Cholesterol deficiency in a mouse model of Smith-Lemli-Opitz syndrome reveals increased mast cell responsiveness

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Mutation of the 3β-hydroxysterol Δ7-reductase gene (Dhcr7−/−) results in Smith-Lemli-Opitz syndrome (SLOS). Patients, and genetically altered mice, are unable to produce cholesterol and accumulate 7-dehydrocholesterol (DHC) in serum and tissue. This causes multiple growth and developmental abnormalities as well as immune system anomalies including allergy. Because cholesterol is a key component of liquid–ordered membranes (lipid rafts) and these domains have been implicated in regulating mast cell activation, we examined whether mast cell responsiveness is altered in this model. Mast cells derived from Dhcr7−/− mice (DHCR KO) showed constitutive cytokine production and hyper-degranulation after stimulation of the high affinity IgE receptor (FcεRI). DHCR KO mast cells, but not wild-type mast cells, accumulated DHC in lipid rafts. DHC partially disrupted lipid raft stability and displaced Lyn kinase protein and activity from lipid rafts. This led to down-regulation of some Lyn-dependent signaling events but increased Fyn kinase activity and Akt phosphorylation. The Lyn-dependent phosphorylation of Csk-binding protein, which negatively regulates Fyn activity, was decreased. This phenotype reproduces some of the characteristics of Lyn-null mast cells, which also demonstrate hyper-degranulation. These findings provide the first evidence of lipid raft dysfunction in SLOS and may explain the observed association of allergy with SLOS.

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive genetic disease with an incidence of 1 in 50,000. It is characterized by mental retardation, microcephaly, syndactyly of the toes, and other dysmorphic features and problems related to abnormal growth and development. SLOS is caused by mutation of the 3β-hydroxysterol-Δ7-reductase gene (DHCR7), which converts 7-dehydrocholesterol (DHC) to cholesterol (1–3). SLOS-affected individuals have increased plasma levels of DHC and abnormally low levels of cholesterol. Many of these patients experience formula and food intolerance. Because mast cells are central in allergy and cholesterol–enriched liquid–ordered domains (lipid rafts) play an important role in their activation (4, 5), it was of interest to study whether the loss of cholesterol altered mast cell sensitivity and activation. Mice carrying a null mutation of the 3β-hydroxysterol-Δ7-reductase gene (Dhcr7−/−, referred to as DHCR KO throughout) were developed as a model for SLOS (6). These mice failed to produce cholesterol and accumulated DHC in serum and tissue. Due to a failure to nurse, newborn mice die shortly after birth. We were able, however, to cultivate mast cells from the liver progenitors of DHCR KO mice.

Cholesterol plays an important role in membrane organization (7, 8). The plasma membrane has specialized regions that are characterized by high concentrations of cholesterol and sphingolipids and spontaneously segregate and are insoluble in nonionic detergents (9). Antigen (Ag)-induced aggregation of FcεRI-bound IgE antibodies causes inclusion of this receptor in these specialized regions commonly referred to as lipid rafts. Lyn kinase is also concentrated in lipid rafts (4, 5) and weakly associates and phosphorylates FcεRI (10). Whether

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Abbreviations used: Ag, antigen; Cbp, Csk-binding protein; DHC, 7-dehydrocholesterol; GC, gas chromatography; LPDS, lipoprotein-deficient serum; MBCD, methyl-β-cyclodextrin; PISK, phosphatidylinositol 3-OH kinase; PLCγ, phospholipase C-γ; SLOS, Smith-Lemli-Opitz syndrome.
the recruitment of FcεRI into lipid rafts is necessary for its interaction with Lyn and for initiation of its phosphorylation, or whether lipid rafts serve to sustain and/or amplify the receptors’ phosphorylation remains unresolved. The mouse SLOS model studied herein provided a system where depletion of cholesterol could be achieved as a consequence of gene deletion. Because cholesterol plays a crucial role in membrane domains with signal transduction potential (8), investigating the molecular consequences of cholesterol deficiency and DHC accumulation in mast cell function could yield new insights for understanding the pathophysiology underlying SLOS and disease treatment.

