Published in final edited form as:

Biochim Biophys Acta. 2013 March ; 1831(3): 642–651. doi:10.1016/j.bbalip.2012.09.018.

Phospholipids: “Greasing the Wheels” of Humoral Immunity

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Abstract

Phospholipids are major structural components of all cellular membranes. In addition, certain phospholipids execute regulatory activities that affect cell behavior, function and fate in critically important physiological settings. The influence of phospholipids is especially obvious in the adaptive immune system, where these macromolecules mediate both intrinsic and extrinsic effects on B and T lymphocytes. This review article highlights the action of lysophospholipid sphingosine-1-phosphate as a lymphocyte chemoattractant, the function of phosphatidylinositol phosphates as signaling conduits in lymphocytes and the role of phospholipids as raw materials for membrane assembly and organelle biogenesis in activated B lymphocytes. Special emphasis is placed on the means by which these three processes push humoral immune responses forward.

Keywords

phospholipid; B lymphocyte; plasma cell; antibody

1. The Basics of Humoral Immunity

In humoral immunity, the host is defended by antibodies that neutralize and eliminate extracellular microbes and microbial toxins. Antibodies are produced by B lymphocytes and are composed of covalently assembled immunoglobulin (Ig) heavy chain (H) and Ig light chain (L) proteins. Each B cell expresses a single type of Ig H chain and L chain and, therefore, produces antibody of single specificity. A highly diverse B cell repertoire provides an arsenal of antibodies specific for a wide array of molecules including proteins, polysaccharides and lipids, thereby affording protection against a variety of foreign agents [1].

There is a division of labor in humoral immunity as different B cell subsets manifest antibody responses to distinct types of antigens [2]. Follicular B cells, the largest B cell subpopulation, reside in the follicles of lymphoid tissue and are primarily responsible for antibody responses against protein antigens [3, 4]. Successful responses to protein antigens require the assistance of helper T cells (T_H) and involve the development of germinal centers, specialized microenvironments within follicles [5]. Germinal center events allow for antibody isotype switching, generation of high-affinity antibodies and development of immunologic memory [6, 7]. Thus, follicular B cells supply long-lasting, protective humoral

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immunity throughout the body against diverse types of pathogens and toxins. In contrast, marginal zone B cells [8] localize adjacent to the splenic marginal sinus and B-1 B cells [9] are enriched in the peritoneal cavity and mucosal tissues. These two smaller B cell subsets are predominant in the recognition of non-protein antigens like polysaccharides and mount rapid, mainly IgM antibody responses to such T cell-independent antigens. As such, marginal zone and B-1 B cells furnish a front-line humoral defense against bacterial pathogens [10].

In the simplest of terms, a successful humoral immune response requires that B cells be in the right place at the right time, become activated by specific antigen, proliferate and differentiate into antibody-secreting cells. A myriad of molecular mechanisms orchestrate these processes including signal transduction cascades, gene expression programs and changes in cellular metabolism. In this article, the spotlight is on different types of phospholipids that play pivotal roles in B cell trafficking, activation and differentiation.

2. B cell Trafficking: Sphingosine-1-Phosphate Shows the Way

Lysophospholipids are minor lipid components as compared to abundant membrane phospholipids like phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) and sphingomyelin (SM). Originally considered as nothing more than metabolic intermediates in the *de novo* biosynthesis of various phospholipids, lysophospholipids have been found to exert regulatory activity, including acting as extracellular signaling factors. One of the most important lysophospholipids is sphingosine-1-phosphate (S1P) which is generated exclusively via the phosphorylation of sphingosine, a central element of all sphingolipids (Fig. 1A) [11]. Ceramidases remove one fatty acid tail from ceramide (Cer), a sphingolipid, yielding the long-chain amino alcohol sphingosine [12]. Sphingosine kinases (SPHK1 and SPHK2) phosphorylate sphingosine, producing S1P [13]. S1P can be de-phosphorylated back to sphingosine by either S1P phosphatases [14, 15] or lipid phosphate phosphatases [16, 17] and then recycled for use in sphingolipid biosynthesis. Alternatively, S1P can be degraded by S1P lyase [18, 19] into hexadecenal and phosphoethanolamine, a precursor for synthesis of PtdEtn. Hence, S1P is produced inside cells and serves as an intermediate in sphingolipid metabolism. However, S1P, an amphiphilic molecule, is also exported outside of cells by a mechanism likely involving ATP-binding cassette transporters and members of the spinster family of transporters [20–22]. Extracellular S1P engages five S1P receptors (S1PR1 – S1PR5), all of which are cell surface heterotrimeric G protein-coupled receptors [23, 24], and has been implicated in several biological processes including lymphocyte trafficking [25].

