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

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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.**

Despite the introduction of new chemotherapeutic agents and high-dose therapy with autologous stem cell transplantation (SCT), multiple myeloma (MM) remains a disease that requires a long-term treatment strategy. Because of the presence of residual multiple myeloma cells (MMCs), almost all patients ultimately relapse. In a fraction of patients, allogenic SCT can induce immune mechanisms (graft versus myeloma effect) leading to molecular complete remission (1). Another possibility to stimulate antitumor immune response is the use of dendritic cell (DC)-based vaccines.

Although tumor-specific T cells can be generated in vivo and in vitro (2–8), vaccinations using the tumor idiotype showed no clinical benefit in patients with MM (2, 5–7). Various hypotheses have been generated to explain this lack of clinical activity of idiotype-pulsed DC vaccination approaches: deletion of tumor-specific T cells (9, 10), altered (reduced) activation of T cells (11), impaired ability of APCs to present tumor Ags to T cells (12, 13), and decreased immunogenicity of tumor cells (14). Decreased expression of the T cell costimulatory molecule CD28 and an increased expression of the CTLA-4 inhibitory molecule was reported in T cells of patients with advanced MM (11). In this context, studies have shown that MMCs transduced with CD40L (15) or 4-1IBB ligand (16) can activate autologous antitumor T cells efficiently, showing no defect in antitumor T cell repertoire in vivo, in particular in patients with terminal disease. In addition, fully functional DCs can be generated from circulating monocytes of patients with MM (17), so that immunotherapy seems to be an interesting topic in the treatment of MM. Whereas almost all DC-based vaccination trials in MM have shown an immunological response (6, 18, 19), a clinical benefit has not yet been observed, showing the need for improvement of these protocols. A promising approach is the implementation of immunomodulatory drugs (IMiDs) in immunotherapeutic protocols. One of the IMiD compounds is lenalidomide (CC-5013; IMiD3; Revlimid), which demonstrated significant clinical activity in patients with newly diagnosed and relapsed MM. However, the exact mechanism of lenalidomide in MM is still unclear. Lenalidomide possesses both tumoricidal and immunomodulatory properties. This combination of direct antineoplastic effects on MMCs (20) and modulation of the host immune system may provide an overall survival benefit in clinical trials (21–23). For example, lenalidomide directly inhibits growth of myelodysplastic syndrome patients' cells and other cells with a deletion in chromosome 5q (24, 25). The tumoricidal activity of lenalidomide was also observed in MM cells from patients and in MM cell lines by a mechanism including expression of tumor suppressor genes, cell cycle arrest, caspase activation, and apoptosis (26, 27). In addition to its tumoricidal activity, lenalidomide shows immunomodulatory potential. Lenalidomide inhibits the secretion of proinflammatory cytokines and increases the secretion of anti-inflammatory cytokines such as TNF-α from PBMCs (mononuclear cells; MNCs) (28). The activity of T cells and NK cells, alone or against tumor cells, is enhanced by lenalidomide (29–37). Lenalidomide enhances Ag-specific expansion

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Abbreviations used in this article: DC, dendritic cell; HD, healthy donor; IMiD, immunomodulatory drug; MM, multiple myeloma; MMC, multiple myeloma cell; MNC, mononuclear cell; PD, plasma cell dyscrasias; SCT, stem cell transplantation.

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 allogenic 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% CO_2 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, Pasching, Austria).

Drug preparation

Lenalidomide (Revlimid; kindly provided by Celgene Corporation, Warren, NY) was dissolved in DMSO (Merck, Darmstadt, Germany) and stored at −80°C until further use. Lenalidomide was used in all experiments in a concentration of 10 μM, with the corresponding DMSO concentration as a negative control. Due to pharmacokinetic data (45), lenalidomide was diluted with culture medium immediately before use resulting in less than 0.01% DMSO in the final specimen. A dose of 10 μM was administered every second/third incubation day as previously published by Galustian et al. (46).

Synthesis of peptides

The MART-1 analogue peptide (ELAGIGILTV, also known as MART-1 peptide) and the HLA-A2–restricted control peptide (LLIIVILGV) were synthesized by the peptide synthesis department of the German Cancer Research Center Heidelberg (Heidelberg, Germany) using standard procedures.

