Sperm-Derived SPANX-B Is a Clinically Relevant Tumor Antigen That Is Expressed in Human Tumors and Readily Recognized by Human CD4+ and CD8+ T Cells

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Abstract

Purpose: The sperm-derived SPANX family proteins can be found expressed in human tumors. Here, we aimed to perform a comprehensive study to evaluate immunotherapeutic relevance of one of its members, SPANX-B. We wanted to test its expression pattern in human tumors and to evaluate CD4+ and CD8+ T-cell responses in healthy humans after in vitro immunizations.

Experimental Design: Expression of SPANX-B in human malignancies, including a multituumor tissue array of 145 primary tumors, was assessed using reverse transcription-PCR, Western blotting, and immunohistochemical analysis. T-cell immunogenicity and immunodominant epitopes of SPANX-B were studied using in vitro immunizations of healthy human donor-derived leukocytes.

Results: SPANX-B was abundantly expressed in melanoma and carcinomas of lung, ovary, colon, and breast. In melanoma, tissue array data indicated that it was expressed in advanced and metastatic disease. Unlike most tumor-associated antigens, SPANX-B was an immunogenic antigen that was recognized by circulating T-cell precursors in healthy humans. Importantly, these T cells were readily expanded to generate SPANX-B-specific helper CD4+ and cytolytic CD8+ T cells that recognized unique immunodominant epitopes: at least one HLA-DR-restricted Pep-9 epitope (SPANX-B12-23) and two HLA-A2-restricted Pep-2 and Pep-4 epitopes (SPANX-B23-31 and SPANX-B57-65, respectively). CD8+ T cells were fully functional to recognize and lyse HLA-A2-expressing tumors, including primary human melanomas.

Conclusions: SPANX-B is an immunogenic sperm-derived antigen that is expressed in several human tumors. SPANX-B is also efficiently recognized by the human T-cell immune arm, indicating its significant value for the development of protective and therapeutic cancer vaccines.

Hominid SPANX family (a sperm protein associated with the nucleus on the chromosome X) consists of highly homologous proteins that share ~40% to 50% similarity with each other. SPANX is encoded by an ancestral SPANX-N and its descendant SPANX-A/D genes (1) that contain two exons separated by a 650-bp intron with an inserted retroviral LTR sequence (2). SPANX-A/D has five members (SPANX-A1, SPANX-A2, SPANX-B, SPANX-C, and SPANX-D) classified based on diagnostic amino acid substitutions. The SPANX-A1, SPANX-C, and SPANX-D genes encode 97-amino acid proteins, whereas the SPANX-B gene, which can have up to a dozen copies, expresses 108-amino acid protein (3). SPANX is not expressed in nongametogenic adult tissues and its biological function is not well understood. However, based on their unique expression and localization in subpopulations of spermatids and spermatooza (4), SPANX proteins were proposed to participate in mammalian spermatogenesis. SPANX-A and SPANX-B are localized in nuclear craters and cytoplasmic droplets of ejaculated spermatozoa, respectively (5). SPANX is a typical cancer/testis-associated antigen, because SPANX genes are also specifically expressed in variety of human tumors and in normal testis (2, 4, 6). To date, the initial observation that SPANX-C (originally designated CTp11) was expressed in human melanoma (6) was expanded showing that the other members (SPANX-A1, SPANX-A2, and SPANX-B) were also present in melanoma, bladder carcinomas, and hematologic malignancies and myeloma (5–8). The SPANX positivity may also indicate the presence of more aggressive skin tumors, particularly in distant, nonlymphatic metastatic...
melanomas (8). SPANX appears to be an immunogenic antigen in humans, as sera of cancer patients contained high titers of SPANX antibody presumably generated during necrosis of malignant cells (7, 8). However, the therapeutic relevance of the presence of SPANX antibody remains unknown, as successful tumor eradication would primarily depend on the activation of antigen/tumor-specific CD4+ helper and CD8+ cytolytic T cells. It is also unclear whether humans contain the SPANX-specific immune effector cells, because tumor-associated antigen (TAA)-reactive cells could be eliminated due to thymic selection. Furthermore, there is no comprehensive study that examines the clinical and therapeutic relevance of these antigens, specifically SPANX-B.

Here, we show that SPANX-B is widely expressed in human malignancies, particularly in melanoma and lung, ovarian, and breast carcinomas. In melanoma specifically, its expression was associated with advanced and metastatic disease. Moreover, we show for the first time that SPANX-B is also recognized by the human T-cell immune arm. Human peripheral blood contains T-cell precursors that recognize SPANX-B, which can be readily expanded to generate the SPANX-B-specific CD4+ and cytolytic CD8+ T cells. We have also mapped their recognition epitopes showing that SPANX-B contains at least one immunodominant HLA-DR-restricted and two HLA-A2-restricted epitopes. These data, taken together, clearly indicate that SPANX-B is a potent and clinically relevant therapeutic antigen that may be successfully exploited in cancer immunotherapy.

