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G2A and LPC: Regulatory functions in immunity

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Abstract

The G2A receptor was originally identified by virtue of its transcriptional induction in murine B lymphoid cells in response to oncogenic transformation and treatment with various DNA-damaging agents. While preliminary characterization of cellular responses to G2A overexpression in fibroblastic cell lines suggested that this receptor may negatively regulate cell growth under conditions of proliferative and genotoxic stress, subsequent studies driven by the discovery of lysophosphatidylcholine (LPC) as a regulator of G2A signaling in immunoregulatory cells point to an important role for this receptor in innate and adaptive immunity.

1. Introduction

Lysophosphatidylcholine (LPC) is a highly abundant bioactive lysolipid present at high concentrations in the circulation where it is predominantly associated with albumin and lipoproteins (1). The production of LPC by phospholipase A₂ (PLA₂)-mediated phosphatidylcholine (PC) hydrolysis, coupled to its re-acylation by LPC-acyltransferases (LPC-ATs), plays an important role in cellular phospholipid homeostasis, maintaining an adequate supply of fatty acid precursors for the generation of important lipid mediators in response to inflammation (2,3). The potentiation of secretory PLA₂ (sPLA₂) activity and the oxidative modification of cell membrane and lipoprotein phospholipids contribute to significant increases in local and circulating levels of LPC and oxidized fatty acids during inflammation and under conditions of oxidative stress (4,5). Based almost exclusively on studies of LPC effects on cultured cells, LPC thus generated is thought to influence the function of immunoregulatory cells to modulate inflammatory processes and immune responses. LPC is also considered to be an etiological factor in certain chronic inflammatory diseases, including atherosclerosis and the autoimmune disease systemic lupus erythematosus (SLE), in which local and systemic increases in LPC levels are a characteristic feature (6–9). Recent studies have demonstrated an important role for the G protein-coupled receptor (GPCR), G2A, in mediating cellular responses to LPC capable of modulating macrophage and T cell migration (10,11), neutrophil and macrophage activation (12–15), and phagocytic clearance of apoptotic cells and activated neutrophils (14,16). These LPC-dependent effects of G2A may contribute to mechanisms controlling the initiation or resolution of inflammation in response to infection and may also modify the susceptibility to sepsis and chronic inflammatory autoimmune disease by facilitating the efficient clearance of bacterial pathogens and apoptotic cells respectively. However, other potentially influential functions of G2A not ascribed to any specific lipid ligand have been revealed in studies with G2A deficient mice, including the regulation of lipoprotein-cholesterol metabolism. This review discusses these immunoregulatory properties of LPC with

focus on the role of the G2A receptor and its potential involvement in chronic inflammatory and autoimmune disease.

2. Discovery of G2A

The G protein-coupled receptor (GPCR), G2A, was originally identified by Owen Witte's group as a transcriptional target of the human leukemogenic tyrosine kinase, BCR-ABL, in murine bone marrow B lymphoid progenitor cells (17). Retrovirus-mediated overexpression of G2A in BCR-ABL expressing bone marrow cells resulted in a significant attenuation of BCR-ABL-induced B lymphoid cell expansion *in vitro* (17). Similarly, overexpression of G2A inhibited the transformation of RAT-1 fibroblasts (a cell-type lacking endogenous G2A expression) to anchorage-independent growth by BCR-ABL (17). Based on the finding that G2A overexpression in NIH 3T3 fibroblasts resulted in an accumulation of cells with a diploid DNA content (ie: G2/M phase of the cell cycle) (17), it was proposed that the transcriptional induction of G2A expression may exert a "tumor suppressive" function by slowing cell cycle progression through the G2 checkpoint. The observation that G2A transcription is also upregulated in B lymphoid cells following treatment with certain DNA-damaging agents (17) further supported the notion that the transcriptional induction of G2A expression may act to attenuate cell growth under conditions of proliferative and genotoxic stress. However, further characterization of G2A signaling in fibroblastic cell lines by Robert Kay's and Owen Witte's groups demonstrated that G2A overexpression results in actin stress fiber formation via $G\alpha_{13}$ heterotrimeric G protein-dependent activation of RhoA and suppressed contact inhibition of fibroblast growth (18,19). Importantly, no inhibitory effect of G2A overexpression on fibroblast proliferation was reported in these studies, suggesting that a slowing of cell cycle progression through the G2 checkpoint may not in fact underlie the previously described accumulation of G2A overexpressing NIH 3T3 cells in the G2/M phase of the cell cycle (17). In light of the important role played by rearrangement of the cellular actin cytoskeleton and microtubule networks in orchestrating mitotic division, it is perhaps worth considering that the afore-mentioned potentiation of actin stress fiber formation in response to G2A overexpression may deregulate these dynamic processes sufficiently to delay cell cycle progression through mitosis rather than G2. Indeed, morphological examination of flow-sorted G2/M fractions from Hoechst 33342-stained G2A overexpressing NIH 3T3 cells revealed a significant increase in the frequency of mitotic cells compared to G2/M preparations flow-sorted from control NIH 3T3 cells (Kabarowski, J.H., unpublished data). Thus, any potential modulatory effect of G2A on cell growth may be mediated indirectly by its effects on the actin cytoskeleton. However, this may not reflect the normal physiological response to increases in G2A expression *in vivo*, as we failed to detect congruous alterations in cell-cycle distribution in primary cells from G2A deficient mice compared to their wild-type counterparts that normally express G2A (Parks, B.W., Kabarowski, J.H., unpublished data). Although T cells from G2A deficient mice have been shown to exhibit hyperproliferative responses to anti-CD3 antibody-mediated antigen receptor crosslinking *in vitro* (20,21), we found no evidence of abnormal proliferative expansion of antigen-specific T cells in G2A deficient mice following immunization (21). It is likely, therefore, that the increased proliferation of G2A deficient T cells observed *in vitro* may not reflect a true physiological function of T cell expressed G2A *in vivo*, and may instead result from the artificial experimental culture conditions employed. Nevertheless, in an elegant study employing micro-positron emission tomography (micro-PET) to quantitatively monitor leukemia development in live mice transplanted with BCR-ABL expressing bone marrow cells, Le *et al* demonstrated that G2A deficiency significantly accelerates BCR-ABL-induced leukemogenesis (22). In this study, leukemic cells emerged at similar anatomical locations in mice irrespective of G2A expression, but did so more rapidly in the absence of G2A. Thus, it remains possible that the transcriptional induction of G2A expression in lymphocytes may provide a growth inhibitory signal as a response to malignant transformation by certain

oncogenes. Additional studies are clearly warranted to further characterize this potential tumor suppressive function of G2A.

