Luteolin Reduces Lipopolysaccharide-induced Lethal Toxicity and Expression of Proinflammatory Molecules in Mice

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Luteolin is a flavonoid that has been shown to reduce proinflammatory molecule expression *in vitro*. In the present study, we have tested the ability of luteolin to inhibit lipopolysaccharide (LPS)-induced lethal toxicity and proinflammatory molecule expression *in vivo*. Mice receiving LPS (*Salmonella enteriditis* LPS, 32 mg/kg, intraperitoneally) exhibited high mortality with only 4.1% of the animals surviving seven days after the LPS challenge. On the contrary, mice that had received luteolin (0.2 mg/kg, intraperitoneally) before LPS showed an increased survival rate with 48% remaining alive on Day 7. To investigate the mechanism by which luteolin affords protection against LPS toxicity we measured intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α) production in response to LPS in the presence or absence of luteolin pretreatment. Treatment of animals with LPS increased serum TNF-α levels in a time-dependent manner. The increase in peak serum TNF-α levels was sensitive to luteolin pretreatment. Luteolin pretreatment also reduced LPS-stimulated ICAM-1 expression in the liver and abolished leukocyte infiltration in the liver and lung. We conclude that luteolin protects against LPS-induced lethal toxicity, possibly by inhibiting proinflammatory molecule (TNF-α, ICAM-1) expression *in vivo* and reducing leukocyte infiltration in tissues.

Keywords: luteolin; tumor necrosis factor-α; intercellular adhesion molecule; lipopolysaccharide; sepsis

Septic shock affects more than 300,000 patients worldwide and has a high mortality of up to 60% despite antibiotic therapy and intensive care support (1, 2). The potentially fatal syndrome of irreversible cardiovascular collapse and critical organ failure was originally attributed to lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria. However, it is the endogenously produced proinflammatory molecules, rather than LPS itself, that mediate the pathophysiological alterations that accompany endotoxemia; endotoxin acts on many different cell types stimulating the expression of cytokines and adhesion molecules that contribute to the inflammatory response (3–5). One cytokine believed to play a major role in sepsis is tumor necrosis factor-α (TNF-α) (6). Serum levels of TNF-α reach nanomolar concentrations in experimental models of sepsis and administration of human recombinant TNF-α to animals induces circulatory collapse and critical organ injury that is similar to that seen in lethal endotoxemia (7). Moreover, an anti-TNF-α monoclonal antibody protects baboons from shock caused by bacteremia and homozygous TNF-α mutant mice, for both p55 and p75 are resistant to sepsis (8, 9).

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily expressed on endothelial cells. ICAM-1 is important for adhesion of leukocytes and transendothelial migration through binding to the β2 integrins present on leukocytes LFA-1 and MAC-1 (10). Exposure of endothelial cells to inflammatory stimuli leads to increased expression of ICAM-1, facilitating leukocyte accumulation in tissues (11, 12). The importance of ICAM-1 in sepsis is underscored by the observation that mice with targeted disruption of the ICAM-1 gene locus show reduced infiltration of neutrophils to the liver and are resistant to the lethal effects of high doses of LPS, in spite of similar increases in circulating levels of cytokines (13, 14).

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom. It is thought that the average western diet contains approximately 1 g of flavonoids per day (15). We and others (16–20) have shown that flavonoids inhibit proinflammatory molecule expression *in vitro*. Luteolin, a flavone found in high concentrations in celery, green pepper, perilla leaf and seeds, and chamomile, is among the most potent and efficacious flavonoid inhibitors of LPS-induced TNF-α, interleukin-6 production and inducible nitric oxide expression (17). In the present study, we extend our *in vitro* findings that luteolin blocks LPS-induced cytokine release by demonstrating that luteolin also attenuates TNF-α production and ICAM-1 expression *in vivo* and abolishes infiltration of leukocytes in the lung and liver of LPS-treated mice. More importantly, pretreatment with luteolin reduces the mortality associated with LPS administration in a mouse model of sepsis.

