**Microenvironment and Immunology**

**MHC-Restricted Phosphopeptides from Insulin Receptor Substrate-2 and CDC25b Offer Broad-Based Immunotherapeutic Agents for Cancer**

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**Abstract**

Cancer cells display novel phosphopeptides in association with MHC class I and II molecules. In this study, we evaluated two HLA-A2–restricted phosphopeptides derived from the insulin receptor substrate (IRS)-2 and the cell-cycle regulator CDC25b. These proteins are both broadly expressed in multiple malignancies and linked to cancer cell survival. Two phosphopeptides, termed pIRS-21097–1105 and pCDC25b38–46, served as targets of strong and specific CD8 T-cell memory responses in normal human donors. We cloned T-cell receptor (TCR) cDNAs from murine CD8 T-cell lines specific for either pIRS-21097–1105 or pCDC25b38–46. Expression of these TCRs in human CD8 T cells imparted high-avidity phosphopeptide-specific recognition and cytotoxic and cytokine-secreting effector activities. Using these cells, we found that endogenously processed pIRS-21097–1105 was presented on HLA-A2+ melanomas and breast, ovarian, and colorectal carcinomas. Presentation was correlated with the level of the Ser1105-phosphorylated IRS-2 protein in metastatic melanoma tissues. The highest expression of this protein was evident on dividing malignant cells. Presentation of endogenously processed pCDC25b38–46 was narrower, but still evident on HLA-A2+ melanoma, breast carcinoma, and lymphoblastoid cells. Notably, pIRS-21097–1105-specific and pCDC25b38–46-specific TCR-expressing human CD8 T cells markedly slowed tumor outgrowth in vivo. Our results define two new antigens that may be developed as immunotherapeutic agents for a broad range of HLA-A2+ cancers. Cancer Res; 74(23); 6784–95. ©2014 AACR.

**Introduction**

Clinical trials using adoptive cellular therapy and vaccination have demonstrated the importance of CD8 T cells in controlling cancer (1–4). A large number of tumor-associated antigens (TAA) recognized by CD8 T cells have been identified in the last 20 years, and clinical tumor regressions have been associated with immunotherapies based on some of them (1, 5). However, clinical response rates to vaccines targeting a range of TAA have been disappointing (6). The repertoire of TAA include (i) neoantigens formed by mutations in cellular proteins; (ii) antigens induced by oncogenic viruses; (iii) cancer-testis antigens normally expressed only in germ-line cells; and (iv) tissue-specific differentiation antigens (7). Only a small number of TAA source proteins have been linked to either initial cellular transformation processes or later tumorigenic processes such as angiogenesis and metastasis (8, 9). Targeting TAA derived from proteins that are vital for a cancer cell’s survival and metastatic potential is attractive, as downregulation and/or mutation of genes encoding these proteins as a means of immune evasion could compromise cellular malignancy (10, 11).

Many signaling pathways that involve protein phosphorylation and dephosphorylation are altered in cancer cells, and some of these have been directly associated with alterations in cellular growth, survival, and metastasis (12, 13). We hypothesized that proteolytic processing of malignancy-associated phosphorylated proteins would yield a pool of phosphopeptides that could be presented by MHC-I and MHC-II molecules on tumor cells, and serve as targets of antitumor adaptive immunity. We have demonstrated that phosphopeptides are presented by many different MHC-I and MHC-II molecules (14–17), and that many phosphopeptides presented by HLA-A2 on melanoma and ovarian cancer cell lines (16), and by HLA-B7 on leukemic malignancies (17) were derived from source proteins that are either overexpressed or dysregulated in cancer cells. We were particularly interested in HLA-A2–restricted phosphopeptides derived from insulin receptor substrate (IRS)-2 and cell division cycle 25b (CDC25b); pIRS-21097–1105 and pCDC25b38–46.
IRS proteins are adapters that link signaling from growth factor and cytokine receptors, including the insulin receptor, insulin-like growth factor receptor, and IL4 receptor, to multiple SH2-containing signaling proteins to modulate cellular growth, metabolism, survival, and differentiation (18). IRS-2 is overexpressed at the gene or protein level in pancreatic cancer (19), hepatocellular carcinoma (20), neuroblastoma (21), breast cancer (22), glioblastoma (23), and colorectal cancer (24). IRS-2 overexpression under a mouse mammary tumor virus promoter causes mammary hyperplasia, tumorigenesis, and metastasis (22, 25–27). The IRS proteins are regulated by phosphorylation of Tyr and Thr residues and to evaluate their immunotherapeutic potential. Our main goal in this study was to evaluate the breadth of expression of pIRS-21097 and pCDC25b38–1105 among different cancer cells has not been evaluated. CDC25 dual-specificity phosphatases regulate the activity of cyclin-dependent kinases by dephosphorylation of Tyr and Thr residues in their active G2–M transition (30). CDC25b overexpression in multiple malignancies is correlated with poor prognosis (29). However, the breadth of expression of pIRS-21097 among different cancer cells has not been investigated. CDC25 dual-specificity phosphatases and Use Committee.

