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## Tumor Necrosis Factor Activity Increases in the Early Response to Trauma

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### ■ ABSTRACT

**Objectives:** 1) To determine whether tumor necrosis factor (TNF) up-regulation occurs in the first hours following severe injury. 2) To determine whether the time from injury to blood sampling affects the probability of detecting TNF.

**Methods:** A prospective, cross-sectional study was performed using a convenience sample of adult major trauma patients ("patients") treated at a university hospital ED (Level-1 trauma center) and 20 healthy volunteers ("controls"). The time interval from injury to specimen collection ( $\Delta T$ ), the injury severity scale (ISS) score, patient demographics, and quantitative cytokine [TNF and interleukin (IL-6, IL-8)] levels were measured. In the patients, cytokine levels were analyzed as a function of  $\Delta T$  (using first hourly cutoff points and then the median T as an arbitrary cutoff point) with and without potential confounders (e.g., ISS, age, gender).

**Results:** The mean  $\Delta T$  was  $92.8 \pm 49.2$  min (range 10-210 min, median 82 min). In the controls, TNF activity was present in 96%, with a mean level of 125 pg/mL. The controls showed no baseline IL-6 activity and only 10% had a measurable baseline IL-8 level. In the patients, TNF was present in 93%, with a mean level of  $628 \pm 138$  pg/mL. When the patients' specimens were divided at the median to obtain roughly equal-sized groups, more TNF levels were elevated  $>2.5$  SD above the controls in the early vs late group (51% vs 30%;  $p = 0.07$ ). The mean levels of TNF and IL-8 also were higher in the early vs late group (756 vs 530 and 287 vs 135, respectively;  $p < 0.05$ ).

**Conclusions:** TNF levels are elevated in the immediate 4 hours post-injury. Previous investigators' inability to detect TNF activity increases may be related to delays in sampling. These results are consistent with the theory that increased TNF activity occurs early after major trauma and may initiate subsequent cytokine activity.

**Key words:** trauma; injury; tumor necrosis factor; cytokines; interleukins; IL-6; IL-8; TNF.

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■ The systemic inflammatory response has been implicated in posttraumatic adult respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS).<sup>1</sup> Injection of tumor necrosis factor (TNF) induces subsequent increases in other inflammatory cytokines and reproduces the pathophysiologic and clinical picture of sepsis and its attendant complications including MODS.<sup>2,3</sup> Previous investigators of the posttraumatic inflammatory response have not detected increases in TNF, despite the presence of "downstream" cytokines such as interleukin (IL)-6, IL-8, and others.<sup>4,5</sup> However, many of these studies collected specimens many hours after injury. Since the half-life of TNF is approximately 90 minutes, we hypothesize that the absence of TNF in prior studies may have resulted from delays in specimen collection. This study was designed to eliminate delays in specimen collection. The study's *null* hypotheses are as follows: 1) TNF levels are not increased in response to trauma, and 2) the time elapsed from injury to sampling does not affect the probability of detecting TNF.

## ■ METHODS

**Study Design:** A prospective cross-sectional study was performed to associate TNF and IL levels with major trauma and interval from injury to specimen collection. This study was approved by the institutional review board of the University of Michigan Medical Center, and the medical control board of Washtenaw and Livingston Counties.

**Setting and Population:** The study was performed at a university hospital ED (Level-1 trauma center) from May 1993 to May 1994. The facility manages about 35,000 ED visits and 1,100 major trauma cases annually. Specimens for analysis were obtained from uninjured healthy volunteers ("controls") who also were employees at the institution and from major *blunt* trauma patients ("patients").

Inclusion criteria for the patients were: adults (age  $\geq 18$  years) with class I or II trauma as defined by the

institutional criteria listed below and specimen drawn  $<4$  hours from the injury.

*Class I* trauma is defined as: Any patient with multi-system injury and *unstable* vital signs, defined as:

- Altered or fluctuating level of consciousness
- Respiratory distress necessitating invasive airway management
- Documented hypotension
- Ongoing fluid requirement to maintain blood pressure

*Class II* trauma is defined as: Any patient with a mechanism suggestive of, or documented, multisystem injury, and *stable* vital signs, defined as the absence of the above features, or a stable patient with  $\geq 1$  open fracture.

Patients were excluded from the study if they were  $<18$  years of age, in cardiopulmonary arrest at the scene, or  $>4$  hours from injury, or if the blood sample was improperly collected. Patients with penetrating injury, burns, inhalational injury, or electric shock were excluded. All patients were followed until hospital discharge or death.

