Presentation of Endogenously Synthesized MHC Class II-Restricted Epitopes by MHC Class II Cancer Vaccines Is Independent of Transporter Associated with Ag Processing and the Proteasome[1]

Samudra K. Dissanayake, Natalia Tuera, and Suzanne Ostrand-Rosenberg[2]

Cell-based vaccines consisting of invariant chain-negative tumor cells transfected with syngeneic MHC class II (MHC II) and costimulatory molecule genes are prophylactic and therapeutic agents for the treatment of murine primary and metastatic cancers. Vaccine efficacy is due to direct presentation of endogenously synthesized, MHC II-restricted tumor peptides to CD4$^+$ T cells. Because the vaccine cells lack invariant chain, we have hypothesized that, unlike professional APC, the peptide-binding groove of newly synthesized MHC II molecules may be accessible to peptides, allowing newly synthesized MHC II molecules to bind peptides that have been generated in the proteasome and transported into the endoplasmic reticulum via the TAP complex. To test this hypothesis, we have compared the Ag presentation activity of multiple clones of TAP-negative and TAP-positive tumor cells transfected with *I-A^k* genes and the model Ag hen egg white lysozyme targeted to the endoplasmic reticulum or cytoplasm. Absence of TAP does not diminish Ag presentation of three hen egg white lysozyme epitopes. Likewise, cells treated with proteasomal and autophagy inhibitors are as effective APC as untreated cells. In contrast, drugs that block endosome function significantly inhibit Ag presentation. Coculture experiments demonstrate that the vaccine cells do not release endogenously synthesized molecules that are subsequently endocytosed and processed in endosomal compartments. Collectively, these data indicate that vaccine cell presentation of MHC II-restricted endogenously synthesized epitopes occurs via a mechanism independent of the proteasome and TAP complex, and uses a pathway that overlaps with the classical endosomal pathway for presentation of exogenously synthesized molecules. *The Journal of Immunology,* **2005, 174: 1811–1819.**

CD4$^+$ T cells are considered an important component of effective immunity against tumors (1). Because of their beneficial role, we are developing cell-based tumor vaccines that specifically target the activation of tumor-reactive CD4$^+$ T lymphocytes. The vaccines are based on the hypothesis that tumor Ag-specific CD4$^+$ T cells will be activated if they receive an MHC class II (MHC II)[3]-restricted tumor Ag-specific signal plus a costimulatory signal. To simultaneously deliver both of these signals, we have generated vaccines that consist of tumor cells that constitutively express MHC class I (MHC I) molecules and are genetically modified to coexpress MHC II and costimulatory molecules. Extensive in vivo studies have demonstrated the protective and therapeutic efficacy of these vaccines in multiple mouse tumor models including sarcomas, melanomas, and mammary carcinomas, and for both primary tumors and spontaneous, metastatic disease (2–6). Additional genetic studies have demonstrated that the cell-based vaccines activate tumor-specific CD4$^+$ T cells by direct presentation of endogenously synthesized tumor Ags via the transfected MHC II molecules and that cross-presentation by host dendritic cells is only minimally involved (7, 8).

We have suggested that these vaccines are efficacious because they activate type 1 CD4$^+$ T cells to multiple tumor Ag epitopes that are not presented by professional APC and to which the recipient is therefore not tolerant (8, 9). Typically, professional APC present MHC II-restricted epitopes that are derived from exogenously synthesized molecules that are endocytosed by the professional APC. The endocytosed molecules are processed in the endocytic pathway where they are degraded to small peptides and bound to newly synthesized and/or recycling MHC II molecules (reviewed in Ref. 10). In contrast, the tumor Ag epitopes of our cell-based vaccines are derived from molecules that are endogenously synthesized within the tumor cells (11, 12). This difference in the source of Ag between professional APC and our vaccine cells (exogenously synthesized vs endogenously synthesized) raises the question of whether the pathways for loading epitopes onto MHC II molecules in the two different cell types is also different. Because efficient Ag presentation by the MHC II molecules is likely to affect vaccine efficacy, it is important to understand the mechanisms and pathways by which tumor Ag epitopes are loaded onto MHC II molecules of the vaccine cells.

The MHC II-associated accessory molecule invariant chain (Ii) plays an important role in MHC II-restricted Ag presentation by professional APC. As newly synthesized MHC II molecules enter the endoplasmic reticulum (ER) of professional APC, their Ag-binding groove is occupied by Ii molecules, thereby preventing the

Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21250

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[2] Address correspondence and reprint requests to Dr. Suzanne Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250. E-mail address: srosenbe@umbc.edu

[3] Abbreviations used in this paper: MHC II, MHC class II; MHC I, MHC class I; Ii, MHC II-associated invariant chain; ER, endoplasmic reticulum; HEL, hen egg white lysozyme; LAMP1, lysosomal membrane glycoprotein 1; TRITC, tetramethylrhodamine isothiocyanate.

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binding of antigenic peptides present in the ER. Endosomal targeting sequences of the Ii chain and the MHC II β-chain then direct the MHC II/Ii complexes to the Golgi compartment and subsequently to the MHC II compartments within the endocytic pathway. Concurrently, endocytosed molecules are degraded within endosomes, producing peptides that bind to the free peptide-binding groove of the trafficking MHC II molecules. Thus, coordinate expression of MHC II with Ii favors the presentation of exogenously synthesized peptides that are generated in endosomal compartments. In contrast, Ii does not bind to the peptide-binding region of MHC I molecules, so newly synthesized MHC I molecules bind peptides in the ER (reviewed in Ref. 13). ER-resident peptides are typically derived from endogenously synthesized proteins that are degraded in proteasomes and transported into the ER by the TAP complex (reviewed in Ref. 14). We have hypothesized that our vaccines present MHC II-restricted epitopes derived from endogenously synthesized proteins because the vaccine cells do not coexpress Ii, and hence the peptide-binding groove of newly synthesized class II molecules is available to bind peptides in the ER. This hypothesis is directly supported by our findings that coexpression of Ii by vaccine cells eliminates both their vaccine efficacy and their ability to present MHC II-restricted endogenously synthesized Ag (8, 11, 12, 15), and suggests that vaccine cell MHC II molecules may bind peptides that are generated by proteasomes and transported by TAP into the ER. In the present report, we have tested this hypothesis using vaccine cells that express MHC II molecules and do not coexpress Ii or TAP. Surprisingly, neither TAP deficiency nor drugs that inhibit proteasome function affect MHC II-restricted vaccine cell Ag presentation, whereas drugs that block the endosomal pathway are potent inhibitors. Therefore, vaccine cell MHC II-restricted Ag presentation is via the endosomal pathway, and the tumor Ag peptides that are presented are generated by a mechanism that is distinct from the mechanism that generates MHC I-restricted epitopes.

