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Role of GM3-enriched microdomains in signal transduction regulation in T lymphocytes

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Gangliosides, sialic acid containing glycosphigolipids, are ubiquitous constituents of cell plasma membranes. Each cell type shows a peculiar ganglioside expression pattern. In human T lymphocytes monosialoganglioside GM3 represents the main ganglioside constituent of cell plasma membrane where it is concentrated in glycosphingolipid-enriched microdomains (GEM). The presence of tyrosine kinase receptors, mono- (Ras, Rap) and heterotrimeric G proteins, Src-like tyrosine kinases (lck, lyn, fyn), PKC isozymes, glycosylphosphatidylinositol (GPI)-anchored proteins and, after T cell activation, the Syk-family kinase Zap-70, prompts these portions of the plasma membrane to be considered as "glycosignaling domains." In particular, during T cell activation and/or other dynamic functions of the cell, such as apoptosis, key signaling molecules are recruited to these microdomains, where they strictly interact with GM3. The association of transducer proteins with GM3 in microdomains suggests that this ganglioside is the main marker of GEM in human lymphocytes and is a component of a cell plasma membrane multimolecular signaling complex involved in cell-cell interaction, signal transduction, and cell activation.
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Introduction

Gangliosides, sialic acid containing glycosphingolipids, are membrane constituents of all cell types, including lymphocytes, and the pattern is peculiar in different cells. These molecules consist of a double chain hydrophobic part (ceramide) and a hydrophilic head and are implicated in a variety of cell surface phenomena [1]. In addition to their well known functions as antigens and as receptors for different molecules and for bacterial toxins [2], gangliosides play an important role in cell adhesion, protein trafficking and transmembrane signaling [3–6].

Gangliosides are synthesized by virtually all the cells of peripheral blood. However, patterns of ganglioside cell expression depend on the species, cell type and age of the individual. In human peripheral blood lymphocytes (PBL) monosialoganglioside GM3 is the major ganglioside constituent (about 72% of total ganglioside content) [7], although not correlated with a particular lymphocyte subpopulation; both CD4+ and CD8+ cells express a similar amount of GM3 [8]. The GM3 content, determined as lipid-bound sialic acid, was 17.5 ± 1.4 μg/mg of protein in GEM fraction, as compared to 0.864 ± 0.1 μg/mg of protein in total lymphocytes.

The identity of the GM3 comigrating band was verified by gas liquid chromatographic (GLC) analysis. In the hydrophilic head galactose, glucose and N-acetylneuraminic acid in a molar ratio of 1:1:1 were found; in the hydrophobic part the main fatty acids were C16:0, C18:0, C18:1, and C15:0. Long-chain bases, analyzed by GLC as O-trimethysilyl derivatives, revealed exclusively the presence of n-C18-sphingenine that accounted for more than 95% of long-chain bases. The mass spectroscopy analysis of GM3 from GEM fraction ganglioside extract conclusively confirmed the identity of the molecule [9].

Minor ganglioside constituents of human PBL include sialosyl paragloboside (neuAcnLc4Cer) (about 14%) and monosialo-lactohexaosyl-ceramide (about 7%). Disialoganglioside GD3 is also a minor component on a small subset of human peripheral blood T cells, where it has been given the cluster designation CDw60, a surface marker of T cell activation [10]. Relatively little is known about the expression of these gangliosides in human lymphocyte subpopulations. GD3 is expressed predominantly on activated memory CD4+

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cells, characterized by their CD4 + CD29 + CD45RA− (11) and CD45RO+ [12] phenotype. In murine T clones two disialogangliosides appeared to be characteristic. They were identified by fast atom bombardment mass spectrometry as IVNeuAc,IINeuAc-GgOse$_4$Cer (GD1a) and IVNeuAc, IIINeuAc-GgOse$_4$Cer (GD1α). GD1a is a specific marker of TH2 cells, whereas GD1α is preferably, but not exclusively, expressed by TH1 lymphocytes [13].

Immunoelectron microscopic observations, using monoclonal antibodies with highly restricted binding specificity, revealed that GM3 molecules, as well as other ganglioside constituents, are distributed in clusters on the cell surface of normal [8] and pathological [14] human PBL. These observations are consistent with previously reported thermodynamic results [15], showing that gangliosides form clusters when their concentration is higher than a critical value.

