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Mice Lacking Serum Amyloid P Component Do Not Necessarily Develop Severe Autoimmune Disease

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Serum amyloid P component (SAP) is a major acute-phase reactant in mice. Recently, it was reported that SAP-deficient mice spontaneously developed anti-nuclear antibodies and severe glomerulonephritis. Because the SAP-deficient mice we generated display no obvious phenotypic abnormalities, we investigated whether our SAP-deficient mice would also spontaneously develop autoimmune responses. In accordance with the report, our mice produced high titers of anti-nuclear antibody but did not develop severe glomerulonephritis. On the other hand, it was recently reported that SAP bound to gram-negative bacteria via lipopolysaccharide (LPS) prevented LPS-mediated activation of a classical complement pathway. Thus, we asked if SAP-deficient mice would show altered responses to an intraperitoneal injection of LPS from *Salmonella typhimirium*. SAP-deficiency did afford resistance to lethality induced by high-dose LPS. Our experiments clearly showed that contrary to documented data, SAP-deficient mice do not develop serious autoimmune disease and we suggest that SAP has a critical role in LPS toxicity. © 2001 Academic Press

Key Words: **acute-phase reaction; autoimmune response; endotoxic shock; knockout mouse; pentraxin; serum amyloid P component.**

Serum amyloid P component (SAP) is a plasma glycoprotein with a characteristic pentameric organization of 10 identical subunits of 25.5 kDa arranged as double annular pentagonal discs interacting face to face. SAP and C-reactive protein (CRP) belong to pentraxins, the family of calcium-dependent ligand binding proteins highly conserved throughout evolution. SAP binds to many ligands such as glycosaminoglycans, pyruvate acetal of galactose, DNA, chromatin, several complement components, calumenin, a calcium binding protein, and lipopolysaccharide (LPS). The physiological functions have remained unclear (1–4). As SAP binds to amyloid fibrils, it accumulates in all types of amyloid deposits. We and others generated SAP-deficient ($sap^{-/-}$) mice through gene targeting and found that the induction of AA amyloidosis in the presence of inflammation is significantly delayed in the $sap^{-/-}$ mice relative to wild-type ($sap^{+/+}$) mice (5, 6). On the other hand, SAP-binding to nuclear components is speculated to play a role in the clearance of cellular debris at sites of inflammation (1–3). In accordance with this speculation, it was reported that the $sap^{-/-}$ mice spontaneously developed anti-nuclear antibodies and severe glomerulonephritis, a phenotype resembling the human serious autoimmune disease, systemic lupus erythematosus (7). Because the $sap^{-/-}$ mice we generated have no obvious phenotypic abnormalities and their longevity is normal, we determined if our $sap^{-/-}$ mice would also spontaneously develop autoimmune responses.

SAP is a major acute-phase reactant in mice (3). It was demonstrated that SAP bound to LPS, or endotoxin, and gram-negative bacteria via LPS prevented LPS-mediated activation of a classical complement pathway (2, 8). On the other hand, SAP bound to C1q was seen to activate a classical complement pathway (9). As a first step toward elucidating the exact role of SAP in LPS toxicity, we researched $sap^{-/-}$ mice for altered responses to an intraperitoneal injection of LPS from *Salmonella typhimirium*.

MATERIALS AND METHODS

Mice. $Sap^{-/-}$ mice were generated with the use of CCE embryonic stem cells, as described (6). The mutant mice were bred for four

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generations back into C57BL/6, and $sap^{-/-}$ and $sap^{+/+}$ littermates from the heterozygote matings were analyzed. Mice were maintained in a conventional environment in the Institute of Experimental Animals of Yamanashi Medical University. Genotype analysis of each mouse was carried out by polymerase chain reaction on DNA from mouse tails and was confirmed by immunoblotting of serum samples with the use of rabbit anti-mouse SAP antibody (Calbiochem–Novabiochem, La Jolla, CA), as described (6). C57BL/6 and MRL/MpJ-*lpr*/*lpr* lupus mice (10) were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Anti-nuclear antibody assay. The titers of anti-nuclear antibody in the sera of mice were measured by indirect immunofluorescence on Hep-2 cells with the use of fluoresceinated goat anti-mouse immunoglobulin antibody (Dako A/S, Glostrup, Denmark) and an assay kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The titers are represented as the end point of twofold serial dilutions of sera giving positive immunofluorescence.

