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EXPERIMENTAL THERAPEUTICS

Protective Effects of Human and Mouse Soluble Scavenger-Like CD6 Lymphocyte Receptor in a Lethal Model of Polymicrobial Sepsis

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ABSTRACT Sepsis still constitutes an unmet clinical need, which could benefit from novel adjunctive strategies to conventional antibiotic therapy. The soluble form of the scavenger-like human CD6 lymphocyte receptor (shCD6) binds to key pathogenic components from Gram-positive and -negative bacteria and shows time- and dose-dependent efficacy in mouse models of monobacterial sepsis. The objective of the present work was to demonstrate the effectiveness of infusing mouse and human sCD6 by different systemic routes, either alone or as adjunctive therapy to gold standard antibiotics, in a lethal model of polymicrobial sepsis. To this end, C57BL/6 mice undergoing high-grade septic shock induced by cecal ligation and puncture (CLP; ≥90% lethality) were infused via the intraperitoneal (i.p.) or intravenous (i.v.) route with shCD6 at different doses and time points, either alone or in combination with imipenem/cilastatin (I/C) at a dose of 33 mg/kg of body weight every 8 h. Significantly reduced mortality and proinflammatory cytokine levels were observed by i.p. infusion of a single shCD6 dose (1.25 mg/kg) 1 h pre- or post-CLP. When using the i.v. route, mice survival was significantly extended by starting shCD6 infusion at later time points post-CLP (up to 6 h after CLP). Significant adjunctive effects on mouse survival were observed by i.p. or i.v. infusion of shCD6 in combination with i.p. I/C post-CLP. Similar results were obtained in mice expressing high sustained levels (5 to 10 μg/ml) of mouse sCD6 in serum by means of transduction with hepatotropic adeno-associated virus (AAV). Taken together, the data support the conserved antibacterial effects of human and mouse sCD6 and their use as adjunctive therapy in experimental models of complex and severe polymicrobial sepsis.

KEYWORDS soluble CD6, cecal ligation and puncture, polymicrobial peritonitis, scavenger receptors, sepsis, septic shock, adjunctive therapy

Sepsis, severe sepsis, and septic shock still present unmet clinical needs with a predicted increase in occurrence and a huge socioeconomic burden as a result of population aging, increases in invasive medical procedures, the emergence of multidrug-resistant (MDR) bacteria, and the increased prevalence of chronic diseases (1). Sepsis is considered a dysregulated systemic inflammatory response syndrome

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caused by an infection, leading to an overwhelming and sustained proinflammatory state and, if unresolved, to multiorgan dysfunction (MOD) and death (2). Such a dysfunctional host inflammatory response is triggered by conserved structures present on microbial cell walls named pathogen-associated molecular patterns (PAMPs). PAMPs are essential compounds for the microbial physiology, among which are lipopolysaccharide (LPS) from Gram-negative (G^-) bacteria, lipoteichoic acid (LTA) and peptidoglycan (PGN) from Gram-positive (G^+) bacteria, β-glucan and mannan from fungi, and single- or double-stranded nucleic acids from viruses (3). Detection of PAMPs is accomplished by germ line-encoded, nonclonally distributed, and nonpolymorphic pattern recognition receptors (PRRs) present on immune cells. PRRs belong to different structural and functional protein receptor families (e.g., Toll-like receptors [TLR], scavenger receptors, or C-type lectins) and contribute not only to pathogen detection but also to engagement and modulation of innate and adaptive immune responses (4).

