Glucose-dependent \textit{de Novo} Lipogenesis in B Lymphocytes

\textbf{A REQUIREMENT FOR ATP-CITRATE LYASE IN LIPOPOLYSACCHARIDE-INDUCED DIFFERENTIATION}\footnote{This work was supported, in whole or in part, by National Institutes of Health Grant 5R01AI074687 (to T. C. C.). This work was also supported by the Margaret Walsh Faculty Research Fund (to T. C. C.)}

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**Background:** The metabolic requirements underlying B lymphocyte differentiation are poorly understood.

**Results:** Differentiation is accompanied by glucose metabolism into fatty acid and cholesterol synthesis, mediated by ATP-citrate lyase (ACLY).

**Conclusion:** ACLY-dependent lipogenesis is required for several phenotypic changes defining plasma cell differentiation.

**Significance:** This study proposes a critical role for ACLY coupled glucose-dependent \textit{de Novo} lipogenesis in LPS-induced B lymphocyte differentiation.

Bacterially derived lipopolysaccharide (LPS) stimulates naive B lymphocytes to differentiate into immunoglobulin (Ig)-secreting plasma cells. Differentiation of B lymphocytes is characterized by a proliferative phase followed by expansion of the intracellular membrane secretory network to support Ig production. A key question in lymphocyte biology is how naive B cells reprogram metabolism to support \textit{de novo} lipogenesis necessary for proliferation and expansion of the endomembrane network in response to LPS. We report that extracellularly acquired glucose is metabolized, in part, to support \textit{de novo} lipogenesis in response to LPS stimulation of splenic B lymphocytes. LPS stimulation leads to increased levels of endogenous ATP-citrate lyase (ACLY), and this is accompanied by increased ACLY enzymatic activity. ACLY produces cytosolic acetyl-CoA from mitochondrially derived citrate. Inhibition of ACLY activity in LPS-stimulated B cells with the selective inhibitor 2-hydroxy-N-arylbenzenesulfonamide (compound-9; C-9) blocks glucose incorporation into \textit{de novo} lipid biosynthesis, including cholesterol, free fatty acids, and neutral and acidic phospholipids. Moreover, inhibition of ACLY activity in splenic B cells results in inhibition of proliferation and defective endomembrane expansion and reduced expression of CD138 and Blimp-1, markers for plasma-like B cell differentiation. ACLY activity is also required for LPS-induced IgM production in CH12 B lymphoma cells. These data demonstrate that ACLY mediates glucose-dependent \textit{de Novo} lipogenesis in response to LPS signaling and identify a role for ACLY in several phenotypic changes that define plasma cell differentiation.

Generating an adaptive immune response places significant bioenergetic demands on lymphocytes (1, 2). In response to antigen challenge, quiescent B lymphocytes transition to a highly activated state characterized by increased transcription and translation necessary for proliferation, effector function, and immunoglobulin (Ig) synthesis (3–5). To support \textit{de Novo} macromolecular synthesis, B lymphocytes must acquire nutrients from extracellular sources (2, 6–9). However, B cells lack the cell-autonomous ability to control nutrient uptake and instead must receive external cues through engagement of a host of membrane receptors, including antigen, cytokine, and Toll-like receptors, in order to acquire exogenous nutrients (6, 9, 10).

Stimulation of lymphocytes and hematopoietic cells is accompanied by a high rate of aerobic glycolysis similar to that displayed by many human cancers (2, 6, 9, 10). It is generally held that the high rate of glycolysis functions to provide ATP, metabolites, and reducing equivalents to support growth and proliferation; however, the metabolic pathways involved in the utilization of glucose beyond glycolysis and their physiologic relevance to B lymphocyte biology have not been defined. Germane to the studies herein, glucose can be metabolized to acetyl-CoA, which is a precursor for both fatty acid and mevalonate synthesis pathways (11–13). Fatty acids are critical metabolic intermediates, because they can be used as precursors to support phospholipid synthesis, protein modification, or degraded to generate acetyl-CoA. In addition to proliferation, the differentiation of B lymphocytes into plasma cells is characterized by expansion of the endomembrane network, particularly the rough endoplasmic reticulum (ER)\footnote{The abbreviations used are: ER, endoplasmic reticulum; ACLY, ATP-citrate lyase; C-9, compound-9 (2-hydroxy-N-arylbenzenesulfonamide); HPTLC, high performance thin layer chromatography; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CM, ceramide; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; Ab, antibody; PG, phosphatidylglycerol.} and Golgi apparatus, where Ig is synthesized and secreted (14–17). Differentiation therefore requires an increased supply of lipids and cholesterol to support both the doubling of membrane content associated with proliferation and to form an endomembrane Ig secretory network (14, 18–20).

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The molecular mechanisms underlying how membrane phospholipid and cholesterol content increases during B lymphocyte differentiation have received little attention (18, 19, 21, 22). Overall rates of fatty acid synthesis in most mammalian tissues are relatively low, in part, because lipids are acquired from the circulation. By contrast, cancer cells and proliferating mammalian cells exhibit high rates of de novo lipogenesis in order to support membrane biogenesis associated with proliferation as well as fundamental cellular processes, such as signal transduction (23–26). Along these lines, one possible biosynthetic fate of glucose is metabolism by fatty acid and mevalonate synthesis pathways, whereby glucose-derived carbon in the form of pyruvate is imported into the mitochondria, decarboxylated to acetyl-CoA, and then condensed with oxaloacetate to form citrate (11, 12). Citrate can be exported from the mitochondria via the malate-citrate shuttle system and used as a substrate for ATP-citrate lyase (ACLY) (13, 27, 28). ACLY catalyzes the formation of acetyl-CoA and oxaloacetate from cytosolic citrate and coenzyme A in the presence of ATP (27–29). ACLY therefore serves as a cross-link between glucose metabolism and the fatty acid and mevalonate synthetic pathways.

