# Release of Proinflammatory Cytokines by Mitogen-Stimulated Peripheral Blood Mononuclear Cells From Critically Ill Multiple-Trauma Victims

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**This study investigated the alterations in circulating proinflammatory cytokines and cytokine production by peripheral blood mononuclear cells (PBMCs) in response to lipopolysaccharide (LPS) or phytohemagglutinin (PHA) after severe trauma. Plasma and PBMCs were collected from 17 severely injured trauma patients and 10 healthy subjects. Plasma was stored at −80°C and analyzed for cytokines. Isolated PBMCs from each subject were stimulated with LPS or PHA and incubated at 5% $CO_2$ for 24 hours. Supernatants were collected and analyzed for cytokines. There was no significant change in the plasma concentration of free TNF-α and IL-1β between healthy subjects and trauma patients. Plasma IL-6, total TNF-α, and total IL-1β were significantly increased in severely traumatized patients compared with healthy control subjects. PBMCs from trauma patients produced higher levels of TNF-α in response to LPS but it showed no significant change in IL-1β and IL-6 production in response to PHA or LPS in comparison to PBMCs from control subjects. We conclude that severe trauma results in a significant increase in plasma proinflammatory cytokine IL-6. Free TNF-α and IL-1β in plasma remain at levels comparable to those in uninjured controls, while plasma free IL-6 levels in trauma patients remain high. Serious injury is associated with an enhanced production of TNF-α by PBMCs stimulated with LPS.**


TRAUMA is the major cause of death for people between the ages of 18 and 45 years in the United States.[1] Most trauma deaths result from direct tissue injury and blood loss; however, sepsis and multiple organ system failure are the major complications contributing to late mortality.[2] Trauma is associated with a depression in immune function including anergy, a decrease in the number of lymphocytes in the circulation, depression of T-cell activation and proliferation, and production and expression of soluble immunomodulatory factors such as cytokines and their receptors.[3-6] The synthesis of cytokines is greatly augmented in response to injury, infections, or inflammatory or other immunological challenges. In addition, the production of acute-phase proteins is under the control of macrophage-derived cytokines.

Proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) have emerged as functional mediators of the response to severe trauma.[7] These cytokines stimulate the production of many mediators of the inflammatory process.[7-9] The moderate synthesis and release of proinflammatory cytokines is necessary for the elimination of pathogens and wound healing.[10] The physiologic response to injury involves an early hyperinflammatory response, which is accompanied by a degree of compensatory antiinflammatory effect.[7,10,11] A prolonged and excessive inflammatory response may progress to further tissue injury, immunosuppression, and finally multiple organ failure and mortality.[12] Antiinflammatory mechanisms are activated after trauma, dependent on the severity of injury. Immediate postinjury care is critical to the clinical management of trauma patients.

Central nervous system injury can also stimulate the production of proinflammatory cytokines in the brain. TNF-α and IL-6 were reportedly undetectable in the normal brain, while there were significant increases in the brain levels of these cytokines[7,13] following head injury. TNF-α was detectable in the brain as early as 1 hour after a closed head injury, peaked at 4 hours postinjury and declined thereafter. IL-6 production lagged behind TNF-α by 2 to 4 hours, peaking at 8 hours after closed head injury.[7] The rapid production of TNF-α and IL-6 following closed head injury is believed to be the initial local inflammatory response in the brain to a primary insult.

TNF-α is a potent cytokine implicated in inflammation and immunity that is produced by activated macrophages/monocytes. TNF-α, a multifunctional cytokine, is an important contributing factor in the inflammatory response mediation of both injury and repair.[7,10] TNF-α induces the in vivo production of IL-1β that initiates a cascade of proinflammatory cytokines leading to neutrophil activation, and further tissue injury.[10] IL-6 is a pleiotropic cytokine that modulates cellular actions such as the proliferation, differentiation, and maturation of progenitor cells and the control of cellular metabolism.[9] Recently, a number of clinical studies have reported increased plasma levels of IL-6 in response to trauma, burns, and surgery.[8,9,14] Measurement of the plasma level of these proinflammatory cytokines could help to identify specific cytokine patterns that have a relationship to posttrauma outcome.

