Cholesterol-Dependent and -Independent CD40 Internalization and Signaling Activation in Cardiovascular Endothelial Cells

Jianjun Chen, Lu Chen, Gang Wang, Hong Tang

Objective—It remains elusive how CD40 endocytosis or clustering on the cell surface is induced by different forms of CD40 agonist. This study aims to investigate whether lipid rafts differentially regulate CD40 traffic and signaling in proinflammatory activation of cardiovascular endothelial cells (ECs).

Methods and Results—Using fluorescent microscopy and flow cytometry, we demonstrated that soluble CD40L and agonistic antibody G28.5 induced CD40 internalization via clathrin-independent pathway. Furthermore, depletion of cholesterol by methyl-β-cyclodextrin (MCD) or siRNA knockdown of caveolin-1 efficiently blocked CD40 internalization, suggesting that caveolae-rafts pathway regulates CD40 internalization. In contrast, a membrane-bound CD40L mimic (megamer) triggered aggregation of CD40 rafts outside of the conventional cholera toxin B subunit-positive lipid rafts resistant to cholesterol depletion. Finally, both G28.5 and megamer induced CD40 translocation to Brij58-insoluble, low buoyant density rafts, a movement insensitive to cholesterol depletion. However, MCD effectively inhibited G28.5 but not megamer-induced CD40 activation, and such inhibition could be alleviated by cholesterol reconstitution, suggesting that 2 different raft structures of CD40 induced by G28.5 or megamer possess differential sensitivity to cellular cholesterol levels in downstream signaling.

Conclusions—Depending on different forms of agonist, CD40 uses either a cholesterol-dependent or -independent mode for trafficking and signaling in ECs. (***Arterioscler Thromb Vasc Biol*. 2007;27:2005-2013.)**

Key Words: CD 40 ■ lipid rafts ■ endocytosis ■ cholesterol ■ endothelial cells

CD40 is constitutively expressed on professional antigen presenting cells (APCs)[1,2] as well as nonprofessional APCs, such as endothelial cells (ECs).[3] Overactivation of CD40 in these cells leads to elevated expression of proinflammatory cytokines and adhesion molecules in chronic inflammatory diseases such as rheumatoid arthritis, graft-versus-host disease, and atherosclerosis.[2,4,5] Besides cell surface expression, CD40L can also exist in a soluble biologically active form (sCD40L) that is shed from activated T-cells[6] or stimulated platelets,[7,8] the latter accounting for 95% of circulating sCD40L.[9] Therefore, it is generally speculated that increased plasma sCD40L levels attributable to abnormal platelet activation most likely plays a role in vascular endothelial inflammation and atherogenesis.[10]

Many receptors are rapidly endocytosed after ligand-induced activation and subsequently move through a series of endosomal compartments. There are 2 major pathways involved in receptor internalization depending on the different plasma membrane moiety engaged. One is the classic clathrin-mediated endocytic pathway, and the other is lipid raft-dependent route either caveolae-dependent[11] or caveolae-independent.[12] Lipid rafts are ≈50 to 100-nm-diameter membrane microdomains that are enriched in cholesterol and glycosphingolipids[13] and invisible under any optical microscopes.[14,15] They serve as an important platform for bringing related signaling proteins together to initiate specific signal pathway.[16] Many important signaling proteins, including glycosylphosphatidylinositol-anchored proteins, Src family proteins, B-cell receptors and T-cell receptors, constitutively reside in or move to lipid rafts on activation.[17] Recent data suggest that lipid rafts play a role in CD40 signaling. Like other TNFR family members, CD40 may reside in these membrane microdomains, and substitution of the CD40 transmembrane domain with that of CD45 excludes the resulting CD40-CD45 chimera from lipid rafts and abolishes the response to CD40 agonists.[18] CD40 engagement by either sCD40L or mCD40L recruits TRAF2 and TRAF3 to lipid rafts.[19] Therefore, disruption of lipid rafts by MCD in dendritic cells abolishes proximal CD40 signal transduction via recruited TRAF2 and TRAF3 and integral raft-associated Src family kinases.[20] However, whether CD40 constitutively resides within lipid rafts or translocates into lipid rafts after stimulation is still controversial, and whether and how lipid rafts play a role in regulation of CD40 endocytosis per se and proinflammatory signaling in ECs remains unknown.

In the present study, we demonstrated that G28.5 and sCD40L efficiently induced CD40 internalization via a nonclathrin, cho-

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lesterol-dependent caveolae rafts pathway, whereas mCD40L mimic, a ribbon-like structure of multimeric sCD40L, induced clustering of lipid rafts on cell surface independently of cholesterol. Moreover, CD40 downstream signaling exhibited a bifurcated mode of cholesterol-dependent and -independent responses to either soluble or membrane bound agonist.

Materials and Methods

Please see supplemental materials (available online at http://atvb.ahajournals.org) for description of reagents, cell lines, and biochemical analysis of caveolae rafts.

Fluorescence Microscopic Analysis of CD40 Endocytosis and Traffic

Where indicated, cells were fixed with 4% papraformaldehyde on ice for 10 minutes before image acquisition with a fluorescence microscopy (Nikon TE2000-E, 60 × oil immersion objective lens and z-step motor) through YFP and Cy5 filter channels as controlled by SlideBook 4.0 workstation (Intelligent Imaging Innovation). Typically, 10 2-dimentional images were obtained from planes above and below focus planes, and deconvoluted after background subtraction using a nearest-neighbor method in SlideBook 4.0. Where indicated, 1 μg/mL CTxB Alexa Fluor 594, 1.2 μmol/L Transferring Alexa Fluor 633 or LysoTracker Green were added to PAE-YFP-CD40 or PAE-CFP-CD40 cells, and incubated at 37°C for indicated time. Inhibition of clathrin-coated pits endocytosis was performed as previously described.[21] In brief, PAE-CD40-YFP cells were exposed to a hypertonic buffer (0.45 mol/L sucrose in serum-free F-12 medium, 37°C) or cytosolic potassium depletion buffer (100 mmol/L NaCl, 50 mmol/L HEPES, pH7.3, 1 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$ and 10 mmol/L glucose, 4°C) for 30 minutes before addition of various CD40 ligands.