Materials and Methods

Cell line care

Breast cancer cell lines were maintained in complete DMEM (containing 10% FBS, 2 mmol/L l-glutamine, 15 mmol/L Hepes, and Pen/Strep). Melanoma, ovarian carcinoma, and colorectal cancer lines were maintained in complete RPMI 1640 (16). Transfectants of the B lymphoblastoid cell line CIR expressing either HLA-A2 (C1R-A2) or a chimeric HLA-A2/Dd chain (CIR-AAD) were maintained in complete RPMI with 300 μg/mL Hygromycin B or G418 (Cellgro), and G418 (Cellgro), respectively (16).

Human CD8 T-cell culture and IFNγ ELISpot

Magnetic bead–enriched (Miltenyi Biotec; 130-096-95) human CD8 T cells were cultured with irradiated, peptide-pulsed dendritic cells (DC) for 7 days in individual 96-well microcultures at a 5:1 ratio (31). In some experiments, enriched CD8 T cells were further magnetic bead–enriched for CD45RO+ cells (Miltenyi Biotec; 130-046-001). An indirect ELISpot was performed as described (32) using 25,000 cells per well with or without 75,000 peptide-pulsed (10 μg/mL) T2 targets. All human protocols were approved by the UVA IRB for Health Sciences Research.

Generation of murine phosphopeptide-specific T cells

Murine CD8 T cells specific for pIRS-21097–1105 (RVA[pS]) or pCDC25b38–46 (GLLG[pS]PVRA) were generated in AAD transgenic mice as described (14, 16), p8-catenin90–99 (YLD[pS]GIIHSGA), Yellow Fever NS4B214–222 (LLWNGPMAV), and M158–66 (Flu (GLGFVFTL) peptides were used as controls. Peptides were synthesized by GenScript or Biosynthesis Inc. All protocols were approved by the UVA Institutional Animal Care and Use Committee.

Cloning of phosphopeptide-specific murine TCR α and β chains

pIRS-21097–1105–specific or pCDC25b38–46–specific murine T-cell lines were magnetically enriched for CD8α (Miltenyi Biotec; 130-049-401). Total RNA was isolated using the PureLink Micro-to-Midi Total RNA isolation kit ( Invitrogen) and cDNA was synthesized using the GeneRacer Kit (Life Technologies) as described (33). 5′RACE PCR was performed using the GeneRacer 5′ primer and one of three specific gene-specific primers: TCR-CDRev (5′-ACTGGACCACAGAGC- CAGGTCCTCAT-3′), TCR-CβRev (5′-TGAAATCTTCCCTTG ACCATAGCCAT-3′), or TCR-CβRev (5′-GGAAATTTTTTTTCT TGACCAGCCCAT-3′). PCR products of the correct size were cloned into the pcR-4 TOPO vector (Life Technologies). TCR sequences were determined and matched to the IMGT database (34). TCR sequences were found under GenBank accession nos. KJ542620, KJ542621, KJ542622, and KJ542623.

Electroporation of in vitro transcribed RNA encoding phosphopeptide-specific TCR chains

In vitro transcribed (IVT) RNA of TCR αβ chains and transfections of OKT3-activated human CD8 T cells was performed as described (35, 36). The 5′ primes included sequence for T7 RNA polymerase binding and transcription, followed by a Kozak sequence, a start codon, and the next 16 to 17 bp of Vα or Vβ region for each TCR gene, whereas the 3′ primes included 66 T residues and 16 to 25 bp of the relevant α or β constant region sequence. pIRS-21097–1105–specific TCRα cDNA was amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and pIRS-21097–1105– specific 3′ primer (5′-T66AACTGGACCACAGGCTCTGTCCT- 3′), whereas the TCRβwas amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAG CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-T66AACTGGACCACAGGCTCTGTCCT-3′), whereas the TCRβwas amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′). pDC25b38–46–specific TCR cDNA was amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′). pDC25b38–46–specific TCR cDNA was amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′) and the 3′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′). pDC25b38–46–specific TCR cDNA was amplified using the 5′ primer (5′-TAATACGACTCACTATAGGGAGACCCATGTTGGCACCAGC CACATCAGCTTGGCACCAGC CACATCAGCTTGGCACCTCCTCCC-3′).
Functional analysis of phosphopeptide-specific murine TCR-expressing human CD8 T cells

