

# Targeting of Key Pathogenic Factors From Gram-Positive Bacteria by the Soluble Ectodomain of the Scavenger-Like Lymphocyte Receptor CD6

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**Gram-positive bacteria cause a broad spectrum of infection-related diseases in both immunocompetent and immunocompromised hosts, ranging from localized infections to severe systemic conditions such as septic and toxic shock syndromes. This situation has been aggravated by the recent emergence of multidrug-resistant strains, thus stressing the need for alternative therapeutic approaches. One such possibility would be modulating the host's immune response. Herein, the potential use of a soluble form of the scavenger-like human lymphocyte receptor CD6 (shCD6) belonging to an ancient family of innate immune receptors has been evaluated. shCD6 can bind to a broad spectrum of gram-positive bacteria thanks to the recognition of highly conserved cell wall components (lipoteichoic acid [LTA] and peptidoglycan [PGN]), which are essential for their viability and pathogenicity and are not amenable to antibiotic resistance. shCD6 has in vitro inhibitory effects on both bacterial growth and Toll-like receptor-mediated inflammatory response induced by LTA plus PGN. In vivo infusion of shCD6 improves survival on mouse models of septic shock by *Staphylococcus aureus* (either multidrug-resistant or -sensitive) or their endotoxins (LTA + PGN) or exotoxins (TSST-1). These results support the use of shCD6 and/or other scavenger-like immune receptors in the treatment of severe gram-positive-induced infectious conditions.**

**Keywords.** gram-positive bacteria; peptidoglycan; lipoteichoic acid; CD6; *Staphylococcus aureus*; superantigens; scavenger receptors; sepsis; toxic shock.

Microbial recognition by the immune system is essential to host defense as it triggers effector responses such as phagocytosis and inflammation to promote pathogen clearance and killing, tissue repair, and induction of adaptive immunity [1]. This is mainly achieved through detection of pathogen-associated molecular

patterns (PAMPs) by soluble and/or membrane-bound pattern recognition receptors (PRRs) expressed by innate immune cells. PAMPs include broadly distributed and highly conserved microbial structures, absent from the host and essential to pathogen viability, such as lipopolysaccharide (LPS) from gram-negative ( $G^-$ ), and lipoteichoic acid (LTA) and peptidoglycan (PGN) from gram-positive ( $G^+$ ) bacteria. PRRs include a relatively small number of nonpolymorphic germ line-encoded receptors belonging to different structural and functional protein families [2], one of which is the ancient and highly conserved scavenger receptor cysteine-rich superfamily (SRCR-SF). This superfamily currently includes >30 members found in all animal phyla [3, 4]. In mammals, SRCR-SF members are found

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expressed on myeloid (SR-AI, MARCO, CD163, Sp $\alpha$ ) [5–7], lymphoid (CD5, CD6) [8, 9], and epithelial cells (SCARA5, DMBT1, S5D-SRCRB) [10–12], where they display multiple functional capabilities, including recognition of bacterial, fungal, viral, and/or parasitic PAMPs [4].

CD6 is a 105–130 kDa transmembrane glycoprotein mainly expressed on lymphocytes (T, B1a, and NK cells) [13–15], hematopoietic precursors [16] and certain brain regions [17]. CD6 has been classically involved in cell-adhesion contacts relevant to lymphocyte activation and differentiation [18–21], through interaction with CD166/ALCAM (activated leukocyte cell adhesion molecule), an adhesion molecule of the immunoglobulin SF [22]. Importantly, CD6 physically associates with the T-cell receptor (TCR)/CD3 complex and colocalizes with it at the center of the immunological synapse [23], where its interaction with CD166/ALCAM results critical for maturation and stabilization of this signaling structure [19, 23].

As reported for other lymphocyte receptors, low levels of a soluble form of CD6 (shCD6) is found in human serum [9, 24]. The exact function of shCD6 is unknown but it could work as a decoy receptor, thus preventing binding of membrane-bound CD6 to endogenous (CD166/ALCAM) or exogenous (PAMPs) ligands. Indeed, membrane-bound CD6 can sense and signal the presence of bacterial PAMPs, and recombinant shCD6 (rshCD6) binds to both intact G<sup>-</sup> (*Escherichia coli*) and G<sup>+</sup> (*Staphylococcus aureus*) bacteria, a fact that is competed by LPS and LTA, respectively [9]. Accordingly, a bacterial-binding motif (VEVXXXXXW) [6, 11] is found in CD6 domains D1 and D3, although its functionality remains to be proven.

The present report further explores the hypothesis that shCD6 could serve as a decoy receptor for G<sup>+</sup> PAMPs. This has been achieved by increasing the circulating levels of shCD6 up to nano/micromolar range. The results show that rshCD6 significantly reduces the systemic inflammatory response induced by live *S. aureus* or their endo/exotoxins, which is of clinical relevance as G<sup>+</sup> bacteria, especially *S. aureus*, are among the main causative agents of sepsis in the United States and Europe [25–27].

## MATERIALS AND METHODS

### Expression, Purification, and Biotinylation of Recombinant Proteins

The rshCD6 and rshCD5 proteins were produced and affinity purified as previously described [9, 23]. Human seroalbumin (HSA; Institut Grifols) and sCD14 (Sigma) were from commercial source. Proteins were labeled with EZ-Link PEO-maleimide-activated biotin (Pierce) following the manufacturer's instructions.

### Bacterial Binding and Viability Assays

Bacterial strains used were clinical isolates from the Microbiology Department of the Hospital Clinic of Barcelona, with the

exception of *S. aureus* ATCC 29213. The minimum inhibitory concentrations of meropenem for the methicillin-susceptible (ATCC 29213) and -resistant *S. aureus* (MRSA clinical isolate) used in this study were 1.5 and 6 mg/L, respectively, as determined by Etest (bioMérieux). Both strains were highly resistant to penicillin.

Binding of biotin-labeled proteins (2.5 ng) to bacteria ( $5 \times 10^7$  colony-forming units [CFU]) was performed as reported elsewhere [9]. Bacterial viability of *S. aureus* 29213 was assessed by plating serially diluted liquid cultures and measuring adenosine triphosphate (ATP) levels with the BacTiter-Glo Microbial Cell viability assay (Promega) of bacterial pellets.

### Dissociation Constant Studies

Steady-state measurements of tryptophan fluorescence emission and further data analysis of rshCD6 (0.12 mM), free and bound to soluble LTA (Mr 14 000) or PGN (Mr 125 000) from *S. aureus* (Sigma), were performed as previously described [8]. The apparent dissociation constant ( $K_d$ ) was calculated from the saturation curve by a nonlinear least-squares fitting procedure.

