Cytokine Secretion via Cholesterol-rich Lipid Raft-associated SNAREs at the Phagocytic Cup

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Lipopolysaccharide-activated macrophages rapidly synthesize and secrete tumor necrosis factor α (TNFa) to prime the immune system. Surface delivery of membrane carrying newly synthesized TNFa is controlled and limited by the level of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin 4 and SNAP-23. Many functions in immune cells are coordinated from lipid rafts in the plasma membrane, and we investigated a possible role for lipid rafts in TNFa trafficking and secretion. TNFa surface delivery and secretion were found to be cholesterol-dependent. Upon macrophage activation, syntaxin 4 was recruited to cholesterol-dependent lipid rafts, whereas its regulatory protein, Munc18c, was excluded from the rafts. Syntaxin 4 in activated macrophages localized to discrete cholesterol-dependent puncta on the plasma membrane, particularly on filopodia. Imaging the early stages of TNFa surface distribution revealed these puncta to be the initial points of TNFa delivery. During the early stages of phagocytosis, syntaxin 4 was recruited to the phagocytic cup in a cholesterol-dependent manner. Insertion of VAMP3-positive recycling endosome membrane is required for efficient ingestion of a pathogen. Without this recruitment of syntaxin 4, it is not incorporated into the plasma membrane, and phagocytosis is greatly reduced. Thus, relocation of syntaxin 4 into lipid rafts in macrophages is a critical and rate-limiting step in initiating an effective immune response.

In response to pathogens, activated macrophages produce and secrete tumor necrosis factor α (TNFa), a proinflammatory cytokine responsible for the activation and recruitment of cells necessary to mount a successful immune response (1). Lipopolysaccharide (LPS), a bacterial membrane component, is a potent activator of macrophages, binding to the CD14–MD2–TLR4 complex at the surface (2) that signals the induction of widespread gene transcription, including that of TNFa (3, 4). This ensures the rapid and abundant synthesis of the 26-kDa transmembrane form of TNFa, which initially accumulates in the Golgi complex (5) and is then trafficked to the cell surface for proteolytic cleavage by the TNFa-converting enzyme (TACE) (6). We have shown recently that TNFa is transported from the trans-Golgi network to the recycling endosome and is then delivered to the cell surface via fusion of the recycling endosome membrane at the site of the phagocytic cup formation (7, 8). Two distinct steps of membrane fusion are therefore required during the post–Golgi/Golgi transport of TNFa.

Membrane fusion of TNFa transport vesicles is mediated by members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein family (7, 8). Cognate pairing of an R-SNARE (generally on the vesicle) and a Q-SNARE complex (generally on the target membrane) is necessary at each trafficking step for specificity in vesicle docking and membrane fusion (9, 10). A variety of SNARE proteins is expressed in macrophages. The trans-Golgi Q-SNARE complex of syntaxin 6/syntaxin 7/Vti1b pairs with VAMP3 on the recycling endosome in the first step of post–Golgi transport of TNFa. Next the VAMP3 pairs with the cell surface Q-SNARE complex of syntaxin 4/SNAP-23 for TNFa delivery to the cell surface (7, 8, 11). Syntaxin 4, syntaxin 6, Vti1b, SNAP-23, and VAMP3 are up-regulated during LPS activation to accommodate the increased trafficking during TNFa secretion (7, 8, 11). During phagocytosis, the Q-SNARE syntaxin 4 concentrates at the phagocytic cup to mediate fusion of the recycling endosome, providing excess membrane for microorganism engulfment and rapidly delivering TNFa to the cell surface for secretion (7).