2.1 Lymphocytes on the move

Adaptive immunity, including humoral immune responses, relies upon the mobility of B and T lymphocytes. Newly made B and T cells must migrate from the bone marrow and thymus, respectively, to secondary lymphoid tissues including spleen, lymph nodes and mucosal Peyer's patches. Lymphocytes enter these tissues from the blood by a complex mechanism involving selectins, chemokines and integrins that coordinate their transmigration across the endothelium [26]. Once inside a secondary lymphoid tissue, naïve B and T cells are in position to encounter foreign antigens. If, after several hours of duty in a given lymphoid tissue, these cells have not been activated by cognate antigen, they will exit the site and move into position in another secondary lymphoid organ. From the spleen, lymphocytes exit back into the blood, whereas from lymph nodes and Peyer's patches, they exit into the lymph. Cells in lymph come back to the blood as lymph drains into the thoracic and right lymphatic ducts. This dynamic process allows the diverse specificities within the B and T cell repertoires to survey more than one anatomic site each day. However, during an infection, this process stops transiently for the involved lymphoid organ, allowing more time

for potential antigen recognition by cognate B or T cells. When activated by antigen, responding lymphocytes no longer quickly exit the secondary lymphoid tissue to patrol another site. Rather, activated lymphocytes remain for a longer period to proliferate and differentiate into effector cells that will eventually leave the lymphoid tissue to combat the infection at peripheral sites. As all of these events unfold, S1P plays a key role in directing lymphocyte movement.

2.2 S1P, S1PR1 and lymphocyte movement

S1P signals lymphocytes to exit lymphoid tissues and influences lymphocyte positioning within lymphoid tissues. These processes require two essential ingredients: S1P receptors with nanomolar affinities for S1P [23, 24] and differential S1P concentrations at sites of lymphocyte movement [18, 25, 27]. The G α i-coupled S1P receptor, S1PR1, is expressed by B and T lymphocytes and is of major importance to both cell types. S1PR1 is required for newly matured T cells to egress from the thymus and for both B and T cells to move out of secondary lymphoid tissues [28, 29]. S1P levels are much higher in blood ($\sim 1\mu\text{M}$) and lymph ($\sim 100\text{ nM}$) than in the interstitial fluid of lymphoid organs ($\sim \text{nM}$) [18, 27]. Thus, the general mechanism involves migration of S1PR1-expressing lymphocytes in lymphoid organs toward an increasing S1P concentration either in blood or lymph (Fig. 1B). In the presence of high S1P levels, lymphocytes internalize S1PR1 and, consequently, lack cell surface S1PR1 when they move back into the S1P-scarce environment of lymphoid organs [18, 27, 30]. The low S1P concentration in tissues is due, at least in large part, to the degradative activity of S1P lyase in these areas [18]. Erythrocytes, which lack S1P lyase and S1P phosphatase, are the primary source of plasma S1P [27, 31], and the lymphatic endothelium supplies S1P to the lymph [32].

The phenomenon of S1P-mediated lymphocyte movement is best understood for lymphocyte egress out of lymph nodes. Upon entering resting lymph nodes from the blood, T cells are drawn to the T cell zone by ligands for the chemokine receptor CCR7 [33] and follicular B cells are attracted to follicles by the CXCR5 ligand CXCL13 [34]. After a period of hours ($\sim 6\text{--}8\text{ h}$ for CD4 $^{+}$ T cells; $\sim 24\text{ h}$ for B cells) in these locales [30, 35, 36], these cells up-regulate expression of S1PR1, exit the S1P-low lymph node tissue and move into the S1P-high lymph in lymphatic vessels [30]. The molecular details of this process are not fully defined, but exiting lymphocytes must move across the lymphatic endothelium. S1PR1-mediated signaling might act by directing lymphocytes to migrate across endothelium and/or by overriding chemokine receptor signals that promote lymphocyte retention [32, 37–39]. Altered cell morphology manifested by several cytoskeletal regulatory proteins downstream of S1PR1 appears to be involved in lymphocyte movement across the lymphatic endothelium [40–42].

2.3 S1P and B cell localization within lymphoid tissues

S1P and various S1P receptors influence the movement and localization of different B cell subsets at distinct stages of development. First, S1P signaling affects developing B cells in the bone marrow. The migration of newly developed, immature B cells into bone marrow sinusoids and peripheral blood involves S1P and S1PR1 [43, 44], albeit to a lesser extent than does T cell emigration from the thymus [45]. Immature B cells also express S1PR3, and this receptor participates in localizing developing, transitional B cells to bone marrow sinusoids [46].

S1P signaling also maintains proper positioning of marginal zone B cells within the spleen. In the splenic marginal zone, this B cell subset is ideally positioned to encounter blood-borne antigens [8]. Both S1PR1 and S1PR3 contribute to the localization of marginal zone B cells in the marginal zone, rather than in follicles [47–49]. Recent studies indicate that when

marginal zone B cells arrive in the marginal zone, the locally high S1P level in this blood-exposed region causes desensitization of S1PR1 [50]. This renders marginal zone B cells subject to CXCR5-dependent attraction to CXCL13 in follicles, a S1P-low environment. Once S1PR1 expression is restored on the cell surface, these B cells respond to S1P and migrate back to the marginal zone. It has been proposed that this movement of marginal zone B cells to and from the marginal zone and follicle facilitates delivery of opsonized antigens by marginal zone B cells to follicular dendritic cells (FDCs) [51], a key event for the maturation of efficient antibody responses to T cell-dependent protein antigens in germinal centers [52].