In vitro generation of DCs

Immature DCs were obtained culturing plastic adherent MNCs for 5 d with culture medium consisting of RPMI 1640, 2 mM L-glutamine, and penicillin/streptomycin, supplemented with 5% heat-inactivated human AB serum, 800 U/ml human GM-CSF (Bayer HeathCare, Seattle, WA), and 500 U/ml human IL-4 (R&D Systems, Abingdon, Oxon, U.K.). Afterward, differentiation into mature DCs was induced by adding 10 ng/ml TNF-α, 1 μg/ml PGE_2 (both from Sigma-Aldrich, Deisenhofen, Germany), 1000 U/ml IL-6 (R&D Systems), and 10 μg/ml MART-1 peptide for 2 d as previously published (3).

PBMCs for in vitro expansion of peptide-specific T cells

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% CO_2. T cell medium and IL-2 were renewed after 7 d.

IFN-γ ELISPOT assay

The generation of *HM1.24*-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 (2×10^4/well) with peptide-loaded T2 cells as targets (1×10^5/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 (15×10^4/ 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), granzyme B, or perforin (both from Mabtech) according to the manufacturer's instructions. Briefly, an mAb specific for IFN-γ, granzyme 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, granzyme 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, granzyme B, and 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 fluorochrome-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 strained 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 CD45RA, CD45R0, CCR7, CD28, and IFN-γ, gating for $CD3^+CD8^+$ and/or $CD3^+CD4^+$ T cells.

Tetramer staining

The generation of *HM1.24*-specific $CD8^+$ T cells was analyzed with HLA-A-0201 peptide-specific tetramers. Tetrameric complexes for HLA-A-201–binding ELAGIGILTV (iTAGTM MHC; Beckman Coulter, Fullerton, CA) were used. The analysis was performed according to the manufacturer's protocol. Briefly, expanded $CD8^+$ T cells were washed twice in PBS and incubated with PE-labeled MART-1–HLA-A2 tetramer, PerCP-labeled anti-CD3, and allophycocyanin-labeled anti-CD8 at room temperature for 30 min. After final washing with PBS, cells were resuspended in 0.5% paraformaldehyde in PBS. The number of tetramer-positive cells was determined by flow cytometry analysis using a FACSCalibur flow cytometer 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-positive cells, gating for $CD3^+CD8^+$ T cells.

Nonspecific activation

Fresh MNCs from patients with PD were isolated by density-gradient centrifugation and incubated with anti-CD3/CD28 beads (Invitrogen Dynal) 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 *HM1.24*-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 $p \leq 0.05$ 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 HM1.24*-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 d 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 ($p \leq 0.05$, Student *t* test) the number of IFN-γ–secreting T cells as determined by IFN-γ ELISPOT assays (Fig. 1*A*); analysis by ELISA (Fig. 1*B*) also showed a statistically significant increase of secreted IFN-γ (Fig. 1*Ba* HDs, $n = 4$; Fig. 1*Bb* PDs, $n = 3$), granzyme B (Fig. 1*Bc* HDs, $n = 5$; Fig. 1*Bd* PD, $n = 6$), and perforin in HDs (Fig. 1*Be* HDs, $n = 3$).

