A novel tumor antigen derived from enhanced degradation of Bax protein in human cancers

Cláudia Trindade Nunes¹, Kelly L. Miners¹, Garry Dolton¹, Chris Pepper², Chris Fegan², Malcolm D. Mason³ and Stephen Man¹

¹Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University; ²Department of Haematology, Cardiff University; ³Section of Oncology and Palliative Medicine, School of Medicine, Cardiff University, Cardiff, Wales, United Kingdom.

Key words: Epitopes, Immunotherapy, T cells,

Running title: Bax as a novel tumor antigen

Keywords: T cells, epitopes, immunotherapy, apoptosis

Financial Support: This research was supported by Cancer Research Wales (C.T.N., K.L.M., M.D.M. and S.M.), the Cardiff Partnership Fund (G.D. and S.M.) and Leukaemia and Lymphoma Research (C.P. and C.F.).

Corresponding author:
Dr. Stephen Man
Department of Infection, Immunity and Biochemistry, Henry Wellcome Building, Heath Park, School of Medicine, Cardiff University
Cardiff CF14 4XN, Wales, UK
mans@cardiff.ac.uk
Tel +44 (0)29 20687056
Fax +44 (0)29 20687303

Conflict of interest disclosure:
Cardiff University has filed a US patent on Bax peptides, with C.T.N., M.M. and S.M. as co-inventors. The remaining authors declare no competing financial interests.
Word Count: Abstract 212; Text 5095; 6 Figures and 1 Table
Abstract

Cancer cells frequently exhibit defects in apoptosis, which contribute to increased survival and chemotherapeutic resistance. For example, genetic mutations or abnormal proteasomal degradation can reduce expression of Bax which limits apoptosis. In cancers where abnormal proteasomal degradation of Bax occurs, we hypothesized that Bax peptides that bind to HLA class I molecules would be generated for presentation to CD8+ T cells. To test this hypothesis, we generated T cells against pooled Bax peptides derived from the blood of healthy human donors. Although T cell responses were of low frequency (0.15%), a CD8+ T cell clone (KSIVB17) was isolated that optimally recognized Bax136-144 peptide (IMGWTLDFL) presented by HLA-A*0201. KSIVB17 was able to recognize and kill a variety of HLA-matched cancer cells including primary tumor cells from chronic lymphocytic leukemia (CLL). No reactivity was seen against HLA-matched, non-transformed cells such as PHA blasts and skin fibroblasts. Furthermore, KSIVB17 reactivity corresponded with the proteasomal degradation patterns of Bax protein observed in cancer cells. Taken together, our findings suggest a new concept for tumor antigens based on regulatory proteins that are ubiquitously expressed in normal cells, but that have abnormally enhanced degradation in cancer cells. Bax degradation products offer candidate immune antigens in cancers such as CLL where increased Bax degradation correlates with poor clinical prognosis.
Introduction

One of the hallmarks of cancer is evasion of apoptosis (1) that is often promoted by the dysregulation of pro- and anti-apoptotic Bcl-2 family proteins (2). The expression of Bax or Bak appears to play a key role in suppressing cancer development and decreased Bax levels have been observed in a number of cancers (3-5). In addition, Bax protein levels are further reduced in advanced chronic lymphocytic leukemia (CLL) and contribute to chemo-resistance in this disease (6-9). Several studies have revealed that Bax has a shortened half-life and low expression in cancer cells due to its abnormal increased degradation by the ubiquitin-proteasomal pathway (3, 8, 9). Enhanced proteasomal degradation of Bax appears to be clinically relevant as it is most evident in the advanced stages of prostate cancer (3), and is correlated with poor prognosis in CLL (8).

Reduced expression and stability of Bax may also have immunological consequences. We hypothesized that the enhanced proteasomal degradation of Bax in cancer cells would generate peptides that can be presented by MHC class I molecules at the cell surface. By contrast, in healthy tissue Bax has a long half-life and is predominantly localized in the cytosol or loosely attached to outer mitochondrial membranes (10, 11). Normal expression and function of Bax is regulated by interactions with other members of the BCL2 family (12) rather than proteasomal degradation. So we would not expect significant quantities of Bax-derived peptides to be presented by MHC class I molecules in healthy cells. Thus, the degraded forms (peptides) of Bax in cancer cells could potentially serve as tumor-specific antigens. Other proteins involved in apoptosis have been proposed as tumor antigens (13), based on over-expression in cancer cells. Here we test the possibility that Bax, a
protein with low or unstable expression in cancer, can be a tumor-specific antigen for human T cells.
Materials and Methods

Blood samples
Healthy volunteer blood samples were collected locally and CLL samples were derived from clinics at the University Hospital of Wales and Llandough Hospital. All samples were collected with informed consent with ethical approval (South East Wales Research Ethics Committee (02/4806)). Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 (Sigma) centrifugation, as previously described (15).

