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ORIGINAL ARTICLE

Efficient tumor cell lysis mediated by a Bcl-X(L) specific T cell clone isolated from a breast cancer patient

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Abstract Based on the detection of spontaneous immune responses in cancer patients with cancer of different origin, Bcl-X(L) was recently described as a highly interesting tumor antigen recognized by CD8 positive cytotoxic T lymphocytes. To further characterize Bcl-X(L) as a tumor antigen we isolated and expanded Bcl-X(L) specific T cells from the peripheral blood of a breast cancer patient hosting a strong Bcl-X(L) specific T cell response. We describe that HLA-A2 restricted Bcl-X(L) specific T cell clones very efficiently lyse peptide pulsed T2 cells. Furthermore, tumor cell lines of different origin, i.e., breast cancer, colon cancer, and melanoma, are efficiently lysed in an HLA-dependent manner. Finally, ex vivo-isolated leukemia cells, but not non-malignant B and T cells are killed by Bcl-X(L) specific T cells. Our data underline Bcl-X(L) as an universal tumor antigen widely applicable in specific anticancer immunotherapy.

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Introduction

The *bcl-x* gene is transcribed into two mRNAs through alternative splicing. The anti-apoptotic protein Bcl-X(L) is produced from the long isoform, while pro-apoptotic Bcl-X(S) is derived from the short isoform mRNA [4]. The protein product of the larger Bcl-X(L) differs from Bcl-X(S) protein by an inserted region (amino acids 126–188). The anti-apoptotic protein Bcl-X(L) plays an important role in cancer as it has been directly linked to resistance to conventional forms of therapies and poor prognosis [13]. Increased expression of Bcl-X(L) has been reported in a variety of different malignancies including acute myeloid leukemia (AML) and multiple myeloma as well as solid cancers like bladder cancer, breast cancer, pancreatic cancer, and melanoma [13]. The functional inhibition of Bcl-X(L) restores the apoptotic process and renders neoplastic cells sensitive to chemical and radiation therapies, whereas manipulation of cancer cell lines to express high levels of Bcl-X(L) results in a multi-drug resistance phenotype. Thus, the attractiveness of using Bcl-X(L) for vaccination purposes is based on the fact that downregulation or loss of expression of this protein as some form of immune escape would impair sustained tumor growth. The combination of immunotherapy targeting Bcl-X(L) with conventional chemotherapy appears to be particularly appealing since expression of this protein is correlated with drug resistance [7, 10]. In that regard, we recently demonstrated that cancer patients with cancer of different origin host spontaneous T-cell responses specifically against Bcl-X(L)-derived peptides presented in the context of the HLA-A2 antigen [3]. In the present study, we isolated and expanded Bcl-X(L) specific T-cell clones and tested the functional capacity of these cells.

Materials and methods

Patient material

Peripheral blood lymphocytes (PBL) from an HLA-A2 positive breast cancer patient (MA41508) were obtained from the University Hospital in Herlev, Denmark. The PBL were isolated using Lymphoprep separation and cryopreserved in FCS with 10% DMSO. Tissue typing was conducted at the Department of Clinical Immunology, The State Hospital, Copenhagen, Denmark. The patient was diagnosed with breast cancer in 2000; ductal carcinoma T1N1M0 and received three cycles of standard FEC (5-fluorouracil, epirubicin, cyclophosphamide) for adjuvant treatment. In 2004 she had disease dissemination with involvement of the lymphatic system, however, no chemotherapy for metastatic disease had been given. The patient is currently included in a cancer vaccination trial but did not receive immunotherapy before sampling of blood. The Institutional Ethical Committees, Copenhagen County, and the Danish Medicines Agency approved the study protocol. Written informed consent was obtained from the patient at study entry.

