

Bone Marrow Contains Melanoma-reactive CD8⁺ Effector T Cells and, Compared with Peripheral Blood, Enriched Numbers of Melanoma-reactive CD8⁺ Memory T Cells¹

Anne Letsch, Ulrich Keilholz, Geraldine Assfalg, Volker Mailänder, Eckhard Thiel, and Carmen Scheibenbogen²

University Hospital Benjamin-Franklin, Medizinische Klinik III, Hematology, Oncology, and Transfusion Medicine, Free University of Berlin, 12200 Berlin, Germany

ABSTRACT

Circulating melanoma-specific T cells can be frequently detected in patients with melanoma. Effective T-cell immunity and tumor surveillance, however, requires the presence of specific T cells in tissues populated by tumor cells. The bone marrow (BM) is a compartment frequently harboring micrometastatic tumor cells. Here, we compared directly *ex vivo* in peripheral blood (PB) and BM frequencies and differentiation phenotypes of T cells reactive with the melanoma-associated antigen tyrosinase and with autologous melanoma cells. Using intracellular cytokine and tetramer staining, we detected tyrosinase- and melanoma-reactive CD3⁺CD8⁺ T cells in the BM in similar or enhanced frequencies as in PB. Additional characterization of the differentiation subset using CD45RA and CCR7 revealed the presence of specific effector and memory T cells in the BM in all five patients analyzed. Remarkably, the frequency of tyrosinase- and melanoma-specific memory T cells was significantly increased in BM compared with PB. Thus, the BM may be an important compartment for tumor surveillance harboring a tumor-specific memory T-cell pool in addition to effector T cells.

INTRODUCTION

In melanoma there is increasing evidence that T cells can mediate tumor control and melanoma-reactive effector T cells with lytic potential are frequently detected in the PB³ of melanoma patients (1, 2). The potential of tumor-reactive T cells to migrate to or expand at distinct organ sites is, however, largely unknown. The BM is a compartment of special interest in melanoma and various solid tumors frequently harboring micrometastatic tumor cells (3–9). Several studies show the presence of melanoma cells in the BM either detected immunocytologically or by reverse transcription-PCR for tyrosinase transcripts in 10–30% of melanoma patients (3–7). This is in accordance with the findings in patients with metastatic breast cancer in whom BM micrometastases have most extensively been studied and its negative prognostic impact on survival has been shown (reviewed in Ref. 9). A detection rate of 1–43% of micrometastatic cells in the BM of patients with breast cancer has been reported from various studies (reviewed in Ref. 9).

No direct functional and phenotypical analysis of tumor-specific T-cell responses in BM has been performed thus far. To investigate T-cell immunity in BM, we used IC and tetramer staining to comparatively analyze T cells specific to tumor cells and a tumor antigen in BM and PB. This was possible in five patients with melanoma with a known tyrosinase-specific T-cell response in PB. Additionally, we had in three of these five patients the rare opportunity to simulta-

neously analyze T cells reactive with autologous melanoma cells from which we had established cell lines (2, 10).

MATERIALS AND METHODS

Patients and Healthy Controls. The characteristics of the four patients with cutaneous melanoma and one patient with ocular melanoma included in this study are shown in Table 1. Patients 1, 3, 4, and 5 had received vaccination with tyrosinase peptides 368–376,370D (HLA-A*0201-binding motif) or 206–214 (HLA-A*2402-binding motif), respectively, as described previously (10). At the time of T-cell analysis, patients 1, 2, and 4 were free of macroscopic disease after resection of melanoma metastases. Patient 3 had complete regression of small cutaneous, lung, and lymph node metastases after repeated vaccination. Patient 5 had stable hepatic and pulmonary metastases of an ocular melanoma after repeated vaccination. The generation of autologous melanoma cell lines in patients 1–3 was performed in our laboratory out of metastases resected from the skin (UKBF-Mel-18, patient 1), the soft tissue of the forefoot (UKBF-Mel-11, patient 2; Ref. 2), or the small bowel (UKBF-Mel12, patient 3; Refs. 2, 10), respectively. MCs from PB and BM were isolated by density gradient centrifugation using Ficoll-Hypaque and cryopreserved. Before analysis, frozen MC samples were thawed and cultured overnight in medium (Iscove's medium supplemented with 10% human serum). This investigation had been approved by the Institutional Ethics Committee and informed consent was obtained.

