Mini Review

The novel neutrophil differentiation marker phosphatidylglucoside is involved in Fas-dependent apoptosis

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A new type of glycolipid, phosphatidylglucoside (PtdGlc), first identified as a component of lipid rafts in the human HL-60 leukemia cell line, has also been detected in human neutrophils during maturation. We show here that PtdGlc forms functional domains different from lactosylceramide (LacCer)-enriched lipid rafts and initiates neutrophil apoptosis via Fas-dependent death signaling. Among the human peripheral blood leukocytes and monocyte-derived dendritic cells (DCs), only neutrophils show high surface expression of PtdGlc, whereas LacCer is expressed on the surfaces of neutrophils, macrophages and DCs. LacCer couples with the Src family kinase Lyn, forming lipid rafts on plasma membranes, and mediates neutrophil chemotaxis, phagocytosis and superoxide generation, whereas PtdGlc does not mediate these functions. In contrast, PtdGlc but not LacCer is involved in neutrophil apoptosis. These observations suggest that PtdGlc and LacCer form distinct lipid domains on the plasma membrane. In addition, PtdGlc-mediated apoptosis was inhibited by specific inhibitors of caspases 8, 9 and 3, but not by the Src family kinase inhibitor PP1, the PI_3 kinase inhibitor LY294002 or catalase. PtdGlc colocalized with Fas on neutrophil plasma membranes, and both the anti-PtdGlc antibody DIM21 and the agonist anti-Fas antibody B-10 induced the formation of large Fas-colocalized clusters of PtdGlc on plasma membranes. Furthermore, the antagonist anti-Fas antibody ZB4 significantly inhibited DIM21-induced neutrophil apoptosis. These results suggest that PtdGlc is specifically expressed on neutrophils and mediates activities of these cells, and that the Fas-associated death signal may be involved in PtdGlc-mediated apoptosis.

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Introduction

Polymorphonuclear neutrophils play critical roles in the innate immune system by recognizing pathogen-associated molecular patterns via pattern recognition receptors (PRR) expressed on the cell surface, and then phagocytosing and eliminating microorganisms[1]. Neutrophils have a very short life span, undergoing apoptosis within 24-48 hours after leaving the bone marrow. Apoptosis is the major mechanism limiting neutrophil content *in vivo*, and may also be important in the clearance of neutrophils from inflamed tissues by tissue macrophages, a clearance critical for limiting inflammatory tissue injury and the subsequent resolution of inflammation[2-4].

Although neutrophils express several kinds of glycolipids, little is known about the biological roles of these molecules. The neutral glycosphingolipid lactosylceramide (LacCer, CDw17; Galβ4Glcβ1Cer) is the most abundant glycosphingolipid in human neutrophils and has been shown to act as a PRR in these cells[5-7]. Among the glycosphingolipids (GSLs), various microorganisms selectively bind to LacCer. LacCer is expressed at high levels on the plasma membrane of human neutrophils, and forms membrane microdomains/lipid rafts with the Src family tyrosine kinase Lyn. Since LacCer-enriched lipid rafts mediate superoxide generation, chemotaxis, and non-opsonic phagocytosis[8,9], LacCer-enriched membrane microdomains are thought to function as pattern recognition receptors (PRRs) in recognizing pathogen-associated molecular patterns (PAMPs) on microorganisms. Although the fatty acid chain structures of LacCer vary greatly, the presence of a C24:0 or C24:1 fatty acid chain has been shown indispensable for the functional connection of LacCer with Lyn in lipid rafts of HL-60 cells[9].

Phosphatidylglucoside (PtdGlc) is a unique cell surface glycophospholipid originally found in human cord RBCs[10]. PtdGlc has been demonstrated to be associated with cell differentiation. For example, treatment with the recombinant anti-PtdGlc Fab antibody rGL-7 induced HL-60 cell differentiation into neutrophilic cells. Since PtdGlc consists exclusively of saturated fatty acid chains, with C18:0 at the sn-1 position and C20:0 at the sn-2 position of the glycerol backbone[11], PtdGlc is thought to form lipid rafts on plasma membranes[12,13]. Indeed, PtdGlc is recovered in the Triton X-100-insoluble fractions of HL-60 cells[12], early erythroblastic leukemia cells[13,14] and astroglial cells from fetal rat brain[13] by sucrose density-gradient ultracentrifugation. Treatment with the anti-PtdGlc antibody induced the phosphorylation of Lyn and Hck, whereas reduction of endogenous cholesterol with methyl-β-cyclodextrin suppressed anti-PtdGlc antibody-stimulated tyrosine phosphorylation, resulting in the up- and down-regulation of CD38 and c-Myc expression, respectively[12]. These findings indicate that PtdGlc may form functional lipid raft-like domains on the plasma membranes of HL-60 cells[11].