Exogenous gangliosides as signal transducer molecules in human peripheral blood lymphocytes

Exogenous gangliosides, as amphiphilic molecules, are incorporated into cellular membranes, as demonstrated by an overall increase of the ion permeation across the plasma membrane and an enhanced polarizability of its hydrophobic region which follows ganglioside treatment [16].

In human T lymphocytes, exogenous gangliosides induce a selective dose-dependent down-modulation of CD4 molecules on the plasma membrane [17], not affecting other surface receptors. Since CD4 is the main receptor for the human immunodeficiency virus type 1 (HIV-1) [18], treatment of the T lymphocytes with gangliosides consequently reduced the *in vitro* infectivity of the virus [19]. Some reports suggested that CD4 modulation by GM1 may be due to the masking of CD4 epitopes [19,20,21] or to the shedding of CD4 molecules [22]. In our work we analyzed the mechanisms responsible for GM3-induced CD4 down-modulation and the early biochemical pathways elicited by GM3 [23], which represents the main ganglioside molecule physiologically expressed on the cell surface of human PBL [7]. Our observations revealed that the decrease of CD4 expression at the cell surface was due to its rapid endocytosis.

CD4 down-regulation on T cell plasma membrane is a well known molecular event triggered by different stimuli which, through activation of PKC, induce the phosphorylation of serine residues in the cytoplasmic tail of CD4 molecule and, consequently, the dissociation of the CD4:p56^{lck} complex [24]. We were able to demonstrate a selective inhibition of the GM3-induced down-modulation in the presence of specific PKC inhibitors [25]. GM3 treatment preferentially promoted translocation of PKC-δ in selective membrane domains in which PKC-δ may interact with CD4. In addition, GM3 exposure induced a significant increase of CD4 phosphorylation on serine residues. As a consequence of this phosphorylation, CD4 dissociated from lck. This is a fundamental step which allows the association of CD4 with uncoated and clathrin-coated pits and subsequently the endocytosis and intracellular degradation of the molecule [26,27]. Regulation of the glycolipid biosynthetic pathways perhaps can regulate this step also [28].

Among the possible upstream pathway triggered by exogenous GM3, the observation of the early activation of cPLA$_2$ with AA release suggested a role for this enzyme in GM3-induced PKC activation and CD4 down-modulation. The hydrolysis products of cPLA$_2$, AA and lyso-PC, are more frequently recognized as lipid second messengers and thought to regulate a number of potential molecular targets. Indeed, these molecules are directly responsible for the regulation of key intracellular players such as PKC isozymes [29], including the Ca^{2+} independent PKC. Moreover, our findings indicated that cPLA$_2$ activation may be mediated by ERKs [30,31], a downstream effector of a Ras-regulated cyoplasmic kinase cascade, which includes RAF-1 and MAPK kinase [32]. Our findings indicated that GM3 treatment leads to rapid ERK phosphorylation in lymphoblastoid T cells, as well as in human lymphocytes [33].

The effect was specific for ERK-2, as revealed by antiphosphotyrosine binding on both cell free lysates and ERK immunoprecipitates. Although a direct stimulation of ERK by GM3 is unlikely, the observation that cell incubation with a MEK-1 inhibitor prevented ERK phosphorylation, as well as CD4 endocytosis, strongly suggests that GM3 stimulates a signaling cascade involving MEK-1 or MAP Kinase-related protein that subsequently activates ERK.

Other biochemical pathways which may underline the cPLA$_2$-dependent PKC-δ activation following GM3 treatment are under investigation. Since we observed the absence of PI-PLC activity in GM3-treated cells, the link between cPLA$_2$ and PKCδ activation can be the induction of phosphatidylcoline (PC) breakdown by PC-specific phospholipases C (PC-PLC) and PLD [34], leading to DAG formation without mobilizing Ca^{2+}. These pathways may represent an alternative route for PKC stimulation, particularly the Ca^{2+} independent isotypes such as PKC-ε and -δ [35].

Gangliosides as components of the "glycosignaling domain" in human peripheral blood lymphocytes

The wide spectrum of biological effects exerted by gangliosides can be explained by a physical and functional connection of gangliosides with signaling molecules (particularly tyrosine kinases) in "glycosphingolipid-enriched microdomains (GEM)," corresponding to "rafts." They can partecipate in signal transduction in at least three ways [36]: (i) by concentrating molecules involved in signaling pathways; (ii) by allowing their molecular interaction; (iii) by modulating signaling function, thus defining the "glycosignaling domain" [37].

