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Docosahexaenoic acid-containing phosphatidylcholine affects the binding of monoclonal antibodies to purified K^b reconstituted into liposomes

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Abstract

Class I major histocompatibility complex (MHC I) molecules are transmembrane proteins that bind and present peptides to T-cell antigen receptors. The role of membrane lipids in controlling MHC I structure and function is not understood, although membrane lipid composition influences cell surface expression of MHC I. We reconstituted liposomes with purified MHC I (K^b) and probed the effect of lipid composition on MHC I structure (monoclonal anti-MHC I antibody binding). Four phospholipids were compared; each had a phosphocholine head group, stearic acid in the *sn*-1 position, and either oleic, α-linolenic, arachidonic, or docosahexaenoic acid (DHA) in the *sn*-2 position. The greatest binding of monoclonal antibody AF6-88.5, which detects a conformationally sensitive epitope in the extracellular region of the MHC I α-chain, was achieved with DHA-containing proteoliposomes. Other epitopes (CTKb, 5041.16.1) showed some sensitivity to lipid composition. The addition of β2-microglobulin, which associates non-covalently with the α-chain and prevents α-chain aggregation, did not equalize antibody binding to proteoliposomes of different lipid composition, suggesting that free α-chain aggregation was not responsible for disparate antibody binding. Thus, DHA-containing membrane lipids may facilitate conformational change in the extracellular domains of the α-chain, thereby modulating MHC I function through effects on that protein's structure. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Docosahexaenoic acid; Omega-3 fatty acid; Liposome; Major histocompatibility complex class I antigen

1. Introduction

Class I major histocompatibility complex (MHC I) molecules are membrane glycoproteins found on the surface of all nucleated cells. Their principal function is to present antigen peptides to T-lymphocytes, a task that is accomplished when a heterotrimer composed of the ~45-kDa MHC I α-chain, a non-covalently associated 12-kDa β2-microglobulin (β2m) protein, and peptide interacts with the antigen receptor (TCR) on T-cells (Fig. 1). If the interaction is sufficiently avid, the antigen-specific T-cell becomes activated. Our current understanding of the molecular interaction between the TCR and MHC I trimer is that the peptide provides one set of contacts with the TCR, and that several critical amino acid residues of the MHC I α-chain also contact the TCR [1], and hence the conformation of the α-chain is

Abbreviations: ATCC, American Type Culture Collection; β2m, β_2-microglobulin; DHA, docosahexaenoic acid; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; MHC I, major histocompatibility complex class I; PBS, phosphate-buffered saline; PC, phosphatidylcholine; TCR, T-cell antigen receptor

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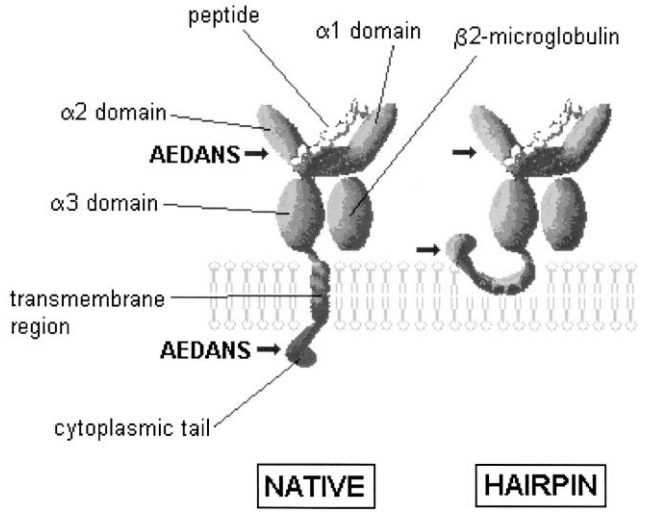

Fig. 1. Cartoons of MHCI in the native and hairpin configurations. The α-chain consists of the α1-, α2-, and α3-domains, transmembrane region, and cytoplasmic tail. The intact heterotrimeric MHCI molecule includes, in addition to the α-chain, the β2m-protein, and a peptide bound in the groove formed by the α1- and α2-domains. In the hairpin configuration, the transmembrane region fails to span the bilayer, i.e. remains in the outer leaflet, causing the cytoplasmic tail to emerge on the 'extracellular' side of the bilayer. The locations of the two free sulfhydryl groups (cysteines) available for IAEDANS labeling are indicated by arrows.

thought to contribute substantially to TCR interaction [2].

MHC I functions naturally while spanning the bilayer of the plasma membrane, and thus it is important to ask whether membrane lipids may control MHC I structure and thereby fine tune T-cell activation. Recently, three-dimensional structure was determined for membrane-bound murine MHC I, i.e. soluble MHC I whose transmembrane and cytoplasmic regions were replaced by a histidine tag to anchor the molecule in a monolayer of nickel-chelating lipids [3]. The extracellular domains of MHC I appear positioned such that β2m contacts the lipid layer and the peptide binding groove is directed away from the lipid surface toward the TCR. However, little else is known about the relationship between membrane lipids and MHC I structure and function. We have directed our efforts to exploring how the omega-3 fatty acid docosahexaenoic acid (DHA, 22:6ω3), an important dietary component that becomes incorporated into membrane phospholipids, may control MHC I properties.

Lateral mobility, clustering, internalization, and protein conformation are critical properties of MHC I molecules to examine for lipid modulation. Lateral mobility of MHC I transmembrane proteins is limited by the proteins' interactions with cytoskeletal components, rather than by membrane lipids as is the case for a few proteins, such as visual rhodopsin [4]. Similarly, clustering of MHC I may not be driven directly by membrane lipid composition. That is, self aggregates of MHC I appear to result from loss or lack of β2m [5–9] and do not correlate with lipid domains detectable in plasma membranes [6]. Aggregation may serve to promote internalization of free α-chains [8,10], thereby removing conformationally altered MHC I from the cell surface. Membrane protein conformation, on the other hand, is influenced by membrane lipids. For example, membrane lipids alter the apparent p*K* for the conformational equilibrium of metarhodopsin I↔metarhodopsin II [11]; DHA-containing phospholipids favor this conformational change purportedly by inducing negative curvature stress in membranes [12]. In the case of calcium ATPase, conformational changes associated with catalytic activity are affected by alterations in membrane phospholipid composition [13], and several indicators of α-fetoprotein conformation (number of estradiol binding sites, estradiol equilibrium dissociation constant, UV absorption spectrum, and monoclonal antibody reactivity) varied with the concentration of free DHA [14]. Albeit limited, there do exist provocative data implicating the membrane and membrane lipids in controlling MHC I conformation. Bene et al. [15] used intramolecular fluorescence resonance energy transfer (FRET) to detect a conformational change in MHC I induced by depolarization of JY (B-lymphoblastoid) cells, and proposed that physical changes in membrane lipids may mediate, directly or indirectly, MHC I's conformational change. Binding of monoclonal antibodies to MHC I on normal mouse lymphocytes is decreased by treatment of the cells with cholesterol hemisuccinate (which increases lipid packing), and increased by a PC preparation (which decreases lipid packing) [16]. Similarly, cholesterol enrichment augments the self-aggregation and reduces the expression of human MHC I on JY cells, whereas cholesterol depletion, which fluidizes the membrane, enhances MHC I expression [17].