Finally, S1P signaling influences the localization and fate of activated B cells. Upon activation by T cell-dependent protein antigens, responding follicular B cells can enter a germinal center near the follicle center [53]. Here, activated B cells undergo proliferation, antibody isotype switching and somatic mutation of Ig genes. In the germinal center, B cells expressing antibody of highest affinity for their cognate antigen survive, whereas the remaining B cells are eliminated by apoptosis. B cells that emerge from a germinal center either differentiate into long-lived antibody-secreting plasma cells or enter the pool of circulating memory B cells [6, 7]. Germinal center B cells down-regulate S1PR1 and up-regulate S1PR2, a G12-G13-linked S1P receptor that appears to restrict B cell movement, thereby promoting retention of responding B cells within the specialized microenvironment of the germinal center [54]. It seems likely that the local S1P concentration is lowest at the follicle center and highest at the follicle edge where it signals S1PR2-expressing B cells to turn back toward the germinal center rather than exiting this unique niche [54]. In addition, S1PR2 signaling inhibits activation of AKT and, consequently, hinders the pro-survival effects of the AKT pathway [54, 55]. Thus, S1P and S1PR2 also play a role in governing B cell numbers in the germinal center [54]. In antibody-secreting cells, S1PR1 again takes center stage as it is required for S1P to guide IgG-secreting cells from secondary lymphoid tissues to the bone marrow and IgA-secreting cells from Peyer's patches to mucosal sites [56, 57].

2.4 Fine-tuning S1P-mediated lymphocyte movement

Lymphocytes carefully regulate the S1P-S1PR1 signaling system. In both B and T cells, S1PR1 is subject to post-translational regulation. Specifically, when the activation marker CD69 [58] associates with S1PR1, the complex is internalized from the cell surface and degraded [43, 59, 60]. Therefore, high levels of CD69 relative to S1PR1 inhibit S1P-directed lymphocyte egress. A variety of pro-inflammatory mediators and innate immunity stimuli, including type I interferons and Toll-like receptor (TLR) agonists, up-regulate expression of CD69 [60, 61]. Such stimuli are often encountered when B and T cells enter a lymph node engaged in an immune response and promote CD69-mediated inhibition of S1PR1, thereby prolonging the stay of B and T cells in the inflamed site [60].

Also, in both B and T cells, S1PR1 is governed by transcriptional control. Activation of B and T cells via their respective antigen receptors down-regulates *S1PR1* transcripts [45, 47, 62], whereas CD69 expression is up-regulated [58]. The transcription factor KLF2 (Kruppel-like factor 2) is critical for *S1PR1* transcription in T cells [63–65], but the factors controlling *S1PR1* transcription in B cells have not been identified. However, it is clear that S1P-mediated lymphocyte egress can be effectively blocked by a combination of transcriptional and post-translational mechanisms that target S1PR1, ensuring that activated B and T cells tarry in secondary lymphoid organs for sufficient time to receive further signals, proliferate and differentiate into effector cells.

Finally, mechanisms exist that can modulate S1P distribution during immune responses. Expression of *SPHK1* is induced in dendritic cells and other myeloid cells by a variety of

inflammatory signals [66–68], enabling increased production of S1P by these cells. Signaling through the high affinity Fc receptor for IgE on mast cells activates SPHK1 and SPHK2, leading to production and secretion of S1P [22, 69]. However, it remains to be determined if these or other mechanisms for inducible synthesis and release of S1P influence its interstitial concentrations within lymphoid organs or alter lymphocyte trafficking. On the other hand, B cells express S1P-degrading enzymes at high levels and are, therefore, well-equipped for S1P degradation [54]. It follows that B cells help maintain S1P scarcity in secondary lymphoid tissues, particularly the very low S1P levels within follicles.

In summary, S1P and its cognate receptors regulate lymphocyte movement from and within lymphoid tissues, positioning B and T cells to respond to foreign materials. Certainly, S1P-mediated signaling exerts influence throughout the lives of both B and T lymphocytes, making this lysophospholipid a critical factor in humoral immune responses.

3. B cell Activation: Phosphatidylinositol Phosphates Fuel the Fire

The membrane phospholipid, phosphatidylinositol (PtdIns), carries an inositol head group, a sugar ring that can be phosphorylated by PtdIns kinases on several positions to generate multiple derivatives. The major pathway by which PtdIns 4,5-bisphosphate [PtdIns(4,5)P₂] is produced involves the sequential actions of PtdIns 4-kinase (PI4K) and PtdIns(4)P-5-kinase (PIP5K) [70]. PtdIns(4,5)P₂ can then be converted into PtdIns 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] by PtdIns 3-kinase (PI3K) [71] (Fig. 2A). Positioned in the inner leaflet of the plasma membrane, these short-lived phosphoinositides are uniquely suited as signaling molecules and play roles downstream of many cell surface proteins, including lymphocyte antigen receptors [72].

