Lenalidomide Enhances Antigen-Specific Activity and Decreases CD45RA Expression of T Cells from Patients with Multiple Myeloma

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Lenalidomide Enhances Antigen-Specific Activity and Decreases CD45RA Expression of T Cells from Patients with Multiple Myeloma

Brigitte Neuber,* Isabelle Herth,* Claudia Tolliver,† Stefan Schoenland,* Ute Hegenbart,* Dirk Hose,‡ Mathias Witzens-Haring,* Anthony D. Ho,* Hartmut Goldechmidt,*§ Bernard Klein,‡§ and Michael Hundemer,*

The aim of this study was to investigate whether the specific T cell response against the multiple myeloma Ag HM1.24 is enhanced by the immunomodulatory drug lenalidomide (Revlimid). Ag-specific CD3⁺CD8⁺ T cells against the HM1.24 Ag were expanded in vitro by dendritic cells in 29 healthy donors and 26 patients with plasma cell dyscrasias. Ag-specific activation was analyzed by IFN-γ, granzyme B, and perforin secretion using ELISA, ELISPOT assay, and intracellular staining, and generation of Ag-specific T cells was analyzed by tetramer staining. Expression of T cell maturation markers (CD45RA, CD45R0, CCR7, and CD28) was investigated by flow cytometry. We found that activation of HM1.24-specific T cells from healthy donors and patients with plasma cell dyscrasias was enhanced significantly by lenalidomide and furthermore that the impact of lenalidomide on T cells depends on the duration of the exposure. Notably, lenalidomide supports the downregulation of CD45RA on T cells upon activation, observed in healthy donors and in patients in vitro and also in patients during lenalidomide therapy in vivo. We showed for the first time, to our knowledge, that lenalidomide enhances the Ag-specific activation of T cells and the subsequent downregulation of CD45RA expression of T cells in vitro and in vivo. The Journal of Immunology, 2011, 187: 1047–1056.
of NKT cells in response to the NKT ligand α-galactosylceramide in both healthy donors (HDs) and patients with MM (38) and induces apoptosis of tumor cells via NK cytotoxicity (39). Recent data indicated that lenalidomide in combination with dexamethasone can increase the frequency of regulatory T cells in MM patients after allogeneic transplantation (40). Corral et al. (28) showed that lenalidomide leads to cytokine modulation and activation of T cells. The stimulation of T cells by lenalidomide was associated with enhanced activation of the transcription factor AP-1, which drives the production of IL-2, IFN-γ, and other T cell-derived cytokines (41). LeBlanc et al. (42) reported that lenalidomide overcomes the inhibitory effect of CTLA-4-immunoglobulin, triggers tyrosine phosphorylation of CD28 on T cells, followed by activation of NF-κB in T cells from HDs. We have recently shown that the HM1.24 B cell differentiation molecule shares a common immunogenic epitope with MART-1 and that MART-1–specific T cells can efficiently lyse HM1.24-expressing MM cells (43). MART-1 shares antigenic peptide sequences with numerous related proteins explaining the high frequency of circulating MART-1–specific naive T cells that can be detected in HDs and cancer patients (44). The aim of our study was to investigate whether the generation of tumor-specific T cells against the HM1.24 Ag can be enhanced by lenalidomide. We therefore analyzed the IFN-γ secretion of T cells after Ag-specific activation upon peptide-pulsed DCs in HDs and in patients with plasma cell dyscrasias (PD) using ELISPOT assay, ELISA, and intracellular staining/flow cytometry.

Materials and Methods

Primary MNCs of patients with PD and of HDs

In this study, after obtaining written informed consent, samples from 29 HDs and 29 patients with PD were used. Approval for the use of MNCs to generate Ag-specific T cells was obtained from the ethics committee of the University of Heidelberg. Data safety management was performed according to the data safety regulations of the University of Heidelberg. The characteristics of patients are given in Table I. HDs were obtained from the IKTZ Heidelberg blood bank (Heidelberg, Germany). In all experiments, only cells of HLA-A2–positive individuals analyzed by flow cytometry were used, except for the experiments in which patient T cells were nonspecifically activated with anti-CD3/CD28 beads (Invitrogen Dynal, Oslo, Norway) displayed in Fig. 4. HLA-A2 typing was performed by flow cytometry as previously published (3). MNCs of peripheral blood and bone marrow aspirates were isolated by Ficoll–Hypaque gradient purification (Biochrom, Berlin, Germany), and some of the MNCs were cryopreserved in FBS supplemented with 10% DMSO.

