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Involvement of glycosphingolipid-enriched lipid rafts in inflammatory responses

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1. ABSTRACT

Glycosphingolipids (GSLs) are membrane components consisting of hydrophobic ceramide and hydrophilic sugar moieties. GSLs cluster with cholesterol in cell membranes to form GSL-enriched lipid rafts. Biochemical analyses have demonstrated that GSL-enriched lipid rafts contain several kinds of transducer molecules, including Src family kinases. Among the GSLs, lactosylceramide (LacCer, CDw17) can bind to various microorganisms, is highly expressed on the plasma membranes of human phagocytes, and forms lipid rafts containing the Src family tyrosine kinase Lyn. LacCer-enriched lipid rafts mediate immunological and inflammatory reactions, including superoxide generation, chemotaxis, and non-opsonic phagocytosis. Therefore, LacCer-enriched membrane microdomains are thought to function as pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) expressed on microorganisms. LacCer also serves as a signal transduction molecule for functions mediated by CD11b/CD18-integrin (αM/β2-integrin, CR3, Mac-1), as well as being associated with several key cellular processes. LacCer recruits PCKα/ε and phospholipase A2 to stimulate PECAM-1 expression in human monocytes and their adhesion to endothelial cells, as well as regulating β1-integrin clustering and endocytosis on cell surfaces. This review describes the organizational and inflammation-related functions of LacCer-enriched lipid rafts.

2. INTRODUCTION

Inflammatory responses are complex biological defense responses to harmful stimuli, such as pathogens, damaged cells, toxins and irritants (1). Pain, fever, redness, swelling, and loss of function are the initial signs of inflammation and are induced by inflammatory mediators. Basically, inflammation is a protective attempt by the organism to remove injurious stimuli, including infectious microorganisms, and initiate the healing process. The initial inflammatory response is called the innate immune system, originally regarded as non-specific. Recent studies, however, have revealed that the innate immune system is not “non-specific”. For example, phagocytic cells express several kinds of pattern recognition receptors (PRRs), such as Toll like receptors and β2 integrins, which recognize pathogen associated molecular patterns (PAMPs) on microorganisms (2-4). The evolutionary process has included the development of immune-surveillance networks to protect against potential pathogens. At the cellular level, glycosphingolipids (GSLs), GPI-anchored proteins and cholesterol, all members of membrane microdomains (also called lipid rafts) (5), have been demonstrated to be involved in those defensive responses (6-11). However, the analogous patterns of GSLs and motifs of PAMPs have been shown to generate autoantibodies against these GSLs, resulting in severe autoinflammatory diseases (12). For example, Guillain-Barre syndrome (GBS), an acute inflammatory demyelinating polyneuropathy, was

found in one patient to be caused by antibodies against a GM1-like lipo-oligosaccharide derived from the lipo-oligosaccharide of *C. jejuni* (12). This review will describe the role of GSL-enriched lipid rafts in inflammatory responses.

3. WHAT ARE GSL-ENRICHED LIPID RAFTS?

The lipid bilayer is a very stable structure, providing a physical boundary between the intra- and extracellular environments. The major components of biological membranes are phospholipids, sphingolipids, cholesterol, and membrane-associated proteins, each of which has different degrees of lateral motility. Non-homogeneous lateral distribution of these components can result in their rearrangement, leading to the formation of membrane areas ("domains") with highly differentiated molecular compositions and supramolecular architectures and stabilized by lateral interactions among the membrane components. The lateral heterogeneity of membrane structure has been detected on the sub-micron and nanometer scales, even in by membrane regions that lack a morphologically distinguishable architecture. GSLs are expressed on the surface of cellular membranes and form defined clusters through cys interaction, because GSLs have many hydroxyl and acetamide groups, which may act as hydrogen bond donors and acceptors. In addition, certain proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins and palmitoylated proteins, tend to go into small domains ("microdomains") (13). The most studied membrane microdomains are membrane lipid domains, defined by their GSL- and cholesterol-rich nature, enrichment in glycosylphosphatidylinositol (GPI)-anchored proteins and membrane-anchored signaling molecules, and cytoskeletal association (14); these domains are called "lipid rafts"(15). The ability of GSLs to form clusters, as shown using artificial membrane models (16), has been confirmed in intact cells by immunoelectron microscopic analysis of ultrathin-sections without organic solvents and by SDS-treated freeze-fracture replication (17, 18). The GSL-associated lipid rafts on plasma membranes have a diameter of 50-100 nm, with some of these rafts closely associated with signal transducer molecules, such as the Src family kinases Lyn and c-Src (17, 18). GSLs are thought to have greater ability to both donate and accept hydrogen bonds through the hydroxyl groups of sphingosine and through acyl amide groups, respectively, than do glycerophospholipids that lack hydroxyl groups. The latter show only the ability to act as hydrogen bond acceptors (19), resulting in *cis* interactions of GSLs within the lipid rafts. Moreover, lipids that contain saturated alkyl chains with higher transition temperatures (20) are excluded from rafts containing unsaturated chains with a lower transition temperature, sometimes below 0°C, resulting in a more ordered, less fluid, liquid phase. Cholesterol is composed of a highly hydrophobic sterol-ring system and a 3-hydroxymoiety, the only hydrophilic part of the molecule. Cholesterol is much smaller than sphingolipids and does not possess a long tail. The ceramide moeities of sphingolipids are thought to interact via hydrogen bonds and hydrophobic van der Waal's interactions (5). Further hydrophilic interactions between GSL head groups promote the lateral association of GSLs and cholesterol. In contrast, because of the low acyl chain melting temperatures and unsaturated acyl chains, phospholipids tend to be loosely packaged in bilayers, forming liquid-disordered membranes that allow rapid lateral and rotational movement of lipids (21). These interactions result in the separation of "GSL and cholesterol domains" from other phospholipids in the cell membrane and the formation of distinct microdomains. Cholesterol is thought to stabilize lipid rafts by filling the voids between the large and bulky GSLs (15).