Lipid rafts were visualized with Vybrant Alexa Fluor 594 Lipid Raft Labeling Kit according to manufacturer's manual. Briefly, CTxB fluorescent conjugate was added to cells on ice for 10 minutes. After cold PBS washes, anti-CTxB antibody was added and further incubated on ice for 15 minutes before image were acquired through RFP channel. CTxB fluorescent conjugate labeling after crosslinking by anti-CTxB enable cluster of CTxB on cell membrane, whereas CTxB alone can internalize with its binding receptor GM1 into cells. To assess formation of large patches on the cell surface induced by megamer, 2 μg/mL megameric CD40L was added PAE-CD40-YFP cells and incubated at 37°C for 30 minutes before addition of anti–IgG-TRITC secondary antibody. Images were acquired through YFP and RFP filter channels, respectively.

Punta overlap as indicative of colocalization in above assays was assessed by mask overlap (individual) using Slidebook software. In brief, 2 different punta masks (eg, CD40 and Transferrin punta) were manually selected using threshold segmentation on acquired images. Mask overlap (individual) was counted and CD40 puncta overlapped with indicated puncta (eg, Transferrin) was represented as a percentage to total CD40 puncta. Ten cells were used in analysis and averaged (mean±SD).

Flow Cytometry

Endocytosis efficiency of CD40 or Transferrin receptor was determined by measuring intracellular mean fluorescence intensities (MFI) of G28.5-Fluorescent Red 646 or Transferrin Alexa fluor 633, respectively. In brief, cells were serum starved for 1 hour before incubated with 2 μg/mL G28.5-Fluorescent Red 646 or Transferrin Alexa Fluor 633 at 37°C for the indicated time. G28.5 or Transferrin not internalized and retained on cell membrane was then acid washed on ice (0.2 mol/L Acetic acid, pH 2.8, 0.5 mol/L NaCl), and cells were dislodged with 0.125% trypsin-EDTA and resuspended in PBS. MFI of internalized G28.5 or Transferrin was analyzed by flow cytometry (10 000 events) with CellQuest Pro software (FACSCalibur or FACSVantage SE, BD Biosciences). Residual fluorescence of G28.5 or Transferrin bound to cells on ice (on ice, the 2 could not internalize) after cold acid wash was measured and treated as background MFI for subtraction. To measure the inhibitory efficiency of sucrose or potassium depletion on CD40 endocytosis, PAE-CD40-YFP cells were first subject to hypertonic buffer or cytosolic potassium depletion buffer for 30 minutes as mentioned above, and intracellular MFI of internalized G28.5-Cy5 was measured similarly. Where indicated, surface MFI was also used to quantify G28.5/EA-5 retained on cell membrane. In brief, after PAE-CD40-YFP cells were incubated with G28.5/EA-5 for the indicated time. Excessive G28.5 was removed by PBS washes, and G28.5 retained on cell surface was labeled with 1 μg/mL anti-mouse IgG-Cy5 at 4°C for 1 hour before fluorescence-activated-cell sorter (FACS) analysis (10 000 events). The MFI of cells incubated with anti-mouse IgG-Cy5 on ice was used as background MFI. Necrotic cells was measured by standard propidium iodide (PI) exclusion staining. In brief, MCD treated or not treated cells were dislodged with trypsin and 2 μg /mL PI was added for flow cytometric analysis (FACSCalibur, BD Biosciences).

All data were the average of 3 independent experiments and represented as mean±SD.

Results

CD40 Endocytosis via Clathrin-Independent but Caveolae-Dependent Pathway

We and others[19,22] have demonstrated that G28.5 mimicks recombinant sCD40L to efficiently and specifically induce internalization of CD40 (supplemental Figure I) independent of TRAF binding; however the exact mechanism remains unclear. Transferrin is usually internalized via clathrin coated pits that mobilizes with intracellular EGFR-GFP vesicles in PAE cells (supplemental Figure IIA-a). However, when we treated PAE-YFP-CD40 cells with G28.5, no apparent colocalization of intracellular CD40-YFP and transferrin-Alexa Fluor 633 occurred (supplemental Figure IIA-a) as revealed by the low percentage of overlapped puncta (supplemental Figure IIA-b). Additionally, internalized receptors via the clathrin pathway usually routes to lysosomes for degradation; however, during the time span we observed (0 to 14 hours), no colocalization of CD40-YFP and LysoTracker occurred (supplemental Figure IIAc-d). Purified sCD40L induced similar internalization and trafficking patterns of CD40 (data not shown). Taken together with the findings that hypertonic media (0.45 mol/L sucrose) or cytosolic potassium depletion did not affect CD40 internalization (supplemental Figure IIB-C), the above evidence strongly indicates that CD40 internalization in response to soluble agonists is clathrin-independent.