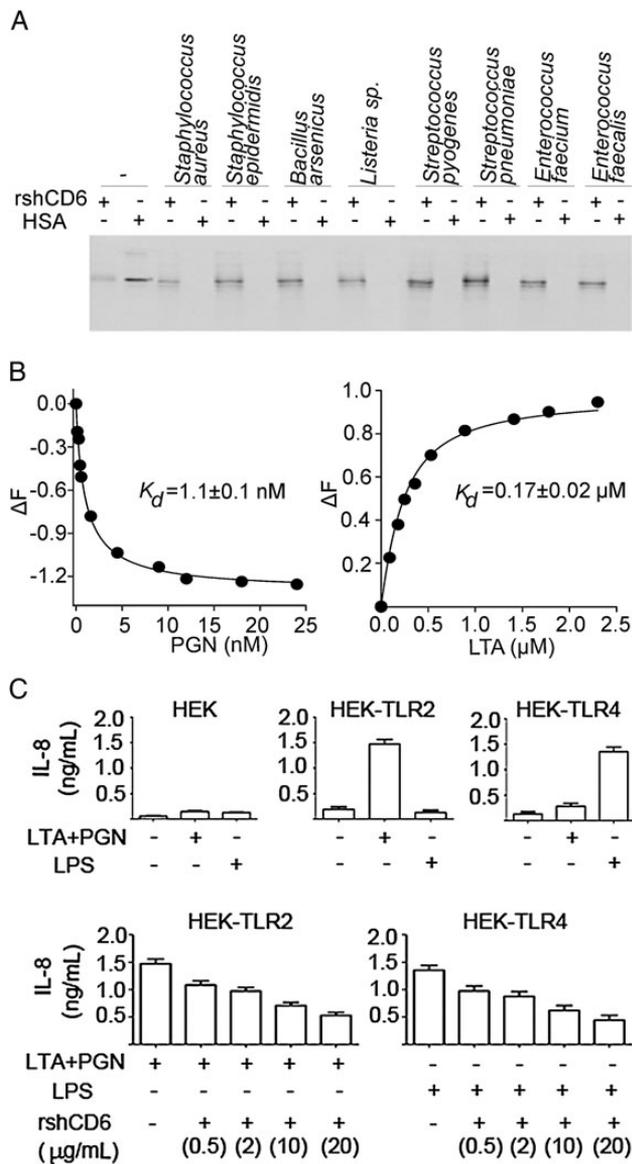
### Mouse Models of Septic Shock

Unless otherwise indicated, 8- to 10-week-old male C57Bl/6J mice (Charles River) were used following approved protocols by the University of Barcelona Animal Experimentation Ethical Committee. Blood samples were taken by facial vein or cardiac puncture in anesthetized mice. For LTA + PGN-induced septic shock, 9 mg/kg LTA plus 30 mg/kg PGN (in saline) were intravenously injected, which was the minimal dose inducing  $\geq 90\%$  lethality at 24–48 hours. For *S. aureus*-induced septic shock,  $3 \times 10^8$  or  $10^{10}$  CFU of *S. aureus* 29213 or MRSA, respectively, were intraperitoneally injected, which were the minimal inocula inducing  $\geq 90\%$  lethality at 24–48 hours. In leukopenic mice, the minimal inoculum of *S. aureus* 29213 rendering  $\geq 90\%$  lethality was determined as  $1 \times 10^6$  CFU. Mice were rendered leukopenic by intraperitoneal administration of 200 mg/kg cyclophosphamide (Baxter) 4 and 2 days before sepsis induction.

For toxic shock induction, 6- to 8-week-old female BALB/c mice (Charles River) were intraperitoneally injected with 0.8 mg/kg toxic shock syndrome toxin 1 (TSST-1; T5662, Sigma) and, 3 hours later, with 5.0 mg/kg LPS (*E. coli* O111:B4; L2630, Sigma).

### Measurement of rshCD6 and Cytokine Plasma Levels

Levels of rshCD6 in plasma samples (1:5 diluted) were determined by sandwich enzyme-linked immunosorbent assay (ELISA) [24], using unlabeled 161.8 and biotin-labeled SPV-L14.2 (2  $\mu$ g/mL each) as capture and detection monoclonal antibodies, respectively, and horseradish peroxidase (HRP)-labeled streptavidin (Roche Diagnostics) and 3,3',5,5'-tetramethylbenzidine TMB Substrate Reagent Set (BD OptEIA, BD Biosciences) as developers.



**Figure 1.** Soluble CD6 binds to live gram-positive ( $G^+$ ) bacteria and purified cell wall components and competes Toll-like receptor (TLR)-mediated interleukin 8 (IL-8) cytokine production. *A*, Biotin-labeled recombinant scavenger-like human lymphocyte receptor CD6 (rshCD6) and human serum albumin (HSA; 2.5 ng each) were incubated with  $5 \times 10^7$  colony-forming units of the indicated  $G^+$  bacterial species. After extensive washings, bacterial pellets were solubilized and separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Biotin-labeled protein was detected by Western blot analysis using horseradish peroxidase–streptavidin. *B*, Changes in Trp fluorescence emission intensity of rshCD6 on addition of either soluble peptidoglycan (PGN) or lipoteichoic acid (LTA). Right-hand panels: Trp fluorescence emission spectra were recorded for a fixed amount of rshCD6 (0.12  $\mu$ M) in the absence or presence of increasing concentrations of PGN (top) or LTA (bottom) at 25°C. Samples were excited at 295 nm, and emission spectra recorded from 300 to 450 nm. One representative experiment of 3 performed is shown. Left-hand panels:  $\Delta F$  is the change in fluorescence intensity at the emission maximum (337 nm) on excitation at 295 nm, in the presence or absence of increasing amounts of PGN (top) or LTA (bottom) at 25°C. All data points are the mean values of experiments performed in triplicate. Recombinant shCD6 binds to PGN and

Mouse cytokine levels in plasma and culture supernatants were determined by commercially available ELISA kits for interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 10 (IL-10), interferon gamma (IFN- $\gamma$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ) (BD OptEIA-Mouse ELISA Sets, BD Biosciences Pharmingen) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) (ELISA Ready-Set Go!, eBiosciences) following the manufacturers' instructions.

#### In Vitro Cell Stimulation Cultures

Splenocytes ( $3 \times 10^5$ ) isolated by density gradient centrifugation over Ficoll-Histopaque (Sigma) were cultured for 48 hours at 37°C in RPMI 1640 medium plus 10% of fetal calf serum containing LTA and PGN (0.5 or 5.0  $\mu$ g/mL each), in the presence or absence of rshCD6. For toxin stimulation, cells were stimulated with 5.0  $\mu$ g/mL staphylococcal enterotoxin A (SEA; S-9399, Sigma), staphylococcal enterotoxin B (SEB; S-4881, Sigma) or TSST-1, also in the presence or absence of rshCD6. Cell viability was assessed by using the PE Annexin V Apoptosis Detection Kit (Immunostep).

