

# Cellular and Molecular Markers of Outcome in Septic Shock

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## 1. Introduction

Sepsis can be defined as a generalized inflammatory response that occurs during infection (Bone et al. 1992) and it seems that a defective host immune system response to a microbiological challenge is pivotal in the pathogenesis of septic shock. The pathogenesis of sepsis is a result of a complex network of events involving inflammatory and anti-inflammatory processes, molecular and cellular reactions and circulatory abnormalities (Hotchkiss & Karl 2003). Signs and symptoms of sepsis are non-specific for the diagnosis of sepsis, but early and appropriate intervention is critical for morbidity and mortality (Levy et al. 2005; Rivers et al. 2001). There is a need to find early markers of infection in patients with systemic inflammatory response syndrome (SIRS), and also to establish procedures for a more accurate risk assessment, predicting patients' outcome, guiding antibiotic therapy or predicting the development of different organ dysfunctions.

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" (Biomarkers 2001). Countless biomarkers of septic shock have been proposed but few or none are currently used in clinical practice or studies. This lack of clinical application of research findings is due to several reasons: the lack of "gold standard" for the diagnosis, the complex pathophysiology of sepsis, which involves many processes as inflammation, immunity, coagulation, etc. Thus, we studied the prognostic value of surface molecule expression on lymphocytes and serum levels of the main cytokines, chemokines and adhesion molecules to classify the survival of the patients with septic shock.

First, we demonstrated a severe redistribution of T lymphocyte subsets in patients with septic shock. A different kinetic pattern of T cell subset involvement is observed in surviving and nonsurviving patients, with lower numbers of circulating CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD62L<sup>+</sup> cells being associated with a better disease outcome (Monserrat et al. 2009b). Second, we have also studied the predictive value for outcome of combining different T-cell, B-cell and NK-cell markers in septic shock patients. We have found a set of five immunophenotypic variables CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>, CD19<sup>+</sup>CD80<sup>+</sup>, CD56<sup>+</sup>CD69<sup>+</sup>, CD3<sup>+</sup>CD11a<sup>br</sup>CD11b<sup>+</sup> cells blood counts that improve the prediction for outcome in septic shock patients to a sensitivity of 94% and a specificity of 100% (Monserrat et al. 2009a).

In another study of prognostic value of serum mediators, we determined by sandwich ELISA the serum levels of proinflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$ , and IL-6) and soluble cytokine antagonists (sTNF-RI, sTNF-RII, and IL-1Ra) in 52 patients with septic shock and in 36 healthy controls at ICU admission and 3, 7, 14, and 28 days later. We demonstrated that serum levels of most of the pro- and anti-inflammatory molecules examined (TNF $\alpha$ , IL-6, sTNF-RI, sTNF-RII, and IL-1Ra) were significantly increased upon admission and during the 28-day observation period in septic patients when compared to controls. Remarkably, the serum levels of anti-inflammatory mediators sTNF-RI, sTNF-RII, and IL-1Ra were better predictors of mortality than the levels of proinflammatory cytokines (de Pablo et al. 2011). We also measured soluble VCAM-1, ICAM-1, ICAM-2 and PECAM-1 levels and we demonstrated that they were significantly higher in septic shock patients at ICU admission and during the follow-up than in healthy controls. Serum soluble ICAM-1 was the best biomarker for separating patients with infection from those with non-infectious SIRS. On the other hand, soluble E-Selectin found to be a sensitive marker of endothelial damage, which may result in multiple organ dysfunction syndrome and death.

## 2. Role of peripheral blood lymphocytes in septic shock outcome

The role of the lymphocytes in the septic shock it's not clear and it has been studied extensively in the last decades (Holub et al. 2000; Holub et al. 2003; Lin et al. 1993; Menges et al. 1999; Nishijima et al. 1986). However, there are not many authors that have proposed to use lymphocytes as biomarkers for the outcome of these patients (Keane et al. 1983; Monserrat et al. 2009a). Thus, we decided study the role of the T, B and Natural Killer (NK) lymphocytes such as biomarkers in the prediction of the outcome of septic shock patients.

The T cell compartment plays a critical role in regulating the effector stage of the immune response. CD3<sup>+</sup>CD4<sup>+</sup> T cells are mainly involved in the regulation of the immune response while CD3<sup>+</sup>CD8<sup>+</sup> T cells are critical in the cytotoxic response (Ochoa & Makarenkova 2005). Several molecules, mainly the T cell receptor/CD3 complex and other co-receptors including CD28, contribute to the activation of T lymphocytes. The expression patterns of other molecules, such as the CD45 isoforms RA and RO, vary during the different T cell activation effector stages (Hermiston et al. 2003). Activated T lymphocytes show several profiles (proinflammatory or anti-inflammatory) of cytokine production (Curfs et al. 1997). Other molecules, such as CD62L, participate in the immune response by regulating the tissue distribution of the T lymphocytes (Sallusto et al. 1999). Therefore, a role for T lymphocytes in severe systemic bacterial infections has been described in several studies, and the findings of other investigations have supported the notion that T lymphocytes are involved in the pathogenesis of septic shock (Holub et al. 2000; Holub et al. 2003; Kabisch et al. 1990; Lin et al. 1993; Nishijima et al. 1986).

The NK compartment is characterized as CD3<sup>-</sup>CD56<sup>+</sup> cells that can be cytotoxic (CD16<sup>+</sup> Bright) or produce high amount of cytokines such as IFN $\gamma$  (CD16<sup>+</sup> Dim) (Romagnani et al. 2007). NK cells are also engaged in crosstalks with other immune cells, such a dendritic cells, monocytes, macrophages (Bellora et al. 2010) and neutrophils (Costantini & Cassatella 2011). Several lines of evidence suggest that NK cells might be involved in key functions during sepsis. During the early stage of septic shock, NK cells may play a key role in the promotion of the systemic inflammation as suggested in mice models, but at later stage, NK cells-acquired dysfunction could favor nosocomial infections and mortality (Chiche et al. 2011).

Defects in T and NK lymphocytes are accompanied by alterations in the humoral response. The functional consequences of these humoral immune defects are an impaired ability to respond to antigens with a rapid and appropriate immune response to microbial antigens (Opal et al. 2005). The role of B cells in septic shock is unclear and weakly studied.

In this first study, we have further characterized the abnormalities of the T cell compartment in septic shock and explore its clinical significance. During the first 28 days of follow up circulating T lymphocytes of 52 patients with septic shock admitted to the intensive care unit (ICU) were analysed and 36 healthy subjects were analysed in parallel. We determined the counts and distributions of the main T cell subsets, as well as their stage of activation (CD3, CD4, CD8, CD28, 45RA, 45RO and 62L antigens).

## **2.1 Patients and methodology**

### **2.1.1 Patients and study design**

Fifty-two consecutive patients admitted to the ICU of the University Hospital "Príncipe de Asturias", Madrid, Spain, with septic shock, diagnosed according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine (Bone et al. 1992), were enrolled in the study. A further requirement was the demonstration of an infectious aetiology through microbiological (Gram stain and/or culture) and/or radiological techniques, or direct observation of the infection focus. The study protocol did not call for a standardised approach to critical care. Exclusion criteria were: anything causing primary or acquired immunodeficiency, previous immunosuppressive or immunomodulation treatment, cancer, or autoimmune or allergic disease. The study was conducted according to the guidelines of the 1975 Declaration of Helsinki, after obtaining the Hospital Universitario Príncipe de Asturias Ethics Committee approval. Written informed consent was obtained from each subject included in the study or surrogate legal representatives. Thirty-six age-matched and sex-matched healthy blood donors were studied in parallel with the patients (0 and 28 days of the follow up). They were studied to control the adequacy of the cytometric techniques as well as to characterise the normal range of the T cell compartment parameters analysed.

