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Rijneveld, A.W.; Florquin, S.; Hartung, T.; Speelman, P.; van der Poll, T.

Published in:
The Journal of Infectious Diseases

DOI:
10.1086/376454

Citation for published version (APA):
Anti–Tumor Necrosis Factor Antibody Impairs the Therapeutic Effect of Ceftriaxone in Murine Pneumococcal Pneumonia

Anita W. Rijneveld,1,2 Sandrine Florquin,3 Thomas Hartung,4
Peter Speelman,5,6 and Tom van der Poll1,5

Departments of 1Experimental Internal Medicine, 2Internal Medicine, and 3Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; 4Department of Biochemical Pharmacology and 5Departments of Infectious Diseases, Tropical Medicine, and AIDS, University of Konstanz, Konstanz, Germany

Treatments aimed at inhibition of tumor necrosis factor (TNF) in patients with sepsis have been unsuccessful. Up to 50% of such patients suffer from pneumonia. To determine the effect that treatment with anti-TNF has on pneumococcal pneumonia, mice were intranasally inoculated with Streptococcus pneumoniae and, 25 h later, treated with 1 of the following: (1) control antibody, (2) anti-TNF, (3) ceftriaxone (CEF) with control antibody, or (4) CEF with anti-TNF. In the absence of treatment with CEF, mice displayed high bacterial loads in lungs, and all of these mice died within 5 days after inoculation. Anti-TNF did not influence these outcomes. In contrast, 60% of mice treated with CEF alone survived. Anti-TNF administered together with CEF reduced survival to 40% and was associated with enhanced bacterial outgrowth. These data suggest that treatment with anti-TNF impairs the therapeutic efficacy of CEF during pneumococcal pneumonia.

Streptococcus pneumoniae is the most frequently isolated organism in patients with community-acquired pneumonia. The majority of cases occur in persons either >55 years old or with underlying chronic illnesses. For nonbacteremic disease, mortality is 5% [1]. In 15%–30% of patients with pneumonia, bacteremia develops, and almost 20% of these patients die [2].

Pneumococcal infection is characterized by an intense inflammatory response that is coordinated mainly by cytokines. Tumor necrosis factor (TNF) is a pluripotent proinflammatory cytokine that exerts powerful effects on the immune system—including the release of other proinflammatory cytokines, activation of neutrophils, and the induction of adhesion molecules—which lead to a rapid attraction of inflammatory cells to the inflammatory site [3]. TNF has been implicated as a central mediator of the host response to bacterial infection, in which it may play a dual role. Systemic and excessive release of TNF into the circulation is harmful to the host, as documented by numerous laboratory studies in which anti-TNF strategies prevented death during otherwise rapidly fatal sepsis [4, 5]. However, local production of TNF, at the site of an infection, is important for adequate antibacterial defense. This importance has been demonstrated, in particular, in murine models of pneumonia, in which treatment with anti-TNF impaired host defense against various respiratory pathogens, including S. pneumoniae [6–9].

In light of its presumed detrimental role in the pathogenesis of overwhelming sepsis, anti-TNF has been evaluated in a fairly large number of controlled clinical trials of patients with sepsis. Although individual trials did not reveal a significant benefit for patients treated with anti-TNF, pooled data from trials that evaluated monoclonal antibodies directed against TNF demonstrated a statistically significant (3.5%) reduction in mortality [10]. Interestingly, ≤50% of patients enrolled in clinical sepsis trials suffer from pneumonia as the primary source of infection [11]. Nonetheless, to our knowledge, investigations examining the effect that treatment with anti-TNF has during either clinical or experimental pneumonia have not been performed. Therefore, in the present study, we sought to determine the effect that anti-TNF therapeutically administered together with antibiotics has in mice with ongoing pneumococcal pneumonia.

Materials and methods. Ten-week-old male BALB/c mice (Harlan Sprague-Dawley) were used in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam, the Netherlands.

