

## Correlation Between Markers of TH2-Oriented Response and SOFA Score in Sepsis

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**Abstract:** A shift from Th1- to Th2-type cell immune response has been suggested to occur during sepsis, contributing to cell-mediated immunity suppression and to poor prognosis. The aim was to study the relationship between old and new Th2 markers and the clinical outcome of sepsis. 30 critically ill patients with sepsis for  $\geq 48$  hours were enrolled in a prospective clinical study. Blood samples were collected at the enrolment, at the 5<sup>th</sup> and 10<sup>th</sup> day. Serum levels of total IgE and soluble chemokines related to Th1- and Th2 responses were evaluated. The percentages and absolute number of CD4+ and CD8+Tcells as well as CRTH2+Tcell subsets were detected by flow cytometry. Sepsis severity was assessed with SOFA score. The mean values of total IgE in septic patients were significantly higher than in controls ( $p < 0.01$ ). Moreover, IgE levels of septic patients who died were higher than those of survived patients ( $p < 0.05$ ). It has been found that IgE levels directly and RANTES inversely correlated with SOFA score at different time points ( $p < 0.01$ ). A significant correlation between the percentages of CRTH2+/CD4+(but not CRTH2+/CD8+)T cells and SOFA at different time points was observed ( $p < 0.05$ ). The direct correlation between total IgE, the percentages of circulating CRTh2+CD4+T cells and the clinical outcome suggests that clinical worsening of sepsis is closely linked to the shift towards a predominant less protective Th2 phenotype. Although these are preliminary results, the longitudinal analysis of these parameters during the disease could be proposed as useful prognostic tools in sepsis.

### INTRODUCTION

Severe sepsis remains to be a major problem in Intensive Care Units (ICU) with a poor outcome and a mortality rate as high as 30-40% [1]. Despite significant improvement in supportive measures, incidence of severe sepsis continues to rise by 1.5% to 8% each year [2].

The release of inflammatory mediators like cytokines, lipid mediators and reactive oxygen species generates a widespread activation of cells responsive to pathogens resulting in uncontrolled systemic inflammation [3,4]. The control over the systemic inflammation is basically a role played by both innate and adaptive immunity. In particular, Toll-like receptors on Antigen Presenting Cells (APC) are triggered by surface components of pathogenic agents to release a panel of cytokines essential for the development of T effector cell subsets, conditioning the efficacy of protection against pathogens. The activated effector T cells are essentially programmed to secrete cytokines with one of two distinct and antagonistic profiles. They either produce cytokines with pro-inflammatory activity (type 1 helper T cells - Th1- such as IFN-g) or cytokines with anti-inflammatory (type 2 helper T cells -Th2- such as IL-4, IL-13, IL-5) or

suppressive (IL-10) properties [5,6]. Factors like type of pathogen, size of bacterial inoculum, site of infection, type of APC influence T effector cells towards a polarized Th1- or a Th2 profile. Patients with sepsis have features of a Th1-oriented response with the production of high levels of proinflammatory cytokines, initially. However, if sepsis persists, there would be a shift towards an anti-inflammatory immunosuppressive state, characterised by anergy of effector T cells (loss of costimulatory and MHC class II molecules by APC), increased apoptosis of T and B cells as well as a switch from a protective Th1- to a less protective Th2 profile of immune response [7,8]. Septic patients with burns or trauma are shown to display reduced levels of Th1 cytokines and an increase of serum IL-4 and IL-10 and reversal of Th2 cytokines improve their survival [9]. To identify Th2 cells in human circulation, a CRTh2 (chemoattractant receptor - homologous molecule expressed on Th2 cells) has been considered to be a reliable tool both in health state and in disease conditions [10, 11]. However, the reliability of such a parameter to identify the state of Th2 in sepsis, in an attempt to foresee patient prognosis, was barely tackled, so far [12]. Therefore, the study of this immune response parameter and the role of immunosuppression in the prognosis of septic patients still need to be explored. Moreover, total IgE serum levels, which are usually considered as a surrogate marker of Th2 response, as well as the serum levels of type 1 and type 2 associated chemokines have been analysed.

