Received: 11 March 2021 | Revised: 6 April 2021 | Accepted: 8 April 2021

DOI: 10.1111/cei.13601

British Society for immunology | Clinical & Experimental Immunology
The Journal of Translational Immunology

ORIGINAL ARTICLE

Intracellular expression of granzymes A, B, K and M in blood lymphocyte subsets of critically ill patients with or without sepsis

M. Isabel García-Laorden[1,2,3] | **Arie J. Hoogendijk**[1] | **Maryse A. Wiewel**[1] | **Lonneke A. van Vught**[1] | **Marcus J. Schultz**[4,5,6] | **Niels Bovenschen**[7] | **Alex F. de Vos**[1] | **Tom van der Poll**[1,8]

[1]Center for Experimental and Molecular Medicine (CEMM), Amsterdam University Medical Centers, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

[2]CIBER de Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain

[3]Research Unit, Hospital Universitario de Gran Canaria Dr. Negrín, Las Palmas de Gran Canaria, Spain

[4]Department of Intensive Care Medicine, and Laboratory of Experimental Intensive Care and Anesthesiology (LEICA), Amsterdam University Medical Centers, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

[5]Mahidol–Oxford Tropical Medicine Research Unit (MORU), Mahidol University, Bangkok, Thailand

[6]Nuffield Department of Medicine, University of Oxford, Oxford, UK

[7]Department of Pathology and Center for Translational Immunology, University Medical Center Utrecht, Utrecht, the Netherlands

[8]Division of Infectious Diseases, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, the Netherlands

Correspondence
M. Isabel García-Laorden, Center for Experimental and Molecular Medicine (CEMM), Amsterdam University Medical Centers, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.
Email: ihalemgl@yahoo.es

Funding information
FP7 People: Marie-Curie Actions, Grant/Award Number: PIEF-GA-2011-300895; Center for Translational Molecular Medicine, Grant/Award Number: grant 04I-201

Summary

Sepsis is a complex syndrome related to an infection-induced exaggerated inflammatory response, which is associated with a high mortality. Granzymes (Gzm) are proteases mainly found in cytotoxic lymphocytes that not only have a role in target cell death, but also as mediators of infection and inflammation. In this study we sought to analyse the intracellular expression of GzmA, B, M and K by flow cytometry in diverse blood lymphocyte populations from 22 sepsis patients, 12 non-infected intensive care unit (ICU) patients and 32 healthy controls. Additionally, we measured GzmA and B plasma levels. Both groups of patients presented decreased percentage of natural killer (NK) cells expressing GzmA, B and M relative to healthy controls, while sepsis patients showed an increased proportion of $CD8^+$ T cells expressing GzmB compared to controls. Expression of GzmK remained relatively unaltered between groups. Extracellular levels of GzmB were increased in non-infected ICU patients relative to sepsis patients and healthy controls. Our results show differential alterations in intracellular expression of Gzm in sepsis patients and non-infected critically ill patients compared to healthy individuals depending on the lymphocyte population and on the Gzm.

KEYWORDS
granzymes, infection, inflammation, sepsis

INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection (1). It is a major public health problem, associated with many hospital admissions and mortality worldwide (1–3). Cells of both the innate and adaptive immune systems play a crucial role in the pathophysiology of sepsis. Besides excessive inflammation, sepsis can be associated with a marked lymphopenia, involving $CD4^+$ and $CD8^+$ T cells and natural killer (NK) cells (4). Apoptosis has been indicated as the cause of this depletion, although migration from peripheral blood to sites of infection has been also proposed for NK cells (5).

Granzymes (Gzm) are a family of serine proteases originally identified in granules of cytotoxic lymphocytes (6). Five different Gzm have been identified in humans (A, B, H, K and M) (7). Gzm are constitutively expressed in several cell types. GzmA, B and M are mainly found in $CD8^+$ T and NK cells, but also in NK T, $CD4^+$ T and γδ T cell receptor (TCR) cells (7–10), and even in non-lymphoid cells (6,7,11–13). GzmK is mainly expressed by $CD8^+$ T cells (9,14). The role of Gzm, particularly GzmB, in eliminating infected, neoplasic or foreign cells via induction of cell death has been widely reported (7,15). However, Gzm have also been proved to play a role in inflammation. Elevated levels of extracellular GzmA, B, K and M have been observed in patients with inflammatory conditions, including several parasitic, viral and bacterial diseases (16–19), chronic inflammatory diseases (17,20) and endotoxemia induced in healthy individuals (21,22). Studies in mouse models have shown that mice deficient in GzmA, B and/or M are relatively protected against endotoxemia (17,23), and that GzmM-deficient mice present lower plasma levels of cytokines than wild-type mice after administration of lipopolysaccharide (LPS) (17). In addition, various extracellular Gzm substrates have been identified (11,24), and the capacity of Gzm to trigger cytokine release and activation has been reported (6). Taking all these studies into account, a role for Gzm in infection and the accompanying inflammatory response seems clear.

Several studies have addressed the expression of Gzm in sepsis patients. Patients with sepsis demonstrate elevated plasma levels of GzmA and B (25) and GzmM (26), as well as increased intracellular GzmA and B levels in cytotoxic T lymphocytes (27). Plasma levels of GzmK have been proposed as a diagnostic marker to stage sepsis (28). In the present study, we aimed to analyse the intracellular expression of GzmA, B, M and K by distinct lymphocyte populations, as well as the extracellular levels of GzmA and B, in patients with sepsis compared to healthy individuals and patients with non-infectious critical illness.

