The Potential Pattern of Circulating Lymphocytes
T_H1/T_H2 Is Not Altered After Multiple Injuries

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Hypothesis: A shift in the balance of helper T cells type 1 (T_H1) toward type 2 (T_H2) has been suggested as a possible mechanism for impaired immune responses after severe trauma. We suggest that major injuries (polytrauma) induce an alteration in the pattern of T_H1/T_H2 cells.

Design, Setting, and Patients: A prospective study of 35 polytraumatized patients (Injury Severity Score >16) admitted to a trauma intensive care unit at a level I trauma center (university hospital).

Interventions: Blood samples were collected from patients at various times during their stay in the intensive care unit and from age- and sex-matched healthy individuals.

Main Outcome Measures: Serial determinations (n=81) of intracellular interleukin (IL)-2 (T_H1 cells) and IL-4 (T_H2 cells) in stimulated CD3+ T cells from patients with polytrauma twice a week during their stay in the intensive care unit accompanied by determination of the cell activation marker CD69 using 3-color flow cytometry. In parallel, the release of IL-2 and IL-4 from stimulated peripheral blood mononuclear cells and systemic plasma IL-4 levels were analyzed by conventional enzyme-linked immunosorbant assay. Healthy donors (n=53) served as the control group.

Data were related to outcome, Injury Severity Scores, and time after trauma.

Results: Expression of the cell activation marker CD69 was similar in stimulated lymphocytes from patients and healthy donors. There were no significant posttraumatic alterations in numbers of CD3+ cells stained for intracellular IL-2 or IL-4, except for a minor decrease in IL-2+ cells during the first week after trauma. Subgroups with high (>24) and lower (<25) Injury Severity Scores or survivors and nonsurvivors revealed no differences in intracellular cytokine staining. In contrast, patients revealed a highly significant decrease in the number of CD3+ T cells. Mean systemic IL-4 levels did not differ in patients compared with healthy donors. Release of IL-2 and IL-4 from peripheral blood mononuclear cell fractions stimulated with phorbolmyristateacetate and ionomycin was significantly increased in patients with trauma but not from those stimulated with toxic shock syndrome toxin-1.

Conclusions: Patients with multiple injuries have no significant alteration in the ratio of circulating T_H1/T_H2 cells. Thus, our results suggest pathomechanisms in posttraumatic T-cell suppression apart from alterations in the T_H1/T_H2 pattern.

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After severe injuries, dysbalanced cytokine synthesis contributes to dysregulated immune functions and to enhanced susceptibility toward microbial infections. During the past decade, the pattern of helper T cells type 1 (T_H1) and type 2 (T_H2) has been recognized as a fundamental principle of T-cell responses. Helper T cells type 1 are potent producers of interleukin 2 (IL-2) and interferon gamma (IFN-γ); in contrast, T_H2 cells produce IL-4, IL-5, or IL-13. Intracellular staining of cytokine generation in activated T cells has emerged as a powerful tool to discriminate T_H1 and T_H2 cells using flow cytometry. Recently, several studies have described distinct alterations in the T_H1-T_H2 ratio of peripheral lymphocytes obtained from severely burned and surgical patients. Whereas after severe burn trauma a clear increase in T_H2 cells but no decrease in T_H1 cells has been reported, different observations were made in surgical patients. No increase in T_H2 cells but a significant decrease in T_H1 cells has been observed after conventional hernia repair. An increased or unaltered release of IL-4 has been reported from isolated lymphocytes after conventional cholecystectomy, accompanied by a decrease in the...
PATIENTS AND METHODS

This study includes 35 patients with polytrauma (Table 1) admitted to the trauma intensive care unit of the BG Klinikum Bergmannsheil, Bochum, Germany, between May 1999 and December 1999 and 33 healthy donors. Procedures in accordance with the Helsinki Declaration of 1975 as revised in 1983 were followed and approved by the local ethics committee. Entry criteria were multiple injuries, Injury Severity Score (ISS) greater than 16, age older than 16 years, primary treatment in our hospital, and surveillance in the intensive care unit for at least 4 days. Excluded were patients with a known history of immunologic disorders such as human immunodeficiency virus infection.

