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FcγRI (CD64) resides constitutively in lipid rafts

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Abstract

Cellular membranes contain microdomains known as 'lipid rafts' or detergent-insoluble microdomains (DRM), enriched in cholesterol and sphingolipids. DRM can play an important role in many cellular processes, including signal transduction, cytoskeletal organization, and pathogen entry. Many receptors like T cell receptors, B cell receptors and IgE receptors have been shown to reside in DRM. The majority of these receptors depend on multivalent ligand interaction to associate with these microdomains. We, here, study association between the high affinity IgG receptor, FcγRI (CD64), and membrane microdomains. FcγRI is a 72 kDa type I glycoprotein that can mediate phagocytosis of opsonized pathogens, but can also effectively capture small immune complexes, and facilitates antigen presentation. We found FcγRI to predominantly reside within detergent-insoluble buoyant membranes, together with FcRγ-chain, but independent of cross-linking ligand. With the use of confocal imaging, FcγRI was found to co-patch with GM1, a microdomain-enriched glycolipid. Depletion of cellular cholesterol, furthermore, modulated FcγRI–ligand interactions. These data indicated FcγRI to reside within lipid rafts without prior triggering of the receptor.

Keywords: Fc receptor; Lipid rafts; Detergent-resistant membranes; Cholesterol depletion

1. Introduction

Cellular membranes are the dynamic structures composed of multiple lipid species that compartmentalize cells in different organelles. Specialized microdomains within the lateral plane of membranes enriched of glycolipids with large hydrophilic caps (such as the sphingolipid GM1), and cholesterol (referred to as detergent-resistant membrane domains (DRM), or lipid rafts), play a crucial role in protein sorting and signaling [1,2]. DRM display resistancy to lysis in non-ionic detergents at low temperatures, and are of low density which allow DRM to float on density gradients upon centrifugation. Isolation of DRM or lipid rafts is however a delicate matter [3,4]. A subset of DRM form specialized invaginations of the membrane by associations with caveolins, and are important signaling and internalization centers [5]. DRM-mediated endocytosis has been found independent from internalization via clathrin-coated pits [5].

Multi-subunit Fc receptors (FcR), as well as T and B cell receptors have transient associations with DRM upon receptor cross-linking (reviewed in Refs. [6–8]). FcR that translocate to DRM upon cross-linking are FcεRI [9], FcαRI [10], and low-affinity FcγR [11–13]. The cytosolic leaflets of DRM are enriched of prenylated proteins such as Src family protein tyrosine kinases (PTK), and DRM were identified as sites for phosphorylation of the intracellular tyrosine-based activation motif (ITAM) present in FcR [14]. Recent studies indicated multiple DRM-subtypes to exist, and some DRM–protein associations to be controlled by cytosolic proteins and the cytoskeleton [15,16].

It is unknown whether the high affinity receptor for IgG, FcγRI, associates with DRM. The class I IgG receptor (FcγRI) is constitutively expressed on monocytes, macrophages and dendritic cells. FcγRI is a 72 kDa glycoprotein expressed as a multimeric complex comprised of an α (ligand binding) chain and a γ chain homodimer. Its in vivo role is illustrated by FcγRI$^{-/-}$ mice that exhibit impaired antibody-dependent

