CD8⁺ T cells Reactive to Survivin Antigen in Patients with Multiple Myeloma

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Abstract
Purpose: Survivin is a member of the inhibitors of apoptosis family and is overexpressed in different types of malignancies. Cytotoxic T cells recognizing survivin epitopes can be elicited in vitro and by vaccination in patients with leukemia, breast cancer, and melanoma. We did this study to investigate whether survivin-specific CD8⁺ T cells occur in patients with multiple myeloma.

Experimental Design: An HLA-A2.1-binding survivin peptide was used to detect peptide-specific T cells by a quantitative real-time PCR to measure antigen-specific IFN-γ mRNA expression in 23 patients with myeloma and 21 healthy volunteers. T cells producing IFN-γ in response to survivin were further analyzed for expression of CD45RA and CCR7 to determine phenotypic characterization. Additional immunohistochemical analyses of survivin antigen expression in bone marrow specimens of patients was done.

Results: T cells recognizing HLA-A2.1-binding survivin peptide were detected in 9 of 23 patients and in 1 of 21 healthy volunteers. Survivin-reactive T cells were identified as terminally differentiated effector T cells (CD8⁺, CD45RA⁺, and CCR7⁻). Positive survivin expression of myeloma cells in bone marrow specimens was shown in 7 of 11 patients.

Conclusion: We provide, for the first time, evidence of T cell reactivity against survivin antigen in patients with multiple myeloma. Our data suggest the immunogenicity of survivin antigen in multiple myeloma and that immunotherapeutic strategies using survivin as a target antigen might be an option for patients with this disease.

Multiple myeloma represents a malignant proliferation of plasma cells derived from a single clone characterized by secreting monoclonal immunoglobulins with a concomitant decrease in normal immunoglobulins and lytic bone lesions (1). It represents the second most frequent hematologic malignancy in the U.S. and accounts for 10% of all such diseases. Despite the considerable progress in understanding its biology and therapy, the disease has essentially remained incurable. The current standard of treatment consists of high-dose myeloablative chemotherapy followed by autologous peripheral stem cell transplantation (PBSCT), and has significantly improved survival of patients compared with conventional chemotherapy (1). However, molecular remission is rare and most of the patients relapse due to minimal residual disease. Allogeneic transplantation and donor lymphocyte infusion offer a curative potential due to the graft-versus-myeloma effect exerted by T lymphocytes, but are associated with a high transplant-related mortality (2). Recent efforts to improve the safety of transplant procedures with reduced intensity or a nonmyeloablative conditioning regimen have shown encouraging results, but longer follow-up is needed to determine the role of this treatment modality (3).

Because current treatment strategies are not expected to offer a cure or long-term survival without considerable toxicity, the development of new therapeutic strategies is required. Recently, several studies have focused on the identification of T-cell target epitopes that are expressed on myeloma cells (4) and on the development of immunotherapeutic strategies (e.g., vaccination and generation of antigen-specific cytotoxic T cells, CTL) to augment the patients’ immune response to eliminate neoplastic cells (5–7).

Survivin, a member of the inhibitor of apoptosis gene family, has recently been suggested as a promising target antigen for immunotherapeutic approaches in different malignancies (8–11) and has not yet been examined in multiple myeloma. It is present during normal fetal development but is undetectable in most terminally differentiated adult tissues except thymus cells, CD34⁺ bone marrow–derived hematopoietic progenitor cells, basal colonic epithelial cells, and activated endothelial cells.

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cells (12). Functionally, survivin is involved in the control of apoptosis (by inhibition of caspase activity) and the regulation of cell division (11). Survivin is highly expressed in most human cancer cells of epithelial and hematopoietic origin, and overexpression is associated with cancer progression, poor prognosis, resistance, and short patient survival (8, 9, 13, 14). Several survivin epitopes could be identified recently, capable of inducing specific cytotoxic T-cell responses in patients with leukemia, breast cancer, and melanoma (15–18). Besides cellular reactivity, the existence of antibodies recognizing survivin antigen has been shown in lung and colorectal cancer (19, 20). A clinical vaccination study using survivin peptide–pulsed dendritic cells has been recently done in melanoma and did not show major toxicities (e.g., autoimmune reaction) after immunization (21).

Recently, it has been shown that survivin seems to play a critical role in the survival and proliferation of human myeloma cells (22, 23) and that in vitro–induced survivin–specific cytotoxic T cells from healthy individuals have the ability to lyse multiple myeloma cell lines in vitro (16). The fact that enhanced expression of survivin is almost completely restricted to malignant tissues, and that manipulating the survivin-mediated antiapoptotic pathway seems to impair tumor cell proliferation (24–27), makes survivin an interesting target molecule for immunotherapeutic strategies.

