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The Novel Lipopolysaccharide-Binding Protein CRISPLD2 Is a Critical Serum Protein to Regulate Endotoxin Function

Zhi-Qin Wang,†* Wen-Ming Xing,†* Hua-Hua Fan,‡† Ke-Sheng Wang,* Hai-Kuo Zhang,* Qin-Wan Wang,* Jia Qi,* Hong-Meng Yang,* Jie Yang,† Ya-Na Ren,‡ Shu-Jian Cui,* Xin Zhang,* Feng Liu,* Dao-Hong Lin,‡ Wen-Hui Wang,‡ Michael K. Hoffmann,§ and Ze-Guang Han3* and Ze-Guang Han3*

LPS is an immunostimulatory component of Gram-negative bacteria. Acting on the immune system in a systemic fashion, LPS exposes the body to the hazard of septic shock. In this study we report that cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2/Crispld2; human and mouse/rat versions, respectively), expressed by multilattices and leukocytes, is a novel LPS-binding protein. As a serum protein, median CRISPLD2 concentrations in health volunteers and umbilical cord blood samples are 607 μg/ml and 290 μg/ml, respectively. Human peripheral blood granulocytes and mononuclear cells including monocytes, NK cells, and T cells spontaneously release CRISPLD2 (range, 0.2–0.9 g/ml) in response to stimulation of both LPS and humanized anti-human TLR4-IgA Ab in vitro. CRISPLD2 exhibits significant LPS binding affinity similar to that of soluble CD14, prevents LPS binding to target cells, reduces LPS-induced TNF-α and IL-6 production, and protects mice against endotoxin shock. In vivo experiments, serum Crispld2 concentrations increased in response to a nontoxic dose of LPS and correlated negatively with LPS lethality, suggesting that CRISPLD2 serum concentrations not only are indicators of the degree of a body’s exposure to LPS but also reflect an individual’s LPS sensitivity. The Journal of Immunology, 2009, 183: 6646–6656.

Plant and animal life evolved in a symbiotic relationship with the microbial world. Higher animals as well as humans are exceedingly conscious of structural and functional microbial components and entrust them with vital roles in the regulation of their life functions (1, 2). Decades ago immunologists identified, in the cell wall of Gram-negative bacteria, a highly bioactive LPS composed of a monomeric lipid core and a polymorphic polysaccharide coat that stimulates immune functions in a systemic rather than local fashion (3). LPS-producing Gram-negative bacteria pose a threat to the health of mammals and may kill them by septic shock (4, 5).

Mammals express an LPS-binding protein (LBP)4 that is considered a critical molecule in LPS-elicted activation cascades. LBP assembles upon reaction with LPS and several molecules on the cell membrane, thus forming a molecular signal transduction complex (6–11) that initiates the clonal expansion of immunocytes or the secretion of immunoregulatory cytokines (11). LPS-binding reagents that down-regulate immunological LPS activities have been reported (12–15). Of note is CD6, belong to the scavenger receptor cysteine-rich superfamily, which is readily expressed on the surface of lymphocytes but maintains a low serum concentration (16). It exhibits significant LPS-binding affinity, and its soluble form has been shown to inhibit the induction of endotoxin shock in mice (17). Passively administered LPS-reactive Abs have also been shown to ameliorate Gram-negative sepsis (18–20).

In this study we present an LPS-binding molecule, cysteine-rich secretory protein (CRISP) LCCL domain containing 2 (CRISPLD2 and Crispld2, representing human and mouse/rat versions, respectively), which was previously known for a variety of other functions. Because it is also known as late gestation lung 1 (Lgl1), it has been implicated in the development of rat lung (21–23) and mouse kidney (24) and is thought to be involved in the development of nonsyndromic cleft lip with or without cleft palate (25). Its possession of two LCCL domains suggests a relationship to LPS. LCCL structures were initially described in the horseshoe crab (Limulus) factor C, which, by binding LPS, initiates the Limulus coagulation cascade to protect the crab against bacterial infection (26–28). It seemed conceivable that mammalian Crispld2, with two LCCL domains, exhibits particular avidity for LPS. Several known LPS-binding components of pulmonary surfactant, such as surfactant proteins A and D, have been implicated in an Ab-independent host defense against pathogens (29, 30). Previously, the analysis of leukocytes from healthy human volunteers

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4 Abbreviations used in this paper: LBP, LPS-binding protein; CHO, Chinese hamster ovary; CRISP, cysteine-rich secretory protein; CRISPLD2/Crispld2, CRISP LCCL domain containing 2 (human/mouse or rat); hTLR4, human TLR4; LTA, lipoteichoic acid; ORF, open reading frame; RU, relative response unit; SPR, surface plasmon resonance.

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i.v. administered bacterial endotoxin has shown the rapidly increased transcript of CRISPLD2 but not CRISPLD1 within 2 h (31), suggesting that transcription of CRISPLD2 in leukocytes is immediately initiated in response to LPS challenge. The present work introduces mammal CRISPLD2 as a major serum protein that acts as a natural LPS antagonist and promises to be of considerable preventative value against endotoxemic shock.

Materials and Methods

Bacterial components, mAbs, and rCRISP-3 protein

Escherichia coli (O127:B8 and O55:B5) LPS was purchased from Sigma-Aldrich and dissolved in PBS at 1 mg/ml. Alexa Fluor 488-labeled E. coli (O55:B5) and Salmonella enterica serovar Minnesota LPS was purchased from Invitrogen and dissolved in PBS at a final concentration of 1 mg/ml. Purified Staphylococcus aureus lipoteichoic acid (LTA), ultra-pure Rhodobacter sphaeroides LPS, and chimeric anti-human TLR4 (hTLR4)-Rhodobacter sphaeroides from Invitrogen and dissolved in endotoxin-free water according to the manufacturer’s instruction. Anti-hTLR4 (HTA125) and OKT3 mAbs were purchased from eBioscience. Human IgA, IgG, and IgM mAbs were purchased from InvivoGen and dissolved in PBS at a final concentration of 1 mg/ml. Purified LPS, and chimeric anti-human TLR4 (hTLR4) were injected into the flow cells at a rate of 20 μl/min. Sterilized and deaerated PBS was used as the running buffer during experiments. The sensorgram and relative response units (RU) for the binding of LPS to immobilized CRISPLD2 were obtained by subtracting the background of the LPS binding to immobilized, unrelated IgG. Following removal of the unbound LPS by injection with PBS, the bound LPS was removed by quick injection with 50 mM NaOH at a flow rate of 50 μl/min. A saturation binding curve was depicted as a function of LPS concentrations vs RU.