Lipid rafts, specific membrane microdomains that are enriched in sterols and sphingolipids, play a number of important roles in immune cells; for instance, they contribute to B cell receptor signaling and antigen uptake (12, 13), ligand-mediated signaling from the T cell receptors (14, 15) and high affinity IgE receptors (16), and play a role in MHCII-mediated antigen presentation (17, 18). In macrophages, phagosomes are enriched in raft proteins (19–21). In other cell types lipid rafts have been shown to organize and regulate SNARE proteins (22, 23). We thus investigated a possible role for lipid rafts in SNARE-mediated delivery of TNFa to the cell surface. We show here that an inducible association of syntaxin 4 with lipid rafts and a cholesterol-rich membrane environment at specific exit sites are required for TNFa secretion.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal and rat monoclonal antimouse TNFa antibodies were purchased from Calbiochem and Auspep (Victoria, Australia), respectively. Anti-SNAP-23 and VAMP2 antibodies were purchased from Synaptic Systems (Goettingen, Germany); anti-Gαq3 antibodies were purchased from DuPont; and antibodies specific for flotillin and syntaxin 6 were purchased from BD Biosciences. Antibodies specific for TACE were purchased from Chemicon (Temecula, CA), and anti-VAMP3 antibodies were purchased from Abcam (Cambridge, UK). Alexa-488 or Alexa-647 conjugated to phalloidin to label F-actin was from Molecular Probes (Eugene, OR), and filipin III was purchased from Sigma. Anti-actin antibody was a gift from Peter Gunning (Children’s Hospital at Westmead, Sydney, Australia), and antibodies specific for syntaxin 4 and Munc18c were the kind gifts from Robert Ashman (University of Queensland, Brisbane, Australia).
Lipid Raft-mediated Cytokine Secretion

Cell Culture, Activation, and Cholesterol Depletion—RAW264.7 murine macrophages were grown in RPMI 1640 medium with 10% serum and 1% L-glutamine as described previously (24). Cells were activated with 100 ng/ml LPS (Salmonella minnesota Re 595, Sigma) in the presence or absence of TACE inhibitor (BB-3103, British Biotech Pharmaceuticals). For cholesterol depletion, cells were incubated for 15–30 min in 7.5 mM methyl-β-cycloextrin (MβCD) (Sigma) in serum-free RPMI 1640.

Phagocytosis—In some experiments macrophages were primed for 18 h in the presence of 500 pg/ml IFNγ (R & D Systems) prior to their incubation with live C. albicans at a ratio of 10:1 (yeast:macrophage) for 10–40 min as described previously (7).

Sucrose Density Gradient Separation—Sucrose density gradient separation of membrane extracts was performed according to published protocols (25). Briefly, macrophages were lysed by 20 passages through a 27-gauge needle in homogenization buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) containing either 0.2 or 1% Triton X-100, and the lysate was centrifuged at 2000 × g for 2 min at 4 °C to pellet unbroken cells and nuclei. The supernatant was loaded onto a 45 to 5% discontinuous sucrose gradient and centrifuged at 200,000 g for 18 h. Eleven fractions (150 μl) were collected from the top of the gradient, plus an additional fraction of 600 μl representing the load fraction. Samples were subjected to SDS-PAGE separation and analyzed by immunoblotting as described previously (5).

Immunofluorescence Staining—Immunofluorescence staining was performed as described previously (5). In some experiments cells grown on coverslips were partially air-dried, and the apical membranes were removed by application (30 s) and removal of damp filter paper (Millipore) (26). The remaining adherent plasma membrane and cell fragments were fixed and immunostained. Cholesterol in cell membranes was stained using 250 μg/ml filipin III in phosphate-buffered saline containing 0.5% bovine serum albumin. Epifluorescence microscopy was performed using an Olympus Provis AX70 microscope equipped with a 100× oil objective and MTI digital camera and NIH Image software. Confocal microscopy was performed using an LSM 510 META (Carl Zeiss Microscope Systems). Three-dimensional reconstructions were generated using LSM 510 META software.

Assays for TNFα Trafficking and Secretion—The trafficking of TNFα from the Golgi complex to the cell surface was measured as described previously using an immunofluorescence-based assay (11). To determine levels of secreted TNFα, a commercial enzyme-linked immunosorbent assay kit (OptEIA, BD Biosciences) was used according to the manufacturer’s instructions.