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Therefore, this study was addressed to evaluate the prognostic value of the abovementioned old and new Th2 markers during the clinical course of sepsis.

## MATERIALS AND METHODOLOGY

**Study population.** Critically ill patients, aged between 40 and 80 years, were consecutively included in this prospective study between January 2004 and December 2006. The enrolled patients had developed a state of severe sepsis/septic shock for more than 48 hours, as defined by the ACCP/SCCM Consensus Conference [13]. A written informed consent was obtained from patients or their relatives. Pregnant women, transplanted patients, patients with cancer, viral hepatitis, known history of allergy, or acquired immunodeficiency syndrome, and patients with uncontrolled bleeding, or under chemotherapy, radiotherapy, hemofiltration, or steroidal/non-steroidal anti-inflammatory agents were excluded. Mortality was defined as death occurring within 28 days after the diagnosis. This study has been carried out according to the ethical standards of the responsible regional Committee on human experimentation and to the Helsinki Declaration principles [14].

**Study design.** Blood samples were collected at the time of their enrolment and thereafter at the 5<sup>th</sup>, and 10<sup>th</sup> day. Sequential Organ Failure Assessment (SOFA) score was used for the daily assessment of patients' disease severity state [15]. 30 age-matched healthy non-allergic volunteers were enrolled as control group to establish the "reference values" for the methods used. The blood samples were collected through an indwelling catheter in tubes containing EDTA, maintained at 20°C, and analyzed within 6 h. The evaluations of sepsis and organ dysfunctions were prospectively done by investigators blinded to the assay results.

**Flow Cytometry.** FITC-, PE- or APC-conjugated mAbs for CD3, CD4, CD8, molecules were purchased from Becton Dickinson (San Jose, CA). anti-CRTH2 mAb was kindly provided by Nagata K. (R & D Center, BML, Inc., Matoba, Kawagoe, Saitama, Japan).

Flow cytometry analysis [16] of cell suspensions was performed on blood samples by a FACSCanto cytofluorimeter using the CellQuest software (Becton Dickinson, San Diego, CA). Absolute number of each lymphocyte subset was automatically calculated, based on the absolute values of lymphocytes detected by CD45+cells count in the total white cells.

**Detection Of Serum Chemokines.** The serum concentration of CCL22 (MDC), CXCL10 (IP-10), CXCL12 (SDF-1), CCL3 (MIP1a), CCL4 (MIP1b), and CCL5 (RANTES) were evaluated by commercial kits (R&D systems, CA) according to the manufacturer's instructions.

**Detection Of IgE Antibody.** Total IgE serum concentration and Ca-specific IgE Ab were evaluated by commercial kits (IgE CapSystems, Pharmacia, Uppsala, Sweden).

### Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation of the mean (SD), and categorical data as median (range). Continuous parametric data were analysed using one-way ANOVA with Bonferroni post-hoc t-test for repeated measurement

comparisons. Non parametric data were analyzed using the Kruskal-Wallis test. Correlation between parameters was analyzed using the Spearman rank correlation test. A value of  $p < 0.05$  was considered statistically significant. Statistical analysis was performed with STATA software 8.0 for Windows (Stata Corporation, College Station, USA).

## RESULTS

30 patients were consecutively enrolled during the study period. Patients' characteristics are shown in Table 1. The mean age for the control group was  $65.4 \pm 9.8$ .

### Increased Th2-Related Serum Markers Correlate with Poor Prognosis in Septic Patients

The mean value (+ SEM) of total IgE Ab in the sera of septic patients ( $134.6 + 35.7$  IU/ml) was significantly ( $p < 0.01$ ) higher than those of control group ( $19 + 13$  IU/ml). Interestingly, total IgE concentration at the admission to ICU of subjects who died ( $165.3 + 64.5$  IU/ml) were higher than those of survived patients ( $60.1 + 32.6$  IU/ml) ( $p < 0.05$ ). Accordingly and more importantly, a significant correlation ( $p < 0.01$ ) was found between IgE serum concentration and the corresponding SOFA score at different time-points of the disease (Fig. 1).