Compared with healthy subjects, lipopolysaccharide (LPS) activated peripheral blood mononuclear cells (PBMCs) from trauma patients within the first day of injury,[15,16] resulting in increased production of TNF-α, IL-1β, and IL-6. Keel et al[17] reported the secretion of IL-1β, TNF-α, and IL-6 into LPS-stimulated whole blood and PBMCs from a group of patients having sepsis with trauma injury. Mitogen-stimulated production of cytokines in the early "flow phase" of injury has not been investigated before. While multiple trauma patients are at high risk to develop a systemic inflammatory response syndrome, sepsis, and multiple organ failure, little is known about the role of cytokines in the development of these syndromes during the initial flow phase after trauma. The present study elucidates the capacity of PBMCs to release proinflammatory

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reacting cytokines in patients with severe trauma during the catabolic flow phase of injury. LPS or phytohemagglutinin (PHA) stimulation of PBMCs as an ex vivo model of inflammation were used to induce activation or altered expression of cell-surface receptors. These polyclonal mitogens induce growth mechanisms (mitosis) of immune cells similar to antigen. LPS mainly stimulates B cells and monocytes-macrophages, while PHA stimulates T helper cells.[18] This study also determines the modulation in circulating plasma levels of proinflammatory cytokines in the early catabolic flow phase of severe trauma.

## SUBJECTS AND METHODS

### *Patient Population*

Seventeen adults (19 to 85 years of age) who sustained multiple injuries were studied in the catabolic flow phase of injury before starting nutritional treatment. The patients were severely injured, with an Injury Severity Score (ISS) of 14 to 41 (mean ± SEM, 28 ± 2). All patients were admitted and treated at the Trauma Intensive Care Unit of the Level I Trauma Center at St. Joseph's Hospital and Medical Center in Phoenix, AZ. Written informed consent was obtained from each patient or legal guardian. The protocol was reviewed and approved by the Institutional Review Board of the Medical Center.

At the time of the study, none of the patients were septic or had multiple organ failure, diabetes, recent weight loss, liver or renal disease, or malignant disease as judged by biochemical determinations and personal history data. No evidence of malnutrition was found. The patients were evaluated and resuscitated on admission according to their individual needs. All patients required mechanical ventilatory support during the study (maximum fraction of inspired oxygen, 40%). The clinical characteristics of the patients are summarized in Table 1. Fourteen patients were involved in motor vehicle crashes and were admitted with multiple bone fractures associated with extensive soft tissue damage. Three patients were victims of multiple gunshot wounds of the face, chest, or abdomen.

### *Experimental Design*

Clinical stabilization was accomplished in the acute stage of injury during which the patients received replacement and maintenance fluids with electrolytes but no calories or nitrogen. At 48 to 60 hours after injury, when the medical status of the patients became stable and resuscitation was complete, the patients were weighed (Flexicair MC3; Support Systems, Charleston, SC). Peripheral blood samples (15 mL) were drawn via an indwelling arterial catheter, and 5 mL was transferred to ice-cooled, heparinized vacutainers (Becton Vacutainer Systems, Rutherford, NJ) and centrifuged at 3,000 rpm for 20 minutes at 5°C to separate the plasma. The plasma samples were stored at −80°C until analysis. The remaining 10 mL blood was used for isolation of PBMCs.

After an overnight fast, morning postabsorptive blood samples were obtained from adult subjects (five men and five women aged 45 ± 3