Materials and Methods

Cells

Media for all cell lines contained 1% gentamicin, 1% penicillin, 1% streptomycin (all from BioSource), and 2 mM Glutamax (Invitrogen Life Technologies). M12C3F6 (*H-2^d* MHC II-deficient B cell lymphoma transfected with *I-A^k* genes (16)), TA3 (B cell hybridoma of M12.4.1 $\times$ B cells from (BALB/c $\times$ A/J)F_1 mice (16)), hen egg white lysozyme (HEL)-specific, I-A^k-restricted hybridomas 3A9, 2B6.3, A2.A2, and 3B11.1, OVA-specific, K^b-restricted hybridoma B3Z, and EL-4/OVA (17), were obtained and maintained as previously described (8, 12, 18, 19). B78H1, B78H1/TAP, and B78H1/TAP/K^b (20) were kindly supplied by Dr. I. Stroynowski (University of Texas Southwest Medical Center, Dallas, TX) and were cultured in IMDM medium (BioSource) supplemented with 10% Fetal Clone I (HyClone). B78H1/TAP and B78H1/TAP/K^b cultures were also supplemented with 400 μg/ml G418 (Sigma-Aldrich).

Transfections were performed with Lipofectin according to the manufacturer's instructions (Invitrogen Life Technologies) with the following modifications: 8×10^5 cells were plated in 4 ml of growth medium in 6-cm petrie dishes the day before transfection. Sixteen hours later, when cells were ~40–60% confluent, the growth medium was removed and 800 μl of serum-free Optimen (Invitrogen Life Technologies) was added. Twenty microliters of Lipofectin reagent was mixed with 2 μg each of I-Aa^k and I-Ab^k plasmids (11) and 1 μg of pSV2neo or pSV2zeo plasmids (Invitrogen Life Technologies) (MHC II transfections), or 5 μg of BCMGhph-erHEL (11) or 4 μg of plasmid pCMV/myc/cytoHEL (8) was mixed with 1 μg of pSV2puro plasmid (Invitrogen Life Technologies), respectively, for the erHEL and cytoHEL transfections, and added to the cells. Lipofectin plus plasmids were in a total volume of 200 μl. Transfectants were selected using G418 (Sigma-Aldrich), hygromycin (Calbiochem), puromycin (Clontech), or zeocin (Invitrogen Life Technologies) and cloned by limiting dilution. All transfectants were tested by flow cytometry approximately once a month to ascertain stable expression of cell surface MHC II and/or internal HEL. Table I lists the B78H1 and B78H1/TAP transfectants used in this study.

Table I. *Cell lines used in these studies*

Cell Line/Transfectant	Clone No.	Drug Selection
B78H1		
B78H1/TAP	1.12	G418[a]
B78H1/A^k	51.3	G418
B78H1/A^k/erHEL	16.1	G418, Hph[a]
	34.9	G418, Hph
	35.1	G418, Hph
B78H1/A^k/cytoHEL	5	G418, puro[b]
	11	G418, puro
	19	G418, puro
	25	G418, puro
B78H1/erHEL	20	Hph
B78H1/TAP/A^k	5.1	G418, zeocin[a]
B78H1/TAP/A^k/erHEL	6	G418, zeocin, Hph
	12	G418, zeocin, Hph
	5.8	G418, zeocin, Hph
B78H1/TAP/A^k/cytoHEL	6	G418, zeocin, puro
	13	G418, zeocin, puro
	15	G418, zeocin, puro
	16	G418, zeocin, puro
B78H1/TAP/erHEL	11.2	G418, Hph

[a] G418, hygromycin (Hph), and zeocin were used at 400 μg/ml.
[b] Puromycin (puro) was used at 3 μg/ml.

Abs and peptides

mAbs 10-2.16 (mouse anti-I-A^k) (21), 3JP (mouse anti-I-A^b) (22), 28.8.6 (mouse anti-H-2K^{b}D^b) (23), hyHEL10 (rat anti-HEL) (24), In-1 (rat anti-invariant chain) (25), 1G10 (BD Pharmingen), and polyclonal K553 (rabbit anti-H-2 DM) (26) were prepared and used as previously described (8). mAb 1D4B against lysosomal membrane glycoprotein 1 (LAMP1) (27) was obtained from the Developmental Hybridoma Bank at the University of Iowa (Ames, IA). Alexa 488-labeled 10-2.16 was prepared using an Alexa 488 Protein Labeling kit (Molecular Probes) according to the manufacturer's directions and used at 2 μg/ml. Alexa 488 isotype control mAb (Pierce/Endogen) was used at 5 μg/ml. Rat anti-mouse IgG-FITC and goat-anti-mouse IgG-FITC were from ICN. Donkey anti-rat IgG (Jackson ImmunoResearch Labs) was used at 15 μg/ml. HEL_{46-61} peptide (NTDG STDYGILQINSR) was prepared in the Biopolymer facility at the University of Maryland (Baltimore, MD). HEL was from Sigma-Aldrich.

Immunofluorescence

Cells were stained by immunofluorescence and analyzed using a Beckman Coulter Epics XL flow cytometer as previously described (12).

Western blots

Western blots were performed as previously described (18) with the following modifications. Following electrophoresis on 12% SDS-PAGE gels, proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences) using a Bio-Rad Mini Trans-Blot cell (100 V for 1 h) and blocked with 2% BSA/TBS-T. I-A^k was detected using the mAb 10.2.16 at 1 μg/ml followed by sheep anti-mouse-HRP (Amersham Biosciences) at 50 ng/ml (1:10,000). Ii was detected using the mAb In-1 at 0.325 ng/ml followed by goat anti-rat HRP (Amersham Biosciences) at 5 ng/ml (1:50,000).

Confocal microscopy

Approximately 0.5–1 $\times$ 10^6 cells in 2 ml of PBS were adhered onto a glass coverslip in a well of a six-well plate. Cultures were incubated for 2 h at 37°C and 5% CO_2, and nonadherent cells were removed by washing the coverslip twice with excess PBS. Adherent cells were then fixed with 1% ice-cold paraformaldehyde, permeabilized with 0.2% saponin (Sigma-Aldrich), and subsequently stained with LAMP1 mAb (3.5 μg/ml), followed by donkey anti-rat IgG2a-tetramethylrhodamine isothiocyanate (TRITC) (18 μg/ml) plus Alexa 488-labeled 10-2.16 mAb (2 μg/ml). Microscopy was performed using a Leica TCS 4D confocal laser-scanning microscope equipped with a $\times$40, 1.0 numerical aperture oil objective. Laser illuminations at 488 and 568 nm (krypton/argon) were dually recorded through a 515- to 540-nm or 589- to 621-nm bandpass filter for Alexa and TRITC, respectively, and the transmission images were collected at the same time.

