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Tumor Cells Present MHC Class II-Restricted Nuclear and Mitochondrial Antigens and Are the Predominant Antigen Presenting Cells In Vivo

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MHC class II-restricted tumor Ags presented by class II+ tumor cells identified to date are derived from proteins expressed in the cytoplasm or plasma membrane of tumor cells. It is unclear whether MHC class II+ tumor cells present class II-restricted epitopes derived from other intracellular compartments, such as nuclei and/or mitochondria, and whether class II+ tumor cells directly present Ag in vivo. To address these questions, a model Ag, hen egg lysozyme, was targeted to various subcellular compartments of mouse sarcoma cells, and the resulting cells were tested for presentation of three lysozyme epitopes in vitro and for presentation of nuclear Ag in vivo. In in vitro studies, Ags localized to all tested compartments (nuclei, cytoplasm, mitochondria, and endoplasmic reticulum) are presented in the absence invariant chain and H-2M. Coexpression of invariant chain and H-2M inhibit presentation of some, but not all, of the epitopes. In vivo studies demonstrate that class II+ tumor cells, and not host-derived cells, are the predominant APC for class II-restricted nuclear Ags. Because class II+ tumor cells are effective APC in vivo and probably present novel tumor Ag epitopes not presented by host-derived APC, their inclusion in cancer vaccines may enhance activation of tumor-reactive CD4+ T cells. The Journal of Immunology, 2000, 165: 5451–5461.

O ptimal antitumor immunity usually requires activation of CD8+ and CD4+ tumor-specific T lymphocytes (1–7). Naive CD8+ and CD4+ T cells are activated by APCs that deliver an Ag-specific signal plus a costimulatory signal to the responding T cells (8). In the case of tumor-specific CD8+ and CD4+ T cell activation, the Ag-specific signal consists of an epitope derived from a tumor-associated Ag bound to an MHC class I or II molecule, respectively. Tumor Ags are either presented by tumor cells themselves (direct Ag presentation) (9–11) or by professional, host-derived APC, such as dendritic cells, macrophages, or B cells (indirect Ag presentation or cross-priming) (9, 12–14).

Numerous MHC class I-restricted tumor-associated Ag epitopes have been identified, and the proteins from which they are derived reside in diverse subcellular compartments, such as nuclei (e.g., CDK4; Ref. 15), cytosol (e.g., β-catenin; Ref. 16), or plasma membrane (e.g., her2/neu; Ref. 17). In contrast, many fewer MHC class II-restricted tumor Ags have been identified, and these molecules are derived from proteins restricted to the cytoplasm (18) or plasma membrane (19) of tumor cells. Whether MHC class II+ tumor cells directly present class II-restricted epitopes from diverse subcellular compartments or are APC in vivo remains unknown.

To study this question we have generated MHC class II+ tumor cells by gene transfection, and further transfected them with a test Ag (hen egg lysozyme, HEL4) targeted to various subcellular sites. The resulting cells were tested as APC for three HEL epitopes in vitro. Ags localized to all tested compartments (nuclei, cytoplasm, mitochondria, and endoplasmic reticulum (ER)) are efficiently presented in vitro in the absence of the class II-associated invariant chain (Ii) and H-2M (DM). In contrast, epitopes derived from these endogenous compartments are differentially inhibited by coexpression of the class II-associated accessory molecules, Ii and DM. In vivo immunization experiments, using the class II+ tumor cells containing HEL localized to the nucleus, demonstrated that the tumor cells are the predominant APC in vivo for priming naive CD4+ T cells.

Materials and Methods

Mice

AJ, BALB/c, C57BL/6, (BALB/c × A/J)F1, and BALB/c nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and/or bred and maintained in the University of Maryland animal facility according to National Institutes of Health guidelines for the humane treatment of laboratory animals. All animal procedures have been approved by the University of Maryland Baltimore County Institutional Animal Care and Use Committee.

Cells

Mouse SaI sarcoma, MHC class II I-Ak-transfected (Sal/Ak), and class II transactivator (CIITA)-transduced (Sal/CIITA, Aa+ Ii+ DM+) cells were maintained as previously described (20). HEL-transfected tumor cells were maintained in the same medium supplemented with 400 μg/ml G418 (Calbiochem, La Jolla, CA) and 1.5 μg/ml puromycin (Clontech, Palo Alto, CA). The following I-Aa-restricted CD4+ T cell hybridomas were used: 3A9 (HEL34–45; Ref. 21), 2B6.3 (HEL28–37; Ref. 22), and 3B11.1 (HEL34–45; Ref. 23). Sal/Aa,erHEL and Sal/CIITA/erHEL cells (Sal/Aa

4 Abbreviations used in this paper: HEL, hen egg lysozyme; Ii, invariant chain; DAPI, 4′,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; MHC, MHC class II compartments; Sal/Aa, Sal sarcoma cells transfected with I-Aa genes; Sal/CIITA, Sal sarcoma cells transduced with the MHC class II transactivator gene CIITA; nciHEL, HEL targeted to mitochondria; cytHEL, HEL targeted to the cytosol; erHEL, HEL targeted to the ER; DM, H-2M; GAMlgG, goat anti-mouse IgG.
and Sal/CITa cells transfected with ER-retained HEL) were previously described (20).