#### Toll-like Receptor–Mediated Interleukin 8 Release Assays

HEK293 transfectants ( $4 \times 10^5$  cells) stably expressing human Toll-like receptor (TLR) 2 or TLR4 [28] were placed in 48-well plated and grown at confluence for 24 hours in serum-free X-VIVO15 medium (Lonza), for further stimulation with optimal doses of LTA + PGN or LPS (250 ng/mL each), in the presence or absence of rshCD6. At 18 hours, interleukin 8 (IL-8) levels were measured by ELISA (BD OptEIA human IL-8 ELISA Set, BD Biosciences Pharmingen).

#### Toxin Binding Assays

F96 Maxisorp plates (Nunc) were coated overnight at 4°C with TSST-1, LPS, or SEB (2  $\mu$ g/well) in Tris-buffered saline (TBS; 10 mM Tris-HCl pH 7.2, 150 mM NaCl). After blocking for 1 hour at room temperature with 5% bovine serum albumin (BSA; Sigma), plates were incubated for 2 hours at room temperature with biotin-labeled rshCD5 or rshCD6 in TBS-BSA. Following extensive washings with 0.05% TBS-Tween 20 (Merck), wells were incubated with HRP-labeled streptavidin in

*Figure 1 continued.* LTA with an apparent dissociation constant ( $K_d$ ) of  $1.1 \pm 0.1$  nM and  $0.17 \pm 0.02$   $\mu$ M, respectively. *C*, Specific and dose-dependent inhibition of TLR-mediated activation by rshCD6. HEK293 transfectants stably expressing TLR2, TLR4, or an empty vector were stimulated in triplicate with LTA + PGN (250 ng/mL each) or lipopolysaccharide (LPS; 250 ng/mL), respectively, in the presence or absence of increasing concentrations of rshCD6 (0.5–20  $\mu$ g/mL). Cell viability was >90% at 24 hours in all experimental conditions, as assessed by flow cytometry with propidium iodide/annexin V staining. Secretion of IL-8 was measured 18 hours later by enzyme-linked immunosorbent assay and expressed as mean  $\pm$  SEM of triplicates.

TBS-BSA for 30 minutes at room temperature, and developed with TMB.

## RESULTS

### Binding of rshCD6 to Highly Conserved Components of G<sup>+</sup> Bacterial Cell Walls

Previous results showed that rshCD6 binds to *S. aureus* and that this binding is competed by LTA, but not LPS [9]. Similarly, direct binding of rshCD6 to LTA but also PGN, a major component of G<sup>+</sup> bacterial cell walls, was also observed [8]. This indicated that rshCD6 would display a broad spectrum of G<sup>+</sup> binding, as supported by data shown in Figure 1A. To validate these studies, and also to find out whether the binding affinity of rshCD6 to G<sup>+</sup> bacterial compounds was high enough to be functionally relevant, the  $K_d$  of the interaction of rshCD6 with PGN and LTA was determined by tryptophan fluorescence emission. The obtained values were  $1.1 \pm 0.1$  nM for PGN and  $0.17 \pm 0.02$  mM for LTA (Figure 1B), indicating that the affinity of rshCD6 for PGN is much greater than for LTA but comparable, or even greater, than the reported affinity of the well-known macrophage PRR CD14 for PGN ( $24.9 \pm 4.7$  nM) [29].

### Effect of rshCD6 in LTA + PGN-Induced Inflammatory Responses

Both LTA and PGN are well-known in vitro and in vivo inducers of immune cell-mediated cytokine release, responsible for systemic inflammation and ultimately multiorgan dysfunction and septic shock [30]. Thus, it was first tested whether rshCD6 could modulate cytokine release of mouse splenocytes stimulated for 48 hours with different concentrations of PGN and LTA. As summarized in Table 1, rshCD6 significantly inhibited the release of typical proinflammatory cytokines such as IL-1 $\beta$

and IL-6, but not of TNF- $\alpha$ . In contrast, significant increases of anti-inflammatory cytokines IL-10 and TGF- $\beta$  were observed. No effects on cell viability were shown for rshCD6 alone.

Next, we further tested the ability of rshCD6 to modulate TLR-mediated cytokine production by PGN + LTA on HEK293 transfectants stably expressing TLR2 or TLR4 [28]. As shown in Figure 1C, rshCD6 specifically inhibited the TLR2-mediated IL-8 release induced by PGN + LTA in a dose-dependent manner. The same was true for TLR4-mediated activation of HEK293 cells, as expected from the previously reported LPS-binding properties of rshCD6 [9].

The in vivo relevance of these modulatory properties of rshCD6 on PAMP-mediated inflammatory responses was further analyzed in a lethal model of LTA + PGN-induced mouse septic shock [30]. As illustrated by Figure 2A, a single intraperitoneal administration of rshCD6 (1.25 mg/kg) indeed improved mouse survival in a time-dependent manner. Maximal efficacy was observed when administered 3 hours prior LTA + PGN administration, which is in agreement with time-course analysis of plasma rshCD6 levels following intraperitoneal infusion (Figure 2B). Dose-dependent experiments evaluated at times when maximal efficacy was achieved (-3 hours and -1 hour) showed that the efficacy of rshCD6 increased when the dose was doubled, but only when the protein was administered 1 hour before LTA + PGN (Figure 2C). Significant reductions in serum levels of proinflammatory cytokines were observed in mice prophylactically treated (-1 hour) with rshCD6 (Figure 2D).