T-Cell Analysis. IC IFN- γ staining was performed as described previously (2). In brief, MCs (2×10^6) were incubated with 10 μ g/ml tyrosinase peptides 368–376, 370D (patients 1, 2, 4, and 5) or 206–214 (patient 3), HIV peptide 476–484 (patients 1, 2, 4, and 5) or without antigen (patient 3) as negative control or autologous tumor cells in a ratio of MC to tumor cells of 10:1. After 2 h, 10 μ g brefeldin A (Sigma, Deisenhofen, Germany) were added, and after additional 16 h, MCs were stained extracellularly with fluorescence-conjugated monoclonal antibodies against CD8, CD3, CD45RA, CCR7, and CXCR4 (BD Bioscience, Heidelberg, Germany) and intracellularly with IFN- γ fluorescence-conjugated monoclonal antibody (BD Bioscience). For tetramer analyses, MCs were incubated for 1 h at room temperature with allophycocyanine-labeled HLA-A*0201 tyrosinase peptide 368–376,370D binding tetramers (Beckman Coulter, San Diego) and after washing for another 20 min at 4°C with fluorescence-conjugated antibodies. Data acquisition was performed on FACSCalibur and analyzed using Cellquest Software (BD Bioscience).

Functional CXCR4 Analysis. For analyzing functional CXCR4 on melanoma-reactive T cells, a secretion assay (Miltenyi Biotec, Bergisch-Gladbach, Germany) was performed as described previously (11). MCs were stimulated for 6 h with autologous tumor cells. Then cells were harvested, washed in RPMI without supplements, and incubated with IFN- γ -Catch-Reagent (Miltenyi Biotec). After an incubation period of 45 min, cells were washed and incubated with IFN- γ detection antibody (Miltenyi Biotec) and a fluorescence-conjugated antibody against CD8. After washing, cells were loaded with 1 μ g of the calcium indicator fluo-3-AM (Molecular Probes, Leiden, the Netherlands) for 45 min at 37°C in the dark. After washing, cells were kept in RPMI medium without supplements before analysis within 1 h by flow cytometry. After establishing a baseline of fluorescence intensity of fluo-3-AM-labeled cells, changes in intracellular Ca release were followed after addition of 1 μ g of SDF-1 (R&D Systems, Wiesbaden, Germany) by gating on CD8⁺ IFN- γ ⁺ T cells. Analyses were performed on a FACSCalibur using Cell Quest software.

mRNA Expression Analysis. Quantitative real-time RT-PCR was performed to assess the expression of tyrosinase and MelanA/MART-1 in tumor

Received 1/16/03; revised 6/16/03; accepted 6/19/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Deutsche Krebshilfe Grant 10-1072.

² To whom requests for reprints should be addressed, at Universitätsklinikum Benjamin Franklin, Medizinische Klinik III, Freie Universität, Hindenburgdamm 30, D-12200 Berlin, Germany. Phone: 49-30-8445-4576; Fax: 49-30-8445-4468; E-mail: carmen.scheibenbogen@ukbf.fu-berlin.de.

³ The abbreviations used are: PB, peripheral blood; BM, bone marrow; CCR, chemokine receptor CC; CXCR, chemokine receptor CXC; IC, intracellular cytokine; MC, mononuclear cell; NED, no evidence of disease; SDF-1, stromal cell-derived factor-1; fluo-3-AM, fluo-3-acetomethylester.

Table 1 Patient characteristics

Patient no.	Union International Contre Cancer stage	Prior systemic treatment	Disease status at time of T-cell analysis in bone marrow
1	IIIb	Adjuvant IFN- α , adjuvant tyrosinase peptide vaccination	NED ^a after resection of skin metastases ^b
2	IV	MAGE-3-protein vaccination, CDDP/DTIC/IFN- α	NED after resection of single site soft tissue/bone metastasis of the forefoot ^b
3	IV	Adjuvant IFN- α , tyrosinase peptide vaccination	Complete remission of skin, lymph node, and lung metastases after vaccination
4	IV	Adjuvant interleukin-2 and IFN- α , adjuvant IFN- α , adjuvant tyrosinase peptide vaccination	NED after resection of lymph node metastases
5	Ocular melanoma IV	Gemcitabine/treosulfan, tyrosinase peptide vaccination	Stable disease of pulmonary and hepatic metastases

^a NED, no evidence of disease.

^b Metastases from which melanoma cell lines were established.

tissue and cell lines as described in detail elsewhere (12). Briefly, RNA extraction was carried out using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany). Random hexamers and avian myeloblastosis virus reverse transcriptase were used for cDNA synthesis. Tyrosinase, MelanA/MART-1, and porphobilinogen deaminase as housekeeping genes were amplified using published primer sequences and conditions using a LightCycler equipment (Roche Diagnostics). A standard curve was established using artificial plasmids for all three RNA species.