Antibodies

Mouse mAbs HyHEL7 and HyHEL.10 specific for HEL (24), 10–2.16 specific for the β-chain of I-Ak (25), 2.43 specific for CD8 (26), and the rabbit polyclonal Ab K553 specific for DM (29) have been previously described (30, 31). Human anti-fibrillarin mAb (32) was used at 1:600. CD3-FITC (145-2C11), CD4-PE (GK1.5), CD8-PE (53-6.7), and H-2Kk-PE (36-7-5) Abs were purchased from PharMingen (San Diego, CA) and were used at 2 μg/ml/2 × 10^5 cells. Secondary Abs used for flow cytometry (goat anti-mouse IgG (GAM IgG) FITC, mouse anti-rat FITC, goat anti-rabbit IgG-FITC), and Fab of GAM IgG used for panning were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Secondary Abs (donkey anti-mouse IgG-FITC and donkey anti-human IgG-tetramethylrhodamine isothiocyanate, minimally cross-reactive to other species) used for microscopy were purchased from Jackson ImmunoResearch (West Grove, PA).

Construction of plasmids encoding nuclear, cytoplasmic, or mitochondrial HEL

For the nuclear and cytoplasmic constructs, a 435-bp HEL fragment was generated by PCR from the pHYK plasmid containing ER-targeted HEL (pHYK/erHEL; Ref. 33); 5′ primer: GAAGTCCTTCTGTAATCTTGTCG; 3′ primer: TGGCAGCCTCTGATCCACGCCTGG). PCR was performed on a GeneAmp PCR system 2400 (Perkin-Elmer, Foster City, CA) using the following conditions: starting temperature of 95°C × 2 min followed by 30 cycles of 95°C × 30 s, 60°C × 30 s, and 72°C × 30 s. The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and, for the nuclear construct (nucHEL), subcloned into the pCMV/nuc/myc vector (Invitrogen, Carlsbad, CA) using the 5′ NcoI and 3′ NotI sites (462-bp fragments). The pCMV/nuc/myc vector (Fig. 1A) contains the 3′ nuclear localization signal from SV40 large T Ag (DPKKKRKV) in triplicate. The mitochondrial HEL construct was generated by PCR amplifying the HEL insert from the pHYK/erHEL plasmid including PstI and XhoI restriction sites at the 5′ and 3′ ends, respectively (5′ primer: AACAGGGTCTTTGCTAATCTTG, PstI site underlined; 3′ primer

![FIGURE 1.](http://www.jimmunol.org/)}
CCGGTCGAGGAGGAGGCTGATCAGCCGCT (XhoI site underlined) using the same conditions as per the nucHEL construct. The 438-bp PCR product was cloned into the pCMV/mito/myc vector (Invitrogen) using the 5′ PstI and 3′ XhoI sites. The pCMV/mito/myc vector contains a 5′ mitochondrial matrix-targeting sequence from human cytochrome c oxidase (MSVLTPLLLRLGKARLPVPRAKHSL). The cytoplasmic HEL construct was generated by inserting the HEL gene from the pGEM-T vector into the 5′ Ncol and 3′ NotI sites of the pCMV/cyto/myc vector (Invitrogen). HEL insert and surrounding bases of all constructs were sequenced in both directions to ascertain correct sequence and reading frame.

Transfections
SaI/A k/nucHEL, SaI/A k/cytoHEL, SaI/CIITA/mitoHEL, SaI/CIITA/nucHEL, SaI/A k/cytoHEL, and SaI/CIITA/mitoHEL stable transfectants were generated by transfecting SaI/A k or SaI/CIITA cells with the appropriate HEL plasmid plus pPUR containing the puromycin resistance gene (Clontech) using lipofectin (Life Technologies, Gaithersburg, MD) as previously described (31).

Immunofluorescence and flow cytometry
Tumor cells and splenocytes were stained by direct or indirect immunofluorescence either internally (Ii, DM, or HEL) or externally (MHC class I, class II, CD3, CD4, CD8) as previously described (11, 31).