### Effect of rshCD6 on *S. aureus*-Induced Septic Shock

Since the LTA + PGN model does not closely resemble the clinical situation, the efficacy of rshCD6 was therefore tested in a lethal model of G<sup>+</sup> sepsis induced by live *S. aureus*. As shown

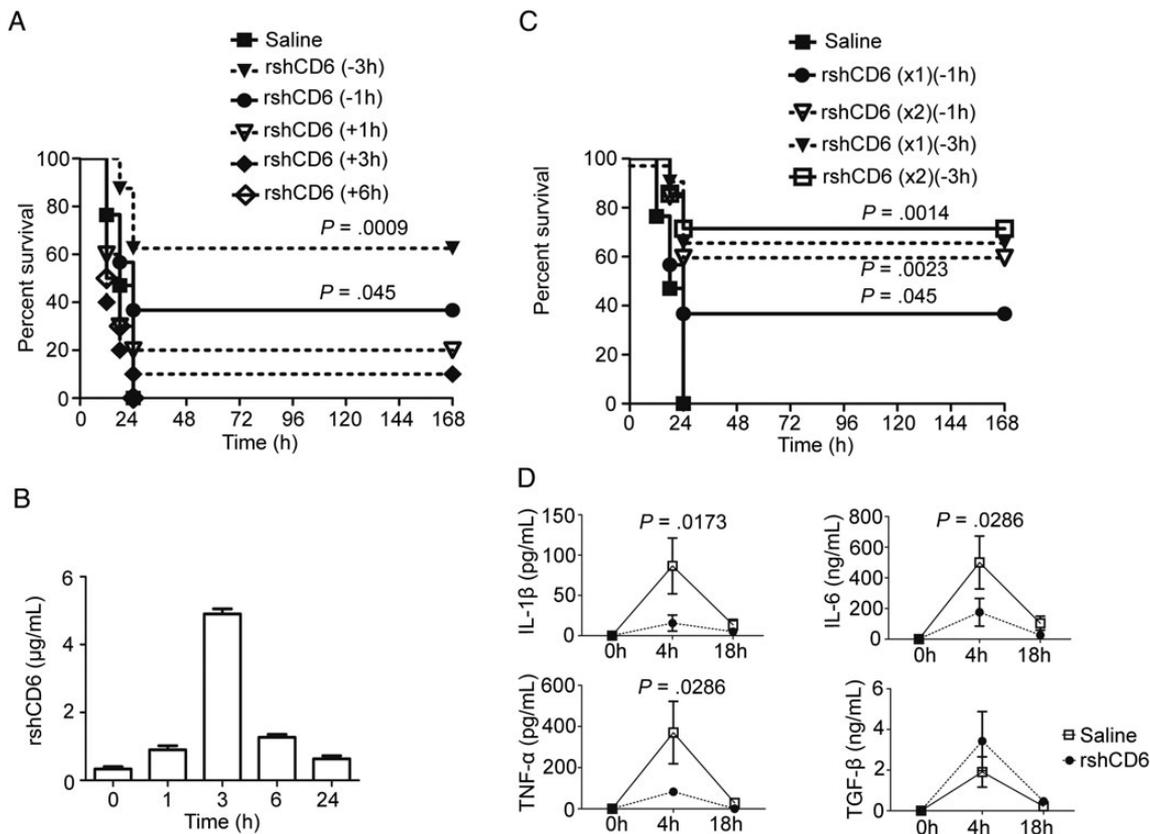
**Table 1. Effect of Recombinant Scavenger-like Human Lymphocyte Receptor CD6 on Cytokine Levels Following In Vitro Stimulation of Mouse Splenocytes With Lipoteichoic Acid Plus Peptidoglycan**

Cytokine	LTA + PGN (0.5 $\mu$ g/mL)			LTA + PGN (5 $\mu$ g/mL)		
	rshCD6 ( $\mu$ g/mL)			rshCD6 ( $\mu$ g/mL)		
	0	2	10	0	2	10
IL-1 $\beta$	138.7 $\pm$ 40.6	53.6 $\pm$ 10.2*	60.3 $\pm$ 23.3*	197.1 $\pm$ 21.2	60.2 $\pm$ 27.2*	85.7 $\pm$ 29.2*
IL-6	625.1 $\pm$ 51.1	256.5 $\pm$ 65.9*	255.1 $\pm$ 93.4*	644.4 $\pm$ 40.6	542.2 $\pm$ 74.2	556.3 $\pm$ 120.9
IL-10	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	2.3 $\pm$ 0.1*	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	1.1 $\pm$ 0.1*
IFN- $\gamma$	0.7 $\pm$ 0.2	1.1 $\pm$ 0.2	2.4 $\pm$ 0.8*	0.9 $\pm$ 0.2	0.9 $\pm$ 0.3	1.2 $\pm$ 0.2
TGF- $\beta$	140.3 $\pm$ 25.3	307.1 $\pm$ 35.4*	454.8 $\pm$ 52.4*	220.3 $\pm$ 13.1	213.7 $\pm$ 25.3	213.7 $\pm$ 12.5
TNF- $\alpha$	125.1 $\pm$ 22.7	128.1 $\pm$ 40.8	156.9 $\pm$ 29.4	150.6 $\pm$ 35.7	168.6 $\pm$ 45.6	161.6 $\pm$ 11.9

Stimulations with LTA + PGN were performed in duplicate for 3 individual mice analyzed. Cytokine results are expressed in picograms per milliliter as mean  $\pm$  SD. Statistical analysis was performed using a 1-tailed Mann-Whitney test, with confidence intervals of 95%.

Abbreviations: IFN, interferon; IL, interleukin; LTA, lipoteichoic acid; PGN, peptidoglycan; rshCD6, recombinant scavenger-like human lymphocyte receptor CD6; TGF, transforming growth factor; TNF, tumor necrosis factor.

\*Represents  $P < .05$  for comparisons between cells stimulated with LTA + PGN in the presence or absence of rshCD6. Viability was  $>75\%$  at 48 hours in all experimental conditions, as tested by flow cytometry with propidium iodide/annexin V staining.



