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Invariant Chain and the MHC Class II Cytoplasmic Domains Regulate Localization of MHC Class II Molecules to Lipid Rafts in Tumor Cell-Based Vaccines

Brian P. Dolan,* Timothy P. Phelan,* Dan Ilkovitch,* Ling Qi,* William F. Wade,† Terri M. Laufer,‡ and Suzanne Ostrand-Rosenberg*‡

Cell-based tumor vaccines, consisting of MHC class I* tumor cells engineered to express MHC class II molecules, stimulate tumor-specific CD4+ T cells to mediate rejection of established, poorly immunogenic tumors. Previous experiments have demonstrated that these vaccines induce immunity by functioning as APCs for endogenously synthesized, tumor-encoded Ags. However, coexpression of the MHC class II accessory molecule invariant chain (II), or deletion of the MHC class II cytoplasmic domain abrogates vaccine immunogenicity. Recent reports have highlighted the role of lipid microdomains in Ag presentation. To determine whether Ii expression and/or truncation of MHC class II localization to lipid microdomains, we examined the lipid raft affinity of MHC class II molecules in mouse M12.C3 B cell lymphomas and SaI/Ak sarcoma vaccine cells. Functional MHC class II heterodimers were detected in lipid rafts of both cell types. Interestingly, expression of Ii in M12.C3 cells or SaI/Ak cells blocked the MHC class II interactions with cell surface lipid rafts. In both cell types, truncation of either the α- or β-chain decreased the affinity of class II molecules for lipid rafts. Simultaneous deletion of both cytoplasmic domains further reduced localization of class II molecules to lipid rafts. Collectively, these data suggest that coexpression of Ii or deletion of the cytoplasmic domains of MHC class II molecules may reduce vaccine efficacy by blocking the constitutive association of MHC class II molecules with plasma membrane lipid rafts.

facilitating MHC class II-mediated signaling (22–24). If MHC class II localization to plasma membrane lipid rafts regulates Ag presentation, then MHC class II-lipid raft associations in the vaccine cells may be important for optimal vaccine efficacy. To test this hypothesis we have studied MHC class II-lipid raft interactions in mouse sarcoma cells that have been genetically engineered to express syngeneic MHC class II molecules (Sal/A<sup>k</sup> cell-based tumor vaccines) and in the I-A<sup>k</sup>-transfected B cell lymphoma cell line, M12.C3. As deletion of the cytoplasmic domains of MHC class II molecules and coexpression of Ii abrogate immunogenicity of the vaccines, we have tested whether these alterations affect MHC class II-lipid raft interactions. Our results demonstrate that truncation of the cytoplasmic domains of MHC class II molecules reduces constitutive localization of MHC class II molecules to lipid rafts of the vaccine cells and M12.C3 cells. Likewise, coexpression of Ii by vaccine cells or M12.C3 cells inhibits constitutive localization of MHC class II molecules to cell surface lipid rafts. Therefore, MHC class II cytoplasmic domains may modulate vaccine efficacy by aiding in the localization of MHC class II molecules to lipid rafts, and Ii expression may block vaccine efficacy by reducing constitutive localization of MHC class II molecules to plasma membrane lipid rafts.

**Materials and Methods**

**Cell lines and transfectants**

Sal is a MHC class I-positive, class II-negative spindle cell sarcoma derived from A/J (H-2<sup>d</sup>) mice. Sal cells transfected with full-length I-A<sup>a</sup> and I-A<sup>b</sup> genes (Sal/A<sup>a</sup>; (3)), transfected with I-A<sup>b</sup> genes with assorted cytoplasmic truncations (Sal/A<sup>b</sup>tr, Sal/A<sup>b</sup>jtr, Sal/A<sup>b</sup>jrt (8, 25, 26)), transfected with the Ii gene (Sal/A<sup>b</sup>/Ii,(9)), transfected with I-2M (Sal/A<sup>b</sup>/DM, (5), or truncated with the MHC class II transactivator (Sal/CITTA, (2)) have been previously described and are listed and referenced in Tables I and II. Sal-derived cells were maintained in IMDM medium supplemented with 1% penicillin, streptomycin, and gentamicin (Biofluids, Rockville MD), 10% Fetal Celine 1 (HyClone Laboratories, Logan, UT), 1% Glutamax (Life Technologies, Rockville, MD), and 400 µg/ml G418 (Calbiochem, La Jolla, CA).

