Definition of an Immunogenic Region Within the Ovarian Tumor Antigen Stratum Corneum Chymotryptic Enzyme

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Abstract  Purpose: The serine protease stratum corneum chymotryptic enzyme (SCCE) is overexpressed by ovarian tumor cells, but is not expressed by normal tissues, suggesting that SCCE may be an attractive target for immunotherapy. In this study, we tested the hypothesis that dendritic cells loaded with SCCE peptides will induce ovarian tumor antigen – specific CD8+ CTL responses and antigen-specific CD4+ helper T-cell responses.

Experimental Design: Computer algorithms were used to identify candidate HLA-A2.1-restricted CD8+ CTL epitopes and HLA-DR-binding CD4+ helper T-cell epitopes within SCCE. CD8+ CTL stimulated with peptide-loaded dendritic cells were tested against targets expressing endogenous SCCE, including HLA-A2.1-matched ovarian tumor cells. Dendritic cells were also loaded with an extended SCCE peptide, SCCE 110-139, which encompassed a defined CD8+ CTL epitope and multiple candidate CD4+ T-cell epitopes.

Results: CD8+ CTL specific for SCCE 123-131 lysed autologous macrophages infected with an SCCE-expressing recombinant adenovirus, and also lysed HLA-A2.1-matched, SCCE-expressing ovarian tumor cells. Dendritic cells loaded with SCCE 5-13 peptide stimulated an HLA-A2.1-restricted CD8+ CTL response, but with a reduced level of lysis against ovarian tumor cells. Dendritic cells loaded with SCCE 110-139 induced antigen-specific CD4+ T-cell and CD8+ T-cell responses. Although SCCE 110-139-loaded dendritic cells processed and presented the 123-131 epitope, the dominant CD8+ CTL response was directed against alternative epitopes within SCCE 110-139.

Conclusions: The 110-139 region of SCCE incorporates multiple CD8+ CTL and CD4+ helper T-cell epitopes, and represents an attractive target antigen for immunotherapy of ovarian cancer.

Ovarian cancer ranks fifth among malignancies affecting women in the United States. The onset of ovarian cancer is insidious and the symptoms are nonspecific, such that two-thirds of women present with advanced disease at diagnosis. Although the initial response rate of patients with advanced disease after treatment with platinum and taxol is 73% to 77%, a large majority develop recurrent disease. These factors contribute to ovarian cancer having the highest mortality rate of all gynecologic tumors. Thus, alternative methods for treatment are a priority for ovarian cancer.

The identification of ovarian tumor – specific antigens that can serve as targets for CD8+ CTLs (1) and the harnessing of dendritic cells that possess the ability to induce CTL responses to those targets (2, 3) strongly suggests that dendritic cell immunotherapy may be of potential therapeutic benefit. Several studies from our laboratory and others have indicated that dendritic cells loaded with different forms of antigen are capable of generating a specific CTL response to gynecologic malignancies. Monocyte-derived dendritic cells loaded with tumor lysate antigen can induce tumor-specific CTL lysis of autologous tumor cells from patients with ovarian cancer or uterine serous papillary carcinoma (4, 5), and dendritic cells pulsed with peptides acid-eluted from HLA class I on the surface of ovarian tumor cells can stimulate CTL killing of autologous tumor (6). Targeting of E7 expressed by human papillomavirus – associated cervical cancer has also been an effective strategy for stimulation of tumor-specific CTL in vitro (7) and for therapeutic dendritic cell vaccination against cervical cancer (8).

The identification of appropriate tumor-specific antigens is a critical component for the development of successful ovarian tumor-specific immunotherapy. Probably the best known ovarian tumor- associated antigen is CA125, the secreted form of which has long been recognized as the gold standard for monitoring patients with ovarian cancer (9). For therapeutic purposes, however, the lack of tissue or disease-specificity of CA125 reduces its appeal. A further potential drawback is that the extensive glycosylation of CA125 may limit antigen processing, presentation, and recognition by HLA class I-restricted CTL (1). HER2/neu is also a well-known tumor antigen against which CTL responses have been described (10–12), but HER2/neu is overexpressed in only a minority of ovarian tumors. In contrast, folate binding protein is expressed by >90% of ovarian tumors and CTL specific for HLA-A2.1-restricted peptides have been

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derived from tumor-associated lymphocytes (13, 14). Other potential T cell targets for ovarian cancer include SART-1, MAGE-1, MUC1, MUC2, MUC4, MUC5AC, and mesothelin (1).

Tumor-associated serine proteases are involved in many biological functions of cancer cells, including activation of growth and angiogenic factors and promotion of invasion and metastasis. Stratum corneum chymotryptic enzyme (SCCE), also known as kallikrein 7, is a serine protease that is overexpressed by ovarian cancer cells but not by normal ovaries or other normal adult tissues, except the outermost cornified layer of the skin. Immunohistochemical analysis of 14 ovarian tumors showed positive staining localized to the cytoplasm and cell membrane, suggesting that SCCE may be expressed as both secreted and membrane forms (15). Quantitative RT-PCR revealed SCCE expression in >88% of serous ovarian tumors, 100% of endometrioid and clear cell tumors, but only 29% (two of seven) mucinous tumors (15).