3.1 Signaling from the B cell antigen receptor

Antigen-specific activation of a B cell is triggered when membrane-anchored antibody on the cell surface, the B cell antigen receptor (BCR), binds antigen. Ultimately, a complex set of signaling pathways culminate in programs of gene transcription that promote cell survival, proliferation and differentiation. Mature, naïve B cells primarily express Ig H chains (μ and δ) containing a carboxyl-terminal, hydrophobic sequence that serves as a membrane-spanning domain. These membrane-bound H chains assemble with soluble Ig L chains in the endoplasmic reticulum (ER), forming H₂L₂ oligomers that function as the BCR. Two additional membrane-anchored proteins, Ig α (CD79a) and Ig β (CD79b), associate with the BCR in the ER, yielding a complete BCR complex that is competent for transport to the plasma membrane and for signal transduction [73]. BCR stimulation initially sparks Src-family tyrosine kinases LYN, BLK and FYN to phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic tails of both Ig α and Ig β . The tyrosine kinase SYK is then recruited to the site via its Src homology 2 (SH2) domain and activated [74, 75]. These BCR-proximal events set the stage for PtdIns(4,5)P₂, to fuel two distinct signaling cascades, the phospholipase C γ 2 (PLC γ 2) and PI3K pathways (Fig. 2B).

3.2 The PLC γ 2 pathway

PLC γ 2 is recruited to the plasma membrane via the interaction of its amino-terminal SH2 domain with the adaptor molecule, BLNK (B cell linker, also known as SH2-containing leukocyte protein of 65 kDa [SLP-65]), a substrate of SYK [76]. BLNK also interacts with BTK (Bruton's tyrosine kinase), thereby facilitating BTK-mediated phosphorylation and activation of PLC γ 2 [77, 78]. Activated PLC γ 2 hydrolyzes PtdIns(4,5)P₂, yielding inositol(1,4,5)-trisphosphate (InsP₃) and diacylglycerol (DAG) [79]. The water-soluble InsP₃ diffuses into the cytosol and binds the InsP₃-receptor, an ER transmembrane complex that functions as an InsP₃-gated Ca²⁺ channel [79]. Thus, InsP₃ stimulates release of Ca²⁺

from the ER into the cytosol. The resulting depletion of the ER Ca^{2+} store causes the ER transmembrane protein STIM1 (stromal interaction molecule 1) to activate Ca^{2+} -release activated Ca^{2+} entry (CRAC) channels on the plasma membrane, allowing entry of extracellular Ca^{2+} [80]. Together, these events cause sustained elevation of cytosolic Ca^{2+} which, in turn, leads to activation of calcineurin, a Ca^{2+} /calmodulin-dependent serine/threonine (Ser/Thr)-specific phosphatase. Calcineurin de-phosphorylates and activates NFAT (nuclear factor of activated T cells) transcription factors to induce genes that promote cell proliferation and survival [80]. Meanwhile, back at the plasma membrane, the DAG resulting from PLC γ 2-mediated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ recruits additional signaling mechanisms into the game. DAG binds directly to PKC β (protein kinase C β) and, together with increased intracellular Ca^{2+} , activates this Ser/Thr kinase to initiate a series of events that lead to the nuclear translocation of the transcription factor NF- κ B (nuclear factor-kappa B) [81, 82]. NF- κ B turns on a variety of genes involved in cell proliferation, survival and differentiation [83]. DAG also interacts with RASGRP (Ras guanyl nucleotide releasing protein), bringing this guanyl nucleotide exchange factor to the plasma membrane where it activates the small G protein RAS [84, 85]. Activated RAS then kicks off the RAF, MEK (mitogen-activated protein kinase/ERK kinase), ERK (extracellular signal-regulated kinase) signaling pathway [86]. This cascade generates additional transcriptional regulators, including AP-1 (activator protein-1), that drive cell activation, proliferation and differentiation [87].

3.3 The PI3K pathway

PI3K is recruited to the plasma membrane via the SH2 domain of its p85 regulatory subunit (p85 α most important in B cells) [88]. In one mechanism, BCR ligation leads to tyrosine phosphorylation of the cytoplasmic tail of CD19, creating a binding site for PI3K [89]. CD19, a B cell-specific transmembrane protein, functions together with CD21 and CD81 as the BCR co-receptor to augment BCR-mediated signaling [90]. Alternatively, PI3K can interact with BCAP (B cell adapter protein), an adapter protein tyrosine-phosphorylated by SYK and BTK at the engaged BCR [91]. Once activated, the p110 catalytic subunit (p110 δ most important in B cells) of PI3K converts $\text{PtdIns}(4,5)\text{P}_2$ into $\text{PtdIns}(3,4,5)\text{P}_3$, which serves as a membrane docking site for pleckstrin homology (PH) domain-containing proteins such as PLC γ 2, BTK and the AKT kinase (also known as protein kinase B) [88]. Binding $\text{PtdIns}(3,4,5)\text{P}_3$ brings PLC γ 2 near its membrane-bound substrate $\text{PtdIns}(4,5)\text{P}_2$. BTK, an activator of PLC γ 2 [78], also escorts PIP5K, a critical kinase for production of $\text{PtdIns}(4,5)\text{P}_2$, to the plasma membrane [92]. Thus, one role of $\text{PtdIns}(3,4,5)\text{P}_3$ is to promote the organization and localization of key BCR signaling components, the so-called signalosome, at the plasma membrane [88]. Equally important, $\text{PtdIns}(3,4,5)\text{P}_3$ interacts with AKT, a Ser/Thr kinase that facilitates cell activation, proliferation and survival by modulating the activity of key regulatory proteins [93]. For example, AKT phosphorylates and inactivates FOXO (Forkhead Box subgroup O) proteins, a family of transcription factors that suppresses proliferation and limits survival of resting B cells [94]. The effects of PI3K signaling are tempered by specific lipid phosphatases that metabolize $\text{PtdIns}(3,4,5)\text{P}_3$. A 3-phosphatase, PTEN (phosphatase and tensin homolog deleted on chromosome ten), changes $\text{PtdIns}(3,4,5)\text{P}_3$ back to $\text{PtdIns}(4,5)\text{P}_2$ [95]. Whereas, the SHIP (SH2-containing inositol phosphatase) 1 and SHIP2 5-phosphatases, convert $\text{PtdIns}(3,4,5)\text{P}_3$ into $\text{PtdIns}(3,4)\text{P}_2$ [96]. $\text{PtdIns}(3,4)\text{P}_2$ is bound by certain PH domain-containing proteins, and this has been observed for additional factors implicated in BCR-mediated signaling [97]. Both the PTEN and SHIP phosphatases are critically important for proper development, activation and function of B lymphocytes [98–100].

