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Monocyte Tumor Necrosis Factor-α–Converting Enzyme Catalytic Activity and Substrate Shedding in Sepsis and Noninfectious Systemic Inflammation*

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*See also p. 1532.

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Objectives: To determine the effect of severe sepsis on monocyte tumor necrosis factor-α–converting enzyme baseline and inducible activity profiles.

Design: Observational clinical study.

Setting: Mixed surgical/medical teaching hospital ICU.

Patients: Sixteen patients with severe sepsis, 15 healthy volunteers, and eight critically ill patients with noninfectious systemic inflammatory response syndrome.

Interventions: None.

Measurements and Main Results: Monocyte expression of human leukocyte antigen-D-related peptide, sol-tumor necrosis factor production, tumor necrosis factor-α–converting enzyme expression and catalytic activity, tumor necrosis factor receptor 1 and 2 expression, and shedding at 48-hour intervals from day 0 to day 4, as well as p38-mitogen activated protein kinase expression. Compared with healthy volunteers, both sepsis and systemic inflammatory response syndrome patients' monocytes expressed reduced levels of human leukocyte antigen-D-related peptide and released less sol-tumor necrosis factor on in vitro lipopolysaccharide stimulation, consistent with the term monocyte deactivation. However, patients with sepsis had substantially elevated levels of basal tumor necrosis factor-α–converting enzyme activity that were refractory to lipopolysaccharide stimulation and this was accompanied by similar changes in p38-mitogen activated protein kinase signaling. In patients with systemic inflammatory response syndrome, monocyte basal tumor necrosis factor-α–converting enzyme, and its induction by lipopolysaccharide, appeared similar to healthy controls. Changes in basal tumor necrosis factor-α–converting enzyme activity at day 0 for sepsis patients correlated with Acute Physiology and Chronic Health Evaluation II score and the attenuated tumor necrosis factor-α–converting enzyme response to lipopolysaccharide was associated with increased mortality. Similar changes in monocyte tumor necrosis factor-α–converting enzyme activity could be induced in healthy volunteer monocytes using an in vitro two-hit inflammation model. Patients with sepsis also displayed reduced shedding of monocyte tumor necrosis factor receptors upon stimulation with lipopolysaccharide.

Conclusions: Monocyte tumor necrosis factor-α–converting enzyme catalytic activity appeared altered by sepsis and may result in reduced shedding of tumor necrosis factor receptors. Changes seemed specific to sepsis and correlated with illness severity. A better understanding of how tumor necrosis factor-α–converting enzyme function is altered during sepsis will enhance our understanding of sepsis pathophysiology, which will help in the assessment of patient inflammatory status and ultimately may provide new strategies to treat sepsis. (*Crit Care Med* 2015; 43:1375–1385)

Key Words: humans; monocytes; sepsis; systemic inflammatory response syndrome; tumor necrosis factor-α; tumor necrosis factor-α–converting enzyme

Sepsis remains a major cause of morbidity and mortality. Current treatment options are limited to prompt source control, appropriate antibiotics, cardiorespiratory resuscitation, and general organ support (1), with attempts to develop new immunological therapies proving unsuccessful (2–6). A better understanding of how sepsis affects leukocyte function may help identify new therapeutic strategies.

Altered leukocyte function occurs in response to inflammation. Monocytes in critically ill patients can display a phase of activation and one of refractoriness. This refractory state has been termed "deactivation" (7) or "reprogramming" (8) and is thought to represent immune-suppression (9–12). It is characterized by reduced expression of human leukocyte antigen-D-related peptide (HLA-DR) and reduced in vitro release of tumor necrosis factor (TNF) (7), but its clinical significance is not fully understood.

TNF mediates a considerable degree of pleiotropy (13) through activation of different receptors; TNF receptor (TNFR)-1 and TNFR-2 (14, 15). TNF-α–converting enzyme (TACE) is a trans-membrane protease enzyme that cleaves membrane-bound (mem)-TNF to soluble (sol)-TNF (16), a process termed "ectodomain shedding." TACE is widely expressed, including on monocytes (17), the largest producers of systemic TNF. In response to inflammatory stimuli, monocyte TACE activity increases rapidly (18, 19) consistent with post-translational modification. TACE can shed proinflammatory (sol-TNF) and anti-inflammatory (TNFR-1 and TNFR-2) substrates (20); hence, modulation of TACE activity may alter cellular inflammatory balance.

Studies measuring TACE expression or indirectly quantifying activity suggest that it may regulate systemic inflammation (21, 22) and potentially be a useful surrogate of inflammation and organ injury in the systemic inflammatory response syndrome (SIRS). TACE and other protease enzymes are thought to form interlinked "networks" that regulate epithelial cell barrier functions and may display altered functionality in inflammatory conditions, such as sepsis (23), whereas conditional murine TACE knockout appears protective in endotoxic shock (24).

We have previously described a method to measure TACE catalytic activity based on TNF cleavage (25) via fluorescence resonance energy transfer (FRET) assay technology and have used this to elucidate upstream TACE signaling in human monocytes. This occurs through reactive oxygen species phosphorylating (activating) p38-mitogen activated protein kinase (MAPK) (18), which activates TACE.

We hypothesized that monocyte TACE activity would be altered by sepsis and may reflect illness severity and/or cellular inflammatory balance. We performed an observational clinical study, obtaining monocytes from patients with sepsis and determining their TACE activity. Activity was determined at several levels: membrane expression, catalytic activity (basal and stimulus induced), as well as expression, and stimulus-induced ectodomain shedding of TNFR-1 and TNFR-2. To provide a baseline for comparison, data were obtained from healthy volunteers and a subgroup of patients with noninfectious SIRS.

MATERIALS AND METHODS

Patients and Controls

An independent research ethics committee approved the study (North London REC 3 reference: 10/H0709/77). Patients were recruited from Charing Cross Hospital between May 2010 and April 2012 within 48 hours of ICU admission. Recruitment was limited to patients who fulfilled two SIRS criteria, in whom sepsis was suspected and were expected to remain intubated for a further 48 hours. Patients with immunosuppression or receiving granulocyte colony-stimulating factor were excluded. Healthy volunteers were recruited from Imperial College London staff. Exclusion criteria for volunteers were immunosuppression, chronic pathology, or intercurrent illness. We calculated that to have 80% power to detect 1 SD difference in TACE activity between two groups (at $p < 0.05$), 24 participants would be required (ie, $n = 12$ in sepsis and volunteer groups). In total, 16 patients with sepsis and 15 healthy volunteers were recruited. Eight additional patients with noninfectious SIRS were recruited to ascertain whether changes in sepsis monocyte TACE activity were present within the wider critical care population.

Data Collection

Baseline demographic data collected included age, sex, Acute Physiology and Chronic Health Evaluation II (APACHE II) score (26) and other clinical parameters. Clinical data were collected daily including Sequential Organ Failure Assessment (SOFA) scores (27), leukocyte counts, microbiological data, and organ support requirements. ICU lengths of stay, as well as ICU and hospital mortality, were also recorded.

