Gene Expression Profiling of Apoptosis Regulators in Patients with Sepsis

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Key Words

Apoptosis · Granulocytes · Immunosuppression · Lymphocytes · Multiplex ligation-dependent probe amplification · Monocytes · Sepsis

Abstract

Introduction: Sepsis is associated with a dysregulation of apoptosis in immune cells, which has been implicated in both immunosuppression and multiple organ failure. We describe the expression profiles of genes encoding key regulators of apoptosis in highly purified monocytes, granulocytes and CD4+ T lymphocytes. **Methods:** Sixteen patients with sepsis were recruited from the intensive care unit and were compared with 24 healthy controls. RNA was isolated from highly purified monocyte, granulocyte and CD4+ T-lymphocyte populations. Gene expression profiles were determined using multiplex ligation-dependent probe amplification for the simultaneous detection of 30 pro- and anti-apoptotic target genes. **Results:** Relative to healthy controls, patients with sepsis showed increased transcription of both pro- and anti-apoptotic genes in peripheral blood leukocytes. Specific monocyte, granulocyte and CD4+ T-lymphocyte mRNA profiles were identified. Anti-apoptotic profiles were found in monocytes and granulocytes, while CD4+ T lymphocytes

displayed a foremost pro-apoptotic mRNA profile. **Conclusions:** These data indicate that in patients with sepsis, the alterations in apoptosis of circulating leukocytes occur in a cell-specific manner.

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Introduction

Sepsis is defined as a systemic inflammatory response to infection and it is one of the leading causes of death in the Western World [1]. An important feature of the host response to sepsis is a dysregulation of apoptosis in immune cells [2–6]. Apoptosis is a physiological and well-controlled process that is essential for the resolution of immune responses and the clearance of inflammation. In sepsis, the disturbed regulation of apoptosis, which involves lymphocytes, dendritic cells, monocytes/macrophages and granulocytes, has been implicated in both immunosuppression and multiple organ failure [2–6].

Patients with sepsis display – after surviving the initial hyperinflammatory phase – features of immunosuppression, which is believed to contribute to the susceptibility of septic patients to nosocomial infections [3–5, 7]. Extensive apoptosis of lymphocytes was found in the circu-

lation of and at autopsy in septic patients [5, 8–10], findings which confirmed observational studies in experimentally induced sepsis in animals [11, 12]. The resulting decrease in the numbers of critical effector cells and the induction of T-helper 2 cell responses in surviving immune cells are considered to impair innate and adaptive immune responses [5, 13]. In support of a pivotal role for lymphocyte apoptosis in septic death, prevention or inhibition of apoptosis of lymphocytes improved survival in experimental sepsis [14–16]. Unlike lymphocytes, granulocytes physiologically undergo constitutive apoptosis within days [6], and their response to sepsis is characterized by a decrease in apoptosis [6, 17]. The extended lifespan of granulocytes in sepsis has been implicated in the pathogenesis of organ injury and failure through their uncontrolled release of oxygen radicals and proteolytic enzymes [6].

Knowledge of the factors which contribute to the altered apoptosis in immune cells during sepsis is limited. Several studies have suggested activation of both mitochondrial (caspase-9) and death receptor (caspase-8) pathways in whole-blood lymphocytes obtained from septic patients [18] and in lymphoid tissue of septic mice [14, 19, 20]. A very recent study, using microarrays with >50,000 transcripts, showed enhanced activation of genes involved in apoptosis in peripheral blood mononuclear cells of sepsis patients [21]. Moreover, in a separate study, sepsis patients displayed gene expression profiles in whole-blood leukocytes compatible with increased apoptosis [22]. These investigations did not provide insight regarding the contribution of apoptotic mediators in purified leukocyte subsets. We here sought to characterize the expression profiles of genes encoding key regulators of apoptosis in purified monocytes, granulocytes and T lymphocytes from patients with sepsis, using multiplex ligation-dependent probe amplification (MLPA) for the simultaneous detection of 30 pro- and anti-apoptotic target genes.

