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B and T Lymphocyte Attenuator Mediates Inhibition of Tumor- Reactive CD8+ T Cells in Patients After Allogeneic Stem Cell Transplantation

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Allogeneic stem cell transplantation (allo-SCT) can cure hematological malignancies by inducing alloreactive T cell responses targeting minor histocompatibility antigens (MiHA) expressed on malignant cells. Despite induction of robust MiHA-specific T cell responses and long-term persistence of alloreactive memory T cells specific for the tumor, often these T cells fail to respond efficiently to tumor relapse. Previously, we demonstrated the involvement of the coinhibitory receptor programmed death-1 (PD-1) in suppressing MiHA-specific CD8+ T cell immunity. In this study, we investigated whether B and T lymphocyte attenuator (BTLA) plays a similar role in functional impairment of MiHA-specific T cells after allo-SCT. In addition to PD-1, we observed higher BTLA expression on MiHA-specific CD8+ T cells compared with that of the total population of CD8+ effector-memory T cells. In addition, BTLA’s ligand, herpes virus entry mediator (HVEM), was found constitutively expressed by myeloid leukemia, B cell lymphoma, and multiple myeloma cells. Interference with the BTLA–HVEM pathway, using a BTLA blocking antibody, augmented proliferation of BTLA+PD-1+ MiHA-specific CD8+ T cells by HVEM-expressing dendritic cells. Notably, we demonstrated that blocking of BTLA or PD-1 enhanced ex vivo proliferation of MiHA-specific CD8+ T cells in respectively 7 of 11 patients. Furthermore, these expanded MiHA-specific CD8+ T cells competently produced effector cytokines and degranulated upon Ag reencounter. Together, these results demonstrate that BTLA–HVEM interactions impair MiHA-specific T cell functionality, providing a rationale for interfering with BTLA signaling in post-stem cell transplantation therapies. The Journal of Immunology, 2012, 189: 000–000.

A lllogeneic stem cell transplantation (allo-SCT) can be a curative treatment for hematological malignancies (1). The therapeutic effectiveness is attributed to the graft-versus-tumor (GVT) effect, which is constituted by donor T cells targeting minor histocompatibility antigens (MiHA) expressed by recipient tumor cells (2). Recipient dendritic cells (DC) present MiHA to donor-derived MiHA-specific CD8+ T cells, whereupon these cells get activated and expand. Subsequently, they migrate to the tumor sites and lyse the cancer cells (3). Adaptive immune responses, including alloreactive responses, terminate in a contraction phase, leaving a small pool of long-lived MiHA-specific memory cells (4). Unfortunately, despite the presence of these memory cells, many patients with a hematological malignancy relapse post-transplant (5, 6), suggesting that MiHA-specific T cell functionality is affected. Tumor cells are known to evade immune attack via multiple mechanisms (7), among which is interference with T cell activation. This is a tightly coordinated event, involving TCR-mediated recognition of the cognate peptide, ligation of coactivating receptors, and cytokine signaling (8). In recent years, the distinct role of coinhibitory receptors in this process has been extensively investigated (9, 10). Expression of these receptors has been linked to functional impairment of T cells in various diseases, such as chronic viral infections (11, 12) and cancer (13–15).

We recently investigated whether MiHA-specific T cells in patients after allo-SCT are functionally impaired by signaling of coinhibitory receptors and demonstrated that blockade of the programmed death-1 (PD-1; CD279) pathway augmented proliferation and cytokine production of MiHA-specific T cells (16). PD-1 is involved in T cell exhaustion, a phenomenon characterized by the gradual loss of T cell functionality (i.e., diminished cytokine production, proliferative capacity, and cytolytic activity) (17). In addition, it was observed that concurrent expression of multiple coinhibitory receptors determines the functional state of T cells (18). One of these coinhibitory receptors is B and T lymphocyte...
attenuator (BTLA; CD272) (19). This receptor is a member of the CD28/B7 family, but in contrast to PD-1 and CTLA-4, BTLA binds to a member of the TNF receptor superfamily, herpes virus entry mediator (HVEM) (20). Other binding partners of HVEM are CD160 and LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes) (21). HVEM ligation to the receptors BTLA and CD160 was shown to be inhibitory, whereas the interaction of HVEM with LIGHT mediates T cell stimulation (22). Recently, a role for BTLA was implicated in the escape of melanoma cells from T cell immunity (23). The authors demonstrated that high BTLA expression on Melan-A+specific CD8+ T cells correlated with inhibition of Ag-specific cytokine production in melanoma patients. Therefore, we investigated the role of BTLA in the functional impairment of MiHA-specific T cells in allo-SCT patients. In this study, we demonstrated that these alloreactive cells had high expression of both BTLA and PD-1. Moreover, we showed that HVEM and BTLA were constitutively expressed by hematological cancer cells, suggesting a role of the BTLA–HVEM pathway in tumor immune evasion in allo-SCT patients. Most importantly, we showed augmented proliferation of MiHA-specific T cells upon ex vivo stimulation after anti-BTLA blockade. Together, these results demonstrate that BTLA interactions contribute to the functional impairment of MiHA-specific T cells, providing a rationale for incorporation of BTLA interference in post-stem cell transplantation therapies.

