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Generation of Cytotoxic Responses in Mice and Human Individuals Against Hematological Malignancies Using Survivin-RNA-Transfected Dendritic Cells

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Survivin is a new member of the inhibitors of apoptosis family and is overexpressed in many types of human cancers, making it an attractive target for T cell-based immunotherapeutic strategies. Recently, HLA-A2-binding peptides derived from the survivin protein were identified as capable of inducing specific T cell responses in cancer patients. Here we demonstrate that human survivin-specific CTLs generated from PBMC by stimulation with autologous dendritic cells transfected with survivin-RNA were cytotoxic for a range of hematopoietic malignant cell lines and primary tumor cells isolated from patients with acute myeloid leukemia. We also show that vaccination of mice with survivin-RNA-transfected dendritic cells leads to long term resistance to challenge by a survivin-expressing lymphoma, demonstrating the potential of survivin as a tumor rejection Ag. Our data provide evidence for the use of survivin as a target structure for immunotherapeutic strategies against hematological neoplasms. The Journal of Immunology, 2003, 170: 5391–5397.

Survivin is a member of the inhibitors of apoptosis gene family (1) that is expressed in mitosis in a cell cycle-dependent manner and localized to components of the mitotic apparatus (2). Potentially involved in both control of cell division and inhibition of apoptosis, survivin is abundantly expressed in adenocarcinomas of lung, pancreas, breast, and prostate as well as in squamous lung cell carcinoma. Survivin is present during normal fetal development but absent in normal adult tissues with only a few exceptions (3, 4). Analysis of human transcriptomes confirmed these findings by demonstrating that survivin was identified as one of the most prominent genes invariably up-regulated in many types of cancer but not in normal tissue (5). In several epithelial and hemopoietic tumor entities, expression of survivin was correlated with abbreviated overall survival and unfavorable prognosis (6–9).

Recently, survivin-derived peptide epitopes could be identified capable of inducing specific cytotoxic T cell responses in both healthy donors (10, 11) and cancer patients (12, 13). Because survivin is also expressed in most of all leukemic blasts of patients with acute myeloid leukemia (AML) (7, 14, 15), we evaluated in the current study whether survivin is a suitable target for T cell-based immunotherapeutic strategies in patients with acute leukemia. We show that CTL generated from healthy HLA-A2 donors by primary in vitro immunization with autologous survivin-RNA-transfected dendritic cells (DC) elicited specific and MHC class I-restricted cytotoxic activity against several hematological tumor lines and primary AML blasts endogenously expressing survivin. In addition, the majority of mice treated with survivin-RNA-transfected DC are capable of rejecting an otherwise lethal dose of a murine B cell lymphoma.

Materials and Methods

Patients and healthy donors

Peripheral blood and tumor samples were collected from healthy donors and patients at the II Department of Internal Medicine (University of Kiel, Kiel, Germany) and cryopreserved. Tumor samples used for cytotoxicity assay were obtained from patients with AML and chronic lymphocytic leukemia (CLL). All specimens were obtained after informed consent, and approval by our institutional review board was provided.

Human tumor cell lines and nonmalignant cell subsets

K562 (erythroleukemia), IM-9 and U-266 (plasmacytoma), REH, Karpas-422, Balm-3, Ramos (B lymphoblastic lymphoma), and T2 cells were obtained from American Type Culture Collection (Manassas, VA). MEC-1, a chronic lymphatic leukemia, was kindly provided by Dr. F. Caligaris-Cappio (University of Torino, Torino, Italy). Leukemic blasts (purity > 80%) from AML patients were obtained by Ficoll (Amersham-Pharmacia, Piscataway, NJ) density gradient centrifugation. CD5⁺CD19⁺ CLL cells were obtained from peripheral blood of previously untreated CLL patients (Benet A). After Ficoll density gradient centrifugation, >95% of resulting cells coexpressed CD19 and CD5. Isolation of CD8⁺ T lymphocytes, CD19⁺ B cells, CD14⁺ monocytes, and CD34⁺ peripheral blood progenitor cells was conducted using MACS technology (Miltenyi, Bergisch-Gladbach, Germany) following the manufacturer’s instructions. FACSCele revealed a cell purity of >90%.