Cell line

The HLA-A2–expressing T–B lymphoblastoid hybrid cell line T2, deficient in transporter for antigenic peptides protein (American Type Culture Collection, Manassas, VA), was cultured at 37˚C in 5% CO2 in complete medium consisting of RPMI 1640, 2 mM L-glutamine, 10 U/ml penicillin/0.1 mg/ml streptomycin, and 10% heat-inactivated FBS (all from PAA Laboratories). To generate Ag-specific T cells, cryopreserved MNCs were thawed and incubated for 12 d together with peptide-pulsed mature DCs (DC/MNC ratio 1:15) in T cell medium consisting of RPMI 1640, 2 mM L-glutamine, 10 U/ml penicillin/0.1 mg/ml streptomycin, 5% heat-inactivated human AB serum (PAA Laboratories), and IL-2 (50 U/ml; Chiron B.V., Amsterdam, The Netherlands) at 37˚C and 5% CO2. T cell medium and IL-2 were renewed after 7 d.

IFN-γ ELISPOT assay

The generation of HLA-A2–specific CD8+ cells was investigated using IFN-γ ELISPOT assay as described (3). Expanded CD3+CD8+ cells were purified with immunomagnetic beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated as effector cells (2 × 10⁶/well) with peptide-loaded T2 cells as targets (1 × 10⁵/well) at an E:T ratio of 1:5 for 48 h in anti–IFN-γ Ab (Mabtech, Nacka, Sweden) precoated 96-well nitrocellulose-plates (Millipore, Eschborn, Germany) in a final volume of 200 μl culture medium. To obtain T2 cells loaded with MART-1 peptide or an HLA-A2–restricted control peptide, T2 cells were preincubated for 2 h with 10 μg/ml of the respective peptide. After detection with biotinylated anti-cytokine Abs (Mabtech) and conjugation with Avidin ALP (Sigma), 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium substrate (Sigma) was added. The spots of the IFN-γ-secreting cells were counted with a computer-controlled microscope (Zeiss Vision, Eching, Germany).

ELISA

The amount of cytokines secreted from activated T cells in the supernatant was analyzed by ELISA. Expanded CD3+CD8+ cells were purified with immunomagnetic beads (MACS system) and incubated as effector cells (15 × 10⁶/well) with peptide-loaded T2 cells as targets (E:T ratio 1:5) in a final volume of 200 μl culture medium for 48 h. To obtain T2 cells loaded with MART-1 peptide or an HLA-A2–restricted control peptide, cells were preincubated for 2 h with 10 μg/ml of the respective peptide. Afterwards, culture supernatants were harvested, and secreted cytokines were quantified using ELISA for IFN-γ (BD OptEIA ELISA Sets; BD Biosciences), IFN-γ, or perforin (both from Mabtech) according to the manufacturer’s instructions. Briefly, an mAb specific for IFN-γ, granulocyte B, or perforin was precoated onto a microplate (Microlon High binding; Greiner Bio One, Frickenhausen, Germany). After blocking and washing, control recombinant proteins or samples were added into the wells, and any cytokine present was bound by the immobilized Ab. After washing to remove the unbound substances, an enzyme-linked mAb specific for IFN-γ, granulocyte B, or perforin was added to the wells. After washing to remove any unbound Ab–enzyme reagent, a substrate solution was added to the wells, leading to different coloring in proportion to the amount of IFN-γ, granulocyte B, or perforin. The intensity of the color was analyzed with an automated plate reader (TECAN; Austria Gesellschaft, Grödig, Austria) using Magellan V2.22 software at OD 450 nm.

