Tumor necrosis factor-α inhibition reduces CXCL-8 levels but fails to prevent fibrin generation and does not improve outcome in a rabbit model of endotoxic shock

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The effects of a monoclonal antibody (mAb) to tumor necrosis factor-α (TNF-α) were examined in a rabbit model of endotoxic shock. Intravenous administration of lipopolysaccharide (100 μg/kg/hr) for 6 hours (*n* = 11) increased TNF-α levels. Fibrinogen was partially consumed, and fibrin deposits were seen in kidney and lungs at 24 hours. Mortality at 24 hours was 64%. Levels of interleukin-8 (aka CXCL-8) were notably increased. Mean arterial pressure (MAP) and leukocyte counts decreased, whereas creatinine levels were enhanced. The anti-TNF-α mAb (20 mg/kg i.v. bolus + 5 mg/kg/h i.v. for the first 90 minutes) (n = 10) efficiently inhibited the TNF-activity. Rabbits exhibited lower CXCL-8 levels; MAP improved, the decrease in leukocyte counts was partially prevented and creatinine levels were lower, but fibrinogen, fibrin deposits in kidneys and lungs and mortality, 55%, were similar to the LPS group. Rabbits that did not survive exhibited lower fibrinogen levels, more fibrin in kidneys and lungs and higher CXCL-8 and creatinine levels than survivors, while there were no differences in TNF-α, MAP and leukocytes. Thus, the inhibition of TNF-α, although beneficial through lowering CXCL-8 levels, is not enough to improve the outcome, which could be partly due to the inability to prevent the fibrin deposits formation in kidneys and lungs. (J Lab Clin Med 2003;141:257-64)

Abbreviations: CXCL-8 = interleukin-8; EDTA = ethylenediaminetetraacetate; IL = interleukin; LPS = lipopolysaccharide; MAP = mean arterial pressure; MIP-2 = macrophage inflammatory protein-2; mAb = monoclonal antibody; NO = nitric oxide; NOx = NO_2 and NO_3-; SEM = standard error of the mean; TNF-α = tumor necrosis factor-α

Sepsis is caused by the immune response of a host to infectious agents. It is characterized by systemic inflammation and activation of coagulation. Sepsis can evolve to septic shock, causing hypotension, coagulopathy, and multiple organ failure.[1,2] Endotoxin — LPS of the outer membrane of Gram-negative bacteria — plays a central role in septic shock. It triggers a set of inflammatory reactions that lead to the generation of a variety of mediators that orchestrate the pathologic response during endotoxic shock through such mechanisms as lipid peroxidation, NO overproduction, and antioxidant depletion, which lead

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to hypotension and tissue damage; or through the induction of a hemostatic imbalance, which leads to the generation of fibrin and thrombi in the capillaries of various organs and subsequent disseminated intravascular coagulation.[1-5] Most of these mechanisms are initiated by cytokines. Among them, TNF-α sets a cascade of inflammatory reactions in motion, leading to the generation of a variety of mediators that orchestrate many pathologic responses during endotoxic shock.[1-3] Therapeutic intervention directing TNF-α should prevent the action of those TNF-α–dependent mediators in the propagation of the inflammatory cascade.[6]

CXC chemokine is a large subfamily of chemokines that attract and activate neutrophils.[7] IL-8 (CXCL8) belongs to the CXC chemokine subfamily and has been detected in human endotoxemia.[8-11] Many CXC chemokines are believed to be involved in the pathogenesis of endotoxic shock.[12,13] CXCL-8 can be induced by LPS in a TNF-α-dependent manner;[14-18] however, its relationship with TNF-α during endotoxic shock remains unclear.

In this study, we infused doses of LPS large enough to induce endotoxic shock into rabbits, along with a mAb raised against rabbit TNF-α[19] that is able to neutralize rabbit TNF-α activity in vivo.[19,20] Although CXCL-8 levels were shown to be reduced by TNF-α, the anti–TNF-α mAb was not able to ameliorate fibrin deposition in kidneys and lungs, which may partly explain the failure of this treatment to improve outcome.