MATERIALS AND METHODS

Patients and controls

Data and samples were prospectively collected as part of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project (ClinicalTrials.gov identifier NCT01905033) (29). Between February and November 2013, patients aged > 18 years admitted to the intensive care unit (ICU) of the Academic Medical Center (Amsterdam, the Netherlands) were evaluated for inclusion. Patients were eligible when they had at least two systemic inflammatory response syndrome (SIRS) criteria on the day of admission to the ICU (body temperature ≤ 36°C or ≥ 38°C, tachycardia > 90/min, tachypnea > 20/min or pCO_2 < 4.3 kPA, leukocyte count < 4×10^9/l or > 12×10^9/l). The presence of infection was established for every affected organ or site using Center for Disease criteria and International Sepsis Forum consensus definitions as described (29). Organ failure was defined as a score of 3 or greater on the sequential organ failure assessment (SOFA) score, except for cardiovascular failure, for which a score of 1 or more was used (30). Shock was defined as the use of vasopressors (noradrenaline) for hypotension in a dose of 0.1 μg/kg/min during at least 50% of the ICU day. Healthy individuals were included as controls. The study was approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam, and written informed consent was obtained from all patients (or legal representative) and healthy subjects.

Flow cytometry

Blood was collected at 9 a.m. on the first morning after ICU admission in tubes with ethylenediamine tetraacetic acid (EDTA) anti-coagulant; erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) and the remaining cells were washed and counted using a hemocytometer. One million cells/well were stained extracellularly by incubation with monoclonal antibodies against CD3-AF700, CD4-peridinin chlorophyll-cyanin (PerCP-Cy)5.5, CD56-phycoerythrin (PE)-Cy7, γδTCR-PE-Cy7 (all from BD Pharmingen, San Diego, CA, USA) and CD8-allophycocyanin (APC) (eBioscience, San Diego, CA, USA) at 4ºC for 25 min in the dark. For the intracellular staining, cells were fixed

and permeabilized for 20 min in Cytofix/Cytoperm (BD Bioscience) at 4ºC in the dark and then washed with Perm/Wash buffer. Subsequently, cells were resuspended in Perm/Wash buffer containing antibodies against GzmA (PE; BD Pharmingen), GzmB (PE-CF594; BD Horizon, San Diego, CA, USA), GzmK [fluorescein isothiocyanate (FITC); Santa Cruz Biotechnology, Dallas, TX, USA] and GzmM (AF488; generated as described previously (31)) and incubated at 4ºC for 25 min in the dark. GzmK staining was not performed for four healthy controls and seven sepsis patients; GzmM staining was not performed in three healthy controls. Samples were analysed by flow cytometry with a FACSCanto (BD Bioscience). FlowJo software (Tree Star Inc, Ashland, OR, USA) and FACSDiva software (BD Bioscience) were used to analyse the data. Lymphocytes were gated in the forward- *versus* side-scatter dot-plots. Within that gate, cells were selected as $CD3^+CD4^+$ ($CD4^+$ T cells), $CD3^+CD8^+$ ($CD8^+$ T), $CD3^+\gamma\delta TCR^+$ (γδ T), $CD3^+CD56^+$ ($CD56^+$ T cells) and $CD3^-CD56^+$ (NK cells), and expression of Gzm was analysed in these populations. NK cells were also subdivided into NK^{bright} and NK^{dim} based on the relative expression of CD56. The results are expressed as a percentage of cells of the specific population expressing the corresponding Gzm. Alternatively, cells were selected as positive for each Gzm and the percentages of the above-mentioned lymphocyte populations were analysed within the Gzm^+ lymphocytes.

Assays

Plasma EDTA samples were obtained from all sepsis patients, 10 non-infected ICU patients and 36 healthy subjects. Plasma was kept at −80ºC until use. GzmA and GzmB were measured by enzyme-linked immunosorbent assay (ELISA) using reagents from Sanquin (Amsterdam, the Netherlands) as previously described (32). The limits of detection of the assays were 18 pg/ml for GzmA and 22 pg/ml for GzmB.

Statistical analysis

Data in the Figures are expressed as box-and-whiskers showing the smallest observation, lower quartile, median, upper quartile and largest observation. Categorical data are presented as numbers (percentage) and comparisons were performed by the use of χ^2 or Fisher's exact tests when needed. Continuous parametric data are presented as means with standard deviations, while continuous nonparametric data are expressed as medians with quartiles 1 and 3 (Q1–Q3), and comparisons between groups were performed using the Mann–Whitney *U*-test. Correlations were calculated using the Spearman's rho test. Analyses were performed using GraphPad Prism version 5.0 (San Diego, CA, USA) and SPSS version 15.0 (Chicago, IL, USA). Statistical significance was defined as p value < 0.05.

RESULTS

Patients and lymphocyte subsets

We performed flow cytometry on blood samples obtained from 22 sepsis patients and 12 non-infected ICU patients within 24 h after admission to the ICU, as well as on blood from 32 healthy controls (46.5 ± 14.2 years, 62.5% male). While enrollment of patients was based on the presence of at least two SIRS criteria, all sepsis patients in retrospect fulfilled the sepsis-3 criteria (1). Table 1 shows the main demographic and clinical characteristics of patient groups. Sepsis patients were older than non-infected ICU patients ($p = 0.006$) and healthy controls ($p = 0.0002$). The most common site of infection among sepsis patients was the lung (50%). No significant differences were found between both groups of patients regarding disease severity or outcome (Table 1). Sepsis and non-infected ICU patients showed reduced absolute numbers of all lymphocyte subsets analysed ($CD4^+$ T, $CD8^+$ T, $CD56^+$ T, NK and γδT cells) when compared with healthy controls (Table 2; for gating strategy see Supporting information, Figure S1). This was largely due to a marked reduction in total lymphocyte numbers relative to healthy individuals; the proportions of $CD4^+$ T, $CD8^+$ T, $CD56^+$ T and γδT cells did not differ significantly between groups, while sepsis and non-infected ICU patients presented lower percentages of NK cells when compared to healthy controls.