In this study, we investigated whether CD8\(^+\) T-cell responses against survivin epitopes occur in patients with multiple myeloma. We detected CD8\(^+\) T cells recognizing a recently described HLA-A2.1–binding peptide in a significant proportion of patients with multiple myeloma and identified them as terminally differentiated effector T cells by analyzing phenotypic expression of CD45RA and CCR7. Additional immunohistochemical staining of bone marrow specimens of patients confirmed survivin expression in neoplastic cells in most of the patients analyzed. In conclusion, we show for the first time that terminally differentiated effector T cells recognizing survivin antigen circulate in patients with multiple myeloma providing evidence for the potential use of survivin as target molecule for immunotherapeutic approaches in multiple myeloma.

**Materials and Methods**

**Patients and healthy controls.** After informed consent, cells from HLA-A2–positive patients (which were enrolled in a clinical investigation approved by the institutional ethics committee) with multiple myeloma (n = 23) and from healthy individuals (n = 21) were obtained from the peripheral blood. One of the patients (patient 4) had an additional history of ovarian cancer, which was in complete remission at the time of analysis. Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque gradient density (PAA Laborataries, Coelbe, Germany) and subsequently frozen in RPMI 1640 complete medium (25 mmol/L HEPES buffer, 2 mmol/L l-glutamine, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin; Life Technologies, Karlsruhe, Germany) containing 20% heat-inactivated FCS and 10% DMSO according to the standard protocols. Before use, cells were thawed, washed, and resuspended in complete medium supplemented with 10% human AB serum and rested overnight.

**HLA typing.** The expression of HLA-A2 in patients and healthy individuals was determined by using phycoerythrin-conjugated mouse anti-human monoclonal antibodies (BB7.2; Serotec, Düsseldorf, Germany). Data acquisition was done on FACSCalibur and analyzed using CellQuest software (Becton Dickinson, Heidelberg, Germany).

**Cell lines.** C1R-A2 cells are a MHC class I–defective LCL cell line that expresses a transfected genomic clone of HLA-A2.A2 (28). The cells were maintained in complete medium supplemented with 10% FCS and used as antigen-presenting cells.

**Peptide synthesis.** The following peptides were used in this study: survivin\(_{95-104}\) (ELTLGEFLKL; ref. 15), gp100\(_{209-217}\) (2M; IMDQVPFSV), MUC1\(_{45-25\text{7}}\) (LLLLTVLTIY), and MUC1\(_{550-558}\) (STAPPVHNV; ref. 29). The peptides were synthesized by Proimmune (Oxford, United Kingdom) to a minimum of 95% purity as measured by high performance liquid chromatography, dissolved in DMSO at a concentration of 5 mg/ml, further diluted in PBS and stored at \(-20^\circ\text{C}\).

**T cell in vitro stimulation.** To determine peptide-specific CD8\(^+\) T-cell reactivity, we measured the IFN-\(\gamma\) mRNA expression by CD8\(^+\) T cells stimulated with candidate peptides. T cells were analyzed without in vitro expansion. Multiple experiments to optimize assay conditions were done previously (30–32). Cryoconserved or fresh PBMC (1 \( \times \) \( 10^6\)) were plated in a 96-well flat-bottomed plate in 200 \( \mu \)l of complete medium supplemented with 10% human serum and incubated overnight at 37\(^\circ\text{C}\) and 5% CO\(_2\) to minimize background expression of IFN-\(\gamma\) mRNA expression due to lymphocyte manipulation. PBMC were then stimulated in vitro with peptides using an adapted protocol from previous studies (33).Briefly, C1R-A2 cells (as APC) were washed thrice in serum-free complete medium and incubated with the test peptide at 10 \( \mu \)g/ml in complete medium at 37\(^\circ\text{C}\) and 5% CO\(_2\) for 2 h. The peptide-loaded cells were then irradiated with 7,500 cGy, washed once, suspended in complete medium containing human serum, and added to the isolated PBMC at a 1:1 ratio. As controls, PBMC were either incubated with unloaded C1R-A2 cells (negative control), or with C1R-A2 cells and 5 \( \mu \)g/ml of staphylococcus enterotxin B (Sigma-Aldrich, Munich, Germany; positive control). After 3 h of coincubation at 37\(^\circ\text{C}\) and 5% CO\(_2\), cells were harvested for RNA isolation and CDNA synthesis. As additional negative controls, PBMC were incubated with C1R-A2 cells pulsed with gp100 (209-2M) as irrelevant peptide.

**RNA extraction and cDNA synthesis.** Total RNA was isolated from test samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at \(-80^\circ\text{C}\). For cDNA synthesis, 1 \( \mu \)g of total RNA was reverse-transcribed into DNA with the Reverse Transcription System cDNA Kit (Promega, Mannheim, Germany) and stored at \(-20^\circ\text{C}\).