The acly gene is expressed in a wide variety of tissues and deletion of the gene in mice results in embryonic lethality (30–33). ACLY is abundantly expressed in the liver and white adipose tissue and is also expressed and catalytically active in pancreatic beta cells (13, 31, 34). Receptor signaling via the PI3K/Akt pathway stimulates ACLY activity predominantly through phosphorylation of ACLY also on serine 454; phosphorylation of ACLY contributes to its protein stabilization (27, 34, 35). Several additional protein kinases have been shown to phosphorylate ACLY, including nucleoside diphosphate kinase and cyclic AMP-dependent protein kinase (34). Alterations in expression or activity of ACLY have been observed in a variety of different metabolic and pathological conditions (13). Notably, ACLY is up-regulated and activated in several types of human cancers, and its inhibition induces proliferation arrest in cancer cells in vitro and in vivo (11, 26, 34–38). We sought herein to investigate whether B lymphocytes stimulated with LPS acquire the capacity to increase cellular lipid content via a pathway that involves ACLY-mediated acetyl-CoA production from extracellularly acquired glucose. Our studies also investigate whether ACLY plays a role in the differentiation of mature B lymphocytes in response to LPS stimulation.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The anti-phospho-Ser-454 ACLY and anti-ACLY antibodies (Abs) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). The anti-β-actin and hsp90 Abs were from Enzo Life Sciences (Farmingdale, NY). Anti-rabbit and anti-mouse IgG-coupled HRP Abs were from Santa Cruz Biotechnology, Inc. Lipopolysaccharide (LPS O111:B4 or Salmonella typhosa), Medica 16 (3,3,14,14-tetramethyl-hexadecanoidic acid), and Percoll were from Sigma-Aldrich. F(ab)2 fragments of goat anti-mouse IgM (anti-Ig) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Murine IL-4 was from R&D Systems, Inc. (Minneapolis, MN). Purified lipid standards were purchased from Matreya Inc. (Pleasant Gap, PA) and Sigma-Aldrich. Lysophosphocholine and cerebrosides were from Avanti Polar Lipids, Inc. (Alabaster, AL). [14C]Citric acid and MicroScint-O were obtained from PerkinElmer Life Sciences. [U-13C]Glucose and [U-14C]glutamine were from Cambridge Isotope Laboratories, Inc. (Andover, MA). [U-14C]Glucose was purchased from MP Biomedicals, LLC (Solon, OH). Immobilon-PVDF membrane was obtained from EMD Millipore Corp. (Bedford, MA). Compound-9 (C-9) was synthesized by AssisChem Inc. (Watertown, MA) according to methods described by Li et al. (39). Enhanced chemiluminescence (ECL) was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). All other reagents were obtained from Fisher.

**B Lymphocyte Isolation and Culture**—BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were cared for and handled at all times in accordance with National Institutes of Health and Boston College Institutional Animal Care and Use Committee guidelines. Splenic B cells from mice at 8–12 weeks were isolated and purified by negative selection using a B cell isolation kit, MidiMACSTM separator, and LS column (Miltenyi Biotec Inc., Auburn, CA). Small dense B cells were isolated following centrifugation through a discontinuous 72:65:50 Percoll gradient. B cells were cultured in RPMI 1640 medium (Mediatech, Inc., Manassas, VA) plus 10% fetal calf serum (FCS), 2 mM l-glutamine, 10 mM HEPES, pH 7.4, 50 μM β-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin as described (6).

**Microscopy and ER Staining**—B cells (1 × 10^6) were collected and washed with HBSS containing 2% FCS. Cells were then incubated with 5 mM Dapoxy ER-TrackerTM dye (Invitrogen) in HBSS containing 2% FCS for 30 min. B cells were washed with HBSS containing 2% FCS and resuspended in RPMI 1640 medium, transferred to chambered coverglass (Lab-Tek II, Nunc Rochester, NY), and incubated for 30 min. B cells were visualized with an Axiovert 200M inverted microscope (×100/1.3 objective, DAPI filter D350/400DCLP) and analyzed with Velocity software (Improvision) version 5.03.

**ELISA**—CH12 B lymphoma cells were cultured in the presence or absence of LPS for the indicated times. Tissue culture supernatant was collected (250 μl, corresponding to 10^4 CH12 B cells), and the amount of IgM was measured by ELISA using a mouse IgM quantitation set (E90-101) according to the manufacturer’s instructions (Bethyl Laboratories, Inc., Montgomery, TX).

**Flow Cytometry and Cell Cycle Measurements**—B cells (10^5 cells) were collected, washed in PBS, and resuspended in PBS containing 0.1% Triton X-100, 50 μg/ml propidium iodide, and 50 μg/ml RNase A for 30 min (37°C). DNA content was measured by flow cytometry using a FACSCanto cytometer (BD Biosciences). Acquired data were analyzed with ModFit LT version 3.0 (Verity Software House, Topsham, ME). To assess viability, B cells (10^5 cells) were incubated in PBS containing 0.5% FCS and 1.0 μg/ml propidium iodide followed by flow cytometry as described (6). Proliferation of B lymphocytes (10^4 cells) was measured by bromodeoxyuridine (BrdU) incorporation and flow cytometry using a Pharmingen FITC-BrdU flow kit according to the manufacturer’s instructions. For CD138 surface staining, 10^6 splenic B cells were washed with ice-cold
staining buffer (PBS containing 1% FCS, 0.1% NaN₃) and then incubated with 100 µl of staining buffer containing 1.5 µl of rat anti-mouse CD15/CD32 Ab (BD Biosciences) for 20 min. Cells were then stained with PE-conjugated rat anti-mouse CD138 Ab or PE-conjugated rat IgG2a, κ isotype control (BD Biosciences) for 45 min (4 °C). Cells were washed three times with staining buffer and analyzed by flow cytometry. B lymphocyte-induced maturation protein-1 (Blimp-1) staining was carried out with 10⁶ splenic B cells that were washed with ice-cold staining buffer and then fixed and permeabilized with the transcription factor buffer set according to the manufacturer’s instructions (BD Pharmingen); cells were stained with Alexa Fluor 647-conjugated rat anti-mouse Blimp-1 (BD Biosciences) and analyzed by flow cytometry. For ER-Tracker™ staining, 10⁶ splenic B cells were washed with ice-cold PBS. Cells were then incubated with 1 µM ER-Tracker™ dye for 30 min (37 °C), washed three times with PBS, and analyzed by flow cytometry.

