Proinflammatory Cytokines in Antilipopolysaccharide Immunity Against *Klebsiella* Infections

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This study was undertaken in order to determine whether proinflammatory cytokines are involved in a previously described protection against *Klebsiella* infection mediated by antilipopolysaccharide antibodies. BALB/c mice were infected intraperitoneally with a lethal challenge of *Klebsiella pneumoniae* Caroli. One group of mice was protected with monoclonal antibodies against lipopolysaccharide prior to infection and the second was not. We determined the number of colony-forming units at different time points in the blood of infected animals and paralleled them with plasma levels of five proinflammatory cytokines measured by enzyme immunoassays. Our results show that the two groups of animals tested expressed different plasma concentrations for all cytokines. The greatest difference was detected 24 hours after infection, with a higher production in the unprotected group. We concluded that a reduced cytokine production is partially responsible for the survival of protected animals.

INTRODUCTION

Klebsiella pneumoniae (*K pneumoniae*) is an important cause of community-acquired and nosocomial infections [1, 2]. In particular, nosocomial pneumonia and septicemia caused by *Klebsiella spp* are a frequent problem in both medical and surgical intensive care units [3, 4]. In spite of the therapeutic efforts to combat severe *Klebsiella* infections, they are still associated with high mortality rates of up to 40% [3, 4]. In order to find alternative strategies to prevent or treat these severe infections, various studies of pathogenicity and characterization of possible virulence factors have been performed. The final goal of these studies is to identify surface antigens that might serve as target molecules for active vaccination or passive immunotherapy. The most promising structure for such an approach is the capsular (K) antigen which plays a significant role in the pathogenicity of *K pneumoniae* [5, 6, 7]. Antibodies specific for K antigen may enhance phagocytosis and protect against experimental *Klebsiella* infections [8, 9, 10, 11]. The obstacle for the successful preparation of such K-antigen-based immunologic tool is the fact that there are more than 70 K antigens expressed on clinical isolates, most of them present in 1%–2% of clinical strains [12, 13]. Indeed, Cryz and coworkers developed a K-antigen-based vaccine containing purified polysaccharides of 24 capsular serotypes [14, 15]. A hyperimmune intravenous immunoglobulin (Ig) preparation made from the postvaccination plasma of volunteers immunized simultaneously with the *Klebsiella* K antigen and *Pseudomonas* vaccines has undergone a randomized clinical trial with intensive care patients [16]. In that study, the *Klebsiella*-capsule-specific Ig exerted significant protection. However, the protective effect was limited to those *Klebsiella* isolates that belonged to capsular serogroups included in the vaccine. In that study the specified capsule-specific vaccine covered only about 70% of the *Klebsiella* clinical isolates examined, indicating a need for broadening the antibacterial activity of this product. Another seroepidemiological study indicated that the addition of 10 more K antigens would broaden the vaccine coverage by only 13% [13]. Therefore, for the preparation of a more effective *Klebsiella* vaccine such product should probably contain some other surface determinants with less complex seroepidemiology than that of the K antigens [17].

Another promising candidate surface molecule for the development of such immunologic tool is the lipopolysaccharide (LPS, O antigen). There are several reasons for such statement. First of all the number of O antigens is relatively low compared to the number of K antigens [18].

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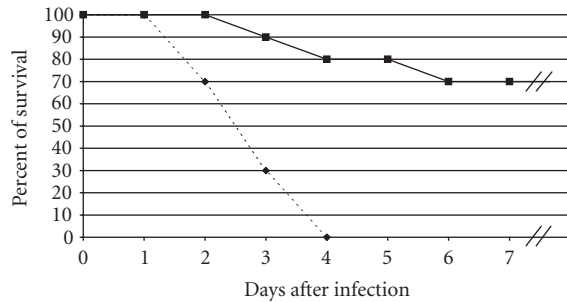
FIGURE 1. Survival curve of BALB/c mice protected with anti-LPS MAb Ru-O1 (full line) and unprotected mice (dashed line) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2).

