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 Han

*J Immunol* 2009; 183:6646-6656; Prepublished online 28 October 2009; doi: 10.4049/jimmunol.0802348

http://www.jimmunol.org/content/183/10/6646

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/10/28/jimmunol.0802348.DC1

References

This article cites 46 articles, 12 of which you can access for free at:

http://www.jimmunol.org/content/183/10/6646.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
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 Han

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 multitissues and leukocytes, is a novel LPS-binding protein. As a serum protein, 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 μg/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 Crispdl2 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 monomorphic 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) that is considered a critical molecule in LPS-elicited 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). Note is CD6, belong to the scavenger receptor cysteine-rich superfamilly, 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 endotoxic 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 Crispdl2, 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...
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 endotoxic shock.

Materials and Methods

**Bacterial components, mAbs, and rCRISPLD-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), a complex *Rhodobacter sphaeroides* lipopolysaccharide LPS, and chimeric anti-human TLR4 (htLR4)-IgA mAbs were purchased from InvivoGen and dissolved in endotoxin-free water according to the manufacturer’s instruction. Anti-hTLR4 (HTA125) and OKT3 mAbs were purchased from eBioscience. Human IgA was purchased from Sigma-Aldrich. Recombinant CRISPLD-3 was purchased from R&D Systems.

**Plasmid constructs and cell transfection**

cDNA with an entire open reading frame (ORF) of human CRISPLD2 was obtained from mixed tissues by PCR using the F1 forward primer 5'-GCTGTCGTCCGCGTCTACCC-3' and the R1 reverse primer 5'-GACGCCCCCTTCCCTGGT-3'. The PCR product was inserted into the plasmid pGEM-T Easy Vector (Promega), which then served as the template in expression vector construction. To construct a recombinant prokaryote expression vector, human partial CRISPLD2 was inserted into pGEX-4T-1 (GE Healthcare) to generate the GST-CRISPLD2 (257–497 aa) fusion protein, which contains two LCCL domains and was used for generating the rabbit anti-CRISPLD2 polyclonal Ab. For mammalian cell expression, human CRISPLD2 with an LCCL domain was cloned into the entire ORF was then subcloned into pcDNA3.1A-myc-His (Invitrogen) to generate the c-myc-His-tagged CRISPLD2. The pcDNA3.1A-CRISPLD2 was transefcted into CHO cells with Lipofectamine (Invitrogen) and then selected with the antibiotic G418 (Invitrogen). These stable CHO cells with human CRISPLD2 were maintained with G418, and many monoclonal recombinant CHO cell lines were further screened out by limited cell dilution and Western blotting assays.

**Anti-CRISPLD2 polyclonal antibody**

The recombinant pGEX-4T-1 containing GST-CRISPLD2 (257–497 aa) with an LCCL domain was transformed into *E. coli* BL21 for prokaryote expression. The GST-CRISPLD2 expression in bacteria was induced by isopropyl-β-D-thiogalactopyranoside at 28°C, followed by centrifugation at 8,000 × g at 4°C for 10 min. The supernatant was discarded and the pellet was resuspended in PBS with 5 mM DTT and 100 μg/ml PMSF. After sonication, the lysate was centrifuged at 8,000 × g at 4°C for 10 min. The GST-CRISPLD2 (257–497 aa) in the supernatant was purified using an affinity column of glutathione-Sepharose 4B (GE Healthcare). Normal rabbit sera were immunized with the purified protein and the polyclonal Abs were purified from the sera using protein G-Sepharose (GE Healthcare).

