Expression of programmed death-1 on lymphocytes in myeloma patients is lowered during lenalidomide maintenance

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Online supplementary data

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Online Supplementary information: Patients and materials and methods

Blood and bone marrow samples

EDTA-anticoagulated peripheral venous blood samples were obtained from healthy donors (n=7; median age: 35 years) and patients diagnosed with multiple myeloma (n=71; *Table 1*) who had provided written informed consent according to the Declaration of Helsinki. The study was approved by the ethical committee of the University Medical Center Wuerzburg (UKW). If concurrently available, heparinized bone marrow (BM) aspirate samples were obtained and analyzed in the same manner (n=15). No material was included from individuals suffering from acute or chronic infections. Diagnosis and assessment of remission were made according to the criteria of the International Myeloma Working Group (IMWG). Comparative values for the healthy adult population were determined by analysis of healthy donor blood samples, or were obtained from the literature[1, 2]. Absolute lymphocyte counts in blood samples were evaluated by local clinical routine laboratories.

Patient cohorts

Initially, peripheral blood samples from four clinical subgroups of myeloma patients were investigated. Samples were taken from patients undergoing lenalidomide (Len) maintenance treatment (LenMT; n=20) as part of the DSMM XII-XIV studies (*Online Supplementary Figure 1A*); from patients at the time of primary diagnosis (Dx, n=15); from patients in complete remission (CR, n=18); and from patients at the time of relapse (PD, n=12) (*Online Supplementary Figure 1B*). Additionally, samples from a group of patients randomly selected from the hospital database and not exposed to immunomodulatory treatment were analyzed (control group, n=6; *Online Supplementary Figure 1B*). BM and serological disease burden were matched to the cohort of patients undergoing LenMT who were not in CR. For evaluation of BM lymphocyte subsets, concurrently available BM aspirate samples with increased numbers of $CD38^+CD138^+$ plasma cells were analyzed (n=15; patients at Dx: n=6; patients with PD: n=8; patients during LenMT: n=1).

Patient characteristics

The myeloma patients under investigation represented a typical patient cohort at a tertiary care center. Half of the patients analyzed at Dx presented with Durie & Salmon stage III

disease; median BM infiltration was 40% [range, 15–90%]. More than three-quarters of the patients in the CR group initially presented with stage III disease; less than one-third displayed adverse cytogenetic alterations upon FISH karyotyping and the last anti-myeloma treatment was administered more than 2 years [median; range, 0–8.5] before analysis. Almost all patients in the PD cohort presented with stage III disease at the time of first diagnosis and more than half featured a high-risk karyotype; at the time of analysis, median BM infiltration was 90% [range, 10–99], even though previous treatment consisted of high-dose chemotherapy with autologous hematopoietic stem cell transplantation (HSCT) up until a median of approximately 2 years [range, 0.42–6.92] prior to analysis, and treatment with other anti-myeloma therapies up until a median of 2 months [range, 0–45] prior to analysis. In the LenMT cohort, 70% of patients presented with stage III disease at the time of first diagnosis, and 45% had a high-risk karyotype. High-dose chemotherapy and consecutive autologous HSCT had been performed according to the study protocols at a median of 14 months [range, 4–41] prior to analysis, and the patients had received continuous Len (starting dose, 10 mg QD) without additional corticosteroids for a median of 12.4 months [range, 1.3–41.4] before analysis. In 11 patients, the Len dose was reduced to 5 mg QD, and 70% (14/20) of the patients receiving LenMT were in CR. The six patients not in CR showed a very good partial response (n=3), a partial response (n=2), or PD (n=1), and BM infiltration rates ranged from 5% to 90%. Accordingly, patients from the matched control group not exposed to Len (n=6) exhibited a disease burden equal to that of the six patients in the LenMT group (evaluated by serological monitoring and immunohistochemistry of the BM).