Table I. Patient characteristics

Patient No.	Entity/Stage[a]	Status	Age (y)	Monoclonal Protein	β_2-Microglobulin (mg/dl)	IgG (g/dl)	IgA (g/dl)	IgM (g/dl)	Experiments
1	MGUS	Newly diagnosed	59	IgG κ	1.39	19.95	4.09	0.81	ELISPOT assay
2	MGUS	Newly diagnosed	70	IgM Λ	1	7.18	1.78	11.39	ELISPOT assay
3	MGUS	Newly diagnosed	59	IgA κ	1.3	4.15	10.17	0.85	ELISPOT assay
4	MM IA	Newly diagnosed	52	IgA κ	1.6	10.34	5.33	0.6	ELISPOT assay; ELISA
5	MM IIA	Newly diagnosed	51	IgG κ	1.3	26.42	0.83	0.71	ELISPOT assay, ELISA
6	MM IA	Newly diagnosed	69	IgG κ	1.9	34.89	0.2	<0.1	ELISPOT assay
7	MM IA	Newly diagnosed	71	IgG Λ	4.1	20.07	2.75	0.36	ELISPOT assay
8	MM IIA	First relapse	60	IgG κ	1.6	44.57	0.72	0.33	ELISPOT assay; ELISA
9	MM IA	Newly diagnosed	58	IgG κ	ND	20.23	0.73	0.35	ELISPOT assay; ELISA
10	MGUS	Newly diagnosed	51	IgG Λ	1.7	12.05	1.65	0.38	ELISPOT assay; ELISA
11	MM IA	Newly diagnosed	45	IgG Λ	1.2	15.6	1.09	0.64	ELISPOT assay
12	MM IA	Newly diagnosed	51	IgG κ	1.9	37.46	0.79	0.41	ELISPOT assay
13	MGUS	Newly diagnosed	52	IgG κ	ND	13.43	1.61	1.74	ELISPOT assay
14	MGUS	Newly diagnosed	47	IgG κ, IgA κ	8.6	8.95	2.58	1.19	ELISA
15	MGUS	Newly diagnosed	48	IgG κ	ND	9.97	ND	ND	ELISA
16	MM IIA	Newly diagnosed	53	IgG κ	3.1	35.25	0.63	0.26	Flow cytometry
17	MM IIIA	Newly diagnosed	52	Asecretory	1.2	16.35	0.9	0.87	Flow cytometry
18	MGUS	Newly diagnosed	61	IgG κ	1.3	12.2	1.7	1.76	Flow cytometry
19	MGUS	Newly diagnosed	74	IgG κ	1.6	10.19	2.47	0.75	Flow cytometry
20	MM IA	Newly diagnosed	49	IgG κ	2.1	48.2	0.44	0.25	Flow cytometry
21	MGUS	Newly diagnosed	70	IgG Λ	21.1	9.6	1.05	0.52	Flow cytometry
22	MM IA	Newly diagnosed	53	IgG κ	1	27.46	0.58	0.37	Flow cytometry
23	MM IIA	Newly diagnosed	84	BJ κ	2.2	5.44	3.03	0.46	Flow cytometry
24	MM	Newly diagnosed	53	ND	ND	ND	ND	ND	Flow cytometry
25	MM IIIA	Newly diagnosed	76	IgA κ	2.6	4.16	6.11	0.1	Flow cytometry
26	MM IIA	Newly diagnosed	70	IgG Λ	1.3	64.92	0.08	0.11	Flow cytometry
27	MM IIIA	During lenalidomide therapy	61	IgG Λ	ND	32.51	0.14	0.33	Flow cytometry in vivo
28	MM IIA	During lenalidomide therapy	70	IgG Λ	2.8	15.36	0.09	0.5	Flow cytometry in vivo
29	MM IIIA	During lenalidomide therapy	62	IgG Λ	ND	36.78	0.09	0.24	Flow cytometry in vivo

[a]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.