3. Ligand specificity of G2A

Early studies aimed at characterizing G2A function employed cell lines engineered to overexpress the receptor in which cellular responses were observed in the absence of exogenously added factors other than fetal calf serum. This suggested that G2A might be a constitutively active GPCR regulated primarily by modulation of its expression, or that its cognate ligand is a naturally abundant component of serum and/or product of cellular metabolism. In accordance with the latter scenario, overexpression of G2A in CHO cells and Swiss 3T3 fibroblasts conferred responsiveness to lysophosphatidylcholine (LPC) (23), a lysolipid present at high concentrations in serum and continually produced by cells as a result of PLA₂-mediated phosphatidylcholine (PC) hydrolysis. When applied to G2A overexpressing cells, LPC at concentrations as low as 100nM elicited rapid activation of ERK2 MAP kinase, while micromolar LPC concentrations stimulated the migration of G2A overexpressing Jurkat T cells in a transwell chamber chemotaxis assay (23). One study subsequently described pro-apoptotic effects of G2A overexpression that were potentiated by LPC treatment in various cell lines, including those representative of T lymphocytes (24). However, we observed no significant effect of G2A on lymphocyte apoptosis by flow cytometric Annexin-V staining in mice engineered to overexpress G2A in hematolymphoid cells by transplantation with bone marrow cells transduced with G2A-encoding retroviruses (Parks, B.W., Kabarowski, J.H., unpublished data). Therefore, as for the proposed anti-proliferative function of G2A in T lymphocytes (20,21), direct evidence is lacking to support a pro-apoptotic function of G2A in T lymphocytes *in vivo*. Nevertheless, in accordance with a potentiating effect of G2A on macrophage apoptosis, our group observed a mild reduction in macrophage apoptosis within early atherosclerotic lesions in G2A deficient hypercholesterolemic low-density lipoprotein receptor knockout (LDLR^{-/-}) mice (25). A similar effect of G2A deficiency was recently reported in hypercholesterolemic apolipoprotein-E knockout (ApoE^{-/-}) mice (26). However, this effect of G2A deficiency was not manifested at later stages of atherosclerotic lesion development in hypercholesterolemic LDLR^{-/-} mice (27) and the results of a recent study by our group suggest that the anti-apoptotic effect of G2A deficiency could be mediated indirectly by a potentiating effect of G2A deficiency on hepatic high-density lipoprotein (HDL) biogenesis (28) (discussed later, 6.).

Secretory phospholipase A₂ (sPLA₂) enzymes released by macrophages and other inflammatory cell-types contribute to local and systemic increases in LPC during inflammation by hydrolyzing membrane and lipoprotein PC (29,30). Stimulatory effects of LPC on cell migration have been widely reported in monocytes/macrophages and T cells (31,32), suggesting that sPLA₂-mediated production of LPC may facilitate leukocyte recruitment at sites of inflammation and/or regulate peripheral lymphocyte trafficking through secondary lymphoid organs under inflammatory conditions (discussed later, 5.1.). Indeed, augmentation of sPLA₂ activity has been shown to mediate LPC liberation and subsequent activation of G2A-dependent responses such as neurite outgrowth in rat pheochromocytoma PC12 cells (33). However, under conditions of oxidative stress, PLA₂ activity can result in the concomitant generation of oxidized free fatty acids from oxidatively modified PC (34). Recent studies demonstrating signaling responses of G2A overexpressing CHO and HEK293 cells to 9-hydroxyoctadecadienoic acid (9-HODE) and other oxidized free fatty acids (35,36) as well as G2A-mediated pro-inflammatory effects of 9-HODE in keratinocytes (37) therefore suggest that G2A activity may be subject to dual regulation by two classes of lipids generated simultaneously in response to inflammation and under conditions of oxidative stress. The reader is directed to an excellent review by Obinata and Izumi on the topic of G2A as a receptor for oxidized free fatty acids in this issue (34).