Although GSL-enriched lipid rafts have been implicated in a number of important membrane events (6, 19, 22), the molecular mechanisms underlying GSL-mediated cell functions remais obscure. GSL metabolism and composition are specifically altered during cell proliferation and differentiation in various cell types (19). Furthermore, the ceramide structure of each GSL is highly variable (23). In mammals, ceramide is synthesized by six ceramide synthases, CerS 1 – 6, each of which uses a relatively restricted subset of fatty acyl-CoAs for N-acylation of the sphingoid long chain base (24). The level of expression of each CerS-encoding gene differs among tissues. These properties suggest that the molecular varieties and expression patterns of GSLs reflect their functions in these cells. Indeed, recent studies have demonstrated that GM1- and GM3-enriched domains are separated in plasma membrane, with each regulated by different mechanisms and the segregation of the two gangliosides dependent on an intact actin cytoskeleton (18), suggesting that each GSL exists in independent lipid rafts in the plasma membrane.

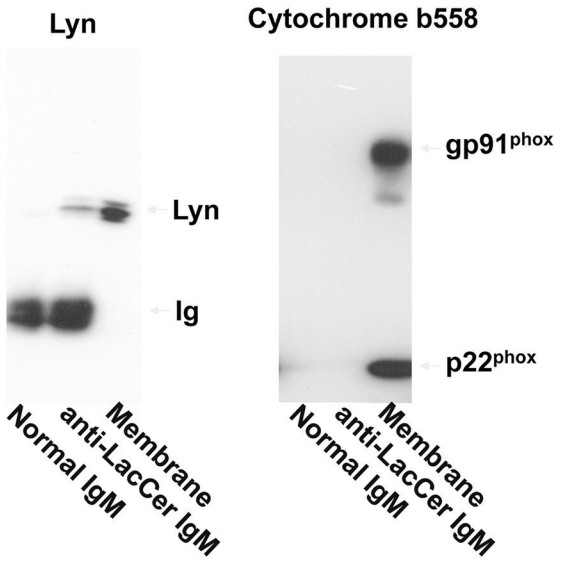

Figure 1. LacCer-enriched lipid rafts are associated with Lyn but not NADPH oxidases on plasma membranes. LacCer-enriched lipid rafts were isolated from plasma membranes of human neutrophils using Triton-x100, and immune precipitated with anti-LacCer antibody. The immunoprecipitates were analyzed by SDS-PAGE/immunoblotting using rabbit anti-Lyn IgG, followed by blotting with rabbit anti-gp91^{phox} and anti-p22^{phox} antibodies.