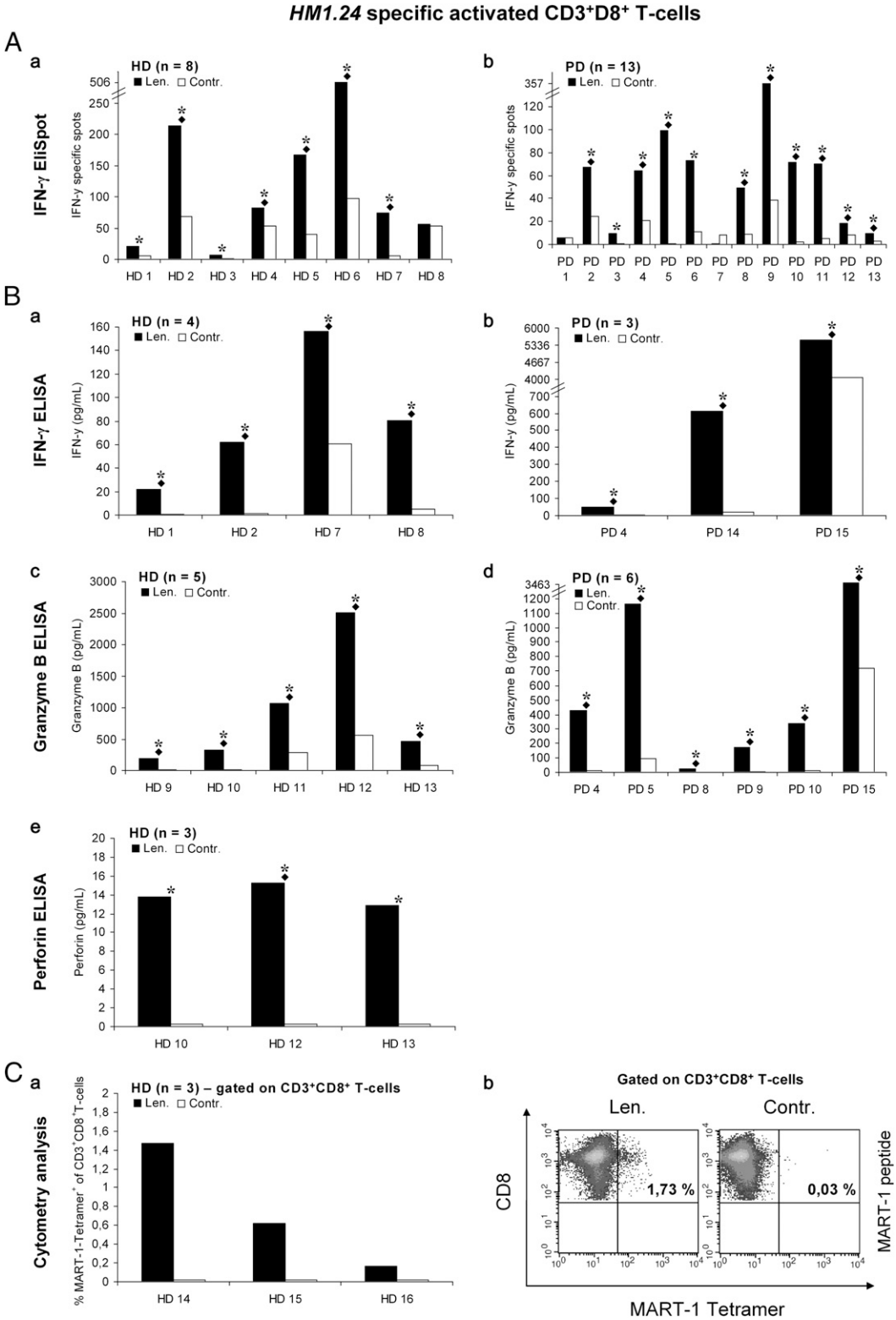

FIGURE 1. Lenalidomide enhances cytokine secretion of *HM1.24*-specific $CD3^+CD8^+$ T cells from HDs and from patients with PD. MNCs were incubated in vitro with MART-1 peptide-pulsed DCs and coincubated with lenalidomide (Len., 10 μM) or with DMSO as negative control (Contr.). After 12 d, expanded $CD3^+CD8^+$ 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 *HM1.24*-specific activations, respectively. Cytokine secretion of *HM1.24*-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 ($n = 13$). *HM1.24*-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. $*p \leq 0.05$ (calculated by Student *t* test, indicates a significant difference in activation of $CD8^+$ T cells in presence or

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. 1*Ca*). Fig. 1*Cb* 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. 2*Aa* (HDs, $n = 3$) and Fig. 2*Ab* (PDs, $n = 4$) show the results of ELISPOT assays and Fig. 2*Ac* (HD, $n = 1$) and Fig. 2*Ad* (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 ($p \leq 0.05$; Student *t* test). Fig. 2*Ba* 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. 2*Bb* 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 performed as negative control. Cells were stained for intracellular IFN-γ and for the T cell surface molecules CD45RA, CD45R0, CCR7, and CD28. The results demonstrate that in HD, a prolonged incubation with lenalidomide leads to an increased amount of IFN-γ–secreting T cells, reflecting an increased specific activation of T cells. The surface molecules CD45RA, CD45R0, CCR7, and CD28 are markers of the T cell maturation process, and notably, the flow cytometry analysis demonstrated that the IFN-γ–secreting $CD3^+CD8^+$ T cells display a $CD45RA^-CD45R0^+CCR7^{+/-}CD28^+$ phenotype. In the PD patient, the highest amount of IFN-γ–secreting T cells is found after a 14-d incubation period, and the T cells also show the $CD45RA^-CD45R0^+CCR7^{+/-}CD28^+$ phenotype. The phenotype was confirmed by a CD28/CCR7 double-staining analysis (data not shown).