According to literature data the inclusion of only four O antigens in such preparation would cover more than 70 percent of all clinical *Klebsiella* isolates [18, 19]. The results of a recent study suggest that as much as 82% of all *Klebsiella* isolates belong to one of the four serogroups: O1, O2ab, O3, and O5 [20]. On the other hand, it seems that a great proportion of clinical isolates share a common epitope located in the core oligosaccharide of the LPS molecule [21]. In addition, antibodies directed against LPS were shown to penetrate the capsule of *K pneumoniae* [22, 23]. Finally, the monoclonal antibody (MAb) against O1 antigen was shown to be protective in a mouse model of lethal systemic *Klebsiella* infection [24].

We have previously described an O-antigen-specific murine MAb (clone Ru-O1, immunoglobulin G2b) directed against an immunodominant epitope expressed on *Klebsiella* O1, O6, and O8 LPS that are mutually highly related [12, 25, 26]. O1 antigen appears to play an important role in clinical strains, being detectable in about one third of isolates [19, 20, 27]. MAb Ru-O1 expressed high specificity for the O1 antigen of *Klebsiella* binding to the outermost partial antigen D-galactan II of the O1 *Klebsiella* LPS molecule [19, 24]. They exerted the ability to protect mice in a murine model of lethal systemic *Klebsiella* infection (Figure 1) [24]. The exact molecular mechanism of this protection remained unknown. A part of their protective effect could be contributed to their ability to enhance opsonization which was demonstrated by *in vitro* experiments [28]. In addition to promoting phagocytosis, they may also exert protection by several other mechanisms. One of the possible mechanisms is the neutralization of circulating free LPS and thereby modulation of cytokine production [29, 30]. Straus et al showed that the release of soluble LPS plays a significant role in the pathogenesis of *Klebsiella*-induced lung injury [31, 32]. Cytokines themselves play the important role in the pathogenesis of *Klebsiella* and other gram-negative infections. A significant part of the pathogenesis is connected with the effect of LPS, which was reported responsible for the production of several cytokines. The data regarding the role of some cytokines in the pathogenesis of infections are often controversial [33].

In the present study, we tried to determine whether the protective effect of anti-LPS Ru-O1 MAb could be a consequence of the modulated production of some proinflammatory cytokines that are known to be important in the pathogenesis of sepsis and septic shock. We analyzed plasma concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) at different time points after a lethal intraperitoneal (IP) bacterial challenge with *K pneumoniae* Caroli (O1:K2). The cytokines production levels were compared with the degree of bacteremia.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old pathogen-free male BALB/c mice weighing 20 to 25 g each were used through study. Animals were obtained from breeding colony at the Medical Faculty, University of Rijeka. They were kept in plastic cages and given standard laboratory food (Standard pellets, Faculty of Biotechnology, Domžale, Slovenia) and water ad libitum. The experiments were conducted according to the laws and principles found in the *International Guiding Principles of Biomedical Research Involving Animals* by the Council of International Organisations of Medical Science. The principles are also in accordance with the Statute for Laboratory Animals of the Croatian Society for Laboratory Animals.

Bacteria

Experimental infections were performed using the highly virulent variant of the strain *K pneumoniae* Caroli (O1:K2) which has been used before by ourselves [24] and by other authors as well [9, 11, 34].

Experimental Klebsiella infection

To ensure the virulence of the challenge strain, bacteria were injected IP into BALB/c mice and reisolated from liver and spleen homogenates 24 hours later. For experimental infection, bacteria grown on blood agar plates for 18 hours were suspended in sterile PBS, pH 7.4. Bacteria were washed two times in PBS to remove loose slime containing extracellular polysaccharides. Bacterial suspensions were adjusted densitometrically at 365 nm to the desired concentration, which was confirmed by colony counts on blood agar plates after serial 10-fold dilutions. LD$_{50}$ of *K pneumoniae* Caroli for IP infected mice is 10 organisms per mouse [24]. The experimental groups of mice were pretreated 4 hours before the infection with an IP injection of purified MAb Ru-O1 at the dose of 40 μg/g, that was previously determined to be protective, or with PBS. Animals were infected IP with an estimated dose of 50 organisms of *K pneumoniae* Caroli, corresponding to five times the LD$_{50}$. This dose was selected because, as described earlier, all animals that were not pretreated with anti-LPS MAb Ru-O1 died within 4 days, with the

mortality of approximately 50% after 2 days (Figure 1). Pretreatment with MAb Ru-O1 resulted in 70 percent survival. The control group of animals was pretreated with PBS because previous experiments showed no difference in mortality between animals pretreated with PBS or irrelevant MAb of the IgG2b subclass [24].

