Interleukin-4 deficiency promotes gallstone formation

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Abstract **Feeding interleukin-4 (IL-4) deficient C57BL/6 LDL receptor (LDLr)$^{-/-}$ mice a modified diet to investigate the role of this cytokine in cholesterol metabolism led to an unexpected phenotype. IL-4$^{-/-}$ → LDLr$^{-/-}$ mice had enlarged gallbladders and an increased mortality that was preceded by acute body weight loss. To determine if IL-4 deficiency accounted for these findings, C57BL/6 IL-4$^{+/+}$ and IL-4$^{-/-}$ mice were fed either a normal or modified diet. IL-4 deficiency did not alter bile composition or cause liver toxicity in mice fed a fat-enriched diet. Following 8 weeks of feeding a fat-enriched diet, no gallstones were detected in IL-4$^{+/+}$ mice, and only 20% had cholesterol crystals. In contrast, IL-4$^{-/-}$ mice had a 100% incidence of gallstones and cholesterol crystals. IL-4$^{-/-}$ deficiency also increased serum concentrations of bilirubin following feeding a fat-enriched diet. Therefore, these studies revealed an unexpected finding that IL-4 deficiency predisposes to gallstone formation.**—King, V. L., S. J. Szilvassy, and A. Daugherty. **Interleukin-4 deficiency promotes gallstone formation. *J. Lipid Res.* 2002.** 43: **768–771.**

Supplementary key words bone marrow transplant • LDL receptor • cholesterol

The deposition of excess cholesterol leads to two prominent pathophysiological conditions: atherosclerosis and gallstones. Atherosclerosis is due to deposition of modified cholesterol in the arterial walls, while gallstone formation in the gallbladder is due to a defect that leads to supersaturation of the bile with cholesterol (1). Diets enriched in cholesterol and cholate promote the development of atherosclerosis and the formation of gallstones in specific strains of mice (2, 3). The differing susceptibilities of specific strains of mice have been used as a method of defining genetic factors that regulate the development of atherosclerosis and formation of gallstones (4–10). This approach has shown that atherosclerotic lesion and gallstone formation are complex traits, in which there are potentially many factors that impact on the disease process (11).

Several specific cytokines have been implicated in cholesterol and lipoprotein metabolism. One of these cytokines is interleukin-4 (IL-4), which appears to have an expression that is restricted to CD4$^+$ T lymphocytes, mast cells, and subtypes of natural killer cells (12). Major functions of IL-4 include regulation of antigen-stimulated naive T cell differentiation and control of the specificity of immunoglobulin class switching (13).

During the course of studies to define the effect of IL-4 deficiency on the development of atherosclerosis in LDL receptor (LDLr)$^{-/-}$ mice, we observed a phenotype consistent with the enhanced development of gallstones (11). Therefore, to further analyze the effect of enhanced gallstone formation in IL-4 deficient mice we used C57BL/6 IL-4$^{-/-}$ and IL-4$^{+/+}$ mice. These studies provided the unexpected result that IL-4 deficiency markedly increased the formation of gallstones during the feeding of a diet that was enriched in cholesterol, saturated fat, and cholate.

MATERIALS AND METHODS

Mice

LDLr$^{-/-}$ (14), IL-4$^{-/-}$ (15), and wild-type C57BL/6 mice were obtained from Jackson Laboratories. LDLr$^{-/-}$ and IL-4$^{-/-}$ mice were backcrossed 10 times onto a C57BL/6 background. Mice were housed under specific pathogen-free conditions and fed either a normal diet or a modified diet that contained 21% saturated fat (w/w), 1.25% cholesterol (w/w), and 0.5% cholate (w/w) (Harlan Teklad). Body weight was monitored weekly. All procedures involving mice were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Bone marrow transplantation

Male LDLr$^{-/-}$ recipients (9–10 months of age) were lethally irradiated with a total of 900 rads from a cesium γ source, administered as two doses of 450 rads for 2 min each separated by a 3 h interval. Bone marrow cells were harvested from femora and tibiae of age-matched IL-4$^{+/+}$ and IL-4$^{-/-}$ donor mice (n = 2 mice/group) and transplanted into irradiated recipients (1×10^7 cells/mouse) by tail vein injection.

Tissue removal

Nonfasting mice were anesthetized by ip injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Terminal blood

Abbreviations: IL-4, interleukin-4; A:G, albumin/total protein; GOT: GPT, glutamic oxalacetic transaminase/glutamic pyruvic transaminase.