Such clathrin-independent endocytosis of CD40 prompted us to ask whether a lipid raft-dependent pathway might be involved in regulation of CD40 internalization in ECs. Lipid rafts can be microscopically visualized by fluorescence labeling of the raft protein ganglioside GM1 with CTxB.[23,24] In resting PAE-CD40-YFP cells on ice, CD40 colocalized with CTxB that was evenly distributed on the cell surface (Figure 1A, a). G28.5 crosslinking caused the comigration of CD40 and CTxB into the cytoplasm, (up to 5 hours, Figure 1A, a-b), suggesting that lipid rafts involve in the endocytosis of CD40. To confirm this finding, we used MCD to deplete cholesterol or filipin to sequester cholesterol in cell membranes, a commonly used method to disrupt lipid rafts.[25,26] Flow cytometric analysis showed that decreased cholesterol levels caused by MCD (Figure 1B, b) correlated with increasing inhibi-

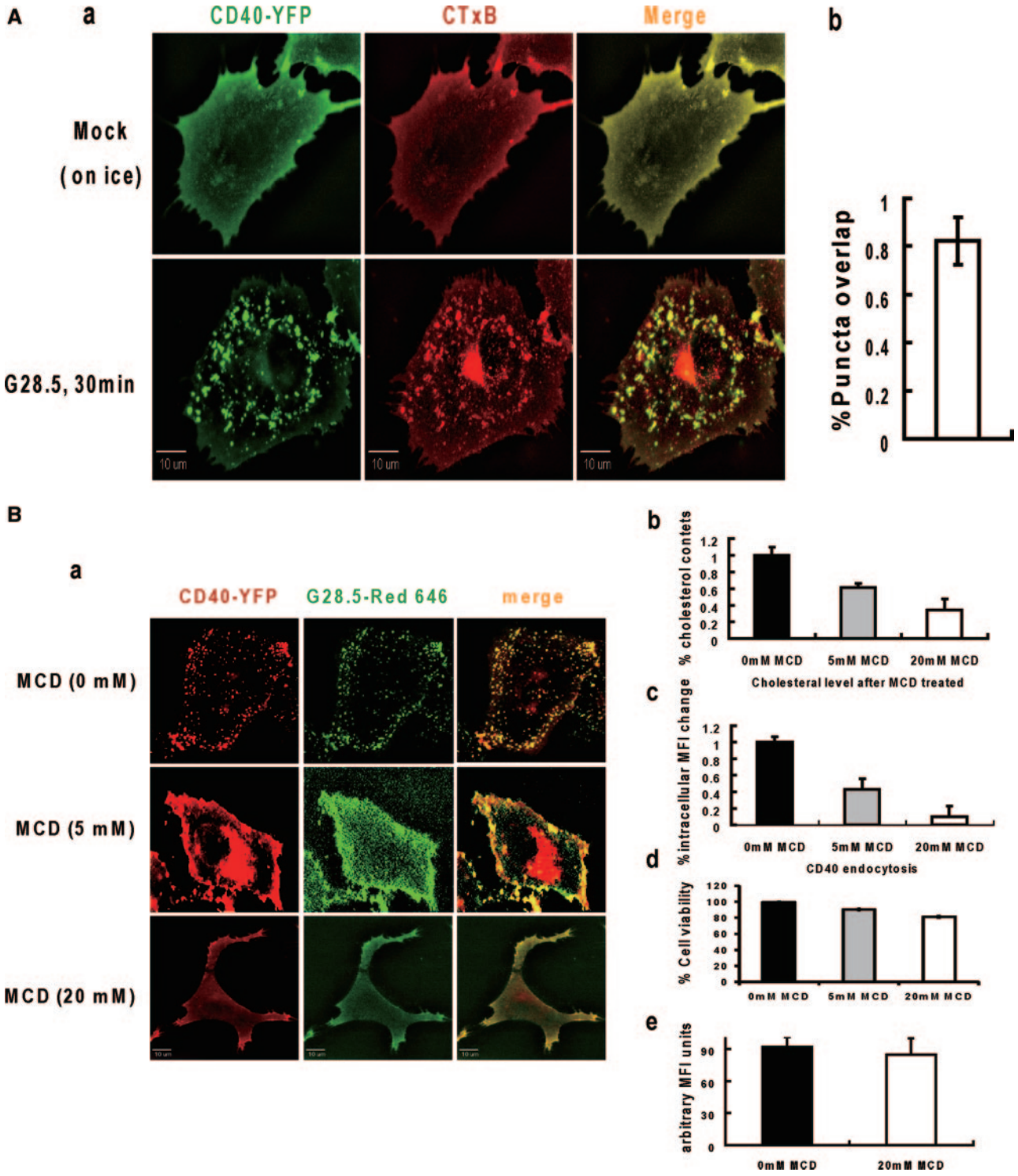

Figure 1. CD40 internalization is lipid-raft dependent. A, Colocalization of CD40 and CTxB. PAE-CD40-YFP cells were incubated with G28.5 and CTxB Alexa Fluor 594 on ice (a, top panel) or further incubated at 37°C (a, bottom panel). Colocalization was depicted by overlaying images acquired through YFP (CD40) and DsRed (CTxB) channels. b, Percentage of CD40 puncta overlap with those of CTxB. Data were the average of 10 cells from 3 independent view fields (mean±SD). B, Cholesterol depletion by MCD inhibits CD40 internalization. a, PAE-CD40-YFP cells were pretreated with 0, 5, or 20 mmol/L MCD at 37°C for 20 minutes before addition of G28.5-Fluorescent Red 646 for additional 30 minutes. Endocytosis of CD40/G28.5 complex was detected with fluorescent microscopy performed as in Figure 1A. b, MCD effectively reduced membrane cholesterol mass as measured by Filipin staining using FACS analysis (λ=568 nm excitation). c, Intracellular MFI of internalized G28.5-Fluorescent Red 646 was reduced by MCD in a dose-dependent manner as compared to that without MCD pretreatment. d, MCD does not cause significant cell death. PI staining was applied to detect necrotic cells at different concentrations of MCD used in a-c. e, Surface MFI of G28.5-Fluorescent Red after PBS wash was measured before or after MCD treatment. All above data were the average of at least 3 independent experiments (mean±SD).