This review shows that, although both LacCer and PtdGlc form lipid rafts on the plasma membranes of neutrophils, the organization and function of LacCer and PtdGlc differ completely different from each other in these cells.

Expression of PtdGlc and LacCer and neutrophil differentiation

PtdGlc plays a role in the formation of signaling domains involved in the cellular differentiation of HL-60 cells. When PtdGlc expression was analyzed along the differentiation pathway of hematopoietic progenitors (Fig.1), this glycophospholipid was found to be preferentially expressed along the neutrophil differentiation pathway of cord blood CD34(+) cells treated with cytokines[15]. Promyelocytes show slight expression of PtdGlc, whereas myelocytes and metamyelocytes express high levels of PtdGlc on their surfaces. PtdGlc expression was found to increase during neutrophil maturation. Among human peripheral leukocytes, PtdGlc is highly expressed on the surfaces of neutrophils (CD14^{low} and CD11b^{low}), but slightly expressed on mono-

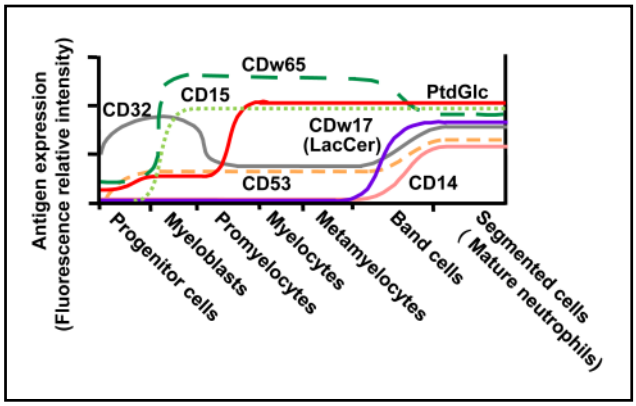

Fig.1 Schematic representation of PtdGlc and LacCer expression in neutrophil differentiation

The expressions of typical neutrophil markers during neutrophil maturation are illustrated based on the data reported by other groups[15, 43, 44]. PtdGlc expression is seen with neutrophil maturation, whereas LacCer is expressed only on matured neutrophils.

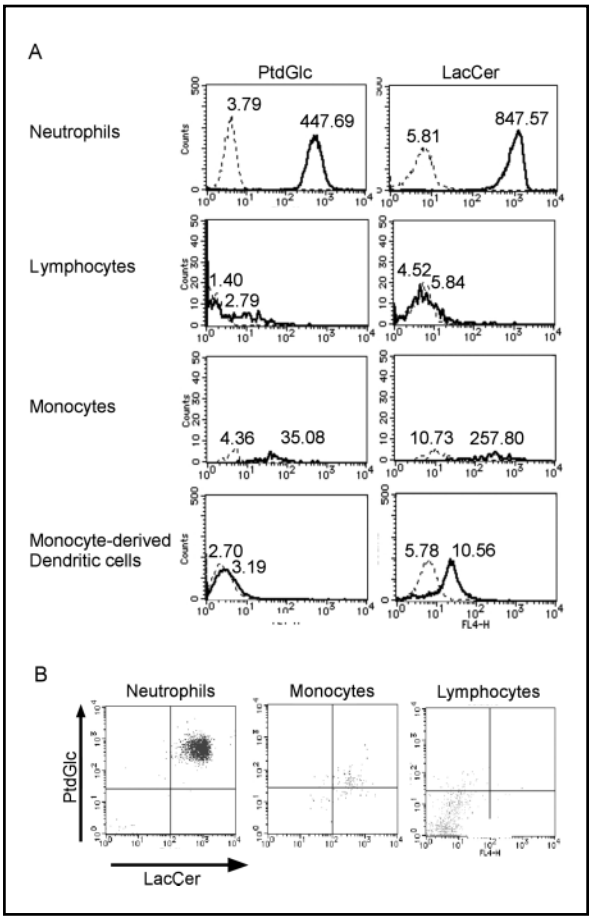

Fig.2 PtdGlc is a neutrophil marker in humans

(A) Expression of PtdGlc and LacCer on leukocytes. Peripheral blood samples were incubated with Alexa488-anti-PtdGlc IgM DIM21, Alexa647-anti-LacCer IgM T5A7 or Alexa-labeled normal IgM (dotted lines).