IFN-γ ELISPOT assay

The ELISPOT assay was used to quantify peptide epitope-specific interferon-γ (IFN-γ) releasing effector cells as described previously [2]. Briefly, nitrocellulosebottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with anti-IFN-γ Ab (1-D1K; Mabtech). The wells were washed, blocked by X-vivo medium and the effector cells were added in duplicates at different cell concentrations, with or without 10 μM peptide. The plates were incubated overnight at 37°C/5% CO_2. The following day, medium was discarded and the wells were washed prior to addition of biotinylated secondary Ab (7-B6-1-Biotin; Mabtech). The plates were incubated at room temperature (RT) for 2 h, washed, and Avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) was added to each well. Plates were incubated at RT for 1 h and the enzyme substrate NBT/BCIP (Invitrogen Life Technologies) was added to each well and incubated at RT for 5–10 min. Upon appearance of dark purple spots, the reaction was terminated by washing with tap water. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers) and the frequency of peptide-specific cells could be calculated from the number of spot-forming cells.

Fluorescence activated cell sorting (FACS)

PBL were analyzed by flow cytometry using FACSAria (BD Biosciences). The T cells were stained with APC-conjugated MHC-dextramers (a kind gift from Jørgen Schøller, Dako, Denmark), followed by antibody staining with the fluorochrome-coupled mAbs: 7-AAD-PerCP, CD3-FITC, CD4-PE and CD8-APC-Cy7 (BD Immunocytometri Systems). Both stainings were performed in RPMI 1640 media (GibcoBRL), for 20 min, 4°C, in the dark. The MHC-dextramer complexes used were: HLA-A2/Bcl-X(L)$_{173–182}$ (YLNDHLEPWI) and HLA-A2/HIV-1 pol$_{476–484}$ (ILKEPVHGV). CD8/Bcl-X(L)$_{173–182}$ positive cells were sorted as single cells for cloning.

Expansion of Bcl-X(L)$_{173–182}$ specific T cell clones

The CD8/Bcl-X(L) $_{173–182}$ positive cells were sorted as single cells into 96 well plates (Nunc) containing 10^5 cloning mix cells/well. The cloning mix was prepared containing 10^6 ml irradiated (20 Gy) lymphocytes from three healthy donors in X-vivo with 5% heat-inactivated human serum, 1 μg/ml PHA (Peprotech) and 120 U/ml IL-2. The plates were incubated at 37°C/5% CO_2. Every 3–4 days 50 μl fresh media was added containing IL-2 to a final concentration of 120 U/ml. Growing clones were further expanded using cloning mix cells (5×10^4 cells/well). The clones were tested for specificity and cytotoxic potential in cytotoxicity assays after expansion.

Cytotoxicity assay

Conventional ^{51}Cr-release assays for CTL-mediated cytotoxicity were carried out as described elsewhere [1]. Target cells were T2 cells, the HLA-A2- positive breast cancer cell line MDA-MB-231, the HLA-A2 positive colon cancer cell line CAMA-1, the HLA-A2-negative breast cancer cell line ZR-75–1 (all available at ATCC) and the HLA-A2-positive melanoma cell line FM3 [8] with or without added mAb W6/32 (Dako). Cancer cell lines were examined for expression of Bcl-X(L) by RT-PCR. In addition, we tested the ability of the Bcl-X(L) specific T cells to lyse malignant cell, as well as B- and T cells, isolated directly ex vivo from a patient with AML. We depleted B and T cells from the bone marrow of the AML patient using CD19+ and CD3+ microbeads (MACS), respectively. The highly enriched AML-blasts (CD3-, CD19-) and the isolated B cells (CD19+) and T cells (CD3+) were used as target cells in a standard ^{51}Cr release assay at effector:target (E:T) ratio 1:1.

Results

Bcl-X(L) response in the breast cancer patient (MA41508)

PBL from a breast cancer patient was analyzed for response against the HLA-A2 restricted peptide epitope Bcl-X(L)$_{173–182}$ in a direct ELISPOT in the absence of pre-stimulation. A spontaneous CTL response against Bcl-X(L)$_{173–182}$ was detectable ex vivo (Fig. 1a). The patient's PBL consisted of 25.2% CD8 T cells and 38.4% CD4 T cells. The response constituted 0.1% of CD8 T cells. In addition, the frequency of Bcl-X(L)$_{173–182}$ specific CTL was evaluated by flow cytometry, using MHC-dextramer staining. Bcl-X(L)$_{173–182}$ specific cells were detectable by flow cytometry directly ex vivo, constituting 0.03% of CD8 positive T cells (Fig. 1b). As control the same cultures were stained with an HLA-A2/HIV-1 pol$_{476-484}$ dextramer.

