**INTRODUCTION**

Severe injury and the subsequent development of a systemic inflammatory response syndrome (SIRS) and sepsis often lead to multiple organ dysfunction and failure, resulting in a substantial morbidity in trauma victims.\(^1\)\(^-\)\(^5\) It is a long-known paradox that excessive immune activation due to vast amounts of endogenous cellular components released after massive tissue trauma and exogenous pathogen-derived danger molecules can be associated with a compensatory anti-inflammatory response which may render the patient susceptible to infections.\(^6\)\(^-\)\(^9\)

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Therefore, close monitoring of the immune function and the eventually occurring alterations in immune status after trauma, possibly also as a remodelling process due to local hyperfunction of the tissue-associated immune system, would be crucial in order to avoid opportunistic infections that can lead to septic complications.\(^3\)\(^5\)\(^\text{10}\) Currently, in contrast to specific parameters that allow monitoring of organ function, the state of the immune system can only be controlled in a static, snapshot-like manner, not allowing conclusions on remaining potency to react to infectious entities.

In this regard, it also seems vital to use a standardized stimulus in order to monitor and assess immune reaction. Interleukin (IL)–6, a highly popular biomarker and mediator stimulus in order to monitor and assess immune reaction. Interleukin (IL)–6, a highly popular biomarker and mediator of inflammation, is released in response to more than 20 damage- and pathogen-associated molecular patterns\(^11\)\(^12\) that are often present in the patients’ circulation in varying amounts after trauma. This underlines the importance of employing one defined molecule as stimulating agent ex vivo in order to master the complex immune response. The resulting information could be of great use especially during the critical early phase after injury when it is imperative to carefully plan necessary surgical interventions which represent ‘second hits’.\(^13\)\(^14\) Such an additional surgical ‘trauma load’ could be applied at a time of a balanced immune status in the trauma patient, to prevent either post-surgery infection or enhanced SIRS. Therefore, it is essential to develop fast testing methods; the so far recommended protocol of stimulating whole blood for at least 24 hours does not allow a close bedside monitoring. For future transfer to widespread clinical application, incubation times will have to be reduced markedly to get results within hours.

The present study was performed to find out whether (a) a whole blood ex vivo system can be used to monitor the pro- and anti-inflammatory immune responsiveness of trauma patients, and (b) whether a 24 hours standard incubation time of this ex vivo system can be reduced to a 4 hours incubation period without loss of discriminatory power.

## 2 | MATERIALS AND METHODS

### 2.1 | Clinical study

The clinical study was performed at the University Hospital Ulm in accordance with the Declaration of Helsinki and its later modifications. The study protocol was approved by the local Ethics Committee of the University of Ulm, approval number 244/11 (ClinicalTrials.gov identifier: NCT00710411; https://clinicaltrials.gov/ct2/show/NCT00710411). Inclusion criteria were an Injury Severity Score (ISS) \(\geq 32\) (estimated after whole-body computed tomography in the emergency room and checked retrospectively) and age \(\geq 18\) years; patients were excluded if one or more of the following criteria were fulfilled: life expectancy <24 hours, participation in other trials, cardiopulmonary reanimation on the accident scene or dying immediately after hospital admission, known or suspected pregnancy, radio- or chemotherapy within the last 3 months. Healthy volunteers served as controls. Patients and healthy volunteers were included between 15 March 2014 and 15 December 2014. All samples were collected with informed written consent of patients or their legal representatives and volunteers.

