The role of HLA-DR expression on monocytes and Sepsis Index as predictive sepsis biomarkers

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KEY WORDS

Sepsis; HLA-DR; Immunoparalysis; Immunosuppresion; Sepsis Index

ABBREVIATIONS

APACHE: Acute Physiology and Chronic Health Evaluation

CARS: Compensatory Anti-inflammatory Response Syndrome

CRP: Reactive-C Protein

HLA-DR: DR isotype of Human Leukocyte Antigens

ICU: Intensive Care Unit

MFI: Mean Fluorescence Intensity

$_{M}$HLA-DR: DR isotype of Human Leukocyte Antigens expressed in the surface of monocytes

$_{N}$CD64: CD64 expressed on neutrophils

PCT: Procalcitonin

ROC: Receiver Operating Characteristic

SOFA: Sequential Organ Failure Assessment

SUMMARY

Sepsis is characterized by a simultaneous imbalance of hyperinflammation and immunosuppression.

The expression of HLA-DR in monocytes ($_{M}$HLA-DR) and CD64 expression in neutrophils ($_{N}$CD64) are considered, respectively, predictive and diagnostic biomarkers of infection. The ratio $_{N}$CD64/$_{M}$HLA-DR has been described as a prognostic biomarker of sepsis.

To evaluate $_{M}$HLA-DR expression and ratio $_{N}$CD64/$_{M}$HLA-DR in patients admitted to the Intensive Care Unit (ICU) and their relationship with the development of infection.

Prospective study of 77 patients admitted to the ICU from our hospital (HUGTiP) due to stroke or severe traumatic brain injury. The $_{M}$HLA-DR and $_{N}$CD64 expression were analyzed in whole blood samples at baseline, +3, +6, +9, +12 and +15 days after admission, using a standardized flow cytometry protocol.

During the follow-up, 71% of patients became infected (infection without sepsis, sepsis or septic shock).

Infected patients showed – already after three days of admission – a lower percentage of $_{M}$HLA-DR+ (85.8 ± 16.22% vs. 92.5 ± 12.13%, p <0.001) than those patients than did not develop it. Interestingly, on day +3, infected patients also had a higher ratio $_{N}$CD64/$_{M}$HLA-DR (0.12 ± 0.19 vs. 0.04 ± 0.08, p <0.001) than the non-infected ones.

The immumonitoring of $_{M}$HLA-DR expression and ratio $_{N}$CD64/$_{M}$HLA-DR may help to evaluate those patients with higher susceptibility to develop infection and sepsis at the ICU.

INTRODUCTION

Among hospitalized patients at Intensive Care Unit (ICU), sepsis admitted patients outnumber patients with acute myocardial infarction or stroke. In addition, despite advances in the field of medicine, sepsis and especially septic shock causes high mortality rates (approximately 39%) (1). In the past, septic shock reached 50% mortality rates (2). The strategy of applying protocols that allow a rapid management has been determinant and significantly influent in the decrease of mortality associated to sepsis (3 - 6).

The diagnosis of sepsis is a clinical challenge because (i) of the non-specificity of the symptoms, and the fact that (ii) it is a time-dependent syndrome which shows an increase of mortality associated with longer time until sepsis diagnostic. Reversible temporal immunodepression (phenomenon known as immunoparalysis) is frequently associated with pathophysiologic events in critical ill patients admitted in ICU. However, there are no clinical signs to indicate its presence and the increased susceptibility to infections. Monitoring the immune status of critical patients is therefore needed in order to recognize immunoparalysis early enough to classify patients with high risk for infectious complications (7).

The research in new biomarkers able to allow us a faster detection of sepsis is essential to reduce mortality and morbidity rates. Numerous molecules of immune failure have been studied. HLA-DR molecules are considered hallmark in the immune response because these molecules play a central role in the

antigen presentation and activation of T lymphocytes. HLA-DR (DR isotype of Human Leukocyte Antigens) is a cell surface protein that is usually present in antigen-presenting cells (B lymphocytes, macrophages, monocytes and dendritic cells). The decrease in the expression of this molecule in the surface of monocytes ($_{M}$HLA-DR) reduces the capacity to respond against infections (8-10). For this reason, it has been proposed as a good predictor of septic complications, in all critical conditions (11-13).