**Materials and Methods**

**Human peripheral blood cell isolation.** Human peripheral blood samples were collected from healthy donors in accordance with Human Subject Protocol 2003054 by the Health Apheresis Unit and the Clinical Core Laboratory of the National Institute on Aging (NIA). CD4+ T cells were isolated from peripheral blood mononuclear cells by negative selection using a human CD4 subset column kit (R&D Systems) after Ficol-Paque (GE Healthcare Bio-Sciences) density gradient separation according to the manufacturer’s instructions. CD8+ T cells were isolated using CD8 beads (Invitrogen); in a ratio 1 μL beads/1 × 10^8 T cells in PBS with 0.1% bovine serum albumin and 2 mmol/L EDTA. Beads were removed from cells using DETACHaBEAD CD4/CD8 reagent (Dynal Biotech/Invitrogen). Cell purity was determined by FACS, which resulted in 94% CD4+ T cells and 98% CD8+ T cells. Cells were cultured in RPMI with 10% fetal bovine serum (FBS) and 5% human serum (clone medium). Monoocyte/macrophage-enriched peripheral blood mononuclear cells by plastic adherence were used for isolation of dendritic cells. Briefly, immature dendritic cells were generated by 5-day culturing adherent cells in RPMI with 10% FBS and 5% human serum containing 20 ng/ml recombinant human interleukin-4 and 30 ng/ml recombinant human granulocyte-macrophage colonystimulating factor. Dendritic cells were matured with overnight treatment with 10 μg/ml lipopolysaccharide (Sigma).

**Reagents and cells.** Recombinant SPANX-B protein fused with MBP was produced and purified from Escherichia coli with the pMAL protein expression and purification system according to manufacturer’s instructions (New England Biolabs). Peptide sequences used in this work were as follows: Pep-1 (SPANX-Ba46-a69), QMQMEEMEP; Pep-2 (SPANX-Ba31-a33), EANENKTM; Pep-3 (SPANX-Ba10-a20), GQQSSVRRL; Pep-4 (SPANX-Ba57-a65), LVRYVRNRV; Pep-5 (SPANX-Ba12-a33), RSPVCES-NEVMETPETPGDSDP; Pep-6 (SPANX-Ba42-a63), QAPKMKTSES-StILVVRYYRN; Pep-7 (SPANX-Ba46-a81), KRTSPEELVNHHAR-NRENPDQME; Pep-8 (SPANX-Ba80-a103), ENRNPDQMEEEFEIT-TERPK; Pep-9 (SPANX-Ba12-a33), RSPVCESNEANEA; Pep-9a-Mod (SPANX-Ba23-a33-scrambled), RASPCASAYNE; Pep-10 (SPANX-Ca16), GGVRKSRPVC; Pep-11 (SPANX-Ba40-a49), EFMEIMEL; Pep-12 (SPANX-Ba15-a23), KTSESTHl; Pep-13 (SPANX-Ba11-a33), KRSPVCESNEANEA;NKT; Pep-14 (SPANX-Ca14), ANEANETKMPETPGDSDP; MOPC (ref. 9, MOPC 1B8b1-a101), ALWERNHFFPGCGGTX; and influenza HLA-A2 peptide M (flu M158-a61), GILG VTFL. All peptides were custom-designed and purchased from AnaSpec. The SPANX-B peptide-specific antibody AEA10117 was generated by immunizing rabbits with SPANX-B-specific synthetic peptides. Antibodies specific to human IFN-γ and to HLA-A, HLA-B, and HLA-C were purchased from BD Pharmingen; antibodies to HLA-DR, HLA-DP, and HLA-DQ were from Leinco.

The majority of cell lines, such as 938 Mel, Jurkat, CCRF-CEM (CEM, CCL-119), and CRL 7002, were purchased from the American Type Culture Collection. UACC1273 cell line was provided by Dr. Ashani T. Weeraratna (NIA). The cells were grown in cRPMI supplemented with 10% FBS. Human embryonic kidney cells (HEK293; American Type Culture Collection) were grown in cDMEM with 10% FBS; ovarian cancer cell lines 2008, BG-1, and OVCAR3 (a generous gift of Dr. Nicholas Restifo, National Cancer Institute) were cultured in cRPMI supplemented with 10% FBS. All cells were maintained in a 37°C humidified 5% CO2. Collection of paraffin non-small cell lung cancer (NSCLC) blocks from seven different patients was supplied by the Department of Pathology, Civile Maggiore Hospital.