In all experiments, analysis was carried out on the gated viable cell population. Flow cytometry was carried with a BD FACSCanto cytometer, and the data were analyzed by FACSDiva software (BD Biosciences).

**Lipid Extraction and High Performance Thin Layer Chromatography (HPTLC)—**B cells (7.5 × 10⁷) were collected and washed in PBS, and total cellular lipid was extracted in methanol/chloroform (1:1) overnight (25 °C). Neutral and acidic lipids were separated by DEAE-Sephadex (A-25, GE Healthcare) column chromatography as described (40). For each condition and/or time point, an equal number of B cells were extracted. To ensure that the same amount of lipid was spotted on the chromatography plate, 10 µg of oleoyl alcohol was added to the initial B cell extract. As an additional control for extraction efficiency, B cell extracts were spiked with 10 µg of lysosphosphocholine and cerebrosides because these lipids were not detected in the bulk lipid isolation of B cells. Lipids were then separated by HPTLC; the chromatography plates were developed first to 4.5 cm (of 10-cm total length) in chloroform/methanol (1:1), then to 10 cm with hexane/diisopropyl ether/acetate (65:35:2, v/v/v) as described (41, 42). Lipids were visualized by charring in the presence of a solution of 3% Cu(OAc)₂ and 8% H₃PO₄. To monitor glucose incorporation into lipid, B cells (7.5 × 10⁷) were cultured in medium containing 1 µCi/ml D-[U-¹³C]glucose and subsequent to 10 cm with hexane/diisopropyl ether/acetate (65:35:2, v/v/v) as described (41, 42). Lipids were visualized by charring in the presence of a solution of 3% Cu(OAc)₂ and 8% H₃PO₄. To monitor glucose incorporation into lipid, B cells (7.5 × 10⁷) were cultured in medium containing 1 µCi/ml D-[U-¹³C]glucose and subsequent to 10 cm with hexane/diisopropyl ether/acetate (65:35:2, v/v/v) as described (41, 42).

**Glycolysis Measurements—**Splen B cells (10⁶ cells/0.5 ml) were incubated with [5-³H]glucose (Amersham Biosciences) for 90 min. B cells (200,000 cells in 100 µl) were then removed and placed in 1.5-ml microcentrifuge tubes containing 50 µl of 0.2 N HCl. ³H₂O was separated from unmetabolized [³H]glucose by evaporation diffusion (25 °C) for 48 h as described (6). The amount of diffused and non-diffused tritium was quantitated by liquid scintillation spectrometry and compared with vials containing [5-³H]glucose only and ³H₂O alone.

**RESULTS**

**Distribution of Neutral and Acidic Lipids in Naive and LPS-stimulated Splenic B Lymphocytes—**We initially evaluated bulk neutral and acidic lipid distribution in control and LPS-stimulated ex vivo splenic B lymphocytes (41). Individual neutral lipids were identified based on their migration distance on HPTLC in comparison with neutral lipid standards and a purified murine brain neutral lipid fraction (Fig. 1A). In the absence of LPS stimulation, B lymphocytes contained several predominant neutral lipids, including PE, phosphatidylcholine (PC), cholesterol, cholesterol esters, and a relatively lower abundance of ceramide (CM) and sphingomyelin. Similarly, acidic lipids were identified based on migration with acidic lipid standards and a purified murine brain acidic lipid fraction (Fig. 1A); the major acidic lipids detected in unstimulated B cells corresponded to phosphatidylglycerol (PG) and cardiolipin (CL) (Fig. 1A). Stimulation of B cells with LPS for 48 and 72 h resulted in elevated levels of several neutral lipids, including PC, PE, CM, and cholesterol (Fig. 1, A and B); this time frame corresponds to the initial endomembrane expansion and induction of IgM production in ex vivo splenic B cells stimulated with mitogenic antigens (15). LPS stimulation

et al. (43). The amount of [¹⁴C]acetyl-CoA produced was then measured by liquid scintillation spectrophotometry.

**Gas Chromatography-Mass Spectroscopy Analysis—**B cells (6.5 × 10⁶) were cultured in modified RPMI 1640 containing 10% dialyzed FCS and 50% [U-¹³C]glucose (1.0 g/liter) or 50% [U-¹³C]glutamine (0.3 g/liter). At the indicated times, B cells were centrifuged at 500 × g, and the supernatant was collected and stored at −80 °C. The resulting cell pellet was washed several times in PBS and then stored at −80 °C. The cell pellets were extracted with a 1:1:1 mix of water/methanol/chloroform. The chloroform extracts were methylated to produce fatty acid methyl esters and analyzed by gas chromatography-mass spectroscopy (GC-MS) as described (44). C17:0 fatty acid was added to the samples to serve as an internal standard. Relative amounts of fatty acids were calculated based on the intensity of methyl ester ions and adjusted for internal standard as needed. Mass spectra of fatty acid methyl esters were corrected for heavy isotopes at all positions in the molecules except for the carbon atoms in the fatty acids (44); these corrected spectra were used to deduce the percentage of fatty acid synthesis and ¹³C labeling of constituent acetyl units in fatty acids using methods from Lee et al. (45), modified as described (46).