4. LACTOSYLCERAMIDE IS A KEY PLAYER IN THE HUMAN INNATE IMMUNOSYSTEM

The innate immune system is one of the most important host defenses against invading microorganisms, including bacteria, fungi, and viruses. Professional phagocytes, such as neutrophils and macrophages, are the primary cells involved in innate immune responses. These responses are initiated by interactions between PAMPs expressed on microorganisms and PRRs on host cells. Several types of PRR, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), and some GSLs, can directly sense PAMPs without opsonization by C3bi or IgG. The binding avidities of microorganisms to several types of GSL (25) suggest that GSL-enriched lipid rafts are involved in host–pathogen interactions. Indeed, microorganisms have been shown to recognize and enter host cells *via* lipid rafts on these cells (26). A neutral GSL, lactosylceramide (LacCer), has been shown to bind specifically to several types of pathogenic microorganisms, including *Escherichia coli*, *Bordetella pertussis*, *Bacillus dysenteriae*, *Propionibacterium freudenreichii*, and *Candida albicans* (27-33), suggesting the importance of LacCer in interactions between these microorganisms and host cells. In human neutrophils, LacCer is highly expressed on plasma membranes (34) and is the most abundant type of GSL (35, 36), forming lipid rafts with the Src family tyrosine kinase Lyn (6, 17) and acting as a PRR, resulting in several immunological functions, such as migration, phagocytosis and superoxide generation (6, 31, 37, 38). CD11b/CD18 integrin collaborates with LacCer-enriched lipid rafts during neutrophil engulfment of non-opsonized microorganisms (37). In contrast, mouse phagocytes express small amounts of LacCer on their cell surfaces (unpublished observation). There are several important differences between mouse and human innate immune systems (39); *e.g.*, differences in expression patterns of the Toll-like receptor family (40) and function of the C-type lectin receptor DC-SIGN (41). Thus, the molecular mechanisms involved in the innate immune system differ between humans and other animals.

5. LACTOSYLCERAMIDE-ENRICHED LIPID RAFTS MEDIATE SUPEROXIDE GENERATION

Inflammation involves the recruitment of many cell types, such as neutrophils, macrophages and lymphocytes, and the production of various inflammatory mediators, such as proinflammatory cytokines, chemokines and reactive oxygen species (ROS). More than 20 years ago, anti-LacCer antibodies were shown to induce superoxide production by neutrophils (42). LacCer was also reported to activate NADPH oxidase to modulate the expression of intercellular adhesion molecule-1 on human umbilical vein endothelial cells (43) and to induce the proliferation of human aortic smooth muscle cells (44). LacCer may therefore directly and/or indirectly activate NADPH oxidase, altering the functions of superoxide producing cells. The transition temperature of LacCer is around 70^{o}C (45), indicating that LacCer, under physiological conditions, is not present as a soluble single molecule in the cytosol of these cells. Indeed, exogenously added LacCer introduced into the plasma membranes of neutrophils was not detected in their cytosol (17).

On plasma membranes of human neutrophils, LacCer forms Lyn-coupled lipid rafts and mediates the generation of superoxide from these cells (6). LacCer-enriched lipid rafts are not associated with gp91^{phox} or p22^{phox} on plasma membranes (Figure 1). LacCer-mediated superoxide generation is blocked by the Src kinase inhibitor PPI; the PI3K inhibitors LY294002 and wortmannin; the p38-MAPK