LPS binding affinity analysis

The dissociation constant of LPS (K_{D}) was calculated by Scatchard plot analysis using the following formula: \[ K_{D} = \frac{1}{slope} = \frac{(RU_{\text{max}} - RU)}{(RU/\text{Conc}_{\text{LPS}})}, \] where RU_{\text{max}} is maximum response and \text{Conc}_{\text{LPS}} is the concentration of LPS. In addition, K_{D} could be calculated as the ratio of these two constants (\text{RU}_{\text{on}}/\text{RU}_{\text{off}}). In brief, the dissociation rate constant \text{k}_{\text{off}} was described by the equation \[ \text{RU}_{\text{off}} = \text{RU}_{\text{on}}(1 - e^{-\text{k}_{\text{off}}t}), \] where RU_{\text{off}} is the concentration of bound LPS at time t. The association rate constant \text{k}_{\text{on}} could be derived when the measured RU_{\text{on}} value using: \[ \text{RU}_{\text{on}} = \frac{\text{RU}_{\text{max}}}{1 + \text{R}_{\text{t}}}, \] where RU_{\text{on}} represents the maximum binding capacity of LPS to immobilized CRISPLD2, and R_{t} represents the amount of LPS bound to the CRISPLD2 at time t. The dissociation rate constant \text{k}_{\text{off}} was determined when the LPS passing over CRISPLD2 on the surface of chip is replaced by PBS alone.

Northern blot analysis and RT-PCR

Northern blotting was performed by using a human multiple tissue Northern blot membrane (Clontech) according to the manufacturer’s instruction. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. Hybridization of a probe to human β-actin in the same membrane was used as a loading control. For RT-PCR, total RNA from human immune cells and mice tissues was extracted by TRizol reagent (Invitrogen). Reverse transcription was performed in 20 μl reactions using 2 μg of total RNA. Each PCR was generally performed with ~30–32 thermal cycles, and the PCR products were detected by electrophoresis on a 2% agarose gel with 1X TAE buffer. The PCR primers and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'. The probe for detecting CRISPLD2 expression was amplified by PCR using the F1 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA-3' and the R2 reverse primer 5'-TGCTCGAGATCATGCCTGACAG-3'.
of TLR4 transcript in T cells, the CD4+ T cells (98% purity) were first isolated by negative selection of CD4+ T cells and then, in second, purified by a CD14 monoclonal negative selection kit (Miltenyi Biotec). To assess CRISPLD2 on cell surfaces, PBMCs (10⁶ cells/ml) in PBS with 1% inactivated human serum were seeded into 96-well plates and incubated with 15 µg/ml rabbit anti-CRISPLD2 polyclonal Ab for 30 min. After washing three times with buffer, the cells were stained with Cy2-labeled goat anti-rabbit IgG (Jackson Immunotechnology) for 30 min. Non-specific rabbit IgG was used as a negative control. To determine intracellular CRISPLD2, immunofluorescence assays were used to simultaneously detect surface cluster of differentiation markers and intracellular CRISPLD2 of these cells. PBMCs were seeded into 96-well plates, stained with PE-labeled (red) anti-CD4, CD8, CD56, or CD14 mAb (BD Biosciences) in the presence of 2% inactivated human serum, and then fixed with 4% parafomaldehyde in PBS. After washing twice with Perm/Wash buffer (BD Pharmingen), the cells were incubated with either rabbit anti-CRISPLD2 polyclonal Ab or normal rabbit IgG for 15 µg/ml for 1 h. The cells were washed three times with 1× Perm/Wash buffer again and stained by Cy2-labeled (green) goat anti-rabbit IgG for 30 min. T cell, NK cell, and monococyte-specific surface markers and the intracellular CRISPLD2 were detected by a FACSCalibur flow cytometer (BD Biosciences). T cells, NK cells, and monocytes were gated according to their characteristic surface marker vs forward scatter characteristics. Granulocytes were gated according to their typical forward vs side scatter characteristics.

**Immunoblotting analysis**

The samples of serum and plasma from human volunteers and mice were diluted 1/10 in PBS, separated by electrophoresis in 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes (GE Healthcare). The blots were blocked at 2% inactivated human serum, and then fixed with 4% parafomaldehyde in PBS. After washing twice with PBS for 1 h, the membranes were incubated with either rabbit anti-CRISPLD2 polyclonal Ab or normal rabbit IgG for 15 µg/ml for 1 h. The membranes were washed three times with 1× PBS and then stained by Cy2-labeled (green) goat anti-rabbit IgG for 30 min. T cell, NK cell, and monococyte-specific surface markers and the intracellular CRISPLD2 were detected by a FACSCalibur flow cytometer (BD Biosciences). T cells, NK cells, and monocytes were gated according to their characteristic surface marker vs forward scatter characteristics. Granulocytes were gated according to their typical forward vs side scatter characteristics.

**Immunofluorescence assay and immunohistochemical staining**

For identification of Crisp2 in mouse and rat tissues, freshly sacrificed mice and rats were transcardially perfused with 4% paraformaldehyde in PBS. The excised tissues were fixed in the same buffer. Paraffin-embedded tissue sections (6 µm) were stained using a microwave oven. The sections were deparaffinized and rehydrated and the mouse placenta tissue was excised and frozen then sectioned with a cryostat at 6  µm. All tissue sections on glass slides were stained for 60 min with anti-CRISPLD2 Ab diluted in PBS containing 1% Tween 20. For immunofluorescent staining, Cy2-labeled secondary Ab was used for staining of the primary Ab. Hoechst dye was used for nuclear staining. Immunofluorescence was visualized using a confocal microscope (LSM 510, Zeiss). For immunohistochemical staining, the HRP-conjugated anti-rabbit secondary Ab (Dako-Cytomation) was used as the secondary Ab, and the signals were detected using a diaminobenzidine substrate kit (Vector Laboratories). Nuclear staining was conducted by hematoxylin.