**Purification of rCRISPLD2**

The serum-free cell medium of CHO cells with stably transfected pcDNA3.1A containing human CRISPLD2 was collected. The rCRISPLD2 in the supernatant was denaturalized by 4 M urea and purified by Ni-NTA metal-affinity chromatography (Qagen). After two washes with 50 μl of washing buffer (50 mM Na2HPO4, 500 mM NaCl, and 4 M urea, pH 7.5), the purified protein was eluted in buffer R1 = R2 = 0.1 M NaOH and R3 = the amplitude of the initial response. The association rate constant kₐ can be derived from the measured kᵢ value using: Rₘₐₓ = kᵢ[1 + e⁻<sup>kₐt</sup> + kₐt – 1], where C is the concentration of LPS, Rₘₐₓ represents the maximum binding capacity of LPS to immobilized CRISPLD2, and Rₘₐₓ represents the amount of LPS bound to the CRISPLD2 at time t. The dissociation rate constant kₐᵢ is 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 F2 forward primer 5'-TGGAAATTCGAGAAGAACCCTACA CTC-3' and the R2 reverse primer 5'-TGGCTGAGATTCGACCGTGAC AGCA-3' labeled with [³²P]dCTP using a random primer DNA labeling kit (GE Healthcare). Hybridization with 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 140 V for 4 h, as compared with that of MD2 (forward) and 5'-TCCCACCTAGAGAAGTCGT-3' (reverse), those used for MD2 were 5'-TCCACCCCTTCTCTTCCTTTCCCA-3' (forward) and 5'-TGCAATACCCCAAAG-3' (reverse). The PCR primers used for human TLR4 were 5'-CTCGAATATGACAGCCG-3' (forward) and 5'-GAGCTTGATACACGACATAC-3' (reverse) and the primers used for CRISPLD2 were 5'-AGT/CTAGACATGAGCTGCTGTT-3' (forward) and 5'-GGCTGCACTGATACACGACAT-3' (reverse). For MD2, those used for TLR4 were 5'-GGAAATTCGAGAAGAACCCTACA CTC-3' and the primers used for CRISPLD2 were 5'-AGT/CTAGACATGAGCTGCTGTT-3' (forward) and 5'-GGCTGCACTGATACACGACAT-3' (reverse). For RT-PCR analysis

**Surface plasmon resonance (SPR) assay**

To measure the affinity between CRISPLD2 and LPS, a SPR assay was performed to determine the recognition of LPS by CRISPLD2 with a Biacore 3000 biosensor instrument (Biacore/GE Healthcare) using a C1 sensor chip (GE Healthcare). Briefly, either rCRISPLD2 or unrelated IgG at 0.18 mg/ml diluted with acetate buffer (pH 4.5) was immobilized to the sensor chip by coupling with the primary amine group. Different concentrations of LPS 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 (Kₒ) was calculated by Scatchard plot analysis using the following formula: Kₒ = 1/slope = (RUₘₐₓ – RU)/RU/ConcLPS, where RUₘₐₓ is maximum response and ConcLPS is the concentration of LPS. In addition, Kₒ can be calculated as the ratio of these two constants (kᵢ/kₐᵢ). In brief, the dissociation rate constant kₐᵢ is described by the equation: Rₘₐₓ/(1 + e⁻<sup>kₐᵢt</sup> + kₐᵢt – 1), where C is the concentration of LPS, Rₘₐₓ represents the maximum binding capacity of LPS to immobilized CRISPLD2, and Rₘₐₓ represents the amount of LPS bound to the CRISPLD2 at time t. The dissociation rate constant kₐᵢ is determined when the LPS passing over CRISPLD2 on the surface of chip is replaced by PBS alone.
of TLR4 transcript in T cells, the CD4+ T cells (98% purity) were first isolated by CD4 T cells negative selection kit and then, in second step, purified by a CD4 monoclonic positive selection kit (Miltenyi Biotech). To assess CRISPLD2 on cell surfaces, PBMCs (10^6 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 Cy5 goat anti-rabbit IgG 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 25% inactivated human serum, and fixed with 4% paraformaldehyde 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 at 15 μg/ml for 1 h. The cells were washed three times with 1× Perm/Wash buffer again and stained with Cy2-labeled (green) goat anti-rabbit IgG for 30 min. T cell, NK cell, and monocyte-specific surface markers and the intracellular CRISPLD2 were detected by a FACS Calibur 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 references of rCRISPLD2 in PBS (0.3, 0.1, 0.03, and 0.01 mg/ml) were used as control for diluted samples. The electrophoresis was performed in loading buffer without DTT for retaining IgG above the 150 kDa position on a gel to avoid overlap of the IgG H chain with CRISPLD2/Crispld2. The membranes were blocked with 5% dry skim milk and incubated with rabbit anti-CRISPLD2 polyclonal Ab at 37°C for 2 h, followed by a secondary Ab coupled with fluorocoe (Rockland) at 37°C for 1 h. The blotting membranes were finally scanned by the Odyssey infrared imaging system (LI-COR Biosciences). The serum CRISPLD2 concentration, represented by the density of each spot, was calculated according to reference of the rCRISPLD2 standard.

Immunofluorescence assay and immunohistochemical staining

For identification of Crispld2 in mouse and rat tissues, freshly sacrificed mice and rats were arterially perfused with 4% paraformaldehyde in PBS. The excised tissues were fixed in the same buffer. Paraffin-embedded tissue sections of 5 μm were mounted on glass slides, 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 recognition of the primary Ab. Hoechst dye was used for nuclear staining. Immunofluorescence was visualized using a confocal microscope (LSM 510, Version 3.5 META; 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 × 10^6 cells/ml in PBS containing 0.3% BSA with different doses of rCRISPLD2 or rCRISP-3 (0, 0.018, 0.054, 0.178, 0.535, 1.78, and 5.35 μg/ml) 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 fluorescent intensity units.

