PD-1 signaling impairs MiHA-specific CD8+ T cells

RESEARCH ARTICLE

PD-1/PD-L1 interactions contribute to functional T cell impairment in patients that relapse with cancer after allogeneic stem cell transplantation


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Abstract
Tumor relapses remain a serious problem after allogeneic stem cell transplantation (SCT), 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 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 leukemic progenitor cells, whereas co-stimulatory molecules such as CD80 and CD86 were not expressed. Thus, immature leukemic progenitor cells appeared 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 co-cultured 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 augments proliferation of MiHA-specific CD8+ memory T cells from relapsed patients. Taken together, our findings indicate that the PD-1/PD-L pathway can be hijacked as an immune escape mechanism in hematological malignancies. Further, they suggest that blocking the PD-1 immune checkpoint offers an appealing immunotherapeutic strategy following allogeneic SCT in patients with recurrent or relapsed disease.
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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) (1). In HLA-matched alloSCT, these alloreactive CD8+ T cell responses are directed against minor histocompatibility antigens (MiHA) (2). Previously, we have characterized CD8+ T cell immunity towards a hematopoietic-restricted MiHA, designated LRH-1, which is presented by HLA-B7 and encoded by the P2X5 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 demonstrated that CD34+ myeloid leukemia progenitor cells can be efficiently targeted in vitro by LRH-1-specific CD8+ cytotoxic T lymphocytes (CTL), indicating that these CTL play a significant role in GVL-specific immunity. However, we have observed that despite the presence of LRH-1-specific CD8+ memory T (T_mem) cells for many years, late relapses do occur in patients with advanced myeloid leukemia. Furthermore, we noticed that LRH-1-specific CD8+ T_mem 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, down-regulation of HLA molecules, and induction of immune suppressive 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 for T cell exhaustion resulting in diminished cytokine production, proliferation and cytolytic activity upon antigen re-stimulation (8). PD-1 binds two ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) (7). While PD-L2 expression is mainly restricted to antigen presenting cells (APC) like dendritic cells (DC) and macrophages, PD-L1 is also expressed by many non-hematological 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-1 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 demonstrated that PD-1/PD-L1 interactions play an important
PD-1 signaling impairs MiHA-specific CD8+ T cells 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 AML cells as well as DC. Together, these findings indicate that the PD-1 signaling pathway suppresses MiHA-specific CD8+ T cell responses and PD-1 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 peripheral blood mononuclear cells (PBMC) obtained from transplanted patients who developed MiHA-specific CD8+ T cell responses. Patient 1 (Pt1) suffered from accelerated-phase (AP) chronic myeloid leukemia (CML) and was successfully treated with therapeutic DLI after allogeneic SCT (3). However, the patient relapsed 4 years after DLI. Patient 2 (Pt2) suffered from AML and developed a LRH-1-specific CD8+ T cell response upon pre-emptive DLI, but developed extramedullary relapses without leukemic involvement in BM (4). Characteristics of these and other transplanted patients are included in Table 2.

DC were generated from monocytes isolated from PBMC of healthy donors by plastic adherence. Immature DC (iDC) were generated by culturing adherent monocytes in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 2% HS, 500 U/ml IL-4 and 800 U/ml GM-CSF (both Immunotools, Friesoythe, Germany). After 3 days, cells were harvested, used for T cell stimulations experiments or further cultured as described before (15). At day 8, mature DC (mDC) were harvested.
PD-1 signaling impairs MiHA-specific CD8+ T cells and used in T cell stimulation experiments. LRH-1-specific CD8+ CTL culture RP1 was isolated from CML-AP Pt1 and was cultured as described previously (4). Before use in T cell stimulation experiments, leukemia samples and DC 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, New York, NY, USA). In lymphocyte-AML reaction assays, allogeneic CD3+ T cells were isolated by direct magnetic labeling with the appropriate MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Subsequently, 10^4 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 LRH-1 peptide TPNQRQNC for 30 minutes at room temperature and co-cultured with LRH-1-specific CTL RP1 at a stimulation ratio of 10:1.

Blocking antibodies were added at a final concentration of 10 µg/ml. Antibodies to PD-1 (BMS-936,558; MDX-1106; ONO-4538) and PD-L1 (BMS-936,559; MDX-1105) and a matching IgG4 isotype control were kindly provided by Dr. Alan Korman (Bristol-Myers Squibb, Biologics Discovery, Milpitas, CA, USA). BMS-936,558 and BMS-936,559 are genetically engineered, fully human IgG4 antibodies currently evaluated in clinical trials for selected tumor treatments (16). All co-cultures were performed in a total volume of 200 µl IMDM/10% HS. After 5 days of co-culture, supernatant was harvested for cytokine analysis. At day 5, 0.5 µCi [³H]-thymidine (Perkin Elmer, Groningen, the Netherlands) was added to each well. After overnight incubation, [³H]-thymidine incorporation was measured using a 1205 Wallac Bepatplate counter (Perkin Elmer).