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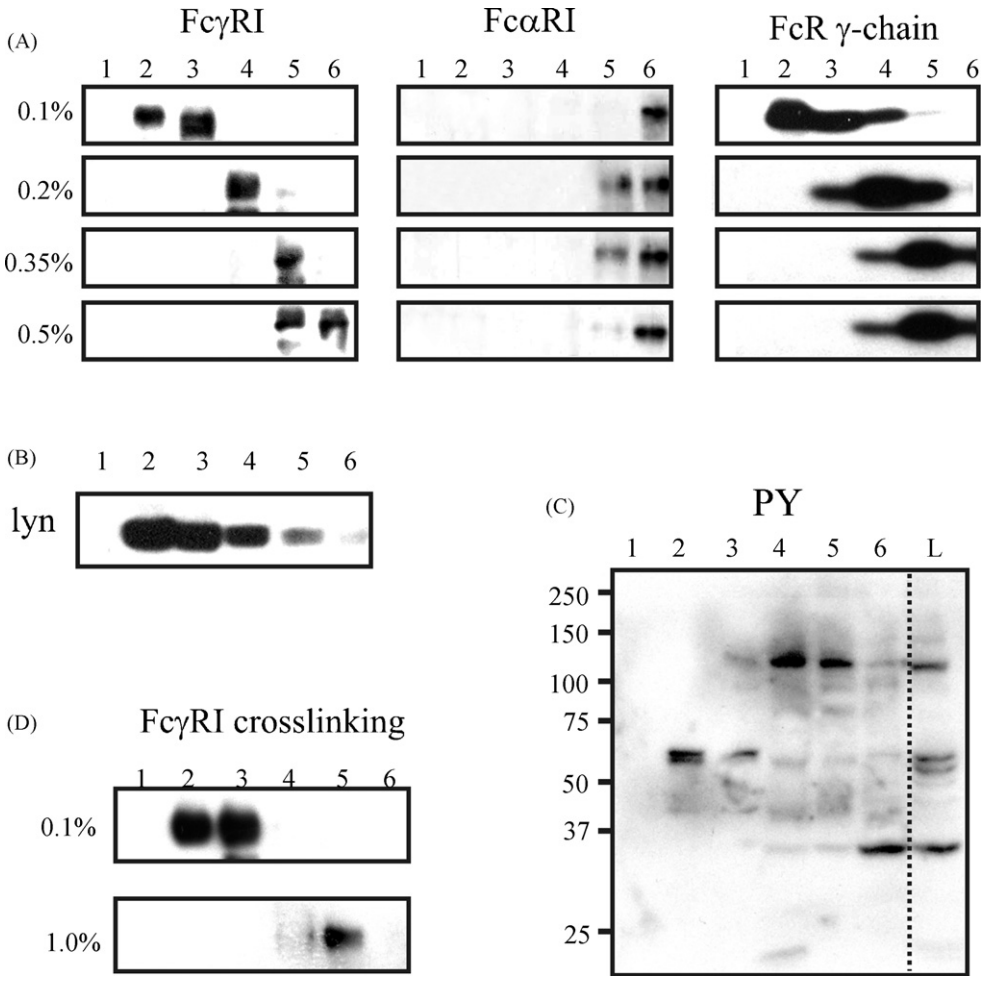

Fig. 1. FcγRI–DRM association upon lysis of U937 cells and density-gradient centrifugation. (A) U937 cells were lysed at 4 °C in buffers containing increasing amounts of Triton, and subjected to density-gradient centrifugation. Six fractions were collected per gradient (density increased from fraction 1 to 6). Fractions were TCA-precipitated, and proteins analyzed by SDS-PAGE and Western blot. Left panels were stained for FcγRI, middle panels for FcαRI, and right panels for FcRγ-chain. (B) Distribution of Lyn over the density-gradient upon lysis of U937 cells in 0.1% Triton. (C) Distribution of phosphotyrosine proteins (recognized by mAb 4G10) in U937 cells upon lysis in 0.1% Triton. (D) FcγRI was cross-linked for 15 min at 4 °C with H22 $F(ab')_2$, followed by goat $F(ab')_2$ anti-human kappa-light chain for 15 min. Cells were lysed at Triton concentrations indicated, and subjected to density-gradient centrifugation. Experiments were repeated thrice, and yielded comparable results.

cellular processes such as bacterial clearance, phagocytosis, antigen presentation, and cytokine production [17,18]. Here, association of FcγRI with DRM is reported. In contrast with other FcR, FcγRI appears constitutively associated with DRM. In addition, we study the cholesterol dependence of FcγRI–ligand interactions.

