

# Spontaneous Immunity against Bcl-x<sub>L</sub> in Cancer Patients<sup>1</sup>

Mads Hald Andersen,<sup>2\*</sup> Sine Reker,\* Pia Kvistborg,\* Jürgen C. Becker,<sup>†</sup> and Per thor Straten\*

It is well-established that peptide epitopes derived from human tumor-associated Ags can be recognized by CTL in the context of the MHC molecule. However, the vast majority of Ags described are not vital for survival and growth of the tumor cells, and immunoselection of Ag-loss variants during immunotherapy has been demonstrated in several cases. Malfunctions in death pathways observed in human cancers are often due to overexpression of antiapoptotic proteins in the Bcl-2 protein family, i.e., Bcl-2, Mcl-1, and Bcl-x<sub>L</sub>. These antiapoptotic proteins are implicated in cancer development, tumor progression, and drug resistance. The general overexpression of the antiapoptotic members of the Bcl-2 family in cancer and the fact that down-regulation or loss of expression of these proteins as a means of immune escape would impair sustained tumor growth makes them very attractive targets for anticancer immunotherapy. Recently, we identified spontaneous T cell responses against Bcl-2- and Mcl-1-derived peptides in patients suffering from cancers of different origin. In this study, we demonstrate that Bcl-x<sub>L</sub> is a target for T cell recognition in cancer patients. Thus, we describe spontaneous HLA-A2-restricted cytotoxic T cell responses against peptide epitopes derived from Bcl-x<sub>L</sub> by means of ELISPOT and flow cytometry stainings, whereas no responses were detected against any of the Bcl-x<sub>L</sub> epitopes in any healthy controls. Moreover, Bcl-x<sub>L</sub>-specific T cells are cytotoxic against HLA-matched cancer cells of different origin. Thus, cellular immune responses against apoptosis inhibitors like the Bcl-2 family proteins appear to represent a general feature in cancer. *The Journal of Immunology*, 2005, 175: 2709–2714.

**A**lmost all malignancies are characterized by defects in apoptosis signaling. This renders the malignant cells resistant to endogenous apoptotic stimuli, as well as exogenous stimuli such as chemotherapeutic drugs and radiation. The defective apoptosis seen in human cancers often result from overexpression of antiapoptotic proteins in the Bcl-2 protein family, i.e., Bcl-2, Mcl-1, and Bcl-x<sub>L</sub>. The antiapoptotic protein Bcl-x<sub>L</sub> is produced from the long alternative splice form of the *bcl-x* gene, while proapoptotic Bcl-x<sub>S</sub> is derived from the short alternative splice form of the same gene (1). Bcl-x<sub>L</sub> plays an important role in cancer as it has been directly linked to resistance to conventional forms of therapies and poor prognosis (2). The functional inhibition of Bcl-x<sub>L</sub> 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<sub>L</sub> results in a multidrug-resistance phenotype. In view of this, strategies aimed at inhibiting the expression or function of anti-apoptotic proteins has gained considerable therapeutic attention, e.g., antisense oligonucleotides or small molecule Bcl-x<sub>L</sub> inhibitors (3, 4).

Increased expression of Bcl-x<sub>L</sub> has been reported in a variety of different malignancies including acute myeloid leukemia and multiple myeloma as well as solid cancers like bladder cancer, breast cancer, pancreatic cancer, and melanoma (2). The identification of tumor Ags whose expression is essential for the survival of tumor cells is a new avenue to prevent Ag-loss variants emerging due to

immunoselection, particularly during immune therapy. In the present study, we examined whether the Bcl-x<sub>L</sub> protein is a target for spontaneous T cell recognition in cancer patients.

## Materials and Methods

### Patients

PBL were isolated using Lymphoprep separation, HLA-typed (Department of Clinical Immunology, University Hospital, Copenhagen, Denmark), and frozen in FCS with 10% DMSO. None of the patients received immunotherapy before sampling of blood. Informed consent was obtained from the patients before any of these measures.

PBLs were collected from 18 HLA-A2-positive breast cancer patients presenting with progressive disease with distant metastases defining stage IV disease. Additionally, PBL were collected from two HLA-A2-positive pancreatic cancer patients presenting with progressive disease with distant metastases defining stage IV disease. Finally, PBL were collected from 6 HLA-A2 patients with advanced malignant melanoma. PBL from 12 HLA-A2-positive healthy individuals served as controls. Informed consent was obtained from the patients before any of these measures.