Intracellular expression of GzmA, B, K and M

Both groups of patients showed reduced absolute numbers of lymphocytes positive for each Gzm when compared with healthy individuals (Table 2; for gating strategy see Supporting information, Figure S2). Sepsis patients presented lower proportions of $GzmA^+$ lymphocytes when compared with healthy controls (Table 2). Non-infected ICU patients showed lower percentages of lymphocytes expressing GzmA, K and M than healthy controls. Differences between the two groups of patients were not significant.

We then determined which lymphocyte subsets expressed each Gzm (Figure 1; Supporting information, Figure S2 shows the gating strategy). NK and $CD8^+$ T cells were the main source of GzmA, B and M in both patients and healthy controls. When compared with health, sepsis and non-infectious critical illness were associated with increases in

TABLE 1 Demographic and clinical characteristics of patients

	Sepsis $n = 22$	Non-infected $n = 12$
Demographics		
Age (years)	62.2 ± 13.5	47.9 ± 15.6*
Gender (male)	11 (50.0)	8 (66.7)
Race (white)	20 (90.9)	9 (75.0)
Charlson Index	1.0 (0–3.0)	0.5 (0–2.0)
Charlson Index age-adjusted	4.0 (2.8–6.0)	2.0 (0.3–5.5)
Severity of disease		
Shock	5 (22.7)	3 (25.0)
APACHE IV score	77.0 (57.8–98.8)	65.0 (55.5–98.3)
SOFA score	8.0 (6.0–12.0)	7.5 (6.3–11.3)
Organs failing	1.0 (1.0–3.0)	2.0 (1.0–2.8)
Mechanical ventilation	13 (59.1)	11 (91.7)
Site of infection		
Pulmonary	11 (50.0)	n.a.
Abdominal	4 (18.2)	n.a.
Urinary	3 (13.6)	n.a.
Other	2 (9.1)	n.a.
Co-infection	2 (9.1)	n.a.
Pathogen		
Gram-positive	5 (22.7)	n.a.
Gram-negative	4 (18.2)	n.a.
Gram-positive and -negative	3 (13.6)	n.a.
Virus	2 (9.1)	n.a.
Unknown	8 (36.4)	n.a.
Admission diagnosis		
Cardiac arrest	n.a.	4 (33.3)
Trauma	n.a.	2 (16.7)
Other	n.a.	6 (50.0)
Outcome of disease		
ICU stay (days)	6.0 (3.0–8.8)	3.5 (3.0–8.8)
ICU mortality	3 (13.6)	3 (25.0)
90-day mortality	7 (31.8)	4 (33.3)

Note: Values are number of individuals (%) except for age, which are mean ± standard deviation, and Charlson Comorbidity Index, APACHE IV score, SOFA score, number of damaged organs and days of stay in ICU, which are medians (Q1–Q3).

Abbreviations: APACHE, acute physiology and chronic health evaluation; n.a., not applicable; ICU, intensive care unit; SOFA, sequential organ failure assessment.

*$p < 0.01$ *versus* sepsis patients by Mann–Whitney *U*-test.

the relative contribution of $CD8^+$ T cells and decreases in the relative contribution of NK cells to intracellular GzmA, B and M expression. Sepsis patients also showed a modest increase in the contribution of $CD4^+$ T cells to intracellular GzmA and B expression. GzmK was primarily expressed by $CD8^+$ T cells and did not differ between patients and healthy controls.

Next, we analysed the percentage of Gzm^+ cells within each lymphocyte subset (Figure 2; see Supporting information, Figure S1 for gating strategy). Patients with sepsis and non-infectious critical illness showed clear decreases in the percentage of NK cells expressing GzmA, B and M relative to healthy controls. Both NK^{bright} and NK^{dim} cells presented this diminution (data not shown). A lower percentage of $CD56^+$ T cells $GzmA^+$ were also observed in patients than in healthy individuals. Conversely, the proportion of $CD8^+$ T and γδT cells expressing GzmB was increased in sepsis patients compared to healthy controls.

Additionally, we analysed co-expression of GzmA, B and M (due to limitations in antibody availability, the four Gzm could not be analysed simultaneously). A high proportion of cells positive for GzmA, B or M co-expressed the three Gzm (Figure 2). Very high positive correlations were found between the percentage of each lymphocyte population expressing GzmA, B or M in patients and healthy controls (Supporting information, Table S1). Lower positive, absent or even low negative correlations were found between expression of GzmA, B or M *versus* K (Supporting information, Table S1).

Intracellular expression of any of the four Gzm did not correlate with disease severity variables such as the presence of septic shock, SOFA or APACHE IV scores (Supporting information, Table S2).

Plasma levels of granzymes A and B

We measured plasma levels of GzmA and B in healthy controls and both groups of patients (Table 3). There were no significant differences between groups regarding GzmA concentration. Plasma levels of GzmB were increased in non-infected ICU patients compared to healthy controls and sepsis patients, and non-infected ICU patients presented a higher percentage of samples with GzmB concentrations above the detection limit than sepsis patients (Table 3).

Only minor correlations were found between plasma levels of GzmA or B and their respective intracellular expression in either patients or controls, and also when taking into account each lymphocyte population (data not shown).