METHODS

Experimental model and treatment groups. Male New Zealand white rabbits (2.8-3.5 kg) were anesthetized by means of intramuscular injection of 30 mg/kg ketamine hydrochloride and 0.002 mg/kg xylazine hydrochloride. Ketamine hydrochloride boosters were given as necessary throughout the experiment. To induce severe endotoxemia, we infused rabbits with LPS (100 μg/kg/hr in 60 mL of saline solution, 10 mL/hr) for 6 hours through the marginal ear vein. The rabbits were divided into the following groups: (1) the LPS group ($n = 11$), in which LPS was administered without additional treatment; (2) A group ($n = 10$) given LPS plus mAb directed against rabbit TNF-α (murine origin, IgG_1)[19] through the opposite marginal ear vein in a bolus of 20 mg/kg before the start of the experiment, followed by a 5 mg/kg/hr infusion for the first 90 minutes of the LPS challenge; additional control groups consisting of (3) 5 rabbits given saline solution; (4) 3 rabbits given saline solution plus mAb against TNF-α; and (5) 3 rabbits given LPS plus nonspecific P3X63Ag8 mAb (murine origin, IgG1).[21] Blood was drawn from the femoral artery before the start of the experiment and 2, 4, and 6 hours afterward. We studied mortality at 24 hours. Surviving rabbits were killed with intravenous injections of 60 mg/kg pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, Ill), after which kidneys and lungs were extracted for histologic analysis. Mortality could not be studied in one mAb-treated rabbit because the animal died as a result of deficient artery ligation once the 6-hour LPS challenge was over.

Measurements. We quantified TNF-α activity in serum using a bioassay based on the cytotoxic effect of TNF-α on the neoplastic murine fibroblast–derived line L929 (ECACC, Salisbury, UK).[22] TNF-α activity was considered to be the inverse of the serum dilution necessary to achieve 50% lysis of the L929 cells. A recombinant human TNF-α (Genzyme, Cambridge, Mass) was used as a standard.

The Clauss method[23] was used to measure fibrinogen levels. Kidneys and lungs were removed 24 hours after the start of the experiment. Sections were fixed in formalin, embedded in paraffin, stained with Masson's trichrome, and analyzed by a pathologist blinded to the experimental groups. In the analysis of the presence of fibrin in kidneys and lungs, severity scores described elsewhere were used. In brief, kidney sections were scored on a scale of 0 to 4 as previously described[24]: 0, no fibrin; 1, partial fibrin deposits in some glomeruli; 2, partial deposits in all glomeruli; 3, large quantities of fibrin in all glomeruli; 4, fibrin thrombi in glomerular capillaries and in noncapillary vessels. Ten fields were examined for each tissue section, after which the average value was calculated. A 0-4 scale was also designed for lung sections.[25] Twenty fields were examined and the fields found to contain intravascular fibrin were counted. Lungs in which 20 out of 20 fields were positive were scored 4; lungs in which none of the fields was positive were scored 0.

To measure CXCL-8 and IL-1β levels in rabbit serum, we used two sandwich enzyme-linked immunosorbent assays.[14,26] In brief, plates were coated with affinity-purified goat anti–rabbit CXCL-8 IgG or purified goat anti–rabbit IL-1β IgG, and detection was performed with the use of biotinylated goat anti–rabbit CXCL-8 IgG or biotinylated goat anti–rabbit IL-1β IgG. Processing was performed in both assays with the use of avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif) and *o*-phenylenediamine. The lower detection limits were 30 and 10 pg/mL in the CXCL-8 and IL-1β assay, respectively. Both tests were specific, and no cross-reaction with other cytokines/chemokines occurred.

We monitored blood pressure with an arterial catheter connected to a pressure transducer (Siemens, Munich, Germany). MAP was determined electronically with the blood-pressure signal (HP1290C; Hewlett-Packard, Palo Alto, Calif). MAP was recorded imme-

diately before the start of the LPS challenge and 2, 4, and 6 hours afterward. MAP value was calculated as follows: MAP (mm Hg) = [2 × diastolic arterial pressure (mm Hg) + systolic arterial pressure (mm Hg)]/3. The value obtained before the start of the experiment was discarded because it was influenced by the anesthesia needed to carry out artery and vein cannulation.

Serum creatinine levels were measured with a direct colorimetric method.[27]

Peripheral leukocytes were quantified in blood drawn into tubes containing K_3-EDTA with a Counter STKS automatic analyzer (Coulter Corp, Hialeah, Fla).

The serum levels of NOx were determined with the use of a commercially available kit (Cayman; Alexis Corp, San Diego, Calif) based on the Griess method.[28]

Statistical analysis. So that we might normalize the CXCL-8 and IL-1β data, we subtracted the baseline value for each rabbit from each value. Fibrinogen, MAP, creatinine, leukocyte count, and NOx data were expressed as percentages with respect to baseline values.