Blood was collected from these patients at baseline (ICU admission) and at 3, 7, 14 and 28 days of follow up, and at baseline and at 28 days in healthy controls. White blood cell differential counts were conducted in a COULTER®LH instrument (Beckman-Coulter Inc).

### **2.1.2 Cell separation and surface immunofluorescence**

Peripheral blood mononuclear cells (PBMC) were obtained from heparinised venous blood by Ficoll-Hypaque (Lymphoprep Nyegaard) density gradient centrifugation. Cells were resuspended ( $1 \times 10^6$  cells/ml) in RPMI-1640 (Biowhittaker Inc.) supplemented with 10% heat-inactivated FBS (Cangera International), 25 mM Hepes (Biochrom KG) and 1% penicillin streptomycin (Difco Lab).

T cells were phenotypically analysed in PBMC by four-colour flow cytometry in a FACScalibur cytometer using CellQuest software (Becton-Dickinson). PBMC were incubated with combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-labelled monoclonal antibodies. The monoclonal antibodies were CD3-PerCP, CD3-FITC, CD8-PerCP, CD45RA-FITC, CD56-PE, CD28-PE, CD62L-PE (Becton-Dickinson), CD8-APC, CD45RO-PE (Caltag Laboratories).

### 2.1.3 Blood lymphocyte count calculation

Blood lymphocyte counts of T lymphocyte subsets were calculated according to standard flow cytometry criteria for lymphocyte subset identification and the lymphocytes counts obtained in conventional haemogram. First, we calculated the percentage of cells expressing CD3 in the total lymphocytes gate defined by forward and side scatter in PBMC. Blood lymphocyte count of circulatory T lymphocytes was calculated by the percentage of CD3<sup>+</sup> cells in peripheral blood lymphocytes multiplied by the total number of lymphocytes per microlitre measured by a Coulter®. Next, we obtained the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes by multiplying the total number of T lymphocytes previously calculated by the percentage of positive cells for each one of both antigens in CD3<sup>+</sup> T cells. We simultaneously stained PBMC with CD3, CD4 and CD8 antibodies to obtain this data. Finally, we calculated the absolute number of the CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells subsets defined by the expression of CD45 isoforms CD45RA<sup>+</sup>CD45RO<sup>-</sup>, CD45RO<sup>+</sup>CD45RA<sup>-</sup>, CD45RA<sup>+</sup>CD45RO<sup>+</sup> and the expression of the antigens CD28<sup>+</sup> and CD62L<sup>+</sup>. To calculate these numbers, we multiplied the percentage obtained of each subset in the parents' CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> populations by the absolute count of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells, respectively. All lymphocyte counts are expressed as cells/ $\mu$ l.

### 2.2 Septic shock patients with different outcomes show different patterns of circulating T cell subsets

Blood counts and distributions of the main circulating T lymphocyte subsets were systematically examined in 52 patients with septic shock at admission to the ICU and at 3, 7, 14 and 28 days of follow up in the ICU. Patients were classified as survivors or nonsurvivors according to their clinical outcome of sepsis during the four weeks of follow up. Thirty-six healthy blood donors who were age-matched and sex-matched ( $60 \pm 3.4$  years, 25 men and 11 women) were studied in parallel with the patients as controls.

Blood lymphocyte count was  $2095 \pm 93$ ,  $1048 \pm 192$  and  $1235 \pm 178$  cells/ $\mu$ l in controls, survivors and non-survivors respectively. Both, surviving and nonsurviving patients, showed significantly lower absolute CD3<sup>+</sup> T lymphocyte counts than controls on admission and during the first 14 days of follow up. In survivors, CD3<sup>+</sup> counts had significantly returned to normal by day 28 (Figure 1A). The CD3<sup>+</sup>CD4<sup>+</sup> T cell subset was also reduced in surviving and nonsurviving patients with respect to healthy controls at baseline and during the first week of follow up. However, this severely decreased CD3<sup>+</sup>CD4<sup>+</sup> T lymphocyte count had normalised in survivors by day 14 (Figure 1B). On admission, CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes were also lower in survivors and nonsurvivors than in controls. In survivors, this T cell subset showed a further drop on day 3 of follow up, followed by a gradual recovery, although numbers failed to reach the count recorded in healthy controls. In addition, CD3<sup>+</sup>CD8<sup>+</sup> T lymphocyte count in survivors was significantly diminished with respect to nonsurvivors on day 3 (Figure 1C).

The activation stage of the CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes was determined by examining the expression of the CD45 RA and RO isoforms. The retraction in circulating CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes observed in the patients could be mainly explained by a decrease in the noneffector CD45RA<sup>+</sup>CD45RO<sup>-</sup> subset (Figures 2a,d). Interestingly, CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> T lymphocytes remained low in survivors at the end of follow up. We detected a significant decrease in CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> T cells on day 3 with respect to the nonsurviving patients. CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup> T cell counts varied over time from a significant

reduction during the first week of follow up to elevated numbers in survivors during the last two weeks of the study (Figures 2a,c). The analysis of effector subsets, characterised by being double positive (CD45RA+CD45RO+) (Hermiston et al. 2003; Najera et al. 2001), showed that were significantly reduced in both CD4+ and CD8+ T lymphocyte subsets at baseline, 3 and 7 days in both groups of patients compared with controls and in survivors compared with nonsurvivors at 3 days. From day 7 of follow up onwards, these values normalised in the surviving patients (Figures 2b,e).

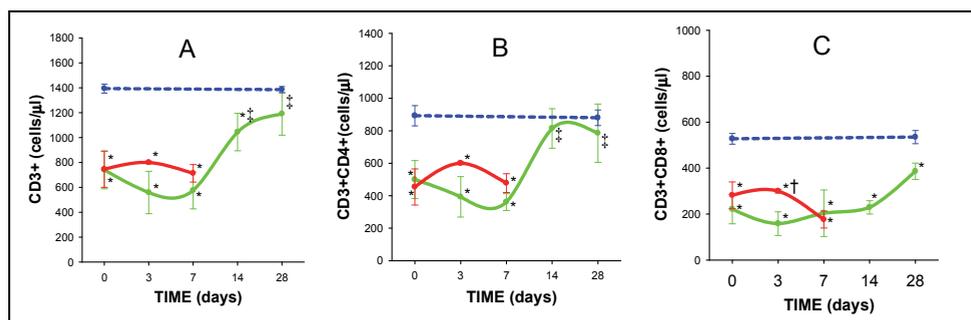


Fig. 1. Kinetic of peripheral blood counts of CD3+, CD3+CD4+ and CD3+CD8+ lymphocyte subsets in patients with septic shock during their stay in the ICU. Symbol represents: (■) Controls line base, (●) Survivors and (●) Nonsurvivors. All values are expressed as the mean of cells/ $\mu$ l  $\pm$  S.E.M. \* $p < 0.05$  for survivors or nonsurvivors versus controls; †  $p < 0.05$  for survivors versus nonsurvivors; ‡  $p < 0.05$  for each follow up time versus admission.

We also analysed the expression of CD28 and CD62L antigens on CD3+CD4+ and CD3+CD8+ T cells. When CD3+CD8+ T cells are activated, CD28 expression is lost (Fiorentini et al. 2001; Labelette et al. 1999). Number of circulating CD3+CD8+CD28+ T cells was significantly and constantly reduced in patients with septic shock compared with the healthy subjects and in survivors compared with nonsurvivors during the first three days of follow up (Figures 3A, 4). Similar behaviour was shown by CD3+CD8+CD62L+ T cells (Figures 3B, 4). Numbers of circulating CD3+CD8+CD28- T cells were normal in both groups of patients.

