Brief report

Enrichment of functional CD8 memory T cells specific for MUC1 in bone marrow of patients with multiple myeloma

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Multiple myeloma (MM) is one of the most common hematologic malignancies. Despite extensive therapeutical approaches, cures remain rare exceptions. An important issue for future immunologic treatments is the characterization of appropriate tumor-associated antigens. Recently, a highly glycosylated mucin MUC1 was detected on a majority of multiple myeloma cell lines. We analyzed bone marrow and peripheral blood of 68 patients with HLA-A2–positive myeloma for the presence and functional activity of CD8 T cells specific for the MUC1-derived peptide LLLLTVLTV. Forty-four percent of the patients with MM contained elevated frequencies of MUC1-specific CD8 T cells in freshly isolated samples from peripheral blood (PB) or bone marrow (BM) compared with corresponding samples from healthy donors. BM-residing T cells possessed a higher functional capacity upon specific reactivation than PB-derived T cells with regard to interferon γ (IFN-γ) secretion, perforin production, and cytotoxicity. (Blood. 2005;105:2132-2134)

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Introduction

Multiple myeloma (MM) is characterized by a clonal proliferation of malignant plasma cells.[1] Since conventional treatments revealed limited efficacy,[2,3] the potential of the patients' immune system to recognize autologous tumor cells has become a focus of investigations.[4] Patients with premalignant monoclonal gammopathy of undetermined significance (MGUS) exerted strong T-cell responses against plasma cells in their bone marrow (BM).[5] In some patients with myeloma, clonal expansion of cytotoxic CD8 T cells has been described and correlated with better prognosis, suggesting the presence of protective tumor-specific T-cell clones.[6-8] Similarly, T cells reactive against idiotype antibodies[9] or unknown antigens derived from plasma cell lysates[10] could be detected in patients' BM either in freshly isolated T cells or after repetitive stimulations in vitro. Other studies reported an inability of the patient's T cells to respond to autologous tumor cells or shared myeloma-associated antigens,[5,11] suggesting a tumor-specific or generalized state of unresponsiveness.[12]

Mucin-1 (MUC1), a glycosylated type I transmembrane glycoprotein has been identified as tumor-associated antigen (TAA) on most myeloma cell lines[13-15] and has been proposed as a candidate for peptide vaccination.[16] A MUC1-derived HLA-A2–restricted peptide (LLLLTVLTV)[14,15] induced the generation of specific cytotoxic T cells from healthy donors upon repeated stimulation. Yet, the presence and frequencies of MUC1-specific T cells in patients with myeloma has not been determined.

In this study involving altogether 68 patients with HLA-A2–positive MM representing a broad variety of tumor stages and pretreatments we analyzed frequencies and functional capacity of MUC1-specific T cells.

Study design

Patients and donors

BM was collected after informed consent from each posterior iliac crest as described.[17] Patient characteristics are summarized in supplemental Table 1 (at the *Blood* website; see the Supplemental Data Set link at the top of the online article). The procedure to make the MM diagnosis was performed according to standard criteria. Mononuclear cells were collected after Ficoll gradient centrifugation.[17]

Cells

For use in functional assays T cells were cultured as described.[17] In brief, cells were cultured for 7 days in RPMI containing 10% AB serum, 100 U/mL interleukin 2 (IL-2), and 60 U/mL IL-4 followed by overnight incubation without interleukins and separation from contaminating cells by anti-CD19, anti-CD15, and anti-CD56 monoclonal antibody (mAb)–conjugated magnetic beads.[17] Dendritic cells (DCs) were generated as described.[18] In brief, adherent cells from peripheral blood (PB) samples were cultured for 7 days in serum-free X-VIVO 20 containing 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhuGM-CSF) and 1000 U/mL IL-4.[17] DCs were enriched using anti-CD3 and anti-CD19–coupled magnetic beads and pulsed for 2 hours with MUC1-derived peptide LLLLTVLTV[15] or with HLA-A2–binding control peptides (HIVgag 77-85, SLYNTVATL,[19] or irrelevant synthetic peptide HLVEALYLV).

The online version of the article contains a data supplement.

IFN-γ ELISPOT assay

Interferon γ (IFN-γ)–producing T lymphocytes were determined as previously described.[17] Briefly, peptide-pulsed DCs were incubated with autologous T cells in a ratio of 1:5 for 40 hours. IFN-γ spots were measured using KS enzyme-linked immunospot (ELISPOT) software (Zeiss, Jena, Germany). Spots induced by control peptides were considered as background. Individuals were designated as responders if spot numbers in the presence of MUC1 peptide were significantly higher ($P < .1$) than in negative control wells. The frequency of tumor-reactive T lymphocytes was calculated as follows: (spots in test wells − spots in control wells)/T-cell numbers per well.

Cytotoxicity assay

The ^{51}Cr-release assay was performed as described.[17] Briefly, DCs were loaded with MUC1 peptide (10 μg/mL) and coincubated for 7 days with autologous T cells (DC/T cell ratio = 1:5). ^{51}Cr-labeled T2 cells loaded with LLLLTVLTV or control peptide HLVEALYLV were used as targets.