**Immunotherapeutic Potency of SPANX-B**

**T-cell stimulation.** To generate CD4+ T-cell lines, CD4+ T cells (2 × 10^6) were stimulated with autologous dendritic cells pulsed with either SPANX-B protein (2 μg/mL) or SPANX-B peptides (10 μg/mL) in the presence of interleukin-2 (20 units/mL) for 7 days. T cells were restimulated once every week for two times. To generate CD8+ CTL lines, 2 × 10^6 CD8+ T cells were stimulated with autologous dendritic cells pulsed with either SPANX-B protein (2 μg/mL) or SPANX-B peptides (10 μg/mL) in the presence of interleukin-2 (20 units/mL) and interleukin-15 (30 ng/mL) for 7 days. T cells were weekly restimulated with peptide-loaded dendritic cells for several times. To test antigen-specific activity of CD4+ T-cell lines, all experiments were done in triplicates in 96-well plates with 1 × 10^5 CD4+ T cells per well. The cells were stimulated with autologous dendritic cells incubated overnight with SPANX-B protein or SPANX-B or control peptides (0.1 and 0.01 μg/mL) in clone medium with interleukin-2 (20 units/mL). T-cell activity is judged by IFN-γ secretion in culture supernatants.
by ELISA after 48 h incubation (see below and ref. 10). For most experiments, unless specified, 1:5 target-to-effector ratio was used.

**Detection of IFN-γ by ELISA.** Briefly, 96-well flat-bottomed plates were coated with 2 µg/mL anti-human IFN-γ antibody (BD Pharmingen) to capture secreted IFN-γ as described elsewhere (10). The captured IFN-γ was detected with 0.5 µg/mL biotin-conjugated mouse anti-human IFN-γ antibody and streptavidin-horseradish peroxidase (BD Pharmingen). The assay was visualized with TMB peroxidase solution B (KPL) and read at 490 nm.

CTL assay and HLA blocking experiments. Tumor cell lines (target cells, 2 × 10⁶) were incubated in 200 µL PBS with 200 µCi Na¹⁵CrO₄ (Perkin-Elmer) for 2 h at 37°C. Cells were washed with RPMI three times and resuspended in RPMI with 10% FBS at 1 × 10⁵/mL. CTL assay was done in triplicates in 96-well round-bottomed plates with 1 × 10⁴ per well 5¹⁵Cr-labeled target cells. The target cells were cocultured at indicated ratios with effector cells (peptide-specific CD8⁺ T cells) for 6 h. The specific 5¹⁵Cr release is calculated using formula: [(test sample release - spontaneous release) / (maximum release - spontaneous release)] × 100. Maximum release is for the target cells alone lysed with 2% Triton X-100.

**MHC class I and class I inhibition assays.** HLA specific monoclonal antibodies or control isotype matched IgG were preincubated with peptide-pulsed dendritic cells at concentration of 10 µg/mL for 1 h at 4°C. Cells were washed with PBS, irradiated at 4,500 rad, and mixed with T cells at indicated ratios. To block HLA class I expression, tumor cell lines were pretreated with 10 µg/mL anti-HLA-A, anti-HLA-B, and anti-HLA-C antibodies (BD Pharmingen) for 1 h at 4°C. Cells were washed with PBS and labeled with 5¹⁵Cr as indicated above. IFN-γ production was determined by ELISA after 48 h incubation as described (10).

**Detection of SPANX-B expression human tumors.** SPANX-B mRNA expression was tested and confirmed using reverse transcription-PCR using combinations of two different sets of primers that amplifies spliced messages, such as forward and reverse primers designed in-house (PRSPANXB-Lar-1 5'-ATGGGCCAACAATCCAGTGT-3' and PRSPANXB-Lar-R1 5'-CTTCTCTGCAGTCAGTCTG-3', respectively) and forward and reverse SPANX-B primers reported by others (ref. 11; 5'-ACCTGTGACATGCAAGAACCC-3' and 5'-TTAGCTCCTGTCCTGCAGGGGC-3').

Total RNA, extracted from frozen cell pellets using RNeasy Mini Kit (Qiagen), was reverse transcribed using MMLV RT (Invitrogen) and amplified using 2 units Taq DNA polymerase (New England Biolabs): 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Control amplification was for expression of GAPDH using PRuGAPDH-1 and PRuGAPDH-R1 (5'-CTTCTCTGCAGTCAGTCTG-3' and 5'-GCCCTGTCCTCACCACTCTGATG-3', respectively).

SPANX-B protein expression was detected using ANEA-10117 anti-SPANX-B antibody (1:5,000) in Western blotting of tumor lysates with 30 µg total protein separated in reducing 14% PAGE gels (Invitrogen). Bands were detected with Enhanced Chemiluminescence Plus Western blotting detection system (GE Healthcare).