**MiHA-specific T_mem cell proliferation assays**

MiHA-specific CD8+ T cells present in PBMC from patients Pt 1, 2, 15-21 (Table 2) were stimulated for one to three 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 U/ml IL-2 and 5 ng/ml IL-15 (Immunotools) was
PD-1 signaling impairs MiHA-specific CD8+ T cells added. At day 7, cells were harvested, counted and the percentage of MiHA-tetramer+ CD8+ T cells was determined.

**Flow cytometry**

Expression of co-signaling ligands on myeloid leukemia cells and DC was analyzed by staining with the following fluorochrome-conjugated antibodies: CD14 (Dako, Glostrup, Denmark), CD3, CD34, CD117, CD54, CD80, CD83, CD86 (all from Beckman Coulter, Fullerton, CA, USA), anti-PD-L1, anti-PD-L2 (both from Becton Dickinson, Franklin Lakes, NJ, USA) and isotype controls IgG1 FITC/PE dual-color control (Dako) and IgG2b PE (Beckman Coulter). PD-1 expression on and percentage of MiHA-specific CD8+ T cells was 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 performed using 10 minutes of boiling in 0.01 M of sodium citrate pH 6.0 followed by incubation with primary antibodies anti-PD-L1 (eBioscience, San Diego, CA, USA), anti-PD-L2 (eBioscience), anti-CD8 (DAKO), anti-CD34 (Klinipath, Duiven, the Netherlands) and anti-FoxP3 (ITK Diagnostics, Uithoorn, the Netherlands). Staining was visualized either by 3,3-Diaminobenzidine (DAB) or aminoethyl carbazole (AEC) staining.

**IFN-γ and granzyme B ELISA**

Production of IFN-γ and granzyme B by stimulated T cells was determined by enzyme-linked immunosorbent assay (ELISA; IFN-γ: Pierce Endogen, Rockford, IL, USA; granzyme B: Mabtech, Nacka Strand, Sweden) according to manufacturer’s protocol.

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

Total RNA was isolated from cell samples using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis and PCR amplification were performed 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 ΔΔCt values and was quantified relative to cell line U266,
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which was set at 1 ΔΔCt value. ΔΔCt was calculated as follows: 2^-([ΔCt_{sample}] - [ΔCt_{U266}]), in which ΔCt was normalized for HMBS by calculating ΔCt = Ct_{gene} - Ct_{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 a >96% pure product, and resuspended in Trizol. RNA extraction, amplification, cDNA generation and microarray analysis were performed as described previously (19).

Statistical analysis

Paired one-tailed student-t test or one-way ANOVA followed by a Bonferroni post-hoc test were used when appropriate.

Results

Myeloid leukemia progenitor cells differentially express PD-L1 compared to 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 two 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 four years after DLI express PD-L1 upon stimulation with IFN-γ (Figure 1A), while expression of co-stimulatory molecules CD80 and CD86 on these CD34+ CML-AP cells is low. Furthermore, we observed high PD-L1 expression on CD34+ leukemia cells in a chloroma biopsy of an AML patient (Pt 2) who relapsed three years after DLI (Figure 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 chloroma, while FoxP3+ regulatory T cells were hardly detectable (Supplementary Figure 1). 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 an 137-fold and 31-fold up-regulation of PD-L1 and PD-L2 mRNA, respectively (Figure 1C).
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Furthermore, consistent with the findings in the two relapsed leukemia patients, PD-L1 cell surface expression was significantly up-regulated (> 20% PD-L1+ cells) on AML cells of 7 out of 10 newly diagnosed patients, while expression of PD-L2 was only slightly induced (Figure 1D). Notably, PD-L1-expressing AML cells displayed very low expression of CD80 and a variable expression of CD86, which was not influenced by IFN-γ/TNF-α treatment.