(B) Comparison of PtdGlc and LacCer expression on leukocytes.

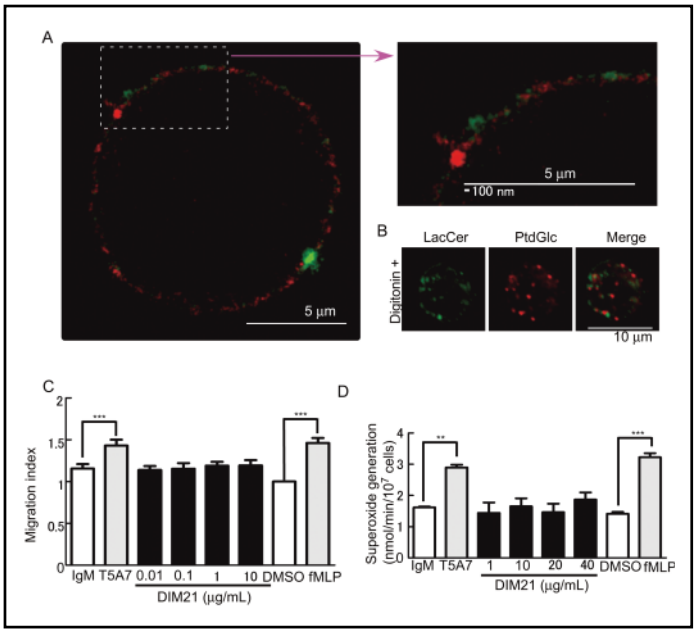

Fig.3 PtdGlc is present in different domains from LacCer-enriched lipid rafts

(A) Neutrophils were stained with Alexa 488-conjugated DIM21 (green) and Atto425-conjugated T5A7 (red) and examined with a TCS SP5 STED CW confocal microscope.

(B) Neutrophils were fixed, permeabilized, stained with Alexa 546-conjugated DIM21 (red) and Alexa-488-conjugated T5A7 (green), and examined with a TCS SP2 confocal microscope.

(C) DIM21, T5A7 and normal IgM were assessed for their chemotactic activities toward neutrophils using the Boyden chamber method. Each bar shows the mean ± SD of 4 independent experiments. ***$p<0.001$

(D) Results of superoxide generating assays. IgM, 10 μg/mL normal IgM. T5A7, 10 μg/mL T5A7. Each bar shows the mean ± SD of 3 independent experiments. **$p<0.01$, ***$p<0.001$.

cytes (CD11b^{high} and CD14^{high} cells) and lymphocytes (CD54^{positive} and CD14^{negative} cells) (Fig.2)[16], suggesting that PtdGlc is a neutrophil differentiation marker.

In contrast, LacCer is expressed only on mature neutrophils[17] and on monocytes, but not on lymphocytes. Peripheral blood-derived unstimulated dendritic cells (DCs) were found to express LacCer, but not PtdGlc, on their cell surfaces, suggesting that LacCer is a differentiation marker not only for human neutrophils, but for monocytes and DCs.

PtdGlc forms domains that differ from LacCer-enriched microdomains

Human neutrophils show abundant surface expression of both LacCer and PtdGlc, suggesting that PtdGlc may be located in LacCer-enriched lipid rafts and be involved in LacCer-mediated functions. Stimulated emission depletion confocal microscopy showed, however, that, although PtdGlc and LacCer both form small clusters on the plasma membranes of neutrophils, they do not colocalize with each other (Fig.3A). Furthermore, PtdGlc containing granules do not contain LacCer (Fig.3B)[16]. Moreover, pretreatment with the anti-LacCer antibody T5A7 or the anti-PtdGlc antibody DIM21 did not affect the binding of the other antibody to neutrophils. As shown for CD11b expression[18], treatment with anti-PtdGlc antibody at 37℃ resulted in the up-regulation of PtdGlc expression, but did not affect LacCer expression. These observations clearly indicate that PtdGlc and LacCer form different domains on plasma membranes and are located in different granules of neutrophils. Although LacCer has been shown to mediate neutrophil