Patients and Methods

Patients and Control Subjects

Patients were recruited prospectively at the Academic Medical Center (Amsterdam, The Netherlands) from 2005 to 2006. Sepsis was defined as (suspicion of) infection (at any location) plus a systemic inflammatory response syndrome [23]. According to the systemic inflammatory response syndrome criteria, patients had to meet at least three of the following four criteria: a core temperature ≥ 38 or $\leq 36^{\circ}\text{C}$; a heart rate of ≥ 90 beats/min; a respiratory rate of ≥ 20 breaths/min, a $\text{PaCO}_2 \geq 32$ mm Hg, or the use of mechanical ventilation for an acute respiratory process, and a

white cell count of $\geq 12 \times 10^9/\text{l}$ or $\leq 5 \times 10^9/\text{l}$. These definitions have been used in large clinical trials and were modified according to the latest revisions [23, 24]. Exclusion criteria were the use of dialysis and/or immunosuppressive therapy (including therapy with steroids), known disorders of coagulation and concomitant infection with human immunodeficiency virus. Blood samples were drawn within 24 h after sepsis was diagnosed. Healthy gender- and age-matched blood donors recruited from a nearby retirement home served as a control population. The study was approved by the institutional ethics and research committees; written informed consent was obtained from all subjects (or their relatives) before enrollment in the study.

Cell-Sorted Populations

Heparinized blood samples were drawn from the antecubital vein and immediately put on ice. Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl , 10 mM KHCO_3 and 0.1 mM ethylenediaminetetra-acetic acid, pH 7.4); the remaining cells were washed twice with PBS. Leukocytes were sorted into monocytes, granulocytes and CD4+ T lymphocytes by FACS. Briefly, unstimulated whole-blood leukocytes were labeled with CD4-PerCP (BD Pharmingen, San Diego, Calif., USA). Subsequently, monocytes (forward scatter high/side scatter low), granulocytes (forward scatter low/side scatter high) and CD4+ T lymphocytes (CD4+ high) were separated by use of a FACSaria instrument (BD Pharmingen). All sorted subsets were >95% pure as determined by their scatter patterns on flow cytometry. After isolation, whole-blood leukocytes and 1×10^5 monocytes, granulocytes and CD4+ T cells were dissolved in TRIzol and stored at -80°C for RNA isolation.

RNA Analysis Using MLPA

RNA was isolated and analyzed by MLPA (MRC-Holland, Amsterdam, The Netherlands) as described previously [25–28] for the simultaneous detection of 30 target genes encoding key mediators of apoptosis (table 1). In short, total RNA is used to prepare cDNA using gene-specific RT primers. MLPA probes consist of two oligonucleotides that anneal to adjacent sites on a target sequence and are then ligated by a heat-stable ligase. Each ligated probe gives rise to an amplification product of unique length. The output is analyzed using capillary sequencer and spreadsheet software [25–28]. All samples were tested with the same batch of reagents. The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of the β_2 -microglobulin (B2M) control gene, resulting in the relative abundances of mRNAs of the genes of interest [25–28]. Sepsis did not influence B2M mRNA expression in whole-blood leukocytes and in the sorted cell subsets (data not shown).

Statistical Analysis

Values are expressed as means $\pm$ SEM. Data were checked for normal distribution and equal variances of the residuals. If Shapiro-Wilk was >0.90 , data were considered normally distributed. All statistical comparisons between sepsis patients and controls were then made by Mann-Whitney U test, since the vast majority of parameters measured were not normally distributed. To account for multiple testing, $p < 0.01$ was considered statistically significant. All analyses were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, Calif., USA).