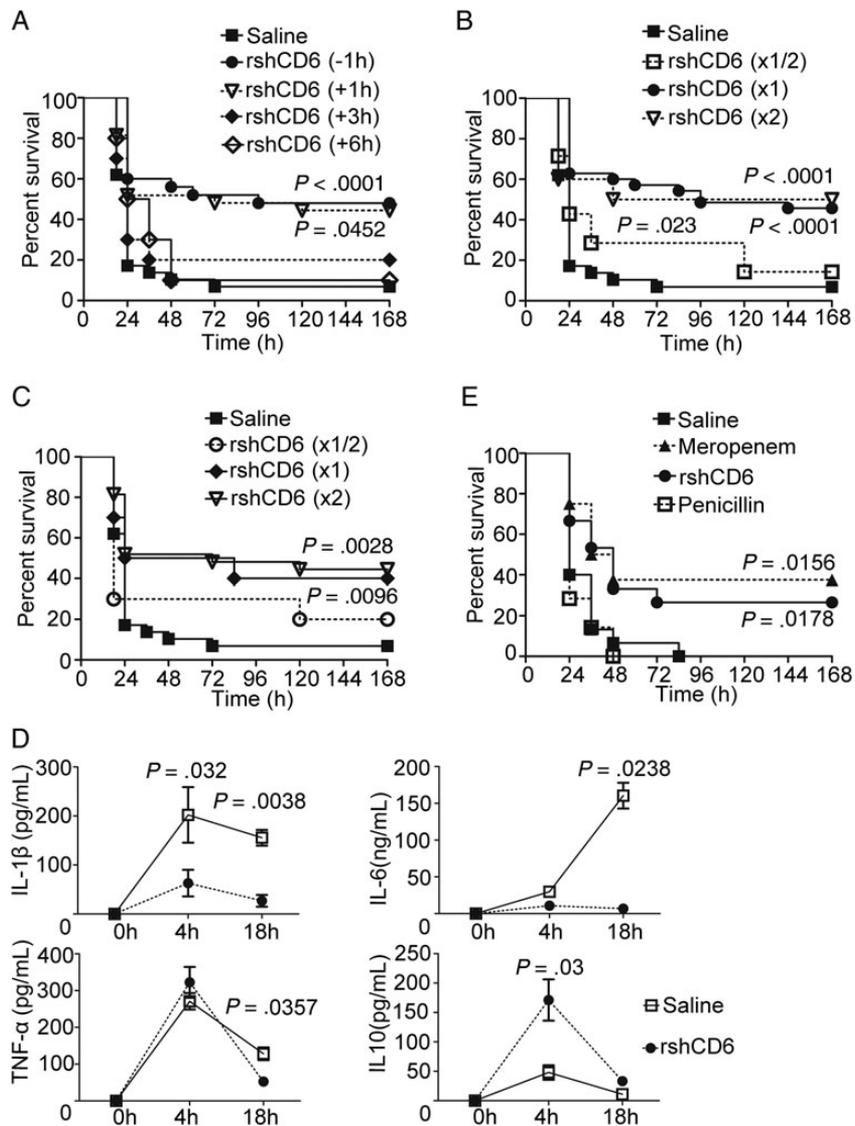
**Figure 2.** Time- and dose-dependent effects of recombinant scavenger-like human lymphocyte receptor CD6 (rshCD6) infusion in a mouse model of sepsis induced by lipoteichoic acid (LTA) + peptidoglycan (PGN). **A**, C57Bl/6J mice were injected intravenously with a lethal dose of LTA (9 mg/kg) plus PGN (30 mg/kg) and then treated with saline ( $n = 17$ ) or rshCD6 (1.25 mg/kg) at different time points before (–3 hours,  $n = 8$ ; –1 hour,  $n = 30$ ) or after (+1 hour,  $n = 11$ ; +3 hours,  $n = 10$ ; +6 hours,  $n = 10$ ) LTA + PGN injection. Average survival was analyzed over time and compared to the saline-treated group using a log-rank  $t$  test. **B**, C57Bl/6J mice ( $n = 3$ ) were infused intraperitoneally with rshCD6 (1.25 mg/kg) and then bled at different time points for enzyme-linked immunosorbent assay (ELISA) determination of plasma levels of rshCD6. **C**, C57Bl/6J mice were injected with LTA + PGN as in (A) and infused at either –3 hours with 1.25 ( $\times 1$ ;  $n = 8$ ) or 2.5 ( $\times 2$ ;  $n = 7$ ) mg/kg rshCD6, or at –1 hour with 1.25 ( $\times 1$ ;  $n = 30$ ) or 2.5 ( $\times 2$ ;  $n = 8$ ) mg/kg of rshCD6. Average survival was analyzed over time and compared to the saline-treated group using a log-rank  $t$  test. **D**, Cytokine plasma levels were monitored by ELISA at different time points following intravenous injection of LTA + PGN (as above), preceded or not by a prophylactic (–1 hour) intraperitoneal infusion of rshCD6 (1.25 mg/kg). Data are expressed as mean  $\pm$  SEM. Statistical differences were evaluated using a 2-tailed Student  $t$  test comparing the rshCD6- and saline-treated groups ( $n = 6$  each). Abbreviations: IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

by Figure 3A, the effect of a single dose of rshCD6 (1.25 mg/kg) on mouse survival was time dependent, with the best results obtained when administered 1 hour before or after bacteria injection. Dose-dependent experiments at times of maximal efficacy (–1 hour and 1 hour) showed no further improvement when the dose was doubled, but administration of half-dose resulted in loss of efficacy (Figure 3B and 3C). It was also showed that therapeutic (1 hour) administration of rshCD6 (1.25 mg/kg) significantly reduced the levels of IL-6 and IL-1 $\beta$ , but not of TNF- $\alpha$  (Figure 3D). Interestingly, a significant increase in the levels of IL-10, a Th2-type anti-inflammatory cytokine, was observed. These results are in agreement with a reduced bacterial load in blood and peritoneum in rshCD6-treated mice (Supplementary Figure 1).

Because the mechanism of antibiotic resistance in MRSA does not involve the highly conserved structures recognized by

rshCD6, it was hypothesized that it could have beneficial effects in septic shock induced by such a strain. As shown by Figure 3E, prophylactic infusion of rshCD6 (1.25 mg/kg) significantly improved survival of mice intraperitoneally injected with a MRSA clinical isolate as compared to the saline-treated group (26.7% vs 0%,  $P = .0178$ ). According to the antibiotic resistance profile of the MRSA strain, prophylactic penicillin (–1 hour) did not show any protective effect, whereas meropenem did result in significant survival improvement. There were no significant differences in survival between the meropenem- and rshCD6-treated groups. Similar results were obtained with a colistin-resistant *Acinetobacter baumannii* strain (Supplementary Figure 2), indicating that the broad spectrum effect of rshCD6 also extends to antibiotic-resistant G<sup>–</sup> bacteria.

The putative involvement of leukocytes in the protective effect of rshCD6 in *S. aureus*-induced septic shock was tested on mice