Sample Collection and Processing

Patient blood (30 mL) was obtained from arterial cannula or central venous catheter at baseline (D0), day 2 (D2), and day 4 (D4) while in ICU. A single peripheral blood sample (30 mL) was taken from healthy volunteers. All samples were collected onto ice and processed immediately.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood using Histopaque-1077 (Sigma-Aldrich, St. Louis,

MO) in conjunction with Leucosep tubes (Greiner, Stonehouse, United Kingdom). The concentration of CD14-positive cells was determined using an anti-CD14 antibody (BD Biosciences, Oxfordshire, United Kingdom), and PBMCs were resuspended at 5×10^6 CD14-positive cells/mL. For stimulation, cells were incubated for 1 hour at 37°C with *Escherichia coli* ultrapure lipopolysaccharide (LPS; Invivogen, San Diego, CA) at 1 μg/mL. To determine p38MAPK activation, PBMCs were fixed, permeated, and stained using antibodies specific for the phosphorylated (phospho)-p38MAPK (Thr180/Tyr182) isoform (Cell Signaling, Danvers, MA). An LPS stimulation of 1 μg/mL was used for 15 or 30 minutes. To measure the response, nonactivated MAPK phosphorylation levels were subtracted from LPS-activated levels. To determine sol-TNF production, PBMCs were stimulated for 4 hours at 37°C ± LPS (1 μg/mL). Supernatants were collected and sol-TNF levels measured by enzyme-linked immunosorbent assay (R&D Systems, Oxfordshire, United Kingdom). Patient interleukin (IL)-6 and sol-TNF levels were both measured by enzyme-linked immunosorbent assay of diluted heparinized plasma samples (Ebioscience, San Diego, CA).

Monocyte Isolation

Monocytes were isolated using magnetic-activated cell selection using a CD14-positive bead selection strategy (Miltenyi Biotech, Surrey, United Kingdom).

"Two-Hit" LPS Model

For chronic LPS stimulus, monocytes were placed in polytetrafluoroethylene inserts (Millipore, Carrigtwohill, Ireland) within 12-well plates (Becton Dickinson, Franklin Lakes, NJ) at 3×10^6 per mL for 16 hours at 37°C ± LPS (1 μg/mL). For acute LPS stimulus, cells were harvested from tissue plates and stimulated as previously described.

Flow Cytometry

Surface marker expression was determined by flow cytometry using anti-HLA-DR (clone L243; Biolegend, San Diego, CA), anti-TNFR-1 (clone 16803), anti-TNFR-2 (clone 22235), and anti-TACE (clone 111633, all R&D Systems) antibodies in conjunction with the manufacturer recommended isotype control. Cells were incubated with fluorophore-conjugated anti-human antibodies for 30 minutes at 4°C. A Cyan ADP fluorescence-activated cell sorter (Beckman Coulter, High Wycombe, United Kingdom) using Summit version 4.3.02 (Beckman Coulter) was used for acquisition and Flowjo V.7.5 (Tree Star, Ashland, OR) for analysis. Expression levels are reported as the geometric mean of the fluorescence intensity (MFI).

FRET Assay

The FRET assay was conducted as previously described (25). In brief, monocytes were resuspended at 2.5×10^4 cells per well in 384-well plates and incubated with a peptide containing two fluorophores conjugated by a TACE-sensitive 13 amino acid mem-TNF sequence. Cleavage of the peptide results in fluorescence due to loss of internal quenching between the donor (fluorescein) and acceptor (tetramethylrhodamine) fluorophores. Measurement of fluorescent signal over time allows TACE activity to be determined and quantified in fluorescence units (FU). For all groups, TACE activity was determined without stimulation (basal activity) and in response to an LPS stimulus of 1 μg/mL for 1 hour (LPS-induced activity).

Data Analysis

A Shapiro-Wilkes test was used to determine normality and appropriate analysis was based on the results of this. For two variables, a *t* test or Mann-Whitney *U* test was used, whereas for three or more variables, a one-way analysis of variance or Kruskal-Wallis test was used as appropriate. For categorical data chi-square analysis was used. Where correlation analysis was performed using Pearson or Spearman *r* values are provided.

RESULTS

Details of the 16 patients with sepsis and eight with noninfectious SIRS are in **Table 1**. Apart from a younger age in the SIRS cohort, the demographics were similar. Vasopressor use was confined to the sepsis group (31.3%), and the predominance of pneumonia as the source of sepsis likely explains the lower Pao_2:Fio_2 ratio within this cohort.

Although the median length of stay was 9.5 days, seven patients died and five were discharged earlier than expected meaning that serial samples were obtained from 14 patients (58%). Day 0 refers to sampling baseline; 78% of patients were sampled within 48 hours of admission and 100% within 72 hours. A single sample was obtained from 15 healthy volunteers; this group was younger (mean age, 33.3 ± 3.6 yr) and 60% ($n = 9$) were men.

Standard Markers of Immune Phenotype Produced Interindividual Variability

We sought to characterize patient inflammatory phenotype and used a cellular expressed marker (HLA-DR) in combination with an inducible marker (sol-TNF release in response to LPS stimulation) to determine whether monocytes displayed evidence of "deactivation" or "reprogramming" as previously described (7).

Human monocytes can be divided into subsets based on their expression of CD14, CD16, and CD64 (28–30). We attempted to subdivide monocytes into subsets for analysis; however, grossly altered expression levels of these surface markers on sepsis patients' monocytes, presumably due to inflammatory modulation of marker expression (31–35) independently of subset type, precluded this. Instead, for consistency, monocytes were analyzed as a single population (CD11b+, CD56–, and CD14+) throughout. As expected, monocytes from patients with sepsis and SIRS displayed decreased HLA-DR expression and sol-TNF release (**Fig. 1, *A*** and ***B***). As these markers behaved similarly across the patient groups, they appear to lack specificity when distinguishing sepsis from SIRS. Two patients in the sepsis group received renal replacement therapy, which has previously been shown to increase monocyte HLA-DR expression in sepsis (36). However, HLA-DR expression data from these two patients lay within the range seen in other patients and their exclusion did not alter the results.