Student's *t* test or the Mann-Whitney U test was applied to compare the LPS group with the anti–TNF-α–treated group and to compare rabbits that survived 24 hours with those that did not. We calculated the Pearson or Spearman correlation coefficient to analyze the association between variables.

Results are expressed as mean ± SEM.

RESULTS

TNF-α activity. When all the rabbits included in the study were taken into account, baseline TNF-α activity was 0.019 ± 0.003 U/μL (n = 32). Anti–TNF-α mAb treatment efficiently inhibited increased serum TNF-α activity after the LPS challenge: TNF-α activity peaked at 2 hours in the LPS group (11.1 ± 2.9 U/μL), and no increase was detected in the sera of the anti–TNF-α–treated rabbits (Fig 1, *A*).

When mortality at 24 hours was analyzed, no differences were detected between the LPS group and the anti–TNF-α–treated group, in spite of TNF-α inhibition: 7 of 11 rabbits (63.6%) died in the LPS group, compared with 5 of 9 (55.5%) in the anti–TNF-α–treated group. When rabbits were grouped according to whether they survived 24 hours, regardless of treatment, serum TNF-α activity in the nonsurvivor animals was not significantly different from that observed in the survivors (6.5 ± 2.3 and 4.8 ± 4.4 U/μL at 2 hours in nonsurvivors and survivors, respectively). Furthermore, we detected no differences in TNF-α activity between survivors and nonsurvivors within each treatment group (Fig 1, *B*).

Hemostasis. Fibrinogen levels demonstrated continuous decreases in the LPS group during the 6 hours of LPS infusion. The anti–TNF-α mAb did not prevent such a decrease; fibrinogen levels were similar to those in the LPS group (Fig 2, *A*). However, fibrinogen consumption was significantly higher in the animals that did not survive 24 hours, regardless of treatment, than in survivors (72.6% ± 4.6% vs 90.2% ± 2.6% at 4 hours, $P < .01$, and 57.5% ± 7.5% vs 83.8% ± 3.7% at 6 hours, $P < .01$, in nonsurvivors and survivors, respectively; Fig 2, *D*).

Fibrin deposits in the kidneys were similar in the LPS-and anti–TNF-α–treated rabbits (scores of 1.80 ± 0.41 in the LPS group and 1.78 ± 0.39 in the anti–TNF-α–treated group; Fig 2, *B*). The animals that did not survive 24 hours exhibited renal fibrin deposits higher than those observed in the survivor animals, regardless of treatment (2.12 ± 0.31 in nonsurvivors vs 1.40 ± 0.37 in survivors; Fig 2, *E*). Moreover, necrotic areas were always seen in kidneys when fibrin was present in glomeruli and tubules.

Lung fibrin deposits were similar in the LPS-and anti–TNF-α–treated rabbits (0.31 ± 0.05 in the LPS group vs 0.29 ± 0.07 in the anti–TNF-α–treated group; Fig 2, *C*). Again the nonsurvivor animals exhibited more extensive lung fibrin deposits than the survivor animals, regardless of treatment (0.40 ± 0.05 in nonsurvivors vs 0.22 ± 0.04 in survivors, $P < .05$; Fig 2, *F*).

CXCL-8 levels. The mean baseline CXCL-8 level for the entire study population was 0.99 ± 0.60 ng/mL (n = 32). Serum CXCL-8 levels increased in both groups and peaked 2 hours after the start of the LPS challenge. However, levels in the rabbits treated with anti–TNF-α mAb rabbits were remarkably attenuated (183.2 ± 24.4 vs 66.1 ± 13.6 Δng/mL at 2 hours [$P < 0.01$], 124.2 ± 37.8 vs 33.2 ± 12.2 Δng/mL at 4 hours [$P < .05$], and 140.5 ± 71.2 vs 22.1 ± 11.3 Δng/mL at 6 hours [$P < .05$] in LPS-and anti–TNF-α mAb–treated groups, respectively; Fig 3, *A*). Accordingly, when data from all the LPS-challenged rabbits (n = 24) were pooled, CXCL-8 levels correlated remarkably well with TNF-α activity at their peak (2 hours): $r = .613$, $P < .01$.

CXCL-8 levels were significantly higher in the rabbits that did not survive 24 hours, regardless of treatment (129.2 ± 33.4 vs 31.9 ± 8.9 Δng/mL at 4 hours [$P < 0.05$] and 138.0 ± 64.5 vs 14.4 ± 5.0 Δng/mL at 6 hours [$P < .05$] in nonsurvivors and survivors, respectively; Fig 3, *C*).