DISCUSSION

Our study aimed to investigate the intracellular expression of GzmA, B, M and K in different lymphocyte subpopulations in patients with sepsis compared to non-infected patients with critical illness and healthy individuals. To our knowledge, this is the first study describing the intracellular expression

TABLE 2 Proportion of lymphocyte populations and granzyme positive cells in patients and controls

	Controls $n = 32$[a]	Sepsis $n = 22$[a]	Non-infected $n = 12$
Lymphocytes			
Cells × 10^5/ml	17.9 (13.9–25.0)	3.6 (2.2–6.3)****	9.5 (5.1–15.8)*##
%	19.8 (15.9–21.9)	2.5 (1.7–4.5)****	6.9 (4.2–9.1)****###
$CD4^+$ T cells			
Cells × 10^4/ml	84.4 (53.8–106.3)	15.3 (7.6–24.6)****	36.0 (22.0–74.4)*##
%	43.5 (36.4–48.8)	37.0 (22.6–48.0)	46.8 (30.9–56.6)
$CD8^+$ T cells			
Cells × 10^4/ml	37.9 (28.4–66.3)	5.6 (2.7–13.8)****	22.1 (6.1–38.9)#
%	21.5 (18.7–26.8)	15.8 (10.9–28.2)	22.3 (12.0–32.9)
$CD56^+$ T cells			
Cells × 10^4/ml	4.7 (1.9–6.0)	0.6 (0.2–1.6)***	1.9 (0.7–3.0)*#
%	2.1 (1.3–3.0)	1.5 (0.9–2.4)	1.4 (1.3–1.8)
NK cells			
Cells × 10^4/ml	23.6 (16.3–39.1)	4.3 (2.0–7.4)****	6.2 (3.5–13.5)**
%	12.7 (10.7–20.8)	11.6 (5.8–14.8)*	5.3 (4.0–10.7)**
γδ T cells			
Cells × 10^4/ml	3.8 (1.5–5.2)	0.6 (0.2–3.1)*	0.8 (0.4–3.2)*
%	2.1 (1.1–4.3)	1.5 (0.8–2.6)	1.1 (0.6–2.3)
$GzmA^+$ cells			
Cells × 10^4/ml	61.3 (38.4–114.4)	6.6 (4.2–19.8)****	15.7 (9.0–25.6)**
%	32.6 (24.0–42.1)	23.9 (15.4–37.2)*	16.2 (11.7–34.5)*
$GzmB^+$ cells			
Cells × 10^4/ml	40.4 (29.7–75.3)	6.2 (4.2–17.6)****	12.6 (6.0–23.3)**
%	24.3 (15.7–30.3)	20.8 (13.6–31.3)	13.5 (7.4–33.0)
$GzmK^+$ cells			
Cells × 10^4/ml	20.9 (11.7–45.8)	3.3 (1.8–6.5)**	9.1 (3.4–13.9)*
%	14.1 (11.4–15.7)	11.7 (3.6–13.7)	8.1 (5.3–12.5)*
$GzmM^+$ cells			
Cells × 10^4/ml	47.8 (30.9–84.8)	7.8 (3.8–19.6)***	14.5 (7.9–22.6)**
%	27.2 (21.7–37.6)	23.5 (13.8–35.4)	12.8 (8.7–34.7)*

Note: Percentages were calculated from total lymphocytes, except for lymphocytes that were calculated from total leukocytes. Values are presented as medians (Q1–Q3).

Abbreviations: NK: $CD3^-CD56^+$; Gzm, granzyme; NK = natural killer.

[a]White blood cell counts for values of cells/ml were available in a subgroup of 13 control individuals and 20 sepsis patients.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ when compared to controls; and #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ when comparing sepsis *versus* non-infected critically ill patients, determined by Mann–Whitney *U*-test.

of GzmM in sepsis patients. We show decreased intracellular expression of GzmA, B and M in NK cells from patients, as well as increased expression of GzmB in $CD8^+$ T cells in sepsis patients. Only minor differences between groups were found for GzmK. Secondarily, we measured plasma levels of GzmA and B in the same groups of individuals. We observed increased extracellular levels of GzmB in non-infected ICU patients relative to sepsis patients and to healthy controls.

Sepsis is often associated with lymphocytopenia (4). In agreement, we observed diminished total lymphocyte counts in sepsis patients, including a marked decrease in $CD8^+$ T and NK cell numbers. Within blood lymphocytes, NK cells and, secondly, $CD8^+$ T cells, were the main source of intracellular GzmA and B, which is consistent with earlier reports (8,10,19). Compared to healthy controls, a higher percentage of $CD8^+$ T lymphocytes expressed GzmA and B in patients,