Cell lines
The following were purchased from ATCC: CaSki, HepG2, U2OS and SAOS and passaged according to ATCC protocols within 6 months. The ATCC authenticates cell lines by DNA fingerprinting (STR analysis). The remaining cell lines used were not authenticated according to AACR guidelines but were assessed regularly (where appropriate) for EBV genes, HPV genes, HLA class I and morphology. These were provided by: BJAB, DG175, BV173 (Prof. M. Rowe via MRC Tissue Culture Facility, Cardiff); DU145 (Prof. W. Jiang, Cardiff); MDA231 (Prof L. Sherman, La Jolla); SK29mel and TK143 (Prof. Cerundolo, Oxford); C33A and C33A-HPV16 (Prof. Dillner, Malmo). Primary skin fibroblasts were obtained from healthy donors as described (14). SiHa-A2 (15), T2 and K562 cells were from laboratory stocks. All cell lines used were mycoplasma negative.

Bax peptides
Twenty-three candidate peptides were identified from the amino acid sequence of Bax using predictive computer algorithms for HLA binding. Fourteen peptides were predicted to bind to HLA-A*0201, with others to HLA-A3, HLA-B7, HLA-A1, HLA-B8 or HLA-B44 (supplemental data, Table S1). Individual Bax peptides (601-623,
Mimotopes) were dissolved in DMSO. Aliquots from these stock peptides were pooled (Bax pool 601-23) and stored at -80°C. Smaller pools of 6-9 peptides were made for epitope mapping. Higher purity peptides Bax p613 (96.4%) and p610 (95.7%) were synthesized (ProImmune) for dose-response experiments.

**T2 binding assay**

Peptide binding was determined by upregulation of HLA-A2 expression on T2 cells as previously described \(^{(15)}\). Briefly, 10^5 T2 cells were incubated with 50µg/ml peptide in serum-free RPMI overnight at 37°C. Upregulation of surface HLA-A2 was assessed by flow cytometry using MA2.1 antibody (IgG1, HLA-A2 specific, ATCC). HLA-A2 increase was calculated as follows: % Increase = [(mean fluorescence with peptide – mean fluorescence without peptide)/ mean fluorescence without peptide x100].

**Induction of Bax-specific T cells**

PBMC were immunomagnetically enriched for CD8^+ T cells with anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). After enrichment (>90% CD8^+), T cells were re-suspended at 2-2.5 x 10^6 cells/ml in RPMI-1640 (Sigma) supplemented with 5% human AB serum, 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 25 mM HEPES (AB-RPMI). The cells were cultured with Bax peptide pool (10µg/ml), irradiated autologous PBMC, 10 IU/ml rIL-2 (Proleukin), 1µg/ml CD28 (Beckman Coulter) and 0.5µg/ml CD49d antibodies (Serotec). Following 3 days of incubation, 1ml AB-RPMI media, supplemented with 40 IU/ml recombinant IL-2, was added to each well. T cell cultures were re-stimulated weekly with the peptide pool with autologous PBMC APC. On day 28, T cells were harvested and tested in ELISpot assays.
Isolation and cloning of peptide-specific T cells

IFN-γ secreting T cells from donor 1 were immunomagnetically enriched (MACS, Miltenyi Biotec). Briefly, 4x10^6 cultured T cells were activated with 10µg/ml of Bax pool plus 4x10^6 irradiated T2 cells for 4 hours. Following enrichment, T cells were cultured in AB-RPMI supplemented with 20 U/ml rIL-2 overnight, before being cloned by limiting dilution (16), and screened for Bax peptide reactivity after 3-4 weeks by ELISpot. Positive cultures were expanded using an antigen-independent protocol (16). Further screening identified one positive culture that was TCR Vβ17+/β5.1+. TCR Vβ17+ cells were selected by cell sorting (MoFlo, Dako) and these cells (KSIVB17) were expanded.

ELISpot assay

These were performed as previously described (17). For detection of peptide-specific responses, cultured T cells were plated out in triplicate at 10^5 (initial screen) or 1-2x10^4 cells (clones/lines) per well. Assay wells were either T cells incubated with APC, or APC plus Bax peptides (10 µg/ml). APC were added to T cells at 1:1 ratio. T cells were also incubated with no APC (negative control) or with mitogens (17) (positive control). The number of spots/well was counted using an inverted stereomicroscope (8x magnification) and verified with an ELISpot reader (AID, Cadama). The specific peptide response was calculated by subtracting the background response (T cells+APC) from the T cells+APC+peptide wells.