Fourteen to 16 hours postelectroporation, human CD8 T cells were cocultured with peptide-pulsed or unpulsed C1R-AAD, C1R-A2, or cancer cells endogenously expressing pIRS-21097–1105 or pCDC25b38–46. Cell surface expression of mouse TCRβ, and human CD3 and CD8 was assessed using antibodies from Becton Dickinson Biosciences or eBioScience. During a 5-hour coculture of stimulator cells with TCR-transfected CD8-T cells at 37°C, anti-human CD107a–Alexa 647 (eBioScience) was added in the presence of 5 μg/mL Brefeldin A (Sigma), 5 μg/mL Monensin (eBioScience), and 300 IU/mL human IL2 (Chiron or R&D Systems). Cells were then stained for surface molecules, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained for intracellular cytokine (anti-IFNγ and anti-TNFα; eBioScience). Immunofluorescence was analyzed using the Becton Dickinson FACSCanto I or Canto II flow cytometers and FlowJo software.

In vitro cytotoxicity assay

Phosphopeptide-specific TCR-expressing CD8 T cells were cocultured for 5 hours with a 1:1 mix of C1R-A2 cells pulsed with 1 μmol/L phosphopeptide and stained with 1 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) and unpulsed C1R-A2 cells stained with 0.1 μmol/L CFSE. Specific killing was assessed by evaluating percentage loss of the peptide-pulsed population relative to the unpulsed population.

Western blot analysis

Lysates were generated as described (16) or using NE-PER protein extraction kit (Thermo Fisher) and separated on 8% to 16% gradient SDS/PAGE gels (ISC Bioexpress or Thermo Scientific). Proteins were transferred to Immobilon FL PVDF (Millipore) membranes, which were blocked and probed with pSer1100-IRS-2- or pCDC25b-specific and GAPDH-specific Ab (Santa Cruz Biotechnology; SC-25778) as described (16). Blots were then stripped with Restore Plus (Thermo Scientific) and reprobed with anti-I RS-2 (Santa Cruz Biotechnology; H-205). Total CDC25b protein was detected with anti-CDC25b (C-20; Santa Cruz Biotechnology). A HEK293T line transfected to express CDC25b (Novus Biologicals) was used as a positive control for anti-CDC25b. Pixel densities were determined using the AlphaEase FC software.

Immunohistochemistry

Formalin-fixed paraffin-embedded cell line pellets and tissue microarrays of metastatic melanoma (UVA Biorepository and Tissue Research Facility) were deparaffinized, rehydrated, counterstained with hematoxylin, incubated with anti-Ser1100-pIRS-2 (16) for 3 hours at 4°C after antigen retrieval, and specific antibody staining was detected using AEC ImmPACT (Vector Labs). Antibodies were removed with ethanol and acidified potassium permanganate and then reprobed with anti-IRS-2 (Santa Cruz Biotechnology). Total intensities and areas of representative fields stained with anti-Ser1100-pIRS-2 (16) in the absence or presence of blocking peptide were evaluated using the "Positive Pixel Count" algorithm on an Aperio Scanner, which accounts for the number of pixels with significant staining and staining intensity of each pixel. Staining densities (total intensity/mm²) were calculated for representative sections in the absence of blocking peptide, and subtracted from staining densities of representative sections from peptide-blocked slides to determine specific staining densities of each metastatic melanoma and adjacent uninvolved tissues.

Tumor control

Seven- to 8-week-old male NOD/SCID/IL-2Rγ−/− (Jackson Immunoresearch) mice were inoculated subcutaneously with 1.4 × 10⁶ AAD+ SLM2 melanoma cells. Three × 10⁶ human CD8 T cells expressing either pIRS-2- or pCDC25b-specific TCR, or 1.5 × 10⁶ of both populations, were adoptively transferred 3 days later. An additional 1.5 × 10⁶ T cells were given 4 days later. All mice received 1,500 CU of IL2 (R&D Systems) i.p. every other day for 10 days. Tumor size was measured every 2 to 3 days with a digital caliper, and calculated as L × W (mm²). Animals were considered tumor free until the evaluation day when the tumor size was measurable (>30 mm³). Nontransfected human CD8 T cells from both cells used did not recognize SLM2 melanoma in vitro (data not shown).

Statistical analysis

Tests performed to determine statistical significance are indicated in the figure legends. P values less than 0.05 were considered significant.