Immunophenotyping

For marker analysis, mononuclear cells (MNC) were incubated with appropriate primary and secondary Abs and analyzed using a flow cytometer (FACScan; BD Biosciences, Mountain View, CA). The following Abs for murine MNC were used: anti-Thy-1.2 (30-H12); anti-NK (DX5); anti-CD19 (ID3); anti-CD11c (HL3); anti-CD45 (30-F11); anti-CD18 (GAM-46); anti-CD80 (1G10); anti-CD86 (GL1); anti-MHC class I (34-2-12); and
anti-MHC class II (AMS-32.1). All mAbs were obtained from BD PharMin- gen (San Diego, CA). For flow cytometric analysis of human MNC, FITC- or PE-conjugated mouse mAbs against CD4, CD3, CD8, CD56, CD14, CD40, CD80, CD86 (BD Biosciences, Heidelberg, Germany), HLA-DR and CD83 (Coulter-Immunotech, Hamburg, Germany) were used.

Intracellular staining of survivin was performed after fixation and permeabilization using the Fix & Perm kit (BD Biosciences, Heidelberg, Germany) following the manufacturer’s instructions. A rabbit polyclonal Ab against mouse survivin (ab8113; Abcam, Cambridge, U.K.) was used. FITC-labeled goat anti-rabbit Ig was used as a secondary reagent. For detection of human survivin, the rabbit polyclonal Ab against human sur vivin (DPC Biermann, Bielefeld, Germany) was used.

**Western blot analysis**

Whole cell protein extracts were prepared from 1×10⁸ cells using a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (Sigma-Aldrich, Diesenhoven, Germany), 2 mM EDTA (pH 8.0), and Complete mini (protease inhibitor; Roche, Basel, Switzerland). Cell lysates were separated by 12% gels in SDS-PAGE under reducing conditions for 50 min at 100 V and blocked in 20 mM Tris/HCl, 20% methanol for 60 min at 100 V and blocked in 20 mM Tris (pH 8.0), 137 mM NaCl, 0.1% Tween 20 (TBST), and 5% powdered milk overnight. Immunoblottings were performed using the rabbit polyclonal anti-survivin (DPC-Biermann, Bad-Naumburg, Germany) Ab at a concentration of 1 µg/ml (in TBST and 5% BSA) as primary and HRP-linked anti-rabbit Ig (diluted to 1/5000 v/v in TBST and blocked milk; Amer sham, Little Chalfont, U.K.) as secondary Ab. All immunoblots were performed using ECL (Amersham).

**Stimulation of B-CLL cells with CD40 ligand**

Primary tumor cells from CLL patients (Binet A) were obtained by Ficoll density centrifugation. Cells were washed twice in PBS (CellConcepts, Umbach, Germany), resuspended in RPMI 1640 supplemented with 10% FCS (Biochrom, Cambridge, U.K.) and 1% penicillin/streptomycin (Sigma-Aldrich), and cultured in six-well plates. For B cell activation, 100 ng/ml CD40 ligand and 1 µg/ml Enhancer (Alexis, Lausen, Switzerland) were added to 1×10⁶ cells/ml and incubated for 3 days at 37°C. FACS revealed that >90% of the stimulated CLL cells were survivin positive (data not shown).

**Peptides and loading of T2 cells**

Survivin1–14, survivin135–140, and Flu1–12, -46 were purchased from Biosyntan (Berlin, Germany) and provided at >90% purity, as verified by HPLC and MS analysis. The HLA-A2* mutant cell line T2 was separately incubated with peptides for 2 h at a concentration of 50 µg/ml, washed three times, and used as target in a 51Cr release assay.

**Isolation of human and murine survivin-RNA**

The coding sequence for human survivin was ligated into BodHI/XohI site of the pcITE-2a(+) vector (Novagen, Madison, WI). The coding sequence for murine survivin was obtained by extraction of total RNA from murine A20 B-cell lymphoma cell line by using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was performed by using the First-Strand cDNA Synthesis Kit (Amersham, U.K.) and visualized on a denaturated agarose gel. Quantity and purity were determined by UV spectrophotometry. The human survivin RNA translation product (SSTP T7 Kit; Novagen, Madison, WI) was visualized on a Western blot.