The tightly limited tissue distribution of SCCE and overexpression in ovarian tumors suggests that it would be a favorable target antigen for immunotherapy. In this study, we used computer analysis to identify potential HLA-A2.1-binding peptides from the SCCE sequence. Of the 12 peptides tested, dendritic cells loaded with SCCE 123–131 stimulated specific CTL that recognized peptide, endogenously expressed full-length protein, and HLA-A2.1-matched ovarian tumor cells that expressed SCCE. Dendritic cells loaded with SCCE 5–13 were also capable of stimulating a limited CTL response against HLA-A2.1-matched ovarian tumor cells. Computer analysis also identified candidate HLA-DR-binding epitopes clustered around the SCCE 123–131 CTL epitope. We found that dendritic cells loaded with an extended SCCE 110–139 peptide stimulated strong antigen-specific CD8+ CTL responses and also stimulated antigen-specific CD4+ helper T cell responses restricted by multiple HLA class II molecules. Dendritic cells loaded with SCCE 110–139 processed and presented the SCCE 123–131 CTL epitope, but the dominant CTL response was directed against other epitopes within the SCCE 110–139 region. Collectively, these results suggest that the 110-139 region of SCCE is an excellent target for dendritic cell immunotherapy of ovarian cancer.

**Materials and Methods**

**Cell lines, antibodies, and cytokines.** The ovarian carcinoma cell line CaOV3 and the natural killer–sensitive K562 cell line were from the American Type Culture Collection (Manassas, VA). CaOV3 cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 1 mmol/L sodium pyruvate, nonessential amino acids, 3 mmol/L l-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (DMEM/10). The OVCAR3 ovarian tumor cell line was provided by Birgit Schultes (AltaRex Corp., Waltham, MA). EBV-transformed lymphoblastoid cell lines were established from HLA-A2.1-positive and -negative donors as described (16). OVCAR3, K562, lymphoblastoid cell lines, and HLA class I-negative CIR cells were grown in RPMI supplemented with 10% FCS, 5 × 10⁻⁵ mol/L 2-β-mercaptoethanol, 3 mmol/L l-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (RPMI/10). Macrophages and dendritic cells were grown in AIM-V (Invitrogen). T cells were grown in RPMI supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA), 5 × 10⁻⁵ mol/L 2-β-mercaptoethanol, 3 mmol/L l-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (RPMI/10 Ilu).

Fluorochrome-conjugated anti-CD4 monoclonal antibody (MAb), anti-CD8 and anti-IL-10 were purchased from Caltag ( Burlingame, CA), Fluorochrome-conjugated MAb specific for interleukin (IL)-4, IFNγ, IL-13, tumor necrosis factor-α, and IL-2 were purchased from BD Immunocytochemistry (San Jose, CA). W6/32 (anti-HLA class I), L243 (anti-HLA class II), and B87.2 (anti-HLA-A2.1) MAb were prepared by hybridomas purchased from the American Type Culture Collection.

Cytokines for the establishment of dendritic cells and T cell cultures included granulocyte macrophage colony-stimulating factor (Immunex, Seattle, WA), IL-4, tumor necrosis factor-α (both from R&D Systems, Minneapolis, MN), prostataglandin E2 (Sigma, St. Louis, MO), IL-1β, and IL-2 (both from the Biological Response Modifiers Program, National Cancer Institute).

**HLA binding motifs.** 9-mer and 10-mer peptides from SCCE with the potential to bind HLA-A2.1 were identified through the use of two computer programs. The first was developed by the Bioinformatics and Molecular Analysis Section of the Center for Information Technology, NIH (http://biomas.dctr.nih.gov/molbio/hla_bind; ref. 17), and the second was formulated by Rammensee et al. (http://134.29.62.221/scripts/hlaserver.dll/EpPredict.htm; ref. 18). To avoid potential T cell cross-reactivity between tumor antigens and proteins expressed in normal tissues, those peptides with significant levels of sequence identity (three or more matched amino acids within a 9- to 10-residue peptide) to ubiquitous serine proteases were not considered as possible candidate epitopes. Peptides were synthesized by Alpha Diagnostic International (San Antonio, TX).

The algorithms of Southwood et al. (19) were used for estimation of peptide binding affinities to HLA-DR1, -DR4, and -DR7. Available data suggest that a large set of DR molecules contain overlapping peptide-binding repertoires and combined analysis of the DR1, DR4, and DR7 motifs has a high probability of predicting degenerate HLA-DR-binding epitopes, or multiple DR-binding clusters.