We have incorporated DHA, esterified to PC, directly into the plasma membrane of viable cells and detected altered surface MHC I expression as monitored by monoclonal antibody binding and recognition by cytotoxic T-lymphocytes [18]. DHA is the longest (22 carbons) and most unsaturated (six double bonds) fatty acid found in abundance in biological systems, and there is substantial evidence to suggest that DHA-containing phospholipids dramatically alter membrane properties, such as domain formation, fusion, and permeability [19–21]. That we found DHA to differentially affect two distinct MHC I epitopes, one epitope increased and one decreased as the membrane's DHA content rose [18], indicated not a global loss of this membrane protein, but rather a DHA-induced change in MHC I conformation or spatial distribution (steric hindrance). To demonstrate that DHA-containing phospholipids directly alter a physical property of MHC I (that monitored by binding of monoclonal antibodies), we have isolated murine MHC I protein, reconstituted liposomes of various phospholipid compositions, and with flow cytometry analyzed MHC I epitope accessibility in different lipid environments. Our results provide direct evidence that DHA-containing phospholipids present in the milieu of this membrane protein affect some physical property, most likely conformation, in turn altering the binding of monoclonal antibodies to epitopes in extracellular protein domains. We predict that DHA-induced changes may ultimately affect MHC I interactions with peptide and TCR, and thereby modulate the activation of T-cells and the outcome of immune responses.

2. Materials and methods

2.1. Cells

The murine T-cell lymphoma EL4 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in serum-free medium HyQ CCM-1 (HyClone Labs, Logan, UT) supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml, and 2 mM glutamine. These culture conditions preserved endogenous murine β2m, i.e. circumvented exchange with serum bovine β2m. Cultures were terminated when the cell density reached $\sim 8\times 10^5$ cells/ml and viability was still ≥90%. Harvested cells were washed twice in cold phosphate-buffered saline (PBS) and stored as a pellet at −80°C.

2.2. Monoclonal antibodies

The hybridoma M1/42.3.9.8 (M1/42; anti-mouse MHC I, all haplotypes) was purchased from ATCC and grown in HyQ CCM-1 medium to confluency. IgG was isolated from culture supernatant by affinity chromatography on Protein G–Sepharose (Sigma, St. Louis, MO). Affinity-purified M1/42 antibodies were covalently linked to CNBr-activated Sepharose 4B (2.2 mg protein/ml Sepharose) as recommended by the manufacturer (Amersham Pharmacia Biotech, Pistcataway, NJ). The hybridoma 28-14-8S (anti-L^dD^b), also purchased from ATCC, was grown in RPMI 1640 medium supplemented with antibiotics, 2 mM glutamine, 25 mM HEPES buffer, and 10% bovine calf serum; IgG was isolated from culture supernatant by affinity chromatography on goat anti-mouse IgG-Sepharose (Cappel/Organon Teknika, West Chester, PA). Fluorophore-conjugated monoclonal antibody AF6-88.5 (anti-K^b) was purchased from PharMingen (San Diego, CA), and fluorescein (FITC)-labeled CTK^b (anti-K^b) and 5041.16.1 (anti-K^bD^b) were obtained from Cedarlane (Accurate, Westbury, NY). FITC-goat anti-mouse IgG was purchased from Kirkegaard and Perry (Gaithersburg, MD).

2.3. Immunoaffinity isolation of MHC I

Frozen EL4 cell pellets were thawed in a 37°C water bath and resuspended in cold PBS to which an equal volume of concentrated lysis buffer was added such that the final suspension equaled 5×10^7 cells/ml of 0.5% NP40, 20 mM Tris, pH 8.0, 140 mM NaCl, and 1.5 mM phenylmethanesulfonyl fluoride. After incubation on ice for 20–25 min, the lysate was centrifuged for 15 min at 4°C at $2400\times g_{max}$, and that supernatant centrifuged for 45 min at 4°C at $165\,000\times g_{max}$. The final supernatant was diluted five-fold with ice-cold water to reduce the detergent concentration, and supplemented to achieve 1.5 mM phenylmethanesulfonyl fluoride. The lysate was loaded onto a 10-ml column of non-

immune IgG2a (Sigma, St. Louis, MO) coupled to Sepharose 4B, and the unbound flow through was channeled directly to the 1-ml M1/42 affinity column at a flow rate of 30 ml/h. The affinity column was washed with 20 column volumes of pre-elution buffer (0.5% deoxycholate, 140 mM NaCl, 15 mM Tris, pH 8.0) and bound MHC I was eluted with 20 column volumes of elution buffer (21 mM *n*-octyl-α-D-glucopyranoside, 20 mM $NaHCO_3$, pH 11.5) at 4°C at 0.5–1.0 ml/min. One-milliliter fractions of both the pre-elution wash and eluant were buffered with 100 μl of 1 M Tris, pH 8.0, and saved for protein and PAGE analyses. Protein analysis was conducted with a BCA protein assay kit (Pierce, Rockford, IL) as described by the manufacturer. Protein in samples for SDS–PAGE was precipitated with cold 12.5% trichloroacetic acid and pelleted by centrifugation at $7800 \times g_{max}$ for 15 min at room temperature. The pellets were washed twice with ice-cold acetone to remove residual detergent, dissolved in sample buffer (1–4 μg protein/20 μl buffer), heated to 100°C for 5 min, and electrophoresed by the method of Laemmli [22]. The gels were stained either with 0.3% w/v Coomassie brilliant blue R-250 or with a silver stain kit (Boehringer Mannheim Biochemicals/Roche, Indianapolis, IN).