**Cell isolation and generation of human immature DC**

Immature DC (iDC) were generated from PBMC of healthy HLA-A2* volunteers following the protocol of Feuerstein et al. (16). Briefly, a concentrated leukocyte fraction was generated through a 2-h restricted peripheral blood leukapheresis processing 6–8 liters of blood with each collection. The leukapheresis product was further separated by density gradient centrifugation over polysucrose-sodium diatrizoate (Histopaque; Sigma-Aldrich, St. Louis, MO), and cells were resuspended in serum-free AIM-V medium (Life Technologies, Gaithersburg, MD). PBMC were incubated at 2×10⁵ cells/ml in T-150 culture flask in a humidified incubator at 37°C to allow plastic adherence. The adherent cell fraction was used for DC culture by incubation in serum-free AIM-V medium supplemented with recombinant human IL-4 (500 U/ml) and recombinant human GM-CSF (800 U/ml; CellConcepts).

**Transfection and maturation of human DC**

Transfection of iDC was performed by using the liposomal transfection reagent dioleoyl-trimethylammonium-propane-methylsulfate (DOTAP; Roche). DOTAP (20 µl) and in vitro-transcribed RNA (10 µg) were mixed in 500 µl of Opti-MEM (Life Technologies, Karlshue, Germany) and incubated for 20 min at room temperature. The RNA-lipid complex was added to 1×10⁶ iDC/ml (in Opti-MEM) and incubated at 37°C for 3 h. Maturation of the transfected iDC was performed by culturing for 2 days in serum-free medium supplemented with GM-CSF, IL-4, IL-1β, TNF-α (10 ng/ml; CellConcepts), IL-6 (1000 U/ml; CellConcepts), and PGE2 (1 µg/ml; Sigma-Aldrich, Diesenhoven, Germany). Flow cytometric analysis revealed that survivin-RNA-transfected and matured DC expressed ~20% of survivin and high levels of CD80, CD83, CD86, MHC class I, and MHC class II molecules.

**Generation of human CTL**

CTL were generated using a protocol adopted from Heiser et al. (17); in brief, the T cell-enriched nonadherent fraction of PBMC obtained after the DC plastic adherence step was used for CTL generation. Nonadherent PBMC were cultured in serum-free medium supplemented with 20 U/ml human IL-2 and 10 ng/ml human IL-7 (CellConcepts). Cells were stimulated weekly at least four times with autologous survivin-RNA-transfected DC.

**CTL assay**

Target cells were labeled with 200 µCi of NaCrO4 (Amersham-Buchler, Braunschweig, Germany) in 0.5 ml of complete medium for 1 h. They were washed three times with complete medium and added at a concentration of 5×10⁵ cells/well in round-bottom microtiter plates (Nunc, Roskilde, Denmark). Effector cells were added at various E:T ratios in a final volume of 200 µl/well. The plates were incubated for 4 h at 37°C in a humid atmosphere with 5% CO₂. Maximum chromium release was ensured by the addition of 10% Triton-X, and spontaneous release was assessed by adding complete medium (RPMI 1640 plus 10% FCS) to the target cells. The culture supernatant was harvested semi automatically with a Scatron Titertek System (Scatron, Suffolk, U.K.) and counted in a gamma counter (Beckmann, Heidelberg, Germany). The percentage of specific lysis was calculated as [[(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100. All determinations were made in triplicate.

**Cold target inhibition assay**

Specificity of tumor cell lysis was determined in a cold target inhibition assay by analyzing the capacity of unlabeled HLA-A2* survivin-negative and HLA-A2* survivin-positive CLL cells to block lysis of tumor cells at a ratio of 20:1 (inhibitor-target ratio).

**Ab blocking inhibition assay**

For Ab-blocking experiments, human CTL were generated as described in “Generation of human CTL.” For blocking experiments, cells were incubated with 10 µg/ml mAbs (anti-HLA-A2 (BB7.2); BD Biosciences, Heidelberg, Germany; CD8 (T8); anti-Pan-TCR (BMA 031), CD4 (T4), isotype- matched controls MAM-6 and HMF-1, Beckman Coulter) for 30 min. Cell suspensions were washed twice with complete medium and tested in a chromium release assay.