Flow cytometry

Expression of intracellular IFN-γ and different surface T cell markers was analyzed by flow cytometry. After activation of expanded CD3+CD8+ T cells with peptide-loaded T2 cells as target cells (E:T ratio 1:5) for 48 h, 10 μg/ml brefeldin A (Sigma) was added to the cell culture 4 h prior to staining. Subsequently, cells were centrifuged and fixed in 4% paraformaldehyde (J.T. Baker, Deventer, The Netherlands) for 10 min at room temperature. After washing, cells were permeabilized with saponin buffer consisting of PBS with 0.5% BSA, 5% FBS (all from PAA Laboratories), 0.1% saponin from quillaja bark (Sigma Laboratories), and 0.07% sodium azide (Merck) and incubated for 10 min at room temperature. Cells were
resuspended in an adequate amount of saponin buffer and incubated with flow-chromate-labeled mAbs according to the manufacturer’s instructions: PerCP-labeled anti-CD3, FITC-labeled anti–IFN-γ, allophycocyanin-labeled anti-CD8, and PE-labeled anti-CD45RA, anti-CD45R0, anti-CCR7, and anti-CD28 (all from BD Biosciences, except for anti-CD45RA and anti-CD45R0, which were from Southern Biotech, Birmingham, AL). Control cells were stained with the corresponding isotype Abs. After a final washing with saponin buffer, cells were resuspended in 0.5% paraformaldehyde in PBS. Flow cytometry analysis was performed on a four-color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and by CellQuest software according to the manufacturer’s instructions. Cells with a lymphocyte profile on the forward scatter, side scatter plot were selected for analysis. Afterwards, cells were examined for tetramer-specific T cells.

**Tetramer staining**

The generation of HML-24-specific CD8+ T cells was analyzed with HLA-A-0201 peptide-specific tetramers. Tetrameric complexes for HLA-A-0201 were selected for analysis. Afterwards, cells were examined for tetramer-positive cells, gating for CD3+CD8+ and/or CD3+CD8+ T cells.

**Non-specific activation**

Fresh MNCs from patients with PD were isolated by density-gradient centrifugation and incubated with anti-CD3/CD28 beads (Invitrogen) in culture medium supplemented with 5% heat-inactivated human AB serum and IL-2 (50 U/ml) for 24 h. After incubation, T cells were used for flow cytometry analysis.

**Statistics**

For ELISPOT assay and ELISA, the statistical difference of results with HML-24-specific T cells incubated with or without lenalidomide was calculated by Student t test. Each test was done in quintuplicate. For flow cytometry analysis, the percentage of intracellular IFN-γ or of T cell surface molecules incubated without lenalidomide was set as 100%. Statistical significance was calculated using paired Student t test, and p ≤ 0.05 was considered statistically significant. Boxplots were calculated using the freeware R-statistic, version 2.10.1 (http://www.r-project.org/).

**Results**

Lenalidomide enhances cytokine secretion by HML-24-specific CD8+ T cells from HDs and from patients with PD

The activity of T lymphocytes can be assessed by their cytokine secretion of IFN-γ, granzyme B, and perforin. In our study, CD3+ CD8+ T cells from peripheral blood of HDs and of untreated patients with PD were used. Patient characteristics are shown in Table I. T cells were activated with MART-1 peptide-pulsed DCs, incubated for 12 h in presence or absence of lenalidomide, and analyzed for their secretion of IFN-γ, granzyme B, and perforin. In seven of eight HDs and in 11 of 13 patients with PD, lenalidomide significantly enhanced (p ≤ 0.05, Student t test) the number of IFN-γ-secreting T cells as determined by IFN-γ ELISPOT assays (Fig. 1A); analysis by ELISA (Fig. 1B) also showed a statistically significant increase of secreted IFN-γ (Fig. 1B, n = 4; Fig. 1Bb PDs, n = 3), granzyme B (Fig. 1Bc HDs, n = 5; Fig. 1Bd PD, n = 6), and perforin in HDs (Fig. 1Be HDs, n = 3).