The CD6 glycoprotein is a lymphocyte surface receptor belonging to the scavenger receptor cysteine-rich superfamily (SRCR-SF)—an ancient and highly conserved group of PRRs characterized by the presence of one or several repeats of a 90- to 110-amino-acid-long cysteine-rich globular domain (5, 6). CD6 is expressed mainly by all T cells but also by a subset of B (B1a) and of natural killer (NK) cells and some hematopoietic cell precursors and brain cells (5, 6). CD6 has an extracellular region composed by three tandem SRCR domains and a cytoplasmic tail suitable for signal transduction. Indeed, CD6 is physically associated with the T-cell receptor (TCR) complex (7) and plays relevant roles in regulating some T-cell developmental and activation/differentiation processes (8, 9). The latter is achieved mainly through interaction with its main reported ligand, namely, CD166/ALCAM (where ALCAM stands for activated leukocyte cell adhesion molecule)—an adhesion molecule of the immunoglobulin superfamily (10). Aside from such an endogenous ligand, the CD6 ectodomain also interacts with certain PAMPs from G^- (LPS) and G^+ (LTA and PGN) bacteria (11, 12). Interestingly, the dissociation constants (K_d) of the CD6-LPS ($2.69 \times 10^{-8} \pm 0.32 \times 10^{-8}$ M), CD6-LTA (0.17 ± 0.02 μM), and CD6-PGN (1.1 ± 0.1 nM) interactions are relatively high (11, 12) and of magnitude similar to that reported for CD14, the main macrophage receptor for those bacterial components (13, 14). Accordingly, the prophylactic infusion of a recombinant soluble form of human CD6 (rshCD6) significantly reduces mortality and levels of proinflammatory cytokines (interleukin-1 beta [IL-1β], IL-6, and tumor necrosis factor alpha [TNF-α]) in serum in mouse models of septic shock induced by G^+ (LTA plus PGN) and G^- (LPS) bacterial endotoxins, as well as whole live G^+ (*Staphylococcus aureus*) and G^- (*Acinetobacter baumannii*) bacteria, independently of their MDR phenotype (11, 12). Several mechanisms of action account for the antibacterial properties of rhsCD6, all of them related to innate immunity. So, rshCD6 interferes with the sensing of bacterial PAMPs by PRRs (e.g., TLR2 and TLR4) broadly expressed on innate immune cells and thus reduces the release of proinflammatory cytokines, as demonstrated by both *in vitro* and *in vivo* evidence (11, 12). Neutralization of PAMP-mediated inflammation by rshCD6 also involves aggregation of whole bacteria and of bacterial PAMPs (11), a very basic mechanism of innate defense interfering with bacterial infection spreading and facilitating engulfment by phagocytic cells. Finally, rshCD6 also reduces viable cell counts and intracellular ATP production following incubation with bacterial cell suspensions (12). This inhibitory effect on bacterial growth should not be taken, however, as fully indicative of direct bactericidal properties of the rshCD6 protein. Indeed, the sepsis survival rate achieved by rshCD6 infusion upon monobacterial infection is greatly reduced in leukopenic mice, a fact that is not observed with the use of bactericidal antibiotics (12).

In the present report, the prophylactic and therapeutic potential of rshCD6 infusion is further explored in the mouse model of septic shock most closely resembling the progression and characteristics of human sepsis: intra-abdominal polymicrobial infection induced by cecal ligation and puncture (CLP) (15). Time- and dose-dependent effects of single or repeated rshCD6 dosage by different routes are investigated, either alone or in combination with broad-spectrum bactericidal antibiotics. By means of

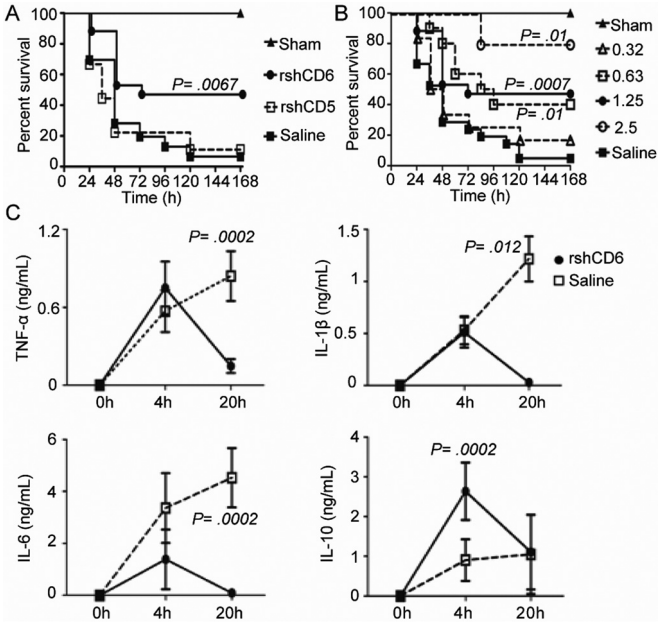

FIG 1 Effects of prophylactic i.p. rshCD6 infusion on CLP-induced polymicrobial septic shock. (A) C57BL/6J mice were infused i.p. with saline ($n = 18$), rshCD5 (1.25 mg/kg; $n = 9$), or rshCDC6 (1.25 mg/kg; $n = 15$) 1 h before induction of CLP-induced septic shock. (B) C57BL/6J mice were infused with either saline ($n = 21$) or increasing amounts of rshCD6 (0.31 mg/kg, $n = 12$; 0.62 mg/kg, $n = 10$; 1.25 mg/kg, $n = 17$; or 2.50 mg/kg, $n = 5$) 1 h before CLP-induced septic shock. In all panels, the average percentage of survival was analyzed over time for each group and compared to that of the saline-treated group using the log rank *t* test. (C) Levels of the indicated cytokines in plasma were monitored at the indicated time points (+4 h, +20 h) post-CLP in C57BL/6J mice prophylactically treated (−1 h) with either saline ($n = 10$) or rshCD6 (1.25 mg/kg; $n = 10$). Data are expressed as means ± standard errors of the means (SEM) in nanograms or picograms per milliliter, and statistical differences were evaluated using the 2-tailed Student *t* test.

adeno-associated virus 8 (AAV8)-mediated gene delivery, the conserved antimicrobial properties of mouse soluble CD6 (msCD6) and its prophylactic potential are also analyzed.