**Dendritic cells and stratum corneum chymotryptic enzyme–specific T cells.** Peripheral blood was drawn from healthy adult volunteer donors, following an Institutional Review Board–approved protocol. Peripheral blood mononuclear cells were recovered by gradient centrifugation (Lymphoprep; Greiner Bio-One, Longwood, FL). The HLA type of donor 1 is A2, A1, B8, B27, Cw1, DR3, DDr52, DQw2, and the HLA type of donor 2 is A2, A29, B44, B61, Cw12, DR1, DR7, DQ1, DQ2, and DR53.

For preparation of dendritic cells, peripheral blood mononuclear cells were placed in six-well plates (Costar, Cambridge, MA) at a concentration of 5 × 10⁶ per well in AIM-V medium. After incubation for 2 to 3 hours at 37°C, nonadherent cells were removed from the culture and the medium was replaced with AIM-V plus 800 units/ml granulocyte macrophage colony-stimulating factor and 500 units/ml IL-4. On days 3 and 5, half the medium was removed and replaced with AIM-V plus 800 units/ml granulocyte macrophage colony-stimulating factor and 500 units/ml IL-4. A mix of maturation cytokines (1 µmol/L/ml prostataglandin E2, 1,000 units/ml tumor necrosis factor-α, and 500 units/ml IL-1β) was added on day 5 or 6. For stimulation of CD8+ T cells specific for HLA-A2.1-binding SCCE peptide epitopes, mature dendritic cells were collected after maturation for 48 hours, and pulsed with 50 µg/mL of peptide for 2 hours in AIM-V at 37°C. The dendritic cells were then washed once with AIM-V medium and used for T cell stimulation at a peripheral blood mononuclear cell/dendritic cell ratio of 30:1. After 7 days, T cells were collected and restimulated with peptide-pulsed dendritic cells. After the second stimulation, CD8+ T cells were recovered by positive selection with anti-CD8 magnetic beads (Dynal Biotech, Brown Deer, WI). During the second and third T cell stimulation and passage, 50 to 100 units/ml IL-2 was added to the medium, and T cells were periodically fed (every 2-3 days) by changing medium, and T cells were periodically fed (every 2-3 days) by changing
stimulation was the same as described above. After the second stimulation, CD4+ and CD8+ T cells were recovered by positive selection with anti-CD4 or anti-CD8 magnetic beads (Dynal). During the second and subsequent T cell passages, 20 to 50 units/mL IL-2 was added to the medium, and the cultures were periodically fed (every 2-3 days) by changing 50% to 70% of the medium and addition of fresh IL-2. Dendritic cells were used as antigen-presenting cells for the first three to five antigen stimulations. Later antigen stimulations were done with autologous lymphoblastoid cell lines pulsed overnight with 50 μg/mL SCCE 110-139.

**Recombinant adenosviruses.** The AdEasy system was used to generate recombinant adenoviruses (20). Briefly, the open reading frame of full-length SCCE (lacking signal peptide) was PCR-amplified from an SCCE cDNA clone (provided by L.I. Underwood, University of Arkansas for Medical Sciences) using the primers SCCE5 (CCGGTGAATTCGGAGAGAAGACCGAGAGG) and SCCE3 (CCGGCGCGCCTAGCCGATGCTTTCGTCG). SCCE5 primer provides a methionine codon immediately upstream of the antigen coding sequence and contains a SalI recognition site. SCCE3 primer contains sequences corresponding to the termination codon and adjacent COOH terminus coding sequences and has a NotI recognition site. The PCR product was digested with SalI and NotI and ligated into SalI- and NotI-cleaved pAdTrack-CMV.

The complete open reading frame (including signal peptide) for a related serine protease, hepsin, was amplified from a cDNA clone (provided by L.I. Underwood) using the primers Hep5 (CCGGTACCCGT-GACATGGCGCAGAAGGAGG) and Hep3 (CCGGTACCCGT-GACATGGCAGAAGGAGG). The Hep5 primer has a KpnI site and the Hep3 primer has a SpeI site. The KpnI and SpeI-digested PCR product was cloned into KpnI- and Xbal-cleaved pAdTrack-CMV. The pAdTrack-cytomegalovirus shuttle vector also contains green fluorescent protein and a kanamycin resistance cassette.

SCCE-pAdTrack-CMV or Hepsin-pAdTrack-CMV were cotransfected into E. coli with the pAdEasy-1 vector. Recombinants were selected by kanamycin resistance, linearized, and transfected into 293 cells for adenovirus packaging. The transfected 293 cells were followed through 7 to 10 days for expression of green fluorescent protein. The cells were then collected, run through two to three freeze/thaw cycles and virus recovered for infection of macrophages, dendritic cells, or tumor cell lines.

**Recombinant stratum corneum chymotryptic enzyme protein.** The Qiagen QIAexpress System was used to generate recombinant SCCE protein. Full-length SCCE cDNA was cloned into the pQE30 expression vector. The M15 strain of E. coli was used for expression of high levels (up to 500 mg/L culture) of recombinant protein. The pQE30 plasmid allows for the expression of proteins with an NH2-terminal 6× histidine tag. His-tagged SCCE (>99% purity) was achieved with the use of Qiagen’s nickel-NTA agarose.