**Table I. Patient characteristics**

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<th>Patient No.</th>
<th>Entity/Stage*</th>
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<th>IgG (g/dl)</th>
<th>IgA (g/dl)</th>
<th>IgM (g/dl)</th>
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*Patients with MM were classified in clinical stage I, II, or III (according to Salmon and Durie [47], a clinical staging system for MM).

BJ, Bence-Jones; MGUS, monoclonal gammopathy of undetermined significance.
Figure 1. Lenalidomide enhances cytokine secretion of HMI.24-specific CD3+CD8+ T cells from HDs and from patients with PD. MNCs were incubated in vitro with MART-1 peptide-pulsed DCs and coincubated with lenalidomide (Len., 10 μM) or with DMSO as negative control (Contr.). After 12 d, expanded CD3+CD8+ T cells were purified and incubated for 48 h with T2 cells as target cells loaded with MART-1 peptide or loaded with an irrelevant peptide as negative control. In the diagrams, black bars represent incubation with lenalidomide and white bars incubation with DMSO. For the different experiments, nonspecific activation formed by irrelevant peptide was subtracted from the results of the HMI.24-specific activations, respectively. Cytokine secretion of HMI.24-specific CD3+CD8+ T cells was analyzed by ELISPOT assay and ELISA.

A, Cumulative results of IFN-γ ELISPOT assays from (a) HDs (n = 8) and (b) patients with PD (n = 13). HMI.24-specific CD3+CD8+ T cells are presented by the number of the IFN-γ spot-forming cells. Results of each experiment represent the median of quintuplicates. HD and PD patient identification numbers are shown under each pair of bars.

B, Cytokine concentration of the supernatant was determined by ELISA. Concentrations are indicated in pg/ml. IFN-γ ELISAs for (a) HDs (n = 4) and (b) patients with PD (n = 3); granzyme B ELISAs for (c) HDs (n = 5) and (d) patients with PD (n = 6); and (e) perforin ELISAs for HDs (n = 3). Results of each experiment represent the median of quintuplicates. *p ≤ 0.05 (calculated by Student t test, indicates a significant difference in activation of CD8+ T cells in presence or absence of lenalidomide).
Lenalidomide enhances generation of HM1.24/MART-1 peptide-specific tetramer-positive T cells

Increased generation of HM1.24-specific T cells induced by lenalidomide was also assessed by tetramer staining. CD3⁺CD8⁺ T cells from three HDs were stained for an analysis by flow cytometry with MART-1–tetramer after T cell expansion with MART-1 peptide-pulsed DCs in presence or absence of lenalidomide for 12 d. In all HDs, lenalidomide increased the number of MART-1⁺ T cells (Fig. 1Ca). Fig. 1Cb shows the tetramer staining for one representative experiment.

Influence of the duration of lenalidomide exposure

To analyze the impact of different incubation intervals with lenalidomide on the generation and activation of T cells, we determined the IFN-γ production of HM1.24-specific CD3⁺CD8⁺ T cells by using IFN-γ ELISPOT assay, ELISA, and intracellular IFN-γ staining with subsequent analysis by flow cytometry. Fig. 2Aa (HDs, n = 3) and Fig. 2Ab (PDs, n = 4) show the results of ELISPOT assays and Fig. 2Ac (HD, n = 1) and Fig. 2Ad (PDs, n = 2) of ELISAs in HDs and patients with PD. We analyzed the impact of different lenalidomide incubation periods in 12-d and 2-d settings. In the 12-d setting, the T cells were incubated with peptide-pulsed DCs for 12 d in the presence of lenalidomide; after the 12-d period, the primed T cells were incubated for 2 d together with peptide-loaded T2 cells in the absence of lenalidomide. For comparison, in the 2-d setting, T cells were incubated with peptide-pulsed DCs for 12 d in absence of lenalidomide. After the 12-d period, the primed T cells were incubated for 2 d together with peptide-loaded T2 cells in presence of lenalidomide. Only the 12-d incubation significantly enhanced the activity of the HM1.24-specific CD3⁺CD8⁺ T cells (p ≤ 0.05; Student t test). Fig. 2Ba shows a representative flow cytometry analysis of T cell maturation markers and intracellular IFN-γ in a HD (HD no. 17, n = 4) and Fig. 2Bb shows that in a PD patient (PD no. 16). T cells were incubated with MART-1 peptide-pulsed DCs for 28 d. During the 28-d incubation, cells were either coincubated with lenalidomide for the entire 28 d or for the last 14 d. Incubation without lenalidomide was also assessed by tetramer staining. CD3⁺CD8⁺ T cells isolated from peripheral blood of patients with PD before and during lenalidomide therapy. Patients received lenalidomide without chemotherapeutic or steroid comedication. The characteristics of patients are shown in Table I (patient no. 27 to no. 29, n = 3). Fig. 4 shows the flow cytometry density plots and the cumulative results. CD3⁺CD8⁺ T cells (Fig. 4A, p = 0.046), and CD3⁺CD4⁺ T cells (Fig. 4B, p = 0.023), which were isolated during therapy, showed a significantly decreased expression of CD45RA in comparison with that of T cells isolated before therapy. Consistent with the specific activation in vitro, lenalidomide enhanced the activation-induced downregulation of CD45RA in vivo.