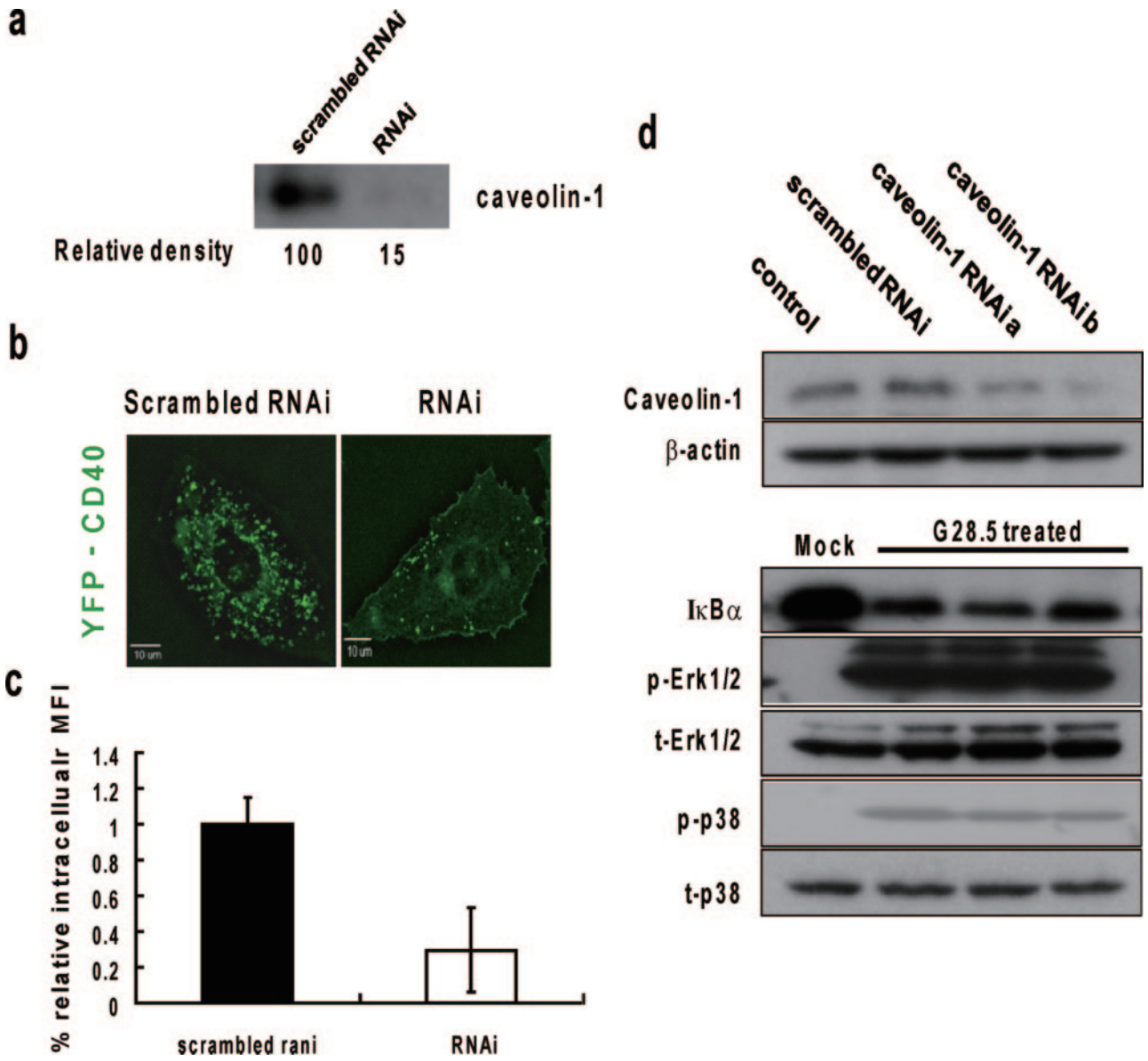

Figure 2. Caveolae are required for CD40 endocytosis but not CD40 signaling. a, Cell lysates from PAE-CD40-YFP cells stably expressing caveolin-1 siRNA or scrambled siRNA were immunoblotted against caveolin-1, with relative densities showing knockdown efficiency. b, PAE-CD40-YFP cells expressing caveolin-1 siRNA or scrambled siRNA were treated with G28.5 at 37°C for 2 hours, and intracellular vesicles were captured using method as in Figure 1A. c, Intracellular MFI of G28.5-Fluorescent Red 646 was FACS quantified as Figure 1B. The results were presented as the average of 3 independent experiments (mean±SD). d, PAE-CD40-YFP cells transfected with scramble siRNA, or caveolin-1 siRNA-a or siRNA-b (top panel indicating knockdown efficiency) were stimulated with G28.5 or mock for 30 minutes, cell lysates were Western blotted for degradation of IκBα, phosphorylation of Erk1/2 (p-Erk1/2) and MAPKp38 (p-p38). Levels of β-actin or total Erk1/2 (t-Erk1/2), MAPKp38 (t-p38) were blotted as the loading controls.

tion of CD40 internalization in a dose-dependent manner (Figure 1B, a and c). MCD at these concentrations did not cause significant cell death (Figure 2B, d), or interfere with G28.5 binding to the cell surface (Figure 1B, e). Filipin treatment of cells also inhibited the endocytosis of CD40 in a dose-dependent manner (supplemental Figure III). Therefore, disruption of cholesterol content, and thus the structure of lipid rafts inhibited CD40 internalization.