METHODS

Reagents and Cell Culture

Luteolin was obtained from Roth Chemicalien (Karlsruhe, Germany). Luteolin was found to contain only trace amounts of endotoxin (25 ng endotoxin/g luteolin) TNF-α and interleukin (IL)-6 ELISA kits were from R&D Systems (Minneapolis, MN). Tissue culture plates were from Nalge Nunc International (Rochester, NY). Protein dye reagent and the chemiluminesence Western blotting system were from Pierce (Rockford, IL). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), and antibiotics were obtained from Gibco BRL (Paisley, UK). The ICAM-1 antibody (H-108/sc-7891) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the secondary peroxidase-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch Lab (West Grove, PA). All other reagents including LPS (*Salmonella enteriditis*) were obtained from Sigma Chemical (St. Louis, MO). RAW 264.7 cells were cultured in low glucose DMEM containing 10% fetal bovine serum supplemented with penicillin and streptomycin at 37° C in a humidified incubator with 5% CO_2.

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LPS Lethality Studies

C57BL/6 male and female mice 10–12 weeks of age were pretreated with vehicle or luteolin (0.2 mg/kg, intraperitoneally) for 30 minutes before the LPS challenge (*Salmonella enteriditis* 32 mg/kg intraperitoneally). Injections were made in a total volume of 100 μl. Animals were observed for seven days.

TNF-α and IL-6 Measurements

Cytokine measurements in RAW 264.7 cells were performed as previously described (17). For serum TNF-α measurements, mice (C57BL/6 10–12 weeks old) were pretreated with luteolin (0.2 or 1 mg/kg, intraperitoneally, for 30 or 120 minutes) or vehicle (dimethylsulfoxide, DMSO) followed by LPS (32 mg/kg intraperitoneally). After the indicated time, blood samples were collected. They were then allowed to clot for two hours at room temperature, centrifuged for 20 minutes at 2,000 × *g*, and stored at −80° C until measured.

Western Blotting

Mice (C57BL/6 10–12 weeks old) were pretreated with luteolin (0.2 mg/kg, intraperitoneally, for 30 minutes) or vehicle (DMSO) followed by LPS (32 mg/kg, intraperitoneally). After eight hours, animals were sacrificed and tissues removed and snap frozen in liquid nitrogen. Proteins were extracted from tissues as previously described with minor modifications (21). Equal amounts of proteins were loaded and separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels. Proteins were blotted to nitrocellulose membrane, incubated with antibodies, and visualized using enhanced chemiluminescence Western blotting detection system.

Histopathological Studies

Mice (three or four per group) were treated with vehicle (DMSO), LPS (32 mg/kg, intraperitoneally), or LPS plus luteolin (0.2 mg/kg, intraperitoneally, 30 minutes pretreatment) and sacrificed 10 hours after LPS exposure by hypercapnia. Lungs and livers were removed immediately by thoracotomy and laparotomy. The right lungs were used for histological studies. The tissue specimens were fixed overnight in 4% buffered formaldehyde, processed by standard methods, and stained for hematoxylin and eosin (H&E). Semiquantitative analysis of tissues was performed by one observer in a blinded fashion.

Data Analysis and Statistics

Data are presented as means ± SEM of the indicated number of observations. For cytokine measurements statistical comparisons between groups were performed using the one-way ANOVA followed by the Dunnett's *post hoc* test. Differences among means were considered significant when $p < 0.05$. Survival as the end point in the lethality experiments was calculated from the time of treatment using the product limit Kaplan–Meier method. Differences in the Kaplan–Meier survival curves were evaluated by the Mantel log-rank test for censored data.

RESULTS

Effects of Luteolin on TNF-α Production from Murine Macrophages

We have previously shown that flavonoids inhibit TNF-α production from RAW 264.7 cells in response to *Escherichia coli* LPS (serotype 0B26) (17). Because for our *in vivo* studies we used LPS derived from *Salmonella enteriditis*, we tested whether luteolin can inhibit TNF-α production from RAW 264.7, in response *S. enteriditis* LPS. Exposure of RAW 264.7 to *S. enteriditis* LPS led to a 6-fold increase in the release of TNF-α in the culture medium. Pretreatment of the macrophages with 10 μM luteolin for 30 minutes led to a significant inhibition of the LPS-induced TNF-α release (Figure 1).