4. Regulation of G2A signaling LPC

Although LPC was reported to activate G2A-mediated cellular responses as a receptor binding ligand (23), attempts by our group and that of Owen Witte failed to reproduce data provided by our collaborators demonstrating G2A/LPC binding in crude cell homogenates prepared from receptor overexpressing cell lines due to high non-specific membrane binding of LPC which precluded detection of ligand/receptor interactions in conventional binding assays (38,39). While a direct interaction between G2A and LPC at the cell surface has not been verified, several studies have since determined that intracellular sequestration and surface expression control G2A signaling in response to LPC. The first such study by Owen Witte's group demonstrated that activation of cellular migratory responses of G2A overexpressing DO11.10 T cells by LPC are mediated by mobilization of intracellular endosomal G2A pools to the plasma membrane resulting in an increased expression of cell-surface receptors (40). Each of the most abundant LPC species in plasma (16:0, 18:0 and 18:1) (25) elicited plasma membrane redistribution of overexpressed G2A linked to green fluorescent protein (G2A-GFP) to comparable extents in DO11.10 T cells as measured by immunofluorescence microscopy (40). Global inhibition of intracellular recycling by monensin inhibited G2A mobilization to the cell surface and suppressed LPC-stimulated ERK2 MAP kinase activation and migration in DO11.10 T cells (40). The failure of LPC to activate G2A-mediated ERK2 signaling when receptor recycling was blocked by monensin suggests that the surface-localized pool of G2A present under resting conditions (~30% of total G2A receptor as measured by immunofluorescence microscopy in untreated DO11.10 T cells) (40) is incapable of responding to LPC and that either movement of additional G2A receptors to the surface, or the subsequent internalization of surface receptors, is obligatory for signaling. The observation that ERK2 signaling by a mutant form of G2A [G2A DRY→DAY (40)] exhibiting constitutive plasma membrane localization is insensitive to monensin treatment is consistent with a model in which elevations in extracellular LPC concentration stabilize the surface expression of G2A to activate receptor signaling. Endogenous G2A receptor was similarly regulated by LPC treatment in DO11.10 T cells (40) and other structurally related lysolipids in addition to LPC, including lysophosphatidylserine (lyso-PS), were subsequently shown by Frasch *et al* to induce G2A plasma membrane redistribution in primary human neutrophils (13). In this study, LPC and lyso-PS treatment of neutrophils stimulated calcium flux in a pertussis toxin sensitive manner, implicating the involvement of Gα_i signaling to phospholipase C, and this signaling response was inhibited by addition of a commercially available polyclonal anti-G2A antibody. Furthermore, flow cytometric assessment of cell surface G2A expression demonstrated that receptor redistribution was evident within 1 minute of lysolipid treatment and was not associated with membrane permeabilization when lysolipids were presented to cells bound to albumin (13). However, an enhanced staining of LPC treated neutrophils with FM 1-43, a compound that preferentially inserts into loosely packed membranes, suggested that stabilization of G2A at the cell surface may be mediated by alterations in the plasma membrane leaflet induced by lysolipid insertion (13). If so, it is unclear how such an effect of LPC could mediate the selective stabilization of G2A at the plasma membrane without evoking a direct lysolipid/receptor interaction which has not been demonstrated. Nevertheless, of the various lysolipids shown to mediate G2A receptor redistribution and signaling in neutrophils, lyso-PS may be of particular physiological significance with respect to neutrophil function as it can be generated on activated neutrophils from NADPH oxidase modified membrane phosphatidylserine (PS) by PLA₂-mediated acyl chain hydrolysis and may act as a recognition signal for their phagocytic clearance by macrophages to prevent excessive inflammation during infection (14) (discussed later, 5.2.).

5. LPC-dependent functions of G2A in immunoregulatory cells

G2A is expressed by a broad range of immunoregulatory cell-types. Major cells of the innate (macrophages, dendritic cells, neutrophils, mast cells) and adaptive (T and B lymphocytes) arms of the immune system express G2A (13,25) and so its potential role in mediating modulatory effects of LPC on inflammation and immunity has become an area of considerable interest. Furthermore, certain chronic inflammatory syndromes and autoimmune diseases are associated with alterations in lipoprotein and phospholipid metabolism which evoke significant elevations in local and systemic LPC levels (discussed later, 7.3.). Focus on LPC in the pathophysiological context therefore extends beyond simply its potential utility as a biomarker for disease, as it is possible that therapeutic modification of its interaction with G2A could be beneficial in the clinical management of diseases with a strong inflammatory component. The results of recent studies have revealed LPC-dependent functions of G2A in immunoregulatory cell-types that may provide a logical rationale for such approaches.

5.1. Chemotactic action of G2A in macrophages and T cells

Experiments in primary macrophages from G2A deficient mice, as well as relevant macrophage and T cell lines expressing G2A-specific small interfering RNA (siRNA) molecules or overexpressing the G2A receptor, have shown that stimulation of macrophage and T lymphocyte chemotaxis by LPC is mediated by G2A (10,11,23,40). In addition, one study reported a potentiating effect of 10–20 μ M concentrations of LPC on primary human T lymphocyte chemotaxis in response to stromal derived factor-1 α (SDF-1 α) that was associated with upregulation of the SDF-1 α receptor, CXCR4, and ameliorated by pretreatment of cells with antisense oligonucleotides specific for G2A sequences (41). Taken together, these observations led to the proposition that G2A might facilitate the recruitment of macrophages and T lymphocytes into, or maintain their retention within, inflammatory foci rich in LPC. However, unlike LPC-dependent macrophage chemotaxis, there is no published evidence that G2A directly mediates chemotactic responses of primary T lymphocytes towards LPC, raising doubt over the physiological significance of data reporting such effects of G2A in T lymphocyte cell lines (Jurkat and DO11.10) (11,23,40). Furthermore, chemotactic responses of DO11.10 T lymphocytes elicited by concentrations of LPC up to 10 μ M appeared weak in comparison to those elicited by classical chemokines such as SDF-1 α , exhibiting a potency less than 3% that of SDF-1 α (40). In addition, although the majority of endogenous G2A was knocked down in DO11.10 T lymphocytes expressing G2A-specific siRNA molecules, their chemotactic response to LPC was only reduced by approximately 50% (11,40), suggesting that certain concentrations of LPC can evoke migratory responses in these cells independently of G2A. Indeed, we have been unable to detect significant differences between wild-type and G2A deficient mouse primary T lymphocytes with respect to their chemotactic responses to LPC in transwell chemotaxis chamber assays similar to those previously employed to demonstrate G2A dependent chemotactic responses to LPC in T lymphocyte cell lines overexpressing G2A or expressing G2A-specific siRNA molecules (11,23) (Parks, B.W., Kabarowski, J.H., unpublished data). Furthermore, current evidence from studies of G2A deficiency in mouse models of inflammatory diseases associated with local over-production of LPC is more consistent with the absence of such an effect in primary T lymphocytes. For example, we did not observe a difference in the frequency of sub-endothelial T lymphocyte infiltrates within the inflammatory milieu of atherosclerotic lesions of G2A deficient hypercholesterolemic LDLR $^{-/-}$ mice compared to their G2A-sufficient counterparts (25,27). Although the fact that a chemotactic function of G2A is not manifested in this mouse model may not be surprising considering the potency of classical chemokine networks in mediating the sub-endothelial recruitment of leukocytes during atherogenesis (42), it is nevertheless noteworthy that G2A does not appear to be involved in mediating their subsequent lesional retention either, despite

evidence that lipid products of lipoprotein oxidation within the arterial wall may play important roles in this process (43).