Statistical Analysis. A Wilcoxon signed rank test was calculated to determine whether the frequencies of tyrosinase-specific memory T cells in BM were significantly increased compared with PB. Because of the small sample size, the *P* value is considered exploratory.

RESULTS

Detection of Tyrosinase- and Melanoma-reactive T Cells in PB and BM. From five patients with known reactivity to the melanoma-associated antigen tyrosinase in PB simultaneous PB and BM samples were collected. In addition in three of these five patients, an autologous melanoma cell line was available that was specifically recognized by the patients PB T cells. BM samples contained between 2.0 and 5.6% of CD34+ cells in contrast to PB samples in which <0.4% CD34+ cells were present. In addition, cytological smears were performed from BM aspirates in all patients showing the presence of typical BM particles and a normal distribution of hematological precursor cells and lymphocytes. Patient characteristics are shown in Table 1. In patients 1, 3, 4, and 5 tyrosinase-specific T cells had been induced by tyrosinase peptide vaccination, whereas patient 2 had a spontaneous T-cell response to tyrosinase.

We analyzed specific T-cell responses to the tyrosinase 369–376,370D HLA-A*0201-binding peptide (patients 1, 2, 4, and 5) or the tyrosinase 206–214 HLA-A*2402-binding peptide (patient 3) and to the autologous melanoma cells (patients 1–3) in parallel in PB and BM by IC IFN- γ staining after brief exposure to antigen. Unstimulated samples or samples stimulated with an irrelevant HLA-A*0201-binding HIV epitope 476–484, respectively, were used as negative controls.

Absolute frequencies of tyrosinase-reactive CD3+CD8+ T cells were rather similar in PB and BM in patients 1–4 with 0.53% (PB) and 0.58% (BM), 0.98% (PB) and 1.04% (BM), 0.87% (PB) and 0.64% (BM), and 0.31% (PB) and 0.30% (BM), respectively, as detected by IC IFN- γ (Fig. 1 shows the data of all patients and the IC IFN- γ staining is illustrated for patient 2 in Fig. 3, A and B). T-cell responses of patients 1 and 2 against the HLA-A*0201-restricted tyrosinase epitope were also analyzed by *ex vivo* staining of lymphocytes with A*0201/peptide tetramers revealing rather similar frequencies as with the IC IFN- γ staining (Figs. 1 and 3, C and D). In patient 5, tyrosinase-reactive CD3+CD8+ T cells were >2-fold higher in BM (1.14%) compared with PB (0.44%) analyzed by tetramers (Fig. 1). A functional T-cell analysis with IC IFN- γ was not possible in this

patient because of a high background secretion of IFN- γ in the absence of antigen.

In patients 1–3, in addition, CD3+CD8+ T cells specifically secreting IFN- γ in response to autologous melanoma cells were analyzed showing 2-fold, similar, or 8-fold higher absolute frequencies, respectively, in the patient's BM samples compared with the PB

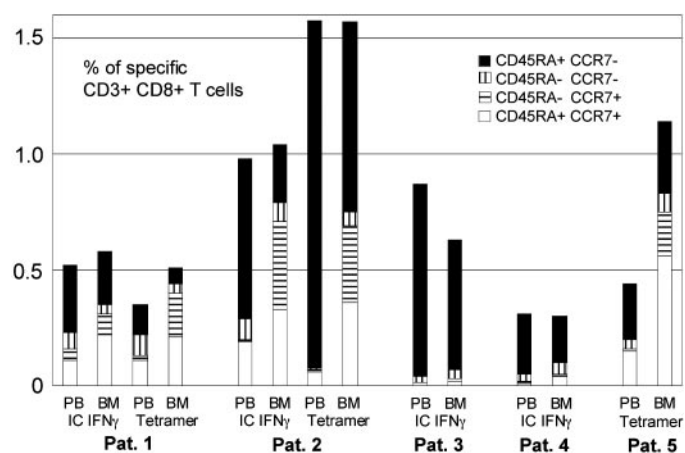


Fig. 1. T-cell responses to tyrosinase peptides in PB and BM of five patients. Frequencies and differentiation phenotypes of specific CD3+CD8+ T cells secreting IFN- γ in response to the antigen (IC IFN- γ) or detected by tetramer staining are shown. The number of IFN- γ -secreting T cells in the absence of antigen or incubated with an irrelevant HIV peptide is subtracted in the experiments performed with IC IFN- γ staining. Specific CD3+CD8+ T cells were separated into distinct subsets based on the expression of CD45RA and CCR7.

