Changes of CD3+CD56+ γδ T cell number and apoptosis during hospital admission are related to mortality in septic patients

J.C. Andreu-Ballester a,*, M.A. Arribas b, M. Rico b, C. García-Ballesteros c, L. Galindo-Regal c, R. Sorando-Serra d, L. Albert b, A. Navarro b, F. López-Chuliá e, F. Peydró b, C. Cuéllar f

a Research Department, Arnau de Vilanova University Hospital, Valencia, Spain

b Critical Care Department, Arnau de Vilanova University Hospital, Valencia, Spain

c Laboratory of Molecular Biology, Arnau de Vilanova University Hospital, Valencia, Spain

d Emergency Department, Arnau de Vilanova University Hospital, Valencia, Spain

e Hematology Department, Arnau de Vilanova University Hospital, Valencia, Spain

f Microbiology and Parasitology Department, Complutense University, Madrid, Spain

* Corresponding author at: Research Department, Arnau de Vilanova University Hospital, c/ San Clemente 12, 46015 Valencia, Spain.

E-mail addresses: jcandreuballester@outlook.com (J.C. Andreu-Ballester), moises@comv.es (M. Rico), garcia_carbar@gva.es (C. García-Ballesteros), lopez_

frachu@gva.es (F. López-Chuliá), cuellarh@farm.ucm.es (C. Cuéllar).

ABSTRACT

Immunoparalysis and apoptosis of T cells are serious problems for the evolution of septic patients. We aimed to relate changes in the number of αβ and γδ T cells during hospital stay to the poor evolution of sepsis. In this prospective study, we recruited a total of 92 septic patients from the Emergency and Intensive Care Departments of two Hospitals, according to the latest criteria for the definition and management of sepsis. According to the severity of the septic process, there was a progressive decrease in T cells, being much more intense in γδ T cells. This decrease recovered in surviving patients, but CD3+CD56+ γδ T cells continued to decreased during hospital stay in non-surviving patients. Apoptosis increased in sepsis. Cell death of CD3+CD56+ γδ T cells progressively increased according to the severity of sepsis, especially in non-surviving patients.

Keywords: Sepsis, αβ T cells, γδ T cell, Apoptosis, Mortality

1. Introduction

Although sepsis survival has improved in the last decade, it remains a serious public health problem, affecting 20 million people per year worldwide, with a hospital mortality rate of close to 20%, and with severe complications after discharge from hospital. [1,2].

Sepsis is characterized by a series of pro- and anti-inflammatory phenomena, in which immunoparalysis is of particular relevance, characterized in part by a decrease in T cells, specifically CD4+ and CD8+ [3]. Increased lymphocyte apoptosis in sepsis is an important factor that induces lymphopenia and increases the risk of infection and mortality [4–8]. Anti-apoptotic agents were experimentally investigated in animals as future therapies to prevent lymphocyte depletion in sepsis, to be applied in future human clinical trials [9]. Therefore, it is important to know the implications of different T-cell subsets on the evolution and prognosis of sepsis.

Most pathogens that cause septic processes enter the host through the mucosas [10]. Lymphocytes are the key cellular elements in mucosal defense, especially γδ T cells. This T cell subset includes 5–10% of peripheral blood T cells, while αβ T cells are the most frequent (90–95%). However, γδ T cells are much more abundant (50–100%) in the mucosal epithelium and the skin as the first line of defense against pathogens, attacking tumor cells and repairing tissues [11].

Due to a hyperinflammatory cytokine storm response and induced immunoparalysis, sepsis can evolve critically, within a few hours, with high mortality. For those reasons, the innate immune response is essential to prevent early deaths [12]. Innate T cells include natural killer T (NKT) cells, mucosa-associated innate T (MAIT) cells and γδ T cells. They are not MHC-restricted and respond much more rapidly than αβ T cells [13]. Although γδ T cells are mainly double negative (CD4- CD8-), a small percentage co-express CD8+ and CD56+, and a lower proportion are CD4+ [14]. Recently, the

designation of the subset of γδ T cells positive for natural killer antigens as γδ NKT cells (to explicitly distinguish them from αβ NKT-cells) has been proposed. The high cytotoxic capacity observed in this cell population suggests important immunological implications in tumor defense. [15]

Persistent lymphopenia in sepsis is associated with poor outcomes and mortality [16,17]. However, most studies do not explain the involvement of non-conventional T lymphocytes, such as γδ T cells, in lymphopenia. We report that the reduction of γδ T cells, specifically CD3+CD56+ γδ T cells, is significantly related to severity and mortality in septic patients [18]. The main objective of this study was to investigate if the αβ and γδ T cell deficit during sepsis is related to mortality.