The cumulative published data highlight macrophage chemotaxis as a principal cellular response to G2A activation by LPC and suggest that this receptor may play a role in controlling the ability of phagocytic cells to recognize and/or subsequently respond to immunogenic and potentially injurious LPC-rich moieties. In this regard, macrophage uptake of oxidized low-density lipoprotein (oxLDL), although initially a protective mechanism designed to eliminate this harmful moiety, leads to macrophage foam cell formation, a key event propagating the development of atherosclerotic lesions. Macrophage-mediated clearance of apoptotic cells, on the other hand, is considered vital to prevent the accumulation of necrotic cells which could otherwise precipitate inflammation. In addition, the process of apoptotic cell clearance ensures efficient “sampling” and subsequent presentation of self-antigens required for the maintenance of immunological tolerance (44). Perturbations in this process can therefore result in a predisposition to the development of autoimmune disease, most notably exemplified by the association between impaired apoptotic cell clearance and the development of systemic lupus erythematosus (SLE), an autoimmune disease characterized by auto-reactive T lymphocyte and auto-antibody responses against nuclear antigens (discussed later, 7.2.).

5.2. G2A function in neutrophils

Neutrophils constitute an important cellular component of the innate immune system and are amongst the earliest cell-type to be recruited to tissues in response to infection and injury. Although extremely short-lived, anti-microbial effects of activated neutrophils are mediated to a large extent by the induction of the NADPH oxidase-dependent oxidative burst, resulting in the rapid production of antibactericidal molecules such as hydrogen peroxide (H_2O_2). A recent elegant and thorough study by Frasch *et al* support a scenario in which LPC and structurally related lysolipids such as lyso-PS produced by PLA₂ during inflammation activate G2A signaling in neutrophils leading to increases in calcium flux (13). Based on the fact that albumin concentrations approaching those naturally found in plasma (~4μM) abolish the capacity of LPC to activate G2A-mediated signaling (13,45), the authors of this study hypothesized that LPC stimulation of neutrophils may be an early inflammatory event, occurring upon their initial recruitment from the circulation into inflamed tissue where albumin concentrations are significantly lower and the “consumption” of locally generated LPC capable of activating G2A is therefore reduced. This is an attractive scenario, as the levels of albumin and other proteins capable of scavenging LPC are modulated during inflammation and the acute phase response (APR) to infection (46,47). Notably, albumin levels are significantly reduced during the APR (46) and this may therefore constitute an additional regulatory mechanism by which G2A-mediated cellular responses can be controlled. However, an earlier study from Richard Ye’s group demonstrated an inhibitory, rather than stimulatory, effect of LPC on the generation of oxidants by activated neutrophils that was partially dependent on Gα_s-mediated elevation of intracellular cyclic AMP (48). Although the involvement of G2A was not addressed in this study, it nevertheless suggests that LPC may elicit different effects on neutrophils depending on their level of activation. For example, perhaps LPC effects on newly immigrated neutrophils at a site of infection promote inflammatory responses designed to eliminate the pathogen, as postulated by Frasch *et al* (13), while the responses of fully activated neutrophils to LPC may act to attenuate NADPH oxidase-mediated inflammatory functions (48) to contribute to the efficient resolution of inflammation as the pathogen is cleared.

Within hours of their activation in inflamed tissue, neutrophils are cleared by phagocytic macrophages in order to ensure the efficient resolution of inflammation. Lysolipids produced by these activated neutrophils have recently been shown to play a role in this process. Frasch

et al demonstrated that lyso-PS, but not LPC, is generated by primary human neutrophils activated by opsonized zymosan via activation of NADPH oxidase and subsequent hydrolysis of oxidized phosphatidylserine (oxPS) by PLA₂ or myeloperoxidase (MPO)-derived hypochlorous acid (14). In a mouse model of peritonitis induced by intraperitoneal (IP) injection of zymosan, IP injection of a commercially available polyclonal anti-G2A antibody retarded the resolution of neutrophilic inflammation. Furthermore, neutrophils with morphological characteristics of apoptotic cells accumulated in the presence of this antibody (14). In an elegant experiment, the authors of this study tested whether IP injection of the same anti-G2A antibody similarly slowed the resolution of zymosan-induced peritoneal neutrophilic inflammation in mice lacking gp^{phox}, a key component of the NADPH oxidase system. Unlike their wild-type counterparts, lyso-PS was not generated during peritonitis in gp^{phox}-deficient mice and no effect of the anti-G2A antibody on peritoneal neutrophil numbers was observed. Further studies in G2A deficient mice are clearly warranted considering the limitations of utilizing antibodies to neutralize G2A signaling *in vivo*.