Immunofluorescence microscopy
 Cells were harvested, washed once with PBS, and fixed with fresh 3–4% paraformaldehyde (Sigma, St. Louis, MO) for 20 min at room temperature. Fixed cells were then washed twice with PBS supplemented with 5% donkey serum (Jackson ImmunoResearch) and 0.1% saponin (Sigma) or 1% Triton X-100 (Sigma) and distributed into 96-well plates at 5 × 10^4 cells per well. Cells were stained for HEL expression with 40 μl mAbs HvHEL7 and HvHEL10 at 0.1 μg/ml for 45 min. After staining, cells were washed twice with PBS, and then incubated with donkey anti-mouse IgG-HRP (1/10,000 in 0.5% BSA/TBST; Amersham). Bands were visualized by staining with 10 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR).

Western blots
Cells were incubated for 40 min on ice in lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1% Nonidet-P-40, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin at 10^4/ml for tumor cells or 10^7/ml for splenocytes). Cell extracts were microfuged at 4°C for 30 min to remove nuclei, and clarified lysates were stored at −80°C until used. Frozen lysates were thawed on ice, resuspended in 2× SDS sample buffer (28 mM 2-ME, 4% SDS, 20% glycerol, 125 mM Tris, pH 6.8, and 0.001% bromophenol blue), and boiled (100°C × 5 min) or unboiled samples run on 12% mini-SDS-PAGE gels under reducing conditions (20 V × the first 40 min; 100 V × 2 h). Following electrophoresis, proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ) at 80 mA for 40 min using a Milliblot Electroblotter-SDE system (Millipore, Bedford, MA). The following procedures were performed at room temperature with gentle agitation: membranes were blocked with 2% BSA/TBST (20 mM Tris-Cl, 130 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h, rinsed twice with double distilled H2O, and probed for 1–1.5 h with mAb 10-2.16 at 1 μg/ml diluted in 0.5% BSA/TBST. Following primary Ab incubation, membranes were washed twice with TBST × 5 min, and sheep anti-mouse IgG-HRP was added (1/10,000 in 0.5% BSA/TBST; Amersham). Bands were developed using ECL detection reagents (Amersham), exposed to X-Omat Blue XB-1 film (NEN Life Science, Boston, MA), and quantified by densitometry using an Alpha ChemiImager (Alpha Innotech, San Leandro, CA).

Ag presentation/priming assays
In vitro APC assays using the T cell hybridomas were performed as previously described (20, 31), and each experiment was repeated at least three times. For presentation of exogenous HEL (Sigma) by SaI/CIITA cells, APC were pulsed with 70 or 300 μM HEL for the 3B11.1 and 2B6.3 hybridomas, respectively. For in vivo Ag priming studies, responder splenocytes were prepared using the following scheme: BALB/c nude (K<sup>A</sup>A<sup>D</sup>D<sup>P</sup>) mice were reconstituted i.p. with 3–3.5 × 10<sup>7</sup> splenocytes from 5– to 8-week-old (BALB/c × A/J)F<sub>1</sub> (K<sup>A</sup>A<sup>D</sup>D<sup>P</sup> × K<sup>A</sup>A<sup>D</sup>D<sup>P</sup>) mice. Before reconstitution, the F<sub>1</sub> splenocytes were depleted of B cells and adherent cells by panning on T flasks coated with GAM IgG as previously described (11). On day 2, the reconstituted mice were injected i.p. with 10<sup>6</sup> live SaI/A<sub>k</sub>/nucHEL tumor cells. On day 10 the reconstituted mice were sacrificed, their spleens removed and depleted of B lymphocytes, and the resulting T cells were used as responder cells in APC assays. Tumor challenge did not affect splenic T cell reconstitution. For some experiments, splenocytes were depleted by panning for CD4<sup>+</sup> or CD8<sup>+</sup> T cells using GK1.5 or 2.43 mAbs (11) before use in the APC assays. APC assays using splenocytes from reconstituted nude mice were performed as follows: 5 × 10<sup>4</sup> responder splenocytes were mixed with 5 × 10<sup>4</sup> presenting cells (splenocytes from A/J, BALB/c, or C57BL/6 mice) in the presence or absence of 1 μg/ml native HEL for 20 h at 37°C. IL-2 release was measured by ELISA using an Endogen IL-2 kit (Cambridge, MA) as previously described (31). SD of triplicate samples was ±10% and is included in the relevant figures.

Results
Endogenously synthesized Ags from the nucleus are presented by MHC class II molecules of tumor cells
To determine whether MHC class II<sup>+</sup> tumor cells present peptides derived from endogenously synthesized Ags localized to the nucleus, a cDNA encoding the HEL gene was subcloned into a vector upstream of three copies of the “DPKKKKKVV” nuclear targeting sequence (Fig. 1A). Tumor cells previously transfected with I<sup>A</sup> genes and not expressing Ii or DM (SaI/A<sub>k</sub> sarcoma cells) were
further transfected with the nucHEL plasmid, and transfectants were selected by drug resistance, screened by flow cytometry for expression of HEL, and cloned by limiting dilution.