2. Material and methods

2.1. Type and population study

This was a prospective study of septic patients (cases) and healthy subjects (controls). We recruited 138 subjects, 92 septic patients and 46 controls. Cases and controls were matched for sex and age ± 5 years (one control for every two cases). All patients were diagnosed according to the latest criteria for the definition and management of sepsis [19]. Septic patients came from the Emergency and Intensive Care departments of two Hospitals, Arnau de Vilanova and Lliria in Valencia (Spain), for a period of one year. Patients had to meet the following criteria: they were not suffering from immunodeficiency or autoimmune or inflammatory diseases, had not been vaccinated in the last 3 months, and were not undergoing immunosuppressive therapy. Control healthy subjects were required to have the same characteristics as the patients in addition to not suffering from any acute infectious diseases.

The study was approved by the Ethics and Investigation Committee of the Arnau de Vilanova University Hospital (Valencia, Spain).

2.2. Methods of blood sample analysis

Blood cell counts were obtained by basal hematimetry, in a cell counter (LH750 Beckman Coulter, Inc., Fullerton, CA), after patient admission to the Emergency and Intensive Care.

2.2.1. Cell isolation

Mononuclear cells (MNCs) were isolated and enriched from EDTA anticoagulated blood by centrifugation on density gradient. We used Lymphoprep™ (Palex Medical SA), spinning at 3500 rpm for 20 min. Cells were then washed twice in phosphate buffered saline (PBS), and resuspended in 0.2 ml of the binding buffer from the ANNEXIN V-FITC/7-AAD Kit (Beckman Coulter, Inc) with the presence of Ca2 + .

The following monoclonal antibodies were used: Tube 1: anti-TCR PANαβ-PE (clon: IP26A), CD56-PC7 (clon N901 (NKH□ 1)), CD4-APC A750 (clon 13B8.2), CD3-APC A700 (clon UCHT1), CD8-PB (clon B9.11), CD45-KRO (clon J33). Tube 2: anti-TCR PANγδ-PE (clon: IMMU 510), CD56-PC7 (clon N901 (NKH-1)), CD4-APC A750 (clon 13B8.2), CD3-APC A700 (clon UCHT1), CD8-PB (clon B9.11), CD45-KRO (clon J33). All of them purchased from Beckman Coulter, Inc., Miami, USA.

After 10 min of incubation, we used the “Fix-and-Lyse” mixture prepared at that time (versalyse plus IOT3, from Beckman Coulter, Inc., Miami, USA): 1 ml of this mix was added to the MNCs pellet, followed by a 20 min incubation at room temperature, protecting from light. Finally, we added 2 ml of PBS and centrifuged for 5 min at 1500 rpm. We removed the supernatant by aspiration and re-suspend the cell button in 0.5 ml of PBS.

2.2.2. Apoptosis evaluation

The apoptosis detection was performed with ANNEXIN V-FITC/7-AAD Kit (Beckman Coulter, Inc), based on the binding properties of annexin V to phosphatidylserine and on the specificity of 7-amino-actinomycin D (7-AAD) for DNA guanine-cytosine base pair, following instructions of the manufacturer. The results described in this work refer to early apoptosis (Annexin V +, 7-AAD -), which accounts for 90–95% of total apoptosis.

2.2.3. Functional analysis of γδ and αβ T cells

Functional analysis of γδ and αβ T cells was evaluated by Flow cytometry. A total of 100.000 events were acquired a multiparameter Navios flow cytometer (Beckman Coulter, Inc) and later analyzed with Kaluza software. Estimated counts of circulating cell subsets were calculated using a hematology counter cell.

As an internal quality assurance, we performed flow cytometer verification with a suspension of fluorescent microspheres that allow the adjustments of SS, FS and lasers (Flow-check Pro Fluorospheres, Beckman Coulter, Inc.). Also, daily monitoring of whole-blood preparation procedures and monoclonal antibody reactivity using Immuno-Trol (Beckman Coulter) control cells was performed.

Gating strategy for T cell subsets and apoptosis (Annexin V/7-AAD) are shown in Supplementary files 1 and 2 respectively.

2.3. Statistical method

To compare numbers and apoptosis percentages of T cell subsets, the Mann-Whitney U test was used. Mean value differences between the first (at admission) and second analysis (after 7–10 days) were analyzed using the Wilcoxon non-parametric test. To compare differences between surviving and non-surviving septic patients, the Mann-Whitney U test was used. P-values <0.05 were considered statistically significant. Data were

analyzed using the Statistical Package for Social Sciences (SPSS 19.0; SPSS Inc., Chicago, IL, USA). Figures were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA.