Isolation and cloning of Bcl-X(L)$_{173–182}$ specific CD8 T cells

To further characterize the functional capacity of Bcl-X(L) positive T cells, HLA-A2/Bcl-X(L)$_{173–182}$ dextramer positive CD8 cells were sorted by FACS. The cells were sorted as single cells into 96 well plates containing a mixture of irradiated PBL from three healthy donors, PHA and IL-2. Out of twenty-isolated dextramer positive CD8 T cells, we were able to expand ten T-cell clones (Fig. 2). The specificity of the expanded CD8 T-cell clones was analyzed using a standard ^{51}Cr-release assay. To this end, T2 cells loaded with either Bcl-X(L)$_{173–182}$ peptide or an irrelevant peptide were used as target cells. Five CD8 T-cell clones (Clone 8, 9, 10, 11, and 12) effectively lysed T2 cells pulsed with Bcl-X(L)$_{173–182}$ without killing of T2 cells pulsed with an irrelevant peptide (Fig. 2). One of these Bcl-X(L)$_{173–182}$ specific CD8 T-cell clones [Clone 9 (*)] were expanded for further analyses. The remaining five expanded clones (Clone 7, 13, 15, 17, and 18) did not show specific lysis against T2 cells pulsed with Bcl-X(L)$_{173–182}$ peptide (Fig. 2).

Lytic capacity of Bcl-X(L)$_{173–182}$ specific CD8 T cells

The lytic capacity of the Bcl-X(L)$_{173–182}$ specific clone 9 was further analyzed in standard ^{51}Cr release assays to assess the E:T ratio and peptide concentration needed for efficient lysis. To this end, T2 cells loaded with