**Cytotoxicity assays.** Standard 51Cr-release assays were done as described previously (16). Autologous lymphoblastoid cell lines were pulsed with 50 μg/mL of appropriate CTL peptide, or left unpulsed, for 1 hour at 37°C in AIM-V medium. Lymphoblastoid cell lines were pulsed overnight with 50 μg/mL of extended SCCE 110-139 or sterile full-length recombinant SCCE protein, whereas dendritic cells were pulsed with 50 μg/mL peptide 110-139 for 48 hours during final maturation. Peptide-pulsed targets were then labeled with 50 μCi Na2[51Cr]O4 for an additional hour and washed thrice before use. The K562 cells, which are sensitive to natural killer cell lysis, were labeled similarly with 51Cr. Autologous macrophages or tumor cell lines were pulsed with 50 μg/mL peptide or infected with recombinant adenosvirus and labeled with 51Cr overnight at 37°C in AIM-V medium. Blocking antibodies were added to target cells at concentrations indicated for each assay immediately prior to plating. Target cells were plated at 1 × 104 per well in 96-well round-bottomed plates with effecter T cells at the ratios indicated for each assay. Assays were done in triplicate wells. The percentage of target cell lysis was calculated as described (16).

**Proliferation assays.** After three rounds of antigen stimulation, CD4+ T cells (5 × 104 per well) were incubated in a 96-well flat-bottomed plate with irradiated, SCCE 110-139 peptide-pulsed dendritic cells (1 × 104 per well) or control unpulsed dendritic cells. After 48 hours, [3H]thymidine (1 μCi) was added to each well for overnight incubation. The plate was then harvested with a Filtermate 196 cell harvester and counted by a Direct Beta Counter (Packard, Meriden, CT). Stimulation indices were calculated as the ratio of [3H]Tdr uptake by antigen-responsive T cells divided by [3H]Tdr uptake by T cells cultured alone. Background counts from control wells containing antigen-presenting cells (dendritic cells or lymphoblastoid cell lines) alone were subtracted from all values prior to calculation.

**Cytokine assays.** Intracellular cytokine expression was measured by flow cytometry after overnight coculture of T cells with peptide-pulsed or unpulsed lymphoblastoid cell lines, dendritic cells, or tumor cell lines. T cells (1.5 × 106) were plated in 12-well Costar plates in 2 mL RPMI/10% FBS. T cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (500 ng/mL) as a positive control. Negative controls included T cells cultured alone, or with unpulsed lymphoblastoid cell lines or dendritic cells. At the onset of coculture, 10 μg/mL of Brefeldin A was added to block cytokine release. Cells were collected after the overnight stimulation and fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature. The cells were washed once in PBS and again in 0.5% saponin and 1% bovine serum albumin in PBS. T cells were labeled with cytokine-specific MAb conjugated to PE or FITC for 30 minutes at room temperature. After staining, the cells were washed twice in 0.5% saponin and 1% bovine serum albumin in PBS, once with 0.5% bovine serum albumin in PBS, and fixed in 2% paraformaldehyde in PBS. Fluorescence was measured with a FACS Calibur (Becton Dickinson, San Jose, CA) and data were analyzed with WinMDI software.

**Results**

**HLA-A2.1-restricted CD8+ CTL responses against stratum corneum chymotryptic enzyme peptides.** Computer algorithms were used to identify candidate HLA-A2 binding peptides from SCCE. High-scoring peptides with little or no sequence homology to other known proteases were chosen and dendritic cells loaded with each of these peptides were tested for their ability to generate a specific CD8+ T cell response. Of the 12 SCCE peptides tested, only 2 peptides, SCCE 123-131 (LLLPQLQILL), and SCCE 5-13 (QLPLQILL), induced a specific CD8+ T cell response to peptide. Donor 1 CD8+ T cells specific for SCCE 123-131 (Fig. 1A) and SCCE 5-13 (data not shown) recognized autologous lymphoblastoid cell lines pulsed with peptide in cytotoxicity assays. Cytotoxicity was inhibited in the presence of a blocking MAb specific for a nonpolymorphic HLA class I determinant (W6/32), and non-HLA restricted natural killer cytotoxicity was ruled out through use of the natural killer–sensitive target, K562, which was not lysed by the peptide-specific CD8+ CTL (data not shown). HLA-A2.1 restriction was shown in cytotoxicity assays using HLA mismatched allogeneic lymphoblastoid cell lines expressing HLA-A2.1, but no other matching HLA class I alleles, or lymphoblastoid cell lines expressing all HLA class I types in common with donor lymphoblastoid cell lines, except for HLA-A2.1. CD8+ CTL only lysed peptide-pulsed lymphoblastoid cell lines expressing HLA-A2.1 and were not able to lyse peptide-pulsed lymphoblastoid cell lines that did not express HLA-A2.1 (Fig. 1A). This result was supported by assays using peptide-pulsed C1R cells and peptide-pulsed A2.1-transfected C1R cells as targets. As C1R cells are deficient in HLA class I expression, lysis of the
A transfection target, but not the parent C1R cells, confirmed that the peptide-specific CD8+ T cell cytotoxicity is A2.1-restricted (Fig. 1B).