On the other hand, CD64 molecule is a type of integral membrane glycoprotein (Fc receptor) that binds to monomeric IgG type antibodies with high affinity. CD64 expression is induced in neutrophils within a few hours after being in contact with bacteria in response to IFN-γ and GM-CSF. Cytokine stimulation induces rapid clustering of CD64 on the cell membrane to facilitate rapid binding and internalization of immune complexes. This contributes to an inflammatory response by triggering release of TNF-α and IL-6, superoxide production, antigen presentation to T cells or lysis of antibody-coated cells. The increase of CD64 expression on neutrophils ($_{N}$CD64) allows to differentiate between resting and activated neutrophils and could be useful as biomarker for infection monitoring. (11, 14-17). In the present study we propose to investigate the role of HLA-DR expression on monocytes and Sepsis Index (ratio between $_{N}$CD64 and $_{M}$HLA-DR) as predictive biomarkers of sepsis.

MATERIALS AND METHODS

Patients

Seventy-seven critic neurological patients, admitted at the Intensive Care Unit of the Germans Trias i Pujol University Hospital without infection were included in a longitudinal prospective study over 24 months.

The inclusion criteria were: 1) Age above or equal to 18 years old; 2) Patients without infection and with serious neurological pathology.

Exclusion criteria were: 1) immunocompromised patients; 2) age of less than 18 years old; 3) patients who died within 24 hours after admission.

Patients were classified as “septic” or “non-septic”, as well as patients who develop septic shock according to the Sepsis-3 definition (18-19). The clinical variables were monitored daily for 28 days after admission. In addition, the severity index was assessed by calculating the acute physiology and chronic health evaluation (APACHE) II score and the sequential organ failure assessment (SOFA) score on admission (Table 1).

This study was approved by the ethics committee of the hospital and the patients or their relatives signed the informed consent prior to their inclusion in the study.

Definitions

Infection was defined as a pathological process caused by the invasion of pathogenic or potentially pathogenic microorganisms to normally sterile tissues, fluids or body cavities.

Sepsis (based on the Sepsis - 3 conference) was defined as a "life threatening organ dysfunction caused by a dysregulated host response to infection". Furthermore, patients were classified according to the development of septic shock defined as “subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality".

Serious neurological pathology was defined as a stroke and traumatic brain injury with decreased level of consciousness that requires admission to an ICU.

Immunocompromised patients were defined as patients diagnosed with any type of primary or acquired immunodeficiency (HIV-positive, immunosuppression, chemotherapy, radiation, steroids during an extended period of time, steroids at high doses), or with advanced enough pathology to suppress defences against infection, e.g. leukaemia or lymphoma.

Samples

Samples were obtained in the first 24 hours after admission to the ICU and the analysis performed within 4 hours after blood extraction. The blood extraction for each patient was repeated every 72 hours for 15 days, unless an early termination was established due to *exitus*.

Flow cytometry staining

Blood samples from the patients were collected with EDTA anticoagulant. 100µl of whole blood was used, which was stained with 15µl CD64 PE, 5µl CD15 APC, 2.5µl HLA-DR BV421, 2.5µl CD14 APCH7 and 2.5µl CD3 BV515 (all from

BD Biosciences, San José, CA, USA). After staining, cells were incubated for 20 minutes in darkness at room temperature followed by erythrocyte lysis, performed using BD FACS Lysing Solution (BD Biosciences, San José, CA, USA) for 7 minutes. After centrifugation and washes with FACSFlow (BD Biosciences, San José, CA, USA), samples were acquired by BD FACSCanto II (BD Biosciences, San José, CA, USA). A minimum of 10,000 monocytes were recorded per sample.

