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An early circulating factor in severe sepsis modulates apoptosis of monocytes and lymphocytes

Ilia Vaki, Hariklia Kranidioti,* Vassiliki Karagianni,* Aikaterini Spyridaki,* Antigone Kotsaki,* Christina Routsi,† and Evangelos J. Giamarellos-Bourboulis*,[1]*

*4th Department of Internal Medicine and †1st Department of Critical Care Medicine, University of Athens, Medical School, Athens, Greece

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ABSTRACT

We hypothesized that a factor may circulate in serum early during sepsis, modulating apoptosis of monocytes and lymphocytes. Serum was collected from 20 healthy volunteers and from 48 patients with severe sepsis/shock within 12 h from signs of the first failing organ. PBMCs were isolated from 20 healthy volunteers and incubated with collected sera. Apoptosis and expression of CD95 were determined by flow cytometry; experiments were run in the presence of caspase-8 and caspase-9 inhibitors and of $CaCl_2$. Activity of caspase-3 was determined in cell lysates by a chromogenic kinetic assay. Incubation with serum of patients induced apoptosis of CD4 lymphocytes and inhibited apoptosis of CD14 monocytes. This was attenuated after diluting serum or mixing with healthy serum. Activity of caspase-3 was consistent with these findings. Induced apoptosis of CD4 lymphocytes was greater among nonsurvivors, and it was inhibited in the presence of caspase inhibitors. Inhibitors did not modify the effect of patients' serum on apoptosis of CD14 monocytes. $CaCl_2$ reversed the inhibitory effect on apoptosis of CD14 moncytes. The above findings support the hypothesis for the existence of an early circulating factor in severe sepsis/shock, modulating apoptosis of CD4 lymphocytes and of CD14 monocytes by interaction with the two apoptotic pathways. *J. Leukoc. Biol.* 89: 343–349; 2011.

Introduction

Severe sepsis and septic shock are among the leading causes of death worldwide. It is estimated that >1.5 million people in North America and another 1.5 people in Western Europe develop severe sepsis/shock annually; mortality ranges between 35% and 50%. Most of them die as a result of MODS [1]. Studies in recent years have suggested that apoptotic cell death may play a role contributing in the immune dysfunction and MODS. Among all categories of white blood cells, apoptosis has been studied extensively in lymphocytes. Apoptosis of lymphocytes leads to immunoparalysis and to the subsequent development of secondary infections [2, 3].

However, current theories for the pathogenesis of sepsis recognize a dominant role of blood monocytes; these cells confer the major bulk of proinflammatory cytokines after stimulation with PAMPs of the bacterial pathogens [4]. Existing data for any role of apoptosis of monocytes in sepsis are limited. In a recently published cohort of patients with sepsis, as a result of VAP by our group [5], a correlation was found between apoptosis of monocytes and final outcome. More precisely, it was shown that whenever the rate of apoptosis of monocytes within the first 24 h from diagnosis was >50%, survival of patients with septic shock was prolonged. It was also shown that under this setting, the potency of monocytes with a rate of apoptosis >50% for the release of proinflammatory cytokines was impaired.

The questions arising from the results of the latter study are: how apoptosis of monocytes and lymphocytes is modulated in sepsis; if a circulating factor mediates this process; and how early this factor appears. We aimed to resolve these questions in a cohort of patients with severe sepsis and/or shock of early presentation.

MATERIALS AND METHODS

Study design

A total of 48 patients with severe sepsis and/or septic shock was enrolled in a prospective study conducted over the period October 2005–April 2006. The study protocol was approved by the Ethics Committee of the Attikon University Hospital of Athens (Greece). Written, informed consent was given by first-degree relatives. Inclusion criteria were: diagnosis of severe sepsis and/or septic shock; HAP, acute pyelonephritis, primary bacteremia, or acute intra-abdominal infection as a cause for sepsis; and blood sampling within 12 h after the first sign of organ failure appeared. Exclusion criteria were: HIV infection;

Abbreviations: $CaCl_2$=calcium chloride, HAP=hospital-acquired pneumonia, MODS=multiple organ dysfunctions, pNS= p value of comparisons non-significant, SIRS=systemic inflammatory response syndrome, TBS=tracheobronhial secretions, VAP=ventilator-associated pneumonia

1. Correspondence: Attikon University Hospital, 1 Rimini St., 124 62 Athens, Greece. E-mail: giamarel@ath.forthnet.gr

TABLE 1. Demographic Characteristics of 48 Patients with Severe Sepsis and/or Septic Shock Enrolled in the Study

Male/Female	26/22
Age (mean±SD)	71.0 ± 2.0 years
APACHE II score (mean±SD)	19.61 ± 9.29
Underlying infection (number, %)	
Acute pyelonephritis	15 (31.3)
Nosocomial pneumonia	6 (12.5)
Acute intra-abdominal infection	4 (8.3)
Primary bacteremia	4 (8.3)
VAP	19 (39.5)
Implicated pathogen (number, %)	
Acinetobacter baumanii	18 (37.5)
Pseudomonas aeruginosa	5 (10.4)
Klebsiella pneumoniae	5 (10.4)
Escherichia coli	4 (8.3)
Proteus mirabilis	3 (6.3)
Other Gram-negatives	3 (6.3)
Enterococcus faecalis	1 (2.1)
Mortality (%)	22 (45.8)

APACHE, Acute Physiology and Chronic Health Evaluation.

neutropenia, defined as <500 neutrophils/mm^3; and intake of corticosteroids, defined as any dose ≥1 mg/kg equivalent prednisone for at least 1 month.