T-cell staining with HLA-A2/peptide tetramers

Tetrameric complexes containing HLA-A*0201 MUC1 peptide LLLLTVLTV were provided by the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility (Bethesda, MD). Tetrameric complexes containing HLA-A *0201 HIV peptide SLYNTVATL were purchased from Proimmune (Oxford, United Kingdom). Freshly isolated HLA-A2–positive (using fluorescein isothiocyanate [FITC] anti–HLA-A2 mAb BB7.2; BD Pharmingen, Heidelberg, Germany) BM and PB mononuclear cells were incubated for 30 minutes on ice with phycoerythrin (PE)–conjugated tetramers (dilution 1:100) and FITC-CD8 (clone HIT8; BD-Bioscience) followed by staining with propidium iodide (PI) for detection of dead cells and flow cytometry. Cells were gated for small lymphocytes on forward/side scatter. Recordings were made only on PI-negative cells. For quantitation of MUC1-specific cells, gates were set on CD8 high cells. Thereby, only $CD8^+CD3^+$ T cells but no $CD3^-$ cells, such as natural killer (NK) cells or DCs were included into the analysis (data not shown).

Intracellular perforin assay

T cells were stimulated as described for the cytotoxicity assay. Cell permeabilization and perforin staining were performed using a cytofix/cytoperm kit and perforin staining kit (BD Bioscience, San Diego, CA). In addition, cells were stained with mAbs against CD8 and CD45RA (clone 4KB5; DAKO, Hamburg, Germany), followed by fluorescence activated cell sorting (FACS) analysis.

Statistical evaluation

Differences between test groups were analyzed using 2-sided Student t test.

Results and discussion

We quantified MUC1-peptide–specific CD8 T cells in 49 patients with MM using HLA-A2 peptide tetramer complexes containing MUC1–derived peptide LLLLTVLTV. Compared with CD8 T cells from PB of 14 and BM of 5 healthy donors (HDs) that contained maximum numbers of 0.27% of CD8 T cells, patients with MM contained elevated frequencies of MUC1-specific CD8 T cells mainly of intermediate affinity in their PB and BM (Figure 1A-B). In total, 44% (22 of 50) of the tested patients had elevated numbers of MUC1-specific T cells (exceeding 0.27% of CD8 T cells) either in their PB (18 of 47) or BM (5 of 16), ranging from 0.3% to 6% of
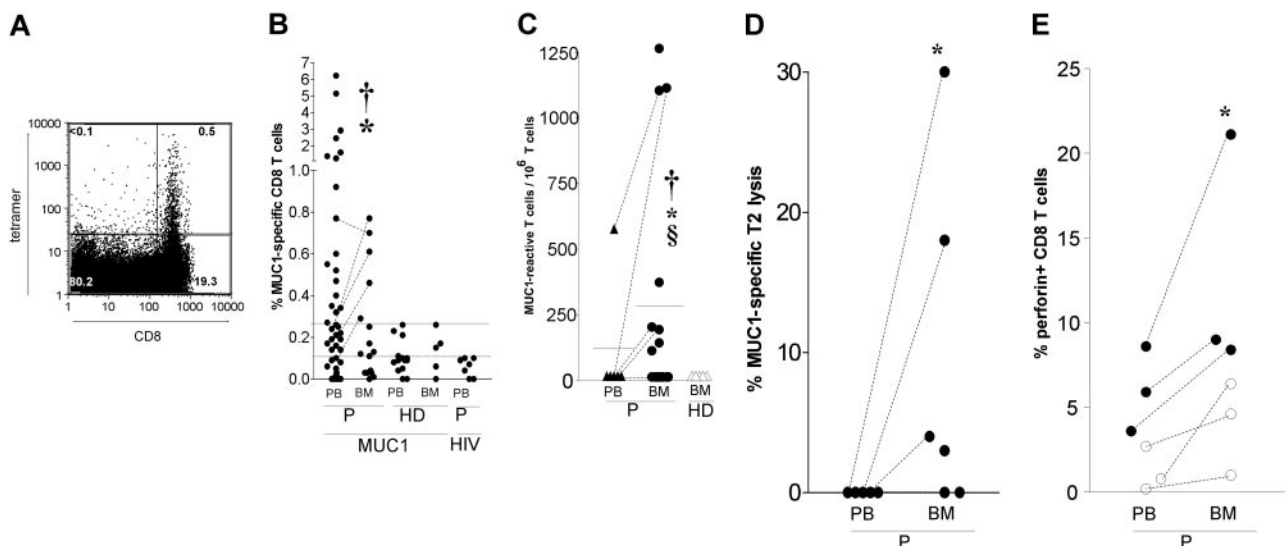