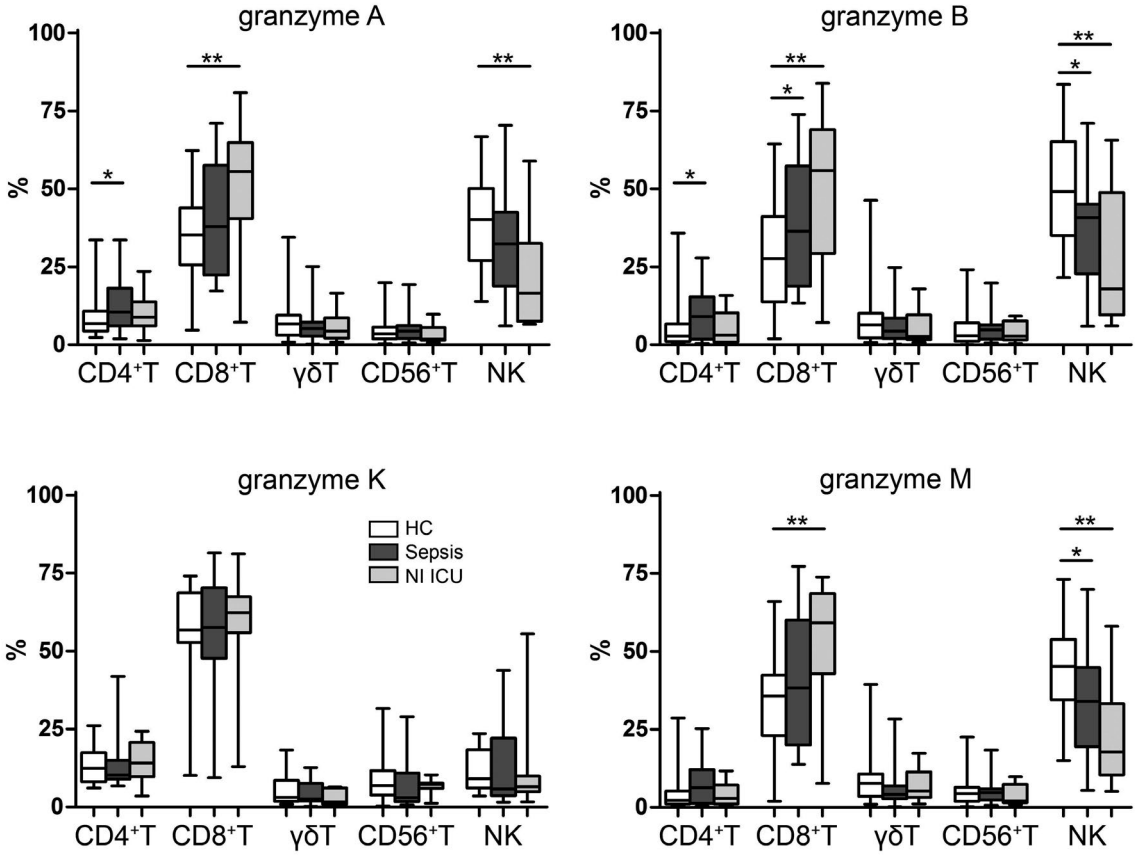

FIGURE 1 Lymphocyte source of intracellular granzymes A, B, K and M in patients and controls. Distribution of Gzm-positive cells among the different lymphocyte populations, expressed as percentage of the total number of lymphocytes positive for that granzyme. Natural killer (NK) = $CD3^{-}CD56^{+}$; Gzm = granzyme; $n = 32$ healthy controls (HC), 22 sepsis and 12 non-infected intensive care unit (NI ICU) patients, except for GzmK (sepsis $n = 15$, HC $n = 28$) and GzmM (HC $n = 29$). Data are box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. $^{*}p < 0.05$ and $^{**}p < 0.01$ determined by Mann–Whitney *U*-test

although this was only significant for GzmB, while a significantly lower percentage of NK cells expressed these Gzm, suggesting differential responses of the two main cellular Gzm sources. Consistently, the relative contribution of the $CD8^{+}$ T cells to GzmA and B expression was increased relative to healthy controls, whereas the relative contribution of NK cells was decreased. In the group of non-infected ICU patients, in which $CD8^{+}$ T cells were not significantly decreased, the relevance of this lymphocyte subset as the source of Gzm, to the detriment of NK cells, was even higher.

When compared to controls, patients presented decreased percentages of NK cells positive for GzmA or B, which may indicate that at the time of blood sampling of patients, NK cells have already released their Gzm content and/or have migrated from blood to the site of infection. Indeed, in response to different activating signals, NK cells become activated (5), and animal studies have suggested that these cells rapidly migrate to sites of infection (5). In this context, investigations on Gzm expression in lymphocyte subsets in infected tissues would be of interest. Studies analyzing samples obtained before sepsis diagnosis and of the activation state of blood NK cells would also be of interest. A study comparing the gene expression profiles of blood leukocytes between sepsis patients with and without HIV infection (33) showed that the genes *GZMA* and *GZMB* were under-expressed in HIV^{-} sepsis patients compared to HIV^{+} sepsis patients and controls; however, this study did not differentiate expression according to the lymphocyte subpopulation.

To the best of our knowledge, the current study is the first to report on the intracellular expression of GzmM in lymphocyte subpopulations in blood showing that, akin to GzmA and B, NK and $CD8^{+}$ T cells are the main GzmM producers. Similar results to those observed for GzmA and B were found for GzmM: in patients (relative to healthy controls) a significantly lower percentage of NK cells expressed GzmM and an increased contribution of $CD8^{+}$ T cells, to the detriment of NK cells, as the lymphocyte source of GzmM, was observed. A previous study (22) reported that the percentage of $GzmM^{+}$ cells did not change in different lymphocyte subpopulations, including NK cells, in human peripheral blood mononuclear cells stimulated with LPS. The difference with our results could be due to the divergences between an *in-vitro* assay with LPS and an infection with different type of