TABLE 1. Demographic, Physiological Variables at Baseline (D0) and Outcomes in the Sepsis and Systemic Inflammatory Response Syndrome Cohorts

	Sepsis ($n = 16$)	SIRS ($n = 8$)	p
Age (yr), mean ± SD	67 ± 16	51 ± 17	0.03
Male sex, *n* (%)	10 (63)	5 (63)	0.73
Surgical admission, *n* (%)	4 (25)	2 (25)	1.0
Acute Physiology and Chronic Health Evaluation II, median + IQR	19 (16–21)	20 (16–24)	0.62
Sequential organ failure assessment score, median + IQR	8 (6–8)	8 (7–11)	0.54
Ventilated, *n* (%)	16 (100)	8 (100)	1.0
Lowest Pa_{O_2}:F_{IO_2} (kPa), median + IQR	20.9 (17.8–34.8)	42.7 (38.4–46.0)	0.005
Inotropes/vasopressor, *n* (%)	5 (31.3)	0 (0)	0.08
Heart rate (bpm), median + IQR	95 (90–108)	110 (95–130)	0.23
Systolic blood pressure (mm Hg), mean ± SD	87 ± 13	97 ± 15	0.1
Lactate (mmol/L), median + IQR	1.45 (1.25–1.9)	1.8 (1.4–2.1)	0.57
Renal replacement therapy, *n* (%)	2 (15.4)	0 (0)	0.29
Sodium (mmol/L), mean ± SD	139.4 ± 5.6	137 ± 7.0	0.36
Creatinine (μmol/L), median + IQR	95 (62–215)	75 (61–109)	0.5
Bilirubin (μmol/L), median + IQR	16 (9–36)	18 (8–28)	0.41
Hemoglobin (g/dL), median + IQR	9.9 (8.6–11.2)	11.8 (8.7–12.7)	0.23
Platelet count (10^9/L), mean ± SD	210 ± 107	163 ± 84	0.3
Temperature (°C), median + IQR	37.1 (35.8–37.7)	37.1 (36.9–38.0)	0.57
Leukocyte count (10^9/L), median + IQR	13.9 (9.4–17.0)	8.0 (6.7–9.9)	0.01
ICU length of stay (d), median + IQR	11.5 (6.5–15.8)	8.0 (4.8–13)	0.37
ICU mortality, *n* (%)	5 (31)	2 (31)	0.75
Hospital mortality, *n* (%)	5 (31)	2 (31)	0.75

bpm = beats per minute, IQR = interquartile range.

There were no significant correlations between APACHE II score and D0 HLA-DR expression (sepsis: $r = –0.37$, $p = 0.16$; SIRS: $r = –0.29$, $p = 0.5$) or in vitro sol-TNF release (sepsis: $r = –0.25$, $p = 0.35$; SIRS: $r = –0.36$, $p = 0.44$) in either group. Because there was a significant difference in age between the patient groups, and when compared with healthy volunteers, we sought to determine whether age had any association with our measured variables. There were no correlations between age and HLA-DR levels (sepsis: $r = –0.18$, $p = 0.34$; SIRS: $r = 0.21$, $p = 0.41$) or sol-TNF release (sepsis: $r = 0.29$, $p = 0.25$; SIRS: $r = –0.11$, $p = 0.73$) in either group. Among patients with sepsis, HLA-DR expression levels and SOFA scores were not correlated ($r = 0.06$; $p = 0.75$) and neither were SOFA scores or in vitro sol-TNF release ($r = 0.24$; $p = 0.22$). HLA-DR levels (MFI, 174.5; interquartile range [IQR], 34.3–312.1 survivors vs MFI, 57.5; IQR, 31–166 nonsurvivors; $p = 0.25$) and in vitro sol-TNF levels (268 pg/mL; IQR, 80–523 pg/mL survivors vs 579 pg/mL; IQR, 115–829 pg/mL nonsurvivors; $p = 0.14$) did not differ between patients with sepsis who survived and those who died.

Plasma levels of IL-6 and sol-TNF were measured at D0 within the patient groups. In contrast to the immune-depressed monocyte phenotype suggested by the HLA-DR and in vitro sol-TNF data, D0 IL-6 levels appeared raised in the sepsis group, suggesting continued inflammation (**Fig. 2*A***). Sepsis IL-6 levels were correlated with APACHE II score ($r = 0.71$; $p = 0.003$) (**Fig. 2*B***), but not with SOFA scores ($r = 0.38$; $p = 0.16$) and did not significantly differ between survivors and nonsurvivors (68 pg/mL; IQR, 3–242 pg/mL survivors vs 85 pg/mL; IQR, 62–433 pg/mL nonsurvivors; $p = 0.43$). IL-6 levels and HLA-DR expression were not correlated ($r = –0.32$; $p = 0.25$).

Plasma sol-TNF levels were elevated in a few sepsis patients only (**Fig. 2*C***) and did not correlate with APACHE II score ($r = 0.17$; $p = 0.57$).

TACE Activity as Determined by FRET Assay

We characterized TACE activity and expression profiles to determine whether they were altered by sepsis. Basal (no stimulation) TACE activity was elevated in the patients with sepsis (**Fig. 3*A***), yet

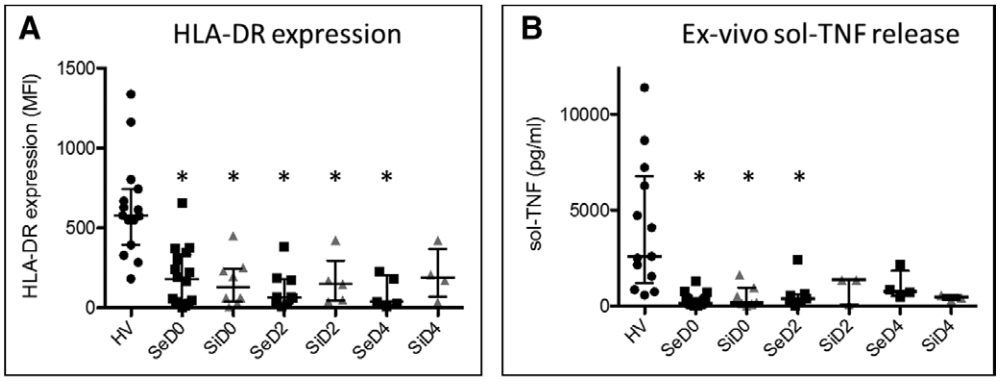

Figure 1. Monocytes from patients with sepsis and systemic inflammatory response syndrome (SIRS) appeared phenotypically similar on the basis of human leukocyte antigen (HLA)-DR expression and sol- tumor necrosis factor (TNF) release. Monocytes were isolated from healthy volunteers (HV; $n = 15$), patients with sepsis (Se; $n = 16$) and patients with SIRS (Si; $n = 8$). Monocyte HLA-DR expression levels, as determined by flow cytometry, were attenuated in sepsis and SIRS when compared with values obtained from healthy volunteer cells (**A**). Cells were then incubated with lipopolysaccharide 1 μg/mL for 4 hr and in vitro sol-TNF release quantified. Sol-TNF release was attenuated across sepsis and SIRS patients when compared with healthy volunteers (**B**). D0 is sampling baseline, D2 is day 2 and D4 is day 4. Data shown as median ± interquartile range. *$p < 0.01$ versus healthy volunteers.

TACE expression and LPS-induced TACE activity appeared unaltered (**Fig. 3, *B* and *C***). No changes in SIRS patient basal and LPS-induced TACE activity or expression were present (Fig. 3, *A–C*).

