunotherapy by DC vaccination using hematopoietic-restricted MiHAs with PD-1 blockade. By inducing a time-limited alleviation of PD-1 signaling combined with an antigen-specific stimulus, we aim to reuscitate the impaired MiHA-specific T cells, without causing autoimmune effects or GVHD. Another strategy is to specifically knock down PD-L1 and/or PD-L2 on MiHA-loaded DC vaccines by siRNA. In a recent article, we showed that stimulation with PD-L1/2 knockdown DCs resulted in specific outgrowth of initially unresponsive MiHA-specific T cells (15). This strategy would minimize off-target stimulatory effects, because the hyperstimulatory DCs are loaded with hematopoietic-restricted MiHA. Results of clinical trials being performed with BMS-936558 and BMS-936559 in parallel with preclinical mouse models using blocking antibodies in a post-SCT setting will determine the ideal therapy combination.

In conclusion, we showed PD-1 expression on myeloid leukemia cells, especially under inflammatory conditions. Interestingly, CD117⁺ early progenitor myeloid leukemia cells express high levels of PD-L1, but low CD80 and CD86 expression. Furthermore, we showed that blockade by human anti-PD-1 or anti-PD-L1 increases proliferation and IFN-γ and granzyme B production by LRH-1–specific CTL incubated with PD-L1⁺ leukemia cells. In addition, LRH-1–specific CD8⁺ T cells exhibit elevated PD-1 expression in vivo. Most importantly, we could specifically resuscitate initially unresponsive MiHA-specific Tₘₑₙₖ cells by PD-1/PD-L1 blockade. Therefore, we postulate that PD-1 blockade could be a powerful addition to post-SCT therapy. Combining MiHA-specific DC vaccination with PD-1 blockade may reinvigorate impaired MiHA-specific Tₘₑₙₖ cells and restore immune control, thereby preventing or attacking leukemia relapses.

Disclosure of Potential Conflicts of Interest

A. Korman is an employee of Bristol-Meyers Squibb. The remaining authors declare no competing financial interests.

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