**Quantitative real-time PCR.** Measurement of IFN-\(\gamma\) mRNA gene expression was done using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer, Foster City, CA) as previously described (34, 35). The feasibility of this approach for the analysis of antigen-specific T-cell responses both in peripheral blood lymphocytes and in tumor tissues has been previously validated (36). Primers for IFN-\(\gamma\), CD8, and TaqMan Probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon-exon junctions to prevent transcription of genomic DNA. To create a standard curve, the cDNA was generated by reverse transcription using the same technique used for the preparation of test cDNA. IFN-\(\gamma\) and CD8 cDNA was amplified by PCR using the same primers designed for the real-time PCR, purified and quantified by UV spectrophotometry. The number of cDNA copies was calculated using the molecular weight of each gene amplicon. Serial dilutions of the amplified gene at known concentrations were tested by real-time PCR. Quantitative real-time PCR reactions of cDNA specimens, CDNA standards, and water as negative control were conducted in a total volume of 25 \( \mu \)l with TaqMan Master mix (Perkin-Elmer), 400 nmol/L primers, and 200 nmol/L probe. Primer sequences were as follows: IFN-\(\gamma\) (forward) 5’-AGCTCTGATCATGTTTGGGTG-3’; IFN-\(\gamma\) (reverse) 5’-GTTCCATTATCGCCATCATCGAA-3’; IFN-\(\gamma\) (probe) FAM-TCTTGCCTGTTACTGCGAGCACCA-TAMRA; CD8 (forward) 5’-CCCTGAGCAACTCCATCATG-3’; CD8 (reverse) 5’-GGCTGCTGGCTGCCGTAAGG-3’; and CD8 (probe) FAM-TCAGCCACTCGTTGCCGCT-TAMRA. The thermal cycler variables were 2 min at 50\(^\circ\text{C}\), 15 s involving denaturation and 60\(^\circ\text{C}\) for 1 min. Standard curve extrapolation of copy number was done for both IFN-\(\gamma\) and CD8. The calculated number of copies of IFN-\(\gamma\) mRNA in each
sample was normalized to the number of copies of CD8 mRNA by dividing the number of copies of IFN-γ transcripts by the number of copies of CD8 transcripts. All PCR assays were done in duplicate and reported as the mean. A 2-fold difference in gene expression was found to be within the discrimination ability of the assay.

Flow cytometric analysis. PBMC were stimulated with peptide-loaded APC and stained for intracellular IFN-γ production using BD Cytofix/Cytoperm Plus Kit (BD, Becton Dickinson) according to the manufacturer’s instructions. Briefly, C1R-A2 cells were washed thrice in serum-free complete medium and incubated with test peptide at 10 μg/ml for 2 h in complete medium at 37°C and 5% CO2. C1R-A2 cells were then washed once, irradiated (7,500 cGy) and suspended with 1 × 10^6 PBMC at a 1:1 ratio in complete medium containing Golgi Stop. Cells were co-incubated for 6 h at 37°C and 5% CO2. Unpulsed C1R-A2 cells and C1R-A2 cells pulsed with gp100 peptide (irrelevant peptide) were used as negative controls. Positive controls were done by stimulating PBMC with 5 μg/ml of staphylococcus enterotoxin B (Sigma-Aldrich). T cells were then stained by incubation with monoclonal antibodies (BD) conjugated with allophycocyanin (CD3), and respectively, phycoerythrin or peridinin chlorophyll protein (PE or PerCP, CD8). The intracellular staining for IFN-γ was done after fixation and permeabilization by using fluoresceinisothiocyanate (FITC) and PE-conjugated monoclonal antibodies (BD), respectively. Data acquisition was done on FACSCalibur and was analyzed using CellQuest Software (BD).

For phenotypic characterization, PBMC were stimulated according to the protocol described above. CD8 T cells were stained by incubation with PerCP-conjugated monoclonal antibodies (BD), CD45RA expression was determined by using allophycocyanin-conjugated monoclonal antibodies (BD). IFN-γ (PE) and CCR7 (FITC) monoclonal antibodies (BD) were added to determine antigen expression after fixation and permeabilization.

Immunohistochemistry. For the determination of survivin expression in multiple myeloma cells, 4-μm sections of routinely processed, EDTA-decalcified and paraffin-embedded bone marrow trephine biopsies were stained using standard procedures. Briefly, immunohistochemical studies used an avidin-biotin peroxidase method with diaminobenzidine chromatogen. After heat-induced antigen retrieval (microwave oven for 30 min at 250 W), immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ). Antibodies were applied to identify survivin (mouse monoclonal, clone 6E4, final dilution 1:200; Cell Signaling Technology, Beverly, MA), and the plasma cell marker CD138 (mouse monoclonal, clone MI15, final dilution 1:100; DAKO, Hamburg, Germany), which served to identify the extent and pattern of bone marrow infiltration. The dilutions had been established using adequate controls. Negative controls were obtained by omitting the primary antibodies. To quantify the immunohistochemical expression of survivin, a scoring system similar to that of Lu et al. was used (36). The mean percentage of survivin-positive cells was estimated as ≤5% (0), 5% to 25% (1), 25% to 50% (2), 50% to 75% (3), and >75% (4). Staining intensity was classified as negative (0), weak (1), moderate (2), or intense (3). In order to identify neoplastic cells for evaluation of survivin expression, serial sections were stained with H&E and CD138. Only bone marrow trephines with an infiltration of plasma cells of >10% were evaluated. The percentage of positive tumor cells and staining intensity were multiplied to produce a final weighted score for each case. Cases with a weighted score of ≤1 were defined as negative, all others were defined as positive.