RESULTS
Mast cells deficient in 3-β-hydroxysterol-Δ7-reductase accumulate DHC
Mast cell progenitors from both DHCR KO and WT mice were cultivated in medium with cholesterol. DHCR KO mast cells cultivated in medium containing 10% FBS proliferated and differentiated similarly to WT mast cells. No substantial differences were found in global tyrosine phosphorylation of proteins, intracellular calcium release, or in degranulation of DHCR KO mast cells cultivated under these conditions (Fig. 1A and not depicted). Cholesterol levels were the same in DHCR KO and WT mast cells (Table I, 0 h), demonstrating that growth of these cells in medium containing cholesterol bypassed the defect of 3-β-hydroxysterol-Δ7-reductase mutation.

To reveal the biosynthetic defect, mature DHCR KO or WT mast cells were cultured in medium supplemented with lipoprotein-deficient serum (LPDS). Cell viability, as determined by propidium iodide staining, and sterol content were measured up to 120 h after depletion. WT mast cells proliferated in medium without cholesterol for 120 h (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051701/DC1) without a significant decrease in cell viability (Table I). Cholesterol content decreased within 48 to 72 h of depletion by ~30% and subsequently increased to reach original levels by 120 h, demonstrating activation of cholesterol biosynthesis. Desmosterol, a precursor of cholesterol, was also detected in WT mast cells at 48 and 72 h but not at 0 h (Table I). Levels of desmosterol reached ~20–30% of total sterol at 72 h. In contrast, DHCR KO cells preferentially accumulated DHC as the cholesterol content decreased (Table I), and DHC levels peaked at ~30% of total sterols between 72 and 96 h of depletion. Total sterol content was relatively constant in WT cells but decreased slightly in DHCR KO cells. DHCR KO mast cells also proliferated poorly (Fig. S1) and cell viability decreased with time (Table I). When grown for 168 h in LPDS, cell viability was <70% (not depicted).

Subsequent experiments were performed with cells grown in LPDS for 72 h, as cell viability was normal and the ratio of DHC or desmosterol to total sterol was maximal (Table I). The numbers of apoptotic cells did not differ substantially between cholesterol-depleted and nondepleted cells and did not exceed 7% of total cells (Fig. 1B). FcεRI expression before or after cholesterol depletion was similar among WT and DHCR KO mast cells (Fig. 1C). Thus, for further experiments, we compared cholesterol-depleted DHCR KO mast cells with cholesterol-depleted or nondepleted WT mast cells.

Loss of cholesterol and accumulation of DHC in mast cells result in FcεRI-dependent hyperresponsiveness
FcεRI-dependent degranulation of cholesterol-depleted DHCR KO cells showed a 2.8-fold increase (P < 0.006, n = 15) over nondepleted WT mast cells in release of the granule marker hexosaminidase (Fig. 2, A and B). Cholesterol-depleted WT cells also showed a twofold increase (P < 0.02,
granule membrane integrity (as determined with toluidine blue staining). Content of cholesterol, DHC, and desmosterol was determined by gas chromatography (GC)/mass spectrometry analysis of extracted sterols. DHC was also measured in cholesterol-depleted WT cells but was nondetectable (sensitivity of assay is pg/mg of protein, n = 5). Total sterols did not differ significantly among genotypes before or after cholesterol depletion (0 vs. 72 h). Data shown are the mean ± SEM for replicate numbers as indicated in parenthesis. nd, not detected.

This suggested that the augmentation of degranulation resulted from cholesterol depletion and/or accumulation of DHC. To test this, cholesterol-depleted WT and DHCR KO mast cells were reconstituted with cholesterol by cultivation in medium containing 30% FBS for 30 min. The concentration of cholesterol in the repleted WT and DHCR KO cells increased to levels equivalent to nondepleted cells as determined by gas chromatography (GC)/mass spectrometry (Table I and not depicted). Cholesterol repletion of WT mast cells reduced degranulation to the level of nondepleted WT mast cells (Fig. 2 C). In contrast, cholesterol-repleted DHCR KO mast cells still had enhanced degranulation relative to cholesterol-repleted or nondepleted WT mast cells. This suggested that the accumulation of DHC in DHCR KO mast cells, but not the accumulation of desmosterol in WT cells, contributed to the maintenance of enhanced degranulation. The concentration of DHC (R² = 0.6094, P < 0.03), but not the concentration of cholesterol (R² = 0.0246, P > 0.70) or of total sterol (R² = 0.0114, P > 0.80; not depicted), correlated with increased degranulation (Fig. 2, D and E) in DHCR KO mast cells. In contrast, cholesterol correlated (R² = 0.740, P < 0.01) with degranulation of cholesterol-depleted WT (Fig. 2 F). Thus, control of WT mast cell degranulation appeared to be dependent on cholesterol, whereas DHC caused an irreversible enhancement of degranulation. The observed effect of cholesterol or DHC on mast cell degranulation was upstream of the exocytic machinery because PMA and calcium ionophore A23187 (a stimulus that bypasses early signaling events) led to similar degranulation from cholesterol-depleted or nondepleted cells (Fig. 2 G). Granule membrane integrity (as determined with toluidine blue) did not differ as a consequence of cholesterol depletion or gene alteration (not depicted).