2.4. Construction of MHC I proteoliposomes

The following phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL): 1-stearoyl, 2-oleoyl-phosphatidylcholine (18:0, 18:1 PC), 1-stearoyl, 2-α-linolenoylphosphatidylcholine (18:0, 18:3 PC), 1-stearoyl, 2-arachidonoylphosphatidylcholine (18:0, 20:4 PC), 1-stearoyl, 2-docosahexaenoylphosphatidylcholine (18:0, 22:6 PC), and egg PC. Solvent was evaporated from the lipids under a stream of nitrogen followed by vacuum pumping, and the lipids were rehydrated to 1 mg/ml of 20 mM $NaHCO_3$, pH 9, for 10 min at room temperature with frequent vortexing. The lipids were then extruded ≥10 times through a 2-μm Nucleopore membrane (Nucleopore Filtration Products, Pleasanton, CA) using a syringe-type extruder (Avestin, Ottawa, Canada). Purified MHC I (2 μg) was added to 20 μg of 2-μm liposomes and 13 mM *n*-octyl-α-D-glucopyranoside (final concentration) in a total volume of 300 μl. The *n*-octyl-α-D-glucopyranoside concentration corresponds to that required to saturate, but not solubilize, the liposomes, and was determined empirically as described by Rigaud et al. [23]. After 1 min at room temperature, complete and rapid detergent removal was achieved with 80 mg of degassed SM-2 Bio-Beads/ml (Bio-Rad, Hercules, CA) for 3 h with agitation in the dark at room temperature followed by a 1-h incubation with a fresh aliquot of Bio-Beads [23].

2.5. Flow cytometry

Proteoliposomes (2 μg of protein in 300 μl total volume) were incubated with 1 μg of fluorescently labeled anti-MHC I monoclonal antibodies for 30 min at room temperature in the dark. Cytometry was accomplished with a Coulter Epics Elite ESP cell sorter equipped with an argon laser. Antibody binding to liposomes, i.e. fluorescence, is described in two ways: the mean and median fluorescence intensity channels (range, 0–1023), and the percent of liposomes having a given fluorescence intensity. In the latter case, fluorescence intensities are divided into four levels corresponding to the four decades (sets of 256 channels) of the log scale on a 1024-channel photomultiplier tube. The gating of forward versus side scatter histograms, to select comparable proteoliposomes for analysis, is shown in the figures. Expo 2.0 software (Beckman Coulter, Fullerton, CA) was used for data analysis and exportation for the creation of graphics.

2.6. Assessment of native vs. hairpin configuration

The fluorescent sulfhydryl probe 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS, Molecular Probes, Eugene, OR), was used to label the two free sulfhydryl groups (Cys-142, Cys-358) present on the MHC I α-chain [24]. IAEDANS (4.3 mg) was dissolved in 1 ml of dimethylsulfoxide, added to 2 μg of purified MHC I, and mixed gently for 1 h at room temperature in the dark. An excess (105 μl) of 2-mercaptoethanol was added to the sample to consume unbound IAEDANS and the sample was dialyzed for 24 h against 1.38 mM *n*-octyl-α-D-glucopyranoside, 20 mM $NaHCO_3$, pH 9 (three changes) in a 100–500 μl Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). The labeled MHC I was then inserted into the

preformed 2-μm liposomes as described above for the construction of proteoliposomes. Proteoliposomes were diluted and placed into an LS50B fluorimeter (Perkin Elmer, Norwalk, CT) and excited at 350 nm; fluorescence emission was monitored from 360 to 600 nm, and then $CoCl_2$ (29–50 mM final concentration) was added to quench [Cys-142]-AEDANS fluorescence from the external surface of the proteoliposomes. Finally, 0.3% Triton X-100 (final concentration) was added to solubilize all proteoliposomes and allow quenching of [Cys-358]-AEDANS in the liposomes' interior.

2.7. Human β2m addition

MHC I proteoliposomes (2 μg protein in 300 μl total volume) were incubated with 30 μl of 0.22-μm filtered human serum (Sigma) or 10 μl of a 0.1 mg/ml solution of purified human β2m (Sigma) for 1 h at room temperature. After incubation, 1 μg of FITC-labeled monoclonal anti-K^b (clone AF6-88.5) was added, and the proteoliposomes were incubated for 30 min at room temperature in the dark and then analyzed by flow cytometry.

2.8. Indirect staining of cells and proteoliposomes for flow cytometry

To assess whether D^b was present in the MHC I protein used for proteoliposome reconstitution, 1 μg of anti-D^b (28-14-8S), purified from hybridoma culture supernatant as described previously in Section 2, was incubated for 30 min at room temperature with proteoliposomes containing 2 μg of MHC I protein and with 1×10^6 live EL4 cells (the original cell source of the MHC I). A secondary FITC-labeled anti-mouse IgG antibody was added in 5-fold molar excess, and after an additional 30-min incubation at room temperature, the cells and proteoliposomes were analyzed for fluorescence by flow cytometry.

2.9. Statistical methods

The Wilcoxon paired sample test was used to assess significant differences between antibody binding to proteoliposomes of different phospholipid composition. A non-parametric test was necessary because proteoliposome fluorescence intensities were not normally distributed. To accommodate day-to-day variations introduced with different MHC I purification lots, proteoliposome preparation, and instrument performance, the statistical method is a paired sample test. The software package used was WinStar (Anderson Bell, Arvada, CO), and $P \leq 0.05$ was accepted as statistically significant.

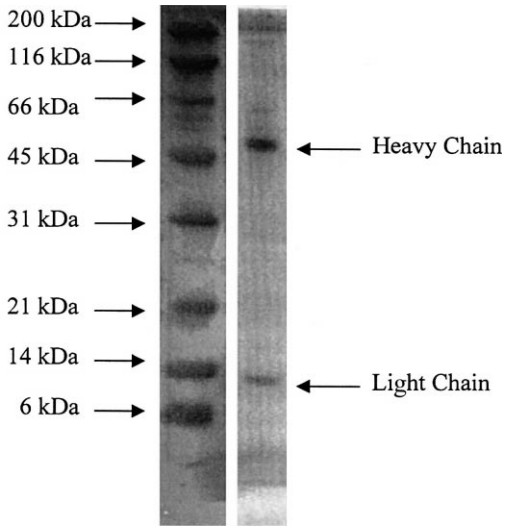

Fig. 2. Electrophoretic properties of immunoaffinity purified MHC I. MHC I purified by affinity chromatography was electrophoresed under denaturing conditions in a 14% polyacrylamide (resolving) gel, and the gel was stained with Coomassie brilliant blue R-250. The left lane provides the molecular weight markers and the right lane shows the trichloroacetic acid-precipitable protein eluted from the M1/42 affinity column.

