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## Roles of Infection, Inflammation, and the Immune System in Cholesterol Gallstone Formation

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### Abstract

Cholesterol gallstone formation is a complex process mediated by genetic and environmental factors. Until recently, the role of the immune system in the pathogenesis of cholesterol gallstones was not considered a valid topic of research interest. This review collates and interprets an extensive body of basic literature, some of which is not customarily considered to be related to cholelithogenesis, describing the multiple facets of the immune system that appear to be involved in cholesterol cholelithogenesis. A thorough understanding of the immune interactions with biliary lipids and cholecystocytes should modify current views of the pathogenesis of cholesterol gallstones, promote further research on the pathways involved, and lead to novel diagnostic tools, treatments, and preventive measures.

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Bile is a complex aqueous colloidal system that is essential for a wide range of physiologic functions, including the excretion of lipids from the organism and intestinal fat absorption.<sup>1</sup> Bile is formed primarily in hepatic canaliculi, small (1–2  $\mu\text{m}$ ) spaces formed between the tight junctions of hepatocytes. It is composed of water, electrolytes, and a variety of lipid solutes dispersed in mixed micelles and vesicles including bile salts, phospholipids (>96% being mixed phosphatidylcholines), and cholesterol, as well as proteins and bilirubin conjugates.<sup>3</sup> Phospholipids and bile salts are essential for the removal from the organism of otherwise insoluble cholesterol molecules in an aqueous environment by solubilizing the sterol in mixed micelles, composed of the catabolic product bile salts, and unilamellar vesicles.<sup>2–4</sup> Bile is transported from the canaliculi along tubules of increasingly greater diameter until it egresses into the gut at the midduodenum. In health, about half the secreted bile is stored, concentrated, and slightly acidified in the gallbladder during the interdigestive interval. The gallbladder is connected to the biliary tree via the cystic duct, which functions simultaneously as a gallbladder filling and emptying conduit.<sup>3</sup>

Alterations in the relative or absolute proportions of cholesterol, phospholipids, and bile salts can lead to phase separation of cholesterol from solution in bile. Most frequently these changes

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result from excess secretion of cholesterol from the liver.<sup>1-3</sup> As the absolute cholesterol concentration increases, the excess cholesterol phase separates, forming unilamellar vesicles with biliary phospholipids (Figure 1). Under suitable physicochemical conditions, these can aggregate to form multilamellar vesicles (lamellar liquid crystals), and eventually cholesterol monohydrate crystals can separate from these and aggregate in the gallbladder.<sup>2,4</sup> These crystals can progress to form cholesterol gallstones by agglomeration within a gallbladder-secreted mucin gel. Formation of cholesterol gallstones invariably occurs adjacent to the gallbladder wall, where cholesterol monohydrate crystals nucleate in the gelled mucin glycoprotein scaffolding.<sup>2,4-6</sup> The majority of cholesterol gallstones form in the gallbladder; however, in rare situations especially and perhaps uniquely from genetic deficiency of biliary phospholipid secretion,<sup>7</sup> the stones can form anywhere along the biliary tree, even intrahepatically.<sup>1-3</sup> Although a relative excess of cholesterol is necessary for formation of cholesterol gallstones, it is not sufficient. Biliary sludge, composed of agglomerated cholesterol crystals of an ultrasonically detectable size ( $\sim 50 \mu\text{m}$ ) suspended in mucin gel, is frequently observed and can progress to form cholesterol gallstones.<sup>8-10</sup> Lee et al found that in 18% of patients studied by ultrasonography for a mean of more than 3 years, biliary sludge disappeared completely; in 60% of the patients, the biliary sludge disappeared and reappeared.<sup>10</sup>

The study of cholesterol gallstones relies on multiple methodologies, including physical-chemical studies of model bile and ex vivo bile systems, biliary lipid secretory studies, animal models with particular focus on cholesterol gallstone (*Lith*) genes, and epidemiologic studies analyzing susceptible and resistant human populations.<sup>2,11-14</sup> These studies provide the basis for our understanding of the epidemiology and pathogenesis of cholesterol gallstones. Formation of cholesterol gallstones is principally a polygenic trait with significant contributions from the environment.<sup>3,15</sup> Although these studies provide a strong basis for our understanding of the etiology and pathogenesis of cholesterol gallstones, many issues remain unresolved. For example, why do so few individuals with phase-separated and aggregated cholesterol monohydrate crystals (such as the biliary sludge that is frequently observed in pregnant women) proceed to gallstones?<sup>8-10</sup> Additionally, why do genetically identical mouse models challenged with the same lithogenic diet but housed at different research institutions display such marked differences in their proclivity to form cholesterol gallstones?<sup>16</sup>

We proposed that the differences in the prevalence of gallstones in genetically identical mice might arise from differences in their colonization status with gastrointestinal microbes. Initially, we analyzed the ability of enterohepatic *Helicobacter* spp to contribute to lithogenicity and identified several enterohepatic *Helicobacter* spp that contributed to cholesterol gallstone nucleation and other *Helicobacter* strains that did not.<sup>16,17</sup> The host immune system might be the primary mechanism whereby these organisms (and perhaps others) promote cholesterol gallstone formation as well as mucin gel and gallbladder inflammation. Subsequent studies showed that the response of the adaptive immune system was important, and probably essential, because Rag-deficient mice, which lack mature T and B cells, rarely (<10%) develop cholesterol gallstones.<sup>18</sup> A number of previous studies showed invariably that gallbladder inflammation occurred concomitantly with cholesterol supersaturation and gallstone formation; none, however, conclusively showed that this process was a primary contributing factor rather than a secondary effect of the lithogenic process.<sup>19-23</sup>

This review focuses on the immune aspects of the pathogenesis of cholesterol gallstones and examines the role of infection, inflammation, and the response of the immune system during the formation of cholesterol gallstones. There is an extensive body of immunity literature related to cholesterol gallstones as well as a number of studies involving mouse models of infection, inflammation, and immunity. This review describes the critical contributions of the gallbladder epithelium and the immune system to modulation of cholesterol gallstone

formation and progression, rather than the nonimmunologic factors (hypersecretion of cholesterol, alterations in cholesterol to bile salt and phospholipid ratios and concentrations, and so on) that are pivotal factors in the pathogenesis of cholesterol gallstones but have been reviewed elsewhere.<sup>3,24</sup> Most of the immunologic factors described in this review occur in conjunction with the fundamental proinflammatory alterations in liver and bile; without these alterations, inflammation would not be sufficient to induce cholesterol supersaturation of bile and gallstone formation. The causes of biliary tree inflammation, as they relate to cholesterol gallstone disease, are far from being fully elucidated; nonetheless, the literature indicates that some noninfective inflammation occurs secondary to physical-chemical alterations of bile (cholesterol supersaturation and early stages in the nucleation and phase-separation sequences; Figure 1), whereas others occur independently of biliary lipid composition.

## Biliary Epithelium and Immunity

Anatomically, the biliary tree is often divided into intrahepatic and extrahepatic portions that include the gallbladder epithelium. However, with respect to biliary tissue and disease, this distinction appears artificial when describing the interactions of the biliary epithelium with the immune system; it appears that biliary epithelial cells from either location have similar responses to immunogenic stimuli.<sup>25,26</sup>

Biliary epithelial cells participate in both innate and adaptive immunity.<sup>27–29</sup> Briefly, the innate immune system induces no immunologic memory to an antigen but responds instead to a variety of evolutionarily conserved patterns present in foreign antigens. In addition to specific cellular subsets that include neutrophils, macrophages, eosinophils, basophils, mast cells, and natural killer cells, a variety of proteins also participate in innate immunity, including the complement cascade, cytokines, and proteins of the acute phase response.<sup>30–32</sup> In contrast, the adaptive immune response induces “memory” to foreign antigens and is mediated by both B and T cells. Following activation, the adaptive immune response can also stimulate the production of a variety of cytokines and the recruitment of inflammatory cells.<sup>33–36</sup> There is overlap in these 2 pathways; perturbation of either can alter the ability of the other to respond appropriately to a stimulus.<sup>36</sup>

Biliary epithelial cells express all known toll-like receptors (TLR), which mediate antigen-pattern recognition—a key response of the innate immune system.<sup>28,37–39</sup> Additionally, myeloid differentiation protein 88 (MyD88), which is an important downstream effector of the TLR pathway, is also present in these cells.<sup>28</sup> Biliary epithelial cells that express dominant-negative forms of MyD88 have increased susceptibility to infection with the intracellular biliary pathogen *Cryptosporidium parvum*, indicating that the TLR/MyD88 system functions in biliary epithelial cells.<sup>39</sup> Additionally, *C parvum* infection up-regulates  $\beta$ -defensins in infected wild-type cells, but  $\beta$ -defensin production is ablated in cells with reduced levels of TLR2, TLR4, or nuclear factor  $\kappa$ B, indicating an essential role for defensins in protection of cholangiocytes and, by implication, bile itself from infection.<sup>38</sup>