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samples were collected by puncture of the right ventricle. Blood was allowed to clot at room temperature for 1 h prior to centrifugation at 2,000 rpm for 20 min to separate the serum. Gallbladders and livers were excised for analysis.

Serum lipid, lipoprotein, and bilirubin analysis

Serum cholesterol, triglyceride, and phospholipid concentrations were measured by enzymatic colorimetric assay (Wako Chemical Company). Lipoprotein-cholesterol distribution was determined by size exclusion chromatography as described previously (16). Serum bilirubin was measured by colorimetric assay (Sigma Diagnostics).

Liver function analysis

Serum albumin/total protein (A:G) (Wako Chemical Company) and glutamic oxalacetic transaminase/glutamic pyruvic transaminase (GOT:GPT) ratios (Sigma Diagnostics) were measured by colorimetric assay.

Bile composition analysis

Bile cholesterol and phospholipid concentrations were measured by enzymatic colorimetric assay (Wako Chemical Company). Bile acids were measured by colorimetric assay (Wako Chemical Company).

Gallstone and cholesterol crystal quantification

The presence and absence of cholesterol crystals and gallstones was determined by gross and microscopic examination of the bile as described by Wang et al. (17, 18).

Statistical analysis

All data are represented as mean ± SEM. Statistical analysis was performed by Student's *t*-test. Non-parametric data was analyzed by Mann-Whitney Rank sum test. Incidence was analyzed by Fisher's exact test. All data analyses were performed using SigmaStat 2.03 software (SPSS, Inc.). Values with $P \leqslant 0.05$ were considered statistically significant.

RESULTS

IL-4$^{-/-}$ → LDLr$^{-/-}$ mice

To determine the effects of IL-4 on the atherogenic process, chimeric mice were created by the repopulation of C57BL/6 LDLr$^{-/-}$ mice with bone marrow cells derived from strain-matched C57BL/6 IL-4$^{+/+}$ or IL-4$^{-/-}$ mice. All mice recovered from bone marrow transplantation. Six weeks after repopulation, mice were placed on a diet enriched in saturated fat, cholesterol, and cholate. For the initial 4 weeks, both groups looked healthy. However, after this interval, mortality began to increase in the group repopulated with IL-4$^{-/-}$ bone marrow. At 8 weeks, no deaths occurred in the LDLr$^{-/-}$ mice repopulated with wild-type bone marrow cells, while there was a 50% mortality in the group repopulated with IL-4$^{-/-}$ bone marrow cells. At the time of death, the mice had lost considerable weight. Although both groups had enlarged gallbladders, mice engrafted with IL-4$^{-/-}$ bone marrow cells had a larger number of gallstones by visual inspection.

IL-4 deficient C57BL/6 mice

The unexpected finding of an increased mortality in IL-4$^{-/-}$ → LDLr$^{-/-}$ mice prevented the quantification of gallstone formation. To determine if IL-4 deficiency altered bile composition and liver function prior to gallstone formation and death, C57BL/6 mice that were IL-4$^{-/-}$ or IL-4$^{+/+}$ were fed either a normal or modified diet for 4 weeks. To determine the effect of IL-4 deficiency on gallstone formation C57BL/6 mice that were IL-4$^{-/-}$ or IL-4$^{+/+}$ were fed either a normal or modified diet for 8 weeks.

There was a modest, but significant increase in plasma cholesterol concentrations in IL-4$^{-/-}$ mice compared with IL-4$^{+/+}$ mice fed the modified diet for 4 weeks (**Table 1**). While the overall serum cholesterol concentrations were not strikingly different, the diet changed the lipoprotein cholesterol distribution with an increasing content in VLDL and LDL and reductions in HDL (**Fig. 1**). In contrast, IL-4 deficiency did not significantly alter total serum cholesterol concentrations and lipoprotein distribution in mice fed a normal diet. Serum triglycerides were not significantly altered by deficiency of IL-4 (Table 1). IL-4 deficiency did not affect concentrations of cholesterol, phospholipid, and bile acid in the bile (Table 1).