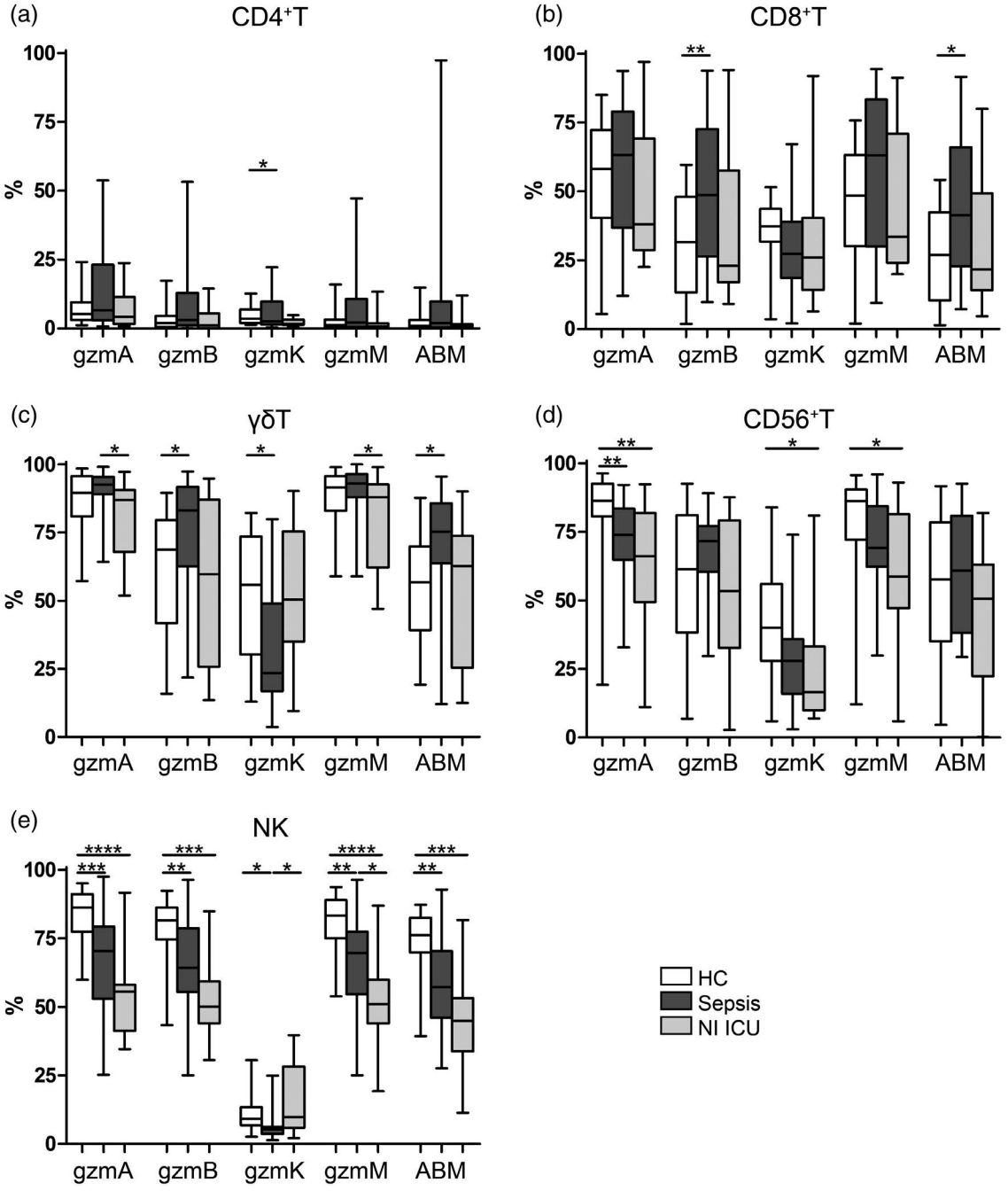

FIGURE 2 Granzymes A, B, K and M expression in lymphocyte populations from patients and controls. Percentage of intracellular expression of the granzymes in each lymphocyte population. Natural killer (NK) = $CD3^{-}CD56^{+}$; Gzm = granzyme; $n = 32$ healthy controls (HC), 22 sepsis and 12 non-infected ICU (NI ICU) patients, except for GzmK (sepsis $n = 15$, HC $n = 28$) and GzmM (HC $n = 29$). ABM indicates cells co-expressing GzmA, B and M. Data are box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ determined by Mann–Whitney *U*-test

live pathogens in humans *in vivo*. In addition, *in-vitro* experiments cannot capture possible migration of cells to the sites of infection. Intracellular expression of GzmK was especially observed in $CD8^{+}$ T cells, which is in accordance with previous studies (14,19); $CD8^{+}$ T cell GzmK expression was relatively unaltered in patients when compared to controls.

Plasma levels of GzmA and B did not differ between sepsis patients and controls. A previous study (25) demonstrated increased plasma levels of GzmA, but not GzmB, in patients with severe sepsis compared to healthy controls. A transient rise in plasma GzmA and B levels was measured after LPS injection into healthy humans (21), and elevated serum or plasma levels of GzmA and/or GzmB were reported in patients with bacteremic melioidiosis (21), typhoid fever (18), tuberculosis (19), malaria (32) or viral infections, such as by Epstein–Barr virus (EBV) or HIV-1 (34). The disparity with

TABLE 3 Plasma levels of GzmA and B in patients and controls

	Healthy controls ($n = 36$)		Sepsis ($n = 22$)[a]		Non-infected ($n = 10$)	
	GzmA	**GzmB**	**GzmA**	**GzmB**	**GzmA**	**GzmB**
Median (Q1–Q3)	33.6 (28.0–40.0)	27.8* (11.0–53.5)	35.7 (24.8–46.3)	23.0** (11.0–38.1)	20.0 (11.5–42.6)	83.4 (45.9–266.0)
Samples above DL (%)	33 (91.7)	25 (69.4)	19 (86.4)	11 (57.9)[#]	7 (70.0)	9 (90.0)

Note: Values are median of granzyme (Gzm) plasma levels (Q1–Q3) or number of samples with values above the detection limit (%). Detection limit (DL) of the assays: 18 pg/ml for GzmA and 22 pg/ml for GzmB. In individuals with Gzm concentration lower than the DL, a value of DL/2 was assigned.

[a]For GzmB, $n = 19$. *$p < 0.05$ and **$p < 0.01$ *versus* non-infected critically ill patients, determined by Mann–Whitney *U*-test. $p < 0.05$ *versus* non-infected critically ill patients, determined by Fisher's exact test.

our present data is uncertain, but may be related to differences in causative pathogens; for example, infections associated with elevated GzmA and B plasma levels are caused mainly by Gram$^-$ bacteria, parasites or virus, which only represent half of our sepsis patients with positive culture. Plasma GzmB levels were elevated in the non-infected ICU patients relative to both sepsis patients and healthy controls. Extracellular levels of Gzm have been also found in other non-infectious diseases, including rheumatoid arthritis, chronic allergic asthma and Behçet's disease, in samples such as plasma, synovial fluid or bronchial lavage fluid (16), indicating that Gzm release can be triggered by a variety of stimuli.

Earlier studies have indicated the involvement of Gzm in the pathophysiology of sepsis (35). GzmA, B, K and M can stimulate the production, release and/or processing of several cytokines (6). Gzm can trigger intracellular signaling through cell–cell interaction leading to target cell killing and/or stimulation of cytokine release. Moreover, Gzm have been implicated in several host response aberrations involved in sepsis, including coagulation activation, platelet function, endothelial barrier and integrity (35). The effect of Gzm (especially GzmB) on extracellular matrix proteins and other cell surface proteins suggest a role in altering cell migration and in tissue remodeling (16,36). We have previously shown role for GzmA and B in limiting bacterial growth in mouse models of pulmonary and abdominal sepsis (37,38). The current investigation does not provide insight into the role of Gzm in critical illness. Nonetheless, it shows that, while the percentage of NK cells expressing GzmA, B and M is decreased in patients relative to controls, only the percentage of those CD8$^+$ T cells expressing GzmB is increased, and there are no relevant differences related to GzmK. This could reflect differential roles of these four Gzm in critical illness.