The lipid raft pathway of receptor endocytosis can be either caveolae-dependent or -independent. Early studies show that CD40 localizes in caveolae, which are required for CD40 signaling in human renal epithelial cells.[27] Indeed, siRNA knockdown of caveolin-1 expression in PAE-CD40-YFP cells (Figure 2a) caused a reduction in CD40 internalization (Figure 2b) by ≈3-fold (Figure 2c) as compared with that in scrambled siRNA treated cells, strongly indicating that endocytosis of CD40 in response to G28.5 uses a lipid raft pathway that is clathrin-independent but caveolae-dependent. Contrary to previous findings in epithelial cells,[27] however, caveolae seemed dispensable for CD40 signaling in ECs, as downregulation of caveolin-1 expression by siRNA did not interfere with CD40 downstream effectors, ie, activation of the NF-κB, Erk1/2, or MAPKp38 pathways (Figure 2d).Thus, inhibition of CD40 internalization attributable to disruption of caveolae does not appear to effect assembly of a functional signaling complex on cell surface.

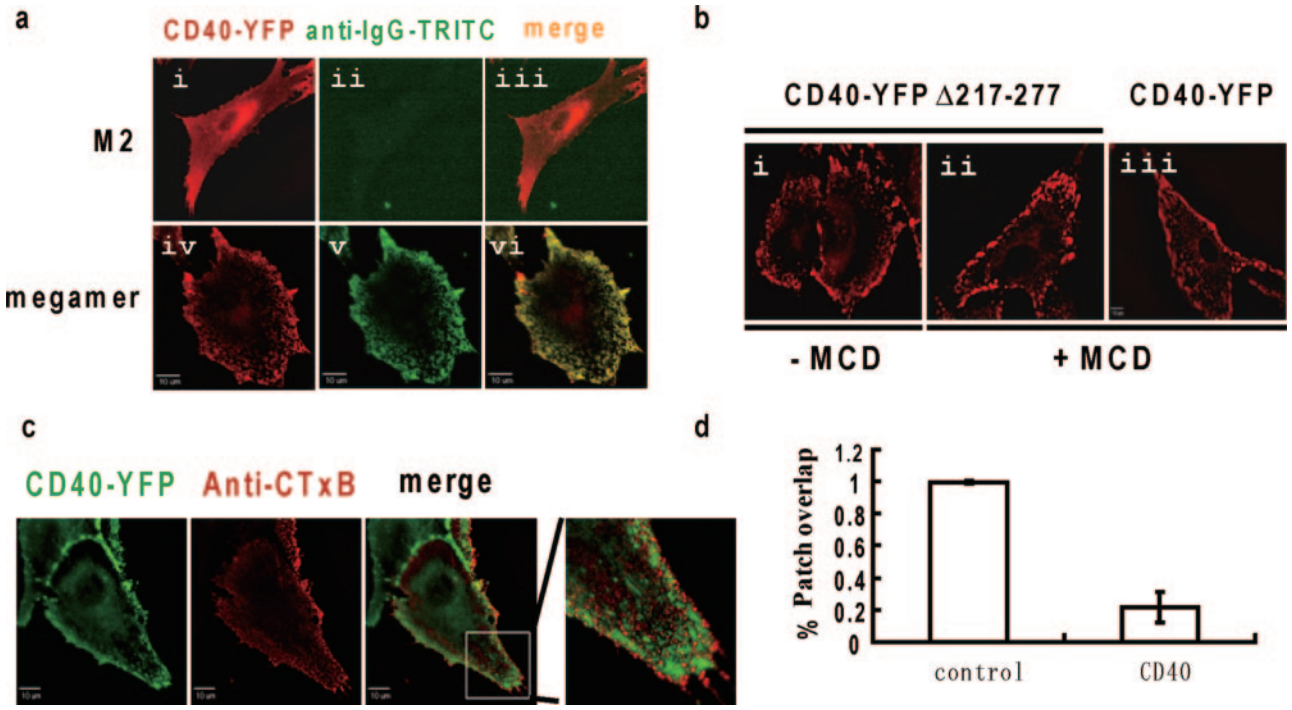

Figure 3. Unique CD40 raft clusters induced by megameric CD40L. A, CD40 patches dissociate from CTxB rafts. a, PAE-CD40-YFP cells were treated with 2 μg/mL anti-FLAG M2 mAb (i-iii) or megamer (iv-vi) at 37°C for 30 minutes, and the large patch clustering of CD40-YFP on live cells was captured through YFP channel (iv). After cells were fixed and immunostained with anti-mouse IgG-TRITC (1:100) against M2 FLAG mAb, surface distribution of CD40L was shown (v). b, Formation of large CD40 clusters is independent of TRAFs binding. PAE cells that stably express CD40-YFP mutant variant without the entire cytoplasmic domain (CD40-YFPΔ217—277, i-ii) or PAE-CD40-YFP (iii) were incubated with megamer without (i) or with MCD pretreatment (ii-iii). Large CD40 patches were depicted by fluorescence microscopy. c, CD40 patches dissociate from CTxB rafts. After PAE-CD40-YFP cells were stimulated with megamer at 37°C for 30 minutes, the reaction was stopped by cooling on ice for 15 minutes. CTxB-containing lipid rafts were visualized with a Lipid Raft Labeling Kit as described in Materials and Methods. Superimposition was enlarged for better judgment of separation of CD40 clusters from CTxB clusters. d, Percentages of CD40 patches overlapping with those of CTxB was plotted as Figure 1A after normalized to total CD40 patches.