Successively, we assessed the correlations between the serum concentration of chemokines associated to type1 (CXCL10, CCL3, CCL4, CCL5) and type 2 (CXCL12, CCL22) T cell responses and SOFA score at different time-points of the disease. Interestingly, whereas no correlation among CXCL10 ( $r = 0.14$ ), CXCL12 ( $r = 0.10$ ), CCL3 ( $r = 0.11$ ), CCL4 ( $r = 0.02$ ), CCL22 ( $r = 0.12$ ) and SOFA score was found, the serum concentration of CCL5/RANTES (a CCR5-ligand, related to Th1 response) were significantly ( $p < 0.01$ ) inversely related to SOFA score (Fig. 2).

### Increased Th2 Cells Correlate with a Poor Prognostic Value in Septic Patients

In order to evaluate the role of functional T cell subsets during different time-points of sepsis, peripheral blood mononuclear cell (PBMC) from septic patients were analysed for the percentages and the absolute values of CD3+CD4+, CD3+CD8+, or CRTH2 (Th2 cells).

A significant ( $p < 0.01$ ) direct correlation between the percentages of CD4+CRTH2+ T cells and SOFA score at different time points was observed (Fig. 3A,B). Of note, an inverse correlation between the percentages of CD4+CRTH2+ T cells and circulating leukocytes ( $r = -0.39$ ,  $p < 0.01$ ) and between CD4+CRTH2+ T cells and CCL5 serum levels ( $r = -0.59$ ,  $p < 0.01$ ) was also seen (data not shown).

## DISCUSSION

The type of immune response during sepsis is determined by factors like pathogens virulence, size of inoculum, patient's conditions, the initial disease, and, finally, the polymorphisms of cytokine genes or other immune effector molecules and their receptors. There is a general consensus that initial immune response in sepsis is characterized by the hyper-expression of proinflammatory mediators, even though it may rapidly progress to a state of hypo-inflammation prevalently due to the downregulation of the