2. Materials and methods

2.1. Antibodies

2.1.1. Antibodies used for DRM analyses

A rabbit serum was used to detect FcγRI (no. 3532, recognizing the FcγRI-cytosolic domain, kindly provided by Dr. R. Kimberly, University of Alabama, Birmingham, AL), FcαRI (recognizing the extracellular domain [19]; a kind gift of Dr. C. van Kooten, LUMC, Leiden, The Netherlands), Lyn (Santa Cruz Biotechnology, Santa Cruz, CA), and FcRγ-chain (Upstate, Lake Placid, NY). Secondary detection was performed by horse radish peroxidase (HRP)-conjugated goat-anti-rabbit (Pierce, Rockford, IL). Tyrosine phosphorylated proteins were detected by monoclonal antibody (mAb) 4G10 (Upstate), and rabbit-anti-mouseIgG-HRP (Dako. Glostrup, Denmark). CD45R/B220 was detected with a rat mAb conjugated to biotin (BD, Franklin Lakes, NJ), and horse radish peroxidase (HRP)-conjugated streptavidin (Immunotech, Marseille, France). FcγRI-cross-linking was performed by incubation of cells for 15 min in the medium containing 10 μg/ml $F(ab')_2$ fragments of H22 (Medarex, Anandale, NJ) [20], washing in medium, and 15 min incubation with 5 μg/ml $F(ab')_2$ fragments of FITC-conjugated goat anti-human kappa light chain (Southern Biotechnology, Birmingham, AL), all at 4 °C, and prior to cell lysis.

2.1.2. Antibodies used for immuno-fluorescence

FITC-conjugated CD64 mAb M22, and anti-CD11b mAb were from Immunotech. FITC-conjugated CD32 mAb IV.3 (a

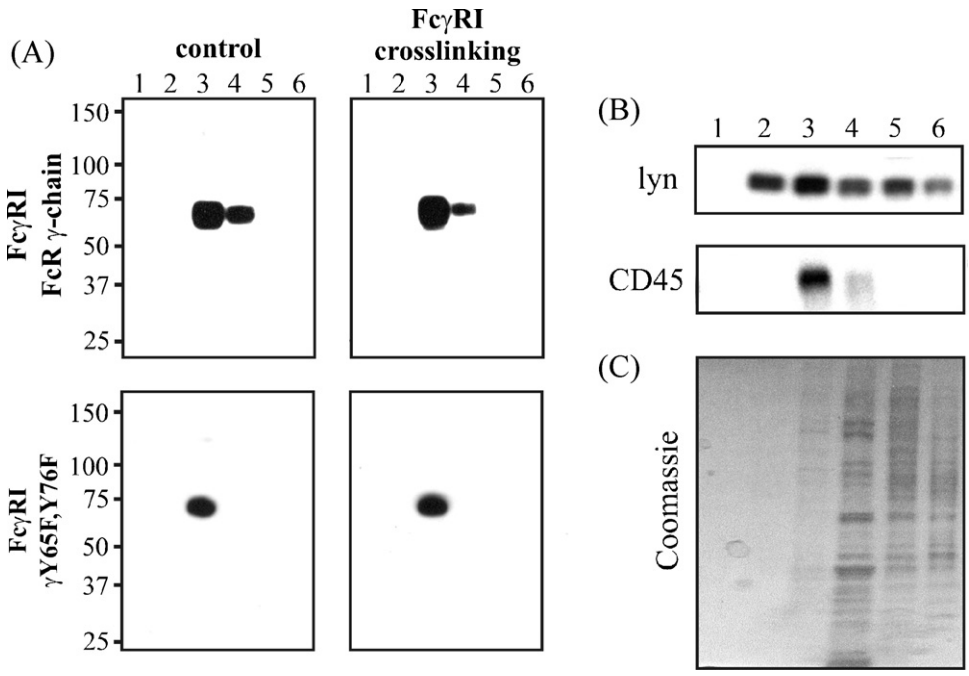

Fig. 2. FcγRI–DRM association upon 0.1% Triton lysis of IIA1.6 cells. (A) IIA1.6 cells transfected with FcγRI, and FcRγ-chain (either wild type, or tyrosine-mutated (γY65F, Y76F)) were lysed in 0.1% Triton, and analyzed by density-gradient centrifugation. FcγRI was cross-linked for 15 min at 4 °C with H22 $F(ab')_2$, followed by goat $F(ab')_2$ anti-human kappa-light chain for 15 min. (B) Distribution of Lyn, and CD45 over denstity-gradient of IIA1.6 cells upon lysis in 0.1% Triton. (C) Total protein distribution of FcγRI/FcRγ-chain-transfected IIA1.6 cells over density-gradient indicated by Coomassie staining.

kind gift of Dr. T. Keler, Medarex, Annandale, NJ), and CD89 mAb A77 were from Medarex. Secondary-detection of A77 was by FITC-conjugated rabbit-anti-mouse IgG (Jackson Laboratories, West Grove, PA).