Because AML clones comprise heterogeneous populations of malignant cells, we studied whether different AML populations exhibited differential expression of co-signaling molecules. Using multi-color FCM, we defined three distinct AML populations defined as CD33+CD117+CD14- AML progenitor cells, CD33+CD117+CD14- AML myelo/monoblasts and CD33+CD117+CD14- AML promonocytes (Supplementary Figure 2A and B). A panel of nine AML patients with different FAB classifications was used for analyzing expression of co-signaling ligands upon IFN-γ ± TNF-α stimulation. Interestingly, the most immature CD33+CD117+CD14- AML cells exhibited high PD-L1 expression (range: 46-94 % PD-L1+ cells) in combination with almost absent or very low expression of PD-L2, CD80 and CD86 under inflammatory conditions (Figure 1E). The CD33+CD117+CD14- AML myelo/monoblasts showed slightly more up-regulation of PD-L1 and CD80 expression as well as higher CD86 expression (Supplementary Figure 2C). Mature CD33+CD117+CD14- AML promonocytes display combined up-regulation of PD-L1, PD-L2, CD80 and CD86 expression (Supplementary Figure 2D).

Collectively, these data demonstrate that immature AML cells which contain the putative leukemic stem cells selectively up-regulate PD-L1 expression following short-term exposure to IFN-γ and TNF-α, enabling these leukemia progenitor cells to inhibit T cell-mediated attack via the PD-1/PD-L1 pathway.

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-936,558 and anti-PD-L1/BMS-936,559 blocking antibodies. Blocking with either anti-PD-1 or anti-PD-L1 antibody significantly increased proliferation of CD3+ T cells upon stimulation with allogeneic PD-L1+ AML cells from AML-M4 Pt 9 (Figure 2A), while
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Allogeneic T cell proliferation stimulated with PD-L1+ and PD-L2+ mDCs could only be inhibited with anti-PD-1 (Figure 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 (Figure 2C) or PD-L1+/L2+ mDC (Figure 2D). These results demonstrate 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-L1 on AML cells in inhibiting the recognition by MiHA-specific CD8+ T cells, we performed antigen re-stimulation experiments using CTL clone RP1 that recognizes the hematopoietic-restricted MiHA LRH-1 on AML progenitor cells. RP1, as well as CTLs against other MiHA, up-regulate expression of PD-1 upon co-culture with MiHA+ AML (Supplementary Figure 3 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-L1-expressing primary AML cells from Pt11 loaded with MiHA-peptide (Figure 2 E and G). As hypothesized, we found that PD-1 blockade strongly elevated the proliferation and IFN-γ production by CTL RP1 when stimulated with peptide-loaded PD-L1+ iDC (Figure 2 F and H). Cytotoxicity of CTL versus AML was also enhanced after PD-1 and PD-L1 blockade (Figure 2 I), whereas no cytotoxicity was observed versus iDC (Figure 2 J). 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 Pt1 and AML-M0 Pt2 who relapsed three and four years, respectively, after the initial DLIs 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 Pt1 and at week 10 for AML-M0 Pt2 (Figure 3A). We observed relatively elevated expression of PD-1 on LRH-1 tetramer-positive T cells compared to tetramer-negative CD8+ T cells in the same patient (Figure 3A and B). After DLI, PD-1 levels at the cell surface of LRH-1-specific CD8+ T
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cells gradually declined, but > 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 (Pt1: 1.8% at week 28; Pt2 2.9% at week 15) and low frequencies of LRH-1-specific CD8+ Tmem cells (Pt1: 0.08% at week 225; Pt2 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 to LRH-1-specific CD8+ Teff cells at the peak of the response for Pt1 and Pt2, respectively (Figure 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 Pt1 containing 0.05-0.10% LRH-1-specific CD8+ Tmem cells several years after the initial response. Stimulation of PBMC of Pt1 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 (Figure 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 three stimulations with peptide-loaded PD-L1+ iDCs (Figure 4B). Consistently, we observed a specific increase to 4.4% LRH-1-specific CD8+ T cells compared to 0.6% with the isotype control after repeated DC stimulations using blockade with anti-PD-1 (Figure 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 Pt2. In these assays, we used mDC in order 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 to 2.0% in the presence of an isotype control (Figure 4D). In addition, during relapse at 36 months post-DLI, upon PD-1 and PD-L1 blockade LRH-1-specific CD8+ T cells increased
PD-1 signaling impairs MiHA-specific CD8⁺ T cells to 1.16% and 0.86%, respectively, compared to 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 MM patient (Pt16) 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 (Figure 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 (Figure 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 non-impaired 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 (Figure 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 (Figure 5C).