Sepsis monocyte D0 basal TACE activity was strongly correlated with APACHE II score ($r = 0.75$; $p = 0.002$) (Fig. 3*C*). Basal TACE activity and age were not correlated within the sepsis cohort ($r = 0.13$; $p = 0.52$). There was no relationship between sepsis monocyte basal TACE activity and ICU mortality (85.9 ± 50.1 FU/min survivors vs 86 ± 38.4 FU/min nonsurvivors; $p = 1.0$) or hospital mortality (88 ± 52.1 FU/min survivors vs 82.8 ± 36.7 FU nonsurvivors; $p = 0.78$).

Basal TACE activity in healthy volunteers was not correlated with age ($r = 0.33$; $p = 0.24$). As expected (18), volunteer monocytes increased their basal TACE activity when LPS stimulated, whereas, in contrast, stimulation-induced activity changes were attenuated in sepsis (**Fig. 4*A***). This attenuated activity negatively correlated with APACHE II and SOFA scores (**Fig. 4, *B* and *C***). Among patients with sepsis, those who survived had larger TACE activity increases on LPS stimulation than those who died. The median fold increase was 1.7 (IQR, 1.4–2.4) for survivors versus 1.2 (IQR, 1.1–1.6) for nonsurvivors, $p = 0.005$. Similarly, the absolute LPS-induced TACE activity values also differed between these groups (146.7 ± 75.3 FU/min survivors vs 98.8 ± 28.7 FU/min for nonsurvivors; $p = 0.05$).

Similar to healthy volunteers, patients with SIRS appeared to display a preserved response to LPS (Fig. 4*A*). No relationship between activity increases and mortality was present (median fold increase, 2.0; IQR, 1.7–2.4 ICU survivors vs 2.4; IQR, 1.7–2.9 ICU nonsurvivors; $p = 0.57$). LPS-stimulated TACE activity values were not correlated with age within either group (sepsis: $r = -0.1$, $p = 0.61$; SIRS: $r = -0.05$, $p = 0.84$).

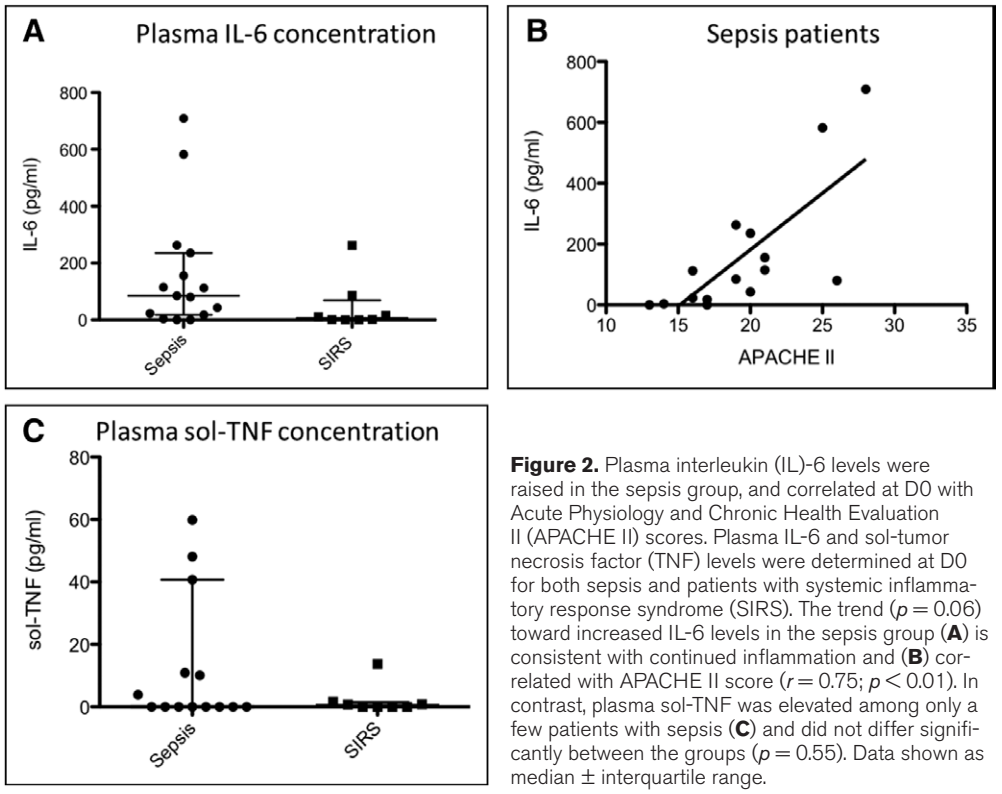

Figure 2. Plasma interleukin (IL)-6 levels were raised in the sepsis group, and correlated at D0 with Acute Physiology and Chronic Health Evaluation II (APACHE II) scores. Plasma IL-6 and sol-tumor necrosis factor (TNF) levels were determined at D0 for both sepsis and patients with systemic inflammatory response syndrome (SIRS). The trend ($p = 0.06$) toward increased IL-6 levels in the sepsis group (**A**) is consistent with continued inflammation and (**B**) correlated with APACHE II score ($r = 0.75$; $p < 0.01$). In contrast, plasma sol-TNF was elevated among only a few patients with sepsis (**C**) and did not differ significantly between the groups ($p = 0.55$). Data shown as median ± interquartile range.

TNFR Shedding

We examined the behavior of TNFR-1 and TNFR-2 using a substrate-shedding index and this confirmed the results obtained using the FRET assay. To determine this index, monocytes were stimulated with LPS and receptor expression measured pre and post stimulation, allowing percentage shedding of each receptor to be calculated and used as a marker of TACE activity: (Baseline expression – LPS-induced expression) × 100/ baseline expression.