Severe sepsis and septic shock were defined according to standard definitions [6]. Patients with HAP could be patients with VAP or patients with nosocomial pneumonia. Diagnosis of VAP was assigned in any patient presenting with all the following: intubation for more than 48 h; new infiltrate in chest X-ray; purulent TBS; and clinical pulmonary infection score >6 [7]. Nosocomial pneumonia was defined as the presence of a new infiltrate in a chest X-ray in any patient hospitalized for >48 h and physical signs compatible with a lower respiratory tract infection [8].

Acute pyelonephritis was defined by the presence of all of the following: two spikes of fever >38°C; lumbar tenderness on examination or compatible ultrasound findings; and pyuria, defined as presence of >10 polymorphs/high power field under a light microscope [9].

Diagnosis of an intra-abdominal infection was made for any patient presenting with two spikes of fever >38°C and radiological findings on abdominal ultrasound or abdominal-computed tomography compatible with an intra-abdominal infection [10].

Diagnosis of primary bacteremia was made in any patient with Gram-negative pathogen or Gram-positive pathogen other than coagulase-negative *Staphylococcus* spp. or skin commensals isolated in at least one blood culture without any other infection site [10].

Quantitative cultures of TBS and quantitative urine cultures were performed and interpreted as described previously [7, 9]. Patients were followed up for 28 days, and survival was recorded.

Experimental design

Whole blood (10 ml) was collected from every patient within ≤12 h from signs of the first organ failure after puncture of one peripheral forearm vein under aseptic conditions and collected into sterile and pyrogen-free tubes. After centrifugation, serum was collected and stored at –70°C until application. Blood was also sampled from nine patients with SIRS and 11 patients with uncomplicated sepsis. SIRS and uncomplicated sepsis were diagnosed according to standard definitions [6]. All patients with SIRS were bearing multiple injuries, and an intense work-out failed to disclose the presence of any infection.

Heparinized venous blood (20 ml) was collected from each of 20 different healthy donors after venipuncture of one forearm vein under aseptic conditions. Blood was layered over Ficoll Hypaque and centrifuged. Isolated mononuclear cells (PBMCs) were washed three times with PBS (pH 7.2; Merck, Darmstadt, Germany). After counting of PBMCs in a Neubauer plate with trypan blue exclusion of dead cells, cells were distributed in a 96-well plate at a density of 5×10^5 cells/well at a volume of 100 μl/well in RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 2 mM glutamine in the presence of 100 U/ml penicillin G and 0.1 mg/ml gentamycin (Sigma-Aldrich, St. Louis, MO, USA). PBMCs were then incubated with 100 μl serum, added in wells, of healthy volunteers or of patients. Experiments were performed on separate days. At every experiment, one sample, coming from one healthy volunteer or from one patient, was added into one well containing PBMCs of one healthy volunteer. Wells without added serum were also used as controls. After

