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Generation of Survivin-specific CD8$^+$ T Effector Cells by Dendritic Cells Pulsed with Protein or Selected Peptides[1]

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ABSTRACT

The identification of tumor-associated antigens recognized by CD8$^+$ cytotoxic T cells paved the way to new concepts in adjuvant anticancer therapy. However, the number of tumor-associated proteins found to be expressed in the majority of human cancers is still rather limited. Recently, the newly identified apoptosis inhibitor protein survivin has been recognized as a widely occurring tumor-associated protein. In the present study, we demonstrate that survivin is capable of inducing specific CD8$^+$ effector T cells *in vitro*. T cells from healthy donors were subjected to several cycles of stimulation by autologous dendritic cells (DCs) pulsed with soluble recombinant survivin protein. Activation of CD8$^+$ cytotoxic T cells by survivin-derived peptides cross-presented by DCs was demonstrated by lysis of autologous survivin-expressing B cell transfectants. Using a peptide-motif scoring system, two survivin peptides (ELTLGEFLKL and TLPPAWQPFL) were predicted and proved to bind to the HLA-A*0201 molecule. Both peptides were shown to induce CD8$^+$ effector T cells when presented on DCs; one peptide could be verified to result from natural intracellular processing of survivin. These findings recommend survivin as a new and widely applicable target for protein- and peptide-based immunotherapy of tumors.

INTRODUCTION

In recent years, immunotherapy of human tumors has gained much impetus by the finding that CD8$^+$ CTLs are capable of recognizing and destroying tumor cells that expose peptides that are derived from TAAs[4] and are bound to MHC class I molecules (1). In addition, clinical studies have shown that the adoptive transfer of tumor-specific CD8$^+$ CTLs can induce regression of established tumors in melanoma patients (2, 3). There is good evidence that also tumor-specific CD4$^+$ T cells are involved in tumor-directed immune reactions (4). Tumor-specific activation of T cells depends on adequate presentation of tumor-associated peptides. The most effective way to present antigenic peptides seems to be the use of DCs, which are known to be crucial for the initiation of primary T-cell responses (5, 6). DCs stand out by their capacity to present peptides derived from exogenous antigens both on MHC class II molecules and on MHC class I molecules, the latter being referred to as cross-presentation (7). In consequence, a number of experimental strategies to induce tumor-specific T-cell responses are based on the appropriate use of DCs. Animal models underline the potential of this approach (8–10). Vaccination of patients with cultured autologous human DCs pulsed with synthetic tumor peptides (11–13) or whole tumor proteins (14) often has been shown to result in partial or complete tumor regression.

The number of well characterized TAAs has steadily increased during the last years and has led to their classification into different groups (15, 16). One major group that seems particularly promising for immunotherapeutic approaches is represented by TAAs encoded by genes that are silent in most normal tissues, except testicular cells, yet are expressed in a variety of tumor types. Members of this group are the genes encoding MAGE (17), BAGE (18), GAGE (19), and RAGE (20). In addition, the telomerase catalytic subunit was identified as a widely expressed TAA, which can also serve as a target for tumor-specific cytotoxic T-cell responses (21). Recently, survivin, a structurally unique new member of the *IAP* gene family, has been described to be selectively expressed in fetal tissue and in tumor cells including carcinomas of the lung, colon, pancreas, prostate, breast, and stomach (22). This observation lately has been confirmed by analysis of human transcriptomes (23), where survivin was described as one of the most prominent tumor-associated transcripts.

In the present study, we investigated whether human CD8$^+$ T lymphocytes can be specifically activated against survivin *in vitro*. A major objective was to find out whether MHC class I-restricted immunogenic peptides derived from survivin can be predicted. Here, we demonstrate that monocyte-derived DCs pulsed with recombinant survivin protein efficiently induce antigen-specific CD8$^+$ CTLs. In addition, two predicted survivin-derived peptides proved to elicit a peptide-specific CTL response; one of the peptides is shown to result from natural intracellular processing of survivin.