**Materials and Methods**

**Patient and donor material**

Mononuclear cells were isolated from healthy donor buffy coats (Sanquin, Nijmegen, The Netherlands) or from blood and bone marrow samples of patients with hematological malignancies. To examine primary tumor cells, bone marrow samples of eight acute myeloid leukemia (AML) patients and 10 multiple myeloma (MM) patients obtained at diagnosis were used. Furthermore, we used PBMC from transplanted patients who developed MiHA- or CMV-specific CD8+ T cell responses. These were obtained 3–13 mo after allo-SCT. Characteristics of these patients are described in Table I. Because no apheresis material of the corresponding donors was available, DC were cultured from apheresis material of allogeneic HLA-A2* or HLA-B7* donors. All cells of healthy donors and patients were obtained after written informed consent.

**Cell culture**

Hematopoietic tumor cell lines were cultured in IMDM (Invitrogen, Carlsbad, CA) supplemented with 10–20% FCS (Integro, Zaandam, The Netherlands). UM-6 cells were cultured in IMDM–10% FCS with 22.5 ng/ml IL-6 (ImmunoTools, Friesoythe, Germany). AML-193 cells were cultured in IMDM–20% human serum (HS; PAA Laboratories, Pasching, Austria) containing 2 ng/ml IL-3 (Cellgenix, Freiburg, Germany) and 2 ng/ml GM-CSF (ImmunoTools).

**Generation of monocyte-derived DC**

Healthy donor monocytes were isolated from PBMC via plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Immature DC were generated by culturing monocytes in X-VIVO-15 medium (Lonza, Verviers, Belgium) supplemented with 2% HS, 500 U/ml IL-4 (ImmunoTools), and 800 U/ml GM-CSF. After 3 d, cells were harvested and cultured in 6-well plates (Corning Costar, Lowell, MA) at 1 × 10^5 DC/well in X-VIVO–15–2% HS containing 500 U/ml IL-4 and 800 U/ml GM-CSF. Maturation of DC was induced at day 6 by culturing in X-VIVO–15–2% HS containing 500 U/ml IL-4, 800 U/ml GM-CSF, 5 ng/ml IL-1β, 15 ng/ml IL-6, 20 ng/ml TNF-α (all ImmunoTools), and 1 μg/ml PGE2 (Pharmacia & Upjohn, Bridgewater, NJ). At day 8, mature DC were harvested and used in T cell stimulation assays.

**Flow cytometry**

BTLA and PD-1 expression on CD8+ T cell subsets of healthy donors and allo-SCT patients was analyzed by staining PBMC with Abs against CCR7 (clone 150503; R&D Systems, Abingdon, U.K.), CD45RA (clone H100; BioLegend, Antwerp, Belgium), CD3 (clone UCHT1; Beckman Coulter, Fullerton, CA), and CD8 (clone 3B5; Invitrogen) in combination with BTLA (clone 116B-340), PD-1 (clone MIH4; both BD Biosciences, Franklin Lakes, NJ), or IgG1 isotype control (Dako, Glostrup, Denmark). In case of PBMC containing MiHA-specific, CMV-p65-specific, or influenza virus (FLU)-specific CD8+ T cells, cells were first labeled with 1.5-2 μg allopurinolcytin conjugated tetramer containing the corresponding peptide. Tetramers were kindly provided by Prof. Dr. Frederik Balkenau (Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands).

**Human AML and MM cell lines, primary patient samples, and healthy donor leukocytes were phenotyped by staining the cells with Abs recognizing MiHA-specific (clone 122; BioLegend), BTLA, CD60 (clone BY55; Beckman Coulter), LIGHT [clone 7-3(7); eBioscience, Vienna, Austria], or IgG1 FITC–PE dual-color isotype control (Dako). Primary AML cells were gated on CD33+ cells using a CD33 Ab (clone D3H10.251; Beckman Coulter). Primary MM cells were gated on CD38+CD138+ cells using Abs against CD38 and CD138 (clone H142; Beckman Coulter). Healthy donor leukocytes were labeled with Abs recognizing CD3 (clone UCHT1), CD8 (clone SFC127/Thy2D3; Beckman Coulter), CD14 (clone HCD14; BioLegend), and CD19 (clone HD37; Dako).