3. Results

Table 1 shows the characteristics of the patients with sepsis. Table 2 describes the relation of comorbidity between septic patients and control healthy subjects. There was a significant relationship between the history of diabetes mellitus and chronic renal failure with sepsis ($p < 0.001$). The mean ages were 68.0 ± 14.1 and 64.4 ± 8.2 years for septic patients and control subjects, respectively ($p = 0.102$).

3.1. Numbers and apoptosis of T cell subsets

The numbers of αβ and γδ T cells were significantly lower in septic patients compared to healthy controls subjects. These values were much lower in the group of patients with septic shock. The lowest values were observed in the γδ T cell population. Apoptosis was higher in septic patients' cell populations than controls and significantly high in CD56+ T cell subsets (Fig. 1B and D). Apoptosis increased progressively according to the severity of the septic process. This phenomenon was especially significant in CD3+CD56+ γδ T cells (Fig. 1F).

3.2. Numbers of T cell subsets and mortality

In-hospital mortality of septic patients was 17.4% (n = 16). It was possible to perform a second analysis 7–10 days after admission in 45 septic patients. Fig. 2 shows the differences in the number of T-cell subsets between admission and 7–10 days after admission, according to mortality. Septic patients who did not survive (red) had lower numbers of all T-cell subsets vs. patients who survived to admission. On the second

analysis, patients who survived had a significant increase in all Tcell subsets ($p < 0.001$). Non-surviving patients did not recover T-cell subsets at 7–10 days post-admission. A significant decrease in the number of CD3+CD56+ γδ T cells was observed at the second análisis (7–10 days post-admission) in non-surviving patients (Fig. 2C).

3.3. Apoptosis of T cells subsets and mortality

Fig. 3 shows the differences in percentages of apoptosis according to the first (at admission) and second analysis (7–10 days post-admission). Differences in apoptosis were observed between surviving and nonsurviving septic patients. In CD3+CD56+ γδ T cells apoptosis was significantly increased at 7–10 days post-admission in non-surviving septic patients during the hospital stay (Fig. 3C).

4. Discussion

This work relates to the first-time changes in T-cell and γδ T-cell subsets related to mortality in septic patients during hospital admission.

As we have previously described, a decrease in all T cell subsets was observed in septic patients compared to healthy subjects at the time of admission. This decrease was related to the severity of sepsis, being more intense in all γδ T cell subsets. In the present work we have used the new sepsis criteria [19]. Despite this, our current study confirms the with the severity of sepsis during hospital admission [18]. Likewise, experimental studies demonstrated the relationship of γδ T cell reduction with poor prognosis of sepsis [20,21].

Vδ1 and Vδ2 are two of the three major Vδ domains of γδ T cells. Vδ1+ γδ T cells constitute a minority of the γδ T population in peripheral blood, while Vδ2+ γδ T cells are widely spread in the epithelial tissues of mucosa and skin [22].

Recently, Wang et al. [23] related the decrease of Vδ2+ T cell percentage in 30 septic patients, with severity of the disease. Conversely, the increase of Vδ1+ T cells was related to sepsis severity. It would be interesting to know the absolute values of Vδ1 and Vδ2 T cells in these patients. The percentages of γδ T cells from our patients (including all subtypes) were also significantly decreased in sepsis (data not shown). We carried out the study in peripheral blood samples where the Vγ9Vδ2 subtype is the most frequent.

As mentioned above, apoptosis of αβ T cells has been related to por prognosis in sepsis [6–8,24]. In our study, apoptosis increased in patients with sepsis compared to healthy subjects, but without changes in αβ T cells. Cell death of CD3+CD56+ γδ T cells was more significant in the most serious cases of sepsis. Likewise, the highest degree of cell death was observed in septic shock.

It is remarkable that when the second analysis was performed (7–10 days of hospitalization), sufficient time had elapsed for the adaptive immunity to begin to act. Thus, during the evolution of sepsis, from the first to the second analysis, we observed an increase in T cells in surviving patients. Likewise, although there was a tendency to decrease in CD3+CD56+ αβ T cells, the decrease of γδ T cells and especially CD3+CD56+ γδ T cells was clearly related to the evolution to death. Besides, increased cell death of CD3+CD56+ γδ T cells in the second analysis was clearly related to mortality of sepsis. This phenomenon was not observed when analyzing the cell death of αβ T cells. All these results continue to highlight the influence of γδ T cells on the course of sepsis.