**LPS binding to immune cells**

PBMCs were seeded into 96-well plate at 2 × 10⁶ cells/ml in PBS containing 0.3% BSA with different doses of rCRISPLD2 or rCRISP-3 in 0.018, 0.054, 0.178, 0.535, 1.78, and 5.35 µM) in the presence of Alexa Fluor 488-labeled E. coli LPS (1.0 and 3.0 µg/ml) or S. enterica serovar Minnesota LPS (9.0 µg/ml) for 30 min. After washing twice, the mean fluorescent intensity of Alexa Fluor 488 on monocytes and lymphocytes was examined by a FACSCalibur flow cytometer (BD Biosciences). Monocyte and lymphocyte regions were distinguished according to their forward vs side scatter characteristics. The fluorescence signal was represented by mean fluorescence intensity units.

**Stimulation of immune cells in vitro**

For assessing CRISPLD2 secretion from cells in vitro, human granulocytes at 1 × 10⁶ cells/ml, PBMCs, purified T cells, and NK cells at 2 × 10⁶ cells/ml, and purified monocytes at 0.5 × 10⁶ cells/ml were seeded into 96-well plates, stimulated by LPS and LTA, respectively, in RPMI 1640 medium containing 0.1% FCS, and incubated in 5% CO₂ at 37°C. To determine the reduction by CRISPLD2 of LPS-induced TNF-α and IL-6, PBMCs at 1 × 10⁶ cells/ml were incubated in the presence of multiple concentrations of rCRISPLD2 or rCRISP-3 in RPMI 1640 medium with 0.3% FCS in 5% CO₂ at 37°C and stimulated by LPS (10 µg/ml). To assess the regulatory function of endogenous and exogenous CRISPLD2, human PBMCs were incubated in RPMI 1640 with 0.1% FCS in 5% CO₂ at 37°C, and the other supplement regents, including human serum, rCRISPLD2, E. coli LPS, anti-CRISPLD2 Ab, and control Ab, were used.

**ELISA**

Commercial TNF-α and IL-6 ELISA kits (R&D Systems) were used to measure human cytokines in culture supernatants and serum according to manufacturer’s instruction. For measuring CRISPLD2/Crispld2 in culture supernatants and serum samples, appropriately diluted samples in sodium carbonate buffer (pH 9.5) were coated 96-well microtiter plates (Nunc MaxiSorp) overnight at 4°C. After washing twice with PBS, the plates were first incubated with 8% FCS in PBS for 60 min and subsequently with rabbit anti-CRISPLD2 Ab (10µg/ml) in PBS with 8% FCS for 2 h. After washing four times, HRP-conjugated donkey anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories) was added. The tetramethylbenzidine substrate in sodium acetate/citric acid buffer (0.1 M; pH 6.0) was used for visualization. The catalytic action was stopped by 2.0 M sulfuric acid. OD was measured in a spectrophotometer at 450 nm with the correction wavelength set at 570 nm. A standard curve of rCRISPLD2 titration (2.0, 1.0, 0.5, 0.25, 0.125, 0.062, 0.031 and 0 µg/ml) was used for the quantification of CRISPLD2 and the concentration of mouse Crisp2. It should be pointed out that the concentration of bovine Crisp2 in FCS is very low, and the incubation with 8% FCS does not significantly increase the background in ELISA (see supplemental Fig. S7).

For measuring anti-LPS Ab in mouse serum, LPS (100 µg/ml) in sodium carbonate buffer (pH 9.5) was plated in 96-well microtiter plates overnight, followed by treatment with 10% FCS in PBS for 60 min. Mouse sera in 1- to 100-fold dilutions in PBS were added to the immobilized LPS, and HRP-conjugated donkey anti-mouse Ab (Jackson ImmunoResearch Laboratories) was used to react with plate-bound anti-LPS Abs.

**Mice and in vivo LPS stimulation**

Six- to 8-week-old BALB/c female mice were purchased from Shanghai Experimental Animal Ltd. All mice were housed in the animal facility of the Shanghai Jiao Tong University Medical School (Shanghai, China). All procedures with mice were performed according to guidelines of the Harvard Medical School Office for Research Subject Protection (Boston, MA) and approved by the ethics committee of the Chinese National Genome Center (Shanghai, China). To determine serum Crisp2 concentration in response to LPS and LTA, 6- to 8-week-old BALB/c female mice were injected i.p. with S. aureus LTA (0.1 mg/mouse) and E. coli-type LPS (0.015, 0.030, and 0.10 mg/mouse), respectively, and blood samples were taken from the tail vein on days 0, 1, 2, 5, 7, 12, and 31. For the prevention of LPS-induced endotoxin shock, 6- to 8-week-old BALB/c female mice were injected i.p. with E. coli (O55:B5) LPS (0.45 mg/mouse; 22.5 mg/kg) in the absence or presence of recombinant human CRISPLD2 (molecular mass, 55 kDa; 1.4 mg/mouse; 70 mg/kg) and CRISP-3 (molecular mass, 25 kDa; 0.64 mg/mouse; 32 mg/kg), respectively. To monitor the serum CRISPLD2 response to repeated LPS challenges, 6- to 8-week-old BALB/c female mice were first injected i.p. with E. coli (O55:B5) LPS (0.03 mg/mouse; 1.5 mg/kg). After 10 days, these mice were separated into four groups and again injected i.p. with LPS at 25, 40, 55, and 70 mg/kg, respectively. After 32 days, surviving mice received a third injection of LPS (45, 60, or 75 mg/kg). Serum Crisp2 and anti-LPS Abs were examined before i.p. injection of high doses of LPS.

**Statistics analyses**

Student’s t test was used for the comparison of two independent groups. Statistical difference of survival rates between two groups with endotoxin shock was analyzed by a Kaplan-Meyer log-rank test. Statistical analyses on the correlation between serum Crisp2 concentrations vs lethal LPS dose were performed by GraphPad Prism 5 software. For all tests, a value of p < 0.05 was considered statistically significant.