A prediction receptor operative curve (ROC) was then used to estimate the value of CD3+CD8+CD28+ and CD3+CD8+CD62L+ T cell counts for predicting death in the patients with septic shock at admission and days 3 and 7. We found that a cutoff value of 136 CD3+CD8+CD28+ T cells/ml on admission to the ICU of a patient with septic shock showed a sensitivity of 70% and 100% specificity for predicting the risk of death, and the area under the ROC curve was 0.84. For the CD3+CD8+CD62L+ T cells, the cut off on admission was 141 cells/ml, with a 60% sensitivity and 100% specificity for predicting the risk of death and an area under the curve of 0.75. The sensitivity and specificity of the data obtained at days 3 and 7 were worse than those found at admission.

The number of circulating CD3+CD4+CD28+ T cells was significantly lower in surviving and nonsurviving patients with septic shock compared with healthy controls on admission and on day 3 of follow-up (Figure 5a). In survivors, this was followed by a gradual recovery of CD3+CD4+CD28+ T cell numbers during the course of follow up. CD3+CD4+CD62L+ T cells showed a similar pattern of behaviour (Figure 5b). In the parallel study performed in healthy blood donors (at 0 and 28 days of the follow up), no significant variations in the absolute counts and distribution of the different subsets of T cells analysed were detected.

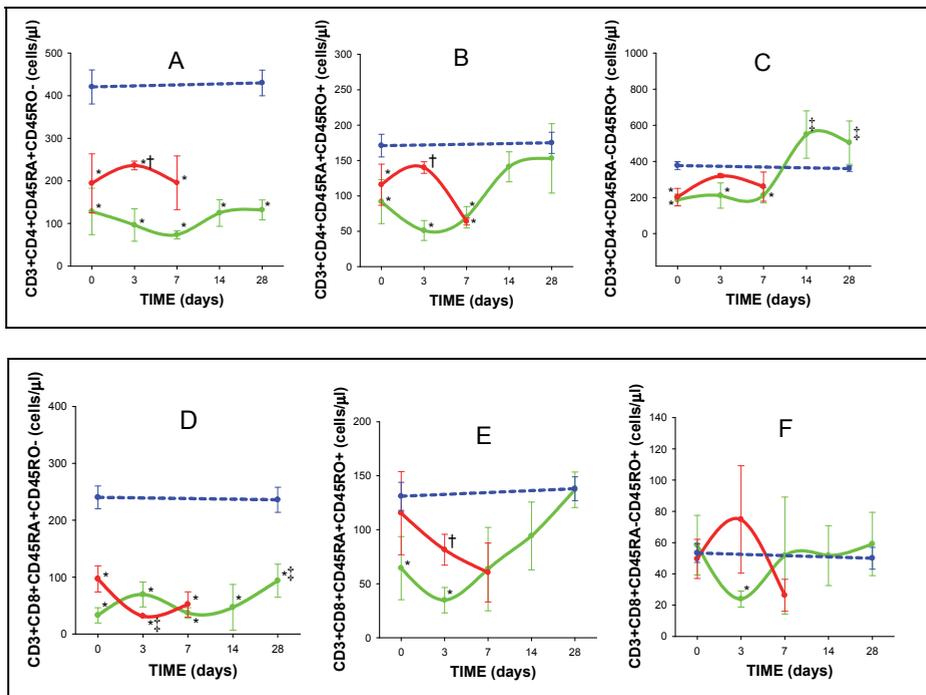


Fig. 2. Kinetic of peripheral blood counts of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> T lymphocyte subsets in patients with septic shock during their stay in the ICU. Symbol represents: (■●■) Controls line base, (—●—) Survivors and (—●—) Nonsurvivors. All values are expressed as the mean of cells/μl ± S.E.M. \*p<0.05 for survivors or nonsurvivors versus controls; †p<0.05 for survivors versus nonsurvivors; ‡p<0.05 for each follow up time versus admission.

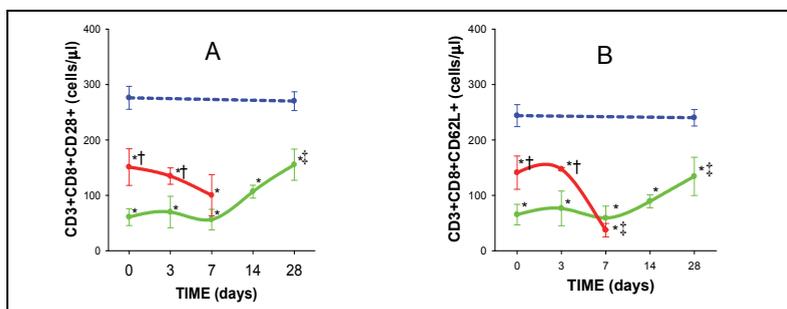


Fig. 3. Kinetic of peripheral blood counts of CD3+CD8+CD62L<sup>+</sup> and CD3+CD8+CD62L<sup>-</sup> lymphocyte subsets in patients with septic shock. Symbol represents: (■●■) Controls line base, (—●—) Survivors and (—●—) Nonsurvivors. All values are expressed as the mean of cells/μl ± S.E.M. \*p<0.05 for survivors or nonsurvivors versus controls; †p<0.05 for survivors versus nonsurvivors; ‡p<0.05 for each follow up time versus admission.