Effect of Luteolin on Survival in a Mouse Model of Sepsis

To test if luteolin can increase survival in a mouse model of sepsis, 10- to 12-week-old animals were injected with DMSO or luteolin (0.2 mg/kg) before LPS (32 mg/kg, intraperitoneally, *S. enteriditis* LPS). The dose of LPS used is close to the LD_{95} and the dose of luteolin chosen for the lethality studies was the one that showed the most promising results in pilot experiments. Mice receiving DMSO or luteolin alone exhibited no lethality, whereas only 4.1% of the mice receiving LPS survived after seven days (Figure 2). Pretreatment with luteolin increased survival rate throughout the time of observation with 48% of the mice pretreated with luteolin remaining alive after seven days. In contrast, a higher dose of luteolin (10 mg/kg) offered no significant protection against LPS-induced lethal toxicity (none of six mice alive in the LPS group after seven days versus one of six in the luteolin + LPS group). Administration of a 50 mg/kg dose of luteolin in the absence of LPS was lethal to three of three mice receiving it.

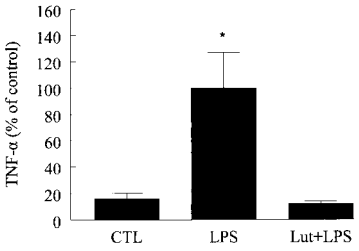

Figure 1. Luteolin inhibits LPS-induced TNF-α release from mouse macrophages. Cells were pretreated for 30 min with vehicle (DMSO:EtOH 1:1 vol/vol) or luteolin (10 μM). At the end of pretreatment, macrophages were incubated with LPS (10 ng/ml) for 24 h and media collected and analyzed by ELISA. Data are presented as means ± SEM; n = 6; *p < 0.05 from CTL.

Effects of Luteolin on LPS-induced Proinflammatory Molecule Expression *in Vivo*

To determine the mechanism by which luteolin protects animals from LPS lethal toxicity we measured its ability to lower serum TNF-α levels in the context of sepsis. Dimethylsulfoxide (vehicle)-treated mice had undetectable levels of circulating TNF-α. Pretreatment of mice with luteolin (0.2 or 1 mg/kg luteolin intraperitoneally) for 30 or 120 minutes before the LPS challenge attenuated the increase in serum TNF-α levels elicited by LPS (Figure 3). However, when luteolin (0.2 mg/kg, intraperitoneally) was given 30 minutes after the LPS, no reduction in serum TNF-α was observed (6.6 ± 1.0 pg/ml for the LPS group versus 10.5 ± 1.4 pg/ml for the luteolin + LPS group; n = 3). In addition, the 10 mg/kg dose of luteolin that failed to protect animals from the lethal toxicity of LPS was effective in reducing serum TNF-α levels (0.9 ± 0.3 pg/ml). Injection of mice with LPS increased serum TNF-α levels in a time-dependent manner, reaching a peak after 60 to 90 minutes (Figure 4A). Luteolin significantly reduced peak TNF-α levels, without affecting the early rise in TNF-α levels at 30 minutes (Figure 4A). In addition, luteolin showed no overall effect in circulating IL-6 levels (Figure 4B).

We next investigated the ability of luteolin to affect ICAM-1 expression, an important regulator of leukocyte trafficking.

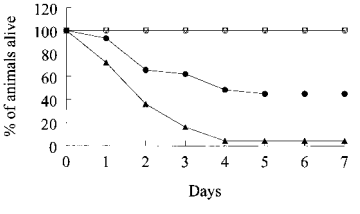

Figure 2. Luteolin protects against LPS-induced lethal toxicity. Mice (10–12 wk of age) were pretreated with vehicle (DMSO; *open circles*; n = 8), 0.2 mg/kg intraperitoneal luteolin (*open triangle*; n = 6), LPS (32 mg/kg, intraperitoneally; *filled triangle*; n = 25), or luteolin for 30 min followed by LPS (*filled circles*; n = 29). Mice were observed for the next 7 d. ○ Vehicle; ▲ LPS; ● Lut + LPS; ▽ Lut.