MATERIALS AND METHODS

Cloning of Survivin cDNA. Total RNA was extracted from Jurkat cells using the guanidinium isothiocyanate method (22). Survivin cDNA of the coding region was generated by RT-PCR using the survivin reverse primer 5′GAGAGAAAGCTTGAGGCCTCAATCCATGGCAGCCAGCTGCTC-3′ and the survivin forward primer 5′-GAGAGAGGATCCATGGGTGC-CCCGACGTTG-3′ (both from Life Technologies, Inc., Karlsruhe, Germany), which contain *Hin*dIII and *Bam*HI restriction sites, respectively, to facilitate directional cloning. The reverse transcriptase reaction was performed with the first-strand cDNA synthesis kit (Clontech, Heidelberg, Germany) according to the manufacturer's advice. PCR amplification was carried out in the presence of 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.4 mM/dNTP, and 5 units of Taq DNA Polymerase (Amersham Pharmacia Biotech, Braunschweig, Germany) for 35 cycles in a Biometra Uno II thermocycler with denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. The generated PCR product was ligated into the corresponding restriction sites of pBluescript KS, and the correct sequence was verified by sequencing the cloned PCR product on both strands using the ALFexpress Auto Read sequencing kit (Amersham Pharmacia Biotech) with cyanine-labeled universal and reverse primers.

Overexpression and Purification of Recombinant His-tagged Survivin. Survivin cDNA was subcloned into the *Bam*HI and *Hin*dIII restriction sites of the procaryotic expression vector pQE30 (Qiagen, Hilden, Germany), which allows the expression of recombinant proteins with a NH_2-terminal 6× His-tag. The correct sequence was checked using the ALF-express Auto Read sequencing kit with cyanine-labeled dATP and pQE30-specific primers. Overexpression was performed in *Escherichia coli* M15[pREP4] (Qiagen), as recommended by the manufacturer. A control culture was grown under the same conditions without induction by isopropyl-1-thio-β-D-galactopyranoside.

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[4] The abbreviations used are: TAA, tumor-associated antigen; DC, dendritic cell; EGFP, enhanced green fluorescent protein; EBV-BLCL, EBV-B lymphoblastoid cell line; IL, interleukin; FACS, fluorescence-activated cell sorting.

Cells were harvested by centrifugation at 2000 × *g* and 4°C for 10 min and resuspended in 7.5 ml of lysis buffer [8 M urea, 100 mM Na_2HPO_4, 10 mM Tris (pH 7.8), 0,1% Triton X-100, and 25 mM imidazole]. After sonication, cellular debris were pelleted at 13,000 × *g* for 30 min. Purification was carried on by mixing the cleared lysate with 2 ml of the equilibrated Ni-NTA resin (Qiagen) on a rotator for 1 h, followed by washing four times with lysis buffer and eluting with the appropriate buffer containing 100 mM imidazole. The His-tagged survivin was refolded by stepwise dialyzing against RPMI 1640 (Biochrom, Berlin, Germany). The protein yield was determined by the Bradford protein assay (Bio-Rad Laboratories, Munich, Germany). The lysates and eluates from both the expression and control culture were analyzed by SDS-PAGE, followed by Coomassie Blue staining or Western blotting. Western blotting was performed according to standard protocols using anti-His antibody (mouse) as primary and alkaline phosphatase-conjugated antimouse IgG as secondary antibody.

Transient Transfection of EBV-transformed B Lymphocytes with Survivin. The coding region of survivin cDNA was amplified by PCR using the forward primer 5′-GAGAGAGAATTCACAACCATGGGTGCCCCGACG-TTGCCC-3′, the reverse primer 5′-GAGAGAGGATCCTCAATCCATG-GCAGCCAGGTGCTC-3′ (both from Genaxis Biotechnology, Spechbach, Germany), and pBsKS/survivin as template under the same conditions as described above. *Eco*RI and *Bam*HI sites in the primers allowed the directional cloning into pIRES2-EGFP (Clontech). This permits both survivin and EGFP to be synthesized from a single bicistronic mRNA.

B lymphocytes were transformed by supernatant of the EBV-producing cell line B95−8 [kindly provided by Dr. J. Endl (Roche, Penzberg, Germany)]. EBV-BLCLs were transfected with pIRES2-survivin/EGFP or pIRES2-EGFP by electroporation. Cell suspension (400 μl ; 5 × 10^6 cells/ml) was transfected with 50 μg of supercoiled plasmid DNA at 280 V/1050 μF (Easyject T Plus; Equibio, Kent, United Kingdom) and then cultivated in RPMI 1640 supplemented with 10% FCS (both from Biochrom). Eighteen to 24 h after transfection, cells expressing EGFP were selected by FACS and used as target cells.