RESULTS

Prophylactic effects of i.p. rshCD6 infusion in CLP-induced septic shock. Given the reported ability of rshCD6 for targeting pathogenic factors from G^+ (LTA, PGN) and G^- (LPS) bacteria (11, 12), its potential use for prevention and/or treatment of polymicrobial sepsis of intra-abdominal origin, namely, CLP, was tested. Based on previous results obtained in a mouse model of monobacterially induced peritonitis (12), we first infused via the i.p. route a single dose of rshCD6 (1.25 mg/kg of body weight) 1 h before (−1 h) CLP induction to C57BL/6 mice. As illustrated by Fig. 1A, survival was significantly increased ($P = 0.0067$) for mice treated with rshCD6 (47.06%, $n = 15$) in comparison to the group treated with saline (6.52%, $n = 18$) or with rshCD5 (11.11%, $n = 9$). These prophylactic effects on mouse survival were dose dependent, with doses above 0.625 mg/kg giving statistically significant results and reaching maximal efficacy at 2.5 mg/kg (Fig. 1B). Prophylactic infusion of rshCD6 also significantly lowered the levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in plasma together with higher levels of anti-inflammatory cytokine IL-10 compared to levels in the saline-treated group (Fig. 1C). Taken together, these results indicate that prophylactic infusion of rshCD6 has specific and dose-dependent effects on mouse survival following CLP-induced septic shock, showing also significant reductions on systemic inflammation parameters.

Therapeutic effects of i.p. rshCD6 infusion in CLP-induced septic shock. To further explore the putative therapeutic effects of rshCD6 infusion, time course experiments were performed. As illustrated by Fig. 2A, a single infusion of rshCD6 (1.25