Table 1. Apoptotic genes analyzed by MLPA

Functional category and gene product	
<i>Bcl2 family</i>	
Bcl2-like, anti-apoptotic	
BCL2A1	BCL2-related protein A1
BCLX	BCL2-like 1
BCL2	B-cell CLL/lymphoma 2
BCL2L2	BCL2-like 2
BCL2L10	BCL2-like 10
MCL-1	myeloid cell leukemia 1
BAX-like, pro-apoptotic	
BAX	BCL2-associated X protein
BH3-only, pro-apoptotic	
BIM	BCL2-interacting protein BIM
BAD	BCL2 antagonist of cell death
BIK	BCL2-interacting killer
BMF	BCL2-modifying factor
BNIP3L	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like
BID	BH3-interacting domain death agonist
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
MAP-1	modulator of apoptosis 1
PUMA	p53-upregulated modulator of apoptosis
HRK	hara-kiri
<i>IAP family</i>	
Anti-apoptotic	
BIRC-6	baculoviral IAP repeat-containing protein-6
BIRC-3	baculoviral IAP repeat-containing protein-3
BIRC-1	baculoviral IAP repeat-containing protein-1
BIRC-2	baculoviral IAP repeat-containing protein-2
<i>Miscellaneous</i>	
Pro-apoptotic	
Apaf-1	apoptotic protease-activating factor 1
AIF	apoptosis-inducing factor
SMAC	second mitochondria-derived activator of caspase
TNFRSF21	tumor necrosis factor receptor superfamily member 21
CDKN1a	cyclin-dependent kinase inhibitor 1a
Anti-apoptotic	
FLIP v11/22	FLICE-inhibitory protein; 2 isoforms
MIL-1	RAF-1; oncogene MIL

Results

Patient Characteristics

Sixteen patients with sepsis and 24 healthy control subjects were enrolled. Patient characteristics are provided in table 2. Sepsis patients displayed an increased amount of granulocytes (control 3.9 ± 0.26 vs. sepsis $9.9 \pm 0.42 \times 10^9/l$; $p < 0.0001$), a decreased amount of CD4+ T cells (control 9.4 ± 0.06 vs. sepsis $3.5 \pm 0.05 \times 10^9/l$;

Table 2. Patient characteristics

Characteristics	Patients with sepsis (n = 16)	Control population (n = 24)
Age, years	57 ± 5	66 ± 5
Male sex	10 (63)	12 (50)
APACHE II	19 ± 2	NA
ICU stay, days	11 ± 3	NA
Sepsis due to		
Peritonitis	3 (19)	
Pneumonia	8 (50)	
UTI	0	
Other	5 (31)	
Pos. blood culture	15 (94)	NA
<i>Klebsiella pneumoniae</i>	1	
<i>Escherichia coli</i>	1	
<i>Pseudomonas aeruginosa</i>	2	
<i>Haemophilus influenza</i>	1	
<i>Enterobacter</i>	4	
<i>Streptococcus pneumoniae</i>	4	
<i>Staphylococcus aureus</i>	1	
Other	7	
Mortality	2 (13)	NA

APACHE = Acute physiology and chronic health evaluation; ICU = intensive care unit; Pos. = positive; NA = not applicable; UTI = urinary tract infection. Data are means $\pm$ SEM or numbers (%) when appropriate.

$p < 0.0001$) and no difference in the amount of monocytes (control 0.5 ± 0.04 vs. sepsis $0.7 \pm 0.11 \times 10^9/l$; nonsignificant) compared to healthy controls.

Profiles of mRNAs for Genes Encoding Pro-Apoptotic Mediators in Whole-Blood Leukocytes

To determine the mRNA profile encoding mediators of apoptosis in sepsis, MLPA was performed on mRNAs isolated from unfractionated leukocytes and sorted monocyte, granulocyte and CD4+ T-lymphocyte populations. In whole-blood leukocytes, sepsis was characterized by an increased expression of mRNAs encoding several pro-apoptotic mediators (BIM, BAX, BID, BNIP3L, APAF1 and CDKN1a; for all, $p < 0.05$ to 0.0001 vs. controls), while the expression of PUMA, PMAIP1, BMF, BAD, MAP1 and SMAC was not altered (table 3). Some pro-apoptotic mediators were not detectable in RNA from unfractionated leukocytes (TNFRSF21, AIF, BIK and HRK; data not shown).