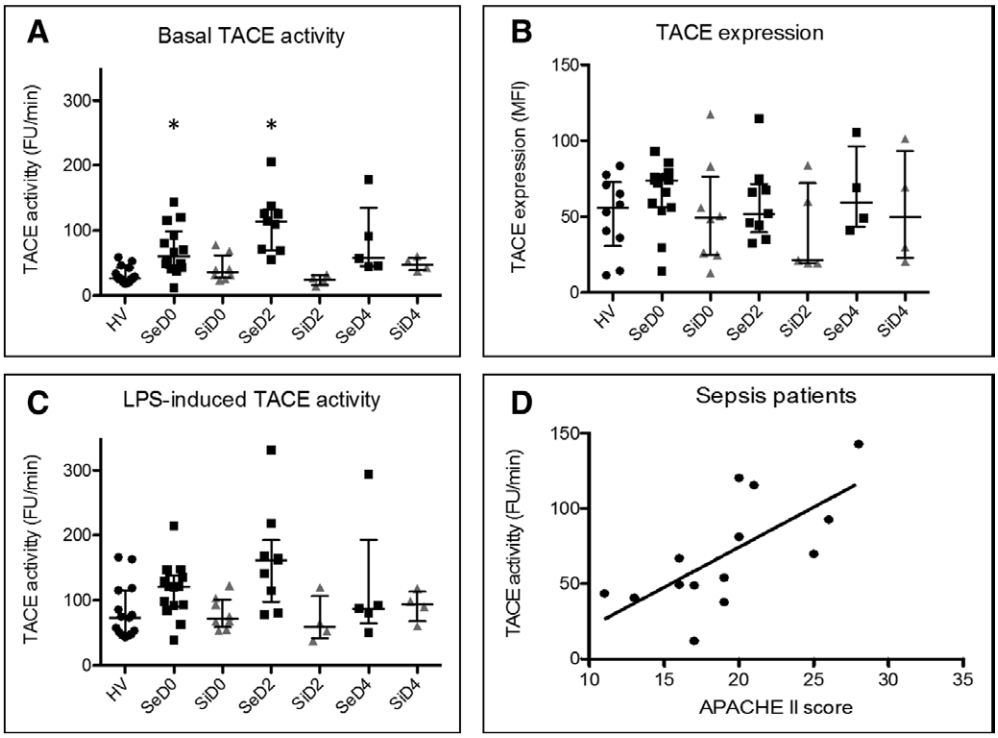

Figure 3. Monocyte basal tumor necrosis factor-α–converting enzyme (TACE) activity was elevated in patients with sepsis compared with healthy volunteers. Monocytes isolated from healthy volunteers (HV), patients with sepsis (Se), and SIRS (Si) had basal (unstimulated) TACE activity determined. Within the sepsis group, significantly increased basal activity was present at D0 and D2 (**A**). Basal TACE activity did not differ in healthy volunteers and patients with SIRS. In sepsis and patients with SIRS, TACE expression (**B**) and lipopolysaccharide (LPS)–induced TACE activity (**C**) were unaltered. Sepsis patient D0 basal TACE activity was positively correlated with Acute Physiology and Chronic Health Evaluation II (APACHE II) score ($r = 0.75$; $p < 0.01$; **D**). Data shown as median + interquartile range. *$p < 0.01$ compared with HVs.

There were no differences in baseline expression of TNFR-1 or TNFR-2 on patient monocytes (**Fig. 5, *A*** and ***B***). LPS-induced shedding of both TNFRs appeared reduced in sepsis (**Fig. 5, *C*** and ***D***). Shedding of TNFR-1 at D0 was negatively correlated with both APACHE II ($r = –0.5$; $p = 0.06$) and SOFA scores ($r = –0.44$; $p = 0.02$). TNFR-1 shedding and age were not correlated ($r = –0.1$; $p = 0.61$), and shedding did not differ between survivors and nonsurvivors (% shedding 52.2 ± 23.6 survivors vs 49.7 ± 22.5 nonsurvivors; $p = 0.82$). There were no correlations between TNFR-2 shedding (D0) and APACHE II score ($r = –0.26$; $p = 0.35$), SOFA scores ($r = –0.20$; $p = 0.33$), or age ($r = –0.28$; $p = 0.16$). TNFR-2 shedding did not differ between survivors and nonsurvivors (% shedding 65.1 ± 20.2 survivors vs 50.2 ± 29.5 nonsurvivors; $p = 0.17$).

In contrast, in response to LPS stimulation, healthy volunteer and SIRS monocytes shed the majority of membrane TNFR-1 and TNFR-2 (Fig. 5, *C* and *D*).

In Vitro Modeling of the Changes in TNF and TNFR Cleavage Present in Sepsis

Differences in TACE behavior between the patient groups may reflect exposure to pathogen-associated molecular patterns (PAMPs). We developed a "two-hit" inflammatory model to reproduce sepsis TACE activity profiles. Volunteer monocytes were cultured with LPS for 16 hours (chronic primary sepsis stimulus), exposed to a second LPS stimulus for 1 hour (acute secondary sepsis stimulus) and their TACE response determined. After untreated 16-hour culture (no chronic sepsis stimulus), cells remained capable of acute TACE activation on LPS stimulation (**Fig. 6*A***). After chronic primary, LPS stimulus volunteer cells displayed increased basal TACE activity (Fig. 6*A*) and unaltered TACE expression (**Fig. 6*B***) but were refractory to acute secondary LPS stimulus (Fig. 6*A*). In addition, shedding of TNFR-1 and TNFR-2 in response to the acute secondary LPS stimulus was also reduced by the chronic primary LPS stimulus (**Fig. 6, *C*** and ***D***). Such changes may indicate that a ceiling of maximal TACE activity has been reached in these cells, with similar behavior observed in sepsis patient monocytes. However, it is important to recognize that substrate shedding will also be affected by TACE-independent factors, such as substrate expression and availability.

To determine whether these altered profiles mirrored the overall changes in monocyte responsiveness, sol-TNF release was determined in the model. After untreated 16-hour culture (no chronic primary sepsis stimulus), monocytes remained capable of releasing sol-TNF on secondary acute LPS stimulus (**Fig. 6**, ***E*** and ***F***). In contrast, this release following acute secondary LPS stimulus was undetectable after chronic primary LPS stimulus (Fig. 6*F*) and was similar to the attenuated sol-TNF release in sepsis and SIRS monocytes.

P38-MAPK Signaling in Patients With Sepsis

We previously demonstrated that upstream LPS-induced TACE activation is dependent on p38-MAPK phosphorylation (18). To investigate the mechanisms responsible for the altered TACE activity seen in patients with sepsis, we measured the p38MAPK response to LPS in the last seven patients with sepsis recruited, and found it to be attenuated (**Fig. 7**). As expected, elevated levels of phospho-p38MAPK were present in healthy volunteer monocytes after LPS stimulation (Fig. 7). The attenuated response in sepsis suggests a direct link between regulation of this pathway and the observed reduction of TACE activation. However, unlike TACE basal activity in sepsis, unstimulated basal ($t = 0$) levels of phospho-p38MAPK were not significantly elevated in the sepsis group compared with healthy volunteers. This difference