2.1.3. Antibodies used for flow cytometry

MIgG2a-FITC was from DAKO, rabbit-anti chicken egg albumin (ovalbumin) was from Sigma (Saint Louis, MO). Goat-anti-rabbit-FITC (Jackson) was applied to detect binding of rabbit-Ig (complexes).

2.2. Density-gradient centrifugation

U937 and transfected IIA1.6 cells (described in Ref. [21]) were maintained in hepes-buffered RPMI 1640 medium (Invitrogen, Paisley, UK) containing 10% fetal bovine serum (HyClone, Logan, UT) and antibiotics (Invitrogen). The protocol was adapted from Ref. [22]. 2×10^6 cells were washed in TNE (Tris 25 mM, NaCl 150 mM, EDTA 5 mM, 1 mM DTT), and protease inhibitor cocktail (Roche, Mannheim, Germany), and lysed in 300 μl TNE containing different concentrations of Triton-X100 (Triton) at 4 °C. 200 μl lysates were subsequently mixed with 400 μl 60% optiprep (Sigma, Steinheim, Germany), transferred to ultra-centrifuge tubes (Beckman, Palo Alto, CA), and subsequently overlaid with 600 μl solution of 35%, 30%, 25%, 20%, and 0% optiprep in TNE. Gradients were spun overnight in a SW60 rotor, and ultracentrifuge of Beckman at 100,000 × *g*. 600 μl fractions were collected, TCA-precipitated, and analyzed by SDS-PAGE, and Western blotting.

2.3. Immuno-fluorescence

Adherent mononuclear cells were prepared from peripheral blood of healthy volunteers, stimulated overnight with or without 300 U/ml IFNγ (IFN-γ1b, Boehringer Ingelheim, Biberach, Germany), and prepared for immuno-fluorescence as in Ref. [23]. Briefly, monocytes were adhered to poly-L-lysine coated glass slides, fixed in PBS with 3.3% paraformaldehyde, blocked and stained in PBS containing 0.2% BSA, 0.1% saponin, 5% mouse serum, 5% goat serum with various mAb. Detection of GM1 was performed by alexa555-conjugated cholera toxin subunit B (Molecular Probes, Leiden, The Netherlands). Stainings were analyzed by confocal scanning laser microscopy.

2.4. Cholesterol depletion and flow cytometry

Cells were cultured as described above. Before each experiment, cells were washed in serum-free medium, and depleted for cholesterol upon head-over-head incubation for 45 min at 37 °C in serum-free medium containing 20 mM methyl-β-cyclodextrin (3×10^6 cells/ml). Cells were then washed, and FcγRI was detected with CD64 mAb M22-FITC, recognizing FcγRI outside the ligand-binding domain [24], via monomeric ligand (mIgG2a-FITC, rabbit IgG), or immune complexes (rabbit IgG-anti-OVA complexes), generated as described [25]. All staining were performed with primary Ab concentrations of ∼10 μg/ml, and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