Measurement of urinary protein levels. The urinary total protein levels of mice were measured, using protein assay kits (Bio-Rad, Hercules, CA).

Histopathological analysis. Kidneys of mice were fixed in 10% formalin, embedded in paraffin, and the deparaffinized tissue sections were stained with hematoxylin and eosin for light microscopy.

LPS-injection. Purified LPS extracted from *Salmonella typhimirium* (Sigma–Aldrich Co., St. Louis, MO) was dissolved in sterile phosphate-buffered saline at 2 mg/ml, then boiled for 5 min prior to freezing in aliquots at −20°C. For analysis of acute-phase protein gene expression, acute inflammation was provoked in groups of two male and two female $sap^{-/-}$ and $sap^{+/+}$ littermates from heterozygote matings by a single intraperitoneal injection of 10 mg/kg (body weight) LPS. The LPS-injected mice were killed following anesthetization with ether, at the time indicated and the excised liver was immediately placed in a liquid nitrogen. $Sap^{-/-}$ and $sap^{+/+}$ littermates were also followed for survival time after a single intraperitoneal injection of 15 mg/kg (body weight) LPS. Survival of LPS-injected female and male mice was determined separately.

Northern blot analysis. Total cellular RNA was extracted from the livers, using 4 M guanidine thiocyanate (Fluka, Neu-Ulm, Switzerland) and was purified by centrifugation through 5.7 M CsCl (11). Ten micrograms of total RNA extracted from the livers was separated by formaldehyde agarose gel electrophoresis, and transferred onto nylon membranes (Hybond-N+, Amersham, Bucks, UK). The RNA was cross-linked to the membrane by exposure to ultraviolet light (UV Stratalinker 1800, Stratagene, La Jolla, CA) and hybridized with the following four ^{32}P-labeled probes; an 835-bp *Pst*I/*Ava*I fragment of mouse CRP cDNA (12), a 404-bp haptoglobin (Hp) cDNA fragment isolated from LPS-treated mouse liver by reverse transcription-polymerase chain reaction with the use of primers (TCGGGCTAATCAAACTCAA and CACCATACTCAGCGACAGC) that amplify mouse Hp cDNA (13), a 546-bp serum amyloid A (SAA) cDNA fragment isolated, as described above, with the use of primers (GAGACACCAGCAGGATGA and TTACCCTCTCCTCCTCAA) that amplify mouse SAA cDNA (14), and a 472-bp α1-acid glycoprotein (α-AGP) cDNA fragment isolated, as described above, with the use of primers (CCCCAACTTGATAAATGACA and CAGACAGAATCAAAGTGC) that amplify mouse α-AGP cDNA (15). The blotted membranes were prehybridized for more than 5 h at 42°C in buffer containing 50% formamide, 5 × standard sodium phosphate EDTA (SSPE) solution (20 × SSPE solution; 3 M NaCl, 0.2 M Na_2HPO_4, 25 mM EDTA), 5 × Denhardt's solution (0.1% polyvinylpyrolidone, 0.1% Ficoll, and 0.1% BSA), and 0.5% SDS in the presence of single stranded salmon sperm DNA, then hybridized for over 16 h at 42°C in prehybridization buffer with one of the ^{32}P-labeled cDNA probes. The membranes were sequentially hybridized to detect the four major acute-phase protein mRNA's. The membranes were washed in 2 × SSPE at room temperature for 10 min, twice in 2 × SSPE/0.2% SDS at 63°C for 15 min each, and in 0.1 × SSPE/0.2% SDS at 63°C for 15 min, then subjected to autoradiography. Transferred RNA on the membranes was stained with methylene blue for loading control. Intensity of the hybridizing band was quantified with use of the BAS 2000 System (Fuji Photo Film Co. Ltd., Tokyo, Japan) and normalized to 28S ribosomal RNA level, estimated from the intensity of methylene blue-staining.