3.4 Crosstalk between the PLC γ 2 and PI3K pathways

While the PLC γ 2 and PI3K pathways play distinct roles downstream of the BCR, these are inter-related signaling mechanisms. PtdIns(4,5)P₂ is the substrate for both PLC γ 2 and PI3K, and the product of PI3K, PtdIns(3,4,5)P₃, can be de-phosphorylated back to PtdIns(4,5)P₂ [95]. Importantly, PtdIns(3,4,5)P₃ brings factors together that are necessary for synthesis of PtdIns(4,5)P₂ and its cleavage by PLC γ 2 [78, 88, 92]. In this way, the PI3K pathway fosters production of the vital second messenger molecules, DAG and InsP₃, by the PLC γ 2 pathway. It is important to note that PLC γ - and PI3K-mediated signal transduction also operates downstream of the T cell antigen receptor (TCR) [101]. Just as in B cells, these pathways are critical for T cell activation, including T_H cells necessary for B cell responses to protein antigens. Therefore, PtdIns phosphates are fundamental to humoral immunity, fueling signal transduction pathways that couple antigen recognition to lymphocyte activation and, ultimately, to the differentiation of antibody-secreting B cells.

4. B cell Proliferation and Differentiation: Phospholipids in Demand

Phospholipids are major building blocks for construction of cellular membranes (Fig. 3A). PtdCho is the most abundant membrane phospholipid in mammalian cells and also serves as a precursor for SM and PtdEtn, the two other most prevalent membrane phospholipids. For all of these, fatty acids are a critical ingredient. For example, fatty acids are incorporated into DAG and Cer, which are then utilized in synthesis of PtdCho and SM, respectively. Cell proliferation and differentiation processes involving organelle biogenesis require membrane assembly and are, therefore, reliant on an increased supply of phospholipids.

4.1 B cell activation and phospholipid demand

Humoral immunity requires that activated B lymphocytes proliferate and differentiate into antibody-secreting cells, often referred to as plasma cells [102]. Proliferation allows for expansion of responding B cell clones, thereby amplifying antibody responses. Each cell division yielding two daughter cells requires a doubling of membrane content, including phospholipids, as the cell cycle proceeds [103]. The differentiation of antibody-secreting cells allows for large scale production of antigen-specific antibodies needed for host defense. During the differentiation process, Ig expression increases substantially and this is highly dependent on the ER. As Ig H and L chains are synthesized, they are co-translationally translocated into the ER, an oxidizing, calcium-rich environment populated by a cast of resident molecular chaperones and folding enzymes [104]. Within the specialized milieu of the ER lumen, Ig chains are assembled into functional antibodies competent for transport to the Golgi where their *N*-linked glycans undergo final maturation. In keeping with the transition to high-rate antibody secretion, the differentiation process includes a marked expansion of rough ER as well as Golgi (at least 3 to 4-fold expansion in surface area and volume for each) [105, 106]. In fact, a highly developed secretory apparatus is the morphologic hallmark of a plasma cell. Phospholipids, therefore, are in high demand to support membrane and organelle biogenesis when activated B lymphocytes proliferate and differentiate into antibody-secreting cells, the effectors of humoral immunity (Fig. 3B).

4.2 Phosphatidylcholine supply in activated B cells

Lipid production as a whole, including *de novo* synthesis of fatty acids and phospholipids, escalates when B cells are activated. Significant increases in PtdCho, the major phospholipid in cellular membranes, are particularly evident in stimulated B cells [105, 107]. In the context of proliferating B cells, PtdCho and other phospholipids are expected to increase during S phase in parallel with DNA synthesis, as has been observed in other types of dividing cells [103]. Modulations in the rates of PtdCho synthesis and degradation during

the cell cycle contribute to the net increase in membrane PtdCho as cells prepare to divide [108–110].