Collectively, these results demonstrate that PD-1 signaling impairs the proliferative capacity of MiHA-specific CD8⁺ 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⁺ T cells targeting MiHA on leukemic blasts and progenitor cells (20). However, MiHA-specific CD8⁺ T cell responses induced after transplantation are in many patients not sufficient to sustain complete remission. Distinct mechanisms are involved in reducing anti-tumor 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⁺ T cells (12,21). Recently, we showed that LRH-1⁺ leukemia can relapse without inducing secondary LRH-1-specific CD8⁺ Tₘₚ cell expansion, suggesting that these Tₘₚ cells are either suppressed or not activated (3,4). In this study,
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we examined the role of PD-1/PD-L1 interactions in functional impairment of LRH-1-specific CD8+ T cells reactive to myeloid leukemia. Interestingly, we showed that PD-L1 and to some extent PD-L2 was expressed by CD34+ progenitor myeloid leukemia cells of two 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 to diagnosis material (22). We investigated this in one CML patient, and indeed PD-L1 expression was higher on relapse tumor cells compared to cells at diagnosis (Table 1). Especially CD117+CD14- early progenitor myeloid leukemia cells, which contain the leukemic stem cells, highly expressed PD-L1. PD-L1 expression increased upon exposure to inflammatory cytokines, while 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 two 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 non-responding 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 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 LRH-1-specific CTL. Most importantly, we demonstrated that blocking PD-1/PD-L1 interactions with human blocking antibodies resulted in increased outgrowth of MiHA-specific Tmem 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 compared to 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 co-inhibitory receptors will be further elucidated. Perhaps combinations of blocking antibodies to PD-1
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and LAG-3 will result in highly re-activated 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-936,558, also used in our study, was administered to patients with solid tumors. Anti-PD-1 was well tolerated and only one serious adverse event, inflammatory colitis, was observed in a melanoma patient. Remarkably, one durable complete response and two 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 resuscitate the impaired MiHA-specific T cells, without causing autoimmune effects or GVHD. Another strategy is to specifically knockdown PD-L1 and/or PD-L2 on MiHA-loaded DC vaccines by siRNA. In a recent paper, 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, since the hyper-stimulatory DCs are loaded with hematopoietic-restricted MiHA. Results of clinical trials being performed with BMS-936,558 and BMS-936,559, in parallel with pre-clinical mouse models using blocking antibodies in a post-SCT setting will determine the ideal therapy combination.

In conclusion, we demonstrated PD-L 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 were able to specifically resuscitate initially unresponsive MiHA-specific T_{mem} 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 T_{mem} cells and restore immune control, thereby preventing or attacking leukemia relapses.
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PD-1 signaling impairs MiHA-specific CD8+ T cells

Legends

Figure 1. Myeloid leukemia cells express PD-L1 under inflammatory conditions. Expression of co-signaling ligands on leukemia cells was determined by flow cytometry, immunohistochemistry and RT-PCR. (A) Flow cytometry 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 Pt2 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 1000x. (C) CD33+ AML cells from 9-12 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 co-stimulatory ligands on CD33+ AML cells, in the absence (-) or presence (+) of IFN-γ and TNF-α, was determined by flowcytometry. (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 one-tailed student-t test was performed. * p<.05; ** p<.01; *** p<.001.

Figure 2. PD-1 blockade enhances T cell responses to stimulation by primary AML cells and DC. Allogeneic CD3+ T cells were co-cultured 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-γ production was evaluated (C and D). LRH-1-specific CTL RP1 was co-cultured 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-γ production (G and H). Cytotoxicity was measured by granzyme B secretion (I and J). One representative experiment out of 3. One way ANOVA was performed, compared to isotype antibody as control. * p<.05; ** p<.01; *** p<.001.

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 Pi2 was determined by flowcytometry. Time post
PD-1 signaling impairs MiHA-specific CD8+ T cells

DLI is indicated in weeks, percentage of LRH-1-specific T cells of total CD8+ 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 to total CD8+ T cells in the same patient. (C) LRH-1 specific T cells were isolated by flowcytometry-assisted sorting. Subsequently, RNA was isolated and PD-1 mRNA levels were determined at the peak of the response, and in LRH-1-specific Tmem 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 Pt1 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 flowcytometry, and numbers at isotype control were set to 1. Results are from three independent experiments from a sample 225 weeks after DLI (▲, ■ and ●) and one 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 flowcytometry. iDC stimulation assays with Pt1 are representative of 3 separate experiments. (D) mDC 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 one week was determined by flowcytometry. (E) mDC 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 one week was determined by flowcytometry.