Statistical analysis. To determine specific response to stimulation, mRNA for IFN-γ from PBMC stimulated with test peptide versus unpulsed APC (C1R-A2, background) was detected by quantitative PCR. The IFN-γ mRNA copy number was first corrected for CD8 mRNA. A cutoff value of 2.0 for the ratio of IFN-γ mRNA obtained from CD8+ T cells stimulated with relevant test peptides to that obtained from PBMC stimulated with unpulsed APC was considered to be evidence of epitope specificity. The cutoff value was derived by analyzing IFN-γ mRNA transcripts detectable in PBMC both from healthy donors and patients stimulated with gp100 (209-2M; irrelevant peptide) to background. Analyses of these PBMC identified a mean ratio of 1.0 (range, 0.95-1.04) with 95% and 99% confidence intervals of 1.0 ± 0.08 and 1.0 ± 1.11, respectively, a SE of 0.04, and a SD of 0.2. The cutoff ratio (stimulation index, SI) was estimated by adding the mean to three SDs, which was equivalent to 1.6. To minimize the possibility of falsely considering CD8 T cells immunoreactive, we accepted a 2-fold increase in stimulated/unstimulated IFN-γ transcript ratio as evidence of epitope-specific reactivity. Wilcoxon’s rank sum test was calculated to determine whether there was a statistically significant difference in T-cell response to test peptides between normal healthy individuals and patients. Statistical significance was achieved at P < 0.05.

Results

Survivin-specific T cells can be detected in patients with multiple myeloma. To determine whether CD8 T cells circulate in patients with multiple myeloma and normal healthy individuals, we analyzed T cells obtained directly from the peripheral blood of individuals without in vitro expansion. IFN-γ mRNA production in peptide-stimulated T cells was determined using quantitative PCR. We analyzed 23 patients with multiple myeloma (16 males, 7 females; median age, 62 years) and 21 normal healthy individuals. Most of the patients were analyzed in advanced disease (stage III, n = 18), the latter in stage II (n = 1) and stage I (n = 4). At the time of analysis, 8 patients showed a progressive disease and 15 patients a stable disease based on classical staging criteria. Clinical data are shown in Table 1. All analyzed individuals were positive for HLA-A2.1 allele. For positive controls, T cells were stimulated with staphylococcus enterotoxin B, negative controls were done using irrelevant peptide (gp100). A positive response was defined as ≥100 IFN-γ mRNA copies/10^4 CD8 copies and a SI of ≥2, where SI = IFN-γ mRNA copies/10^4 CD8 copies in peptide-pulsed C1R-A2 cell cultures/unpulsed cultures. Analyzing T cell reactivity of individuals at least at two different time points showed a high degree of reproducibility. Survivin-specific T-cell responses were detected in 1 of 21 (4.7%) healthy individuals (SI 7; Fig. 1; Table 2). In contrast, 9 of 23 (39.1%) patients had a positive response to survivin antigen with a SI range from 2.9 to 83 (P = 0.0026). The mean SI in the patients was 8.61 and 1.32 in normal individuals. None of the patients or healthy individuals had a positive response to gp100 (Table 2). One of the patients having a T-cell response to survivin antigen (patient 4) had an additional history of ovarian cancer in complete remission at the time of analysis. Five of nine (56%) patients having a positive T-cell response to survivin antigen showed progressive disease and four of nine (44%) patients showed stable disease. In contrast, patients without T-cell response to survivin antigen showed progressive disease in 3 of 14 patients (21%) and stable disease in 11 of 14 (79%) patients.