Epitope Prediction. Epitope prediction was done as described (24). Briefly, potential HLA-A*0201 ligands from the sequence of survivin were selected using a matrix pattern suitable for the calculation of nonamer or decamer peptides fitting to the HLA-A*0201 motif. Such motif predictions are available on our web page.[5]

Peptides. Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy. After removal from the resin by treatment with trifluoroacetic acid/phenol/ethanedithiol/thioanisole/water (90:3.75:1.25:2.5:2.5 by vol) for 1 h or 3 h, arginine-containing peptides were precipitated from methyl-tert. butyl ether, washed once with methyl-tert. butyl ether and twice with diethyl ether, and resuspended in water prior to lyophilization. Synthesis products were analyzed by high-performance liquid chromatography (system gold; Beckman Instruments, Munich, Germany) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (G2025A; Hewlett-Packard, Waldbronn, Germany). Peptides of <80% purity were purified by preparative high-performance liquid chromatography.

MHC Stabilization Assay. The MHC stabilization assay was performed in 96-well microtiter plates with 10^5 T2 cells (25) per well incubated overnight in 100 μl of RPMI 1640 without FCS. Peptide concentration during incubation was 100 μM or 10 μM. Unbound peptide was washed away, and surface MHC molecules were stained by incubation with the HLA class I-specific antibody W6/32, followed by incubation with a FITC-labeled mouse immunoglobulin-specific antibody (Dianova, Hamburg, Germany). Fluorescence intensities were recorded in a FACSCALIBUR flow cytometer (Becton Dickinson, Heidelberg, Germany).

***In Vitro* Generation of Survivin-specific T Lymphocytes.** Blood was obtained from three healthy donors with the following HLA types: JB: HLA-A*0201/0101, HLA-B*0801/1501, HLA-Cw7, HLA-Cw3, HLA-DRB1*0301/0401, HLA-DQA1*0501/0303, HLA-DQB1*0201/0301; FS: HLA-A*0201/2301, HLA-B*4403/2705, HLA-C*0102/0103, HLA-DRB1*0101/0701, HLA-DQB1*0501/0202; MS: HLA-A*0206/0101, HLA-B*4402/0801, HLA-C*0701–0712, HLA-DRB1*09012/1501, HLA-DQB1*0303/0602. Peripheral blood mononuclear cells were purified from 100 ml of peripheral blood by Ficoll Hypaque (Pharmacia, Freiburg, Germany) density centrifugation with the informed consent of blood donors. Monocytes were isolated by immunomagnetic cell separation with anti-CD14 antibody coupled to magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). To generate immature DCs, monocytes were cultured in the presence of 1000 units/ml granulocyte macrophage colony-stimulating factor and 1000 units/ml IL-4 (both from Strathmann Biotech, Hannover, Germany) in X-VIVO medium (BioWhittaker, Walkersville, MD) supplemented with 10% human serum (CC pro; Neustadt, Germany) for 7 days. After the addition of 5–10 μg/ml recombinant survivin protein, DCs were cultured for an additional 3 days in the presence of 1000 units/ml granulocyte macrophage colony-stimulating factor, 1000 units/ml IL-4, 10 ng/ml IL-1β, 10 ng/ml tumor necrosis factor-α (all from Strathmann Biotech), and 1 μg/ml PGE_2 (Sigma-Aldrich, Steinheim, Germany) in X-VIVO medium supplemented with human serum for further maturation. For T-cell activation, $CD8^+$ T lymphocytes were enriched by positive selection using an anti-CD8 antibody coupled to magnetic microbeads (Miltenyi Biotech). Protein-loaded DCs (5 × 10^5) were cocultured with 2 × 10^6 $CD8^+$ T cells in 2 ml of RPMI 1640 supplemented with 10% human serum per well of a 24-well tissue culture plate (Greiner, Frickenhausen, Germany). Seven days later, cultures were washed and restimulated with freshly prepared survivin-loaded DCs at a responder to stimulator ratio of 2:1 and supplemented with 25 units/ml recombinant human IL-2 and 10 ng/ml recombinant human IL-7 (both from Strathmann Biotech). After six to eight cycles of stimulation, the cultures were tested for survivin-reactive T cells.

For activation of $CD8^+$ T cells against selected survivin peptides, mature autologous monocyte-derived DCs were pulsed with a mixture of two predicted peptides, each at a concentration of 50 μg/ml in serum-free X-VIVO medium for 4 h at 37°C. Stimulation of enriched $CD8^+$ T cells was performed as described above. After four to six cycles of stimulation, the cultures were assayed for survivin peptide-reactive T cells.