Fig. 1B shows the flow cytometry histograms of three Sal/A\(^{4}\)/nucHEL clones (7.4, 16.1, and 16.3) and control parental Sal/A\(^{4}\) and class II\(^{+}\) Sal tumor cells. The three Sal/A\(^{4}\)/nucHEL clones present mitochondrial and nuclear antigens.

**FIGURE 3.** A, Schematic outline of in vivo Ag presentation experiments. On day 1, BALB/c nude mice were reconstituted with B cell-depleted (BALB/c × A/J)\(_{F_{1}}\) splenocytes. Twenty-four hours later, mice were immunized with live Sal/A\(^{4}\)/nucHEL cells. On day 10, mice were sacrificed and their spleens were removed. The resulting spleenocytes were depleted of B cells and used as responders in Ag presentation assays with A/J, BALB/c, or C57BL/6 splenocytes as APCs. In some experiments, responding spleocytes were also depleted of CD4\(^{+}\) or CD8\(^{+}\) T cells. B, Reconstituted nude mice contain F\(_{1}\) donor T cells. Splenocytes from donor, recipient, day 2 reconstituted recipient, and day 10 reconstituted/immunized recipients were double stained for CD3 plus CD4 or CD3 plus CD8. Day 10 splenocytes were also stained following CD4 or CD8 T cell depletion. The percentages of each population are indicated within each quadrant.
express varying levels of HEL, whereas I-A<sup>k</sup> levels on all MHC class II<sup>1</sup> clones (Sai/A<sup>k</sup> and Sai/A<sup>k</sup>/nucHEL clones) are similar. Localization of HEL to the nucleus was ascertained by immunofluorescence microscopy. Fig. 1C shows the HEL staining of one Sai/A<sup>k</sup>/nucHEL clone (16.3) and, as a comparison, a clone in which HEL is localized to the ER (Sai/A<sup>k</sup>/erHEL cells; a and d, respectively). Nuclei are visualized by DAPI staining (b and e, respectively). The HEL and DAPI images for each clone are overlaid in c and f, respectively. As seen in a–c, HEL in the Sai/A<sup>k</sup>/nucHEL cells is localized within nuclei, but is not evenly distributed. Because the HEL staining pattern resembles the distribution of nucleoli, the Sai/A<sup>k</sup>/nucHEL cells were double stained for HEL and fibrillarin, a nucleolar protein (34). However, the staining patterns for HEL and fibrillarin do not overlap (data not shown), indicating that nucHEL is not restricted to nucleoli. Sai/A<sup>k</sup> cells transfected for HEL targeted to nuclei, therefore, express HEL in the nuclei and not in other intracellular compartments.

To ascertain that the MHC class II heterodimers are functional and bind high affinity peptides in the absence of li and DM, Western blots were performed. As shown in Fig. 1D, Sai/A<sup>k</sup> and Sai/A<sup>k</sup>/nucHEL transfecteds both contain SDS stable dimers (~55-60-kDa bands) if the samples are not boiled before electrophoresis. Densitometry quantitation of the bands shows that 45 and 43% of the class II of Sai/A<sup>k</sup> and Sai/A<sup>k</sup>/nucHEL cells, respectively, are compact dimers, as compared with 73% of control A/J splenocytes. Therefore, transfecteds contain properly conformed MHC class II molecules, despite the absence of li and DM and in agreement with other studies with I-A<sup>k</sup> molecules (35, 36).

In vitro presentation of three independent HEL epitopes by the Sai/A<sup>k</sup>/nucHEL transfectants was tested using the 3A9, 2B6.3, and 3B11.1 T cell hybridomas that secrete IL-2 in response to presentation of the HEL 46-61, 25-43, and 34-45 epitopes, respectively. As shown in Fig. 2, the three Sai/A<sup>k</sup>/nucHEL clones present the three HEL epitopes, although the efficiency of presentation varies among the clones. HEL-negative Sai/A<sup>k</sup> cells do not present HEL. Therefore, class II<sup>1</sup> I<sup>i</sup>/DM<sup>2</sup> tumor cells efficiently present multiple MHC class II-restricted peptides derived from endogenously synthesized Ags localized to the nucleus.