There is a heterogeneous population of CD8 + CD56+ cells that were produced by ex vivo incubation of human peripheral blood lymphocytes with an anti-CD3 antibody, IFN-γ, and IL-2, named Cytokine-induced killer cells (CIK). These cells have immunological effector characteristics and, specifically, CD3+CD56+ are the most powerful [25]. They have characteristics of cells of innate immunity, such as natural killer cells (CD3-CD56+),

because they recognize tumor epitopes directly without MHC restriction. For this reason, these cells have been called natural killer T cells (NKT cells) and are good candidates for use in adoptive immunotherapy, especially against cancer [26]. Until now,

NKT cells had not been studied in relation to the αβ and γδ TCR. However, von Lilienfeld-Toal M et al. have described the expression of a specific NK marker (NKp44) in polyclonal γδ T cells isolated according to the CIK protocol, with marked cytotoxic activity against myeloma [27]. It is possible that a large part of the CD3+CD56+ γδ T cells in our study have the characteristics of these CIK-NKT cells. This hypothesis is supported by the strong association of changes in this cell subset with sepsis's severity and poor prognosis. Future works should investigate this hypothesis.

5. Conclusions

In this study, a progressive decrease in T cells was observed depending on the severity of the septic process. This reduction was more intense in the case of γδ T cells. Surviving patients had improved T cell numbers at 7–10 days after admission. In contrast, there was a significant decrease in CD3+CD56+ γδ T cells in non-surviving patients during hospital stay.

T cell death increased in patients with sepsis. Specifically, cell death of CD3+CD56+ γδ T cells progressively increased with the severity of sepsis in non-surviving septic patients.

The present work demonstrates the great importance of γδ T cells – and specifically CD3+CD56+ γδ T cells and their early apoptosis – in the poor prognosis of sepsis.

Contributors

Conception and design of the work (Andreu-Ballester JC); collecting data and recruitment of patients (Sorando-Serra R, Arribas MA, Rico M, Albert L, Navarro A, Peydr´o F); Flow cytometry analysis of T cell subsets and apoptosis (Galindo-Regal L, García-Ballesteros C and L´opez-Chuli´a F); Statistical method (Andreu-Ballester JC); interpretation of data for the work (Andreu-Ballester JC, Cuellar C); drafting the work and revising it critically for important intellectual content (Andreu-Ballester JC and Cuellar C).

Declarations of interest

None.

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Table 1

Characteristics of patients with sepsis.

	No. (%) of patients	*Organic Failure*	
Stages of sepsis		Cardiovascular	63 (68.5)
Sepsis	29 (31.5)	Acute Respiratory Failure	39 (42.9)
Septic Shock	63 (68.5)	Acute Renal Failure	37 (40.2)
Diagnosis		Metabolic	34 (38.6)
Urinary Tract Infections	32 (34.8)	Acute Hepatic Failure	32 (35.2)
Pneumonia	15 (16.3)	Acute Heart Failure	25(27.2)
Peritonitis	13 (14.1)	Hematologic	21 (23.1)
Primary bacteremia	8 (8.7)	Neurologic	(19.8)
Acute Cholangitis	7 (7.6)	*Analytical and score parameters*	Mean ± S. D.
Acute Cholecystitis	5 (5.4)	APACHE II score	18.9 ± 7.4
Abscess	4 (4.3)	SOFA score	6.4 ± 3.3
Meningitis	3 (3.3)	C-reactive protein (mg/l)	225.8 ± 132.4
Cellulitis	2 (2.2)	Procalcitonin (ng/ml)	33.7 ± 49.4
Acute pancreatitis	1 (1.1)	Lactic acid (mg/dl)	3.2 ± 2.7
Endocarditis	1 (1.1)	*In-hospital death*	16 (17.4)
Gastroenterocolitis	1 (1.1)	*Positive Cultures*	75 (81.5)

Table 2

Relation of comorbidity between septic patients (n = 92) and control healthy subjects (n = 46).

	Sepsis n (%)	Control n (%)	OR (CI95%)	Sig (p)
Gender				
- Male	56 (60.9)	28 (60.9)	–	NS
- Female	36 (39.1)	18 (39.1)	–	NS
Comorbidity				
- Hypertensive Disease	49 (53.3)	21 (45.7)	1.4 (0.7–2.8)	NS
- Diabetes Mellitus	33 (35.9)	2 (4.3)	12.3 (2.8–54.0)	<0.001
- Dislipemia	35 (38.0)	14 (33.3)	1.2 (0.6–2.6)	NS
- COPD	21 (22.8)	5 (10.9)	2.4 (0.9–6.9)	NS
- Congestive Heart Failure	8 (8.7)	0 (0.0)	1.1 (1.0–1.2)	0.052
- Chronic Renal Failure	12 (13.0)	0 (0.0)	1.2 (1.1–1.3)	0.009
- Obesity	13 (14.1)	2 (4.3)	3.6 (0.8–16.8)	NS

COPD: Chronic Obstructive Pulmonary Disease. NS: Not significant.