As shown in Fig. 2 H, constitutive secretion of both IL-6 and TNF was observed after cholesterol depletion. Constitutive TNF secretion by DHCR KO mast cells was considerable (105 ± 6.3 pg/ml/10⁶ cells), reaching ~40% of the response of stimulated WT control cells (246 ± 17.1 pg/ml/10⁶ cells). FcεRI stimulation did not substantially augment this secretion. IL-6 secretion was also constitutive after cholesterol depletion of DHCR KO (223 ± 9 pg/ml/10⁶ cells), reaching approximately one third the level of stimulated control WT mast cells (643 ± 37 pg/ml/10⁶ cells). For cholesterol-depleted WT mast cells, IL-6 secretion was also constitutive and significant (P < 0.05, n = 4) relative to nondepleted cells. For TNF, a consistent but not significant (P < 0.09) increase was observed. Thus, the higher constitutive secretion of IL-6 and TNF from cholesterol-depleted DHCR KO cells suggested that the accumulation of DHC played an important role in the enhanced cytokine response.

### Table I. Effect of cholesterol depletion on mast cell sterol content and viability

| Time of cholesterol depletion (h) | Cholesterol (μg/mg of protein) | DHC (μg/mg of protein) | Viability (%) | Cholesterol (μg/mg of protein) | Desmosterol (μg/mg of protein) | Viability (%) |
|----------------------------------|-------------------------------|------------------------|---------------|-------------------------------|-------------------------------|---------------|
| 0                                | 3.6 ± 0.3 (10)                | 0.3 ± 0.1 (10)         | 96.4 ± 0.3 (6) | 3.9 ± 0.3 (10)                | nd                           | 96.9 ± 0.3 (6) |
| 48                               | 2.6 ± 0.2 (3)                 | 0.5 ± 0.1 (3)          | 95.1 ± 0.1 (3) | 2.7 ± 0.2 (3)                 | 0.6 ± 0.3 (3)                | 95.8 ± 0.2 (3) |
| 72                               | 2.2 ± 0.3 (10)                | 0.9 ± 0.4 (10)         | 93.6 ± 0.3 (6) | 2.9 ± 0.3 (10)                | 1.3 ± 0.5 (3)                | 95.9 ± 0.1 (6) |
| 96                               | 1.9 ± 0.1 (3)                 | 1.1 ± 0.2 (3)          | 89.7 ± 0.6 (3) | 3.3 ± 0.1 (3)                 | nd                           | 95.2 ± 0.3 (3) |
| 120                              | 2.4 ± 0.2 (4)                 | 1.1 ± 0.1 (4)          | 89.3 ± 0.8 (3) | 3.4 ± 0.1 (5)                 | nd                           | 95.0 ± 0.2 (3) |