Table 1. Patients' Characteristics

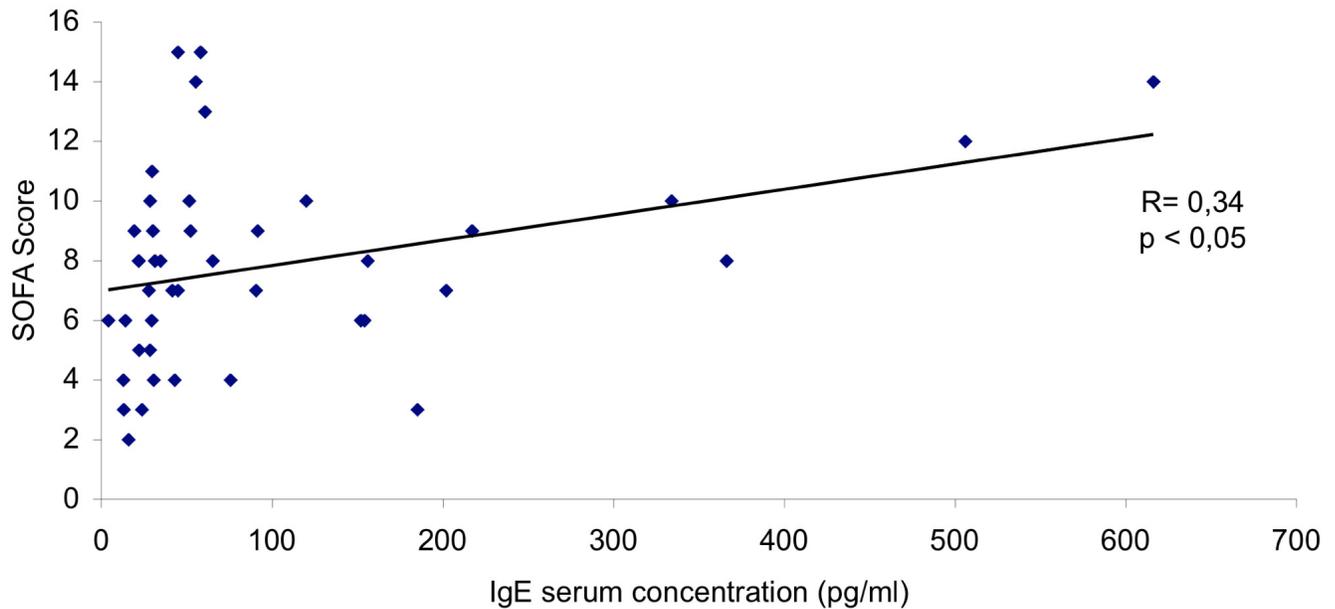
Pts	Age (Years)	Gender	Infection Source	ACCP/SCCM Diagnosis	SOFA Score	Blood Cultures	Outcome
LP	44	F	Pneumonia	Septic shock	11/7/4	Staph. NMRSA	Survived
MG	60	M	Peritonitis	Severe sepsis	13/8/13	<i>Enteroc. faecalis</i>	-
ML	77	M	Cellulitis	Severe sepsis	7/15/4	<i>Staph. epidermidis</i>	survived
SG	64	M	Pneumonia	Septic shock	14/13/13	Sterile	-
ST	76	F	Peritonitis	Severe sepsis	5/9/15	Sterile	-
PU	76	F	Cholecystitis	Severe sepsis	8/13/16	Sterile	-
LE	50	M	Peritonitis	Severe sepsis	8/4/1	<i>Esch. coli</i>	survived
FC	82	F	Peritonitis	Septic shock	8/11/15	Staph. MRSA	-
CR	68	M	Pneumonia	Severe sepsis	8/12/15	Candida (BAL)	-
BP	58	M	Pneumonia	Severe sepsis	6/12/15	staph. MRSA	-
ML	74	M	Pneumonia	Septic shock	7/4/1	Sterile	survived
DA	60	F	Lung abscess	Severe sepsis	11/6/3	Pseudom. (BAL)	survived
NM	37	M	Peritonitis	Severe sepsis	6/2/1	Sterile	survived
LF	46	F	Cellulitis	Severe sepsis	6/10/10	Sterile	-
LC	62	M	Pneumonia	Septic shock	10/12/12	Acitenobacter (BAL)	-
PP	74	F	Lung abscess	Septic shock	12/10/8	Staph. MRSA	survived
MA	63	F	Peritonitis	Severe sepsis	10/14/13	Sterile	-
MN	55	M	Peritonitis	Septic shock	8/4/4	Sterile	survived
CB	80	F	Pneumonia	Severe sepsis	6/4/2	Sterile	survived
SB	52	F	Pneumonia	Severe sepsis	8/10/10	Sterile	-
MM	82	M	Peritonitis	Severe sepsis	4/6/5	<i>Enteroc. faecium</i>	survived
PL	68	F	Peritonitis	Septic shock	10/8/6	Sterile	survived
PA	58	M	Pneumonia	Severe sepsis	6/8/6	Sterile	survived
LL	63	M	Pneumonia	Severe sepsis	8/12/12	Staph. VREF	-
GB	60	M	Peritonitis	Septic shock	6/10/10	<i>Esc. coli</i> (peritoneal fluid)	-
MG	41	M	Pneumonia	Severe sepsis	6/5/5	Staph. MRSA	survived
MF	67	M	Pneumonia	Severe sepsis	5/6/4	Sterile	survived
TC	58	F	Pneumonia	Septic shock	14/14/14	Sterile	-
MC	79	M	Peritonitis	Severe sepsis	10/8/8	<i>Enteroc. faecalis</i>	survived
GN	72	M	Peritonitis	Severe sepsis	12/8/4	Sterile	survived
<b>Mean ± SD</b>	63,5 ± 12	M/F: 18/12					mortality 46,8%

Pts: patients, F female, M male; SD: standard deviation, NMRSA: non methicillin-resistant *Staphylococcus aureus* MRSA: methicillin-resistant *Staphylococcus aureus*; VREF: Vancomycin resistant staphylococcus