Discussion

The aim of this study was to evaluate the impact of lenalidomide on the Ag-specific activation of T cells in HDs and patients with PD. We analyzed the generation/activation of specific T cells against the MART-1 peptide, which shares immunogenic epitopes with numerous “self” proteins (48). Notably, the immunogenic MART-1 peptide is also shared by HM1.24, a B cell differentiation marker

absence of lenalidomide, *p ≤ 0.05 (calculated by Student t test, indicates a significant difference in activation of HM1.24-specific CD3⁺CD8⁺ T cells compared with nonspecific activation by irrelevant peptide). C. Number of MART-1–tetramer-positive T cells was analyzed by flow cytometry after gating on CD3⁺CD8⁺ T cells: (a) cumulative results of HDs (n = 3) and (b) representative density plot of one HD (HD no. 14). The percentage of CD3⁺CD8⁺ MART-1–tetramer-positive T cells is shown in each plot.
FIGURE 2. Prolonged incubation period of lenalidomide enhances the number and activation of HMI.24-specific CD3⁺CD8⁺ T cells. A, Different incubation intervals with lenalidomide: 12 d (12d) versus 2 d (2d). In the 12-d setting, MNCs were incubated with MART-1 peptide-loaded DCs in presence of lenalidomide (Len.; 10 μM) or with DMSO as negative control (Contr.) for a 12-d period, and subsequently the purified CD3⁺CD8⁺ T cells were restimulated with MART-1 peptide-loaded T2 target cells in absence of lenalidomide for 2 d. For comparison, in the 2-d setting, MNCs were incubated with peptide-loaded DCs without any coincubation with lenalidomide or DMSO for a 12-d period. After 12 d, CD3⁺CD8⁺ T cells were incubated with peptide-loaded T2 cells in presence of lenalidomide or DMSO as negative control for 2 d. Cumulative results of IFN-γ ELISPOT analysis are shown for (a) HDs (n = 3) and for (b) patients with PD (n = 4) and ELISA results for IFN-γ for (c) HD (n = 1) and granzyme B for (d) patients with PD (n = 2). Black bars indicate incubation with lenalidomide and white bars incubation with DMSO; nonspecific activations by irrelevant peptide were subtracted from the specific activation. The results represent the median of quintuplicates. *p ≤ 0.05 (calculated by Student t test, indicates a significant difference in activation of CD3⁺CD8⁺ T cells in presence or absence of lenalidomide). ♦p ≤ 0.05 (calculated by Student t test, indicates a significant difference in activation of HMI.24-specific CD3⁺CD8⁺ T cells compared with nonspecific activation by irrelevant peptide).