M12.C3 is a MHC class II negative B cell lymphoma derived from BALB/c (H-2<sup>d</sup>) mice (27). Previously reported M12.C3 transfectants with I-A<sup>a</sup> cytoplasmic domain truncations are listed and referenced in Table III. Clone M12.C3.5C2 contains 12 and 10 aa deletions of the carboxyl ends of I-Ab k genes (SaI/A k ; (3)), transfected with I-A<sup>k</sup> genes with assorted cytotoxic tumor vaccines) and in the I-A<sup>k</sup>-transfected B cell lymphoma cell line, M12.C3. As deletion of the cytoplasmic domains of MHC class II molecules and coexpression of Ii abrogate immunogenicity of the vaccines, we have tested whether these alterations affect MHC class II-lipid raft interactions. Our results demonstrate that truncation of the cytoplasmic domains of MHC class II molecules reduces constitutive localization of MHC class II molecules to lipid rafts of the vaccine cells and M12.C3 cells. Likewise, coexpression of Ii by vaccine cells or M12.C3 cells inhibits constitutive localization of MHC class II molecules to cell surface lipid rafts. Therefore, MHC class II cytoplasmic domains may modulate vaccine efficacy by aiding in the localization of MHC class II molecules to lipid rafts, and Ii expression may block vaccine efficacy by reducing constitutive localization of MHC class II molecules to plasma membrane lipid rafts.

**Abs and reagents**

As previously described, mAbs 10–2.16 (mouse anti-A<sup>a</sup>), and IN-1 (rat anti-Ii) were purified on protein A- or protein G-Sepharose (2). Anti-caveolin mAb, CD45 mAb, AF6-120.1-FITC (anti-A<sup>a</sup>), and 10-3-6-FITC mAb (anti-A<sup>b</sup>) were purchased from BD Biosciences (San Diego, CA). Anti-β tubulin Tub 2.1 mAb, saponin, and methyl-β-cyclodextrin (MCD) were purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin-coupled Sepharose, sheep anti-mouse HRP conjugate, and mouse anti-rat HRP conjugate Abs were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-mouse FITC and mouse anti-rat FITC Abs were purchased from ICN Pharmaceuticals (Irvine, CA). HRP-labeled cholera toxin-B was purchased from List Biological Laboratories (Campbell, CA). Sulfo-NHS-biotin was purchased from Pierce (Rockford, IL) and used according to manufacturer’s recommendations.

**Isolation of lipid rafts**

Approximately 1.5–2.5 × 10<sup>7</sup> M12.C3 cells were harvested and washed in excess PBS and lysed in 1 ml buffer A (150 mM NaCl, 2 mM Tris, pH 7.5, and 5 mM EDTA) containing 0.05% Triton X-100 for 30 min on ice. Nuclei and insoluble matter were removed from lysates by centrifugation at 12,000 × g for 5 min at 4°C. Cleared lysates were mixed with an equal volume of cold 85% sucrose in buffer A, layered on the bottom of 14 × 89 mm Beckman polyallomer centrifuge tubes, and overlaid with 6 ml of 35% sucrose followed by 3 ml of 5% sucrose to form an 11 ml gradient. Gradients were ultracentrifuged in a Beckman SW40Ti swinging bucket rotor at 200,000 g for 20 h at 4°C. One milliliter fractions were manually collected from the top of the gradient. Raft isolation from Sal cells was as described for M12.C3 cells except 1.5–3 × 10<sup>7</sup> cells were lysed in 0.5 ml of 0.25% Triton X-100 in buffer A and lipid rafts isolated in a 4.5 ml sucrose gradient using a Beckman SW55Ti swinging bucket rotor. One-half milliliter fractions were collected from the top of the gradient.

**Ab cross-linking, MCD treatment, and biotin-labeling of cell surface proteins**

In some experiments, cells were cross-linked with I-A<sup>a</sup> specific Abs, MCD treated, or biotinylated before cell lysis and lipid raft isolation. For MHC class II Ab-mediated cross-linking, cells were incubated with 10 µg/ml of 10-2.16 mAb and 10 µg/ml of goat anti-mouse IgG for 10 min at 37°C in PBS, followed by two washes with excess PBS. For MCD treatment, cells were washed with excess PBS, resuspended in 10 mM MCD in OptiMEM (Life Technologies) to a concentration of ~2 × 10<sup>7</sup> cells/ml, and incubated at 37°C for 10 min followed by two washes with excess PBS. Viability of cells after MCD treatment was >85%. For biotinylation of cell surface proteins, 1.5–3 × 10<sup>7</sup> cells were washed in excess PBS, resuspended in 0.5 mg/ml sulfo-NHS-biotin in PBS at a concentration of 1 × 10<sup>7</sup> cells/ml, incubated on ice for 30 min, and washed with excess PBS.