**MiHA-specific T cell cultures were incubated with PE-labeled tetramer and, subsequently, labeled with FITC-conjugated CD8 (clone LT8; Proimmune, U.K.) and PE-Cy7–conjugated CD3. After washing with PBS–0.5% BSA (Sigma, St. Louis, MO), cells were resuspended in washing buffer containing 0.1% 7-aminoactinomycin D (Sigma). Cells were analyzed using the Coulter FC500, Navios, or the Cyan-ADP flow cytometer (all Beckman Coulter).

**BTLA and PD-1 blocking Abs**

Mouse anti-human BTLA IgG1 blocking Ab, BTLA-8.2, was kindly provided by Prof. Dr. Daniel Olive (INSERM Unité Mixte de Recherche 991, Institut Paoli Calmettes, Marseille, France). Mouse IgG1 isotype control (clone MOPC21) was obtained from BioXCell (West Lebanon, NH). Human PD-1 blocking Ab (BMS-936,558; MDMX-1106; ONO-4538) and a matching IgG4 isotope control were kindly provided by Dr. Alan Korman (Bristol-Myers Squibb, Biologics Discovery, Milpitas, CA).

**Ex vivo Ag-specific T cell expansion and degranulation assays**

CMV-p65–specific or MiHA-specific CD8+ memory T cells present in PBMC from respectively healthy donors or patients (patients 1–11, Table I) were stimulated for 1 wk with peptide-loaded DC in the presence of Ag and/or PD-1 blocking Ab. Mature allogeneic DC, cultured from apheresis material of HLA-A2* or HLA-B7* donors, were loaded with or without 10 μM MiHA or CMV-p65 peptide (LRH-1: TPQRQGCVNQ; HA-1: VLHDDLEA; HA-2: YIGEVLVSV; HA-8: RTLDKVLVEY; HY.A2: FYD-SYICQV; CMV-p65: NLYVMVATVP or RPHERNQFTVL) for 30 min at 37˚C. PBMC were washed with IMDM–10% HS supplemented with 50 U/ml IL-2 and 5 ng/ml IL-15 (ImmunoTools) and a matching IgG4 isotype control were kindly provided by Prof. Dr. Alan Korman (Bristol-Myers Squibb, Biologics Discovery, Milpitas, CA).

**Intracellular cytokine staining**

CMV-p65–specific or MiHA-specific CD8+ memory T cells present in PBMC were stimulated with DC loaded with 10 μM peptide, as described earlier. After 7 d, cells were restimulated with 5 μM Ag peptide. After 1.5 h, brefeldin A (BD Biosciences) was added to inhibit protein transport within the cell. The next day, cells were first labeled with allopurinolcytin conjugated tetramer, CD3 PE–CY7, and CD8 Alexa Fluor 700, as described previously. After washing in PBS–0.5% BSA, cells were fixed during 15 min at room temperature with Reagens A (Life Technologies, Grand Island, NY). Cells were washed, resuspended in Reagens B (Life Technologies), and stained for 10 min at room temperature. After 1.5 h, FITC AB (clone B27) and TNF-α PE AB (clone MAB11; both BD Biosciences) or IgG1 FITC–PE isotype control (Dako). Finally, cells were washed and analyzed using flow cytometry.
Statistics
To determine statistical differences, a two-sample two-tailed $t$ test assuming independent samples, one-way ANOVA with a Bonferroni post hoc test, or a Kruskal–Wallis analysis followed by a Dunn’s post hoc test was used, as indicated. The $p$ values <0.05 were considered significant.

Results
MiHA-specific CD8$^+$ EM T cells show high BTLA and PD-1 expression
Flow cytometric analysis was performed on CD8$^+$ T cells from allo-SCT patients and healthy donors to determine BTLA and PD-1 expression levels on MiHA-, CMV-, and FLU-specific CD8$^+$ T cells in comparison with distinct CD8$^+$ T cell differentiation stages. CD8$^+$ T cell subset composition was analyzed by gating on CCR7 and CD45RA (Fig. 1A, 1B). In healthy donors, the total CD8$^+$ T cell pool was composed of naive, effector-memory (EM), and central-memory (CM) cells. Allo-SCT patients, on the contrary, had very low numbers of naive and CM CD8$^+$ T cells, and their CD8$^+$ T cells predominantly consisted of EM cells (84.2 ± 2.3%). Similar to CMV-pp65–specific CD8$^+$ T cells in healthy donors (94.1 ± 3.0%), MiHA-specific and CMV-pp65–specific CD8$^+$ T cells in allo-SCT patients were mainly of EM phenotype (95.7 ± 2.1% and 95.7 ± 1.3%, respectively). However, the FLU-