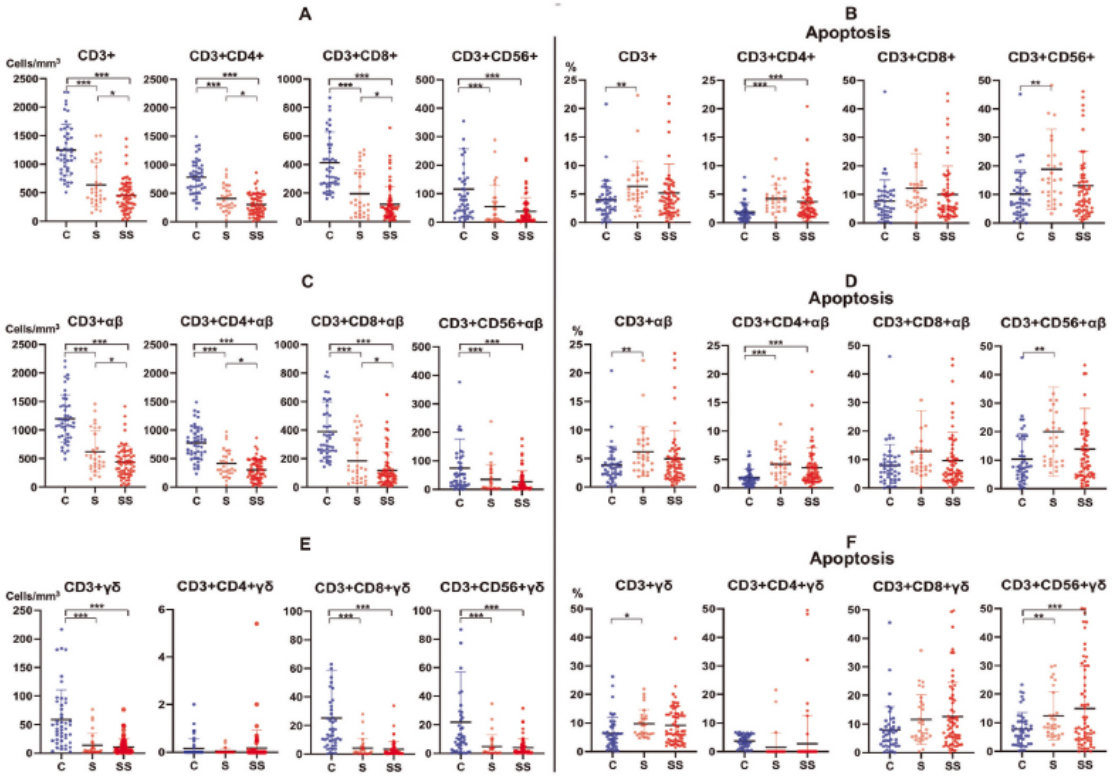


Fig. 1. Numbers (means) and apoptosis percentages of T cell subsets at admission according to Sepsis (S, n = 29), Septic Shock (SS, n = 63) and control healthy subjects (C, n = 46). T cell subsets (Panel A and B); αβ T cells subsets (panel C and D); and γδ T cell subsets (panel E and F). Values are expressed as means (cells/mm3), and double T bars denote standard deviation. (***p < 0.001, **p < 0.01 and *p < 0.05). Differences between C, S and SS, Mann-Whitney U test was used.