**Results**

**Molecular analysis and LPS reactivity**

To examine the possibility that CDRISPLD2 is a LPS-binding protein, we isolated the whole ORF of human CRISPLD2 and stably

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transfected it into CHO cells using the mammalian expression plasmid pcDNA3.1 for expressing the recombinant c-myc-His tagged CRISPLD2. rCRISPLD2 was purified from culture medium and evaluated by electrophoresis on SDS-polyacrylamide gels and by immunoblotting assays using c-Myc Abs and home-made anti-CRISPLD2, respectively (supplemental Fig. S1). To assess potential CRISPLD2 LPS-binding activity, we used an EMSA on native polyacrylamide gel using purified rCRISPLD2 together with LPS from E. coli or S. enterica serovar Minnesota. The electrophoretic mobility of the CRISPLD2 band was significantly shifted by E. coli and S. enterica serovar Minnesota LPS (Fig. 1A), indicating that CRISPLD2 did interact physically with LPS. To further confirm the finding, we performed a SPR assay by using a BIAcore instrument when E. coli LPS (1.0, 3.0, 10, and 30 μM) was passed over immobilized CRISPLD2 chip. E. Scatchard plot analysis and the K_D of E. coli LPS binding to CRISPLD2 (mean ± SD).

Table 1

<table>
<thead>
<tr>
<th>Immobilized</th>
<th>LPS K_D (M)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRISPLD2</td>
<td>2.46 ± 0.84 μM</td>
<td>Fig. 1D</td>
</tr>
<tr>
<td>CRISPLD2</td>
<td>1.33 ± 0.90 μM</td>
<td>Fig. 1B</td>
</tr>
<tr>
<td>LBP</td>
<td>1.40 ± 10^-8</td>
<td>8</td>
</tr>
<tr>
<td>CD14</td>
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<td>CD14</td>
<td>0.87 ± 0.2 μM</td>
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<tr>
<td>MD-2</td>
<td>2.33 ± 0.90 μM</td>
<td>32</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.41 ± 0.70 μM</td>
<td>32</td>
</tr>
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* All K_D values were measured by SPR assay.
* Data from three independent experiments (mean ± SD).

FIGURE 1. CRISPLD2 binds LPS but not S. aureus LTA. A, Reaction with S. enterica serovar Minnesota (S. minnesota) and E. coli LPS shifts the mobility of CRISPLD2 in a native PAGE mobility shift assay. B and C, Sensorgrams of E. coli LPS (B) and S. aureus LTA (C) binding to immobilized CRISPLD2. Either E. coli LPS or S. aureus LTA (0.3, 1.0, 3.0, 10, and 30 μM) was passed over immobilized CRISPLD2. The dissociation constant of LPS, K_D, was calculated as the ratio of these two constants (k_off/k_on) according to the binding parameters from a sensogram. D, RU that reflects E. coli LPS binding activity was measured by a BIAcore instrument when E. coli LPS (1.0, 3.0, 10, 30, and 100 μM) was passed over the CRISPLD2 chip. E, Scatchard plot analysis and the K_D of E. coli LPS binding to CRISPLD2 (mean ± SD).

To assess the molecular phylogeny of CRISPLD2, we also compared CRISPLD2 with homologous protein sequences deposited in GenBank by using bioinformatic tools. We found that CRISPLD2 is highly conserved throughout evolution (supplemental Fig. S2).

Tissue expression and serum concentration

RT-PCR technology was applied to demonstrate the occurrence of Crispld2 mRNA in six mouse tissues (supplemental Fig. S3A). In addition, Crispld2 was immunofluorescently identified in tissue sections of mouse lung, placenta, and small intestine (supplemental Fig. S3B), and also immunohistochemically identified in rat lung and intestine (supplemental Fig. S3C). Northern blot analysis revealed that the CRISPLD2 gene is transcribed in leucocytes and many human organs; it is most pronounced in the placenta and also in heart, lung, small intestine, and blood leukocytes, but far less pronounced in liver, kidney, spleen, thymus, colon, muscle, and brain (supplemental Fig. S3D).

CRISPLD2 was identified and quantified in healthy human serum (range, 384–790 μg/ml; n = 7) and plasma (range, 402–998 μg/ml; n = 3) by using an immunoblotting assay (Fig. 2A). However, the concentrations of CRISPLD2 in infant umbilical cord blood plasma (range, 149–426 μg/ml; n = 5) are lower than those in adult blood plasma (Fig. 2B). As compared with published serum concentrations of known LBPs, the serum levels of CRISPLD2 are substantially higher than those of LBP, soluble CD14, bactericidal/permeability-increasing protein, and soluble CD6 (supplemental Table I, Fig. 3) by using an immunoblotting assay (Fig. 2A). Moreover, human peripheral blood granulocytes and mononuclear cells, including monocytes, NK cells and T cells, express CRISPLD2 that was identified by RT-PCR (Fig. 2C) in concordance with published microarray data for gene expression (35–38).

CRISPLD2 was readily seen in the cytoplasm of PBMCs (Fig. 2, D and F), including monocytes, NK cells, and T cells (supplemental Fig. S4F), by using intracellular Ab staining, but we were unable to detect it on the cell surface by using extracellular Ab staining (Fig. 2E). Interestingly, intracellular Crispld2 was also found in lamina propria lymphocytes in the small intestines of mouse and rat (supplemental Fig. S3, B and C).

Soluble rCRISPLD2 interferes with LPS target cell binding

The ability of soluble rCRISPLD2 to interfere in the interaction of LPS with its target cells was tested under several experimental conditions. Fig. 3, A and B, revealed that the presence of CRISPLD2, but not CRISP-3, in the culture medium inhibited the reaction of two LPS preparations with cellular LPS receptors on monocytes in a dose-dependent fashion. Accordingly, the release of the cytokines TNF-α (Fig. 3C) and IL-6 (Fig. 3D) subsided in
Spontaneously, the cells emitted lower quantities of CRISPLD2. LPS up-regulates the release of CRISPLD2 by granulocytes and CRISPDL2 curtail LPS bioregulatory function, but also in turn. In the course of these experiments, it was noted that not only does secretion LPS and an anti-hTLR4-IgA mAb up-regulate CRISPLD2 consolidation of LPS binding. a prior commitment of target cells to LPS response rather than reduced CRISPLD2 inhibitory activity at 6 h after LPS may reflect altered even when CRISPLD2 was added after LPS. However, the reduced CRISPLD2 inhibitory activity at 6 h after LPS may reflect a prior commitment of target cells to LPS response rather than consolidation of LPS binding.