Immunohistochemistry stainings for SPANX-B were done on multi-tumor tissue arrays (T-MTA-5, Tissue Array Research Program, National Cancer Institute) or paraffin-embedded biopsies of human with melanoma and NSCLC lung cancer (pathology collection of Ospedale Borgo Trento Piazzale Stefani). Briefly, the tissue slides were treated with series of xylene (three times for 5 min), 100% ethanol (twice for 2 min), 90% ethanol, 80% ethanol, 75% ethanol (one time for 2 min), PBS, and water. Antigen retrieval was done with citrate buffer in a steamer for 25 min. Slides were rinsed with PBS and blocked for 5 min with V block solution (Lab Vision) and 1 h with blocking buffer (0.2% Triton X-100, 0.2% casein, 0.2% bovine serum albumin, 5% normal goat serum, 0.2% gelatin, and 0.02% NaN₃). To perform immunohistochemistry staining on cultured cells, 3 × 10⁴ tumor cell lines were seeded in an 8-well chamber slide (Lab-Tek II chamber slide system). Cells were washed twice with PBS and fixed with 300 µL methanol/H₂O₂ 3% for 30 min. After several PBS washes, the cells were blocked with ultra V block solution (Lab Vision), rinsed with PBS, and blocked with 200 µL blocking buffer for 1 h. The slides were treated with ANEA-10117 anti-SPANX-B antibody (1:500) or control rabbit Ig for 2 h at room temperature. Slides were washed three times in PBS, incubated for 30 min with biotinylated goat anti-rabbit antibody (Lab Vision), washed three times with PBS, and incubated with streptavidin-peroxidase (Lab Vision) for 20 min. Protein expression was visualized with 3,3'-diaminobenzidine (Lab Vision) and counterstained with hematoxylin.

**Data analysis of the Mannheim data set.**Scaled gene expression data were generated from the Affymetrix HG-U133 microarray chips for 45 primary cultures of melanoma biopsies using accession no. GSE4843 (Mannheim data set). These data were loaded into GeneSpring GX 7.3 (Agilent Technologies) and normalized as described previously (12). Normalized data for the Motif 2 set of genes (averaged within each sample expression pattern; ref. 12) was compared with the expression pattern for SPANXB1 (probe set 220921_at) and the correlation coefficient was calculated. Statistical significance of variation of SPANX-B between the cohorts was calculated using Student’s two-tailed t test.

**Results**

**SPANX-B protein is uniquely expressed in human tumors.** SPANX-B can be often found in the list of highly expressed genes in microarray study of human tumors, including hematologic malignancies and breast cancer (11, 13). To confirm this, we have tested its expression using two antibodies, a rabbit 3ANEA antibody and mouse SPANX-B antibody. The 3ANEA antibody was reported to be specific for the amino-terminal portion of SPANX-B in human spermatozoa (14), and it specifically detected an expected size (~ 15 kDa) band in SPANX-B-transduced murine RL5 cells in Western blotting (Fig. 1A). The second SPANX-B antibody was produced from mice DNA immunized with SPANX-B-expressing plasmid. It was also specific for SPANX-B, as it preferentially recognized Pep-G1 peptide (Fig. 1B) encoding the amino terminus of SPANX-B (Fig. 1C) but not other region-specific peptides, such as Pep-G2, Pep-2, Pep-3, and Pep-6 (Fig. 1B and C). In support, it failed to recognize Pep-5 peptide that encoded the overlapping Pep-G1, but homologous with SPANX-C, portion (Fig. 1B and C). In contrast, it reacted with a second peptide Pep-2 that only represented unique for SPANX-B insert of Pep-G1 (Fig. 1B and C).

In concordance with reverse transcription-PCR data (data not shown), SPANX-B was detected in lysates of immortalized human tumor lines, such as melanoma cells (UACC1273 and 938 Mel; Fig. 1D) and carcinomas of colon (HCC2998), ovarian (IGROV 1), renal (786-O and UO-31), and NSCLC (NCI H226; data not shown). In contrast, SPANX-B was not detected in lysates of normal human peripheral blood mononuclear cells (Fig. 1D) and cell lines such as HCT116 and HCT15 (colon cancer), OVCAR3 and OVCAR4 (ovarian cancer), and A498 and ACHN (renal cancer), and HOP62 and NCI H522 (NSCLC; data not shown). These data were also

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Fig. 1. Specificity of antibody and expression of SPANX-B in human immortalized tumor cell lines. 

A, ANEA-I0117 antibody recognized SPANX-B expressed in mouse erythroleukemia RL5 cells. Western blotting with ANEA-I0117 anti-SPANX-B antibody on lysates of RL5 cells (2, 5, and 10 µL, respectively) transiently transduced with plasmid expressing SPANX-B. Control lysates were made from untransduced parental RL5 cells (Control). 