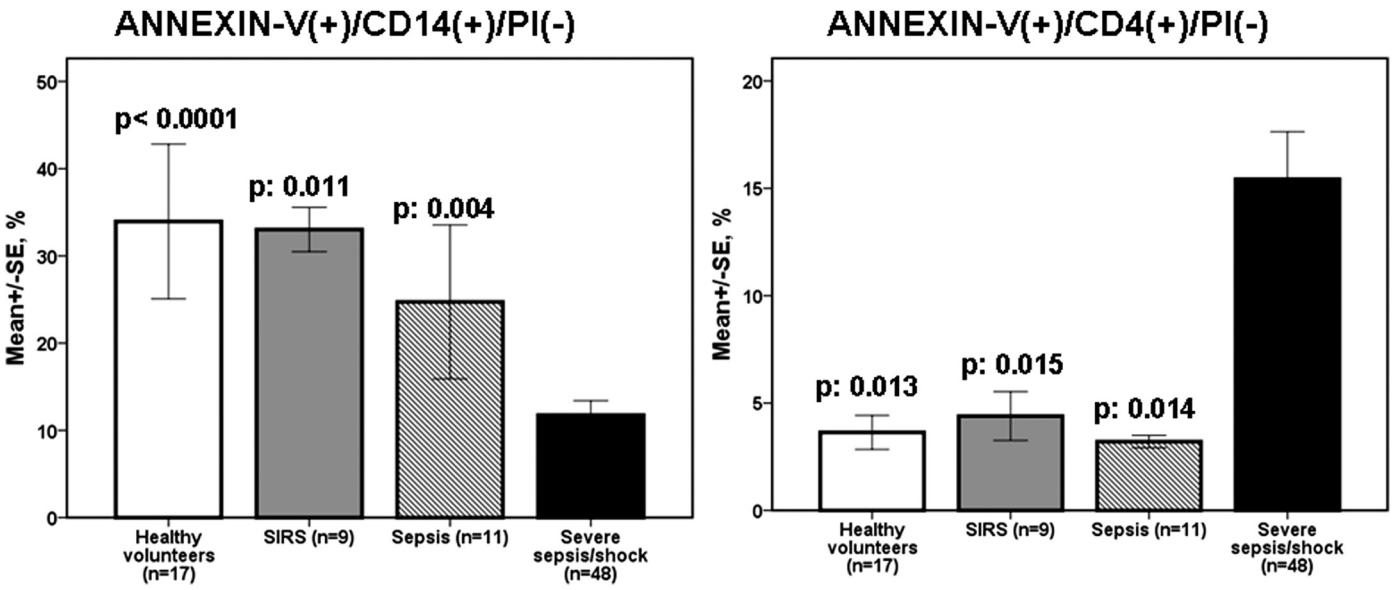

Figure 1. Apoptosis of CD14 monocytes and CD4 lymphocytes after incubation of cells isolated from healthy volunteers with sera collected from healthy volunteers and patients with SIRS, uncomplicated sepsis, and severe sepsis and/or septic shock. The represented number of patients is indicated in parentheses. *P* values indicate the level of statistical significance compared with patients with severe sepsis/shock.

24 h of incubation at 37°C in 5% CO_2, the lymphocyte-rich supernatant of each well was collected and centrifuged. Adherent monocytes were harvested with 0.25% thrypsin/0.02% EDTA solution (Biochrom) and centrifuged. One-half nonadherent cells and one-half adherent cells were lysed by a buffer containing 50 mM HEPES, 0.1% CHAPS, 5 mM DTT, and 0.1 mM EDTA (pH 7.4). After centrifugation for 10 min at 10,000 g under 4°C, activity of caspase-3 was estimated in the cytosolic extract by an enzymatic chromogenic assay (Biomol Research Laboratories, Plymouth Meeting, PA, USA). It was based on the rate of hydrolysis at 37°C of a substrate releasing p-nitroaniline over time, as assessed by sequential photometry at 410 nm. One caspase-3 inhibitor was also used in this assay as an internal control. The activity of caspase-3 in cell extracts was expressed as pmol/min.

The remaining cells were stained for 15 min in the dark with Annexin-V and the mAb anti-CD95 at the fluorochrome FITC (emission 520 nm; Immunotech, Marseille, France); with the mAb anti-CD4 and anti-CD14 at the fluorochrome PE (emission 575 nm; Immunotech); and with PI at the fluorochrome ECD (emission 613 nm; Immunotech). Stained cells were analyzed through the EPICS XL/MLS flow cytometer (Beckman Coulter Co., Miami, FL, USA). IgG isotypic negative controls at the fluorocolors FITC and PE (Immunotech) were applied before the start of analysis.

We chose to conduct experiments after incubation of PBMCs with sera for 24 h, as preliminary experiments with shorter times of incubation presented several technical difficulties in isolation of cell-rich fractions of nonadherent and adherent cells.

Some experiments were repeated with incubation of PBMCs with 20 μl (1:4 dilution) and with 5 μl of one patient's serum (1:20 dilution) and after mixing 50 μl of one patient's serum with 50 μl serum of one healthy volunteer.

In an attempt to investigate the type of pathway involved in the stimulation of apoptosis (extrinsic or intrinsic), experiments were repeated in the absence or presence of 3 μM of an inhibitor of caspase-8 (N-acetyl-Ile-Glu-Thr-Asp-CHO; Biomol Research Laboratories); 3 μM of an inhibitor of caspase-9 (N-acetyl-Leu-Glu-His-Asp-CHO; Biomol Research Laboratories); and 5 mM $CaCl_2$ (Ward Chemical, Canada), which is a stimulant of the intrinsic pathway of apoptosis [11]. The applied inhibitor of caspase-8 may also inhibit caspase-6, whereas the inhibitor of caspase-9 is specific for the enzyme, according to the instructions of the manufacturer.

Statistical analysis

Results were expressed as means ± SE. Comparisons between two groups were done by Student's *t* test. Comparisons between more than two groups were done with ANOVA with post hoc Bonferroni adjustments for multiple comparisons. Time intervals from signs of the first organ failure until blood sampling were noted for every single patient; quartiles were estimated. Comparisons of