Impact of lenalidomide on the expression of CD45RA in vitro

We analyzed the expression of CD45RA on the surface of DC-activated T cells (Fig. 3). MNCs from HDs ($n = 11$) and from untreated patients with PD ($n = 10$) were incubated with peptide-pulsed DCs in presence or absence of lenalidomide for a period of 12 d. The expression of CD45RA was determined by flow cytometry on $CD3^+CD8^+$ T cells and on $CD3^+CD4^+$ T cells (Fig. 3*Ab–d*). In all HDs (Fig. 3*Aa*: Len., median 44.6%; Contr., median 54.4%; $p < 0.001$; Fig. 3*Ab*: Len., median 30.2%; Contr., median 42.3%; $p < 0.001$) and in 9 of 10 patients with PD (Fig. 3*Ac*: Len., median 39.6%; Contr., median 48.0%; $p = 0.023$; Fig. 3*Ad*: Len., median 31.9%; Contr., median 44.0%; $p < 0.001$), we found a significantly decreased expression of CD45RA induced by lenalidomide for $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells. In Fig. 3*B*, T cells were incubated with lenalidomide during T cell expansion with peptide-pulsed DCs for 28 d, 14 d, or in absence of lenalidomide (as mentioned earlier). The 28-d incubation with lenalidomide enhanced the downregulation of CD45RA compared with an incubation period of 14 d or an incubation completely without lenalidomide for $CD3^+CD8^+$ T cells (Fig. 3*Ba*: 28d-Len., median 13.3%; 14d-Len., median 28.7%; Contr., median 47.5%; $p \leq 0.001$) and for $CD3^+CD4^+$ T cells (Fig. 3*Bb*: 28d-Len., median 11.3%; 14d-Len., median 19.2%; Contr., median 35.7%; $p \leq 0.001$). Fig. 3 shows also representative density plots of 28-d incubation compared with 14-d incubation, stained for CD45RA of $CD3^+CD8^+$ T cells, and of $CD3^+CD4^+$ T cells. The lenalidomide-enhanced downregulation of CD45RA might indicate a maturation of T cells toward a memory type, holding true for $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells. For the expression of CCR7 and CD28 on T cells, we found divergent results (data not shown).

We analyzed four PD patients in this setting, but three patients did not have an Ag-specific activation. Analysis of the patient with a specific activation (PD no. 16) is shown in Fig. 2*Bb*. In this patient, the strongest downregulation of CD45RA was found in the 14-d setting, where of special interest, we also observed the highest IFN-y secretion, indicating that CD45RA downregulation, respectively a memory immunophenotype of T cells, might be important for Ag-specific IFN-γ secretion.

Impact of lenalidomide on the expression of CD45RA in vivo

To analyze the impact of lenalidomide on the expression of CD45RA on nonspecifically activated T cells in vivo, we performed flow cytometry analysis of CD3/CD28 bead-activated $CD3^+CD8^+$ and $CD3^+CD4^+$ 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. 4*A*, $p = 0.046$), and $CD3^+CD4^+$ T cells (Fig. 4*B*, $p = 0.023$), 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), ♦$p \leq 0.05$ (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.