Table 3. Apoptotic gene profile of whole-blood leukocytes

	Control	Sepsis
Anti-apoptotic genes		
BCLX	0.021 ± 0.005	0.070 ± 0.015***
BCL2A1	0.658 ± 0.082	2.013 ± 0.342***
MCL-1	1.149 ± 0.055	1.760 ± 0.152***
MIL-1	0.054 ± 0.010	0.099 ± 0.010*
BIRC1	0.151 ± 0.037	1.067 ± 0.241***
BIRC3	0.527 ± 0.048	0.255 ± 0.035***
BIRC6	0.121 ± 0.017	0.133 ± 0.009
FLIP V11	0.401 ± 0.026	0.854 ± 0.065***
FLIP V22	0.081 ± 0.019	0.066 ± 0.007
Pro-apoptotic genes		
CDKN1a	0.034 ± 0.008	0.077 ± 0.013*
PMAIP1	0.061 ± 0.012	0.057 ± 0.008
BAX	0.252 ± 0.032	0.401 ± 0.039**
BIM	0.225 ± 0.031	0.366 ± 0.020**
BID	0.268 ± 0.030	0.519 ± 0.025***
PUMA	0.052 ± 0.010	0.083 ± 0.012
BNIP3L	0.122 ± 0.020	0.246 ± 0.021***
APAF1	0.480 ± 0.048	1.107 ± 0.122***
BAD	0.001 ± 0.001	0.008 ± 0.004
BMF	0.040 ± 0.008	0.058 ± 0.014
MAP-1	0.021 ± 0.006	0.022 ± 0.006
SMAC	0.044 ± 0.010	0.065 ± 0.005

Expression of apoptotic mRNAs from sepsis patients and control subjects in whole-blood leukocytes (normalized to β 2M mRNA; mean $\pm$ SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. controls. BCL2L2, BCL2, BCL2L10, BIRC2, AIF, TNFRSF21, BIK and HRK expression was not detectable (data not shown).

Profiles of mRNAs for Genes Encoding Anti-Apoptotic Mediators in Whole-Blood Leukocytes

Concurrent with the upregulation of pro-apoptotic mediators, a similar upregulation of anti-apoptotic mediators was observed in whole-blood leukocytes of sepsis patients. Patients showed increased levels of FLIP V11, Bcl2A1, MCL-1, BIRC1, Bcl-X and MIL mRNA in whole-blood leukocytes compared to controls (MIL: $p < 0.05$; others: $p < 0.0001$ vs. controls), while BIRC3 mRNA was decreased compared to controls ($p < 0.0001$ vs. controls; table 3). mRNA levels of FLIP isoform V22 and BIRC6 were not altered in septic patients compared to control subjects (table 3), and Bcl2, Bcl2L2, Bcl2L10 and BIRC2 were below the detection level (data not shown).

Apoptotic Gene Profiles of Isolated Cell Populations

Gene expression profiling of whole-blood leukocytes does not provide further insight regarding the alterations

Table 4. Apoptotic gene profile of isolated monocytes

	Control	Sepsis
Anti-apoptotic genes		
BCLX	0.048 ± 0.007	0.058 ± 0.013
BCL2A1	0.591 ± 0.067	0.909 ± 0.160
MCL-1	1.119 ± 0.119	1.390 ± 0.077
MIL-1	0.176 ± 0.018	0.160 ± 0.031
BIRC1	0.636 ± 0.065	1.079 ± 0.190*
BIRC3	0.338 ± 0.029	0.156 ± 0.031***
BIRC6	0.213 ± 0.024	0.173 ± 0.031
FLIP V11	0.354 ± 0.031	0.483 ± 0.056*
FLIP V22	0.151 ± 0.038	0.131 ± 0.021
BCL2L2	0.008 ± 0.001	0.007 ± 0.002
Pro-apoptotic genes		
CDKN1a	0.294 ± 0.050	0.231 ± 0.047
PMAIP1	0.206 ± 0.034	0.060 ± 0.016***
BAX	0.488 ± 0.040	0.622 ± 0.069
BIM	0.418 ± 0.035	0.444 ± 0.048
BID	0.588 ± 0.034	0.583 ± 0.072
PUMA	0.067 ± 0.010	0.058 ± 0.012
BNIP3L	0.390 ± 0.031	0.356 ± 0.047
APAF1	0.772 ± 0.063	0.835 ± 0.080
BAD	0.014 ± 0.002	0.011 ± 0.003
BMF	0.190 ± 0.022	0.123 ± 0.027*
AIF	0.050 ± 0.007	0.050 ± 0.012
MAP-1	0.052 ± 0.008	0.036 ± 0.008
SMAC	0.117 ± 0.015	0.084 ± 0.016*
TNFRSF21	0.007 ± 0.003	0.000 ± 0.000