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**A. Neutral Lipids**

|       | LPS 48 hr | LPS 72 hr |
|-------|-----------|-----------|
| C     | 1.1       | 2.0       |
| CM    | 0.4       | 2.3       |
| PE    | 1.7       | 3.6       |
| PC    | 1.1       | 2.1       |

**Acidic Lipids**

|       | LPS 48 hr | LPS 72 hr |
|-------|-----------|-----------|
| CL    | 0.6       | 1.3       |
| PG    | 0.0       | 1.0       |
| PS    | 0.5       | 1.0       |
| PI    | 0.8       | 1.4       |

**FIGURE 1. Bulk neutral and acidic lipid distribution in ex vivo B lymphocytes.** B cells (10⁷ cells) were cultured in the absence (M) or presence of 50 μg/ml LPS for 48 and 72 h. A, lipids were isolated and spotted (50 μg B cell dry weight per lane) and separated by HPTLC; the plate was developed as described under "Experimental Procedures," and lipids were visualized by charring. **Left,** HPTLC of neutral lipids. Purified murine brain neutral lipids (B) and neutral lipid standards (Std) corresponding to 1 μg were also separated on the chromatogram for comparison and identification of neutral lipids. CE, cholesterol esters; TG, triglycerides; C, cholesterol; CB, cerebrosides; SM, sphingomyelin; LPC, lysocephatidylcholine. **Right,** HPTLC of acidic lipids. Murine brain (B) and acidic lipid standards (Std) corresponding to 1 μg were separated on the chromatogram for comparison and identification of acidic lipids. FFA, free fatty acids; PA, phosphatidic acid; S, sulfatides. B, quantification of individual band density for the major lipids species induced by LPS at 48 and 72 h was obtained by densitometric scanning of HPTLC plates and analysis using Camag Wincan software as described under "Experimental Procedures." Numbers represent -fold increase in individual lipid species from LPS-stimulated B cells in comparison with the corresponding lipids in unstimulated B cells. The data are representative of three independent experiments.

LPS also resulted in the production of triglycerides (Fig. 1A) as well as increased levels of the acidic lipids, PI, PS, and CL in comparison with unstimulated B cells (Fig. 1A and B). The HPTLC also identified free fatty acids in the acidic lipid fraction; however, we observed that the migration distance of the free fatty acid band relative to the origin on HPTLC varied over several experiments, as did the relative -fold increase in free fatty acids in response to LPS.

**Stimulation of B Lymphocytes with LPS Leads to Glucose-dependent de Novo Fatty Acid and Lipid Synthesis—**We next determined if extracellular glucose was directly utilized to support de novo lipid synthesis in B lymphocytes. For these experiments, B cells were cultured in the absence (24 h) and presence of LPS (24, 48, and 72 h) in medium supplemented with [¹⁴C]glucose. B cells were then collected, neutral and acidic lipid fractions were isolated, and the amount of [¹⁴C]glucose incorporation into individual lipids was determined by autoradiography following HPTLC. As shown in Fig. 2A, under the experimental conditions used, unstimulated B cells exhibited no detectable incorporation of [¹⁴C] label into the predominant neutral and acidic lipids previously identified by bulk lipid analysis (see Fig. 1A). Unstimulated B cells exhibited incorporation of [¹⁴C] label into two slower migrating neutral lipid species (Fig. 2A), which was increased at 24 h and then declined at 48 and 72 h following LPS stimulation (Fig. 2A). The identity of these lipid species is currently unknown. In contrast to unstimulated B cells, LPS treatment led to increased [¹⁴C] label incorporation into PE (1.4-7.6- and 11.1-fold at 24, 48, and 72 h, respectively) and PC (5.3-26.5- and 29.2-fold at 24, 48, and 72 h, respectively) and to a lesser extent CM (1.1-, 1.6-, and 2.1-fold at 24, 48, and 72 h, respectively) and cholesterol (1.0-, 2.1-, and 2.6-fold at 24, 48, and 72 h, respectively) (Fig. 2, A and B). LPS also induced [¹⁴C] label incorporation into triglycerides at 48 h (1.4-fold) and 72 h (9-fold) (Fig. 2, A and B). In a similar manner, LPS treatment led to increased [¹⁴C] label incorporation into several acidic lipids, including PS (1.9-, 10.1-, and 29.5-fold at 24, 48, and 72 h, respectively), PI (4.8-, 27.1-, and 35.2-fold at 24, 48, and 72 h, respectively), CL (2.1-, 9.1-, and 14.1-fold at 24, 48, and 72 h, respectively), and PG (2.2-, 6.8-, and 7.3-fold at 24, 48, and 72 h, respectively) (Fig. 2, A and B). It should be noted that under these experimental conditions, the presence of [¹⁴C] label incorporation into long chain polyunsaturated fatty acids, such as arachidonic acid (C20:4) and C22:6, was not detected. The level of arachidonic acid (C20:4) in the total unlabeled lipid fraction was low and similar in naive B cells and in the LPS-stimulated B cells after 72 h (~5.9 mol%).

We next sought to determine the contribution of glucose metabolism to de novo fatty acid synthesis by GC-MS. B cells were cultured in the presence of [¹³C]glucose or [¹³C]glutamine during LPS stimulation for 24 h. Mass spectra for [¹³C]-labeled fatty acids were assumed to be dependent on the random incorporation by fatty acid synthase of [¹³C]-labeled or unlabeled acetyl units from acetyl-CoA into the fatty acid molecule (up to C16:0 palmitate, incorporating 8 acetyl units) and could therefore be fitted to a binomial model. This fitting process (described in detail in Refs. 45 and 46) yielded values for percentage of [¹³C] labeling of acetyl-CoA units and percentage of de novo synthesis for fatty acids. The percentage of de novo
extension of palmitate to C18:0 stearate (versus salvage) by elongation was calculated based on the extra mass labeling in stearate, corresponding to the incorporation of an extra labeled acetyl unit, compared with the expected mass labeling based on the mass distribution in palmitate (46). Upon LPS stimulation for 24 h, incorporation of 13C label from glucose to acetyl-CoA increased to 36% (Fig. 3A), whereas the incorporation of 13C label from glutamine to acetyl-CoA was not affected with LPS stimulation. At 48 h after LPS stimulation, the percentage contribution of [13C]glucose (36%) and [13C]glutamine (10%) to the acetyl-CoA pool combined to be equivalent to 92% of the total acetyl-CoA content (because input of 13C label is 50% of either substrate). These results suggest that in response to LPS, the majority of acetyl-CoA used for fatty acid synthesis is derived from glucose; most of the remainder comes from glutamine, and only a small fraction (the remaining 8%) is derived from unlabeled sources, such as turnover of (unlabeled) fatty acids or other metabolites. The labeling of free fatty acids was also evaluated in control and LPS stimulated B cells. The total relative amount of free fatty acids incorporating 13C label increased with LPS stimulation at 24 h and to a greater extent at 48 h (Fig. 3B). In each case, the amount of 13C label converted to fatty acids was normalized to extracts of unstimulated B cells cultured with [13C]glutamine for 24 h. Without stimulation, the percentage of de novo synthesis of free cellular palmitate (C16:0) was less than 4% but increased to 60% at 48 h after LPS stimulation (Fig. 3C). A similar increase was observed in stearate (C18:0) synthesis, whereas myristate (C14:0) synthesis could not be measured in unstimulated B cells because of low amounts and low labeling but was also ~60% newly synthesized after 48 h in stimulated B cells (Fig. 3C). We also observed varying increases in the relative amounts of free fatty acids (C14:0, C16:0, C16:1, C18:0, and C18:1 (two species corresponding to a major and minor peak eluting from the GC), C18:2, C20:0) in LPS-stimulated B cells (Fig. 3B).