### Assembly assay for peptide binding to MHC class I molecules

The binding affinity of the synthetic peptides (Invitrogen Life Technologies) to HLA-A2 molecules, metabolically labeled with [<sup>35</sup>S]methionine, was measured in the assembly assay, as described previously (5). The assay is based on stabilization of the class I molecule after loading of different concentrations of peptide to the TAP-deficient cell line T2. Stably folded HLA molecules were immune-precipitated using the HLA class I-specific, conformation-dependent mAb W6/32, and separated by isoelectric focusing gel electrophoresis. MHC H chain bands were quantified using the ImageGauge Phosphorimager program (FUJI Photo Film). The intensity of the band is directly related to the amount of peptide-bound class I MHC complex recovered during the assay. Subsequently, the extent of stabilization of HLA-A2 is directly related to the binding affinity of the added peptide. The recovery of HLA-A2 was measured in the presence of 100, 10, 1, 0.1, 0.01 μM of the relevant peptide. The C<sub>50</sub> value was calculated for each peptide as the peptide concentration sufficient for half maximal stabilization.

### Ag stimulation of PBL

To extend the sensitivity of the ELISPOT assay, PBL were stimulated once in vitro before analysis (6, 7). At day 0, PBL were thawed and plated in 2 ml/well at a concentration of 2 × 10<sup>6</sup> cells in 24-well plates (Nunc) in X-vivo medium (BioWhittaker) with 5% heat-inactivated human serum in the presence of 10 μM peptide (GenScript). Two days later 20 IU/ml rIL-2

\*Tumor Immunology Group, Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark; and <sup>†</sup>Department of Dermatology, University of Würzburg, Würzburg, Germany

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<sup>2</sup> Address correspondence and reprint requests to Dr. Mads Hald Andersen, Tumor Immunology Group, Danish Cancer Society, Strandboulevarden 49, Dk-2100 Copenhagen, Denmark. E-mail address: mha@cancer.dk

(Chiron) was added to the cultures. The cultured cells were tested for reactivity in the ELISPOT on day 10.

#### IFN- $\gamma$ ELISPOT assay

The ELISPOT assay was used to quantify peptide epitope-specific IFN- $\gamma$ -releasing effector cells as described previously (8). Briefly, nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with anti-IFN- $\gamma$  Ab (1-D1K; Mabtech). The wells were washed, blocked by X-vivo medium before adding 10<sup>4</sup> stimulator T2 cells (with or without 10  $\mu$ M peptide) and effector cells at different concentrations. The plates were incubated overnight. The following day, medium was discarded and the wells were washed before addition of biotinylated secondary Ab (7-B6-1-Biotin; Mabtech). The plates were incubated for 2 h, washed, and Avidin-enzyme conjugate (AP-Avidin; Calbiochem/Invitrogen Life Technologies) was added to each well. Plates were incubated at room temperature (RT)<sup>3</sup> 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. The reaction was terminated by washing with tap water upon the appearance of dark purple spots. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers) and the peptide-specific CTL frequency could be calculated from the numbers of spot-forming cells. All assays were performed in triplicates for each peptide Ag. Responders are defined as average number of Ag-specific cells  $\pm$  0.5 SD >25 per 10<sup>5</sup> cells. The definition is based on a study of CTL responses against cancer/testis Ags in cancer patients and healthy controls by means of ELISPOT (9).

#### Granzyme B (GrB) ELISPOT

The Granzyme B (GrB) ELISPOT assay was used for measuring Ag-specific CTL cytotoxicity as described (10). Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45; Millipore) were coated with GrB Capture Ab (BD Biosciences). The wells were washed and blocked by X-vivo medium with 5% human serum. The cells were added at different cell concentrations. T2 cells and peptides were then added to each well and the plates were incubated 4 h, medium was discarded, and the wells were washed before addition of GrB Detection Ab (BD Biosciences). The plates were incubated for 2 h, washed, and Avidin HRP (BD Biosciences) was added to each well. Plates were incubated at RT for 1 h; AEC Substrate Reagent (BD Biosciences) was added to each well and incubated at RT for 5–10 min. The reaction was terminated by washing with tap water upon the appearance of red spots. The spots were counted and the peptide-specific CTL frequency was calculated as it was for the IFN- $\gamma$  ELISPOT. All assays were performed in duplicate or triplicate for each peptide Ag.

#### Flow cytometry (FACS)

CD8 stainings were performed with fresh PBL from cancer patients. For pentamer stainings, PBL from breast cancer patients were stimulated once in vitro with Bcl-x<sub>L173–182</sub> peptide. In some experiments the CD8<sup>+</sup> cells were isolated from PBL using the Dynal CD8 negative isolation kit (Dynal Biotech) at day 7. The resulting T cell cultures were stained with PE coupled Pro5 MHC pentamers (ProImmune), followed by Ab staining with the fluorochrome-coupled mAbs: CD8-allophycocyanin and CD3-FITC (BD Immunocytometry Systems). Both stainings were performed in PBS + 2% FCS, for 30 min, 4°C, in the dark. The Pro5 MHC pentamer complexes used were: HLA-A2/Bcl-x<sub>L173–182</sub> (YLNDHLEPW) and HLA-A2/HIV-1 pol<sub>476–484</sub> (ILKEPVHGV). The samples were analyzed on BD FACS aria, using DIVA software (BD Biosciences). CD8/pentamer-positive cells were sorted and stimulated with Dynabeads CD3/CD28 T cell expander beads (Dynal Biotech) and IL-2 before analysis.