5.3. G2A and LPC: protective effects against sepsis

The rapid and robust recruitment of neutrophils to sites of bacterial infection is a vital innate immune process designed to clear the pathogen. Subsequent clearance of these neutrophils following elimination of the bacterial pathogen is a key step in the resolution of the inflammatory response to microbial infection and the avoidance of neutrophil-mediated tissue injury. However, potentially life-threatening syndromes such as sepsis or endotoxemia characterized by systemic inflammation and organ dysfunction can arise if the bacterial pathogens are not properly eliminated. G2A has been linked both to the initial activation of neutrophil bactericidal function in response to infection (12), as well as the subsequent clearance of activated and dying neutrophils by macrophages required for the successful resolution of inflammation (5.2.) (14). Consistent with an important role for G2A as a mediator of neutrophil activation, studies in mice have demonstrated that G2A mediates potentiating effects of LPC on bacterial clearance and provides protection against sepsis and endotoxemia. When mice were treated by subcutaneous (SC) injection of 4 doses of 18:0 LPC (1–10 mg/Kg with 2% albumin) at 12 intervals beginning at time-points as late as 10 hours (but not 16 hours) after cecal ligation and puncture (CLP, a clinically relevant model of bacterial sepsis), peritoneal bacterial clearance was enhanced and lethality was reduced (12). Similar LPC treatment also reduced mortality in mice caused by IP injection of the endotoxin, lipopolysaccharide (5mg/Kg), and attenuated plasma levels of TNF α and interleukin-1 (IL-1), causative mediators of sepsis. These protective effects of 18:0 LPC were blunted by intravenous injection of a goat polyclonal anti-G2A antibody (80 μ g per mouse), pointing to G2A as the mediator of LPC-induced protection against sepsis. Indeed, high concentrations of 18:0 LPC (30 μ M) potentiated the generation of H₂O₂ by neutrophils to enhance their bactericidal activity *in vitro* and the same anti-G2A polyclonal antibody inhibited this effect (12). However, there is as yet no published data reporting whether these protective effects of LPC are also ameliorated in G2A deficient mice.

Lyso-PS and other LPC species, including 16:0 LPC, were ineffective at protecting against sepsis or endotoxemia and failed to stimulate H₂O₂ generation by neutrophils *in vitro* (12). It may be noteworthy in this regard that although both 16:0 LPC and 18:0 LPC (presented with albumin) stimulated calcium flux by neutrophils, only that induced by 18:0 LPC occurred in the absence of cell permeabilization (13). Acyl chain specificity and concentration are therefore both critical determinants of the protective action of LPC. Furthermore, other effects of LPC have been reported that could contribute to its protective action against sepsis. For example, high-mobility group box 1 (HMGB1) is secreted by monocytes/macrophages and is considered a late mediator of sepsis (49). HMGB1 stimulates the release of proinflammatory cytokines from other immunoregulatory cell-types and injection of anti-HMGB1 antibodies, or treatment

with agents that inhibit its release, which include 18:0 LPC (15), protect mice against sepsis (15,50,51). IP administration of 18:0 LPC (20mg/Kg) to mice inhibited HMGB1 release during lipopolysaccharide-induced (10mg/Kg) endotoxemia and CLP-induced sepsis (15). Furthermore, the inhibitory effect of 18:0 LPC (30 μ M) on lipopolysaccharide-induced HMGB1 release from cultured macrophages was attenuated in the presence of a commercially available anti-G2A polyclonal antibody (1–5 μ g/ml) (15). While the mechanism by which 18:0 LPC inhibits HMGB1 secretion by macrophages through G2A has not been established, one could speculate that LPC-induced mobilization of the G2A receptor to the plasma membrane may be mechanistically relevant (40). Considering the fact that both 18:0 LPC and 16:0 LPC induce G2A redistribution to the plasma membrane (40), yet only the former is effective at protecting against sepsis and endotoxemia (12), it may be worthwhile examining whether 16:0 LPC inhibits HMGB1 release in lipopolysaccharide-treated macrophages.

6. Modulation of lipoprotein metabolism and atherosclerosis by G2A

A key pathophysiological process in the development of atherosclerosis is the generation of bioactive lipids as a result of the oxidative modification of low-density lipoprotein (LDL) particles trapped in the vascular intima (52). Pro-atherogenic effects of such bioactive lipids, which include lysolipids and oxidized fatty acids, are mediated to a large extent by their ability to promote the sub-endothelial recruitment of inflammatory cells (monocytes/macrophages and T lymphocytes) and the formation of macrophage foam cells within the vascular wall (53). LPC is generated in the arterial wall at atherosclerotic foci by platelet activating factor-acetylhydrolase (PAF-AH) mediated hydrolysis of oxidized PC decomposition products of LDL oxidation (54) and by the hydrolysis of lipoprotein and membrane PC by sPLA₂ enzymes released by inflammatory cells (29). A principal mechanism counteracting the excessive accumulation of LDL-derived lipids/cholesterol and macrophage foam cell formation in the vascular wall is the process of reverse cholesterol transport (RCT) whereby high-density lipoprotein (HDL) particles, synthesized predominantly by hepatocytes (55), transport excess lipids and cholesterol from peripheral tissues back to the liver for excretion (56). In addition to the beneficial effect of increasing circulating HDL particle numbers, the composition of HDL also influences its athero-protective properties. For example, HDL-associated enzymes such as paraoxonase (PON) exert anti-oxidant and anti-inflammatory effects capable of attenuating the generation of bioactive lipids produced during LDL oxidation and reducing local inflammation in the vascular wall (57). Both quantitative and qualitative alterations in HDL can therefore modulate inflammation and it is widely accepted that identifying new therapeutic targets capable of modulating HDL biogenesis and/or composition will have significant benefits in atherosclerosis and perhaps also in other chronic inflammatory diseases.

Studies by our group demonstrated that G2A deficient mice on the hypercholesterolemic LDL receptor knockout (LDLR^{-/-}) background develop atherosclerosis at a significantly reduced rate compared to their G2A-sufficient counterparts (27). Although this athero-protective effect of G2A deficiency was proposed to be due, at least in part, to the loss of G2A-mediated chemotactic responses of monocytes/macrophages and/or T lymphocytes to LPC generated in the vascular wall resulting in the attenuation of inflammatory cell recruitment, no reduction in the number of T lymphocytes in atherosclerotic lesions was detected (25,27) and a subsequent study revealed that G2A deficiency in bone marrow-derived cells (which include monocytes/macrophages and T lymphocytes) of LDLR^{-/-} mice had no effect on atherosclerosis (28). These data showed that the chemotactic function of G2A does not modulate atherosclerosis in LDLR^{-/-} mice. However, elevated concentrations of plasma HDL in hypercholesterolemic G2A deficient LDLR^{-/-} mice (27) suggested that modulation of HDL metabolism might contribute to the athero-protective effect of G2A deficiency. Consistent with this scenario, bone marrow transplantation revealed that deletion of G2A in resident tissues alone is sufficient to raise plasma HDL concentrations and suppress atherosclerosis in LDLR^{-/-} mice (28).