IL-1β levels. The baseline IL-1β level for the entire study population was 16.5 ± 5.2 pg/mL (n = 32). We noted a tendency for the level of IL-1β to decrease after treatment with anti–TNF-α mAb (Fig 3, *B*), although this trend was not statistically significant. IL-1β levels in nonsurvivor rabbits, regardless of treatment, were

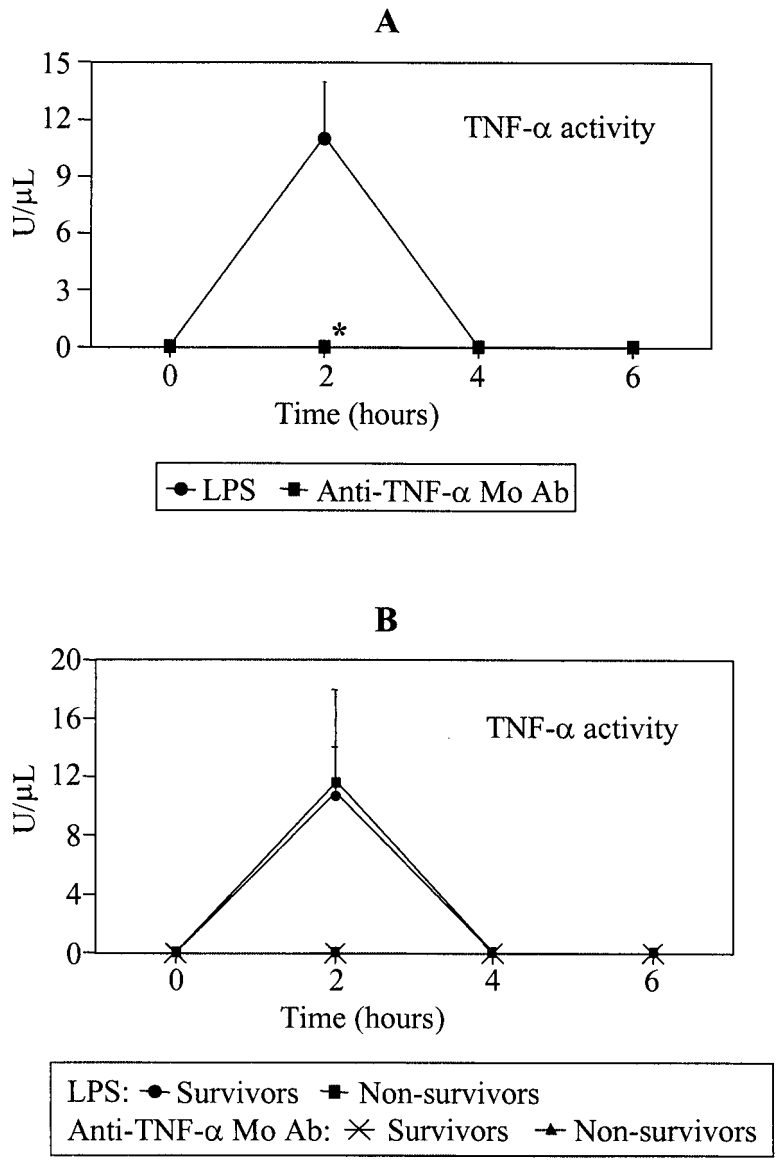

Fig 1. Serum TNF-α activity (U/L) at 2, 4, and 6 hours: **A**, in the LPS group and the anti–TNF-α mAb–treated group; **B**, in survivors and nonsurvivors within each group. Data expressed as mean ± SEM. *$P < .001$ with respect to the LPS group.

not significantly higher than those observed in survivors (Fig 3, *D*), although we did note such a trend.

MAP, renal dysfunction, and peripheral leukocyte count. MAP was significantly improved at 4 and 6 hours by treatment with anti–TNF-α mAb (58.2% ± 5.9% vs 74.6% ± 3.4% at 4 hours [$P < .05$] and 54.2% ± 4.6% vs 83.6% ± 3.9% at 6 hours [$P < .05$] in LPS and anti–TNF-α mAb–treated animals, respectively; Fig 4, *A*).

Creatinine levels in the rabbits treated with anti–TNF-α mAb were significantly lower than those observed in the LPS group at 2 and 4 hours (115.9% ± 3.3% vs 101.3% ± 4.8% at 2 hours [$P < .05$] and 150.7% ± 6.1% vs 130.1% ± 7.8% at 4 hours [$P < .05$] in the LPS and anti–TNF-α mAb–treated groups, respectively; Fig 4, *B*). We detected no differences at 6 hours.