Quantification of K pneumoniae Caroli in blood

The degree of bacterial dissemination was detected by enumeration of *K pneumoniae* Caroli in blood samples. The animals were euthanized by inhalation of CO_2. The blood was obtained by cardiac puncture 2, 6, 12, and 24 hours after infection. Serial 10-fold dilutions in sterile PBS were plated in duplicates on blood agar plates (100 μL per plate). After incubation at 37°C for 24 hours, colonies of *K pneumoniae* were counted. Bacterial counts are presented as mean values $\pm$ SE of the mean (SEM) colony-forming units (CFU)/mL.

Plasma cytokine analyses

Plasma levels of IL-1β, IL-6, IL-12, IFN-γ, and TNF-α were also determined at the time points specified above. The blood was obtained by cardiac puncture as described above. Plasma samples were separated and stored at -20°C until assayed. Cytokine concentrations were determined by commercially available mouse cytokine ELISA kits (Bender MedSystems, Austria) according to the manufacturer's instructions. According to data supplied by the manufacturer, detection limits for specified kits were as follows: 1.2 pg/mL for IL-1β, 12 pg/mL for IL-6, 6 pg/mL for IL-12, 8 pg/mL for IFN-γ, and 4.5 pg/mL for TNF-α. The overall interassay and interassay reproducibility, expressed by coefficient of variation was declared to be less than 10% for all kits specified. The results are presented as mean values $\pm$ SEM of cytokine concentration.

Statistical analyses

Statistical significance of the difference between bacterial counts and cytokine concentrations of unprotected, protected, and uninfected control groups were determined by two-tailed Student *t* test.

RESULTS

Bacteremia

In order to determine the degree of bacteremia in unprotected and protected groups of animals, at designated time points blood bacterial counts were determined (Figure 2). Unprotected animals had at all time points higher values than protected animals. The bacteria appeared in blood 2 hours after the infection in a relatively small amount; 50 $\pm$ 21 CFU/mL of blood in the unprotected group while bacteremia was not detected in the protected group. Furthermore, 6 hours after the infection bacteria were detected in both groups with a significantly higher number in the unprotected group. Similar results were recorded also 12 and 24 hours after in-
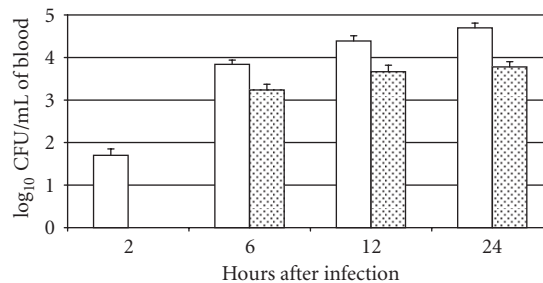

FIGURE 2. The bacterial counts in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as $\log_{10}$ of mean values $\pm$ SEM CFU/mL of blood. Six animals in each group were infected. Statistical significance was found between CFU counts from the groups at all analyzed time points ($P < .01$).

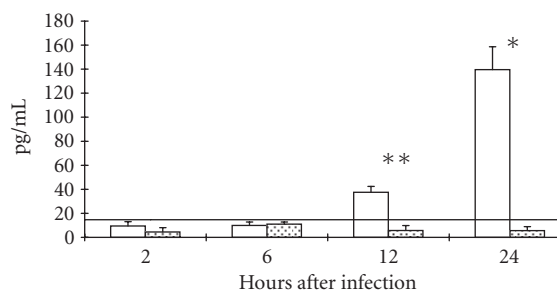FIGURE 3. Plasma concentration of IL-1β in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as mean values $\pm$ SEM pg/mL. $*$ and $**$ marks above bars represent the statistical significance between the groups at particular time point at the level of $P < .05$ and $P < .01$, respectively.

fection. Bacterial counts continuously increased in both groups with approximately 8-fold higher values in the unprotected group 24 hours after infection (57500 $\pm$ 6657 versus 6875 $\pm$ 1023 CFU/mL of blood; $P < .001$).