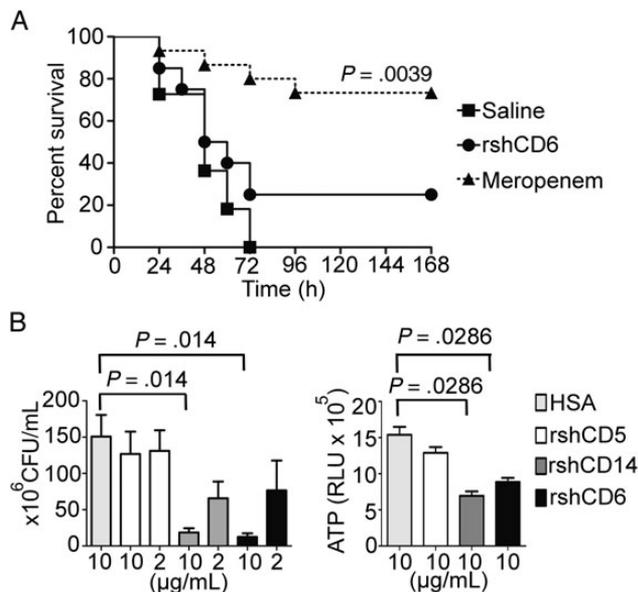


**Figure 3.** Time- and dose-dependent effects of recombinant scavenger-like human lymphocyte receptor CD6 (rshCD6) infusion in a mouse model of sepsis induced by live *Staphylococcus aureus*. **A**, C57Bl/6J mice were injected intraperitoneally with a lethal dose of *S. aureus* ATCC 29213 ( $3 \times 10^8$  colony-forming units [CFU]), and then infused with rshCD6 (1.25 mg/kg) at different time points either before (–1 hour,  $n = 25$ ) or after (+1 hour,  $n = 25$ ; +3 hours,  $n = 10$ ; +6 hours,  $n = 10$ ) bacterial injection. Average survival was analyzed over time and compared to the saline-treated group ( $n = 29$ ) using a log-rank  $t$  test. **B**, C57Bl/6J mice were intraperitoneally injected with *S. aureus* 29213 as above and infused 1 hour before (–1 hour) with 0.625 ( $\times 1/2$ ;  $n = 7$ ), 1.25 ( $\times 1$ ;  $n = 25$ ) or 2.5 ( $\times 2$ ;  $n = 10$ ) mg/kg rshCD6. Average survival was analyzed over time and compared to the saline-treated group using a log-rank  $t$  test. **C**, The same experiment as in (**B**) was performed except that rshCD6 was infused 1 hour after (+1 hour) bacterial injection with 0.625 ( $\times 1/2$ ;  $n = 10$ ), 1.25 ( $\times$ ;  $n = 18$ ) or 2.5 ( $\times 2$ ;  $n = 10$ ) mg/kg of rshCD6. **D**, Cytokine plasma levels were quantified by enzyme-linked immunosorbent assay at different time points following *S. aureus* injection and further therapeutic (+1 hour) infusion of rshCD6 (1.25 mg/kg). Data are expressed as mean  $\pm$  SEM. Statistical differences were evaluated using a 2-tailed Student  $t$  test comparing the rshCD6- and saline-treated groups ( $n = 8$  each). **E**, C57Bl/6J mice were injected intraperitoneally with a lethal dose ( $10^{10}$  CFU) of methicillin-resistant *S. aureus* before (–1 hour) intraperitoneal infusion of sterile saline solution ( $n = 15$ ), 1.25 mg/kg rshCD6 ( $n = 15$ ), 7.15 mg/kg/12 hours meropenem ( $n = 8$ ), or 41.5 IU/g/8 hours penicillin ( $n = 8$ ). Average survival was analyzed over time and compared to the saline-treated group using a log-rank  $t$  test. Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

rendered leukopenic by prior cyclophosphamide treatment. As shown in Figure 4A, the efficacy of rshCD6 administered prophylactically (–1 hour) was importantly reduced in leukopenic mice, dropping from >50% survival in nonleukopenic mice (see Figure 3A) to <30%, thus indicating the requirement of intact

leukocyte counts for maximal efficacy. In contrast, treatment with the bactericidal antibiotic meropenem retained its efficacy.

The fact that leukopenia did reduce but not fully abrogate the effect of rshCD6 on mice survival suggested that an additional mechanism of action was still operative. It was then tested



**Figure 4.** Recombinant scavenger-like human lymphocyte receptor CD6 (rshCD6) has both leukocyte-dependent and direct inhibitory bacterial growth effects. *A*, C57BL/6J mice were rendered leukopenic by intraperitoneal administration of 200 mg/kg cyclophosphamide 4 and 2 days before intraperitoneal injection of *Staphylococcus aureus* ATCC 29213 ( $1 \times 10^6$  colony-forming units [CFU]). Leukopenic mice were prophylactically (–1 hour) infused with saline ( $n = 11$ ), rshCD6 (1.25 mg/kg;  $n = 20$ ), or meropenem (7.15 mg/kg/12 hours;  $n = 15$ ) before bacteria injection. Average survival was analyzed over time and compared to the saline-treated group using a log-rank *t* test. *B*, *S. aureus* ATCC 29213 cells ( $4 \times 10^4$  CFU/mL) were cultured in Dulbecco’s modified Eagle medium in the presence of human seroalbumin (HSA), rshCD5, rshCD14, or rshCD6 (2 or 10 μg/mL each) at 37°C for 18 hours. Bacteria were then either plated on Luria-Bertani agar for determination of CFU numbers (left-hand panel) or tested for adenosine triphosphate (ATP) content (right-hand panel). Results are expressed as relative luminescence units (RLU) and represented as mean  $\pm$  SE of 3 independent experiments performed. Statistical differences were evaluated using a 2-tailed Student *t* test.

whether rshCD6 exhibited direct inhibitory effects on bacterial growth in a way similar to that recently reported for soluble CD14 [31]. As shown by Figure 4*B*, addition of rshCD6 to liquid cultures inhibited *S. aureus* growth in a dose-dependent fashion, as measured by viable bacterial cell counts and ATP production. Similar inhibitory effects were observed for shCD14, but not for HSA or rshCD5, the latter being a structurally related protein used as negative control for bacterial binding. The rshCD6-mediated inhibitory effects were also observed when *E. coli* was used (data not shown), suggesting that they are extensible to  $G^-$  bacteria as also reported for shCD14 [31].

#### Effect of rshCD6 in Superantigen-Induced Mouse Inflammatory Responses

In addition to classical sepsis, some  $G^+$  strains (staphylococci and streptococci) secrete exotoxins responsible of sepsis-like

syndromes. These toxins behave as superantigens and trigger simultaneous and rapid polyclonal T-cell activation with subsequent massive cytokine release, shock, and eventually death [32]. Because rshCD6 inhibits cell–cell interactions mediated by bacterial superantigens [19, 23], its protective effect on toxic shock mediated by *S. aureus* exotoxins was tested. Indeed, rshCD6 significantly reduced the in vitro release of IL-1 $\beta$ , IL-6, and TGF- $\beta$  induced by different subfamilies of *S. aureus* superantigens (TSST-1, SEB, and SEA; Table 2). Significant reductions in TNF- $\alpha$  and IFN- $\gamma$  production, as well as induction of IL-10, could also be observed for only some superantigens (SEA and TSST-1). These results were in agreement with those obtained in a lethal mouse model of TSST-1–induced toxic shock [33]. As shown by Figure 5*A*, both prophylactic and therapeutic intraperitoneal administration of rshCD6 significantly improved mice survival; maximal efficacy (100% survival) was observed when administered 1 hour after TSST-1. Similar effects were observed when mice were pretreated with rshCD6 1 hour before SEB-induced toxic shock (data not shown). Interestingly, ELISA assays demonstrated the direct and dose-dependent binding of biotin-labeled rshCD6 to both TSST-1 and SEB (Figure 5*B*).