Ag presentation assays

Ag presentation assays were conducted as previously described in 96-well flat-bottom plates in a total volume of 200 μl per well (12) with the following modifications: Assays with the T cell hybridomas A2.A2 (28), 2B6.3 (29), and 3B11.1 (30) were performed in RPMI 1640 medium supplemented with 10% FCS (HyClone), 1% penicillin, 1% streptomycin, and 1% Glutamax. Medium for assays with the 3A9 hybridoma (16) contained IMDM instead of RPMI 1640. Hybridoma and APC cells were irradiated with 2300 and 5000 rad, respectively. 3A9 and 2B6.3 hybridoma cells were used at 1×10^5 cells/well. For assays using erHEL transfectants, A2.A2 and 3B11.1 hybridoma cells were used at 1×10^4 cells/well. For assays using cytoHEL transfectants, A2.A2 and 3B11.1 hybridoma cells were used at 4×10^4 cells/well. All HEL APC assays included positive-control wells consisting of hybridoma cells plus 1×10^4 TA3 cells pulsed with 500 ng/ml exogenous HEL. Values ranged from 20 to 25 ng/ml IL-2. For assays using a mixture of APCs, 2×10^4 B78H1/erHEL cells were mixed with 2×10^4 B78H1/TAP/A^k or 1×10^4 TA3 cells and the combination incubated with 4×10^4 A2.A2 hybridoma cells. Exogenous HEL protein and HEL_{46-61} peptide were used at 500 and 50 μg/ml, respectively. B78H1/erHEL supernatants were used at 100 μl/well and were taken from cultures that were at confluence for 20 h. For assays of OVA presentation, 1×10^5 EL-4/OVA cells were cocultured with an equal number of B3Z hybridoma cells. All APC cultures were incubated for ~16–20 h at 37°C in 5% CO_2, after which 20–50 μl of supernatant were removed from each well and assayed by ELISA for IL-2 and IFN-γ activity using a kit according to the manufacturer's directions (Pierce/Endogen). The data presented are the average of triplicate wells ± SD.

RT-PCR

Total RNA was isolated, and first-strand cDNA synthesis was performed according to the manufacturers' directions using an RNeasy Mini kit (Qiagen) and a Retroscript RT-PCR kit (Ambion), respectively. PCR was performed as follows: 0.5–1 μg of cDNA was combined with one pellet of puReTaq Ready-To-Go PCR beads containing stabilizers, BSA dNTPs, 2.5 U of puReTaq DNA polymerase, and reaction buffer (Amersham Biosciences), 1 μl each of 20 μM upstream (5′-GATCAACCTGCGGATAC GAGAG-3′) and downstream (5′-CGCAGTTCAGAATCAGCACC-3′) TAP primers, or 0.25 μl of control rig/S15 (a small ribosomal subunit protein) primers (Retroscript RT-PCR kit; Ambion), in a total volume of 25 μl of water. DNA was amplified in a PTC-200 Peltier Thermal Cycler (MJ Research) under the following conditions: denature at 94°C for 15 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final incubation at 72°C for 7 min. PCR products were analyzed on a 1.0% agarose gel stained with ethidium bromide.

Drug treatments

Assays with drug-treated erHEL or cytoHEL APC used 4×10^4 APC/well and TA3 at 1×10^4 cells/well. A2.A2 and 3B11.1 were used at 4×10^4 cells/well. 2B6.3 cells were used at 1×10^5 cells/well. HEL protein was added concurrently with drug. Working stocks of chloroquine (Sigma-Aldrich; 100 mM in water), epoxomicin (A.G. Scientific; 100 μM in DMSO), and 3-methyladenine (Sigma-Aldrich; 1M in DMSO boiled at 70°C for 10 min and diluted in growth medium to 100 mM) were prepared.

Chloroquine and 3-methyladenine treatments. Acid-stripped (12) APC were plated at $1.5–2 \times 10^6$ cells in 10 ml of growth medium in 10-cm dishes. Chloroquine or 3-methyladenine was added at the indicated doses, and the cells were cultured for 16–18 h at 37°C in 5% CO_2. Treated cells were washed twice with excess, ice-cold PBS, fixed for 10 min with 1% ice-cold paraformaldehyde (Sigma-Aldrich), and then washed twice with excess ice-cold T cell hybridoma growth medium. Treated cells were >90% viable after drug treatment and before fixation.

Epoxomicin treatment. APCs were striped with mild acid (12) and plated in 6-cm dishes at 1.5×10^6 cells in 4 ml of growth medium supplemented with the indicated amount of epoxomicin for 12–16 h at 37°C in 5% CO_2. Treated cells were washed with excess PBS as per the chloroquine and 3-methyladenine-treated cells. Treated cells were >90% viable after epoxomicin treatment and before fixation.

Percent response = 100% × ([IL-2 release from drug-treated APC]/[IL-2 release from untreated APC]).

Results

B78H1 and B78H1/TAP transfectants express I-A^k, the model Ag HEL, and do not express Ii or DM

To determine whether TAP and the proteasome are involved in presentation of MHC II-restricted endogenously synthesized Ags by vaccine cells, we have generated TAP-positive and TAP-negative tumor cell transfectants that express MHC II molecules and tested their ability to present an endogenously synthesized, model tumor Ag. The parental tumor line for the transfectants is the C57BL/6-derived B78H1 melanoma, which is a poorly metastatic, amelanotic variant that was originally derived from the melanotic, B16 melanoma (31). B78H1 cells do not contain functional *TAP2* and *LMP7* genes and do not express cell surface MHC I H-2K^b or H-2D^b molecules. However, transfection of B78H1 cells with the *TAP2* gene under a CMV promoter (B78H1/TAP cells) is sufficient to restore MHC I expression (20, 32). Because coexpression of Ii inhibits presentation of MHC II-restricted endogenously synthesized Ag in the tumor vaccines, we have tested whether B78H1 cells express Ii. B78H1, B78H1/TAP, and positive-control M12C3F6 cells, a BALB/c-derived B cell lymphoma (16), were permeabilized, stained with the Ii-specific mAb In-1, and analyzed by flow cytometry. As shown in Fig. 1, neither B78H1 nor B78H1/TAP cells contain Ii, whereas M12C3F6 cells contain high levels of Ii. Likewise, the B78H1 cells do not contain H-2-DM, another MHC II-associated accessory molecule. Therefore, B78H1 and B78H1/TAP cells appear to be an appropriate set of cells to use to determine whether TAP expression is required for presentation of MHC II-restricted endogenously synthesized Ag.