Hepatocytes were found to express G2A, albeit at considerably lower levels compared to macrophages (28). By comparing the functional characteristics of hepatocytes from G2A deficient and G2A-sufficient hypercholesterolemic LDLR^{-/-} mice, we found that the absence of G2A resulted in a significantly greater secretion of apolipoprotein A1 (ApoA1), the principal constituent of all HDL particles. While the attenuation of hepatocyte ApoA1 secretion by G2A and resulting reductions in the numbers of circulating HDL particles may contribute to elevating the risk for atherosclerosis development in hypercholesterolemic LDLR^{-/-} mice, the resulting decreases in HDL-associated anti-inflammatory and immunomodulatory effects could also conceivably influence the development of autoimmunity (discussed later, *section 7.3.*).

Although the mechanism by which G2A regulates ApoA1 secretion by hepatocytes is not presently understood, it is tempting to speculate that lysolipids such as LPC present on lipoproteins within the hepatic milieu (1) may regulate ApoA1 recycling/secretion by hepatocytes through G2A to achieve appropriate modulatory effects on HDL biogenesis in response to hypercholesterolemia. Approximately 60% of plasma LPC is found in chromatographically separated fractions containing HDL and albumin in human plasma, with the remainder present on VLDL/LDL fractions (1). A major portion of the LPC present on HDL is derived from lecithin:cholesterol acyltransferase (LCAT)-mediated deacylation of PC during cholesterol esterification (58). This LPC may therefore act as an “indicator” of esterified cholesterol levels on HDL particles, “sensed” by hepatocyte G2A receptors to coordinate fluctuations in peripheral tissue cholesterol accumulation with necessary changes in HDL production. However, it is important to note that plasma HDL elevations were the result of G2A deficiency in hypercholesterolemic LDLR^{-/-} mice, supporting a scenario in which LPC-mediated redistribution of endosomal G2A receptor pools to the plasma membrane may relieve their inhibitory effect on ApoA1 secretion, rather than actually augmenting ApoA1 secretion. We therefore favor a model in which, through a direct or indirect mechanism, G2A localized to hepatocyte endosomes acts as a “brake” on ApoA1 secretion that is constantly modified in response to fluctuating local levels of LPC as a means of regulating plasma HDL levels. Future studies aimed at testing such a model and establishing the mechanism of G2A action will undoubtedly have to take into account several important issues. For example, it is possible that one or more G2A-mediated signaling pathways activated by 9-HODE act to control hepatocyte ApoA1 recycling and/or secretion. Studies examining the molecular interactions involved in hepatocyte G2A action must therefore address the potential involvement of a broad range of lipid activators as well as strive to recapitulate *in vitro* their modes of production in the hepatic environment in response to hypercholesterolemia.

7. Role of G2A in autoimmunity

7.1. T lymphocyte proliferation and chemotaxis

The finding that LPC stimulates chemotaxis of T cells *in vitro* through activation of the G2A receptor (11,23,40) led to the proposition that G2A may contribute to the recruitment of T cells to sites of inflammation and thus promote autoimmune diseases such as multiple sclerosis (MS) in which the generation and subsequent tissue infiltration of autoreactive T cells play important pathophysiological roles. In support of this notion, PLA₂ activity is high in MS lesions and its inhibition in mice leads to a significant reduction in the onset and progression of experimental autoimmune encephalomyelitis (EAE), a well-characterized mouse model of MS in which autoreactive T cells are generated in secondary lymphoid organs following immunization with myelin oligodendrocyte glycoprotein (MOG) peptide and subsequently infiltrate the central nervous system (CNS) (59,60). The LPC-sensitive chemotactic action of G2A, if penetrant *in vivo*, may therefore be expected to exacerbate EAE by promoting autoreactive T cell infiltration. However, G2A was described in an earlier study as an “anti-proliferative” receptor in T cells whose absence in mice predisposes them to the development of a systemic

autoimmune syndrome resembling human systemic lupus erythematosus (SLE) (20). Although mice with heterogenous genetic backgrounds were used in this study, it was postulated by the authors that an increased proliferative expansion of autoreactive T cells contributed to the development of SLE in these G2A deficient mice based on the fact that T lymphocytes from G2A deficient mice exhibited hyperproliferative responses to anti-CD3 antibody-mediated antigen receptor crosslinking *in vitro* (20). We recently addressed the relative contribution of these proposed chemotactic and anti-proliferative functions of G2A to the pathophysiology of EAE (21). G2A-sufficient and G2A deficient mice backcrossed the same number of generations onto the C57BL/6J background exhibited a similar incidence and onset of disease following immunization with MOG peptide. Furthermore, the numbers of MOG-specific T cells generated in secondary lymphoid organs of MOG immunized G2A deficient mice was comparable to that in their G2A-sufficient counterparts, despite the fact that T lymphocytes from these G2A deficient mice retained hyperproliferative responses to antigen receptor crosslinking *in vitro* (21). Although disease severity was mildly reduced in G2A deficient mice, comparable numbers of T cells were detected in spinal cords of G2A-sufficient and G2A deficient mice with EAE (21), demonstrating that the proposed “anti-proliferative” and chemotactic functions of G2A are incapable of modifying the generation of autoreactive T cells or their subsequent recruitment into the CNS to significantly alter the course of EAE. However, distinct effects of G2A on the susceptibility to other autoimmune diseases may be manifested in appropriate mouse models, a possibility currently being investigated by our group.