We additionally analyzed the T-cell reactivity to mucin 1 (MUC1), a glycosylated type I transmembrane glycoprotein that has recently been identified as a tumor-associated antigen on most myeloma cell lines, and that has been proposed as a candidate for peptide vaccination (29, 37). Neither patients nor healthy individuals showed a positive response to MUC1 peptides (Table 2). To confirm the specificity of survivin-reactive T cells detected by quantitative PCR, intracellular detection of IFN-γ by flow cytometry was done. Peptide
specificity determined by quantitative PCR was previously confirmed by independent studies (including our group) using intracellular cytokine assays showing strong correlation between both assays (31, 38, 39). In multiple experiments optimizing quantitative PCR assay conditions in our study, we compared IFN-γ mRNA expression following cytomegalovirus pp65495-503 peptide stimulation (in cytomegalovirus-positive individuals) with intracellular IFN-γ production confirming strong correlation ($R^2 = 0.97$; data not shown). A response was considered positive in intracellular cytokine assays if the percentage of IFN-γ–producing T cells (CD3+, CD8+ gated) cultured with peptide-pulsed APC was 2-fold or more higher compared with T cells cultured with unpulsed APC. Data from intracellular cytokine assays and corresponding data from quantitative PCR of three patients [patients 4 (A), 8 (B), and 15 (C)] with a positive response to survivin antigen are shown in Supplementary Fig. S1 and S2. IFN-γ production of unpulsed APC was detected in 0.07%, 1.87%, and 0.05% of CD3+, CD8+ gated cells (patients 4, 8, and 15), whereas a specific IFN-γ production of peptide-pulsed APC was observed in 0.98%, 7.56%, and 0.46%, respectively. IFN-γ production of gp100-peptide (negative control) was detected in 0.1%, 1.8% and 0.03% of CD3+, CD8+ gated cells, respectively.

Survivin-reactive T cells can be identified as terminally differentiated effector T-cells (CD8+ CD45RA+, CCR7−). To further characterize survivin-reactive T cells, we determined the expression of CD45RA and CCR7 of specifically IFN-γ–producing CD8+ T cells. Figure 2 displays representative data from two patients [patients 4 (A) and 15 (B)] demonstrating that most of the T cells that specifically produced IFN-γ after survivin peptide stimulation had the phenotype CD8+, CD45RA+, CCR7−. T cells with this functional phenotype were recently described as terminally differentiated effector T cells exerting direct lytic activity (40, 41).

Survivin expression in myeloma cells from patients after bone marrow trephine biopsies. In order to determine survivin expression in myeloma cells, we analyzed formalin-fixed paraffin-embedded bone marrow trephine biopsies from patients with myeloma. Biopsies from 13 of 23 patients were

### Table 1. Patients and clinical properties

| Patient no. | Sex | Age (y) | Stage* | Treatment | Years after diagnosis |
|-------------|-----|---------|--------|-----------|-----------------------|
| 1           | M   | 66      | IIIa, P| aHSCT     | 0                     |
| 2           | F   | 52      | IIIa, P| ID        | 0                     |
| 3           | F   | 66      | IIIa, P| aHSCT     | 4                     |
| 4’          | F   | 66      | Ia, S  | None      | 3                     |
| 5           | M   | 68      | Ia, S  | aHSCT, IFN| 3                     |
| 6           | M   | 68      | Ia, S  | None      | 10                    |
| 7           | M   | 64      | IIb, P | CAD       | 0                     |
| 8           | M   | 64      | IIIa, P| aHSCT, IFN, TCD | 3         |
| 9           | F   | 54      | IIIa, P| aHSCT, TCD| 3                     |
| 10          | M   | 62      | IIIa, S| CAD       | 0                     |
| 11          | M   | 64      | Ia, S  | None      | 0                     |
| 12          | M   | 68      | IIIa, S| aHSCT, IFN, TCD| 2         |
| 13          | M   | 69      | IIIa, S| aHSCT, IRD, CAD, TCD| 7         |
| 14          | M   | 56      | IIb, S | aHSCT, IFN| 1                     |
| 15          | M   | 67      | IIa, P | aHSCT     | 0                     |
| 16          | F   | 54      | Ia, S  | 0         | 4                     |
| 17          | M   | 61      | IIIa, P| aHSCT     | 0                     |
| 18          | M   | 58      | IIb, S | ID        | 0                     |
| 19          | F   | 65      | IIIa, S| aHSCT     | 0                     |
| 20          | M   | 61      | IIIa, S| VAD, IEV  | 0                     |
| 21          | M   | 57      | IIIa, S| ID        | 6                     |
| 22          | M   | 62      | IIIa, S| aHSCT     | 0                     |
| 23          | M   | 70      | IIIa, S| aHSCT, IFN| 3                     |

**Abbreviations:** None, not on active treatment at the time of analysis; aHSCT, autologous stem cell transplantation; TCD, thalidomide/clarithromycin/dexamethasone; VAD, vincristine/doxorubicin/dexamethasone; IEV, ifosfamide/epirubicin/etoposide; CAD, cyclophosphamide/adriamycin/dacarbazine; ID, idarubicin/dexamethasone; IFN, IFN-γ; IRD, idarubicin, ribosumin, and dexamethasone.

*Salmon/Durie stage at time of analysis (P, progressive; S, stable, based on classical staging criteria).