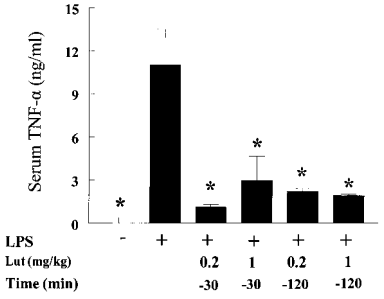

Figure 3. Luteolin reduces serum TNF-α in LPS-treated animals. Mice were treated with vehicle (DMSO) or luteolin (0.2 or 1 mg/kg, intraperitoneally) for the indicated time followed by LPS (32 mg/kg, intraperitoneally). After 90 min, blood samples were collected and processed as described in the METHODS section. Data are presented as means ± SEM; n = 4; *p < 0.05 from LPS.

Western blot analysis showed that ICAM-1 protein expression was markedly up-regulated by the LPS challenge in liver homogenates after eight hours. Pretreatment of animals with 0.2 mg/kg luteolin prevented the up-regulation of the protein (Figure 5).

Effects of Luteolin on Leukocyte Accumulation in the Lung and Liver

To test the functional relevance of the reduced ICAM-1 expression in the face of luteolin pretreatment, we studied the inflammatory infiltration in organs that constitute major targets of LPS. Histopathologic analysis of H&E-stained tissue sections from LPS-treated mice revealed a marked peribronchiolar and perivascular infiltration of leukocytes in the lung and around portal or central spaces in the liver (Figures 6B and 6E). Pretreatment with luteolin abolished the accumulation of leukocytes in both organs (Figures 6C and 6F and Table 1). However, luteolin did not prevent more subtle changes in tissue architecture caused by LPS, such as the thickening of alveolar septa and the hyperplasia of bronchial epithelium in the lung or the hyperplasia of vascular endothelium in the liver, as well as hyperplasia of the epithelium in the bileary ducts (Figures 6C and 6F).

DISCUSSION

In sepsis, challenge of cells with LPS stimulates cascades leading to phosphorylation and activation of multiple mitogen-activated protein kinase family members (Erk1, Erk2, p46/p54 JNK/SAPK, and p38), as well as phosphoinositide-3 kinase/Akt and nonreceptor tyrosine kinases of the Src family (Lyn, Fgr, and Hck) (22–26). Blocking LPS-stimulated phosphorylation cascades has been proposed as a promising target for the development of novel therapeutic agents for patients with sepsis (27). For example, tyrphostins, a class of agents with tyrosine kinase blocking activity, increase survival of mice injected with LPS (28).

The major finding of the present study is that the flavonoid luteolin protects mice from LPS-induced lethal toxicity. The increased survival of mice pretreated with luteolin is not entirely surprising as flavonoids are known inhibitors of serine/threonine and tyrosine protein kinases (29). Indeed, flavonoids have been described to act as competitive inhibitors with respect to the ATP binding site on a variety of kinases, a region of considerable homology among the kinase family (30, 31). This property enables flavonoids to block the activity of both lipid and protein kinases, such as PI3-K and PKC (32). Inhibition of a broad spectrum of kinases could be advantageous as it could interfere with many cascades involved in sepsis. Moreover, in addition to inhibiting phosphorylation events, flavonoids block the activity of a variety of enzymes involved in inflammation, including lipo- and cyclooxygenases (29, 33, 34). We have previously shown that luteolin blocks LPS-stimulated tyrosine phosphorylation and Akt activation in cultures of the macrophage cell line RAW 264.7,