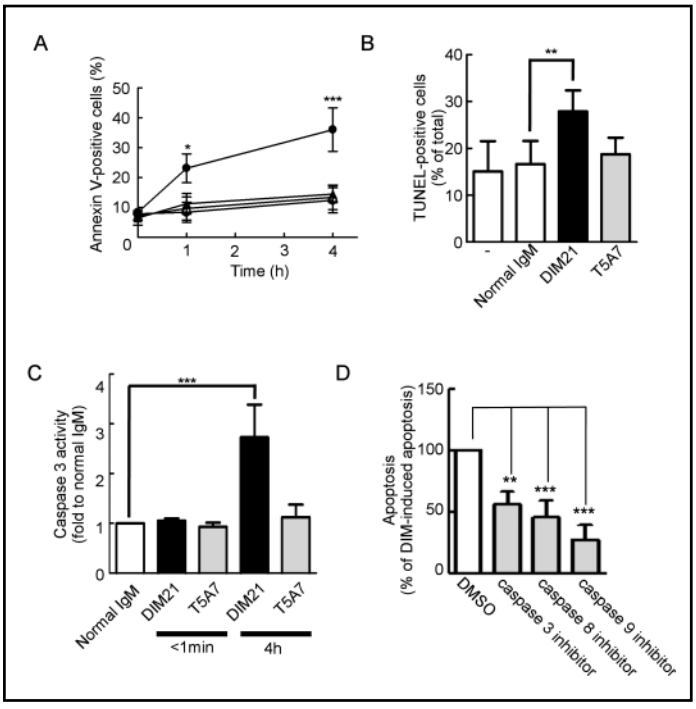

Fig.4 PtdGlc involvement in neutrophil apoptosis

(A)Neutrophils were incubated without (Control) or with DIM21, T5A7, and normal IgM (IgM) at 37℃ for 1 min, 1 h, and 4 h, and the numbers of annexin V positive cells were determined. Each point represents the mean ± SD of three independent experiments. ● DIM21, ○ without antibody, ▲ Normal IgM, △ T5A7, *p<0.05, ***p<0.001.

(B)Neutrophils were incubated with DIM21, T5A7, or normal IgM at 37℃ for 4 h. Cells with chromatin fragmentation were analyzed by TUNEL assay. n=3, **p<0.01.

(C)Neutrophils were incubated with DIM21, T5A7, or normal IgM, and the caspase 3 activity of the cells was analyzed. n=3, *** p<0.001.

(D)Neutrophils were preincubated with caspase 3 inhibitor VII, caspase 8 inhibitor II, or caspase 9 inhibitor III, and the apoptosis ratios relative to the absence of these inhibitors were determined. n=3, **p<0.01, ***p<0.001.

chemotaxis and superoxide generation[8, 9, 19], PtdGlc did not mediate either neutrophil chemotaxis (Fig.3C) or superoxide generation (Fig.3D). Therefore, it is likely that PtdGlc is neither located in LacCer-enriched lipid rafts nor involved in LacCer-mediated functions.

PtdGlc is involved in neutrophil apoptosis

A series of provocative discoveries led to the hypothesis that neutrophil programmed cell death is the result of an apoptosis-differentiation program, a final stage of transcriptionally regulated PMN maturation or hematopoietic differentiation[20]. Treatment with recombinant anti-PtdGlc Fab fragment, which preferentially reacts with PtdGlc, induced the differentiation of HL60 cells[12], suggesting that PtdGlc is involved in neutrophil differentiation. Moreover, treatment of neutrophils with the DIM21 resulted in the appearance of cells positive for annexin V, a sensitive probe used to quantitate cellular apoptosis (Fig.4A)[21]. In contrast, treatment with anti-LacCer antibody did not induce annexin V-positive cells. The terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) reaction is also used to measure apoptosis, because DNA cleavage generally occurs at much higher frequency in apoptotic than in non-apoptotic cells. TUNEL positive cells were significantly increased by DIM21 but not by T5A7 treatment (Fig.4B). Moreover, DIM21 but not T5A7 activated caspase-3, a hallmark event leading to apoptosis (Fig.4C)[20], and specific inhibitors of caspases 3, 8, and 9 significantly inhibited DIM21-induced neutrophil apoptosis (Fig.4D). These observations clearly suggest that PtdGlc clusters form functional domains leading to neutrophil apoptosis, and that these clusters differ from Lyn-coupled LacCer-enriched lipid rafts.