B, Four-color flow cytometry analysis of (a) a HD (no. 17) and of (b) a patient with PD (no. 16) with different incubation intervals with lenalidomide (Len.). During the 28-d incubation with peptide-pulsed DCs, cells were...
that is highly expressed on primary myeloma cells of patients with MM (3, 49). We previously showed that MART-1 peptide-specific T cells are able to recognize and kill \( \text{HM1.24} \) + myeloma cells (43). We report here that lenalidomide increases the generation and activity of MART-1 peptide-specific T cells in HDs and in patients with PD. The increased activation of specific T cells was improved with longer duration of lenalidomide exposure in vitro. This has been shown in ELISPOT assay, ELISA, and flow cytometry analysis (intracellular IFN-\( \gamma \) and tetramer staining). The methodologies make it possible to show that lenalidomide increases both the single-cell rate of IFN-\( \gamma \), granzyme B, and perforin secretion (analyzed by ELISA) and the frequency of MART-1 peptide-specific T cells (analyzed by ELISPOT assay and flow cytometry). We found this lenalidomide-induced increase of cytokine secretion in HDs and in patients with PD except for perforin. No sufficient secretion of perforin could be detected in any PD patient by perforin ELISA (data not shown). As only very low levels of perforin were detected in HDs (Fig. 1B), one could speculate that

either coincubated with lenalidomide (10 \( \mu \)M) for the entire 28-d period (28d) or only for the last 14 d (14d). Incubation without lenalidomide was performed as negative control (Contr.). During this 28-d incubation, restimulations with MART-1 peptide-loaded T2 cells were performed on days 7, 14, and 21, along with culture medium and IL-2 renewal. After the 28-d incubation, PBMCs were incubated with T2 cells loaded with MART-1 peptide for 24 h and subsequently stained for intracellular IFN-\( \gamma \) and for the surface molecules CD45RA, CD45R0, CCR7, and CD28. Density plots were gated on CD3\(^+\)CD8\(^+\) T cells and CD3\(^+\)CD4\(^+\) T cells. A \( p \) value \( \leq 0.05 \) (calculated by paired Student \( t \) test) indicates a significant difference of the CD45RA expression of T cells in presence or absence of lenalidomide.

\( \text{FIGURE 3.} \) Lenalidomide decreases the expression of CD45RA of \( \text{HM1.24} \)-specific activated CD8\(^+\) and CD4\(^+\) lymphocytes in vitro. Expression of CD45RA was analyzed by flow cytometry, gated on CD3\(^+\)CD8\(^+\) T cells and CD3\(^+\)CD4\(^+\) T cells. Cumulative results are shown in boxplots displaying medians of the expression of CD45RA. A representative density plot for each approach, the percentage of the CD45RA\(^+\)CD3\(^+\)CD8\(^+\) cells and CD45RA\(^+\)CD3\(^+\)CD4\(^+\) cells is shown. A, MNCs from (a, b) HDs (\( n = 11 \)) and (c, d) patients with PD (\( n = 10 \)) were incubated in vitro with MART-1 peptide-pulsed DCs in presence of lenalidomide (Len., 10 \( \mu \)M) or presence of DMSO (Contr.) for a 12-d period, subsequently incubated with T2 cells loaded with MART-1 peptide for 2 d, and stained for the surface molecule CD45RA. B, MNCs from (a, b) HDs (\( n = 5 \)) were incubated with peptide-pulsed DCs for 28 d and coincubated with lenalidomide for the entire 28 d (28d) versus the last 14 d (14d). Incubation with DMSO was performed as negative control (Contr.), as described for Fig. 2B. After 28-d incubation, MNCs were incubated with T2 cells loaded with MART-1 peptide for 24 h and subsequently stained for the surface molecule CD45RA for flow cytometry analysis. Boxplots and representative density plots for (a) CD3\(^+\)CD8\(^+\) T cells and (b) CD3\(^+\)CD4\(^+\) T cells. A \( p \) value \( \leq 0.05 \) (calculated by paired Student \( t \) test) indicates a significant difference of the CD45RA expression of T cells in presence or absence of lenalidomide.
in PD patients, the perforin secretion is even weaker than in HDs so that we were unable to detect perforin in this setting. Another explanation is that due to the differentiation of the T cells into CD45RA^2CD45R0^CCR7^/CD28^ T cells, these cells are unable to secrete high amounts of perforin according to Andersen et al. (50). In previous studies (data not shown), we observed that lenalidomide can enhance the polyclonal activation of T cells, in agreement with data of LeBlanc et al. (42). Regarding the expression of CD45RA on CD3^+CD4^+ and CD3^+CD8^+ T cells, lenalidomide decreased the expression of CD45RA in T cells stimulated by MART-1 peptide-pulsed DCs in vitro indicating a switch from a naive state into a more mature state of T cells, but for the expression of CCR7 and CD28, we found divergent results (data not shown). Remarkably, the decrease of CD45RA expression also takes place in patients under lenalidomide therapy in vivo compared with the CD45RA expression before lenalidomide therapy. This effect was observed in CD8^+T cells and CD4^+ T cells, indicating that lenalidomide enhances a "maturation" of T cells irrespective of the method of activation.