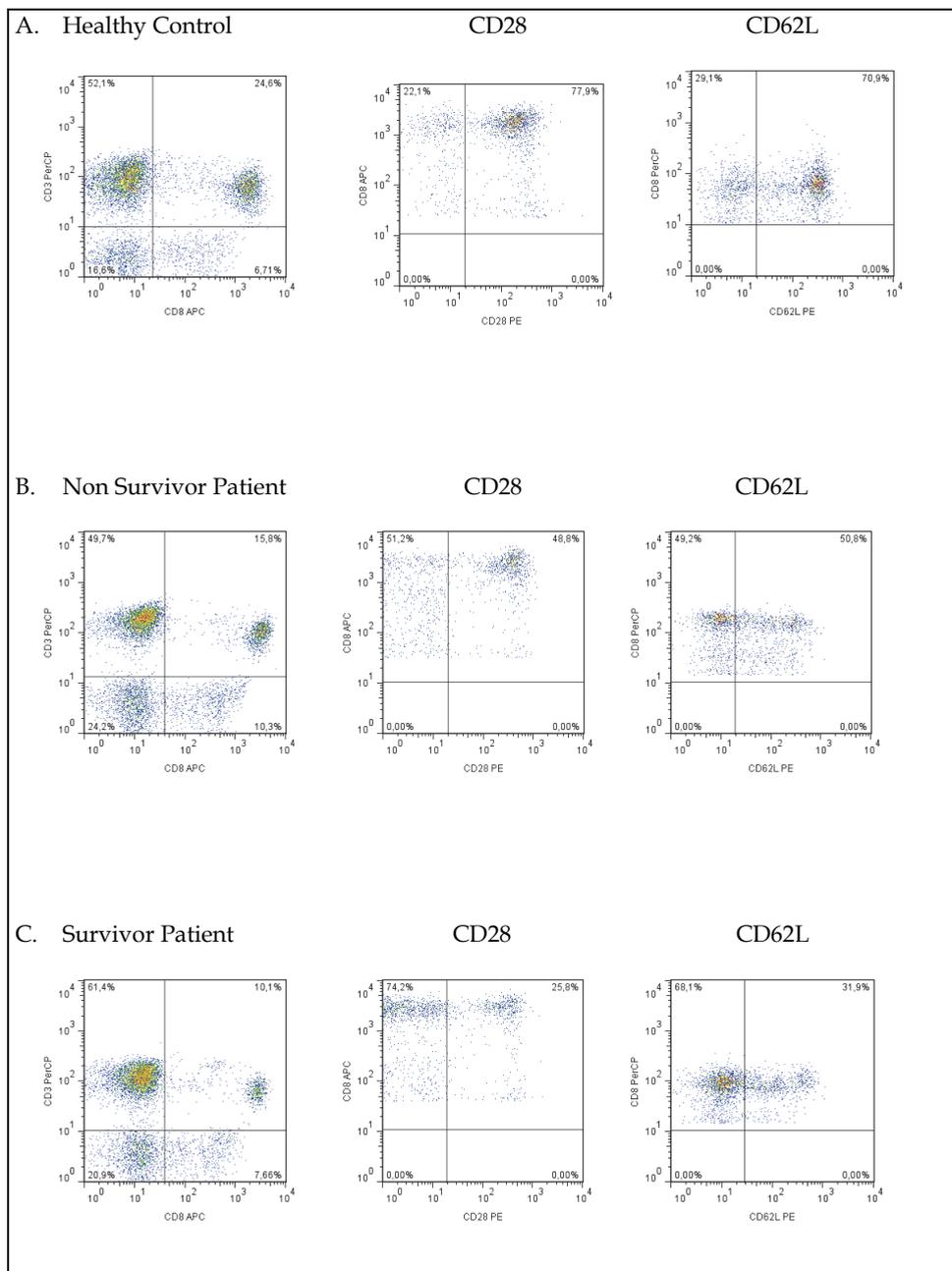


Fig. 4. Flow cytometry data analysis of the CD28 and CD62L surface expression in CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes from peripheral blood of septic shock patients

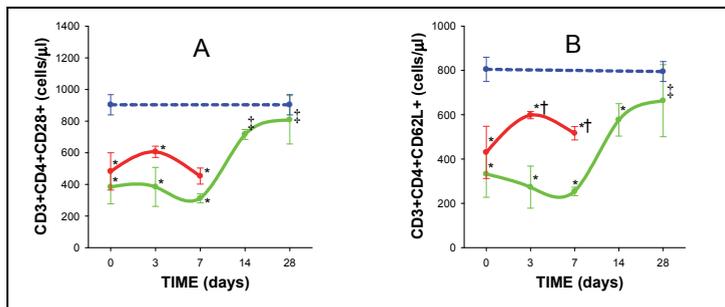


Fig. 5. Kinetic of peripheral blood counts of  $CD3^+CD8^+CD62L^+$  and  $CD3^+CD8^+CD62L^+$  lymphocyte subsets in patients with septic shock. Symbol represents: (■●■) Controls line base, (●—●) Survivors and (●—●) Nonsurvivors. All values are expressed as the mean of cells/ $\mu$ l  $\pm$  S.E.M. \* $p < 0.05$  for survivors or nonsurvivors versus controls; † $p < 0.05$  for survivors versus nonsurvivors; ‡ $p < 0.05$  for each follow up time versus admission.

### 2.3 Interpretation of blood lymphocyte count alterations in patients with septic shock

In this study, we show that surviving and nonsurviving patients with septic shock have different patterns of involvement in circulating T lymphocyte compartment. A drop in circulating  $CD3^+CD4^+$  and  $CD3^+CD8^+$  T cells has been described in patients with severe sepsis or septic shock at admission to the ICU (Holub et al. 2000; Holub et al. 2003; Kabisch et al. 1990; Lin et al. 1993; Nishijima et al. 1986). In our kinetic study, we also observed that this T lymphopenia persists during the first week of follow up and is independent of the outcome. Moreover, by the end of the second week of follow up, the absolute number of circulating  $CD3^+CD4^+$  T cells had clearly normalised. In contrast, after four weeks of follow up, there was still no return to normal circulating numbers of  $CD3^+CD8^+$  T cells.

In a mouse sepsis model of caecal ligation and puncture, the depletion of  $CD3^+CD8^+$  T and natural killer cells was associated with a survival benefit with decreased blood bacterial concentrations, improved physiological function and an attenuated proinflammatory response (Sherwood et al. 2004). It has been reported that mice infected with *Plasmodium berghei* develop a syndrome similar to septic shock and the depletion of  $CD3^+CD8^+$  T cells also significantly ameliorates the complications that induce shock (Chang et al. 2001). In addition, in patients with trauma and multiple organ failure (MOF), nonsurvivors showed  $CD3^+CD8^+$  T cell numbers that were two-folds higher than those recorded in survivors (Menges et al. 1999). In agreement with these experimental and clinical findings, we observed significantly lower  $CD3^+CD8^+$  T cell counts in survivors compared with nonsurvivors on day three of follow up. Thus, diminished circulating  $CD3^+CD8^+$  T cells might have a protective pathogenic role in the outcome of septic shock.

The expression patterns of the RA and RO isoforms of CD45 by T lymphocytes serve to identify subsets associated with different stages of T cell activation. When noneffector  $CD45RA^+CD45RO^-$  T lymphocytes are activated by inflammatory agents, such as bacterial infection, CD45RO is up-regulated and CD45RA down-regulated (Hermiston et al. 2003). Thus, our patients showed a persistent reduction in circulating noneffector  $CD4^+CD45RA^+CD45RO^-$  and  $CD8^+CD45RA^+CD45RO^-$  T cells. In contrast, numbers of  $CD8^+CD45RA^-CD45RO^+$  T cells remained normal and  $CD4^+CD45RA^-CD45RO^+$  T cell counts initially fell yet had returned to normal by the second week of follow up in surviving patients. An

increased percentage of CD3<sup>+</sup> cells expressing CD45RO has been reported in patients with sepsis (Roth et al. 2003). Our findings could be the consequence of abnormal polyclonal activation of circulating T lymphocytes. It has been proposed that the switch of T cells from a CD45RA<sup>+</sup>CD45RO<sup>-</sup> to a CD45RA<sup>-</sup>CD45RO<sup>+</sup> phenotype may have a functional effect in halting the sustained immune response in an effort to avoid tissue injury (Hermiston et al. 2003). Thus, the expansion of CD4<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup> T lymphocytes observed here during the follow up of surviving patients might be considered a compensatory anti-inflammatory mechanism that develops in these patients with septic shock.

CD28 is a costimulatory molecule that plays a key role in regulating the activation and survival of T lymphocytes (Sansom & Walker 2006). Activation of CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes has been related to the loss of CD28 expression (Fiorentini et al. 2001; Labalette et al. 1999). In effect, it has been reported that patients with severe sepsis showed a significant reduction in T lymphocyte CD28 expression (Manjuck et al. 2000). Our data indicate diminished circulating CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup> T cell numbers in patients with septic shock with respect to healthy subjects on admission to the ICU and at least during the first 28 days of follow up. Interestingly, a reduced CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup> T cell count during the first three days of admission to the ICU was of prognostic value for predicting the survival of a patient. Conversely, an elevated number of circulating CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup> T cells was associated with a worse prognosis for the patient. Recently, it has been reported that the stimulation of CD28 by a monoclonal antibody in healthy volunteers is followed by severe multiple cytokine-release syndrome (Suntharalingam et al. 2006). Our results support a relevant role for CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>+</sup> T cells in the pathogenesis of septic shock. Future studies should address the potential clinical relevance of this cell variable.

The migration of circulating T lymphocytes to peripheral lymph nodes depends on the expression of the CD62L homing receptor (Tang et al. 1998). We found here that the down-regulation of L-selectin expression on CD3<sup>+</sup>CD8<sup>+</sup> cells in patients with septic shock was associated with a better outcome. Hence, the rapid migration of CD8<sup>+</sup> T cells to peripheral lymph nodes may be a mechanism contributing to patient survival.