The anti–TNF-α mAb significantly attenuated the decrease in leukocyte counts observed in the LPS group (27.0% ± 2.5% vs 35.6% ± 4.1% at 4 hours [$P < .05$] and 31.4% ± 3.0% vs 49.2% ± 8.3% at 6 hours [$P < .05$] in the LPS and anti–TNF-α–treated groups, respectively; Fig 4, *C*).

Although MAP and leukocyte counts were similar in survivors and nonsurvivors, regardless of treatment

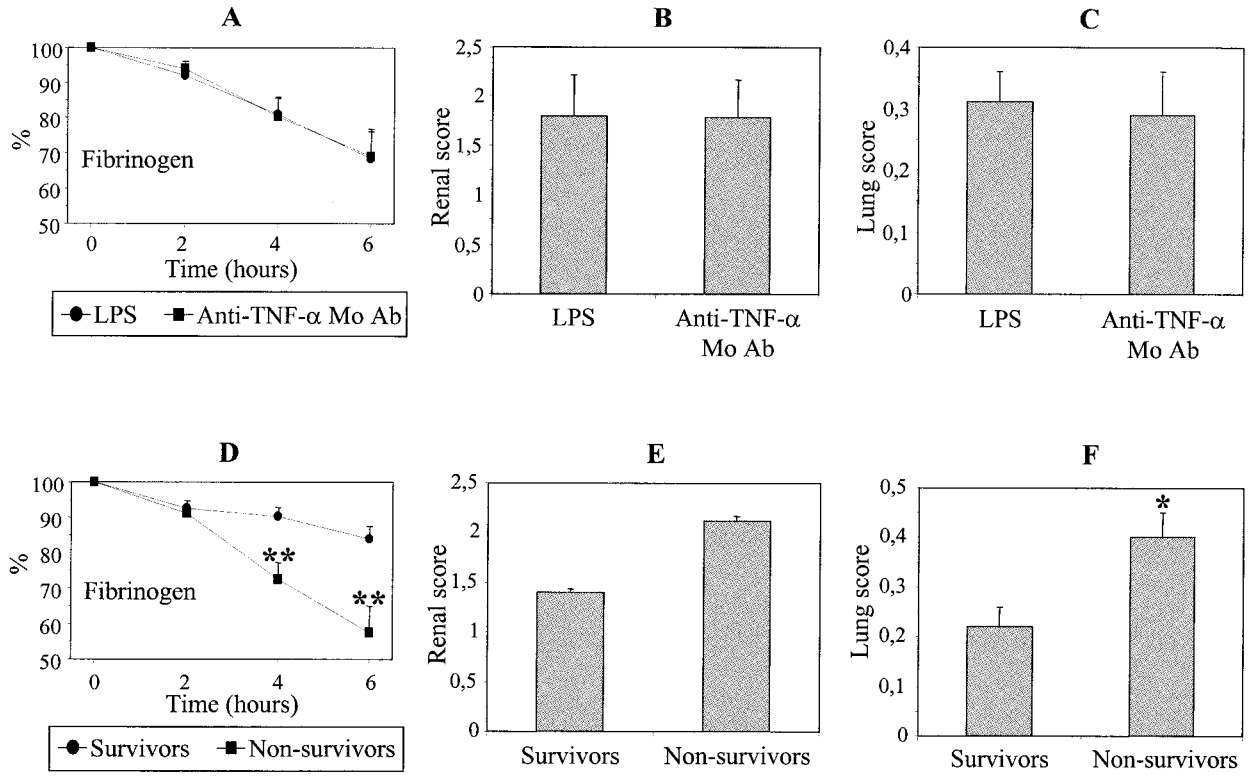

Fig 2. Fibrinogen levels at 2, 4, and 6 hours: in the LPS and anti–TNF-α mAb–treated groups (**A**); in survivors and nonsurvivors (**D**). Fibrin deposits in kidneys (**B**) and lungs (**C**) in LPS and anti–TNF-α mAb–treated group. Kidneys (**E**) and lungs (**F**) in the survivors and nonsurvivors. Data expressed as mean ± SEM. *$P < .05$. **$P < .01$ with respect to survivors.

(Fig 4, *D* and *F*), creatinine levels were significantly higher in nonsurvivors at 6 hours (177% ± 19% and 133% ± 7% [$P < .05$] in nonsurvivors and survivors, respectively; Fig 4, *E*).