**Table 1. Clinical Characteristics and Diagnoses of the Trauma Patients**

| Patient No. | Sex | Age (yr) | Weight (kg) | BMI (kg/m²) | ISS | GCS | APACHE II | Injury Pattern |
|---|---|---|---|---|---|---|---|---|
| 1 | M | 27 | 95 | 30.0 | 14 | 15 | 23 | GSW, multiple, bilateral thigh |
| 2 | M | 23 | 77 | 23.0 | 26 | 8 | 31 | MVC ejection, CHI, pulmonary contusion, hemothorax, rib Fx |
| 3 | M | 79 | 79 | 25.6 | 16 | 6 | 29 | MVC, CHI, cervical spine injury ($C^1$-$C^2$) |
| 4 | M | 18 | 60 | 18.9 | 26 | 14 | — | GSW, S/P craniotomy |
| 5 | M | 51 | 98 | 30.5 | 34 | 13 | 35 | Motorcycle crash, CHI, flail chest |
| 6 | M | 37 | 77 | 25.8 | 34 | 12 | 24 | Auto pedestrian accident, flail chest, femur Fx, liver laceration |
| 7 | F | 38 | 52 | 28.6 | 27 | 15 | — | MVC, CHI, MFX, ruptured spleen, pulmonary contusion |
| 8 | M | 31 | 71 | 25.3 | 29 | 3 | 29 | MVC, CHI, complete spinal cord injury, blunt abdominal injury |
| 9 | M | 28 | 76 | 24.9 | 26 | 4 | 24 | MVC, craniotomy, zygomia Fx, complex eyelid laceration |
| 10 | M | 38 | 74 | 23.3 | 31 | 8 | 16 | MVC, MFX, pneumothorax, flail chest, pulmonary insufficiency |
| 11 | M | 31 | 91 | 26.5 | 25 | 7 | 20 | MVC, CHI, basilar skull Fx, subdural hematoma, craniotomy |
| 12 | M | 22 | 60 | 25.4 | 38 | 4 | 24 | MVC, subdural hematoma; pulmonary contusion, pneumothorax, facial Fx |
| 13 | M | 85 | 72 | 29.7 | 24 | 14 | 30 | MVC, CHI, multiple rib Fx, flail chest, clavicle Fx, orbital Fx |
| 14 | M | 19 | 79 | 23.5 | 41 | 8 | 13 | MVC, CHI, occipital skull Fx, epidermal hematoma, maxillar Fx |
| 15 | M | 49 | 84 | 25.0 | 30 | 3 | 23 | MVC, CHI, MFX, C-2 Fx, spinal cord injury |
| 16 | M | 31 | 96 | 26.4 | 25 | 15 | 39 | GSW, arm, abdomen, humerus open Fx, hemothorax |
| 17 | F | 57 | 56 | 25.8 | 35 | 10 | 25 | Auto/bicycle crash, CHI, MFX, scalp laceration |
| Mean ± SEM | 15M/2F | 39 ± 5 | 76.3 ± 3.4 | 25.8 ± 0.7 | 28 ± 2 | 10 ± 1 | 26 ± 2 | |

Abbreviations: BMI, body mass index; APACHE II, Acute Physiologic and Chronic Health Evaluation II score; CHI, closed head injury; MVC, motor vehicle crash; GSW, gunshot wound; MFX, multiple fractures; Fx, fracture.

years; weight, 66 ± 1 kg), recruited from the laboratory and office personnel, all of whom were in good health and consuming a normal diet without any medication. No dietary or activity restrictions were imposed on the control subjects during this period.

### *Isolation of PBMCs for Proliferation Study*

Heparinized peripheral blood was diluted 1:2 with sterile solution. PBMCs were isolated from the blood by the Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density centrifugation method. Following centrifugation at 900 × *g* for 20 minutes at room temperature, the interface was removed. The cells were washed twice with RPMI cell culture medium (Cellgro; Mediatech, Heindon, VA) and counted, and viability was determined by trypan blue exclusion (>90%). Cells were adjusted to a concentration of $1 \times 10^7$/mL in RPMI with 10% fetal bovine serum (Sigma, St Louis, MO). PBMCs were cultured in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA; $1 \times 10^6$ cells/well). PBMCs were incubated in the presence of either LPS (from *E. Coli* Serotype 0111, B4; Sigma; 10 μg/mL) or PHA (from *Phaseolus Valgaris;* Sigma; 4 μg/mL) for 24 hours at 37°C in 5% $CO_2$. The supernatants were collected after a 24-hour incubation and stored at −80°C. Plasma and supernatant concentrations of TNF-α, IL-1β, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) kits.