B, murine anti-SPANX-B antibody, which was generated from mice DNA immunized with plasmid expressing SPANX-B, recognized unique SPANX-B-specific insert of SPANX-B (encoded in Pep-G1 and Pep-2 peptides; C). ELISA assay on plates coated with various peptides that are depicted in C. 

C, protein sequence of SPANX-B and locations of the synthetic peptides used. Complete list of peptides used in the work is shown in Materials and Methods. Pep-5 does not contain 6-amino-acid insert of SPANX-B (underlined). 

D, expression of SPANX-B protein was evaluated by Western blotting with ANEA-I0117 antibody (1:5,000) in lysates of cell lines, such as HEK293 (HEK), Jurkat, CRL 7002, UACC1273, 938 Mel, CCRF-CEM (CEM), and normal human donor peripheral blood mononuclear cells (top). The blot was stripped and hybridized with anti-actin antibody (bottom). E and F, immunohistochemistry and immunofluorescent assay, respectively, of melanoma (UACC1273) and ovarian carcinoma (2008 and OVCAR3) cell lines. Cells were stained with ANEA-I0117 antibody (left and bottom in E and F, respectively) and control IgG (right and top in E and F, respectively).
supported by the immunohistochemistry staining of the cells. For example, SPANX-B-expressing cells, such as UACC1273 and 2008 cells [Fig. 1D], but not SPANX-B-negative OVCA3 cells, were also positive in both immunohistochemistry (Fig. 1E) and immunofluorescence (Fig. 1F) stainings. The staining of primary human tumor samples revealed that it was expressed in human melanoma (2 of 2) and lung carcinoma (5 of 5; NSCLC; Fig. 2A). Furthermore, the survey of a panel of human primary 145 tumors on a multitumor tissue microarray has revealed that SPANX-B was expressed in ovarian, colon, breast, and lung cancers besides melanoma (see a representative picture in Fig. 2B). Although we did not intend to study normal human tissues, SPANX-B was not detected in the limited numbers of normal tissues included in multitumor tissue microarray, such as normal endometrium and colon (Fig. 2C). However, it was abundantly expressed in Sertoli cells of normal testis (Fig. 2C), indicating that SPANX-B is a typical cancer/testis-associated antigen. The expression of SPANX-B was variable, ranging from an abundant production in melanoma and ovarian carcinomas to low or almost undetectable levels in T-cell tumors (Jurkat and CEM, respectively; Fig. 1D). At least in melanoma, the presence of SPANX-B may indicate a metastatic stage of the disease, as its expression was associated with a group of genes expressed in melanoma with the highest metastatic potential (cohort C in Mannheim data set; ref. 12; correlation coefficient \( r = 0.503 \); Fig. 2D). In contrast, we did not find any association in cohort A that consisted of highly proliferative cells with low metastatic potential.

Characterization of SPANX-B–specific CD4+ T-cell responses. The clinical and therapeutic relevance of TAA depends on its ability to induce and expand the antigen-specific T-cell precursors. To test this, human peripheral blood CD4+ T cells were stimulated with autologous dendritic cells incubated with SPANX-B protein. As a result, this allowed to generate SPANX-B–specific CD4+ T-cell lines from almost every human donor; the cells specifically recognized SPANX-B–treated dendritic cells, but not control antigen-treated dendritic cells, by secreting IFN-\( \gamma \) (SPANX-B protein; Fig. 3A). To determine an immunodominant epitope, CD4+ T cells were stimulated with dendritic cells pulsed with synthetic peptides to various parts of SPANX-B (Fig. 1C). Among them, two peptides from the amino-terminal portion of SPANX-B, SPANX_{11-31} or SPANX_{12-35} (Pep-G1 and Pep-5, Fig. 1C), activated the CD4+ T-cell lines to secrete IFN-\( \gamma \) (Fig. 3A and B). In contrast, the CD4+ T cells failed to respond to dendritic cells pulsed with other SPANX-B peptides, including peptides SPANX_{21-41} (Pep-G2) and SPANX_{12-65} (Pep-6), mixture of peptides 1 to 4 (Pep-1, Pep-2, Pep-3, and Pep-4; Fig. 3A), or control MOPC peptide (Fig. 3B and C). Together, the MHC class II immunodominant region (epitope) is probably located within overlapping portion of Pep-G1 and Pep-5 between residues 11 and 35 of SPANX-B. The region was recognized by almost every normal human donor CD4+ T cells tested (9 of 9), indicating that humans contain a preexisting pool of precursor T cells specific for SPANX-B. In support, SPANX-B–specific CD4+ T-cell lines were also independently and readily generated using peptides Pep-5 and Pep-G1 (Fig. 3C and D, respectively) that also specifically and in a dose-dependent manner recognized dendritic cells incubated with SPANX-B protein.