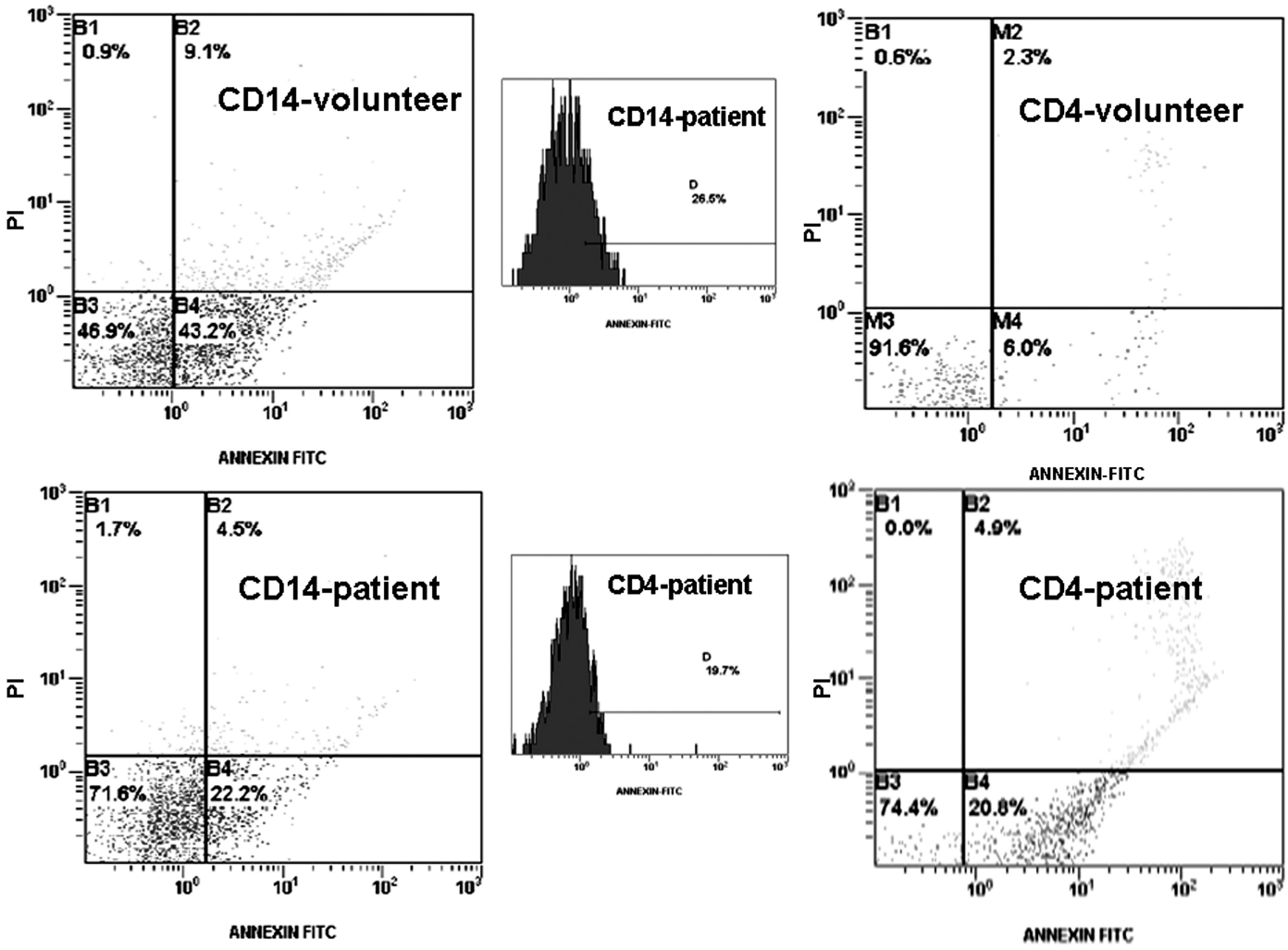

Figure 2. Flow charts of CD14 monocytes and CD4 lymphocytes stained for the protein Annexin-V and for PI of one healthy volunteer after incubation with serum of one healthy volunteer and one patient with severe sepsis.

apoptosis of monocytes and of apoptosis of lymphocytes between quartiles were done with ANOVA with post hoc Bonferroni adjustments for multiple comparisons, and comparisons between patients without septic shock and those with septic shock were done by Student's *t* test. Comparisons of apoptosis of monocytes and of apoptosis of lymphocytes before and after mixing patients' serum with healthy volunteers' serum as well as similar comparisons before and after serum dilution were done by Wilcoxon's rank sum test. Any value of $P < 0.05$ was considered significant.

RESULTS

Demographic characteristics of enrolled patients with severe sepsis/shock are given in **Table 1**. Mean ± SD age of the nine patients with SIRS was 50.3 ± 22.5 years and of the 11 patients with uncomplicated sepsis, 61.8 ± 14.2 years. Five patients with SIRS were male and four female; four patients with uncomplicated sepsis were male and seven female. The causes of uncomplicated sepsis were acute pyelonephritis (three patients), HAP (three patients), primary bacteremia (three patients), and VAP (two patients). Isolated microorganisms among patients with uncomplicated sepsis were *K. pneumoniae* (two patients), *Enterobacter aerogenes* (two patients), *A. baumannii* (one patient), and *P. aeruginosa* (one patient). None of patients with SIRS or uncomplicated sepsis died.

Mean ± SE apoptosis of CD14 monocytes of wells without any added serum was 34.55 ± 12.90% and of wells with 100 μl serum of healthy volunteers, 26.91 ± 8.04 (pNS). Mean ± SE apoptosis of CD4 lymphocytes of wells without any added serum was 3.11 ± 1.08% and of wells with 100 μl serum of healthy volunteers, 4.16 ± 1.20 (pNS).

Incubation with serum of patients with severe sepsis/shock inhibited apoptosis of CD14 monocytes and induced apoptosis of CD4 lymphocytes, whereas this was not the case for patients with SIRS and for patients with uncomplicated sepsis (**Fig. 1**). Indicative flow charts are given in **Fig. 2**. As a consequence, the study was focused on the phenomenon that takes place in the event of severe sepsis/shock.