7.2. G2A in macrophage-mediated apoptotic cell clearance

In addition to elimination of pathogen, macrophage recruitment to inflammatory sites is critical for efficient clearance of necrotic cell debris and apoptotic cells. Recognition and uptake of apoptotic cells by macrophages and immature dendritic cells is also important for maintaining peripheral tolerance to self antigens (44). By “sampling” and subsequently presenting self antigens derived from apoptotic cells, these monocyte-derived cells constitute a critical homeostatic mechanism contributing to the suppression of autoimmunity. Impaired apoptotic clearance can promote the development of autoimmunity (61). Indeed, abnormal accumulation of apoptotic cells associated with a reduced frequency of engulfed apoptotic nuclei within macrophages has been described in SLE-prone mouse strains (62). In addition to alterations in surface expression of phospholipids such as PS, PC oxidation is also a characteristic feature of apoptotic cells, resulting in the expression of oxLDL-specific ligands on their surfaces that are recognized by macrophage scavenger receptors to mediate their engulfment (63). Although increased PLA₂-mediated LPC production in the outer plasma membrane leaflet of injured and apoptotic cells has been suggested to potentiate binding of the C-reactive protein (CRP) and natural immunoglobulin M (IgM) antibodies leading to complement activation and their consequent phagocytosis (64–66), recent studies suggest that this LPC is also released from apoptotic cells to provide an attraction signal for the recruitment of macrophages via G2A to facilitate efficient apoptotic cell clearance (16,67). Lauber *et al* demonstrated that LPC is released from apoptotic cells by the caspase-3-dependent activation of calcium-independent PLA₂ and that culture supernatants from apoptotic cells applied to the lower chamber of a transmigration plate can stimulate the chemotaxis of macrophages from the upper chamber (16,67). Knockdown of macrophage G2A expression by G2A-specific siRNA molecules inhibited their chemoattraction towards apoptotic cell culture supernatants as well as LPC (16). Addition of LPC to the macrophages in the upper chamber of the transmigration plate in order to selectively ameliorate the LPC gradient resulted in the inhibition of macrophage chemotaxis (16), thus establishing a key role for LPC in mediating this chemotactic response via G2A. Interestingly, when various lysolipids and oxidized free fatty acids were tested in a similar fashion, it was found that lyso-PS partially blocked the chemoattraction of macrophages towards apoptotic cell culture supernatant (16). However, unlike LPC (10), lyso-PS did not stimulate macrophage chemotaxis in a conventional transwell chemotaxis assay (16). As both

LPC and lyso-PS have been shown to similarly induce G2A redistribution to the plasma membrane, albeit in different hematopoietic cell-types (T lymphocytes and neutrophils respectively) (4.) (13,40), this suggests that these two structurally related lysolipids may subsequently exert different effects on cell surface receptor signaling to mediate distinct cellular responses. If so, the observation by Frasch *et al* that lyso-PS treatment results in cell surface “patching” of the G2A receptor following its mobilization to the plasma membrane (13) may be mechanistically relevant. The authors of this study hypothesized that the lyso-PS-induced cell surface G2A “patching” occurs as a result of receptor coalescence accompanying desensitization to subsequent lyso-PS stimulation after the initial formation of signaling receptor dimers/oligomers. Finally, it is additionally noteworthy that oxidized free fatty acids capable of activating G2A (34) also failed to elicit chemotactic responses of macrophages in transwell chemotaxis chamber assays (16).

Although G2A mediates LPC-dependent migration of macrophages towards apoptotic cells, there is no direct evidence that this facilitates their engulfment. It is unlikely that apoptotic cell uptake by macrophages in conventional *in vitro* liquid culture-based assays would be affected in the absence of the G2A-mediated chemoattractive signal. Indeed, we have been unable to detect differences in apoptotic cell uptake employing such methods and *in vivo* experimental approaches are currently in progress. However, it should perhaps also be considered that other lipid mediators released from apoptotic cells in addition to LPC have been implicated as attraction signals for macrophage apoptotic cell engulfment. For example, caspase-dependent sphingosine kinase 1 (SphK1) upregulation leading to increased sphingosine-1-phosphate (S-1-P) secretion has been demonstrated to occur during apoptosis and can potently stimulate the chemotaxis of monocyte/macrophage cell lines (68). This raises the possibility that S-1-P secretion may account for a proportion of the macrophage chemotaxis towards apoptotic cell culture supernatants observed in the study by Lauber *et al* described earlier. However, it was not determined whether, similarly to LPC, a competitive effect of S-1-P on macrophage chemotaxis to apoptotic cell culture supernatant occurs following its application to macrophages in the upper chamber of a transmigration plate.

7.3. Lipoprotein-cholesterol metabolism and autoimmunity

The acute phase response (APR) to infection and inflammation is a protective reaction orchestrated largely by modulation of hepatic synthesis of specific plasma proteins leading to alterations in their circulating levels (46). Proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) that are increased during the APR are termed positive acute phase (AP) proteins, while those that are reduced are negative AP proteins and include albumin as well as certain proteins involved in lipoprotein-cholesterol and lipid metabolism (46,69). These changes in AP protein synthesis are mediated by cytokines such as tumor necrosis factor- α (TNF α), interleukin-1 (IL-1) and interferon- γ (IFN- γ) produced by immunoregulatory cells (monocytes/macrophages, T lymphocytes) and endothelial cells. Although changes in AP proteins collectively serve to facilitate the elimination of pathogens, modulate immune responses and attenuate tissue damage, if prolonged due to a failure to eradicate the infection, it can elevate the risk for developing chronic inflammatory disease.