Chromium Release Assay. Cytotoxic activity of the *in vitro*-stimulated CTLs was tested against survivin-transfected autologous EBV-BLCLs as targets in a 4-h standard ^{51}Cr− release assay. Briefly, EBV-BLCLs were transfected with pIRES2-survivin/EGFP or pIRES2-EGFP and were selected by FACS. After washing, 1 × 10^6 EBV-BLCLs were labeled for 1 h at 37°C with 100 μCi ^{51}Cr (sodium chromate; NEN, Zaventem, Belgium) in 1 ml of RPMI 1640. To assay the cytotoxic activity of cultured T cells against predicted survivin peptides, the HLA-A2-positive mutant cell line T2 was incubated for 4 h with individual peptides at a concentration of 50 μg/ml, washed three times, and then used as target. Chromium-labeled target cells were washed three times and plated in round-bottomed 96-well plates at 5 × 10^3 cells/well. Effector cells were added as triplicates at various E:T ratios. After 4 h of incubation, 100 μl of supernatant was collected from each well and the released ^{51}Cr was determined in a β-plate scintillator (Wallac, Freiburg, Germany). Maximal and spontaneous release were measured by treating labeled cells with 1% NP40 or medium alone, respectively. The specific cytotoxicity was calculated according to the formula:

Percent specific lysis = 100 × [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)].

RESULTS

Generation of Survivin-reactive $CD8^+$ CTLs from Blood of Healthy Donors. To explore whether survivin-specific CTLs can be generated *in vitro*, $CD8^+$ T cells were enriched from peripheral blood mononuclear cells of healthy donors and subjected to stimulation with survivin-pulsed DCs. To this end, recombinant His-tagged survivin protein was prepared. After separation on SDS-PAGE, purified survivin appeared as a single band at ~20 kDa, as detected by Coomassie Blue staining and by Western blotting using anti-His antibody (Fig. 1). In this experimental setting, DCs exposed to survivin were supposed to take up and process the protein for cross-presentation as MHC class I-bound peptides. To prove that CTLs were raised against survivin peptides resulting from intracellular processing and not against contaminating peptides contained in the survivin preparation, we had to choose target cells exposing peptides that are bona fide derived from survivin by intracellular processing. To verify survivin

[5] http://www.uni-tuebingen.de/uni/kxi.

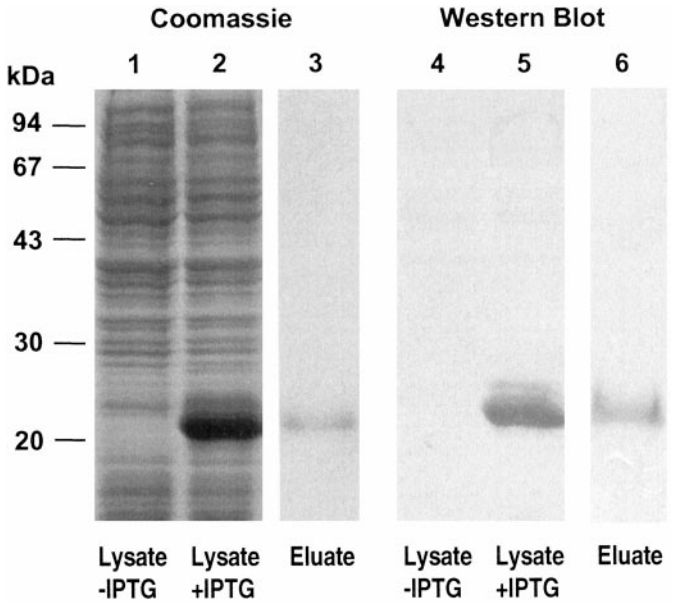

Fig. 1. Overexpression and purification of recombinant His-tagged survivin. *E. coli* M15[pREP4] cells were transformed with the expression vector pQE30 containing the coding region of the survivin gene. To verify the expression of recombinant survivin, expression cultures were grown either in the presence or absence of the inductor IPTG and cell extracts were compared by SDS-PAGE and Coomassie Blue staining *(Lanes 1* and *2*) or Western blotting using anti-His-tagged antibodies *(Lanes 4* and *5*). Purity and integrity of recombinant survivin after purification on Ni-NTA resin is shown in *Lanes 3* and *6*.