Immunodominant epitope of SPANX-B is recognized in HLA-DR–restricted fashion. To fine map the epitope, a shorter peptide Pep-9 (SPANX-B_{12-23}; Fig. 1C), which represents an overlapping portion of peptides Pep-5 and Pep-G1, was tested. Autologous dendritic cells pulsed Pep-9 were indeed able to activate the SPANX-B–specific CD4+ T-cell lines (independently generated to SPANX-B protein, Pep-1, or Pep-G1) to secrete IFN-\( \gamma \) (a representative result on the Pep-G1–specific CD4+ T cells is shown in Fig. 3D). In contrast, the CD4+ T cells did not respond when stimulated with scrambled Pep-9 peptide (Pep-9-Mod; Fig. 3D). Together, the CD4+ T cells recognized an immunodominant epitope encoded “RSVPCESNEANE” sequence of Pep-9 (Fig. 1C).

Next, to characterize HLA specificity, the CD4+ T-cell lines were stimulated with autologous dendritic cells pulsed with Pep-9 in the presence of antibody that blocked HLA-DR, HLA-DQ, or HLA-DP molecules. The stimulation of the SPANX-B–specific CD4+ T cells was not affected by the presence of control antibody or antibodies that block HLA-DQ, HLA-DP, or anti-MHC class I (Fig. 4A-C). In contrast, anti-HLA-DR antibody completely abrogated the Pep-9–induced IFN-\( \gamma \) secretion from all CD4+ T-cell lines specific to SPANX-B protein (Fig. 4A), Pep-5 (Fig. 4B), or Pep-9 (Fig. 4C), respectively. Thus, the peptide Pep-9 represents an immunodominant epitope recognized by human CD4+ T cells in HLA-DR–restricted fashion.

SPANX-B is also recognized by CD8+ T cells in HLA-A2 context. To exploit the full therapeutic value of TAA, the induction of CD8+ T cells is required. Computer analysis of SPANX-B protein has revealed two 9-mer peptides designated Pep-2 and Pep-4 (SPANX-B_{23-31} and SPANX-B_{57-65}, respectively) that contained moderate Parker binding scores for HLA-A2.10 To test whether they are recognized by human CD8+ T cells, HLA-A2-positive peripheral blood CD8+ T cells were stimulated with autologous dendritic cells incubated with SPANX-B protein. As a result, SPANX-B–specific CD8+ T-cell lines were generated from every donor peripheral blood lymphocyte (3 of 3). The CD8+ T cells secreted IFN-\( \gamma \) on incubation with irradiated autologous dendritic cells that were pulsed with SPANX-B protein (Fig. 5A), Pep-4 (Fig. 5A), or Pep-2 (data not shown). Reciprocally, the CD8+ T cells that were independently generated by Pep-2- or Pep-4–pulsed dendritic cells also recognized SPANX-B protein–pretreated dendritic cells (data not shown). The recognition was specific, as no IFN-\( \gamma \) was produced when the CD8+ T cells were stimulated with dendritic cells pulsed with control HLA-A2–positive M1 influenza peptide (Fig. 5A). Furthermore, this was the MHC class I–restricted recognition, as SPANX-B–induced activation was specifically and significantly abrogated by the presence of anti-MHC-I, but not control, antibody (Fig. 5B).