Expression of apoptotic mRNAs from sepsis patients and control subjects in fractionated monocytes (normalized to β 2M mRNA; mean $\pm$ SEM). * $p < 0.05$, *** $p < 0.001$, vs. controls. BCL2, BCL2L10, BIRC2, BIK and HRK expression was not detectable (data not shown).

in mRNA levels in distinct leukocyte subsets. Therefore, we analyzed the mRNA profiles of genes encoding apoptosis regulators in sorted cell populations of highly purified monocyte, granulocyte and CD4⁺ T-lymphocyte populations separately. Monocytes of septic patients were shown to display a foremost anti-apoptotic mRNA profile, with upregulated mRNA levels of the anti-apoptotic regulators FLIP isoform V11 and BIRC1 (both $p < 0.05$) and downregulated pro-apoptotic genes SMAC, PMAIP1 and BMF ($p < 0.05$, $p < 0.0001$ and $p < 0.05$, respectively; table 4). Simultaneously, anti-apoptotic mRNA of BIRC3 was downregulated in monocytes. Moreover, granulocytes also displayed a mainly anti-apoptotic profile, with enhanced mRNA levels of anti-apoptotic mediators FLIP V11, MCL-1, Bcl2A1 and BIRC1 (first two: $p < 0.05$; latter two: $p < 0.0001$; table 5). In contrast, CD4⁺ T lympho-

Table 5. Apoptotic gene profile of isolated neutrophils

	Control	Sepsis
Anti-apoptotic genes		
BCL2A1	0.660 ± 0.082	1.991 ± 0.364***
MCL-1	1.408 ± 0.135	1.899 ± 0.155*
BIRC1	0.043 ± 0.025	0.875 ± 0.235***
FLIP V11	0.526 ± 0.066	0.782 ± 0.089*
FLIP V22	0.179 ± 0.060	0.231 ± 0.060
Pro-apoptotic genes		
PMAIP1	0.053 ± 0.028	0.027 ± 0.020
BAX	0.111 ± 0.041	0.082 ± 0.026
BIM	0.129 ± 0.052	0.182 ± 0.063
BID	0.238 ± 0.051	0.291 ± 0.054
APAF1	0.432 ± 0.082	0.579 ± 0.113
BAD	0.111 ± 0.041	0.082 ± 0.026

Expression of apoptotic mRNAs from sepsis patients and control subjects in fractionated neutrophils (normalized to β 2M mRNA; means $\pm$ SEM). * $p < 0.05$, *** $p < 0.001$, vs. controls. BCLX, MIL-1, BIRC3, BIRC6, BCL2L2, BCL2, BCL2L10, BIRC2, CDKN1a, PUMA, BNIP3L, BMF, AIF, MAP-1, SMAC, TNFRSF21, BIK and HRK expression was not detectable (data not shown).

cytes of septic patients displayed an overall pro-apoptotic phenotype with upregulated mRNA levels of apoptotic mediators BIM, BAX, PMAIP1 and BNIP3L ($p < 0.05$). Additionally, mRNA expression of the anti-apoptotic mediator MCL-1 was enhanced in T lymphocytes of these patients ($p < 0.05$; table 6).

Discussion

Sepsis remains a major challenge for clinicians. Recent evidence indicates that the host response to sepsis involves immunosuppression, which contributes to the susceptibility of septic patients to nosocomial infections [3]. Enhanced lymphocyte apoptosis, resulting in decreased numbers of effector lymphocytes and more pronounced anti-inflammatory cytokine release, has been shown to play a major role in immune dysfunction [2–5, 10]. On the other hand, reduced granulocyte apoptosis may be involved in tissue injury due to prolonged activity of oxygen radicals and proteinases [6]. We here sought to obtain insight into which factors attribute to the altered apoptosis in different immune cells during sepsis. Our study is the first to perform an analysis of the mRNA profile of genes encoding pivotal regulators of apoptosis in highly purified monocyte, granulocyte and CD4+ T-lymphocyte cell