**Western Blot analysis, whole cell lysate, and SDS-stable dimer preparation**

Western blots were done as previously described (7) with the following modifications. Samples were run on 12% SDS-PAGE gels, blotted onto PVDF membranes (Amersham Pharmacia Biotech) using a Bio-Rad Mini Trans-Blot cell (100 V for 1 h), blocked with 3% nonfat dry milk (Safeway brand) in TBS-T or 2% BSA/TBS-T, probed, and developed using Pierce Super Signal (Rockford, IL). MHC class II bands were quantified by densitometry using a Bio-Rad Gel Doc 2000. Contents of lipid rafts were quantified using the following formula: % MHC class II in rafts = 100% × ([MHC class II in raft fractions]/(total MHC class II in all fractions)). For analysis of whole cell lysates, cells were washed in excess PBS, resuspended to a concentration of 1 × 10<sup>7</sup> cells/ml in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 and a protease inhibitor mixture (Roche, Mannheim, Germany)), and incubated for 45 min on ice. Lysates were centrifuged for 10 min at 4°C to pellet insoluble debris. Supernatants were stored at ~80°C until used. In some experiments, cells were treated with MCD before lysis. To analyze MHC class II heterodimers, whole cell lysates or fractions from sucrose gradients were mixed with SDS-sample buffer on ice and loaded onto SDS-PAGE gels without boiling of the sample. Compact stable dimers were detected as bands at ~55 kDa.

<table>
<thead>
<tr>
<th>Table I.</th>
<th>Ii expression inhibits MHC class II localization to cell surface lipid rafts in cell-based cancer vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line (clone)</td>
<td>Ii Expression</td>
</tr>
<tr>
<td>Sal/A&lt;sup&gt;a&lt;/sup&gt; (19.6.4)</td>
<td>No</td>
</tr>
<tr>
<td>Sal/A&lt;sup&gt;a&lt;/sup&gt;/DM</td>
<td>No</td>
</tr>
<tr>
<td>Sal/A&lt;sup&gt;a&lt;/sup&gt;/Ii (36.9)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sal/CITTA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are the average of two to three independent experiments.
Ab. The ratio of I-Ak to Ii was determined by dividing the mean channel fluorescence value for I-Ak by the mean channel fluorescence value for Ii.

Flow cytometry

Cells were stained by immunofluorescence and analyzed by flow cytometry for cell surface (I-Ak) and internal (Ii and I-Ak) molecules as previously described (2). Cell surface and intracellular I-Ak molecules were detected using the 10-3.6 mAb directly coupled to FITC. Intracellular Ii molecules were detected using the rat mAb IN-1 followed by a mouse-anti rat FITC. Intracellular Ii molecules of detergent-resistant membranes were isolated from the cellular fraction on discontinuous sucrose gradients at 4°C. To ascertain whether MCD treatment decreases MHC class II expression, treated and untreated M12.C3.F6 cells were stained by immunofluorescence, and IBs of boiled and nonboiled cell extracts were probed with the I-Ak-specific mAb. As shown in Fig. 1D, neither MHC class II expression levels nor the ratio of stable dimer to free a-chain changed with MCD treatment. Therefore, functional MHC class II molecules associate with cholesterol-containing lipid rafts in M12.C3.F6 cells.

Results

MHC class II molecules are constitutively present in lipid rafts of professional and nonprofessional APC

Previous reports have established that MHC class II molecules can be detected in lipid rafts isolated from several cell types, including professional APC (18–21, 24, 28). To determine whether MHC class II molecules of the MHC class II vaccines are also localized to lipid rafts, we have compared MHC class II molecules in lipid rafts with lipid rafts of the professional APC M12.C3.F6 (I-Ak-expressing murine B cell lymphoma) and of SaI/Ak vaccine cells. M12.C3.F6 and SaI/Ak cells were lysed in buffers containing 0.05% and 0.25% Triton X-100, respectively. These concentrations are the lowest levels of Triton X-100 that give separation of raft and nonraft markers for each cell line (see below). Further reduction in detergent levels resulted in detection of raft markers in detergent-resistant membranes (data not shown). Lipid rafts in the form of detergent-resistant membranes were isolated from the cellular lysates by flotation on discontinuous sucrose gradients at 4°C. Individual fractions from the sucrose gradients were run on SDS-PAGE gels and blotted onto nitrocellulose.

For M12.C3.F6 cells, supernatants were run on 11 ml sucrose gradients and 11 1-ml fractions were collected. Western blot analysis of the fractions revealed that the raft marker GM1 (29) localized to the 5–35% interface of the sucrose gradient, identifying fractions 3–5 as the lipid raft fractions (Fig. 1A), CD45 (data not shown) and c-tubulin (Fig. 1A), which are nonraft markers (29, 30), were found in fractions 9–11, identifying these fractions as containing nonraft membranes. To determine whether MHC class II molecules localize to lipid rafts, immunoblots (IBs) were probed with an I-Ak-specific mAb (Fig. 1B, top panel). In M12.C3.F6 cells, this Ab detects I-Ak as a doublet at ~30 kDa. As quantified by densitometry, ~20% of the total I-Ak molecules in M12.C3.F6 cells localized to the detergent resistant membranes. A hallmark of functional MHC class II molecules that demonstrates their proper conformation is their stability in SDS (31). To determine whether MHC class II molecules of M12.C3.F6 cells are properly conformed, proteins of nonboiled (NB) fractions were separated by SDS-PAGE and the resulting blots probed with the I-Ak-specific mAb (Fig. 1B, bottom panel). Compact SDS-resistant heterodimers were detected as bands of ~55 kDa in the raft fractions of M12.C3.F6 cells, indicating raft-associated MHC class II is properly conformed.