RESULTS

SNARE Recruitment to Lipid Rafts—Cholesterol-rich membrane domains and their associated proteins, typically insoluble in nonionic detergents, can be isolated by flotation on sucrose density gradients (27). Macrophage membranes extracted with 1% Triton X-100 were investigated by sucrose density gradient separation and immunoblotting to analyze the distribution of SNARE proteins involved in TNFα secretion (Fig. 1, A and B). Cholesterol-rich lipid raft domains (fractions 5–8) were characterized by the enrichment of known raft marker proteins flotillin (25, 28) and the heterotrimeric Goαi subunit (29) (Fig. 1A). The cell surface Q-SNARE protein SNAP-23 distributed throughout both raft and nonraft fractions, although its Q-SNARE partner syntaxin 4 was recovered almost entirely in nonraft fractions (Fig. 1A). Munc18c, a protein known to bind and regulate syntaxin 4 (30), also partitioned in nonraft fractions (Fig. 1A). The trans-Golgi Q-SNARE syntaxin 6 (8) segregated into nonraft fractions, although the R-SNAREs VAMP2 and VAMP3 were detected in discrete fractions from both raft and nonraft fractions (Fig. 1A).

LPS activation of macrophages induces increased expression of syntaxin 4 (11) and initiates a 4-fold recruitment of syntaxin 4 to raft domains, resulting in over 40% of cellular syntaxin 4 now being concentrated in lipid rafts (Fig. 1, B and C). The distributions of other proteins analyzed were unchanged after LPS activation (Fig. 1, B and C). Thus, the differential distributions of the Q-SNARE components SNAP-23 and syntaxin 4 in lipid raft domains and the LPS-induced recruitment of syntaxin 4 to rafts may represent a mechanism for modulating vesicle-mediated delivery to the cell surface during TNFα secretion.

Syntaxin 4 Localizes to Cholesterol-dependent Puncta with LPS Activation—We next examined the distribution of syntaxin 4 on the plasma membrane by immunostaining. Because SNARE proteins are typically on the cytoplasmic face of the plasma membrane, staining was performed on patches of ripped open cells exposing the cytoplasmic face of the plasma membrane (26). Staining of F-actin provided orientation for identifying and viewing cell patches. Prior to macrophage activation, syntaxin 4 was localized to distinct puncta (~0.5–1 μm diameter) randomly distributed on the cytoplasmic face of the plasma membrane (Fig. 2A). Upon macrophage activation, syntaxin 4-stained puncta on the cell body increased in size (~1–2 μm), whereas smaller syntaxin 4 puncta (less than 500 nm) also appeared de novo on filopodia (Fig. 2B). Treatment of cells with MβCD depletes cholesterol from cell membranes disrupting cholesterol-dependent lipid raft domains (31, 32). Cholesterol depletion with MβCD resulted in a significant loss of syntaxin 4 puncta from the cell body in activated cells and a complete loss of small syntaxin 4 puncta on filopodia (Fig. 2C). Disruption of cholesterol with MβCD in LPS-activated macrophages also resulted in depletion of syntaxin 4, and the raft markers flotillin and Goαi from sucrose density
Surface Delivery of TNFα Is Cholesterol-dependent—Treatment of LPS-activated macrophages with MβCD revealed that secretion of TNFα, but not its synthesis, is cholesterol-dependent. Secretion of soluble TNFα, as measured by an enzyme-linked immunosorbent assay, was greatly reduced (>60%) after cholesterol depletion in activated cells (Fig. 3A). This reduction in surface delivery of TNFα was confirmed by using an immunofluorescence-based assay that measures the transport of newly synthesized TNFα from the Golgi complex to the cell surface (11). TNFα is labeled on the surface of LPS-activated macrophages (Fig. 3B), although depletion of cholesterol effectively blocks this surface delivery of TNFα (Fig. 3B). LPS signaling through the CD14-MD2-TRR4 complex occurs in lipid rafts, and pretreatment of monocytes with MβCD has been shown to inhibit TNFα secretion (33); however, the p38 and c-Jun N-terminal kinase signaling pathways are reportedly initiated (34). Consistent with this, TNFα staining is present at the level of the Golgi complex, suggesting TNFα surface delivery rather than its synthesis is disrupted (Fig. 3C). In addition, depletion of cholesterol had no effect on the total levels of TNFα present in LPS-activated cells (Fig. 3D). Thus, disruption of cholesterol-dependent lipid rafts effectively reduced TNFα secretion by blocking its surface delivery, without affecting earlier steps in its synthesis or trafficking.