**Experimental Protocol:** A 5-mL specimen of blood was drawn from the controls and the patients. The patients had specimens obtained at the scene of the incident, in transport to the trauma center, or upon arrival at the trauma center. Investigators were notified through the medical center's Trauma Notification System of all in-bound severely injured patients. After confirming that the patient met study criteria, the investigator on call came to the ED to collect specimens obtained in the out-of-hospital setting or in the ED. Specimens were placed into a sterile syringe containing 25  $\mu$ L of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ). Pre-hospital specimens, upon arrival to the ED, were transferred into a 5-mL sterile Eppendorf tube (Baxter Scientific, Romulus, MI).

All plasma samples were assayed for TNF, IL-6, and IL-8 levels. The specimens were logged in, given a study number, and centrifuged immediately at 4,200 rpm for 5 minutes at 4°C. The plasma was transferred to a sterile 5-mL polypropylene test tube and stored at  $-70^{\circ}\text{C}$  until assayed for cytokine activity (see below for details).

Data for each patient were collected after hospital discharge. Clinical data collected included the time interval from injury to specimen collection ( $\Delta T$ ), the injury severity scale (ISS) score, and patient demographics. If patients were unable to report the time of injury, the injury was presumed to have occurred 5 minutes prior to the 9-1-1 call for motor vehicle crashes in populated areas and witnessed industrial injuries. The time of specimen collection was obtained either by the on-call investigator's direct observation or by the time documented on the emergency medical services run sheet or nursing trauma flow

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sheet. The ISS score was calculated using the 1985 Abbreviated Injury Scale (AIS 85).<sup>6</sup>

**TNF Assay.** The WEHI (from the Walter and Elizabeth Hall Institute of Melbourne, Australia) 164 subclone 13 cell line was used to assess TNF concentration. Plasma samples were serially diluted in 96-well plates using a multichannel pipette. Dilutions were made in RPMI (Ruswell Park Memorial Institute) 1640 wells (Gibco Laboratories, Grand Island, NY) containing 1% fetal bovine serum (FBS) and 1 mmol L-glutamine in a final volume of 100  $\mu$ L. A standard curve of serially-diluted human recombinant TNF also was included in each assay. WEHI cells were suspended at a concentration of  $5 \times 10^5$  cells/mL in RPMI 1640 containing 10% FBS, 30  $\mu$ g/mL gentamycin, 1 mmol L-glutamine, and 0.5  $\mu$ g/mL Actinomycin D (Calbiochem, LaJolla, CA); 100  $\mu$ L was added to each sample-containing well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 18–20 hours. MTT-Tetrazolium (20  $\mu$ L of a 5 mg/mL solution, Sigma Laboratory, St. Louis, MO) was added to each well, and the plates were allowed to incubate for an additional 4 hours. Finally, 150  $\mu$ L of fluid was aspirated from each well using a Bio-Tek plate washer (Bio-Tek Instruments, Inc., Winooski, VT), and 100  $\mu$ L of 0.04 N HCl/isopropanol was added to dissolve the dark blue Tetrazolium crystals. The plates were protected from light and allowed to stand overnight at room temperature. Absorbance was read at 550 nm on a Bio-Tek ELISA reader (Bio-Tek Instruments, Inc.), and TNF concentrations were calculated based on the recombinant TNF standard.<sup>7</sup>

**IL-6 Assay.** The B-9 cell line was used to assess IL-6 concentrations. Similar to the TNF bioassay, samples were serially diluted in 96-well plates using Iscove's Modified Dulbecco's Medium (IMDM, Gibco Laboratories) containing 5% FBS, 1 mmol L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. A standard of serially-diluted human recombinant IL-6 was run in each assay. The cultured B-9 cells were washed twice and resuspended at a concentration of  $5 \times 10^4$  cells/mL in the above media supplemented with 100  $\mu$ mol 2-mercaptoethanol. Then 100  $\mu$ L of the cell suspension was added to each sample well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for approximately 72 hours. The plates were then processed as described above for the TNF assay, except that they were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium for 6 hours. IL-6 concentrations were calculated based on the recombinant IL-6 standard.<sup>7</sup>