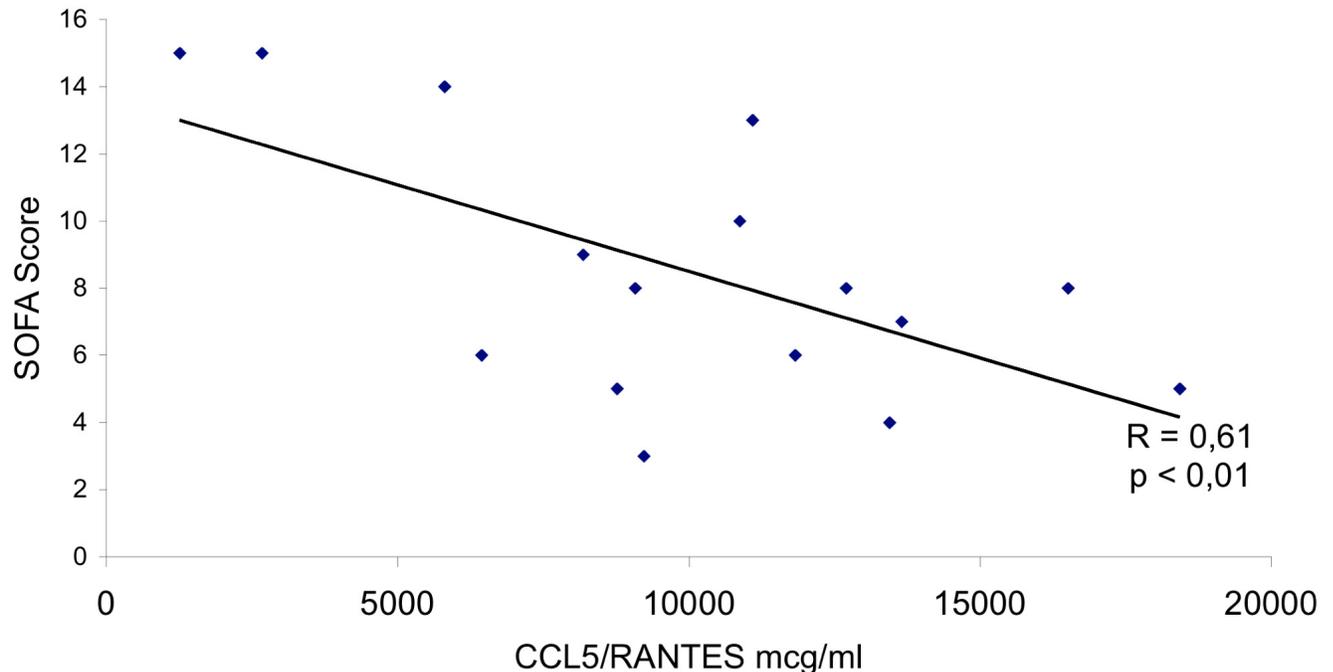
major protective mechanisms. Indeed a shift to a less protective T (namely Th2 cells) cells secreting anti-inflammatory cytokines have been described to be relevant for a poor prognosis [7, 17]. Even though timing of these alterations are not fixed and the involved mechanisms may be different in each patient, however measurement of circulating inflammatory mediators may prove to be useful in evaluating the stage of sepsis and in adopting the most appropriate treatment [18].

Aim of this study was to evaluate the prognostic value of old and new Th2 cell markers and to analyse their relationship with the clinical outcome of severe sepsis.

We initially examined the total IgE serum levels which are usually considered as a surrogate marker of Th2 response since the type 2 cytokines (such as IL-4 and IL-13) are the major switching factors for ε isotype. Total IgE levels in the sera of septic patients were significantly higher than those of the control group. In addition, total IgE levels, measured at