Cells were depleted of cholesterol by incubation for the indicated times in medium containing LPDS. Cell viability was monitored by trypan blue exclusion and propidium iodide staining. Content of cholesterol, DHC, and desmosterol was determined by GC/mass spectrometry analysis of extracted sterols. DHC was also measured in cholesterol-depleted WT cells but was nondetectable (sensitivity of assay is pg/mg of protein, n = 5). Total sterols did not differ significantly among genotypes before or after cholesterol depletion (0 vs. 72 h). Data shown are the mean ± SEM for replicate numbers as indicated in parenthesis. nd, not detected.
Cholesterol depletion by MBCD can alter the localization of proteins associated with lipid rafts (13). We proposed that the cholesterol to DHC shift in cholesterol-depleted DHCR KO mast cells might cause or enhance alterations in protein localization. A substantial decrease in the previously described (4) lipid raft localization of FcεRI was observed in cholesterol-depleted DHCR KO mast cells (29.6 ± 4.1%) when compared with cholesterol-depleted (40.7 ± 2.4%) or nondepleted (43.4 ± 4.1%) WT mast cells (Fig. 4 A). These changes were not due to decreased FcεRI expression (Fig. 1 C) or to decreased recovery of soluble FcεRI as determined by bound radiolabeled IgE (WT/WT-/DHCR KO- = 1.0:1.02 ± 0.07:0.987 ± 0.09). A large fraction of Lyn kinase is also associated with lipid rafts in mast cells (14). This association may well depend on lipid raft stability given that the...
detectable fraction of Lyn within these domains can vary depending on the solubilization conditions (15). Solubilization of WT cells in 0.1% Triton X-100 showed that 51 ± 5% of total cellular Lyn was associated with lipid rafts (Fig. 4, B and C; n = 6). Depletion of cholesterol from WT cells decreased the amount of Lyn associated with lipid rafts to 36 ± 6%. Localization of Lyn to lipid rafts, however, was dramatically reduced (20 ± 8%, P < 0.009) when cholesterol was depleted from DHCR KO mast cells. Total recovery of cellular Lyn, as determined by densitometry of immunoblots, did not differ substantially (Fig. 5 A, WT/WT-/DHCR KO-, 21 ± 7.7; n = 6). Recovery of total cellular Lyn was similar among the genotypes (WT/WT-/DHCR KO-, 1.0:0.936 ± 0.11:0.947 ± 0.17). Collectively, the findings demonstrate that decreased lipid raft stability causes selective loss of some proteins (like Lyn kinase) from these domains, which might alter cellular signaling and responses.

**Cholesterol depletion and DHC accumulation alter FcεRI-mediated early signaling upstream of calcium responses**

The selective delocalization of Lyn kinase from lipid rafts in cholesterol-depleted DHCR KO mast cells led us to explore its cellular activity. To formally address the issue of the effect of Lyn displacement on its activity, we isolated Lyn from whole cell lysates as well as from the lipid raft fractions of nondepleted or cholesterol-depleted WT and DHCR KO mast cells. Fig. 5 A shows that the total Lyn kinase activity in cholesterol-depleted WT and DHCR KO mast cells did not differ substantially from that in nondepleted WT mast cells. In contrast, Lyn activity in lipid rafts was dramatically decreased upon cholesterol depletion of WT and DHCR KO mast cells relative to nondepleted WT mast cells (Fig. 5 B). The loss of Lyn activity in these domains led us to assess the phosphorylation of FceRI in cholesterol-depleted DHCR KO cells. Fig. 5 C shows that the overall phosphorylation of FceRI was decreased by cholesterol depletion. Densitometric quantitation demonstrated significant differences (*, P < 0.05; **, P < 0.01; n = 4). Although the kinetics were unaltered, the extent of FceRIβ and FceRIγ phosphorylation was reduced by ~30–40%.

Downstream of Lyn kinase, Syk kinase is activated by binding the ITAM sequences in the cytoplasmic domain of the γ subunit of FceRI. Tyrosine (Y)346 in the linker region of Syk has been reported to be involved in the interaction and/or tyrosine phosphorylation and activation of phospholipase C-γ (PLCγ; reference 16). The linker region tyrosines are also possible targets of Lyn kinase activity (17). Phosphorylation of Syk Y346 in nondepleted WT mast cells was rapid (1 min) and increased sixfold after Ag stimulation (Fig. 5 D). In contrast, Syk Y346 phosphorylation in cholesterol-depleted WT and DHCR KO cells was delayed (>3 min) and reduced in magnitude (~50% inhibition). LAT, the adaptor that scaffolds a signaling complex that includes PLCγ, is a target of Syk activity (18). Because we previously showed that Y195 of LAT contributes to stable PLCγ binding (19), we used a phosphospecific antibody to assess its phosphorylation. As shown in Fig. 5 D, phosphorylation of LAT in nondepleted WT mast cells increased by eightfold after FceRI stimulation with maximal phosphorylation at 3 min. Depletion of cholesterol significantly prolonged
LAT Y195 phosphorylation for both WT and DHCR KO mast cells with peak responses reaching sixfold that of non-stimulated WT cells. Interestingly, DHCR KO mast cells showed a delay in maximal Y195 phosphorylation, demonstrating that the accumulation of DHC altered the kinetics. The sustained LAT phosphorylation suggested that PLCγ phosphorylation was unaltered, and no substantial differences were observed in the phosphorylation of PLCγ1 or PLCγ2 (not depicted). This was consistent with the small differences in the calcium response of nondepleted and cholesterol-depleted cells when normalized to the actual calcium concentration of each cell type after Fura 2 loading (Fig. 5 E). Thus, the findings demonstrate that the delocalization of a considerable fraction of Lyn protein after cholesterol depletion of DHCR KO mast cells reduced FcεRI phosphorylation, delayed Syk activation, and delayed LAT phosphorylation. However, the extent of LAT phosphorylation did not differ substantially from that of WT cells, consistent with no detectable alteration in PLCγ phosphorylation and calcium responses.