### 2.2 | Samples and assays

Polytrauma patients were analysed 4 hours, 24 hours and 5 days after admission. 1 mL of venous blood was drawn into TruCulture\textsuperscript{®} blood collection tubes prefilled by the manufacturer under standardized conditions with 2 mL medium, with unfractionated heparin as an anticoagulant at a final concentration of 50 IU/mL and with or without 100 ng/mL lipopolysaccharide (LPS, from Escherichia coli, O55:B5; HOT Screen GmbH). Using these tubes allow direct blood withdrawal into the culture system without any pipetting steps by the clinician which strongly reduces the risk of contamination and intra-individual variation.\(^15\) Tubes were incubated at \(37^\circ\)C for 24 hours; in order to analyse whether a shorter incubation time with better clinical applicability was sufficient for considerable cytokine production, a second set of tubes \(\pm\)LPS taken at the second time point (24 hours after trauma) was incubated for only 4 hours. After incubation, the cellular sediment was separated from the supernatant using a valve, and supernatants were stored at \(-80^\circ\)C until further analysis. After inclusion of all patients and volunteers, samples were analysed using a multiplexed sandwich immunoassay on a Bio-Plex\textsuperscript{®} 200 platform (both BioRad) according to the manufacturer’s recommendations. Since most samples were out of range for the central pro-inflammatory marker IL-6, they were re-analysed using the IL-6 Quantikine kit (BD Biosciences).

### 2.3 | Statistics

Results after LPS stimulation were compared using one-way analysis of variance followed by Student-Newman-Keuls post hoc test. SigmaPlot (Version 11.0, Systat Software) was used as analytic software. For the comparison of incubation for 24 hours and 4 hours, stimulated and unstimulated samples were compared using paired \(t\) testing. Pearson Product Moment Correlation was employed to assess associations between cytokine production upon LPS stimulus with clinical parameters. A \(P < .05\) was considered as significant.

## 3 | RESULTS

### 3.1 | Patients

Seven patients with a median ISS of 41 (34-47) and five healthy volunteers were included in the study. The median
Glasgow Coma Scale at the time of hospital admittance was three. A median of two units of blood were transfused on the first day after injury. Further clinical parameters are summarized in Table 1; individual patient characteristics are included in Table S1.

3.2 | Endogenous cytokine secretion

In contrast to healthy volunteers who did not have detectable endogenous IL-6 concentrations, polytrauma patients displayed IL-6 concentrations of 21 pg/mL (12-66 pg/mL) 4 hours after trauma which increased to around 274 pg/mL (40-866 pg/mL) and 39 pg/mL (0-1054 pg/mL) 24 hours and 5 days after injury, respectively (Figure 1A). Similarly, endogenous secretion of the early pro-inflammatory cytokines tumour necrosis factor and IL-1β, produced mainly by activated monocytes and macrophages, was elevated in unstimulated samples from patients especially 24 hours and 5 days after trauma (Figure 1B,C).

3.3 | Central pro-inflammatory cytokines with unaltered or decreased LPS response after trauma

In order to evaluate how the reaction to a defined endotoxin stimulus was altered after severe trauma, peripheral whole blood was stimulated with LPS. Blood samples from healthy volunteers and trauma patients displayed highly increased IL-6 concentrations in response to the LPS stimulus, but the amounts secreted by leucocytes after trauma did not differ significantly from those of the healthy controls at all time points (Figure 1A). Regarding tumour necrosis factor and IL-1β, patients displayed significantly lower concentrations in response to LPS compared to the healthy individuals (Figure 1B,C). Interestingly, the same was true for the IL-1β/IL-1RA ratio; all patients displayed a significantly decreased ratio after LPS stimulus compared to healthy controls (Figure 1D). Furthermore, TNF concentrations secreted in response to LPS (the difference between stimulated and unstimulated sample) in patient blood drawn 4 hours after injury correlated with the length of stay on the intensive care unit (Pearson correlation coefficient \( r = .954, P = .0031 \)).

3.4 | Mediators unaltered by polytrauma after LPS challenge

Endogenous concentrations of interferon-γ, an important activator of macrophages secreted by lymphocytes, IL-10 produced by monocytes and lymphocytes, IL-12, secreted by lymphoid and myeloid cells, IL-17 (T helper cells), eotaxin (also known as eosinophil chemotactic protein), basic fibroblast growth factor, an inducer of angiogenesis, and granulocyte-macrophage colony-stimulating factor as the central regulator of myeloid proliferation and maturation were all visibly higher in patients especially 24 hours and 5 days after trauma. However, the amounts released by leucocytes from patients after severe trauma in response to LPS incubation...
remained very similar compared to healthy volunteers (Figure 2A-E, G, H). Endogenous granulocyte colony-stimulating factor as a specific activator of neutrophil proliferation remained largely unaffected by trauma and concentrations produced after LPS stimulus were even slightly decreased in patients (Figure 2F).