**IL-8 Assay.** The ELISA for IL-8 was developed as reported by DeForge and Remick.<sup>8</sup> Briefly, a rabbit polyclonal antibody to recombinant mononuclear cell-derived (72 amino acid form) human IL-8 was raised, and the IgG was isolated from the antisera using a protein A-agarose column (Pierce, Rockford, IL). ELISA plates (Nunc-Im-

muno Plate Maxisorb, Neptune, NJ) were coated with 50  $\mu$ L/well of anti-IL-8 diluted to 1  $\mu$ g/mL and incubated overnight at 4°C. The plates were washed 3 times using phosphate-buffered saline (PBS) containing 0.05% v/v polysorbinate 20 (Tween-20), blocking solution [PBS containing 2% bovine serum albumin (BSA)] was added, and the plates were incubated for 1 to 2 hours at 37°C. An IL-8 standard curve was prepared using rIL-8, and the samples and standards were added to the plates and incubated for 1 hour at 37°C. The plates were then washed, biotinylated rabbit anti-human IL-8 antibody was added, and the plates were incubated at 37°C for 30 min. Avidin-horseradish peroxidase (Dako, Carpinteria, CA) was diluted to 1:5,000 and added. The plates were again incubated for 30 min at 37°C. The plates were washed, and substrate solution was added [0.67 mg/mL orthophenylenediamine dichloride (Dako, Santa Barbara, CA), 0.0125% H<sub>2</sub>O<sub>2</sub> in 0.25 mmol citrate phosphate buffer, pH 5.0]. Color development proceeded for 4 to 5 min at room temperature before being stopped by the addition of 50  $\mu$ L 3 mol H<sub>2</sub>SO<sub>4</sub>. The absorbance was then measured at 490 nm on an ELISA reader (Biotek, Inc.), and the concentration of IL-8 was calculated based on the standard curve of rIL-8. The lower limit of sensitivity for the assay was 45 pg/mL.<sup>7</sup>

**Data Analysis:** Characteristics of the patients vs the controls and between the patients subgroups were compared using the unpaired t-test for interval variables (data expressed as mean  $\pm$  SD) and  $\chi^2$  for categorical variables. The patients' cytokine data were grouped by hourly  $\Delta$ T intervals for comparison of mean levels of TNF, IL-6, and IL-8 using multivariate analysis of variance (MANOVA).

The patients' specimens also were empirically subdivided at the observed median  $\Delta$ T into "early" and "late" specimen collections. The mean cytokine levels for the early and late specimens were compared with the mean levels for the controls. As there are no established normal ranges for these cytokines, values  $>2.5$  SD above the mean level for the controls were considered elevated. The level of significance was  $p < 0.05$  throughout; all statistical tests were 2-sided.

## RESULTS

A total of 86 patient specimens were studied over a 1-year period. After sample collection, 16 specimens were subsequently excluded due to contamination ( $n = 7$ ), collection  $>4$  hours past injury ( $n = 6$ ), or patient age  $<18$  years ( $n = 3$ ). Thus, 70 patients' specimens were analyzed. The mean age and ISS score for the patients were  $37 \pm 2$  years and  $19 \pm 2$ , respectively. The mean  $\Delta$ T was  $92.8 \pm 49.2$  min (range 10–210 min, median 82 min). As has been found previously, there was no correlation between severity of injury, trauma class (class I or II), age, or gen-

■ **TABLE 1** Percentage of Subjects with Measurable Cytokine Levels and Mean ( $\pm$ SD) Levels for Controls vs Patients

| Cytokine*        | Controls      | Patients      | p-value |
|------------------|---------------|---------------|---------|
| TNF% measurable  | 96%           | 93%           | NS      |
| IL-6% measurable | 0%            | 63%           | <0.001  |
| IL-8% measurable | 10%           | 46%           | <0.05   |
| TNF (pg/mL)      | 125 $\pm$ 149 | 628 $\pm$ 877 | <0.05   |
| IL-6 (pg/mL)     | 0 $\pm$ 0     | 215 $\pm$ 679 | <0.001  |
| IL-8 (pg/mL)     | 2 $\pm$ 7     | 201 $\pm$ 657 | <0.01   |

\*TNF = tumor necrosis factor; IL = interleukin.

der of patients and presence of measurable cytokine levels. Since the controls were healthy volunteers employed at the medical center, no demographic data were kept for comparison with the patients.

There was detectable TNF activity in 96% of the controls and in 93% of the patients. In both groups, TNF was detected more frequently than IL-6 or IL-8. The mean levels of TNF, IL-6, and IL-8 were all increased in the patients compared with the controls' mean levels ( $p < 0.05$ ). The frequencies of detection of TNF were not significantly different, but the mean level of TNF was significantly higher in the patients vs the control group (Table 1).