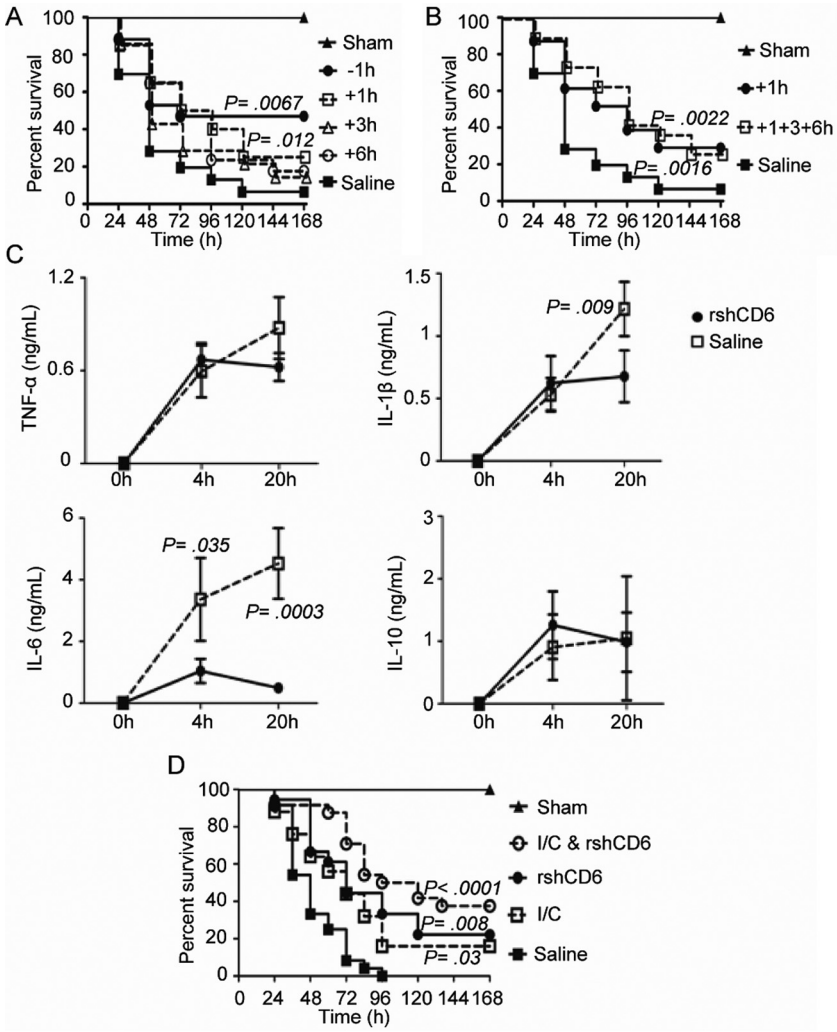

FIG 2 Effects of therapeutic i.p. rshCD6 infusion on CLP-induced polymicrobial septic shock. (A) C57BL/6J mice were infused i.p. with either saline ($n = 46$) or rshCD6 (1.25 mg/kg) at different time points either before (−1 h; $n = 19$) or after (+1 h, $n = 31$; +3 h, $n = 14$; +6 h, $n = 17$) CLP-induced septic shock. (B) C57BL/6J mice were i.p. infused with a single dose of saline ($n = 46$) or rshCD6 (1.25 mg/kg) 1 h post-CLP (+1 h, $n = 31$) or a repeated dosage of rshCD6 (1.25 mg/kg) at +1 h, +3 h, and +6 h (1+3+6h; $n = 19$) after CLP-induced septic shock. In panels A and B, average percent survival was analyzed over time, and statistical comparisons were made with regard to the saline-treated group by using the log rank *t* test. (C) Levels of proinflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory (IL-10) cytokines in plasma were monitored by ELISA at the indicated time points (+4 h, +20 h) after CLP-induced septic shock in C57BL/6J mice therapeutically treated (+1 h) i.p. with either saline ($n = 10$) or rshCD6 (1.25 mg/kg; $n = 10$). Data are expressed as means ± standard deviations (SD) in nanograms or picograms per milliliter, and statistical comparisons were made by using the 2-tailed Student *t* test. (D) C57BL/6J mice were therapeutically (+1 h) infused i.p. with saline ($n = 24$), rshCD6 (1.25 mg/kg; $n = 18$), imipenem/cilastatin (I/C; 33 mg/kg/8 h; $n = 25$), or a combination of the last two ($n = 24$), and the percent average of mouse survival was represented over time. Statistical comparisons were made with regard to the saline-treated group by using the log rank *t* test.

mg/kg) i.p. at different times pre- and post-CLP showed statistically significant effects ($P = 0.012$) on mouse survival when administered at +1 h (29.03%, $n = 31$) but not +3 h (14.28%, $n = 14$) or +6 h (17.65%, $n = 17$) post-CLP, compared to the saline-treated group (6.52%, $n = 46$). The survival achieved by infusing rshCD6 at +1 h was, however, lower than that obtained at −1 h pre-CLP and was not improved by repeated dosage at +1 h, +3 h, and +6 h post-CLP (26.32%, $n = 19$), although the last group still reached statistical significance ($P = 0.0016$) compared with the saline-treated group (Fig. 2B). Monitoring of cytokine levels in plasma at 4 and 20 h post-CLP showed that the mice treated with rshCD6 via i.p. +1 h post-CLP presented lower proinflammatory

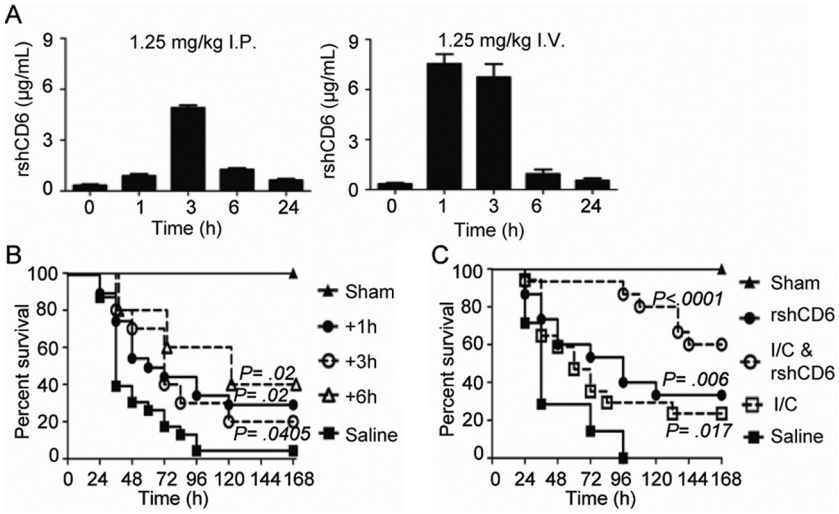

FIG 3 Effects of therapeutic i.v. rshCD6 infusion on CLP-induced polymicrobial septic shock. (A) C57BL/6J mice were infused i.p. ($n = 6$, left) or i.v. ($n = 6$, right) with rshCD6 (1.25 mg/kg), and levels of rshCD6 in plasma were determined by ELISA as mean (± SD) micrograms per milliliter. (B) C57BL/6J mice were infused i.v. with saline ($n = 18$) or rshCD6 (1.25 mg/kg) at different time points (+1 h, $n = 20$; +3 h, $n = 10$; and +6 h, $n = 5$) after CLP-induced septic shock. (C) C57BL/6J mice were therapeutically (+1 h) infused i.v. with saline ($n = 24$), rshCD6 (1.25 mg/kg, $n = 18$), imipenem/cilastatin (I/C; 33 mg/kg/8 h; $n = 25$), or a combination of the last two ($n = 24$). In panels B and C, average survival was analyzed over time for each group, and all the statistical comparisons were made with regard to the saline-treated group by using the log rank *t* test.

cytokine levels, which reached statistical significance for IL-1β and IL-6, but not TNF-α, with no differences observed for the anti-inflammatory cytokine IL-10, than in the saline-treated group (Fig. 2C). In conclusion, these results indicate that therapeutic effects of rshCD6 infusion via the i.p. route are achieved only at early (+1 h) but not at later (+3 h or +6 h) time points post-CLP induction, even when administered at repeated dosage.