Results

Immunogenicity of phosphopeptides for human donors in vitro

The pIRS-21097–1105 and pCDC25b38–46 phosphopeptides were initially identified on two melanomas and an ovarian carcinoma (16), but their ability to induce T-cell responses in humans was not evaluated. Thus, we cultured T cells from normal human donors in replicate microwells with autologous mature DCs pulsed with either phosphopeptide. After 7 days, T cells in these cultures produced IFNγ when restimulated with phosphopeptide-pulsed HLA-A2+ targets (Fig. 1A and B). They did not recognize targets pulsed with the unphosphorylated homologous peptide (Fig. 1B). The magnitude of these responses was surprisingly high. Donor 44’s phosphopeptide-specific responses were significantly greater than that to a yellow fever virus peptide (LLWNGPMAV), to which this donor had not been previously exposed. Donor 54 had been immunized with yellow fever vaccine and this individual’s phosphopeptide-specific responses were somewhat lower than the yellow fever response although still strong (Fig. 1A). We recently established that immunity to some leukemia-associated phosphopeptides in normal individuals resides in the central memory compartment, suggesting prior exposure in conjunction with immune surveillance (17). Thus, we isolated CD45RO+ memory CD8 T cells from 4 different donors and stimulated them with autologous DCs pulsed with either pIRS-21097–1105 or pCDC25b38–46 for 7 days. Using a cutoff of >50 spots/25,000 cells, all 4 donors showed moderate to strong preexisting memory
responses to the pCDC25b38–46 peptide, and two of four donors responded to pIRS21097–1105 (Fig. 1C). In all cases, the T cells were specific to the phosphorylated peptide and did not recognize the unphosphorylated homolog (not shown). The magnitude of these memory responses was quite variable among peptides and donors, but was in some cases equivalent to or greater than memory responses to influenza and/or yellow fever epitopes. (Note that donors 54 and 62 had been immunized with a yellow fever vaccine. Donors 43 and 44 are yellow fever naïve.) This is inconsistent with the development of self-tolerance to these phosphopeptides. Combined, the strength of the responses in Fig. 1 is consistent with the possibility that these 4 normal human donors have been previously exposed to both phosphopeptides. However, none of these donors have indications of autoimmune disease, consistent with the possibility that these phosphopeptides are not displayed on normal tissue.

**Functional activity of phosphopeptide-specific murine TCR upon expression in human CD8 T cells**

Adoptive transfer of human T cells transfected with cloned high-affinity tumor-reactive TCR can lead to positive clinical responses in patients with cancer (2, 37–39). These TCRs also enable the expression of endogenously processed and presented TAA on cancers of multiple types to be determined. In addition to the constraints of growing human T cells in vitro (40), tolerance mechanisms are believed to purge most of the high-affinity CD8 T cells that would mediate effective tumor regression (39). A method to isolate human TAA-specific T cells with TCRs of sufficient functional avidity to mediate tumor regression is through the immunization of HLA transgenic mice (39, 41). Murine TCRs are of similar structural homology to human TCRs such that they can be incorporated into the human CD3 complex and preferentially pair upon expression in human T cells (37, 41). We previously demonstrated that...
these phosphopeptides were immunogenic following in vivo immunization of HLA-A2 transgenic mice (16). To avoid the generation of unintended cross-reactivities through pairing of transfected and endogenous human TCR chains, we used HLA transgenic mice to elicit phosphopeptide-specific murine T cells, from which TCR cDNAs were cloned. AAD mice, expressing a class I MHC molecule that contains the α1 and α2 domains from HLA-A2, and the α3, transmembrane, and cytoplasmic domains from H-2Dd, were immunized with autologous DCs pulsed with either pIRS-21097–1105 or pCDC25b38–46. CD8 T-cell lines derived from these animals secreeted IFNγ when cultured with AAD+ targets pulsed with the phosphorylated forms of these epitopes but not their nonphosphorylated counterparts (Fig. 1D). However, they failed to recognize phosphopeptide-pulsed targets expressing fully human HLA-A2, most likely due to the low affinity of murine CD8 for the human α3 domain (37, 42). cDNAs encoding the TCR α and β chains from pIRS-21097–1105–specific (Supplementary Fig. S1) or pCDC25b38–46–specific (Supplementary Fig. S2) T-cell lines were molecularly cloned and used as templates to produce IVT RNA (33, 36). Electroporation of IVT RNA into either TCR-deficient SupT1 cells or human CD8 and CD4 T cells resulted in surface expression as detected by staining for mouse TCRβ (Supplementary Fig. S3A and S3B). TCR expression was detected at high levels at 9 hours (Supplementary Fig. S3B) with some TCR still detectable 5 days postelectroporation (Supplementary Fig. S3C).