1 Patient with multiple myeloma and additional history of ovarian cancer in complete remission at the time of analysis.
available for analysis. Diagnosis was based on standard clinical and histopathologic criteria. It was possible to assess 11 of 13 bone marrow trephines for the immunohistochemical expression of survivin (Table 3). Two biopsies showed a plasma cell infiltrate of ≤10% for all cells and were therefore omitted. The remaining trephines showed a clearly recognizable infiltration by neoplastic plasma cells (mean, 61%; range, 15-90%) as proven by conventional H&E staining and additional immunohistochemical detection by CD138. Based on the weighted scores, survivin expression in myeloma cells was detected in 7 of 11 (63.6%) cases of multiple myeloma (survivin expression in myeloma cells of patient 9; Fig. 3). Survivin signals were predominantly localized in the nucleus. In two of the seven patients (patients 9 and 15), a survivin-specific T-cell response was detected in the peripheral blood (Tables 2 and 3). In one patient having a survivin-specific T-cell response, the bone marrow biopsy showed a plasma cell infiltrate of ≤10% and had to be omitted. Analyzing peritumoral T-cell infiltration in bone marrow trephines from patients 9 and 15 by immunostaining of CD3+ cells showed a T-cell infiltration of 5% to 10% (patient 9) and 10% to 30% (patient 15), respectively (data not shown). Three of seven (42%) patients with survivin expression in myeloma cells showed progressive disease and four of seven (58%) patients showed stable disease.

Analyzing patients with no detectable survivin expression in myeloma cells showed progressive disease in one of four individuals (25%) and stable disease in three of four (75%) individuals.

**Discussion**

Survivin is a member of the inhibitor of apoptosis gene family that has recently been suggested as a promising target antigen in different malignancies (8, 9, 11, 42, 43). Both spontaneous specific T-cell reactivity and antibody response to survivin have been recently detected in patients with leukemia, melanoma, breast, lung, and colorectal cancer (15–20). It has been shown that survivin plays a critical role in the survival and proliferation of human myeloma cells (22, 23), and that in vitro – induced survivin-specific cytotoxic T cells from healthy individuals have the ability to lyse multiple myeloma cell lines (16). To our knowledge, no other groups have thus far studied the T cell–mediated immunity to survivin in patients with myeloma. This study provides, for the first time, direct evidence that CD8+ T cells recognizing survivin antigen circulate in patients with multiple myeloma. Using quantitative PCR, we found that CD8+ T cells reactive to a recently described HLA-A2–restricted survivin peptide are present in 39% of the patients and in only 4.7% of the healthy controls, suggesting that specific T-cell responses were induced in response to myeloma cells. Our data are in concordance with a recent study in which spontaneous T-cell reactivities to survivin were almost completely restricted to patients with cancer (18). To investigate whether there is a relationship between survivin-specific immune response and active disease, we analyzed the clinical course of patients and found that those with a T-cell response to survivin antigen more often showed a progressive than stable disease (56% versus 44%) compared with patients without a T-cell response who, for the most part, showed stable disease (79%). Although data are not significant and further studies analyzing a larger number of patients are required, it is suggested that T-cell reactivity is elicited in response to tumor cells (to immunologically counteract tumor progression) which could be due to increased antigen expression by tumor cells or to increased antigen presentation by antigen-presenting cells in the bone marrow. The analysis of two patients having survivin expression in myeloma cells and T-cell response to survivin antigen in the peripheral blood showed T-cell infiltration

### Table 2. CD8+ T cell reactivities to peptides in patients and healthy individuals

| Patients| Survivin | gp100 | MUC1a | MUC1b | SEB |
|---------|----------|-------|-------|-------|-----|
|         | SI*      |       |       |       |     |
| 1       | 0.7      | 0.4   | 0.9   | 0.4   | 134 |
| 2       | 1        | 1     | 1.1   | 0.9   | 99  |
| 3       | 4.1      | 0.9   | 1.1   | 1     | 138 |
| 4       | 63       | 0.9   | 1.7   | 1.2   | 117 |
| 5       | 0.9      | 1.1   | 0.9   | 0.9   | 139 |
| 6       | 0.7      | 0.9   | 1     | 0.7   | 156 |
| 7       | 5.8      | 0.9   | 1.1   | 1.1   | 126 |
| 8       | 83       | 1.1   | 1.2   | 1.8   | 128 |
| 9       | 9.5      | 1.3   | 1.3   | 1.3   | 130 |
| 10      | 1        | 0.8   | 1     | 0.8   | 138 |
| 11      | 2.9      | 0.8   | 0.9   | 0.7   | 128 |
| 12      | 1.1      | 0.9   | 1     | 0.9   | 145 |
| 13      | 0.6      | 0.9   | 0.8   | 0.9   | 150 |
| 14      | 1.5      | 1     | 1     | 0.9   | 137 |
| 15      | 3.8      | 0.9   | 0.9   | 1     | 120 |
| 16      | 1.1      | 0.7   | 0.9   | 0.9   | 142 |
| 17      | 5.5      | 1     | 0.9   | 1     | 134 |
| 18      | 1.4      | 0.7   | 0.8   | 0.7   | 115 |
| 19      | 0.9      | 0.9   | 0.8   | 0.9   | 124 |
| 20      | 7        | 1.2   | 0.9   | 1     | 130 |
| 21      | 0.9      | 1.1   | 0.8   | 0.9   | 114 |
| 22      | 0.7      | 1.1   | 1     | 0.9   | 120 |
| 23      | 1.1      | 1.3   | 1.1   | 1.2   | 136 |