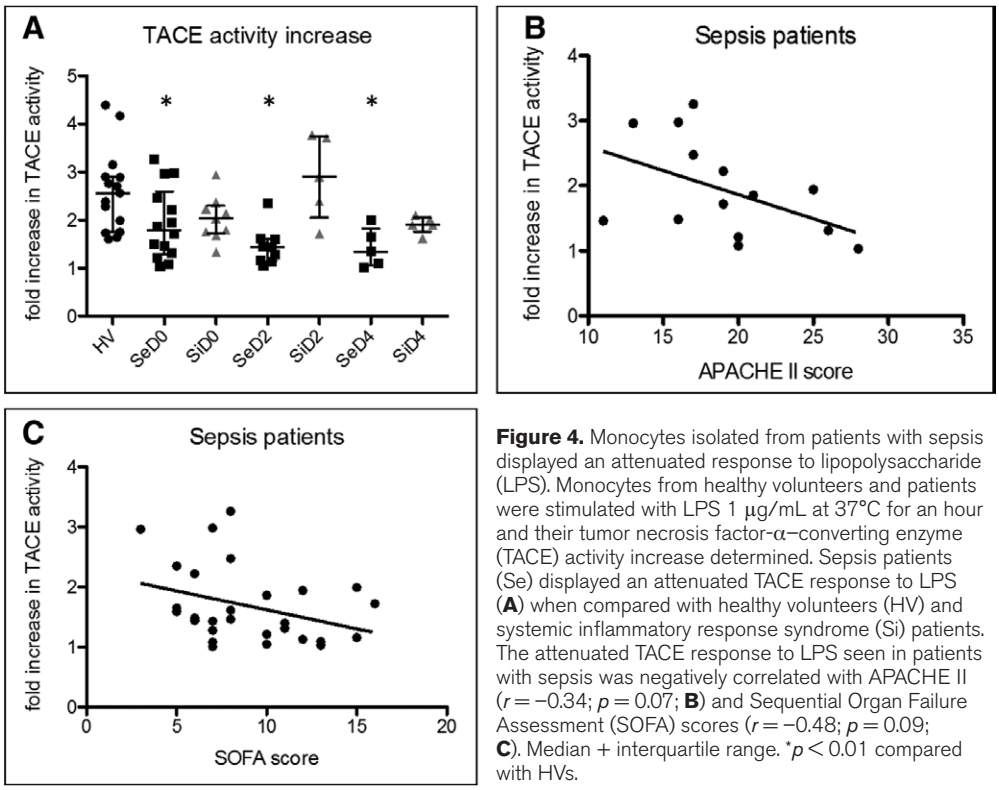

Figure 4. Monocytes isolated from patients with sepsis displayed an attenuated response to lipopolysaccharide (LPS). Monocytes from healthy volunteers and patients were stimulated with LPS 1 μg/mL at 37°C for an hour and their tumor necrosis factor-α–converting enzyme (TACE) activity increase determined. Sepsis patients (Se) displayed an attenuated TACE response to LPS (**A**) when compared with healthy volunteers (HV) and systemic inflammatory response syndrome (Si) patients. The attenuated TACE response to LPS seen in patients with sepsis was negatively correlated with APACHE II ($r = -0.34$; $p = 0.07$; **B**) and Sequential Organ Failure Assessment (SOFA) scores ($r = -0.48$; $p = 0.09$; **C**). Median + interquartile range. *$p < 0.01$ compared with HVs.

suggests that TACE can be maintained in an activated state without the need for prolonged up-regulation of phospho-p38MAPK levels.

DISCUSSION

We hypothesized that TACE activity profiles would be altered by severe sepsis and may reflect illness severity and/or cellular inflammatory balance. In this observational study, comparing and contrasting patients with sepsis with healthy volunteers and a small cohort of noninfected critically ill patients, we found that basal TACE activity was increased and LPS-induced TACE activity changes appeared attenuated among patients with sepsis. Although patient numbers were limited, the increase in basal activity was correlated with illness severity and was associated with attenuated TNFR shedding within our cohort. A two-hit inflammatory in vitro model could reproduce the observed changes in TACE activity profiles and, by examining upstream signaling, we found alterations in sepsis patients' p38-MAPK signaling that were characterized by a reduced response to LPS. It is plausible that prolonged exposure to inflammatory stimuli result in increased basal TACE activity and an attenuated TACE response to further LPS stimulation, through altered p38MAPK signaling. By demonstrating that TACE is in a higher activation state but refractory to further stimulation, these results suggest a resetting of the monocyte phenotype rather than simple deactivation as part of the response to sepsis.

Monocytes obtained from both patient groups showed reduced HLA-DR expression and sol-TNF release as expected. Consistent with previous work (37, 38), there appeared to be no differences in the magnitude and kinetics of these changes between sepsis and SIRS. We found no association between HLA-DR expression and patient outcomes, but it should be noted that our study was not powered to detect such an association. Relating HLA-DR expression to patient outcomes has produced conflicting results, and a review of studies in this area failed to find a threshold in HLA-DR expression that successfully predicted unfavorable outcomes in sepsis (12). There is no accepted consensus around threshold levels or in its measurement and thus the clinical significance of HLA-DR expression remains uncertain.

Plasma IL-6 levels were elevated among the patients with sepsis at D0 in a manner that correlated with APACHE II scores. Given that the half-life of IL-6 is under 6 hours (39), elevated levels indicate continued inflammation at the same time that other markers (HLA-DR and sol-TNF) suggest immune-suppression. It should be noted that IL-6 levels represent responses from a number of different cells, hence may be different from a monocyte-only response. Therefore, the relationship between pro- and anti-inflammatory processes appears complex, with both potentially occurring concurrently, and not necessarily consecutively, as has been suggested (40, 41). Given this complex picture, it may be beneficial to assess inflammatory balance at a cellular level, comparing basal and inducible responses in the same individual. Such an approach may help resolve some of the intrinsic interindividual variability when other markers such as HLA-DR, in vitro sol-TNF, and plasma IL-6 levels are used in conjunction with each other.

TACE activity has not previously been directly measured in critical illness. Our data provide the first direct functional insight into monocyte inflammatory function in this context. Within the sepsis group, basal TACE activity appeared increased and correlated with APACHE II score. Combined with this increase in basal activity, LPS-induced up-regulation of enzyme activity decreased and these changes were not seen in patients with SIRS. The refractoriness to LPS seen in sepsis may represent tolerance in a specific pathway, or a general inability to respond to PAMPs (cross-pathway tolerance), and a state of apparent cellular immune-suppression.

It is interesting to note that in vitro sol-TNF release was attenuated across both groups, whereas TACE activity profiles appeared altered only by sepsis. This may reflect a lack of substrate availability as, if mem-TNF expression was reduced