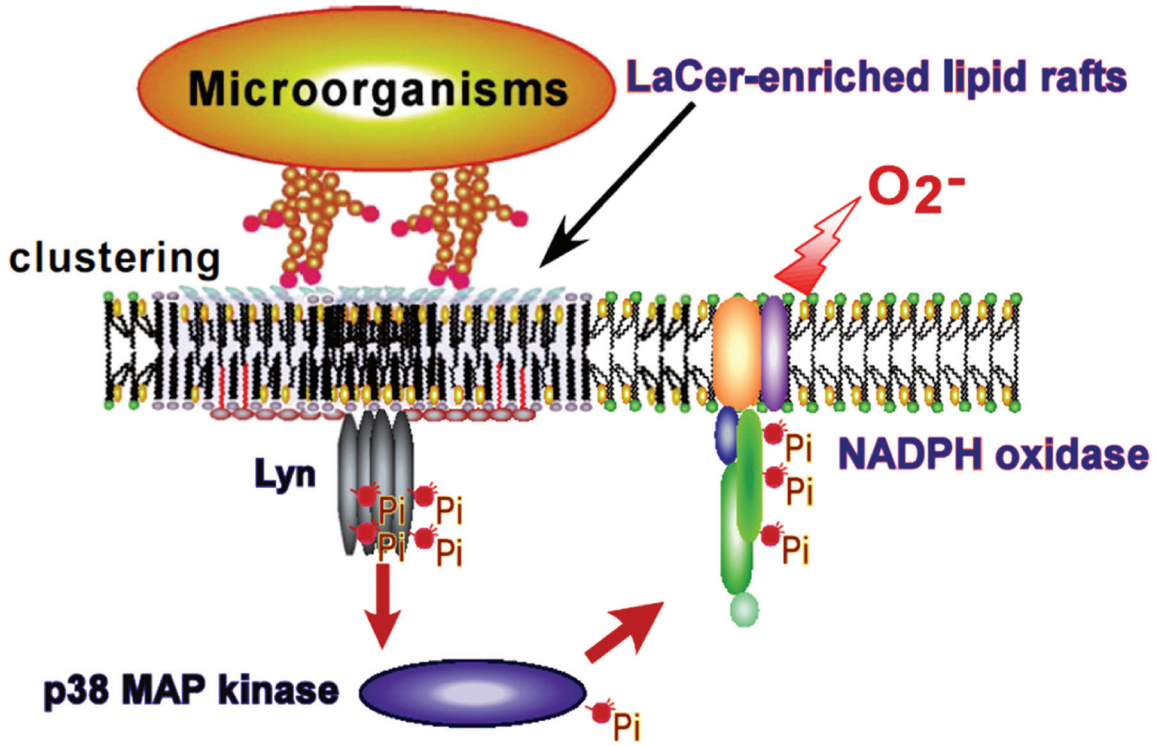

Figure 2. LacCer-enriched lipid rafts mediate superoxide generation. LacCer clusters on the plasma membranes of human neutrophils to form lipid rafts, which contain Lyn molecules. When PAMPs expressed on microorganisms, such as *Candida albicans*-derived β-glucan, bind to LacCer, Lyn is phosphorylated, and the subsequent p38MAPK-ERK signaling pathway is activated, resulted in superoxide generation through NADPH oxidase.

inhibitor SB203580, and the protein kinase C inhibitor H7. Although neutrophilic differentiated HL-60 cells (D-HL-60 cells) express almost the same amount of LacCer as neutrophils, D-HL-60 cells do not have Lyn-associated LacCer-enriched lipid rafts and lack LacCer-mediated superoxide generating ability. The very long fatty acid chains C24:0 and C24:1 were found to be the main components of LacCer of neutrophil plasma membranes. In contrast, plasma-membrane lipid rafts of D-HL-60 cells included over 70% C16:0-LacCer, but little C24-LacCer. D-HL-60 cells loaded with C24:0 or C24:1-LacCer acquired LacCer-mediated superoxide generating ability. Moreover, precipitation with anti-LacCer antibody resulted in the coimmunoprecipitation of Lyn. Immunoelectron microscopy revealed that LacCer clusters were closely associated with Lyn molecules in neutrophils and C24:1-LacCer-loaded D-HL-60 cells, but not in D-HL-60 or C16:0-LacCer-loaded cells. Thus, the C24 fatty acid chains of LacCer are specifically necessary for coupling of Lyn with LacCer-enriched lipid rafts and for LacCer-mediated superoxide generation by neutrophils.

6. LACTOSYLCERAMIDE-ENRICHED LIPID RAFTS MEDIATE INFLAMMATION

LacCer has been associated with a number of key cellular processes. For example, LacCer was shown to recruit PCKα/ε and phospholipase A2 to stimulate PECAM-1 expression in human monocytes, resulting in their adhesion to endothelial cells (46); and to regulate β1-integrin clustering and endocytosis on cell surfaces (47). LacCer was found to be a receptor activator of NF-κB ligand and to be essential for osteoclastogenesis mediated by macrophage colony stimulation factor (48).

GSLs, especially gangliosides, are abundant in the central nervous system (CNS) and defects in their catabolism are known to induce neurodegenerative and neuroinflammatory diseases, such as Gaucher and Sandhoff diseases (49). In primary rat astrocytes, LPS/IFN-γ caused a rapid increase in the activity of galactosyltransferase GalT-2 and the synthesis of LacCer, resulted in up-regulation of iNOS (50). In