Healthy individuals

|         | SI*      |       |       |       |     |
|---------|----------|-------|-------|-------|-----|
| 1       | 1.3      | 1.1   | 0.7   | 0.7   | 124 |
| 2       | 1.1      | 0.7   | 1.3   | 1.2   | 135 |
| 3       | 0.7      | 0.6   | 0.6   | 0.3   | 150 |
| 4       | 1        | 1     | 0.8   | 1.5   | 118 |
| 5       | 1.1      | 1.5   | 1.2   | 1     | 150 |
| 6       | 1.1      | 1.8   | 0.9   | 0.7   | 128 |
| 7       | 1.1      | 1.1   | 1.2   | 1.1   | 145 |
| 8       | 1        | 1.1   | 1.4   | 1.2   | 161 |
| 9       | 1.2      | 0.7   | 1.2   | 0.7   | 110 |
| 10      | 1.5      | 1.3   | 1.5   | 0.8   | 116 |
| 11      | 0.6      | 0.5   | 0.9   | 0.6   | 134 |
| 12      | 0.8      | 0.8   | 0.8   | 0.7   | 119 |
| 13      | 1.5      | 1.3   | 1.8   | 1.5   | 131 |
| 14      | 1        | 1     | 1     | 1     | 119 |
| 15      | 1.2      | 1.3   | 1.2   | 1.5   | 137 |
| 16      | 0.8      | 0.7   | 0.8   | 1     | 111 |
| 17      | 0.9      | 0.9   | 0.9   | 0.7   | 135 |
| 18      | 0.6      | 0.9   | 1.5   | 1.3   | 116 |
| 19      | 1.6      | 1.7   | 1.2   | 1.5   | 126 |
| 20      | 1.1      | 1.3   | 1.2   | 1.1   | 116 |
| 21      | 0.7      | 0.7   | 0.8   | 0.9   | 136 |

*SI determined by the ratio of IFN-γ mRNA copy number obtained from PBMC stimulated with relevant test peptides to that obtained from CD8+ T-cells stimulated with unpulsed antigen-presenting cells. A cutoff value of 2.0 was considered to be evidence of epitope specificity. SI displayed in boldface represent positive reactivities.

*Staphylococcus enterotoxin B used as positive control.

*Corresponding numbers of patients/healthy individuals were maintained throughout this article.
(up to 30%) in bone marrow areas affected by myeloma cells, which supports the hypothesis that specific T-cell stimulation occurs at the location of the tumor. Survivin is highly expressed in most human cancer cells of epithelial and hematopoietic origin and overexpression is associated with cancer progression, poor prognosis, resistance, and short patient survival (8, 9, 13, 14). It is supposed that the overexpression of survivin in the cytoplasm of tumor cells and rapid degradation by the proteasome-related mechanism results in an increased expression of survivin-derived epitopes on the surface of tumor cells leading to the induction of a specific T-cell response.

The cytolytic abilities of survivin-reactive T-cells detected by measuring specific IFN-γ production cannot directly be assessed \textit{ex vivo}. To provide more insight into that question, we characterized the differentiation phenotype of survivin-reactive CD8⁺ T-cells. Survivin-reactive CD8⁺ T-cells displayed the phenotype CD45RA⁺, CCR7⁻, corresponding to terminally differentiated effector T-cells. It has recently been shown that these cells have the highest cytotoxic potential and directly mediate tumor cytotoxicity (40, 41). However, long-term follow-up analysis to determine the effect of survivin-specific T-cell response on the course of disease is needed.

Because survivin-specific T-cell responses were mostly restricted to the patients, we did immunohistochemical analyses of patients' bone marrow biopsies to determine the survivin expression in tumor cells. Potential immunotherapeutic

![Flow cytometric analysis of the differentiation phenotype of CD8⁺ T cells specifically producing IFN-γ in response to survivin in patients with multiple myeloma.](Fig. 2)
was found in tumor cells from in accordance with recent studies in which survivin expression myeloma cells in almost 64% of the patients analyzed. This is expression in tumor cells. Survivin expression was detected in strategies using survivin as a target antigen requires its bone marrow–derived hematopoietic progenitor cells, colonic