Chromium-51 (⁵¹Cr) release cytotoxicity assays

The cytotoxic activity of Bax specific T cells was tested in ⁵¹Cr release assays as previously described (16). For cold target inhibition experiments, an E:T ratio of 50:1
was used. Along with the labeled targets (T2, T2+Bax p613, tumor cells), non-labeled T2, T2+irrelevant peptide, T2+Bax p613 and the same tumor cell type were included into the cytotoxicity assay at cold target:hot target ratios of 20:1, 10:1, 5:1. For antibody blocking assays, labeled targets were incubated with varying doses of HLA-specific antibodies for 30 minutes at 37°C before addition of a fixed number of effector cells. HLA-DR antibody (L243, IgG2a, ATCC), and anti-HLA-A23/24 (0041HA, IgG2b, One Lambda) were used for controls.

**LiveCount killing assay**

The cytotoxicity of Bax specific T cells against primary CLL cells was assessed using a modified flow cytometry based assay (18). Briefly, untreated or Bax p613-pulsed CLL cells from HLA-A2+ and HLA-A2- patients were labeled with 0.1 μM CFSE (target, CFSE low) for 10 minutes at 37°C. Daudi cells were used as reference controls by labeling with 2μM CFSE (CFSE high). A 1:1 mixture of 3x10^4 CFSE low and CFSE high cells were incubated overnight at 37°C, and the percentage of specific lysis was calculated as previously described (18).

**Treatment of cells with proteasome inhibitors**

*Clasto*-Lactacytin β-lactone (Calbiochem) or bortezomib (Millenium Pharmaceuticals) were used as proteasome inhibitors. *Clasto*-Lactacytin β-lactone was reconstituted in DMSO and bortezomib in H2O and stored in aliquots at -20°C. Cell lines were split 48 hours prior to assay and then proteasome inhibitor was added 18 hours before the assay. For PBMC cultures, cells at 2x10^6/ml were incubated overnight with proteasome inhibitor at 37°C. Thereafter, the cells were harvested, counted and used in further experiments.
Western blotting

Cells were resuspended in 50 µl PBS per 10^6 cells, and test samples prepared as previously described (19). Proteins were then separated by SDS-PAGE and transferred onto PVDF membranes (Amersham Biosciences) for immunoblotting using a chemiluminescent detection protocol (19). Specific antibody-protein complexes were detected using alkaline phosphatase-conjugated secondary antibodies and CDP-Star (Tropix) chemiluminescence reagent. The anti-human Bax antibody (R&D systems) was used as a 1:1000 dilution and the anti-human actin antibody was used as a 1:10000 dilution of the stock supplied.
Results

Peptide binding assay

Fourteen peptides with predicted binding to HLA-A2 were tested in the T2 peptide-binding assay. Of these, only four peptides showed measurable binding to HLA-A2 molecules (Supplementary Table S1). Bax p614 (ALCTKVPEL) was the strongest HLA-A2 binding peptide (150% increase) followed by Bax p613 (IMGWTLDFL, 115% increase). Bax p612 (KLSECLKRI) and p610 also exhibited binding (49% and 5% respectively). Based on their HLA-A2 binding, only these four Bax peptides would be predicted to be candidate T cell epitopes. However we included all the candidate HLA-A2 peptides in subsequent experiments because we have previously demonstrated that even peptides with poor or low binding in the T2 assay can be T cell epitopes (16, 20).

Bax peptide-specific T cell responses detected in healthy donors

A Bax peptide pool (601-23; including 14 candidate HLA-A2 peptides) was used to stimulate CD8\(^+\) T cells from the blood of five HLA-A2\(^+\) healthy donors, before testing in IFN\(\gamma\) ELISpot assays. Positive but variable Bax T cell responses (23-160 spots/10\(^5\) cells) were detected from all donors after four weeks of stimulation with the Bax peptide pool (Figure 1A). All of the T cell cultures were capable of responding to mitogens (PHA, PMA, concanavalin, ionomycin) in the ELISpot assay (>200 spots/10\(^5\) cells) (data not shown). These results suggest that peptides derived from the ubiquitously expressed Bax protein are immunogenic to human T cells.
Definition of T cell epitopes from Bax

Donor 1 showed the strongest peptide response (Figure 1A), thus T cells from this donor were used for detailed characterization. Bax peptide-specific T cells were immunomagnetically enriched on the basis of IFNγ secretion and cloned to derive KSIVB17. These T cells were tested by ELISpot to map epitopes, first using three smaller pools (Bax p601-609, Bax p610-615 and Bax p616-623) to narrow down the response, followed by individual peptides to allow epitope identification. KSIVB17 was able to strongly respond to the complete Bax peptide pool (p601-23) and to the split pool Bax p610-615 (Figure 1B). Of the peptides in the Bax p610-615 pool, only p610 and p613 induced an ELISpot response. Interestingly, these two peptides are almost identical: Bax p610 is a 10-mer (Bax135-144; TIMGWTLDFL) and Bax p613 is a 9-mer (Bax136-144; IMGWTLDLF). Further testing showed that KSIVB17 T cells were also cytotoxic against HLA-A2+ target cells (T2) in the presence of either p610 or p613 peptides (Figure 1C). KSIVB17 CTL did not kill T2 targets pulsed with irrelevant peptide (Flu M1) (data not shown).