**LPS and an anti-hTLR4-IgA mAb up-regulate CRISPLD2 secretion**

In the course of these experiments, it was noted that not only does CRISPLD2 curtail LPS bioregulatory function, but also in turn LPS up-regulates the release of CRISPLD2 by granulocytes and PBMCs, including purified monocytes, T cells, and NK cells. Spontaneously, the cells emitted lower quantities of CRISPLD2 into the culture medium, but quite substantial amounts accumulated in the presence of CRISP-3, a cysteine-rich secretory protein without LCCL domain (Fig. 3, C and D).

TLR4 has been found on surface of NK cells (39). Fluorescence-labeled E. coli and S. enterica serovar Minnesota LPS were detectable on CD56+ cells and CD56− lymphocytes in our experiment (supplemental Fig. S4, D and E), although the LPS fluorescence intensity on these cells was far lower than that on monocytes (supplemental Fig. S4, A–E). Expectedly, rCRISPLD2 prevented LPS binding to CD56+ cells and CD56− lymphocytes (supplemental Fig. S4, D and E).

To determine whether CRISPLD2 is capable of dislodging LPS after it has reacted with cellular surface receptors, PBMCs were incubated with labeled LPS for 30 min before unbound LPS was removed and CRISPLD2 was added. Fig. 3, E and F, suggest that in sufficient doses, competitive removal of bound LPS from monocyte surfaces occurs in the presence of CRISPLD2. Fig. 3G revealed that LPS-induced TNF-α production was significantly altered even when CRISPLD2 was added after LPS. However, the reduced CRISPLD2 inhibitory activity at 6 h after LPS may reflect a prior commitment of target cells to LPS response rather than consolidation of LPS binding.

**Rhodobacter sphaeroides** LPS is the potent antagonist of toxic LPS in both human and murine cells and prevents LPS-induced shock in mice (40). In the present study we found that ultra-pure R. sphaeroides LPS alone up-regulated CRISPLD2 secretion and induced significantly lower TNF-α production (Table II). To determine whether the LPS-induced up-regulation of CRISPLD2 secretion could be blocked by a neutralizing mAb against TLR4, we used an anti-TLR4 (HTA125) mAb and a humanized chimeric anti-hTLR4-IgA mAb in vitro experiments. According to the reference data from the manufacturer (InvivoGen), the efficacy of the humanized chimeric IgA mAb is 100-fold higher than that of the HTA125 mAb for blocking LPS-induced intracellular activation. In concordance with the reference data, the anti-hTLR4-IgA mAb effectively suppressed E. coli LPS-induced TNF-α and IL-6 production in our experimental system (Table II). Unexpectedly, both anti-TLR4 mAbs were failed to block E. coli LPS-induced up-regulation of CRISPLD2 secretion. Interestingly, we found that the anti-hTLR4-IgA mAb alone, but not anti-TLR4 (HTA125) mAb or anti-hTLR4-IgA mAb alone, but not anti-TLR4 (HTA125) mAb or...
control human IgA, was capable of up-regulating CRISPLD2 secretion (Table II and supplemental Fig. S5A). Moreover, the chimeric anti-hTLR4-IgA mAb synergized the E. coli LPS-induced up-regulation of CRISPLD2 release (Table II). Together, the results in Table II suggested that the up-regulation of CRISPLD2 secretion can be triggered by TLR4-mediated signaling that seems to be irrelevant to TNF-α/H9251 production.

It is known that purified S. aureus LTA is a ligand of CD6 (17) that can activate TLR2 but no other TLRs, including TLR4 (41). We investigated whether purified S. aureus LTA up-regulates CRISPLD2 release in vivo and in vitro. Fig. 4, A and B, and supplemental Fig. S5B show that purified S. aureus LTA (1 μg/ml) failed to up-regulate CRISPLD2 release from PBMCs, purified CD4 T cells, and monocytes. Furthermore, Crispld2 serum levels of purified S. aureus LTA-treated mice (100 μg/per-mouse) did not significantly rise (Fig. 4F), implying that S. aureus LTA binding to CD6 and S. aureus LTA-induced activation of TLR2 is irrelevant to the up-regulation of CRISPLD2 release. Whether the activation of other TLRs, except TLR4 and TLR2, up-regulate CRISPLD2 release is to be further investigated.

To determine whether the TLR4/MD2 receptor complex is expressed on T lymphocytes, RT-PCR was applied to demonstrate the occurrence of TLR4 and MD2 mRNA in PBMCs, purified CD4 T cells (98% pure), and CD14 monocytes (96% pure), and PE-labeled anti-TLR4 mAb was also used to determine whether TLR4 anchors on the surfaces of CD4 and CD8 T cells and CD14 monocytes in PBMCs. Supplemental Fig S4G revealed that TLR4 and MD2 transcripts were detected.
FIGURE 4. LPS promotes human granulocytes and PBMCs to secrete CRISPLD2 and increases the CRISPLD2 serum levels. A and B, Human PBMCs (A) and monocytes (B) were stimulated with E. coli LPS and S. aureus LTA, respectively, and CRISPLD2 in supernatants were quantitated at various time points. C–E, Human granulocytes, NK cells, and T cells were stimulated with or without E. coli LPS, and the CRISPLD2 concentrations in culture medium were quantitated by ELISA (mean of three experiments ± SD). F and G, Up-regulation of Crispld2 expression in mouse serum after administration of a nontoxic dose of LPS. BALB/c mice were injected i.p. with S. aureus LTA (100 μg/mouse) and E. coli type LPS (15, 30 and 100 μg/mouse), respectively. Western blotting (IB, immunoblotting) was used to determine Crispld2 in mice sera at days (D) 0, 1, 2, 5 and 7. The mice sera were diluted 1/10 in PBS for PAGE separation, so real concentrations of Crispld2 are ~10 times higher. G, Serum Crispld2 from mice treated with S. aureus LTA (100 μg/mouse) or E. coli LPS (15, 30 and 100 μg/mouse) was measured by ELISA at different time points. Recombinant human CRISPLD2 was used as reference for western blot and ELISA.