Pneumonia was induced as described elsewhere [8, 9]. In brief, S. pneumoniae serotype 3 obtained from American Type Culture Collection (ATCC 6303) was grown for 6 h at 37°C, to midlogarithmic phase, by use of Todd Hewitt broth (Difco); were harvested by centrifugation at 1500 g for 15 min at 4°C; and were washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline, at ~6 × 10^5 cfu/mL, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott) and were inoculated intranasally with 50 µL of bacterial suspension (~5 × 10^5 cfu).
Twenty-five hours after induction of pneumonia, mice received a single intraperitoneal injection (total volume, 200 µL) of 1 of the following treatments: sterile saline (100 µL) with preimmune sheep serum (Sigma) (100 µL) (group 1); sterile saline (100 µL) with polyclonal sheep anti-mouse anti-TNF antiserum (100 µL) (group 2) [12, 13]; ceftriaxone (CEF) (Roche) (20 mg/kg) in saline (100 µL) with normal sheep serum (100 µL) (Sigma) (group 3); or CEF (20 mg/kg) in saline (100 µL) with anti-mouse TNF antiserum (100 µL) (group 4). The timing of CEF administration was based on preliminary studies (not discussed here) in which we aimed to establish an antibiotic-treatment schedule that would rescue ~50% of the mice. We argued that this design would allow the evaluation of effects of immunomodulatory treatments (such as anti-TNF) in the context of antibiotic treatment. We chose to administer anti-TNF at the same time that we administered CEF, since we sought to assess the effect that anti-TNF has in a therapeutic setting.

After fixation in 10% buffered formalin for 24 h, lungs were embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin-eosin. All slides were coded and scored by 1 pathologist who had no knowledge regarding the treatment of the mice.

Whole lungs were harvested and were homogenized at 4°C, in 5 volumes of sterile isotonic saline, with a tissue homogenizer (Biospect) that, after each homogenization, was carefully cleaned and disinfected by use of 70% ethanol. Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates (and blood), and 50-µL volumes were plated onto sheep-blood agar plates and were incubated at 37°C. After incubation for 16 h, colony-forming units were counted. For interleukin (IL)–6 measurements, lung homogenates were lysed in lysis buffer (300 mmol NaCl/L, 15 mmol TRIS/L, 2 mmol MgCl/L, 2 mmol Triton X-100/L, 20 ng pepstatin A/mL, 20 ng leupeptin/mL, and 20 ng aprotinin/mL [pH 7.4]) and were centrifuged at 1500 g for 15 min at 4°C; the supernatant was frozen at −20°C, until IL-6 measurement by ELISA (Pharmingen).

Data were analyzed by use of the SPSS statistical package (SPSS) and are expressed as mean ± SEM. Comparisons between groups were made by Mann-Whitney U test. Survival curves were compared by log-rank test. P < .05 was considered a statistically significant difference.

**Results.** All mice that did not receive CEF died soon after inoculation with *S. pneumoniae* (0 survivors after 5 d after inoculation), irrespective of concurrent anti-TNF treatment (figure 1). Treatment with CEF resulted in survival in 60% of the mice, whereas concurrent treatment with anti-TNF reduced survival to 40% (P = .09, vs. treatment with CEF alone). Mice surviving for 14 days after inoculation were assumed to be permanent survivors.

To evaluate antibacterial host defense after the different treatment strategies, we determined the number of colony-forming units in lungs and blood 40 h after inoculation (i.e., 15 h after treatment). Mice not treated with CEF displayed a high number of pneumococci in their lungs, a result that was not influenced by treatment with anti-TNF. Among these mice, results of blood cultures were positive in 87% injected with normal sheep serum and in 50% injected with anti-TNF. As expected, treatment with CEF strongly reduced the number of *S. pneumoniae* colony-forming units recovered from lungs; remarkably, mice treated with CEF with anti-TNF had significantly more *S. pneumoniae* colony-forming units than did mice treated with CEF alone (P < .001) (figure 1). *S. pneumoniae* could not be cultured from the blood of mice treated with either CEF alone or CEF with anti-TNF.

At 40 h after inoculation, mice not treated with either CEF or anti-TNF presented interstitial inflammatory infiltrates predominantly composed of mononuclear cells (figure 2A). Mice treated with anti-TNF showed an exacerbated inflammation in the lungs, compared with mice treated with control antibody
Figure 2. Histopathology of lungs of mice treated with either (A) control antibody (group 1), (B) anti–tumor necrosis factor (TNF) (group 2), (C) ceftriaxone (CEF) (group 3), or (D) CEF with anti-TNF (group 4), 40 h after intranasal inoculation with $3 \times 10^6$ cfu of Staphylococcus pneumoniae. Hematoxylin-eosin staining. ×33.