Because B78H1 cells do not constitutively express MHC II molecules or have a known tumor Ag, we have stably transfected them with genes encoding the class II molecule I-A^k and HEL, respectively. The allogeneic I-A^k molecule was used because expression of functional I-A^k is known to be independent of Ii expression, whereas expression of the functional syngeneic I-A^b allele is thought to be Ii dependent (33, 34). Two forms of the *HEL* gene have been used. One form contains a KDEL signal, which localizes HEL to the ER. The second form contains no targeting sequence so HEL localizes to the cytoplasm (8). These two alternative HEL constructs have been used so we can analyze the role of TAP and the proteasome in the presentation of Ag from different subcellular compartments. Table I lists the various transfectants and their clones that have been generated and shows the drugs that have been used for their selection. Fig. 2 shows the expression of I-A^k and HEL by the transfectants as measured by immunofluorescence and flow cytometry of live and fixed cells, respectively. MHC II transfectants express comparable levels of I-A^k, whereas HEL transfectants express roughly equivalent levels of HEL, although the erHEL transfectants as a group (Fig. 2*A*) tend to express higher levels of HEL than do the cytoHEL transfectants (*B*). Autologous MHC I (H-2K^{b}D^b; 28-8-6 mAb) and class II (I-A^b; 3JP

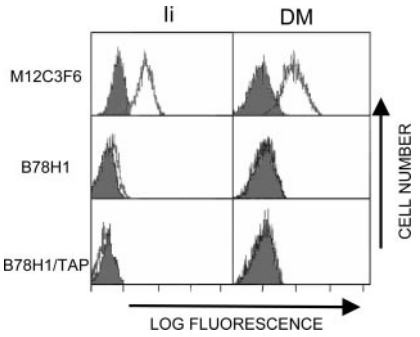

FIGURE 1. B78H1 and B78H1/TAP cells do not express Ii chain or DM. Cells were fixed and permeabilized and stained by indirect immunofluorescence for Ii or DM. Unfilled peaks are Ab-specific staining; filled peaks represent staining with fluorescent conjugate alone. These data are representative of three independent experiments.

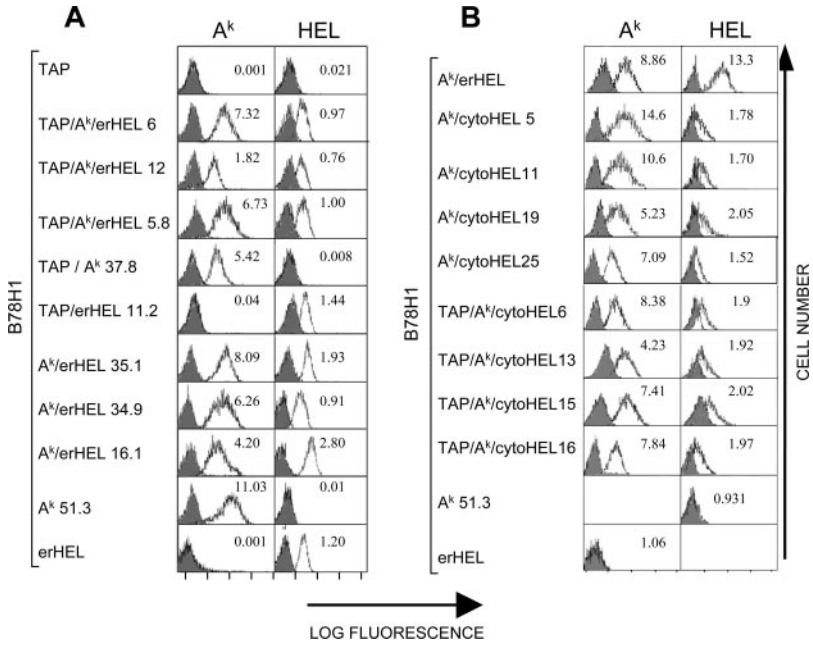

FIGURE 2. B78H1 and B78H1/TAP transfectants express high levels of surface MHC II and internal HEL. B78H1 and B78H1/TAP cells transfected with I-A^k and erHEL (*A*) or cytoHEL (*B*) were stained for surface I-A^k or internal HEL. Unfilled peaks denote Ab-specific staining; filled peaks represent staining with fluorescent conjugate alone. Numbers in the *upper-right corner* of each histogram are the mean channel fluorescence values. These data are representative of two to five independent experiments.

mAb) expression in these cells is absent as measured by immunofluorescence and flow cytometry (data not shown).

To ascertain that the MHC II heterodimers are functional and bind peptides in the absence of Ii and DM, Western blots were performed. Cell lysates were prepared from B78H1, B78H1 transfectants, and control M12C3F6 cells. If the MHC II α- and β-chains are properly conformed and bind peptide, then the $\alpha\beta$ heterodimer forms a stable complex of ~55 kDa that dissociates with boiling (35). The I-A^k molecules of B78H1/A^k/erHEL and B78H1/TAP/A^k erHEL (Fig. 3*A*) and cytoHEL (*B*) transfectants form stable dimers in the nonboiled samples. Stable dimer formation in the transfectants is independent of HEL expression, because I-A^k transfectants without HEL also contain stable dimers (Fig. 3*A*). To confirm that MHC II stable dimers are formed in the absence of Ii, the B78H1/erHEL transfectants were also analyzed by Western blots for Ii expression using the Ii-specific In-1 mAb. As shown in Fig. 3*C*, neither B78H1/A^k/erHEL nor B78H1/TAP/A^k/erHEL cells contain Ii, although it is present as a 31-kDa band in the control M12C3F6 cells. Therefore, the transfectants contain properly conformed MHC II molecules, and these I-A^k molecules are expressed in the absence of Ii and DM, in agreement with our previous studies (8, 36) and those of others (34, 37).

Presentation of MHC II-restricted endogenous HEL is TAP independent

To determine whether TAP is required for presentation of class II-restricted endogenous Ag, the B78H1 and B78H1/TAP transfectants were used as APC to HEL-specific, I-A^k-restricted T cell hybridomas, and IL-2 production was monitored to assess Ag presentation. Four hybridomas that react to three different HEL peptides have been used: A2.A2 (HEL_{46-61}), 2B6.3 (HEL_{25-43}), 3B11.1 (HEL_{34-45}), and 3A9 (HEL_{46-61}). Figs. 4 and 5 show the results of Ag presentation assays using three independent clones of the TAP-negative and TAP-positive erHEL (Fig. 4) and cytoHEL (Fig. 5) transfectants. Positive-control wells using TA3, an I-A^k-expressing B cell hybridoma, pulsed with exogenous HEL were included in all assays and gave values of 20 to 25 ng/ml IL-2. Although there is some variation between the clones of each transfectant line, Ag presentation activity of the TAP-negative and TAP-positive lines is very similar.

The similarity in Ag presentation between the TAP-positive and TAP-negative B78H1 lines could be the result of up-regulation of TAP2 during the Ag presentation assays. To test this possibility, supernatants from the APC cultures were assayed by ELISA for IFN-γ, a known inducer of TAP2 expression in B78H1 cells (32).