Stimulation of immune cells in vitro

For assessing CRISPLD2 secretion from cells in vitro, human granulocytes at 1 × 10^6 cells/ml, PBMCs, purified T cells, and NK cells at 2 × 10^6 cells/ml, and purified monocytes at 0.5 × 10^6 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 × 10^6 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 mouse serum, appropriately diluted solutions in sodium carbonate buffer (pH 9.5) were used for the quantification of CRISPLD2 and for the reference of mouse Crispld2. It should be pointed out that the concentration of bovine Crispld2 in FCS is very low, and the incubation with 8% FCS does not significantly increase the background in ELISA (see supplemental Fig. 5).

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. A standard curve of rCRISPLD2 titeration (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 for the reference of mouse Crispld2. It should be pointed out that the concentration of bovine Crispld2 in FCS is very low, and the incubation with 8% FCS does not significantly increase the background in ELISA (see supplemental Fig. 5).

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. A standard curve of rCRISPLD2 titeration (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 for the reference of mouse Crispld2. It should be pointed out that the concentration of bovine Crispld2 in FCS is very low, and the incubation with 8% FCS does not significantly increase the background in ELISA (see supplemental Fig. 5).

Mice and in vivo LPS stimulation

Six- to 8-week BALB/c female mice were purchased from SIPPR-BK Experimental Animal Ltd. All mice were housed in the animal facility of 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 Crispld2 concentration in response to LPS and LTA, 6- to 8-week-old BALB/c mice were injected i.p. with S. enterica 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 Crispld2 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. Statistics analyses on the correlation between serum Crispld2 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

The online version of this article contains supplemental material.
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 homemade 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 LPS or S. enterica LTA, indicating that CRISPLD2 did interact physically with LPS. To further confirm the finding, we performed a SPR assay by using a BIAcore 3000 biosensor to quantitatively assess the recognition of 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 sensorgram. 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).

**Table I. Affinity of LPS to its receptors and soluble binding proteins**

<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 ± 0.8 × 10^{-6}</td>
<td>Fig. 1D</td>
</tr>
<tr>
<td>CRISPLD2</td>
<td>1.33 ± 0.9 × 10^{-6}</td>
<td>Fig. 1B</td>
</tr>
<tr>
<td>LBP</td>
<td>1.40 × 10^{-8}</td>
<td>8</td>
</tr>
<tr>
<td>CD14</td>
<td>0.98 × 10^{-5}</td>
<td>8</td>
</tr>
<tr>
<td>CD14</td>
<td>0.87 ± 0.2 × 10^{-5}</td>
<td>32</td>
</tr>
<tr>
<td>MD-2</td>
<td>2.33 ± 0.9 × 10^{-6}</td>
<td>32</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.41 ± 0.7 × 10^{-5}</td>
<td>32</td>
</tr>
</tbody>
</table>

* All K_D values were measured by SPR assay.

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 CRISPDL2 gene is transcribed in leukocytes 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 CRISPDL2 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 LBP s, the serum levels of CRISPDL2 are substantially higher than those of LBP, soluble CD14, bactericidal/permeability-increasing protein, and soluble CD6 (supplemental Table I, Fig. 2, A and B, and Refs. 16, 33, and 34). Moreover, human peripheral blood granulocytes and mononuclear cells, including monocytes, NK cells and T cells, express CRISPDL2 that was identified by RT-PCR (Fig. 2C) in concordance with published microarray data for gene expression (35–38). CRISPDL2 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 Crispdl2 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 CRISPDL2, 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 PBMCs, including purified monocytes, T cells, and NK cells. 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.

**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).

**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...
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 \textit{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-\alpha production.

It is known that purified \textit{S. aureus} LTA is a ligand of CD6 (17) that can activate TLR2 but no other TLRs, including TLR4 (41). We investigated whether purified \textit{S. aureus} LTA up-regulates CRISPLD2 release in vivo and in vitro. Fig. 4, A and B, and supplemental Fig. S5B show that purified \textit{S. aureus} LTA (1 \mu g/ml) failed to up-regulate CRISPLD2 release from PBMCs, purified CD4 T cells, and monocytes. Furthermore, Crispld2 serum levels of purified \textit{S. aureus} LTA-treated mice (100 \mu g/per-mouse) did not significantly rise (Fig. 4F), implying that \textit{S. aureus} LTA binding to CD6 and \textit{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.
Moreover, supplemental Fig S4 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 further 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 \textit{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-\(\alpha\) 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.