## DISCUSSION

The present work reports on the in vitro and in vivo effects of a recombinant soluble form of the scavenger-like lymphocyte receptor CD6, in different experimental settings of  $G^+$  bacterial aggression. This form showed inhibitory effects on cytokine release induced by *S. aureus* endotoxins (LTA + PGN) and exotoxins (TSST-1, SEA, SEB) on mouse splenocytes. Moreover, single-dose administration of rshCD6 reduced mortality, bacterial load, and plasma cytokine levels in mouse models of septic shock by live *S. aureus* in a time-, dose-, and leukocyte-dependent manner. These effects very likely relate to the direct binding of rshCD6 to LTA and PGN, as well as to its ability of inhibiting TLR2-mediated cytokine release induced by those PAMPs. However, rshCD6 also showed inhibitory effects on bacterial growth, suggesting a more complex and multifactorial mechanism of action.

Soluble members of the SRCR-SF have been shown to similarly bind to and aggregate  $G^+$  and/or  $G^-$  bacteria [5–7, 10–12]. However, there is no reported evidence on the in vivo functional relevance of any of these proteins in mouse models of infection. Thus, the present report opens new pathways to explore the in vivo anti-infectious and/or anti-inflammatory properties of other soluble SRCR-SF members during microbial aggression.

Outside the SRCR-SF, a well-documented LPS- and PGN-binding PRR whose in vivo efficacy in sepsis has been explored is CD14, a macrophage receptor that also exists in soluble form in serum. CD14 shares with rshCD6 a similar  $K_d$  for both PAMPs [9], as well as similar capacity to inhibit bacterial

**Table 2. Effect of Recombinant Scavenger-like Human Lymphocyte Receptor CD6 on Cytokine Levels Following In Vitro Stimulation of Mouse Splenocytes With Staphylococcal Enterotoxin A, Staphylococcal Enterotoxin B, or Toxic Shock Syndrome Toxin 1**

Stimulation	rshCD6, $\mu\text{g}/\text{mL}$	IL-1 $\beta$	IL-6	IL-10	IFN- $\gamma$	TGF- $\beta$	TNF- $\alpha$
SEA	0	479.2 $\pm$ 14.6	742.7 $\pm$ 152.0	2772.6 $\pm$ 146.1	1547.4 $\pm$ 201.1	481.3 $\pm$ 59.6	130.3 $\pm$ 13.8
	2	137.2 $\pm$ 38.8*	311.9 $\pm$ 45.1*	4433.3 $\pm$ 648.2*	965.4 $\pm$ 83.3*	246.8 $\pm$ 107.6*	42.3 $\pm$ 10.6*
	10	150.6 $\pm$ 26.9*	337.8 $\pm$ 76.2*	4620 $\pm$ 1159.1*	1179.4 $\pm$ 407.9	1135.9 $\pm$ 269.9*	44.6 $\pm$ 30.4*
SEB	0	71 $\pm$ 12.5	375.6 $\pm$ 21.1	2124.6 $\pm$ 762.2	1284 $\pm$ 63.8	500.6 $\pm$ 30.2	57.3 $\pm$ 4.0
	2	14.5 $\pm$ 11.4*	291.6 $\pm$ 20.5*	1966 $\pm$ 748.0	1269 $\pm$ 111.3	330.7 $\pm$ 66.3*	46 $\pm$ 4*
	10	15.2 $\pm$ 10.1*	271.3 $\pm$ 27.7*	2216 $\pm$ 1031.1	1162.6 $\pm$ 464.1	299.1 $\pm$ 87.6*	67.6 $\pm$ 67.9
TSST-1	0	834.8 $\pm$ 34.6	420.4 $\pm$ 25.6	348.1 $\pm$ 25.1	1050.3 $\pm$ 149.4	271.3 $\pm$ 28.6	42 $\pm$ 4.5
	2	331.2 $\pm$ 70.6*	281.7 $\pm$ 52.4*	1347.3 $\pm$ 124.2*	1167.6 $\pm$ 139.5	156.7 $\pm$ 12.6*	40.3 $\pm$ 5.5
	10	323.6 $\pm$ 115.6*	266.3 $\pm$ 63.0*	1467.3 $\pm$ 293.8*	1135.9 $\pm$ 269.9	150.8 $\pm$ 51.6*	46 $\pm$ 25.3

Stimulations with the indicated superantigens (5  $\mu\text{g}/\text{mL}$  each) were performed in duplicate for 3 individual mice analyzed. Cytokines results are expressed in picograms per milliliter as mean  $\pm$  SD. Statistical analysis was performed using a 1-tailed Mann–Whitney test, with confidence intervals of 95%.

Abbreviations: IFN, interferon; IL, interleukin; rshCD6, recombinant scavenger-like human lymphocyte receptor CD6; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; TGF, transforming growth factor; TNF, tumor necrosis factor; TSST-1, toxic shock syndrome toxin 1.

\*Represents  $P < .05$  for comparisons between superantigen-stimulated cells in the presence or absence of rshCD6. Viability was  $>75\%$  at 48 hours in all experimental conditions, as tested by flow cytometry with propidium iodide/annexin V staining.

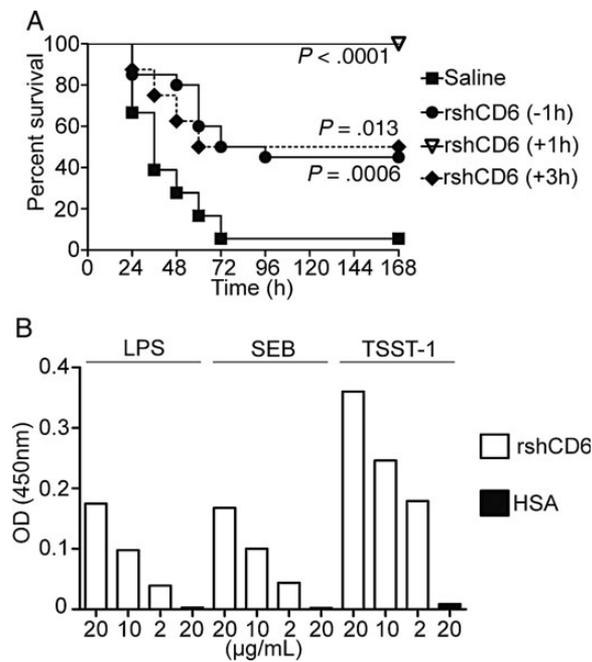
growth [31]. Transgenic expression of or treatment with sCD14 is known to decrease mortality and proinflammatory cytokine levels in LPS-induced sepsis [34, 35] or Shwartzman reaction [36]. However, several reports show neutral or even detrimental effects in mouse models of  $G^-$  [37–39] and  $G^+$  infection [40, 41], and strategies addressed to block CD14 are being considered [42]. This different in vivo efficacy suggests that the mechanism of action of sCD14 and rshCD6 might differ considerably despite sharing similar PAMP-binding spectra.