Peptide dose response experiments confirmed that KSIVB17 could recognize both Bax peptides at high peptide concentrations (10-0.1 µM), but had greater avidity for the p613 nonamer as shown by the amount of peptide required for 50% maximal lysis (0.5 nM for p613 vs 10.9 nM for p610, Figure 1D). These results, together with the T2 peptide binding data (Supplementary Table S1), suggest that the Bax p613 sequence (IMGWTLDFL) is the optimum epitope for KSIVB17 T cells.

Bax-specific T cells can kill a range of human tumor cells

The underlying hypothesis for this study was that abnormal proteasomal degradation of Bax protein should generate peptides that bind to HLA class I for presentation to
CD8+ T cells. The KSIVB17 T cells were therefore used to probe for the presence of degradation products of Bax on the surface of cancer cells.

The KSIVB17 T cells were tested for killing activity against a panel of HLA-A*0201+ human cells that included cancerous and non-cancerous cell lines (Table 1). KSIVB17 T cells were able to kill two HLA-A2+ HPV16-expressing cervical carcinoma cell lines above background levels (Table 1). There was no lysis of HPV16+ SIHA-A2 cells or the HPV- C33A cells. KSIVB17 T cells were also able to lyse the HLA-A2+ osteosarcoma cell line, TK143, with weaker lysis seen against two other osteosarcomas, Saos-2 and U2OS. This weak lysis was also observed against an HLA-A2+ hepatocellular carcinoma HepG2, but not against a breast carcinoma MDA-231 or a melanoma SK29Mel (both HLA-A2+). There was no obvious connection between target cell lysis and HLA-A2+ expression (Table 1).

Since Bax protein is also expressed in normal, non-transformed cells, KSIVB17 T cells were tested for cytotoxicity against autologous and allogeneic healthy PHA blasts, and allogeneic healthy skin fibroblasts. KSIVB17 did not induce specific lysis of either autologous or allogeneic PHA blasts (Figure 2A). However, there was lysis when the Bax p613 was added exogenously to the HLA-A2+ PHA blasts demonstrating that these targets were competent at presenting HLA-A2 binding peptides. No specific lysis by KSIVB17 was observed against two HLA-A2+ allogeneic skin fibroblasts (Figure 2B). NK cell-like activity was also excluded since KSIVB17 T cells did not kill NK-sensitive K562. Specific killing was seen only against Bax p613-pulsed T2 cells (Figure 2B). These results indicate that specific killing by KSIVB17 is directed against tumor cells and not healthy non-transformed cells.
It was possible that the killing of allogeneic tumor cells was not mediated by Bax peptide-specific T cells, but by subpopulations of alloreactive T cells (21) or T cells with unknown specificity. Therefore to confirm the antigenic specificity and MHC restriction of tumor cell killing, cold-target competition and monoclonal antibody blocking assays were performed. KSIVB17 killing of labeled (hot) TK143 target cells was decreased in a Cold:Hot target ratio-dependent manner, whereas unlabeled T2 alone or T2 pulsed with Flu M158-66 peptide showed no effect on TK143 target lysis (Figure 3A). Similar cold target inhibition of KSIVB17 cytotoxicity against “hot” C33A-HPV16 targets was also observed (Figure 3B). These results indicate that killing of the TK143 and C33A-HPV16 tumor cell targets was specifically mediated by Bax p613-specific CTL.

MHC restriction of the tumor cell recognition was tested by blocking experiments using an anti-HLA-A2 antibody (Ma2.1). KSIVB17 killing of T2 target cells pulsed with Bax p613 peptide or TK143 tumor cells was inhibited by Ma2.1 antibody but not by a control anti-HLA-DR (L243) antibody (Figure 3C). Similarly KSIVB17 lysis of C33A-HPV16 tumor cells was also inhibited by Ma2.1 antibody but not by a control anti-HLA-A23/24 antibody (Figure 3D). Collectively, these results indicate that the KSIVB17 T cells specifically recognize Bax p613 peptide presented by HLA-A2 on the tumor cells.

**Proteasome inhibition affects MHC class I presentation of Bax peptides to T cells**

We predicted that blocking proteasome function would prevent generation of Bax peptides in cancer cells. To test this, KSIVB17 T cells were tested against tumor cell lines in the presence or absence of a proteasome inhibitor (bortezomib). The lysis of all the tumor cell lines was partially inhibited by bortezomib (Figure 4A). No
inhibition of peptide-pulsed target T2 cells was seen, ruling out any non-specific toxicity effect (Figure 4A). Using C33A-HPV16 target cells, the inhibitory effects of bortezomib were seen to be potent at concentrations as low as 0.001 µM (Figure 4B). Addition of exogenous p613 peptide to target cells treated with an inhibitory dose of bortezomib, restored lysis by KSIVB17 T cells (data not shown). These results support the concept that the proteasome pathway is generating Bax p613 in tumor cell lines.