To further confirm the association of MHC class II with lipid rafts, M12.C3.F6 cells were depleted of cholesterol using MCD before raft isolation. Cholesterol is a key component in stabilizing raft-protein interactions and its removal de-stabilizes a protein’s ability to associate with rafts (32). Following a 10-min treatment with MCD, levels of MHC class II molecules in lipid rafts were reduced by ~50% (Fig. 1C). To ascertain whether MCD treatment de-stabilizes MHC class II aβ heterodimers or decreases MHC class II expression, treated and untreated M12.C3.F6 cells were stained by immunofluorescence, and IBs of boiled and nonboiled cell extracts were probed with the I-Ak-specific mAb. As shown in Fig. 1D, neither MHC class II expression levels nor the ratio of stable dimer to free a-chain changed with MCD treatment. Therefore, functional MHC class II molecules associate with cholesterol-containing lipid rafts in M12.C3.F6 cells.

Gradient fractions from M12.C3.F6 cells were also probed for the MHC class II accessory molecule Ii. As quantified by densitometry, <2% of the p31 isoform (Fig. 1E) and no detectable p41 isoform (data not shown) were detected in raft fractions. Since MHC class II is bound to Ii as it traffics from the ER to early

Table II. Truncation of the MHC class II cytoplasmic domains diminishes MHC class II-lipid raft interactions in cell-based vaccines

<table>
<thead>
<tr>
<th>Cell Line (clone)</th>
<th>MHC Class II Cytoplasmic Domain</th>
<th>Immunogenic Reference</th>
<th>Percentage of MHC Class II in Lipid Raft ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaI/Ak (19.6.4)</td>
<td>wt/wtβ</td>
<td>Yes</td>
<td>38 ± 11</td>
</tr>
<tr>
<td>SaI/Ak tr (8.7.1)</td>
<td>−12α/−12β</td>
<td>Yes</td>
<td>25 ± 12</td>
</tr>
<tr>
<td>SaI/Ak tr (12.11.1)</td>
<td>wt/−10β</td>
<td>Yes</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>SaI/Ak tr (6.11.8)</td>
<td>−12α/−10β</td>
<td>No</td>
<td>3 ± 6</td>
</tr>
<tr>
<td>SaI/Ak tr (6.11.5)</td>
<td>−12α/−10β</td>
<td>No</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are the mean ± SD of a minimum of three experiments.

Table III. Deletion of MHC class II cytoplasmic domains diminishes localization of MHC class II molecules to lipid rafts in M12.C3 B cell lymphomas

<table>
<thead>
<tr>
<th>Clone Designation</th>
<th>MHC Class II Cytoplasmic Domain</th>
<th>Reference</th>
<th>Percentage of MHC Class II in Lipid Raft ± SD</th>
<th>Percentage of Decrease of MHC Class II in Rafts Following MCD Treatment ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12.C3.F6</td>
<td>wt/wtβ</td>
<td>13</td>
<td>20 ± 7</td>
<td>60</td>
</tr>
<tr>
<td>M12.C3.9D4</td>
<td>wt/wtβ</td>
<td>13</td>
<td>23 ± 9</td>
<td>40</td>
</tr>
<tr>
<td>M12.C3.10B3</td>
<td>−12α/−12β</td>
<td>13</td>
<td>12 ± 8</td>
<td>66</td>
</tr>
<tr>
<td>M12.C3.5A2</td>
<td>wt/−12β</td>
<td>13</td>
<td>14 ± 9</td>
<td>49</td>
</tr>
<tr>
<td>M12.C3.5B2</td>
<td>−12α/−12β</td>
<td>13</td>
<td>6 ± 9</td>
<td>Not tested</td>
</tr>
<tr>
<td>M12.C3.5C2</td>
<td>−12α/−10β</td>
<td>This report</td>
<td>2 ± 4</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

* Values are the averages of a minimum of three independent experiments.

* Values are significantly different (p < 0.05) from the other clones tested.

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endosomes (reviewed in Ref. 33), this result suggests that the raft-associated MHC class II molecules are not transiting from the ER to endosomal compartments.