LacCer-mediated signal transduction is highly dependent on the activation of Lyn and PI_3 kinase[22]. PtdGlc was shown to form lipid raft-like domains and to be involved in rapid tyrosine phosphorylation of the Src family protein kinases Lyn and Hck[12]. However, several studies demonstrated that Src family kinases, including Lyn, suppressed apoptosis by inhibiting caspase-8 activity[23]. Many factors that promote neutrophil survival are known to activate PI_3 kinase[24]. PtdGlc-mediated neutrophil apoptosis was not inhibited by the Src family kinase inhibitor PP1 or by the PI_3 kinase inhibitor LY294002 (Fig.5A), further indicating that LacCer and PtdGlc form different domains on neutrophil plasma membranes and mediate different functions via different molecular mechanisms.

Neutrophils from patients with chronic granulomatous disease (CGD), who have a deficiency in superoxide generation, undergo spontaneous apoptosis but have a prolonged life span[25]. Reactive oxygen species (ROS) have been demonstrated to mediate neutrophil apoptosis[26]. SOD enhances the spontaneous apoptosis of neutrophils, whereas

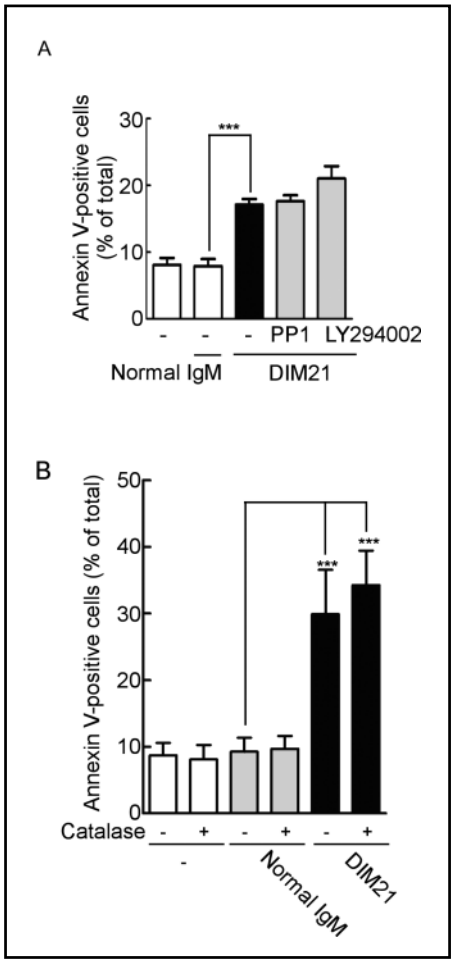

Fig.5 Effect of signal transduction inhibitors on PtdGlc-mediated apoptosis

(A) Neutrophils were preincubated with PP1 or LY294002, and incubated without (-) or with (+) normal IgM or DIM21 for 4h at 37℃. The specific binding of Alexa 488-conjugated annexin V to neutrophils was analyzed by flow cytometry. n=3
(B) Neutrophils were incubated without (-) or with (+) normal IgM or DIM21 in the absence (catalase -) or presence (catalase +) of catalase. Specific binding of Alexa Fluor488-conjugated annexin V to neutrophils was analyzed by flow cytometry. The number in each panel represents the percentage of Annexin V positive cells. n=3