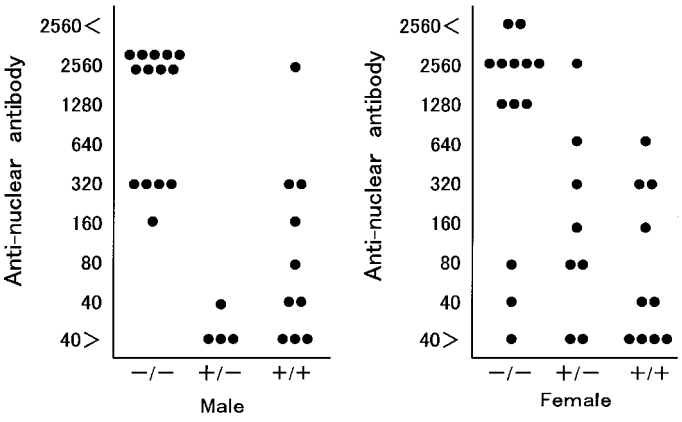

FIG. 1. Anti-nuclear antibody levels in sera of 11- to13-month-old $sap^{-/-}$ (−/−), $sap^{+/-}$ (+/−), and $sap^{+/+}$ (+/+) mice. The titers are represented as the end point of twofold serial dilutions of sera giving positive immunofluorescence. Circles represent one mouse.

Statistical methods. The difference in the titers of serum anti-nuclear antibody between mutant and wild-type mice was examined using the Mann–Whitney test. The difference in the susceptibility to lethal effects of LPS between mutant and wild-type mice was examined using the Kaplan–Meier analysis. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Autoimmune phenotype. It was reported that $sap^{-/-}$ mice generated through gene targeting spontaneously produced high titers of anti-nuclear antibodies and autoantibodies against chromatin, DNA, and histones and had a high incidence of severe proliferative glomerulonephritis, a phenotype resembling human systemic lupus erythematosus. The frequency and titer of anti-nuclear antibody in the mice increased with age. At age 8 months, a high titer of anti-nuclear antibody was present in 63.3% of male and 82% of female $sap^{-/-}$ mice, compared with 17.3% of male and 50.9% of female $sap^{+/+}$ mice. Severe glomerulonephritis was detected in 3.9% of male and 41.8% of female $sap^{-/-}$ mice compared with 1.9% of male and 9.3% of female $sap^{+/+}$ mice at the same age (7). Because we had also generated $sap^{-/-}$ mice (6), we wanted to determine if the level of anti-nuclear antibody in sera of our 11- to 13-month-old $sap^{-/-}$ mice was higher than that of age-matched $sap^{+/+}$ mice. The titers of anti-nuclear antibodies in the serum of 27 $sap^{-/-}$ mice were significantly higher than that in 20 $sap^{+/+}$ or 12 heterozygous ($sap^{+/-}$) mice (Fig. 1) ($P < 0.05$; Mann–Whitney test). However, the longevity and fertility of our $sap^{-/-}$ mice appeared to be much the same with that of $sap^{+/+}$ mice.

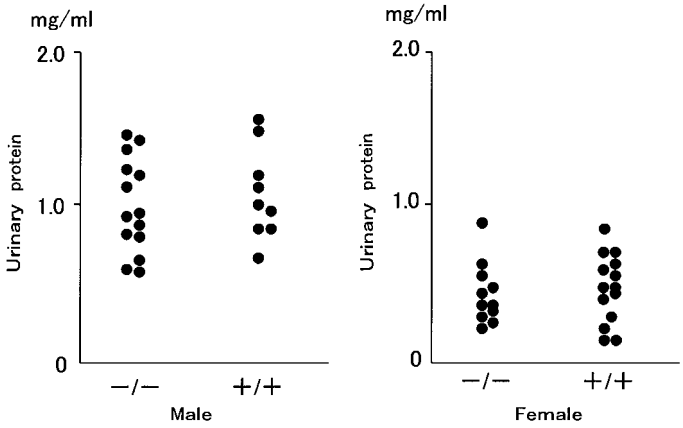

FIG. 2. Total urinary protein levels of 11- to 20-month-old male and female *sap*$^{-/-}$ (−/−), and *sap*$^{+/+}$ (+/+) mice. Circles represent one mouse.