3. Results and discussion

3.1. Immunoaffinity purified MHC I is incorporated into large unilamellar vesicles in a native conformation

MHC I isolated from EL4 (H-2^b) cells by affinity chromatography on M1/42-Sepharose consisted, under denaturing conditions, of a heavy chain (i.e. α-chain) with an apparent molecular weight ~50 kDa and a 12-kDa light chain (β2m) (Fig. 2). Densitometry of the Coomassie blue-stained gel indicated the relative densities of these two bands to be 1.3–2.5 (α-chain/β2m), which varied with the purification lot and elution fraction. This relative recovery of the

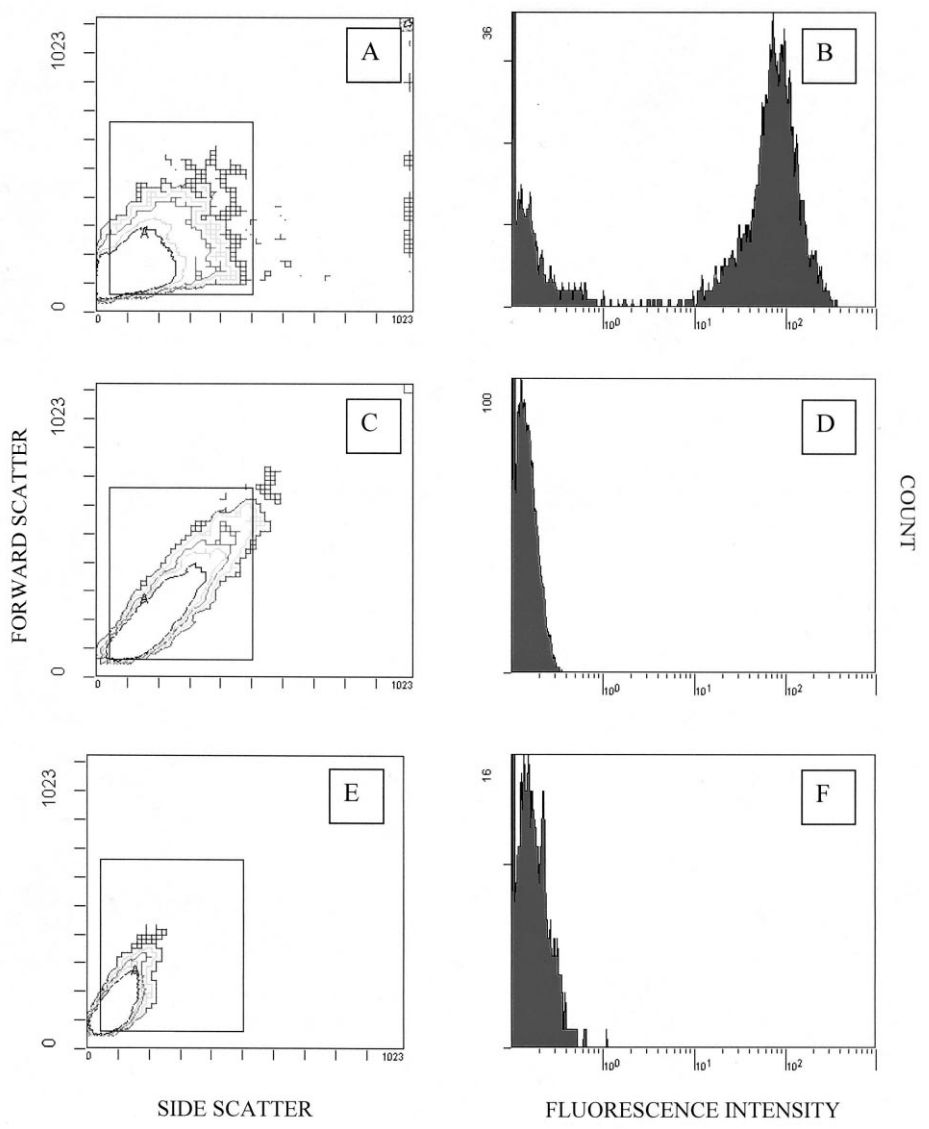

Fig. 3. MHC I reconstituted into proteoliposomes displays specific staining. Egg PC liposomes were reconstituted with MHC I and stained with FITC-labeled AF6-88.5 (anti-K^b) (A, B) or with FITC-labeled anti-Thy-1 (an irrelevant antibody) (E,F). In C and D, egg PC liposomes lacking MHC I were stained with FITC-labeled AF6-88.5. A, C, and E display the forward (*y*-axis) and side (*x*-axis) scatter characteristics of the liposomes and illustrate the gating (box) for the corresponding fluorescence histograms in B, D, and F (*x*-axis, fluorescence channel; *y*-axis, number).

two chains is similar to that presented in the illustrations of Stallcup et al. [25], whose MHC I purification procedure is the model upon which our immunoaffinity method is based. Contaminating bands constituted 4.5–6.6% of the total mass detected densitometrically, and thus were unlikely to interfere significantly with proteoliposome reconstitution. The incorporation of protein into liposomes was highly efficient (80% of added protein was incorporated) and equivalent for different liposome preparations (Table 1). Equivalent protein incorporation is important (it is assumed in subsequent assays), but not surprising; saturation of the bilayers with detergent prior to MHC I addition obviates the influence of the phospholipids on protein insertion.

Fig. 3 demonstrates that fluorescent monoclonal antibodies directed against K^b will intensely stain liposomes reconstituted with affinity purified MHC I (Fig. 3B), whereas liposomes lacking MHC I are not stained (Fig. 3D), nor are MHC I-reconstituted

proteoliposomes treated with FITC-anti-Thy-1, an irrelevant antibody (Fig. 3F). The corresponding plots of liposome size versus complexity (forward scatter versus side scatter) are provided in Fig. 3A, C and E; variations in size (forward scatter) and trapped internal membranes (side scatter) were likely introduced during initial liposome formation as well as subsequent manipulations to insert protein. The gates shown in Fig. 3A, C, and E are those assigned to the fluorescence histograms (Fig. 3B,D,F). Although different numbers of liposomes were counted in the three samples (ranging from 2187 to 9641 events), it is clear that intense staining is only achieved for MHC I-reconstituted proteoliposomes treated with fluorescent anti-MHC I antibodies.

The reconstitution method, insertion of protein into pre-formed detergent-saturated liposomes followed by rapid detergent removal with Bio-Beads, was chosen to maximize MHC I rightside-out orientation and non-hairpin conformation. Earlier reconstitution methods utilized extensive dialysis starting with mixed micelles of protein and lipid, and resulted in proteoliposomes with heterogeneous protein–lipid ratios and orientations [23] and a putative hairpin conformation for K^k [24]. In contrast, when preformed liposomes and rapid detergent removal were used, the protein–lipid ratios were more homogeneous and the protein orientation was highly asymmetrical, with the protein hydrophobic domain inserted first into the membrane (i.e. 85–95% of protein molecules inserted rightside-out) [23]. Although we cannot completely exclude the possibility that some MHC I is oriented inside-out, this possibility seems remote. Furthermore, antibodies, which are large (>150 kDa) globular proteins, do not penetrate the liposomes to stain MHC I and therefore MHC I with an inside-out orientation would not be analyzed by flow cytometry.