After feeding a modified diet for 4 weeks, serum concentrations of bilirubin were significantly increased in

TABLE 1. Serum and bile concentrations of selected lipid and protein parameters

Diet	Normal		Modified	
IL-4 Genotype	+/+	−/−	+/+	−/−
Serum				
Cholesterol (mg/dl)	72 ± 3	70 ± 3	61 ± 3	77 ± 3[a]
Triglycerides (mg/dl)	67 ± 4	63 ± 3	33 ± 1	34 ± 2
Total Bilirubin (mg/dl)	ND	ND	0.04 ± 0.04	0.121 ± 0.06[b]
Albumin (g/dl)	2.7 ± 0.3	2.9 ± 0.2	2.8 ± 0.1	3.0 ± 0.3
Total Protein (g/dl)	5.1 ± 0.4	5.8 ± 0.5	4.9 ± 0.3	6.4 ± 0.6
GOT (SF units/ml)	113 ± 6	112 ± 9	118 ± 8	105 ± 8
GPT (SF units/ml)	15 ± 2	11 ± 1	43 ± 7	59 ± 13
Bile				
Cholesterol (mg/dl)	110 ± 28	164 ± 59	232 ± 19	239 ± 10
Phospholipids (mg/dl)	ND	ND	1444 ± 175	1618 ± 245
Bile acids (μmol/L)	ND	ND	3231 ± 279	3851 ± 330

Values are represented as mean ± SEM. n = 10 for each measurement. ND = Not determined.
[a] $P = 0.003$.
[b] $P = 0.009$ (compared to wild-type control).

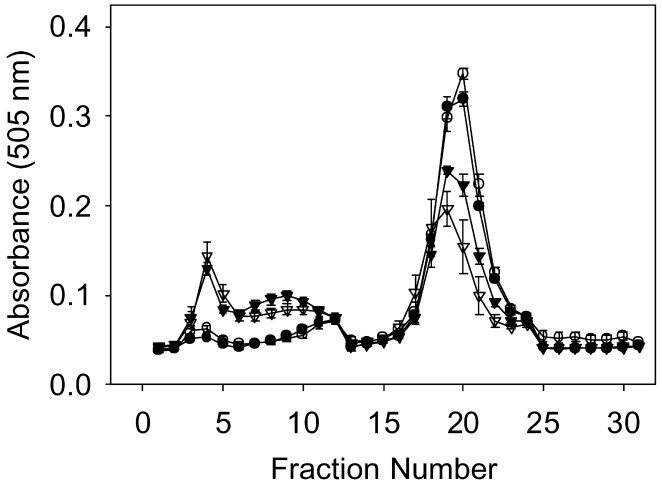

Fig. 1. Cholesterol-lipoprotein distributions are not altered by interleukin-4 (IL-4) deficiency in C57BL/6 mice fed either a normal or modified diet. Lipoprotein cholesterol distribution was determined in serum from five individual mice per group. IL-$4^{+/+}$ (open shapes) or IL-$4^{-/-}$ (closed shapes) mice were fed a normal (circles) or modified (triangles) diet for 4 weeks. Points represent the mean of five observations and bars are SEM.

IL-$4^{-/-}$ mice. To determine if liver function was altered in IL-$4^{-/-}$ mice, serum GOT:GST and A:G ratios were measured. These markers (Table 1) were not altered in IL-4 deficient mice fed either a normal or modified diet.

After 8 weeks of consuming normal or modified diets, the gallbladders were examined in the IL-$4^{-/-}$ and IL-$4^{+/+}$ mice. No discernable cholesterol crystals or gallstones were present in the gallbladders from either group that

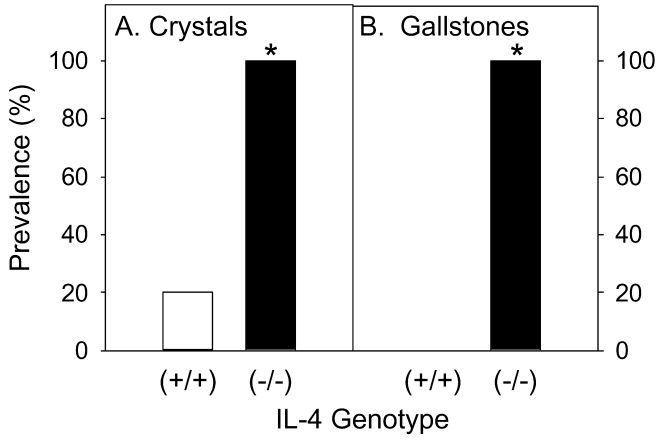

Fig. 2. IL-4 deficiency resulted in (A) cholesterol crystal and (B) gallstone formation during feeding of a modified diet. No discernable cholesterol crystals or gallstones were present in either group fed a normal diet. A: Following 8 weeks on a modified diet, cholesterol crystals were present in gallbladders of all IL-$4^{-/-}$ mice (n = 5) and in one mouse in the IL-$4^{+/+}$ group (n = 5; $P = 0.048$). B: No gallstones were present in the IL-$4^{+/+}$ mice fed a modified diet; however, all mice in the IL-$4^{-/-}$ group fed a modified diet had gallbladders that were filled with gallstones ($P = 0.008$ denoted as *). Histobars represent the percent incidence.

was fed a normal diet for 8 weeks. In IL-$4^{+/+}$ mice fed the modified diet, 20% of gallbladders contained cholesterol crystals, but no gallstones were observable. In marked contrast, all gallbladders from IL-$4^{-/-}$ mice fed the modified diet were engorged with both cholesterol crystals and white gallstones (**Fig. 2**).