When the data were grouped by hour following injury ( $\Delta T$ ), mean TNF levels declined over time (Fig. 1;  $p = NS$ ). Although these differences did not obtain statistical significance, division of the data into 2 nearly equal

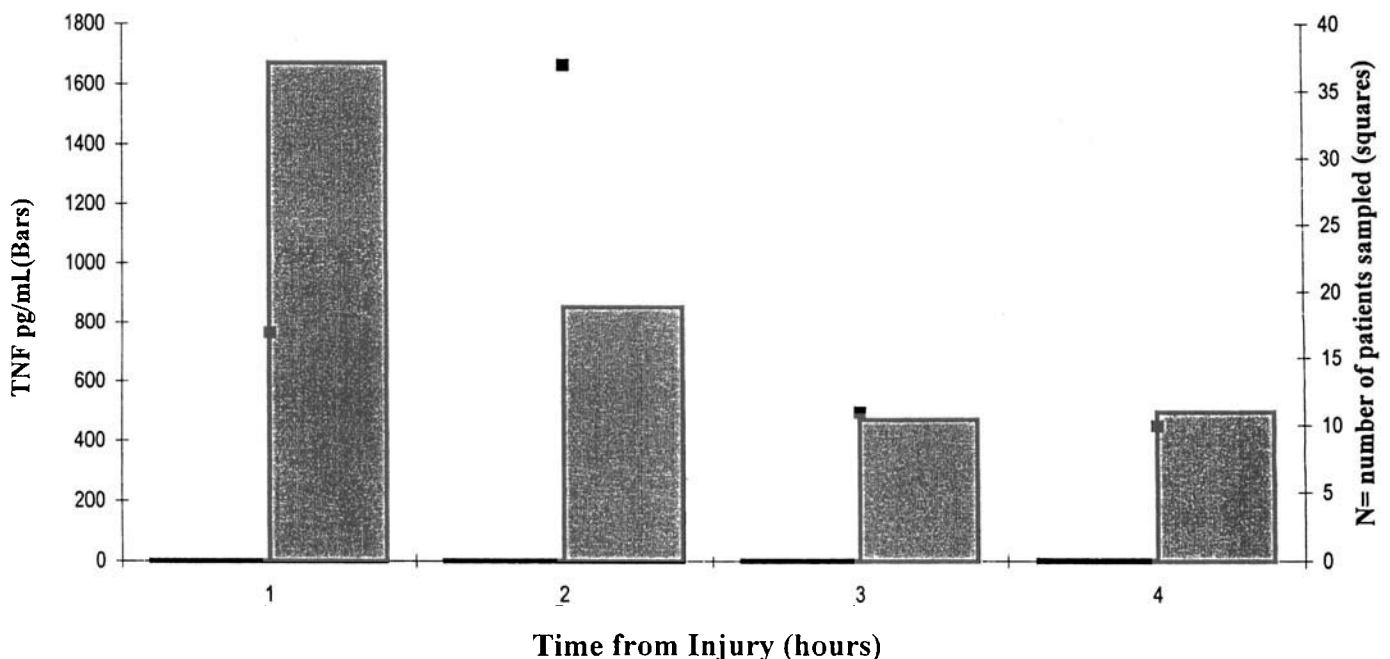
groups using the median  $\Delta T$  demonstrated that the late patients had significantly lower TNF values (Table 2).

## ■ DISCUSSION

It is clear from our data that TNF, IL-6 and IL-8 activity is detectable in the immediate posttraumatic period. Mean levels of all 3 cytokines were higher in the patients than in the controls. Absolute TNF levels (756 vs 530 pg/mL;  $p < 0.05$ ) and the proportion of abnormally elevated levels of TNF (51% vs 30%;  $p < 0.07$ ) were higher in the early vs late patient groups. This finding is consistent with TNF levels declining with longer intervals from injury until measurement. Hence, our data are consistent with the theory that TNF is an active early cytokine in trauma.

Our results are consistent with the described inflammatory cascade in the sepsis model.<sup>2,3,9</sup> Injection of TNF initiates the same pathophysiologic response as does injection of gram-negative endotoxin lipopolysaccharides (LPSs).<sup>2,3</sup> Further, pretreatment with anti-TNF antibodies is protective from the inflammatory response to injection of both TNF and LPS.<sup>10,11</sup> That injury and sepsis would use the same mechanism to create the same end response is not unexpected; teleologically it makes more sense than the presence of 2 different inflammatory mechanisms. Our results also are consistent with previous reports of admitted patients with major trauma, which report no direct correlation between the magnitude of the injury (ISS), age, or gender and injury-associated cytokine levels.<sup>12-14</sup>

Trauma patients who survive their initial injury and



■ **FIGURE 1.** Bar graph showing the decline in tumor necrosis factor (TNF) levels vs time from over 1,600 pg/mL in the first hour grouping to just over 500 pg/mL in the fourth hour grouping (left vertical axis). The filled squares indicate the number of specimens included in each hourly grouping (right vertical axis).