Peripheral venous blood samples were collected daily at 8 AM for analysis of systemic IL-4 (2.7 mL of EDTA) (Monovette; Sarstedt, Nürnberg, Germany) and twice a week for analysis of intracellular formation or cytokine release (8 mL of sodium heparin) (Vacutainer CPT; Becton Dickinson, Heidelberg, Germany). Blood samples from patients and the control group were processed identically within 30 minutes of withdrawal. EDTA monovettes were centrifugated for 5 minutes at 2000 g at room temperature (RT). Plasma was aspirated and stored at −80°C until analysis. Peripheral blood mononuclear cells were isolated from the Vacutainer CPT by density centrifugation at 1600g at RT for 30 minutes. The PBMC fraction was washed twice in RPMI 1640 medium (Sigma, Deisenhofen, Germany) supplemented with 1-glutamine, 2 mmol/L; HEPES, 25 mmol/L (Sigma); and 10% heat-inactivated fetal calf serum (Life Technologies, Egggenstein, Germany). Viability of the isolated cells always exceeded 95% as determined using the trypan blue exclusion test. In addition, smears from each PBMC fraction were stained using the modified Pappenheim procedure to differentiate cellular compositions. Washed PBMCs were divided in aliquots of $1 \times 10^6$ cells/mL supplemented RPMI 1640 medium; transferred to 24-well tissue culture plates (Falcon; Becton Dickinson); and differentially stimulated with ionomycin, 1 µmol/L (Sigma), in combination with phorbolmyristateacetate (PMA), 1 ng/mL (Sigma), in the presence or absence of brefeldin A, 1 µg/mL (Sigma), or with 10 ng of toxic shock syndrome toxin-1 (TSST-1) (Sigma) for 22 hours using cell culture conditions (5% carbon dioxide, humidified atmosphere, 37°C). Subsequently, cell aliquots stimulated with PMA and ionomycin or TSST-1 in the absence of brefeldin A were harvested and centrifugated at 2000g at 4°C for 5 minutes. Supernatants were stored for extracellular cytokine analysis at −80°C. The remaining PMA and ionomycin-stimulated cell aliquots were transferred to 5-mL fluorescent activated cell sorter (FACS) tubes (Becton Dickinson) and analyzed for expression of surface CD69 (stimulations without brefeldin A) or intracellular cytokine generation (stimulations with brefeldin A).12,14

All specific antibodies and isotype control antibodies used in this study were of IgG1 isotypes purchased from Becton Dickinson. For antibody labeling, cells underwent centrifugation at 400g for 5 minutes at RT. Supernatants were discarded and the vortexed pellets were differentially processed. For detection of CD69 expression, cells were incubated with phycoerythrin-labeled anti–human CD69 or with the phycoerythrin-labeled isotype control antibody for at least 30 minutes at RT in the dark. For detection of intracellular cytokines, cells were initially subjected to 1 mL of FACS Lysing Solution (Becton Dickinson) for 10 minutes at RT to fix the cells and to optimize cell permeabilization. In addition, any contaminating erythrocytes were lysed during this procedure. Subsequently, cells were

release of IL-2 or IFN-γ, respectively. In addition, it was shown7 that cytokine release from T_h1 and T_h2 cells was concomitantly diminished in severely injured patients with trauma. Furthermore, in vivo exposure of healthy individuals to endotoxin led to reduced production of T_h1 cytokines but not to an increase in T_h2 cytokines from stimulated whole blood.13 Because of similar conflicting results obtained in animal trauma models,11,12 the effect of severe injuries on the T_h1/T_h2 pattern remains unclear.

Thus, we launched this study to determine alterations in the T_h1/T_h2 pattern of patients with polytrauma using intracellular analysis of IL-2 and IL-4 in CD3+ T cells using flow cytometry and respective cytokine release from isolated peripheral blood mononuclear cell (PBMC) fractions using conventional enzyme-linked immunosorbant assay. In addition, we analyzed expression of the cell activation marker CD69 to assess optimal cell activation.