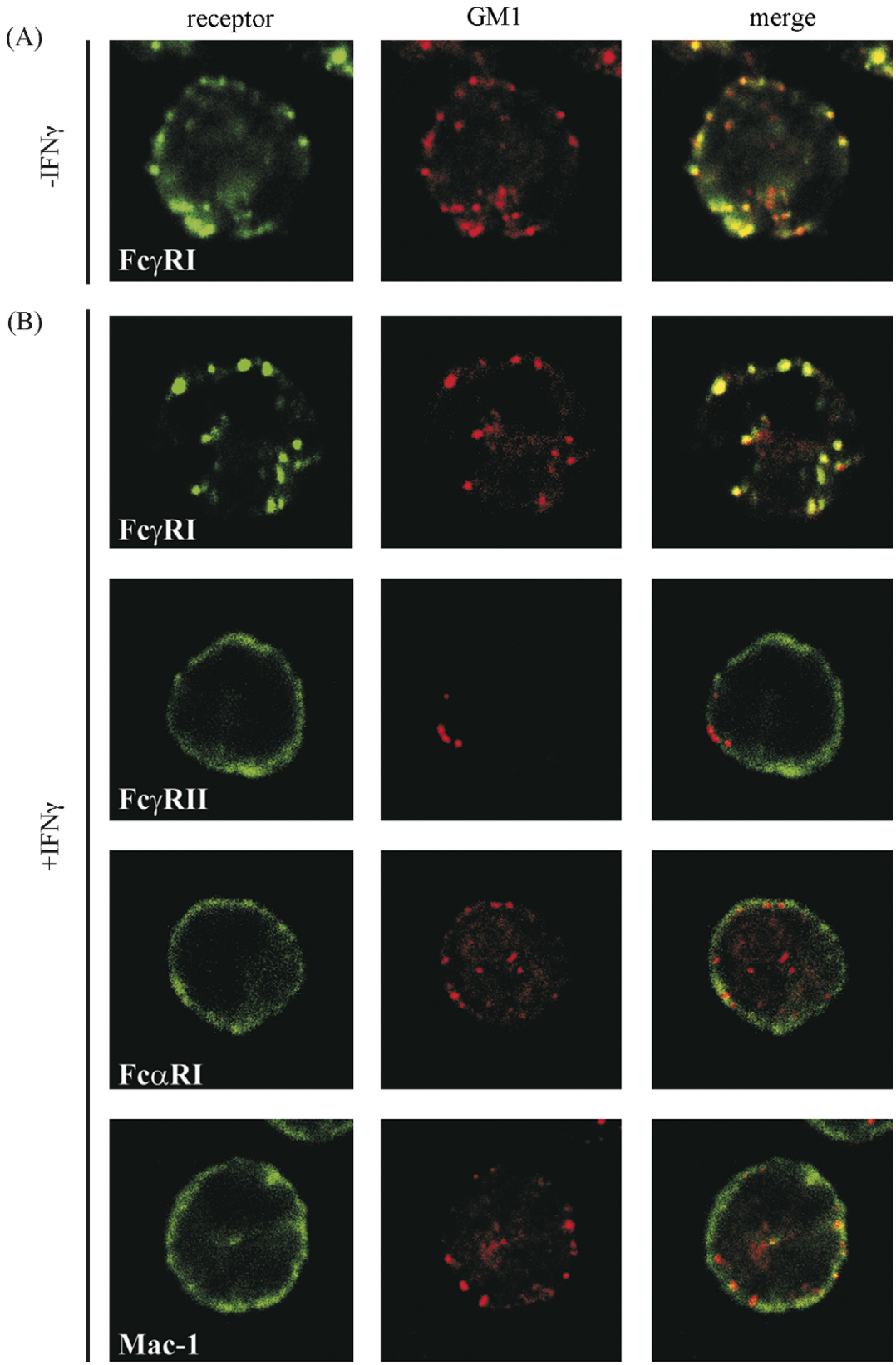

Fig. 3. Subcellular distribution of FcR and GM1 in monocytes. (A) Monocytes were prepared from healthy human volunteers, fixed, permeabilized, and stained for FcγRI (by FITC-conjugated CD64 mAb M22, left panel) and GM1 by cholera toxin subunit B conjugated to alexa 555 (middle panel). Right panels indicate merged images. (B) Monocytes were prepared, stimulated overnight with IFN-γ, and fixed. Co-stainings of GM1 and FcγRI, FcγRII (mAbIV.3-FITC), FcαRI (mAb A77-FITC), and CD11b (FITC) are shown ($n = 3$).