We pooled all data from rabbits challenged with LPS so that we might analyze the correlation between CXCL-8 levels, MAP, and renal dysfunction. CXCL-8 levels correlated remarkably well with MAP ($r = -.729$ at 2 hours, $P < .01$; $r = -.851$ at 4 hours, $P < .001$) and with creatinine concentration ($r = .654$ at 2 hours, $P < .01$; $r = .678$ at 4 hours, $P < .001$; $r = .729$ at 6 hours, $P < .001$).

NOx levels. Anti–TNF-α mAb treatment did not alter serum NOx levels, which were 109.0% ± 19.7%, 91.2% ± 6.7%, and 85.8% ± 6.5%, respectively, at 2, 4 and 6 hours with respect to those observed in the LPS group. The nonsurvivor animals exhibited NOx levels similar to those observed in the survivor animals (116.6% ± 14.0%, 105.5% ± 2.9%, and 111.4% ± 4.7% at 2, 4, and 6 hours, respectively, with respect to survivor levels).

Control rabbits. Rabbits not challenged with LPS but administered saline solution or saline solution plus anti–TNF-α mAb tolerated femoral catheter and ear vein cannulation well and did not demonstrate alterations in the variables included in the study (data not shown). Rabbits given LPS plus nonspecific P3X63Ag8 mAb demonstrated changes similar to those observed in the LPS group (data not shown).

DISCUSSION

The important role played by TNF-α in sepsis is unquestionable.[1-3] However, LPS elicits a wide range of responses that involve many different mediators. For this reason, in spite of the efficiency of the anti–TNF-α mAb in inhibiting TNF-α activity, the inability of this treatment to improve outcome in our model of severe sepsis should not be surprising. The inhibition of TNF-α activity notably prevented CXCL-8 and, to a lesser extent, IL-1β production and improved MAP, leukocyte count, and renal dysfunction during the LPS-infusion period. However, the similar mortality rates in control and treated rabbits suggest the existence of LPS-induced mechanisms that influenced outcome and were not controlled by the anti–TNF-α mAb. One important contributor to multiorgan failure in sepsis is hemostatic imbalance leading to disseminated intravascular coagulation. The inability of the anti–TNF-α