Expression and Activity of ACLY in B Lymphocytes—ACLY catalyzes a key regulatory step in de novo lipogenesis in mammalian cells by cleaving mitochondrially derived citrate to generate cytosolic acetyl CoA plus oxaloacetate (28, 29). To investigate the role of ACLY in B lymphocytes, endogenous ACLY protein expression was measured by Western blot in ex vivo splenic B cells culture in medium alone or stimulated with LPS (Fig. 4). ACLY was expressed in unstimulated B cells, and the level of ACLY protein increased over time following LPS stimulation (Fig. 4). ACLY undergoes phosphorylation on serine 454 in response to growth factor stimuli, and this modification has been reported to increase its enzymatic activity (27, 47). We found a relatively low level of phosphorylated ACLY in unstimulated B lymphocytes (Fig. 4). In response to LPS stimulation, ACLY phosphorylation on serine 454 was increased at 6 h following LPS stimulation but more substantially between 12 and 72 h (Fig. 4). To determine if ACLY enzymatic activity was...
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FIGURE 3. Acetyl-CoA levels and fatty acid synthesis in LPS-stimulated B lymphocytes. A, B cells were cultured in medium containing 50% [13C]glucose (gln) or [13C]glutamine (gln) for 24 and 48 h in the absence (M) or presence (LPS) of 50 μg/ml LPS. Cells were then collected, and 13C-labeled acetyl units were analyzed in comparison with metabolite label by GC-MS as described under "Experimental Procedures." The average percentage of 13C label incorporation into total acetyl-CoA was quantified and is represented on the y axis. Error bars, S.D. of duplicate measurements. The data were compared using Student's two-tailed t test. The p values comparing B cells cultured in the absence of LPS with B cells stimulated with LPS for glutamine labeling at 24 and 48 h correspond to 0.64 and 0.62, respectively. The p values comparing B cells cultured in the absence of LPS with B cells stimulated with LPS for glucose labeling at 24 and 48 h correspond to 0.0071 and 0.038, respectively. B, B cells were extracted to recover fatty acids. Samples were methylated to produce fatty acid methyl esters and subsequently analyzed by GC-MS, as described under "Experimental Procedures." Fatty acid species were measured based on methyl ester ion signal and are presented as the relative amount of fatty acids normalized to values of 24 h unstimulated B cells cultured in [13C]glutamine. C, de novo fatty acid synthesis of C14:0 and C16:0 and the elongation of C18:0 were evaluated in [13C]glucose and [13C]glutamine B cell extracts, and the pooled percentage of de novo synthesis was calculated. Measurements were based on methyl ester ion signal. unstim (B and C), B cells cultured in medium alone; LPS, B cells stimulated with LPS. Error bars, S.D. of duplicate measurements. In B, the p values comparing B cells cultured in the absence of LPS with B cells stimulated with LPS at 24 h correspond to 0.309 (C14:0), 0.414 (C16:1), 0.282 (C16:0), 0.128 (C18:2), 0.289 (C18:1), 0.049 (C18:1), 0.355 (C18:0), and 0.438 (C20:0), whereas values at 48 h correspond to 0.015 (C14:0), 0.048 (C16:1), 0.080 (C16:0), 0.114 (C18:2), 0.104 (C18:1), 0.002 (C18:1), 0.045 (C18:0), and 0.047 (C20:0). The two C18:1 species are shown in the order they elute from the GC, with the major peak first and the minor peak second. In C, the p values comparing B cells cultured in the absence of LPS with B cells stimulated with LPS at 24 and 48 h correspond to 6.6 × 10−3 and 6.0 × 10−2 for C16:0, respectively, and 5.2 × 10−3 and 5.8 × 10−4 for C18:0, respectively.

FIGURE 4. Expression of ACLY in B lymphocytes. B cells were cultured in medium alone (M) or stimulated with 50 μg/ml LPS for the indicated times. Cells were then collected, cellular lysates were prepared in Triton X-100 buffer, and equivalent amounts of total protein were examined by Western blot for total ACLY (ACLY), ACLY phosphorylated in serine 454 (pACLY(Ser454)), and β-actin as a loading control for electrophoresis, as described previously (6). Individual bands were subjected to densitometric scanning using ImageQuant software. Numbers represent -fold increase in pACLY(Ser454) and ACLY bands at each time point following LPS stimulation in comparison with B cells cultured in medium alone. The data are representative of two independent experiments.

modulated in response to LPS stimulation of B cells, we measured ACLY activity in whole cell extracts prepared from unstimulated and LPS-stimulated B cells. ACLY enzymatic activity was measured as a function of the amount of [13C]acetate produced from the conversion of [14C]citrate by endogenous ACLY (43).