**Murine tumor cell lines**

A20 is a nonimmunogeneic B cell leukemia/lymphoma that occurred spontaneously in a 15-mo-old BALB/c mouse and was used for in vivo tumor challenge experiments. The murine EL-4 thymoma cell line derived from C57BL/6 mice were grown in RPMI 1640 (Life Technologies, Karlsruhe, Germany) containing 5% FCS (Biochrom) and was used as a fully MHC-mismatched target for CTL analysis. Both cell lines were obtained from American Type Culture Collection (Manassas, VA).
Isolation of total A20-RNA
A total RNA was obtained by using the RNeasy Kit following the manufacturer’s instructions. Isolated RNA was visualized on agarose gel. Quantity and purity of RNA were determined by UV spectrophotometry. Total RNA samples were stored at \(-80^\circ\)C.

Generation of murine survivin-RNA-transfected DC
For in vitro restimulation, bone marrow-derived DC were used as professional APC and generated as previously described (18). iDC were transfected using 50 \(\mu\)g of DOTAP (Roche) and 25 \(\mu\)g of in vitro-transcribed RNA or total RNA mixed in 500 \(\mu\)l of Opti-MEM. The complex was incubated for 20 min at room temperature and then added to 5 \(\times\) 106 immature DC. DC Maturation was achieved by adding LPS (2 \(\mu\)g/ml for 5 \(\times\) 106 iDC/ml; Sigma-Aldrich, Deisenhofen, Germany) for 4 h at 37\(^\circ\)C. Afterward, mature DC were washed three times and applied for vaccination studies. Flow cytometric analysis demonstrated that these matured DC expressed 15–25\% of survivin and high levels of CD45 (96 ± 1.9\%), CD11c (92 ± 2.9\%), CD18 (95 ± 1.8\%), CD80 (89 ± 2.2\%), CD86 (83 ± 2.9\%), class I MHC (98 ± 0.3\%), and class II MHC (92 ± 1.0\%) Ags.

Tumor challenge experiments
Healthy BALB/c mice 8–12 wk old received three s.c. injections of 5 \(\times\) 105 syngeneic DC transfected with murine survivin-RNA at weekly intervals. Control groups were treated with untransfected DC or PBS (tumor control group). One week after the last vaccination, animals were inoculated s.c. with 1 \(\times\) 105 A20 lymphoma cells.

Marine CTL assay
BALB/c mice were treated as described above. On day 100, DC-treated animals were killed under ether anesthesia. The spleens of two mice per group were removed, pooled, and pressed through wire mesh screens to obtain single-cell suspensions. MNC were isolated by Lympholite-M (Cedarlane, Hornby, Ontario, Canada) gradient centrifugation. Splenocytes were restimulated at a concentration of 2 \(\times\) 106 cells/ml with 5 \(\times\) 104/ml DC transfected with survivin-RNA or total A20-RNA in 24-well plates. After 5 days, viable cells were harvested and tested in a \(^{51}\)Cr release assay for their ability to lyse A20 and EL-4 cells.

Statistical analysis
Survival of animals was calculated according to the method of Kaplan and Meier. Group comparisons were made by the Wilcoxon test. The calculations were done on a PC with Statistica data analysis software (StatSoft, Tulsa, OK).

Results
Survivin is expressed in primary AML blasts but not in newly diagnosed CLL
The expression of survivin on malignant hemopoietic cells was assessed by FACS and Western blot using the polyclonal survivin-specific Ab. As illustrated in Tables I and II, survivin expression could be detected in all tumor cell lines and primary AML blasts tested (Fig. 1). Fig. 1A gives an example of survivin expression on AML blasts from two patients. In contrast, in none of the CLL cells obtained from newly diagnosed B-CLL patients (Binet A) was expression of survivin protein found (Table II). We could not detect any expression of survivin on cell subpopulations purified from peripheral blood like CD19+ (B lymphocytes), CD3+ CD8+ (T lymphocytes), CD14+ (monocytes) cells. In addition, we observed that purified CD34+ progenitor cells were survivin negative, as well as iDC and matured DC (Table II).