In mammalian cells, PtdCho is synthesized predominantly by the enzymatic activities of the cytidine diphosphocholine (CDP-choline) pathway [111]. In the first step, the soluble, cytosolic choline kinase (CK) phosphorylates choline in the presence of ATP to yield phosphocholine [112]. Next, choline cytidyltransferase (CCT), associated with the cytosolic face of ER membranes, utilizes phosphocholine and CTP to produce CDP-choline [113]. Notably, in all cell types examined, the CCT-catalyzed step is rate-limiting in the CDP-choline pathway [114]. Finally, the phosphocholine moiety of CDP-choline is transferred to DAG, yielding PtdCho; thus, the supply of CDP-choline and DAG determine the output of PtdCho. This final step can be catalyzed by either cholinephosphotransferase (CPT1) or choline/ethanolaminephosphotransferase (CEPT1), a bifunctional enzyme that can synthesize both choline- and ethanolamine-containing phospholipids. Both CPT enzymes are integral membrane proteins, with CPT1 localizing to the Golgi and CEPT1 residing in the ER [115, 116]. Here, the combined actions of CPT1 [117] and CEPT1 [118] are referred to as CPT activity. In regard to PtdCho turnover in mammalian cells, two phospholipases have been linked to the regulation of PtdCho degradation during cell cycle progression, the group VIA calcium-independent phospholipase A₂ [119] and the neuropathy target esterase [120]. PtdCho turnover can be down-regulated near the G1/S transition, thereby contributing to a net increase in membrane PtdCho [103].

Studies of the mechanisms regulating PtdCho supply in activated B cells have primarily been performed using lipopolysaccharide (LPS)-stimulated mouse B cells. LPS signals through TLR4 and drives mouse B cells to proliferate and to differentiate into antibody-secreting cells [121], making LPS-treated B cells an excellent *in vitro* experimental system. When splenic B cells are activated by LPS, CK activity changes very little, CCT activity modestly elevates ~2-fold, CPT activity increases ~6-fold and PtdCho synthesis ramps up 6 to 7-fold [105]. Similar studies using the CH12 B cell lymphoma revealed that, as expected, an increase in CCT activity plays a central role in augmenting PtdCho output by the CDP-choline pathway in LPS-stimulated B cells [107]. In B cells, like most cell types, CCT activity is primarily provided by the CCT α isoform [122]. Indeed, CCT α -deficient B cells are unable to up-regulate PtdCho synthesis upon stimulation, and this correlates with poor proliferation and abnormal antibody responses *in vivo* [122], demonstrating essential roles for CCT α and PtdCho in humoral immunity.

Interestingly, in CH12 B cells, evidence indicates that allosteric activation of CCT by membrane lipids increases following LPS stimulation and correlates with increased amounts of DAG in microsomal lipid preparations [107]. Thus, in activated B cells, DAG may play dual roles in PtdCho synthesis, serving as an activator of CCT and as a substrate for the CPT enzymes. In contrast to PtdCho synthesis, PtdCho degradation in activated B cells has not been studied extensively. Based on other cell types [119, 120], it seems likely that diminished turnover helps increase the pool of PtdCho in proliferating B cells, but the status of these mechanisms during the differentiation of antibody-secreting cells remains to be investigated.

4.3 Phospholipids, the unfolded protein response transcription factor XBP1 and antibody secretion

When activated B cells differentiate into antibody-secreting cells, the ER and the entire secretory pathway expands and is equipped for high-rate antibody production. Construction of this “antibody factory” is directed by X-box binding protein 1 (XBP1), a transcription factor generated by the unfolded protein response (UPR) [123]. Comprised of three signaling pathways emanating from the ER membrane, the UPR allows cells to adapt to

increased demands on the protein folding capacity of the ER. This adaptation to ER stress involves translational control and increased transcription of genes encoding ER resident molecular chaperones and folding enzymes as well as factors involved in ER-associated degradation of mis-folded proteins [123]. The UPR also influences lipid supply and ER size as evidenced by the link between XBP1, phospholipid synthesis and ER biogenesis [124–127].

In the UPR, XBP1 is activated by the IRE1 (inositol requiring enzyme 1) signaling pathway [123]. IRE1 α is ubiquitously expressed as an integral ER transmembrane protein containing a Ser/Thr kinase module and a carboxyl-terminal endoribonuclease domain in its cytoplasmic region [128, 129]. Upon its activation, IRE1 α executes site-specific cleavage of *XBPI* mRNA and excises a 26-nt intron. An undefined mechanism then ligates the resulting 5' and 3' fragments, yielding a spliced *XBPI* mRNA with an altered reading frame. Both unspliced and UPR-spliced *XBPI* transcripts encode basic leucine zipper transcription factors, XBP1(U) and XBP1(S), respectively. The two forms of XBP1 have identical DNA-binding regions, but only XBP1(S) possesses a carboxyl-terminal transactivation domain [130–132]. XBP1(S) is essential for optimal induction of genes encoding proteins that function throughout the secretory pathway. These include proteins involved in the targeting and translocation of nascent polypeptides into the ER, ER resident chaperones and folding enzymes, ERAD components and factors involved in vesicular transport [126, 133]. In addition, XBP1(S) drives lipid biosynthesis and ER biogenesis [126, 127].