The relationship between biliary epithelial cells and the adaptive immune response requires a variety of receptor-ligand interactions that occur between epithelial cells, T cells, and “professional” antigen-presenting cells such as dendritic cells. Human biliary epithelium cell lines constitutively express HLA class I (major histocompatibility complex class I) antigens but only low levels of class II antigens (Figure 2).<sup>25,26</sup> However, after stimulation with proinflammatory cytokines, the cells express a variety of HLA class II antigens, including HLA-DR, HLA-DP, and HLA-DQ (Figure 2).<sup>25</sup> Essentially, this expression profile shows that biliary epithelial cells are likely to interact with both CD8<sup>+</sup> T cells (cytotoxic T cells) and CD4<sup>+</sup> T cells (helper T cells). In addition to these primary immune receptors, biliary epithelial cells also possess a variety of costimulatory molecules necessary for functional interaction with

these immune cells, including CD40, intercellular adhesion molecule 1, lymphocyte function-associated antigen 3, and FAS (Figure 2).<sup>29,40–45</sup> These protein expression profiles indicate that the biliary epithelium actively participates in adaptive immunity through interaction with T cells and local antigen-presenting cells.

The second aspect of adaptive immunity involves the B-cell response, mediated through immunoglobulins (Igs). All Ig classes, including IgA, IgG, and IgM, are present in low concentrations in bile.<sup>29</sup> However, biliary epithelial cells have only minimal participation in Ig secretion. In humans, IgA is synthesized by plasma cells that line the bile ducts and then is bound to the polymeric Ig receptor at the basolateral surface of cholangiocytes.<sup>46–48</sup> IgA is then transcytosed to the apical surface of the cells and secreted into bile. This intracellular transport system has an important role in protecting the biliary tree from intracellular pathogens by binding these antigens in transit.<sup>49</sup> Similar to IgA secretion, IgM is transported via the polymeric Ig receptor, but in contrast, IgG is secreted using the FcRn.<sup>49–51</sup> Igs that reach intraluminal bile aid in protecting bile from colonization with gastrointestinal pathogens.<sup>52,53</sup>

Aside from expressing the necessary receptors and downstream activators for participation in the immune response, biliary epithelial cells also secrete a variety of cytokines and undergo phenotypic changes in response to these inflammatory mediators. Cultured murine cholecystocytes (gallbladder epithelial cells) express *Tnf- $\alpha$* , *Ccl5* (RANTES), and *Mip-2* (CXCL2) (Figure 2).<sup>54</sup> Following treatment with lipopolysaccharide (LPS), these cells increase expression of *Mip-2* and *Tnf- $\alpha$* . Additionally, expression of *Il-1 $\beta$* , *Il-6*, and *Mcp1* is induced following exposure to LPS (Figure 2).<sup>54</sup> Interestingly IL-10, the Th2 and T-regulatory cytokine, is not expressed in resting or LPS-stimulated gallbladder epithelium.<sup>54</sup> It has been established that the biliary epithelium possess receptors for some of these cytokines and chemokines (including tumor necrosis factor [TNF]- $\alpha$ ), indicating that these secreted cytokines can act in both an autocrine and a paracrine manner (Figure 2).<sup>29,55</sup> The addition of exogenous cytokines to cultured biliary epithelial cells induces phenotypic changes in these cells, supporting an autocrine role for some of these cytokines (Figure 2). For example, the addition of TNF- $\alpha$ , LPS, interleukin (IL)-1 $\alpha$ , and prostaglandin E<sub>2</sub> to cultured human cholecystocytes alters the transport of sodium and chloride and diminishes the absorptive function of the gallbladder epithelium.<sup>56</sup> Studies in guinea pigs (*Cavia porcellus*) with ligated bile ducts support these in vitro findings.<sup>57</sup> Specifically, the instillation of IL-1 or LPS into the gallbladder induces gallbladder wall inflammation and stimulates the release of myeloperoxidase, prostaglandin E<sub>2</sub>, and water into the gallbladder lumen.<sup>57</sup> Systemic administration of cytokines during chemotherapeutic treatment is also associated with changes in the biliary epithelium. For example, IL-2 administration to humans causes acalculous cholecystitis.<sup>58–60</sup> The administration of transforming growth factor (TGF)- $\beta$  to cultured rabbit (*Oryctolagus cuniculi*) gallbladder epithelium induces a mesenchymal, fibrotic phenotype.<sup>61</sup> This in vitro phenomenon appears to have an in vivo correlate because TGF- $\beta$  expression is associated with fibrosis and inflammation during formation of cholesterol gallstones in humans.<sup>62</sup> Finally, histamines released during mast cell degranulation induce gallbladder smooth muscle contraction.<sup>63</sup>

There are several disease states associated with immune-mediated destruction of the biliary epithelium: primary biliary cirrhosis, primary sclerosing cholangitis, and graft-versus-host disease.<sup>40,42,64–67</sup> The biliary epithelium clearly participates in cellular and humoral immunity through antigen presentation, cytokine and chemokine production, and Ig transport. Moreover, biliary epithelial cells possess all of the necessary cellular components required to participate in the innate immune system; perturbations of innate immunity appear to predispose to biliary infection.<sup>28,37–39</sup>

## Role of Infection, Inflammation, and the Immune System in Formation of Cholesterol Gallstones

### Igs

In vitro studies with model bile created with various concentrations of purified lipids have shown that biliary Igs, particularly IgM and IgG, promote the nucleation of solid cholesterol crystals.<sup>68,69</sup> Interestingly, the source of the Igs appears to influence the ability of these proteins to nucleate solid cholesterol crystals from bile. IgM and IgA from commercial sources are incapable of nucleating supersaturated bile, whereas commercially available IgG has pronucleating activity.<sup>68,70</sup> The anatomic source of the Igs also appears to influence the ability of these molecules to cause cholesterol crystal phase separation. Specifically, biliary Igs seem to promote solid cholesterol nucleation more than Igs isolated from blood.<sup>70</sup> Of the biliary Igs, IgM most potently nucleates supersaturated bile, whereas IgG has slightly less facility and IgA has very little activity.<sup>68–70</sup>

Prospective studies utilizing animal models generally do not support a role for Igs in the pathogenesis of cholesterol gallstones. AKR or C57L mice fed a lithogenic diet undergo an initial decrease, followed by a temporary increase, in biliary IgM and IgA levels.<sup>22</sup> In both strains of mice, the total amount of IgG remained relatively unchanged. Van Erpecum et al concluded that Igs are not likely to participate in formation of cholesterol gallstones because both gallstone-resistant (AKR) and gallstone-susceptible (C57L) mice displayed alterations in their Ig levels when placed on lithogenic diets.<sup>22</sup> Actually, gallstone-resistant AKR mice had higher concentrations of biliary Igs than the gallstone-susceptible C57L mice.<sup>22</sup> Although these changes appeared to negate any important role that Igs had in formation of cholesterol gallstones, data interpretation was complicated by pronounced differences in cholesterol saturation index (a quantifiable measurement of the relative amount of cholesterol in bile) values between C57L mice (~2.0) and AKR mice (~1.5).<sup>71</sup> Therefore, the variable contributions of differing cholesterol saturation indexes of biliary lipids most likely confounded these results. Moreover, results of human studies indicated that Igs did not contribute to formation of cholesterol gallstones; in ex vivo bile samples from individuals with or without gallstones, there was no correlation between biliary Igs and cholesterol crystal detection times.<sup>72</sup>

One limitation of these studies is that the investigators focused primarily on the effect of Ig concentrations on solid cholesterol monohydrate crystal nucleation. Although Ig concentrations could be important, it is likely that other factors are relevant to this physical-chemical interaction. For example, the antigen specificity of the Igs may be crucial. Indeed, Fab fragments isolated from patients with cholesterol gallstones accelerated cholesterol crystal observation times in vitro, whereas commercially available Fab fragments did not.<sup>73</sup> These data indicate that antigen-specific Fab fragments might contribute to cholesterol nucleation in vitro.<sup>73</sup> Therefore, merely quantifying Ig concentrations in bile appears to provide limited functional information.