Cholesterol-dependent Syntaxin 4 Puncta Represent the Delivery Sites for TNFα—Additional sucrose density experiments were performed using 0.2% Triton X-100 (35), producing a cleaner separation of the raft and nonraft fractions. Lipid raft fractions 5–8 are concentrated on the surface of filopodia. TNFα and syntaxin 4 staining are seen to colocalize in adjacent images, both appearing at the same points and on the same surface features (arrows).
TACE inhibitor and is partially lipid raft-associated (Fig. 4A). Confocal imaging was used to examine the relationship between syntaxin 4 and newly synthesized TNFα delivery to the cell surface. To capture and stain TNFα at the surface, its proteolytic release was prevented by using a TACE inhibitor, and the external aspect of the cell surface was imaged on intact macrophages shortly after LPS activation. Three-dimensional composite confocal images were enhanced by surface rendering to remove background staining and reveal TNFα surface staining (Fig. 4B). Under these conditions, the earliest appearance of newly synthesized TNFα was observed in discrete patches on the macrophage surface. TNFα patches (Fig. 4B, red labeling) were notably concentrated on filopodia where they colocalized with the syntaxin 4 puncta (Fig. 4B). At later times (1–2 h), TNFα staining becomes more widely spread across the cell surface (data not shown and Fig. 3B), either because of additional delivery sites being recruited or because of retention and spreading of uncleaved TNFα. Thus, these syntaxin 4 patches correspond to the cholesterol-dependent syntaxin 4 puncta labeled on filopodia in R-SNAREs delivered open cells and can now be pinpointed as the initial delivery sites for TNFα on the macrophage surface. Interestingly, TACE, the enzyme responsible for cleaving TNFα at the cell surface (6), is totally excluded from lipid raft fractions (36) (Fig. 4A), suggesting that surface delivery and TNFα cleavage occur in different membrane domains.

**Cholesterol Dependence of SNARE Accumulation and TNFα Delivery to the Phagocytic Cup**—During the phagocytosis of C. albicans, TNFα is delivered to the actin-rich phagocytic cup (7) (Fig. 5A) where TACE also accumulates (7) (Fig. 5C). Disruption of cholesterol-dependent lipid rafts inhibited TNFα surface delivery to the phagocytic cup without affecting TNFα delivery (Fig. 5B). Macrophages were stained for cholesterol using filipin III revealing the presence of cholesterol-rich membrane domains in the phagocytic cups (Fig. 5D). Syntaxin 4 also concentrates in the phagocytic cups (Fig. 6A). Syntaxin 4 localization to the actin-rich phagocytic cup is dependent upon cholesterol. Bar, 10 μm. 8, IFNγ-primed macrophages were incubated as in A and stained for VAMP3 and F-actin. VAMP3 localization to the actin-rich phagocytic cup is dependent upon cholesterol. 36) (Fig. 6B). Like-
TNFα. TACE, the enzyme responsible for cleavage of TNFα at the cell surface, is excluded from these raft domains suggesting that TNFα is translocated to other membrane subdomains prior to its cleavage. During the initial stages of phagocytosis, syntaxin 4 recruitment to lipid rafts is concentrated at the phagocytic cup and is crucial for the delivery of the excess membrane required to engulf a microorganism in addition to the rapid delivery and secretion of TNFα.

It has been suggested that spatial distribution of SNAREs in the plasma membrane may play a prominent role in regulating exocytosis. In other cell types (35, 39–42) a number of SNARE proteins are located in cholesterol-dependent lipid raft domains. Previous studies in macrophages have shown that syntaxin 4 and SNAP-23 are also associated with lipid rafts in these cells (21, 22). SNAREs display different levels of raft association depending on the cell type (23); however, to date no other cell type has shown the stimulus coupled relocation of SNARE proteins into lipid rafts that we demonstrate occurs with syntaxin 4 in LPS-activated macrophages. The precise role of lipid rafts in SNARE-mediated fusion is currently unclear. The lipid rafts may act as sites for transmembrane pairing and membrane fusion, or they may function in a regulatory fashion by spatially separating the Q-SNARE partners in the membrane until they are required for fusion. Consistent with lipid rafts as fusion sites in PC12 cells (39, 41), neutrophils (43), synaptosomes (44), and now macrophages, disruption of lipid rafts leads to a decrease in secretion. In contrast, the decreased lipid raft association of SNAP-23/25 increased the secretion of recombinant growth hormone from PC12 cells (45) suggesting a more regulatory role for lipid rafts in this case. There are of course many other examples of molecules being recruited to lipid rafts in immune cells. The relocation of receptors for antibody complex or antibody in B cells, T cells, mast cells, and basophils to lipid rafts coordinates signaling (46).