#### Cytotoxicity assay

Conventional <sup>51</sup>Cr-release assays for CTL-mediated cytotoxicity was conducted as described elsewhere (11). Target cells were T2 cells, the HLA-A2-positive breast cancer cell line MDA-MB-231 (available at American Type Culture Collection (ATCC)), the HLA-A2-positive melanoma cell line FM3 (12), the HLA-A2-negative breast cancer cell line ZR-75-1 (available at ATCC) and the HLA-A2-negative melanoma cell line FM79 (12). All cancer cell lines expressed Bcl-x<sub>L</sub> as examined by RT-PCR (data not shown).

## Results

### Binding of Bcl-x<sub>L</sub>-derived peptides to HLA-A2

The *bcl-x* gene is transcribed into two mRNAs through alternative splicing. The antiapoptotic protein Bcl-x<sub>L</sub> is produced from the

long alternative splice, while proapoptotic Bcl-x<sub>S</sub> is derived from the short alternative splice form of this gene (1). The protein product of the larger BCL-x<sub>L</sub> differs from the Bcl-x<sub>S</sub> protein by an inserted region (aa 126–188). Thus, to investigate whether Bcl-x<sub>L</sub> is a natural target for T cells in cancer patients, we scrutinized this inserted region (including 9 aa at each end) for putative HLA-A2 epitopes using the main HLA-A2-specific anchor residues (13). Subsequently, we synthesized seven Bcl-x<sub>L</sub> deduced peptides and examined these for binding to HLA-A2 by comparison with the HLA-A2 high affinity-positive control epitope from HIV-1 pol<sub>476–484</sub> (ILKEPVHGV) by the assembly assay. The peptide concentration required for half maximal recovery of class I MHC molecules (C<sub>50</sub> value) was 0.6  $\mu$ M for the HIV-1 pol<sub>476–484</sub> (Table I). None of the peptides bound with similar high affinity as the positive control. The best binding peptides Bcl-x<sub>L173–182</sub>, Bcl-x<sub>L118–126</sub>, and Bcl-x<sub>L169–178</sub> bound with 5- to 7-fold less affinity compared with the positive control (C<sub>50</sub> = 3, 4, and 4  $\mu$ M, respectively) (Table I). Two peptides Bcl-x<sub>L165–174</sub> and Bcl-x<sub>L161–170</sub> bound only HLA-A2 very weakly (C<sub>50</sub> > 100  $\mu$ M), whereas Bcl-x<sub>L158–166</sub> and Bcl-x<sub>L141–150</sub> did not show any measurable binding to HLA-A2 (Table I).

### Spontaneous CTL responses against Bcl-x<sub>L</sub>-derived peptides

To examine the natural immunity of Bcl-x<sub>L</sub> in cancer patients, we scrutinized PBL from HLA-A2<sup>+</sup> cancer patients of different origin by means of ELISPOT against the HLA-A2-binding peptides. This method has previously been shown to be highly effective to identify tumor-specific CTL in cancer patients (14–16). Indeed, strong and frequent CTL responses were detected against the HLA-A2-binding peptide Bcl-x<sub>L173–182</sub> (responders are defined as average number of Ag-specific cells  $\pm$  0.5 SD >25 per 10<sup>5</sup> cells). Thus, in 9 of 18 examined breast cancer patients we detected an immune response against Bcl-x<sub>L173–182</sub>, whereas in 2 of 6 examined HLA-A2<sup>+</sup> melanoma patients we detected an immune response against this peptide (Fig. 1A). As control, PBL from 12 healthy HLA-A2<sup>+</sup> individuals were examined. Importantly, no responses were detected against Bcl-x<sub>L173–182</sub> in any of the control individuals. More surprisingly, we additionally identified a few spontaneous responses in cancer patients of different origin against the weak HLA-A2-binding peptides Bcl-x<sub>L161–170</sub>, and Bcl-x<sub>L165–174</sub>. We detected a response in PBL from six breast cancer patients, and one examined pancreatic cancer patient against Bcl-x<sub>L161–170</sub> (Fig. 1B). Finally, in four breast cancer patients, two melanoma patients, and one pancreatic cancer patient, we detected a response against Bcl-x<sub>L165–174</sub> (Fig. 1C). No responses were detected against either Bcl-x<sub>L161–170</sub>, or Bcl-x<sub>L165–174</sub>, peptide in any of the healthy individuals. Overall, we were able to detect an immune response against at least one Bcl-x<sub>L</sub> peptide in 15 of 18 HLA-A2<sup>+</sup> breast cancer patients, 4 of 6 examined melanoma patients and 1 of 2 examined pancreatic cancer patients.