Recent studies have shown that mice that have only T cells (that lack B cells and Ig) develop gallstones at a greater frequency than wild-type mice. In contrast, mice possessing only B cells do not recapitulate the prevalence of gallstones observed in wild-type mice.<sup>18</sup>

### Mucin Glycoproteins

Biliary mucin glycoproteins are the most thoroughly analyzed biliary proteins involved in the pathogenesis of cholesterol gallstones. Mucin genes that are expressed in human biliary epithelia include *MUC1*, *MUC2*, *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, and *MUC6*.<sup>74</sup> Mucin gel accumulation appears to be important for formation of cholesterol gallstones in humans and

in animal models.<sup>5,75–79</sup> Moreover, these proteins promote solid cholesterol-phase nucleation in vitro.<sup>76,80–83</sup> In vitro and in vivo data suggest that mucin gel accumulation precedes and promotes formation of cholesterol gallstones by nucleating cholesterol crystals from (liquid crystal) phase-separated bile.

In cultured cholangiocytes, many mucin genes are regulated by inflammatory mediators. Studies in 2 different cell culture systems have shown that mucin gel accumulation and mucin gene expression increased following exogenous addition of LPS and TNF- $\alpha$ . In cultured cholangiocytes, expression of *Muc2* and *Muc5ac* increased following exposure to LPS or TNF- $\alpha$  (Figures 3 and 4).<sup>84</sup> Utilizing the same cell culture system, the investigators showed that TNF- $\alpha$  stimulated protein kinase C, which appeared to signal increased production of *Muc2* and *Muc5ac* (Figures 3 and 4).<sup>84</sup> In the case of *Muc3*, its production was increased by LPS, but not TNF- $\alpha$ , possibly due to an interaction of these molecules with CD14 (Figure 5).<sup>84</sup> In another study, LPS and other pathogen-associated molecular patterns were found to interact with TLR2 or TLR4 on the surface of biliary epithelium.<sup>85</sup> This interaction induced expression of *Cdx2*, a gene whose product is associated with intestinal metaplasia of epithelial cells; CDX2 in turn induced expression of *Muc2* (Figure 3).<sup>85</sup> Furthermore, MUC2 protein expression is increased in the gallbladders of humans with cholesterol gallstones.<sup>86</sup>

Choi et al<sup>87</sup> obtained similar results utilizing cultured canine (*Canis familiaris*) gallbladder epithelial cells. The addition of LPS from any of 3 bacterial species stimulated mucin production in cultured dog cholecystocytes. There appeared to be differences in the ability of LPS from different bacterial species to induce mucin secretion. For example, LPS from *Escherichia coli* produced the greatest stimulation, whereas LPS from *Pseudomonas aeruginosa* stimulated the least mucin production. Furthermore, the addition of TNF- $\alpha$  to the culture media promoted mucin production, albeit to a lesser degree than LPS. The diminished response to TNF- $\alpha$  could have been due to the use of human rather than canine TNF- $\alpha$ . The investigators noted that the changes in mucin accumulation were not due to cytotoxicity of either LPS or TNF- $\alpha$ , because cell viability assays demonstrated survival of the cultured cells.<sup>87</sup>

Recent studies have shown that TNF- $\alpha$  regulates MUC5AC expression via an epidermal growth factor (EGF)-mediated pathway (Figure 4).<sup>88</sup> Stimulation of gallbladder epithelium with TNF- $\alpha$  induced production of the EGF receptor (EGFR). EGF then binds to the EGFR and this interaction induces expression of *Muc5ac*, presumably through a second messenger protein<sup>88</sup>; inhibition of the EGF-EGFR pathway prevented production of MUC5AC.<sup>88</sup>

Findings from these in vitro and in vivo studies indicate that production and secretion of the mucin glycoproteins are influenced by inflammatory mediators. TNF- $\alpha$ , pathogen-associated molecular patterns, and their second messengers are likely to be only “the tip of the iceberg” with regard to inflammatory mediators that stimulate mucin production by the gallbladder. Perhaps a more important question to ask is not whether inflammatory mediators alter mucin production, but rather under what pathophysiological situations do inflammatory mediators exert their effects on the biliary epithelium?

### Other Putative Pronucleating Biliary Proteins

Many other proteins nucleate cholesterol monohydrate crystals in vitro.<sup>72,89,90</sup> Some of these putative pronucleating agents include haptoglobin, phospholipase C,  $\alpha_1$ -acid glycoprotein, albumin, and aminopeptidase N.<sup>72,89,90</sup> In general, in vivo studies that analyzed the role of putative pronucleating proteins have been hampered by a number of factors: (1) large-scale proteomic analysis has only recently been undertaken to systematically analyze the protein complexity of gallbladder bile,<sup>91</sup> (2) prior methods used to identify quantitative differences in these biliary proteins were relatively insensitive and might be inadequate to demonstrate

significant differences in concentration, and (3) concentrations of individual proteins might not be important, but rather a cumulative or cooperative effect of multiple proteins could be required to promote phase separation of crystals or liquid crystals in bile and lead to formation of cholesterol gallstones. Moreover, there is little understanding of the physical-chemical interactions between these proteins. Large-scale, sensitive proteomic screens are becoming easier to perform and more widely used in pathophysiology and translational research; utilization of these techniques will likely increase our understanding of what role, if any, the myriad of secreted biliary proteins may play in formation of cholesterol gallstones.

### Gallbladder Inflammation

In both animal models and humans, formation of cholesterol gallstones is preceded by histopathologic alterations in the gallbladder wall that indicate inflammation. These changes include edema, increased gallbladder wall thickness, the presence of inflammatory cells, and remodeling of the epithelium with concomitant increases in TGF- $\beta$  production.<sup>19–21,62,92,93</sup> These alterations are accompanied often by changes in gallbladder contractility and modifications in the ability of the gallbladder epithelium to transport a variety of substances.<sup>2,21,23,94,95</sup> Furthermore, lithogenic bile can alter mechanisms associated with cytoprotection in the gallbladder muscle, demonstrated by the ability of the gallbladder muscle to respond to reactive oxygen species.<sup>96</sup>

A systematic histopathologic study<sup>23</sup> showed that C57L mice, which are susceptible to gallstones, increase gallbladder wall thickness progressively when fed a lithogenic diet. In contrast, AKR mice, which are resistant to gallstones, display only a mild increase in thickness. Both strains accumulate subepithelial inflammatory cells and edema; however, C57L mice do so to a significantly greater degree. These changes coincide with decreases in the gallbladder expression of the aquaporin genes *Aqp1* and *Aqp8*.<sup>23</sup> These findings suggest that during formation of cholesterol gallstones, the gallbladder undergoes progressive changes that ultimately result in decreased motility, increased edema, and altered transport functions. The farnesoid X receptor (FXR) is a critical transcription factor that alters biliary lipid composition.<sup>92</sup> In *Fxr*<sup>-/-</sup> mice, the gallbladder becomes thickened, edematous, and infiltrated with granulocytes following 1 week of lithogenic diet feeding; these changes were not observed in wild-type mice.<sup>92</sup> Additionally, levels of potentially cytotoxic hydrophobic bile salts, such as deoxycholate conjugates, were significantly increased in *Fxr*<sup>-/-</sup> mice.<sup>92</sup>

It is likely that phase-separated cholesterol crystals and hydrophobic bile salts are responsible, in part, for the histopathologic changes noted in the murine gallbladder during formation of cholesterol gallstones. Ex vivo analysis of gallbladders from patients with cholesterol gallstones revealed smooth muscle cells that contained excess cholesterol in their plasma membranes.<sup>96</sup> Furthermore, the isolated muscle tissues displayed less contractility and decreased levels of scavengers of reactive oxygen species.<sup>96</sup> These changes appeared to be associated with dysfunction of the receptor for prostaglandin E<sub>2</sub>; prostaglandin E<sub>2</sub> binding to its receptor usually has a cytoprotective effect. Others have reported correlations between human gallbladder muscle dysfunction, increases in the cholesterol saturation index of bile, and the histopathologic inflammation score of the gallbladder.<sup>97</sup> Additionally, oxysterols are capable of inducing apoptosis in cultured gallbladder epithelial cells.<sup>98,99</sup> The mechanism that mediates apoptosis appears to involve incorporation of oxysterols into the mitochondria of epithelial cells and subsequent cytochrome *c* activation.<sup>99</sup>

Based on these studies, it is not possible to determine whether the functional and histopathologic changes that occur in the gallbladder are due to cytotoxicity or inflammation; however, studies in the prairie dog model of cholelithogenesis indicated that inflammation is an essential element of cholesterol gallstone formation.<sup>100</sup> Specifically, prairie dogs fed high doses of acetylsalicylic acid (aspirin) failed to form gallstones, whereas 100% of controls

developed gallstones.<sup>100</sup> Because both groups supersaturated their gallbladder biles, this study shows that cholesterol supersaturation is necessary but not sufficient for formation of cholesterol gallstones. Therefore, the proinflammatory effects of solid cholesterol itself, and likely other proinflammatory mediators, have an integral role in cholelithogenesis. However, subsequent studies failed to fully recapitulate these findings; it is difficult to make direct comparisons between these types of studies because they all used different doses of acetylsalicylic acid, dosing regimens, parameters of measured success, and lithogenic diets.<sup>101–105</sup>