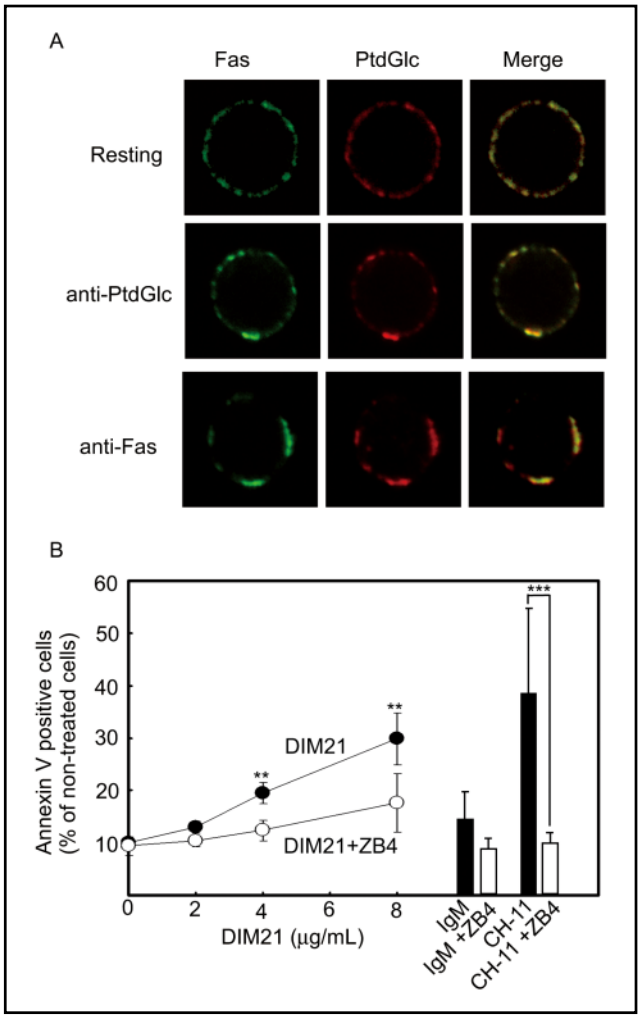

Fig.6 Association of PtdGlc and Fas

(A) Neutrophils were incubated without (Resting) or with anti-Fas IgG DX2, DIM21 at 37℃ for 5 min. The cells were washed with 10 volumes of ice-cold PBS, fixed, and treated sequentially with primary DX2 or DIM21 antibodies. The stained cells were examined with a TCS-SP2 Leica confocal microscope.
(B) Neutrophils were incubated with or without anti-Fas IgG ZB4, followed by the addition of DIM21, anti-Fas IgG CH-11(CH-11) or normal IgM. ● DIM21, ○ DIM21+ZB4, $**p<0.01$, $***p<0.001$.

catalase inhibits their spontaneous apoptosis and the activation of caspase-3[27], suggesting that hydrogen peroxide may mediate ROS-induced neutrophil apoptosis in a caspase-dependent manner. In contrast, other groups could not detect a difference in the rate of spontaneous apoptosis between neutrophils from normal individuals and patients with CGD[28]. Importantly, catalase did not affect DIM21-induced neutrophil apoptosis (Fig.5B), and DIM21 alone did not induce superoxide generation. These observations make it unlikely that ROS is involved in PtdGlc-mediated neutrophil apoptosis.

Fas-mediated death signal plays a key role in PtdGlc-induced apoptosis

Spontaneous apoptosis of neutrophils has been shown to be mediated by Fas-dependent signaling[29,30]. Fas-induced neutrophil apoptosis is sequentially mediated by the

activation of caspases 8, 9 and 3[31]. Specific inhibitors of each of these caspases significantly downregulated DIM21-induced neutrophil apoptosis (Fig.4D), suggesting that PtdGlc induces neutrophil apoptosis by activating caspases 8, 9 and 3.

Fas has been associated with lipid rafts during Fas mediated apoptosis[32-34]. PtdGlc and Fas colocalize on the plasma membranes of resting neutrophils (Fig.6A). Upon stimulation with DIM21, PtdGlc and Fas molecules form large clusters on the cell surfaces. Moreover, treatment with anti-Fas IgG DX2, which can induce apoptosis, also causes the formation of Fas clusters, which colocalize with PtdGlc. These observations suggest that PtdGlc forms large clusters with Fas and that cells are activated through the crosslinking of PtdGlc or Fas.