specificity of activated CTLs, survivin-negative homologous target cells had to be included. To fulfill these requirements, we decided to transfect autologous EBV-BLCLs with the cDNA for survivin because in nontransfected EBV-BLCLs survivin-mRNA was not detectable by PCR (data not shown). To evaluate the transfection efficiency and to enrich the survivin-expressing cells, cDNA coding for EGFP was included by using the bicistronic vector pIRES2-survivin/EGFP. Transfection efficiencies were between 10% and 20%, as determined by flow cytometry (Fig. 2*A*), and survivin-positive target cells could be enriched to a purity of 80–90% by FACS (Fig. 2*B*). EBV-BLCLs transfected with the control vector pIRES2-EGFP were treated in the same way, resulting in similar transfection and sorting efficiencies (Fig. 2, *C* and *D*). The transfected cells served as target cells in a chromium release cytotoxicity assay. Fig. 3 shows marked lysis of pIRES2-survivin/EGFP-transfected and enriched EBV-BLCLs by survivin-activated $CD8^+$ CTLs of both donors, whereas only background activity was seen against pIRES2-EGFP-transfected EBV-BLCLs. These results reveal that survivin-specific CTLs can be generated from T-cell populations of healthy donors when cultured with monocyte-derived DCs pulsed with soluble survivin.

Selection of HLA-A*0201-binding Survivin Peptides and Generation of CTL Responses. The sequence of survivin (accession no. U75285) was screened for peptides containing the HLA-A*0201 peptide motif. The five high-scoring peptides LTLGEFLKL and TLPPAWQPFL (both score 23), and ELTLGEFLKL, KVRRAIEQL, and RAIEQLAAM (all score 19) were synthesized and tested in an HLA stabilization assay after loading onto T2 cells. Only the 10mer peptides ELTLGEFLKL and TLPPAWQPFL showed significant stabilization of HLA-A*0201, most probably due to the presence of the optimal HLA-A*0201 anchor residue Leu both at position 2 and at the COOH terminus. The other peptides without optimal anchor residues did not bind (data not shown). To evaluate the capacity of the HLA-A*0201-binding survivin peptides to induce CTL-enriched $CD8^+$ T lymphocytes were activated by autologous DCs loaded with both survivin peptides. Specific cytotoxicity was determined in a chromium release assay with peptide-pulsed T2 cells as targets. As shown in Fig. 4, peptide-stimulated $CD8^+$ T lymphocytes from donor JB efficiently lysed T2 target cells loaded with either peptide ELTLGEFLKL or peptide TLPPAWQPFL. CTLs from donor FS showed marked lysis of T2 cells only when pulsed with peptide ELTLGEFLKL, but failed to recognize peptide TLPPAWQPFL. To determine whether the predicted survivin peptides were endogenously produced, $CD8^+$ T cells were tested against EBV-BLCLs transfected