Although SCCE peptide-specific CTL recognize peptide-pulsed targets, it is unclear whether those CTL will recognize targets expressing endogenously processed SCCE antigen. We asked whether SCCE 123-131 and SCCE 5-13 peptide-specific CTL were cytotoxic against macrophages infected with recombinant adenovirus that expressed SCCE (Ad-SCCE) or a control recombinant adenovirus expressing another ovarian tumor antigen, hepsin (Ad-hepsin). SCCE 123-131 peptide-specific CTL were able to lyse Ad-SCCE-infected targets, but did not recognize Ad-hepsin-infected targets (Fig. 2A). Ad-SCCE-infected targets were not recognized or killed by SCCE 5-13 peptide-specific CTL, even though these CTL were able to kill peptide-pulsed autologous lymphoblastoid cell lines (data not shown).

To determine if the SCCE 123-131 epitope is presented by SCCE-expressing ovarian tumor cells, SCCE 123-131 peptide-specific CTL were further evaluated for their ability to recognize and kill HLA-A2.1-matched ovarian tumor cell lines. The SCCE 123-131 CTL recognized and killed SCCE-expressing CaOV3 tumor cells (Fig. 2B). As seen in previous assays, autologous lymphoblastoid cell lines pulsed with peptide 123-131 were lysed, but unpulsed lymphoblastoid cell lines or the natural killer-sensitive cell line, K562, were not lysed. The specific recognition by SCCE 123-131 CTL of CaOV3 cells could be partially blocked by MAb specific for HLA class I (W6/32) or HLA-A2.1 (BB7.2).

SCCE 5-13 CTL lysed CaOV3 tumor cells at a low level, whether or not the tumor cells were peptide-pulsed (Fig. 3), even though SCCE 5-13 CTL did not recognize Ad-SCCE-infected
targets. The lysis of CaOV3 tumor cells was comparable to the level of cytotoxicity against SCCE 5-13 peptide-pulsed autologous lymphoblastoid cell lines. SCCE 5-13-specific CTL did not lyse a second tumor cell line, OVCAR-3, unless the OVCAR-3 tumor cells were peptide-pulsed (Fig. 3).

Identification of an extended stratum corneum chymotryptic enzyme 110-139 peptide with the potential to induce CD4+ helper T cell responses. To identify potential CD4+ helper T cell epitopes, we used the algorithms of Southwood et al. (19) for estimation of peptide binding affinities to HLA-DR1, -DR4, and -DR7. Available data suggests that a large set of DR molecules contain overlapping peptide binding repertoires, such that combined analysis of the DR1, DR4 and DR7 motifs has a high probability of predicting degenerate HLA-DR-binding epitopes, or multiple DR-binding clusters. Analysis of the SCCE sequence revealed clusters of candidate HLA-DR-binding epitopes in proximity to the known SCCE 123-131 CD8+ CTL epitope (Fig. 4). To test the hypothesis that this region would be a potent immunogen for both CD4+ helper T cell and CD8+ CTL responses, we synthesized the SCCE 110-139 peptide, and tested for antigen-specific T cell responses following autologous stimulation with peptide-loaded dendritic cells from a donor whose HLA type included DR1, DR7, and A2.1 (donor 2).

Donor 2 CD4+ T cells specifically proliferated in response to dendritic cells pulsed with SCCE 110-139 in a 3H-thymidine uptake assay (data not shown). In flow cytometric assays for cytokine expression at the single cell level, a high proportion of SCCE 110-139-specific CD4+ T cells expressed IFNγ after overnight stimulation with peptide-pulsed autologous dendritic cells (Fig. 5). In contrast, the expression of IL-4 was remarkably lower than that of IFNγ (Fig. 5). A high proportion of 110-139-specific CD4+ T cells also expressed tumor necrosis factor-α, but only limited expression of IL-4 and IL-13 was observed (data not shown), suggesting a strong type 1 bias in cytokine expression.

SCCE 110-139-specific CD4+ T cells from donor 2 were also cytotoxic against SCCE 110-139 pulsed targets. CD4+ T cells lysed autologous lymphoblastoid cell lines pulsed with SCCE 110-139, but not lymphoblastoid cell lines alone, in a standard...
chromium release assay (Fig. 6). Lysis of SCCE 110-139 pulsed lymphoblastoid cell lines was partially blocked by MAb L243, specific for a nonpolymorphic HLA class II determinant (data not shown).