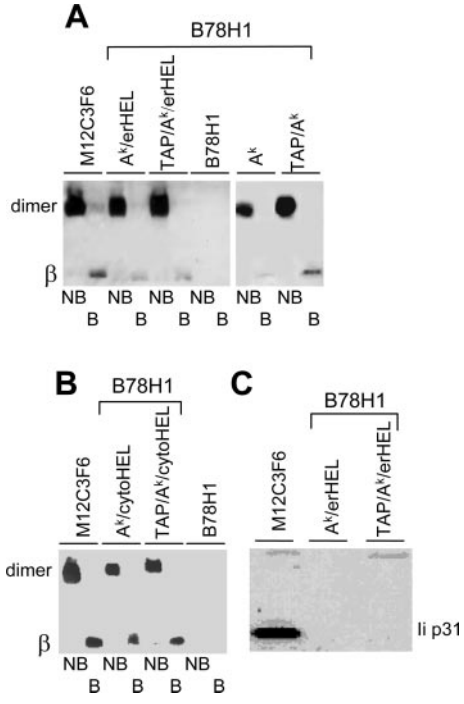

FIGURE 3. I-A^k-transfected B78H1 and B78H1/TAP cells express MHC II stable dimers and do not contain Ii. *A* and *B*, Extracts of detergent-lysed erHEL-transfected (*A*) or cytoHEL-transfected (*B*) B78H1/A^k, B78H1/TAP/A^k, B78H1, B78H1/A^k, or control M12C3F6 cells were boiled or not boiled, electrophoresed on SDS-PAGE gels, transferred to nitrocellulose, and probed for I-A^k. *C*, Boiled extracts were blotted onto nitrocellulose and probed for Ii. "Dimer" indicates stable dimer of ~55 kDa; "β" indicates free β-chain. These data are from one of three independent experiments.

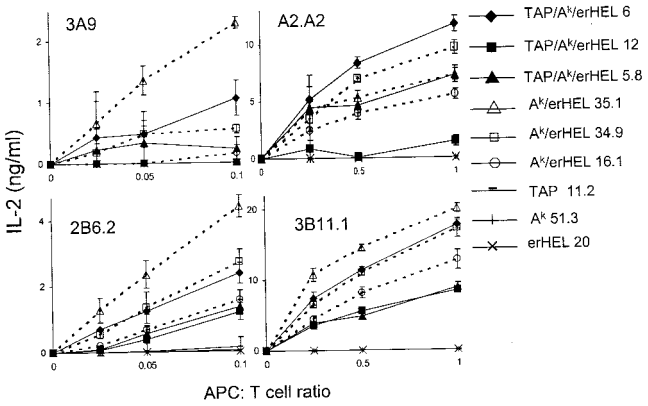

FIGURE 4. MHC II-restricted presentation of endogenously synthesized ER-localized HEL is TAP independent. Three independent clones of B78H1/A^k/erHEL (dotted lines; △, □, ○) and B78H1/TAP/A^k/erHEL (solid lines; ◆, ■, ▲) transfectants were cocultured with each of four I-A^k-restricted, HEL-specific $CD4^+$ T cell hybridomas (3A9, A2.A2, 2B6.3, or 3B11.1), and T cell hybridoma activation was determined by measuring IL-2 production. Presentation of exogenous HEL by TA3 cells gave 20–25 ng/ml IL-2. These data are representative of five independent experiments.

No detectable IFN-γ was present (assay detected >1 pg/ml IFN-γ). B78H1 cells were also cocultured for 16 h with supernatants from the APC assays and subsequently tested by RT-PCR for TAP2, to ascertain whether there were other factors in the APC assays that could up-regulate TAP2. As shown in Fig. 6, B78H1/TAP/A^k/erHEL cells and control A2.A2 cells express TAP2, but neither B78H1/A^k/erHEL, B78H1, nor B78H1/A^k/erHEL cocultured with APC supernatant contain TAP2 message. Therefore, presentation of endogenously synthesized erHEL and cytoHEL is not regulated by the TAP complex.

Processing of MHC II-restricted endogenous Ag is proteasome independent and does not involve autophagy

The proteasome is the site of degradation of most cellular proteins and is responsible for the generation of MHC I-restricted peptides. Drugs that selectively block proteasomal degradation limit the

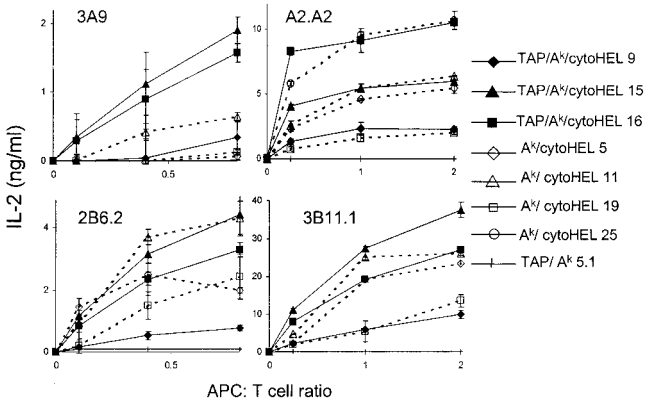

FIGURE 5. MHC II-restricted presentation of endogenously synthesized cytoplasmically localized HEL is TAP independent. Four independent clones of B78H1/A^k/cytoHEL (dotted lines; ◇, △, □, ○) and three clones of B78H1/TAP/A^k/cytoHEL (solid lines; ◆, ■, ▲) transfectants were cocultured with each of four I-A^k-restricted, HEL-specific $CD4^+$ T cell hybridomas (3A9, A2.A2, 2B6.3, or 3B11.1), and T cell hybridoma activation was determined by measuring IL-2 production. Presentation of exogenous HEL by TA3 cells gave 20–25 ng/ml IL-2. These data are representative of three to five independent experiments.

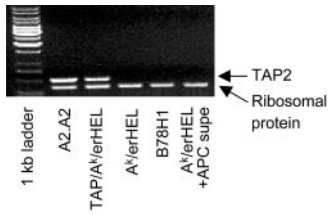

FIGURE 6. B78H1 transfectants are not induced to express TAP2 during the Ag presentation assays. RNA was isolated from the indicated cell populations and from B78H1/A^k/erHEL cells cocultured with supernatant from an APC assay of A2.A2 plus B78H1/A^k/erHEL, reverse transcribed, and PCR amplified using primers specific for TAP2 (*upper band*) and a positive control ribosomal gene (*lower band*). Supernatant from the A2.A2 plus B78H1/A^k/erHEL culture contained 25 ng/ml IL-2.

generation of ER-resident peptides and reduce the stability of MHC I molecules (38). To determine whether proteasomal degradation is involved in the generation of MHC II-restricted peptides, we have used the drug epoxomicin. Epoxomicin is a highly selective inhibitor of the proteasome because it reacts with both the hydroxyl and amino groups of the catalytic N-terminal threonine of the proteasome. It is highly specific for the proteasome and does not have other intracellular targets, whereas lactacystin, a commonly used proteasomal inhibitor, also affects some nonproteasomal proteases. Epoxomicin is also highly stable in cells, whereas lactacystin is rapidly hydrolyzed by water at physiological pH (39, 40).