ACLY Activity Is Required for Glucose-dependent de Novo Lipogenesis in B Lymphocytes—We next determined the contribution of ACLY to de novo lipogenesis by inhibiting ACLY with the pharmacological inhibitor compound-9 (C-9) (39). C-9 is a cell-permeable 2-hydroxy-N-arylsulfonamide analog that specifically inhibits ACLY activity in mammalian cells with an IC50 = 0.13 μM and demonstrates efficacy in vivo mouse models (39). C-9 inhibited endogenous B cell ACLY activity (data not shown), in keeping with previous reports in mammalian cells (39). In control experiments, treatment of splenic B
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FIGURE 5. Increased ACLY enzyme activity in response to LPS stimulation of B lymphocytes. A, B cells were cultured in medium alone (0) or stimulated with 50 μg/ml LPS for the indicated times. Whole cell extracts were prepared, and ACLY enzyme activity was measured as a function of the amount of [14C]acetetyl-CoA produced from the conversion of [14C]citrate by ACLY according to the method of Ma et al. (43); enzyme activity is represented as the amount of [14C]acetetyl-CoA produced (in cpm/μg cellular protein/min) as described under "Experimental Procedures." Error bars, S.D. of triplicate measurements. The data are representative of three independent experiments. The p values comparing B cells cultured in medium alone (0) with B cells stimulated with LPS at several time points (*) correspond to p < 0.05. B, B cells were cultured in the absence (Medium) or presence of 50 μg/ml LPS for 12 h (open bars); parallel B cell cultures were incubated in the presence of 50 μM compound-9 (closed bars); glycosylation was measured as described under "Experimental Procedures." Error bars, S.D. of triplicate measurements. Data are representative of three independent experiments. The p value comparing B cells cultured in medium alone with B cells stimulated with LPS (*) corresponds to p < 0.001.

cells with the ACLY inhibitor C-9 did not block LPS-induced glycolysis when measured at 12 h (Fig. 5B). We next determined if culture of LPS-stimulated B cells with [14C]glucose in the presence or absence of C-9 blocked the incorporation of 13C label into the major neutral lipids at 24 and 48 h, as detectable by autoradiography and HPTLC (Fig. 6, A and B). Incorporation of 13C label into the acidic lipids was also blocked by C-9 in LPS-stimulated B cells treated at 24 and 48 h (Fig. 6, A and B). Treatment of B cells with C-9 also eliminated the increase in de novo synthesis incorporation of palmitate or stearate fatty acid species in LPS-stimulated B cells when measured at 36 h by GC-MS (Fig. 7A) and completely reversed the increase in the amounts of various free fatty acids observed with LPS stimulation (Fig. 7B). As mentioned above, 13C label arachidonic acid (C20:4) was not detectable by HPTLC and autoradiography following incubation of B cells with [14C]glucose; however, GC-MS revealed the presence of 13C-labeled arachidonic acid (C20:4) in unstimulated splenic B cells cultured with [13C]glucose (Fig. 7C). LPS stimulation of B cells led to a statistically significant, albeit relatively small, increase in 13C-labeled arachidonic acid (C20:4) of ~0.7-fold above that of unstimulated B cells (Fig. 7C). The increase in 13C label incorporation into arachidonic acid (C20:4) following LPS stimulation was blocked by C-9 (Fig. 7C). Collectively, these data suggest that ACLY activity is required for glucose-dependent de novo lipid synthesis in B lymphocytes.

We also determined if activation of naive B lymphocytes by other surface receptor-linked pathways led to glucose-dependent de novo lipogenesis. Activation of B cells via cross-linking the B cell antigen receptor with anti-Ig together with IL-4 led to increased 13C label incorporation into several neutral and acidic lipids (Fig. 8, A and B). Qualitatively, a similar profile of neutral and acidic lipids incorporated [14C]glucose; however, the relative amounts of [14C]glucose incorporation into individual lipids differed in B cells stimulated with anti-Ig plus IL-4 in comparison with LPS. This may reflect that anti-Ig plus IL-4 induces proliferation of naive B cells, whereas LPS induces both proliferation and differentiation, the latter requiring additional lipid production (to support endomembrane expansion for Ig secretion) beyond that needed for cell growth and division. The increase in [14C]glucose label incorporation into neutral and acidic lipids following stimulation of B cells with anti-Ig plus IL-4 was also reduced following inhibition of ACLY activity with C-9 (Fig. 8, A and B). Whereas treatment of LPS-stimulated B cells with C-9 reduced 13C label incorporation into neutral and acidic lipids to levels not detectable by HPTLC and autoradiography (Fig. 6), treatment of anti-Ig plus IL-4-stimulated B cells with C-9 led to reduced 13C label incorporation that varied with respect to individual neutral and acidic lipids (Fig. 8, A and B). Our studies also revealed that culture of B lymphocytes with the combination of anti-Ig plus IL-4 resulted in increased ACLY phosphorylation on serine 454 (Fig. 8C). Moreover, inhibitors of PI3K blocked the increase in ACLY serine 454 phosphorylation, suggesting the involvement of PI3K in regulating ACLY activity (Fig. 8C).

ACLY Activity Is Required to Maintain Survival of ex Vivo B Lymphocytes in Response to LPS—Proliferation of mammalian cells requires a doubling of membrane phospholipid content (20). We therefore determined whether ACLY activity was necessary for B cell proliferation in response to LPS. Inhibition of ACLY activity with C-9 was accompanied by reduced BrdU incorporation into DNA in LPS-stimulated B cells, corresponding to 0.9% (48 h) and 0.4% (72 h) in comparison with BrdU incorporation in parallel B cells cultured in the absence of C-9 (8.2% at 48 h and 15.3% at 72 h) (Table 1). B lymphocytes stimulated with LPS in the presence of C-9 also exhibited a reduction in cell viability corresponding to 31.2 and 13.5% at 48 and 72 h, respectively (Table 1). The decrease in cell viability was accompanied by an increased percentage of hypodiploid DNA content, corresponding to 28.3 to 35.0% at 48 and 72 h, respectively (Table 1).