**Fig. (1).** Correlation between SOFA score and total IgE serum levels ( $R\ 0.34$ ;  $p < 0.05$ ).



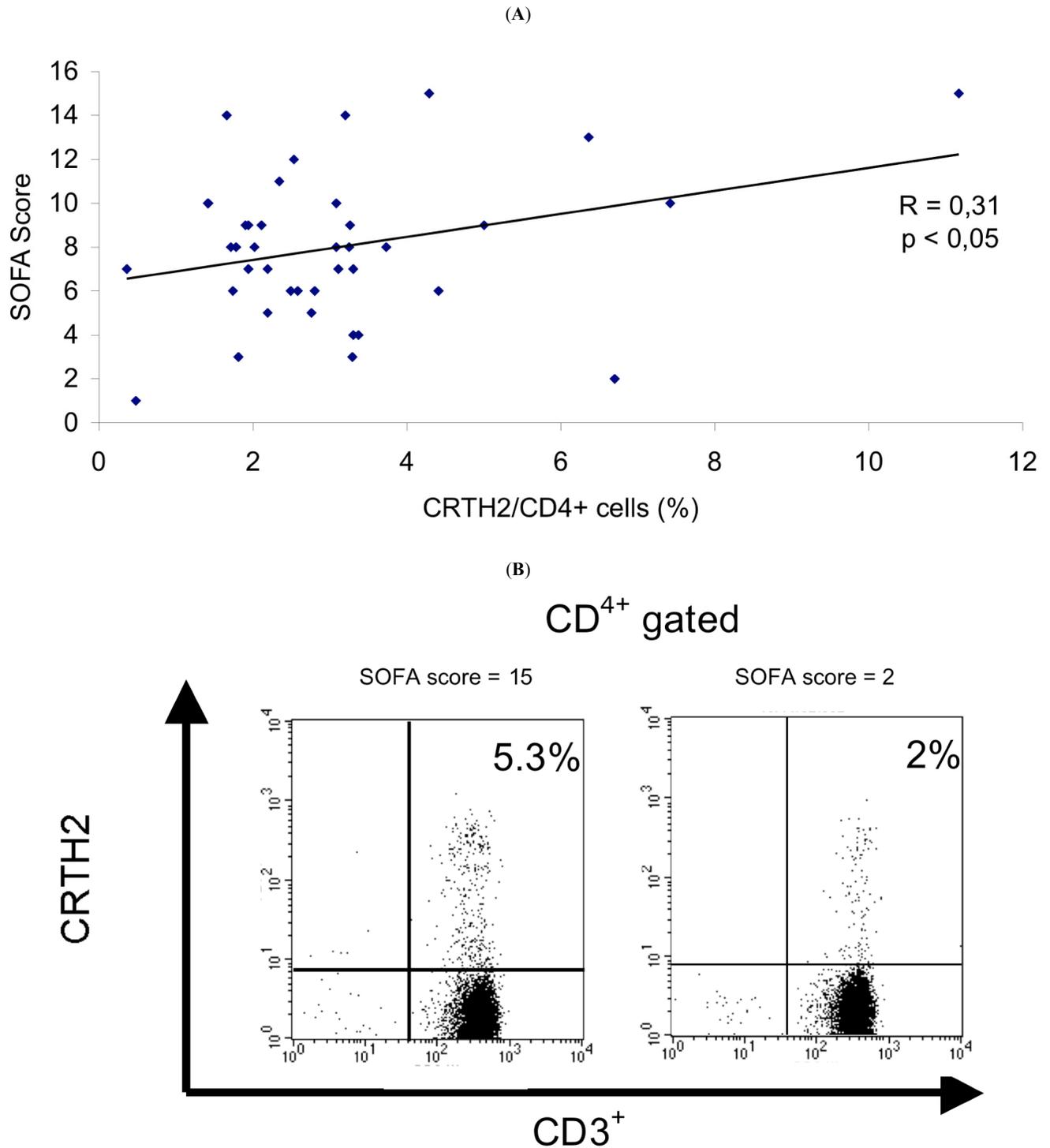
**Fig. (2).** Correlation between SOFA score and serum levels of CCL5/RANTES ( $R\ 0.61$ ;  $p < 0.01$ ).

the enrolment in the study, of subjects who died were higher than those of survived patients. More importantly, IgE serum levels were correlated to the corresponding SOFA scores at different time-points of the disease. All these findings suggest that a Th2-oriented response is frequently observed in septic patients and that it correlates with a poor prognosis. Even though the increased IgE serum levels and hyper-eosinophilia have been found associated with sepsis in traumatic patients [19,20], this parameter has not been extensively evaluated in the majority of studies on septic patients.