Figure 5. The effect of PD-1 blockade on proliferation of MiHA-specific T cells in relapsed patients is higher than in patients in remission. PBMC containing MIHA-specific T cells were stimulated with DC containing their cognate peptide with or without anti-PD-1 or anti-PD-L1. (A) PD-1/PD-L1 blockade enhances expansion of MiHA-specific T cells in relapsed patients. Pt 2 and 16 experienced relapse after initial MIHA-specific responses. PBMC prior to additional therapy to treat relapse were investigated. (B) PD-1/PD-L1 blockade moderately enhances expansion of MIHA-specific T cells in patients in long-term remission. Pt 15 and 17 remained in remission after initial immune
PD-1 signaling impairs MiHA-specific CD8+ T cells responses. (C) The effect of PD-1 blockade is significantly higher on MiHA-specific T cells in relapsed patients. Ratio was calculated by dividing the absolute number of tet+ T cells in the presence of anti-PD-1 antibody by the absolute number of tet+ T cells in the presence of isotype control. One-tailed student t-test was performed, * p<.05.
Figure 1

A

CML-AP Pt1

B

AML-M0 Pt2

CD34

isotype

CD86

PD-L1

PD-L2

C

mRNA expression (L-G)

IFNγ + TNFα

PD-L1

PD-L2

D

Surface expression [%]

100

50

25

10

0

PD-L1

PD-L2

CD80

CD86

E

Surface expression [%]

100

50

25

10

0

PD-L1

PD-L2

CD80

CD86

CD86

CD80

CD86

CD86
Figure 2
Figure 3

A

Pt1
+ week 9 (0.33%)
MFI 23.5
Pt2
+ week 6 (0.12%)
MFI 27.1
+ week 28 (1.80%)
MFI 24.8
+ week 10 (6.90%)
MFI 19.9
+ week 39 (0.54%)
MFI 19.3
+ week 50 (0.38%)
MFI 17.7
+ week 15 (2.91%)
MFI 8.3
+ week 20 (0.69%)
MFI 8.5

B

Pt1
CD8⁺ LRH1⁺
CD8⁺
CD8⁺ Isotype

C

Pt1
Pt2
CD8⁺ LRH1⁺
CD8⁺
CD8⁺ Isotype

arbitrary expression units
0 10 20 30 40 50
Time (weeks)
0 10 20 30 40 50
arbitrary expression units
0 10 20 30 40 50
arbitrary expression units
0 10 20 30 40 50
20 25 30 35 40 45 50
20 25 30 35 40 45 50
20 25 30 35 40 45 50
20 25 30 35 40 45 50
Figure 4

A week 1 peptide

B week 3 IDC

C Pt 1 week 2 IDC

D Pt 2 week 1 mDC

E Pt 16 week 1 mDC
Figure 5

A. Pt 2 Absolute number of LRH-1-specific T cells

B. Pt 15 Absolute number of HA-1-specific T cells

C. Increase of MiHA-specific T cells upon PD-1 blockade

- Number of cells
- Fold increase
- Relapse remission
### Table 1. Myeloid leukemia patient characteristics and PD-L1 expression.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease-FAB classification</th>
<th>Sample type</th>
<th>WBC count at sample date</th>
<th>PD-L1 mRNA expression unstim</th>
<th>IFN-γ</th>
<th>TNF-α</th>
<th>PD-L1 surface expression (MFI) unstim</th>
<th>IFN-γ</th>
<th>TNF-α</th>
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<td>CML-CP</td>
<td>PB</td>
<td>11</td>
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<td>ND</td>
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<td>0.6</td>
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<td>2-relapse</td>
<td>AML-M0 chloroma</td>
<td>BM</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>+^a</td>
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<td>259</td>
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<td>AML-M2</td>
<td>BM</td>
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<td>254.2</td>
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<td>AML-M5</td>
<td>BM</td>
<td>ND</td>
<td>28.3</td>
<td>800.2</td>
<td>3.5</td>
<td>29.5</td>
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Characteristics of myeloid leukemia patients which 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; PB Peripheral Blood; BM Bone Marrow; unstim unstimulated; MFI Mean Fluorescence Intensity; ND not determined; WBC White Blood Cell Count in 10^9/L peripheral blood; ^a Determined by immunohistochemistry.
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>
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<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, 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, 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 gr/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>
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<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>
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<tr>
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<td>AML</td>
<td>HA-8</td>
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<td>0.7</td>
<td>Remission; alive at 213 mo post-SCT</td>
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<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>
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<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>

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 Donor Lymphocyte Infusion; tDLI therapeutic Donor Lymphocyte Infusion; SCT Stem Cell Transplantation; MiHA-specific response: MiHA for which a response was observed; Effect PD-1 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;
PD-1/PD-L1 interactions contribute to functional T cell impairment in patients that relapse with cancer after allogeneic stem cell transplantation


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