Human CD8 T cells electroporated with IVT RNA encoding either TCR produced IFNγ and/or upregulated CD107a, a marker of cytotoxic activity, in a dose-dependent manner after coculture with phosphopeptide-pulsed AAD+ targets (Fig. 2A and B). Both TCR conferred half-maximal recognition at a peptide dose of approximately 400 to 800 pmol/L. In contrast with the murine T cells expressing these TCR (Fig. 1D), the human CD8 T cells recognized phosphopeptide-pulsed targets...
expressing HLA-A2 at least as well as those expressing AAD (Fig. 2A and B). Neither cell produced IFNγ or upregulated CD107a in response to HLA-A2+ targets pulsed with high levels of the nonphosphorylated peptide. Human CD8 T cells expressing the pIRS-2–specific murine TCR also killed pIRS-21097–1105–pulsed, but not pCDC25b38–46–pulsed, targets in vitro (Fig. 2A), whereas those expressing the pCDC25b–specific TCR killed pCDC25b38–46–pulsed but not pIRS-21097–1105 or pβ-catenin30–39–pulsed targets (Fig. 2B). Thus, the expression of these murine TCRs in human CD8 T cells imparts

Figure 3. pIRS-21097–1105 is endogenously processed and presented by cancer cells of multiple histologic types. A, immunoblots showing expression of pSer1100-IRS-2 (top), total IRS-2 (middle), and GAPDH (bottom) in extracts representing 1.5 × 10^6 cell equivalents of the indicated cell lines. Anti-pSer1100-IRS-2 and anti-GAPDH staining was done simultaneously for each blot, which was then stripped and reprobed with anti-IRS-2 (5 min exposure for all blots). Because lysates were loaded on the basis of equivalent cell numbers instead of protein, the evident variation in GAPDH protein indicates differences in expression of this housekeeping gene in different cancer cells. Data are from a single experiment representative of four experiments. B, total responding pIRS-2–specific human CD8 T cells (mTCRb+) following coculture with indicated human cancer cells was determined as in Fig. 2A. HLA-A2–negative cancer cell lines. Data are representative of three to five experiments. C, immunoblots of pSer1100-IRS-2 (top) and total IRS-2 (bottom) in extracts representing 50 μg total protein of the indicated cancer cells. D, correlation between pSer1100-IRS-2 protein and recognition of HLA-A2+ cancer cells by pIRS-2–specific mTCRb+ human CD8 T cells (data in A and B). Linear regression analysis (solid line) with 95% confidence intervals (dashed lines) is shown. *P value indicates best-fit value for the slope of the curve being significantly nonzero.
phosphopeptide-specific, high-avidity recognition and both cytotoxic and cytokine-secreting effector activities.

Expression of pIRS-2\(_{1107-1105}\) and pCDC25b\(_{38-46}\) phosphopeptides on cancer cells

We next evaluated whether these transfected CD8 T cells could recognize endogenously processed and presented pIRS-2\(_{1107-1105}\) or pCDC25b\(_{38-46}\) phosphopeptide on HLA-A\(^2\) cancer cell lines. To correlate pIRS-2\(_{1107-1105}\)-specific T-cell recognition with phosphoprotein expression, we used an antibody specific for the Ser\(_{1100}\)-phosphorylated IRS-2 protein (pSer\(_{1100}\)-IRS-2), as well as an antibody that recognizes total IRS-2 protein (16). A substantial fraction of pIRS-2\(_{1107-1105}\)-specific T cells upregulated CD107a, and a subset of these also produced IFN-\(\gamma\) upon coculture with two HLA-A\(^2\) melanoma cell lines, MelSwift and 1102Mel (Fig. 2C). These two cell lines also expressed high levels of pSer\(_{1100}\)-IRS-2 (Fig. 3). However, there was no recognition of an HLA-A\(^2\) pSer\(_{1100}\)-IRS-2 melanoma, SK-Mel-28, or an HLA-A\(^2\)-pSer\(_{1100}\)-IRS-2 low to negative ovarian carcinoma, OV-90. Although there is no specific antibody for Ser\(_42\)-phosphorylated CDC25b, human CD8 T cells transfected to express the pCDC25b\(_{38-46}\)-specific TCR recognized two HLA-A\(^2\) melanomas that expressed high levels of total CDC25b (MelSwift and 1102Mel), and failed to recognize either an HLA-A\(^2\)-pSer\(_{1100}\) CDC25b melanoma, SK-Mel-28, or an HLA-A\(^2\)-CDC25b\(_{58}\) ovarian carcinoma, OV-90 (Figs. 2C and 4).