To determine whether proteasomal activity is required for presentation of endogenously synthesized Ag by MHC II molecules, B78H1 transfectants were acid stripped, treated with epoxomicin, and tested as APCs to the HEL-specific hybridomas. As shown in Fig. 7*A*, epoxomicin slightly impairs presentation by B78H1/A^k/cytoHEL to A2.A2, but has no effect on presentation to the 3B11.1 or 2B6.3 hybridomas or by B78H1/A^k/erHEL to A2.A2. In contrast, epoxomicin inhibits >90% of the Ag presentation activity of EL-4/OVA transfectants to the OVA-specific, H-$2K^b$-restricted B3Z hybridoma, demonstrating its ability to block presentation of proteasome-generated epitopes.

Epoxomicin also blocks the generation of MHC I-restricted epitopes in B78H1 cells. B78H1 cells transfected with TAP2 and the *H-2K*^{b} gene under a viral promoter (B78H1/TAP/K^b) (20, 32) were incubated with epoxomicin and tested for H-$2K^b$ and H-$2D^b$ expression by flow cytometry. As shown in Fig. 7*B*, epoxomicin treatment results in decreased MHC I expression, but does not affect MHC II expression. These experiments are in full agreement with our earlier findings in which lactacystin was shown to inhibit presentation of MHC I epitopes, but did not affect presentation of MHC II epitopes by a SaI sarcoma tumor cell-based vaccine (12). Therefore, although the proteasome is involved in processing of MHC I-restricted epitopes, it is not a significant factor in generating MHC II-restricted peptides.

The process of autophagy, or the trafficking of degraded cytosolic proteins from the cytoplasm to the endosomal compartment, has also been suggested as a possible mechanism for generating peptides for presentation by MHC II molecules. To determine whether autophagy is responsible for peptide generation in our vaccines, we have used the drug 3-methyladenine, which specifically inhibits autophagy (41). B78H1 transfectants were treated with 3-methyladenine, fixed, and used as APC to the A2.A2 hybridoma. No reduction in Ag presentation activity was seen relative to untreated cells (data not shown), indicating that autophagy is not involved in the processing and presentation of MHC II-restricted epitopes.

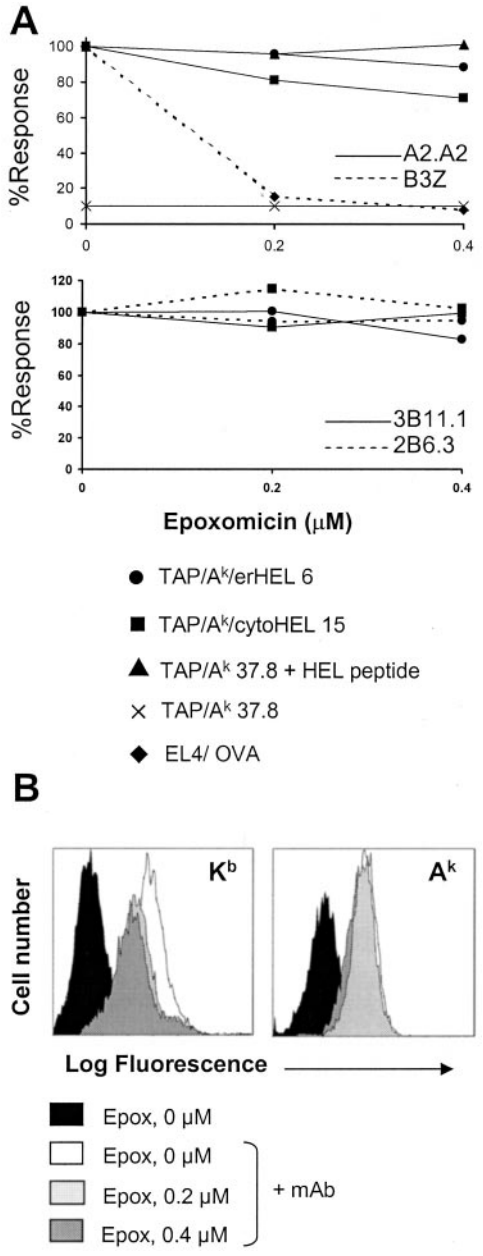

FIGURE 7. MHC II-restricted endogenously synthesized epitopes are not processed in the proteasome. *A*, B78H1/TAP/A^k/erHEL, B78H1/TAP/A^k/cytoHEL, and EL-4/OVA transfectants were treated with the indicated amounts of epoxomicin, fixed, incubated with A2.A2, 3B11.1, or 2B6.3 (HEL transfectants) or B3Z (EL-4/OVA) hybridoma cells, and IL-2 release was determined by ELISA. HEL_{46-61} peptide was added to some cultures as a control. *B*, B78H1/TAP/K^b and B78H1/TAP/A^k transfectants were untreated or treated with 0.4 or 0.2 μM epoxomicin, fixed, stained for H-2K^b or I-A^k expression, and analyzed by flow cytometry. These data are representative of two to three independent experiments.

Presentation of endogenously synthesized MHC II-restricted epitopes requires a functional endosomal compartment and does not involve recycling

Binding of exogenously synthesized, endocytosed Ag to MHC II molecules occurs in the MHC II compartments and requires functional endosomal compartments (42). To determine whether the endosomal pathway is also involved in presentation of MHC II-restricted endogenously synthesized peptides, we have used the drug chloroquine, which inhibits endosomal processing by blocking acidification of endosomal/lysosomal compartments. Despite its targeted effects on the endocytic pathway, chloroquine is a pleiotropic agent and can also affect the secretory pathway. To ascertain that, at the dosages used, chloroquine specifically affects the endosomal compartment and does not interfere with MHC I peptide generation or the class I secretory pathway, B78H1/K^b cells were acid stripped, treated with varying doses of chloroquine, and monitored for cell surface MHC I expression by immunofluorescence and flow cytometry. As shown in Fig. 8*A*, chloroquine treatment has no effect on MHC I expression, indicating that it is not affecting class I peptide generation, loading of peptides onto MHC I molecules, or the secretory pathway. To determine whether chloroquine affects MHC II presentation of endogenously produced Ag, B78H1/TAP/A^k cells with endogenous erHEL or cytoHEL were acid stripped, treated with chloroquine, and used as APCs to the HEL-specific A2.A2 hybridoma. As a control, chloroquine-treated TA3 cells were pulsed with HEL peptide. As shown in Fig. 8*B*, presentation of exogenous Ag by TA3 cells is chloroquine sensitive, as is presentation of endogenously synthesized HEL by the B78H1 transfectants. Therefore, presentation of endogenous Ag by MHC II molecules requires functional endocytic compartments, suggesting that the MHC II/peptide complexes traffic via the endocytic pathway on their way to the cell surface.