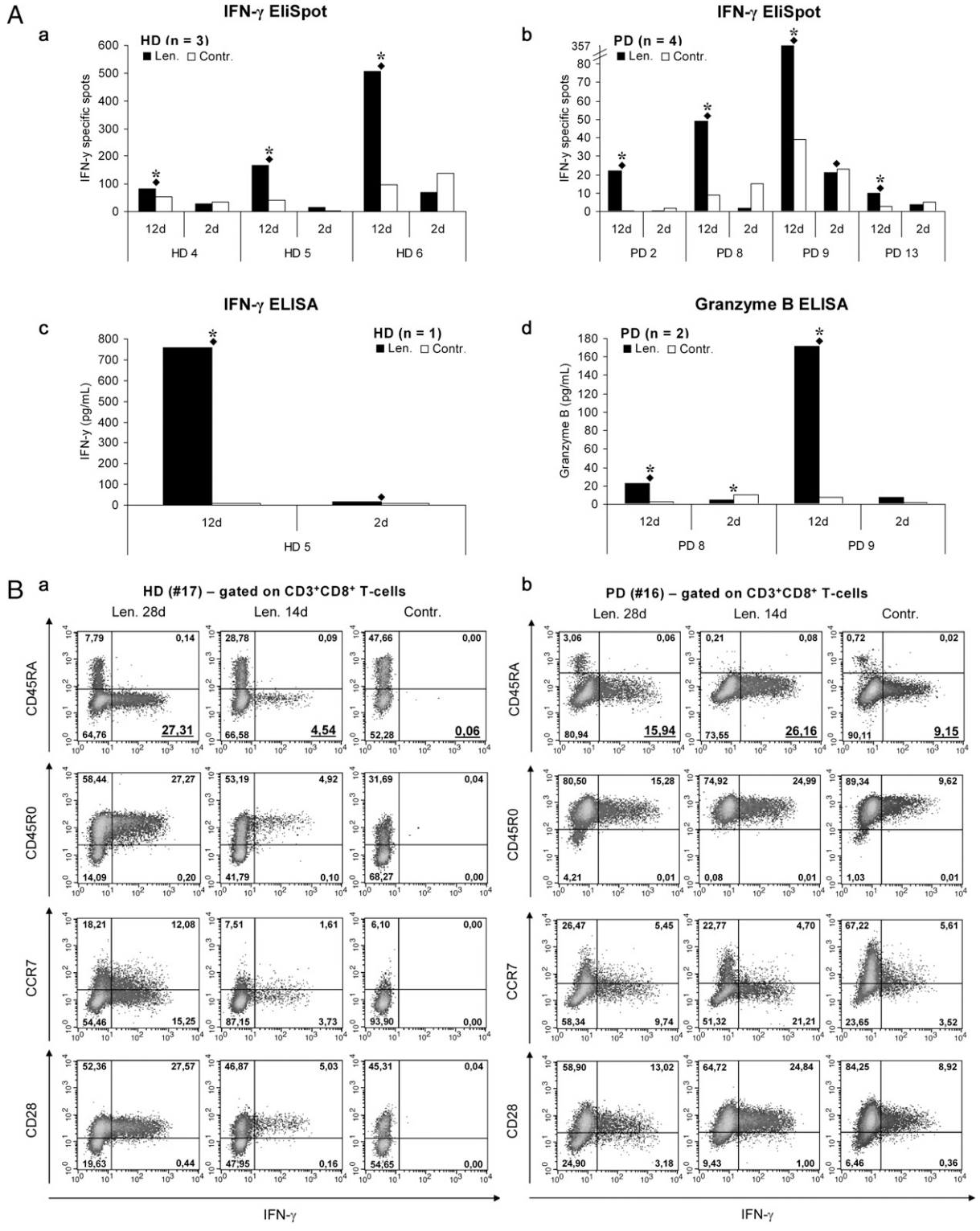

FIGURE 2. Prolonged incubation period of lenalidomide enhances the number and activation of *HM1.24*-specific $CD3^+CD8^+$ T cells. *A*, Different incubation intervals with lenalidomide: 12 d (12d) versus 2 d (2d). In the 12-d setting, MNCs were incubated with MART-1 peptide-loaded DCs in presence of 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. $*p \leq 0.05$ (calculated by Student *t* test, indicates a significant difference in activation of $CD3^+CD8^+$ T cells in presence or absence of lenalidomide), $^\blacklozenge p \leq 0.05$ (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). *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