RESULTS

Optimal cell activation is the prerequisite for intracellular cytokine analysis. To assess whether successful activation was achieved, we analyzed surface expression of CD69 on lymphocytes, which reaches a maximum under the cell stimulatory conditions we used.16 There were no differences in the expression of CD69 on lymphocytes of patients (99.3%±0.7% positive cells) and controls (98.3%±1.4% positive cells). In contrast, we observed a significant reduction in T-cell numbers in PBMC fractions obtained from patients compared with controls (Figure 1) mainly due to the occurrence of immature and mature myeloid cells as determined by differentiation of stained cell smears.

There were only minor differences in the percentages of IL-2+ T cells or IL-4+ T cells obtained from patients and healthy individuals (Figure 2 and Figure 3). Numbers of IL-4+ T cells from patients and healthy donors were mainly detected as less than 1% of CD3+ cells, which was also recently reported15; however, there was

![Table 1. Characteristics of 35 Patients With Polytrauma](image)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Sex, M/F</td>
<td>31/4</td>
</tr>
<tr>
<td>Age, mean (range), y</td>
<td>45.8 (18-76)</td>
</tr>
<tr>
<td>Injury Severity Score, mean (range)</td>
<td>28.2 (16-51)</td>
</tr>
<tr>
<td>Stay in intensive care unit, mean (range), d</td>
<td>13.1 (3-37)</td>
</tr>
<tr>
<td>Mortality, No. (%)</td>
<td>4/35 (11)</td>
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a trend toward elevated numbers of IL-4+ T cells in the trauma group. The relative mean fluorescence values of IL-2+ T cells, which serve as quantitative markers for intracellular IL-2 content, showed minor increases in the patient group vs healthy donors (110.7±27.9 vs 107±25.8); however, these differences reached statistical significance (*P<0.05*). A positive correlation has been demonstrated between T-cell receptor to gate T cells activated by PMA and ionomycin. A positive correlation has been demonstrated between TH1 and TH1-type or TH2 and TH2-type cells in combination with CD3–peridinin chlorophyll protein or fluorescence isothiocyanate and phycoerythrin isotype controls in combination with CD3–peridinin chlorophyll protein and left for 30 minutes in the dark at RT. After antibody labeling, 3 ml of wash buffer was added and the cells were centrifugated at 470g for 5 minutes at RT. Subsequently, cells were washed in phosphate-buffered saline solution and fixed with ice-cold paraformaldehyde (1% wt/vol in phosphate-buffered saline solution). After centrifugation (470g for 5 minutes at RT), 500 μL of sheath fluid (FACS-Flow; Becton Dickinson) was added to the vortexed pellets and the cells were immediately analyzed by multiparameter flow cytometry.

Surface and intracellular fluorescence was measured using a flow cytometer (FACSCalibur; Becton Dickinson), and fluorescence data were analyzed with computer software (CELLQuest 1.2.2; Becton Dickinson). Calibration reagents and solutions for flow cytometry (sheath fluid, rinsing fluid, and cleaning fluid) were from Becton Dickinson. After achieving appropriate instrument settings and compensations (FACScanComp 2.0, Three-Color Applications; Becton Dickinson), the instrument settings were not changed during the study. For analysis of CD69 expression, 10,000 cells were acquired. For intracellular cytokine detection, cell acquisition proceeded until 5000 cells were collected in the CD3+ gate. Analysis of intracellular fluorescence was performed by double gating on the CD3+ gate and a lymphocyte scatter gate. Regions of positive fluorescence were determined by respective isotype control antibodies (Becton Dickinson). Fluorescence signals of the isotype antibodies were adjusted as negative for 99% of gated cells. Fluorescence values were expressed as a percentage of positive cells after subtraction of respective isotype control values or as mean fluorescent intensity. Reference experiments using fixed but nonpermeabilized cells showed a reduction in the intracellular detection of IL-2 by more than 85%, which did not differ between cells obtained from patients and controls. Specificity of the used antibodies were additionally confirmed using reference cells (human intracellular cytokine control cells) (Becton Dickinson).

For the analysis of IL-4 in plasma or IL-2 and IL-4 released from activated PBMCs, commercially available enzyme-linked immunosorbent assay systems were used (R & D Systems, Wiesbaden, Germany). The sensitivity of these assays were 5 pg/mL (IL-4) and 10 pg/mL (IL-2). Data were adjusted to lymphocyte count in the PBMC fractions according to cell numbers in the lymphocyte gate (forward scatter/side scatter).