Megamer-Induced Formation of Large Patches of CD40 on Plasma Membrane Outside of CTxB Positive Lipid Rafts

We have previously shown that CD40L expressed on the surface of HEK293A cells (mCD40L) induces CD40 clustering at the site of contact between the cell junctions.[22] To further investigate the mechanism of CD40 clustering, we used multimeric CD40L (hereafter designated as megamer) as a surrogate form of mCD40L.[28,29] Megamer is made of recombinant FLAG-tagged sCD40L crosslinked with anti-FLAG M2 antibody, which yields a ribbon-like structure of sCD40L, and induced cell-surface clustering of CD40 on literally every cell (Figure 3a, compare i and iv for enlarged patches). Immunostaining showed that CD40L did not form intracellular vesicles (Figure 3a, iv) but colocalized with CD40-YFP on cell surface (Figure 3a, vi). These patches were functionally equivalent to those induced by mCD40L because megamer activated CD40 signaling as efficiently as mCD40L (see also Figure 5A). CD40 lacks intrinsic catalytic activity, but it uses the cytoplasmic tail to recruit various TRAFs to activate Jun N-terminal protein kinase (JNK), phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and NF-κB signaling pathways in a cell type–dependent fashion.[3,30] Binding of TRAF2 to the CD40 cytoplasmic tail plays a role in formation of CD40 microdomain structure.[19] However, when we stably expressed a CD40 mutant whose TRAF binding sites are completely removed in PAE cells (PAE-CD40Δ217 to 277-YFP), megamer still induced formation of the large CD40 aggregation (Figure 3b-i). These patches were resistant to cholesterol depletion by MCD (Figure 3b-ii) similar to wild-type CD40-YFP (Figure 3b-iii). More surprisingly, such large patches seemed to cluster in microdomains different from the conventional, CTxB positive lipid rafts in PAE-CD40-YFP cells (Figure 3c), with less than 30% overlap (Figure 3d). Therefore, megameric CD40L clusters and partitions its cognate receptor into a unique liquid-ordered phase independent of cholesterol (see also Figure 4) or TRAF binding. The detailed mechanism remains to be understood. That binding of mCD40L to the extracellular domain of CD40 per se would be sufficient for patch assembly is very reminiscent of recent finding that membrane FasL-induced Fas clustering in lipid rafts is independent of the cytoplasmic tail and resistant to cholesterol depletion.[31]

Cholesterol-Dependent and -Independent Signaling by CD40

The observation of CTxB-associated and nonassociated CD40 signaling complex formation triggered by G28.5 and megamer, respectively, raised the question of whether CD40 constitutively resides in or translocates into lipid rafts after stimulation. Previous studies on this issue yielded rather opposite interpretations attributable to different detergents and density cushions used in gradient centrifugation. We assessed the localization and

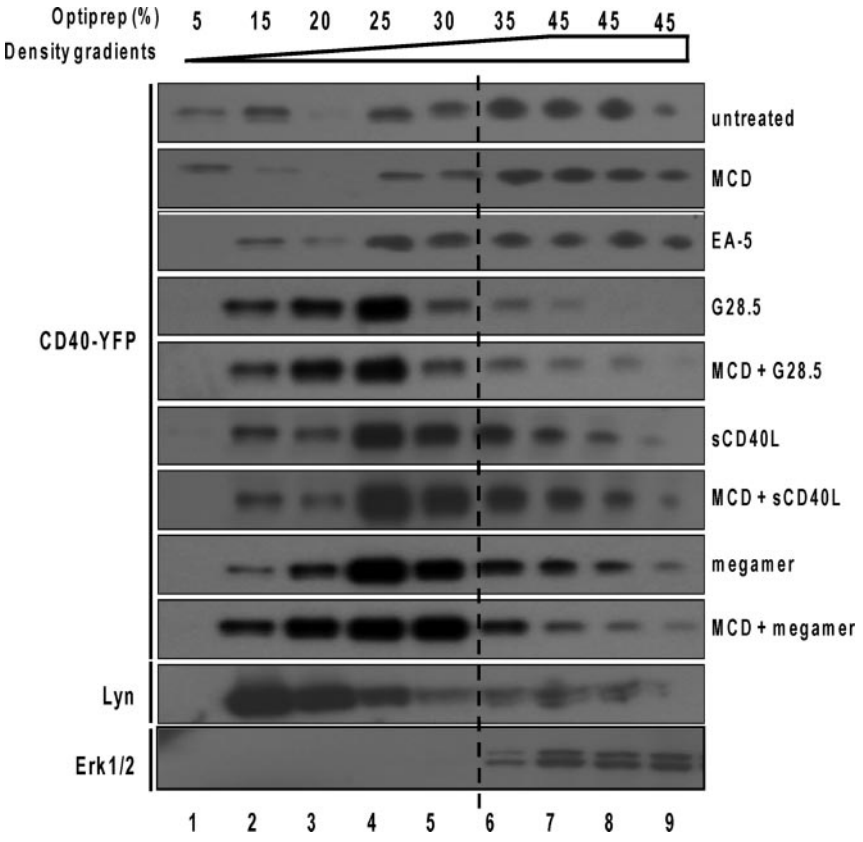

Figure 4. Cholesterol-independent translocation of activated CD40 into low-buoyant density rafts. PAE-CD40-YFP cells were untreated or treated with 5 mmol/L MCD for 30 minutes, followed by addition of various CD40 agonists (G28.5, EA5, sCD40L, or megamer). Brij58-insoluble (lanes 1—5) and -soluble fractions (lanes 6—9) were separated by Optiprep gradient centrifugation as described in Methods. The dash line in between densities 30 and 35% was set arbitrarily based on separation of Lyn (a marker for Brij58 insoluble rafts) and Erk1/2 (a marker for Brij58-soluble fractions). Distribution of CD40-YFP in different liquid phases was tracked by immunoblotting with anti-GFP antibody. The result was representative of at least 5 independent experiments.