We next measured total urinary protein levels of 11 female and 14 male *sap*$^{-/-}$ and 14 female and 9 male *sap*$^{+/+}$ mice (11 to 20 months old) to identify mice which developed severe glomerulonephritis. For this we used the urine of 19-week-old female MRL/MpJ-*lpr*/*lpr* lupus mice which have a high incidence of lethal glomerulonephritis resembling human lupus nephritis (10), as a positive standard. In both genotypes, the males tend to have higher urinary protein levels relative to females but there was no significant difference in mean levels between *sap*$^{-/-}$ and gender-matched control *sap*$^{+/+}$ mice (Fig. 2). Urinary protein levels of all the 25 *sap*$^{-/-}$ and 23 *sap*$^{+/+}$ mice examined were lower than that of MRL/MpJ-*lpr*/*lpr* female mouse which was 1.93 mg/ml. Although the daily urinary protein excretion of mice was not measured, the finding of no significant difference in the urinary protein levels between the *sap*$^{-/-}$ and *sap*$^{+/+}$ mice and the levels of none of the *sap*$^{-/-}$ and *sap*$^{+/+}$ mice examined higher than that of 19-week-old female MRL/MpJ-*lpr*/*lpr* lupus mouse suggested that our *sap*$^{-/-}$ mice did not spontaneously develop severe glomerulonephritis. To confirm the absence of severe glomerulonephritis in the kidneys of *sap*$^{-/-}$ mice, we histopathologically examined the kidneys of 13 female and 14 male *sap*$^{-/-}$ mice and 8 female and 5 male *sap*$^{+/+}$ mice (11 to 20 months old), and the 19-week-old female MRL/MpJ-*lpr*/*lpr* lupus mouse. As shown in Fig. 3, although typical proliferative glomerulonephritis was present in kidneys from 19-week-old female MRL/MpJ-*lpr*/*lpr* lupus mouse, it was absent in those from any of 27 *sap*$^{-/-}$ and 13 *sap*$^{+/+}$ mice. These data clearly show that the *sap*$^{-/-}$ mice we generated do not develop severe glomerulonephritis. The reason for the discrepancy between data of other authors and our data is not clear. The genetic background of mice (129/Sv × C57BL/6; F2) examined by Bickerstaff *et al.* differed from that examined in the present study (see Materials and Methods), and the specific name of the embryonic stem cell line used to generate the *sap*$^{-/-}$ mice analyzed in a previous report was not mentioned (5). This may reflect minor genetic differences between the two lines of mutant mice used. There have been several reports of the strain background affecting the phenotypical effect of a gene knockout. For example, the epidermal growth factor-deficient mice were growth-retarded and died at different stages of development depending on their genetic background (16, 17). Similarly, D2 dopamine receptor-deficient mice exhibited different ability to balance and walk on a rotating cylinder depending on their genetic background (18).

In accordance with the previous report (7), our *sap*$^{-/-}$ mice produced high titers of anti-nuclear antibody. Paradoxically, although *sap*$^{-/-}$ mice degraded exogenous chromatin more rapidly than did *sap*$^{+/+}$ mice, they spontaneously generated autoantibodies against chromatin (7, 19). Therefore, SAP-deficiency may not be a direct cause of high titers of anti-nuclear antibodies. As suggested by Bickerstaff *et al.*, the 129/Sv or 129/Sv//Ev mice may carry a gene located close to the deleted *sap* gene thus conferring susceptibility to anti-nuclear autoimmunity when expressed in the C57BL/6 genetic background. Analyses of *sap*$^{-/-}$ mice carrying

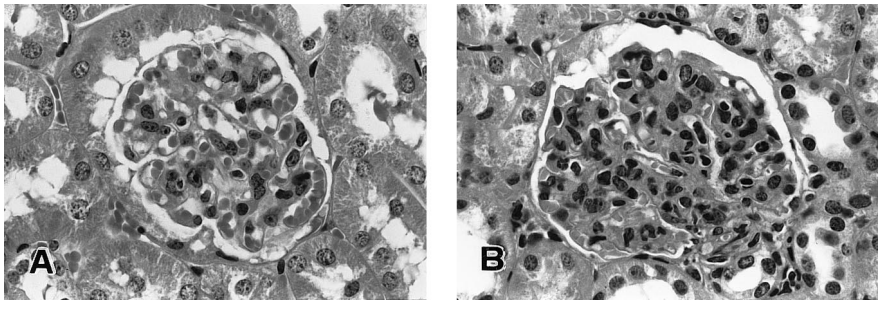

FIG. 3. Microscopic findings of the glomerulus of a 13-month-old female *sap*$^{-/-}$ mouse (A) and of a 19-week-old female MRL/MpJ-*lpr*/*lpr* lupus mouse (B). (A) The glomerulus in the *sap*$^{-/-}$ mouse showed no remarkable changes (H & E, ×200). (B) MRL/MpJ-*lpr*/*lpr* lupus mouse showed characteristic diffuse cell-proliferative glomerular changes with an accumulation of mononuclear and polymorphonuclear cells (H&E, ×200).