We used these T cells to evaluate expression of pIRS-2\(_{1107-1105}\) and pCDC25b\(_{38-46}\) on HLA-A\(^2\) cancer cell lines of different types. For the HLA-A\(^2\) cancer cells, we loaded the Western blots based on cell equivalents rather than protein equivalence, so we could directly compare per cell level of expression of Ser\(_{1100}\)-phosphorylated IRS-2 with T-cell recognition, which also occurs on a per cell basis. Although the amount varied, Ser\(_{1100}\)-phosphorylated IRS-2 was detected by Western blot analysis in the majority of melanoma, ovarian cancer, colorectal adenocarcinoma, breast cancer, bladder cancer, and non-small cell lung cancer (NSLC) lines evaluated, but was poorly expressed in prostate cancer cells (Fig. 3A and C). None of the bladder, prostate, or NSCL cancer cells were HLA-A\(^2\) and their recognition by pIRS-2\(_{1107-1105}\)-specific T cells could not be tested. However, of the HLA-A\(^2\) cell lines evaluated, pIRS-2\(_{1107-1105}\) was presented by 10 of 10 melanomas, 3 of 4 ovarian carcinomas, 2 of 2 colorectal carcinomas, and 2 of 3 breast carcinomas (Fig. 3B). Cancer cells that were better recognized by pIRS-2\(_{1107-1105}\)-specific T cells also expressed higher amounts of pSer\(_{1100}\)-IRS-2 by Western blot analysis (Fig. 3D). pCDC25b\(_{38-46}\)-specific T cells also did not recognize the HLA-A\(^2\)-pSer\(_{1100}\)-IRS-2 melanoma SK-Mel-28 (Figs. 2C and 4B). They did recognize 3 of 4 HLA-A\(^2\) EBV-transformed lymphoblastoid cell line JY (not shown). However, although pCDC25b-specific T cells showed high avidity and high-level recognition of peptide-pulsed targets (Fig. 2B), their recognition of these cancer cells was relatively low to negative ovarian carcinoma, OV-90.

Figure 4. pCDC25b\(_{38-46}\)-specific TCR-expressing human CD8 T cells recognize endogenously processed and presented phosphopeptide on human melanoma and breast cancer cells. A, immunoblots showing expression of total CDC25b (top) and GAPDH (bottom) in indicated cancer cells (30 \(\mu\)g cytoplasmic protein). Only 1 \(\mu\)g of a CDC25b-transfected HEK293T cell lysate was loaded to not overexpose blot, resulting in an almost undetectable GAPDH signal. Representative blots from two experiments are shown. B, total responding pCDC25b-specific human CD8 T cells (mTCR\(^{1b}\)-human CD8\(^{+}\)) following coculture with indicated human cancer cells was determined as described in Fig. 2. C, correlation between CDC25b protein level and recognition of HLA-A\(^2\)-cancer cells by pCDC25b-specific mTCR\(^{1b}\)-human CD8 T cells in melanoma cell lines (left) and all other cancer cell lines evaluated (right; data in A and B). Analysis completed as in Fig. 3D.
low (Fig. 4B). They also did not recognize the two colorectal adenocarcinomas and four ovarian cancer cell lines evaluated. In melanoma cells, there was a direct correlation between pCDC25b_{38-46}–specific T-cell recognition and total CDC25b protein levels (Fig. 4C). However, good pCDC25b_{38-46}–specific T-cell recognition was associated with high-level expression of CDC25b source protein in breast, ovarian, and colorectal cancer cells but low level expression in others. This suggests that there are differences in the level or the turnover of pSer42-CDC25b in relation to total CDC25b protein in different histologic types of cancer cells. In sum, pIRS-2_{1097-1105} is endogenously processed and presented by numerous malignancies of different types, and this display elicits strong effector responses from pIRS-2–specific TCR-expressing human CD8 T cells. In contrast, pCDC25b_{38-46} is presented by melanoma, breast cancer, and EBV-transformed lymphoblastoid cell lines, but its overall expression on these and other cells is more limited.

Immunohistochemical analysis of pSer_{1100}-IRS-2 expression in metastatic melanoma and normal tissues

The expression of pSer_{1100}-IRS-2 in human melanoma explants and normal tissues has not been previously evaluated. We compared sections from cell blocks containing the pSer_{1100}-IRS-2–stained tissue sections from the melanoma cell line SLM2 (A), ovarian carcinoma OV-90 (B), and lung melanoma metastasis 1 (see Table 1; C) without (left) or with (right) blocking peptide added to antibody before staining. All images are ×100 magnification. Arrows, mitotic cells.

Figure 5. pSer_{1100}-IRS-2 staining is highest in mitotic cancer cells. pSer_{1100}-IRS-2–stained tissue sections from the melanoma cell line SLM2 (A), ovarian carcinoma OV-90 (B), and lung melanoma metastasis 1 (see Table 1; C) without (left) or with (right) blocking peptide added to antibody before staining. All images are ×100 magnification. Arrows, mitotic cells.
densities did not extend beyond the epithelium to involve deeper portions of the colonic tissue (Supplementary Fig. S4). Pulmonary epithelia also demonstrated relatively high staining densities, although these were not as high as those seen in the colonic epithelia (Table 1). These findings may relate to the normal proliferation and turnover of epithelial cells. Interestingly, peritumoral stroma (fibroblasts and blood vessels) surrounding lung metastases show increased staining relative to adjacent "normal" pulmonary parenchyma (Table 1). Overall, these data suggest that successful immunotherapy based on pSer\textsuperscript{1100}-IRS-2 may target dividing malignant cells, and may also target peritumoral stroma. Each of these may support tumor control (43, 44). However, the data also raise the possibility that there is some risk of adverse effects on