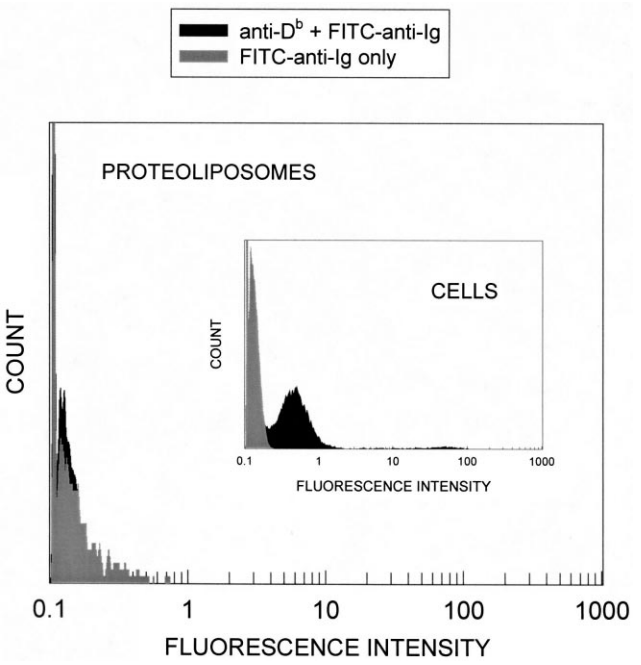

Fig. 4. D^b, predicted to be lost during affinity chromatography, is not detected in proteoliposomes. Proteoliposomes reconstituted with immunoaffinity purified MHC I were treated with anti-D^b monoclonal antibodies (clone 28-14-8S, black histogram) plus FITC-labeled anti-mouse IgG, or with the secondary antibody alone (gray histogram). Positive staining of the cells from which the MHC I was obtained is shown in the inset.

Our principal concern was that MHC I may assume a hairpin configuration (extracellular domains and cytoplasmic tail all facing out, Fig. 1); this conformation may obscure the effects of membrane lipids and have little biological relevance. Hence, to assess the likelihood of a hairpin configuration for reconstituted MHC I we used the approach described

Table 1
Protein is incorporated efficiently into preformed 2-μm liposomes[a]

Liposomes	Protein added (μg)	Protein recovered (μg)	Protein incorporated[b]
18:0, 18:1 PC	2.0	1.7 (Expt. 1)	83%
		1.6 (Expt. 2)	(80–85%)
18:0, 22:6 PC	2.0	1.7 (Expt. 1)	80%
		1.5 (Expt. 2)	(75–85%)

[a]Preformed liposomes were incubated with immunoaffinity isolated MHC I at a saturating *n*-octyl-α-$_D$-glucopyranoside concentration. After detergent removal with Bio-Beads, the liposomes were washed in 15 vols. of PBS and harvested by centrifugation at $100\,000 \times g_{max}$ for 30 min at 4°C, and the protein was quantified with a CBQCA protein assay kit.
[b]The values for protein incorporated are means of two experiments, with the range shown in parentheses.

by Cardoza et al. [24] in which MHC I is fluorescently labeled on two free sulfhydryl groups (Cys-142 in the extracellular α2-domain and Cys-358 in the cytoplasmic tail of K^b; accession no. P01901, The National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Once MHC I is inserted into liposomes, fluorophore molecules (Cys-AEDANS) facing out of the liposomes are quenched by cobalt ion in the bathing solution, whereas fluorophores facing into the liposome interior are not quenched. Thus, proteins in the non-hairpin configuration will display partial (50%) quenching in this assay, although the method does not distinguish rightside-out from inside-out orientation. Table 2 demonstrates that approximately half of the total AEDANS fluorescence is external to the liposome, consistent with a non-hairpin configuration. The low fluorescence intensities made it difficult to achieve exacting precision with this assay, but clearly only partial quenching occurred with intact proteoliposomes. This is in stark contrast to the results of Cardoza et al. [24], who found all fluorescence from proteoliposomes formed by dialysis to be quenched externally, i.e. in their study all MHC I was in the hairpin configuration.

EL4 cells were the source for MHC I, and these cells express both MHC I proteins K^b and D^b. The monoclonal antibody (M1/42) used for affinity purification of MHC I molecules binds MHC I of all mouse haplotypes, but reportedly does not isolate D^b [26]. Our results, shown in Fig. 4, agree with [26]; D^b is not detectable on MHC I-reconstituted proteoliposomes stained with the 28-14-8S antibody, which recognizes an epitope in the α3-domain of D^b. This antibody is a good choice to monitor D^b expression because it detects a stable epitope whether β2m is present or absent [27–29] and will bind independently of peptide's influence on the α1/α2 domains [2]. Thus, we believe it very likely that failure of 28-14-8S to stain proteoliposomes indicates that most if not all MHC I reconstituted into liposomes is K^b. Further analysis focused on K^b alone.

3.2. Among phospholipids, DHA-containing PC shows the greatest impact on MHC I expression measured by monoclonal antibody binding

K^b was reconstituted into liposomes; each liposome preparation contained a single phospholipid species. With this simplified model membrane representing annular lipids or those within lipid microdomains we were able to compare unambiguously the effect of different phospholipid species on MHC I. The left panels in Fig. 5 show the forward scatter and side scatter characteristics of proteoliposomes composed of PC with stearic acid (18:0) in the *sn*-1 position and either oleic acid (18:1ω9) or DHA (22:6ω3) in the *sn*-2 position. In the right panels are flow cytometric histograms of phycoerythrin-labeled AF6-88.5 monoclonal antibody (anti-K^b) binding to MHC I in 18:0, 18:1 PC (Fig. 5B) and 18:0, 22:6 PC (Fig. 5D) proteoliposomes. Two independent experiments were performed with phycoerythrin-labeled AF6-88.5 and each demonstrated that bind-

Table 2
MHC I in liposomes assumes a native, not hairpin conformation

Experiment	Fluorescence intensity[a]				% Quench[b]
	Unquenched	29 mM $CoCl_2$	50 mM $CoCl_2$	Triton X-100	
1	167	53	47	9	76
2	130	80	77	7	43
				Average =	60

[a]Immunoaffinity purified MHC I (K^b) was labeled with IAEDANS, which reacted with free sulfhydryl groups, one in the extracellular α2-domain and the other in the cytoplasmic tail. The labeled protein was then reconstituted into liposomes composed of egg PC and unquenched fluorescence was monitored. Quenching of AEDANS external to the bilayer was achieved by the addition of $CoCl_2$ to the bathing solution. To demonstrate quenching of all AEDANS, Triton X-100 was added to dissolve the liposomes in the presence of $CoCl_2$.