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** BTLA and PD-1 are highly expressed by MiHA-specific CD8$^+$ T cells. CD8$^+$ T cell subsets of healthy donors and allo-SCT patients were examined for BTLA and PD-1 expression using flow cytometry. (A and B) T cell subset composition was analyzed by gating on CCR7 and CD45RA expression within the total or Ag-specific CD3$^+$CD8$^+$ T cell populations. Naive (N), CCR7$^+$CD45RA$^+$; CM, CCR7$^+$CD45RA$^-$; EM, CCR7$^-$ (A) Representative examples of the CD8 subset composition in one healthy donor (HD) and one allo-SCT patient (Pt) are given. (B) Combined data on CD8 subset composition for healthy donors and allo-SCT patients is shown (HD CD8, $n=8$; HD CMV and FLU, $n=5$; Pt CD8, $n=6$; Pt CMV and MiHA, $n=5$). Data are expressed as mean ± SEM. (C) Percentage of BTLA$^+$ and PD-1$^+$ cells was examined within the different CD8$^+$ T cell subsets. (D) Within the EM subset of the total or Ag-specific CD3$^+$CD8$^+$ T cell population, the percentage of BTLA$^+$ and PD-1$^+$ cells was examined. Statistical analysis was performed using a two-sample two-tailed $t$ test assuming independent samples (C) or one-way ANOVA followed by a Bonferroni post hoc test (D). White bars, healthy donors; gray bars, allo-SCT patients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 

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specific CD8+ T cell population in healthy donors comprised both CM (35.0 ± 9.8%) and EM (62.9 ± 10.3%) cells.

In healthy individuals, BTLA was highly expressed on naive CD8+ T cells and gradually decreased on more differentiated T cells, with EM CD8+ T cells showing markedly lower BTLA levels than the other T cell subsets (Fig. 1C, Supplemental Fig. 1A). When comparing CD8+ T cell subsets of healthy donors with those of allo-SCT patients, the number of naive BTLA+ T cells was significantly lower in allo-SCT patients. In contrast to BTLA, PD-1 expression by naive T cells was low and markedly increased upon T cell differentiation into effector and memory cells (Fig. 1C, Supplemental Fig. 1). In allo-SCT patients, the percentage of PD-1+ cells within each CD8+ T cell subset was significantly higher than that in healthy individuals.

As most of the MiHA-specific and CMV-specific CD8+ T cells displayed an EM phenotype, we compared the expression of BTLA and PD-1 within the EM subset of tetramer+ and total CD8+ T cells (Fig. 1D, Supplemental Fig. 1B, 1C). Both in healthy donors and allo-SCT patients, the percentage of BTLA+ cells was significantly higher for CMV-specific CD8+ EM T cells than for total CD8+ EM T cells. The percentage of BTLA-positive FLU-specific T cells was similar to that in the total CD8+ EM T cell population of healthy donors, although BTLA levels on these T cells were slightly lower (Supplemental Fig. 1C). Notably, MiHA-specific CD8+ T cells of allo-SCT patients highly expressed both BTLA and PD-1. Also CMV-specific and FLU-specific T cells in healthy donors showed increased PD-1 expression, however to a lesser extent than in allo-SCT patients. Collectively, these data demonstrate that MiHA-specific EM T cells of allo-SCT patients have elevated BTLA and PD-1 expression.

**HVEM and BTLA are highly expressed by human hematopoietic tumor cells**

To determine the expression of HVEM, BTLA, CD160, and LIGHT on hematopoietic malignancies, we analyzed various tumor cell lines, patient bone marrow samples, and healthy donor leukocytes for cell surface expression using flow cytometry. First, we analyzed myeloid leukemia [both AML and chronic myeloid leukemia (CML)], B cell non-Hodgkin lymphoma (B-NHL), and MM cell lines (Fig. 2A). HVEM levels were constitutively high on all of these malignancies, although the membrane expression was slightly lower on the immature (FAB type M0-M2) leukemia cell lines compared with the more mature (FAB type M5) leukemia cell lines (Supplemental Table I, Supplemental Fig. 2A). In contrast to HVEM, BTLA was restricted to the B cell malignancies and showed a more heterogeneous expression pattern. No expression of CD160 was observed for any of the tumor cell lines examined. LIGHT expression levels, in contrast, were highest on some leukemia cell lines and modest in B-NHL and MM tumor cell lines. To confirm these expression patterns in primary human tumor cells, we analyzed bone marrow samples of 8 AML and 10 MM patients obtained at time of diagnosis. AML and MM cells
were gated on the CD33+SSC\textsuperscript{low} and CD38+CD138+ populations, respectively. Similar expression patterns were found for HVEM and LIGHT in the primary tumor samples compared with the tumor cell lines (Fig. 2B, 2C, Supplemental Table II, Supplemental Fig. 2B). Although CD160 was again not expressed by the malignant cells, BTLA expression was substantially higher on the primary AML and MM cells than on the corresponding tumor cell lines. We also examined healthy donor control cells and observed high HVEM and BTLA expression on T cells, monocytes, and B cells (Fig. 2D). However, expression levels were significantly higher on the monocytes and B cells (Supplemental Fig. 2C). Furthermore, monocytes also displayed significantly higher LIGHT expression than the other cell types. Together, these data show that human AML and MM cells display high levels of HVEM, whereas BTLA is highly expressed on MM cells and moderately on AML cells.