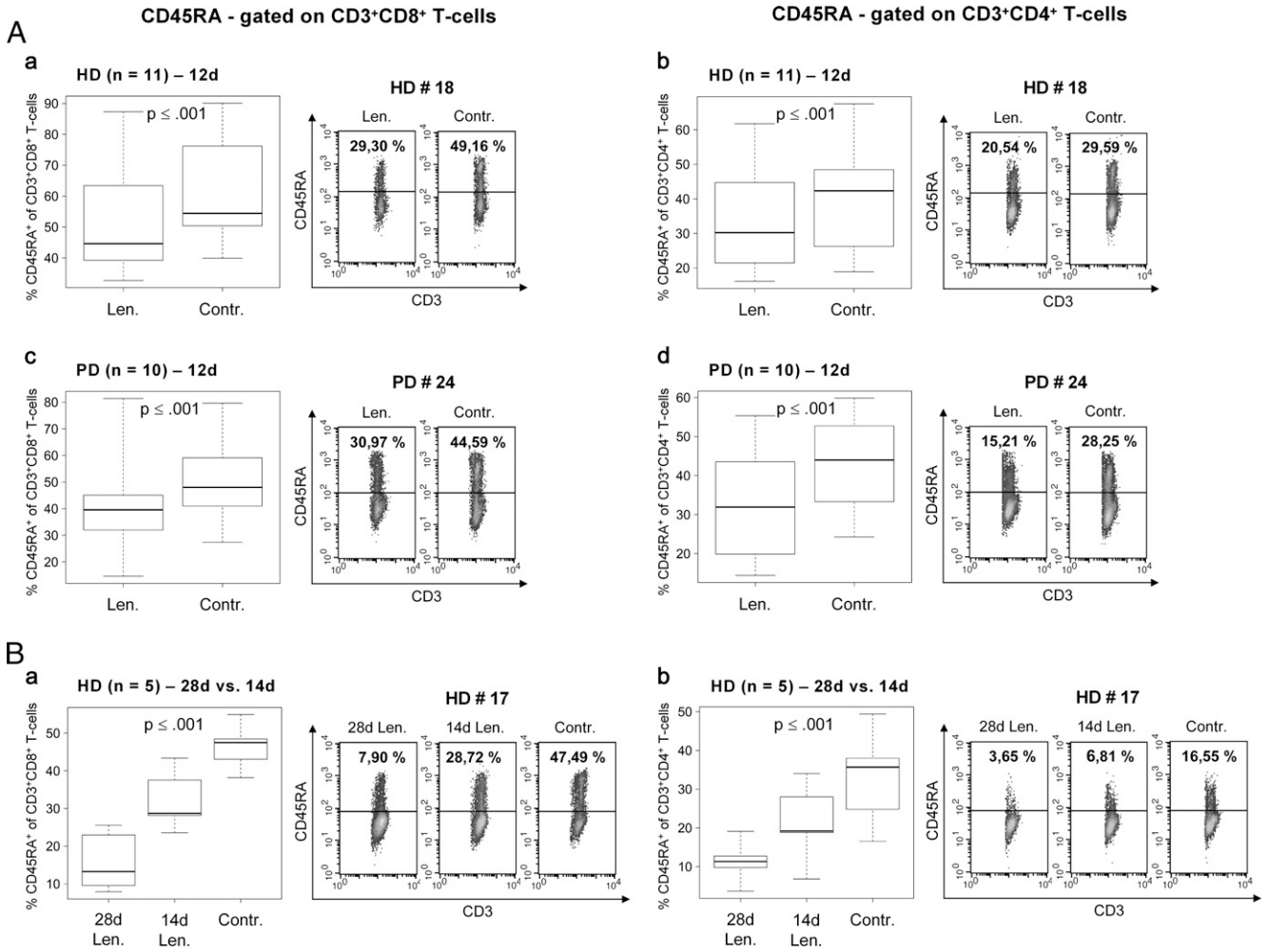

FIGURE 3. Lenalidomide decreases the expression of CD45RA of *HM1.*24-specific activated $CD8^+$ and $CD4^+$ lymphocytes in vitro. Expression of CD45RA was analyzed by flow cytometry, gated on $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells. Cumulative results are shown in boxplots displaying medians of the expression of CD45RA. In 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 μM) 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. 2*B*. 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 ≤ 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.