### Table 1. Specific anti-Ser\textsuperscript{1100}-pIRS-2 staining densities (positive pixel count \(\times 10^7/mm^2\)) for melanoma metastases and surrounding tissues

<table>
<thead>
<tr>
<th>Tissue specimen</th>
<th>Melanoma metastasis</th>
<th>Adjacent <em>normal</em> tissue</th>
<th>Pseudostratified respiratory epithelium within normal tissue</th>
<th>Peritumoral stroma</th>
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<td>Colon with melanoma metastasis</td>
<td>3.3</td>
<td>1.6–34</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mean in mitotic cells (lung with melanoma metastasis 2)(^a)</td>
<td>27</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mean in mitotic cells (SLM2 melanoma)(^b)</td>
<td>419.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE:** Specific staining densities were calculated as described in Materials and Methods. Abbreviation: ND, not done.

\(^a\)Mean for mitotic cells \((n = 5)\) in lung melanoma metastasis 1 (Fig. 5C).

\(^b\)Mean for mitotic cells \((n = 5)\) in SLM2 melanoma cells in vitro.

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**Figure 6.** Ser\textsuperscript{1100}-phosphorylated IRS-2 expression in metastatic melanoma sections involving vital organs. pSer\textsuperscript{1100}-IRS-2–stained sections from melanoma metastases in lung (sample 3 in Table 1), heart, and liver (A, C, and E, respectively), together with adjacent uninvolved tissues (B, D, and F, respectively). Arrows in A, mitotic cells with intense staining. All images are \(\times 100\) magnification.
Phosphopeptide-specific TCR-expressing T cells can slow tumor outgrowth

We next determined whether these two phosphopeptides could serve as immunotherapeutic targets for treatment of cancer. NOD/SCID/IL-2Rγ−/− mice were inoculated subcutaneously with SLM2 melanoma cells, and 3 days later were injected with human CD8 T cells expressing either the pIRS-2-specific or pCDC25b-specific TCR, or both populations, together with IL2. A second infusion of transfected CD8 T cells was given 4 days later. Animals that received any of these phosphopeptides remained tumor free (tumor size less than 30 mm²) for significantly longer than control animals that only received IL2 (Fig. 7A–C). Tumors in animals that received any of these phosphopeptide-specific murine TCR-expressing cells were significantly smaller on day 10 and 15 than those of control animals that received only IL2 (Fig. 7D). On day 18, tumors in treated animals were still smaller, but only those in mice given pCDC25b-specific T cells were still significant. No significant differences in outgrowth in any groups were evident beyond day 22, most likely due to loss of expression of phosphopeptide-specific murine TCR (Supplementary Fig. S3C). Indeed, at the end of the experiment, we identified persisting human T cells that no longer expressed murine TCR in tumor and spleens of treated animals (data not shown). Nonetheless, this demonstrates that the endogenous levels of pIRS-21097–1105 and pCDC25b38–46 phosphopeptide on melanoma are sufficient for T-cell recognition and allow some control of tumor growth in vivo.

Discussion

In this study, we characterized two phosphopeptide TAA that are endogenously processed and presented on multiple HLA-A2+ cancers. To explore the display on cancer cells, we used cloned murine TCR specific for each phosphopeptide and an antibody specific for the phosphorylated IRS-2 source protein. pIRS-21097–1105 is displayed on multiple HLA-A2+ melanomas and breast, ovarian, and colorectal carcinomas, and this display is correlated with the level of Ser1100-phosphorylated IRS-2 source protein. Mitotically active tumor cells expressed very high levels of Ser1100-phosphorylated IRS-2 protein. On the basis of immunologically relevant levels of display of pIRS-21097–1105, we expect to be able to immunologically target a variety of distinct malignancies. In contrast, pCDC25b38–46 display is restricted to melanoma and breast cancer. Although it is also displayed on lymphoblastoid cell lines, it was not found on a set of hematologic malignancies (17). Nonetheless, this peptide adds to the otherwise small number of antigenic epitopes that have been defined for breast cancer.

Both phosphopeptides are strongly immunogenic in vitro for human T cells and in vivo for HLA-A2 transgenic mice, lending credence to their utility as immunotherapeutics. Indeed, CD8 T-cell responses to these phosphopeptides in many normal healthy donors were evident primarily in the CD45RO+ and pCDC25b38–46 phosphopeptide on melanoma are sufficient for T-cell recognition and allow some control of tumor growth in vivo.