Kinetics of proinflammatory cytokine concentrations

The levels of different proinflammatory cytokines in the plasma of unprotected animals and animals protected with anti-LPS Ru-O1 MAb, IP infected with *K pneumoniae* Caroli were observed during 24 hours after infection.

Interleukin-1β

Two and six hours after the infection IL-1β concentrations were almost the same in both groups (Figure 3). Plasma levels did not differ significantly from values in uninfected control animals (15 $\pm$ 1.9 pg/mL). Twelve and twenty-four hours after infection, IL-1β production in the protected group of animals did not change. On the
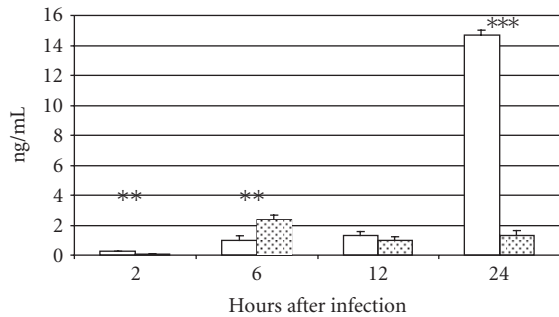
FIGURE 4. Plasma concentration of IL-6 in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as mean values ± SEM ng/mL. ∗∗ and ∗∗∗ marks above bars represent the statistical significance between the groups at particular time point at the level of $P < .01$ and $P < .001$, respectively.

contrary, plasma concentrations in unprotected animals increased significantly after 12 and 24 hours to the levels of $38 ± 4.8$ pg/mL ($P < .05$) and $140 ± 5.6$ pg/mL ($P < .05$), respectively. Plasma levels of IL-1β differed significantly also between unprotected and protected group of infected animals 12 and 24 hours after infection ($P < .01$ and $p < .05$, respectively). Namely, values in the protected group remained within the levels in the uninfected control animals ($6 ± 1.8$ and $3 ± 1.1$ pg/mL, respectively).

Interleukin-6

IL-6 was not detectable in the plasma of the uninfected control group of animals. In both infected groups (Figure 4), 2 hours after the infection plasma levels of IL-6 started to increase. The concentration was significantly higher in the unprotected group compared to protected animals ($252 ± 20.3$ versus $79 ± 6.7$ pg/mL; $P < .01$). The amount of IL-6 further raised in both groups after 6 hours. The plasma of the protected group contained higher concentration compared to the unprotected animals ($2329 ± 155.5$ versus $991 ± 144.3$ pg/mL; $P < .01$). Twelve hours after the infection, plasma level of unprotected animals continuously increased to the level of $1330 ± 225$ pg/mL. IL-6 in this group reached maximal concentration 24 hours after the infection ($14.7 ± 0.3$ ng/mL). On the contrary, the concentration in the protected group slightly decreased and remained almost unchanged during the next 12 hours.

Interleukin-12

IL-12 production in both infected groups was almost identical with no statistically significant differences between them (Figure 5). The dynamics of production showed peak values 24 hours after infection. The differences for both groups at all time points were statistically significant compared to values of uninfected control mice ($P < .01$).
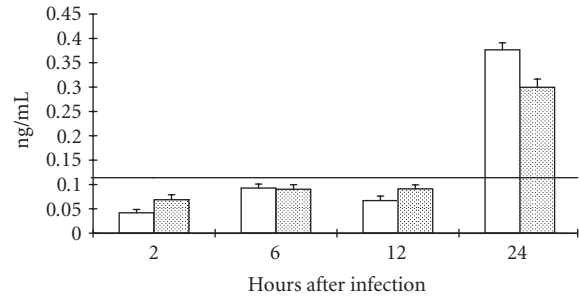