Further evidence for the role of the immune system in the pathogenesis of cholesterol gallstones arose from studies with Rag-deficient mice, which do not have mature T or B cells (Rag mice). Both wild-type and Rag-deficient mice exhibited supersaturated gallbladder biles, evidenced by the phase separation of liquid crystals; however, only the wild-type mice had a high prevalence of cholesterol gallstones.<sup>18</sup> Gallstone formation in this case appeared to be due in part to a potent Th1 immune response, which accompanied the formation of solid cholesterol monohydrate crystals in the gallbladder. These data indicate that most likely solid cholesterol crystals promote inflammation via the adaptive immune system, specifically via T-cell function.<sup>18</sup>

Hydrophobic bile salts promote inflammation and apoptosis in vitro and are likely to do so in vivo.<sup>106–108</sup> In contrast, the hydrophilic bile acid ursodeoxycholic acid decreases mucosal inflammation and biliary protein concentrations.<sup>109</sup> In C57L mice infected with *Helicobacter* spp, the relative concentration of hydrophobic bile salts (such as deoxycholate conjugates) increased in hepatic bile whereas that of hydrophilic bile salts (such as  $\alpha$ - and  $\beta$ -muricholate conjugates) decreased compared with uninfected mice.<sup>110</sup> It appears that one downstream effect of hydrophobic bile salts is the induction of proinflammatory cytokines.<sup>108</sup> An increase in hydrophobic bile salts, principally deoxycholate conjugates, is a common finding in patients with cholesterol gallstone disease and in animal models; it is likely that bile salt-mediated inflammation is also important in cholelithogenesis.<sup>111–114</sup>

### Bacterial Colonization of the Biliary Tract and Formation of Cholesterol Gallstones

Bacteria have historically been implicated in the pathogenesis of cholesterol gallstones; however, a definitive cause-and-effect relationship has never been established.<sup>115–117</sup> A major factor contributing to this confusion is the chronic nature of cholecystolithiasis. The formation of cholesterol gallstones requires a long time, often decades. When gallstones do form, they do not disappear spontaneously except by migration into the duodenum, which is invariably followed by reformation.<sup>118–120</sup> Furthermore, after cholesterol gallstones form in a patient, they do not always lead to symptoms; when they do, clinical expression can also take years.<sup>2,4,121</sup> In prospective mouse models, organisms need not colonize the gallbladder to promote formation of cholesterol gallstones.<sup>16</sup> Therefore, the identification of bacteria in bile or gallbladder tissue of patients with cholesterol gallstones does not mean that these organisms caused the gallstones. More likely, the bile, gallbladder mucosa, and biliary motility might have been altered to allow for subsequent bacterial colonization. Furthermore, bacteria that might have been present at the time of initial stone formation could have been cleared and therefore not detected by conventional culture techniques. Another issue is that some bacteria alter the composition of bile directly via  $\beta$ -glucuronidase, cholyl-glycyl hydrolase, phospholipase A1, or urease activities, thereby promoting calcium bilirubinate (brown pigment stone) formation.<sup>122</sup>

Despite these deficiencies, numerous studies have identified bacteria in bile, cholesterol stones, and gallbladder tissue from patients with cholesterol gallstones.<sup>123–127</sup> Some propose that bacteria, through their  $\beta$ -glucuronidase activity, are responsible for nucleation and calcium salt deposition.<sup>128,129</sup> Although this hypothesis is biologically plausible, no studies have shown



conclusively that it occurs. In fact, in vitro studies utilizing *E coli* or calcium salts have failed to induce cholesterol crystal nucleation from supersaturated bile.<sup>130</sup>

Recently, there has been a great deal of interest in a possible connection between the gastric pathogen *Helicobacter pylori* and cholesterol gallstones.<sup>17,131–134</sup> Interestingly, growth of this organism is inhibited by bile salts both in vivo and in vitro, and chemotactic assays show that bile salts actually repel the organism,<sup>17,135,136</sup> findings that contradict the ability of *H pylori* to colonize a healthy gallbladder. Nonetheless, a number of groups claimed to have identified *H pylori* DNA in biliary tissue and gallstones.<sup>131,132,134,137</sup> However, there are several problems with these studies. First, some samples were collected at endoscopic retrograde cholangiopancreatography<sup>134</sup>; because *H pylori* colonizes the gastric mucosa of more than half the world's population, the statistical chances for sample contamination by gastric *H pylori* are high. Additionally, 16S ribosomal RNA genus-specific primers are commonly used to identify these organisms in polymerase chain reaction (PCR) analysis, despite the fact that these primer sets can amplify other non-*H pylori* helicobacters.<sup>16,17</sup> Furthermore, sequence evaluation of these primer products is generally inadequate to speciate *H pylori* properly, because of high homology with non-*H pylori* *Helicobacter* spp.<sup>138</sup> For example, Avenauid et al<sup>139</sup> identified *H pylori*-like organisms based on sequencing of the 16S ribosomal RNA gene from human liver samples; however, rigorous follow-up tests proved that these organisms were an unclassified *Helicobacter* spp.<sup>139</sup> The development of species-specific primers to other regions of the genome may alleviate some of these concerns. Nonetheless, the efficacy of species-specific primers depends on their precise level of specificity. Finally, these organisms could invade the biliary tree following alterations in the biliary microenvironment and especially biliary dysmotility. Supporting this hypothesis is a recent study showing that *H pylori* DNA was present in gallbladder bile of patients with chronic cholecystitis, consistent with obstruction, but not in asymptomatic patients with cholesterol gallstones.<sup>131</sup>

In addition to the gastric pathogen *H pylori*, a variety of enterohepatic helicobacters (eg, *Helicobacter bilis* and *Helicobacter hepaticus*) exist.<sup>140,141</sup> These reside in the small and large intestines and canalicular spaces of the liver of experimentally and naturally infected animals, including humans.<sup>140,141</sup> DNA from these organisms was detected in nonwhite patients with chronic cholecystitis, gallstones, and malignant biliary tract diseases.<sup>142,143</sup> Studies with experimental animal models indicate that these enterohepatic helicobacters promote formation of cholesterol gallstones in vivo.<sup>16</sup> In contrast, in the same experimental model, *H pylori* did not promote formation of cholesterol gallstones.<sup>17</sup> Interestingly, a follow-up study showed that urease-positive *Helicobacter* spp can precipitate calcium salts in vitro.<sup>144</sup> The investigators of this study proposed that this activity might contribute, at least in part, to the ability of these organisms to promote cholesterol gallstones by inducing the formation of a calcium salt nidus, because mono-infection with urease-positive helicobacters or coinfection with at least one urease-positive helicobacter is necessary to induce cholesterol gallstones.<sup>16,144</sup>

Because PCR was used to identify *Helicobacter* spp and other bacteria in many of these studies, data interpretation can be challenging. PCR can detect relatively small quantities of bacterial DNA, a marker for colonization with organisms that might not be identified with standard culture methods. Unfortunately, the presence of bacterial DNA at the time of examination does not mean that it contributed to formation of gallstones. Theoretically, analyzing the core of stones might provide more reliable data on the inciting cause; however, due to the sensitive nature of PCR, the mere act of trying to isolate the core can contaminate it with nucleic acid from the external stone surface or from other sources. Additionally, no studies have analyzed the ability of cholesterol gallstones to absorb DNA.<sup>145</sup> If stones are capable of imbibing DNA, then regardless of the analysis (be it core or external), the DNA detected might represent only the most recent DNA encountered. Furthermore, when multiple DNA fragments are present and nonspecific primers are used (designed to amplify 16S ribosomal RNA genes from multiple

bacteria), there is the possibility that the DNA present at the highest concentration was preferentially amplified.<sup>146</sup> Because PCR amplification is nonlinear, even a small bias in initial amplification can lead to logarithmic differences in the final DNA concentration. Unfortunately, the most prevalent bacteria are not necessarily the most important bacteria or might not be important at all. The use of more discriminatory PCR techniques such as those utilized to analyze complex environmental samples could circumvent this problem in the future.<sup>147,148</sup>