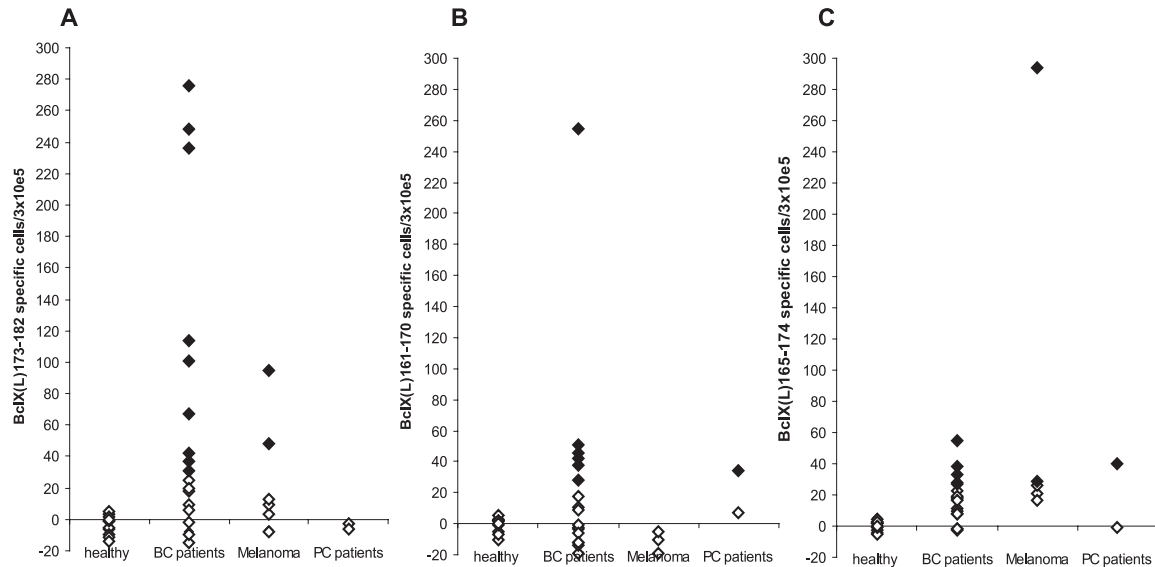
Table I. Peptides examined in this study

Peptide <sup>a</sup>	Sequence	C <sub>50</sub> ( $\mu$ M) <sup>b</sup>
HIV-1 pol <sub>476–484</sub>	ILKEPVHGV	0.6
Bcl-x <sub>L158–166</sub>	EMQVLVSRI	Not binding
Bcl-x <sub>L118–126</sub>	TAYQSFEQV	4
Bcl-x <sub>L173–182</sub>	YLNDHLEPWI	3
Bcl-x <sub>L165–174</sub>	RIAAWMATYL	>100
Bcl-x <sub>L169–178</sub>	WMATYLNDHL	4
Bcl-x <sub>L161–170</sub>	VLVSRIAAWM	>100
Bcl-x <sub>L141–150</sub>	VAFFSFGGAL	Not binding

<sup>a</sup> The subscripted value range indicates the position of the amino acids in the protein sequence.

<sup>b</sup> The C<sub>50</sub> value is the concentration of the peptide required for half maximal binding to HLA-A2.

<sup>3</sup> Abbreviations used in this paper: RT, room temperature; GrB, Granzyme B.



**FIGURE 1.** HLA-A2-restricted T cell responses against Bcl- $x_L$  as measured by IFN- $\gamma$  ELISPOT. PBL from 12 healthy individuals, 18 patients with breast cancer (BC patients), 6 melanoma patients, and 2 pancreatic cancer patients (PC patients) were analyzed. All individuals were HLA-A2 positive. The peptides Bcl- $x_{L173-182}$  (YLNDHLEPW1) (A), Bcl- $x_{L161-170}$  (VLVSRIAAMW) (B), and Bcl- $x_{L165-174}$  (RIAAMWATYL) (C) were examined. T lymphocytes were stimulated once with peptide before being plated at  $10^5$  cells per well in triplicates either without or with the relevant peptide. The average number of peptide-specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). Responders (defined as average number of Ag-specific spots  $\pm$  0.5 SD  $>$ 25 per  $10^5$  lymphocytes) are marked as  $\blacklozenge$ , whereas nonresponding individuals are marked as  $\diamond$ .

#### Direct ELISPOT

To examine whether the responses against Bcl- $x_{L173-182}$  was detectable in direct ELISPOT in the absence of prestimulation, we scrutinized PBL from six breast cancer patients in whom we had detected the strongest responses against this peptide with prestimulation. The direct ELISPOT was not sufficiently sensitive to detect responses against Bcl- $x_{L173-182}$  in two of the examined patients. However, in four patients responses against Bcl- $x_{173}$  were readily detectable. This enabled us to include a quantitative estimate of the actual numbers of Bcl- $x_{L173-182}$ -specific T cells in these patients (Fig. 2). We also included six healthy controls, but were not able to detect any responses in these individuals (Fig. 2).