As was observed in control experiments, the CD4 antigen and partly the CD8 antigen are down-regulated by PMA and ionomycin activation to varying degrees; therefore, we used the CD3 antigen that is stably associated with the T-cell receptor to gate T cells activated by PMA and ionomycin. A positive correlation has been demonstrated between Tn1 and Tn1-type or Tn2 and Tn2-type cells in human PBMC fractions.

Nonparametric data were analyzed using the Mann-Whitney U test. The minimum level of significance was 

\[ P<0.05 \]

correlations were calculated using the Spearman rank test (Statistica 5.0; StatSoft Inc, Tulsa, Okla).
more IL-2 compared with patients in the lower ISS group. Regarding outcome, there was no difference in IL-2 release; however, a significantly reduced release in IL-4 was observed in nonsurvivors.

On the other hand, when patients were selected who revealed an individual major decrease in IL-2+ cells (<35%, n=8) or a major increase in IL-4+ cells (>1%, n=6), there were no significant differences in stay in the intensive care unit or therapeutic interventions compared with other patients. None of these patients belonged to the group of nonsurvivors.

**COMMENT**

In the present study we observed neither a profound shift in T_{H1}/T_{H2} cells in circulating CD3+ lymphocytes ob-
tained from patients with polytrauma, including clinically different subgroups, nor any significant reduction in expression of the cell activation molecule CD69 on respective lymphocytes after cell stimulation using PMA and ionomycin. We observed a highly significant reduction in patients’ CD3+ lymphocyte counts in isolated PBMCs, which reflects the reported decline in absolute lymphocyte counts after major injuries, mainly due to the occurrence of mature and immature myeloid cells. A decrease in lymphocyte counts has also been seen after hernia or vascular surgery. Such typical alterations in cellular compositions of isolated PBMC fractions or whole blood samples from patients with trauma will obviously affect variables of functional analysis such as cell proliferation or cytokine release. Thus, interpretation of study results on lymphocyte dysfunctions after severe injuries that were not accompanied by precise cellular differentiation remains critical. The heterogeneity of isolated PBMC fractions, especially from patients with trauma, might be a major reason why extracellular cytokine release is poorly correlated to intracellular staining technique. Basophils in PBMC fractions are an additional potent source of IL-4, and myeloid cells might modulate the cytokine response of T cells via priming factors such as cytokines or lipid mediators. Furthermore, our results revealed stimulus-dependent differences in extracellular cytokine release. Whereas patients’ PBMC fractions released significantly more IL-2 and IL-4 after stimulation with PMA and ionomycin, this was not observed using TSST-1 as cell activator. Optimal T-cell activation by TSST-1 depends on monocyte counts and expression of major histocompatibility complex II molecules, which are decreased on monocytes of severely injured patients.

Flow cytometry allows the gating of selective leukocyte subpopulations by the use of cell surface markers and improves functional analysis of heterogeneous cell populations. In contrast, cytokine release from stimulated PBMC fractions depend strongly on respective cellular compositions. Our results do not support therapeutic regimens designed to elevate T helper (TH1) cell levels using, eg, IFN-γ or IL-12 after polytrauma injuries. However, the commonly used flow cytometric technique for differentiation of T helper 1 (TH1) from TH2 cells on a single cell level still requires artificial activation (PMA and ionomycin) of lymphocytes because more physiologic stimuli were inferior in the ability to induce intracellular cytokines. Thus, results obtained using standard stimulation by PMA and ionomycin reflect the potential production of intracellular cytokines. Posttraumatic alterations in polarized T-cell patterns are obviously driven by a multitude of regulatory factors, such as toxins from Gram-positive and Gram-negative bacteria, unusual antigens, complement factors, cytokines, or lipid mediators. Especially an extensive challenge of bacterial toxins in severely burned patients might result in a different TH1/TH2 pattern compared with mechanically induced injuries. Furthermore, different sequestration of lymphocytes into organs such as lung or lymphoid tissues may lead to a TH1/TH2 pattern different from lymphocytes in circulation. In addition, alterations in T-cell functions after severe injury obviously involve mechanisms not related to T-cell phenotypes, such as transient phases of unresponsiveness. Overcome of membrane-associated alterations of lymphocytes from trauma patients by the use of PMA has been demonstrated. Thus, intracellular cytokine analysis does not detect membrane-associated altered cell signaling after trauma. The high levels of extracellular cytokine release induced by PMA and ionomycin are obviously also due to the bypass of posttraumatically disturbed signal transduction.