Survivin-specific CTL killed several types of hematological cell lines
We next generated polyclonal CTL in vitro using autologous survivin-transfected DC as Ag-presenting cells obtained from healthy HLA-A2* individuals. CTL obtained after four weekly restimulations elicited efficient T cell responses recognizing and lysing endogenously survivin-expressing tumor cell lines. As depicted in Fig. 2A, the survivin-positive, HLA-A2 -expressing cell lines Karpas-422, Balm-3, IM-9, REH, and U266 were killed whereas no lysis of the hematological cell lines K562, Ramos, and Mec-1 (survivin+/HLA-A2*) occurred (Fig. 2B). In addition, survivin-specific CTL elicited significant cytotoxic activity against survivin RNA-transfected autologous DC (Fig. 2A) but showed no lysis of untransfected DC, purified monocytes, or CD34+ hemopoietic progenitor cells (Fig. 2C).

Ab-blocking experiments (Fig. 3A) and cold target inhibition assays (Fig. 3B) using the Karpas-422 lymphoma cell line and different types of HLA-A2* CLL cells as cold targets revealed an HLA-A2-restricted killing induced by specific cytotoxic CD8+ T lymphocytes.

Table I. Expression of survivin on malignant hematological cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>Survivin Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARH77</td>
<td>B-ALL</td>
<td>++</td>
</tr>
<tr>
<td>Balm-3</td>
<td>B-ALL</td>
<td>++</td>
</tr>
<tr>
<td>Balm-5</td>
<td>B-ALL</td>
<td>++</td>
</tr>
<tr>
<td>NALM 6</td>
<td>B-ALL</td>
<td>++</td>
</tr>
<tr>
<td>REH</td>
<td>B-ALL</td>
<td>++</td>
</tr>
<tr>
<td>BJAB</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>CA46</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>DAUDI</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>IM-9</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>Karpas-422</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>Mec-1</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>Ramos</td>
<td>B cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>U-266</td>
<td>Plasmacytoma</td>
<td>+</td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>Plasmacytoma</td>
<td>+</td>
</tr>
<tr>
<td>Jurkat</td>
<td>T-ALL</td>
<td>++</td>
</tr>
<tr>
<td>Molt-4</td>
<td>T-ALL</td>
<td>++</td>
</tr>
<tr>
<td>K562</td>
<td>Erythroleukemia</td>
<td>+</td>
</tr>
<tr>
<td>HL60</td>
<td>AML</td>
<td>+</td>
</tr>
<tr>
<td>Mono-Mac-6</td>
<td>AML</td>
<td>+</td>
</tr>
<tr>
<td>THP-1</td>
<td>AML</td>
<td>+</td>
</tr>
</tbody>
</table>

Table II. Expression of survivin on nonmalignant hematological subpopulations and on primary malignant cells from patients with various hematological malignancies

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>n</th>
<th>Survivin Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CD14+</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CD19+</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CD56+</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CD34+</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Immature DC</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mature DC</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>EBV blasts</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>AML blasts</td>
<td>21</td>
<td>++</td>
</tr>
<tr>
<td>ALL blasts</td>
<td>4</td>
<td>++</td>
</tr>
<tr>
<td>CLL</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>LGL</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CML-BCm</td>
<td>2</td>
<td>+</td>
</tr>
</tbody>
</table>

* Cell surface staining of neoplastic cell lines was assessed by flow cytometry using the anti-Survivin Ab. Intensity of staining is denoted as follows: 0, absent; +, low; ++, medium; ++++, high. ALL, acute lymphatic leukemia; LGL, large granular leukemia; CML-BCm, chronic myelogenous leukemia in myeloid blast crisis.
Polyclonal survivin-specific CTL efficiently kill primary AML blasts

To examine whether T cell lines generated against survivin protein could kill not only tumor cell lines but also fresh malignant targets from patients, cytotoxicity assays were performed by using survivin-specific CTL against survivin-expressing blasts from AML patients. In four cases, T cell lines were capable of lysing HLA-A2\(^+\)/survivin-expressing AML blasts (Fig. 4A), whereas HLA-A2\(^-\) targets were not lysed (Fig. 4B).