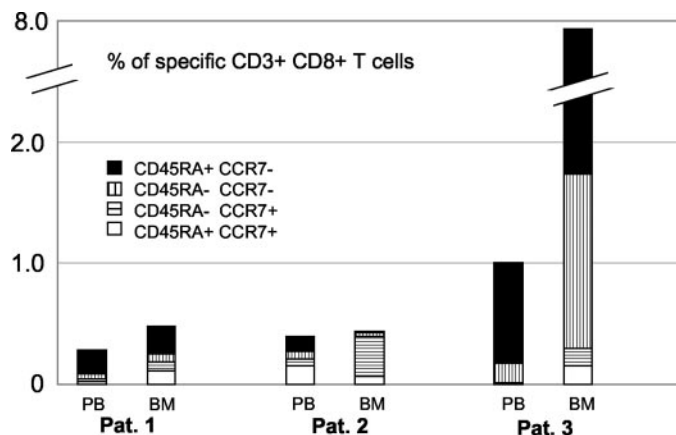
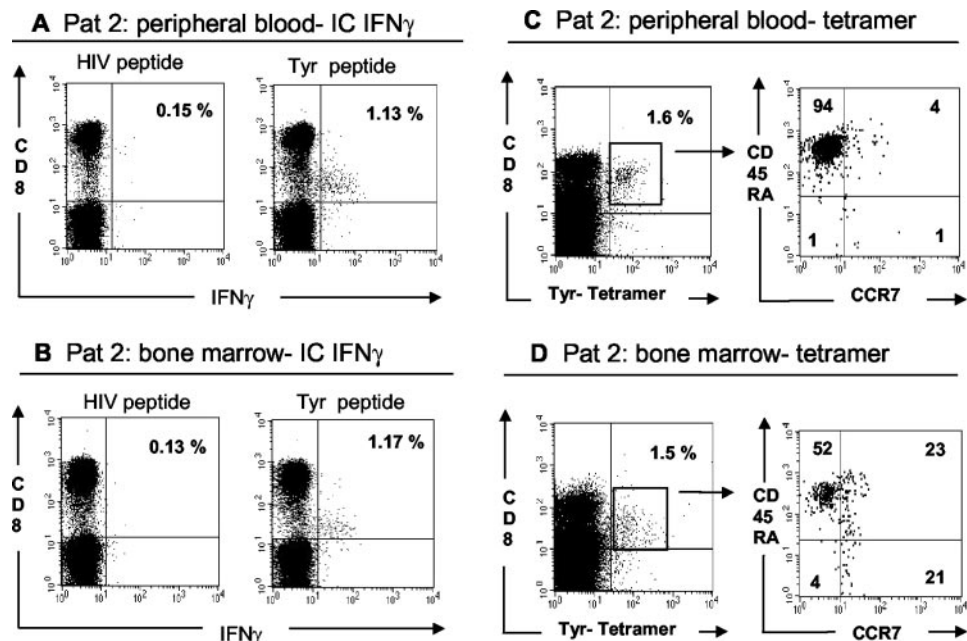


Fig. 2. T-cell responses to the autologous melanoma cells in PB and BM of three patients. Frequencies and differentiation phenotypes of specific CD3+CD8+ T cells secreting IFN- γ in response to the antigen (IC IFN- γ) are shown. The number of IFN- γ -secreting T cells in the absence of antigen is subtracted. Specific CD3+CD8+ T cells were separated into distinct subsets based on the expression of CD45RA and CCR7.

Fig. 3. CD3+CD8+ T-cell responses to the tyrosinase 368–376, 370D peptide detected by IC IFN- γ staining (A and B) or by tetramer staining (C and D) in PB and BM of patient 2. A and B show the CD8/IFN- γ profile of CD3-gated lymphocytes in samples incubated with HIV peptide as negative control (left) or tyrosinase peptide (right). Frequencies of IFN- γ +CD3+CD8+ T cells are indicated. C and D show the tetramer staining of CD3-gated T cells and the CD45RA/CCR7 profile of the CD3+CD8+tetramer+ T cells. Frequencies of CD3+CD8+tetramer+ T cells and their distribution among CD45RA/CCR7 subsets are indicated.



samples, as summarized in Fig. 3 and illustrated for patient 3 in Fig. 4, A and B. The melanoma cell line UKBF-Mel-11 of patient 2 had lost tyrosinase expression during *in vitro* culture, whereas the original melanoma lesion had expressed high levels of tyrosinase (2). The frequency of the T-cell response detected against UKBF-Mel-11 in patient 2 therefore most likely underestimates the magnitude of the antimelanoma response present *in vivo*.