Recently, the selective expression of chemokine receptors (CCR and CXCR) on TH1 (CXCR3) or TH2 (CCR3) cells has been reported. Analysis of such cell surface markers and of local lymphocyte patterns may further contribute to our understanding of functional T-cell polarization after severe injuries.

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### REFERENCES


Table 4. Relation of Cytokine Pattern to Clinical Variables

<table>
<thead>
<tr>
<th>Patient Subgroup</th>
<th>TISS</th>
<th>CD3+ Cells, %</th>
<th>IL-2+ Cells, %</th>
<th>IL-4+ Cells, %</th>
<th>Released IL-2, pg/mL</th>
<th>Released IL-4, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISS &lt;25</td>
<td>35 ± 10</td>
<td>33.8 ± 13.9</td>
<td>40.4 ± 11.7</td>
<td>0.34 ± 0.31</td>
<td>2954 ± 1859</td>
<td>1290 ± 951</td>
</tr>
<tr>
<td>ISS &gt;24</td>
<td>36 ± 7</td>
<td>28.4 ± 12.4†</td>
<td>43.3 ± 11.9</td>
<td>0.29 ± 0.35</td>
<td>5071 ± 7120‡</td>
<td>1016 ± 1483</td>
</tr>
<tr>
<td>Survivors</td>
<td>32 ± 9</td>
<td>31.0 ± 12.9</td>
<td>42.8 ± 11.7</td>
<td>0.32 ± 0.35</td>
<td>4305 ± 6334</td>
<td>1300 ± 1416</td>
</tr>
<tr>
<td>Nonsurvivors</td>
<td>43 ± 3‡</td>
<td>30.9 ± 20.0</td>
<td>42.5 ± 14.3</td>
<td>0.31 ± 0.39</td>
<td>5744 ± 5069</td>
<td>185 ± 83‡</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SD. TISS indicates therapeutic interventions; IL-2 and IL-4, interleukins 2 and 4; and ISS, Injury Severity Score.

†‡P < .05.
D r Wick and colleagues add welcome data to ongoing evaluations of the immunologic effects of severe injury. Using a refined flow cytometric analysis, these investigators further define the effects of blunt trauma on the pattern and function of circulating lymphocytes: T\(_1\) vs T\(_2\) CD3\(^+\) cells. Prior studies have produced extremely variable results in the shift from T\(_1\) to T\(_2\) phenotype consistent with an increased risk for infectious and other immunologic complications.

A major contribution of the current data is the appropriate warning that previous results derived from either artifically defined murine models or highly selected aspects of alterations in humans are insufficient and potentially harmful if extrapolated to deriving therapeutic targets for clinical trials. While the study confirms a decrease in the CD3\(^+\) T-lymphocyte cell, this is exaggerated when subpopulations are combined, which is magnified when subpopulations are analyzed. Analysis of the potential effect of sex (recently linked to immunosuppression following injury) is not possible with only 4 female patients. In addition, the most severely injured are most likely to have immunologic derangements; however, only 4 patients died and the number with ISS greater than 25 is not stated; however, while the mean score is 28, the median score appears to be near 16. Thus, most of the patients are only moderately injured. Importantly, the stimulus used for cytokine production is not physiologic. Phorbol myristate acetate bypasses any alterations or impairment in cell surface receptor status, producing normal cytokine production despite potential suppression in vivo. Similarly, the potential effects of alterations in adherence and aberrant up-regulation in lymphocytes recruited to areas of inflammation are not assessed. Consequently, as to the presence or absence of significant immunosuppression following severe injury dependent on alteration in the lymphocyte phenotype remain to be proven.

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