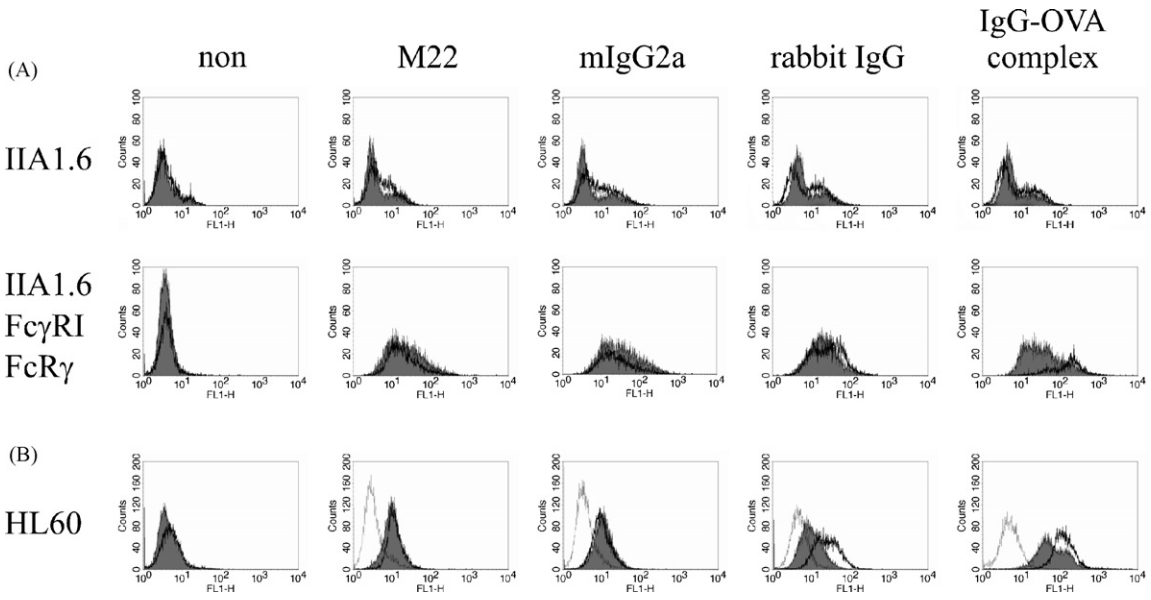

Fig. 4. Sensitivity of FcγRI–ligand interactions for cholesterol depletion. (A) Interactions between untransfected IIA1.6 cells, or FcγRI/FcRγ-chain-transfected IIA1.6 cells with CD64 mAb M22-FITC, mIgG2a-FITC, anti-OVA rabbit IgG, and anti-OVA rabbit IgG-OVA-complexes (solid grey-filled histograms) were analyzed by flow cytometry. Transparent histograms (thick black lines) indicate interactions between cells and FcγRI-recognizing antibodies upon depletion of cholesterol with 20 mM methyl-cyclodextrin. Non indicates unstained cells. One-out-of two representative experiments is shown. (B) Interactions between a panel of FcγRI-binding molecules and HL60 cells. Cells were incubated with (thick black lines) or without (solid grey-filled histograms) 20 mM methyl-*b*-cyclodextrin. For M22 and mIgG2a stainings, the dotted line indicate control IgG1 staining. For rabbit IgG stainings (monomeric, or in complex), the dotted line indicate secondary antibody staining.

3. Results

The association of FcγRI with buoyant Triton-X100 (Triton)-resistant membranes was investigated upon density-gradient centrifugation of lysed U937 cells without addition of FcγRI ligands. We observed the bulk of FcγRI to reside within low-density membrane fractions upon lysis of cells in 0.1% Triton (Fig. 1A; fractions 2 + 3). Upon lysis in 0.2% Triton, however, FcγRI shifted towards higher density fractions (fraction 4), and FcγRI was found in fractions 5 and 6 upon lysis in >0.35% Triton concentrations. Under these conditions, however, no association of FcαRI was observed with low-density membranes. Triton-dependent association of the FcR γ-chain with DRM correlated well with the distribution of FcγRI (Fig. 1A). The low-density DRM marker Lyn was selectively enriched in similar fractions as FcγRI at 0.1% Triton (Fig. 1B). Phosphotyrosine proteins were distributed over the gradient, and suggested phophorylated Lyn (a doublet of ∼53–56 kD that migrated identically as Lyn in Fig. 1B) to accumulate in low-density membrane fractions (Fig. 1C). FcγRI cross-linking did not affect its distribution (Fig. 1D). These data demonstrated FcγRI to selectively associate with DRM at low Triton concentrations.

Murine IIA1.6 cells transfected with FcγRI and the FcRγ-chain (wild type, or ITAM-mutated) were assayed for FcγRI–DRM association. FcγRI was found in low-density fractions (fraction 3), irrespective of receptor cross-linking, or an intact ITAM in the FcRγ-chain in FcγRI-expressing cells (Fig. 2A). Significant amounts of Lyn and CD45 were also present in fraction 3 (Fig. 2B). However, Coomassie staining indicated the majority of proteins to reside within fractions 4–6 (Fig. 2C).