To determine whether MHC class II molecules associate with lipid rafts in tumor cell-based vaccines, we isolated lipid rafts from SaI/Ak vaccine cells. Lysates were run on 4.5 ml sucrose gradients and nine 0.5 ml fractions were collected. In SaI/Ak cells, the raft marker caveolin (24) was used to identify fractions 2–4 as lipid raft fractions, while /H9252-tubulin identified fractions 7–9 as soluble membrane (Fig. 2A). To determine the location of MHC class II molecules, IBs of SaI/Ak gradient fractions were probed with the I-Ak-specific mAb. As quantified by densitometry, 35% of I-Ak molecules were detected in lipid raft fractions 2–4 of SaI/Ak cells (Fig. 2B). To ascertain whether the MHC class II molecules of SaI/Ak cells are conformationally correct, lysates were not boiled before running on SDS-PAGE, and IBs were probed with the I-Ak-specific mAb. SDS-stable dimers were detected in lipid raft fractions (Fig. 2C), although the ratio of free-β-chains to stable dimers was much higher in raft fractions than in nonraft fractions. High m.w. forms of MHC class II were also present in lipid rafts (data not shown), in agreement with previous studies (24). Therefore, functional MHC class II molecules of SaI/Ak tumor cell vaccines localize to lipid rafts.

Expression of Ii blocks constitutive MHC class II localization to cell surface lipid rafts

Lipid rafts are present in plasma membranes as well as the membranes of intracellular organelles (reviewed in Ref. 34). To determine whether MHC class II molecules detected in detergent resistant membranes resided in plasma membrane rafts, M12.C3.F6 and SaI/Ak cells were labeled with sulfo-NHS biotin before raft isolation. This technique allows biotin labeling of cell surface proteins only. Following lysis in Triton X-100 and sucrose gradient isolation of rafts, lipid raft fractions and soluble membrane fractions were precipitated using streptavidin-coupled Sepharose beads. Precipitates were electrophoresed by SDS-PAGE, blotted, and probed with the I-Ak-specific mAb. As is shown in Fig. 3A, M12.C3.F6 cells had no detectable MHC class II molecules in cell surface lipid rafts. These findings suggest that the raft-associated MHC class II of M12.C3.F6 cells (see Fig. 1B) resides in lipid rafts of intracellular membranes. In contrast, 30% of the total surface MHC class II localized to rafts in SaI/Ak cells (Fig. 3A). Therefore, the MHC class II molecules of M12.C3.F6 B lymphoma cells appear to constitutively associate with rafts of intracellular membranes, whereas MHC class II molecules of the vaccines constitutively associate with plasma membrane lipid rafts.

Professional APC such as M12.C3.F6 cells express MHC class II-associated accessory molecules that are not expressed by non-professional APC such as SaI/Ak cells. These accessory molecules facilitate the presentation of exogenously synthesized Ags, and include molecules such as DM (H-2M) and Ii. Since M12.C3.F6 and SaI/Ak cells differ in their constitutive localization of MHC class II to cell surface lipid rafts and also differ in their content of Ii and H-2M, we have determined whether the expression of H2-M or Ii modulates the localization of MHC class II molecules to plasma membrane rafts. To address this issue, we used SaI/Ak cells

FIGURE 1. Functional MHC class II molecules of M12.C3.F6 cells associate with lipid rafts. M12.C3.F6 cells were lysed in buffer containing 0.05% Triton X-100 and lipid rafts were isolated by flotation on discontinuous sucrose gradients. Gradient fractions were analyzed by SDS-PAGE and Western analyses. A, IBs were stained for GM1 (raft fractions) or for β-tubulin (nonraft, soluble fractions). B, Before running on SDS-PAGE, lysates were boiled (B, upper panel) or not-boiled (NB, lower panel). IBs were probed with an I-Ak-specific mAb. C, Before lysis, M12.C3.F6 cells were treated with MCD to disrupt lipid rafts. IBs were probed for I-Ak. D, MCD treated and untreated cells were stained by indirect immunofluorescence for I-Ak and analyzed by flow cytometry. B and NB lysates were analyzed by SDS-PAGE and Western analysis for stable dimer formation. E, Gradient fractions were probed for Ii with a rat mAb.
expressing combinations of Ii and DM. Cell surface proteins were labeled with biotin before raft isolation, precipitated with streptavidin-Sepharose beads, and analyzed by Western blotting with the I-A\(^{\kappa}\)/H9252\(^{k}\)-specific mAb. As is shown in Fig. 3B, MHC class II-expressing Sal cells that coexpress Ii or Ii+I-H-2M do not have MHC class II molecules in cell surface raft fractions (Sal/A\(^{k}\)/Ii and Sal/CIITA, respectively). In contrast, MHC class II-expressing Sal cells that coexpress I-H-2M without Ii contain MHC class II in cell surface rafts (Sal/A\(^{k}\)/DM). Therefore, coexpression of Ii correlates with the absence of MHC class II molecules in plasma membrane lipid rafts.