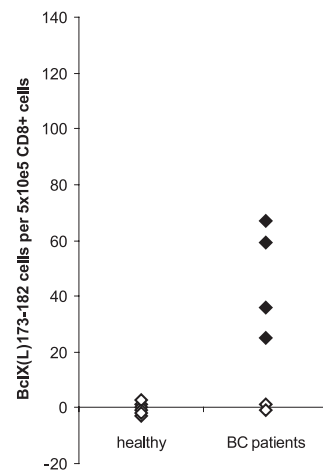
#### Bcl- $x_L$ -specific GrB release in PBL

Using the GrB ELISPOT we assessed whether the Bcl- $x_L$ -specific T cells detected in PBL exhibit cytotoxic function. Thus, PBL from two of the Bcl- $x_L$ -reactive breast cancer patients (nos. 35 and 36) were analyzed for reactivity against Bcl- $x_{L173-182}$ . In both patients, responses against Bcl- $x_{L173-182}$  could be detected with a frequency at  $\sim$ 50–100 peptide-specific CTL per  $3 \times 10^5$  cells (data not shown). As a control we included a patient (no. 17), in whom we could only detect a response against Bcl- $x_{L141-150}$  but not against Bcl- $x_{L173-182}$  in the IFN- $\gamma$  ELISPOT. As expected, no GrB release was detected against Bcl- $x_{L173-182}$  in breast cancer patient no. 17.

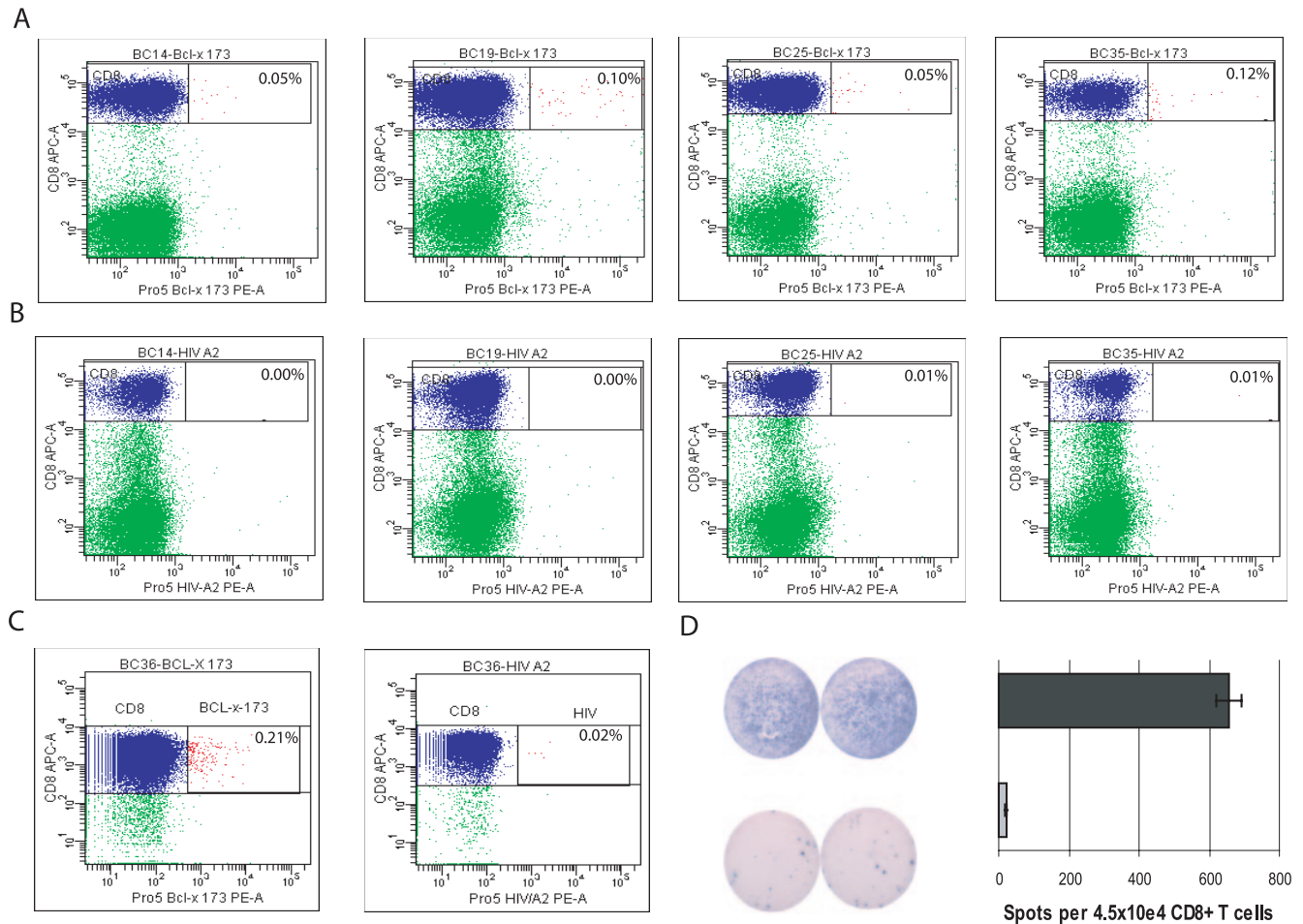
#### FACS analyses of Bcl- $x_L$ -specific T cells