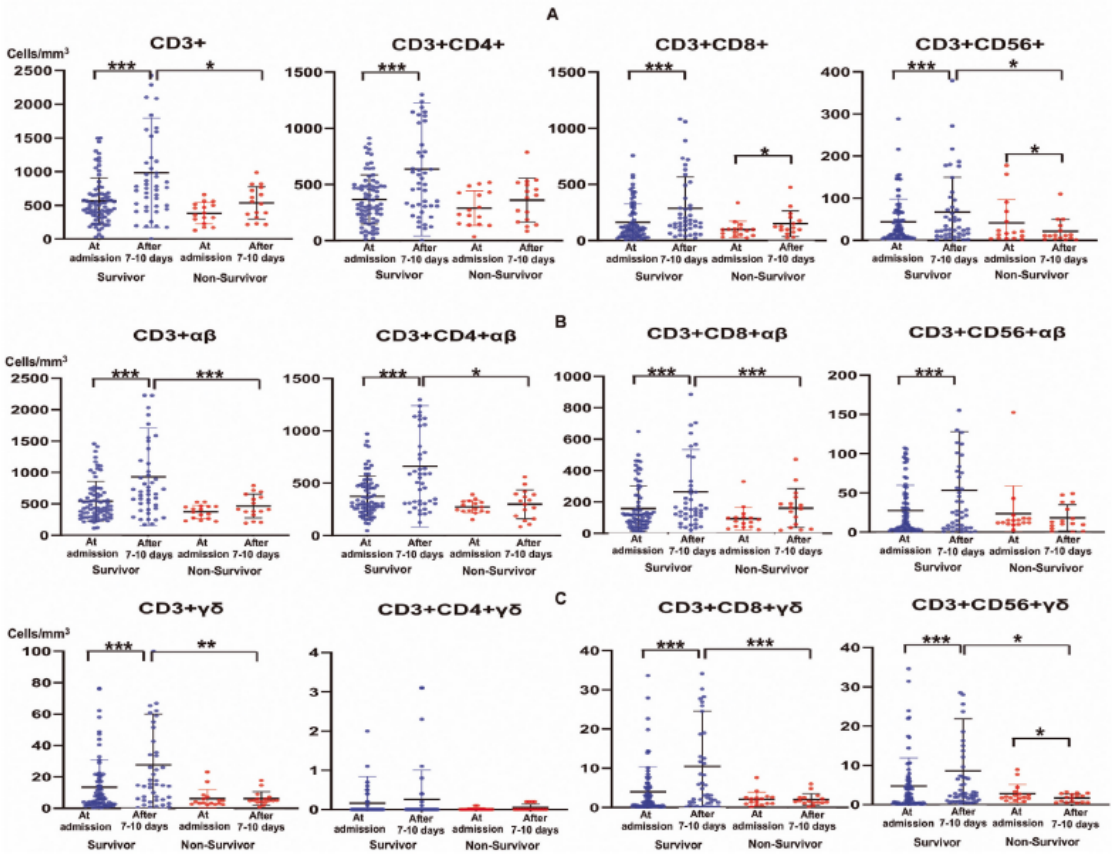


Fig. 2. Numbers of T cell subsets according to surviving (blue) and non-surviving (red) (n = 16) septic patients and evolution time: at admission and after 7–10 days. T cell subsets (Panel A); αβ T cell subsets (panel B) and γδ T cell subsets (panel C). Values are expressed as means (cells/mm3), and double T bars denote standard deviation (***p < 0.001, **p < 0.01 and *p < 0.05). Differences between first analysis (at admission) and second analysis (after 7–10 days) Wilcoxon test was used. Differences between surviving (blue) and non-surviving (red) patients, Mann-Whitney U test was used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