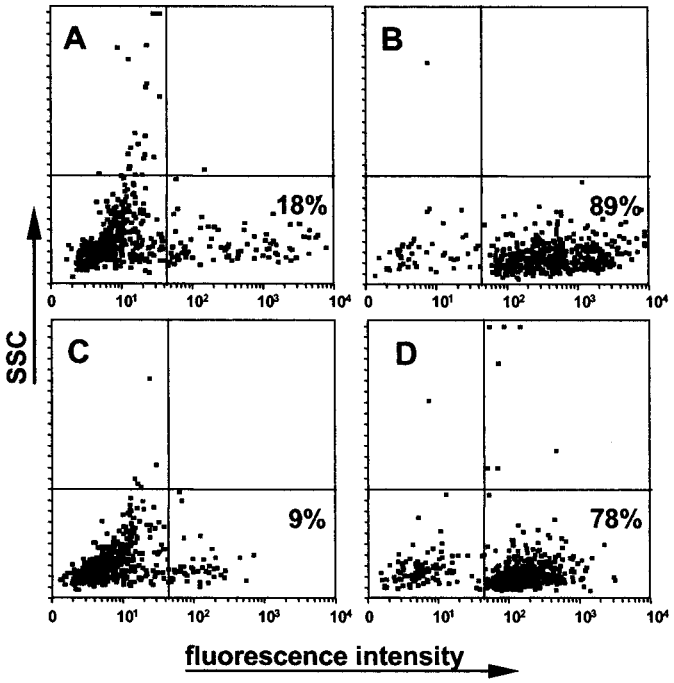

Fig. 2. Transfection of autologous EBV-BLCLs with pIRES2/survivin-EGFP and pIRES2-EGFP and enrichment by FACS. EBV-BLCLs from donor JB were transfected with pIRES2/survivin-EGFP. *A*, FACS analysis of EBV-BLCLs 18 h after transfection with pIRES2/survivin-EGFP. *B*, FACS analysis of EBV-BLCLs transfected with pIRES2/survivin-EGFP after FACS. *C* and *D*, transfection and sorting efficiencies of EBV-BLCLs transfected with pIRES2-EGFP.

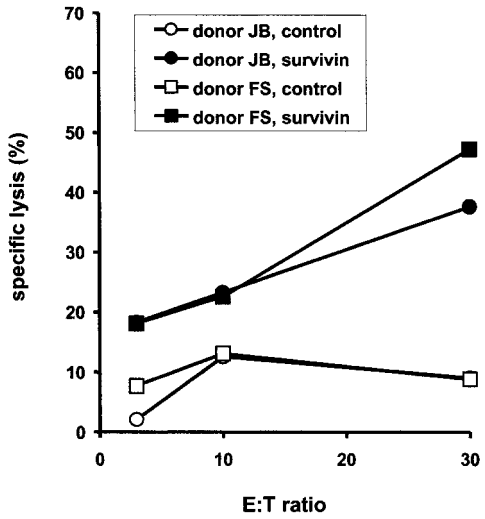

Fig. 3. Generation of survivin-reactive $CD8^+$ CTLs. CTLs of two healthy donors were activated against the tumor-associated protein survivin presented by DCs. Autologous EBV-BLCLs were transfected with pIRES2-survivin/EGFP and labeled with ^{51}Cr. EBV-BLCLs transfected with pIRES2-EGFP served as controls. After 4 h of incubation with CTLs at various E:T ratios (3:1, 10:1, 30:1), chromium release was measured. *Symbols* represent the means of triplicate chromium release determinations, SE being <10% in all determinations.

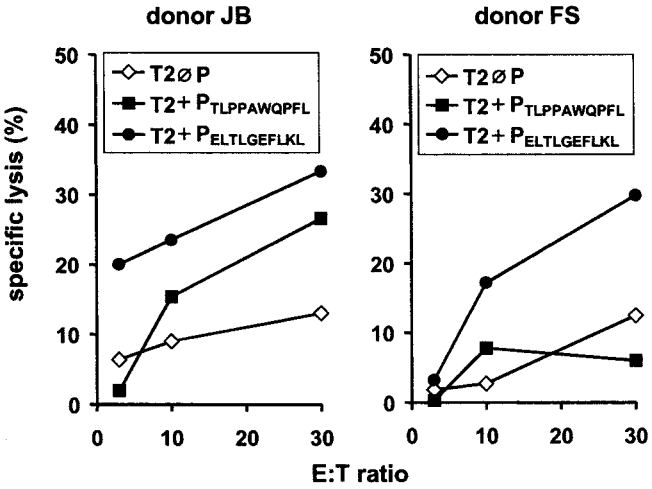

Fig. 4. Generation of survivin-reactive CTLs by peptide-loaded DCs. CTLs of two healthy donors were activated against the two survivin-derived peptides (*P*) ELTLGEFLKL and TLPPAWQPFL presented by autologous DCs. T2 cells loaded with either of the two peptides were labeled with ^{51}Cr. T2 cells without survivin-derived peptides served as negative controls. After 4 h of incubation with CTLs, chromium release was measured. *Symbols* represent the means of triplicate chromium release determinations, SE being <10% in all determinations.