To ascertain whether MHC class II molecules of Ii positive cells are present in rafts of intracellular organelles, raft and soluble fractions of whole cell lysates of Sal/A\(^{k}\)/Ii cells were analyzed by Western blots. As is shown in Fig. 3C, I-A\(^{k}\) molecules were detected in both raft and soluble membrane fractions. Ii was also detected in soluble, but not lipid raft fractions (Fig. 3C). Therefore, coexpression of Ii blocks the constitutive localization of MHC class II molecules to cell surface rafts, but not to rafts of intracellular organelles.

Proteins expressed by transfection can be over-expressed, giving rise to artificial results. To ascertain that neither I-A\(^{k}\) nor Ii are overexpressed relative to each other or to another cell line, I-A\(^{k}\) and Ii levels of Sal/A\(^{k}\)/Ii, Sal/CIITA, and M12.C3.F6 cells were compared. Cells were harvested, fixed, permeabilized, stained for Ii and I-A\(^{k}\), and analyzed by flow cytometry. All cell lines had similar levels of Ii expression (data not shown). Likewise, the ratio of I-A\(^{k}\) to Ii was similar for the three cell lines (1.08, 0.89, and 1.50 for M12.C3.F6, Sal/CIITA, and Sal/A\(^{k}\)/Ii, respectively.) Therefore, invariant chain expression levels and the relative ratios of MHC class II to Ii molecules are comparable for the three cell lines used in this study.

We also examined the effect of cross-linking of plasma membrane MHC class II molecules on MHC class II-lipid raft interactions. Others have reported that Ab cross-linking of MHC class II molecules causes translocation of class II from the soluble membrane into lipid rafts to induce MHC class II mediated signaling (22–24). M12.C3.F6, Sal/A\(^{k}\), and Sal/CIITA cells were labeled with biotin and then subjected to Ab cross-linking with the I-A\(^{k}\)-specific mAb before raft isolation, precipitation with streptavidin-Sepharose beads, and immunoblotting for MHC class II. As shown in Fig. 3D, ~30–50% of the cell surface MHC class II molecules of M12.C3.F6 and Sal/CIITA cells reside in lipid rafts following Ab cross-linking. Cross-linking of MHC class II molecules of Sal/A\(^{k}\) cells also increases the levels of MHC class II localized to
plasma membrane rafts. Therefore, plasma membrane I-Ak molecules are in lipid rafts following Ab cross-linking, regardless of expression of Ii.

**MHC class II cytoplasmic domains aid in localization to lipid rafts**

Because Sal/Ak transfectants with truncated α- and β-chains are ineffective vaccines and are highly tumorigenic (8), we have studied the effects of cytoplasmic domain deletion on MHC class II lipid raft interactions. Lipid rafts were isolated from M12.C3 and Sal cells expressing full-length MHC class II molecules or with truncations of one or both chains. Fig. 4 and Tables II and III show the results of these experiments. M12.C3 clones with full-length MHC class II chains (clones 9D4 and F6), had 23 ± 9% and 20 ± 7% of their MHC class II localized to lipid rafts, respectively. M12.C3 clones with deletions of the cytoplasmic portion of the α-chain, (clone 10B3), or the β-chain, (clone 5A2), had less MHC class II localized to lipid rafts (12 ± 8% and 14 ± 9%, respectively). Truncation of both chains (clones 5C2 and 5B2) further reduced the percentage of MHC class II constitutively localized to rafts (2 ± 4 and 6 ± 9%, respectively). To confirm the raft-association of the truncated MHC class II molecules, the various M12.C3 cell lines were MCD treated before analysis. Following MCD treatment, raft localization of MHC class II molecules was reduced by 40–66% (Table III). Therefore, truncation of either the α- or β-chain of MHC class II molecules of M12.C3 cells reduces MHC class II molecule association with lipid rafts, and concurrent truncation of both chains further reduces MHC class II association with rafts.

To ascertain whether cytoplasmic domain truncations similarly affected MHC class II localization in the vaccine cells, we analyzed the distribution of truncated MHC class II molecules in Sal-derived cells. Lipid rafts were isolated from Sal cells with full-length class II molecules, truncated α- or β-chains, or truncated α- and β-chains. The results of these experiments are shown in Fig. 4B and Table II. Sal/Ak cells with two full-length chains have 38 ± 11% of their MHC class II in lipid rafts. Similar to M12.C3 cells, truncation of either the α- or β-chain in Sal cells reduced the levels of raft-associated MHC class II (25 ± 12% and 26 ± 9%, respectively for Sal/Aαtr and Sal/Aβtr). Concurrent truncation of both α- and β-chains reduced MHC class II levels to 0–3%. Collectively, these data demonstrate that MHC class II cytoplasmic domains are essential for lipid raft localization of I-Ak molecules in both professional APC and Sal/Ak vaccine cells.

