Pneumonia: an investigation of host defence mechanisms
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Anti-tumor necrosis factor antibody impairs the therapeutic effect of ceftriaxone in murine pneumococcal pneumonia
Therapies aimed at inhibition of tumor necrosis factor-α (TNF) in patients with sepsis have been unsuccessful. Up to 50% of such patients suffer from pneumonia. To determine the effect of anti-TNF therapy in pneumococcal pneumonia, mice were intranasally inoculated with Streptococcus (S.) pneumoniae, followed 25 hours later with one of the following therapies: (I) control antibody, (II) anti-TNF, (III) ceftriaxone (CEF) with control antibody, or (IV) CEF with anti-TNF. In the absence of CEF treatment, mice displayed high bacterial loads in lungs, and all of these mice died within 5 days; anti-TNF did not influence these outcomes. In contrast, 60% of mice treated with CEF alone survived. Anti-TNF given together with CEF reduced survival to 40% (P=0.09 vs. CEF) and was associated with an enhanced bacterial outgrowth (P<0.001 vs. CEF). These data suggest that anti-TNF therapy impairs the therapeutic efficacy of CEF during pneumococcal pneumonia.

Introduction

*S. pneumoniae* is the most frequently isolated organism in patients with community acquired pneumonia. The majority of cases occur in persons over 55 years of age or with underlying chronic illnesses. For non-bacteremic disease the mortality rate is 5%. In 15-30% of patients bacteremia develops and almost 20% of these patients die.

Pneumococcal infection is characterized by an intense inflammatory response that is mainly coordinated by cytokines. TNF is a pluripotent pro-inflammatory cytokine that exerts powerful effects on the immune system including the release of other pro-inflammatory cytokines, activation of neutrophils and the induction of adhesion molecules, leading to a rapid attraction of inflammatory cells to the inflammatory site. 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 clearly is harmful to the host, as documented by numerous laboratory studies in which anti-TNF strategies prevented mortality during otherwise rapidly fatal sepsis. However, local production of TNF, at the site of an infection, is important for adequate antibacterial defense. This has in particularly been demonstrated in murine models of pneumonia, in which anti-TNF treatment impaired host defense against various respiratory pathogens including *S. pneumoniae*.

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

**Animals.** Ten week old male BALB/c mice (Harlan Sprague Dawley Inc. Horst, the Netherlands) 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.

*Induction of pneumonia.* Pneumonia was induced as described previously. Briefly, *S. pneumoniae*, serotype 3, obtained from American Type Culture Collection (ATCC 6303; Rockville, MD), were grown for 6 hours to midlogarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x g at 4°C for 15 minutes, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at approximately 6 x 10^6 colony forming units (CFU)/ml, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott, Queensborough, Kent, UK), and 50 μl of bacterial suspension (approximately 3 x 10^5 CFU) was inoculated intranasally.

*Experimental design.* 25 hours after induction of pneumonia, mice received a single intraperitoneal injection (total volume 200 μl) with one of the following treatments: Group I, 100 μl sterile saline with 100 μl pre-immune sheep serum (Sigma, St.Louis, MO); group II: 100 μl sterile saline with 100 μl polyclonal sheep anti-mouse anti-TNF antiserum; group III: ceftriaxone (CEF) 20 mg/kg (Roche Nederland B.V., Mijdrecht, the Netherlands) in 100 μl saline with 100 μl normal sheep serum (Sigma); group IV: CEF 20 mg/kg in 100 μl saline with 100 μl anti-mouse TNF antiserum. The timing of CEF administration was based on preliminary studies not shown here in which we aimed to establish a treatment schedule with antibiotics that would rescue approximately 50% of the mice. We argued that this design would allow the evaluation of effects of immunomodulatory therapies (such as anti-TNF), in the context of antibiotic therapy. We chose to administer anti-TNF at the same time as CEF since we sought to assess the effect of anti-TNF in a therapeutic setting.

*Histologic examination.* After 24 hours fixation in 10% buffered formaline, lungs were embedded in paraffin. Four μm thick sections were stained with haematoxylin and eosin. All slides were coded and scored by one pathologist without knowledge of the treatment of the mice.

*Preparation of lung homogenates.* Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK) which was carefully cleaned and desinfected with 70% ethanol after each homogenization. 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 incubated at 37°C. CFU were counted after 16 hours incubation. For interleukin (IL)-6 measurements lung homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Trition (X-100), Pepstatin A, Leupeptin, Aprotinin (20 ng/ml), pH 7.4) and spunned at 1500 x g at 4°C for 15 minutes; the supernatant was frozen at -20°C until IL-6.
measurement by ELISA (Pharmingen, San Diego, CA).

Statistical analysis. Data are expressed as means ± SEM. Comparisons between groups were conducted using the Mann Whitney U test. Survival curves were compared by log-rank test. P-value <0.05 was considered to represent a statistically significant difference.

Results

Survival. All mice that did not receive CEF, rapidly died after inoculation with S. pneumoniae irrespective of concurrent anti-TNF treatment (0% survivors after 5 days; [Figure 1]). CEF prevented mortality in 60% of the mice, whereas concurrent treatment with anti-TNF reduced survival to 40% (P=0.09 vs. CEF alone). Mice, surviving for 14 days post inoculation, appeared to be permanent survivors. 