SPANX-B–specific CD8+ T cells kill human tumor in HLA-A2 context. Next, we have tested whether the CD8+ T cells can recognize human tumors that express SPANX-B. As shown in Fig. 6, the CD8+ T cells were able to kill melanoma cells. For example, they recognized and lysed SPANX-B-expressing and HLA-A2-positive UACC1273 melanoma cells (Fig. 6A-C). Several lines of evidence indicate that it is indeed a HLA-A2–restricted response. First, the CD8+ T cells did not lyse HLA-A2–negative but SPANX-B-expressing melanoma cells 938 Mel (data not shown). Second, all three types of CD8+ T-cell lines (independently generated to SPANX-B protein, Pep-2, or Pep-4) not only killed
Fig. 2. SPANX-B is expressed in human primary tumors. A, immunohistochemistry staining of slides from paraffin-embedded human primary melanoma (top) and NSCLC (bottom) and multitumor tissue microarray slides with human tumors (B) and normal tissues (C). ANEA-10117 antibody (SPANX-B) and control isotype-matched antibody (control IgG) were used at 1:500 dilution. D, SPANX-B is mostly expressed in metastatic melanomas. Normalized signal intensity data for 77 genes previously identified as being associated with increased metastatic potential were averaged in each of 45 melanoma sample data sets (Mannheim data set; ref. 12). This averaged profile (gray shaded line) was plotted against that of SPANX-B1 (black solid) across all samples and a positive correlation coefficient of 0.503 was calculated. Melanoma lines are labeled according to their cohort membership (12). Dotted lines, 95% confidence interval for the averaged profile. *P* value is for comparisons between cohorts A and C.
UACC1273 melanoma cells but also lysed HLA-A2-expressing HEK293 cells that were pulsed with Pep-2 (HEK-A2; Fig. 6A-C). In contrast, the CD8+ T cells did not kill HLA-A2-negative parental HEK293 cells (Fig. 6A-C). Lastly, the CD8+ T cells could not lyse HLA-A2-positive OVCAR3 cells that did not express SPANX-B unless pulsed with Pep-2 (Fig. 6D). Furthermore and importantly, all three CD8+ lines also recognized and lysed primary tumors from melanoma patients that expressed HLA-A2 (TC526, TC624, and TC2492; Fig. 6D), whereas HLA-A2-negative tumors were not affected (TC2547 and TC938; Fig. 6E). Thus, humans contain a readily available proportion of circulating CD8+ T cells (at least precursors) that recognize two epitopes of SPANX-B, Pep-2 and Pep-4, presented on the MHC class I molecules in the HLA-A2 context.

**Discussion**

TAAs are the main targets of cancer immunotherapy. TAAs, as self-antigens, are usually poorly immunogenic and their immune effector cells may have been subjected to thymic selection and elimination. In contrast, a group of TAAs that are expressed in embryonic cells (OFA-iLRP; ref. 15) or in various immunoprivileged sites (cancer-testis antigens designated by their unique expression in the named tissues) are highly immunogenic in humans. Importantly, their antigen-specific precursor and effector T cells appear to be preserved in adult humans and cancer patients, which can be associated with a favorable cancer outcome. The induction of embryonic OFA antigen-recognition CTLs was used as a positive control in renal carcinoma patients immunized with renal tumor RNA-transfected dendritic cells (16). Hence, OFA-iLRP-specific CD4+ and CD8+ T cells are shown to be readily expanded from the peripheral blood of healthy donors or patients with B-cell malignancies and renal cell carcinoma (16–18). The CD8+ cells were fully active and able to kill antigen-expressing tumors, such as acute myeloid leukemia and chronic lymphocytic leukemia cells (17, 18).

SPANX family proteins, specifically SPANX-C (CTp11), are usually produced in normal human sperm cells but was found also expressed in melanoma and cancer cells of kidney, bladder, and prostate (2, 4, 6). However, due to the lack of specific antibodies that discriminate each member of the family, no systemic study on the expression of SPANX-B in human tumors was reported. To date, its expression in melanoma, testicular germ cell tumors, and hematopoietic malignancies was detected.

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**Fig. 3.** SPANX-B induces CD4+ T-cell responses. A and B, SPANX-B-specific CD4+ T-cell lines were generated by repeated stimulations of human T cells with irradiated autologous immature dendritic cells treated with SPANX-B protein. The immunodominant region of SPANX-B is located in the overlapping portion of synthetic peptides Pep-G1 and Pep-5, as the T-cell line can be also activated to secrete IFN-γ with irradiated mature dendritic cells pulsed with 1 μg/mL Pep-G1 or Pep-5 but not with individual peptides (Pep-G2 and Pep-6) or mixture of peptides (Pep-1, Pep-2, Pep-3, and Pep-4) specific to other regions of SPANX-B. CD4+ T-cell lines generated to SPANX-B protein (B), Pep-5 (C), or Pep-G1 (D) specifically and reciprocally recognize dendritic cells pulsed with titrated amounts (μg/mL) of SPANX-B protein, Pep-5, Pep-G1, or Pep-9 and secrete IFN-γ (pg/mL). T cells were not activated with dendritic cells pulsed with control murine class II peptide (MOPC) or scrambled Pep-9 (Pep-9-Mod; D).