Cell phenotyping

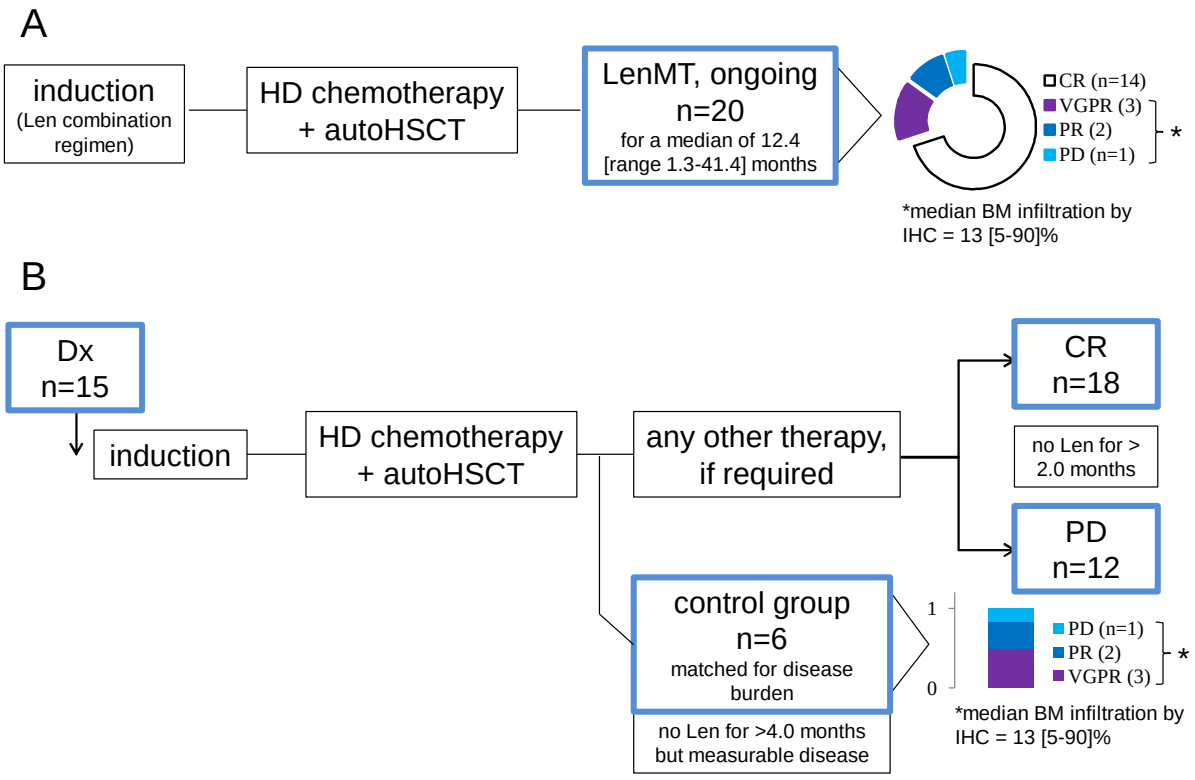
Lymphocyte subset composition and cell surface expression of PD1 (CD279) by $CD4^+$ T cells, $CD8^+$ T cells, $CD45RA^+CD45RO^-CD62L^+$ (naïve) T cells, $CD45RO^+CD62L^+$ (central memory) T cells, $CD45RO^+CD62L^-$ (effector memory) T cells, $CD45RA^+CD62L^-$ (terminally differentiated effector memory) T cells, $CD3^-CD56^+$ natural killer (NK) cells, $CD3^+CD56^+$ NKT cells, $CD3^-CD19^+$ B cells, and $CD3^+\gamma/\delta TCR^+$ T cells were determined in whole blood lysates using multi-parameter flow cytometry. The gating strategy is depicted in *Online Supplementary Figure 2.* Cells were stained with the following conjugated monoclonal antibodies: PD1 FITC (eBioscience, San Diego, CA), CD3 Pacific blue, CD3 PerCP, CD4 PerCP, and CD62L V450 (all BD Biosciences, San Jose, CA), CD8 PECy7, CD45RA APC, γδTCR APC, CD19 PECy7, CD56 APC, IgG1κ FITC, CD38 PerCP, and CD138 FITC (all

BioLegend, San Diego, CA), and CD45RO PE (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry was performed using a FACSCanto II cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software version 9 (Tree Star Inc., Ashland, OR).

Statistical analysis

Where a normal distribution was confirmed by the Kolmogorov-Smirnov test, statistical significance was evaluated using Student's t test; where normal distribution was rejected, statistical significance was evaluated using the nonparametric Mann-Whitney U (MWW) test. Significance levels of $p<0.05$ (*), $p<0.01$(**), and $p<0.001$ (***) were defined. Analysis and artwork was performed using IBM SPSS v24 (Chicago, IL). Further artwork was created using GraphPad Prism version 5 (La Jolla, CA).