Next, the therapeutic effect of combining rshCD6 and the broad-spectrum bactericidal carbapenem imipenem/cilastatin—a first-choice treatment in critically ill patients with sepsis—on mouse survival post-CLP was investigated. To this end, mice subjected to CLP were i.p. infused at +1 h with rshCD6 (1.25 mg/kg), imipenem/cilastatin (33 mg/kg/8 h), or a combination of the two. As illustrated in Fig. 2D, each of rshCD6 (26.4%, $n = 34$; $P = 0.008$) and imipenem/cilastatin (19%, $n = 21$; $P = 0.03$), given alone, showed similar significant effects on mouse survival compared to what was seen in the saline-treated group. Importantly, the simultaneous administration of rshCD6 plus imipenem/cilastatin showed additive effects on mouse survival (37.5%, $n = 24$), which were statistically significant not only compared with the saline-treated group ($p < 0.0001$) but also compared with the group treated with only imipenem/cilastatin ($P = 0.032$). These additive effects would be indicative of different mechanisms of action for the two treatments.

Therapeutic effects of i.v. rshCD6 infusion in CLP-induced septic shock. The i.p. route is widely used for the systemic administration of compounds to animals (16), though the route of choice for human clinical purposes is the i.v. one, a matter that can influence systemic drug bioavailability. Accordingly, rshCD6 plasma levels differed when a single dose (1.25 mg/kg) was administered to C57BL/6 mice either via i.p. or i.v. As illustrated in Fig. 3A, maximal rshCD6 levels were achieved at +3 h following i.p. infusion, and then the levels decreased to nearly basal values by +6 h. In contrast, i.v. infusion achieved maximal and sustained levels in plasma at +1 h to +3 h, which then declined to nearly basal levels by +6 h. Interestingly, maximal levels achieved via i.v. administration were higher than those obtained via the i.p. route, thus supporting a higher and more sustained systemic bioavailability for the i.v. route than for the i.p. route.

In light of these results, time course experiments exploring the therapeutic efficacy of i.v. rshCD6 infusion on CLP-induced mouse survival were performed. As shown in Fig. 3B, mouse survival was significantly improved over that of the saline-treated group by a single dose of i.v. rshCD6 (1.25 mg/kg) at +1 h (30%, $n = 20$, $P = 0.02$), but also at +3 h (20%, $n = 10$; $P = 0.0405$) and +6 h (40%, $n = 5$; $P = 0.02$) post-CLP. Although there were not statistical significant differences between rshCD6-treated groups at +1 h, +3 h, and + 6 h, the results indicate that the i.v. route extends the therapeutic effects of rshCD6 over time. Similarly, the above-mentioned additive effects of rshCD6 plus imipenen/cilastatin infused i.p. at +1 h post-CLP on mouse survival were also extended by administering rshCD6 via the i.v. route at the same dose and time point. As shown in Fig. 3C, the mouse survival achieved by individual administration of imipenem/cilastatin given i.p. at 33 mg/kg/8 h (23.5%, $n = 17$, $P = 0.017$) or rshCD6 given i.v. at 1.25 mg/kg (33.3%, $n = 15$; $P = 0.006$) was greatly improved by the combined administration of the two agents (60%, $n = 15$; $P = 0.0001$) compared with survival of the saline-treated group. Importantly, the differences in mouse survival observed between groups receiving imipenen/cilastatin alone or combined with rshCD6 were also statistically significant ($P = 0.008$).

Prophylactic effect of liver-specific AAV-mediated expression of mouse soluble CD6 in CLP-induced septic shock. In order to achieve high and sustained circulating levels of sCD6, a recombinant AAV8 was engineered to express a mouse sCD6 form (AAV-rmsCD6) under the transcriptional control of a liver-specific promoter. The rationale behind using not the human but the msCD6 was that the latter would be less immunogenic for mice and its long-term expression and function would not be interfered with by newly appearing neutralizing antibodies. Transduction of C57BL/6 mice with AAV-rsmCD6 virus resulted in detectable rmsCD6 in plasma from day 10 on, reaching maximal levels at days 15 to 20 (Fig. 4A). This expression system allowed sustained expression levels of rmsCD6 in the range of 5 to 15 μg/ml for periods of time longer than 3 months. According to these results, mice were subjected to CLP-induced septic shock 2 weeks after transduction with 1×10^{11} viral particles of AAV-rmsCD6 or control AAV-Luc, and their survival was monitored over time. As illustrated in Fig. 4B, mice treated with AAV-rmsCD6 showed a statistically significant higher survival rate (42.85%, $n = 14$; $P = 0.0145$) than did control mice treated with AAV-Luc (7.14%, $n = 13$). This effect was concomitant with significant reductions in the levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in serum and increments in those of IL-10 (Fig. 4C). Importantly, therapeutic infusion of imipenem/cilastatin (33 mg/kg/8 h, i.p.) at +1 h post-CLP induced significant increments of mouse survival in both the AAV-rsmCD6-treated (78.57%, $n = 14$) and AAV-Luc-treated (56.25%, $n = 16$) groups.