**Stratum corneum chymotryptic enzyme 110-139 is immunogenic in the context of HLA class II molecules other than those identified by the predictive algorithm for class II-binding peptides.**

A key feature of Southwood’s algorithm is that if sequences match a stringent threshold for binding at least two of the three DR-identified molecules, there would be at least an 81% probability that those sequences would also bind other DR molecules, and thus have the potential to be immunogenic on diverse class II backgrounds (19). This is important for vaccine construction, since the HLA class II type would thus impose fewer constraints on immunogenicity. To test whether SCCE 110-139 is immunogenic on a divergent HLA-DR background, we attempted to generate SCCE 110-139 specific CD4+ T helper cells from donor 1, which does not express DR1, DR4, or DR7.

SCCE 110-139-specific CD4+ T cell lines were derived by stimulation with peptide-pulsed autologous dendritic cells. In microwell proliferation assays, we observed a strong response to dendritic cells pulsed with SCCE 110-139 relative to dendritic cells alone (data not shown). For two-color flow cytometric assays for cytokine expression, SCCE 110-139 CD4+ T cells were stimulated with dendritic cells or dendritic cells pulsed overnight with SCCE 110-139. Consistent with previous assays, some CD4+ T cells produced a type 1 cytokine only (either IFNγ, tumor necrosis factor-α or IL-2), some expressed both type 1 and type 2 cytokines, and a small minority expressed only a type 2 cytokine (either IL-4, IL-10, or IL-13; data not shown). The overall bias was clearly in favor of a type 1 pattern of cytokine expression.

We also tested donor 1 SCCE 110-139 peptide-specific CD4+ T cells in cytotoxicity assays against SCCE 110-139-pulsed dendritic cells. As shown in Fig. 7, SCCE 110-139 CD4+ T cells lysed dendritic cells pulsed with SCCE 110-139, but showed minimal lysis of control dendritic cells. Therefore, SCCE ovarian tumor antigen-specific CD4+ T cells not only have the potential for helper responses but also for cytotoxic responses. Furthermore, the immunogenicity of SCCE 110-139 extended peptide is not limited to the DR1, DR4, and DR7 haplotypes, since SCCE 110-139 specific CD4+ T helper cell responses are readily elicited from a donor that does not express DR1, DR4, and DR7. From these results, we conclude that vaccination with SCCE 110-139-loaded dendritic cells is likely to be immunogenic in the majority of individuals, and would not be restricted to a limited set of HLA class II haplotypes.

**Dendritic cells loaded with stratum corneum chymotryptic enzyme 110-139 stimulate strong CD8+ CTL responses that are at least partially HLA-A2.1-restricted.** We also examined the ability of SCCE 110-139-loaded dendritic cells to stimulate CD8+ CTL, since the SCCE 110-139 peptide contains the SCCE 123-131 CTL epitope. Donor 2 CD8+ T cells stimulated with SCCE 110-139-pulsed autologous dendritic cells were tested for their ability to recognize 110-139-loaded targets. SCCE 110-139-stimulated CD8+ T cells lysed 110-139 peptide-loaded autologous lymphoblastoid cell lines and this interaction could be reduced by the addition of a Mab specific for HLA class I molecules, W6/32 (Fig. 8A). SCCE 110-139-stimulated CD8+ T cells also lysed peptide-loaded HLA-A2.1-matched, allogeneic lymphoblastoid cell lines (donor 1), but did not fully lyse class I-mismatched allogeneic peptide-loaded lymphoblastoid cell lines (Fig. 8B), or control peptide-free lymphoblastoid cell lines from any donor. Although A2.1-positive lymphoblastoid cell lines were recognized and lysed by SCCE 110-139 peptide-specific CTL, lysis of allogeneic A2.1-matched lymphoblastoid cell lines was markedly lower than that of the peptide-loaded autologous lymphoblastoid cell lines, suggesting that peptide 110-139-specific CD8+ CTL recognize additional epitopes processed and presented by HLA class I molecules other than A2.1. SCCE 110-139 specific CD8+ CTL from donor 2 did not lyse A2.1-matched ovarian tumor cells, even when the tumor cells were infected with Ad-SCCE (data not shown).

We further wished to determine whether SCCE 110-139 peptide-specific CD8+ T cells could recognize targets expressing
endogenously processed full-length SCCE antigen. As the dominant HLA restriction molecules for 110-139-specific CD8+ CTL have not been determined, we do not have a suitable HLA class I-matched tumor target available. Consequently, we tested SCCE 110-139 peptide-specific CTL against lymphoblastoid cell lines pulsed with full-length recombinant SCCE protein. SCCE 110-139 peptide-specific CTL recognized and lysed both peptide-pulsed and full-length recombinant SCCE protein pulsed lymphoblastoid cell lines at significantly higher levels than background lysis of lymphoblastoid cell lines alone (Fig. 9). The SCCE 110-139 peptide-specific CTL had recently been passaged by restimulation with peptide-pulsed lymphoblastoid cell lines instead of dendritic cells, which may account for the slightly elevated background lysis of lymphoblastoid cell line targets in these experiments.