(figure 2B). When mice were treated with CEF, the inflammatory reaction was almost abolished in the lungs (figure 2C). Addition of anti-TNF to treatment with CEF was associated with enhanced inflammation in the lungs, with more-intense and more-diffuse inflammatory infiltrates, compared with treatment with CEF alone (figure 2D). To obtain insight into the effect that treatment with CEF and/or anti-TNF has on the lung inflammatory response, we measured concentrations of IL-6 in lung homogenates. Treatment with CEF strongly reduced levels of IL-6 in lungs, levels that were not further influenced by concurrent treatment with anti-TNF (control antibody, 3.3 ± 0.9 ng/mL; CEF, 0.9 ± 0.2 ng/mL; CEF with anti-TNF, 0.6 ± 0.2 ng/mL; $P < .05$, both for CEF vs. control antibody and for CEF with anti-TNF vs. control antibody). Treatment with anti-TNF tended to reduce IL-6 levels in lungs (1.7 ± 0.7 ng/mL; $P = .08$, vs. control antibody).

Discussion. In patients with sepsis, the success of treatment with anti-TNF is limited, in spite of an abundance of experimental data indicating that, during overwhelming sepsis in animals, elimination of endogenous TNF activity exerts strong protective effects [3–5]. One possible explanation for this paradox is that preclinical-sepsis models using intravenous administration of live bacteria inadequately reproduce the clinical situation. Using murine models of pneumonia, which is the most frequent source of sepsis in recent clinical trials, we and others have demonstrated that anti-TNF administered before bacterial inoculation via the airways impairs local host defense and survival, a finding indicating that TNF produced at the site of infection is required for an adequate antibacterial defense during pneumonia [6–9]. These studies, which focus on the role that TNF plays in the pathogenesis of pneumonia, do not explain how treatment with anti-TNF (i.e., treatment administered to animals with already-ongoing respiratory-tract infection) influences the course of pneumonia in the context of treatment with antibiotics. We have shown here that anti-TNF administered 25 h after induction of pneumonia reduces the therapeutic effect of concurrently administered CEF, as reflected by a trend toward diminished survival, more pneumococci recovered from lung tissue, and enhanced destruction of lung tissue, compared with mice treated with CEF alone.

In a recent study, treatment with the anti-inflammatory cytokine IL-10 was reported to improve the efficacy of CEF during pneumococcal pneumonia in mice [14]. IL-10 reduced both the extent and the duration of inflammation, the outgrowth of pneumococci, and mortality, findings suggesting that a decrease of the inflammation, by adjunctive anti-inflammatory immu-
notherapy, may result in a beneficial effect on outcome [14]. However, the results of the present study, in which anti-TNF (another anti-inflammatory strategy, directed at the cytokine network) was combined with CEF, do not support these findings. Of note, both IL-10 and anti-TNF impaired host defense against pneumococcal pneumonia when they were administered either shortly before or simultaneously with the infectious challenge in mice not treated with antibiotics [8, 9, 15]. The present finding—that, in the absence of concurrent treatment with CEF, postponed administration of anti-TNF does not influence bacterial outgrowth—suggests that, in these mice, a point of no return has already been reached 25 h after infection and/or that only in the early phases of pneumococcal pneumonia is TNF essential for host defense.

Treatment with anti-TNF was associated with both an enhanced inflammatory response in lung tissue and unaltered or modestly reduced (IL-6) cytokine levels, a finding that contrasts with the strong anti-inflammatory effects of anti-TNF prophylactically administered to animals with severe sepsis [4, 5]. However, the current findings are in line with previous studies that used anti-TNF in the same model of murine pneumococcal pneumonia that we used [8, 9], a finding indicating that, in animals treated with anti-TNF, the bacterial load (which increases, relative to that in control animals) determines the extent of the inflammatory response in the lungs. This finding further suggests that the interplay between TNF and IL-6 is important mainly during the early phases of the infection.

Several anti-TNF strategies have been evaluated in clinical trials involving patients with severe sepsis, many of whom suffered from pneumonia. We have demonstrated here that anti-TNF administered 25 h after the induction of pulmonary infection with S. pneumoniae in mice is associated with a diminished effect of CEF. These data add to our knowledge of potentially adverse effects of treatment with anti-TNF in patients with severe infections, particularly in patients with bacterial pneumonia. This raises the question whether there is a clinical parallel between the subset of patients with pneumonia in the clinical studies and anti-TNF treatment in patients with sepsis.

Acknowledgments

We would like to thank I. Kop and J. B. Daalhuijsen, for expert technical assistance.

References