The subcellular distribution of the sphingolipid GM1, which is enriched in DRM [22], was studied with respect to FcγRI and other receptors in primary monocytes. FcγRI co-patched with GM1 at both the plasma membrane, as well as intracellularly (Fig. 3A). Similar staining patterns were observed for interferon-γ stimulated monocytes (Fig. 3B). However, other receptors such as FcγRIIA (CD32), FcαRI (CD89), and Mac-1 (CD11b) did not co-localize with GM1 (although some was observed for Mac-1), suggesting these receptors to predominantly be found outside GM1-positive DRM.

DRM–protein associations are sensitive to cholesterol-sequestering agents such as methyl-β-cyclodextrin [1]. To functionally address FcγRI–DRM associations, cholesterol was depleted from FcγRI-expressing cells, and interactions with FcγRI ligands were assessed by flow cytometry. Upon depletion of cholesterol, IIA1.6 cells transfected with FcγRI and FcRγ-chain selectively increased interactions with rabbit IgG-ovalbumin (IgG-OVA) complexes, but not with monomeric mIgG2a and CD64 mAb M22 (Fig. 4A, $n = 2$). As negative control, untransfected IIA1.6 cells were used, and found predominantly negative. Promyeloblastic HL60 cells expressed some FcγRI, and displayed a ∼2-fold increased interaction with monomeric rabbit IgG and IgG-OVA (Fig. 4B, $n = 2$). Upon incubation of HL60 cells with IFNγ, a similar cholesterol-dependency of FcγRI–ligand interaction was observed (data not shown, $n = 2$).

4. Discussion

In this report, FcγRI–DRM association was assessed by density-gradient centrifugation upon cell lysis in Triton,

and co-localization studies with DRM-markers by immunofluorescence. The bulk of FcγRI was found associated with DRM upon lysis of U937 cells, and transfected IIA1.6 cells in low (0.1%) Triton concentrations, irrespective of FcγRI cross-linking (Figs. 1 and 2). FcαRI, however, was not found in DRM as was expected for the non-cross-linked receptor [26]. Constitutive FcγRI–DRM association was, furthermore, supported by the examination of the subcellular distribution of FcγRI and GM1, a DRM-associated glycolipid [22], on monocytes (Fig. 3). Patches of GM1 were observed to co-cluster with FcγRI (but not with FcγRII, and FcαRI) at the plasma membrane, and at intracellular sites. DRM are formed in Golgi, and may sort FcγRI to compartments such as the plasma membrane, and the endosomal compartment [1,2].

The FcR γ-chain was also associated with DRM at 0.1% Triton. Both FcγRI and FcαRI have been described to associate with FcRγ-chain [27,28], and were suggested to be stronger for FcαRI and FcRγ-chain due to the electrostatic interactions between their transmembrane domains. However, the data presented here are inconsistent with such model. FcαRI was found in high-density fractions, and suggested most FcαRI molecules not to associate with FcRγ-chain positive membrane compartment in unstimulated cells. A proportion of FcαRI has already been observed not to associate with FcRγ-chain in neutrophils and monocytes (partly) supporting the present data [29,30].

We functionally addressed FcγRI–DRM association by cellular cholesterol depletion, followed by assessment of FcγRI–ligand interactions. Unexpectedly, FcγRI binding to select ligands such as rabbit IgG (monomeric, as well as in complex), but not mIgG2a or a CD64 mAb, were increased upon cholesterol depletion (Fig. 4). This suggested cholesterol depletion to disrupt FcγRI-organization in the membrane, leading to increased recognition of immune complexes. Possibly, factors that co-patch with FcγRI in DRM may prevent interactions of immune-complexes by steric hindrance. Similarly, TCR recognition of MHC ligands required the heavily glycosylated CD43 to be excluded from the TCR area [31]. The organization of DC-SIGN in membranes, and interaction with multivalent ligands has also been shown to depend on cholesterol [32].

We published periplakin to affect FcγRI ligand-binding capacity, and downstream effector functions such as antigen presentation via interactions with the FcγRI cytosolic domain [21]. It is tempting to speculate that intracellular proteins that bind FcγRI-CY may control its association with DRM. A role of the constitutive FcγRI–DRM association in FcγRI signal transduction, and sorting upon internalization, however, remains to be elucidated.

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