Fas is activated by inducing its trimerization[35]. Activated Fas recruits adaptor molecules, such as Fas-associating protein with death domain (FADD)[36]. In addition to activating FADD, Fas complexes recruit procaspase 8, where it undergoes autocatalytic activation. The apoptotic effects of the Fas agonistic antibody CH-11 are thought to be mediated through the cross-linking of Fas receptors, in a manner analogous to Fas ligand[37, 38]. The Fas antagonistic antibody ZB4 has been shown to block CH-11 induced apoptosis by interfering with the binding of the latter to its epitope on cells[39]. Since ZB4 significantly inhibited DIM-21 as well as CH-11- induced neutrophil apoptosis (Fig.6B), the Fas mediated pathway is likely responsible for PtdGlc-mediated neutrophil apoptosis.

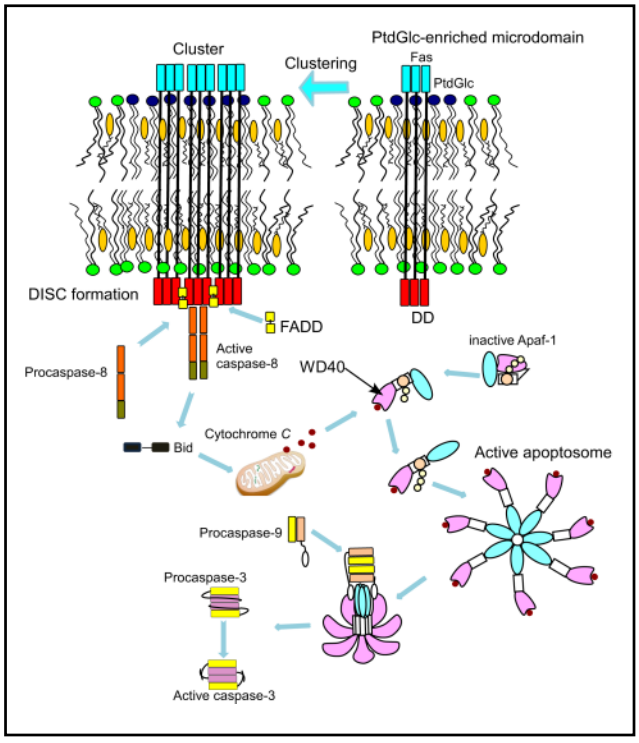

Fig.7 PtdGlc forms lipid rafts and is involved in Fas-dependent neutrophil apoptosis

PtdGlc can form large clusters with Fas, with cells activated through the crosslinking of PtdGlc or Fas. Activated Fas recruits adaptor molecules such as Fas-associating protein with death domain (FADD), which recruit procaspase 8 to the receptor complex, where it undergoes autocatalytic activation[36,45]. LacCer-enriched domains are more tightly packed than the surrounding ordered domains of the lipid bilayer, which contains complex lipid species carrying 16-18 carbon atom acyl chains. Interdigitation of C24:0- and C24:1-LacCer hydrophobic chains could occur in lipid rafts of neutrophils, causing the association of LacCer-enriched domains of external and cytoplasmic leaflets and the interaction of alkyl chains of LacCer and Lyn.

Conclusion

PtdGlc is a highly specific neutrophil marker expressed on human neutrophil progenitor cells and mature neutrophils. On mature neutrophils, PtdGlc forms functionally different domains from LacCer-enriched lipid rafts (Fig.7). PtdGlc but not LacCer is involved in Fas-dependent neutrophil apoptosis. The most important unresolved issue involving PtdGlc-mediated neutrophil apoptosis is to identify the natural ligands of PtdGlc *in vivo*. PtdGlc-enriched domains form glucose clusters on the neutrophil plasma membrane. Glucose residues can be recognized by certain kinds of glucose-binding lectins, such as glucose/mannose-specific lectins and C-type animal lectins. Macrophage mannose receptors, which are expressed on human macrophages, can bind to glucose residues[40, 41]. Apoptosis, the final step in the differentiation of neutrophils, is necessary to maintain a certain number of neutrophils *in vivo* and to allow macrophages to remove neutrophils from inflamed tissues, steps critical in limiting inflammatory tissue injury and the subsequent resolution of inflammation[2-4,42]. Spontaneous apoptosis of neutrophils may be initiated by the binding of PtdGlc to natural ligands. Further studies are required to elucidate the biological significance of PtdGlc in neutrophils.

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Disclosure of conflict of interests

There is no conflict of interests in this study.

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