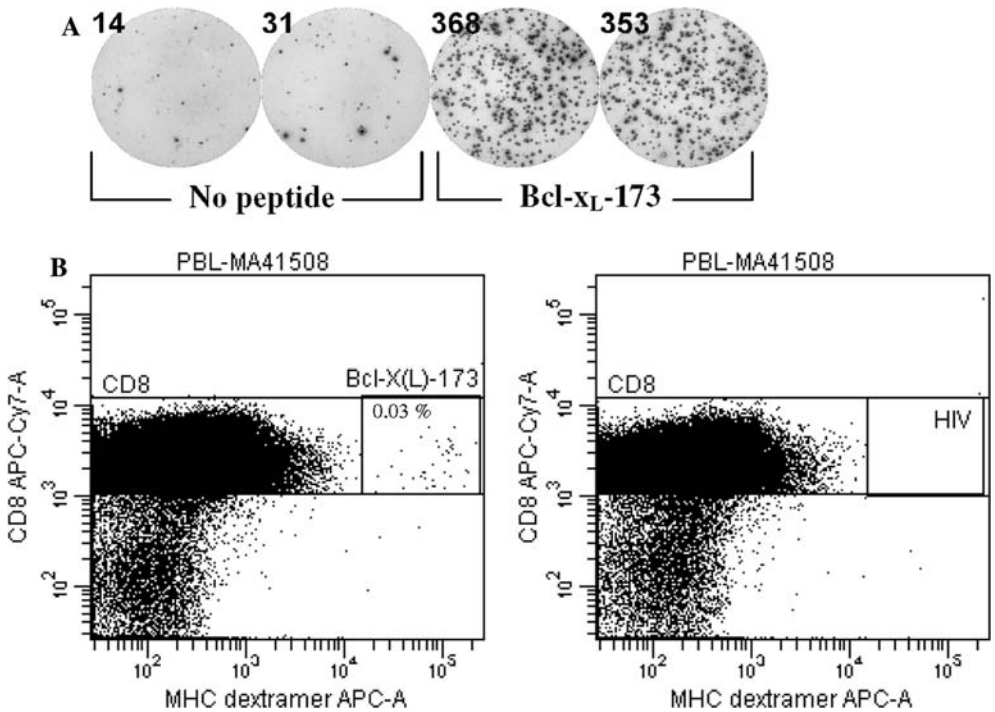

Fig. 1 Analysis of Bcl-X(L)-specific, CD8 positive T cells in PBL from a breast cancer patient. **a** PBL from a breast cancer patient was analyzed by ELISPOT ex vivo either with or without the Bcl-X(L)$_{173–182}$ peptide, 10^6 PBL/well in doublets. The number of spots was counted using the Immunospot Series 2.0 Analyzer (CTL Analysers). **b** PBL from the patient was analyzed ex vivo by flow cytometry to identify Bcl-X(L)$_{173–182}$ specific CD8 T cells using the dextramer complex HLA-A2/Bcl-X(L)$_{173–182}$-APC, 7-AAD-PerCP, CD3-FITC, and CD8-APC-Cy7. The detectable population of dextramer positive CD8 T cells was sorted as single cells. The dextramer complex HLA-A2/HIV-1 pol$_{476–484}$-APC was used as negative control

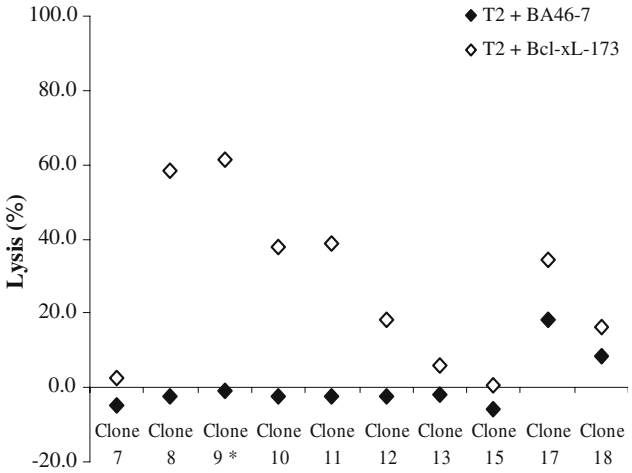

Fig. 2 Specificity and cytolytic capacity of expanded CD8 T-cell clones. HLA-A2/Bcl-X(L)$_{173–182}$ dextramer positive CD8 T cells were sorted as single cells by FACS and expanded for 3 weeks. The expanded T-cell clones were analyzed for specificity in a ^{51}Cr-release assay measuring cell lysis of T2 cells pulsed with Bcl-X(L)$_{173–182}$ peptide or an irrelevant peptide (BA46$_{97–105,}$ GLQHWVPEL)

either Bcl-X(L)$_{173–182}$ peptide or BA46$_{97–105}$ peptide served as targets. This assay revealed that only T2 cells pulsed with Bcl-X(L)$_{173–182}$ were killed. Furthermore, the clone efficiently lysed T2 cells at an E:T ratio of 1:1 pulsed with down to 10^{-7} mM peptide (Fig. 3 a, b).

In addition, clone 9 was further tested for the capacity to kill cancer cell lines of different origin (Fig. 4). Thirty cancer cell lines (breast cancer, melanoma, colon and AML) were examined for Bcl-X(L) expression by RT-PCR and all expressed Bcl-X(L) (data not shown). Subsequently, the HLA-A2 positive breast cancer cell line MDA-MB-231, the HLA-A2 positive colon cancer cell line CAMA-1, the HLA-A2-positive melanoma cell line FM3 and the HLA-A2 negative breast cancer cell line ZR-1-75 were used as target cells. The Bcl-X(L)$_{173–182}$ specific clone 9 efficiently lysed all HLA-A2-positive cells, whereas no cytotoxicity was observed against the HLA-A2-negative breast cancer cell line ZR-1-75 (Fig. 4a). To further examine the antigen specificity and HLA restriction of clone 9 we examined the effect of blocking HLA-class I by addition of the specific monoclonal antibody W6/32 to the melanoma cell line FM3. Lysis could be completely blocked by pre-incubation of target cells with W6/32 (Fig. 4b). Likewise, the addition of cold (unlabeled) T2 cells pulsed with the Bcl-X(L)$_{173–182}$ peptide blocked the lysis of FM3 tumor cells, whereas the addition of T2 cells without peptide only showed a limited dilution effect.