Differentiation Phenotypes of Tyrosinase-specific and Melanoma-specific T Cells in PB and BM. The classification of specific T-cell differentiation subsets is based on the expression of CD45RA and CCR7 as described previously (13). Results of the phenotypical analyses of the tyrosinase- and melanoma-specific CD3+CD8+ T-cell differentiation subsets in PB and BM are summarized in Figs. 1 and 2 and illustrated for patient 2 in Fig. 3. Because naive T cells do not usually secrete IFN- γ under the assay conditions used, we considered the specific CD45RA+CCR7+ T cells detected by IC IFN- γ as early memory T cells (14). In all five patients, tyrosinase-specific T cells and in patients 1–3 also melanoma-specific T cells belonging to memory [CD45RA+CCR7+, CD45RA-CCR7+ (central memory), CD45RA-CCR7- (effector memory)] and effector (CD45RA+CCR7-) T-cell subsets were found in PB and BM. Although the quantitative distribution of specific T cells among the four different subsets varied in the five patients, one finding was consistent in all five patients for both targets and for both assays: The proportion of the tyrosinase-specific memory T cells was increased 1.5–4.1-fold (as determined by IC IFN- γ in patients 1–4 and by tetramers in patients 1, 2, and 5) and of the melanoma-specific memory T cells 1.6–10-fold in BM compared with PB. The increase of tyrosinase-specific memory T cells in BM compared with PB was statistically significant ($P = 0.04$, $n = 5$, comparing frequencies of all tyrosinase-specific memory T-cell subsets in BM *versus* PB in the five patients). The enrichment of specific memory T cells in BM was restricted to the early and central memory subset (CD45RA+CCR7+ and CD45RA-CCR7+) in patients 1, 2, 4, and 5 (Figs. 1 and 2, illustrated for patient 2 in Fig. 3D), whereas the melanoma-specific memory T cells detected in patient 3 were mostly of the effector memory subset (CD45RA-CCR7-, Figs. 1 and 2). Remarkably, this was the only patient in whom we could also observe a strong enrichment of melanoma-reactive effector T cells in BM.

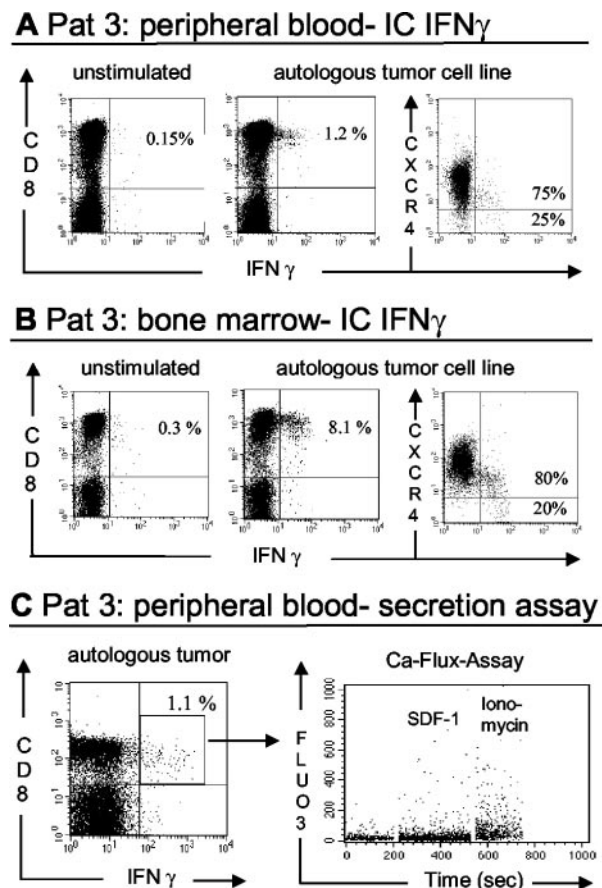


Fig. 4. T-cell responses to the autologous tumor cell line in PB and BM detected by IC IFN- γ staining and expression and functional analysis of CXCR4 on tumor-specific T cells in patient 3. A and B show the CD8/IFN- γ profile of CD3 gated lymphocytes in samples incubated in the absence of antigen (left) and after exposure to autologous tumor cell line (middle). The right figures show the CXCR4/IFN- γ profile of tumor specific CD3/CD8 gated lymphocytes. C shows the functional CXCR4 analysis that was performed in PB on CD8+ T cells secreting IFN- γ in response to the autologous melanoma cell line detected by IFN- γ secretion assay. Melanoma-reactive CD8+/IFN- γ + positive T cells show elevation of intracellular Ca levels indicated by the increase of fluorescence intensity of fluo-3-AM after exposure to the CXCR4 ligand SDF-1 or Ionomycin, which was used as positive control.