One of the classic bacterial pathogens of the biliary tree in humans is *Salmonella enterica* ser Typhi, which causes typhoid fever.<sup>149,150</sup> This organism crosses the intestinal epithelial barrier, invades macrophages, and spreads systemically.<sup>151</sup> After colonizing the liver, the organism can be shed into the gallbladder and chronically colonize the gallbladder wall (3%–5% of those infected).<sup>149,150</sup> Although chronic colonization with *S typhi* is associated with gallstones and gallbladder cancer,<sup>149,150</sup> it is not clear whether this organism contributes to stone formation or alternatively if the presence of gallstones promotes chronic colonization. Recent evidence indicates that *S typhi* forms bacterial biofilms on the surface of cholesterol gallstones.<sup>151</sup> This biofilm formation would allow for chronic colonization and protect the organism during antibiotic treatment. Biofilm formation is dependent on the presence of bile because organisms cultured without bile do not readily form biofilms.<sup>151</sup> This indicates that this organism is exquisitely adapted to survival in a gallbladder that contains cholesterol gallstones. Furthermore, biofilm formation is reduced when pebbles or glass beads are utilized instead of cholesterol gallstones, indicating that both bile and cholesterol gallstones are essential for biofilm formation. Moreover, colonization in the presence of cholesterol gallstones requires expression of several bacterial genetic components.<sup>151,152</sup> These data argue that cholesterol gallstones promote chronic colonization with *S typhi* rather than *S typhi*–promoting gallstones. Regardless of whether gallstones promote colonization or vice versa, the concomitant presence of gallstones and *S typhi* markedly promotes the risk of gallbladder cancer, making the interaction between *S typhi* and cholesterol gallstones an important topic of clinical interest.<sup>149,151</sup>

It is not clear whether there is a causative role for bacteria in formation of cholesterol gallstones in humans. It is likely that some bacteria identified in humans with cholesterol gallstones are at least partially responsible for those gallstones due to their ability to promote inflammation and precipitate calcium salts. Unfortunately, most of these studies are primarily descriptive and fail to thoroughly analyze the organisms identified. For example, it is important to determine whether any of the organisms possess virulence factors that would allow for their invasion or colonization of a nondiseased biliary tree. Additional information could be garnered by culturing these organisms and utilizing them to prospectively infect mouse models. For example, when the C57L/J mouse model is acquired directly from The Jackson Laboratory (Bar Harbor, ME) and the mice are housed in microisolator cages under specific pathogen-free conditions and free of enzootic *Helicobacter* spp, they rarely (~10%) develop cholesterol gallstones. However, purposeful infection with some enterohepatic strains of *Helicobacter* spp significantly increases the prevalence of gallstones to a high of 80%.<sup>16,17</sup> It will prove valuable to test other putative lithogenic organisms utilizing this model system approach, bearing in mind the caveat that for the assay to be effective the organisms must be capable of colonizing the mouse strain being utilized.

### Chronic Inflammatory Conditions and Gallstones

Chronic hepatitis C virus (HCV) infection is associated with cholesterol and pigment gallstone formation.<sup>153–156</sup> Chronic hepatic damage might be responsible for this effect, leading to deranged liver function and cirrhosis.<sup>155,156</sup> However, recent studies have shown that HCV infection, independent of liver cirrhosis, is associated with increased prevalence of cholesterol

gallstones.<sup>153,154</sup> HCV can replicate in the gallbladder epithelium,<sup>157,158</sup> and damage of the biliary tree was noted in 22%–91% of biopsy specimens from patients with HCV.<sup>154</sup> It is therefore possible that the association between noncirrhotic patients with HCV and gallstones depends on HCV replication in biliary epithelia, leading to local inflammation, alterations in gallbladder contraction, and inflammation-mediated alterations in mucin gene production. Interestingly, one such study reported a correlation between HCV and peaks in the prevalence of gallstones at 2 time points. The first peak occurred in patients between the ages of 31 and 40 years (7% gallstone prevalence) and the second peak occurred much later, at 61–70 years of age (16% gallstone prevalence).<sup>154</sup> Perhaps the first peak represents the direct effect of HCV infection itself, whereas the second peak represents the effect of chronic liver dysfunction and hepatic cirrhosis. Future studies using biochemical and molecular techniques that focus on changes in the gallbladder itself could provide greater insight into the mechanistic relationships between HCV infection and formation of cholesterol gallstones.

Patients with Crohn's disease (CD) are at increased risk for the development of gallstones.<sup>159–162</sup> Results vary regarding which factors correlate with pigment or cholesterol gallstone formation. Factors mentioned include age, female sex, site of disease, surgical resection, and duration of disease (independent of age).<sup>160,161,163</sup> Some investigators noted that patients with CD with ileal disease or colonic resection supersaturated their bile with cholesterol, probably because of alterations in cholesterol and bile acid absorption from the small intestine.<sup>164,165</sup> However, several studies have described a decrease in the cholesterol saturation in bile of patients with CD, especially when the ileum is involved.<sup>166,167</sup> Furthermore, bile acid composition is altered in patients with CD, and these alterations are often characterized by a decrease in deoxycholate and an increase in ursodeoxycholate conjugates.<sup>164,166,167</sup> These changes seem paradoxical, because ursodeoxycholate enrichment would seem to favor prevention of cholesterol gallstones.<sup>118,168,169</sup> Unlike patients with CD, patients with ulcerative colitis are not at increased risk for cholesterol gallstones.<sup>159,161</sup> Gallstone formation might vary between patients with ulcerative colitis and patients with CD because of differences in inflammatory changes.<sup>170–172</sup> Although not true in all cases, CD is most commonly associated with a Th1 proinflammatory response whereas ulcerative colitis is more often associated with a Th2 response.<sup>170–172</sup> No studies have described the gallbladder inflammatory profile of patients with either ulcerative colitis or CD; this would provide insight into the relationship of CD, inflammation, and gallstones.

### **Inflammation, Cholesterol Metabolism, and Gallstones**

Inflammation, and more specifically the acute phase response, produces marked alterations in the metabolism of a variety of proteins and lipids; these changes are extensive and beyond the scope of this review (see Heller et al<sup>172</sup> for a leading account). However, metabolic changes of the liver that alter cholesterol and bile acid metabolism can directly alter the composition of bile. Most studies of these phenomena were conducted using exogenous, acute administration of cytokines or LPS,<sup>173–177</sup> which causes rapid (minutes to hours), relatively short-lived (24 hours or less) alterations in hepatic gene transcription and translation. Although these changes are significant, it is not clear if they are maintained for prolonged periods (days to weeks).<sup>176</sup> In the case of cholesterol gallstone formation this could prove to be very important, because short-lived (hours to days) alterations in bile composition are less likely to promote disease than prolonged and sustained alterations.

With this proviso in mind, it is clear from these studies that acute inflammation does alter the hepatic metabolism of both cholesterol and bile salts. In rodents, administration of LPS or proinflammatory cytokines (IL-1, TNF) raises serum cholesterol levels and increases the production of HMG-CoA reductase at both the transcription and translational levels.<sup>173,175,178</sup> This change augments de novo cholesterol synthesis. However, the increase in cholesterol

production is rather modest because other enzymes involved in cholesterol biosynthesis, including squalene synthase and enzymes downstream of HMG-CoA reductase in the mevalonate pathway, are down-regulated.<sup>173,177–179</sup> Some investigators propose that these changes maintain adequate cholesterol synthesis while redirecting mevalonate metabolites into non-sterol pathways.<sup>176</sup> This hypothesis is supported by the observed increases in dolichol phosphate and glycosylated plasma proteins during the acute phase response.<sup>180,181</sup> In contrast, primates decrease their serum cholesterol levels in response to a variety of proinflammatory mediators.<sup>182–184</sup> Unfortunately, the mechanism underlying this change is less well understood but might arise from alterations in cholesterol secretion or ApoB synthesis.<sup>185,186</sup>

Profound alterations in bile salt production also occur under these conditions. Specifically, LPS down-regulates the classic and alternative pathways of bile salt synthesis by decreasing production of CYP7A1 (classic) and CYP7B1 or CYP27A1 (alternative) proteins.<sup>187,188</sup> Additionally, exogenous administration of LPS decreases hepatocellular uptake and secretion of bile salts by down-regulating basolateral and apical bile salt transporters.<sup>189–193</sup> Not surprisingly, because bile salts stimulate the biliary secretion of both phospholipids and cholesterol, administration of LPS also decreases expression of phospholipid (MDR2) and cholesterol transporters (ABCG5/ABCG8) on the canalicular (apical) membranes of the hepatocytes.<sup>190,193,194</sup>

These observations indicate that acute infections would decrease cholesterol secretion into bile due to intrahepatic cholestasis. Because formation of cholesterol gallstones requires only a relative excess of cholesterol, these changes may or may not be lithogenic. Additionally, the studies do not necessarily reflect the events following chronic immune and inflammatory stimulation. In fact, during chronic (10 weeks) infection with enterohepatic *Helicobacter* spp, hepatic bile salt secretion rate and bile flow increase.<sup>110</sup> Interestingly, infection also alters the composition of the bile salt pool, rendering it more hydrophobic consistent with a proinflammatory response. These findings highlight the differences between acute studies involving exogenous administration of cytokines and LPS and those studies involving chronic infection. Furthermore, these data highlight the need for studies with pathogens that cause either or both chronic and acute infections, which could provide entirely different results than studies utilizing exogenous cytokine and LPS administration.