Since 1972, the existence of areas (domains) of cell plasma membranes has been shown where peculiar components are concentrated [38]. These small and highly dynamic structures are envisaged as lateral assemblies of specific lipids and proteins in cellular membranes proposed to function in processes

Table 1. Association of some human PBL proteins with GEMs

Protein	*Molecular mass (kDa)*	*Leukocyte*
GPI-Proteins		
CD14	55	B, G, Mo
CD16 (FcγRIII)	48	NK
CD24	35–45	B, G
CD44	80–90	T, NK, B
CD48	43	T, B, null cell
CD55	70	T
CD5	19	T
PrP^c	33	T, B, M, Mo, G
Src family kinases		
p56	56	T, NK
Lyn	56	B, G, Mo, P
Fyn	59	T
Other proteins		
CD4	58	T, M
LAT	36–38	T
Raf-1	72–76	T
CXCR4	40	T
After activation		
ZAP-70	70	T
TCR	83	T
CD3	73	T
CD122	75	T, B, NK, Mo
SKAP55	55	T
Grb2	24	T
After Fas triggering		
CD95/Fas	43	T, B, NK, Mo
FADD	30	T, B, NK, Mo
Caspase 8	55	T, B, NK, Mo
Ezrin	80	T, B, NK, Mo

Abbreviations: B: B cells; T: T cells; G: granulocytes; M: mast cells; Mo: monocytes; NK: natural killer cells; P: platelets.

such as membrane transport, signal transduction, and cell adhesion [37]. They are specifically enriched in certain lipids (sphingolipids, including gangliosides, sphingomyelin, and cholesterol), whereas other lipids (*e.g.,* glycerophospholipids) are selectively depleted from the extracts. In particular, GM1 ganglioside has been proposed as a marker for these membrane structures [39] in cells where this glycolipid is expressed. The variety of proteins detected in these domains isolated from different cell types is extremely wide (see Table 1 below). In fact, tyrosine kinase receptors such as EGF-R [40], mono- (Ras, Rap) [41] and heterotrimeric G proteins [42], Src-like tyrosine kinases (lck, lyn, fyn) [43,44], PKC isozymes [45], GPI anchored proteins [46,47], and others have been found in these plasma membrane microdomains. Interestingly, multiple classes of cell adhesion proteins are GPI-anchored, *e.g.,* F3, LAMP, NCAM120, TAG1, and BIG-1 [48,49] and some of them are localized in detergent-resistant domains [50,51]. Thus, ganglioside domains have been suggested to participate directly in the mechanism of cell adhesion. In particular, in T lymphocytes GEM play a key role in regulating integrin function. Without stimulation, integrin LFA-1 is excluded from lipid rafts, but following cell activation LFA-1 is mobilized to GEM. This correlation between integrin activation and GEM extends to a second integrin, $\alpha 4\beta 1$, since the clustering of $\alpha 4\beta 1$ is also GEM-dependent [52]. In addition, chemokine signaling through a common Gi-protein activation of G-protein-coupled chemokine receptor to different integrins coexpressed on PBL can take place at different lipid microdomains [53]. At the end, GEM may play a role in the mechanism of protein and lipid sorting at the trans-Golgi network level, for apical delivery in polarized cells [54]. Thus, the general function of GEM in signal transduction may be to concentrate receptors for interaction with ligands and effectors on both sides of the membrane, thus speeding up binding during signaling and preventing inappropriate crosstalk between pathways [55] although the different signaling pathways may depend on the cell type.