### *Free Cytokine (IL-6, TNF-α, and IL-1β) Assay*

Cytokine levels were measured in plasma samples and cell supernatants by a commercially available solid-phase ELISA kit using the sandwich technique (Endogen, Woburn, MA). First, 50 μL biotinylated antibody reagent was added to each well of a precoated stripwell plate with anti-IL-6, -TNF-α, or -IL-1β monoclonal antibody. Duplicate 50-μL standards, plasma samples, and a 1:20 dilution of cell supernatants were pipetted into the wells. The microtiter plate was incubated for 2 hours at room temperature. After three washes in buffer solution to remove unbound protein, 100 μL streptavidin horseradish peroxidase–conjugated polyclonal antibodies specific for IL-6, TNF-α, or IL-1β, respectively, were added to each well and incubated for 30 minutes under the previous conditions. The plate was again washed three times, and 100 μL premixed tetramethylbenzidine substrate solution was added to each well. Color development, which is directly proportional to the bound amount of the respective cytokine, proceeded for 30 minutes at room temperature in the dark. The color reaction was stopped by adding 100 μL stop solution to each well. Optical densities were measured within 15 minutes using a Vmax microplate reader (Molecular Devices, Menlo, CA) set at 450 nm with a 550-nm wavelength correction. The concentration of IL-6, TNF-α, or IL-1β in each sample was calculated on the basis of the standard curve-fitting SoftMax statistical software (Molecular Devices). Detection limits for all of these cytokines were less than 1 pg/mL with a coefficient of variation less than 10%. All samples and standards were assayed in duplicate.

### *Total Human Plasma TNF-α and IL-1β Assay*

This "competitive" enzyme immunoassay is designed to measure bound and unbound (total) levels of human TNF-α and IL-1β in plasma and other biological fluids. Total human TNF-α and IL-1β kits (Intergen, Purchase, NY) were used. Briefly, 100 μL of the standards (200 ng/mL) were dispensed in the appropriate wells. To each of the designated wells, 50 μL plasma and 50 μL assay diluent were added. TNF-α antibody dilution solution (25 μL) was dispensed into each well of the TNF-α plates, while IL-1β plates did not require antibody solution since they were precoated with polyclonal antibody. All plates were sealed and incubated at room temperature for 3 hours. After removal of the plate sealer, 25 μL TNF-α or IL-1β conjugate was added to each well of the appropriate plates, which were then resealed and incubated at room temperature for an additional 30 minutes. Fluid was removed from the wells by inverting the plate over a sink. The plates were washed four times with diluted wash buffer and blotted dry over clean paper towels. Into each well, 50 μL streptavidin-alkaline phosphatase dilution solution was placed. The plate was again resealed and incubated for 30 minutes at room temperature. After four washes with wash buffer, 50 μL of the substrate solution was dispensed into each well. Once again, the plates were resealed and incubated for 20 minutes at room temperature. Amplifier solution (50 μL) was then dispensed into each well. After 5 minutes of incubation, the optical density (OD) was determined using a plate reader at 490-nm wavelength. The concentration of the sample was calculated with reference to a standard curve. The detection limit of total TNF-α or total IL-1β is 195 pg/mL with an intraassay variation of ±8% and interassay variation of ±12%. This method has been validated by parallelism and quantitative recovery studies to ensure reliability.

### *Statistical Analysis*

Quantitative variables are expressed as the mean ± SEM. Statistical significance was ascertained by an unpaired Student's *t* test. The significance level was set at a *P* value less than .05.

## RESULTS

Clinical characteristics of the subjects are summarized in Table 1. These trauma patients were severely injured (ISS, 28 ± 2; GCS, 10 ± 1). The majority (14 of 17) had motor vehicle crashes either as a driver, passenger, or pedestrian. Three patients had penetrating wounds in multiple organs. Studies were initiated by drawing blood samples within 48 to 60 hours after the patients sustained multiple injuries, when they were receiving maintenance fluid and electrolytes but no calories or protein. This sampling period corresponds to the early catabolic stage of the flow phase of the metabolic response to injury.