DISCUSSION

Studies in our laboratory investigating the effect of IL-4 deficiency on the development of atherosclerosis in C57BL/6 LDLr$^{-/-}$ mice produced an unexpected phenotype. In response to a diet enriched in cholesterol, saturated fat, and cholate, LDLr$^{-/-}$ mice repopulated with IL-$4^{-/-}$ bone marrow cells had increased mortality and morbidity. Strikingly, gallbladders were grossly enlarged and filled with gallstones. To determine if IL-4 deficiency affected gallstone formation, we examined C57BL/6 IL-$4^{+/+}$ and IL-$4^{-/-}$ mice. C57BL/6 IL-$4^{+/+}$ mice did not develop gallstones at the interval studied, although 20% developed cholesterol crystals in the bile. In contrast, all C57BL/6 IL-$4^{-/-}$ mice developed both cholesterol crystals and gallstones, despite any profound changes in serum cholesterol concentrations. These findings suggest that IL-4 does not exert its effect on gallstone formation through a discernable effect on peripheral cholesterol metabolism.

Synthesis and subsequent excretion of bile acids is a major mechanism for the elimination of excess cholesterol. Additionally, bile acids and phospholipids solubilize cholesterol in the bile, preventing the precipitation of cholesterol in gallbladder. During the early stages of cholesterol crystal and gallstone formation, inflammatory mediators are present (19, 20). Additional evidence for a role of inflammatory mediators is the finding that IL-1 and tumor necrosis factor α may regulate the negative feed back loop for bile acid synthesis (21). IL-4 deficiency did not alter concentrations in the bile of cholesterol, phospholipid, or bile acid in mice fed the modified diet. However, our studies would not necessarily have determined if hypersecretion of cholesterol and subsequent incorporation into cholesterol crystals and gallstones was occurring in IL-$4^{-/-}$ mice.

Suppression of the principal enzymes involved in bile acid production, cholesterol 7-α hydroxylase (C7AH), and sterol 27-hydroxylase is associated with gallstone formation in susceptible inbred mice fed a cholate containing diet (8, 22). While specific cytokines have been demonstrated to play a role in the regulation of these enzymes during cholestasis, the mechanism of this effect has not been defined. Recently, Miyake et al. (23) demonstrated that the PPAR-γ agonist, rosiglitazone, blocked repression of C7AH in HepG2 cells (23). IL-4 can generate endogenous PPAR-γ agonists by the enhanced activity of 15-lipoxygenase on linoleic and arachadonic acid (24). Therefore, a potential mechanism underlying the effect of IL-4 deficiency may be the decreased ability to generate endogenous derepressors of enzymes involved in bile acid

synthesis. This suppressed ability to generate bile acid would lead to cholesterol supersaturation and gallstone formation. However, we were not able to discern an effect on bile acid concentrations.

The IL-4 gene is located on chromosome 11. Quantitative trait loci analysis has demonstrated the presence of loci on chromosome 11 which contribute to the regulation of C7AH (25) in addition to *Lith1* and *Lith2* genes localized to chromosomes 2 (8, 9) and 19 (11), respectively. The loci on chromosome 11 also regulate HDL-cholesterol (HDL-C) levels (25). The *Lith1* and *Lith2* genes are associated with increased susceptibility of gallstone formation in C57BL/6 mice (11). Moreover, the loci regulating C7AH and HDL-C levels exhibited a significant association with gallstone formation in C57BL/6 mice fed an atherogenic diet (25).

In conclusion, IL-4 deficiency resulted in enhanced gallstone formation in mice fed a diet enriched in cholesterol, saturated fat, and cholate. This may be due to IL-4 deficiency resulting in hypersecretion of cholesterol into the bile or decreased bile acid synthesis resulting in enhanced gallstone formation. However, other mechanisms, such as regulation of a host of genes involved in cholesterol metabolism, may play a role in IL-4 deficiency enhancing gallstone formation.

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