**PD-1 blockade augments the proliferative capacity of fully functional CMV-specific CD8\textsuperscript{+} T cells**

First, we investigated the effect of BTLA and/or PD-1 blockade on CMV-specific T cell responses. As in healthy donors, a high level of PD-1 and BTLA on pp65-specific T cells was observed. Therefore, we stimulated PBMC of three healthy donors with CMV-pp65 peptide-loaded DC in the presence or absence of blocking Ab. After 1 wk, CMV-specific CD8\textsuperscript{+} T cell proliferation and function was analyzed. In Fig. 3A, the percentage of tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells is shown for one representative healthy donor. Blockade of BTLA resulted in a slightly higher percentage of CMV-specific T cells after 1 wk. In contrast, PD-1 blockade clearly boosted the percentage and absolute number of CMV-specific CD8\textsuperscript{+} T cells (Fig. 3B). The combination of BTLA and PD-1 blockade had no synergistic effects in this donor. To combine data of all healthy donors, the fold expansion was calculated as the absolute number of MiHA-specific CD8\textsuperscript{+} T cells in the presence of Ab blockade relative to isotype control treatment (Fig. 3C). Notably, only PD-1 blockade and the combination with anti-BTLA treatment significantly enhanced the expansion of CMV-specific CD8\textsuperscript{+} T cells. To visualize the proliferation of the CMV-specific T cells, we labeled PBMC of donor 2 with CFSE\textsuperscript{+} and performed a 1-wk stimulation assay. After overnight restimulation with 5 \(\mu\)M CMV peptide in the presence of anti-CD107a Ab, the proliferation and degranulation capacity of the CMV-specific CD8\textsuperscript{+} T cells was analyzed. Upon stimulation in the presence of PD-1 blocking Ab, more cells had a CFSE\textsuperscript{−} phenotype compared with isotype control-treated cells (Fig. 3D). Furthermore, >66% of the tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells showed high CD107a staining upon overnight restimulation with CMV peptide (Fig. 3E). Notably in this donor, BTLA blockade did not further increase the degranulation capacity of the CMV-specific CD8\textsuperscript{+} T cells, but blocking of PD-1 resulted in a higher percentage of CD107a\textsuperscript{+} Ag-specific T cells. Moreover, combined BTLA and PD-1 blockade further boosted CMV-specific T cell degranulation. Finally, we examined the production of effector cytokines IFN-\(\gamma\) and TNF-\(\alpha\) of 1-wk expanded CMV-specific T cells. After overnight restimulation, most of the CMV-specific CD8\textsuperscript{+} T cells produced either IFN-\(\gamma\) alone or in combination with TNF-\(\alpha\) (Fig. 3F). Blockade of BTLA did not improve the percentage of Ag-specific T cells producing cytokines. On the contrary, blockade of PD-1 alone or in combination with BTLA enhanced the percentage of IFN-\(\gamma\) and IFN-\(\gamma\)-TNF-\(\alpha\) cells within the CMV-specific CD8\textsuperscript{+} T cell population of this donor. These results indicate that blockade of PD-1, but not of BTLA, results in increased proliferation of CMV-specific CD8\textsuperscript{+} T cells of healthy donors and that these Ag-specific T cells are highly functional upon restimulation with the cognate peptide.

**Both BTLA blockade and PD-1 blockade augment expansion of MiHA-specific CD8\textsuperscript{+} T cells ex vivo**

To investigate the role of BTLA in the inhibition of MiHA-specific CD8\textsuperscript{+} T cell responses, we performed functional assays using PBMC from 11 patients with a hematological malignancy who developed MiHA-specific T cell responses after allo-SCT. Previously, we demonstrated that reinvigoration of MiHA-specific CD8\textsuperscript{+} T cell responses requires mature DC loaded with the cognate MiHA (16, 24). Therefore, we first analyzed expression levels of HVEM and its binding partners on mature monocyte-derived donor DC (Fig. 4A). Similarly to hematological tumor cells, mature DC displayed high HVEM expression and moderate levels of BTLA and LIGHT. CD160, in contrast, was not expressed. Subsequently, these DC were pulsed with MiHA peptide and used for ex vivo stimulation of MiHA-specific memory T cells in the presence of BTLA and/or PD-1 blocking Ab. In Fig. 4B, results of three representative patients are shown. At start, PBMC of patients 4, 8, and 10 contained 0.09, 0.76, and 0.11% CD8\textsuperscript{+} T cells recognizing either the MiHA LRH-1 or HA-1. In these patients, we observed augmented percentages of MiHA-specific CD8\textsuperscript{+} T cells after blockade of BTLA and/or PD-1 compared with isotype control-treated cells. To combine data of all patients, the fold expansion was calculated as the absolute number of MiHA-specific CD8\textsuperscript{+} T cells after Ab blockade relative to isotype control treatment. In 7 of 11 patients, BTLA blockade augmented MiHA-specific T cell expansion >2-fold (range, 2.1- to 11.7-fold; Fig. 4C, Table I). Furthermore, PD-1 blockade enhanced MiHA-specific T cell numbers >2-fold in 9 of 11 patients (range, 2.5- to 12.5-fold; Fig. 4D). Notably, in 3 of the 7 BTLA responders, the effect of BTLA blockade was more prominent than that of PD-1 blockade. However, no significant difference was observed when comparing the effects of BTLA and PD-1 blockade for all patients. Moreover, combined blockade of BTLA and PD-1 boosted MiHA-specific CD8\textsuperscript{+} T cell expansion, although when compared with single receptor blockade, no additive effect was observed.