Blood was sampled in 10 patients within 1.0–3.5 h after signs of the first failing organ, in 15 patients within 4.0–7.5 h after signs of the first failing organ, in 10 patients within 8.0–11.0 h after signs of the first failing organ, and in 13 patients 12 h after signs of the first failing organ. Mean ± SE apoptosis of CD14 monocytes after incubation of PBMCs of healthy volunteers with serum sampled at these respective time intervals was 4.06 ± 1.72%, 11.15 ± 3.15%, 15.56 ± 5.50%, and 15.58 ± 2.78% (pNS between them). Mean ± SE apoptosis of CD4 lymphocytes after incubation of PBMCs of healthy volunteers with serum sampled at these respective time intervals was 20.19 ± 4.60%, 17.68 ± 5.05%, 10.06 ± 3.02%, and 9.92 ± 3.23% (pNS between them). No differences were found regarding apoptosis of CD14 monocytes and apoptosis of CD4 lymphocytes after incubation with serum derived from patients without septic shock and after incubation with serum derived from patients with septic shock.

Inhibition of apoptosis of CD14 monocytes after incubation with serum of patients with severe sepsis/shock did not differ between survivors and nonsurvivors (12.49 ± 1.99% for survivors and 10.62 ± 2.87% for nonsurvivors; pNS). However, that of CD4 lymphocytes was greater among nonsurvivors compared with survivors (10.23 ± 2.20% for survivors and 20.80 ± 3.69% for nonsurvivors, P: 0.015).

We hypothesized that a circulating factor may exist in serum modulating the rate of apoptosis of monocytes and of lymphocytes. This hypothesis was confirmed after repeating experiments using dilutions of patients' sera. Inhibition of apoptosis of CD14 monocytes disappeared when serum was diluted or after mixing

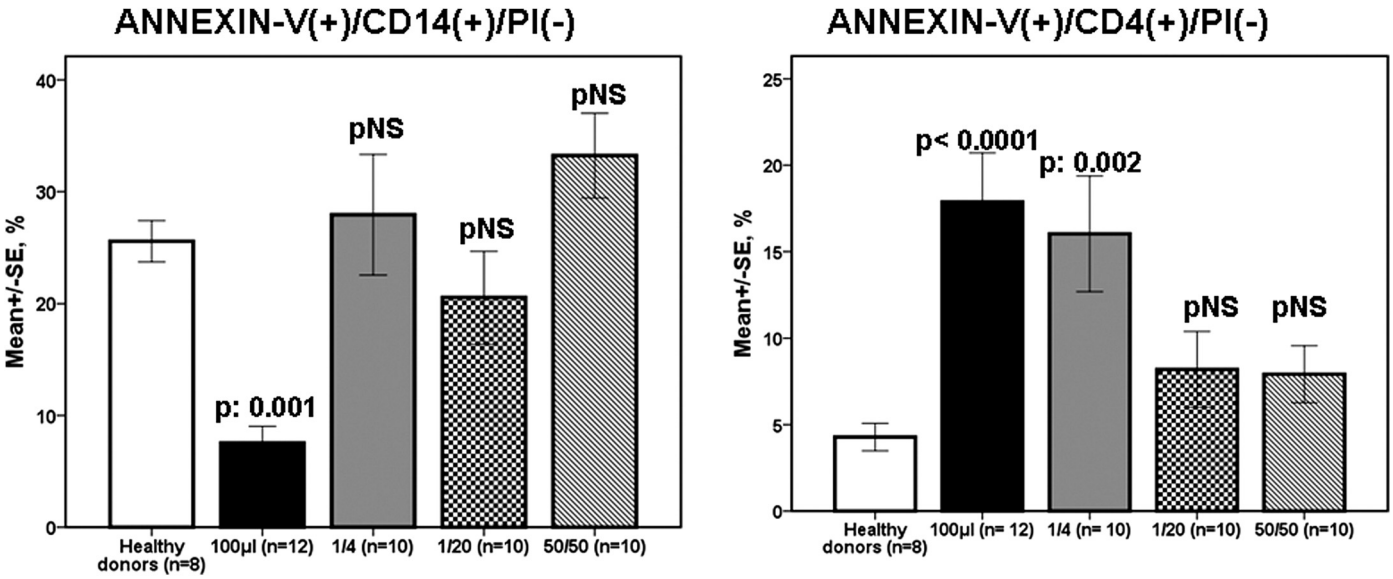

Figure 3. Apoptosis of CD14 monocytes and CD4 lymphocytes of cells isolated from healthy volunteers after incubation with sera collected from patients with severe sepsis and/or septic shock. Incubation was done with 100 μl patients' serum in every well, with 20 μl patients's serum in every well (1/4 dilution), with 5 μl patients' serum in every well (1/20 dilution), or with mixing 50 μl patients' serum with 50 μl healthy donors' serum in every well. The represented number of patients is indicated in parentheses. *P* values refer to comparisons with healthy donors.

serum of patients with serum of healthy donors (**Fig. 3**). In a similar way, induction of apoptosis of CD4 lymphocytes was decreased upon dilution of patients' serum and after mixing serum of patients with serum of healthy volunteers.