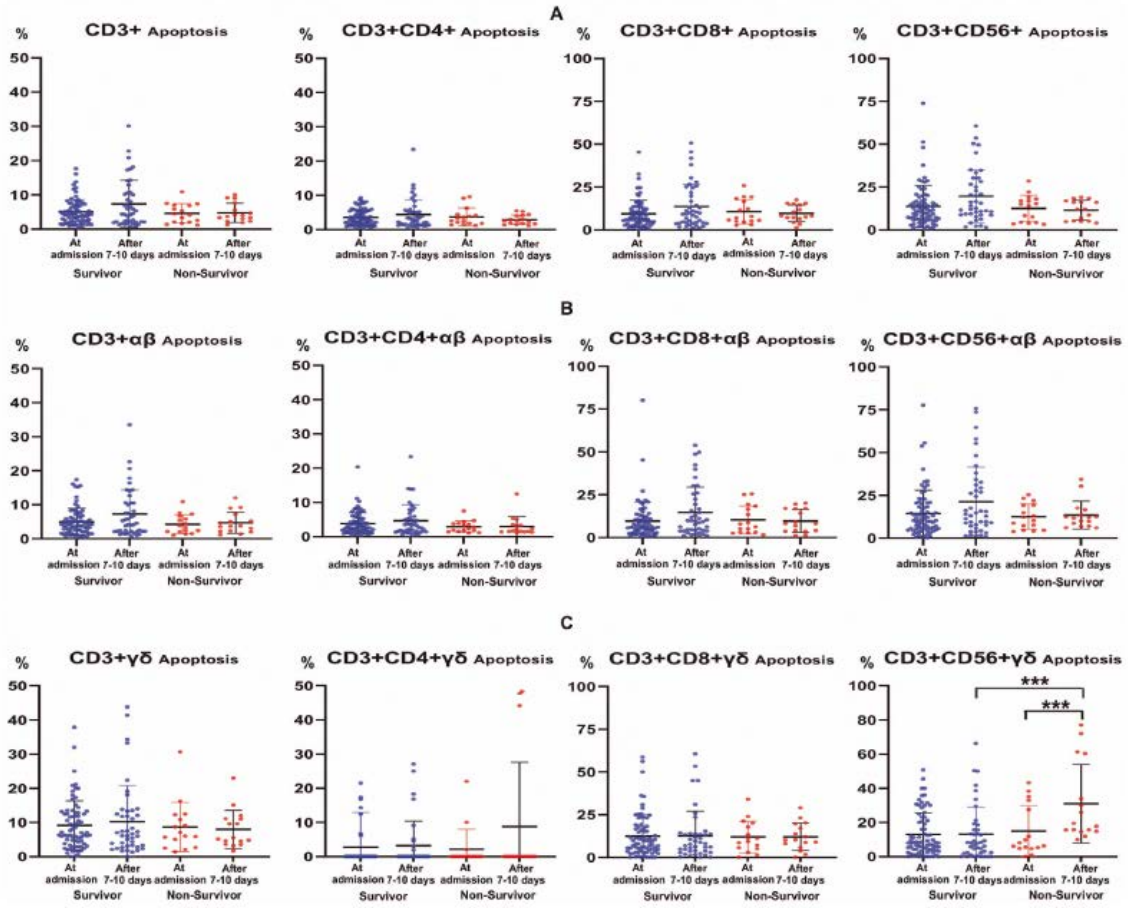
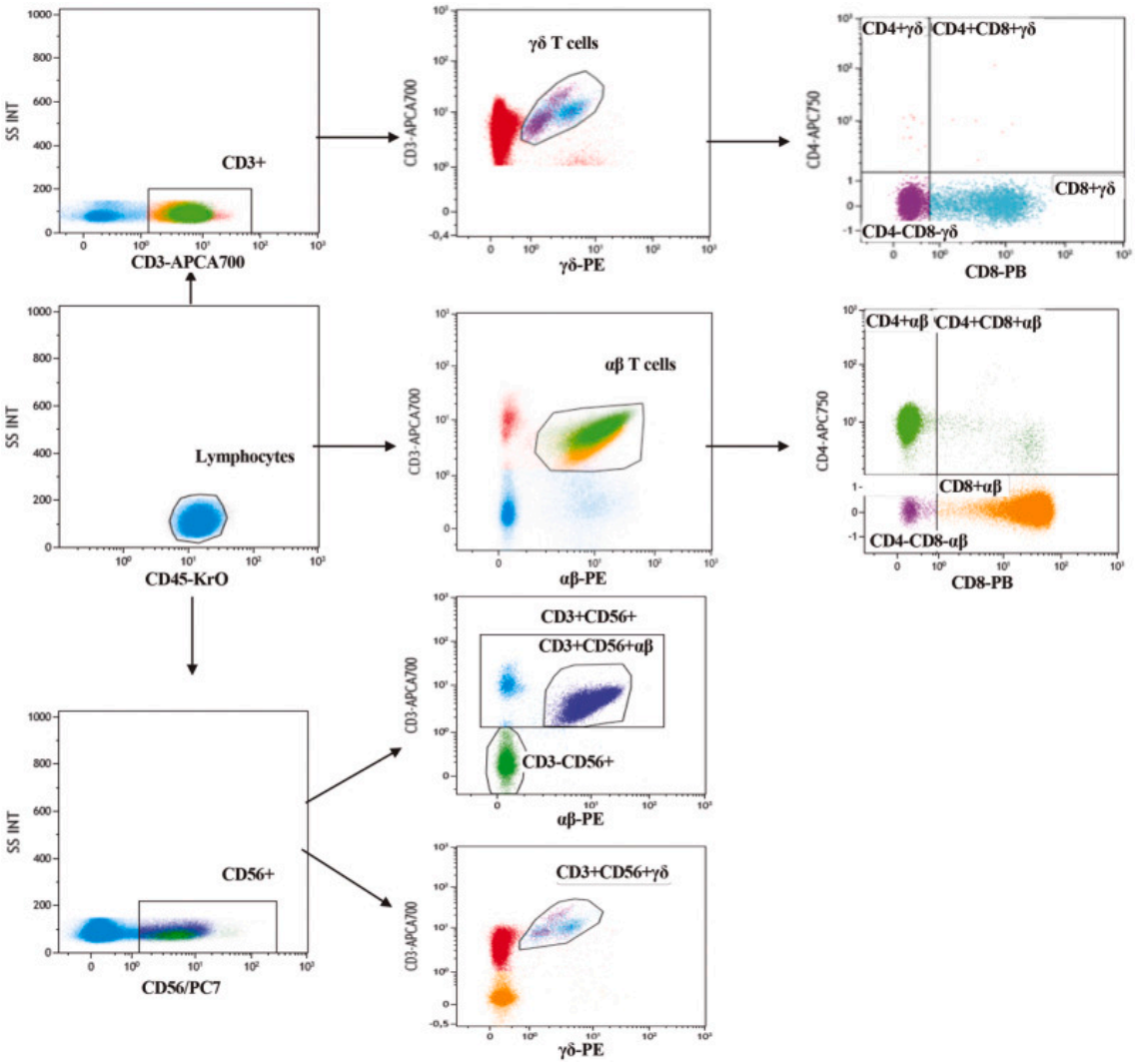
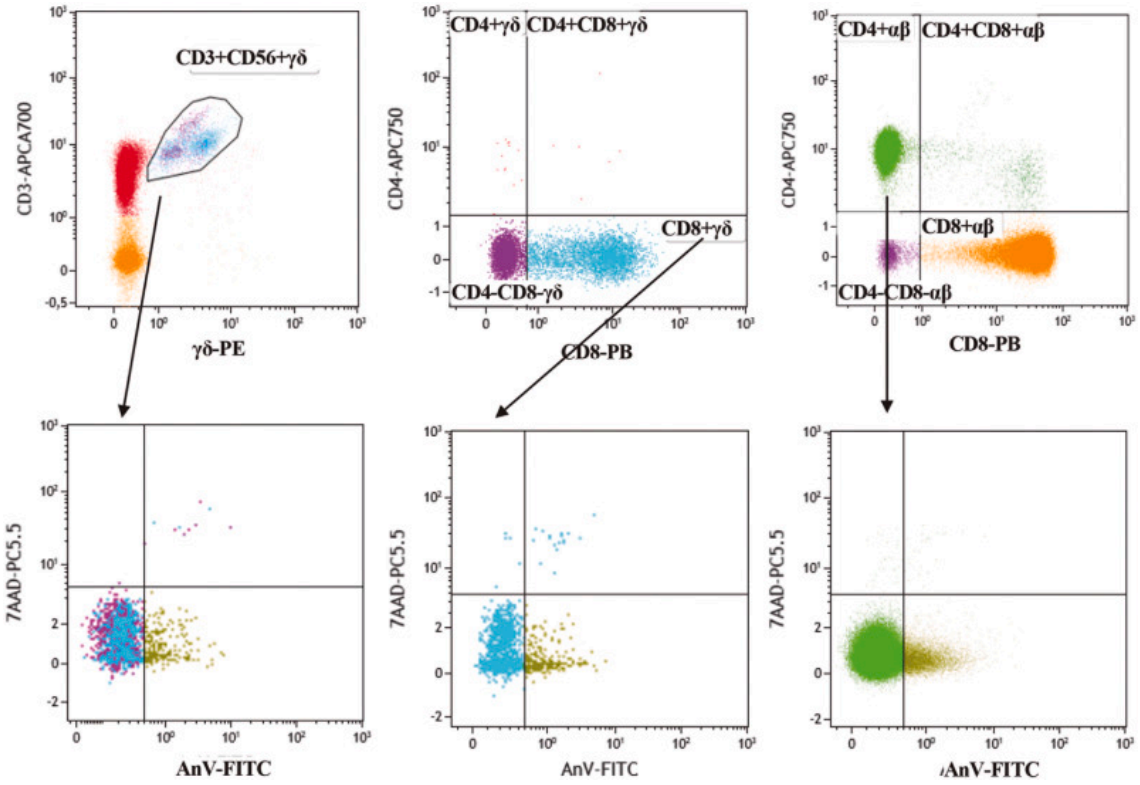