DISCUSSION

Overall mortality of sepsis and septic shock remains high (35% and 60%, respectively) despite significant advances in supportive care and the availability of potent broad-spectrum antibiotics (17). Although antibiotics constitute a necessary part of the treatment of sepsis, they are probably not sufficient to substantially reduce the mortality associated with the MOD accompanying sepsis and septic shock. This, together with the increase in MDR bacteria, makes mandatory the search for alternative nonantibiotic (adjunctive) strategies. Aside from improvements in supportive care, those strategies include treatments aimed at bacterial virulence factors and/or host inflammatory and immune mediators (1, 2, 17, 18). Accordingly, the present work reports the *in vivo* prophylactic and therapeutic effects of the soluble ectodomain from the scavenger-like lymphocyte receptor CD6 (sCD6) in a lethal model of polymicrobial infection of intra-abdominal origin. The results show time- and dose-dependent effects of sCD6 infusion on mouse survival post-CLP induction, which are concomitant with reduced systemic inflammatory response. Interestingly, both human and mouse sCD6 infusion showed additive effects on mouse survival when combined with the broad-spectrum bactericidal antibiotic imipenem/cilastatin.

Previous studies from our group indicate that sCD6 acts mainly through direct

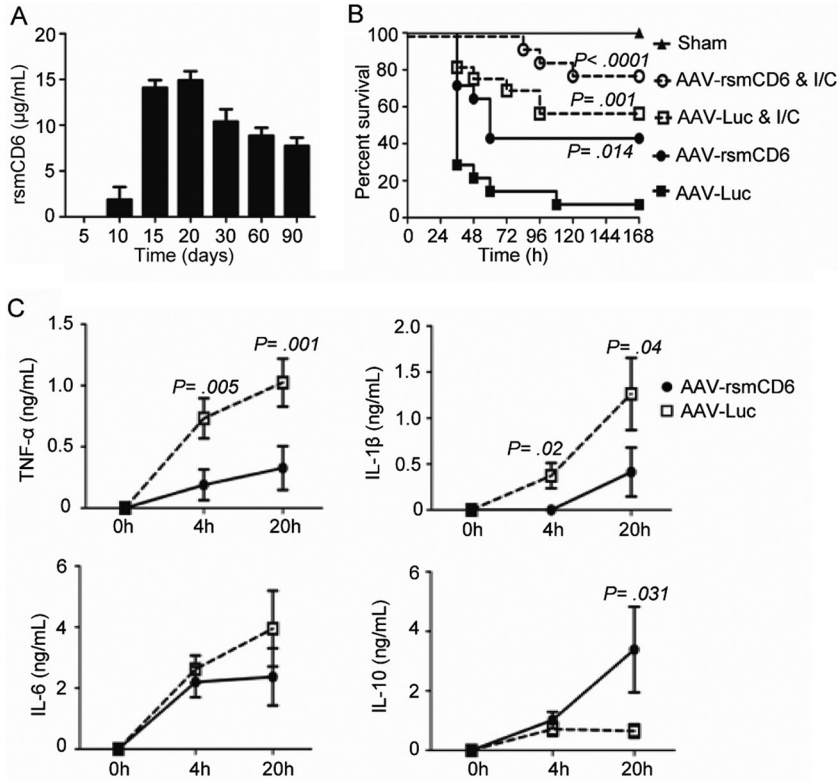

FIG 4 Effects of AAV-mediated gene delivery of rsmCD6 on CLP-induced polymicrobial septic shock. (A) C57BL/6J mice ($n = 6$) were treated i.v. with hepatotropic AAV coding for rsmCD6 (AAV-rsmCD6) and bled at different time points for monitoring of rsmCD6 levels in plasma by ELISA. Data are expressed as mean (± SD) micrograms per milliliter. (B) C57BL/6J mice i.v. treated with 1×10^{11} AAV-rsmCD6 or AAV-Luc viral particles 2 weeks before CLP-induced septic shock were therapeutically (+1 h) infused i.p. with ($n = 15$ and $n = 16$, respectively) or without ($n = 14$ and $n = 13$, respectively) imipenem/cilastatin (I/C; 33 mg/kg/8 h). Average percent survival was analyzed over time, and statistical comparisons were made with regard to the saline-treated group by using the log rank *t* test. (C) Levels of the indicated cytokines in plasma were monitored by ELISA at the indicated time points (+4 h, +20 h) after CLP-induced septic shock in C57BL/6J mice treated 2 weeks before with 1×10^{11} AAV-rsmCD6 ($n = 10$) or AAV-Luc ($n = 10$) viral particles. Data are expressed as mean (±SD) nanograms or picograms per milliliter, and statistical comparisons were made using the 2-tailed Student *t* test.