### Lith (LITH) Genes

Large genetic screens have identified a variety of mouse *Lith* loci; products of genes at this loci promote cholesterol cholelithogenesis.<sup>3,93</sup> Most of these alleles span large chromosomal regions, and the genes responsible for gallstone proclivity have not been identified (see Lyons et al<sup>93</sup> for a detailed review of *Lith* alleles and their potential to encode inflammatory mediators). Unfortunately, the *Lith* loci identified to date occur on virtually every mouse chromosome and span relatively large regions encompassing numerous genes<sup>14</sup>; the proinflammatory genes are likely to be located in many, if not most, of these loci. Therefore, a more plausible and productive approach to study the role of inflammation in gallstone pathogenesis would be to utilize currently available transgenic and knockout mouse models, with alterations in genes important in inflammation and immune responses, and compare these with their wild-type counterparts. With the creation of these new models, the genetic loci involved in gallstone pathogenesis will become more refined and can be used to study the influence of potential roles of inflammation and immunity on formation of cholesterol gallstones.

### Conclusions

The gallbladder, despite its simple structure and function, is a complex organ. Like most organs, when it becomes inflamed and damaged, it loses its ability to perform concentrative, pH

modification, absorptive, and contractive functions. Unlike most other organs in the body, the gallbladder can be exposed to large concentrations of free (unesterified) cholesterol and potentially cytotoxic detergent-like bile salts. These molecules, as well as other proinflammatory molecules, can induce potent inflammatory responses under certain circumstances.<sup>23,106–108</sup> Such inflammatory damage, through a variety of undetermined mediators, then induces gallbladder hypomotility and promotes the production of pronucleating agents.<sup>23,84,87</sup> These effects appear to be due, at least in part, to T cells and a proinflammatory Th1 immune response.<sup>18</sup> Furthermore, inflammation, at least in short-term studies, modifies hepatic lipid metabolism and secretion, which could promote cholelithogenesis by altering biliary cholesterol secretion and bile concentration.<sup>173,175,178</sup> These findings raise the possibility that inflammatory mediators or their subsets are serologic biomarkers of cholesterol gallstone formation. If identified, these biomarkers could prove valuable for diagnosis and interventional therapy. Specifically, abatement of prolonged immune stimulation might prove useful in the prevention or treatment of cholesterol gallstones, either alone or in conjunction with other lipid-altering therapies.

It is important to note that many of the studies described in this review relied principally on observation using genetically homozygous mice (>99% identify among genomes). In contrast, even the most consanguineous of human populations are relatively heterogeneous. The role of inflammation in human cholesterol gallstone disease is therefore likely to be subtle and involve a wide variety of overlapping factors. Both environmental (exposure to pathogens and proinflammatory conditions) and genetic immune factors (polymorphisms in genes that promote the response to immune stimuli) are likely to contribute to pathogenesis. Clearly, much remains to be learned in this regard, and further *in vitro*, animal model, and human studies are likely to clarify many of these exciting possibilities.

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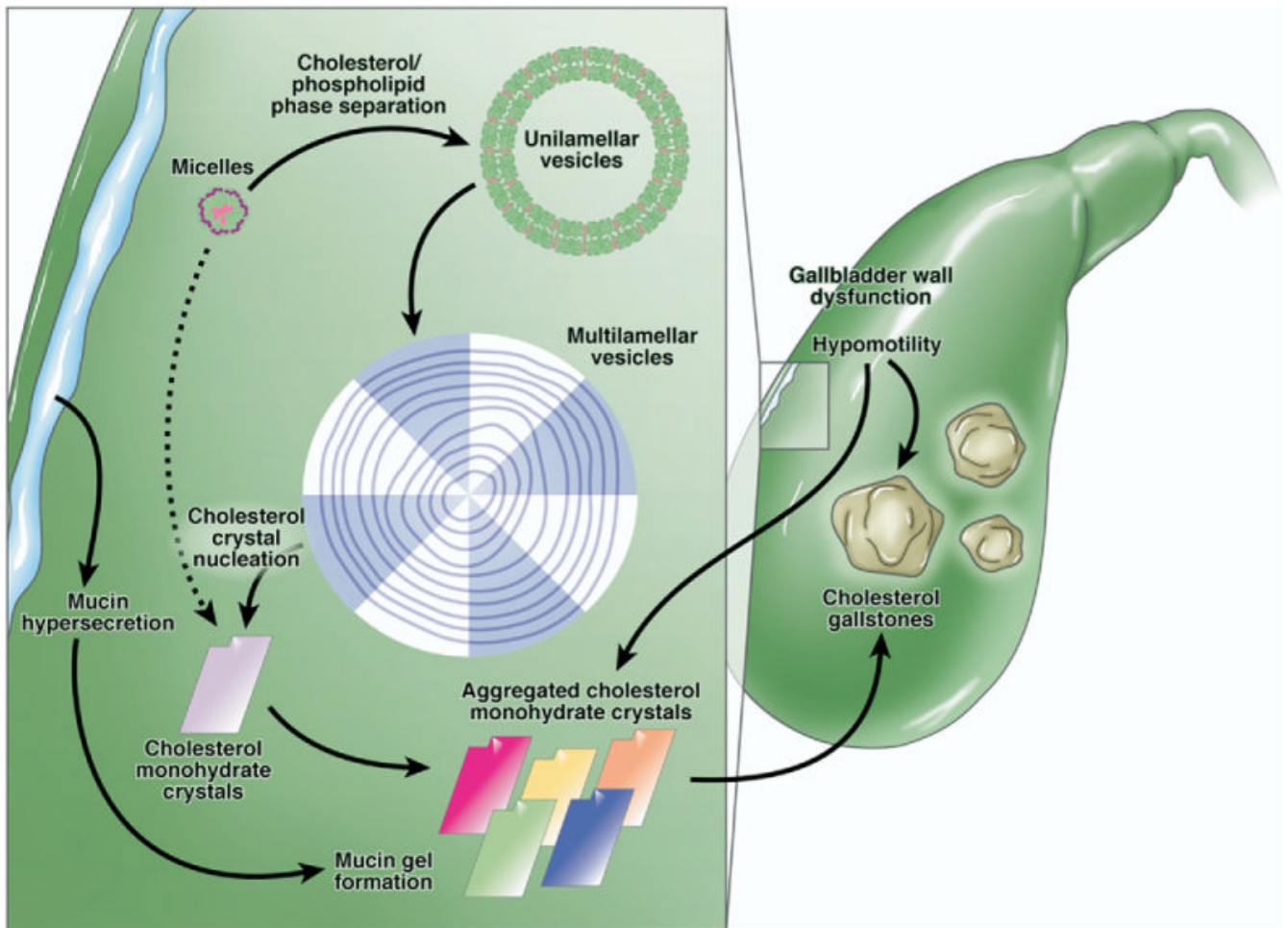
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### Abbreviations used in this paper

<b>EGF</b>	epidermal growth factor
<b>EGFR</b>	epidermal growth factor receptor
<b>FXR</b>	farnesoid X receptor
<b>IL</b>	interleukin
<b>LPS</b>	lipopolysaccharide
<b>MyD88</b>	myeloid differentiation protein 88
<b>TGF</b>	transforming growth factor
<b>TLR</b>	toll-like receptor
<b>TNF</b>	tumor necrosis factor
<b>PCR</b>	polymerase chain reaction