**SaI/A<sup>k</sup>/nucHEL cells are APCs for HEL in vivo**

To determine whether nuclear localized molecules are directly presented by the MHC class II<sup>1</sup> tumor cells in vivo or whether presentation is via cross-priming (37, 38), we modified a nude mouse system originally adapted by (9) for assessing presentation of MHC class I-restricted Ags. The design of these experiments is shown in Fig. 3A. BALB/c nude mice were reconstituted on day 1 with 3–3.5 × 10<sup>7</sup> (BALB/c × A/J)F<sub>1</sub> splenocytes that were depleted for B cells and adherent cells. One day later, the chimeric mice were immunized i.p. with 10<sup>6</sup> live Sai/A<sup>k</sup>/nucHEL 16.3 cells.
Splenocytes were harvested on day 10, and their restriction pattern was tested using BALB/c, A/J, or negative control C57BL/6 APC. Following reconstitution, the chimeric mice have CD4<sup>+</sup>T cells that potentially recognize I-A<sub>k</sub> and/or I-A<sub>d</sub>-restricted Ag, but only have professional APC of the I-A<sub>d</sub> genotype (i.e., host-derived cells). Therefore, if professional APC are the principal APC for tumor-derived HEL, then splenocytes of immunized chimeras will be restricted to the host genotype APC (I-A<sub>d</sub>). In contrast, if the tumor cells directly present HEL to CD4<sup>+</sup>T cells, then the chimeric splenocytes will be predominantly restricted to the genotype of the immunizing tumor cells (I-A<sub>k</sub>). If both cell types present Ag, then T cells will respond to both APC genotypes (I-A<sub>d</sub> and I-A<sub>k</sub>).

To ascertain that the reconstitution is successful, splenocytes from nude mice were tested for CD4<sup>+</sup> and CD8<sup>+</sup>T cells on days 2 and 10. As shown in Fig. 3B, spleens of unreconstituted mice contain 1.4 and 0.6% CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively, whereas 1 day after reconstitution, splenocytes are 5.8 and 3.3% CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively. On day 10, splenocytes are 8.2 and 5% CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively. Day 2 splenocytes were also tested for donor MHC class I genotype to ascertain that the T cells are of the donor genotype (H-2<sup>k</sup>). If both cell types present Ag, then T cells will respond to both APC genotypes (I-A<sub>d</sub> and I-A<sub>k</sub>).

To identify the responding T cells, day 10 splenocytes were depleted for CD4<sup>+</sup> or CD8<sup>+</sup>T lymphocytes before performing the Ag presentation assay. As shown in Fig. 3B, depleted splenocytes have 0.1 and 0.5% CD4<sup>+</sup> or CD8<sup>+</sup>T cells, respectively, demonstrating that the depletions have been effective. HEL-specific Ag presentation by the depleted populations is shown in Table I. Depletion of CD4<sup>+</sup>T cells eliminates 100% of IL-2 production for A/J APC. Depletion of CD8<sup>+</sup>T cells modestly reduces IL-2 production, probably due to nonspecific effects of the depletion procedure. Therefore, HEL-specific CD4<sup>+</sup>T cells are restricted to the tumor genotype (I-A<sub>k</sub>), demonstrating that Sal/A<sup>k</sup>nucHEL tumor cells are the exclusive APC in vivo for activating tumor-specific CD4<sup>+</sup>T cells, and that nucHEL presentation by the tumor cells is direct and not via cross-priming. It is unlikely that this result reflects a preference for presentation of a high affinity I-A<sub>k</sub> peptide (e.g., HEL<sub>46–61</sub>; Ref. 39) because the assay measures all HEL peptide, including I-A<sub>k</sub> high affinity peptides (e.g., HEL<sub>11–25</sub>; Ref. 40), which could be presented during cross-priming.

To identify the responding T cells, day 10 splenocytes were depleted for CD4<sup>+</sup> or CD8<sup>+</sup>T lymphocytes before performing the Ag presentation assay. As shown in Fig. 3B, depleted splenocytes have 0.1 and 0.5% CD4<sup>+</sup> or CD8<sup>+</sup>T cells, respectively, demonstrating that the depletions have been effective. HEL-specific Ag presentation by the depleted populations is shown in Table I. Depletion of CD4<sup>+</sup>T cells eliminates 100% of IL-2 production for A/J APC. Depletion of CD8<sup>+</sup>T cells modestly reduces IL-2 production, probably due to nonspecific effects of the depletion procedure. Therefore, HEL-specific CD4<sup>+</sup>T cells are restricted to the tumor genotype (I-A<sub>k</sub>), demonstrating that Sal/A<sup>k</sup>nucHEL tumor cells are the exclusive APC in vivo for activating tumor-specific CD4<sup>+</sup>T cells, and that direct presentation is the predominant route of CD4<sup>+</sup>T cell activation.

Coexpression of Ii and DM inhibits presentation of HEL<sub>25–43</sub> and HEL<sub>34–45</sub>, but not presentation of HEL<sub>46–61</sub> derived from Ag localized to the nucleus

In previous studies we demonstrated that Sal/A<sup>k</sup> cells efficiently present ER-retained HEL in vitro to hybridoma cells and in vivo to naive CD4<sup>+</sup>T cells (11, 20). However, in the presence of Ii (either
Sal/A²/iI or Sal/CIITA cells) ER-retained HEL was not presented. DM expression did not affect eHEL presentation (31). To determine whether presentation of nuclear localized Ag is also blocked by Ii, Sal/CIITA cells were further transfected with the nucHEL construct to generate Sal/CIITA/nucHEL cells.