in CD4⁺ T cells as compared with CD14 monocytes and PBMCs. Moreover, supplemental Fig S4H demonstrated that TLR4 was found on the cell surfaces of CD4 and CD8 T lymphocytes via immunofluorescence assay, which is consistent with the published literature (42–44). To confirm LPS binding to TLR4 on T cells, the anti-hTLR4-IgA mAb was used to interfere with the binding of FITC-labeled E. coli LPS to PBMCs stained with PE-conjugated anti-CD4 and CD8 mAbs, respectively. The results revealed that anti-hTLR4-IgA mAb, but not control IgA, partially blocked the binding of E. coli LPS-FITC to CD4⁺ and CD8⁺ T lymphocytes (supplemental Fig. S4I), suggesting that LPS can bind to TLR4 on the surface of T cells. To exclude the possibility that the up-regulation of CRISPLD2 secretion from T cells is a secondary response or side effect of contamination that could stimulate T lymphocytes, in the present work CD4⁺ T cells from PBMCs were purified twice by negative selection to reach high purity (95%), and the anti-hTLR4-IgA mAb was extensively dialyzed to exclude possible contamination. Also, a stimulatory anti-CD3 mAb (OKT3) was used to mimic contamination for activating the TCR/CD3 complex. The results showed that the stimulation of anti-hTLR4-IgA mAb, but not pure S. aureus LTA or human IgA, effectively increased the CRISPLD2 secretion from purified CD4 T cells in a short time, as compared with
that of the ultra pure *R. sphaeroides* LPS (supplemental Fig. S5B), whereas the OKT3 mAb-induced activation of T cells did not increase CRISPLD2 secretion although the proinflammatory mediators, including TNF-α from PBMCs, were induced (supplemental Fig. S5A), suggesting that the activation of TLR4 on T cells can directly lead to the up-regulation of CRISPLD2 secretion. The collective results, as shown in Fig. 4 and supplemental Fig S5, A and B, seem to exclude the possibility that the up-regulation of CRISPLD2 secretion is a secondary response, such as proinflammatory mediator-induced response, or a side effect of contamination, such as the direct stimulation of Ags via the TCR/CD3 complex or unknown molecules (<20 kDa).

However, we cannot exclude the possibility that the LPS-induced up-regulation of CRISPLD2 secretion can be mediated by the activation of other LPS-receptors such as CD6, which binds to LPS and LTA, respectively, via TLR4-independent and nonoverlapping extracellular sites and then delivers intracellular signaling in the presence of LPS (17). The question of whether LPS-activated CD6 can up-regulate CRISPLD2 secretion should be further investigated in the future.

**CRISPLD2 is a major biological regulator of LPS function**

It is possible that endogenous CRISPLD2 may down-regulate LPS immunostimulatory activities. If indeed the LPS sensitivity of monocytes in PBMCs was to correlate negatively with the availability of endogenous CRISPLD2 in culture medium, then experimentally interfering with the interaction between LPS and CRISPLD2 should increase LPS sensitivity.

To test this hypothesis, human PBMCs were incubated with increasing concentrations of LPS in the presence or absence of CRISPLD2-specific Abs, and then the release of CRISPLD2 and TNF-α were examined 18 h later. The experiment revealed that PBMCs spontaneously released CRISPLD2 into culture medium and that the higher doses of LPS, >10 ng/ml, up-regulated the release (Fig. 5A). In concordance with our hypothesis, the efficacy of LPS was increased in the presence of Ab against CRISPLD2 as compared with the control Ab, suggesting indeed that the anti-CRISPLD2 Ab can remove an effective safety device against LPS (Fig. 5B).

Intruding germs reach their cellular targets via the bloodstream. Previous analysis revealed substantial but varying CRISPLD2 quantities in the serum of humans (Fig. 2A). It seemed of interest to determine whether CRISPLD2 molecules contained in the serum provide animals and humans with a natural shield against the effects of LPS. Human PBMCs were challenged under culture conditions with 0.1% FCS in the presence or in the absence of 10–15% human serum. Using three different samples, Fig. 5C shows that human serum suppressed LPS-induced TNF-α release readily. However, the suppressive effect was reversed by the addition of the Ab against CRISPLD2, but not the control Ab. The evidence revealed that the endogenous CRISPLD2 in sera down-regulated LPS-induced TNF-α production. To further confirm this result, rCRISPLD2 was added into the culture system in the presence or absence of 10% human serum. Fig. 5D demonstrates that the increased concentrations of rCRISPLD2 inhibit LPS-induced TNF-α production in a dose dependent manner and that the anti-CRISPLD2 Ab reverses the inhibitory effects of not only endogenous CRISPLD2 but also those of additional rCRISPLD2 in culture medium.

**CRISPLD2 protects mice against endotoxic shock**

As the above experiments demonstrated that CRISPLD2 specifically inhibits biological functions of LPS, we examined whether CRISPLD2 can ameliorate the devastating effects of systemic LPS action in generating toxic shock in mice. Fig. 6A revealed that one i.p. injection of 450 μg of *E. coli* LPS killed 80% of the treated animals within a few days. Injection of *E. coli* LPS in combination with 1.4 mg of rCRISPLD2 reduced the death rate to <20%, suggesting that CRISPLD2 exerts life-saving effects of anti-endotoxin in vivo (Fig. 6A). By contrast, CRISP-3 as a control has no protective effect.