![Figure 7](image-url). Enhanced tumor-free survival and delayed tumor outgrowth following adoptive transfer of phosphopeptide-specific TCR-expressing human CD8 T cells. SLM2AAD melanoma tumor-bearing NOD/SCID/IL-2Rγ−/− mice were injected with phosphopeptide-specific TCR-expressing human CD8 T cells on days 3 and 7, together with 1,500 CU/mL IL2 every other day for 10 days. Control animals received only IL2. Combo TCR animals received equal amounts of pIRS-2 and pCDC25b-TCR expressing human CD8 T cells. (n = 5 per group). A–C, P values were determined by the log-rank (Mantel–Cox test) analysis of control animals to the indicated experimental group through day 25. D, data points are mean tumor volumes for each group ± SD. P values were determined by ANOVA with post hoc comparisons and no adjustment for multiple comparisons for each day and are in the sequence pCDC25b-TCR, pIRS2, and combo TCR. * P = 0.034; +, P = 0.025; ns, nonsignificant, P > 0.05.
memory compartment. In contrast, immunologic responses to cancer-testes antigens and tissue-associated differentiation antigens are usually only seen in patients with cancer (45, 46). Although high-level responses to an HLA-A2-restricted epitope from MART-1 are seen in normal individuals, these are primarily evident in the naïve T-cell compartment (46). Although it is difficult to prove in humans, we hypothesize that the memory responses to the pIRS-21105-1105 and pCDC25b38-46 phosphopeptides in normal individuals are evidence of previous encounters with nascent tumors that have dysregulated phosphorylation cascades. Although we have seen expression of pSer1100-IRS-2 protein in some normal colonic and pulmonary epithelia by immunohistochemistry, there is no evidence of autoimmunity in these healthy human donors that we have evidence of memory phosphopeptide-specific T-cell responses. There may be self-tolerance directed against very high avidity human TCR for these antigens, thus emphasizing the utility of high-affinity murine TCRs for adoptive therapy.

One approach currently showing some success for the treatment of patients with cancer involves the adoptive transfer of tumor-specific CD8 T cells, generated through vaccination or by genetic modification via expression of TCR chains specific for an appropriate TAA (1). Clinical benefit following infusion of such transfected T cells has been observed in melanoma and synovial cell sarcoma (35, 39). Most of the TCR chains currently cloned and studied in human clinical trials for melanoma have been specific for melanocyte differentiation proteins (1, 6, 39). Although of obvious importance for melanoma, extending this form of immunotherapy to antigens that are broadly expressed on other types of cancers offers the possibility of broadening adoptive cell therapy to multiple cancer patients. In addition, we have identified phosphopeptides presented on cancer cells by other HLA alleles besides HLA-A2 (data not shown; ref. 17), extending the number of patients that can be treated with phosphopeptide-specific immunotherapy.

The utilization of murine TCR minimizes concerns about formation of unintended allo- or self-reactive TCRs through pairing with human endogenous TCR α and β chains while allowing isolation of chains of sufficient avidity to mediate tumor regression (37, 41). We have not seen alloreaction of human cancer cells expressing other alleles than HLA-A2 when these murine TCR chains are expressed in human CD8 T cells. Although we have not directly compared the avidities of these cloned murine TCR with those of bulk short-term human CD8 T cells, we believe that the salient issue is that the avidities of the cloned murine TCR are high and in a therapeutically useful range based on peptide dose–response curves (47). Thus, we now have new tools that can be utilized to evaluate and treat patients with cancer: the phosphopeptides themselves for use in active vaccination, and murine TCR chains specific for either pIRS-21105-1105 or pCDC25b38-46 peptides that can be utilized as immunotherapeutic agents to target a patient's T cells to these posttranslationally modified epitopes.

Disclosure of Potential Conflicts of Interest

A.L. Zarling, R.C. Obeng, and K.L. Cummings have ownership interest (including patents) in Phosimmune, Inc. C.L. Slighluff Jr. reports receiving a commercial research grant from GlaxoSmithKline; has ownership interest (including patents) with UVA Licencing and Ventures Group; and is a consultant/ advisory board member for Immutas and Polynoma. V.H. Engelhard is employed as Scientific Founder of, and has ownership interest (including patents) in, Phosimmune, Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.L. Zarling, R.C. Obeng, A.N. Desch, J. Pinczewski, K.L. Cummings, C.L. Slighluff Jr
Writing, review, and/or revision of the manuscript: A.L. Zarling, R.C. Obeng, J. Pinczewski, K.L. Cummings, M. Conaway, C.L. Slighluff Jr, V.H. Engelhard
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MHC-Restricted Phosphopeptides from Insulin Receptor Substrate-2 and CDC25b Offer Broad-Based Immunotherapeutic Agents for Cancer

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