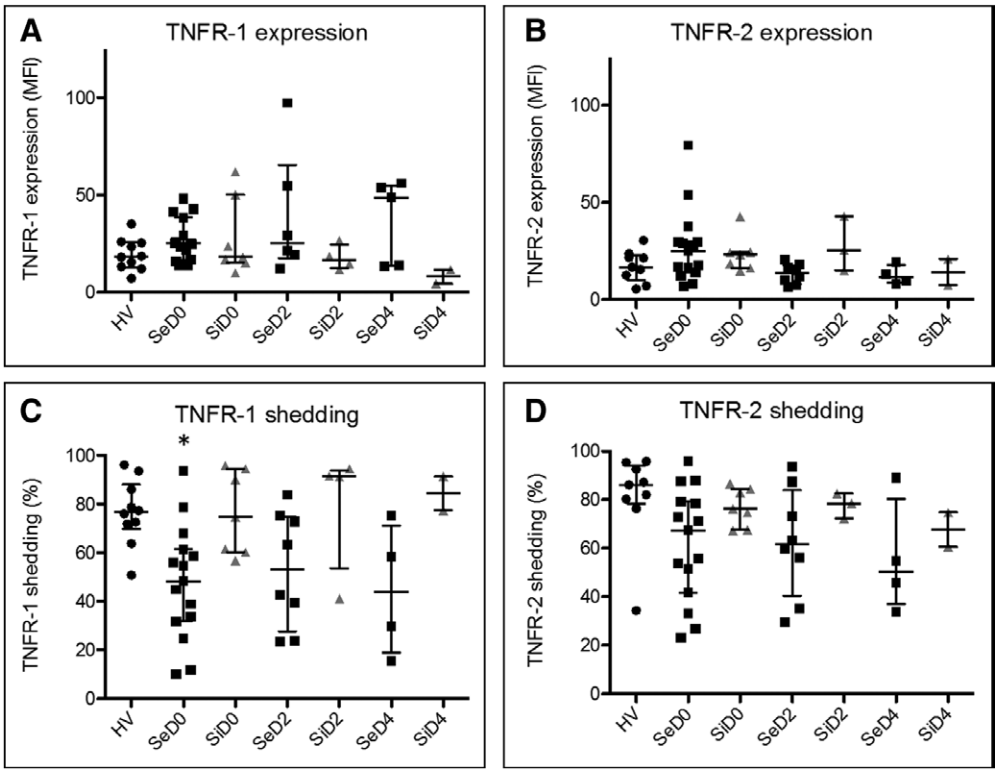

Figure 5. Attenuation of tumor necrosis factor receptor (TNFR)-1 and TNFR-2 shedding in patients with sepsis. Monocyte expression of TNFR-1 and TNFR-2 was determined for healthy volunteers (HV), sepsis (Se), and systemic inflammatory response syndrome (SIRS [Si]) patients (**A** and **B**). No significant alterations were detected in expression across the two patient groups. Percentage shedding of each substrate (in response to lipopolysaccharide 1 μg/ml for an hour) was then determined. In patients with sepsis, shedding was attenuated at D0 for TNFR-1 and TNFR-2, but did not appear altered by noninfectious SIRS (**C** and **D**). Data shown as median ± interquartile range. *$p < 0.05$ compared with HVs.

equally in both groups, but the shedding mechanism suppressed only in the sepsis group, it would explain these results.

We examined expression levels and shedding profiles of both TNFRs to assess for any relationship with TACE behavior changes and association with clinical status. Because the FRET assay uses a TNF-specific cleavage sequence peptide, extrapolating TACE catalytic activity values from this assay directly to proteolytic cleavage of alternate substrates is not possible, and a shedding index was used as a substrate-specific surrogate marker of activity. Distinct from any implications of TACE in the inflammatory process (42–44), enhanced release of TNFRs represents an anti-inflammatory process by reducing TNF signaling in cells and neutralizing sol-TNF (20), and therefore is of importance when attempting to draw conclusions as to cellular inflammatory balance. Within the sepsis group, there was attenuated shedding of TNFR-1 at D0, but no changes in expression. Although not statistically significant, attenuated TNFR-2 shedding was also present in the sepsis group. Overall, therefore, LPS-inducible TNFR-1 and TNFR-2 shedding was attenuated in patients with sepsis, but not in patients with SIRS, indicating a similar pattern to LPS-induced TNF cleavage. The TACE activity and shedding profiles obtained from the two patients who received renal replacement therapy were comparable with data from other patients within the sepsis group.

Because we found no alterations in the surface expression of either TNFR-1 or TNFR-2, it is difficult to draw firm conclusions on the implications of this reduced shedding, but it is likely that this will affect paracrine signaling. Together with the TACE activity changes, altered shedding provides further information that TACE-mediated functions are altered in sepsis. It has been shown that plasma membrane microdomains regulate TNFR-1 shedding in human endothelial cells by colocalizing TACE to membrane expressed TNFR-1 (45), and this process may be altered in sepsis. Altered colocalization of protease enzymes and substrates may be of importance in the regulation of protease networks. Alternatively, it is plausible that the reduced shedding of TNFR-1 may result from the mitochondrial dysfunction seen in sepsis (46) because some investigators have implicated mitochondria in TNFR-1 ectodomain shedding (47).

The p38MAPK pathway appears altered in sepsis and the attenuated signaling we report is temporally related to changes in TACE activity. Therefore, TACE and/or p38MAPK may be of benefit when determining any evolving inflammatory response in longitudinal patient studies. MAPK signaling has been implicated in TNFR-1 and TNFR-2 downstream signaling in humans (48) and murine TNFR-2 shedding is p38MAPK dependent (49). Hence, the reduced TNFR shedding in sepsis monocytes is likely related to the attenuated p38MAPK activation that we observed.

Our data would suggest that sepsis may "reprogram" cells, altering their response state. As we could reproduce sepsis TACE behavior in vitro using an LPS model, it is possible that reprogramming may be mediated through exposure to PAMPs. The fact that these changes are not seen within the SIRS cohort may reflect an absence of PAMPs and suggest a lack of single- or cross-pathway tolerance therein. In our septic cohort, both basal TACE activity and TNFR-1 shedding correlated with APACHE II scores. Data from a larger patient population may help delineate whether TACE (and/or other protease) enzyme behavior better reflects illness severity than HLA-DR or in vitro sol-TNF release in sepsis. Although TACE knockout mice are nonviable (50, 51), mice with myeloid cell–inactivated TACE have a survival benefit in endotoxic shock (24), and knockouts of other protease enzymes also confer a survival benefit in