To investigate if this factor is acting on the cellular level through the extrinsic pathway or through the intrinsic pathway of apoptosis, experiments were repeated in the absence or presence of one caspase-8 inhibitor and one caspase-9 inhibitor, respectively (**Fig. 4**). Addition of both inhibitors did not affect the rate of apoptosis of monocytes. However, the addition of $CaCl_2$ reversed a serum-induced decrease of apoptosis of CD14 lymphocytes. Results on CD4 lymphocytes showed a decrease of serum-induced apoptosis by the addition of both inhibitors.

Incubation with patients' serum induced expression of CD95 on CD14 monocytes and CD4 lymphocytes (**Fig. 5**).

The activity of caspase-3 was estimated in cell lysates (**Fig. 6**). In lysates of lymphocyte-rich, nonadherent cells, the activity of caspase-3 was greater for cells incubated with serum of patients than cells incubated with serum of healthy volunteers. The opposite finding was shown for the activity of caspase-3 of cell lysates of the monocyte-rich, adherent cells.

DISCUSSION

The present study was based on previous findings showing that sepsis was accompanied by apoptosis of white blood cells and mainly of lymphocytes [3, 12, 13]. The salient finding was that serum collected early, i.e., ≤12 h from signs of the first failing organ when incubated with PBMCs of healthy volunteers, induced apoptosis of CD4 lymphocytes and inhibited apoptosis of monocytes (Fig. 1). Although sepsis-induced apoptosis is a well-described phenomenon involving a variety of cells such as CD4 lymphocytes, B lymphocytes, splenocytes, and gut epithelia, it is presented for the first time that serum of patients, collected early after signs of severe sepsis/shock appeared, modulates apoptosis of monocytes and CD4 lymphocytes of healthy cells. Serum samples exert a completely opposite effect on monocytes and on CD4 monocytes, probably depending on the type of failing organ.

Aiming to investigate the probable mechanism of that phenomenon, we hypothesized that one or several factors might circulate in the sera of patients with severe sepsis/shock, which when applied ex vivo on healthy cells, induced these phenomena. This factor should be of major pathophysiological importance, as its presence is related to final outcome. The presence of that factor was rendered more probable after finding that serum dilution decreased the effect on healthy cells. Inhibition of the effect of serum coming from patients after 1:1 mixing with serum coming from healthy volunteers suggested that another factor may circulate in normal serum that blocks the phenomenon and that is depleted in sepsis (Fig. 3).