Thereafter, the serum levels of type1- (CXCL10, CCL3, CCL4 and CCL5) or type 2-(CXCL12, CCL22) associated chemokines (prevalently recruiting CXCR3+CCR5+Th1- or

CXCR4+CCR4+ Th2 cells) have been analysed. Interestingly, we observed that the serum levels of CCL5/RANTES (one of the ligands for CCR5+Th1 cells) were inversely related to SOFA score. A decreased CCL5 level has been shown in umbilical serum of preterm neonates with pneumonia or sepsis [21] and it has been associated with mortality in children with cerebral malaria [22]. Also the early effect of a systemic administration (in bolus) of endotoxin in healthy volunteers revealed a significant impairment of CCL5 serum levels [23].

Based on these previous studies, our result acquires a stronger relevance since, for the first time, it defines an in-



**Fig. (3).** Correlation between CRTh2 expression and SOFA score ( $R=0.31$ ;  $p<0.05$ ). **(A)** CRTh2 expression on CD4<sup>+</sup> gated T cells was evaluated by flow cytometry. **(B)** Representative experiment of CRTh2 expression by CD4<sup>+</sup>CD3<sup>+</sup> T lymphocytes as detected by flow cytometry in two patients showing high (left panel) or low (right panel) SOFA score.

verse correlation between a clinical index and a Th1-associated chemokines in septic patients.

In this contest the absence of any correlation with other type 1- or type 2-chemokines is intriguing and difficult to explain. It certainly reflects the complexity of mechanisms underlying the immune response, elicited also in the same

phase of sepsis, which in turn may influence and annul each other.

Lastly, our study has been addressed to define possible correlations among clinical indexes and circulating Th2 cells which may condition the protection against infections.

As regards the circulating Th2 cells in septic patients, a chemokine receptor has been recently described on such cell

subset. Such a molecule, which is called chemoattractant receptor for Th2 cells (CRTH2) [24], is also expressed by eosinophils and basophils. We have shown that CRTH2 is the only molecule among those described, so far, which was selectively expressed by Th2 and type 2 cytotoxic CD8+ T cells (Tc2) *in vitro* and by circulating Th2 and Tc2 cells *in vivo*, whereas it was never detectable on Th1, Tc1, type 0 Th (Th0) or type 0 Tc (Tc0) cells [11]. The ligand for CRTH2 is produced by mast cells and has recently been identified as prostaglandin D2 [24,25]. The results of this paper clearly show a high correlation between the percentages of circulating CRTH2+CD4+ T cells and the clinical course. This suggests that the clinical worsening of septic patients is often induced and maintained by the specific shift towards a predominant Th2 phenotype. This result is in agreement with previous published data indicating a lower Th1/Th2 ratio of cytokine-producing T cells (or an increased IL-4-producing CD4+ T cells) in PBMC of septic or polytraumatic patients [8,17]. On the other hand, it has been shown that in the murine model of sepsis obtained with the cecal ligation and puncture, the release of IL-4 was markedly increased and the IL-4-induced activation of the STAT6 pathway contributed to the immunosuppression and death in sepsis [26]. By contrast the *in vivo* treatment with neutralising anti-IL-4 Ab markedly increased the survival rates in septic animals [26].

In addition, there are clearcut evidences showing that the cytokines IL-17A/F are crossregulated by IL-4 *in vitro* and *in vivo* models [27,28]. Furthermore, it has been shown that a novel subset of T cells producing these cytokines (now called Th17) is associated to chronic inflammation sustained by neutrophil infiltration since IL-17A/F triggers several inflammatory cells to secrete pro-inflammatory molecules (as CCL5) and neutrophil chemoattractants [29,30]. In this context it is relevant that the percentages of CRTH2+CD4+ T cells of our septic patients are inversely correlated to both CCL5 serum levels and to total circulating leukocytes.

## CONCLUSION

On the whole, these data suggest that the correlations between the clinical course of sepsis versus total IgE levels (direct), CCL5 serum levels (inverse) and the percentages of circulating CRTH2+CD4+T cells (direct) confirm that the clinical worsening in septic patients may be linked to a Th2 shift of immune responses. Since several other (immunologic and non immunologic) mechanisms may contemporarily operate in these patients, it is likely that the Th2 shift can be evident at the tissue but not always at the systemic level. Although these results need to be validated on a more large scale of population, the longitudinal analysis of all these parameters during the disease course may be considered as an useful prognostic tool in sepsis.

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