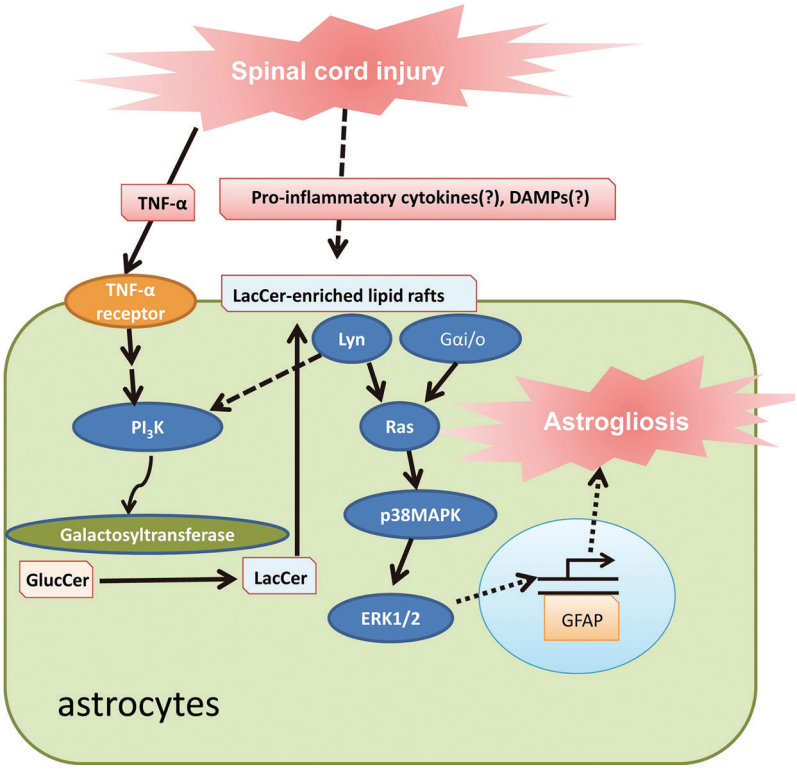

Figure 3. Schematic diagram showing the regulation of TNFα-induced GFAP expression and proliferation of primary astrocytes resulting in astrogliosis. TNFα may activate the ERK1/2 pathway through the direct activation of Lyn present associated with the LacCer-enriched lipid rafts, resulting in astrocyte proliferation and GFAP expression and triggering astrogliosis. Pro-inflammatory cytokines and damage-associated molecular pattern molecules (DAMPs) may activate cells through the direct activation of LacCer-enriched lipid rafts.

rat primary astrocytes, TNFα stimulation increased LacCer production and induced expression of the gene encoding glial fibrillary acidic protein (GFAP), a protein characteristic of astrogliosis (51). *In vivo* treatment with the GSL synthesis inhibitor PDMP was found to attenuate spinal cord injury (SCI)-induced astrocyte proliferation and to improve functional recovery after SCI. TNFα, by activating PI3K, induces LacCer biosynthesis. Increased LacCer biosynthesis thought to increase the expression of LacCer-enriched lipid rafts, which activate the p38MAPK-ERK signaling pathway (6). The activities mediated by LacCer-enriched lipid rafts are also inhibited by the Gαi/o inhibitor pertussis toxin (31), suggesting that these LacCer-enriched lipid rafts also act as G protein-coupled receptors. A novel assay for GTP loading showed that the activation of ERK1/2 correlates with the activation of p21ras by both tyrosine kinase and G-protein-coupled receptors. Thus, TNFα-induced activation of the ERK1/2 pathway may be through the direct activation of Src kinases, such as Lyn, associated with LacCer-enriched lipid rafts (51), resulting in astrocyte proliferation and GFAP expression and triggering astrogliosis (50) (Figure 3). Interestingly, human plasma membrane-associated sialidase (NEU3) specifically hydrolyzes GM3, resulting in the formation of LacCer (52). IL-6 treatment of human RCC ACHN cells was found to significantly enhance *NEU3* promoter activity and endogenous sialidase activity. *NEU3* transfection or IL-6 treatment resulted in both the suppression of apoptosis and the promotion of cell motility. NEU3 activated by IL-6 is involved in IL-6-mediated signaling, largely via the PI3K/Akt cascade, in a positive feedback manner and contributes to the malignant phenotype of RCCs. Increased LacCer expression on cell surfaces may enhance LacCer-mediated functions.

7. CONCLUSION

LacCer-enriched lipid rafts play important roles in innate immunity and inflammatory reactions.

However, the physiological roles of other GSL-enriched lipid rafts in inflammatory reactions are largely undetermined. In addition, the analogous patterns of GSLs and motifs of PAMPs results in the generation of autoantibodies against these GSLs, inducing severe auto-immune inflammatory diseases (12). Antibodies against neuronal tissues are involved in immune-mediated neurological disorders, with expression of several of these antibodies found to correlate with the pathophysiology of the disease (53, 54). The key to establishing the immunopathogenic role of auto-antibodies in neuropathies is to determine their effects on specific CNS systems. Gangliosides are thought to play important roles in memory formation, neuritogenesis, synaptic transmission, and other neural functions (55, 56). It is likely that the crosslinking of the gangliosides or other GSLs by their specific antibodies excessively stimulate neuronal functions mediated by GSL-enriched lipid rafts, resulting in neuronal inflammation. Therefore, the elucidation of the molecular mechanisms of inflammatory disease caused by autoantibodies against GSL-enriched lipid rafts may shed light on the physiological functions of GSL-enriched lipid rafts.

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