Fig. 4A shows the flow cytometry profiles of three Sal/CIITA/nucHEL clones (clones 29, 40, and 53), control Sal/CIITA, and Sal/A² cells stained for I-A², Ii, DM, and HEL. To evaluate the effect of the level of nucHEL on presentation, transfectants expressing similar quantities of I-A², Ii, and DM (data not shown), but with different levels of HEL, were selected. Immunofluorescence microscopy showed that HEL staining was restricted to the nucleus in a pattern similar to that seen for Sal/A²/nucHEL cells (data not shown). Western blot analysis using mAb 10–2.16 revealed that ~80–90% of I-A² molecules form SDS-stable compact dimers (Fig. 1D), demonstrating proper conformation.

In vitro presentation of endogenously synthesized, nuclear-localized HEL was tested using the three T cell hybridomas and the Sal/CIITA/nucHEL transfectants. As shown in Fig. 4B, the three Sal/CIITA/nucHEL transfectants present the HEL₄⁶–₆¹ epitope, but do not present the HEL₂⁵–₄₃ or HEL₃₄–₄₅ epitopes. However, parental Sal/CIITA cells present the HEL₂⁵–₄₃ and HEL₃₄–₄₅ epitopes when pulsed with exogenous HEL. Therefore, coexpression of Ii and DM differentially affects presentation of epitopes derived from nuclear-localized molecules.

Class II⁺ tumor cells present Ags localized to mitochondria
To determine whether MHC class II⁺ tumor cells present Ag localized to mitochondria, a mitoHEL construct containing the 5⁹ mitochondrial targeting sequence of cytochrome c oxidase was generated (Fig. 5A). Sal/A² and Sal/CIITA cells were transfected with the construct to generate Sal/A²/mitoHEL and Sal/CIITA/mitoHEL cells, respectively. Fig. 5B shows flow cytometry profiles of two Sal/A²/mitoHEL and two Sal/CIITA/mitoHEL clones stained for HEL. Immunofluorescent confocal microscopy showed colocalization of HEL and mitochondria (data not shown). Clones were chosen for consistent I-A², Ii, and DM expression (data not shown) and varied HEL expression.

MHC class II-restricted Ag presentation of endogenously synthesized Ag localized to mitochondria is shown in Fig. 5C. Sal/A²/mitoHEL clones present all three HEL epitopes, whereas Sal/CIITA/mitoHEL clones only present HEL₄⁶–₆¹. Therefore, class II⁺ Ii⁻ DM⁻ tumor cells present peptides derived from molecules localized to mitochondria, and coexpression of Ii and DM differentially affects epitope presentation in a pattern identical with that of nuclear localized Ag.

Class II⁺ tumor cells present Ag localized to the cytosol
Previous studies by others demonstrated presentation of cytoplasmically localized tumor Ags by MHC class II⁺ tumor cells (18,
These studies examined class II+ tumor cells that coexpress Ii. To determine whether Ii expression is required for presentation of cytoplasmic Ags in tumor cells, Sal/Ak and Sal/CIITA cells were transfected with a plasmid targeting HEL to the cytoplasm (cytoHEL; Fig. 6A). Fig. 6B shows the flow cytometry profiles of two Sal/Ak/cytoHEL and four Sal/CIITA/cytoHEL clones. Clones were chosen for consistent I-Ak, Ii, and DM expression (data not shown), and variation in HEL expression.

Ag presentation of endogenously synthesized, cytoplasmic HEL by Sal/Ak/cytoHEL and Sal/CIITA/cytoHEL cells is shown in Fig. 6C. The two Sal/Ak/cytoHEL clones present all three HEL epitopes. In contrast, none of the four Sal/CIITA/cytoHEL clones presents HEL25–43 or HEL34–45, whereas only one Sal/CIITA/cytoHEL clone (no. 1) presents HEL46–61.

Coexpression of Ii differentially inhibits presentation of endogenous Ag from diverse compartments

Table II summarizes the Ag presentation activity and mean channel fluorescence of HEL for the transfectedants containing HEL localized to nuclei, mitochondria, cytosol, or ER. Coexpression of Ii completely inhibits presentation of HEL25–43 and HEL34–45. In contrast, presentation of HEL46–61 by Sal/CIITA cells with nuclear or mitochondrial localized Ag is not inhibited by Ii and is comparable to presentation by Sal/Ak cells. However, Sal/CIITA/erHEL cells and three of four Sal/CIITA/cytoHEL clones do not present endogenous HEL. Interestingly, the single Sal/CIITA/cytoHEL clone that presents HEL46–61 (clone no. 1) has a very high level of endogenous HEL.