traffic of CD40 using 1% cold Brij58 extraction[19] followed by Optiprep buoyant density centrifugation[32] before and after MCD depletion of cholesterol. The results showed that CD40 was evenly distributed in cold Brij58-soluble and -insoluble fractions in resting or MCD-treated cells (Figure 4, top 2 panels). CD40 agonists (G28.5, sCD40L, and megamer) except for EA5, induced CD40 to translocate to low-density Brij58-insoluble lipid rafts (Figure 4, lanes 2 to 4), which was reminiscent of previous findings.[32] This was different from other findings which show constitutive residence of CD40 in lipid rafts[19] or even outside of raft microdomains.[33] Moreover, cholesterol depletion by MCD did not alter the distribution or translocation pattern of CD40 in the lipid-ordered phase after CD40 engagement (Figure 4). This method was validated by the observation that Lyn, a protein that constitutively resides in lipid rafts,[34] and cytoplasmic Erk separated well in Brij58-insoluble and -soluble phases, respectively (Figure 4). Lipid rafts are heterogeneous in protein and lipid contents, and not equally sensitive to cholesterol depletion.[13] That CD40 rafts induced by G28.5 or megamer were insensitive to MCD, and that megamer-induced CD40 rafts were intrinsically different from conventional CTxB-positive microdomains, suggests distinct microdomains for activated CD40 on the cell membrane.

To further characterize how cholesterol-independent CD40 rafts transduce signaling, we assessed the activation of CD40 downstream effectors after MCD treatment. Western blotting showed that the different agonists possessed qualitatively similar activities because they activated CD40 signaling well in terms of NF-κB activation (degradation of IκBα) and phosphorylation of Erk1/2 in PAE-CD40-YFP (supplemental Figure IV). Megameric CD40L in general yielded more sustainable and potent activation of these signaling pathways than G28.5 or sCD40L in a time course analysis (data not shown). However, after PAE-CD40-YFP cells were pretreated with MCD, G28.5, and sCD40L failed to activate NF-κB and Erk1/2 (Figure 5A). More surprisingly, cholesterol depletion by MCD did not affect megamer or mCD40L-induced NF-κB and Erk1/2 activation at all (Figure 5A). MCD itself did not activate the NF-κB or Erk1/2 pathways. MCD possesses multiple functions other than cholesterol depletion (Pizzo et al, 2002). To confirm that CD40 signaling activated by G28.5 requires cholesterol, we added water-soluble cholesterol back into MCD-treated cells and found that reconstitution of membrane lipid content restored CD40 internalization (data not shown) and NF-κB and Erk1/2 activation (Figure 5B). These results therefore indicated that, although formation of Brij58-resistant CD40 rafts was independent of cholesterol, G28.5 required cholesterol to activate CD40 internalization and appropriate CD40 downstream signaling. mCD40L, on the other hand, apparently induced clustering of CD40 rafts that were stable enough to resist cholesterol dissipation in downstream signaling.

Discussion

We and others[19,22] have demonstrated that G28.5 mimicks sCD40L in activation of CD40 internalization and corresponding endosomal signaling, whereas mCD40L triggers assembly of CD40 signaling complex on the cell surface. In this study, we show that, unlike most TNFR superfamily receptors,[35–37] CD40 endocytosis is clathrin-independent but caveolae-dependent. Caveolae are flask-shaped membrane