Role of GEMs in T cell activation

Since 1994, detergent-insoluble GEMs have been identified in lymphocytes in the absence of caveolae [56]. The finding that GPI-anchored proteins (*i.e.* CD55) are present in these detergent-insoluble complexes [57], together with several tyrosine kinases of the Src family (lck, lyn, fyn), indicates that these complexes represent membrane microdomains involved in the transduction of signals. Since lck is linked to CD4, the presence of these proteins in GEM was investigated. Interestingly, in a lymphoblastoid T cell line (CEM), it was found that CD4 and lck colocalized in these domains [44]. The CD4-lck complex represents one of the most important receptor systems in the T cell function, and CD4 is considered to be the TCR co-receptor in thymic selection, T cell activation, and cellular response. The existence of an interaction between gangliosides and transducer proteins, including tyrosine kinases, within microdomains was supported by the observation that anti-CD4 coimmunoprecipitated GM3, as revealed by both Western blot and TLC analysis [8]. Thus, GM3 directly and tightly binds to CD4 in a manner that is sodium dodecyl sulfate-resistant, analogous to the binding between GM1 and Trk A [58] and between GM3 and prosaposin [59]. The GM3-CD4 association was also demonstrated by a novel approach, showing that CD4 and $p56^{lck}$ were immunoprecipitated by IgG anti-GM3 [60]. In order to verify whether GM3 association with CD4 molecules may depend on the presence of lck, we analyzed this association in U937, a CD4+ and $p56^{lck}$ negative cell line. The immunoprecipitation with anti-GM3 revealed the presence of a 58 kDa band immunostained with anti-CD4 Ab, suggesting that the GM3-CD4 interaction does not require its association with lck. Another important receptor which was also immunoprecipitated by the IgG anti-GM3 is CXCR4 (fusin) [61]. It is a chemokine receptor involved as a coreceptor in gp120 binding to the cell plasma membrane. In particular, binding of gp120 on cell plasma membrane triggers CXCR4

recruitment to GEM, as revealed by both scanning confocal microscopic observations and coimmunoprecipitation experiments. Gp120 binding induces the lateral reorganization of GEM, bringing the CD4-gp120 complexes together with GEM containing the chemokine receptor. Indeed, it was demonstrated that GEM integrity is required for lateral assembly of CD4-gp120 complexes with the CXCR4 and for fusion of the HIV-1 envelope and the cell plasma membrane. The role of GM3 can be to facilitate the migration of the CD4-gp120 complex to an appropriate coreceptor, such as CXCR4, since CD4 and coreceptor are not physically associated in the absence of HIV-1.

An important confirmation of the role of GM3 within GEM in signal transduction in human PBL also derives from the evidence that phorbol myristate acetate (PMA)-induced downregulation of the cell surface pool of CD4 occurs within these microdomains [45]. In addition, PMA induced the rapid translocation of PKCs, particularly PKCα, to GEM [45]. This finding was independently confirmed by (i) *in vivo* inhibition of CD4 downmodulation by a PKCα selective inhibitor; (ii) a series of *in vitro* phosphorylation experiments using purified microdomains; (iii) evidence that PKCα, GM3, and lck are strictly associated after PMA treatment, constituting a cell membrane multimolecular signaling complex, including lyn, which may be involved in, or contribute to lymphocyte activation. It became obvious after the demonstration that T cell activation by costimulation through CD28 led to the redistribution and clustering of membrane and intracellular kinase-rich raft microdomains at the site of TCR engagements [62]. The hypothesis for the role of GEM in initiating of TCR signaling gained further support from the demonstration that disruption of GEM inhibits CD3-induced ζ phosphorylation and association with the cytoskeleton [63]. In addition, a large variety of studies indicate the role of GEM in the initiation of TCR signaling. They demostrate the recruitment of TCR to GEM upon receptor stimulation and the redistribution and clustering of plasma membrane and intracellular kinase-rich microdomains at the site of TCR engagement [62,64]. The IL-2 and the TCR are similar in several aspects, including their shared association with signaling components, such as non-R tyrosine kinases, *e.g.,* lck or adapter molecules, such as Grb-2, which is recruited to these domains during T cell activation [65]. Indeed, the IL-2/IL15R β-chain has been shown to be enriched in GEM, and this enrichment may be relevant as a possible mechanism to ensure cytokine selectivity and specificity [66]. On the other hand, T cell stimulation induces Grb-2 interaction with another transducer, the adapter protein SKAP55 [65]. Mutational analysis revealed that tyrosine 271 in SKAP55 plays a pivotal role for interaction with both fyn and Grb-2, indicating that the fyn-phosphorylated SKAP-55 transiently associates with Grb-2 to mediate MAP-kinase activation. TCR engagement induces the translocation of SKAP-55 to GEM where it interacts with fyn, indicating that the function of SKAP55 via its association with fyn and other signaling components may be involved in GEM-mediated T cell activation. Furthermore, in GEM two other crucial molecules, phosphatidyl-inositol 4,5 biphosphate, a substrate of PLCγ, and LAT [67,68] have been identified. It is one of the earliest and major tyrosine-phosphorylated proteins following TCR-triggering. LAT phosphorylated following Zap-70 activation. Zap-70, a Syk family kinase, is activated via both a self- and a lck-dependent phosphorylation mechanism and translocates from cytoplasmic compartment to the cell surface. It phosphorylates substrates which in turn lead to the subsequent docking and activation of other Src homology 2 (SH2)-containing molecules involved in the amplification and diversification of TCR-initiated signaling. Zap-70 becomes GEM-associated [9,64], binds PLCγ which, after phosphorylation, cleaves phosphatidyl-inositol 4,5 biphosphate. In this concern, we recently provided evidence that GM3 tightly binds to the phosphorylation transducer protein Zap-70 after CD3 and CD28 engagements [9]. The GM3-Zap-70 association was demonstrated by both Western blot and TLC analyses, showing that GM3 was coimmunoprecipitated by anti-Zap-70 MoAb. These findings demonstrated that CD3 and CD28 engagements induce not only Zap-70 translocation to the cell surface, but also its high affinity binding with the GEM component GM3. This SDS-resistant interaction is similar to that described between Trk A receptor and GM1 [58]. Specific high affinity SDS-resistant ganglioside-protein interactions reported in different cell types [59,69] appear, in fact, to be involved in transducing stimulatory and/or inhibitory signals [37], although the precise mechanism involved remains to be elucidated. In this concern, it has been hypothesized that interaction of ganglioside with some transducers (*e.g.,* c-Srk, Rho) may be due to the presence of aliphatic chain (fatty acyl or farnesyl group) linked to the transducer, although other transducers present in glycosignaling domains (*e.g.,* FAK, Ras) may require different mechanisms [37]. Interestingly, T cell activation does not modify GM3 distribution, revealing that costimulation through CD28 does not promote a redistribution of GEM, as suggested [62], but induces a preferential translocation of Zap-70 to discrete microdomains of the plasma membrane in which it may interact with GM3 [9].