### Discussion

Renewed enthusiasm for cancer vaccines has focused interest on MHC class II+ tumor cells as potential APC for tumor-encoded, endogenously synthesized Ag. There is compelling evidence demonstrating that MHC class II+ APC present epitopes derived from endogenously synthesized proteins localized to the cytosol (44), nuclear fluorescence of HEL for the transfectedants containing HEL localized to nuclei, mitochondria, cytosol, or ER. Coexpression of Ii completely inhibits presentation of HEL25–43 and HEL34–45. In contrast, presentation of HEL46–61 by Sal/CIITA cells with nuclear or mitochondrial localized Ag is not inhibited by Ii and is comparable to presentation by Sal/Ak cells. However, Sal/CIITA/erHEL cells and three of four Sal/CIITA/cytoHEL clones do not present endogenous HEL. Interestingly, the single Sal/CIITA/cytoHEL clone that presents HEL46–61 (clone no. 1) has a very high level of endogenous HEL.

#### Table II. MHC class II+Ii-DM- tumor cells (Sal/Ak) present a larger repertoire of epitopes derived from nuclear, mitochondrial, cytosolic, and ER Ags than class II+Ii+DM+ tumor cells (Sal/CIITA)

<table>
<thead>
<tr>
<th>HEL Location</th>
<th>Clone</th>
<th>MCF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HEL&lt;sub&gt;25–43&lt;/sub&gt;</th>
<th>HEL&lt;sub&gt;34–45&lt;/sub&gt;</th>
<th>HEL&lt;sub&gt;46–61&lt;/sub&gt;</th>
<th>HEL&lt;sub&gt;25–43&lt;/sub&gt;</th>
<th>HEL&lt;sub&gt;34–45&lt;/sub&gt;</th>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>29</td>
<td>2.4</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<sup>a</sup> APC activity is defined as the amount of IL-2 released in APC assays at a 1:1 ratio of APC to T cell hybridoma cells as follows: ——, <100 pg/ml; ++, 100–300 pg/ml; +++, 300–600 pg/ml; ++++, >600 pg/ml.

<sup>b</sup> MCF and APC ability for erHEL cells taken from data in (31) and L. Qi and S. Ostrand-Rosenberg, unpublished data; all other values were obtained from this study.

<sup>c</sup> MCF and APC ability for erHEL cells taken from data in (31) and L. Qi and S. Ostrand-Rosenberg, unpublished data; all other values were obtained from this study.
and several epitopes derived from cytosolic-resident proteins of tumor cells have been reported as tumor Ags (18, 41–43). In this study, we show that epitopes derived from Ags localized to nuclei and mitochondria are also presented by MHC class II tumor cells, and that the tumor cells, rather than host-derived cells, are the principle APC in vivo for priming naive CD4+ T cells to nuclear-derived Ags. These results are in agreement with earlier studies assessing in vivo presentation of ER-retained Ag using bone marrow chimeras (10, 11); however, they are unexpected because presentation of tumor-encoded class I-restricted epitopes is via cross-priming and host-derived APC (12, 13). Combined with our earlier studies with ER-retained and membrane-anchored Ag (11, 20), these results demonstrate that MHC II tumor cells play an important role in CD4+ T cell activation and are potential APC for epitopes derived from many subcellular locales.

MHC II-restricted exogenous Ags are principally processed by cathepsin proteases (45), and the resulting epitopes bound to MHC class II molecules in endosomal/lysosomal compartments or MIIC (46). In contrast, it is not clear where endogenous Ag is degraded and loaded onto MHC class II molecules in class II tumor cells. If peptides from all subcellular compartments are loaded onto class II molecules in the MIIC, then Ii expression should equally affect presentation of peptides regardless of their source. However, the present data demonstrate that Ii expression differentially affects peptide presentation. The differential effect of Ii is unexpected and suggests that processing and/or loading of Ag occurs in multiple cellular compartments, and possibly not in endosomes. This hypothesis is supported by the observations that 1) SaI/CIITA cells pulsed with exogenous HEL efficiently present HEL25–43 and HEL34–45, whereas SaI/CIITA cells do not present these epitopes when HEL is endogenously encoded (Table II) 2) in the SaI sarcoma system, cathepsin inhibitors such as E64 (47) and the cysteine protease inhibitor leupeptin (48) block presentation of endocytosed HEL, but do not inhibit presentation of endogenously synthesized erHEL (31) or nuHEL (L. Q. and S. O.-R., unpublished results); 3) recent studies by Blum and colleagues (49) demonstrate that presentation of the cytosolic endogenous Ag glutathione decarboxylase by B lymphoblastoid tumor cells requires cytosolic proteases that are not required for presentation of exogenous glutathione decarboxylase; and 4) studies by van der Bruggen and colleagues (50) indicate that dendritic cells pulsed with the MAGE-3 tumor Ag generate DR13-restricted epitopes, whereas MAGE-3 melanoma cells do not generate these peptides. Therefore, at least some MHC II-restricted endogenous Ags are not processed by endosome-resident proteases, suggesting that some processing occurs in compartments other than the MIIC. The presence of proteases in other cellular compartments, including the cytosol (proteosomes, calpains), mitochondria, and nuclei, provides ample alternative locales for Ag degradation (51–54).