Figure 1. Frequencies and functional activity of MUC1-specific CD8 T cells in PB and BM of MM patients or healthy donors. (A) One representative dot plot from a patient's PB showing tetramer binding cells among PI$^-$ mononuclear cells (2.5% tetramer binding cells among CD8$^+$ T cells). (B) Frequencies of MUC1-specific CD8 T cells indicated as percent of total CD8 T cells from each individual patient (P; n = 50) and healthy donor (HD; n = 14) analyzed. The solid line indicates the maximum frequency measured in the group of healthy donors. The dotted line indicates maximum frequency of CD8 T cells binding HIV tetramers (n = 7). Significant differences between myeloma samples and corresponding samples from healthy donors are depicted for PB (†, $P < .02$) and BM (*, $P < .05$). Values from patients with elevated numbers of MUC1 specific CD8 T cells in either PB or BM are indicated by interrupted lines connecting corresponding values of PB and BM. As control, frequencies of the patient's CD8 T cells binding irrelevant tetramers containing HIV-derived peptide SLYNTVATL are shown (HIV). (C) Frequencies of IFN-γ secreting MUC1 peptide–reactive memory T cells in PB (▲; n = 10) and BM (●; n = 19) of altogether 21 myeloma patients (P) and of BM samples of 5 healthy donors (HD) (△). Numbers of MUC1-reactive cells were calculated as difference between mean spot numbers in test wells and control wells and indicated as numbers of 10^6 purified T cells. †, overall significant difference between BM samples from myeloma patients and healthy donors ($P < .05$). *, overall significant difference between PB and BM of myeloma patients ($P < .01$). Values from patients with elevated numbers of MUC1-reactive T cells in either PB or BM are indicated by dotted lines connecting corresponding values of PB and BM. §, overall significant difference between PB and BM in paired samples from patients containing MUC1-reactive memory T cells ($P < .02$). (D) Percent of MUC1-specific lysis quantifies the significant difference between specific lysis of LLLLTVLTV-loaded T2 cells and lysis of T2 cells loaded with control peptide HLVEALYLV measured at an effector-target ratio of 25:1. No MUC1-specific cytotoxicity was observed in PB T cells at different effector-target ratios (6:1 vs 50:1). *, overall significant difference between cytotoxicity of BM T cells compared to PB T cells ($P < .03$). (E) Percent of perforin content of CD45RA$^-$ (●) and CD45RA$^+$ CD8 T cells (○) from PB and BM samples of 3 myeloma patients after 7 days of coculture with autologous DCs pulsed with MUC1-derived peptide LLLLTVLTV. *, significant difference between perforin content of BM T cells compared to PB T cells ($P = .04$).

CD8 T cells. Since slightly higher frequencies in PB samples compared with BM samples were not statistically significant and in a subgroup of paired BM and PB samples in 4 of 5 patients the frequencies were higher in the BM (Figure 1B), we summarize that in our limited study group no apparent difference was detectable regarding frequencies of MUC1-specific CD8 T cells between PB and BM. Using IFN-γ ELISPOT assay we analyzed the functional properties of freshly isolated T cells from 21 patients with MM after stimulation with peptide-loaded autologous DCs (Figure 1C). Forty-two percent (8 of 19) of BM samples but only 10% (1 of 10) of PB samples of patients with MM ($P < .01$) and 0% (0 of 5; $P < .05$) of BM samples from healthy donors contained MUC1-reactive T cells. In patients containing MUC1-reactive T cells, these were enriched in BM compared with paired PB samples ($P < .02$; Figure 1C). Furthermore, we detected MUC1-specific cytotoxicity in BM T cells from 4 of 5 patients but not in corresponding PB T cells (Figure 1D). Interestingly, the intracellular perforin content of MUC1-stimulated CD8 T cells was consistently higher in BM compared with PB (Figure 1E). In both compartments perforin was found mainly in the fraction of CD45RA⁻ memory T cells.

Our data demonstrate the presence of TAA-specific memory T cells in about 40% of all patients with MM. In contrast to previous studies, we here used as model TAA the HLA-A2–restricted MUC1-derived HLA-A2–binding peptide LLLLTVLTV from the leader sequence. Therefore, HLA-A2–independent antigen recognition, as described previously,[20] is very unlikely in our experimental setting. Our data demonstrate for the first time that MUC1 is also recognized as autologous TAA during the course of disease in patients with myeloma and results in the generation of memory T cells.

Functional MUC1-specific T cells were mainly detected in BM and to a lesser extent in the PB of the patients. This may reflect the fact that myeloma cells are located in the BM and may provide a source of antigen for the presentation by BM-resident DCs. The presence of BM-resident DCs capable of priming naive T cells and restimulating memory T cells against TAAs has recently been demonstrated.[21] In a mouse lymphoma model such antigen-presenting cells (APCs) led to tumor dormancy in the BM and long-term protective immune memory based on specific T cells.[22-24] Enrichment of tumor-reactive memory T cells in BM has been described for other tumor entities such as breast cancer.[17,25]

Since short-term reactivation of BM T cells by antigen-laden DCs resulted in rapid generation of tumor-reactive effector T cells, our data suggest that functional efficacy of TAA-specific T cells is either maintained in patients with MM or can be rapidly restored upon appropriate reactivation. Thus, MUC1-derived peptides may be suitable candidates for either DC vaccination or for reactivation of patient-derived BM T cells ex vivo, followed by their adoptive retransfer.

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