Reactivity against cancer cells and normal cells ex vivo

We tested the ability of Bcl-X(L)$_{173–182}$ specific CTL to lyse AML-blasts enriched directly ex vivo from the bone marrow of an HLA-A2 positive AML patient (Fig. 5). To this end, we depleted T cells (CD3+) and B cells (CD19+) from the bone marrow. The Bcl-X(L)$_{173–182}$ specific CTL showed tumor specificity by selectivity lysing the enriched leukemia cells, but not the non-malignant B cells (CD19+) and T cells (CD3+) from the same patient (Fig. 5).

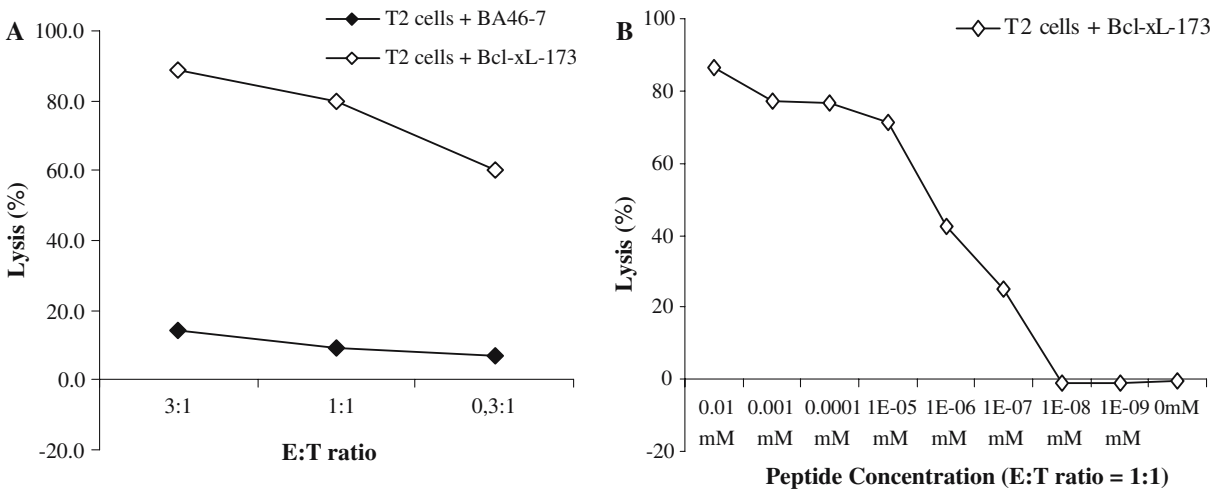

Fig. 3 The cytotoxic capacity of a Bcl-X(L)$_{173–182}$ specific CD8 T-cell clone (Clone 9). The Bcl-X(L)$_{173–182}$ specific clone 9 was expanded for additional 2 weeks before the cytotoxic potential was examined further in ^{51}Cr-release assays. **a** Cell lysis of T2 cells pulsed with Bcl-X(L)$_{173–182}$ peptide or an irrelevant peptide (BA46$_{97–105,}$GLQHWVPEL) in three E:T ratios. **b** Cell lysis of T2 cells pulsed with different concentrations of Bcl-X(L)$_{173–182}$ peptide at the E:T ratio 1:1