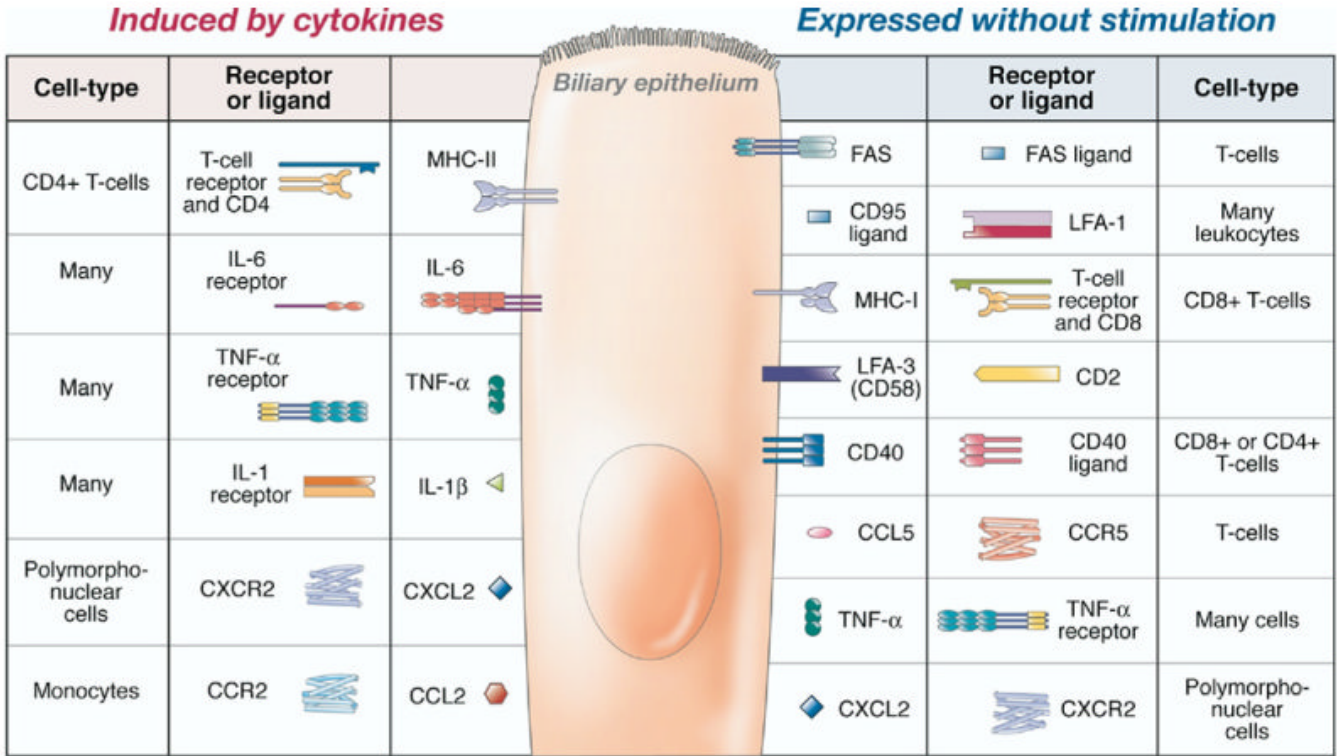


**Figure 1.**

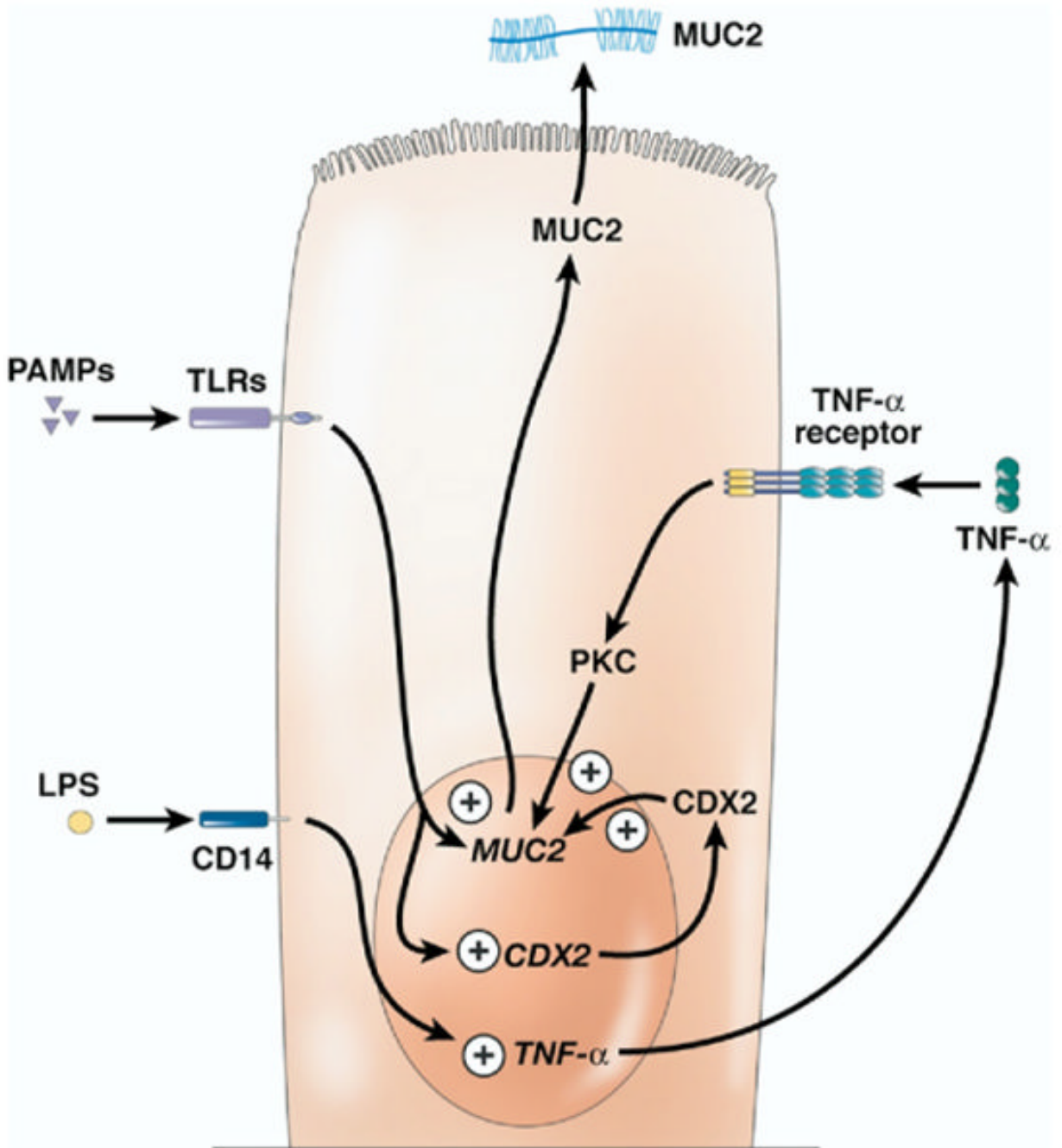
The physical-chemical processes involved in formation of cholesterol gallstones. The classic physical-chemical symbols for cholesterol (*pink*), phospholipid (*green*), and bile salt (*purple*) molecules are shown, along with the macromolecular structures they form. Cholesterol, phospholipids, and bile salts combine by hydrophobic interactions to form mixed micelles (micelles) and cholesterol and phospholipids form unilamellar vesicles. Normally the unilamellar vesicles would be ~5–10 times larger than micelles (~40 Å in radius), but for illustration purposes they are depicted here nonproportionately. As cholesterol concentration in gallbladder bile increases principally from hepatic hypersecretion of cholesterol, the true supersaturated state forms transiently. Supersaturated bile usually implies that phase separation of excess cholesterol from micelles has occurred, forming unilamellar vesicles with biliary phospholipids (mostly >95% phosphatidylcholine). In the most common nucleation sequence, unilamellar vesicles fuse to form multilamellar vesicles, or liquid crystals, which are visible by low-power polarizing microscopy. From these, plate-like cholesterol monohydrate crystals (solid cholesterol crystals) nucleate heterogeneously, usually in a mucin gel. The *dotted arrow* indicates how cholesterol can occasionally phase separate directly from supersaturated micelles. The solid resulting cholesterol monohydrate crystals are a polymorph of the classic cholesterol monohydrate plates into which they transform with passage of time. Once the nucleation sequence has occurred and solid cholesterol crystals have formed, the phase sequence is not repeated if bile remains continuously supersaturated. Gallbladder dysmotility and mucin gel formation also contribute to the aggregation of the plate-like cholesterol

monohydrate crystals and contribute to their agglomeration and growth into macroscopic cholesterol gallstones.