Lysis of survivin-peptide-loaded T2 cells by polyclonal survivin-specific CTL

In a next step, we investigated whether polyclonal survivin-specific CTL contain T lymphocytes capable of recognizing two recently identified HLA-A2-binding peptides (survivin\(_{5-14}\) and survivin\(_{95-104}\)) derived from the survivin protein (3, 5). As shown in Fig. 5, T2 cells pulsed with the cognate survivin peptides could be recognized and efficiently killed by survivin-specific CTL, whereas T2 cells coated with an irrelevant control peptide (FluM158\(_{5-66}\)) were not lysed. These findings suggest that at least two clonotypes were induced specific for different survivin epitopes.

Induction of antitumoral immunity in mice using murine survivin-RNA-transfected DC

To evaluate the therapeutic potential of survivin as a tumor rejection Ag healthy BALB/c mice were vaccinated with syngeneic DC transfected with RNA coding for the murine survivin protein. After three weekly s.c. injections of \(5 \times 10^5\) DC, animals were subsequently challenged with an otherwise lethal dose of \(1 \times 10^5\) A20 lymphoma cells. As depicted in Fig. 6, vaccination of animals with unpulsed DC did not significantly improve survival compared with the tumor control group. In contrast, animals receiving vaccinations with DC transfected with survivin-RNA or with total RNA derived from the A20 leukemia experienced tumor protection in 10 of 12 (83%) and 11 of 12 (91%) animals being tumor free after an observation period of 140 days (Fig. 6A). CTL analysis from animals treated with survivin-RNA- or A20-RNA-transfected DC demonstrated efficient tumor-specific killing of the A20 leukemia, whereas the fully MHC-mismatched control target EL-4 was not lysed (Fig. 6B).

Tumor rechallenge experiments were performed separately. At day 140, animals treated with syngeneic total A20-RNA- or murine survivin-RNA-transfected DC received a second otherwise

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**FIGURE 1.** Survivin expression in human leukemias. Intracellular FACS of survivin expression on AML blasts obtained from two different AML patients. A. Open histograms, staining with survivin Ab; filled histograms, isotype control. B. Detection of survivin expression by Western blot analysis in some primary AML blasts, human tumor cell lines, and purified subpopulations obtained from peripheral blood of healthy volunteers.

**FIGURE 2.** Induction of survivin-specific CTL using autologous DC transfected with survivin (Surv.)-RNA. DC from HLA-A2\(^+\) individuals were transfected with survivin-RNA and used to stimulate CTL responses. Cytotoxic reactivity was determined in a 4-h \(^{51}\)Cr release assay against HLA-A2\(^+\)/survivin\(^+\) hematological tumor cell lines (A), HLA-A2\(^-\)/survivin\(^+\) targets (B), and nonmalignant HLA-A2\(^+\)/survivin\(^+\) target cells (C). PBPC, peripheral blood progenitor cells; EGFP, enhanced green fluorescence protein. One representative experiment is shown.
lethal tumor inoculation of $1 \times 10^5$ A20 lymphoma cells. All treated animals (five animals/group) rejected the tumor and survived the observation period of 220 days (data not shown).

**Discussion**

In this study, we have shown that survivin-specific CTL obtained from healthy volunteers elicit potent cytotoxic reactivity against several hematological malignant cell lines and against primary AML blasts from different patients. Furthermore, treatment of animals with survivin-RNA-transfected DC induced potent antitumor immunity.

First experimental evidence that survivin can be regarded as a potential tumor-associated Ag came from Schmitz et al. (10), demonstrating that survivin-peptide-specific cytotoxic T cell lines generated from healthy HLA-A2 donors can recognize and selectively kill autologous EBV-transformed B lymphocytes transfected with survivin-coding cDNA. Using an ELISPOT assay, Andersen et al. (12, 13) detected CTL responses against two survivin-de-duced peptide epitopes in three of six melanoma patients and three of four CLL patients, whereas no T cell reactivity could be measured in PBL from six healthy HLA-A2* individuals. In a more recent report, the same author isolated survivin-peptide-specific T cells from HLA-A2* melanoma patients capable of eliciting cytotoxic reactivity against some established HLA-A2-matched tumor cell lines (13).