It has previously been shown that pretreatment of mice with small, nontoxic doses of LPS render recipients resistant to a lethal challenge with LPS (45, 46). This finding was consistent with our notion that LPS stimulates the generation of endogenous CRISPLD2. We therefore considered the possibility that CRISPLD2 elevation induced by a low dose of LPS may account for the protection against the lethal effect of a high dose of LPS. We found that the serum of mice that have been treated with a small dose of LPS more than doubled its CRISPLD2 expression (Fig. 4G and Table I). At the same time, we also noticed that CRISPLD2 was not the only LBP that accumulated in the serum. LPS-binding IgG rose by an even higher margin (supplemental Fig. S6). Table III showed that under such conditions mice also noticed that CRISPLD2 was not the only LBP that accumulated in the serum. LPS-binding IgG rose by an even higher margin (supplemental Fig. S6).

Table II. Both *R. sphaeroides* LPS and anti-hTLR4-IgA Ab up-regulate CRISPLD2 secretion and reduce *E. coli* LPS-induced TNF-α and IL-6 production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μg/ml)</th>
<th>CRISPLD2 (μg/ml)</th>
<th>TNF-α (ng/ml)</th>
<th>IL-6 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.37 ± 0.10</td>
<td>0.04 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td><em>E. coli</em> LPS</td>
<td></td>
<td>1.59 ± 0.26</td>
<td>8.56 ± 0.62</td>
<td>10.5 ± 0.28</td>
</tr>
<tr>
<td><em>R. sphaeroides</em> LPS</td>
<td></td>
<td>1.40 ± 0.06</td>
<td>3.45 ± 0.27</td>
<td>7.98 ± 0.22</td>
</tr>
<tr>
<td><em>E. coli</em> LPS + <em>R. sphaeroides</em> LPS</td>
<td></td>
<td>1.65 ± 0.09</td>
<td>3.29 ± 0.07</td>
<td>7.25 ± 0.59</td>
</tr>
<tr>
<td>Anti-hTLR4-IgA</td>
<td></td>
<td>1.66 ± 0.04</td>
<td>0.08 ± 0.03</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Anti-hTLR4-IgA</td>
<td></td>
<td>1.93 ± 0.21</td>
<td>0.13 ± 0.01</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td><em>E. coli</em> LPS + anti-hTLR4-IgA</td>
<td></td>
<td>2.25 ± 0.24</td>
<td>3.17 ± 0.09</td>
<td>7.06 ± 0.45</td>
</tr>
<tr>
<td><em>E. coli</em> LPS + anti-hTLR4-IgA</td>
<td></td>
<td>2.55 ± 0.43</td>
<td>2.63 ± 0.07</td>
<td>5.27 ± 0.68</td>
</tr>
</tbody>
</table>

* Human PBMCs were treated by the reagents as indicated in table for 12 h.
* CRISPLD2, TNF-α, and IL-6 were measured by ELISA in culture supernatants.
* Ten microliters of PBS was added in culture as control. Data (mean ± SD of triplicate wells) are from two independent experiments.
* Value of p < 0.005 as compared with that from *E. coli* LPS treatment alone.
* Value p < 0.05 as compared with that from *E. coli* LPS treatment alone.
CRISPLD2 is a molecule recognized in the literature, but no LPS-related functions are attributed to it (21–25). We were intrigued by the fact that it contains two LCCL domains, which are characteristic LPS-binding structures (26). Our data indeed show, for the first time, that CRISPLD2 is a potent LPS-binding protein that exhibits significant LPS-binding affinity.

A major LPS-binding protein in the mammalian body, one known to guide the transduction of signals involved in eliciting LPS-dependent immune functions, is LBP (6, 7). CRISPLD2 shares with LBP none of the tasks examined here. On the contrary, the postulate that it could oppose LBP-mediated immunostimulation is supported by the finding that the polyclonal Ab against the CRISPLD2 region containing LCCL domains unleashes LPS immunostimulatory activities (Fig. 5). The mechanism of this antagonism is unclear. CRISPLD2 could inhibit LBP-mediated immune functions by competing for the target molecule, by facilitating negative signaling, or by using both mechanisms.

CRISPLD2 is not the only molecule the body may engage in the fight against bacterial LPS. We noted in our experiments that LPS-reactive Igs have a similar effect. Recently, it was also reported that CD6 acts as negative regulator of LPS function and endotoxic shock induction (17). However, it may be argued, in favor of CRISPLD2, that CD6 is highly expressed on cell membranes and poorly present in serum (16), whereas CRISPLD2 lacking membrane expression is amply represented in the serum. The downside of LPS-reactive Abs is the fact that they are primarily directed against the immunodominant species-specific oligosaccharide side chains, in contrast to CRISPLD2, and protect selectively, namely against the immunizing strain. The immunological effects of LPS are associated with the core glycolipid region in which there is little strain variation. Experimental vaccines have been produced using an E. coli J5 mutant that lacks side strains to its core. J5-specific Abs have been demonstrated to provide some protection against endotoxic shock (20).

Given the fact that CRISPLD2 effectively blocks LPS-induced immune functions, the uneven organ distribution of the molecule in the body may indicate that organs expressing high levels of CRISPLD2 would benefit from a persistent down-modulation of LPS immunostimulatory activity. The desirability of an abundant presence of CRISPLD2 in the placenta would seem evident from the fact that LPS is a strong inducer of fetal abortion (47).