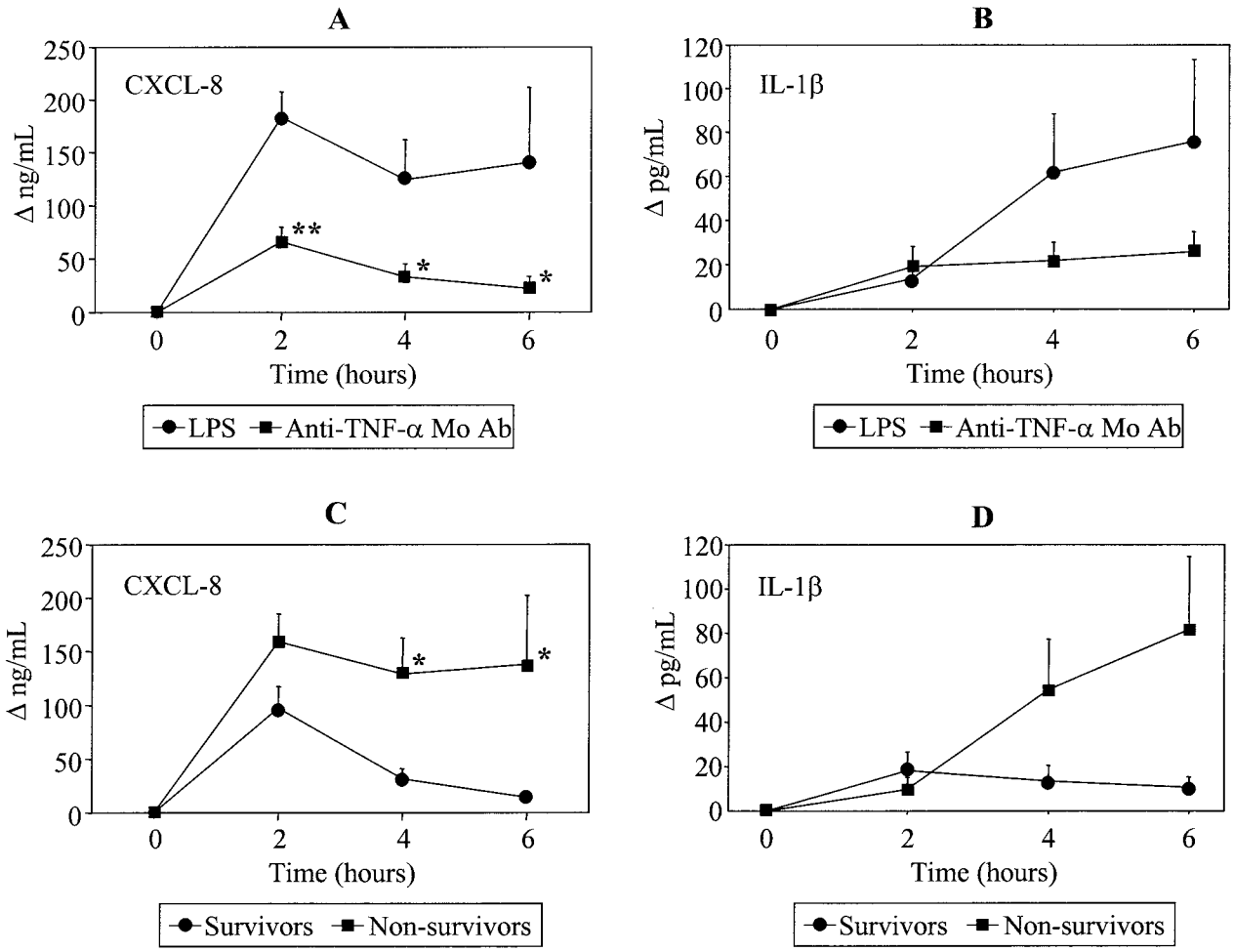

Fig 3. Serum CXCL-8 (Δng/mL) and IL-1β (Δpg/mL) increments at 2, 4, and 6 hours. CXCL-8 (**A**) and IL-1β (**B**) in the LPS and anti–TNF-α mAb–treated groups. CXCL-8 (**C**) and IL-1β (**D**) in survivors and nonsurvivors. Data expressed as mean ± SEM. *$P < .05$. **$P < .01$ with respect to the LPS group (**A**) or the survivor group (**C**).

mAb to prevent the consumption of fibrinogen and the subsequent LPS-induced generation of fibrin does not support the notion that TNF-α plays a pivotal role in triggering coagulation in sepsis[2,29] and could partly explain the failure of this treatment to prevent death; fibrin deposits in kidneys and lungs were more extensive in rabbits that did not survive the experiment. IL-1β seems to play a more preponderantly prothrombotic role in sepsis.[29,30] Nevertheless, factors other than TNF-α/IL-1β that were not corrected by the treatment must also be involved in the generation of fibrin; renal and pulmonary fibrin deposits were similar in LPS-treated and mAb-treated rabbits in spite of the TNF-α and IL-1β decreases after treatment.

CXCL-8 increased in serum after LPS infusion, peaking at 2 hours, when TNF-α activity had also reached its peak. Anti–TNF-α mAb treatment efficiently decreased serum levels of CXCL-8. Because levels of TNF-α and CXCL-8 correlated to a high degree during the experiment, we conclude that the up-regulation of CXCL-8 in severe endotoxemia is driven by TNF-α, as is the case in other LPS-based in vivo models.[16,18,31] However, a down-regulatory effect of some CXC chemokines on TNF-α has been described[32]; a down-regulation of the TNF-α activity by CXCL-8 could also contribute to this high correlation. Even a regulation of both TNF-α and CXCL-8 by some other common mediator cannot be ruled out.

It should be noted that CXCL-8 levels were significantly higher in the rabbits that did not survive 24 hours than in survivors, whereas no such difference was found with regard to TNF-α activity. The involvement of CXCL-8 in sepsis is indubitable [33-35]: CXCL-8 is a potent contributor to neutrophil infiltration and neutrophil-mediated tissue injury in a variety of LPS-induced models of inflammation.[14-18] However, some of the