We acknowledge some limitations to our study. The number of patients included was relatively low, precluding subanalyses by origin of the infection or causative pathogen. We did not measure extracellular levels of Gzm M and K. Wensink *et al*. (22) reported a transient increase in plasma GzmM and, to a lesser extent, GzmK levels after injection of LPS into healthy humans (22).

In summary, the present study shows a marked decrease in intracellular expression of GzmA, B and M by NK cells in both sepsis and non-infected ICU patients and an increased intracellular expression of GzmB by CD8$^+$ T cells in sepsis patients, compared to healthy controls. Further studies in sepsis patients are warranted to provide insight into intra- and extracellular Gzm expression in different body compartments, including the primary site of infection.

ACKNOWLEDGEMENTS

This study was partially supported by a Marie Curie Intra European Fellowship within the 7th Framework Programme – People (PIEF-GA-2011-300895), and by the Center for Translational Molecular Medicine (http://www.ctmm.nl), project Molecular Diagnosis and Risk Stratification of Sepsis (grant no. 04I-201). The funding agencies have no role in the design of the study, collection, analysis or interpretation of data, or in the writing of the manuscript. The authors are grateful to the patients and the healthy volunteers for their trust and cooperation. The authors also thank the Department of Immunopathology at Sanquin Research, especially to Gerard J. van Mierlo, for providing the GzmA and B ELISAs.

CONFLICT OF INTEREST

The authors declare that they have no competing interests regarding the publication of this article.

AUTHOR CONTRIBUTIONS

M. I. G. L. obtained funding, designed the study, performed the experiments, analysed and interpreted the data and drafted the manuscript. A. J. H. performed experiments and interpreted data. M. A. W., L. A. V and M. J. S. provided clinical samples and data. N. B. tested and provided experimental material. A. F. d. V. designed the study and interpreted the data. T.v. d. P. obtained funding, designed and supervised the study, interpreted the data and drafted the manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

M. Isabel García-Laorden https://orcid.org/0000-0001-6270-6306

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus definitions for sepsis and septic shock (Sepsis-3). JAMA 2016;315:801–10. https://doi.org/10.1001/jama.2016.0287.
2. Reinhart K, Daniels R, Kissoon N, Machado FR, Schachter RD, Finfer S. Recognizing sepsis as a global health priority – a WHO resolution. N Engl J Med. 2017;377:414–7. https://doi.org/10.1056/NEJMp1707170.
3. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. Lancet 2020;395:200–11.
4. Rimmelé T, Payen D, Cantaluppi V, Marshall J, Gomez H, Gomez A, et al. ADQI XIV Workgroup. Immune cell phenotype and function in sepsis. Shock 2016;45:282–91. https://doi.org/10.1097/SHK.0000000000000495.
5. Guo Y, Patil NK, Luan L, Bohannon JK, Sherwood ER. The biology of natural killer cells during sepsis. Immunology 2018;153:190–202. https://doi.org/10.1111/imm.12854.
6. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. J Immunol. 2015;194:491–7. https://doi.org/10.4049/jimmunol.1401214.
7. Susanto O, Trapani JA, Brasacchio D. Controversies in granzyme biology. Tissue Antigens. 2012;80:477–87.
8. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood 2004;104:2840–8. https://doi.org/10.1182/blood-2004-03-0859.
9. Bratke K, Kuepper M, Bade B, Virchow JC Jr, Luttmann W. Differential expression of human granzymes A, B, and K in natural killer cells and during CD8+ T cell differentiation in peripheral blood. Eur J Immunol. 2005;35:2608–16. https://doi.org/10.1002/eji.200526122.
10. de Poot SA, Bovenschen N. Granzyme M: behind enemy lines. Cell Death Diff. 2014;21:359–68. https://doi.org/10.1038/cdd.2013.189.
11. Anthony DA, Andrews DM, Watt SV, Trapani JA, Smyth MJ. Functional dissection of the granzyme family: cell death and inflammation. Immunol Rev. 2010;235:73–92. https://doi.org/10.1111/j.0105-2896.2010.00907.x.
12. Vernooy JHJ, Möller GM, van Suylen RJ, van Spijk MP, Cloots RHE, Hoet PH, et al. Increased granzyme A expression in type II pneumocytes of patients with severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2007;175:464–72. https://doi.org/10.1164/rccm.200602-169OC.
13. Campbell RA, Franks Z, Bhatnagar A, Rowley JW, Manne BK, Supiano MA, et al. Granzyme A in human platelets regulates the synthesis of proinflammatory cytokines by monocytes in aging. J Immunol. 2018;200:295–304. https://doi.org/10.4049/jimmunol.1700885.
14. Bovenschen N, Kummer JA. Orphan granzymes find a home. Immunol Rev. 2010;235:117–27. https://doi.org/10.1111/j.0105-2896.2010.00889.x.
15. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. Nat Rev Immunol. 2015;15:388–400.
16. Buzza MS, Bird PI. Extracellular granzymes: current perspectives. Biol Chem. 2006;387:827–37. https://doi.org/10.1515/BC.2006.106.
17. Anthony DA, Andrews DM, Chow M, Watt SV, House C, Akira S, et al. A role for granzyme M in TLR4-driven inflammation and endotoxicosis. J Immunol. 2010;185:1794–803. https://doi.org/10.4049/jimmunol.1000430.
18. de Jong HK, Garcia-Laorden MI, Hoogendijk AJ, Parry CM, Maude RR, Dondorp AM, et al. Expression of intra- and extracellular granzymes in patients with typhoid fever. PLOS Negl Trop Dis. 2017;11:e0005823.
19. Garcia-Laorden MI, Blok DC, Kager LM, Hoogendijk AJ, van Mierlo GJ, Lede IO, et al. Increased intra and extracellular granzyme expression in patients with tuberculosis. Tuberculosis 2015;95:575–80. https://doi.org/10.1016/j.tube.2015.05.016.
20. Shah D, Kiran R, Wanchu A, Bhatnagar A. Soluble granzyme B and cytotoxic T lymphocyte activity in the pathogenesis of systemic lupus erythematosus. Cell Immunol. 2011;269:16–21. https://doi.org/10.1016/j.cellimm.2011.03.004.
21. Lauw F, Simpson A, Hack C, Prins J, Wolbink A, van Deventer S, et al. Soluble granzymes are released during human endotoxemia and in patients with severe infection due to Gram-negative bacteria. J Infect Dis. 2000;182:206–13. https://doi.org/10.1086/315642.
22. Wensink AC, Wiewel MA, Jongeneel LH, Boes M, van der Poll T, Hack CE, et al. Granzyme M and K release in human experimental endotoxemia. Immunobiology 2016;221:773–7. https://doi.org/10.1016/j.imbio.2016.02.006.
23. Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, et al. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity 2008;29:720–33. https://doi.org/10.1016/j.immuni.2008.08.014.
24. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev. 2010;235:105–16. https://doi.org/10.1111/j.0105-2896.2010.00908.x.
25. Zeerleder S, Hack C, Caliezi C, Vanmierlo G, Eerenbergbelmer A, Wolbink A, et al. Activated cytotoxic T cells and NK cells in severe sepsis and septic shock and their role in multiple organ dysfunction. Clin Immunol. 2005;116:158–65. https://doi.org/10.1016/j.clim.2005.03.006.
26. Hollestelle MJ, Lai KW, van Deuren M, Lenting PJ, de Groot PG, Sprong T, et al. Cleavage of von Willebrand factor by granzyme M destroys its factor VIII binding capacity. PLOS ONE. 2011;6:e24216. https://doi.org/10.1371/journal.pone.0024216.
27. Napoli AM, Fast LD, Gardiner F, Nevola M, Machan JT. Increased granzyme levels in cytotoxic T lymphocytes are associated with disease severity in emergency department patients with severe sepsis. Shock 2012;37:257–62. https://doi.org/10.1097/SHK.0b013e31823fca44.
28. Rucevic M, Fast LD, Jay GD, Trespalcios FM, Sucov A, Siryaporn E, et al. Altered levels and molecular forms of granzyme k in plasma from septic patients. Shock 2007;27:488–93. https://doi.org/10.1097/01.shk.0000246905.24895.e5.
29. Klouwenberg PMCK, Ong DSY, Bos LDJ, de Beer FM, van Hooijdonk RTM, Huson MA, et al. Interobserver agreement of