3.5 | Cytokines increased after trauma and LPS stimulus

When comparing patients with healthy volunteers, secretion of several cytokines was significantly increased upon stimulus, especially 24 hours after trauma. Concentrations of IL-2, IL-4, IL-5 and IL-9, all secreted mostly by T cells, as well as IL-7, a stimulator of lymphocyte maturation, were unaltered or only slightly elevated 4 hours after injury compared to controls, but increased significantly in blood drawn 24 hours after polytrauma. Concentrations in stimulated blood from patients remained high until day 5 (Figure 3A-E). IL-13, also secreted mainly by T helper cells, did not change over time (Figure 3F). Endogenous levels of the inducer of natural killer cell proliferation IL-15 were increased in some patients 24 hours and 5 days after injury, and the secretion in response to endotoxin challenge was significantly higher in PT patients at all time points (Figure 3G). Similarly, monocyte chemoattractant protein 1 was higher in some patients after 24 hours and 5 days, and the secretion after LPS incubation was significantly elevated 4 hours and 24 hours after trauma (Figure 3H).

3.6 | Shortened incubation time to improve clinical applicability

In order to test whether blood stimulation for 24 hours is necessary to detect the response to LPS stimulation, we drew a second set of tubes from patients 24 hours after trauma and performed a shortened incubation time of 4 hours before supernatants were collected. As shown in Figure 4, the median concentrations of secreted cytokines in response to LPS were partly lower, but clearly detectable and showed a pattern greatly similar to the results after incubation for 24 hours. A comparison of stimulated and unstimulated samples after different incubation is shown in Table S2.

4 | DISCUSSION

This study was performed with two aims: (a) to define the alterations in immune reaction to a defined microbial stimulus after severe trauma, and (b) to test the applicability of a standardized whole blood model for clinical monitoring of the immune function. To our knowledge, this is the first study performing standardized functional immune monitoring in polytraumatized patients using whole blood to reflect
the complexity of the immune reaction and may improve diagnostic opportunities to assess the immune functional capacity.

We were able to confirm that pro-inflammatory cytokines predominantly secreted by monocytes and macrophages, but also by lymphocytes, are reduced when whole blood is challenged with a pathogen-associated molecular pattern early after severe trauma.\textsuperscript{3,4,16-18} However, it was surprising that 24 hours after injury, the TNF response was not as profoundly impaired as at the other time points. Whether these alterations are due to systemic suppression of the inflammatory response or rather represent a remodelling process simultaneous to tissue-specific hyperactivation of immune cells remains an open question. In contrast to previous publications and a recently published study in septic patients,\textsuperscript{19} we did not observe an inhibition of T cell function. In our cohort, cytokines secreted by or involved in the activation of T cells, such as interferon-\(\gamma\), IL-2, IL-4 and IL-9 were either unaltered or even increased in blood from polytrauma patients. Endogenous production of IL-10 with its dual anti- and pro-inflammatory features was also increased, but amounts secreted after LPS challenge were comparable to healthy controls.

The present study has some limitations. The use of heparin as an anticoagulant may have interfered with complement activation by thrombin.\textsuperscript{20,21} However, the high amounts of released inflammatory mediators suggest that this inhibition is only of minor importance, if at all, in the employed model. Furthermore, cytokine release is a biomarker of immune responsiveness; other aspects of cellular immune function such as chemotactic and phagocytic activity which may also have