**Figure 5. Reduced Lyn kinase activity and altered Syk, LAT, and FcεRI phosphorylation, but not calcium mobilization, in cholesterol-depleted WT and DHCR KO mast cells.** In vitro kinase activity of immunoprecipitated Lyn (autophosphorylation) isolated from total cell lysates (A) or from the lipid raft fractions (B). For total and lipid raft–localized Lyn, activity is normalized to that activity in nondepleted WT cells. One representative of four individual experiments is shown. (C) Cholesterol-depleted WT and DHCR KO and nondepleted WT mast cells were activated with 100 ng Ag for the indicated time. IgE–FcεRI complexes were immunoprecipitated from the postnuclear supernatant using antibodies specific to IgE. Blots were probed with anti-phosphotyrosine–specific antibody 4G10 and anti-γ antibody. Fold increase is shown as a graph and was determined from three individual experiments. (D) Phosphorylation of Syk (Y346) and LAT (Y195) in cholesterol-depleted WT and DHCR KO mast cells as well as in nondepleted WT cells as detected with phosphospecific antibody. Fold induction reflects the increase in phosphoprotein/protein normalized to nonstimulated WT cells (1.0) in all panels. LAT (Y195) phosphorylation in cholesterol-depleted WT and DHCR KO mast cells as well as in nondepleted WT cells was detected with phosphospecific antibody. (E) Cholesterol-depleted WT and DHCR KO mast cells as well as nondepleted WT cells were loaded with Fura 2, and calcium mobilization was measured by fluorescence spectroscopy. Calcium mobilization is shown as the intracellular calcium concentration over time. Arrow indicates the addition of 20 ng Ag.

**Cholesterol depletion enhances Fyn-mediated signaling; similarities with Lyn deficiency in mast cells**

We and others previously demonstrated that the absence of Lyn in mast cells resulted in increased Fyn kinase activity and degranulation (20, 21). Failure to phosphorylate the Csk-binding protein (Cbp/PAG) and recruit the negative regulatory kinase, COOH-terminal Src kinase (Csk), was demonstrated as contributory to increased Fyn kinase activity (20). Because Cbp is present in lipid rafts, we speculated that Cbp phosphorylation might be impaired in cholesterol-depleted WT and DHCR KO mast cells. Phosphotyrosine analysis of immunoprecipitated Cbp demonstrated that cholesterol depletion of WT and DHCR KO mast cells resulted in decreased phosphorylation (Fig. 6 A). For cholesterol-depleted DHCR KO mast cells, the extent of phosphorylation was more appreciably reduced (greater than twofold difference relative to cholesterol-depleted WT cells). The activity of Fyn was increased three- to fivefold in cholesterol-depleted WT and DHCR KO mast cells relative to the nondepleted WT mast cells (Fig. 6 B).
Our previous studies established that Fyn kinase is able to regulate the phosphorylation of Gab2 (22), an adaptor molecule demonstrated by others to directly modulate the activity of phosphatidylinositol 3-OH kinase (PI3K) and found to be essential for mast cell degranulation (23). To determine whether enhanced PI3K activity played a role in the enhanced degranulation phenotype observed upon cholesterol depletion, we treated cholesterol-depleted DHCR KO and WT cells with the PI3K-specific inhibitor, LY 294002. Cholesterol depletion of DHCR KO and WT mast cells caused increased resistance to this inhibitor (Fig. 6 C). At the suboptimal concentration of 10 μM, the degranulation of nondepleted WT mast cells was inhibited by 15.2 ± 2.1%, whereas degranulation of cholesterol-depleted WT and DHCR KO mast cells was inhibited by 5.1 ± 2.1% and 5.9 ± 1.8% (P < 0.001, n = 3), respectively. To further verify increased PI3K activity, we measured the phosphorylation of Akt, a kinase whose activity is dependent on PI3K-generated PIP3. Phosphorylation of S473 of Akt in FceRI-stimulated cholesterol-depleted DHCR KO cells was increased almost threefold compared with nondepleted WT and twofold relative to cholesterol-depleted WT mast cells (Fig. 6 D). Importantly, cholesterol depletion led to a two-fold increase in constitutive phosphorylation of Akt in DHCR KO mast cells. This increase in Akt phosphorylation was consistent with our prior findings of increased constitutive S473 phosphorylation in Lyn-null bone marrow–derived mast cells (22).