Centers for Disease Control and Prevention criteria for classifying infections in critically ill patients. Crit Care Med. 2013;41:2373–8. https://doi.org/10.1097/CCM.0b013e3182923712.
30. Kaukonen KM, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000–2012. JAMA 2014;311:1308–16. https://doi.org/10.1001/jama.2014.2637.
31. de Koning PJA, Tesselaar K, Bovenschen N, Çolak S, Quadir R, Volman TJH, et al. The cytotoxic protease granzyme M is expressed by lymphocytes of both the innate and adaptive immune system. Mol Immunol. 2010;47:903–11. https://doi.org/10.1016/j.molimm.2009.10.001.
32. Hermsen CC, Konijnenberg Y, Mulder L, Loé C, van Deuren M, van der Meer JW, et al. Circulating concentrations of soluble granzyme A and B increase during natural and experimental Plasmodium falciparum infections. Clin Exp Immunol. 2003;132:467–72.
33. Huson MAM, Scicluna BP, van Vught LA, Wiewel MA, Hoogendijk AJ, Cremer OL, et al. The impact of HIV co-infection on the genomic response to sepsis. PLOS ONE. 2016;11:e0148955. https://doi.org/10.1371/journal.pone.0148955.
34. Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer JA, Swaal AJ, et al. Extracellular granzymes A and B in humans: detection of native species during CTL responses *in vitro* and *in vivo*. J Immunol. 1998;160:3610–6.
35. Garzón-Tituaña M, Arias MA, Sierra-Monzón JL, Morte-Romea E, Santiago L, Ramirez-Labrada A, et al. The multifaceted function of granzymes in sepsis: some facts and a lot to discover. Front Immunol. 2020;11:1054. https://doi.org/10.3389/fimmu.2020.01054.
36. Hiebert PR, Granville DJ. Granzyme B in injury, inflammation, and repair. Trends Mol Med. 2012;18:732–41. https://doi.org/10.1016/j.molmed.2012.09.009.
37. García-Laorden MI, Stroo I, Blok DC, Florquin S, Medema JP, de Vos AF, et al. Granzymes A and B regulate the local inflammatory response during *Klebsiella pneumoniae* pneumonia. J Innate Immun. 2016;8:258–68. https://doi.org/10.1159/000443401.
38. García-Laorden MI, Stroo I, Terpstra S, Florquin S, Medema JP, van Veer TC, et al. Expression and function of granzymes A and B in *Escherichia coli* peritonitis and sepsis. Mediat Inflamm. 2017;2017:4137563. https://doi.org/10.1155/2017/4137563.

SUPPORTING INFORMATION

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

How to cite this article: García-Laorden MI, Hoogendijk AJ, Wiewel MA, et al. Intracellular expression of granzymes A, B, K and M in blood lymphocyte subsets of critically ill patients with or without sepsis. *Clin Exp Immunol*. 2021;205:222–231. https://doi.org/10.1111/cei.13601