\textbf{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-\(\alpha\) 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-\(\alpha\) 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-\(\alpha\) 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-\(\alpha\) 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.

\textbf{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 \(\mu\)g of \textit{E. coli} LPS killed 80% of the treated animals within a few days. Injection of \textit{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 III). 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 experienced substantial protection against LPS-induced lethal shock. Plotted against a lethal LPS dose, it was shown that CRISPLD2 serum levels as well as LPS-reactive IgG serum levels correlate similarly and reciprocally with LPS lethality (Fig. 6B and supplemental Fig. S6). However, it has been known preadministration of the J5 LPS vaccine increases the concentration of serum anti-LPS core Abs in individuals (20). Whether both LPS-binding structures synergistically account for enhanced LPS resistance is to be further determined.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
Treatment & Concentration (\(\mu\)g/ml) & CRISPLD2 (\(\mu\)g/ml) & TNF-\(\alpha\) (ng/ml) & IL-6 (ng/ml) \\
\hline
Control & 0.37 \(\pm\) 0.10 & 0.04 \(\pm\) 0.01 & 0.11 \(\pm\) 0.02 & 0.06 \(\pm\) 0.04 \\
\textit{E. coli} LPS & 1.59 \(\pm\) 0.26 & 8.56 \(\pm\) 0.62 & 10.5 \(\pm\) 0.28 & 0.76 \(\pm\) 0.28 \\
\textit{R. sphaeroides} LPS & 1.40 \(\pm\) 0.06 & 3.45 \(\pm\) 0.17 & 7.98 \(\pm\) 0.22 & 0.59 \(\pm\) 0.22 \\
\textit{E. coli} LPS + \textit{R. sphaeroides} LPS & 1 \(\pm\) 1 & 1.65 \(\pm\) 0.09 & 3.29 \(\pm\) 0.07 & 7.25 \(\pm\) 0.59 \\
Anti-hTLR4-IgA & 1 & 1.66 \(\pm\) 0.04 & 0.08 \(\pm\) 0.03 & 0.11 \(\pm\) 0.03 \\
Anti-hTLR4-IgA & 5 & 1.93 \(\pm\) 0.21 & 0.13 \(\pm\) 0.01 & 0.07 \(\pm\) 0.03 \\
\textit{E. coli} LPS + Anti-hTLR4-IgA & 1 \(\pm\) 1 & 2.25 \(\pm\) 0.24 & 3.17 \(\pm\) 0.09 & 7.06 \(\pm\) 0.45 \\
\textit{E. coli} LPS + Anti-hTLR4-IgA & 1 \(\pm\) 5 & 2.55 \(\pm\) 0.43 & 2.63 \(\pm\) 0.07 & 5.27 \(\pm\) 0.68 \\
\hline
\end{tabular}
\caption{Both \textit{R. sphaeroides} LPS and anti-hTLR4-IgA Ab up-regulate CRISPLD2 secretion and reduce \textit{E. coli} LPS-induced TNF-\(\alpha\) and IL-6 production}
\end{table}
Discussion

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 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) 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 alone (open bars), 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).

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 selectivly, 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).
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 transcrip
tion 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 ensure 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 immunostimulatory 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 guarding 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
We thank Q. N. Zhuang (GE Healthcare) for technical assistance on SPR.

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>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>Yes</td>
<td>0.323–0.446</td>
<td>0.8</td>
<td>4/6</td>
</tr>
<tr>
<td>Yes</td>
<td>0.338–0.386</td>
<td>1.1</td>
<td>4/4</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. CRISPLD2 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 recorded. 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 R² indicated on the graph.


38. Hyczra, M. D., C. Kovacs, M. Loutfy, R. Halpeny, L. Heisler, S. Yang, O. Wilkins, M. Ostrowski, and S. D. Ber. 2007. Distinct transcriptional profiles in ex vivo CD4+ and CD8+ T cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon-γ response as well as extensive transcriptional changes in CD8+ T cells. J. Virol. 81: 3477–3486.


42. Cairns, B., R. Maile, C. M. Barnes, J. A. Feingold, and A. A. Meyer. 2006. Increased Toll-like receptor 4 expression on T cells may be a mechanism for enhanced T cell response late after burn injury. J. Trauma 61: 293–299.