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 *HM1.24*$^+$ myeloma cells (43). We report here that lenalidomide increases the generation and activity of *HM1.24* 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-γ and tetramer staining). The methodologies make it possible to show that lenalidomide increases both the single-cell rate of IFN-γ, granzyme B, and perforin secretion (analyzed by ELISA) and the frequency of *HM1.24*-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. 1*Be*), one could speculate that

either coincubated with lenalidomide (10 μM) 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-γ and for the surface molecules CD45RA, CD45R0, CCR7, and CD28. Density plots were gated on $CD3^+CD8^+$ T cell population. Numbers represent percentages of positive cells in each quadrant. The percentage of IFN-γ–secreting $CD45RA^+CD3^+CD8^+$ T cells for the different incubation intervals is underlined and shown in the first row of *B*.

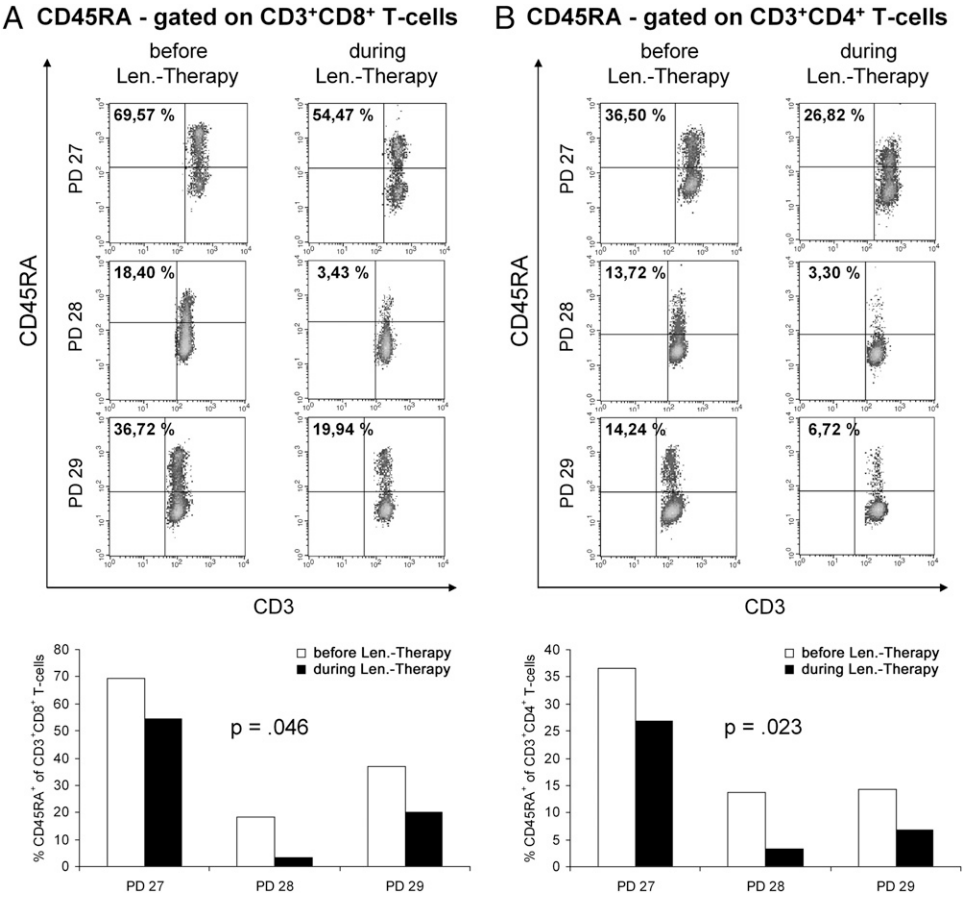

FIGURE 4. Lenalidomide decreases the expression of CD45RA on activated $CD8^+$ and $CD4^+$ lymphocytes in vivo. The influence of lenalidomide therapy on the CD45RA expression of T cells was analyzed by flow cytometry. PBMCs from three patients with PD before (white bars) and during (black bars) lenalidomide 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.

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 $CD45RA^-CD45R0^+CCR7^{+/-}CD28^+$ T cells, these cells are unable to secret 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 $CD8^+$ and in $CD4^+$ 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 $HLA\text{-}DR^+$ T cells as well as $CD4^+$, $CD25^+$, and $CD127^{low}$ regulatory T cells in patients receiving lenalidomide salvage therapy after allogenic SCT. Regarding maintenance therapy with lenalidomide and the control of tumor cells in a minimal residual disease situation, 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

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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