**Table 3. Immunohistochemical analysis of survivin expression in bone marrow trephines of patients with multiple myeloma**

| Patient no.* | Plasma cell infiltration (%) | Mean percentage | Staining intensity | Final scoring |
|--------------|-----------------------------|-----------------|-------------------|--------------|
| 2            | 20                          | 1               | 3                 | 3            |
| 5            | 40                          | 0               | 1                 | 1            |
| 6            | 15                          | 3               | 2                 | 6            |
| 9*           | 70                          | 2               | 3                 | 6            |
| 10           | 90                          | 1               | 3                 | 3            |
| 11*          | 5                           | n.a.            | n.a.              | n.a.         |
| 12           | 10                          | n.a.            | n.a.              | n.a.         |
| 13           | 80                          | 3               | 1                 | 3            |
| 14           | 90                          | 0               | 0                 | 0            |
| 15*          | 90                          | 1               | 3                 | 3            |
| 18           | 60                          | 0               | 0                 | 0            |
| 21           | 90                          | 0               | 1                 | 1            |
| 22           | 30                          | 3               | 3                 | 9            |

Abbreviation: n.a., not analyzed due to low plasma cell infiltration (<10%).

* Corresponding numbers of patients and healthy individuals were maintained throughout this article.

† Mean percentage of survivin expressing plasma cells estimated as <5% (0), 5% to 25% (1), 25% to 50% (2), 50% to 75% (3), and >75% (4).

‡ Intensity of immunohistochemical survivin staining classified as negative (0), weak (1), moderate (2), or intense (3).

§ The percentage of survivin expressing plasma cells and staining intensity were multiplied to produce a final weighted score for each case. Cases with weighted score ≤1 were defined as negative, all others were defined as positive.

* Patients with a survivin-specific T-cell response.

epithelial cells, and activated epithelial cells (12). One might raise the hypothesis that autoimmune reactions will be induced after performing immunotherapy (either adoptive or by vaccination) using survivin as a target antigen. The patients in our study, having a specific T-cell response to survivin, did not show clinical signs of gastrointestinal disease or vascular involvement. Some of the patients had decreased levels of hemoglobin and/or white blood count, but due to the biology of multiple myeloma affecting hematopoiesis and due to potential therapy effects, it might be difficult to distinguish between the effects caused by survivin-reactive T-cells or by disease respectively therapy. However, a vaccination study that was recently done in patients with melanoma in which survivin-specific T-cells could be induced by survivin peptide–pulsed dendritic cells did not show any clinical or histochemical changes (21). We additionally tested T cell reactivity to MUC1, a glycosylated type I transmembrane glycoprotein that has been identified as a tumor-associated antigen on most myeloma cell lines and that has been proposed as a candidate antigen for vaccination treatments (29). In contrast to recent studies in which T cells recognizing MUC1-derived peptide epitopes have been found in the peripheral blood of healthy individuals (29, 37) or in the peripheral blood and/or bone marrow of patients with myeloma (47, 48), we could not detect any response in our patients or healthy individuals. In the studies mentioned, MUC1-specific T-cells were mainly detected after in vitro T-cell expansion, suggesting that the MUC1-specific T-cells circulate in very low frequencies. We used a highly sensitive assay to detect spontaneous antigen-specific T-cells; however, in the individuals tested, MUC1-specific T-cells might be circulating in frequencies below the detection limit of the assay or might even be absent. Further studies using in vitro T-cell expansion or analyzing patients’ bone marrow could be helpful to clarify the existence of MUC1-specific T-cells in the patients analyzed.

![Fig. 3. Immunohistochemical analysis of survivin expression in the bone marrow of a patient with multiple myeloma. Immunohistochemical staining of bone marrow was done using an avidin–biotin peroxidase method with diaminobenzidine chromagen. To quantify the immunohistochemical expression of survivin, a scoring system similar to that of Lu et al. was used (36). The mean percentage of survivin-positive cells was estimated as ≤5%, 5% to 25%, 25% to 50%, 50% to 75%, and >75%. Staining intensity was classified as negative, weak, moderate, or intense. In order to identify neoplastic cells for evaluation of survivin expression, serial sections were stained with H&E and CD138. Only bone marrow trephines with an infiltration of plasma cells of >10% were evaluated. Survivin expression in 25% to 50% of myeloma cells with an intense staining pattern (brown; magnification, ×200).](www.aacjrournals.org)
In conclusion, we provide the first time, evidence for T-cell reactivity against survivin antigen in patients with multiple myeloma. Our data suggest the immunogenicity of survivin antigen in multiple myeloma and that survivin could serve as useful target antigen for T-cell–based immunotherapeutic strategies in the treatment of multiple myeloma. Because the bone marrow was recently identified as being important for tumor surveillance, containing a pool of antigen-specific T cells (49), and because multiple myeloma represents a disease that is mainly located in the bone marrow, we plan further investigations analyzing survivin-specific T-cell reactivities in patients’ bone marrows to get more information about the relevance of that compartment in controlling tumor cells.

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