Xbp1 is required for embryonic development in mice [134]; thus, the critical role of this factor in antibody secretion was elucidated by methods of selective deletion in B lymphocytes. XBP1-deficient B cells fail to become normal antibody-secreting cells both *in vivo* in response to immunization and *in vitro* in response to TLR agonists [135–137]. Similarly, XBP1 is required for proper development and function of other specialized secretory cell types including salivary gland and pancreatic cells [138]. In response to LPS stimulation *in vitro*, XBP1-deficient B cells exhibit a poorly developed rough ER and secrete antibody at low levels [125, 139]. This phenotype correlates with weak expression of many secretory pathway genes [126] and reduced levels of phospholipids, particularly PtdCho and SM [124, 125]. SM is produced when the SM synthase transfers the phosphocholine headgroup of PtdCho to Cer [140], so a limited supply of PtdCho would equate with a reduced pool of SM. Synthesis of Cer, like PtdCho, increases in LPS-stimulated B cells [124]. Induction of ceramide synthesis is attenuated in XBP1-deficient B cells, and pharmacologic inhibition of *de novo* ceramide formation hinders ER expansion in LPS-stimulated B cells [124]. These data reveal a complex relationship between XBP1, PtdCho, Cer and SM and illustrate the critical role of phospholipids in the development of antibody-secreting B cells. Interestingly, in LPS-stimulated CCT α -deficient B cells, the inability to up-regulate PtdCho synthesis correlates with enhanced induction of XBP1(S), providing further evidence of a link between phospholipid supply and XBP1(S) in activated B cells [122].

XBP1(S), but not XBP1(U), effectively restores the ability of XBP1-deficient B cells to secrete antibody in response to LPS *in vitro* [139] and is sufficient to drive ER expansion [126, 127]. Importantly, another UPR-regulated transcription factor, termed activator of transcription factor 6 α (ATF6 α) [142], has the capacity to drive lipid synthesis and ER proliferation independent of XBP1(S) [143, 144]. ATF6 α also upregulates expression of a cohort of genes that collectively promote ER quality control processes [145, 146]. Like XBP1(S), ATF6 α is activated in LPS-stimulated B cells [147–149], raising the possibility that ATF6 α might play a role in equipping activated B cells for efficient antibody production. However, recent studies utilizing gene-targeted mice demonstrated that ATF6 α is dispensable for normal development of antibody-secreting B cells both *in vitro* and *in*

vivo [150]. While the mechanistic details are not fully understood, it seems that activated B cells rely on XBP1(S) to coordinate increases in certain phospholipids and proteins necessary for expansion of the rough ER and the entire exocytic pathway [141], thereby enhancing cellular biosynthetic and secretory capacity. Thus, phospholipids, produced on demand downstream of XBP1(S), help pave the way for plasma cell development and effective humoral immunity.

5. Conclusion

The multi-dimensional roles of phospholipids are clearly evident in the generation of humoral immune responses. The lysophospholipid S1P directs lymphocyte trafficking, steering B and T cells into position to encounter antigens and to mount efficient antibody responses. Phosphoinositides propagate signals downstream of antigen-engaged B and T cell antigen receptors, igniting lymphocyte activation, proliferation and differentiation. Membrane phospholipids, such as PtdCho and SM, are major building blocks for organelle biogenesis during plasma cell differentiation, facilitating expansion of the secretory apparatus necessary for copious antibody production. In these ways, phospholipids help turn the wheels of humoral immunity.

Acknowledgments

The author thanks collaborator, Dr. Suzanne Jackowski (St. Jude Children's Research Hospital, Memphis, TN), and Ileana V. Aragon (University of South Alabama) for critically reading the manuscript and Jason P. Clark (University of South Alabama) for assistance with artwork. J. W. B. is supported by a grant from the US National Institutes of Health (GM061970).

Abbreviations

AP-1	activator protein-1
BLNK	B cell linker protein
BTK	Bruton's tyrosine kinase
CCT	choline cytidyltransferase
CDP-choline	cytidine diphosphocholine
CEPT1	choline/ethanolamine phosphotransferase 1
Cer	ceramide
CK	choline kinase
CPT1	choline phosphotransferase 1
CRAC	Ca ²⁺ -release activated entry channel
DAG	diacylglycerol
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FA	fatty acid
FOXO	Forkhead Box subgroup O
FDC	follicular dendritic cell
H	heavy chain
T_H	helper T cell

Ig	immunoglobulin
InsP₃	inositol(1,4,5)-trisphosphate
IRE1	inositol requiring enzyme 1
ITAM	immunoreceptor tyrosine-based activation motif
KLF	Kruppel-like factor
LPS	lipopolysaccharide
MEK	mitogen-activated protein kinase/ERK kinase
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-kappa B
PH	pleckstrin homology
PLCγ2	phospholipase C γ 2
PKCβ	protein kinase C β
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdIns(4,5)P₂	PtdIns 4,5-bisphosphate
PtdIns(3,4,5)P₃	PtdIns 3,4,5-trisphosphate
PI-4K	PtdIns 4-kinase
PIP5K	PtdIns(4)P-5-kinase
PtdIns 3-kinase	PI3K
PTEN	phosphatase and tensin homolog deleted on chromosome ten
RASGRP	Ras guanyl nucleotide releasing protein
SM	sphingomyelin
S1P	sphingosine-1-phosphate
S1PR	S1P-receptor
Ser	serine
SH2	Src-homology 2 domain
SLP-65	SH2-containing leucocyte protein of 65 kDa
SHIP	SH2-containing inositol phosphatase
SPHK	sphingosine kinase
STIM1	stromal interaction molecule 1
TCR	T cell antigen receptor
Thr	threonine
TLR	Toll-like receptor
UPR	unfolded protein response
XBP1	X-box binding protein 1