[b]The percent quench was calculated as follows: [1-(fluorescence with 50 mM $CoCl_2$-fluorescence after Triton X-100 dissolution)/(unquenched fluorescence-fluorescence after Triton X-100 dissolution)]×100%. Under ideal conditions, 50% quenching will occur when all MHC I molecules are in the rightside-out, non-hairpin configuration.

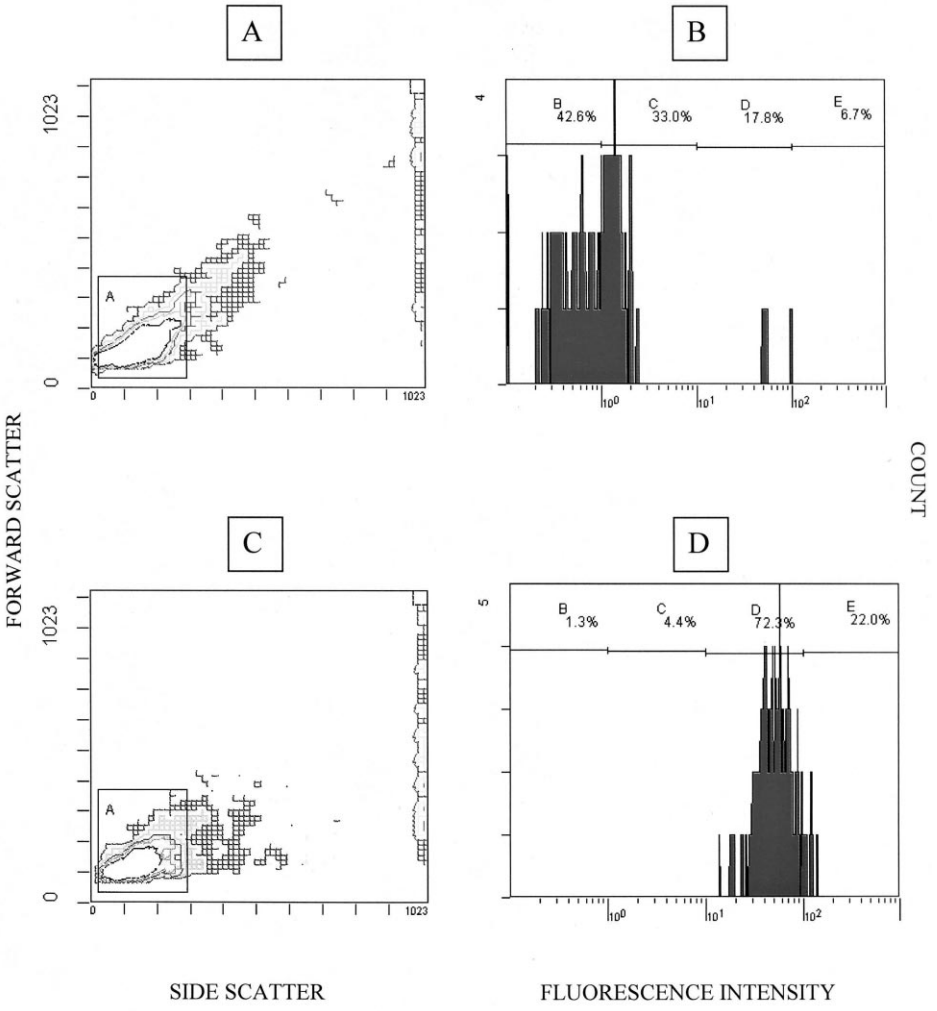

Fig. 5. MHC I expression in proteoliposomes is dependent upon the lipid composition of the bilayer. MHC I was reconstituted into liposomes composed of PC with stearic acid (18:0) in the *sn*-1 position and either oleic acid (18:1) or DHA (22:6) in the *sn*-2 position, and analyzed by flow cytometry. Forward and side scatter characteristics of the proteoliposomes are shown in the left panels: (A) 18:0, 18:1 PC proteoliposomes; (C), 18:0, 22:6 PC proteoliposomes. The corresponding fluorescence histograms for the boxed (gate A) proteoliposomes are shown in B (18:0, 18:1 PC proteoliposomes) and D (18:0, 22:6 PC proteoliposomes). Shown on these histograms are linear gates B–E representing the four decades of fluorescence intensity, as well as the percent of liposomes in each gate. Proteoliposome staining was with phycoerythrin-labeled AF6-88.5 (anti-K^b) monoclonal antibody.

ing of monoclonal antibody is greater when K^b is present in a DHA-containing phospholipid environment. Fluorescence intensity (antibody binding) was divided into four levels, from dull to bright, corresponding to the four decades (sets of 256 channels) of the 1024-channel photomultiplier tube. The percents of liposomes expressing fluorescence intensities at these four levels (decades) were then calculated. The percent of proteoliposomes fluorescing in the third and fourth (brightest) decades ranged from 58 to 72% (decade 3) and 22 to 35% (decade 4) for 18:0, 22:6 PC-proteoliposomes, but only 18% (decade 3) and 7 to 15% (decade 4) for 18:0, 18:1 PC-proteoliposomes. Accordingly, median fluorescence channels (median fluorescence intensity in all decades) ranged from 51 to 64 for the DHA-containing and 1 to 4 for the oleic acid-containing proteoliposomes.

Because we can rule out differing amounts and orientations of protein present in the two liposome populations, the most reasonable explanations for the lipid dependence of K^b expression (shown in Fig. 5) are, first, that the lipid environment has

Table 3
β2m addition does not equalize monoclonal antibody binding to proteoliposomes of disparate lipid composition

Liposome	β2m[a]	% of Liposomes in:				Relative % increase[b]
		Decade 1	Decade 2	Decade 3	Decade 4	
Experiment 1						
18:0, 18:1 PC	−	62.7	4.8	13.6	19.0	
18:0, 18:1 PC	+	50.1	9.9	18.2	21.6	14
18:0, 22:6 PC	−	43.8	8.9	21.3	26.3	
18:0, 22:6 PC	+	27.9	10.9	22.1	38.9	48
Experiment 2						
18:0, 18:1 PC	−	78.2	2.6	4.3	14.9	
18:0, 18:1 PC	+	75.2	0.6	2.3	21.9	47
18:0, 22:6 PC	−	64.4	3.2	5.2	27.2	
18:0, 22:6 PC	+	51.4	1.3	5.8	41.6	53