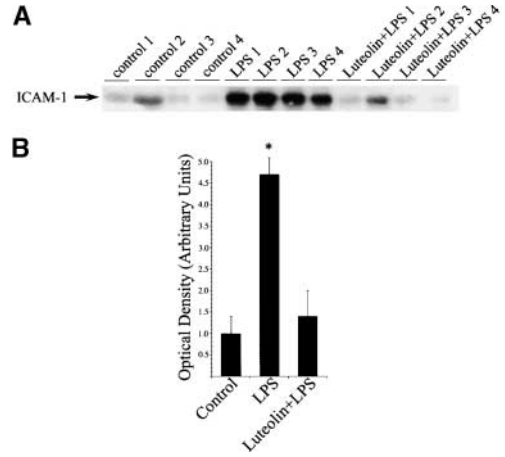

Figure 5. Luteolin attenuates LPS-induced intercellular adhesion molecule-1 in the liver of LPS-treated animals. (*A*) Western blot analysis showing inhibition of LPS-induced ICAM-1 protein expression by luteolin (0.2 mg/kg, intraperitoneally, 30 min before LPS challenge) in mouse liver from four different animals. Tissues were isolated at 8 h following either injection with vehicle, LPS (32 mg/kg, intraperitoneally), or luteolin and LPS. The level of expression was compared with mice receiving vehicle (DMSO). (*B*) Densitometric analysis of ICAM-1 expression in the liver. Data are presented as means ± SEM; n = 6; *p < 0.05 from CTL.

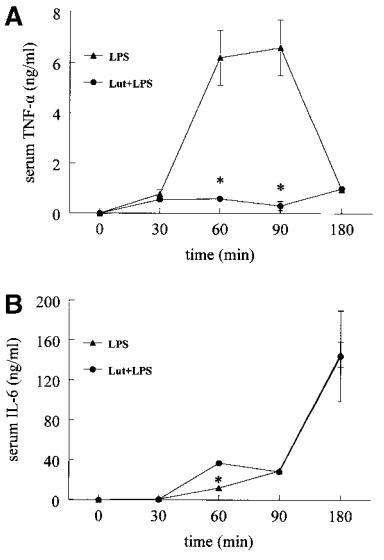

Figure 4. Time dependence of luteolin's effect on serum TNF-α and IL-6 levels. Mice were treated with vehicle (DMSO) or luteolin (0.2 mg/kg, intraperitoneally) for 30 min followed by LPS (32 mg/kg, intraperitoneally). After the indicated time, blood samples were collected and TNF-α (*A*) or IL-6 (*B*) concentration was determined in the serum. Data are presented as means ± SEM; n = 4; *p < 0.05 from LPS.

TABLE 1. TREATMENT WITH LPS VERSUS LUT + LPS*

	Treatment			
	LPS		Lut + LPS	
Animal	Liver	Lung	Liver	Lung
A	++	++	0	0
B	+++	+++	0	0
C	++	+++	0	+
D			0	+

Definition of abbreviations: LPS = lipopolysaccharide; Lut = luteolin.

* Semiquantitative scoring of histological sections from LPS-treated mice: 0 = no leukocyte infiltration; + = one leukocyte aggregate of 10–20 cells/4 mm^2 of tissue section; ++ = one to two leukocyte aggregates of 40 cells/4 mm^2 of tissue section; +++ = more than two leukocyte aggregates of 40 cells/4 mm^2 of tissue section.