Role of GEM in T cell apoptosis

Apoptosis of T lymphocytes limits immune and inflammatory responses, is involved in elimination of autoreactive cells, and shapes acquisition of the immune memory. Triggering of cell apoptosis is strictly regulated by ligand-receptor systems, including Fas ligand-CD95/Fas. The binding of CD95/Fas by its ligand results in trimerization of the receptor, recruitment of Fas-associated death domain (FADD) protein to the death domain of CD95 and binding of caspase-8 to the death-effector domain of FADD [70]. This process induces the formation of the death-inducing signaling complex (DISC). Binding and activation of caspase-8 results in transmission of the activation

signal to other caspases, in particular caspase-3, involvement of mitochondria with release of cytochrome c and membrane depolarization, and release of apoptosis-inducing factor (AIF) [71]. Evidence suggests that many receptors aggregate in distinct plasma membrane microdomains or rafts [72]. This notion is supported by the finding that disruption of microdomains prevents clustering of many receptors, including TNF-R [73] and CD95/Fas [74]. In addition, recent studies pointed out the role of GEM in initiating of CD95/Fas triggered cell death in lymphoblastoid T cells or in mouse thymocytes [75,76]. We recently demonstrated that in T cells gangliosides GM3 and GD3 coimmunoprecipitated with the cytoskeletal protein ezrin [77], which, in turn, was complexed with CD95/Fas after triggering through anti-Fas Ab, but not after treatment with other drugs, such as staurosporine [77]. Following this finding, we analyzed the possible association of DISC components with GM3 during CD95/Fas triggered apoptosis [78]. Scanning confocal microscopic observations showed the association of caspase-8 with GEM, as revealed by nearly complete colocalization areas between caspase-8 and GM3 after triggering through CD95/Fas. Interestingly, T cell stimulation did not modify the ganglioside distribution, revealing that CD95/Fas engagement did not promote a redistribution of GEM, but induced a preferential translocation of DISC to discrete microdomains of cell plasma membrane in which it associates with GM3. This association was supported by coimmunoprecipitation experiments which demonstrated that GM3 was immunoprecipitated by anti-caspase 8 after triggering through CD95/Fas. In addition, the analysis of linear sucrose gradient fractions further clarified the recruitment of caspase-8, as well as of Fas, to GEM upon CD95/Fas engagement. Thus, we provided evidence that CD95/Fas triggering induces the lateral organization of rafts, bringing the CD95/Fas receptor together with GEM and demonstrating that the DISC associates with GEM [78]. This finding strongly suggested a role for GEM in triggering of T cell apoptosis, since binding and activation of caspase-8 results in transmission of the activation signal to other caspases and involvement of mitochondria. The key role of GEM in initiating of Fas signaling gained further support from the demonstration that disruption of GEM prevents DNA fragmentation as well as CD95/Fas clustering on the cell surface [78].