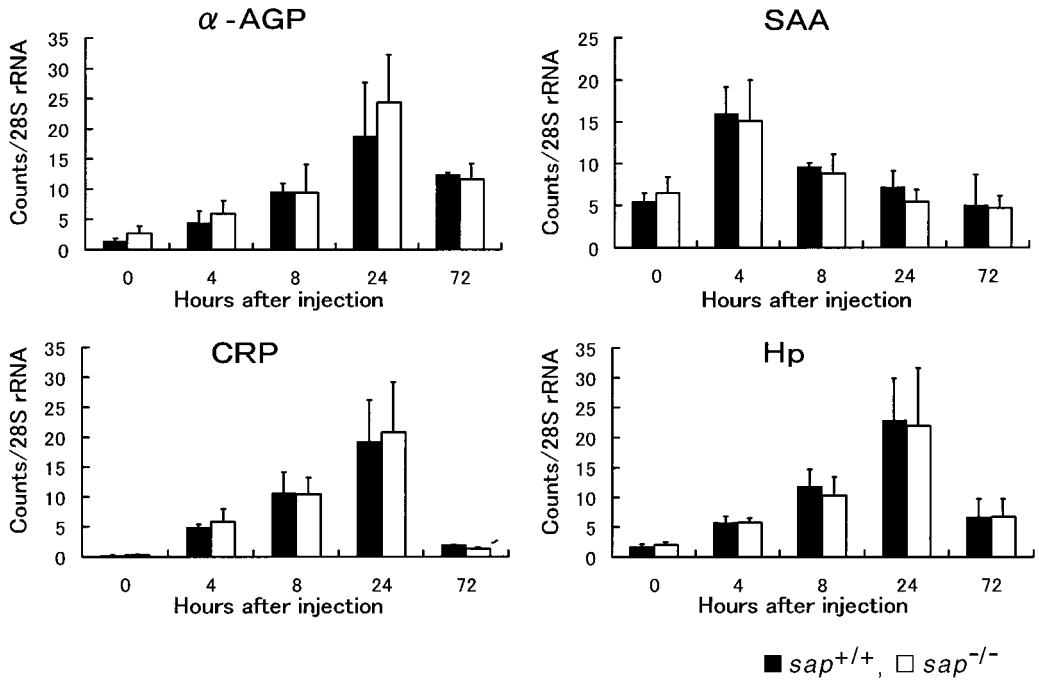

FIG. 4. Acute-phase protein gene expression following LPS administration. Acute inflammation was provoked in groups of two male and two female $sap^{-/-}$ and $sap^{+/+}$ littermates from heterozygote matings by giving a single intraperitoneal injection of 10 mg/kg (body weight) LPS. At 0, 4, 8, 24, and 72 h after LPS injection, the mice were killed, total liver RNA's were isolated, and sizes and relative levels of acute-phase protein mRNA's were determined by Northern blot hybridization to the respective cDNA probes. Values are the averaged expression of each acute-phase protein mRNA normalized with 28S rRNA.

and expressing the mouse *sap* gene as a transgene are needed to further investigate the exact role of SAP in the development of autoimmune response.

Induction of major acute-phase reactant genes in the liver of SAP-deficient and wild-type mice following LPS injection. Although SAP is a major acute-phase serum reactant in mice (3, 20), the exact function in acute-phase is unclear. As a first step toward determining the function of SAP in acute-phase, we investigated whether or not the induction-pattern of several major acute-phase reactant genes during acute-inflammation was altered in the $sap^{-/-}$ mice relative to $sap^{+/+}$ mice. The kinetics of change in expression of liver acute-phase proteins, including α-AGP, SAA, CRP, and Hp were determined by quantitating the RNA levels following an intraperitoneal injection of 10 mg/kg (body weight) *Salmonella typhimirium* LPS, as described under Materials and Methods. At 0, 4, 8, 24, and 72 h after LPS injection, the mice were killed, total liver RNA's were isolated, and sizes and relative levels of acute-phase protein mRNA's were determined by Northern blot hybridization to the respective cDNA probes. As shown in Fig. 4, significant differences were never detected in the amount of mRNA induced for any of the acute-phase proteins examined between the $sap^{-/-}$ and $sap^{+/+}$ mice. SAP and CRP are members of the pentraxin family of proteins conserved through evolution and are characterized by pentameric assembly and Ca-dependent high affinity for specific ligands (1, 21). These findings suggest that SAP and CRP may have similar essential functions during acute-phase. However, we suggest that the function of SAP during acute-phase, as induced by LPS-injection may not be compensated for by other acute-phase proteins, including CRP, α-AGP, Hp, and SAA.