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Highlights

- Diverse roles of phospholipids in the generation of humoral immunity are discussed.
- Sphingosine-1-phosphate is a major chemoattractant for B and T cells.
- Phosphatidylinositol phosphates are vital signaling conduits in B and T cells.
- Phospholipids are key for proliferation and differentiation of activated B cells.

Fig. 1. The lysophospholipid sphingosine-1-phosphate (S1P) is a lymphocyte chemoattractant. A. Phosphorylation of sphingosine by the sphingosine kinase produces S1P, which can dephosphorylated by either lipid phosphate phosphatases or a S1P phosphatase. S1P can also be metabolized by S1P lyase, yielding hexadecenal and phosphoethanolamine. Sphingosine is derived from ceramide and used in ceramide synthesis by the actions of ceramidase and ceramide synthase, respectively. B. B and T lymphocytes express S1P receptors (S1PR), most prominently the G α i-coupled S1PR1. In S1P-low environments, such as the bone marrow, thymus and secondary lymphoid tissues, S1PR1 is expressed on the cell surface and lymphocytes are attracted to increasing S1P concentrations in the blood and lymph.

Fig. 2.

The phosphatidylinositol (PtdIns) phosphates, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, are key signaling molecules in lymphocyte activation. A. PtdIns(4,5)P₂ is primarily produced by sequential phosphorylation of PtdIns by the PtdIns 4-kinase (PI4K) and PtdIns(4)P-5-kinase (PIP5K). Phosphorylation of PtdIns(4,5)P₂ by PtdIns 3-kinase (PI3K) yields PtdIns(3,4,5)P₃. B. Crosslinking of the B cell antigen receptor (BCR, membrane-anchored antibody) by cognate antigen triggers B cell activation. Phosphorylation of the BCR-associated proteins Igα and Igβ initiates a cascade of signaling events that engage the phospholipase Cγ2 (PLCγ2) and PI3K pathways. PLCγ2 hydrolyzes PtdIns(4,5)P₂, yielding soluble InsP₃ and membrane-associated diacylglycerol (DAG). InsP₃ stimulates the release of calcium (Ca²⁺) from the endoplasmic reticulum, which in turn, leads to entry of extracellular Ca²⁺ into the cytosol. Elevated cytosolic Ca²⁺ results in the activation of the NFAT (nuclear factor of activated T cells) transcription factor. DAG binds protein kinase Cβ (PKCβ) and, together with increased cytosolic Ca²⁺, activates this Ser/Thr kinase to initiate a series of events that lead to nuclear translocation of the NF-κB (nuclear factor-kappa B) transcription factor. DAG also binds RASGRP (Ras guanyl nucleotide releasing protein), recruiting this guanyl nucleotide exchange factor to the plasma membrane where it activates the small G protein RAS. This event initiates the RAF, MEK (mitogen-activated protein kinase/ERK kinase), ERK (extracellular signal-regulated kinase) signaling pathway, generating additional transcriptional regulators that, along with NFAT and NF-κB, induce genes that promote lymphocyte activation, proliferation and survival. PI3K phosphorylates PtdIns(4,5)P₂, yielding PtdIns(3,4,5)P₃, which recruits pleckstrin homology domain-containing proteins such as the AKT kinase. AKT mediates regulatory events such as the phosphorylation and inactivation of FOXO (Forkhead Box subgroup O) transcription factors that suppress cell proliferation and survival. Thus, AKT-mediated signaling is one way the PI3K pathway promotes lymphocyte activation, proliferation and survival.

Fig. 3. Phospholipids are in demand for membrane assembly and organelle biogenesis during the differentiation of antibody-secreting plasma cells. **A.** When B cells are stimulated to secrete antibody, lipid synthesis is up-regulated. Fatty acid (FA) synthesis increases and these are incorporated into diacylglycerol (DAG) and ceramide (Cer). DAG is used in synthesis of phospholipids, most prominently phosphatidylcholine (PtdCho), which is the product of the CDP-choline pathway and the most abundant membrane phospholipid. PtdCho can be converted into phosphatidylserine (PtdSer), which in turn, can be converted into phosphatidylethanolamine (PtdEtn). PtdEtn is also generated by the CDP-ethanolamine pathway. Cer and the polar headgroup of PtdCho are used to produce sphingomyelin (SM). All of these phospholipids are building blocks for cellular membranes. **B.** The differentiation of B cells into antibody-secreting plasma cells is marked by expansion of the endoplasmic reticulum (ER) and the entire secretory apparatus. XBP1(S), a transcriptional activator generated by the unfolded protein response, plays a key role in up-regulating expression of ER protein folding factors and secretory machinery, enhancing lipid synthesis and driving ER biogenesis.