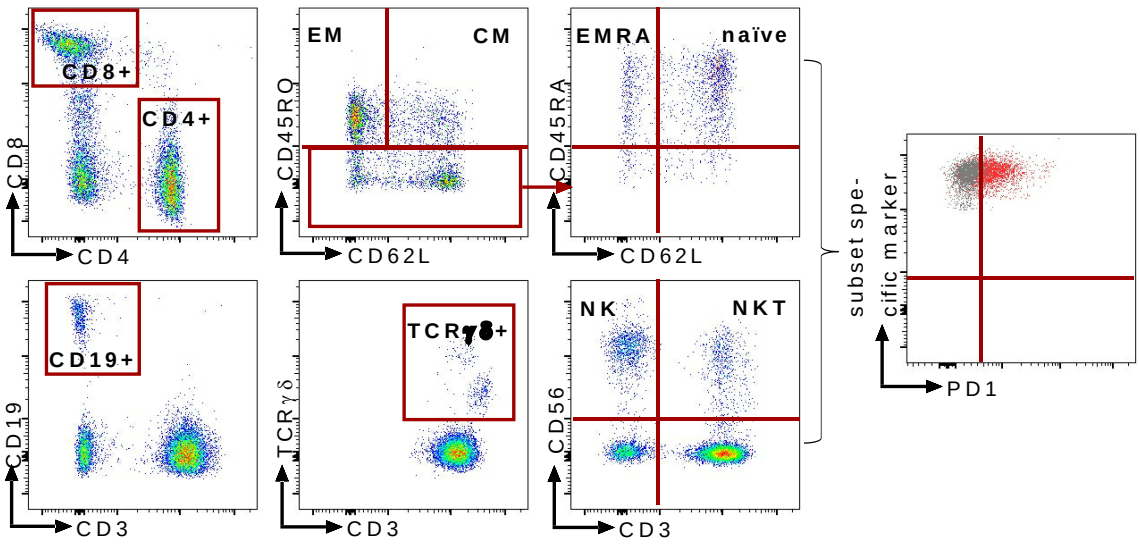
Online Supplementary Figures



Online Supplementary Figure S1: Schematic overview of analyzed patient cohorts.

(A) After a lenalidomide (Len)-based induction therapy and high-dose (HD) chemotherapy with melphalan, followed by single or tandem autologous hematopoietic stem cell transplantation (autoHSCT), patients treated within the DSMM XII-XIV studies received continuous Len maintenance treatment (LenMT). Analysis of lymphocyte subset composition and PD1 phenotype was performed ≥40 days after initiation of LenMT. Most patients experienced optimal disease control; however, malignant plasma cells were detected by immunohistochemistry (IHC) of bone marrow (BM) samples from six patients. The patient with progressive disease (PD) at the time of sample acquisition was excluded from the study at the time of confirmed PD (according to the protocol) and underwent intensified anti-myeloma treatment thereafter. (B) For comparison, lymphocyte subset composition and PD1 phenotype were analyzed in myeloma patients at Dx (primary diagnosis), CR (complete remission), and PD. Furthermore, a control group not recently exposed to Len was matched (in terms of disease burden) to the subgroup of patients with measurable disease during LenMT.

Abbreviations: Len, lenalidomide; LenMT, lenalidomide maintenance therapy; HD, high-dose; auto-HSCT, autologous hematopoietic stem cell transplantation; BM, bone marrow; IHC, immunohistochemistry; Dx, primary diagnosis; CR, complete response; VGPR, very good partial response; PR, partial response; PD, progressive disease.



Online Supplementary Figure S2: Gating strategy

Representative dot plots from flow cytometric analysis of a patient's whole blood sample after red blood cell lysis. The gating strategy for identification and proportionate quantification of lymphocyte subsets is depicted. In addition to PD1 (or isotype control), the surface markers CD4, CD8, CD45RA, CD45RO, and CD62L were used for T-cell analysis (upper panel), CD3 and CD19 for B-cell analysis, CD3 and TCRγδ for γδ T-cell analysis, and CD3 and CD56 for NK(T)-cell analysis (lower panel). CD4, CD8, CD19, TCRγδ, and CD56 were used as subset-specific markers. dMFI PD1 was defined as the difference in mean fluorescence intensity (MFI) between PD1 and isotype control staining.

Abbreviations: EM, effector memory; CM, central memory; EMRA, terminally differentiated effector memory; TCR, T-cell receptor; NK, natural killer; dMFI, differential mean fluorescence intensity; PD1, programmed death-1.

Supplementary references

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