The spontaneous occurrence of Bcl- $x_{L173-182}$ -specific CTL in PBL from breast cancer patients was further evaluated using FACS analyses and Pro5 MHC pentamer staining. PBL from four breast cancer patients were stained with the HLA-A2/BCL- $x_{L173-182}$  pentamer complex. FACS analyses revealed readily detectable populations of pentamer-positive T cells constituting between 0.05% and 0.12 of the CD8 $^+$  T cells (Fig. 3A). As a control the same cultures were stained with a HLA-A2/HIV pol pentamer

(Fig. 3B). In addition, PBL from a breast cancer patient (no. 36) was stimulated once in vitro with peptide and the CD8-positive cells were isolated. This culture was stained with the HLA-A2/BCL- $x_{L173-182}$  pentamer complex. FACS analyses revealed an easily detectable population of pentamer-positive T cells constituting 0.24% of the CD8 $^+$  T cells (Fig. 3C). In comparison, the same CD8 $^+$  T cells showed around 1.4% Bcl- $x_{L173-182}$ -specific, IFN- $\gamma$ -secreting CD8 $^+$  T cells when analyzed by means of ELISPOT (Fig. 3D).



**FIGURE 2.** Direct ELISPOT. PBL from six healthy controls and six breast cancer patients was analyzed directly in ELISPOT either without or with the peptide Bcl- $x_{L173-182}$ . The average number of peptide specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers). Responders (defined as average number of Ag-specific spots  $\pm$  0.5 SD  $>$ 25 per  $5 \times 10^5$  CD8 $^+$  cells) are marked as  $\blacklozenge$ , whereas nonresponding individuals are marked as  $\diamond$ .



**FIGURE 3.** Analysis of Bcl-x<sub>L</sub>-specific, CD8-positive T cells in PBL from breast cancer patients. *A*, PBL from four breast cancer patients were stimulated once in vitro with Bcl-x<sub>L</sub>173–182 and analyzed by flow cytometry to identify Bcl-x<sub>L</sub>173–182-specific CD8<sup>+</sup> T cells using the pentamer complex HLA-A2/Bcl-x<sub>L</sub>173–182-PE, CD3-FITC, and CD8-allophycocyanin. *B*, Negative controls, using the pentamer complex HLA-A2/HIV pol<sub>476–484</sub>-PE, and CD8-allophycocyanin. *C*, PBL from the patient BC36 were stimulated once with Bcl-x<sub>L</sub>173–182 in vitro and the CD8<sup>+</sup> T cells were isolated before analysis by flow cytometry using the pentamer complex HLA-A2/Bcl-x<sub>L</sub>173–182-PE, and CD8-allophycocyanin. The HLA-A2/HIV pentamer was used as a negative control. *D*, The T cell culture was additionally analyzed by means of ELISPOT. A total of  $4.5 \times 10^4$  CD8<sup>+</sup> T cells were plated in duplicates with or without the Bcl-x<sub>L</sub>173–182 peptide.