To determine whether the T-cell response toward the autologous melanoma cells described above was also present at the time the patients were bearing tumor metastases, additional peripheral blood mononuclear cell samples were analyzed in patients 1–3, which had been drawn 3–35 months earlier, directly before resection of the melanoma lesion from which the melanoma cell lines had been generated. Similar frequencies and phenotypes of melanoma-reactive T cells were found in patients 1–3 in these PB samples with 0.49% (68% CD45RA+CCR7–), 0.34% (27% CD45RA+CCR7–), and 1.3% (92% CD45RA+CCR7–)-specific CD3+CD8+T cells, respectively, measured by IC IFN- γ (graphic data not shown).

Melanoma-reactive T Cells Express Functional CXCR4. The chemokine receptor CXCR4, mediating migration to the ligand SDF-1, which is expressed at high levels in BM, was analyzed on the melanoma-reactive T cells in PB and BM in patients 1–3. Melanoma-reactive T cells were CXCR4^{high} in PB and BM in patients 1 and 2 and CXCR4^{low} with ~25% CXCR4-negative T cells in patient 3 (see Fig. 4, A and B, for patient 3, data from patient 1 and 2 not shown). In patient 3 in whom 1% of the CD3+CD8+ T cells in PB recognized the autologous melanoma cells, we tried to determine the CXCR4 response to the ligand SDF-1 on melanoma-reactive CXCR4^{low} T cells in PB. In these experiments, melanoma-reactive T cells were detected by trapping secreted IFN- γ on the cell surface using a bispecific monoclonal antibody to avoid permeabilization of the cell membrane. CXCR4 expression on the melanoma-reactive IFN- γ +CD8+ T cells was functional as could be shown by elevation of intracellular Ca levels after exposure to SDF-1 (Fig. 4C), suggesting their potential to migrate to the BM.

Melanoma Cells Express Functional CXCR4 but Are Absent from BM. Functional CXCR4 expression could be detected on all three melanoma cell lines derived from the patients studied here (data not shown), suggesting that the melanoma cells should be able to migrate to BM. To analyze whether melanoma cells were present in the BM samples, a quantitative real-time PCR for tyrosinase and MelanA/MART-1 was performed, which is known to be one of the most specific and sensitive markers for occult melanoma cells. Tyrosinase and MelanA/MART-1 were expressed in all three patients melanoma tissues from which the cell lines had been generated. No tyrosinase and MelanA/MART-1 expression could be detected in any of the three patients BM samples nor in PB by quantitative real time PCR.

DISCUSSION

In this study, we provide a first direct functional and phenotypical analysis of tumor-reactive T cells in BM. We could show that similar or higher frequencies of melanoma antigen-reactive T cells are present in BM in all 5 patients analyzed. The characterization of the differentiation phenotype of the melanoma-antigen-specific T cells revealed that the BM contained both melanoma-specific effector T cells and memory T cells in all five patients. T cells exhibiting an effector phenotype are those with the highest cytotoxic potential and have been shown to directly mediate tumor cytotoxicity (2, 13). Importantly, specific memory T cells providing long-term immunity were present in all five patients and were constantly found at higher frequencies in BM as in PB. Taken together, these findings suggest that the BM is a compartment where effective tumor surveillance can take place.

The enrichment of specific memory T cells in BM is in accordance with investigations in breast cancer patients in whom tumor-reactive T cells could be expanded from BM but not from PB in the majority of patients (15). In mice, specific T-cell responses against viral epitopes have been found in BM during acute and chronic infection