Although we found that dendritic cells pulsed with SCCE 110-139 readily induced antigen-specific CD8+ CTL responses that are at least partly A2.1-restricted, these CTL did not recognize the known SCCE 123-131 CTL epitope (Fig. 10A), suggesting that the SCCE 123-131 epitope is not a major component of the A2.1-restricted CD8+ CTL response to the longer 110-139 peptide, even though it is a naturally processed epitope from the full-length SCCE protein. To determine whether dendritic cells pulsed with SCCE 110-139 were capable of processing and cross-presenting the 123-131 CTL epitope, we tested the sensitivity of dendritic cells pulsed with SCCE 110-139 to lysis by CD8+ T cells specific for peptide 123-131. SCCE 123-131-specific CD8+ T cells lysed dendritic cells pulsed with SCCE 110-139, indicating that dendritic cells pulsed with SCCE 110-139 were able to cross-present the SCCE 123-131 epitope (Fig. 10B). However, the degree of specific lysis was relatively low, suggesting that dendritic cell cross-presentation of the 123-131 CTL epitope from peptide 110-139 is relatively inefficient.

**Discussion**

Despite recent advances in post-surgical chemotherapy for ovarian cancer, nearly 90% of advanced cases will develop progressive disease that is refractory to further treatment. In response to the need for alternative treatments that prevent disease recurrence or progression, tumor-specific immunologic intervention has received considerable recent attention. This interest is based in part on the identification of novel target antigens, and in part on the application of dendritic cells as powerful inducers of cellular immune responses against tumor antigens. SCCE is one of a group of serine proteases that are highly expressed in ovarian cancer, suggesting that it may be an excellent target antigen for dendritic cell-based vaccination protocols. This notion is strengthened by the identification in this report of two HLA-A2.1-restricted CTL epitopes, SCCE 5-13
CD8+ T cell responses. It is well-established that T cell help provide essential help for dendritic cell induction of effective responses between CD40 ligand expressed by CD4+ T cells and CD40 alone (by stimulation with matured dendritic cells pulsed with SCCE peptide 110-139 expressed by dendritic cells (21, 22). CD4+ T cell–induced peptides to induce CD8+ CTL that can lyse ovarian tumor expressed SCCE.

A2.1-matched ovarian tumor cells, showing that these peptides for induction of CD8+ CTL responses and generation of cells loaded with 50 μg/mL SCCE123-131. Dendritic cells loaded with either of these antigen-specific CD4+ helper T cell responses, which may restriction of such peptides, and the failure to stimulate However, this approach is limited by the tight HLA antigens in dendritic cell immunotherapy for ovarian cancer. CD4+ helper sequences and CD8+ CTL epitopes could induce durable peptide-specific CD8+ CTL responses that were lytic against tumor cells (30). Induction of supporting helper CD4+ T cell responses by this approach was confirmed by predominant CD4+ T cell infiltrates in biopsies from positive delayed-type hypersensitivity sites (31). A further remarkable feature of the helper/CTL multiepitope peptide vaccine is that immunization was associated with epitope spreading, i.e., induction of immune responses to epitopes that were not components of the vaccine antigen (31, 32). In contrast, vaccination with a peptide representing a CD8+ CTL epitope alone induced immunity of limited duration and specific only for the peptide (33). Peptide-loaded dendritic cell immunotherapy that is capable of inducing both CD8+ CTL responses and CD4+ helper T cell responses thus offers greater potential for inducing durable immune responses and clinical benefit than treatment with dendritic cells loaded with CD8+ CTL peptide epitopes alone, and provides a rationale for the second phase of our analysis, which sought to identify CD4+ helper T cell epitopes within SCCE.

The algorithm for prediction of HLA-DR-binding epitopes was built on motifs for DR1, DR4, and DR7. However, DR molecules frequently share common peptide-binding motifs, with the result that many peptides show degenerate binding to multiple DR molecules. Consequently, combined analysis of the DR1, DR4, and DR7 motifs has a high probability of predicting degenerate HLA-DR-binding epitopes, or multiple DR-binding clusters (19). In support of this hypothesis, we found that SCCE 110-139-loaded dendritic cells not only stimulated a CD4+ T cell response from an individual expressing DR1 and DR7, but also efficiently stimulated an antigen-specific CD4+ T cell response from an individual that expressed disparate HLA class II molecules (DR3 and DRw52). Although our results do not distinguish between the presence of one or more degenerate DR-binding epitopes, or multiple independently restricted epitopes within the 110-139 sequence (possibilities that are not mutually exclusive), we can conclude that vaccination with SCCE 110-139-loaded dendritic cells is likely to be immunogenic in the majority of individuals, and would not be restricted to a limited set of HLA class II haplotypes.

SCCE 110-139 peptide-loaded dendritic cells also processed and cross-presented epitopes on HLA class I, efficiently priming a CD8+ CTL response. Peptide-specific CD8+ CTL lysed autologous lymphoblastoid cell lines loaded with SCCE 110-139, showed reduced but significant lysis against HLA-A2.1-matched lymphoblastoid cell lines, and failed to lyse HLA class I-mismatched, and SCCE 123-131. Dendritic cells loaded with either of these peptides could stimulate CD8+ CTL responses against HLA-A2.1-matched ovarian tumor cells, showing that these peptides represent naturally processed epitopes from endogenously expressed SCCE.