Tumor cells transfected with MHC class II and costimulatory molecule genes were originally designed as tumor vaccines to stimulate tumor-specific CD4+ T cells in tumor-bearing individuals (55) and have been shown in animal models to be effective against established, dispersed metastatic disease (56–58). Their therapeutic efficacy may be due to several factors. First, class II (II or Iit) tumor cells may present a novel set of MHC II-restricted tumor epitopes that are not presented by professional APC. If tumor Ags are processed in nonendocytic compartments by proteases other than cathepsins, it is likely that class II tumor cells present different epitopes than those presented by professional APC via cross-priming. Therefore, class II tumor cells may stimulate a different repertoire of CD4+ T cells than professional APC presenting tumor Ags via cross-priming. Second, as shown in this study, Sal/A and Sal/CIITA cells process and present endogenous Ag differently (Table II). Therefore, class IIi tumor cells may present a novel set of tumor Ags and epitopes that are not presented by professional APC due to the absence of lii expression. The finding that Sal/A cells are immunogenic and not tumorigenic, whereas Sal/CIITA cells are highly tumorigenic and not immunogenic (20, 31, 59), supports this hypothesis. Third, the vaccine may present novel tumor epitopes to which the tumor bearer is not tolerant. Because many tumor Ags are self-Ags (18, 60), tumor-bearing individuals may be tolerant or anergized to epitopes derived from these Ags and presented by professional APC. In contrast, if the class II-restricted epitopes presented by the vaccines are novel epitopes, then tumor-bearing individuals may not be tolerant to them or ignore them, and the vaccines may activate previously quiescent tumor-reactive CD4+ T cells.

These studies also have implications for CD4+ T cell activation by wild-type tumor cells. Most human tumors do not express MHC class II molecules, and the direct presentation pathway for activation of CD4+ T cells, therefore, is not available. CD4+ T cell activation for these tumors depends exclusively on indirect presentation via professional APC, and limited CD4+ T cell responses to such tumors may reflect the inefficiency of cross-priming. However, a subset of human tumors constitutively expresses MHC class II molecules (e.g., melanoma and mammary carcinoma). Furthermore, these and other tumor types are frequently induced for class II expression by agents such as IFN-γ (61). Human tumor cells that constitutively express MHC class II or are induced by IFN-γ usually coexpress lii and DM (V. Clements, L. Qi, and S. Ostrand-Rosenberg, unpublished results). Because these accessory molecules block presentation of some class II-restricted endogenous Ag, wild-type tumor cells may effectively present fewer epitopes than class IIi/DM− vaccine cells. Whether class II wild-type tumor cells activate CD4+ T cells depends on the availability of costimulatory signals. In the presence of costimulation, either by the tumor cells themselves or by third party APC, CD4+ T cells may be activated. In contrast, if costimulation is not available, then class II wild-type tumor may tolerate/anergy potently tumor-reactive T cells. For some tumors, class II expression correlates with a more favorable prognosis (e.g., larynx and breast carcinomas; Refs. 62, 63), whereas for other tumors MHC class II expression correlates with a more aggressive malignancy (e.g., melanoma; Ref. 64). In the case of mouse Sal sarcoma cells, even if the tumor cells do not constitutively express costimulatory molecules, they are induced to express CD80 and CD86 during the immunization process (65) and can deliver both of the signals necessary for activation of naive CD4+ T cells. It is not clear whether other tumor cells are similarly induced to express costimulatory molecules. If not, then MHC class II-positive, costimulatory signal-negative tumor cells may tolerate, rather than activate, naive CD4+ T cells.

As demonstrated here, class II-transfected tumor cells are effective APC for endogenously synthesized Ags derived from nuclear, mitochondrial, cytosolic, or ER compartments, and class IIi/DM− tumor cells present a broader repertoire of endogenous epitopes than class IIi/DM+ tumor cells. Therefore, immunotherapy strategies relying on professional class IIi/DM+ APC to process and present intact tumor Ags or epitopes identified using professional APC may not include the broadest repertoire of tumor Ag epitopes. In contrast, MHC II cell-based vaccines capable of direct presentation of tumor epitopes may induce a wider repertoire of tumor-reactive T cells and thereby stimulate a more efficacious tumor-specific CD4+ T cell response.
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References