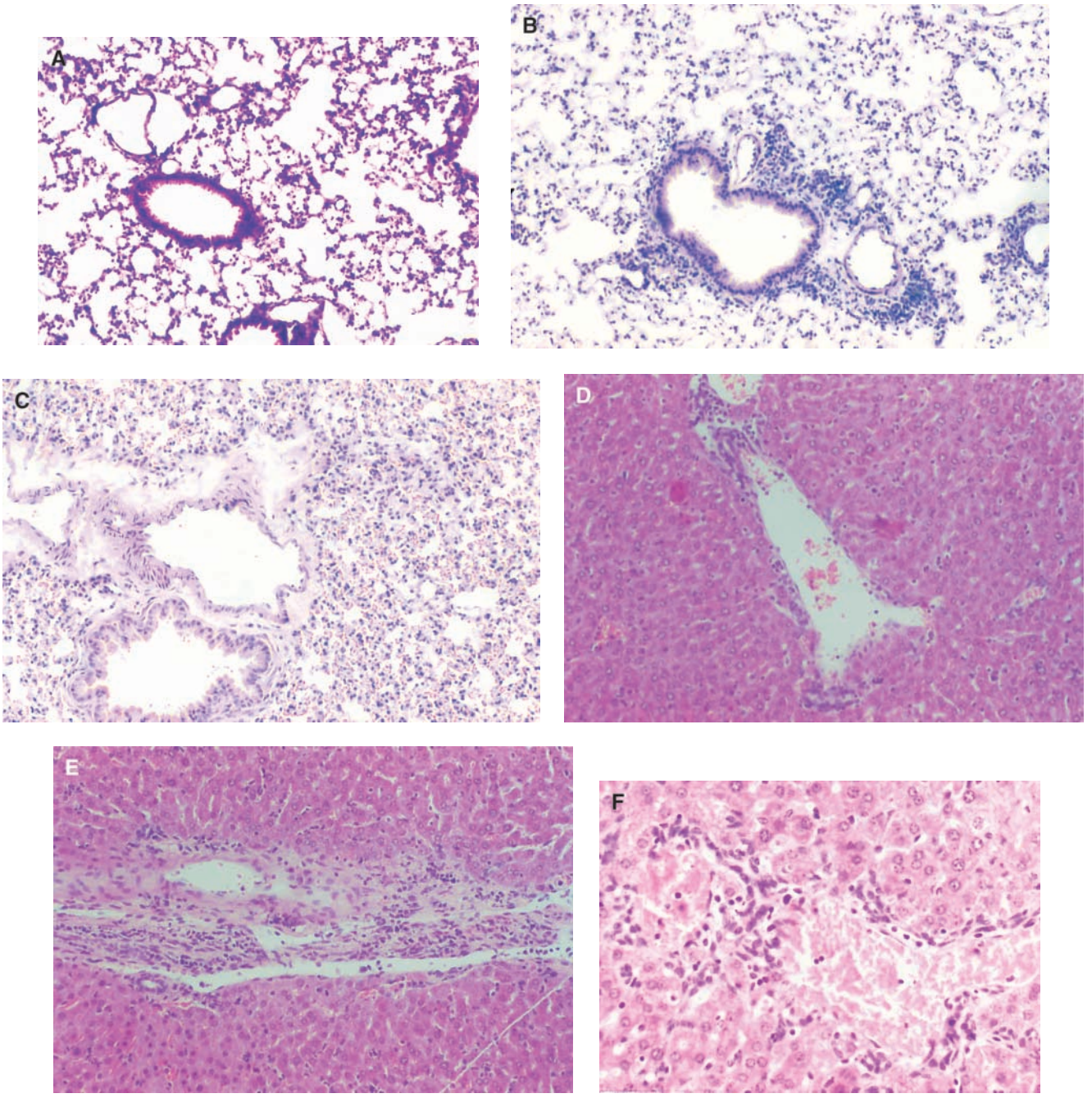

Figure 6. Luteolin abolishes leukocyte infiltration in the lung and liver of LPS-treated animals. Representative photomicrographs of hematoxylin and eosin-stained sections from control (*A*, *D*), LPS (*B*, *E*), or luteolin + LPS (*C*, *F*)-treated mice. *A*, *B*, and *C* are lung sections and *D*, *E*, and *F* are liver sections. Treatments for control, LPS, and Lut + LPS are identical to those described in Figure 2.

leading to inhibition of NF-κB activation (17), a transcription factor known to mediate expression of many inflammatory genes (35). Recently, Takahashi and coworkers (36) compared a series of flavonoids (quercetin, rutin, baicalein, baicalin, daidzein, catechin, and hesperetin) in two models of sepsis. Pretreatment of mice with the flavonoids (4 mg/kg) protected against the lethal response to a high dose of LPS, as well as the lethal response that follows LPS injection in D-galactosamine-sensitized mice. These findings are in line with our observations on the protective effects of luteolin and strengthen our hypothesis that flavonoids can be used to block the cascades triggered by endotoxin *in vivo*.