To our knowledge, this is the first description of a decreased CD45RA expression induced by lenalidomide, although for the IMiD thalidomide, Direskeneli et al. (51) showed in patients with Behçet disease a maturation of T cells during thalidomide therapy, and Lioznov et al. (52) described an enhanced presence of HLA-DR^+ T cells as well as CD4^+, CD25^+, and CD127^low regulatory T cells in patients receiving lenalidomide salvage therapy after allogeneic SCT. Regarding maintenance therapy with lenalidomide and the control of tumor cells in a minimal residual disease setting, it might be essential to induce a mature immunophenotype of antitumor-specific T cells for an effective antitumor immune response. Also for Ag-specific immunotherapy, like vaccination with peptide-pulsed DCs, peptides, or tumor lysates, lenalidomide seems to be a promising adjuvant, possibly enhancing the generation of effective antitumor-specific T cells. Although we found in our study the aforementioned impact on T cells in vitro and in vivo and also the enhanced activation of T cells by lenalidomide in vitro, it is absolutely necessary to analyze further the tumor-specific T cell responses in patients with MM during therapy with lenalidomide and to confirm that a maturation of T cells leads to a stronger Ag-specific immune response in vivo.

Based on these results, new questions arise: does lenalidomide also augment tumor-specific T cell responses in MM patients? Does lenalidomide show these effects on T cells in vivo, for example in patients receiving a maintenance therapy with lenalidomide after high-dose chemotherapy and autologous transplantation in a minimal residual disease setting? Does this contribute to the clinical benefit of lenalidomide therapy? What is the role of regulatory T cells regarding the immunomodulatory effects of lenalidomide? Whereas Minnema et al. (40) described an increased frequency of regulatory T cells upon lenalidomide and dexamethasone exposure in an allogenic setting in vivo, Galustian et al. (46) showed that lenalidomide inhibits the proliferation and function of regulatory T cells. This difference may be attributable to the effect of dexamethasone, a potent immunosuppressant, on the patient’s immune system. To answer these questions, it is necessary to analyze the T cells from MM patients receiving lenalidomide and

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**FIGURE 4.** Lenalidomide decreases the expression of CD45RA on activated CD8^+ and CD4^+ lymphocytes in vivo. The influence of lenalidomide therapy on the CD45RA expression of T cells was analyzed by flow cytometry. PBMCs from three patients with PD before (white bars) and during (black bars) lenalidomide therapy were activated with CD3/CD28 beads and incubated for 24 h. Density plots and results for (A) CD8^+ T cells and (B) CD4^+ T cells. The percentage of the CD45RA^−CD3^+CD8^+ cells and CD45RA^−CD3^+CD4^+ cells is shown in each plot.
to investigate in detail the influence of lenalidomide on the cross-talk of T cells and APCs.

In summary, our data show for the first time, to our knowledge, that lenalidomide significantly increases in vitro tumor-specific CD8+ T cell responses of patients with MM, which are enhanced by prolonged exposure to lenalidomide. Furthermore, we found an additional new property of lenalidomide: it decreases, irrespective of the method of activation, the CD45RA expression of T cells in vitro and in vivo, suggesting the induction of a more mature immunophenotype of the T cells. Our results may provide further elucidation of the mechanism of action of lenalidomide in the incurable disease MM.

Disclosures

The authors have no financial conflicts of interest.

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