ACLY Activity Is Required for Several Phenotypic Changes Defining Plasma Cell-like Differentiation—The results above suggest that inhibition of ACLY activity blocks glucose-dependent synthesis of neutral and acidic lipids, including PC and CM (Fig. 6A); both of these lipids are required for ER expansion in B lymphocytes (17–19). It therefore seemed likely that ACLY
activity might contribute to expansion of the endomembrane secretory network in response to LPS stimulation. To assess the expansion of the ER compartment, we stained ex vivo B lymphocytes with Dapoxyl ER-Tracker™, an ER-specific probe. Intracellular staining of B cells cultured in the absence of LPS revealed a small area of staining that expanded substantially following stimulation with LPS for 24 h (Fig. 8A). Treatment of LPS-stimulated B cells with C-9 resulted in decreased intracellular staining (Fig. 8A). Flow cytometry of ER-Tracker™ stained B cells also revealed a significant reduction in mean fluorescence intensity in LPS-stimulated B cells treated with C-9 (Fig. 9B). For these experiments, the viable B cell population was gated and subsequently analyzed for fluorescence intensity by flow cytometry.

To investigate further the involvement of ACLY activity in plasma-like B cell differentiation, we evaluated whether ACLY activity was required for LPS-induced surface CD138 (Syndecan-1) expression in splenic B lymphocytes; CD138 is expressed upon B cell differentiation into plasmablasts and plasma cells (15). LPS stimulation of splenic B cells increased surface CD138 expression (Fig. 10A). To minimize the confounding effects of decreased viability at later time points observed with C-9, B cells were instead treated with Medica 16, a small molecule inhibitor of ACLY that is structurally unrelated to C-9 and has been used both in vitro and in vivo (33). Treatment of LPS-stimulated B cells with Medica 16 resulted in a significant reduction in CD138 expression in viable B cells analyzed by flow cytometry (Fig. 10A). We also evaluated the expression of Blimp-1, which is required for development of immunoglobulin-secreting cells and for maintenance of long lived plasma cells (16). We found that LPS-induced Blimp-1 expression was reduced in splenic B cell cultures incubated with Medica 16 (Fig. 10B).

We also sought to determine the effects of ACLY inhibition on IgM production. For these studies, the CH12 B lymphoma cell line was used as a model system to evaluate the role of ACLY in the differentiation of B cells into IgM secreting cells.
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(17, 18, 49). CH12 B lymphoma cells express surface IgM and MHC II and undergo morphological differentiation and IgM secretion in response to LPS, similar to splenic B cells (17, 49). CH12 B lymphoma cells stimulated with LPS for 24 h exhibited an increase of $[^{14}C]$glucose incorporation into neutral and acidic lipid species that was blocked following inhibition of ACLY activity with C-9 (data not shown). To test whether ACLY activity was necessary for IgM production, CH12 B lymphoma cells were stimulated with LPS in the presence and absence of C-9 and IgM levels in the tissue culture supernatant following exposure to C-9 (data not shown). Viability following exposure to C-9 (data not shown). Tissue culture supernatant from these experiments was analyzed by HPLC to determine the levels of neutral and acidic lipids that were blocked following inhibition of ACLY activity with C-9 (data not shown). Tissue culture supernatant from these experiments was analyzed by HPLC to determine the levels of neutral and acidic lipids that were blocked following inhibition of ACLY activity with C-9 (data not shown).

**DISCUSSION**

LPS induces the proliferation and differentiation of B lymphocytes into Ig-secreting plasma cells. A doubling of membrane phospholipid content precedes proliferation, whereas differentiation involves a major morphological change of the cell interior, including a substantial expansion of the rough ER and Golgi network (17–19). Distinct changes in the ER network are evident as early as 24 h after exposure of naive B lymphocytes to LPS (14, 15). B cells are capable of anticipating their secretory role via enacting a sequential and multistep program of protein expression to accommodate the massive Ig production that follows (4, 5). A recent metabolomic study identified several bioenergetic pathways that discriminate between the proliferation and Ig-secreting phases (50). By contrast, the metabolic reprogramming necessary to support de novo phospholipid synthesis has not been described in depth. Lymphocytes and hematopoietic cells must access extracellular nutrients, such as glucose in order to support growth and effector function (1, 2). PI3K/Akt signaling represents a mechanism that links antigen receptor engagement to glucose uptake and glycolytic metabolism in B and T lymphocytes (2, 6, 9, 10). Germaine to the studies herein, one of the biosynthetic fates of glucose metabolism in highly proliferating cancer cells is its conversion to fatty acids and cholesterol (11, 13, 51, 52). We reasoned therefore that LPS signaling might promote de novo lipogenesis, at least in part, by reprogramming glucose utilization.

Our results indicate that the levels of neutral and acidic lipids detected by HPTLC increase after LPS stimulation of B lymphocytes, but the bulk composition does not change substantially, consistent with a recent report in CH12 B lymphoma cells.

![FIGURE 7. Inhibition of ACLY activity reduces glucose-dependent de novo fatty acid synthesis in LPS-stimulated B lymphocytes.](image-url)
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**FIGURE 8. Glucose-dependent de novo lipid synthesis in B lymphocytes stimulated with anti-Ig plus IL-4 requires ACLY activity.** A, B cells (10^7 cells) were cultured in medium supplemented with [14C]glucose (1 μCi/ml) in the absence (M) or presence of 10 μg/ml anti-Ig plus 4 ng/ml IL-4 for 24 and 48 h (lanes αlg+IL-4). Parallel B cells were cultured in the presence of 50 μM compound-9 (+C9). At the indicated times, viable B cells were recovered via centrifugation through a Lympholyte M gradient, and lipids were isolated, separated by HPTLC, and then visualized by charring and autoradiography. The HPTLC plates were then subjected to autoradiography. Autoradiography of [14C]glucose incorporation into neutral (top) and acidic (bottom) lipids in B lymphocytes. Note that B cells cultured in medium alone (M) were incubated with [14C]glucose for 24 h. The corresponding autoradiograms were subjected to densitometric scanning using ImageQuant software. Numbers represent -fold increase in individual neutral (top) and acidic (bottom) lipids from LPS-stimulated B cells in comparison with the corresponding lipids in unstimulated B cells, normalized for HPTLC loading as described under “Experimental Procedures.”