Modification of lipoprotein and lipid metabolism also occurs during the APR, including alterations in very low-density lipoprotein (VLDL) synthesis and clearance leading to hypertriglyceridemia, increased hepatic cholesterol synthesis and lipoprotein oxidation (5, 69). The APR also results in reduced levels and altered composition of plasma HDL, resulting in an attenuation of reverse cholesterol transport, increased cholesterol delivery to immunoregulatory cells and a decrease in activity of the HDL-associated anti-oxidant enzyme PON. PON activity makes a significant contribution to the anti-inflammatory properties of HDL by suppressing lipoprotein oxidation and eliminating bioactive lipids generated under

conditions of oxidative stress. Circulating levels of LPC are therefore increased in chronic inflammatory diseases with prolonged acute phases as a result of lipoprotein oxidation and the conversion of normal plasma HDL into pro-inflammatory “acute-phase HDL” (70). These increases in LPC may have systemic effects on immune function. Indeed, one study suggests that lipoprotein oxidation and concomitant LPC generation during the APR to infection and inflammation may provide signals of “danger” that are detected by monocyte-derived dendritic cells to potentiate antigen presentation and subsequent adaptive immune responses (71). Increased LPC generation associated with the pathogenesis of autoimmune diseases such as SLE (7,9,62) may therefore contribute mechanistically to the breakdown of immune tolerance. Although a role for G2A in mediating such effects of LPC has not been demonstrated, the effects of this GPCR in T lymphocytes (5.1.) and hepatocytes (6.) described earlier have the potential to do so. For example, based on its G2A-dependent effects on T lymphocyte migration *in vitro*, elevations in circulating levels of LPC may modulate the trafficking of T lymphocytes through secondary lymphoid organs (lymph nodes) by augmenting their migratory potential through G2A. A potentiation of T lymphocyte migration into lymph nodes would be expected to facilitate efficient antigen surveillance required for the generation of effective adaptive immune responses during inflammation, while sustained increases in plasma LPC levels may deregulate this G2A-mediated mechanism and thereby promote immune responses to auto-antigens, including those relevant to SLE. Such a scenario could also be postulated to contribute to the synergistic inter-relationship between atherosclerotic and autoimmune diseases under hypercholesterolemic conditions in which circulating LPC levels are chronically elevated (25,62,72,73). Although imaging of cell migration using two-photon laser-scanning fluorescence microscopy did not demonstrate any effect of G2A deficiency on T lymphocyte locomotion within lymph nodes explanted from normal mice (74), the consequences of G2A deficiency on T lymphocyte homing into lymph nodes in the whole animal in the face of sustained LPC elevation as a result of chronic inflammation or hypercholesterolemia have not been addressed.

Finally, similarly to the lipoprotein modifications during the APR described above, reductions in circulating levels of ApoA1/HDL are a common feature in SLE patients (75,76). Auto-antibodies against ApoA1 generated in SLE patients are thought to contribute to these decreases in ApoA1 and to impair HDL-associated PON activity (77–79). The resulting attenuation of HDL-mediated cholesterol transport and anti-inflammatory/anti-oxidant functions contribute to promoting pro-inflammatory and autoimmune processes in SLE (80–82). Indeed, lymphocyte expansion and production of SLE-associated auto-antibodies was recently reported by Mary Sorci-Thomas’s group in hypercholesterolemic ApoA1-deficient LDLR^{−/−} mice (82). The ability of G2A to modulate HDL levels in hypercholesterolemic LDLR^{−/−} mice (6.) (27,28) therefore suggests that G2A may similarly exert an influence on the susceptibility to SLE independently of its direct effects in immunoregulatory cells (5.), an issue currently being addressed by our group.

8. Concluding remarks

Based on the amphipathic nature of LPC, the ubiquitous presence of lipoproteins and other “LPC scavenging” proteins (1,47), and its susceptibility to a range of enzymatic modifications, it is likely that the actions of this lysolipid *in vivo* are subject to significant spatial and temporal constraints. Such restriction of LPC action may be imposed by the abundance of enzymatic activities, such as autotaxin (ATX) and LPC-acyltransferases (LPC-ATs), capable of converting this lysolipid to other lipid mediators (such as lysophosphatidic acid, LPA) or “inert” phospholipids (such as PC) (83,84). Furthermore, LPC generated at sites of inflammation and in the circulation can be consumed by lipoproteins and albumin, so that the effective level of LPC capable of mediating G2A activation may be governed by the balance between its continual production versus its consumption or enzymatic modification. Indeed,

the responsiveness of cells to LPC could be controlled by the availability of LPC-binding proteins such as albumin and lipoproteins whose concentrations may set a threshold for G2A activation by LPC generated during inflammation and under oxidative stress. Furthermore, reductions in circulating albumin concentrations and concomitant increases in oxLDL-derived plasma LPC associated with the APR to infection and inflammation may result in the exposure of peripheral leukocytes to LPC pools capable of activating G2A to modulate their activity. These are all important considerations when extrapolating observations made in cell culture systems in which LPC is added exogenously to infer biological functions *in vivo* where LPC is continually generated together with other lipid mediators during the protracted course of an inflammatory response or under oxidative stress. In addition, the cumulative data suggest that G2A has pleiotropic effects on a broad range of cell-types, some of which may have opposing influences on inflammatory processes and immunity. The range of immunoregulatory processes influenced by G2A is likely determined in part by its ability to be activated by oxidized free fatty acids as well as lysolipids. However, it is worth considering that as both these classes of lipids can be generated concomitantly during inflammation and under conditions of oxidative stress, one may influence the activation of G2A by the other. For example, LPC-induced mobilization of the G2A receptor to the plasma membrane may act to facilitate robust receptor-mediated signaling in response to 9-HODE by increasing the number of cell surface receptors available for 9-HODE binding. Future studies aimed at determining the physiological significance of G2A-mediated effects in inflammation and chronic inflammatory disease, and establishing the lipid specificity of such effects, should therefore employ mouse models with cell-specific deficiency or transgenic expression of G2A in combination with genetic modification of key enzymes (such as sPLA₂) that are responsible for LPC generation. These studies should also incorporate accurate methodologies for quantification of LPC and other lipids regulating G2A activity (lyso-PS, oxidized free fatty acids). Such approaches will be required to determine if targeting G2A activity or its interaction with LPC in specific immunoregulatory cell-types is a viable strategy to beneficially modulate inflammation and immune processes in chronic inflammatory and autoimmune disease.

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