Table 6. Apoptotic gene profile of isolated CD4+ T lymphocytes

	Control	Sepsis
Anti-apoptotic genes		
BCLX	0.080 ± 0.008	0.075 ± 0.014
BCL2A1	0.153 ± 0.012	0.163 ± 0.030
MCL-1	1.135 ± 0.055	1.538 ± 0.148**
MIL-1	0.129 ± 0.011	0.167 ± 0.014
BIRC1	0.115 ± 0.013	0.158 ± 0.034
BIRC3	1.730 ± 0.098	1.314 ± 0.179
BIRC6	0.317 ± 0.021	0.370 ± 0.052
FLIP V11	0.300 ± 0.016	0.284 ± 0.041
FLIP V22	0.117 ± 0.016	0.146 ± 0.023
BCL2	0.084 ± 0.007	0.064 ± 0.012
Pro-apoptotic genes		
PMAIP1	0.031 ± 0.006	0.078 ± 0.028*
BAX	0.305 ± 0.026	0.468 ± 0.067*
BIM	0.452 ± 0.026	0.604 ± 0.048*
BID	0.084 ± 0.009	0.101 ± 0.017
PUMA	0.057 ± 0.008	0.041 ± 0.012
BNIP3L	0.116 ± 0.011	0.178 ± 0.031*
APAF1	0.199 ± 0.016	0.297 ± 0.076
AIF	0.043 ± 0.006	0.045 ± 0.010
MAP-1	0.125 ± 0.013	0.131 ± 0.025
SMAC	0.123 ± 0.010	0.124 ± 0.018

Expression of apoptotic mRNAs from sepsis patients and control subjects in fractionated CD4+ T cells (normalized to β 2M mRNA; mean $\pm$ SEM). * $p < 0.05$, ** $p < 0.01$, vs. controls. BCL2L2, BCL2L10, BIRC2, BAD, CDKN1a, BMF, TNFRSF21, BIK and HRK expression was not detectable (data not shown).

fractions. Our results indicate that in the circulation of patients with sepsis, monocytes and granulocytes predominantly display an anti-apoptotic gene expression profile, whereas CD4+ T cells demonstrate a pro-apoptotic gene expression profile.

Our findings indicate that isolated monocytes from septic patients display a predominantly anti-apoptotic phenotype, as reflected by the downregulation of pro-apoptotic mRNA levels of PMAIP1, BMF and SMAC, and the upregulation of anti-apoptotic mRNA levels of BIRC1 and FLIP. In contrast, blood monocyte dysfunction in sepsis was suggested to be associated with enhanced apoptosis, which was accompanied by decreased mRNA and protein expression of BCL2 and no obvious effect on the expression of BAX [29, 30]. Of note, in these earlier studies [29, 30] peripheral blood mononuclear cells, containing a mixture of monocytes and lymphocytes, were examined; mRNA levels of BCL2 and BAX were not detectable in highly purified monocytes in the

present study. Similarly, a recent study demonstrated enhanced expression of pro-apoptotic genes with concurrent reduced expression of the anti-apoptotic gene BCL2 in peripheral blood mononuclear cells of sepsis patients, using microarrays with >50,000 transcripts [21]. Our current study strongly suggests that lymphocytes (and not monocytes) drive this pro-apoptotic gene expression profile in unseparated circulating mononuclear cells. In accordance, BCL2 protein expression was reported to be downregulated in CD4+ T cells of patients with sepsis [22].

Neutrophils play a pivotal role in innate defense against infection by eliminating invading pathogens and typically use their constitutive programmed cell death machinery in the resolution of inflammation and cell turnover [6]. Previously, decreased apoptosis of peripheral blood neutrophils has been described in a sepsis model induced by cecal ligation and puncture in mice [31]. Furthermore, peripheral blood neutrophils showed decreased apoptosis in patients with systemic inflammatory response syndrome compared to healthy controls, and the plasma of these patients suppressed apoptosis of control neutrophils *ex vivo* [17]. It has been suggested that decreased apoptosis of neutrophils in an inflammatory state serves to fortify neutrophil-mediated killing, but can ultimately lead to organ dysfunction and failure [2, 6, 17]. *In vitro* experiments have led to the suggestion that lipopolysaccharide-induced upregulation of the inhibitor of apoptosis (IAP)-2 causes accelerated degradation of activated caspase-3 and might be responsible for reduced apoptosis in neutrophils during sepsis [32]. The current study underlines and extends these findings by showing that not only the IAP family member BIRC1, but also the BCL2 family members BCL2A1 and MCL-1 are strongly upregulated in neutrophils of septic patients. Whether induction of FLIP mRNA in neutrophils from septic patients is the result of activation of the death receptor-driven pathway or retaliatory activation of caspase-8 by activated caspase-3 remains to be investigated.