Survival of mice following LPS injection. It was reported that SAP binds to all forms of LPS via the lipid A part of the molecule *in vitro* and neutralizes the biologic effects of LPS (22). To examine the role of SAP in LPS toxicity or endotoxic shock, we monitored $sap^{-/-}$ mice for survival after administering a single intraperitoneal injection of 15 mg/kg (body weight) LPS from *Salmonella typhimirium*. Fourteen female and thirteen male $sap^{-/-}$ mice (3 to 4 months old) and 12 female, and 13 male $sap^{+/+}$ mice were monitored. In both genotypes, females were more resistant than males to the lethal action of LPS (Fig. 5). Both female and male $sap^{-/-}$ mice were less susceptible than gender-matched $sap^{+/+}$ mice to LPS-induced endotoxic shock but a statistically significant difference was observed only between $sap^{-/-}$ and $sap^{+/+}$ males ($P < 0.05$; Kaplan–Meier analysis). With a higher-dose of LPS (30 mg/ml), the difference was nil (data not shown).

As noted above, SAP bound to LPS neutralized the biologic effects of LPS. The inhibitory effect of SAP was

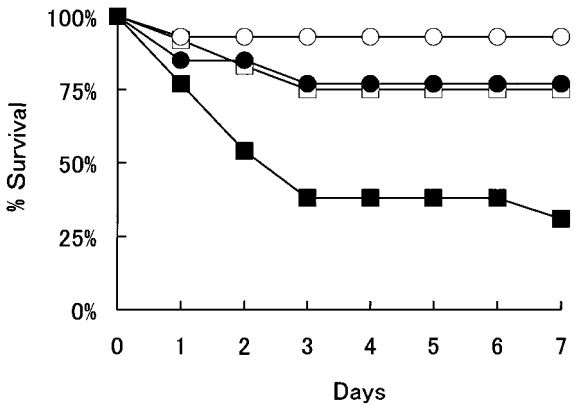

FIG. 5. Survival after a single intraperitoneal administration of 15 mg/kg (body weight) LPS. Fourteen female (open circle), and 13 male (open square) $sap^{-/-}$ mice and 12 female (closed circle), and 13 male (closed square) $sap^{+/+}$ mice were monitored.

reported to be strongly reduced in the presence of serum concentrations over 0.1% (22). Thus SAP may not directly neutralize LPS *in vivo.* Besides binding to LPS in its isolated form, SAP binds to gram-negative bacteria via LPS and prevents LPS-mediated activation of classical complement pathway (2, 8). On the other hand, SAP bound to C1q was found to activate a classical complement pathway (9). Therefore, it is difficult to speculate on the net outcome of *in vivo* functions of SAP in LPS toxicity or infection with gram-negative bacteria. It was recently reported that although $sap^{-/-}$ mice survived lethal infection with *Streptococcus pyrogenes* and *E. coli* J5, they were more susceptible than controls to lethal infection with *E. coli* O111:B4 (23). Although activation of a host defense system is essential during acute-phase to fight infectious agents, it has to be restrained in order to avoid self-injury or endotoxic shock. The finding that $sap^{-/-}$ mice are less susceptible than controls to endotoxic shock suggests that SAP may play an important role in modulating the inflammatory responses in LPS toxicity. This finding seems in contrast with the recent finding of Noursadeghi *et al.*, who could not demonstrate any significant difference in lethality induced by high-dose LPS between $sap^{-/-}$ and $sap^{+/+}$ mice (23). The difference may also be due to subtle variations in genetic background of the two lines of $sap^{-/-}$ mice examined (5, 6, 23). Analyses of $sap^{-/-}$ mice expressing the mouse *sap* transgene should confirm the exact role of SAP in modulation of LPS toxicity. $Sap^{-/-}$ mice will be useful to determine the exact role of SAP in acute-phase reactions.

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