**DISCUSSION**

Herein, we describe a genetic model in which mutation of the 3β-hydroxysterol Δ7-reductase gene caused reduction of cholesterol in the absence of an exogenous agent. This SLOS model provides the first in vivo evidence in a genetic model that DHC can partition to lipid raft domains and cause lipid raft dysfunction. Previous findings, using artificial membranes or pharmacological inhibitors of 3β-hydroxysterol Δ7-reductase, demonstrated that DHC is able to incorporate into lipid rafts and partitions more effectively than cholesterol (24). We now find that DHC is able to maintain the structural integrity of lipid rafts, albeit with clear differences in domain stability and cell signaling. In contrast, desmosterol (the sterol accumulating in cholesterol-depleted WT cells) partitions poorly to lipid rafts but has been demonstrated to partly alter cell signaling (25). However, accumulation of desmosterol in cells was more variable and together with the reduced levels of cholesterol in WT cells (at 72 h of depletion) could provide an explanation for the milder and more variable phenotype (degranulation, cytokine production, lipid raft instability, and Lyn displacement) of the cholesterol-depleted WT mast cells. Regardless, the reversal of the hyper-degranulation in cholesterol-depleted WT cells by repletion of cholesterol demonstrated that this sterol principally accounts for the alteration of degranulation observed for WT cells. This is consistent with the prior finding that acute lowering of cholesterol enhances mast cell degranulation (26). The implication is that altered localization of signaling proteins, as a consequence of
either cholesterol deficiency and/or DHC accumulation, contributes to the SLOS phenotype.

Several studies promote the view that cholesterol is key in compartmentalizing signaling during growth, differentiation, and function. In the immune system, tolerance-sensitive transitional immature B cells with low levels of cholesterol showed impaired localization of the B cell Ag receptor in lipid rafts after engagement of this receptor, as well as altered signaling (27). In embryonic development, Hedgehog signaling requires cholesterol for its appropriate function. Sonic, Indian, and Desert Hedgehogs, which are involved in limb and midline pattern formation, bone mineralization, and genital development, respectively, are covalently modified by the addition of cholesterol to their amino-terminal signaling domain, which is required for their appropriate function (28, 29). The work of Drs. Baird and Holowka has provided ample evidence of a key role for cholesterol-enriched lipid rafts in FcεRI phosphorylation and mast cell activation. The evidence is multifaceted: (a) the fraction of FcεRI found in cholesterol-rich membranes is rapidly increased upon receptor engagement, and these regions also contain a large fraction of Lyn kinase (4, 14); (b) the active fraction of Lyn kinase is primarily found in cholesterol-rich membranes, and this environment protects included proteins from dephosphorylation by phosphatases (30); (c) a large proportion of phosphorylated FcεRI is also found within these domains relative to that outside of these regions (5); and (d) depletion of cholesterol by pharmacological agents caused reduced phosphorylation of FcεRI (5). However, considerable evidence to the contrary also exists. Collectively, this apparently contradictory evidence suggests that the presence of FcεRI or Lyn in lipid rafts is not essential for FcεRI phosphorylation (10, 15, 31, 32). We now demonstrate that phosphorylation of FcεRI is altered by cholesterol depletion, arguing in favor of a role for lipid raft–localized Lyn in this event. However, the kinetics of FcεRI phosphorylation were unaltered, suggesting that Lyn outside of lipid rafts may also contribute to receptor phosphorylation. This latter view is also supported by the fact that with 80% of Lyn activity lost from lipid rafts of cholesterol-depleted DHCR KO mast cells, the inhibition of receptor phosphorylation was at best 40%. Recent studies also demonstrate that mutation of the FcεRIβ ITAM, which ablates the interaction of Lyn with receptor, inhibited the initiation of FcεRI phosphorylation (33, 34), arguing that protein–protein interaction of Lyn with FcεRI is required for either initiation or sustaining phosphorylation. Thus, a cohesive model is one in which protein interaction of FcεRI with Lyn is important for the initiation of receptor phosphorylation, whereas the cholesterol-enriched environment, which includes a significant fraction of Lyn, may function to amplify this interaction and sustain receptor phosphorylation. This model incorporates the protective phosphatase-deficient environment of lipid raft domains (30) as important in maintaining the phosphorylated state of the receptor.