Lymphocyte apoptosis is considered to be an important pathophysiological consequence of severe sepsis [4, 5]. The sepsis-induced loss of lymphocytes is thought to contribute strongly to the immunosuppressive state in septic patients by means of extensive depletion of immune effector cells and the immunosuppressive effects of apoptotic cells on the immune system [4, 5, 13, 33]. As such, animal studies indicate that prevention of lymphocyte apoptosis may improve survival [14–16]. The present results underline the concept of massive apoptosis of lymphocytes during sepsis by demonstrating a pro-apoptotic

mRNA expression profile in CD4+ T lymphocytes as manifested by increased expression of BIM, PMAIP1 and BNIP3L in addition to increased BAX mRNA expression. Lymphocyte apoptosis in sepsis is suggested to occur by both death receptor and mitochondrial stress pathways, as shown by the involvement of both active caspases 8 and 9 in apoptotic cell death of circulating lymphocytes in patients with sepsis [18]. This concept is supported by studies in animal models of sepsis indicating activation of both death pathways [34, 35]. The current study did not show any sign of activation of the death receptor pathway, since no alterations were found in death receptor TNFRSF21 expression. Moreover, cFLIP, an inhibitor of death receptor signaling, appeared to be upregulated in CD4+ T lymphocytes. Noteworthy, although a decrease in BCL2, an inhibitor of mitochondrial-mediated apoptosis, is considered to contribute to the mitochondrial-mediated death in circulating lymphocytes of septic patients, BCL2 was not decreased in septic CD4+ T lymphocytes in the current study.

We investigated the mRNA expression profile of 30 apoptotic genes in whole-blood leukocytes and in sorted granulocytes, monocytes and CD4+ T lymphocytes. Both pro- and anti-apoptotic genes were altered in whole-blood leukocytes, whereas each isolated cell type displayed a rather specific apoptotic gene expression profile, emphasizing that measuring the expression of an array of genes in whole-blood leukocytes is not a vigorous reflection of the actual profile of these genes in different cell types. Since the majority of whole-blood leukocytes consist of granulocytes – especially in patients with sepsis – the contribution of the monocyte and CD4+ T-lymphocyte fraction to the mRNA profile of whole-blood leukocytes is limited and the mRNA extracted from the isolated cell types is much more enriched and concentrated. This might explain why some apoptotic genes were expressed in the isolated cell types, but not in the mRNA profile of whole-blood leukocytes, such as BMF, SMAC and BCL2L2 (for monocytes) and FLIP isoform V22 and AIF (for CD4+ T lymphocytes). Notably, our study indicates that gene expression profiles studied in whole-blood leukocytes to obtain information on the expression of apoptosis regulators do not provide adequate information on gene expression profiles in leukocyte subsets purified from the same sample. This knowledge is important considering that many investigations have made use of whole-blood leukocytes to study gene expression profiles in critically ill and/or sepsis patients.

The current study has several limitations. First, although the procedure for the isolation of the individual

cell populations and the subsequent storage of RNA was standardized for each individual (sepsis patient/control), we cannot exclude artifactual changes in gene expression during the cell-sorting process. Second, a kinetic analysis over multiple time points in a larger population would have increased the value of our observations. Third, gene expression profiles in purified B cells and possibly dendritic cells were not examined here and would have been of interest. Fourth, inclusion of other patient populations, such as critically ill patients without infection, would have increased the value of our findings. Last, phenotypic confirmation of the gene expression profiles at the protein level would have been of value. Unfortunately, sam-

ples for measuring proteins by flow cytometry or Western blot were not obtained.

This study is the first to report on the expression of genes encoding apoptosis regulators in purified granulocytes, monocytes and CD4⁺ T lymphocytes in patients with sepsis, focusing on the early phase of the septic host response. Our data underline the notion that apoptosis, specifically of T lymphocytes, is an important feature in patients with sepsis, providing insight into potential factors responsible for initiating cell suicide. Knowledge of the regulation of apoptosis may allow for the development of more targeted therapies, which in turn may result in more individualized therapeutic interventions.

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