**TABLE 1** Evaluation of ACLY activity on B lymphocyte proliferation and survival

| Culture conditions | Cell viability | Sub-G0 (%) | BrdU (%) |
|--------------------|----------------|------------|----------|
| Medium             | 95.0           | 0.1        | 0.2      |
| LPS, 24 h          | 93.0           | 3.2        |          |
| LPS, 48 h          | 87.8           | 5.8        | 8.2      |
| LPS, 72 h          | 81.6           | 9.0        | 15.3     |
| LPS + C-9, 24 h    | 89.2           | 2.7        |          |
| LPS + C-9, 48 h    | 31.2           | 28.3       | 0.9      |
| LPS + C-9, 72 h    | 13.5           | 35.0       | 0.4      |

* B cells were cultured in medium alone or stimulated with 50 μg/ml LPS for the indicated times in the presence or absence of 50 μM C-9. B cells were collected, and sub-G0 DNA content was measured by flow cytometry as described under “Experimental Procedures.” The data are presented as the percentage of cells with sub-G0 DNA content and are representative of three independent experiments.

* B lymphocytes were cultured in medium alone or stimulated with 50 μg/ml LPS for the indicated times in the presence or absence of 50 μM of C-9. At 24 h post-LPS-stimulation, BrdU was added to the B cell cultures. Cells were then incubated for an additional 24 and 48 h and collected, and then incorporation of BrdU was measured by flow cytometry as described under “Experimental Procedures.” The data are presented as the percentage of B cells incorporated BrdU and are representative of three independent experiments.

(18) LPS leads to an increase in neutral and acidic lipid biosynthesis that is dependent on the acquisition of extracellular glucose. The predominant neutral lipids whose de novo synthesis derives carbon, in part, from glucose, measured by [13C]glucose in response to LPS stimulation of B cells. The predominant fatty acids identified by GC-MS include eicosanoid acid (20:4), palmitate (16:0), and oleate (18:1), which are derived from the desaturation and/or elongation of palmitate. Collectively, these findings support a role for glucose in de novo lipogenesis in splenic B cells.

In addition to de novo synthesis, we cannot rule out the possibility that the cellular levels of lipids in B lymphocytes may also result from the uptake of fatty acids from external sources. On this point, polyunsaturated fatty acid species (C20:4 or C22:6) were present in the total unlabeled lipid fraction;
however, we were unable to detect (by HPTLC and autoradiography) de novo synthesis of long chain polyunsaturated fatty acids (C20:4 or C22:6) from [14C]glucose. By contrast, GC-MS analysis revealed the presence of detectable [13C]-labeled arachidonic acid (C20:4) in naive B cells cultured with [13C]glucose; LPS stimulation led to a statistically significant, albeit modest, increase in [13C]-labeled arachidonic acid (C20:4). It is possible that acquisition of polyunsaturated fatty acids under the experimental conditions used herein is derived from an exogenous source (e.g. serum in the culture medium). A similar finding was reported in a previous study of concanavalin A-stimulated lymphocytes (53).

GC-MS analysis revealed a time-dependent decrease in several C16:0 and C18:0 fatty acids in control B lymphocytes devoid of LPS stimulation. Because these analyses were carried out with viable B cells, we do not believe the findings reflect cell death. Lymphocytes require the continuous presence of extrinsic signals (e.g. pro-survival cytokines) from their in vivo microenvironment to maintain cell viability. Ex vivo T cells cultured in medium devoid of these signals undergo atrophy and eventually died. Germane to the studies herein, atrophy is characterized in reduced cell size and decreased glycolytic rate (54). It is possible that the time-dependent decrease in glucose-dependent C16:0 and C18:0 de novo synthesis observed in ex vivo B lymphocytes devoid of LPS reflects decreased glycolysis over time in culture and thus reduced glucose-derived metabolites available to support de novo fatty acid synthesis.

We cannot rule out the possibility that other metabolites, in addition to glucose, may be used to support de novo fatty acid synthesis. Fatty acid synthesis precursors can be produced via other metabolic pathways, such as glutamine (55, 56). For example, in tumor cells with defective mitochondria, reductive carboxylation of glutamine-derived α-ketoglutarate produces citrate to support de novo lipogenesis (55). The findings herein indicate that the majority of acetyl-CoA used to support de novo fatty acid synthesis is derived from glucose and not glutamine when measured at 24 h following LPS stimulation, whereas at 48 h, a relatively smaller fraction of acetyl-CoA is derived from glutamine. Interestingly, analysis of metabolites consumed and released during B lymphocyte differentiation in ex vivo cultures revealed recruitment of distinct metabolic pathways during the proliferation phase (i.e. lactate production increased), whereas the onset of Ig secretion was parallel with glutamine being used primarily as carbon and energy sources (50).

Our findings suggest that ACLY activity is required for glucose-dependent de novo lipogenesis, as evidenced by inhibition of glucose incorporation into fatty acid, cholesterol, and neutral and acidic lipids following treatment of B cells with the ACLY inhibitor C-9. The results also indicate that ACLY is regulated at several levels by LPS. LPS stimulation of B lymphocytes promotes an increase in ACLY enzymatic activity. This is probably attributable, in part, to increased endogenous ACLY protein levels following LPS stimulation; however, we also found that phosphorylation of ACLY on serine 454 is increased; phosphor-
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The role of ACLY activity was also evaluated in CH12 B lymphoma cells, which represents a model for studying LPS-induced IgM production in B cells (17, 18, 49). We found that LPS-stimulated CH12 B lymphoma cells undergo glucose-dependent de novo lipogenesis and that inhibition of ACLY activity blocks glucose incorporation into neutral and acidic lipids (data not shown). Our results revealed that ACLY activity is required for LPS-induced IgM production in CH12 B lymphoma cells, as evidenced by a significant reduction in IgM secretion following treatment with the ACLY inhibitor C-9. In summary, our study provides many new insights into the molecular pathways underlying the reprogramming of glucose metabolism that accompanies B lymphocyte differentiation. The results suggest a previously unknown role for glucose utilization and ACLY activity in the de novo lipogenesis during LPS-induced B lymphocyte differentiation. Taken together, the results from primary B lymphocytes and CH12 B lymphoma cells suggest that ACLY plays a role in several phenotypic changes that define B lymphocyte differentiation. This pathway
may provide new targets for therapeutic exploration in treating plasma cell dyscrasias.

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