The work described herein also argues that Lyn kinase has functions outside as well as inside lipid raft domains. For example, one might attribute Cbp phosphorylation as a lipid raft localized function of Lyn because Cbp is lipid raft localized and Lyn-null mast cells were equally defective in Cbp phosphorylation (20). The phosphorylation of FcεRI and Syk, however, is seemingly a function of Lyn both inside and outside of lipid rafts. Syk activation, which depends on FcεRI phosphorylation, peaked at ~50–60% of that in nondepleted WT cells (as measured by phosphorylation) but still caused sustained LAT phosphorylation and thus normal PLCγ phosphorylation and calcium responses. In contrast, Lyn-null mast cells showed a marked defect in calcium responses (22, 35, 36), which is consistent with a more appreciable defect in Syk and LAT phosphorylation.

Although the phenotype of cholesterol depletion in WT and DHCR KO mast cells is not entirely consistent with Lyn-null mast cells, significant similarities exist. Increased activity of Fyn in DHCR KO cells, relative to cholesterol-depleted WT cells, was consistent with Lyn-null mast cells. Loss of Cbp phosphorylation was demonstrated to cause increased Fyn activity in Lyn-null bone marrow–derived mast cell (20). Now we find that cholesterol-depleted DHCR KO mast cells showed a pronounced loss of Cbp phosphorylation relative to that seen in cholesterol-depleted or nondepleted WT mast cells. This would indicate that the DHC-mediated maintenance of a hyperresponsive mast cell is, at least in part, dependent on the loss of Cbp phosphorylation causing increased Fyn kinase activity. It is notable that Fyn kinase activity was also increased in cholesterol-depleted WT mast cells in conjunction with a reduction in Cbp phosphorylation. The increased Fyn activity and constitutive activation of Akt in both cholesterol-depleted WT and DHCR KO mast cells are also consistent with the constitutive secretion of cytokines. Our recent study (37) demonstrates that Fyn is required for cytokine production and deficiency in Fyn kinase results in reduced Akt phosphorylation and nuclear NFκB activity, an activity required for both IL-6 and TNF production. Although the constitutive activity of Fyn upon cholesterol depletion of WT and DHCR KO mast cells was modest (WT/WT-/DHCR KO− = 1.0±1.2 ± 0.04±1.5 ± 0.06; P < 0.05, n = 3), it was significant and sustained. Thus, the constitutive signaling in the absence of cholesterol could drive the cytokine production observed and contribute to the sensitivity of SLOS patients to allergic inflammation.

Clinical treatment of SLOS patients includes placing these patients on a cholesterol-rich diet, which allows better management of the multiple aberrant behavioral characteristics and enhances patient quality of life, but does little to alter the fixed developmental abnormalities (38). Reports of allergies for these patients are for the most part anecdotal, but an increase in diet cholesterol allows better food tolerance and may decrease the frequency of other allergic-like and inflammatory manifestations. Unlike for WT mast cells, cholesterol depletion of DHCR KO mast cells did not completely reverse the hyper-degranulation. This suggests that SLOS patients possess and maintain (even in the presence of a cholesterol-rich diet) lipid raft dysfunctions and increased mast cell
sensitivity relative to normal individuals. This is consistent with the continued accumulation of DHC in patients on a cholesterol-rich diet. Thus, one might speculate that a weak allergic stimulus may be sufficient to elicit unwanted mast cell responses in SLOS. This does not appear to be mediated through increased total IgE production, as our initial screening of patient serum did not reveal abnormal levels (not depicted). Whether increased allergen-specific IgE occurs in these patients remains to be determined. Regardless, the findings point to a dysregulation of lipid raft function and cell signaling as a possible underlying cause of some of the developmental and neurological problems in SLOS. The observation of lipid raft dysregulation due to DHC strongly supports the use of a therapeutic strategy designed to lower DHC levels in addition to cholesterol supplementation.