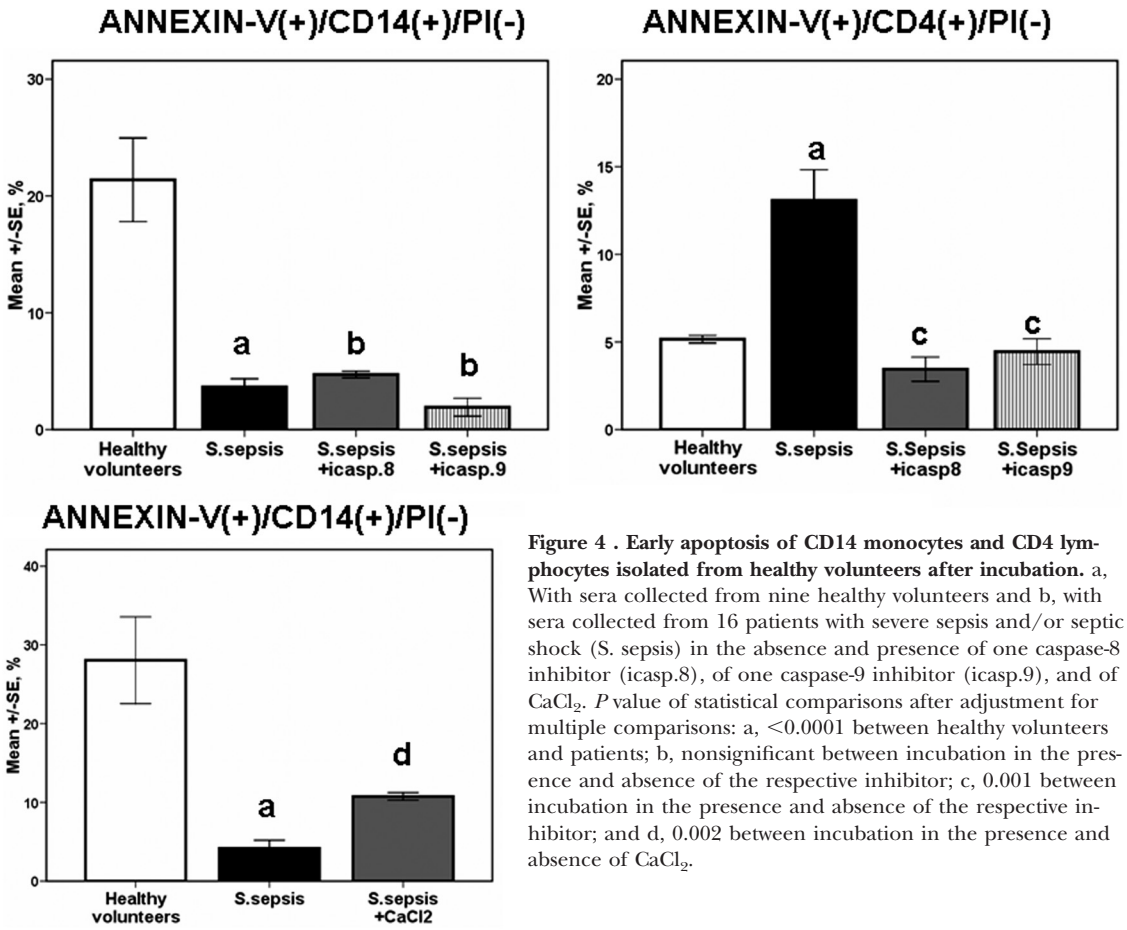

Figure 4 . Early apoptosis of CD14 monocytes and CD4 lymphocytes isolated from healthy volunteers after incubation. a, With sera collected from nine healthy volunteers and b, with sera collected from 16 patients with severe sepsis and/or septic shock (S. sepsis) in the absence and presence of one caspase-8 inhibitor (icasp.8), of one caspase-9 inhibitor (icasp.9), and of $CaCl_2$. *P* value of statistical comparisons after adjustment for multiple comparisons: a, <0.0001 between healthy volunteers and patients; b, nonsignificant between incubation in the presence and absence of the respective inhibitor; c, 0.001 between incubation in the presence and absence of the respective inhibitor; and d, 0.002 between incubation in the presence and absence of $CaCl_2$.

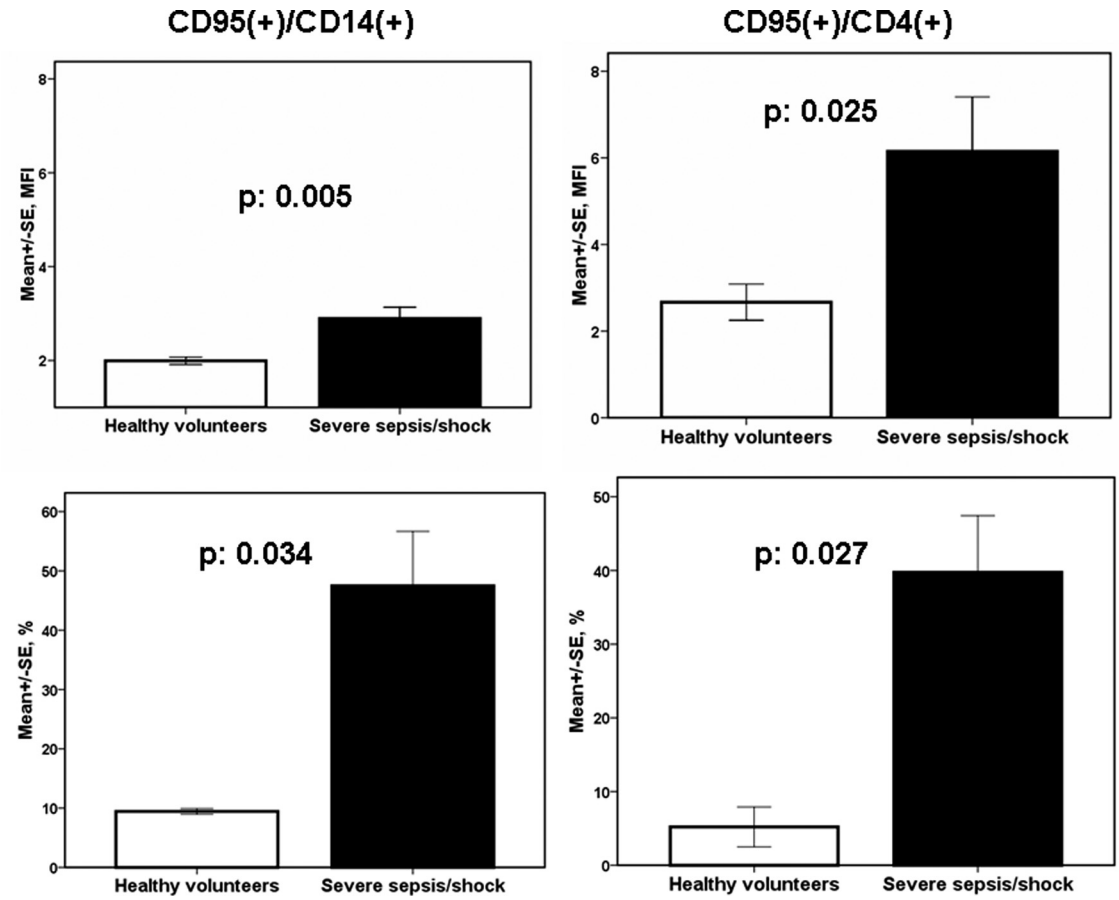

Figure 5. Expression of CD95 on CD14 monocytes and CD4 lymphocytes isolated from healthy volunteers after incubation with sera collected from 10 healthy volunteers and from 15 patients with severe sepsis and/or septic shock. *P* values indicate the levels of statistical significance between groups. MFI, Mean fluorescence intensity.

This factor seemed to stimulate the intrinsic and extrinsic pathways of apoptosis in CD4 lymphocytes, as suggested by the observed decrease after addition of caspase-8 and caspase-9 inhibitors and by the observed increase of Fas (CD95) expression on CD4 lymphocytes (Figs. 4 and 5). As a result of stimulation of both apoptotic pathways, the activity of caspase-3 was increased in the lymphocyte-rich lysates (Fig. 6). In a previous work, Hotchkiss et al. [14] showed that apoptosis of lymphocytes in sepsis may occur by both pathways. However, the present study proved that serum of patients with severe sepsis/shock may stimulate the intrinsic and extrinsic pathways of apoptosis in CD4 lymphocytes isolated from healthy volunteers.