To confirm that MHC II molecules in the B78H1 transfectants traffic via the endosomal compartment, we have used confocal microscopy to visualize I-A^k in B78H1/A^k cells. B78H1/A^k/erHEL and B78H1/A^k/cytoHEL cells were fixed, permeabilized, and stained for I-A^k and the endosomal compartment marker LAMP1. As shown in Fig. 9, in B78H1/A^k/erHEL and B78H1/A^k/cytoHEL cells, much of the internal I-A^k colocalizes with LAMP1. Cells incubated with an irrelevant isotype-matched mAb (1G10) or Alexa-labeled isotype control Ab alone were unstained (data not shown). Therefore, MHC II molecules traffic through endosomal compartments.

We have hypothesized that the MHC II molecules of our vaccine cells bind self peptides derived from Ags produced within the vaccine cells. Because the MHC II molecules traffic via endosomal compartments, it is possible that the transfectants secrete Ag,

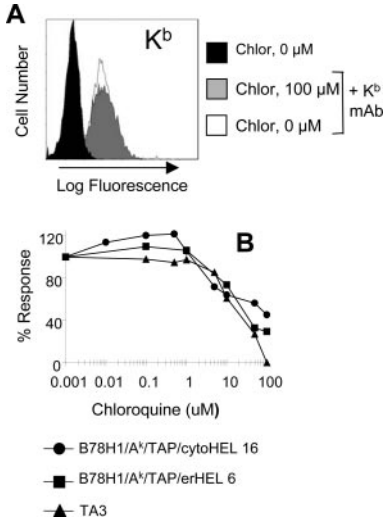

FIGURE 8. Presentation of MHC II-restricted epitopes derived from endogenous erHEL or cytoHEL requires functional endosomal compartments. *A*, B78H1/TAP/K^b cells were untreated or treated with 100 μM chloroquine, fixed, stained for H-2K^b expression, and analyzed by flow cytometry. *B*, B78H1/TAP/A^k/erHEL, B78H1/TAP/A^k/cytoHEL, or control TA3 cells were treated with the indicated amounts of chloroquine, fixed, and cocultured with A2.A2 hybridoma cells, and IL-2 release was measured by ELISA. These data are representative of three independent experiments.

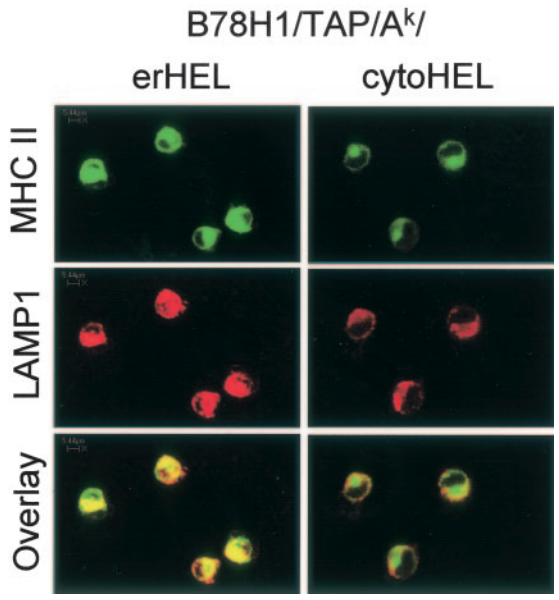

FIGURE 9. MHC II traffics through the endosome. B78H1/TAP/A^k/erHEL (*left panels*) and B78H1/TAP/A^k/cytoHEL (*right panels*) transfectants were fixed, permeabilized, and stained for MHC II (Alexa 488) and the endosomal marker LAMP1 (TRITC), and visualized by confocal microscopy.

which they subsequently endocytose and re-present via the classical MHC II endosomal presentation pathway. To test this hypothesis, we have performed mixing experiments in which Ag presentation can only occur if the vaccine cells release HEL and it is endocytosed by other vaccine cells. For these experiments, B78H1/TAP/A^k cells were mixed with B78H1/erHEL cells or supernatant, and cocultured with A2.A2 hybridoma cells. As shown on the *left* side of Fig. 10, IL-2 release only occurs if I-A^k and HEL are associated with the same vaccine cell, suggesting that the HEL-expressing cells do not secrete HEL and/or that they are incapable of endocytosing soluble HEL. To distinguish whether the vaccine cells are not secreting HEL vs not able to endocytose HEL, the professional APC, TA3, was mixed with B78H1/erHEL cells and subsequently cocultured with A2.A2 hybridoma cells. As shown in the *right* side of Fig. 10, TA3 cells stimulate IL-2 release when

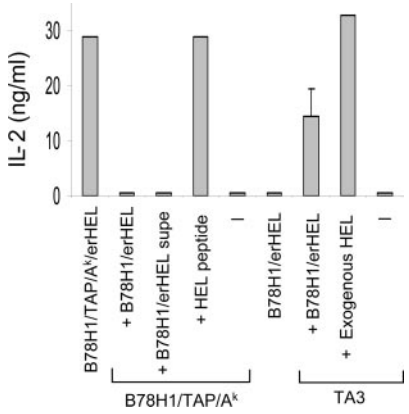

FIGURE 10. Presentation of erHEL by B78H1 transfectants does not involve endocytosis of secreted HEL. B78H1/TAP/A^k or TA3 cells were mixed with B78H1/erHEL cells, supernatants from B78H1/erHEL cells, or HEL_{46-61} peptide, and the mixture was cocultured with I-A^k-restricted, HEL-specific A2.A2 hybridoma cells. Culture supernatants were assayed by ELISA for IL-2 production. Error bar indicates the SD. These data are representative of three independent experiments.

mixed with B78H1/erHEL cells, indicating that the B78H1 cells secrete HEL. Therefore, although the vaccine cells release HEL, they do not endocytose it, so vaccine presentation of MHC II-restricted epitopes is not a re-presentation of endocytosed material.