Finally, we examined CFSE dilution, degranulation, and cytokine production by the expanded MiHA-specific CD8\textsuperscript{+} T cells after overnight restimulation with the cognate peptide. After 1 wk of stimulation with peptide-loaded DC, >90% of the MiHA-specific CD8\textsuperscript{+} T cells were CFSE\textsuperscript{−} (Fig. 5A). However, no differential effect of BTLA or PD-1 blockade could be observed. Furthermore, these expanded MiHA-specific EM T cells efficiently degranulated upon Ag reencounter, as demonstrated by CD107a staining (Fig. 5B). This in contrast to the tetramer\textsuperscript{+} CD8\textsuperscript{+} T cell population, of which <5% of the cells stained positive for CD107a (data not shown). In some patients, single PD-1 blockade resulted in somewhat increased percentages of CD107a\textsuperscript{+} Ag-specific T cells; however, this was not observed for the other treatments. In Fig. 5C, intracellular IFN-\(\gamma\) and TNF-\(\alpha\) expression levels by MiHA-specific T cells of one representative patient (patient 2) are shown. After Ag restimulation, >90% of the Ag-specific CD8\textsuperscript{+} T cells produced the effector cytokines IFN-\(\gamma\) and/or TNF-\(\alpha\) (Fig. 5C–E). Although the percentage of cytokine-producing T cells did not further increase, the expression levels of IFN-\(\gamma\) and TNF-\(\alpha\) within the MiHA-specific CD8\textsuperscript{+} T cells seemed to be higher after prior BTLA and/or PD-1 blockade. Especially, TNF-\(\alpha\) seemed differentially up-regulated in MiHA-specific T cells treated with BTLA blocking Ab. In conclusion, we demonstrate that next to PD-1, BTLA is also involved in MiHA-specific T cell inhibition and that the relative contribution of these two receptors to functional impairment differs between patients.
Discussion

Alloreactive MiHA-specific CD8+ T cells play a pivotal role in GVT responses after allo-SCT and donor lymphocyte infusion (25, 26). Unfortunately, many patients develop relapses despite the long-term presence of MiHA-specific memory T cells, suggesting that these cells become functionally impaired. Tumor cells deploy distinct mechanisms to evade immune attack (7), including expression of ligands for coinhibitory receptors on T cells (27). Previously, we demonstrated that the coinhibitory PD-1–PD-L1 pathway is involved in the functional impairment of MiHA-specific responses after allo-SCT and that these could be reinvigorated by DC stimulation in combination with PD-1 blockade (16). Another coinhibitory receptor, BTLA, was recently shown to be involved in dysfunction of melanoma-specific T cells (23). This prompted us to investigate the potential inhibitory role of BTLA on MiHA-specific T cells in cancer patients after allo-SCT.
First, we investigated BTLA and PD-1 expression on the different CD8+ effector/memory subsets in healthy donors and alloSCT patients. In concordance with the work from Derré et al. (23), we found that BTLA expression in healthy donors is highest on naive CD8+ T cells and is gradually reduced in CM and EM cells. This phenomenon was similar for CD8+ T cells of patients, al-
Table I. Characteristics of transplanted patients and effect of BTLA and/or PD-1 blockade on MiHA-specific CD8+ T cell proliferation

<table>
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<tr>
<th>Patient No.</th>
<th>Disease Status at GVHD at ISD at</th>
<th>Clinical Outcome</th>
<th>ISD at</th>
<th>Effect Anti-BTLA*</th>
<th>Effect Anti–PD-1</th>
<th>Effect Combination</th>
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<td>1</td>
<td>All, post-SCT</td>
<td>Remission; alive at 7d mo post-SCT</td>
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<td>2.5</td>
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<td>3</td>
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<td>MM, post-SCT</td>
<td>Hematologic relapse due to GVHD at 57 mo post-SCT</td>
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<td>7.0</td>
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<tr>
<td>5</td>
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<td>4.6</td>
<td>2.7</td>
<td>5.7</td>
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Characteristics of patients with hematological malignancies displaying MiHA-specific CD8+ T cell responses.