Inflammatory cytokine levels in the circulating plasma and in the supernatant of mitogen-induced PBMCs are reported in Table 2. The plasma levels of free TNF-α and IL-1β are near the detection limit, and there was no significant difference between healthy subjects (n = 10) and trauma patients (n = 17). Plasma IL-6 levels were significantly increased (13-fold) in severely traumatized patients compared with healthy control subjects. Plasma total (free + bound) TNF-α and IL-1β levels showed a

**Table 2. Proinflammatory Cytokine Levels (pg/mL)**

| Cytokine | Normal Controls (n = 10) | Trauma Patients (n = 17) |
|---|---|---|
| TNF-α | | |
| Plasma (total) | 1,654 ± 106 | 2,443 ± 160* |
| Plasma (free) | 1.77 ± 0.91 | 1.34 ± 0.35 |
| PHA | 187 ± 50 | 329 ± 68 |
| LPS | 340 ± 69 | 1,006 ± 209* |
| IL-1β | | |
| Plasma (total) | 2,328 ± 397 | 15,178 ± 3,522* |
| Plasma (free) | 1.37 ± 0.27 | 1.13 ± 0.28 |
| PHA | 246 ± 40 | 294 ± 101 |
| LPS | 612 ± 145 | 858 ± 159 |
| IL-6 | | |
| Plasma | 6.3 ± 1.6 | 81 ± 17* |
| PHA | 4,141 ± 622 | 6,336 ± 877 |
| LPS | 7,999 ± 704 | 8,457 ± 652 |

NOTE. Results are the mean ± SEM.
*$P < .05$ *v* control.

significant ($P < .01$) increase (50% for TNF-α and 650% for IL-1β) due to trauma. TNF-α secretion by LPS-stimulated PBMC supernatant from patients with severe trauma was significantly increased in comparison to the uninjured control group. There was no difference in the secretion levels of IL-1β and IL-6 in LPS- or PHA-stimulated PBMC supernatants.

## DISCUSSION

The present study describes the modulation of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in severe trauma patients. Previously, investigators indicated that circulating levels of plasma TNF-α and IL-1β were undetectable both in control subjects and in patients with severe trauma.[8,19-23] No increase in the expression of any of the proinflammatory cytokines (TNF-α, IL-1β, or IL-6) was obtained after trauma.[23] It is known that local immunocyte activation may result in the production of cytokines despite their being undetectable in serum.[22] In our study, the concentration of these cytokines is barely within the detection limits of the sandwich ELISA kits (TNF-α limit, <1 pg/mL; IL-1β limit, <1 pg/mL). We observed no significant difference in the plasma levels of free, unbound TNF-α and IL-1β in trauma patients in comparison to control subjects, but plasma IL-6 and total (free and bound) TNF-α and IL-1β levels were consistently higher in trauma patients (Table 2). Our finding of an increase in IL-6 concentrations without any significant change in circulating concentrations of IL-1β or TNF-α after severe injury is consistent with findings in previous studies.[20] However, our trauma patients had significantly elevated total (bound and unbound) plasma TNF-α and IL-1β levels in comparison to control subjects (Fig 1). Free TNF-α represents the biologically active fraction, while circulating receptor-bound TNF-α is a potential reservoir for active TNF-α; measurements of both free and total levels may be relevant. It is also possible that we missed the peak levels of these free cytokines in the circulation, which would occur at 4 to 8 hours postinjury and decline to a steady state thereafter. This may also indicate that the levels of antiinflammatory mediators such as soluble TNF receptors (sTNFRs), IL receptors, and IL-1 receptor antagonist (IL-1ra) were increased in the circulation after severe trauma compared with the control group. These

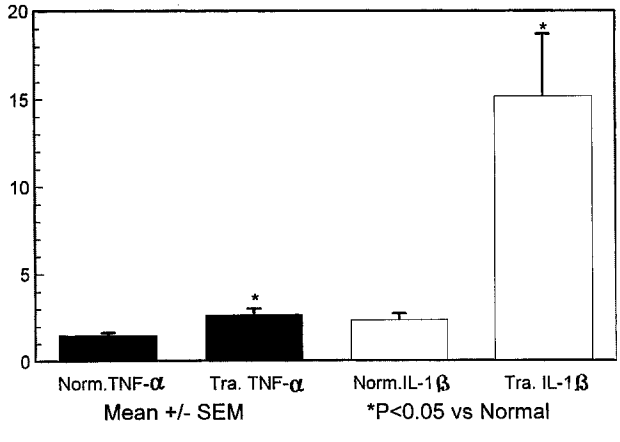


**Fig 1. Plasma levels of total TNF-α and IL-1β (ng/mL) in trauma patients (Tra.) compared with uninjured controls (Norm.).**

mediators which are bound to free circulating TNF-α and IL-1β may interfere with the accurate measurement of these cytokines. Keel et al[22] reported significant increases in the plasma concentration of sTNFR p55, sTNFR p75, and IL-1ra after severe trauma compared with a control group. Other antiinflammatory cytokines such as IL-10 and IL-4 are associated with multiple trauma.[11,24,25] IL-10 induces the production of the endogenous TNF inhibitor TNFRs from monocyte cultures while also downregulating surface TNFR expression.[25] IL-4 induces the production of IL-1ra, which inhibits native IL-1.[25]