Fig. 3. Apoptosis percentages of T cell subsets according to surviving (blue) and non-surviving (red) (n = 16) septic patients and evolution time: at admission and after 7–10 days. T cell subsets (Panel A); αβ T cell subsets (panel B) and γδ T cell subsets (panel C). Double T bars denote standard deviation (***p < 0.001, **p < 0.01 and *p < 0.05). Differences between first analysis (at admission) and second analysis (after 7–10 days), Wilcoxon test was used. Differences between surviving (blue) and non-surviving (red) patients, Mann-Whitney U test was used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Supplementary Fig. S1. Supplementary file 1. Gating strategy for T cell subsets. Gating strategy for T cell subsets. Peripheral blood events were measured against forward and side scatter parameters, and total lymphocytes were selected. From CD3 we differentiate the αβ and γδ T cell subpopulations, and from them we face the expression of CD4 against CD8. CD3 + CD56+ cells were analyzed in parallel from total lymphocytes.



Supplementary Fig. S2. Supplementary file 2. Gating strategy for apoptosis (Annexin V/7-AAD). Flow cytometry analysis showing annexin positive cells from CD3 + CD56 + γδ + gate, CD3 + CD8 + γδ + gate and CD3 + CD4 + αβ + gate. After select the target population, we obtain the apoptotic cells in the AnV/7-AAD histogram. AnV+/7-AAD- cells are defined as cells in early apoptosis.