**Discussion**

MHC class II+ tumor cell-based cancer vaccines were designed to facilitate presentation of endogenously encoded tumor Ag peptides (3). In vivo and in vitro studies confirmed the hypothesis that the vaccine cells directly present Ag to tumor-reactive CD4+ T cells, thereby activating them, and enhancing tumor immunity against wild-type MHC class II negative tumor cells (2, 6, 7). In previous studies we have demonstrated that coexpression of Ii in the Sal/Ak vaccine cells restores tumorigenicity and blocks the vaccine effect (2, 9). Our original interpretation of this result was that coexpression of Ii inhibits Ag presentation by blocking binding of endogenously synthesized tumor Ag peptides, thereby inhibiting activation of tumor-reactive CD4+ T cells. This hypothesis is consistent with the known function of Ii as an inhibitor of MHC class II/peptide binding in the ER (reviewed in Ref. 17). It is also consistent with the function of Ii as a chaperone that directs newly synthesized MHC class II molecules to the endosomal pathway where MHC class II molecules bind exogenously synthesized peptides (reviewed in Ref. 33). The findings reported here demonstrating that Ii inhibits constitutive association of MHC class II molecules with cell surface lipid rafts, suggest that Ii may also inhibit vaccine efficacy by preventing constitutive localization of MHC class II molecules in cell surface lipid rafts. If localization of MHC class II molecules into plasma membrane lipid rafts is required for CD4+ T cell activation as proposed by others (18, 19), then Sal/Ak/Ii and Sal/CIIA cells may be poor APC for tumor Ags and ineffective vaccines because their MHC class II molecules are not constitutively present in cell surface lipid rafts.

In earlier studies we reported on the efficacy of cell-based vaccines with truncated MHC class II α- and/or β-chains. These studies demonstrated that vaccines with concurrent α- and β-chain truncations were ineffective, while vaccines with one wild type chain and one truncated chain retained some vaccine efficacy (8, 25). We concluded from these studies that vaccine cell Ag presentation was diminished because of inadequate signaling to upregulate costimulatory molecules and/or incorrect intracellular trafficking of truncated class II molecules. Both of these conclusions were supported by other studies demonstrating a signaling or trafficking role for the MHC class II cytoplasmic region (10–15). However, the present data demonstrating the absence of constitutively localized MHC class II molecules in lipid rafts of double

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**FIGURE 4.** Truncation of MHC class II cytoplasmic domains of M12.C3.F6 and Sal/Ak cells minimizes localization to lipid rafts. Sucrose gradient fractions of whole cell lysates of M12.C3 cells (A) and Sal-derived vaccine cells (B), expressing wild-type or truncated MHC class II α- and/or β-chains were analyzed by SDS-PAGE and Western blot for I-Ak. Levels of MHC class II in raft fractions are quantified in Tables II and III.

TABLE II. Localization of truncated MHC class II molecules in lipid rafts

<table>
<thead>
<tr>
<th>Clone</th>
<th>Fraction</th>
<th>Raft (%)</th>
<th>Soluble (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9D4</td>
<td>1</td>
<td>32 ± 6</td>
<td>68 ± 6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19 ± 6</td>
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<tr>
<td></td>
<td>3</td>
<td>16 ± 6</td>
<td>84 ± 6</td>
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<tr>
<td></td>
<td>4</td>
<td>10 ± 6</td>
<td>90 ± 6</td>
</tr>
</tbody>
</table>

**TABLE III. Localization of truncated MHC class II molecules in lipid rafts**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Fraction</th>
<th>Raft (%)</th>
<th>Soluble (%)</th>
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<tbody>
<tr>
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truncated cells, provides yet another possible mechanism for reduced immunogenicity of these cells.

Tables I and II summarize previous vaccine efficacy studies and show the status of MHC class II – lipid raft associations for each vaccine. Note the striking correlation between constitutive localization of MHC class II molecules in lipid rafts and vaccine cell immunogenicity/tumorigenicity. These findings strongly suggest that Ii and MHC class II cytoplasmic domains may regulate MHC class II-mediated Ag presentation by several mechanisms, one of which is constitutive localization of MHC class II molecules to lipid rafts.

Bouillon et al. (21) reported that only a small fraction of MHC class II molecules of HLA-DR-transfected HeLa cells was constitutively present in lipid rafts (4.4%) and that deletion of the MHC class II cytoplasmic domains reduced the level to 2.5%. Although this apparent 43% reduction agrees with our results reported here, these authors concluded that truncation had no effect, presumably because their quantification did not yield statistically significant differences.