Most of the studies on luteolin's actions have been performed *in vitro* (isolated organ or cell culture) (17, 20, 37, 38). *In vivo*, luteolin has been reported to exhibit antifertility, antiallergic, and radioprotective properties (39–41). To investigate the mechanisms through which luteolin reduces LPS-induced lethal toxicity we determined the ability of luteolin to decrease serum TNF-α levels in mice challenged with LPS. Treatment of animals with LPS resulted in a time-dependent increase in circulating TNF-α that peaked at 60 to 90 minutes. Pretreatment of mice with luteolin (0.2–1 mg/kg) reduced peak TNF-α production, whereas administration of luteolin after the LPS did not inhibit TNF-α levels. Interestingly, pretreatment of mice with a higher dose of luteolin (10 mg/kg), which failed to protect from the lethal toxicity of LPS, decreased serum levels of TNF-α, suggesting that at high concentrations

luteolin, in addition to inhibiting inflammatory cytokine production, inhibits pathways that offer protection against LPS lethal toxicity. Rutin, another flavonoid, reduced the ability of LPS to increase serum TNF-α levels in D-galactosamine-sensitized mice, but was ineffective in reducing serum TNF-α in mice receiving a high dose of LPS (36).

Unlike serum TNF-α, luteolin did not reduce circulating IL-6 in LPS-treated animals. This is in contrast to our observation that luteolin inhibits LPS-induced IL-6 production from RAW 264.7 and that apigenin, a related flavone, inhibits IL-6 release from endothelial cells (17, 19). IL-6 is produced by a variety of cells *in vivo* in response to LPS, but the relative contribution of each cell type remains unclear. Although there is no evidence showing that luteolin selectively inhibits IL-6 production in some cell types, it is possible that the inability of luteolin to block IL-6 release results from a lack of effect of luteolin on certain IL-6-producing cells. Alternately, luteolin may not be distributed in compartments in which most of the IL-6 production occurs.

Expression of leukocyte-specific adhesion molecules on the endothelial cell surface is induced by LPS or inflammatory cytokines. *In vivo*, during sepsis, up-regulation of leukocyte adhesion molecules promotes leukocyte accumulation in tissues, which enhances inflammation and contributes to the multiple organ dysfunction syndrome (13, 14). Inhibiting the expression of these molecules represents a major target for antiinflammatory therapy. One molecule known to be important for leukocyte trafficking through endothelial monolayers is ICAM-1 (10). Flavonoids, such as apigenin and luteolin, reduce ICAM-1, vascular cell adhesion molecule-1, and E-selectin expression induced by TNF-α and interferon-γ treatment of human endothelial cells (19). Apigenin inhibits neutrophil and peripheral blood lymphocyte adhesion to cytokine-stimulated EC monolayers and blocks TNF-α-induced ICAM-1 expression *in vivo* (12). To test if luteolin is capable of blocking ICAM-1 expression in the context of sepsis, we determined ICAM-1 expression in the liver of LPS-treated mice with or without luteolin pretreatment. The increase in ICAM-1 expression due to LPS was abolished by luteolin. Moreover, leukocyte infiltration in the lung and liver was reduced in the face of luteolin pretreatment, providing evidence that reduced expression of ICAM-1 in luteolin-treated mice correlates functionally with a decreased leukocyte migratory response.

In summary, we have demonstrated that the flavonoid luteolin can protect mice from LPS-induced lethal toxicity. Moreover, luteolin inhibits LPS-induced TNF-α production, ICAM-1 expression, and leukocyte accumulation in tissues. More extensive screen of this class of compounds and preparation of synthetic analogues might yield molecules with increased potency and efficacy that could be useful for patients with sepsis.

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