**Notes:** *, *P* < 0.01; ***, *P* < 0.001 for comparison with the group indicated by line. Mean ± SE of representative and reproducible results of at least three independent experiments done in triplicates.
mostly by the use of reverse transcription-PCR analysis (2, 7, 19, 20). Here, we reevaluated reverse transcription-PCR results using two different SPANX-B-specific antibodies that were specific to a unique 6-amino acid insert only present in SPANX-B. We have found SPANX-B in several different tumors besides melanoma. It is worth noting that the SPANX-B-specific exon 1 fragment was also amplified by reverse transcription-PCR in all 10 randomly chosen positive samples. Together, the antibodies used in this study are specific, although due to a high homology between SPANX-A/D members the issue of cross-reactivity may not be fully resolved. Overall, the survey of a panel of tumors on a multitumor tissue microarray indicated that SPANX-B was expressed in carcinomas of breast, lung, colon, and ovary. A functional relevance of our results remains unknown, as SPANX should not be expressed in nongametogenic adult tissues. It is tempting to speculate that SPANX-B may facilitate motility and metastasis of tumors and lead to more aggressive stage of the disease. First, Westbrook et al. suggested that the presence of SPANX (family members not specified) could be an indicator for the presence of more aggressive skin tumors, particularly in distant, nonlymphatic metastatic melanomas (8). Furthermore, SPANX-B was among genes that were overexpressed in human breast cancer cell lines that metastasize to lung (see ref. 13). Lastly, we have also found that SPANX-B was also preferentially expressed in cohort C of the Mannheim data set that represented the pattern of genes associated with highly metastatic motif 2 (12).

SPANX proteins, including SPANX-B, appear to be immunogenic in humans, as the sera of patients with hematopoietic malignancies that express SPANX-B also contained SPANX-B-reactive antibodies (7). Despite this, it remains unknown...
whether SPANX-B can elicit T-cell responses in humans. It is hardly arguable fact that the successful cancer therapy would require induction of the antigen-specific cellular immune responses. The therapeutic value/potency of TAAs is closely linked with their ability to elicit long-lasting CD4+ T helper and CD8+ CTLs. As such, our data suggest that SPANX-B is indeed a potentially valuable therapeutic antigen. First, healthy humans contain circulating SPANX-B-specific T-cell precursors. Second, these T cells can be readily expanded to generate both helper CD4+ T cells and cytolytic CD8+ T cells using in vitro immunizations with SPANX-B-treated dendritic cells. Third, SPANX-B is not only expressed in human melanomas but also processed and presented on their MHC class I molecules. SPANX-B has at least two immunodominant HLA-A2-restricted Pep-2 and Pep-4 epitopes that alone can be used to elicit SPANX-B-specific CD8+ CTLs. Fourth, and importantly, the SPANX-B-specific CD8+ T cells generated from normal human peripheral blood lymphocytes can recognize and efficiently kill the HLA-matched human primary melanomas. Lastly, we have determined at least one HLA-DR-restricted and

![Image](https://example.com/image.png)

**Fig. 6.** SPANX-B-specific CD8+ T cells recognize and kill HLA-A2-expressing melanoma cells. Effector CD8+ T cells (E) generated by stimulating with SPANX-B protein (A), Pep-2 (B), or Pep-4 (C) were mixed with target (T) UACC1273 melanoma cells at indicated ratio (Y axis) to perform cytoytic 6 h 51Cr release assay. Control targets were parental (HEK) or HLA-A2-expressing (HEK-A2) HEK293 cells that were pulsed with 1 μg/ml Pep-2. Autologous dendritic cells alone were used as a negative control group. D, CD8+ T cells did not lyse SPANX-B-negative but HLA-A2-positive OVCAR3 cells unless pulsed with Pep-2. A to D, percentage of cytoxicity (Y axis) of a representative experiment done in triplicate and repeated at least twice. E, effector CD8+ T cells also recognize and kill HLA-A2-expressing (Tc526, Tc524, and Tc2492) but not HLA-A2-negative (Tc938) primary human melanoma cells. Mean ± SE (%; Y axis) of 51Cr release of the Pep-2-specific CD8+ T cells at target-to-effector ratio of 1:10. Comparable cytolytic was also detected from the SPANX-B protein-specific and Pep-4-specific CD8+ effector cells (data not shown).
immunodominant Pep-9 epitope against which the SPANX-B-specific CD4+ T cells can be readily generated from normal human peripheral blood lymphocytes. Thus, it is tempting to speculate that this ability would provide an additional important benefit for the immunotherapy of cancers as a source of T helper cells. In fact, CD8+ T cells primed in the absence of CD4+ T-cell help are shown to undergo TRAIL-dependent death on antigen restimulation despite their ability to mediate effector functions such as cytotoxicity (21). At present, this possibility is not testable in vivo primarily due to the fact that SPANX-B is only expressed in higher primates and no proper animal models exist thus far. Our attempts to establish a murine model that expresses SPANX-B in poorly immunogenic tumor cells failed, as SPANX-B rendered the cells immunogenic and inhibited tumor progression in mice.5

Taken together, SPANX-B is not only a cancer/testis-associated antigen expressed in a majority of human tumors but also a very attractive therapeutic target antigen. Our data indicate that SPANX-B is an immunogenic antigen that can readily expand and activate both arms of T-cell immunity, helper CD4+ T cells and CD8+ CTLs, to recognize and kill HLA-matched SPANX-B-expressing melanomas.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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