a Effect anti-BTLA: ratio of absolute number of tetramer+ CD8+ cells after stimulation with DC plus peptide plus anti-BTLA/absolute number of tetramer+ CD8+ cells after stimulation with DC plus peptide plus isotype control.

b Transplantation; ISD, immunosuppressive drugs; NHL, non-Hodgkin lymphoma; pre-T ALL, pre-T cell acute lymphoblastic leukemia; SCT, stem cell transplantation.


e Graft-versus-host disease; no, absent; minimal; early; grade III/IV.

f Hematologic relapse; no, absent; minimal; early.

g Active disease; no, absent; minimal; early.

h Active disease in MM, active disease at 6 mo post-SCT. Plasma cells 5%; M-protein 7 g/L.

Though overall BTLA expression was lower. In contrast, PD-1 expression shows a reversed profile, with low expression on naive T cells and high presence on CM and EM cells, which was also reported by Duraiswamy et al. (28). In allo-SCT patients, PD-1 levels in the distinct subsets were evidently higher than those in corresponding healthy donor T cells. This is probably because of the presence of a long-lasting inflammatory milieu due to the pre-SCT conditioning regimen (29), development of alloreactive T cell responses (30), occurrence of graft-versus-host disease (GVHD), and reactivation of viruses and subsequent immune responses (31, 32). The differential expression profiles of BTLA and PD-1 indicate that there is a distinct role for these molecules to exert their physiological function. Because peripheral blood of allo-SCT patients predominantly contains EM CD8+ T cells with low numbers of naïve and CM T cells, analysis of BTLA and PD-1 expression was limited to the EM subset. Skewing of the CD8+ T cell population to the EM subset is a common phenomenon after allo-SCT, as many T cells become activated as mentioned before. When focusing on the MiHA-specific T cells, these are also mainly of the EM type, as expected due to in vivo alloergic activation.

Subsequently, we investigated BTLA and PD-1 expression specifically on CMV-, FLU-, and MiHA-specific T cells of healthy donors and allo-SCT patients, respectively. In accordance with findings by others (23, 33), healthy donor CMV-specific T cells showed relatively increased BTLA and PD-1 expression, which is probably due to frequent activation by latent CMV. On the contrary, lower BTLA expression was observed in case of cleared viral infections, like influenza. In general, BTLA levels on total CD8+ T cells in patients were slightly lower compared with those in healthy individuals. Notably, CMV- and MiHA-specific T cells of allo-SCT patients were highly BTLA positive despite their EM phenotype. PD-1 levels, on the contrary, were already high on the total CD8+ T cell pool, as well as on CMV- and MiHA-specific EM T cells. These data confirm our recent observations (6) and can be attributed to the high numbers of activated alloreactive and microbial-reactive T cells as mentioned before. Notably, MiHA-specific T cells of allo-SCT patients showed high expression levels of both PD-1 and BTLA.

To investigate whether BTLA ligation could be a potential evasion mechanism, we next examined ligand expression levels on tumor cells. Therefore, we analyzed hematopoietic tumor cells for the presence of BTLA, HVEM, CD160, and LIGHT. Notably, both HVEM and its binding partner BTLA were expressed on primary leukemia and myeloma cells, whereas LIGHT and CD160 were barely present. Our data correspond with the literature describing HVEM to be constitutively expressed on primary myeloma cells (34). Furthermore, we report here for the first time, to our knowledge, that primary AML cells highly express HVEM. Notably, in contrast to the ligands for PD-1 (16), HVEM is constitutively expressed, and further upregulation under inflammatory conditions was not observed (data not shown). Because BTLA can also be expressed on the tumor cells, we also investigated whether T cells were positive for HVEM. Indeed, HVEM was observed on human T cells, indicating that a bidirectional HVEM–BTLA signaling route between tumor cell and T cells can occur. As yet, the effect of BTLA signal transduction into the tumor cell is unknown. Furthermore, besides BTLA, HVEM itself has been reported to function as a receptor as well (35, 36). Altogether, this results in a complex network of interactions for BTLA, HVEM, and their binding partners (37), which needs to be elucidated in future experiments.