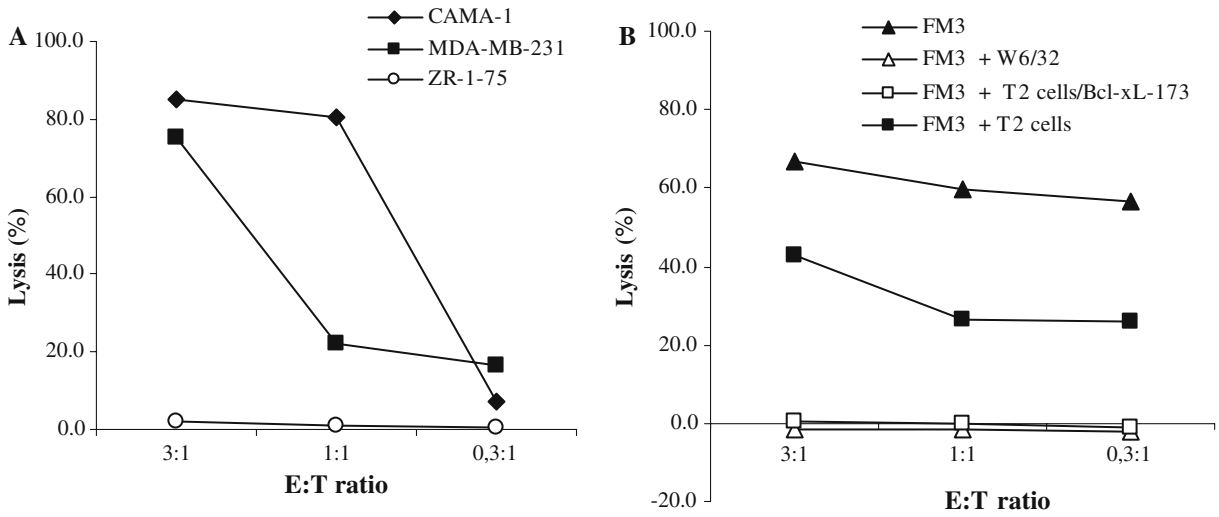

Fig. 4 Antigen-specific lysis of human tumor cell lines endogenously expressing Bcl-X(L). **a** Cell lysis of the HLA-A2-positive breast cancer cell line MDA-MB-231, the HLA-A20-positive colon cancer cell line CAMA-1, and the HLA-A2-negative breast cancer cell line ZR-1–75. **b** Cell lysis of the HLA-A2 positive melanoma cancer cell line FM3 without and with the HLA specific antibody W6/32. In addition, unlabeled T2 cells either with or without the Bcl-X(L)$_{173–182}$ peptide were added to FM3 cells at a ratio of inhibitor to target cells of 20:1. All^{51}Cr-release assays were performed in three E:T ratios

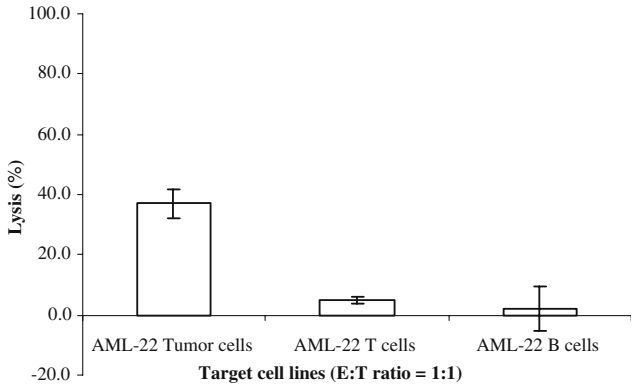

Fig. 5 Lysis of ex vivo enriched AML cells and non-malignant cells cell lysis of AML-blasts enriched from an HLA-A2 positive AML patient. AML-blasts, B cells, and T cells were depleted from the bone marrow of the AML patient using CD19+ and CD3+ microbeads, respectively. The highly enriched AML-blasts (CD3-, CD19-) and the isolated B cells (CD19+) and T cells (CD3+) were used as target cells in a standard ^{51}Cr release assay at E:T ratio 1:1