Our T lymphocyte phenotype data show a time difference, or shift, in the recirculation of T lymphocytes between patients who survive septic shock and those who do not. Taken together and analysing the phenotype of the circulating T cells according to the activation criteria (CD45RA<sup>+</sup> and CD45RO<sup>+</sup>) related to CD28 (activation and co-stimulation) and CD62L (activation and migration) expression point to a slower migration of naive and effector cells in nonsurviving patients. This different T lymphocyte kinetics would mean a delayed tissue response that could determine the failure of the immune system and the fatal prognosis of the patient. In particular, the delay in the disappearance of CD45RA<sup>+</sup>, CD45RA<sup>+</sup>CD45RO<sup>+</sup>, CD28<sup>+</sup>, CD62L<sup>+</sup>, T CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes observed between days 3 and 7 of follow up in the nonsurvivors appears to be crucial to the final outcome. Accordingly, in surviving patients, effectors cells would migrate more rapidly to tissues and this would in turn trigger the quick action of the immune system in combating the infection and thus determine the survival of the patient. It is known that cellular immune responses play a critical role in the defense against viral infections and strong T-cell responses have been reported in patients who clear infection (Sarobe et al. 2006a). If the immune response is late or less efficient against microorganism viral epitopes, the outcome of the disease worsens (Hermiston et al. 2003; Lim et al. 2006; Sarobe et al. 2006b). Not surprisingly, the survivor group had lower APACHE II, MODS and SOFA scores than nonsurvivors. An increasing APACHE II score reflects an increasing severity of illness and escalating risk of

hospital death for multidagnostic ICU patient groups. However, an APACHE II score cannot be directly equated with a specific risk of lower mortality than the same score for a patients with septic shock (Wagner et al. 1986). In this group of patients, we found that a cut-off value of 136 cells/ml for CD3+CD8+CD28<sup>+</sup> T cells and 141 cells/ml for CD3+CD8+CD62L<sup>+</sup> T cells on ICU admission showed high specificity for predicting the risk of death. However, it is known that the positive predictive value for APACHE II for the validation study population was only 69.6% and the negative predictive value was 87.9% (Joseph E.Parrila & Roger C.Bone 1995). Moreover, SOFA, MODS and APACHE II scores require at least 24 hours of monitoring to be performed and lymphocyte phenotyping can be performed in a short time, approximately 2 hours. It is not possible to replace clinical score in septic shock patients by immunological markers. However, these analytical parameters may help to make clinical decisions in these patients and to establish new potential therapeutic targets. Future studies will need to study in more depth the mechanisms involved in the severe abnormality found on the T cell compartment in patients with septic shock.

#### **2.4 Cytomics: In the future of the study of cellular biomarkers**

Advances in the knowledge of the immune system has demonstrated the great complexity of the T, B and Natural Killer (NK) lymphocyte subsets (Appay et al. 2008). T cell subsets display defined immunophenotypic markers at different stages of activation and when they have different patterns of tissue migration (Lanzavecchia & Sallusto 2005;Sallusto et al. 2004). Several pathologic conditions have been associated to different alterations of T cell subset distribution (Appay et al. 2008;Wong et al. 2008). In critically ill patients, global T-cell responsiveness is typically reduced, reflected in the inability to respond to recall antigens in vivo or decreased (Christou et al. 1995) and in impaired lymphocyte proliferation to mitogens in vitro (Keane et al. 1983). However, the response to a T-cell-independent antigens is preserved or even enhanced (Nohr et al. 1986). Abnormal redistribution of NK and B lymphocytes subsets have also been found to be involved in the pathogenesis of other diseases (Cerwenka & Lanier 2001;Sanz et al. 2008) but the evidence reported in critical illness is less compelling.

The simultaneous study of different cell subsets distribution can help to translate basic knowledge on immune system alterations into clinically and therapeutically relevant markers. Our study demonstrates that deeply characterizing abnormalities on T cell subsets can have clinical significance in septic shock (Monserrat et al. 2009b). Thus, we hypothesized that the combination of the simultaneous analysis of different immune system cell subsets would improve the prediction of outcome in septic shock patients. In addition to the results described previously in our work and following a cytomics analysis, we have also studied the predicting value for outcome of combining different T, B and NK cell markers in the 52 septic shock patients reported in our article (Monserrat et al. 2009b).

Cytomics is an innovative mathematic and statistic bioinformatics analysis procedure (similar to data mining studies) of multiple cellular variables obtained from a population of patients (Valet G.V. et al. 1998;Valet et al. 1993;Valet 2002). Cytomics allows to select a set of multiple quantitative biological variables able to improve the accuracy of patient prognosis and/or establish a predictive value of response to an optional treatment (Valet & Tarnok 2003). Cytomics studies have been previously applied to different fields in medicine: intensive care (Rothe et al. 1990;Valet G.V. et al. 1998), oncology/hematology (Valet et al. 2003;Valet & Hoeffkes 2004), or autoimmune diseases (Jacobi et al. 2003); moreover a human cytoome project is underway (Valet et al. 2004;Valet & Valet 2005).

Following the methods and flow cytometry technique described previously (section 2.1.2-2.1.3), we quantified the absolute number of circulating CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup> and CD56<sup>+</sup> lymphocytes and their subsets defined by the co-expression of one, two or three of the following antigens: CD69<sup>+</sup>, HLA-DR<sup>+</sup>, CD25<sup>+</sup>, CD26<sup>+</sup>, CD38<sup>+</sup>, CD45RA<sup>+</sup>, CD45RO<sup>+</sup>, CD71<sup>+</sup>, CD23<sup>+</sup>, CD57<sup>+</sup> as activation markers, CD80<sup>+</sup>, CD86<sup>+</sup>, CD40L<sup>+</sup> and CD40<sup>+</sup> as co-stimulation markers and CD11a<sup>+</sup>, CD11b<sup>+</sup>, CD11c<sup>+</sup>, CD31<sup>+</sup>, CD62L<sup>+</sup>, CD29<sup>+</sup> as adhesion markers and CD95<sup>+</sup> as apoptosis marker. This cellular quantification was performed in each patient at ICU admission and the clinical outcome was analyzed over a 28-day period. The absolute numbers of the different lymphocyte subsets obtained in each patient were introduced in a database. Symmetrical upper and lower threshold percentile values (low percentile 33 and high percentile 67) were selected according to the mortality in our series of patients. These thresholds were used to re-express individual data-base values into +, 0 or - according to their position above, within or below the respective reference percentile thresholds as described (Valet G.V. et al. 1998; Valet et al. 1993). Receiver operating characteristics (ROC) curves were built for each phenotypic variable. The sensitivity and specificity of each variable to predict the real outcome was thus obtained (McNeil & Hanley 1984; Metz 1978). The variables with higher sensitivity values were selected and combined to create multiple variable combinations or masks. The mask with the highest sensitivity and specificity prediction ability for outcome was selected to define the combinations of cut-off values for each variable. According to this methodology we have found a set of five variables and their cut off values (showed in table 1) that are able to improve the prediction for outcome on septic shock patients to a sensitivity of 94 per cent and a specificity of 100 per cent.

MASK SUBSET	N°/μl
CD56 <sup>+</sup> CD69 <sup>+</sup>	>114
CD3 <sup>+</sup> CD8 <sup>+</sup> CD45RA <sup>+</sup> CD45RO <sup>-</sup>	>114
CD3 <sup>+</sup> CD8 <sup>+</sup> CD28 <sup>+</sup>	>163
CD19 <sup>+</sup> CD80 <sup>+</sup>	>67
CD3 <sup>+</sup> CD11A BR <sup>+</sup> CD11B <sup>+</sup>	>250

Table 1. Final Mask results after a cytomics study: represent the cut off count of lymphocytes subsets by microlitter that can be able to predict the fatal outcome of the shock septic patients.

These results support the notion that the immune phenotypical analysis of different circulating lymphocyte subsets in septic shock patients has a relevant prognostic value. Leukocyte phenotyping might also have predictive value for the development of immune-supportive or immuno-stimulatory therapies in the management of septic shock patients. The analysis of the abnormalities in the distribution of the T cell compartment in critical patients outside septic shock could also be of great interest (McDunn et al. 2009). We have applied in parallel the immunophotypical T lymphocyte protocol described here to severe acute pancreatitis and ischemic myocardial infarct patients. Our results showed that the patterns of alteration of the distribution and activation stage of T cell subsets are not homogenous in these different acute severe diseases. Thus, the potential involvement of T cell compartment in the pathogenesis of septic shock requires further research and raises the development of innovative diagnostic and therapeutic strategies in these patients.