(16–18). Although during the acute infection frequencies of virus-specific T cells were lower in BM than in PB, memory responses were ~2-fold higher in BM as compared with PB (18). The finding of an enrichment of melanoma-specific memory T cells in BM raises the question whether they have been generated *in situ* upon direct antigenic stimulation or have entered the BM from PB. The detection of melanoma cells in the BM by PCR has been reported in up to one-third of patients with metastatic disease (3–7). Because melanoma cells of all three patients analyzed in this study express functional CXCR4 (which we find in ~50% of melanoma cell lines, unpublished observation), they should have the potential to migrate to the BM. The failure to detect melanoma cells by quantitative PCR in the BM of these three patients does not argue against the possibility that their melanoma cells migrate to the BM because they may become rapidly destroyed by cytolytic T cells present in BM. Thus, we are unable to conclude from our data whether the specific T cells we find in the BM are attracted by specific antigens or whether alternatively the BM is a place where memory T cells preferentially migrate to. The clinical significance of specific T-cell responses in relation to the presence of micrometastatic or metastatic disease could only be clarified by a larger study simultaneously analyzing the presence of tumor-specific T cells and of tumor cells in BM.

The chemokine receptor CXCR4 plays an important role in the migration of cells to the BM, and there is evidence that CXCR4 mediates the metastatic spread of tumor cells to the BM (19). CXCR4 mRNA and surface expression were described in melanoma, and the ligand SDF-1 is expressed at high levels in BM, lung, liver, and lymph nodes, organs that are frequently involved in melanoma metastases (20, 21). Although the majority of PB T cells express CXCR4, it was shown to be functional in response to SDF-1 only in a subpopulation of T cells (22). The demonstration of functional CXCR4 on melanoma-reactive T cells in the PB in one patient suggests their potential to migrate to the BM and other compartments with high levels of SDF-1. Recently, it was shown that stimulation of the adhesion molecule L-selectin (CD62L) enhances functional expression of CXCR4 on T cells, resulting in enhanced T-cell migration to SDF-1 (23). Remarkably, L-selectin is expressed on the majority of CCR7+ memory CD8+ T cells but only on a small subpopulation of the CCR7– effector memory and effector CD8+ T-cell subsets (13). The differential expression of L-selectin on T cells could result in the preferential migration to and retention of CCR7+ memory T cells in the BM. This hypothesis would fit well to our observation that in four of five patients the enrichment of tyrosinase- and melanoma-specific memory CD8+ T cells in BM is restricted to the CCR7+ subset.

Our finding that the BM contains melanoma-specific effector T cells and enriched numbers of memory T cells has important implications for immunotherapeutic concepts. It provides a rationale for T-cell vaccination strategies in patients with micrometastatic disease in the BM. Furthermore, it suggests that T-cell monitoring in PB may underestimate the type and magnitude of tumor-specific T-cell responses. Finally, the tumor-reactive memory T-cell pool present in BM may be a valuable source for adoptive T-cell therapies.

REFERENCES

- Letsch, A., Keilholz, U., Schadendorf, D., Nagorsen, D., Schmittel, A., Thiel, E., and Scheibenbogen, C. High frequencies of melanoma-reactive CD8+ T cells in peripheral blood of patients with advanced melanoma. *Int. J. Cancer*, 87: 659–664, 2000.
- Valmori, D., Scheibenbogen, C., Dutoit, V., Nagorsen, D., Asemisen, A. M., Rubio-Godoy, V., Rimoldi, D., Guillaume, P., Schadendorf, D., Lipp, M., Dietrich, P. Y., Cerottini, J. C., Thiel, E., Liénard, D., Romero, P., and Keilholz, U. Circulating tumor-reactive CD8+ T cells in melanoma patients contain a CD45RA+CCR7– effector subset exerting *ex vivo* tumor-specific cytolytic activity. *Cancer Res.*, 62: 1743–1750, 2002.
- Schadendorf, D., Dorn-Beineke, A., Borelli, S., Riethmuller, G., and Pantel, K. Limitations of the immunocytochemical detection of isolated tumor cells in frozen