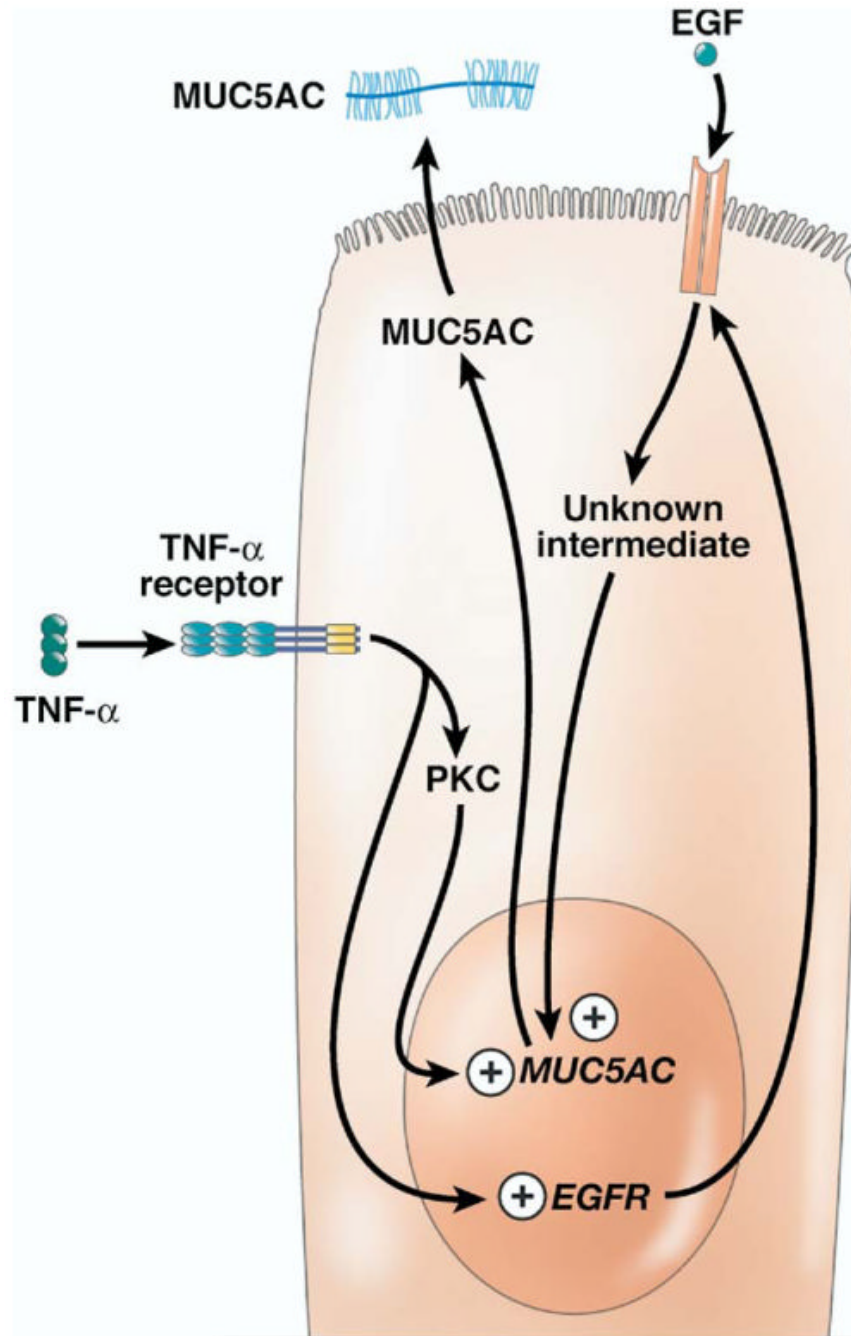


**Figure 2.** Receptors and ligands expressed by biliary epithelial cells. The receptors (displayed on the basolateral surface of the cell) and ligands (displayed extracellularly) expressed by biliary epithelial cells are shown in the unstimulated (*right panel*) and stimulated (*left panel*) state. The receptors, ligands, and cells that interact with these biliary expressed proteins are also shown. CCL, chemokine CC motif ligand; CCR, chemokine CC motif receptor; CXCL, chemokine CXC ligand; CXCR, chemokine CXC receptor; LFA, lymphocyte function-associated antigen; MHC, major histocompatibility complex.



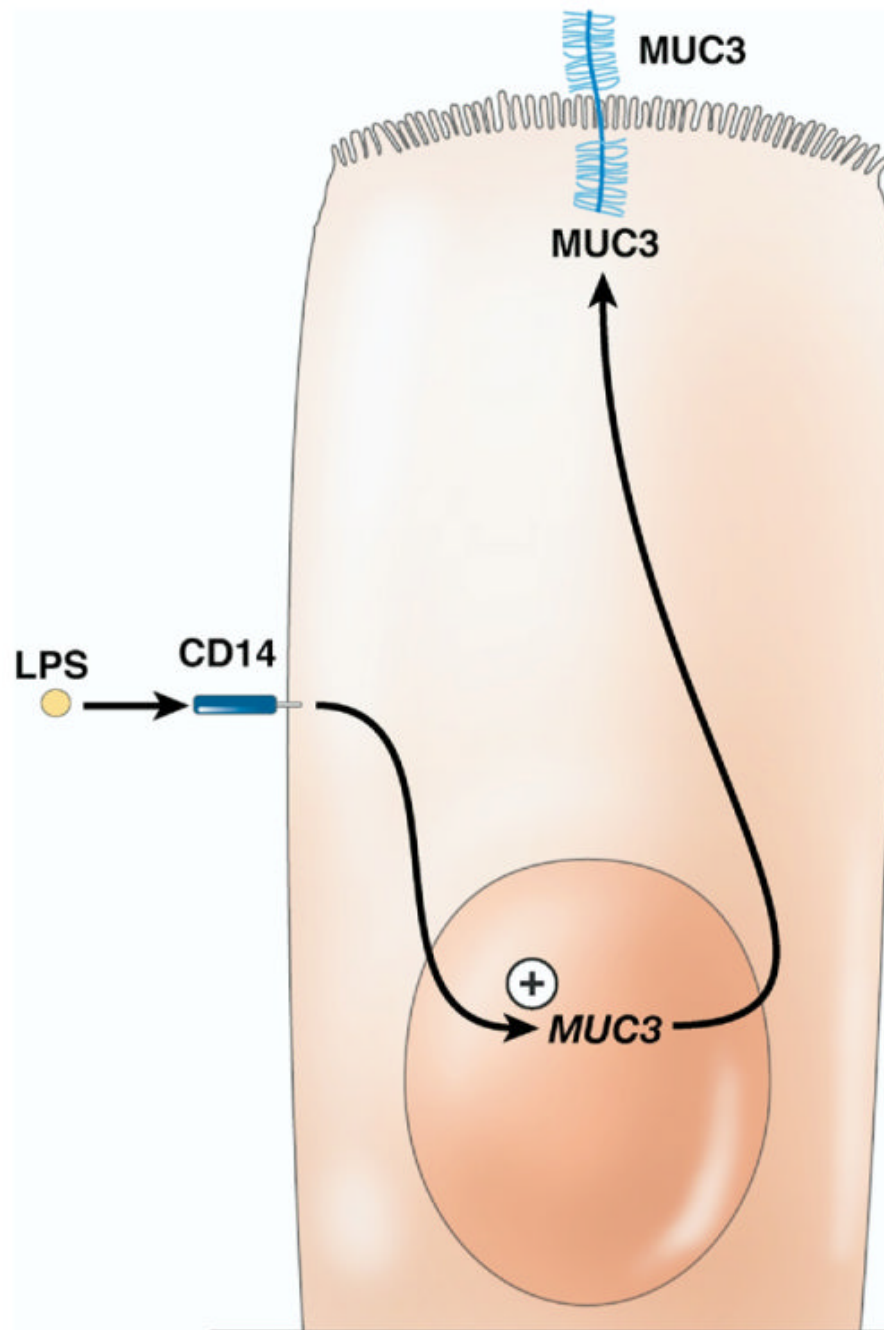
**Figure 3.**

Immune system–stimulated production of MUC2. Various pathogen-associated molecular patterns (PAMPs), through their interaction with TLR molecules, increase the production of mucin 2 (MUC2). A portion of this increase seems to be due to increased production of caudal type homeobox transcription factor 2 (CDX2), which feeds back to stimulate MUC2 production. Further, LPS and TNF- $\alpha$  stimulate the production of MUC2. In the case of LPS, this stimulation seems to be mediated through the TNF- $\alpha$  pathway because inhibition of TNF- $\alpha$  blocks the effects of LPS treatment on MUC2 production.



**Figure 4.**

The points at which the immune system stimulates production of MUC5AC. As described for mucin 2, LPS stimulation leads to production of mucin 5AC (MUC5AC) in a TNF- $\alpha$  dependent manner (not shown). TNF- $\alpha$  acts via protein kinase C (PKC)-dependent and EGF-mediated pathways to up-regulate MUC5AC production. In the case of the EGF pathway, this up-regulation results from the production of apical EGF receptors and subsequent binding to apical EGF.



**Figure 5.** Stimulation of mucin 3 (MUC3) production by LPS via CD14 and likely TLR molecules. Stimulation of MUC3 by LPS is independent of  $\text{TNF-}\alpha$ , in contrast to MUC2 and MUC5AC. It is currently unclear what downstream pathways are important in LPS stimulation of MUC3 production.