### 3. Role of the serum molecules in septic shock

#### 3.1 Diagnosis of sepsis

Numerous putative markers of sepsis have been studied: microbial products, physiologic parameters, hematopoietic cells, cell surface markers, soluble receptors, cytokines, acute phase reactants, mediators of coagulation, cellular processes and others like procalcitonin (Marshall et al. 2003). Probably, procalcitonin is the most extensive laboratory marker used for the discrimination of sepsis in patients with SIRS. In 2001, the SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference included plasma procalcitonin above 2SD of the normal value into the diagnostic criteria for sepsis (Levy et al. 2003). They also included plasma C-reactive protein as a criterium for sepsis, but most studies found procalcitonin to be superior to C-reactive protein (Uzzan et al. 2006). We also found these findings and we demonstrate that procalcitonin in combination with Sequential Organ Failure Assessment (SOFA Score) (Vincent et al. 1998) was useful to diagnose infection (Ruiz-Alvarez et al. 2009). However, in newborns, immunosuppressed or elderly patients, procalcitonin had not been shown to be a great asset in the diagnosis of sepsis. Moreover, little is known about serum procalcitonin levels in renal failure and evidence suggests that it is dialyzed (Dahaba et al. 2003).

The release of inflammatory cytokines, such TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IFN $\gamma$  in response to infection leads to SIRS and MOF. Probably, the cytokine that longer has been investigated as a marker of infection is IL-6. IL-6 can be measured reliably in the blood after insult to the host, but it is relatively nonspecific of infection and is elevated in other inflammatory states. When serum IL-6 levels were measured to assess the diagnostic value of infection in patients with SIRS, procalcitonin was superior to IL-6 (Harbarth et al. 2001).

Vascular endothelium injury is widely recognized to play a critical role in many systemic inflammatory diseases as sepsis. However, the ability of the measurement of soluble adhesion molecules to distinguish infection in SIRS patients has been poorly investigated. Boldt et al. demonstrated that trauma patients showed lower plasma levels of circulating adhesion molecules than did sepsis patients indicating more pronounced endothelial activation or damage in sepsis (Boldt et al. 1996). Cummings et al. reported that soluble E-selectin levels are higher in serum of patients with microbiologically documented sepsis than in other critically ill patients (Cummings et al. 1997). Recently, we also studied circulating soluble adhesion molecules levels in 92 patients with SIRS and demonstrated that sICAM-1 was a better biomarker of infection than sE-Selectin, sVCAM-1 and sICAM-2.

An acute-phase proteins has been defined as one whose plasma concentrations increases or decreases by at least 25% during inflammatory disorders (Gabay & Kushner 1999). Lipopolysaccharide-binding protein (LBP) is considered an acute-phase protein. It is synthesized by hepatocytes and intestinal epithelial cells and its concentrations rising up after induction of an acute-phase response. A very interesting issue by Albillos et al. analyzed the risk factors associated with a first episode of severe bacterial infection in 84 ascitic cirrhotics. Increased LBP was the only factor independently associated with severe bacterial infection in a multivariate analysis (relative risk 4.49, 95% CI 1.42-14.1). The authors suggested that monitoring of serum LBP could, therefore, help to target cirrhotic patients with ascites for antibiotic prophylaxis (Albillos et al. 2004). However, LBP measurements in ICU patients with SIRS and sepsis have not been found any benefit (Prucha et al. 2003), except in selected patient populations as heart surgery, neonates or neutropenic patients (Pavcnik-Arnol et al. 2004; Sablotzki et al. 2001).

Phagocytic cells are the primary mediators of the innate immune response to bacterial and fungal infections. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a member of the immunoglobulin family and is upregulated in response to infection acting synergistically with Toll-like receptor-4 to augment the immune response. TREM-1 may be not upregulated in noninfectious inflammatory disorders. A soluble form of TREM-1 (sTREM-1) is shed from the membranes of activated phagocytic cells and can be quantified. Studies conducted in ICUs indicated that sTREM-1 is more specific and sensitive than either procalcitonin or C-reactive protein (Bouchon et al. 2001; Gibot et al. 2005). However, in a recent study in patients admitted in a surgical ICU, measurement of sTREM-1 did not distinguish between patients with SIRS, severe sepsis, or septic shock (Bopp et al. 2009). Finally, a recent study only 10 biomarkers have been assessed for their ability to distinguish septic from non-septic patients with SIRS, but no biomarker was clearly identified as being able to differentiate infection in SIRS from other causes (Pierrakos & Vincent 2010).

### 3.2 Risk assessment and severity

A severity marker provides information about whether a patient with sepsis will experience an adverse outcome. Classical illness scores, such as Acute Physiology and Chronic Health Evaluation (APACHE) and Simplified Acute Physiology Score (SAPS), can predict the severity and outcome of sepsis. Relatively few biomarkers have been evaluated to establish risk assessment and severity in sepsis. Plasma procalcitonin concentrations correlated to sepsis-related organ failure scores and may be useful in risk assessment (Meisner et al. 1999). In 2007, the FDA approved the use of procalcitonin in conjunction with other laboratory findings and clinical assessments to aid in the risk assessment of critically ill patients on their first day of ICU admission for progression to severe sepsis and septic shock.

On the other hand, it has been reported that IL-2 and IL-8 increased in parallel with disease severity (Balci et al. 2003) and anti-inflammatory cytokines as IL-4, IL-10 and IL-13 have higher concentrations in patients with sepsis than in patients with septic shock (Collighan et al. 2004; Heper et al. 2006). It is important to remark that the production of anti-inflammatory cytokines during septic shock correlates positively with the intensity of the inflammatory response and, as we will describe later, with fatal outcome.

The severity of septic reaction varies a lot in course and outcome depending on host predisposing factors, the infection characteristics, the intensity of host response and, finally, the number of organs failing. The PIRO staging model based on these factors could better predict outcomes of patients with sepsis (Rubulotta et al. 2009). The risk of dying from an infectious disease is much more depend on genetic than on environmental factors (Sorensen et al. 1988). Genetic polymorphisms in innate immunity result in significant interindividual variability in response to infection. In the last years, more than 30 genes have had their respective polymorphisms studied for relations to sepsis and critical infection or inflammation (Namath & Patterson 2009). Sepsis-related polymorphism studies have most commonly focused on one or more polymorphisms for specific genes whose protein products are implicated in sepsis. Current studies include genome-wide approaches, which analyze large representative sets of genetic markers derived from the human haplo-type map in search of those markers associated with a chosen phenotype. New techniques enable detection of thousands of single nucleotide polymorphisms in a single patient, which promise new insights into genetics variations (Marshall & Reinhart 2009).

### 3.3 Guide of therapy

A biomarker may also aid to guide therapy. First, we need a reliable diagnostic laboratory test that indicates that a clinical syndrome of systemic inflammatory response is likely due to a bacterial or fungal infection. Prompt appropriate empirical antibiotic therapy, searching and controlling infection source, hemodynamic and adjuvant therapy must be initiated as soon as possible for improving survival in patients with sepsis. Conversely, if infection is unlikely to be present permits the clinician to discontinue antibiotics and therefore a minimization of the adverse events. Second, a biomarker may help us to administer therapies to the right patients at the right time. A biomarker whose levels changes during ICU stay may provide information to monitor the response to treatment. In patients with sepsis, an increase or decrease in one marker levels may reflect that the patients responds or fails to respond. Third, critically ill patients with SIRS are a heterogeneous population of patients. A biomarker may also aid to distinguish patterns of a homogeneous group of patients, in whom the benefit of a therapy is known or could be investigated. Fourth, rapid diagnosis of reduced levels of a critical factor or elevated levels of a specific target may be of interest for the rational use of therapies (Cohen et al. 2001).