**Bax protein expression in cancer cells and non-malignant cells**

Given that the use of Bax as a tumor antigen would appear to depend on enhanced Bax protein degradation via the proteasome, we next investigated Bax expression in cancer and normal cells in the absence and presence of proteasome inhibitors.

An increase of Bax (21 kDa) was seen in the cervical carcinoma CaSki (3-fold increase, Supplementary Table S2) after treatment with bortezomib (Figure 5A). While this was not seen with C33A-HPV16 or C33A cells, bortezomib treatment did reveal a band (24 kDa) that corresponded to the Bax-β isoform (22). Increased expression of Bax (21 kDa, Bax-α) was also observed in both U2OS (2.6 fold) and TK143 osteosarcoma (1.5 fold) cells treated with bortezomib (Figure 5A, Supplementary Table S2). Additionally, accumulation of a lower molecular weight form of Bax (18 kDa) was seen in the bortezomib-treated TK143 cells. This 18 kDa form corresponds to a truncated version of Bax that is formed during apoptosis by calpain cleavage of the full-length form (23).

These data indicate proteasomal degradation of several different forms of Bax in tumor cells. These patterns of degradation were not generally seen after PBMC samples or PHA activated T cells blasts from healthy donors (HD) were treated with a
proteasome inhibitor (Figure 5B). There was however, some donor variation, for example an increase in Bax 21 kDa expression was seen in HD7 and HD8 (Figure 5B, Supplementary Table S3). There was also some weak but detectable increase in the Bax 18 kDa cleavage product in some samples e.g. HD1 (Figure 5B, Supplementary Table S3). But, overall there was no significant increase in the Bax 18 kDa expression levels after proteasome inhibitor treatment in healthy donors (Supplementary Figure S2). This data indicates that there is enhanced proteasomal degradation of Bax in tumor cells compared to non-transformed cells.

**Bax peptide-specific T cells recognize primary malignant cells from CLL patients**

The KSIVB17 T cell clone was shown to kill human cancer cell lines, and this appeared to correspond with proteasomal degradation of Bax. However, such established cell lines might not be representative of primary cancer cells. For ethical and practical reasons it was not possible to obtain primary epithelial cancer tissue from patients. Therefore we tested primary cancer cells obtained from patients with a haematological malignancy, CLL. This was based on previous reports that Bax was targeted by the ubiquitin-proteasome pathway in CLL (9), and this activity was correlated with poorer clinical prognosis (8). As a prelude to T cell assays, the expression of Bax after treatment with proteasome inhibitor lactacystin was assessed in primary samples obtained from 13 CLL patients. There was an increased expression (1.5 to 2.4 fold) of the Bax 21 kDa form after lactacystin treatment in 3/13 patients (Supplementary Table S4), but overall this was not significantly different to results obtained with HD (Supplementary Figure S2). By contrast, there was significant upregulation of the Bax 18 kDa form by lactacystin in 10/13 CLL patients (Figure 5C, Supplementary Table S4) and this upregulation was significant when
compared to untreated CLL samples (P=0.0041) or lactacystin treated HD samples (p=0.0092). These results confirm previous findings in CLL (8) and suggest that enhanced proteasomal degradation of Bax 18 kDa form occurs in most CLL patients.

Based on the evidence for degradation of Bax in CLL cells, the KSIVB17 clone was tested for its ability to recognize CLL cells (HLA-A2+). ELISpot assays and a LiveCount killing assay (18) were used because of difficulties encountered in labeling primary cells with 51Cr (data not shown). The KSIVB17 clone was able to respond against the majority of HLA-A2+ CLL samples (11/14), but not the HLA-A2- CLL samples (0/5) in ELISpot assays (Figure 6a). There was a significant difference between T cell responses against HLA-A2+ versus HLA-A2- CLL samples (p=0.0008, T-test). Furthermore KSIVB17 was able to lyse HLA-A2+ CLL cells, but not HLA-A2- CLL cells (Figure 6B). This recognition appeared to be specific for CLL because testing of other malignant B cell lines (BJAB, BV173, DG75, Daudi) by 51Cr release assays, did not reveal any reactivity (Table 1). It was not possible to test the effects of proteasome inhibitors in this assay because of the high levels of bortezomib-induced cytotoxicity in CLL cells (data not shown) (24).
Discussion

There has been an intense drive to define human tumor antigens for use in immunotherapy and as surrogate markers of anti-tumor immunity. Human tumor antigens can be divided into several overlapping categories including but not restricted to: cancer testis antigens (25), oncogenic viral antigens (26) and tissue specific antigens (27). There is also a category of human tumor antigens based on proteins that have a low expression in normal tissue but have over-expression in cancers (28,29). Here we define a novel epitope derived from Bax that is presented to T cells on variety of human cancer cells, including primary CLL cells. We propose that this epitope is derived from proteasomal degradation of Bax, a process that is absent or occurring at lower levels in normal cells. This represents the first definition of a human tumor epitope based on enhanced proteasomal degradation as opposed to over expression of protein in cancer cells.