**TABLE 2** Proportion of Patients with Abnormally Elevated Cytokine Levels (i.e., >2.5 Times the Mean Value for Controls) and Absolute Mean ( $\pm$ SD) Values for Early vs Late Subgroups of Patients, Using the Median Interval from Injury until Presentation as the Cutoff Point

| Cytokine*      | Early (<82 min) | Late (>82 min) | p-value |
|----------------|-----------------|----------------|---------|
| TNF% elevated  | 19/37 (51%)     | 10/33 (30%)    | NS      |
| IL-6% elevated | 23/37 (62%)     | 21/33 (64%)    | NS      |
| IL-8% elevated | 15/37 (47%)     | 14/33 (42%)    | NS      |
| TNF (pg/mL)    | 756 $\pm$ 1,208 | 530 $\pm$ 860  | <0.05   |
| IL-6 (pg/mL)   | 201 $\pm$ 977   | 205 $\pm$ 631  | NS      |
| IL-8 (pg/mL)   | 287 $\pm$ 906   | 135 $\pm$ 644  | <0.05   |

\*TNF = tumor necrosis factor; IL = interleukin.

resuscitation phase face delayed complications associated with the inflammatory response, thus resulting in significant morbidity and mortality. ARDS alone carries at best a 40% mortality rate.<sup>15,16</sup> While emergency physicians (EPs) are generally not managing these complications, they may perform an important protective role for their patients by modulating the inflammatory response in patients with diagnoses known to carry a risk for delayed complications. The setting of major trauma is one in which the EP is generally aware of the interval from insult until presentation. Given a brief interval from injury until medical evaluation, EPs may be able to intervene in the ED or potentially in the out-of-hospital setting, using agents to block the inflammatory response to trauma.

## LIMITATIONS AND FUTURE QUESTIONS

This report represents one of the largest series of early cytokine levels reported to date for major trauma patients. However, by attempting to collect as many specimens as close as possible to the point of injury, our  $\Delta T$  is skewed toward the first hour after injury. This selection bias, while helpful for demonstrating that TNF is elevated shortly after injury, may have limited our ability to demonstrate the reduction in TNF following acute injury. Subsequent studies with serial measurements of cytokines following injury would be valuable for delineating the effects of time and other confounders on these mediators of the inflammatory response. Other potential confounders such as patient body mass, degree of hypotension, and preexisting illness also should be evaluated for their effect on cytokine activity as a function of time.

Another problem we experienced was the exclusion of 15 specimens for various reasons. Seven consecutive specimens were lost to contamination, which was not recognized until the time of specimen assay. This phenomenon is due to the nature of the bioassay. Until the clone is incubated with the plasma, contamination is not evident. Bacterial contamination ruins the utility of that specimen.

The other specimens were assayed but their results were dropped from data analysis because after review of patient charts, the subjects did not meet study entry criteria. Overall, about 18% of the specimens had to be excluded.

The precise sequence and duration of cytokine activity, direct precipitating causes and associated effects, have yet to be described in humans. While we have demonstrated TNF activity in the response to trauma, serial cytokine measurements from individual patients will be needed to define normal and pathologic cytokine responses to trauma. Given that elevated TNF activity appears early and presumably for a short duration, monitoring of cytokine levels and the physiologic response to these agents must begin during the initial patient resuscitation.

## CONCLUSION

While there is baseline TNF activity in healthy individuals, TNF activity increases significantly in response to trauma. Following major trauma, there also is a significant increase in "downstream cytokines," IL-6 and IL-8. These cytokines may increase after TNF levels peak. Previous investigators' inability to detect this early cytokine activity is probably due to delayed specimen collection. Further investigation into the early phase of cytokine response to trauma should begin in the earliest phase of care.

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### Further Thoughts from the Reviewers

■ The authors provide important preliminary data related to cytokine levels in injured patients; however, their data analysis likely understates the time dependency of these markers. Given the reported large SDs about the mean hourly cytokine levels, the authors' assumption of data distribution normality must be called into question. Logarithmic transformation of their data may have yielded a more normal data distribution and demonstrated a time dependency when the transformed data were analyzed using a repeated-measures ANOVA. Alternatively, a non-parametric analysis (e.g., Friedman 2-way ANOVA by ranks) may have provided a more accurate analysis. Future studies in this area should pay close attention to the distribution of the raw data and base the analytical approach accordingly.