These findings strongly suggest a role for gangliosides as structural components of the membrane multimolecular signaling complex involved in initiating of Fas-triggered signal transduction pathway.

Many lines of evidence reveal the existence of GM3-enriched microdomains in human periheral blood lymphocytes. GEM

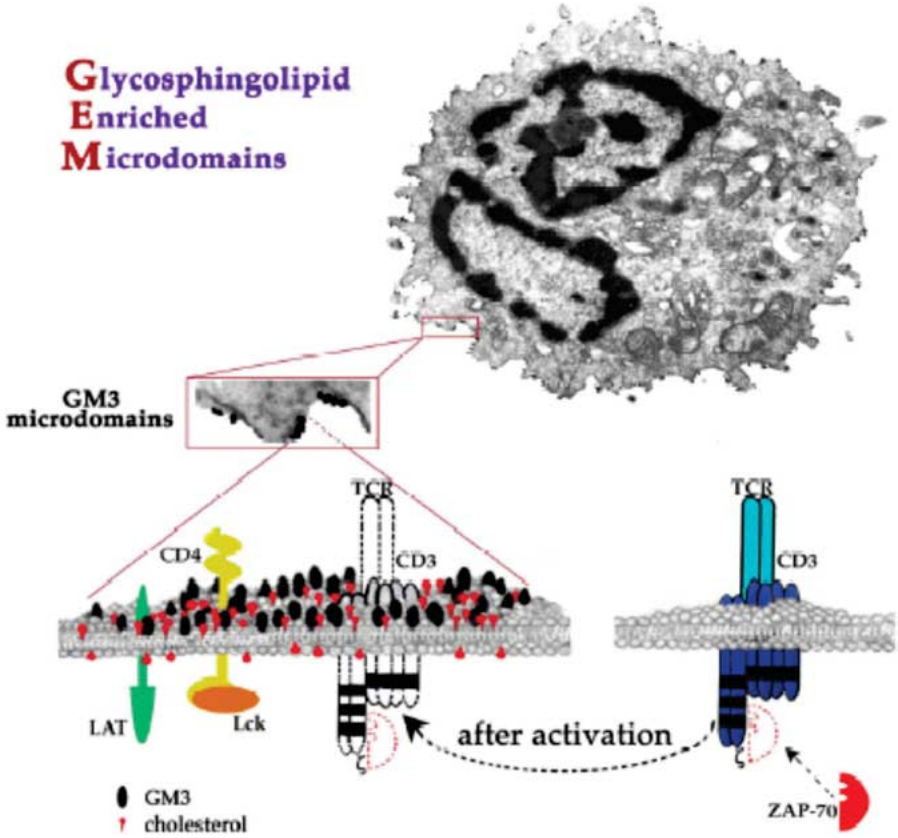

Figure 1. The figure shows the distribution and composition of GM3 microdomains on lymphocyte plasma membrane. Immunogold labeling was obtained using the GMR6 anti-GM3 MoAb. T cell activation by costimulation through CD3 and CD28 does not promote a redistribution of GM3 microdomains but induces CD3 complex and Zap-70 translocation in the microdomains.

may play a general role in signaling via immunoreceptors such as TCRs, B-cell receptors and FcRs. Indeed, the phosphorylated immunoreceptor may associate with other protein-tyrosine-kinases (Syk family), inducing activation of downstream members of signaling cascades such as PLCγ, phosphatidylinositol 4,5 biphosphate, and proteins regulating the activity of the small G-protein Ras. In particular, during T cell activation and/or other dynamic functions of the cell, key signaling molecules are recruited to these microdomains, where they strictly interact with GM3. The association of transducer proteins with GM3 in microdomains suggests that this ganglioside is the main marker of GEM in human lymphocytes and is a component of a cell plasma membrane multimolecular signaling complex involved in cell-cell interaction, signal transduction, cell activation, and apoptotic pathway (see Figure 1).

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