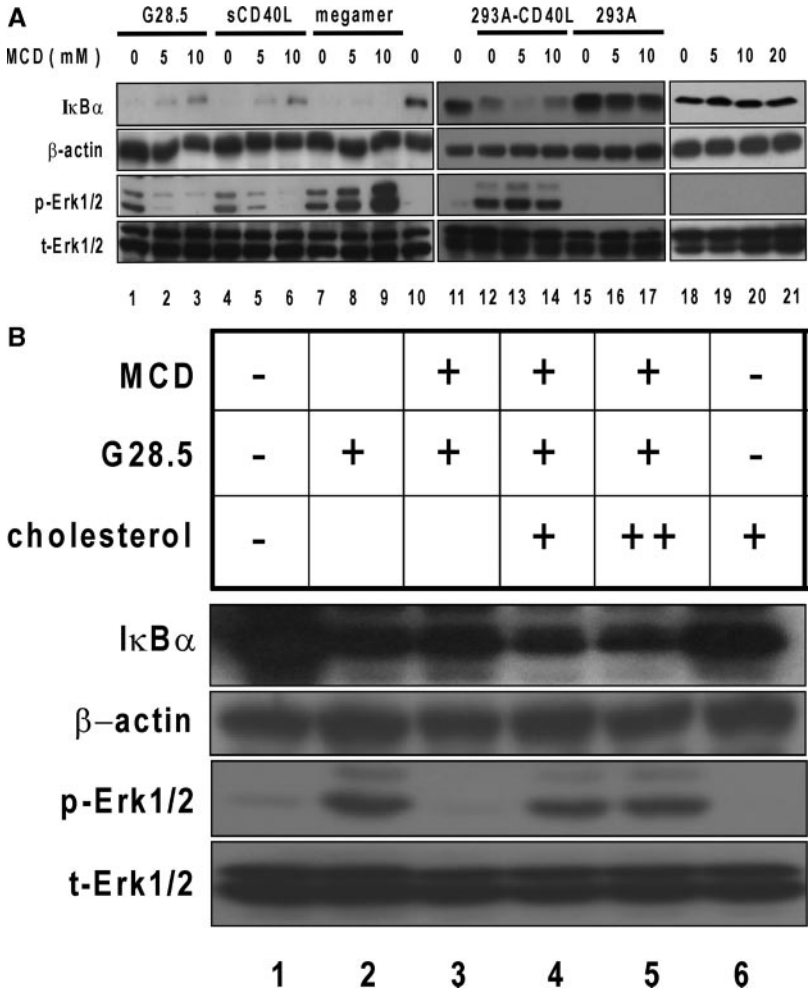

Figure 5. Cholesterol-dependent and -independent activation of CD40 signaling. A, Cholosterol levels differentially affect activation of CD40 signaling. PAE-CD40-YFP cells (1×10^6) were preincubated with increasing concentrations of MCD for 30 minutes, 2 μg/mL G28.5 (lanes 1 to 3), recombinant sCD40L (lanes 4 to 6), megamer (lanes 7 to 9), 5×10^6 HEK293A-CD40L (lanes 12 to 14), or HEK293A (lanes 15 to 17) cells were then added for 30 minutes. Excessive agonists were thoroughly washed off with ice-cold PBS, especially to remove HEK293A cells (within incubation for 30 minutes, 293A cells did not adhere to PAE cells). Activation of NF-κB and Erk1/2 was detected by Western blotting as in Fig 2. Basal levels of CD40 activation in cells not treated with G28.5 or MCD were shown (lanes 10 to 11). The effect of MCD on CD40 activation was performed in parallel (lanes 18 to 21). B, Cholesterol-dependent activation of CD40 signaling by G28.5. After PAE-CD40-YFP cells were treated with MCD (lanes 3 to 5), cellular cholesterol contents were reconstituted by addition of 50 (lane 4) or 100 μg/mL (lane 5) water-soluble cholesterol into culture medium at 37°C for 1 hour. G28.5 was then added for 30 minutes, and activation of NF-κB and Erk1/2 were performed exactly as that in A.

invaginations of the plasma membrane that serve as an alternative raft route for receptor endocytosis.[38] Such caveolae-dependent internalization agrees with previous findings that CD40 and its major downstream signaling components are assembled in caveolae.[27] However, dependence on caveolae for CD40 downstream signaling exhibits cell-type specificity, being required in epithelial[27] but not in cardiovascular ECs.

We further show that CD40 engagement by soluble or membrane-bound agonist induces its translocation to Brij58-resistent membrane fractions. This agrees with early observations that lipid rafts mediate CD40 signaling in lymphocytes.[19,20,32] Our observation also suggests that G28.5 and megamer induce the formation of different lipid rafts in terms of different CTxB association patterns and sensitivity to cholesterol depletion. The translocation of engaged CD40 to low buoyant density domains is unique in that it is insensitive to cholesterol depletion. Although cholesterol depletion abrogates phase segregation, one should be cautious to interpret this data because, besides its perturbation of liquid-ordered phase, MCD also has global inhibition of the lateral mobility of plasma membrane lipid/proteins irrespective of their putative association with liquid-ordered domains.[39] More careful experiments should also be performed to determine whether CD40 in detergent-resistant membrane fractions (Brij58 in this study) is truly associated with liquid-ordered domains in intact cells, by using more advanced imaging techniques that can explore short distances and timescales.[39] Nevertheless, cholesterol is required for signaling activation of CD40 by soluble but not membrane-bound CD40L, suggesting a drastic difference in CD40 raft structure and function.

Whereas G28.5-induced CD40 rafts exhibit conventionally small and short-life feature of liquid-ordered microdomains that are limited by endocytosis,[39] megamer is able to cluster CD40 rafts to form much larger surface patches. In fact, such receptor activation induced raft aggregation into micronsized quilt-like patches[26] has been shown to critically involve in various biological processes, such as T cell receptor[40] and Fas/CD95.[31] Such a larger scale platform would inevitably provide more stable rafts for spatial-temporal regulation of complex signaling. Moreover, it is rather intriguing that supermolecular raft clusters of CD40 induced by megamer are resistant to cholesterol depletion in signaling. One would speculate that ribbon-like multimeric CD40L crosslink CD40 lipid rafts to facilitate protein segregation by reducing its diffusion in plasma membrane that can counteract the partial cholesterol depletion (5 mmol/L MCD). On the other hand, CD40 raft patches might enable preferential association of lipid-anchored and transmembrane proteins for lipid organization and boundary determination. Such protein-protein

interaction within patches would further stabilize raft domains and render insensitivity to cholesterol depletion.

Our results suggest that cellular cholesterol levels differentially regulate CD40 internalization and signaling by the soluble or membrane-bound CD40L. Our previous work[22] and that of others[8,41] indicate that mCD40L but not sCD40L activates ECs with characteristic upregulation of adhesion molecules, proinflammatory cytokines and chemokines in NF-κB–independent manner. It is therefore reasonable to speculate that cholesterol- and caveolae-raft dependent CD40 endocytosis and signaling would be dispensable for proinflammatory activation of ECs. Rather, stable raft clusters induced by mCD40L may be required for activation of EC inflammation, reminiscent of previous findings of constitutive signaling activation of B-cell lymphoma by the so-called CD40 signalosome on the cell surface.[42] Such speculation is supported by preferential association of TRAF6 with raft-clustering CD40 on cell surface,[22] a critical adaptor in mediating CD40 proinflammatory signaling. TRAF6 is believed to be a facilitator of Caspase-8 recruitment to lipid rafts on TCR activation.[43] Whether TRAF6 association with aggregated CD40 is preferentially regulated by cholesterol remains to be understood. Further characterization is needed to verify whether cholesterol-independent patch formation contributes to pathophysiological functions of mCD40L in vascular endothelial activation.

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Disclosures

None.

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