**FIGURE 2** Secretion of several inflammatory mediators after LPS challenge is unaltered by trauma. Whole blood from healthy volunteers and polytrauma patients 4 h, 24 h and 5 d after trauma was incubated with and without LPS. Concentrations of (A) IFN-\(\gamma\), (B) IL-10, (C) IL-12, (D) IL-17, (E) eotaxin, (F) basic FGF, (G) G-CSF, and (H) GM-CSF were detected in the supernatant. Values are shown as mean and standard error of the mean; \(n = 5\) for controls, \(n = 6\) for PT 4 h; \(n = 7\) for PT 24 h; \(n = 5\) for PT 5 d. Results for blood stimulated with LPS were compared using one-way analysis of variance with Student-Newman-Keuls post hoc test; unstimulated values are shown as a reference.
profound effects on organ dysfunction and ability to clear infection are not captured using this technique. As a major limitation, we were only able to include seven patients during a 9-month period due to the high preset ISS limit since our study was aimed at most severely injured patients. In addition, the assays used are currently associated with a high cost/sample ratio which limited extensive testing such as adding a shorter incubation time of samples taken on the first day after injury. We were also unable to assess the extent of subtle, but clinically meaningful alterations in T cell function.\(^9,22-24\)

It was all the more unexpected that despite the small cohort, we were able to detect several significant differences between the groups.

Several previous studies have investigated the influence of shock, (surgical) trauma and sepsis on the ability of peripheral leucocytes to produce pro-inflammatory mediators upon LPS stimulus.\(^18,25-34\) However, those studies either used isolated cells, disregarding the role of fluid-phase mediators, granulocytes, and platelets, or employed stimulation protocols which are not feasible in clinical routine. Using our approach has the advantage of stimulation under highly standardized conditions and rapid assessment of the complex immune response in whole blood. A larger patient cohort including those with less severe injuries as planned by the REALISM study group (NCT02638779) may allow the identification of the markers with best sensitivity and specificity. However, the group is performing stimulation of whole blood for 24 hours\(^35\); considering the substantial amounts of mediators we were able to detect after 4 hours of incubation, shorter incubation times are both feasible and advisable. This would enable a bedside

**FIGURE 3** Lipopolysaccharide-induced release of interleukins regulation T cell proliferation and maturation and of MCP-1 is increased after severe trauma. After incubation of blood from healthy volunteers and polytrauma patients 4 h, 24 h and 5 d after trauma with and without LPS, secreted concentrations of (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-7, (E) IL-9, (F) IL-13, (G) IL-15 and (H) MCP-1 were measured. Values are shown as mean and standard error of the mean; n = 5 for controls, n = 6 for PT 4 h; n = 7 for PT 24 h; n = 5 for PT 5 d. Results for blood stimulated with LPS were compared using one-way analysis of variance with Student-Newman-Keuls post hoc test; unstimulated values are shown as a reference. *, \(P < .05\) compared to healthy controls; #, \(P < .05\) compared to 4 h post-trauma.
monitoring similar to blood gas analysis and monitoring of the coagulation system during surgeries and in intensive care units. Shorter stimulation and clinical routine point-of-care cytokine measurements of a small number of parameters with established thresholds could offer a protocol to determine within less than 24 hours whether secondary surgery is recommended or not. Apart from trauma victims, the described approach may also be highly useful for the surveillance of sepsis or other intensive care patients and their stratification for clinical trials.36

After validation in a larger cohort, our approach can be of high clinical interest as a practical tool for monitoring the immune status in severely injured patients in order to better time necessary procedures and avoid lethal post-injury complications such as excessive inflammatory reactions or life-threatening infections.

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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS
RH and SK collected the samples and supported the study design. RH performed readout analysis, interpretation, and drafted the manuscript. FS analysed clinical patient data. SW, MF, EB, HB and MK recruited healthy volunteers and polytrauma patients and informed volunteers, patients or their legal representatives. FG, MSHL and MP designed the study. All authors critically revised the manuscript for important intellectual content, approved the final version to be published and agree to be accountable for all aspects of the work.

ORCID
Rebecca Halbgebauer https://orcid.org/0000-0001-8060-2076
Markus S. Huber-Lang https://orcid.org/0000-0003-2359-6516

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.