[a]Proteoliposomes were pretreated with either purified human β2m (Expt. 1) or filtered human serum (Expt. 2) and then stained with FITC-AF6-88.5.
[b]To assess an effect of β2m addition, appearance of proteoliposomes with the brightest fluorescence (Decade 4) was monitored, and the relative % increase calculated as 100%×[(% in Decade 4 with β2m)−(% in Decade 4 without β2m)]/(% in Decade 4 without β2m).

changed the conformation of K^b thereby affecting the expression of the AF6-88.5 epitope and, second, that the MHC I proteins are self aggregating in the 18:0, 18:1 PC bilayer, thereby obscuring the binding of antibodies. Of course, these two processes are not mutually exclusive and may both be operative. Self aggregation of free MHC I heavy chains (α-chains dissociated from β2m) has been reported, and having K^b epitopes in close proximity may sterically hinder binding of the monoclonal antibodies or bring together fluorophore molecules, such that fluorophore self quenching occurs. Phycoerythrin is relatively resistant to self quenching [30] and thus self quenching is unlikely to be at play in the experiment shown in Fig. 5. Fluorescein, on the other hand, is quite sensitive to self quenching, and fluorescein-conjugated antibodies were used in various aspects of this study. We therefore addressed the participation of MHC I self aggregation in our system. Chakrabarti et al. [9] reported that human MHC I reconstituted into dimyristoylphosphatidylcholine (14:0, 14:0 PC) displayed self aggregation (measured by FRET), which could be blocked with exogenous β2m. Note, however, that 14:0, 14:0 PC is not typically found in mammalian plasma membranes and does not produce the usual surface bilayer thickness; this results in a hydrophobic mismatch between the bilayer and the protein's transmembrane region and promotes protein oligomerization to minimize exposed hydrophobic area [31]. EL4 cells (our source for K^b) reportedly express ∼one-third of their surface MHC I molecules as free α-chains, which are created on the cell surface by β2m loss [32]. It is possible that 18:0, 18:1 PC proteoliposomes have a higher proportion of free α-chains, which in turn may cause less antibody to bind to 18:0, 18:1 PC compared to 18:0, 22:6 PC proteoliposomes. To test this hypothesis, exogenous human β2m (which binds avidly to murine MHC I [33]), was added to the proteoliposomes prior to staining with FITC-AF6-88.5. When human β2m, either as a purified protein or in human serum, was added to the proteoliposomes, FITC-AF6-88.5 binding was enhanced 14–53% (consistent with some exchange between endogenous and added β2m, or addition of β2m to free α-chains), however, this treatment did not equalize antibody binding to K^b in 18:0, 18:1 PC and 18:0, 22:6 PC, nor was the greatest enhancement associated with the 18:0, 18:1 PC environment (Table 3). The presence of β2m in the protein used for reconstitution (Fig. 2) and the failure of β2m addition to equalize fluorescence provided no compelling reason to pursue self aggregation of MHC I free α-chains as the reason for disparate antibody binding.

We explored the expression of K^b in various lipid environments, namely, PC with stearic acid in the *sn*-1 position and either oleic acid (18:1ω9), α-linolenic acid (18:3ω3), arachidonic acid (20:4ω6) or DHA (22:6ω3) in the *sn*-2 position. The 'control' bilayer is formed from 18:0, 18:1 PC, which is a phospho-

lipid molecular species among the most abundant in leukocyte membranes [34] and which contains oleic acid, a monounsaturated fatty acid. In contrast, DHA has 22 carbons and six double bonds, the last of which is three carbons from the methyl (omega) end, hence the name 'omega-3'. It is the longest and most unsaturated fatty acid found in abundance in biological systems and its tissue levels are altered substantially by diet [35]. α-Linolenic acid, like DHA, is an omega-3 fatty acid; it is a precursor for the synthesis of longer, more polyunsaturated omega-3 fatty acids, such as DHA and typically does not accumulate in substantial amounts in membranes. Arachidonic acid is a long chain polyunsaturated fatty acid, but with two fewer carbons and double bonds than DHA, and its last double bond is six carbons from its methyl end ('omega-6'). Like DHA, arachidonic acid is found in phospholipids, such as phosphatidylserine, phosphatidylethanolamine, and PC [36,37] and enrichment of membranes with DHA typically produces a concomitant decrease in arachidonic acid content [34,38,39]; thus, these two fatty acids may compete for the same position in cellular membrane phospholipids. Fluorescein-labeled AF6-88.5 was used to probe for differences in expression of MHC I in the different liposomes. As shown in Table 4, DHA-containing PC provided for significantly greater binding of fluorescein-labeled AF6-88.5 to K^b when compared to all other lipids tested. Parenthetically, the experimental procedures represented by the data in Tables 3 and 4 are not identical and thus trends, rather than absolute fluorescence intensities, are compared; all statistical analyses were performed with a non-parametric, paired sample test. The mean (30.2) and median (22.5) fluorescence channels for 18:0, 22:6 PC proteoliposomes were significantly higher than the other proteoliposomes, resulting from a significantly greater percentage of proteoliposomes with fluorescence in the third decade and concomitantly fewer in the first decade. Proteoliposomes composed of 18:0, 18:3 PC and 18:0, 20:4 PC displayed fluorescence intensities that were not significantly different from each other, but that showed distinct differences from 18:0, 22:6 PC. As each proteoliposome population is composed of only the immunoaffinity-isolated K^b and a single lipid species, it must be a direct effect of the lipid on the protein causing differences in antibody binding and fluorescence.

3.3. *Effects of 18:0, 22:6 PC on other MHC I epitopes*

Two other FITC-conjugated monoclonal antibodies were tested for their ability to bind K^b in the control (18:0, 18:1 PC) and the DHA-containing (18:0, 22:6 PC) lipid environments. Based on haplotype specificity, each antibody detects an epitope distinct from AF6-88.5. That is, whereas AF6-88.5 reacts with an epitope expressed by the b haplotype and weakly expressed by $H\text{-}2^k$, CTKb reacts with a private K^b specificity and 5041.16.1 detects a specificity shared by K^b and D^b (specificities provided by the manufacturers). Proteoliposome staining by CTKb and 5041.16.1 is approximately 10-fold less than with AF6-88.5, although live EL4 cells stain