The enhanced proteasomal degradation of key regulatory proteins contributes to the malignant phenotype in several human cancers (30-33). In this study, Bax was chosen as a potential tumor target because decreased protein stability and expression had been correlated with cancer progression (3) or poorer clinical prognosis (8). Decreased stability and/or increased proteasomal degradation of intracellular proteins can also have immunological consequences. For example, CTL recognition of endogenously processed proteins can be governed by instability or susceptibility to degradation, rather than high levels of protein expression (34-38). Enhanced T cell recognition of epitopes from p53 appears to correlate with low p53 expression, as a result of increased degradation (37,38). Our results suggest that a similar effect is occurring with Bax in cancer cells.
Mass spectrometry has been used to detect the presence of tumor-specific peptides presented by MHC class I molecules (39) but requires large-scale culture of cancer cells. Therefore, we tested our hypothesis by using T cells as sensitive probes for Bax peptide presentation by cancer cells. The KSIVB17 T cell clone was able to recognize HLA-A2+ CLL, cervical, osteosarcoma and hepatocellular carcinoma cells in functional assays. Cold-target competition and antibody blocking assays support the notion that this was specific T cell recognition of the Bax p613 peptide on cancer cells. These cancer cells had increased levels of Bax following treatment with proteasome inhibitors. This was particularly evident with CLL cells, with significant increases in the Bax 18 kDa form after proteasome inhibition. Our immunoblot analysis only provided a snapshot of Bax expression, so we cannot absolutely rule out proteasomal degradation of Bax in normal cells. However, our results do suggest an enhanced degradation of Bax in cancer cells relative to normal cells; a finding supported by previous studies (8, 9). Collectively, these results support the concept that proteasomal degradation of Bax in cancer cells generates the p613 peptide for presentation by HLA-A2 to T cells.

Despite the ubiquitous expression of Bax we propose that enhanced proteasomal degradation of Bax preferentially occurs in tumor cells, therefore Bax epitopes will not be available for inducing tolerance during normal development. We speculate that T cell responses against Bax in healthy individuals will therefore be generated from naïve T cells. In support of this, detectable T cell responses against pooled Bax peptides were obtained from all donors tested, suggesting a lack of tolerance to Bax. The low frequency (<0.15%) and kinetics (>4 weeks) of the response also suggested a primary T cell response. Despite the low frequency, it was possible to clone T cells against Bax p613. To our knowledge this is the first T cell
epitope defined from the Bax-α protein. However, T cell epitopes from the Bax-δ isoform have also been defined (40). Those epitopes were distinct from the ones investigated in the current study and by contrast to Bax-α the functional significance of the δ isoform remains unclear (41).

Allogeneic hematopoietic stem cell transplants have the potential to be curative for a subset of CLL patients based on the T cell-mediated graft versus leukemia effect (42). On the basis of our results, we propose that peptides from Bax could serve as T cell targets in CLL. This is an attractive concept because Bax instability is more common in poor prognosis CLL patients (8). In preliminary experiments in CLL patients (n=6), we have been unable to directly detect memory T cells against Bax p613 using tetramers (data not shown). Although further experiments are needed, this was not entirely surprising, because it is well documented that CLL cells are poor APC and exert potent immunosuppression (42). Thus it has proved difficult to detect T cell responses against any tumor antigens, even in vaccinated patients (43). It is also possible that the enhanced proteasomal degradation of Bax proteins prevents the cross-presentation mechanism that usually primes T cell responses against tumor antigens (44). While this may prevent generation of memory T cell responses, it could be advantageous in preventing generation of peripheral tolerance, thereby allowing access to the naïve T cell repertoire against Bax. However vaccine priming of naïve T cell responses in advanced cancer patients will be challenging. Therefore the most effective utilization of Bax as a target may be through adoptive cell therapy protocols, generating Bax-specific T cells from healthy donors. The data presented here shows that the latter is possible, and support the novel concept of targeting proteins with abnormal or enhanced proteasomal degradation in cancer cells for T cell immunotherapy.
Acknowledgments:
We thank Dr Paul Brennan for helpful advice on western blotting, Sian Llewellyn-Lacey for help with cell lines, and all the healthy volunteers and patients who provided blood samples.