#### Functional capacity of Bcl-x<sub>L</sub>-specific T cells

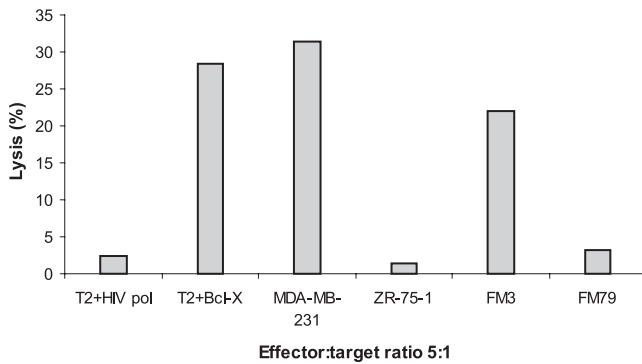
To further characterize the functional capacity of Bcl-x<sub>L</sub>-reactive CTL, HLA-A2/BCL-x<sub>L</sub>173–182 pentamer-positive CD8 cells from the breast cancer patient no. 36 were isolated by flow cytometry. The cells were stimulated once with CD3/CD28 expander beads and IL-2 and on day 8 the capacity of the cells to kill peptide-loaded T2 cells was tested in standard <sup>51</sup>Cr-release assays. To this end, T2 cells loaded with either Bcl-x<sub>L</sub>173–182 peptide or HIV-1 pol<sub>476–484</sub> served as targets. This assay revealed that only T2 cells pulsed with Bcl-x<sub>L</sub>173–182 were killed (Fig. 4). The isolated and in vitro-stimulated Bcl-x<sub>L</sub>173–182-reactive T cells were further used to test the capacity to kill the HLA-A2-positive breast cancer cell line MDA-MB-231 and the HLA-A2-positive melanoma cell line FM3. The enriched T cells efficiently lysed both HLA-A2-positive cells, whereas, in contrast, no cytotoxicity was observed against either the HLA-A2-negative breast cancer cell line ZR75-1 or the HLA-A2-negative melanoma cell line FM79 (Fig. 4).

#### Discussion

To test the hypothesis of Bcl-x<sub>L</sub> as a broadly expressed T cell target we initially followed a “reverse immunology” approach. We scanned the inserted region of Bcl-x<sub>L</sub> protein for the presence of

HLA-A2 peptide-binding motifs and used these to search for specific T cell responses in cancer patients. To this end, natural T cell reactivity was detected against Bcl-x<sub>L</sub> in PBL from patients suffering from unrelated tumor types, i.e., breast cancer and melanoma. These data indicates that CTL-defined Bcl-x<sub>L</sub> epitopes might be broadly applicable in therapeutic vaccinations against cancer and consequently of substantial immunotherapeutic value. We identified CTL responses against three peptide epitopes from Bcl-x<sub>L</sub> by means of IFN-γ ELISPOT. Taken together, PBL from 15 of 18 of the breast cancer patients, 4 of 6 melanoma patients, and 1 of 2 pancreatic cancer patients possessed Bcl-x<sub>L</sub>-specific CTL. Notably, the patients without measurable response might host a Bcl-x<sub>L</sub> response below the detection limit of the ELISPOT or restricted to other HLA molecules. However, it would be highly interesting to investigate the correlation between the expression of Bcl-x<sub>L</sub> and the immune response. Unfortunately, we have not been able to perform such studies due to lack of tumor material.

It has been shown that restimulation of the patients’ lymphocytes in vitro with peptide before the ELISPOT assay significantly amplifies the immune activity (17). However, this approach, as compared with ELISPOT analyses conducted ex vivo, is highly dependent on the growth capacity of the T cells being analyzed.



**FIGURE 4.** Cytolytic capacity of Bcl- $x_L$ -specific CTL. HLA-A2/BCL- $x_{L173-182}$  pentamer-positive CD8 cells from breast cancer patient no. 36 were isolated by flow cytometry and expanded for a week. The cultured T cells were analyzed for specific lysis of T2 cells pulsed with HIV pol<sub>476-484</sub> or BCL- $x_{L173-182}$  peptide, the HLA-A2-positive breast cancer cell line MDA-MB-231, the HLA-A2-negative breast cancer cell line ZR75-1, the HLA-A2-positive melanoma cell line FM3, and the HLA-A2-negative melanoma cell line FM79.

Clearly, responses measured without prestimulation of PBL may reflect in vivo conditions more accurately.

Subsequently, we examined whether the responses against Bcl- $x_{L173-182}$  was detectable in direct ELISPOT in the absence of prestimulation in six breast cancer patients in whom we had detected strong responses against this peptide after an in vitro stimulation. The direct ELISPOT was not sufficiently sensitive to detect responses against Bcl- $x_L$  in two of the examined patients. However, in four patients responses against Bcl- $x_L$  were readily detectable. Therefore, it appears that, using the less quantitative prestimulation approach, we have detected responses that otherwise would have been left undetected without changing the general picture of spontaneous immune responses against Bcl- $x_L$  in cancer patients.