MATERIALS AND METHODS

Antibodies and reagents. mAbs to Syk (clone 4D10), phosphotyrosine (clone 4G10), and polyclonal anti-pY191 LAT were from Upstate Biotechnology. Rabbit polyclonal antibody to Lyn, Fyn, and goat anti-Cbp (P-18) were from Santa Cruz Biotechnology, Inc. Phosphospecific antibodies to Syk, Akt, and ERK, as well as mAbs Act and ERK were from Cell Signaling Technology. Rabbit polyclonal anti-Cbp and mouse mAbs anti-LAT were provided by T. Yamashita (Hokkaido University, Sapporo, Japan) and P. Draber (Institute of Molecular Genetics, Prague, Czech Republic), respectively. Chicken anti-Fyn antibody was prepared by Aves Labs, Inc. using peptide sequence CKDKEAALKLTEDGLNQSQ from the unique domain of Fyn. FITC-conjugated monoclonal rat anti-mouse IgE and FITC-conjugated rat IgG1 monoclonal Ig isotype standard (both used in flow cytometry) were from BD Biosciences. Cy5.5-conjugated anti–chicken, avidin, and anti–rabbit antibody, as well as IRDye800-conjugated anti–mouse were from Rockland, Inc. Mouse DNP-specific IgE was produced as described previously (39). Cell culture media (EMEM, DMEM, and RPMI 1640) was prepared as described previously (22, 40). β-octyl-glucoside was from Pierce Chemical Co., [32P]-ATP was from AP Biomedical, and FURA 2 was from Invitrogen. Other described reagents were from Sigma-Aldrich.

Cell culture, cell stimulation, degranulation, and cytokine assays. Cell cultures were maintained from liver progenitors. Cells from the livers of newborn mice were isolated using a cell strainer (40). Mast cells were cultured from liver progenitors. Cells from the livers of 2-wk-old newborn mice were isolated using a cell strainer (40)Mast cells were cultured from liver progenitors. Cells from the livers of 2-wk-old newborn mice were isolated using a cell strainer (40) and cultured as described previously (37). Polyclonal anti-CD115/c-Kit and mAbs anti-LAT were provided by P. Draber (Institute of Molecular Genetics, Prague, Czech Republic), respectively. Chicken anti-Fyn antibody was prepared by Aves Labs, Inc. using peptide sequence CKDKEAALKLTEDGLNQSQ from the unique domain of Fyn. FITC-conjugated monoclonal rat anti-mouse IgE and FITC-conjugated rat IgG1 monoclonal Ig isotype standard (both used in flow cytometry) were from BD Biosciences. Cy5.5-conjugated anti–chicken, avidin, and anti–rabbit antibody, as well as IRDye800-conjugated anti–mouse were from Rockland, Inc. Mouse DNP-specific IgE was produced as described previously (39). Cell culture media (EMEM, DMEM, and RPMI 1640) was prepared as described previously (22, 40). β-octyl-glucoside was from Pierce Chemical Co., [32P]-ATP was from AP Biomedical, and FURA 2 was from Invitrogen. Other described reagents were from Sigma-Aldrich.

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Protein loading was determined by probing the membranes with appropriate antibodies, and quantification was performed by densitometry.

For calcium measurements, 2 × 10^6 cells were loaded with IgE (1 μg/10^5 cells) and 2 μM FURA-2/AM (Invitrogen) in RPMI 2% FBS media for 45 min at 37°C. Cells were washed twice in Tyrodes-BSA, and changes in dye fluorescence with time were determined by Fluostar Optima spectrometer (BMG Labtechnologies) with excitation wavelengths of 340 and 380 nm and with a constant emission at 510 nm. Calcium concentrations were calculated as described previously (45).

Online supplemental material. Fig. S1, which shows the kinetics of affinity receptor phosphorylation, was available at http://www.jem.org/cgi/content/full/jem.20051701/DC1.

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