binding to key pathogenic factors from G^- (LPS) and G^+ (LTA and PGN) bacterial cell walls and consequently interferes with TLR2- and TLR4-mediated cytokine release induced by them, aside from reducing bacterial growth and viability (12). Such a broad set of bacterial binding properties makes sCD6 a suitable candidate for treating complex polymicrobial infections as in the case of that induced by CLP. In this model, a massive seeding of indigenous gut flora (mainly bacteria, from anaerobic to facultative aerobic or aerobic) into peritoneum and systemic circulation develops the symptoms characteristic of peritonitis and sepsis, allowing testing of the broad-spectrum antibacterial properties of sCD6. Not withstanding, PAMPs targeted by sCD6 are conserved constituents of bacterial cell walls, which are essential for bacterial survival and are not easily amenable to mutation without losing viability and/or pathogenicity. All these properties make sCD6 a good target for alternative/adjunctive antibacterial strategies.

The results show the induction of both prophylactic and therapeutic effects of sCD6 infusion and how they are influenced by the route of administration. By using the i.p. route, the best results are achieved in the prophylactic mode (at −1 h pre-CLP), although therapeutic effects were still observed at early (+1 h) but not later (+3 h) time points post-CLP. Interestingly, the use of the i.v. route allowed extending the therapeutic effects of sCD6 up to +3 h or +6 h post-CLP. This would support the notion that sCD6 acts mainly in early phases of the sepsis process and that there is no benefit in

delaying and/or repeating its infusion over time. This situation recalls the reported relationship between the delay in starting antibiotics and the eventual outcome (19). Thus, when antibiotics are started within 1 h of documented hypotension, survival is high (79.9%) and each subsequent hour of delay over the next 6 h is associated with an average decrease in survival of 7.6%. This means that sCD6 and antibiotics in no way have the same mechanism of action but rather share a similar therapeutic window. Accordingly, when imipenem/cilastatin was coadministered with sCD6 (i.p. or i.v.), clear additive therapeutic effects were evidenced, a fact indicative of different mechanisms of action. Indeed, the broad-spectrum bactericidal antibiotic used, imipenem/cilastatin, is a member of the carbapenem class that acts by inhibiting cell wall synthesis of various G^- and G^+ bacteria, thus reducing the amount of PAMPs released during bacteriolysis. This makes it a first-choice treatment in critically ill patients with sepsis. It remains, however, to explore whether the observed additive effects are shared by other bactericidal or bacteriostatic antibiotics from the same or a different class, since rshCD6 protein-mediated interactions could putatively interfere with their pharmacodynamics or mechanisms of action.

Lifelong broad-spectrum antibiotic therapy is also a suitable choice for prophylaxis or treatment of infections in some clinical settings such as congenital or acquired immune deficiencies. However, clinicians must balance the benefits of prophylactic antibiotic usage with problems such as compliance, adverse reactions, and development of antibiotic resistance. In light of these circumstances, the prophylactic effects achieved by inducing sustained sCD6 levels in plasma through liver-specific AAV-mediated expression could be considered for future interventions. The possibility of sustained high circulating sCD6 levels having deleterious consequences by compromising certain immune cell functions would need to be first totally excluded. In this regard, preliminary characterization of a transgenic mouse line constitutively expressing circulating levels of shCD6 generated by our laboratory does not reveal significant harmful immune cell defects.

In order to minimize immunogenicity-related problems related with human proteins, AAV8 coding for mouse sCD6 was used. This allowed to demonstrate that human sCD6 and mouse sCD6 share similar antibacterial properties, which have been conserved through evolution. The bacterial binding site/s responsible for such an activity has not been identified yet. However, SRCR domains 1 and 3 of both human and mouse CD6 hold a motif (VEVxxxxxW) previously found in other members of the SRCR-SF as responsible for their reported bacterial binding properties (20, 21), and this possibility should be investigated. Similarly, there is no reported evidence on the *in vivo* functionality in mouse models of infection or sepsis for soluble members of the SRCR-SF with reported binding and aggregating ability to G^+ and G^- bacteria (22–25), and this should be further explored.

In conclusion, the results of the present report open new pathways to explore the antimicrobial and/or anti-inflammatory properties of sCD6 and other soluble SRCR-SF members in experimental models of mono- or polybacterial infection. Moreover, they show that sCD6 targeting is a feasible adjunctive strategy to antibiotic therapy as well as to other nonantibiotic therapies targeting late components of the host inflammatory cascade (e.g., TREM-1 and HMGB-1).

MATERIALS AND METHODS

Expression, purification, and biotinylation of recombinant proteins. Production of purified rshCD6 and rshCD5 proteins (phosphate-buffered saline [PBS] with 10% glycerol, pH 7.4) was done based on previously reported methods (25) but using SURE CHO-M Cell line clones from the Selexis SURE-technology Platform (Geneva, Switzerland) and subjecting serum-free supernatants to size exclusion chromatography protocols developed at PX'Therapeutics (Grenoble, France).