Grant Support
This research was supported by Cancer Research Wales (C.T.N., K.M., M.M. and S.M.), Cardiff Partnership Fund (G.D., S.M.) and Leukaemia and Lymphoma Research (C.P., C.F.).
References

22. Fu NY, Sukumaran SK, Kerk SY, Yu VC. Baxbeta: a constitutively active human Bax isoform that is under tight regulatory control by the proteasomal degradation mechanism. Mol Cell 2009; 33: 15-29.
Figure Legends

Figure 1. T cell responses against Bax peptides in healthy volunteers and mapping the specificity of KSIVB17 clone. (A) Purified CD8+ T cells were cultured with irradiated autologous PBMC and Bax peptides 601-23 for 4 weeks before testing in IFNγ ELISpot assay. APC were autologous PBMC. Numbers shown are spots/10^5 T cells (Mean±SD of triplicates). Background responses (T cells+APC) were subtracted from the data (Donor1: 17; Donor2: 46; Donor3: 19; Donor4: 69; Donor5: 76 SFC), and a positive response was considered ≥ 20 spots/10^5 cells after background subtraction. SFC = Spot Forming cells. (B) KSIVB17 (from donor 1) was tested by IFNγ ELISpot against the Bax peptide pool 601-23, split pools and individual peptides. T cells were plated (2x10^4/well) in triplicate and incubated with nothing, or T2 or T2+peptides at a 1:1 ratio. Background responses, T cells+T2 (5 SFC/10^5 cells), were subtracted. Positive responses were considered ≥600 SFC as this was the maximum number of countable spots (*). Images represent one of the ELISpot wells for each condition. (C) Cytotoxicity of KSIVB17 against peptide-pulsed targets was tested in ^51Cr release assay; a positive result was considered >20% lysis. (D) KSIVB17 was assayed against T2 cells pulsed with varying concentrations of purified Bax p613 and Bax p610 peptides at an E:T of 10:1 in ^51Cr release assays. The EC_{50} value was calculated using the fitted curve.

Figure 2. KSIVB17 do not kill healthy, non-transformed cells or K562. (A) KSIVB17 was assayed against either autologous, allogeneic HLA-A2+, allogeneic HLA-A2- PHA blasts or PHA blasts pulsed with Bax p613. (B) KSIVB17 was assayed against T2+Bax, K562, and allogeneic HLA-A2+ skin fibroblasts (SF) in a ^51Cr release assay. The dashed line represents the 20% lysis cut-off for a positive result.

Figure 3. KSIVB17 recognizes Bax p613 presented by HLA-A2 on the tumor cells. (A) KSIVB17 was tested in the presence of varying numbers of unlabeled cold targets: TK143, T2 alone or pulsed with Bax p613 or control peptide (Flu M158-66) and incubated with
\(^{51}\)Cr-labelled TK143 (hot targets) (B) KSIVB17 was tested in the presence of varying numbers of unlabeled cold targets and incubated with \(^{51}\)Cr-labeled C33A-HPV16 (hot targets). (C-D) HLA-A2 restriction was confirmed by antibody blocking experiments. \(^{51}\)Cr-labeled TK143 (C) or C33A-HPV16 (D) or T2 pulsed with p613 (C and D) were incubated with different dilutions of HLA-specific antibodies before adding T cells at E:T of 40:1. Ma2.1 supernatant was used at 50% concentration downwards, while L243 and anti-HLA-A23/24 were used at top concentrations of 10\(\mu\)g/ml and 20\(\mu\)g/ml respectively.

**Figure 4. Bortezomib inhibits presentation of Bax in tumor cell lines.** (A) KSIVB17 was assayed against T2 pulsed with Bax p613, TK143 and C33A-HPV16 which were either untreated (white bars) or treated with 0.1\(\mu\)M proteasome inhibitor bortezomib (grey bars) overnight at 37\(^\circ\)C. Viability of the cell lines after treatment was 70-80%. (B) KSIVB17 was assayed against C33A-HPV16 untreated (white bar) or treated with varying concentrations of bortezomib (\(\mu\)M) overnight at 37\(^\circ\)C (grey bars). Cytotoxicity was measured by \(^{51}\)Cr release assay at E:T of 30:1 (mean of triplicates). The dashed line represents the 20% lysis cut-off for a positive result.

**Figure 5. Expression of Bax in cancer and non-cancer cells in the presence or absence of proteasome inhibitors.** (A) Cervical and osteosarcoma cancer cell lines were treated with or without bortezomib overnight. (B) Freshly isolated PBMC or PHA blasts from healthy donors were treated with or without clasto-lactacystin overnight. (C) CLL cells were treated with or without clasto-lactacystin overnight and analysed by western blotting for Bax expression. The blots were incubated with specific antibodies to Bax or Actin (42 kDa; loading control). HD = healthy donor. Representative blots from the 13 patients studied are shown.