The IFN- $\gamma$  ELISPOT assay is one of the most useful techniques for immunological monitoring of CTL responses and has gained increased application as a measure of specific T cell activation. However, it does not assess cell-mediated cytotoxicity directly as IFN- $\gamma$  secretion is not limited to only cytolytic cells. Thus, although it has been shown that IFN- $\gamma$  ELISPOT reactivity in most cases correlates with the capacity to exhibit cytotoxic function, the formal proof for this notion can only be obtained directly. GrB is a key mediator of target cell death via the granule-mediated pathway and the GrB ELISPOT assay was recently demonstrated to provide an estimation of cytotoxic effector cell frequency (18). Additionally, unlike the IFN- $\gamma$  ELISPOT assay, the GrB ELISPOT directly measures the release of a cytolytic protein. In the present study we took advantage of the GrB ELISPOT assay to demonstrate that the Bcl- $x_L$ -specific CTL in the patients PBL are indeed cytotoxic effector cells. This was confirmed by the conventional chrome release assay. Moreover, the Bcl- $x_L$ -specific T cells isolated from a breast cancer patient were capable of lysing HLA-matched tumor cells of different origin, i.e., melanoma and breast cancer. To further prove this notion it would be exciting to grow Bcl- $x_L$ -specific T cells in vitro and test the effector function of these cells by cold targets consisting of T2 cells loaded with the Bcl- $x_{L173-182}$  peptide. Finally, the clean demonstration of this point may consist of using Bcl- $x_L$  low target cells transfected with a plasmid directing expression of the *Bcl-x<sub>L</sub>* gene. The authors currently address these issues.

In addition, the presence of Bcl- $x_L$ -specific CD8<sup>+</sup> cells in PBL from cancer patients was confirmed by CD8/pentamer FACS stainings. Interestingly, the frequency of Bcl- $x_L$ -specific T cells mea-

sured by multimer labeling was 0.24% and that measured in the same culture by ELISPOT was 1.4%. Tetramers detect both Ag-experienced and naive T cells whereas the ELISPOT assay only measures the number of memory and effector cells. Furthermore, it is well-described that not all specific T cells secrete IFN- $\gamma$  upon short-term stimulation with peptide. Although, tetramer staining and ELISPOT, at least in certain settings, yielded the same outcome in numbers of Ag-specific T cells, this is certainly not always the case. For instance, in patients with advanced cancer, tumor-reactive T cells may have become anergic. Such T cells would still be visible with tetramers, but not through cytokine staining. In that regard, Whiteside et al. (19) have reported that the tetramer assay consistently detected the highest numbers of peptide-specific CD8<sup>+</sup> T cells compared with ELISPOT. Still, the sensitivity of the tetramer staining are more dependent on the binding affinity of the peptide to the HLA molecule (the stability of the tetramer) and on the avidity of the specific T cells. These might be the reasons for the observed higher sensitivity of the ELISPOT in our hands. Recently, Speiser et al. (20) examined a standardized approach for immune monitoring of Ag-specific CD8<sup>+</sup> T cells within PBLs. The detection limit for multimer-based flow cytometry identification of Ag-specific CD8<sup>+</sup> T cells was 0.01% (100 in 1 million). This value is similar to that determined independently by other groups. The number of background spots in the IFN- $\gamma$  ELISPOT assay was below 0.003% (30 in 1 million) CD8<sup>+</sup> T cells in the majority of assays, suggesting that the sensitivity could be about 3-fold higher than that of multimer-based assays. However, due to the larger background fluctuation, the experimentally determined detection limit for IFN- $\gamma$  ELISPOT assays turned out to be ~0.01%.

The selection of Ag-deficient tumors due to an immune-generated selection pressure is a well-recognized limitation when targeting Ags that play an insignificant role in relation to tumor cell growth and survival (21–23). The attractiveness of using Bcl- $x_L$  for vaccination purposes relies on the fact that down-regulation or loss of expression of this protein will impair sustained tumor growth, because survival of Bcl- $x_L$ <sup>+</sup> tumor cells requires functionally active Bcl- $x_L$ . In addition, because elevated expression of Bcl- $x_L$  in cells is correlated with drug resistance, the combination of a Bcl- $x_L$ -based immunotherapy with cytotoxic chemotherapy is a very appealing way to treat cancer. This includes acute myeloid leukemia patients with poor prognosis and in advanced and relapsed cases of multiple myeloma (2, 24). In multiple myeloma patients, expression of Bcl- $x_L$  in plasma cells has been found to correlate with decreased response to chemotherapy (4). In nonhematological cancers, elevated levels of Bcl- $x_L$  have been demonstrated in bladder transitional cell carcinoma, correlated with poor remission after radiotherapy. Likewise, in pancreatic cancer and melanoma, overexpression of Bcl- $x_L$  is associated with shorter patient survival (25).

Recently, we described that both Mcl-1 and Bcl-2 are the target for spontaneous T cell reactivity in patients with various cancers (26, 27). We believe that our reports taken together prove that cellular immune responses against this group of proteins represent a general feature in cancer. In an attempt to maximize the impact of immunotherapy, an exciting strategy would be to consider the expression profile and prognostic significance of the chosen target in the particular disease, or disease stage, being treated. Thus, while coexpression of Bcl-2, Mcl-1, and Bcl- $x_L$  is seen in some cancers, or a particular stage of disease, other cancers exhibit exclusive expression of one or the other protein.

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M. H. Andersen, J. C. Becker, and P. thor Straten are all shareholders in Survac ApS, which holds a patent application describing the use of Bcl-x<sub>L</sub> in immunotherapy of cancer.

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