Although the precise structural requirements for localizing integral membrane proteins to lipid rafts are unknown, there are several hypotheses. Posttranslational modifications of proteins by GPI linkages or saturated fatty acid acylation can mediate raft association (35). Other data suggest that the structure of a protein’s transmembrane domain impacts raft localization (36, 37). Cytoplasmic domains could be responsible for either of these mechanisms. For example, the cytoplasmic domain of influenza hemagglutinin protein contains palmitoylated cysteine residues that are required for raft localization (35, 38). In contrast, the cytoplasmic domain of influenza neuraminidase protein does not contain sites for posttranslational modifications, but it is required for raft localization and is thought to mediate its effect by altering the structure of the influenza neuraminidase transmembrane region (37, 38). Because MHC class II molecules do not contain sites for lipid raft-favorable posttranslational modifications, the class II cytoplasmic domains may affect raft association by altering the class II transmembrane region.

There are many known functions of Ii; however, its role in localization of proteins to lipid rafts has not been previously established. This Ii-mediated reduction in MHC class II association with cell surface lipid rafts may not have been observed by other investigators because they have only surveyed professional APC that constitutively express both MHC class II and Ii. The data of Anderson et al. (18) demonstrating class II in cell surface rafts of B cells appear to contradict our findings. However, these authors did not test Ii-negative B cells, so one cannot evaluate the role of Ii in class II localization to cell surface rafts in their experiments. Indeed, other studies using Ii positive cells show very low levels of MHC class II molecules in lipid rafts (21, 23, 28), consistent with our findings that coexpression of Ii minimizes raft-localized MHC class II.

The finding that Ii prevents class II from associating with plasma membrane lipids rafts has several implications for the role of Ii in intracellular trafficking of MHC class II molecules. Extensive studies have demonstrated that in Ii+ cells newly synthesized MHC class II molecules immediately associate with Ii in the endoplasmic reticulum and traffic via the Golgi to endosomes and the MHC class II compartment (MIC). In the MIC, Ii is degraded, and the remaining CLIP peptide is replaced with nominal peptide (reviewed in Ref. 33). Because Ii itself is not in lipid rafts, it is likely that MHC class II molecules that are tethered to Ii are also not in rafts, and that Ii mediates its inhibitory affect by direct binding to MHC class II molecules. Therefore, newly synthesized MHC class II molecules that are associated with Ii are probably not raft-associated. In contrast, class II molecules of endosomal compartments in which Ii has been degraded may be raft-associated, and are likely to be the class II molecules that are detected in the raft fractions of whole cell lysates of Ii+ cells. MHC class II molecules may also associate with lipid rafts of recycling endosomes, as these compartments have been shown to have lipid raft components (39, 40). Because these raft-associated MHC class II molecules are free of Ii, it is not clear why they do not remain in lipid rafts as they traffic from the MIC to the plasma membrane. Perhaps there are sufficient Ii-MHC class II complexes in the plasma membrane to inhibit MHC class II localization to lipid rafts, or alternatively, there may be conformational changes in the membranes during this period that prevent MHC class II molecules from remaining in rafts.

In contrast, newly synthesized MHC class II molecules of Ii− cells may enter lipid rafts of the Golgi, trans-Golgi network, and endosomal membranes because Ii is not present to inhibit the association. In our cell-based tumor vaccines, previous data have established that in the absence of Ii, MHC class II molecules traffic via the endosomal pathway (5). Therefore, as MHC class II molecules of Ii− cells traffic through the secretory pathway, they remain localized to lipid rafts. Although the overall trafficking pattern in terms of raft localization is unclear at present, coexpression of Ii effectively prevents association of MHC class II molecules with cell surface lipid rafts.

The mechanism by which constitutive localization of MHC class II molecules to lipid rafts enhances vaccine efficacy is unclear. Although cross-linking of MHC class II molecules initiates lipid-raft dependent signaling cascades (21–24), resting SaI/Aκ cells do not constitutively signal (24), despite our observation that MHC class II molecules constitutively associate with cell surface lipid rafts. Perhaps constitutive localization to rafts allows for more rapid recruitment of additional MHC class II molecules to rafts, or the increased stability of raft/MHC II complexes once CD4+ T cells and vaccine cells interact. Association with cell surface lipid rafts may also be a prerequisite for signal initiation, as both full-length and double-truncated MHC class II molecules localize to rafts following Ab cross-linking; however, only full-length MHC class II molecules mediate lipid raft-dependant signals in vaccine cells (24).

Regardless of the underlying mechanism, there appears to be a strong relationship between lipid raft localization of MHC class II molecules and increased immunogenicity of tumor cell-based vaccines. If constitutive MHC class II-lipid raft associations are responsible for these effects, then further polarization of MHC class II into rafts may yield more potent vaccines.

**Note added in proof.** Experiments with I-Aα4-transfected 4T1 mammary carcinoma cells demonstrate the coexpression of Ii also reduces the amount of MHC class II in cell-surface lipid raft fractions relative to Ii-negative 4T1 cells.

**Acknowledgments**

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**References**