- samples of bone marrow obtained from melanoma patients. *Exp Dermatol.*, *12*: 5–171, 2003.
4. Ghossein, R. A., Coit, D., Brennan, M., Zhang, Z. F., Wang, Y., Bhattacharya, S., Houghton, A., and Rosai, J. Prognostic significance of peripheral blood and bone marrow tyrosinase messenger RNA in malignant melanoma. *Clin. Cancer Res.*, *4*: 419–428, 1998.
 5. Waldmann, V., Deichmann, M., Bock, M., Jackel, A., and Naher, H. The detection of tyrosinase-specific mRNA in bone marrow is not more sensitive than in blood for the demonstration of micrometastatic melanoma. *Br. J. Dermatol.*, *140*: 1060–1064, 1999.
 6. Blaheta, H. J., Paul, T., Sotlar, K., Maczey, E., Schittek, B., Paul, A., Moehrl, M., Breuninger, H., Bueltmann, B., Rassner, G., and Garbe, C. Detection of melanoma cells in sentinel lymph nodes, bone marrow and peripheral blood by a reverse transcription-polymerase chain reaction assay in patients with primary cutaneous melanoma: association with Breslow's tumour thickness. *Br. J. Dermatol.*, *145*: 195–202, 2001.
 7. Ghossein, R. A., and Bhattacharya, S. Molecular detection and characterization of circulating tumor cells and micrometastases in prostatic, urothelial, and renal cell carcinomas. *Semin. Surg. Oncol.*, *20*: 304–311, 2001.
 8. Zippelius, A., and Pantel, K. R. T. PCR-based detection of occult disseminated tumor cells in peripheral blood and bone marrow of patients with solid tumors. An overview. *Ann. N. Y. Acad. Sci.*, *906*: 110–123, 2000.
 9. Ozbas, S., Dafydd, H., and Purushotham, A. D. Bone marrow micrometastasis in breast cancer. *Br. J. Surg.*, *90*: 290–301, 2003.
 10. Scheibenbogen, C., Nagorsen, N., Seliger, B., Schmittel, A., Bauer, S., Max, N., Bock, M., Atkins, D., Thiel, E., and Keilholz, U. Long-term freedom from recurrence in two high-risk melanoma patients following adjuvant vaccination with tyrosinase peptides. *Int. J. Cancer*, *99*: 403–408, 2002.
 11. Asemussen, A. M., Nagorsen, D., Keilholz, U., Letsch, A., Schmittel, A., Thiel, E., and Scheibenbogen, C. Flow cytometric determination of intracellular or secreted IFN- γ for the quantification of antigen reactive T cells. *J. Immunol. Methods*, *251*: 101–108, 2001.
 12. Max, N., Willhauck, M., Wolf, K., Thilo, F., Reinhold, U., Pawlita, M., Thiel, E., and Keilholz, U. Reliability of PCR-based detection of occult tumour cells: lessons from real-time RT-PCR. *Melanoma Res.*, *11*: 371–378, 2001.
 13. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature (Lond.)*, *401*: 708–712, 1999.
 14. Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A., Lechner, F., Spina, C. A., Little, S., Havlir, D. V., Richman, D. D., Gruener, N., Pape, G., Waters, A., Easterbrook, P., Salio, M., Cerundolo, V., McMichael, A. J., and Rowland-Jones, S. L. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.*, *8*: 379–385, 2002.
 15. Feuerer, M., Beckhove, P., Bai, L., Solomayer, E. F., Bastert, G., Diel, I. J., Pedain, C., Oberniedermayr, M., Schirmacher, V., and Umansky, V. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nat. Med.*, *7*: 452–458, 2001.
 16. Kuroda, M. J., Schmitz, J. E., Seth, A., Veazey, R. S., Nickerson, C. E., Lifton, M. A., Dailey, P. J., Forman, M. A., Racz, P., Tenner-Racz, K., and Letvin, N. L. Simian immunodeficiency virus-specific cytotoxic T lymphocytes and cell-associated viral RNA levels in distinct lymphoid compartments of SIVmac-infected rhesus monkeys. *Blood*, *96*: 1474–1479, 2000.
 17. Marshall, D., Turner, S., Belz, G., Wingo, S., Andreansky, S., Sangster, M., Riberty, J., Liu, T., Tan, M., and Doherty, P. Measuring the diaspora for virus-specific CD8+ T cells. *Proc. Natl. Acad. Sci. USA*, *98*: 6313–6318, 2001.
 18. Masopust, D., Vezy, V., Marzo, A. L., and Lefrancois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science (Wash. DC)*, *291*: 2413–2416, 2001.
 19. Geminder, H., Sagi-Assif, O., Goldberg, L., Meshel, T., Rechavi, G., Witz, I. P., Ben-Baruch, A. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J. Immunol.*, *167*: 4747–4757, 2001.
 20. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature (Lond.)*, *410*: 50–56, 2001.
 21. Payne, A., and Cornelius, L. The role of chemokines in melanoma tumor growth and metastasis. *J. Investig. Dermatol.*, *118*: 915–922, 2002.
 22. Rabin, R. L., Park, M. K., Liao, F., Swofford, R., Stephany, D., and Farber, J. M. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J. Immunol.*, *162*: 3840–3850, 1999.
 23. Ding, Z., Issekutz, T. B., Downey, G. P., and Waddell, T. K. α -Selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration. *Blood*, *101*: 4245–4252, 2003.