**Figure 6. KSIVB17 recognizes HLA-A2\(^+\) CLL cells.** (A) KSIVB17 clone was tested in IFN\(\gamma\) ELISpot assay against either nothing or HLA-A2\(^+\) (1-14) or HLA-A2\(^-\) CLL samples (15-19). T cells were plated (triplicates) and incubated with CLL cells at a 1:1 ratio. The dotted line
denotes Mean+3xSD of T cell only SFC, used for assigning positive responses. (B) KSIVB17 was assayed against CFSE-labeled targets in a LiveCount cytotoxicity assay. Primary CLL targets were tested in the presence/absence of Bax p613, at an E:T of 1:1 (mean of duplicates/triplicates).
Table 1. Lysis of human HLA-A2 positive tumor cells by Bax peptide-specific CTL

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Tissue type</th>
<th>% Lysis*</th>
<th>HLA-A2#</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>Lymphoid (TxB)</td>
<td>5%</td>
<td>20</td>
</tr>
<tr>
<td>T2+p613 peptide</td>
<td>Lymphoid (TxB)</td>
<td>89%</td>
<td>40</td>
</tr>
<tr>
<td>CaSki</td>
<td>Cervical cancer (HPV16)</td>
<td>38%</td>
<td>73</td>
</tr>
<tr>
<td>C33A-HPV16</td>
<td>Cervical cancer (HPV16)</td>
<td>47%</td>
<td>28</td>
</tr>
<tr>
<td>C33A</td>
<td>Cervical cancer</td>
<td>16%</td>
<td>35</td>
</tr>
<tr>
<td>SiHa-A2</td>
<td>Cervical cancer (HPV16)</td>
<td>16%</td>
<td>350</td>
</tr>
<tr>
<td>TK143</td>
<td>Osteosarcoma</td>
<td>61%</td>
<td>74</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Osteosarcoma</td>
<td>23%</td>
<td>206</td>
</tr>
<tr>
<td>U20S</td>
<td>Osteosarcoma</td>
<td>28%</td>
<td>64</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatocyte carcinoma</td>
<td>22%</td>
<td>88</td>
</tr>
<tr>
<td>BJAB</td>
<td>B cell lymphoma (EBV)</td>
<td>11%</td>
<td>146</td>
</tr>
<tr>
<td>BV173</td>
<td>Pre-B leukemia (CML)</td>
<td>12%</td>
<td>523</td>
</tr>
<tr>
<td>DG75</td>
<td>B cell lymphoma (EBV)</td>
<td>18%</td>
<td>214</td>
</tr>
<tr>
<td>SK29mel</td>
<td>Melanoma</td>
<td>17%</td>
<td>43</td>
</tr>
<tr>
<td>MDA231</td>
<td>Breast cancer</td>
<td>2%</td>
<td>394</td>
</tr>
<tr>
<td>DU145 (Bax null)</td>
<td>Prostate cancer</td>
<td>1%</td>
<td>20</td>
</tr>
<tr>
<td>K562 (HLA class I negative)</td>
<td>Myelogenous leukemia</td>
<td>1%</td>
<td>nil</td>
</tr>
<tr>
<td>Daudi (HLA class I negative)</td>
<td>Burkitt’s Lymphoma</td>
<td>7%</td>
<td>nil</td>
</tr>
</tbody>
</table>

The % specific lysis from $^{51}$Cr release assays shown is an average of at least duplicate experiments at an E:T ratio of 40-50:1. The results considered as positive killing (>20% lysis) are in bold. #HLA-A2 Mean Fluorescence Intensity as measured by flow cytometry using MA2.1 antibody, nil= no expression. Representative graphs showing lysis of targets over a full range of E:T ratios can be seen in Supplemental figure S1.
Figure 1
Figure 2

A

- □ Auto PHA blasts
- ■ Auto PHA blasts + p613
- ▲ Allo A2+ PHA blasts
- ◇ Allo A2+ PHA blasts + p613
- ◇ Allo A2- PHA blasts
- ▼ Allo A2- PHA blasts + p613

E:T ratio

% Lysis

40 20 10 5 2.5 1.3 0.6 0.3

B

- ▲ Healthy A2+ GW SF
- ▼ Healthy A2+ SB SF
- ■ K562
- ◇ T2+Bax p613

E:T ratio

% Lysis

50 25 12.5 6.3 3.1 1.6 0.8 0.4
A novel tumor antigen derived from enhanced degradation of Bax protein in human cancers

Claudia T. Nunes, Kelly L. Miners, Garry Dolton, et al.

Cancer Res  Published OnlineFirst June 22, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-0393

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/06/22/0008-5472.CAN-11-0393.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.