**FIGURE 5.** Endogenous CRISPLD2 interferes with LPS-induced TNF-α response. A, PBMCs were stimulated with the increased concentration of E. coli LPS as indicated. The endogenous CRISPLD2 released in the culture supernatants was quantified at 18 h by ELISA, and the medium cultured without cells was used as a negative control. B, In the same experiment, anti-CRISPLD2 polyclonal Abs (■) and unrelated polyclonal Abs (□) were added into cell cultures as compared with cultures without Abs (○). TNF-α released in the culture supernatants at 18 h was measured by ELISA. C, Human serum (HS) down-regulates LPS-induced TNF-α production. Sera from three healthy volunteers were used in the experiment. PBMCs were stimulated by E. coli LPS (100 ng/ml) with anti-CRISPLD2 polyclonal Ab or a control Ab (both 500 μg/ml) in the presence or absence of 15% human serum. TNF-α in culture supernatants were measured at 18 h by ELISA according to the protocol provided by the manufacturer (R&D Systems); LPS alone (open bars), LPS with control IgG (gray bars), LPS with the anti-CRISPLD2 polyclonal Ab (black bars) are shown; **, p < 0.05; ***, p < 0.005 vs absence of human serum. D, Anti-CRISPLD2 polyclonal Abs against endogenous CRISPLD2 and rCRISPLD2 unleashes LPS immunostimulatory activities. In the same culture system with or without human serum (HS) 10% (■11), PBMCs were stimulated by E. coli LPS (10 ng/ml) in the presence of anti-CRISPLD2 polyclonal Ab or a control Ab (both 500 μg/ml) with additional rCRISPLD2 at the indicated concentrations. TNF-α released in the culture supernatants at 18 h was measured by ELISA. LPS with human serum (open bars), LPS with human serum plus control IgG (gray bars), LPS with human serum plus anti-CRISPLD2 Ab (black bars), and LPS with only 0.1% FCS (diagonal bars) are shown; **, p = 0.048; and ***, p = 0.00036; vs presence of anti-CRISPLD2 Ab (mean of three experiments ± SD).
The observations in mice regarding endotoxic shock prevention through CRISPLD2 treatment may have implications for improving strategies in treating this devastating clinical event in humans. Our data show that CRISPLD2 serum levels fluctuate in considerable amplitude, possibly due to communicating with a sensed microbial environment. Current results in this present work indicate that a large majority of white blood cells spontaneously release CRISPLD2 and, upon stimulation with LPS, enhance release of the LPS-binding molecule that would occur in vivo and may contribute to the serum CRISPLD2 level. We do not exclude the possibility that the heart, small intestine, and lung also release CRISPLD2 into body fluid because of apparent CRISPLD2 trans-cription in these tissues. Given the fact that CRISPLD2 blocks the immunostimulatory activity of LPS in cell culture, it would seem obvious that CRISPLD2 protects the body as well against the ex-

cess of a systemic functional presence of LPS in Gram-negative bacteria. The prevention of endotoxin shock by CRISPLD2 is unequivocally demonstrated by our findings in the experimental mouse model.

A drawback in conventional sepsis treatment is the fact that, once the first indications of an immanent shock are detected, the avalanche of immunological events is already in full motion and it can no longer be stopped. The most effective protection against these avalanches lies in preventing their initiation. If we were to draw the conclusion from our experiments that the risk of acquiring Gram-negative sepsis is high when CRISPLD2 serum levels are low and, vice versa, the risk is low when CRISPLD2 serum levels are high, it would logically follow that the best precaution a physician can take against the onset of Gram-negative sepsis is to assure that a patient possesses sufficient amounts of CRISPLD2 in his or her serum. We noted in these mice (Table III) that small nontoxic doses of LPS boost the serum expression of CRISPLD2 and protect animals against the lethal effect of the immunostimu-latory agent. We recognize that E. coli LPS itself can hardly be considered a desirable therapeutic reagent, but it is conceivable that other less toxic microbial products or preparations may also up-regulate CRISPLD2 serum levels. A clinical concept of guard-ing against Gram-negative sepsis would thus entail the careful tracking of CRISPLD2 serum levels in patients with increased risk of developing Gram-negative septic shock and attempts to boost CRISPLD2 expression once its level falls below a critical threshold.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflict of interest.

**References**


**Table III. Correlation between serum Crispld2 level and LPS sensitivity**

<table>
<thead>
<tr>
<th>LPS Pretreatment (0.03 mg)</th>
<th>Serum Crispld2 (OD 450–570 nm)</th>
<th>LPS Administration (mg/mouse)</th>
<th>Outcome (Survival/total mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>0.134–0.166</td>
<td>0.5</td>
<td>0/4</td>
</tr>
<tr>
<td>Yes</td>
<td>0.338–0.386</td>
<td>1.1</td>
<td>4/4</td>
</tr>
<tr>
<td>No</td>
<td>0.130–0.201</td>
<td>0.8</td>
<td>0/4</td>
</tr>
<tr>
<td>Yes</td>
<td>0.223–0.327</td>
<td>0.5</td>
<td>3/4</td>
</tr>
<tr>
<td>No</td>
<td>0.323–0.446</td>
<td>0.8</td>
<td>3/4</td>
</tr>
<tr>
<td>Yes</td>
<td>0.351–0.371</td>
<td>1.4</td>
<td>0/2</td>
</tr>
</tbody>
</table>

a Serum Crispld2 was detected by ELISA assays on the 8th day after LPS pretreatment.
b LPS was injected i.p. on the 10th day after LPS pretreatment.
c Outcome on the 15th day after LPS pretreatment.

**FIGURE 6.** rCRISPLD2 protects mice against endotoxin shock. A, Mice were injected i.p. with *E. coli* LPS (450 μg/mouse, 22.5 mg/kg; n = 22) alone or in combination with rCRISPLD2 (1.4 mg/mouse, 70 mg/kg; n = 17) or CRISP-3 (0.64 mg/mouse, 32 mg/kg; n = 7). The percentages of surviving animals are plotted in a Kaplan-Meyer survival curve; p < 0.001 vs LPS with PBS or CRISP-3. B, The lethal LPS doses correlate positively with serum Crispld2 concentrations. Serum CRISPLD2 was measured by ELISA on the 8th day after i.p. preinjection with or without a nontoxic dose of LPS (0.03 mg/mouse). Lethal doses of LPS (0.5, 0.8, 1.1, or 1.4 mg/mouse) were injected i.p. on the 10th day after i.p. preinjection. The outcome was accounted on the 15th day after LPS pretreatment. Serum Crispld2 in surviving mice was examined again on the 30th day, surviving mice received a third injection of LPS (0.9, 1.2 or 1.5 mg/mouse) on the 32nd day, and the outcome was accounted again on the 37th day. The data of lethal LPS dose vs serum Crispld2 concentration from each mouse were measured. Together, all data were plotted by lethal LPS doses vs serum Crispld2 concentrations. Value of p = 0.00009 and value of F = 35.4. GraphPad Prism 5 software was used for statistic analysis. In regression analysis, logarithmic trendline program in Excel was best-fit to the values array in the plot, with the correlation coefficient $R^2$ indicated on the graph.


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