There are many more potential biomarkers for sepsis than are currently used in clinical studies. IL-6 levels of more than 1000 ng/ml were reported to be highly predictive of sepsis-related death (Harbarth et al. 2001). This finding was used to identify patients more severe who would benefit from adjuvant treatments. However, the results from a large multicenter study randomized patients with severe sepsis to treatment with antibody against TNF or placebo, stratifying patients on the basis of baseline levels of IL-6 have proven disappointing (Panacek et al. 2004).

Several studies have investigated the use of procalcitonin as a marker of treatment strategies. Recently, a meta-analysis which included seven randomized controlled studies reporting on antibiotic use and clinical outcomes of 1131 ICU patients managed with a procalcitonin-guided algorithm or according to routine practice has been published. The main results are: 1) decreased antibiotic exposure; 2) similar mortality rates and ICU or hospital length of stay; and 3) comparable rates of superinfection and persistent or relapsed infection.

C-reactive protein is often reported as inferior compared with procalcitonin as a marker, but it is frequently used in clinical practice because of its greater availability. Serial monitoring of C-reactive protein levels may have some value in predicting infection and response to antibiotics in the ICU (Povoa et al. 2006; Schmit & Vincent 2008).

### 3.4 Predicting development of organ dysfunction

It is known that the severity of organ failure has significant impact on the prognosis of patients with sepsis. Numerous biomarkers were assessed for their ability to predict the development of MOF. We focused our investigation on the possible association between different organ failures and serum concentrations of soluble cytokines and their soluble receptors in patients with septic shock (de Pablo et al. 2011). The overall prevalence of organ dysfunction at ICU admission was 46% for acute respiratory distress syndrome (ARDS), 36% for acute renal failure (ARF) and 19% for coagulation failure (DIC). Circulating IL-10, sTNF-RI, sTNF-RII, IL-1Ra concentrations were significantly higher in patients with ARDS, ARF and DIC at the time of patients' entry in the study. Serum TNF $\alpha$  levels were found higher in sepsis-induced ARF or DIC. IL-6 was higher in ARDS patients than in patients with septic shock without ARDS. However, there were no significant differences in serum

levels of IL-1 $\beta$ , IFN $\gamma$  or TGF- $\beta$  than septic patients without these organ failures. TGF- $\beta$  is a cytokine that plays a pivotal role mainly in the tissue repair reaction subsequent to inflammatory response. It is interesting to remark that in patients with septic shock and persistent ARDS, TGF- $\beta$  levels are increased. These high concentrations at day 7 of follow-up are related to MOF and death (Figure 6)(de Pablo et al. 2006c).

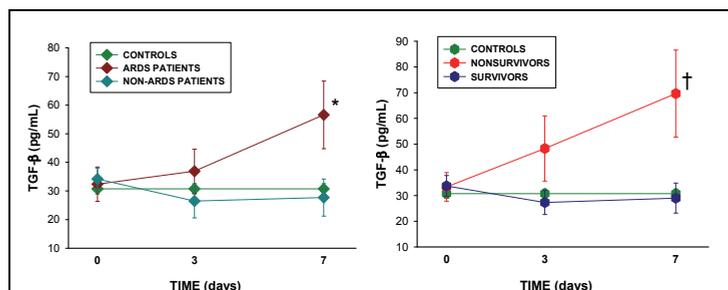


Fig. 6. Serum TGF- $\beta$  levels in septic shock patients.\*  $p < 0.05$  between patients with or without ARDS, †  $p < 0.05$  between survivors and nonsurvivors. Data are expressed as mean  $\pm$  S.E.M.

When the patients had failure in two or more organ system, they were considered as multiple organ dysfunction syndrome (MODS). Once again, anti-inflammatory molecules such as IL-6, IL-10, sTNF-RI, sTNF-RII, IL-1Ra and sIL-2R were elevated in patients with MODS, but not pro-inflammatory cytokines: TNF $\alpha$ , IL-1 $\beta$ , IFN $\gamma$  or chemokines: IL-8, MCP-1, MIP-1 $\alpha$  or RANTES (table 2).

	With MODS	Without MODS		With MODS	Without MODS
IFN $\gamma$	9.17 $\pm$ 3.01	15.13 $\pm$ 5.59	IL-10	5.57 $\pm$ 1.98	35.95 $\pm$ 20.42*
TNF $\alpha$	14.42 $\pm$ 2.97	36.12 $\pm$ 10.83	IL-2R	4584 $\pm$ 235	5724 $\pm$ 417*
IL-1 $\beta$	2.10 $\pm$ 0.84	2.15 $\pm$ 0.80	TGF- $\beta$	40.06 $\pm$ 6.67	31.04 $\pm$ 4.18
IL-6	1267 $\pm$ 672	2737 $\pm$ 663*	IL-8	206 $\pm$ 157	339 $\pm$ 116
sTNF-RI	3673 $\pm$ 500	5042 $\pm$ 617*	MCP-1	2797 $\pm$ 1049	2997 $\pm$ 572
sTNF-RII	4984 $\pm$ 558	6373 $\pm$ 724*	MIP-1 $\alpha$	61.94 $\pm$ 15.50	66.42 $\pm$ 13.90
IL-1 ra	7099 $\pm$ 1464	11052 $\pm$ 2030*	RANTES	20177 $\pm$ 3831	15325 $\pm$ 2140

Table 2. Serum cytokines and chemokines levels in septic shock patients with or without MODS at ICU admission.\*  $p < 0.05$  between patients with or without MODS. Data are expressed as mean  $\pm$  S.E.M.

### 3.5 Prognosis

Sepsis, severe sepsis and septic shock are the leading cause of death in critically ill patients (Hotchkiss & Karl 2003). The ICU mortality rates increase with the severity of the syndrome: 27% for sepsis, 32% for severe sepsis and 54% for septic shock (Vincent et al. 2006). Outcome from sepsis in terms of mortality is not only associated with the amount of organ dysfunction developed (Alberti et al. 2003). Other variables as age, genetic backgrounds, comorbidities, reasons for ICU admission, and related to infection (acquisition, extension, site and agent) were observed in outcome of patients with sepsis (Gustot 2011). PIRO system emerged from the Fifth Toronto Sepsis Roundtable. It includes Predisposition, Injury, Response and Organ Dysfunction (Marshall et al. 2003). However, this approach remained

up to now virtually conceptual and other research groups empirically developed in cohorts of patients a scale for predicting mortality in sepsis (Moreno et al. 2008).

Sepsis biomarkers may contribute to this model of classification. However, at this moment the majority of the biomarkers have been evaluated as prognosis markers and in our search, we assessed the ability of a biomarker to differentiate patients likely to survive from those likely to die. We studied by sandwich ELISA serum levels of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IFN $\gamma$ ), anti-inflammatory cytokines (IL-10 and TGF $\beta$ ) and soluble cytokine antagonist (sTNF-RI, sTNF-RII and IL-1Ra) during the first 28 days of ICU admission in 52 patients with septic shock. We found that serum an early response to continuously elevated soluble sTNF-RI, sTNF-RII and IL-1Ra serum levels was associated with an enhanced risk of fatal outcome (table 3). ROC analysis revealed that sTNF-receptor I or sTNF receptor II concentrations over 2767 or 4619 pg/ml, respectively, determinate a high risk of death. The sensitivity-specificity for sTNF-RI was 100%-57% and for sTNF-RII was 100%-71%. IL-1Ra concentrations below 7033 pg/ml determined a high probability of survival (sensitivity-specificity of 60-100%).