Table 4
DHA-containing PC favors the binding of monoclonal antibody AF6-88.5

Proteoliposome	Fluorescence channel		% of Liposomes in:			
	Mean	Median	Decade 1	Decade 2	Decade 3	Decade 4
18:0, 18:1 PC	22.4 ± 13.1^a	15.2 ± 12.6^a	28.2 ± 15.9^a	$33.2 \pm 12.5^{a,b}$	33.3 ± 12.3^a	5.6 ± 4.6^a
18:0, 18:3 PC	7.8 ± 0.5^a	4.0 ± 0.7^a	$13.5 \pm 4.6^{a,b}$	59.0 ± 7.8^c	22.9 ± 7.1^a	0.4 ± 0.4^b
18:0, 20:4 PC	18.9 ± 10.6^a	14.0 ± 9.0^a	8.5 ± 3.3^b	$51.6 \pm 13.9^{b,c}$	37.2 ± 13.7^a	$3.2 \pm 2.9^{a,b}$
18:0, 22:6 PC	30.2 ± 12.2^b	22.5 ± 12.0^b	5.3 ± 2.2^b	33.2 ± 12.3^a	56.4 ± 11.7^b	5.4 ± 3.9^a

MHC I-reconstituted liposomes of various phospholipid compositions were stained with fluorescein-conjugated AF6-88.5 (anti-K^b) and analyzed by flow cytometry. Antibody binding is expressed as mean and median fluorescence intensity channel, and the percent of liposomes having fluorescence in Decade 1 (channels 0–255), decade 2 (256–511), decade 3 (512–767), and decade 4 (768–1023) on a four-decade, 1024-channel log scale.

[a–d]Significantly different values *within a column* are denoted by different superscript letters ($P \leq 0.05$, Wilcoxon paired sample test). The data are presented as means ± S.E. ($n = 4$ independent experiments for all lipids except 18:0, 18:3 PC where $n = 3$).

Table 5
Binding of monoclonal antibodies to other MHC I epitopes is modestly but significantly affected by DHA-containing PC

Proteoliposome	Fluorescence channel		% of Liposomes in:			
	Mean	Median	Decade 1	Decade 2	Decade 3	Decade 4
Monoclonal antibody CTKb						
18:0, 18:1 PC	2.0 ± 0.9	0.8 ± 0.7	61.4 ± 12.4	34.7 ± 9.7	3.8 ± 2.9	0.0 ± 0.0
18:0, 22:6 PC	5.0 ± 1.5[a]	4.6 ± 1.6[a]	19.0 ± 4.3[a]	65.9 ± 3.2[a]	14.9 ± 7.2[a]	0.0 ± 0.0
Monoclonal antibody 5041.16.1						
18:0, 18:1 PC	2.7 ± 0.7	0.2 ± 0.1	78.1 ± 10.2	13.4 ± 6.0	8.0 ± 6.0	0.2 ± 0.1
18:0, 22:6 PC	4.6 ± 1.6[a]	2.7 ± 2.5	62.7 ± 23.6	23.5 ± 14.7	12.1 ± 9.4[a]	0.4 ± 0.3

MHC I-reconstituted liposomes were stained with fluorescein-labeled monoclonal antibodies CTKb and 5041.16.1, and analyzed by flow cytometry. Decades are defined in the legend to Table 3.
[a]Significant difference ($P \leq 0.05$) between 18:0, 18:1 PC and 18:0, 22:6 PC proteoliposomes, as tested with the non-parametric Wilcoxon paired sample test. The data are means ± S.E. ($n = 3$ for each monoclonal antibody).

equivalently with all antibodies (data not shown). However, even moderately stained, DHA-containing proteoliposomes treated with FITC-conjugated CTKb antibodies were significantly more fluorescent than the 18:0, 18:1 PC-proteoliposomes, as judged by mean and median fluorescence intensity and the percent of proteoliposomes distributed in the four decades of fluorescence intensity (Table 5). The difference between 18:0, 22:6 PC and 18:0, 18:1 PC proteoliposomes stained with FITC-labeled 5041.16.1 antibodies was equivocal, with statistical significance achieved only for comparisons of mean fluorescence intensity and percent of proteoliposomes with decade 3-level fluorescence intensity. It would thus appear that three epitopes (detected by three distinct monoclonal antibodies) are not affected identically by the DHA-containing lipid environment, hinting that DHA may induce a conformational change in K^b wherein some portions of the molecule, i.e. epitopes, are altered while others are not.

The epitope to which the AF6-88.5 monoclonal antibody binds was reported to be in the α2-domain of K^b [40], but more recently it was found to be a conformational determinant sensitive to amino acid substitutions in all three α-chain extracellular domains as well as the source of β2m [41]. It is therefore not surprising to us that the greatest effect of DHA-containing PC was on this conformationally sensitive epitope. The α1 and α2 extracellular domains most distal from the membrane are particularly prone to change conformation, consistent with their role in antigen binding and presentation. For example, the α1/α2-epitope on D^d detected with monoclonal antibody 34-5-8S disappears more quickly than an epitope in the α3-domain (detected by 34-2-12S) upon loss of β2m [42]. However, a key consideration is whether interactions between a membrane protein (presumably at its transmembrane region) and phospholipids in the membrane bilayer can produce conformational changes in distant protein domains. Dissociation of β2m from the extracellular domains of either murine or human MHC I produces a conformational change that exposes epitopes in the cytoplasmic tail [10,32]. These are important findings because they imply that conformational changes can be telegraphed through the α-chain.

Can changes in MHC I conformation induced by the lipid composition of membranes affect MHC I function, i.e. presentation of antigen? Lipids added to MHC class II molecules in detergent affected radiolabeled peptide binding by MHC II, suggesting that lipids may induce, perhaps indirectly, a conformational change in the peptide binding groove of this MHC molecule [43]. Early studies germane to MHC I noted that resistance to killing by cytotoxic T-lymphocytes correlated with reduced PC synthesis and increased cholesterol content [44], conditions that reduced the apparent expression of certain MHC I epitopes [16,17]. Because the α1/α2 surface of the MHC I contacts the TCR, the propensity for conformational change in that portion of the MHC I could sway the repertoire of T-cell clones activated during an immune response. Relatively minor conformational changes in MHC I are thought to mask epitopes recognized by, and which inhibit, activated natural killer cells [45]. Recently, the transmembrane

region of human MHC I was found to be responsible for inhibiting certain natural killer clones through an undetermined mechanism likely to involve control of protein conformation rather than MHC I oligomerization, association with other proteins, or cellular localization [46]. We therefore consider it an essential next step to test the T-cell activating ability of MHC I proteoliposomes of various lipid compositions. Additionally, we are currently undertaking additional assays of conformational change of MHC I reconstituted into liposomes. By understanding the direct effect of DHA-containing phospholipids on MHC I properties, such as conformation and antigen presentation, we hope not only to better predict the effects of DHA in vivo (e.g. when provided in the diet), but also to devise novel immunotherapies that utilize DHA's abilities to influence antigen presentation.

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