Discussion

Most peptides that are presented by MHC II molecules are exogenously synthesized molecules that are either endocytosed in the fluid phase or following binding to cell surface receptors. Following endocytosis, these exogenously synthesized Ags associate with MHC II molecules within endocytic compartments. Endogenously synthesized proteins are also presented by MHC II molecules; however, their pathway for presentation is not as well defined (reviewed in Ref. 43). In the present report, we have examined endogenous presentation of MHC II-restricted Ags in tumor cells that have been genetically modified as tumor vaccines. It is important to understand the mechanisms underlying Ag presentation by the vaccine cells because they are effective prophylactic and therapeutic agents for activating tumor-specific $CD4^+$ T lymphocytes and for facilitating rejection of primary and metastatic tumor cells (3–6). In contrast to most other cells in which endogenous MHC II Ag presentation has been examined, our vaccine cells are not professional APC and they do not express MHC II-associated accessory molecules (e.g., Ii and DM) that have previously been shown to affect exogenous and endogenous Ag presentation (reviewed in Ref. 10). Because our vaccine cells do not coexpress Ii, the peptide-binding cleft of newly synthesized MHC II molecules may be accessible to peptides. We have therefore proposed that MHC II-restricted tumor peptides presented by the vaccine cells traffic via a pathway similar to that taken by peptides presented by MHC I molecules (11, 44). Accordingly, MHC II-restricted peptides are generated in the proteasome from endogenously synthesized, cytosolic proteins and transported via the TAP complex into the ER. In the current report, we demonstrate that our earlier hypothesis is not correct, because neither TAP nor the proteasome is involved in the presentation of three peptides derived from either cytosolic or ER-retained Ags of the vaccine cells.

The multicatalytic proteasome degrades cytosolic proteins, which are subsequently translocated by the TAP complex into the ER where they are bound by newly synthesized MHC I molecules (reviewed in Ref. 45). Because the proteasome generates peptides of variable length (46), including ones appropriate for binding to MHC II dimers, we hypothesized that it may supply ligands for MHC II molecules as well as for MHC I molecules. However, epoxomicin does not significantly inhibit presentation of either cytoplasmic or ER-tethered Ag, indicating that the proteasome is not involved in the generation of the peptides presented by the vaccine cells. The absence of LMP7 (IFN-γ-inducible 20S proteasome subunit) in B78H1 cells (20), a critical component of the immunoproteasome, further supports our conclusion that peptide generation does not require the proteasome. Our results are in agreement with studies of others (41), and our own earlier studies in which the less-specific proteasome inhibitor, lactacystin, also showed no inhibition of MHC II Ag presentation (12), but differ from those of Lich et al. (47), who showed that the proteasomal protease calpain is required for the generation of a cytosolic peptide. Collectively, these studies could be interpreted as showing that different peptides are generated via divergent pathways, some of which involve proteasomal degradation. However, our results with multiple peptides and with molecules targeted to multiple compartments suggest that, in the Ii negative vaccine cells, proteasomal degradation is not involved in the generation of endogenously synthesized MHC II peptides.

The TAP complex is essential for the transport of peptides into the ER where they are subsequently bound by newly synthesized

MHC I molecules (48–53). Deficiencies and/or mutations of TAP impair MHC I expression and protect tumor cells against CD8-mediated T cell lysis (reviewed in Ref. 54). Although some investigators have found that TAP is not required for presentation of endogenously synthesized MHC II-restricted peptides (41, 55, 56), others have found that presentation is TAP dependent (57, 58). It has been suggested that this discrepancy in the apparent requirement for TAP is due to differences in Ag stability, because rapidly degraded, but not long-lived cytosolic Ag requires TAP for Ag presentation (59). However, our results showing that presentation of cytoHEL, which is very rapidly degraded in the cytoplasm (Ref. 60; L. Qi and S. Ostrand-Rosenberg, unpublished data), is TAP independent, contradict this explanation. The discrepancy between these studies could be due to the different APC that have been used. With the exception of our vaccine cells, the APC used in previous studies were professional APC that contain both Ii and DM. In addition, most of the earlier reports drew their conclusions from studies with a single epitope derived from an Ag localized to a single subcellular compartment (e.g., cytosol or plasma membrane). In contrast, we have examined three epitopes from two subcellular locations and consistently find no role for TAP. Therefore, although there are conflicting reports of the role of TAP in presentation of MHC II-restricted peptides by professional APC, there appears to be no role for TAP in our vaccine cells.

In contrast to the lack of involvement of the proteasome and the TAP complex, a functional endosomal pathway is required for presentation of MHC II-restricted, ER- and cytosolic-localized Ags by the vaccine cells. This finding agrees with our previous studies (12) for ER-localized tumor Ag, and with many other studies that have examined presentation of endogenous Ag by professional APC (30, 61–63); however, it differs from the results of Lich et al. (47), who found that endosomal acidification was not required for presentation of a cytosolic Ag by Ii-positive B lymphoblastoid cells. Inconsistency with this latter study cannot be easily explained, other than the superficial conclusion that a small percentage of Ags are processed through an alternative mechanism.

The finding that a functional endocytic pathway is required raises the possibility that vaccine cell MHC II Ag presentation is via the classical route in which Ag is endocytosed, and subsequently degraded to peptides and bound to MHC II molecules in endosomal compartments. This pathway could be active if the vaccine cells release soluble or microvesicle-contained tumor-encoded molecules that are subsequently endocytosed by the same or other vaccine cells. However, the mixing experiments reported here indicate that, although the vaccine cells release some endogenously synthesized Ag, they do not endocytose that Ag and present it. Autophagy has also been proposed as a mechanism by which cytosolic molecules access endosomal compartments (41). However, vaccine cells treated with 3-methyladenine, a drug that inhibits autophagy, retain their Ag presentation activity, indicating that autophagy is not involved.

Cytoplasmic and ER-resident Ags in our vaccines may be degraded to peptides and presented via MHC II through several potential mechanisms. MHC II-restricted peptides could be generated via cytoplasmic hydrolytic enzymes that are independent of the proteasome and that have been shown to be active in generating MHC I peptides (64, 65). The ER also contains peptides that have not been transported into the ER by TAP. These peptides are presumably derived from partially or incorrectly folded proteins that access the ER through channels. The complete processing to peptides could occur in the ER by ER-resident enzymes, or alternatively, partially degraded protein could bind to newly synthesized MHC II molecules in the ER, and final trimming of the peptide occurs after it is bound to MHC II, either in the ER or in endosomal compartments, as suggested by Sercarz and Maverakis (66). Such complexes between MHC II molecules and partially folded proteins have been observed in Ii-negative cell lines (Refs. 67 and 68; reviewed in Ref. 43). Alternatively, empty class II dimers may traffic from the ER to endosomal compartments and pick up peptides along the way. Such peptides could access endosomes by chaperone-mediated trafficking from the cytosol such as via heat shock proteins (69, 70). Because the β-chain of MHC II molecules contains an endosomal targeting sequence, even in the absence of Ii, MHC II dimers will enter endosomal compartments (71–73).

The studies reported here demonstrate the critical role of endosomal compartments and the lack of involvement of the proteasome and TAP complex in Ag presentation of endogenously synthesized MHC II-restricted peptide, and suggest various scenarios by which Ag processing and presentation could occur in the vaccine cells. Because vaccine potency correlates with Ag presentation activity, a complete understanding of the Ag processing and presentation mechanisms of the vaccine cells may lead to more efficacious vaccines.

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