Aside from PAMP-binding, rshCD6 binds to CD166/ALCAM, although with a lower affinity [18]. This would allow interfering with heterotypical (CD6-ALCAM) and/or homotypical (ALCAM-ALCAM) interactions established by ALCAM-expressing cells (activated T and B cells, macrophages, dendritic cells, fibroblasts, epithelial cells, and endothelial cells) [22], likely affecting diverse inflammation-related events. These effects might take place in our models as the CD6-ALCAM binding site is evolutionarily conserved and allows for the reported cross-species binding of mouse and human CD6 and CD166/ALCAM [22]. It cannot therefore be excluded that rshCD6 may impair inflammation by one or more of these mechanisms, which might explain its broader efficacy when compared to sCD14. The possibility also exists that, by interfering with the function of CD166/ALCAM or other still uncharacterized CD6 ligands [43], rshCD6 may exert immunomodulatory effects that could be detrimental in the short or long term. Nevertheless, single-dose use of rshCD6 limits its presence in time, and, in the context of excessive inflammation occurring in sepsis, the effects appear to incline toward the beneficial. In this regard, no improvement is observed by repeated infusions of rshCD6, the beneficial effect being mainly dependent on the time of administration as reported for antibiotics [44].

Expression of CD166/ALCAM in leukocytes with phagocytic function could also explain the partial dependence of rshCD6 on intact leukocyte populations. In light of these data, it can then be proposed that bacteria or endotoxins bound to rshCD6 might be aggregated [9] and then phagocytized in a ligand-dependent manner (eg, mediated by CD166/ALCAM), thus being eliminated from the tissues and reducing their proinflammatory potential.

Another relevant aspect of the present work is that rshCD6 not only operates against whole  $G^+$  bacteria and their endotoxins, but also against exotoxins. Indeed, rshCD6 showed direct binding to different *S. aureus* superantigens (TSST-1, SEB, SEA) as well as inhibited cytokine release induced by them on mouse splenocytes. Moreover, single-dose administration of rshCD6 improved mouse survival upon TSST-1- (and SEB-) induced toxic shock. The underlying mechanism of action might relate to the known destabilizing effects of rshCD6 on superantigen-induced immunological synapse maturation [19, 23]. Superantigens bypass recognition of peptide-loaded major histocompatibility complex (MHC) molecules by the TCR, but other cell–cell interactions are also needed to stabilize the immune synapse, among them CD6-CD166/ALCAM. Blocking by rshCD6 would reduce the ability of superantigens to induce polyclonal T-cell activation. It is also possible that direct interaction between rshCD6 and superantigens could also impair their association with the TCR and/or the MHC. Both of these mechanisms might be active at the same time.

Altogether, the experimental results reported here might be of clinical relevance for the treatment of  $G^+$  septic shock. It should be noted that experimental models used in this work are much more rapid and lethal ( $>90\%$  mortality in 24–48 hours) than what is usually found in clinical settings (30%–50%



**Figure 5.** Effects of recombinant scavenger-like human lymphocyte receptor CD6 (rshCD6) infusion in a mouse model of sepsis induced by toxic shock syndrome toxin 1 (TSST-1). *A*, BALB/c mice were injected intraperitoneally with TSST-1 (0.8 mg/kg) and 3 hours later with a nonlethal potentiating dose of lipopolysaccharide (LPS; 4.2 mg/kg). Mice were then treated with saline (−1 hour;  $n = 18$ ) or 1.25 mg/kg rshCD6 at different time points (−1 hour,  $n = 20$ ; +1 hour,  $n = 8$ ; +3 hours,  $n = 8$ ). Average survival was analyzed over time and compared to the saline-treated group using a log-rank  $t$  test. *B*, Enzyme-linked immunosorbent assay plates were sensitized with 2 µg/well of TSST-1, staphylococcal enterotoxin B (SEB), or LPS in triplicate. Different concentrations of biotin-labeled rshCD6, rshCD5, or human seroalbumin (HSA) were then added to wells and excess unbound protein was washed off. Bound biotinylated proteins were detected with horseradish peroxidase-labeled streptavidin plus 3,3',5,5'-tetramethylbenzidine (TMB) and monitored by optical density (OD) at 450 nm.

mortality). Results shown here indicate that the maximum efficacy for rshCD6 occurs when this protein is administered prophylactically, which may suggest a limited use in the clinical setting, where patients usually present long after the infection has been initiated. However, it should also be noted that administration in our experimental model is intraperitoneal, which delays rshCD6 access to the bloodstream (serum rshCD6 peaks at 3 hours following intraperitoneal administration); intravenous administration in either an experimental or a clinical situation would make rshCD6 readily available, and would shift the window of efficacy toward later times, thus making its therapeutic use possible.

Currently, sepsis of  $G^+$  origin is the most prevalent, particularly caused by *Staphylococcus* species [26, 27]. The incidence of sepsis has been steadily on the rise, paralleling population aging, increased use of invasive surgical procedures, increased

microbial resistance to antibiotics, and immunosuppressive treatments such as those used in transplanted patients, autoimmune diseases, or hematological malignancies. Mortality figures range from 25% to 50% [44]. The triggers of  $G^+$  sepsis are multiple (endotoxins, exotoxins, drug-sensitive/-resistant live bacteria) and they normally act in combination to cause the clinical symptoms. Recombinant shCD6 displayed in vitro and in vivo beneficial effects against each and every one of these triggers, suggesting that it can act at different levels of mouse sepsis pathophysiology. Importantly, the effect of rshCD6 was independent of the antibiotic-resistance profile of the sepsis-inducing microorganism. Furthermore, results indicate that all these beneficial effects of rshCD6 are also extensible to sepsis by live  $G^-$  bacteria (eg, colistin-resistant/sensitive *A. baumannii*). This broad-spectrum efficacy of rshCD6 also supports its potential use in models of sepsis of polymicrobial origin, although this remains to be proven.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Potential conflicts of interest.** This work is the subject of a patent application (WO2008119851A1). M. M.-F. and M. C.-F. are employees of ImmunNovative Developments, a spinoff company from the University of Barcelona interested in clinical applications of soluble scavenger-like lymphocyte receptors. F. L. is an ad honorem scientific advisor at ImmunNovative Developments. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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