The concurrent expression of BTLA on MiHA-specific T cells and HVEM by malignant cells suggested that this coinhibitory pathway contributed to the functional inhibition of MiHA-specific
T cells in vivo. Therefore, we performed ex vivo blocking experiments using DC as APCs. Based on their strong stimulatory potential, DC are attractive vaccines for ex vivo (24) and in vivo (38) stimulation of MiHA-specific T cell responses. Importantly, DC also express HVEM, BTLA, and to some extent LIGHT, providing a rationale for combined DC stimulation and BTLA blockade. In addition, by interfering with the ligation of HVEM to BTLA, an additive effect could be realized by increased interaction of HVEM with LIGHT (22), yielding a more costimulatory signal. After blockade of BTLA interactions, we observed enhanced proliferation of MiHA-specific T cells in 64% of the patients, indicating that BTLA ligation also contributes to impairment of alloreactive T cells in transplanted cancer patients. On the basis of our previous findings, where we identified the involvement of PD-1 in MiHA-specific T cell dysfunction, we next compared the relative contribution of both coinhibitory receptors in this process. Compared to BTLA blockade, PD-1 blockade exerted evident boosting of Ag-specific T cell expansion in 82% of the patients. Notably, some patients responded better to BTLA blockade, whereas others showed more pronounced effects after PD-1 blockade. Whether the increase in number of cells is due to enhanced cell cycling was investigated in a CFSE dilution assay. For CMV-specific CD8+ T cells, we observed a higher percentage of CFSE-diluted dividing cells after PD-1 blockade. However, we did not observe these effects in MiHA-specific CD8+ memory T cells. The increased number of Ag-specific T cells may here be attributed to less apoptosis after BTLA and/or PD-1 blockade. In addition, we examined whether the boosted Ag-specific T cells exhibited competent functionality. Upon Ag reencounter, both CMV- and MiHA-specific CD8+ T cells efficiently degranulated and produced effector cytokines. Notably, blocking of BTLA seemed to mediate increased TNF-α production in MiHA-specific T cells.

Possibly, the differences observed between PD-1 and BTLA blocking can be attributed to inherent differences between the Abs. The anti–PD-1 Ab is fully humanized and has proved its functionality in phase I clinical trials (39), whereas the anti-BTLA Ab is murine and still in the preclinical stage. Although few immune-related adverse events have been reported for PD-1 blockade, dosing and timing of the BTLA blocking Ab has to be carefully examined in clinical trials, as both molecules are involved in the main

**FIGURE 5.** Expanded MiHA-specific CD8+ memory T cells are highly functional. PBMC of patients containing MiHA-specific CD8+ T cells were stimulated for 1 wk with mature DC loaded with the cognate peptide at a ratio of 1:0.1. To visualize the effect on T cell proliferation and function by BTLA and/or PD-1 blockade, PBMC labeled with or without 1.25 μM CFSE were after 1 wk restimulated overnight with 5 μM Ag peptide in the presence of CD107a Ab or brefeldin A. The following day, the percentage of CFSE- (A) and CD107a+ (B) cells within the MiHA-specific CD8+ T cell population was analyzed. Data are depicted as mean + SEM, n = 3. (C) Intracellular IFN-γ and TNF-α levels within the MiHA-tetramer+ CD8+ T cell population of one representative patient (patient 2) out of three patients. Numbers in the plot indicate the percentage of cells in each quadrant. (D and E) Intracellular IFN-γ (D) and TNF-α (E) levels within the tetramer+ CD8+ T cell population. Data are depicted as mean + SEM, n = 2 to 3.
tenance of self tolerance. Especially, in the allo-SCT setting, interference with coinhibitory molecules could deteriorate GVHD. In mouse models, however, BTLA has been reported to have a differential role in GVT responses, and not in GVHD (40, 41). Although we found high expression of both BTLA and PD-1 on MiHA-specific CD8+ T cells, we could not correlate their expression levels to the effect on T cell proliferation upon blocking of either of these pathways (data not shown). Perhaps the impairment via these receptors depends more on the amount of signaling it has experienced than on the expression of the receptor as such, as has been suggested previously for PD-1 (17, 42). Furthermore, no evident correlation in BTLA effect and disease status could be observed, although patients in long-term remission after allo-SCT seemed to respond better. This is in contrast to PD-1, which was shown to be more involved in the impairment of MiHA T cell responses in relapsed patients (16). It could be that the inhibitory effect of BTLA and PD-1 on MiHA-specific T cells has been influenced by the treatment with immunosuppressive drugs; however, in our study only few patients received immunosuppressive drugs at time of analysis, and no firm conclusions could be drawn. As the reversed expression profiles of BTLA and PD-1 on normal T cells already demonstrate, these molecules are likely to have distinct roles in hampering MiHA-specific T cell responses. Finally, we examined whether an additive effect could be observed by combined blockade of BTLA and PD-1. However, we did not observe an unambiguous additive effect on MiHA-specific T cell proliferation. This might suggest that the two receptors share common intracellular signaling pathways. It has been reported that both PD-1 and BTLA relay their coinhibitory signal via both of their ligands associated with the negative regulation and coinhibition.