This circulating factor seemed to modulate the two apoptotic pathways in a different way in monocytes of healthy volunteers than the one described for lymphocytes. More precisely (Figs. 4 and 5), it inhibited activation of the intrinsic pathway, as apoptosis was unaffected after addition of the caspase-9 inhibitor and increased after stimulation with $CaCl_2$, and it inhibited activation of the extrinsic pathway, as apoptosis was unaffected after addition of caspase-8 inhibitor. However, expression of CD95 was expressed on CD14 monocytes. This may imply that although this circulating factor may induce expression of CD95, whatever is necessary for the priming of the extrinsic apoptotic pathway [15], stimulation failed to be intracellularly linked. The end result was a decrease of caspase-3 activity in cell lysates (Fig. 6).

Activation of the mitochondrian pathway of apoptosis has been reported previously in a study enrolling only 18 patients [16]. In a previous study of our group in patients with septic shock as a result of VAP, it was found that patients could be divided into two groups regarding the rate of apoptosis of monocytes within the first 24 h: those with >50% and those with ≤50%. Survival of the former was prolonged compared with the latter. The existence of an early circulating factor modulating apoptosis of monocytes may help to understand the great variability of the rate of apoptosis of monocytes in our former study of patients with VAP [5].

Three main limitations of this study should be addressed: most data are based on the expression of Annexin-V on cell membranes assessed by flow cytometric analysis, however findings are also confirmed through estimation of the caspase-3 activity in cell lysates; some of the apoptosis findings may be attributed to cell starvation in the medium, however the use of appropriate controls makes this unlikely; and no data are provided for the exact nature of the circulating factor that modulates apoptosis. This factor may be part of the offending pathogens or released during the septic process. LPS of the cell wall of Gram-negative may

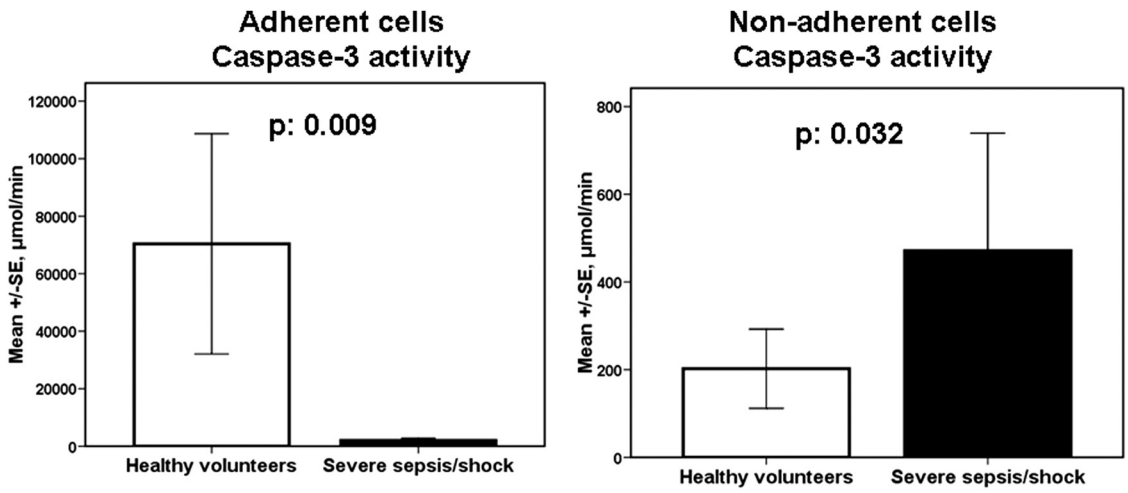

Figure 6. Caspase-3 activity of lysates of monocyte-rich, adherent cells and of lymphocyte-rich, nonadherent cells isolated from healthy volunteers after incubation with sera collected from 15 healthy volunteers and from 48 patients with severe sepsis and/or septic shock. *P* values indicate the levels of statistical significance between groups.

stimulate apoptosis of lymphocytes [17]. LPS may be one modulator of apoptosis circulating in serum, as isolated pathogens were Gram-negative bacteria (Table 1).

The presented findings indicate the existence of a circulating factor in serum of patients with severe sepsis/shock potent to modulate apoptosis of mononuclear cells. This factor already exists in serum of patients as early as the first 12 h after signs of the first failing organ. When applied ex vivo on healthy cells, this factor induces apoptosis of CD4 lymphocytes and inhibits apoptosis of monocytes. These findings may create a background of future therapeutic developments to modulate apoptosis of CD4 lymphocytes and monocytes in critically ill septic patients.

AUTHORSHIP

I.V. performed incubation assays, performed assays for caspase-3, and wrote the manuscript. H.K., V.K., A.S., and A.K. contributed to flow cytometry analysis and in the collection of clinical data. C.R. contributed in the enrollment of patients and in the collection of clinical data. E.J.G-B. designed the study, performed statistical analysis, and drafted the manuscript.

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