FIGURE 5. Plasma concentration of IL-12 in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as mean values ± SEM ng/mL.
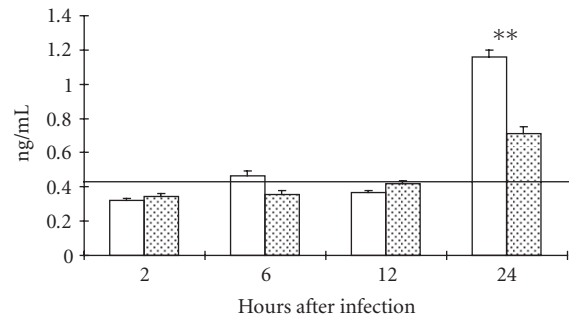

FIGURE 6. Plasma concentration of IFN-γ in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as mean values ± SEM ng/mL. ∗∗ mark above bars represents the statistical significance between the groups at particular time point at the level of $P < .01$.

Interferon-γ

The moderate rise of IFN-γ in unprotected animals was noticed 6 hours after infection while the level of protected mice remained within values of the uninfected control group ($428 ± 21.2$ pg/mL) (Figure 6). The production in both groups reached its maximum 24 hours after infection. The concentration levels of both groups were significantly higher compared to uninfected animals ($P < .01$). A significant difference was recorded also for IFN-γ concentrations in the plasma from unprotected versus protected animals with almost 2-fold higher concentration in unprotected animals compared to the protected group ($P < .01$).

Tumor necrosis factor alpha

In the unprotected group TNF-α concentration reached the first peak 6 hours after infection and then slightly decreased (Figure 7). The maximum level was reached 24 hours after infection. In comparison with the protected group the concentration of TNF-α was 4 and
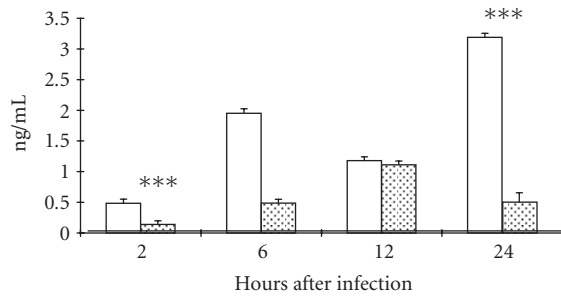
FIGURE 7. Plasma concentration of TNF-α in the blood of BALB/c mice protected with anti-LPS MAb Ru-O1 (gray bars) and unprotected mice (white bars) after the IP infection with 50 CFU of *K pneumoniae* Caroli (O1:K2) at different time points. Results are expressed as mean values $\pm$ SEM ng/mL. $* * *$ mark above bars represents the statistical significance between the groups at particular time point at the level of $P < .001$.

6 fold higher 6 and 24 hours after infection, respectively. During the first stage of infection the level of TNF-α increased to a much lesser extent in the protected group. In this group TNF-α production reached maximum value 12 hours after infection and then gradually decreased. TNF-α levels were significantly higher compared to uninfected control mice level at all time points for both infected groups ($P < .001$).

DISCUSSION

We have previously described that anti-LPS MAb Ru-O1 exerts protection in the model of lethal systemic *Klebsiella* infection [24]. The exact mechanism of this protection remained unknown. According to our previous research, such effect can be explained partly by enhancement of opsonophagocytosis [28] but we presumed that the exact molecular mechanism is much more complex.

There is evidence that several proinflammatory cytokines play an important role in the pathogenicity of septic events. As a part of the innate immunity, their role is to orchestrate an anti-infectious process by enhancing the microbicidal activities of phagocytic cells, contributing to the recruitment of leucocytes towards the site of infection, enhancing hematopoiesis, and inducing fever [33]. Many bacterial compounds activate the production and release of cytokines, such as LPS. Straus et al reported the importance of LPS-containing extracellular toxic complex in the pathogenesis of *Klebsiella* infections [31]. We presumed that this effect is at least partly mediated by the modulated proinflammatory cytokine response.

In the present study, we tried to determine whether the protective effect of Ru-O1 MAb could be the consequence of the modulated production of cytokines. We included some of the most important proinflammatory cytokines in order to determine their kinetics during the first 24 hours after a lethal IP *Klebsiella* challenge. We did not analyze later periods since animals in the unprotected group begun to die between 24 and 48 hours after infec-

tion (Figure 1). We have also analyzed the relation between cytokine production and the degree of bacteremia.