Cecal ligation and puncture procedure. All animal procedures were approved by the Animal Experimentation Ethical Committee, University of Barcelona. CLP-induced septic shock was induced in 8- to 10-week-old C57BL/6J male mice (20 to 25 g; Charles River) as in previously reported methods (26). The severity of the CLP model was adjusted to achieve a high-grade mortality (≥90% mortality in the first 48 to 72 h) by ligating 75% of cecum. Mice were anesthetized with 100 mg/kg ketamine (Imalgéne 1000; Merial) and 10 mg/kg xylazine (Rompun; Bayer) and subsequently resuscitated with 1 ml saline serum

intraperitoneally (i.p.). To mitigate pain effects, 0.05 mg/kg/12 h buprenorphine was administered subcutaneously. When indicated, imipenem/cilastatin (33 mg/kg/8 h; Actavis, Iceland) and rshCD6 (0.32 to 2.5 mg/kg) were administered i.p. or i.v. The final experimental outcome was observation of spontaneous mortality, which was represented as the percentage of surviving animals over time. Daily weight loss monitoring (usually ≤10 to 15% in the first 48 h post-CLP) allowed exclusion of animals not undergoing clinical sepsis.

ELISA measurement of rshCD6 and cytokine levels in plasma. Levels of rshCD6 in plasma were determined by sandwich enzyme-linked immunosorbent assay (ELISA), using unlabeled 161.8 and biotin-labeled SPV-L14.2 (2 μg/ml, each) as capture and detection anti-CD6 monoclonal antibodies, respectively. Plasma samples were diluted 1:5, and serial dilutions of purified rshCD6 were used as a standard. Plates were developed with horseradish peroxidase-labeled streptavidin (Roche Diagnostics) and the TMB substrate reagent set (BD OptEIA; BD Biosciences), and optical density at 450 nm (OD_{450}) was measured. Diluted or undiluted plasma samples were used for monitoring levels of IL-6 (1:70; mouse IL-6 ELISA set; catalog no. 555240; BD Bioscience), TNF-α (1:10; mouse TNF-α ELISA set; catalog no. 558534; BD Bioscience), IL-10 (neat; mouse IL-10 ELISA set, catalog no. 555252; BD Bioscience), and IL-1β (neat; mouse IL-1β ELISA set, catalog no. 559603; BD Bioscience) by commercially available ELISA kits, according to the manufacturer's instructions.

Recombinant AAV vector construction and virus production and purification. Recombinant AAV8 vectors expressing msCD6 or luciferase protein under the control of the chimeric liver-specific promoter formed by the albumin enhancer and human α-1-antitrypsin promoter (EalbAAT) were constructed (27). The cDNA coding for signal peptide from immunoglobulin κ light chain variable region fused in frame with amino acids Gly25-Thr398 from msCD6 (28) was cloned as SalI-NotI into the AAV2 genome, obtaining the plasmid pAAV-EalbAAT-rmsCD6. For AAV-rmsCD6 virus production, a mixture of pAAV-EalbAAT-rmsCD6 (20 μg) and pDP8.ape (55 μg; PlasmidFactory GmbH & Co. KG, Bielefeld, Germany) plasmids was transfected into 15-cm plates seeded with semiconfluent 293 T cells using linear polyethyleneimine (PEI) 25 kDa (Polysciences, Warrington, PA). Cells were harvested 72 h posttransfection, and virus was released by three rounds of cell freeze-thawing. Crude lysates were treated with Benzonase (50 U/ml) for 1 h at 37°C and kept at −80°C until purification by using iodixanol gradients according to a previously described method (29). The purified viral batches were concentrated by cross-flow filtration and then filtered (0.22 μm) and stored at −80°C. Viral titers (in terms of genome copies per milliliter) were determined by real-time quantitative PCR using primers specific to the human α-1-antitrypsin promoter (AAT-Forward, 5′-CCCTGTTTGCTCCTCCGATAA-3′, and AAT-Reverse, 5′-GTCCG TATTTAAGCAGTGGATCCA-3′). For AAV biodistribution studies, mice received a single i.v. dose of 1×10^{11} viral particles/mouse (final volume, 200 μl), and msCD6 plasma levels were monitored by ELISA (DuoSet mouse CD6; R&D Systems) at different time points.

Statistical analysis. Statistical analyses were performed using the Prism 5.00 computer program (GraphPad Software Inc., San Diego, CA). The survival data were represented in Kaplan-Meier graphs and analyzed by the log rank χ^2 test. Statistically significant differences ($P < 0.01$) were determined by the 2-tailed nonparametric Mann-Whitney test.

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ERRATUM

Erratum for Martínez-Florensa et al., "Protective Effects of Human and Mouse Soluble Scavenger-Like CD6 Lymphocyte Receptor in a Lethal Model of Polymicrobial Sepsis"

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