Discussion

Recently, we identified spontaneous immune responses against Bcl-X(L) in cancer patients with cancer of different origin [3]. We were able to detect spontaneous HLA-A2-restricted cytotoxic T-cell responses against peptide epitopes derived from Bcl-X(L) by means of ELISPOT and flow cytometry stainings in PBL from breast cancer patients, melanoma patients and pancreatic cancer patients. In contrast, no responses were detected against any of the Bcl-X(L) epitopes in any healthy controls. In addition, we were able to enrich Bcl-X(L) specific T cells by flow-cytometry and show that the resulting T-cell culture were weakly cytotoxic against a HLA-matched melanoma cell line and a breast cancer cell line. However, the critical issue in the reverse immunology strategy for CD8 T cell target antigen identification is the clear demonstration that the peptide(s) is (are) processed and naturally presented in the tumor cells. Thus, to make a clean demonstration of this point we cloned Bcl-X(L) specific T cells and examined the specificity and immunogenicity of such CD8 T cells cells. First, we identified a breast cancer patient hosting a strong spontaneous response against Bcl-X(L) as confirmed by ELISPOT and flow cytometry. The frequency of Bcl-X(L)$_{173–182}$ specific T cells determined by flow cytometry was comparable to the level of IFN-γ secretion detected when analyzing the same culture by means of ELISPOT, suggesting that the majority of the Bcl-X(L)$_{173–182}$ specific T cells posses functional capacity.

The Bcl-X(L)$_{173–182}$ specific clone 9 was obtained after isolation and in vitro expansion of T cells from the patient. These T cells not only killed target cells pulsed with the antigenic peptide but also recognized tumor cells endogenously expressing the Bcl-X(L) protein in an antigen specific and HLA-A2 restricted manner. Thus, whereas the clone very effectively lysed HLA-A2 positive cancer cell lines of different origin, there was no lysis of the HLA-A2 negative breast cancer cells ZR-1-75. Subsequently, the presentation of

Bcl-X(L) peptide in context of HLA-A2 molecules on the tumor cells is necessary for the efficient lysis of target cells. The HLA-restriction of clone 9 was confirmed using monoclonal antibodies in an inhibition assay. The killing of tumor cells of different origin underlines the universal characteristics of this tumor antigen.

Importantly, the Bcl-X(L) restricted clone did not only lyse in vitro generated tumor cell lines, but in addition lysed ex vivo enriched AML cells but spared purified B- and T cells from the same AML patient. Since T cells and B cells normally express Bcl-X(L) following activation, Bcl-X(L) cannot be considered to be a cancer-specific protein and caution is required when targeting this protein in vaccination therapies. However, our findings indicate that although non-malignant, B cells, and T cells express Bcl-X(L), they are not lysed by Bcl-X(L) specific T cells. Similar findings have been reported for another anti-apoptotic protein, survivin. Thus, although activated B and T cells express survivin, survivin-specific CTL did not recognize and kill such cells ex vivo [12].

Harnessing of the immune system for the battle against cancer has been the focus of tremendous research efforts over the past two decades. Multiple means to achieve this goal have been investigated, including adoptive transfer of anti-tumor-reactive T cells, and systemic or localized administration of immune modulating cytokines. Likewise, the use of 'therapeutic' vaccines has been scrutinized aiming at inducing CTL specific for tumor-associated antigens presented by cancer cells in the context of HLA molecules. Surprisingly, until recently only limited attention has been focused on elucidating the most suitable targets for induction of clinically relevant anti-cancer immune responses. In this regard, anti-apoptotic molecules that enhance the survival of cancer cells and facilitate their escape from cytotoxic therapies represent prime candidates. Indeed, most malignancies are characterized by defects in apoptotic signaling cascades, e.g. an overexpression of proteins from the Bcl-2 family (e.g. Bcl-2, Bcl-X(L), or Mcl-1). Attempts to overcome the cytoprotective effects of Bcl-2 and Bcl-X(L) in cancer include three strategies: (a) shutting off gene transcription, (b) inducing mRNA degradation with antisense oligonucleotides, and (c) directly attacking the proteins with small-molecule drugs [14, 15]. In addition to the above-mentioned means of targeting, immune-mediated tumor destruction is emerging as an interesting modality to treat cancer patients. Notably, lymphoablation, e.g., induced by chemotherapy, enhances the efficacy of adoptive T-cell transfer. Subsequently, it may also increase a vaccination induced T-cell response [5, 6, 9, 11] further emphasizing the possible synergic effects of Bcl-X(L) based immunotherapy and traditional therapy.

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