After the IP challenge, bacteria started to appear in the blood within the first few hours after infection (Figure 2). Our results indicate a difference between the protected group (treated previously with Ru-O1 MAb) and the unprotected group with respect to the dynamics of bacterial appearance. Namely, a low degree of bacteremia was detectable in the unprotected group 2 hours after infection while in the protected group it was noticeable after 6 hours (according to our experimental design). We speculate that the delayed onset of bacteremia in the protected group is the consequence of certain events (such as opsonophagocytosis) in the peritoneal cavity since the route of MAb administration and the route of infection were similar (IP). After the onset, bacteremia continuously increased in both groups and reached the highest level 24 hours after infection with almost 10-fold greater CFU/mL values in the unprotected group. We reasonably assumed that the quantity of bacterial compounds released in the circulation is proportional with the bacterial load. Therefore, it can be assumed that the unprotected group was exposed to higher levels of these substances.

Our cytokine assays have shown that the first cytokine that begin to rise in the blood is TNF-α. Two hours after infection its concentration was significantly higher in the protected group of animals compared with the unprotected group. The fact that it increases first among all the tested cytokines is not a surprise. There is evidence that TNF, after the LPS challenge in experimental animal models [35] or in human volunteers [36], appears in the blood before any other cytokine. Moreover, it seems that TNF is a prerequisite for the production of many other proinflammatory cytokines. Experiments conducted with anti-TNF antibodies indicated that blocking TNF in bacterial or endotoxin-induced shock models led to a dramatic decrease in the levels of other cytokines such as IL-1β, IL-6, and IL-8 [37, 38]. The explanation for different TNF levels between the groups in our experiment could be the difference in the bacterial blood counts and possibly the neutralization of released LPS and consequently the neutralization of its effects at the cellular level at the early stage of infection. We speculate that in the protected group the stimulation of some receptor structures by the circulating LPS, such as toll-like receptors, was reduced and that it led to the reduced TNF production. The TNF level in the unprotected group continues to increase and reaches the first peak after 6 hours. Such event preceded the increase of other analyzed cytokines. In the protected group, the increase of TNF level was not so high, reached the peak 12 hours after infection, and then gradually decreased. It is well documented that TNF production could have beneficial [39, 40, 41, 42, 43, 44, 45] or detrimental effects [46, 47, 48] in different experimental infectious models. We think that our results are in accordance with these facts and that TNF levels in each experimental group can be considered responsible for the different outcome of infection.

All other cytokines tested started to appear in greater amounts after TNF. IL-1β, IL-12, and IFN-γ remained within or near the limits of the uninfected control group of mice until 24 hours after infection with the exception of the moderate increase of IL-1β, 6 hours after infection in the unprotected group. The increase of IL-6 concentration is more pronounced. Its concentration reaches, interestingly, after 6 hours more than 2-fold higher values in the protected group. That result may be of importance and may contribute to the survival of mice from the protected group since IL-6 was also described to have anti-inflammatory properties via its capacity to induce the release of acute-phase proteins [33].

Twenty-four hours after the infection all cytokines included in our study reached their highest levels in the unprotected group, while in the protected group their levels were much lower. Moreover, IL-1β remained within normal limits during the whole experiment in the protected group. The high level of certain proinflammatory cytokines alone is associated with a poor outcome of the infection [33, 49]. On the other hand, some of these proinflammatory cytokines combined seem to express deleterious synergistic effect [50]. We speculate that such synergy between the two or even more proinflammatory cytokines included in the study is a possible triggering factor that led to the mortality in the unprotected group of animals. On the contrary, the reduction of such synergism can be considered as contributing factor to the survival of the protected group of animals.

Our results led us to a conclusion that the mechanism of action of Ru-O1 MAb can be explained partially by the modulation of proinflammatory cytokines response to *Klebsiella* infection. Their mode of action is certainly much more complex and further research is necessary for a better understanding of this phenomenon. Our data emphasize the need for broadening the research of anti-LPS immunity against *Klebsiella* infections in order to find appropriate strategies for the design of a second-generation *Klebsiella* vaccine.

ACKNOWLEDGMENTS

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