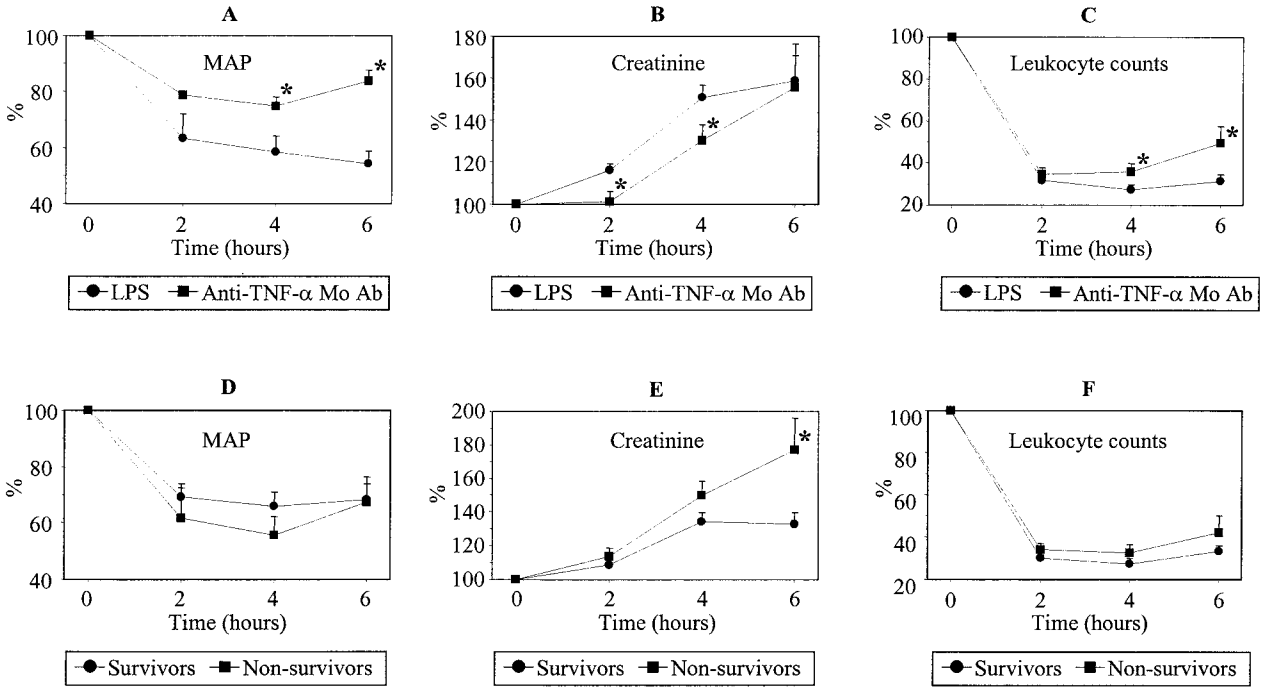

Fig 4. MAP, creatinine, and leukocyte counts (percentage of baseline value) at 2, 4, and 6 hours: MAP (**A**), creatinine (**B**), and leukocyte counts (**C**) in the LPS and anti–TNF-α mAb–treated groups. MAP (**D**), creatinine (**E**), leukocyte counts (**F**) in survivors and nonsurvivors. Data expressed as mean ± SEM. $*P < .05$ with respect to LPS group (**A**, **B**, and **C**) or survivors (**E**).

actions induced by CXCL-8 in sepsis remain unclear. We found an important correlation of serum CXCL-8 levels with MAP and renal dysfunction. A direct relationship between CXCL-8 and MAP/creatinine cannot be inferred from correlation analysis. However, it is tempting to speculate that CXCL-8 is involved in hypotension and the subsequent kidney failure in severe sepsis; a recent study by Matsukawa et al in a sepsis model showed that therapeutic control of the levels of two CXC chemokines, MIP-2 and KC, improved kidney function without notably changing renal neutrophil influx.[36] The fact that renal injury in sepsis can be at least partly caused by renal hypoperfusion rather than inflammation would support this hypothesis.[37] Nevertheless, the mechanism by which CXCL-8 may hamper hemodynamics in endotoxemia is unclear, and further work will be required to test this hypothesis.

Finally, the finding that the NOx values observed in the LPS and anti–TNF-α mAb–treated groups were similar suggests that TNF-α is not the only cytokine resposible for NO overproduction in sepsis. Nevertheless, the fact that NOx levels were also similar among survivors and nonsurvivors does not support a key role for NO in the outcome with this model.

We thus conclude that successful inhibition of TNF-α activity was not enough to prevent fatality in this rabbit model of severe sepsis. Although inhibition of TNF-α improved MAP, renal dysfunction, leukocyte counts, and CXCL-8 levels, the similar mortality rates in control and treated rabbits may have been at least partially caused by the inability of the mAb to prevent the fibrin deposition and subsequent tissue damage revealed on histologic analysis. Because the range of inflammatory mechanisms triggered by LPS is so wide, treatments should not be limited to single targets.

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