![Figure 1](image)

Figure 1. Influence of anti-TNF therapy on survival and bacterial outgrowth in mice with pneumococcal pneumonia with and without concurrent antibiotic treatment. (A) Survival after intranasal inoculation with $3 \times 10^5$ CFU S. pneumoniae in mice treated with control Ab (group I: closed squares), with anti-TNF (group II: open squares), with ceftriaxone (CEF) (group III: closed circles) or with CEF and anti-TNF (group IV open circles). Mortality was assessed twice daily for 14 days. N=20 per group. (B) CFU S. pneumoniae in lungs of mice treated with control Ab (Group I), with anti-TNF (Group II), with CEF (group III) or with CEF and anti-TNF (group IV) 40h after intranasal inoculation with $3 \times 10^5$ CFU S. pneumoniae. Data are mean ± SEM. N=9 per group. *P<0.05 vs. control Ab treated mice (group I). $^\#$P<0.05 vs. anti-TNF treated mice (group II). $^\#\$P<0.05 vs. ceftriaxone treated mice (group III).

Bacterial outgrowth. To evaluate antibacterial host defense after the different treatment strategies, we determined the number of CFUs in lungs and blood 40h after inoculation (i.e. 15h after treatment). Mice not receiving CEF displayed a high number of pneumococci in their lungs, which was not influenced by anti-TNF therapy. In these mice blood cultures were positive in 87% of animals injected with normal sheep serum, and 50% of animals injected with anti-TNF. As expected, CEF strongly reduced the number of S. pneumoniae CFUs recovered from lungs. Remarkably, mice treated with CEF in combination with anti-TNF had
significantly more *S. pneumoniae* CFU than mice treated with CEF alone (P<0.001)(Figure 1). *S. pneumoniae* could not be cultured from the blood of mice treated with CEF or CEF with anti-TNF.

**Figure 2** Histopathology of lungs. (A) Lungs of mice treated with control Ab (group I) 40h after infection. (B) Lungs of mice treated with anti-TNF (group II) 40h after infection. (C) Lungs of mice treated with CEF (group III) 40h after infection. (D) Lungs of mice treated with CEF and anti-TNF (group IV) 40h after infection. HE staining magnification x 33.

**Pulmonary inflammation.** At 40h after inoculation mice not treated with CEF or anti-TNF presented interstitial inflammatory infiltrates composed of predominantly mononuclear cells (Figure 2A). Anti-TNF treated mice showed an exacerbated inflammation in the lungs compared to control Ab treated mice (Figure 2B). When mice were treated with CEF, the inflammatory reaction was almost abolished in the lungs (Figure 2C). Addition of anti-TNF to CEF treatment was associated with an enhanced inflammation in the lungs with more intense and diffuse inflammatory infiltrates compared to treatment with CEF alone (Figure 2D). To obtain insight into the effect of CEF and/or anti-TNF therapy on the lung inflammatory response, we measured concentrations of IL-6 in lung homogenates. CEF therapy strongly reduced lung IL-6 levels, which was not further influenced by concurrent anti-TNF therapy (control: 3.3 ± 0.9 ng/ml; CEF: 0.9 ± 0.2 ng/ml; CEF plus anti-TNF: 0.6 ± 0.2 ng/ml; P<0.05 for difference between CEF and CEF plus anti-TNF vs. control). Anti-TNF therapy tended to.
reduce IL-6 levels in lungs (1.7 ± 0.7 ng/ml; P=0.08 vs. control)

Discussion
The success of anti-TNF therapy in patients with sepsis is limited, in spite of an abundance of experimental data indicating that elimination of endogenous TNF activity exerts strong protective effects during overwhelming sepsis in animals.\(^3\)\(^-\)\(^5\) 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, the most frequent source of sepsis in recent clinical trials, we and others demonstrated that anti-TNF given before bacterial inoculation via the airways impaired local host defense and survival, indicating that TNF produced at the site of infection is required for an adequate antibacterial defense during pneumonia.\(^6\)\(^-\)\(^9\) These studies which focussed on the role of TNF in the pathogenesis of pneumonia, left unanswered how anti-TNF therapy, i.e. administered to animals with already ongoing respiratory tract infection, influences the course of pneumonia in the context of antibiotic treatment. We here show that anti-TNF given 25h after induction of pneumonia reduced the therapeutic effect of concurrently administered CEF, as reflected by a diminished survival, more pneumococci recovered from lung tissue and enhanced destruction of lung tissue when compared to mice treated with only CEF.

Recently, treatment with the anti-inflammatory cytokine IL-10 was reported to improve the efficacy of CEF during pneumococcal pneumonia in mice. IL-10 reduced both the extent and the duration of inflammation, reduced the outgrowth of pneumococci and decreased mortality suggesting that decrement of the inflammation by adjunctive anti-inflammatory immunotherapy 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. Notably, both IL-10 and anti-TNF impaired host defense against pneumococcal pneumonia when administered 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 CEF therapy, postponed administration of anti-TNF did not influence bacterial outgrowth, suggests that in these mice a point of no return has already been reached at 25h postinfection and/or that TNF is essential for host defense only in the early phases of pneumococcal pneumonia.

Anti-TNF therapy was associated with an enhanced inflammatory response in lung tissue and unaltered or modestly reduced cytokine levels (among which IL-6), which contrasts with the strong anti-inflammatory effects of anti-TNF given prophylactically to animals with severe sepsis.\(^4\)\(^,\)\(^5\) However, the current findings are in line with our previous studies with anti-TNF in this model of murine pneumococcal pneumonia,\(^8\)\(^,\)\(^9\) indicating that in anti-TNF treated animals the bacterial load (which is increased relative to control animals) determines the extent of the inflammatory response in the lung.

Several anti-TNF strategies have been evaluated in clinical trials involving patients with
severe sepsis, many of whom suffered from pneumonia. We here demonstrate that anti-TNF given 25h after the induction of pulmonary infection with S. pneumoniae in mice is associated with a diminished effect of CEF. These data add new information to our knowledge of potential adverse effects of anti-TNF therapy in patients with severe infections, in particular with bacterial pneumonia.

References