The Sec/Munc18 family of SNARE-binding proteins regulates SNARE complex formation, and because in macrophages Munc18c is completely excluded from raft domains, it may function to restrict the movement of syntaxin 4 into lipid rafts for SNARE complex formation (47, 48). Similar results are seen in mast cells where syntaxin 3/Munc18b complexes were found in nonraft fractions, whereas syntaxin 3-containing SNARE complexes were found within lipid rafts (49). Lipid rafts may also orchestrate the fate and biological activities of TNFα. TNFα can be retained on cells as an active 26-kDa transmembrane protein at the cell surface, otherwise it is cleaved by the enzyme TACE and released as a soluble 17-kDa cytokine for other roles in immunity (50–52). We found TACE in nonraft fractions regardless of the activation state of the macrophages, implying that TNFα must first exit the lipid raft domains in order to be cleaved and released from the membrane, although this remains to be formally shown. Retaining TNFα in lipid rafts could potentially preserve the 26-kDa transmembrane form at the surface, and the release from lipid rafts would allow cleavage and release of the 17-kDa form, thus dictating the physiological functions of TNFα.

Upon activation, macrophages assume a highly ruffled cell surface with the extension of exaggerated filopodia (53) rich in cholesterol and lipid rafts (54). We now show syntaxin 4 in resting macrophages localizes to discrete puncta mainly on the cell body similar to the syntaxin 1 cholesterol-dependent puncta found in neuroendocrine cells (41) and the punctate staining of SNAP-23 on adipocytes (35). Upon activation, additional cholesterol-dependent syntaxin 4 puncta appear on the surface, particularly on filopodia forming the sites for the initial delivery of TNFα. Larger cholesterol-dependent clusters also emerge on the surface of macrophages after LPS activation, the significance of which is not yet clear. Individual lipid raft microdomains are typically below 50 nm diameter (55, 56), and TNFα delivery occurs at the many smaller syntaxin 4-labeled clusters; nevertheless, raft clustering has been demonstrated at sites of T cell receptor stimulation during T cell activation (57).

The formation of preferential fusion sites on filopodia is important in the context of macrophages where excess membrane is required for the formation of the nascent phagocytic cup (58, 59). We have recently shown the delivery of recycling endosome membrane containing TNFα to the nascent phagocytic cup serves to provide extra membranes to engulf a microbe while simultaneously delivering TNFα to surface delivery for rapid secretion (7). The relocation of syntaxin 4 to lipid rafts in the phagocytic cup ensures the recycling endosome and its cargo TNFα are delivered to these specific sites. Efficient phagocytosis of particles, such as mycobacteria, opsonized red blood cells, and now yeast, requires cholesterol (20, 60). Our data suggest this reliance on cholesterol is because of the requirement of SNAREs to be associated with lipid rafts for delivery of extra membrane to the phagocytic cup.

The family of serum cholesterol-reducing drugs termed statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) has been reported to have anti-inflammatory properties, such as reducing inducible nitric-oxide synthase and proinflammatory cytokines, including TNFα, in macrophages treated with lovastatin (61–64). However, other reports show that statins can increase the pro-inflammatory response; for example, simvastatin can increase the promoter activity and production of IL-12p40 and TNFα in macrophages (65–67). How the requirement of cholesterol for the delivery to the cell surface of cytokines is involved in the statin influence on inflammatory response has yet to be determined.

In conclusion, the data presented here show that the delivery of TNFα to the plasma membrane in LPS-activated and phagocytosing macrophages is dependent upon the movement of syntaxin 4 into cholesterol-dependent lipid rafts to be fusion-competent. Our findings are in keeping with the participation of lipid rafts in forming surface delivery and exit sites for cytokine secretion.

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