	Survivors	Nonsurvivors		Survivors	Nonsurvivors
<b>IFN<math>\gamma</math></b>	16.59 $\pm$ 5.90	7.16 $\pm$ 2.07	<b>IL-10</b>	7.38 $\pm$ 3.43	57.39 $\pm$ 35.71*
<b>TNF<math>\alpha</math></b>	23.81 $\pm$ 7.42	40.42 $\pm$ 17.91	<b>IL-2R</b>	4830 $\pm$ 183	6177 $\pm$ 661
<b>IL-1<math>\beta</math></b>	1.78 $\pm$ 0.51	2.75 $\pm$ 1.38	<b>TGF-<math>\beta</math></b>	33.74 $\pm$ 4.06	33.38 $\pm$ 5.57
<b>IL-6</b>	1960 $\pm$ 609	2811 $\pm$ 931	<b>IL-8</b>	265 $\pm$ 113	356.7 $\pm$ 167.7
<b>sTNF-RI</b>	3910 $\pm$ 396	6050 $\pm$ 1043*	<b>MCP-1</b>	3020 $\pm$ 670	2774 $\pm$ 771.7
<b>sTNF-RII</b>	4862 $\pm$ 430	8005 $\pm$ 1074*	<b>MIP-1<math>\alpha</math></b>	67.7 $\pm$ 12.9	59.3 $\pm$ 18.6
<b>IL-1 ra</b>	7331 $\pm$ 1351	3819 $\pm$ 2880*	<b>RANTES</b>	14758 $\pm$ 2105	20714 $\pm$ 3624

Table 3. Serum cytokines and chemokines levels in septic shock patients at ICU admission.

\*p<0.05 between survivors and nonsurvivors. Data are expressed as mean  $\pm$  S.E.M.

Serum levels of TNF $\alpha$ , IL-1 $\beta$ , IL-6 were significantly elevated on admission and during the 28 days of follow-up in septic patients when compared with healthy controls, but were not predictors of mortality (table 3). Interestingly, IFN $\gamma$  levels were significantly higher in survivors than in controls during the initial two weeks of observation (de Pablo et al. 2011). Serum IL-10 concentrations were elevated in the first 3 days in non-survival patients. TGF $\beta$ , an anti-inflammatory cytokine capable of converting an active site of inflammation into one dominated by resolution and repair (Letterio & Roberts 1997), discriminated patients with fatal outcome at day 7 of the follow-up (Figure 6). All these findings together suggest that mortality in patients with septic shock correlates more with anti-inflammatory molecules than with pro-inflammatory immunomodulatory molecules. Many previous clinical trials have failed to show benefit using anti-inflammatory agents, because the inflammatory status of the patients is a dynamic process where it may be reasonable to test the hypothesis of using immunoinflammatory stimulation therapy on patients with septic shock with high risk of death determined by the presence in blood of high levels of sTNF-RI, sTNF-RII and IL-1Ra or low of IFN $\gamma$ . We also studied the prognostic value of chemokines and soluble adhesion molecules. Chemokines are a large superfamily of small peptides that are key participants to not only control of leukocyte trafficking, but necessary for the linkage between innate and adaptative immunity. No significant differences were found between survivors and nonsurvivors at any time of the follow-up in serum levels of IL-8, MCP-1, MIP-1 and RANTES (table 3) (de Pablo et al. 2006b).

Next, we focused our investigation in the prognostic value of fatal outcome of the circulating soluble adhesion molecules in the population of patients with septic shock admitted at the ICU. Mortality was defined as death occurring within 28 days after study enrolment. At ICU admission, serum soluble E-Selectin concentrations showed significantly higher levels in the nonsurvival group ( $110.08 \pm 8.00 \text{ ng/ml}$ ) than in survival group ( $88.73 \pm 4.92 \text{ ng/ml}$ ;  $p=0.041$ ). We also analysed serum E-Selectin levels at ICU admission as a predictor of outcome, determining the area under the receiver operating characteristics curve (AUC). The AUC value for baseline measurements of E-Selectin was 0.728 ( $p=0.049$ ; 95% confidence interval, 0.516-0.939). Thus, an E-Selectin concentration of  $106.8 \text{ ng/ml}$  was identified as the optimum threshold to distinguish the patients for outcome with a sensitivity of 60.0% and specificity of 85.7%. Over the study period, no significant change in circulating soluble VCAM-1, ICAM-1, ICAM-2 and PECAM-1 concentrations was observed between survival and nonsurvival patients. Thus, soluble E-Selectin is a marker of endothelial damage, which may result in failure of the different organs, multiple organ dysfunction syndrome and death (de Pablo et al. 2006a).

Numerous biomarker have been reported in prognosis like other cytokines as IL-12 or IL-18, cell markers as soluble HLA-DR, receptors as TREM-1 or as urokinase type plasminogen activator receptor, related to vascular endothelial damage as von Willebrand factor and antigen or like acute phase proteins as ferritin or procalcitonin (Pierrakos & Vincent 2010). However, no biomarker has established itself sufficiently to be of help in clinician practice. Each biomarker has limited sensitivity and specificity; therefore, it may prove more useful to combine various markers. Biomarker panels or composite markers may prove most useful in examining a particular immunologic pathway, identifying at-risk individuals who require aggressive intervention, predicting organ response and determining the ability to differentiate patients likely to survive from those likely to die (Ventetuolo & Levy 2008).

#### 4. Conclusion

Many biomarkers have been evaluated for the use in sepsis, many more than in other disease processes. At present, around of 178 different biomarkers were actually evaluated in the 3370 studies, 77 in experimental studies and 101 in clinical studies only, 58 in both experimental and clinical studies (Pierrakos et al. 2010). However, hardly a few of them are able to report sensitivity and specificity values greater than 90%. We demonstrated that septic shock patients show a severe redistribution of circulating T lymphocyte subsets and CD62L and CD28 expression on circulating T cells at ICU admission are good markers to predict the outcome of shock septic patients. We found that T lymphocyte phenotype data show a time difference in the recirculation of T cells between survivors and nonsurvivors that might provoke a delayed tissue response of the immune system. Moreover, we demonstrated that the simultaneous analysis of different immune system cell subsets in combination like ( $\text{CD56}^+\text{CD69}^+$ ,  $\text{CD3}^+\text{CD8}^+\text{CD45RA}^+\text{CD45RO}^-$ ,  $\text{CD3}^+\text{CD8}^+\text{CD28}^+$ ,  $\text{CD19}^+\text{CD80}^+$ ,  $\text{CD3}^+\text{CD11A}^+\text{BR}^+\text{CD11B}^+$ ) would improve the prediction of outcome in septic shock patients. With respect to serum cytokines, we found that antiinflammatory and adhesion molecules are good markers for the prognosis of septic shock patients. We propose that the blood counts of circulating cells of the immune system are good candidates to study for value as biomarkers. In this book chapter we attempt to resume the continuous effort by clinician and researchers to find new biomarkers that going to allow us to improve the prognostic of septic shock patients. New experiments and clinical studies are necessary to achieve this goal.

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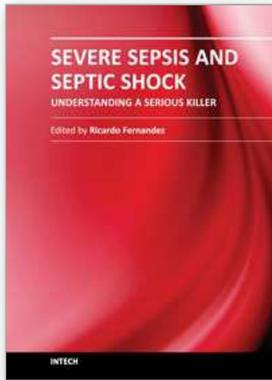
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## **Severe Sepsis and Septic Shock - Understanding a Serious Killer**

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Despite recent advances in the management of severe sepsis and septic shock, this condition continues to be the leading cause of death worldwide. Some experts usually consider sepsis as one of the most challenging syndromes because of its multiple presentations and the variety of its complications. Various investigators from all over the world got their chance in this book to provide important information regarding this deadly disease. We hope that the efforts of these investigators will result in a useful way to continue with intense work and interest for the care of our patients.

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