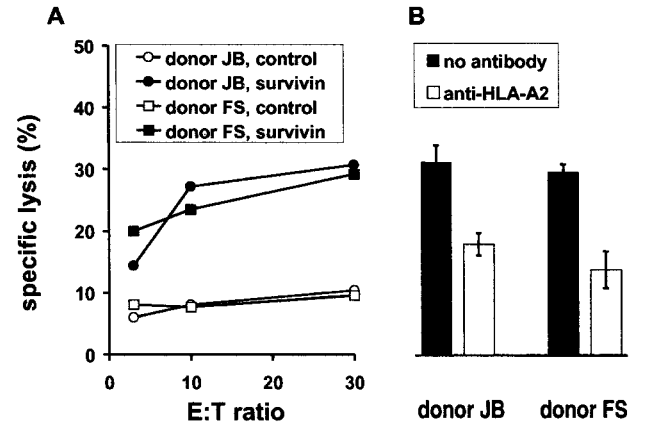

Fig. 5. Recognition of endogenously produced survivin-derived peptides by survivin-reactive CTLs. *A*, CTLs of two blood donors induced by DCs pulsed with the two predicted survivin peptides were cocultured for 4 h with chromium-labeled EBV-BLCLs transfected with pIRES2-survivin/EGFP or pIRES2-EGFP as control at various E:T ratios. *Symbols* represent the means of triplicate chromium release determinations, SE being <10% in all determinations. *B*, inhibition of T cell-mediated cytotoxicity against survivin-expressing EBV-BLCLs at an E:T ratio of 30:1 in the presence of anti-HLA-A2 monoclonal MA 2.1. The *columns* represent the means of triplicate chromium release determinations. *Bars*, SE.

with pIRES2-survivin/EGFP or pIRES2-EGFP. As shown in Fig. 5*A*, responder T cells of both donors efficiently lysed EBV-BLCLs transfected with pIRES2-survivin/EGFP, whereas no cytotoxicity was observed against EBV-BLCLs transfected with pIRES2-EGFP. Because CTLs of both donors recognized the survivin peptide ELTLGEFLKL it can be concluded that at least this peptide results from intracellular processing. Its presentation by HLA-A*0201 molecules is evidenced by partial inhibition of lysis in the presence of HLA-A2-specific monoclonal antibody MA 2.1 (Fig. 5*B*). In addition, we tested whether the survivin-reactive CTLs induced by protein-loaded DCs can recognize the two survivin-derived peptides. As shown in Fig. 6, CTLs of donor JB efficiently lysed T2 cells loaded with peptide ELTLGEFLKL, but not T2 cells loaded with the peptide TLPPAWQPFL. In contrast, CTLs of donor FS failed to recognize survivin peptide-pulsed T2 cells. The lysis of T2 cells pulsed with peptide ELTLGEFLKL by CTLs of donor JB after induction by protein-pulsed DCs confirm the endogenous processing and MHC class I-restricted presentation of this survivin peptide.

DISCUSSION

In the present study, we provide for the first time evidence that the recently described inhibitor of apoptosis protein survivin, which was found to be selectively expressed in various cancer tissues (22, 23), can induce $CD8^+$ T-cell immune responses *in vitro* when presented by autologous monocyte-derived DCs.

After incubation with whole tumor proteins, DCs process the endocytosed proteins to peptides that are bound to MHC class II molecules and are presented on the cell surface for stimulation of $CD4^+$ T helper lymphocytes. In addition to this classical mechanism of antigen presentation, DCs turned out to be capable of processing exogenous proteins by an alternative pathway leading to peptide presentation on MHC class I molecules, which is referred to as cross-presentation (7, 26). The efficient induction of antigen-specific CTL responses by protein-pulsed DCs was demonstrated by recent *in vivo* experiments (27, 28). In the present study, DCs incubated with soluble recombinant survivin were shown to induce specific MHC class I-restricted CTLs. To prove that these CTLs were induced by survivin peptides cross-presented by DCs, we chose autologous EBV-BLCLs as targets that had been transfected with cDNA for both survivin and EGFP using a bicistronic vector construct. We reasoned that this approach might be of 2-fold advantage. First, it provided appropriate control target cells because EBV-BLCLs not transfected or only transfected with the EGFP-cDNA did not express survivin. Second, the transiently transfected EBV-BLCLs could be enriched by FACS to obtain a suitable survivin-expressing target cell population. Transient transfection with the bicistronic EGFP-cDNA-containing vector combined with FACS seems to be particularly useful in experiments where high-frequency expression of proteins is attempted without establishing a stably transfected cell line. When assayed against these transfected EBV-BLCL targets, CTLs generated by stimulation with survivin-pulsed DCs performed their cytotoxicity in a survivin-dependent manner. From these results we conclude that monocyte-derived DCs cross-present survivin peptides and are capable to efficiently induce survivin-specific CTLs from lymphocytes of healthy donors.