In this study, PBMCs from the trauma patients stimulated with LPS ex vivo produced significantly higher levels of TNF-α than cells from healthy subjects. There was a nonsignificant difference in TNF-α levels between PBMCs isolated from severely traumatized patients and control subjects stimulated with PHA. There were no significant differences in IL-6 and IL-1β levels in PBMCs isolated from trauma patients and stimulated with LPS or PHA in comparison to PBMCs from control subjects.

Keel et al[17] reported that the secretion of TNF-α, IL-1β, and IL-6 into LPS-stimulated whole blood and PBMCs from patients with severe trauma was significantly decreased in comparison to the control group. In their study, TNF-α and IL-6 levels were measured by bioassay, and half of their patients developed sepsis. None of our patients were septic, and cytokine levels were measured by a more sensitive method using an ELISA. Two other studies, Kim et al[15] and Kelly et al,[16] demonstrated that in vitro LPS-activated monocytes-macrophages or PBMCs from trauma patients early after severe injury (within the first day) displayed a significant increase in supernatant TNF-α, IL-1β, and IL-6, as compared with supernatant from LPS-stimulated monocytes-macrophages or PBMCs from healthy subjects. However, in the present study, samples were collected 48 to 60 hours posttrauma, which may partly account for our different observations. Keel et al[17] hypothesized that potential imbalances between inflammatory T helper ($Th_1$) and $CD4^+$ T helper ($Th_2$) lymphocytes are responsible for the responsiveness of PBMCs from traumatized patients to mitogen. Tolerant (unresponsive) states resulting from depletion are induced in the precursor $CD4^+$ ($Th_0$) cell before it diverges into the $Th_1$ or $Th_2$ subsets. IL-4 and IL-10 produced by $Th_2$ and PBMCs of severely traumatized patients suppress the development of naive $CD4^+$ cells ($Th_0$) into $Th_1$ cells and the differentiation of $Th_0$ into $Th_2$.[18,26] $Th_2$ cells are associated with the production of IL-4, IL-10, IL-6, and TNF-α.[26] The reason we did not observe a reduction in the responsiveness of PBMCs to LPS and PHA and a suppression of their cytokine production may be the lack of sepsis and the lower ($28 \pm 2$) ISS than the study by Keel et al ($39 \pm 3$) and the time of blood sampling.

Immediately after trauma, the monocyte-macrophage lineage becomes hyperactive with the release of excessive proinflammatory cytokine. This development is followed by substantial impairment of the cell immune function. This immune suppression is usually short-lived (less than 3 to 5 days) because human monocytes circulating in the bloodstream have a half-life of approximately 3 days.[27] Monocytes-macrophages play crucial roles in antigen presentation to lymphocytes, so any potential

shift in monocyte phenotyping could result in the functional alteration of immune cells. The delay of 48 to 60 hours in the posttrauma sample collection may result in a shift toward a state of homeostasis that existed prior to the injury.

In conclusion, this study shows that severe trauma causes elevated levels of plasma proinflammatory cytokines, as shown by increases in total TNF-$\alpha$, total IL-1$\beta$, and free IL-6. PBMC responsiveness to mitogen 48 to 60 hours posttrauma had a tendency to return to control values. Anti-TNF antibody strategies may have a potential role in the short-term treatment of acute trauma. Elucidation of the role of cytokines and their interactions will result in an increased understanding of the metabolic responses following injury. Modulating this response may hold promise for decreasing the morbidity and mortality in trauma patients. Future clinical investigations into the role of inflammatory cytokines in trauma patients will need to focus on the exact kinetics of their production and its relationship to antiinflammatory cytokines and receptors.

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