To develop a vaccination strategy for the treatment of human hematological neoplasms potentially representing multiple human survivin-epitopes, we investigated the capability of human DC transfected with human survivin-RNA to stimulate primary CTL responses in vitro. We were able to demonstrate powerful cytotoxic responses against a broad range of both hematological tumor cell lines and primary AML blasts, whereas nonmalignant cell sub-populations derived from the hemopoietic system were not affected (monocytes, DC, CD34+ cells). HLA-A2-matched CD34-purified peripheral blood progenitor cells obtained from an healthy donor were not lysed by survivin-specific CTL, suggesting that non-malignant hemopoietic cells might not be the target for T cell-based immunotherapeutic approaches using survivin as a target structure. However, because in nonmalignant CD34+ peripheral blood progenitor cells survivin is detectable at low levels (14), but can be up-regulated in the presence of hemopoietic growth factors (3), induction of autoimmune reactions cannot be completely excluded.

In the current study, polyclonal survivin-specific CTL generated with autologous survivin-RNA-transfected DC contain T lymphocytes capable of recognizing two recently described epitopes (survivin5-14 and survivin95-104) as demonstrated by their potential to

**FIGURE 3.** Ab-blocking experiments and cold target inhibition assays using survivin-specific CTLs. To investigate MHC-dependent cytotoxicity, Ab blocking experiments (A) were performed in a 4-h $^{51}$Cr release assay against the IM-9 target. Cold target inhibitions assays were conducted in a 4-h $^{51}$Cr release assay using the Karpas-422 lymphoma cell line (HLA-A2* survivin*). The specificity of the CTL lines was tested in the presence of unlabeled cold targets, i.e., unmanipulated CLL cells (HLA-A2* survivin*) or CLL cells (HLA-A2* survivin*) pretreated with the soluble CD40 ligand (see Materials and Methods). Cold targets were added at an inhibitor-target ratio of 20:1.

**FIGURE 4.** Survivin-specific CTL lyse primary tumor cells from leukemia patients. HLA-A2* survivin* leukemic blasts (A, AML 1, 3, 5, and 6) and HLA-A2* survivin* blasts (B, AML 2 and 4) were used as targets in a 4-h $^{51}$Cr release assay.

**FIGURE 5.** Lysis of survivin (Surv)-peptide-loaded T2 cells by polyclonal survivin-specific CTL. T2 cells were separately pulsed with survivin5-14, survivin95-104, FluM158-66 (see Materials and Methods) and used as target cells in a 4-h $^{51}$Cr release assay. One of two experiments is presented.
kill survivin-peptide-pulsed T2 cells. Using DC loaded with whole survivin protein, Schmitz et al. (10) generated CTL from one HLA-A2-matched donor unable to recognize either one of the two survivin peptides, whereas T cells obtained from another healthy donor revealed a specific reactivity against survivin95–104 but not against survivin5–14.

In an established murine B cell leukemia model, we were able to demonstrate efficient antitumoral CTL responses in vitro and a complete tumor rejection in most of the treated animals receiving survivin-RNA-transfected DC. Although survivin is expressed at low levels on normal epithelial cells of the gastrointestinal tract (4) and on hemopoietic progenitor cells (3, 14), no visible signs of any autoimmune manifestations occurred after this type of DC therapy.

Taken together, these findings advance the notion that survivin could serve as a useful Ag for T cell-based immunotherapeutic strategies in the treatment of hematological malignancies, especially of AML. Similar to the catalytic subunit of telomerase (19), the use of survivin as an Ag for vaccination purposes has the advantage that down-regulation or Ag loss as a mechanism for immune escape would hinder the progression of the tumor. Future studies will have to investigate this as well as from what proportion of cancer patients it is possible to induce survivin-specific CTL and whether immunization against survivin could lead to autoimmune reactions.

References