The ability of dendritic cells loaded with HLA class I-binding peptides to induce CD8+ CTL that can lyse ovarian tumor cells suggests that CTL peptides may have utility as target antigens in dendritic cell immunotherapy for ovarian cancer. However, this approach is limited by the tight HLA restriction of such peptides, and the failure to stimulate antigen-specific CD4+ helper T cell responses, which may provide essential help for dendritic cell induction of effective CD8+ T cell responses. It is well-established that T cell help for induction of CD8+ CTL responses and generation of effective tumor immunity is dependent on the interaction between CD40 ligand expressed by CD4+ T cells and CD40 expressed by dendritic cells (21, 22). CD4+ T cell–induced maturation of dendritic cells is thus the essential link in providing help for CD8+ CTL (23–25). Although recent experiments have shown that injection of dendritic cells matured in vitro can circumvent the need for CD4+ T cell help for dendritic cell maturation and induce effective CD8+ CTL responses to class I-restricted peptides (26), CD4+ T cell responses fulfill other important functions, including long-term maintenance of the CD8+ T cell responses in vivo (27), and promotion of the recruitment and accumulation of endogenous antigen-presenting cells at the tumor site (28). In addition, recent evidence suggests that helper CD4+ T cells can directly interact with tumor-reactive CD8+ CTL, enhancing their proliferation and survival (29).

Clinical experience with breast and ovarian cancer patients has indicated that peptide-based vaccines that incorporated both helper sequences and CD8+ CTL epitopes could induce durable peptide-specific CD8+ CTL responses that were lytic against tumor cells (30). Induction of supporting helper CD4+ T cell responses by this approach was confirmed by predominant CD4+ T cell infiltrates in biopsies from positive delayed-type hypersensitivity sites (31). A further remarkable feature of the helper/CTL multiepitope peptide vaccine is that immunization was associated with epitope spreading, i.e., induction of immune responses to epitopes that were not components of the vaccine antigen (31, 32). In contrast, vaccination with a peptide representing a CD8+ CTL epitope alone induced immunity of limited duration and specific only for the peptide (33). Peptide-loaded dendritic cell immunotherapy that is capable of inducing both CD8+ CTL responses and CD4+ helper T cell responses thus offers greater potential for inducing durable immune responses and clinical benefit than treatment with dendritic cells loaded with CD8+ CTL peptide epitopes alone, and provides a rationale for the second phase of our analysis, which sought to identify CD4+ helper T cell epitopes within SCCE.

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peptide-loaded lymphoblastoid cell lines. These observations provide an important confirmation that CTL epitopes are cross-presented by dendritic cells, and that the CD8+ CTL response to peptide 110-139 is restricted by multiple class I molecules, including HLA-A2.1. Intriguingly, our results also suggest that the target lymphoblastoid cell lines are capable of cross-presenting soluble antigen. This conclusion is supported by the ability of SCCE 110-139-specific CD8+ CTL to lyse lymphoblastoid cell lines loaded with recombinant full-length SCCE protein.

SCCE 110-139-specific CD8+ CTL did not lyse SCCE 123-131-pulsed targets, suggesting that although SCCE 123-131 is a naturally processed epitope, it isn't a major component of the SCCE 123-131-pulsed targets, suggesting that although SCCE 123-131 is a major component of the target lymphoblastoid cell lines capable of cross-presenting epitopes from SCCE 110-139 and CD8+ CTL response to the extended 110-139 peptide. Rather, the data indicate that peptide 110-139-specific CD8+ CTL recognize other epitopes processed from SCCE 110-139 and presented by A2.1 and other HLA class I molecules. Nevertheless, CD8+ CTL specific for SCCE 123-131 lysed peptide 110-139-loaded dendritic cells, indicating that the SCCE 123-131 epitope is cross-presented by dendritic cells loaded with SCCE 110-139. However, the low level of lysis suggests that peptide 123-131 is not processed as a major epitope from the extended SCCE 110-139 sequence, which possibly explains why CD8+ T-cell responses from diverse HLA-DR backgrounds suggests that immunotherapy targeted at SCCE 110-139 would not be subject to the limitations of HLA type that are usually applicable to a broad patient population.

In conclusion, the SCCE 110-139 peptide is a promising vaccine antigen candidate for dendritic cell immunotherapy of ovarian cancer. The combined ability of dendritic cells loaded with SCCE 110-139 to stimulate CD8+ CTL restricted by HLA-A2.1 and other class I molecules, as well as to stimulate CD4+ helper T cell responses from diverse HLA-DR backgrounds, suggests that immunotherapy targeted at SCCE 110-139 would not be subject to the limitations of HLA type that are usually associated with peptide-based strategies, and would thus be applicable to a broad patient population.

References


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Definition of an Immunogenic Region Within the Ovarian Tumor Antigen Stratum Corneum Chymotryptic Enzyme

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