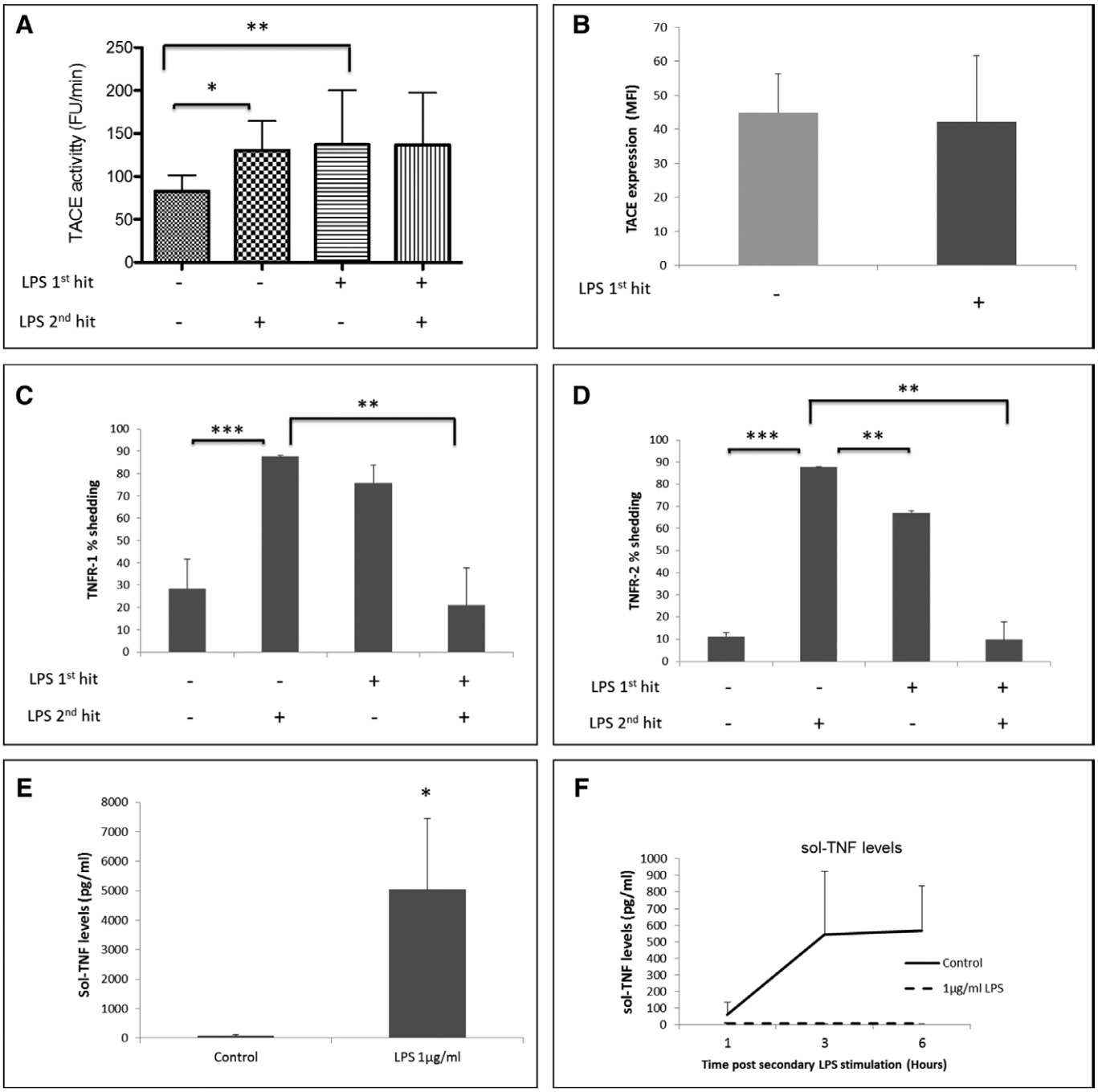

Figure 6. The tumor necrosis factor (TNF)-α–converting enzyme (TACE) profiles seen in the patients with sepsis could be induced in healthy volunteer monocytes through the use of a two-hit inflammatory model. A two-hit inflammatory model was used to induce changes in TACE activity profiles in healthy volunteer cells. Monocytes were cultured with lipopolysaccharide (LPS) for 16 hours (primary chronic LPS stimulus) and then further stimulated with LPS (secondary acute LPS stimulus) and their TACE activity profiles determined. Changes elicited were consistent with those seen in sepsis patients: basal activity was increased and refractory to further LPS stimulus (**A**). No differences in TACE expression after primary LPS stimulus were present (**B**). Shedding of the TNF receptors (TNFRs)-1 (**C**) and TNFR-2 (**D**) were attenuated by LPS exposure. Sol-TNF was released after primary chronic LPS stimulus with no secondary acute stimulus (**E**), but not after secondary acute stimulus in cells exposed to both primary and secondary LPS stimuli (**F**). $n = 8$ (**A** and **B**), $n = 4$ (**C**–**F**), data shown as mean + SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **E**, Control refers to monocytes cultured without primary chronic LPS stimulus. **F**, Control refers to monocytes cultured without primary chronic LPS stimulus subsequently exposed to secondary acute LPS stimulus.

murine models of sepsis (52). Thus, an enhanced understanding of how enzyme function is altered in sepsis may ultimately lead to new therapeutic strategies.

There are some limitations of this observational study that should be considered. There is considerable heterogeneity within the sepsis group, with patients presenting with infection in different tissues and with different organisms. In future, it may be more appropriate to try and study a more homogenous population by concentrating on specific pathologies/organisms. Also the groups differ in their demographic

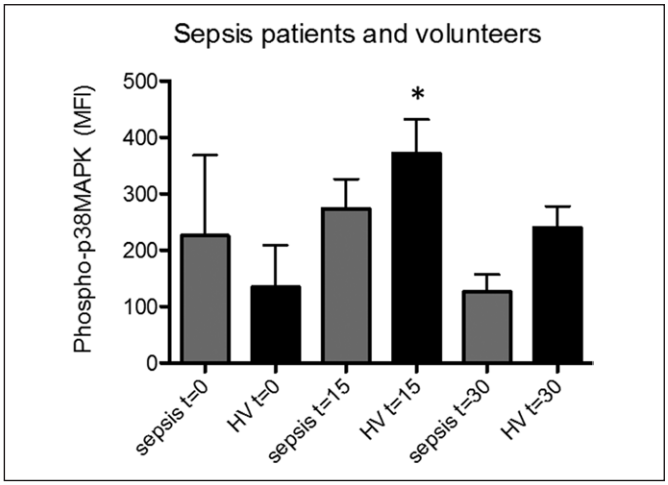

Figure 7. Alterations in basal and lipopolysaccharide (LPS)–induced p38-mitogen activated protein kinase (MAPK) signaling were seen in sepsis. Cells from a subgroup of patients with sepsis ($n = 7$) were LPS stimulated (1 μg/mL) and had levels of phospho-p38MAPK determined at baseline ($t = 0$) min, 15 ($t = 15$) min, and 30 ($t = 30$) min. Levels of phospho-p38MAPK were not significantly elevated at 15 or 30 min after LPS stimulation. In contrast, when cells taken from healthy volunteers (HVs) were exposed to the same protocol, LPS-induced phospho-p38MAPK levels were significantly increased at $t = 15$ (*$p < 0.01$ vs HV $t = 0$). Data shown as mean + SD.

data and therefore potential confounding effects cannot be excluded. Despite performing a power calculation and targeting patient recruitment based on this, numbers are low (especially within the SIRS subgroup) and some nonsignificant correlations may reflect this limited power. As patients improved and were discharged, or deteriorated and died, they were lost from the study with consequent reduction in our sample size over time. Although not the major focus of the study, we attempted to analyze individual monocyte subsets but were unable to clearly identify distinct populations in all patients with the markers we used. The lack of follow-up to determine whether TACE basal activity changes indicate resolution or chronic low-grade inflammation should also be considered a limitation.

CONCLUSIONS

Monocyte TACE activity appeared altered by sepsis in a manner that reflects illness severity, could be reproduced in vitro through exposure to LPS, and may be mediated through attenuated p38MAPK signaling. These data support the reprogrammed monocyte concept and future work should focus on determining the functional capacity (ie, migratory and phagocytic capability) of this inflammatory phenotype in a larger patient cohort. Real-time measurements of enzymatic function, or other dynamic responses involving post-transcriptional protein modification, may provide a more immediate and complete determination of monocyte immune phenotype, revealing both the underlying inflammatory status and the response to further stimulation. This approach would allow a more sophisticated determination of cell signaling, differentiating sepsis from sterile inflammation, potentially identifying therapeutic targets and stratifying patients for immune-modulating therapies.

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