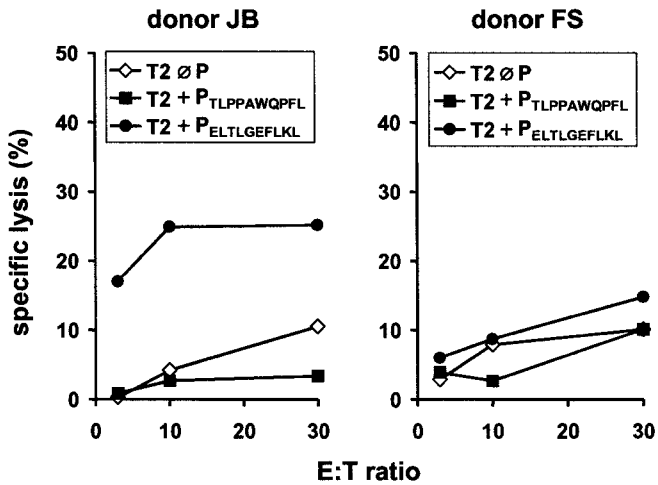

Fig. 6. Recognition of survivin peptide-loaded T2 cells by specific CTLs induced with protein-pulsed DCs. Activated CTLs were cocultured with chromium-labeled T2 cells loaded with survivin-derived peptides (*P*) ELTLGEFLKL or TLPPAWQPFL at various E:T cell ratios (3:1, 10:1, 30:1). T2 cells without survivin-derived peptides served as negative controls. After 4 h of incubation, chromium release was measured. *Symbols* represent the means of triplicate chromium release determinations, SE being <10% in all determinations.

A whole array of strategies in experimental immunotherapy of cancer are based on peptide vaccination. Because survivin seems to be an almost universal TAA (22, 23) it seemed an important task to identify relevant HLA class I-restricted survivin peptides. In a first approach we made a search for survivin peptides displaying the HLA A*0201 peptide motif, because HLA A*0201 is the most frequent HLA-A allele in Caucasian individuals. Among five scoring peptides, two peptides (ELTLGEFLKL and TLPPAWQPFL) were identified that showed significant stabilization of HLA A*0201 molecules at the surface of T2 cells. Both peptides proved to be immunogenic because they induced specific CTLs *in vitro*. The peptide-induced CTLs of both donors included in the experiments were found to lyse T2 cells pulsed with the peptide ELTLGEFLKL as well as EBV-BLCLs transfected with pIRES2-survivin/EGFP. From these data we conclude that at least this peptide is generated by natural processing of endogenously synthesized survivin. The finding that $CD8^+$ T cells of one donor (FS) were activated only against one of the predicted peptides (ELTLGEFLKL) and not against the other peptide (TLPPAWQPFL) is in line with the general observation that individual healthy blood donors and also tumor patients often fail to show an *in vitro* CTL response against a selected peptide. This selective unresponsiveness might be due either to the low frequency of cognate precursors in the blood or to an overall affinity of the T-cell receptors being too low to induce T-cell activation. Finally, the failure of T cells to be activated by a peptide *in vitro* can be caused by a specific T-cell anergy or tolerance that may be induced by homologous or altered peptides derived from constitutively expressed proteins.

When $CD8^+$ T cells of donor FS were activated by DCs loaded with whole survivin protein they turned out to be unable to recognize either one of the two peptides presented on T2 cells, whereas T cells from donor JB subjected to an identical activation procedure revealed a specific reactivity against the peptide ELTLGEFLKL, but not against the second predicted peptide TLPPAWQPFL. Because CTLs of both donors stimulated by survivin-loaded DCs were able to lyse survivin-expressing target cells the failure of donor FS to recognize the two selected HLA A*0201-bound peptides may be due to an individually different pattern of survivin-derived peptides, which are presented by various HLA-class I molecules on DCs after processing for cross-presentation.

To further substantiate the survivin peptide ELTLGEFLKL as a potent candidate antigen for immunotherapy of tumors it remains to be shown that it is naturally exposed on tumor cells for recognition by CTLs. Because most prepared tumor tissues (22) and all tumor cell lines (29) seem to express survivin, a survivin-negative target cell that could serve as an appropriate specificity control is missing. For this reason, specific recognition of survivin on tumor cells can only be proven by the use of cloned CTLs that are not yet available.

In summary, we show here for the first time the specific activation of $CD8^+$ CTLs against the widely occurring tumor-associated protein survivin. Thus, survivin recommends itself as a universal antigen for the design of protein- and peptide-based anticancer vaccines.

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