Flow cytometry calibration

To standardize the analysis, we use the Rainbow Calibration Particles 6 peaks (BD Biosciences, San José, CA, USA) which contain a mixture of particles of similar size with different fluorescence intensities. The particles were used according to the manufacturer's protocol. The particles were reconstituted with phosphate buffered saline (PBS) (BD Biosciences, San José, CA, USA). Equipment voltages and the mean fluorescence intensity (MFI) were adjusted for each fluorochrome at a daily basis in order to standardize the protocol reducing the existing inter-test variability.

Flow cytometry analysis

MFI of CD64 on neutrophils ($_{N}$CD64), MFI of HLA-DR on monocyte ($_{M}$HLA-DR) and on lymphocytes ($_{L}$HLA-DR) were measured (Fig. 1). In addition, the HLA-DR expression rate on monocytes was measured. For the analysis, we used HLA-DR expression on lymphocytes as negative control and CD64 on monocytes as a positive control. The analysis of cell subpopulations was

performed using the FACS Diva version 6.1.2 software (BD, San José, CA, USA).

Plasma analysis

Reactive-C protein was analyzed in plasma samples of patients at each time point using AU-5800 (Beckman Coulter) immunoanalysers.

Statistical Analysis

Quantitative variables are presented as the mean ± SD and qualitative variables as percentages and numbers. Normality criteria were determined using the Shapiro-Wilk test. Mann-Whitney U-test was used to compare differences in CRP, lymphocyte count, HLA-DR expression rate, MFI of $_{M}$HLA-DR, HLA-DR Index, and Sepsis index between outcome groups at each timepoint. In order to evaluate more stable biomarkers, we assessed the HLA-DR Index which was defined as the ratio between MFI of $_{M}$HLA-DR and $_{L}$HLA-DR. Also, we analyzed the Sepsis Index, defined as the ratio between $_{N}$CD64 and $_{M}$HLA-DR, as previously reported by other authors.

Analyses of HLA-DR Index and Sepsis Index were performed after logarithmic transformation given that the distribution was evaluated as log-normal. Unadjusted and adjusted linear regression were used to evaluate $_{M}$HLA-DR in relation to different timepoints before and after infection. Linear mixed models for repeated measurements were used to evaluate the dynamic variation in

$_{M}$HLA-DR and sepsis index at different time points unadjusted and adjusted for gender and age.

Predictive values of the candidate biomarkers were investigated through Receiver Operating Characteristic (ROC) curves. Based on these curves, cut-off values for relapse prediction were assessed for each potential biomarker. P-Values <0.05 were considered significant.

Figures show means ± SEM. The Statistical Package for Social Sciences (SPSS/Windows version 15.0; SPSS Inc, Chicago, IL, USA) and the software program GraphPad Prism (5.0 version; GraphPad, La Jolla; CA, USA) were used to perform statistical analyses.

RESULTS

Patients

Seventy-nine patients were selected for the study but 2 of them were not eligible for further analysis due to *exitus* before 72h from admission. Clinical and demographical characteristics of patients included in the study are shown in Table 1. The severity indicators showed a high APACHE II score which was 21 (SD: 7) and SOFA score was 7 (SD: 4). According to the sepsis stratification, patients who developed sepsis during the follow-up, had a higher basal APACHE II and SOFA score than patients who remained without infections (APACHE II score: septic patient: 22±6, non-septic patient: 19±9; p=0.05; SOFA score: septic patient: 8±3, non-septic: 4±3; P<0.001). Regarding the days

staying in the ICU, patients who developed sepsis showed a longer time admitted in ICU than those patients who did not develop sepsis during the follow-up (septic: 25±15 days; non-septic patients: 10±7 days, p<0.001). In addition, mechanic ventilation was required longer for septic patients (septic: 17±13, non-septic:4±8, p<0.001). There were no differences in gender, age, comorbidities or mortality between groups. The septic group showed blood culture positive in 36 patients (71%) and adequate antibiotic treatment was assessed in 41 patients (79%). Nine out of 55 septic patients developed septic shock during the follow-up (Table 1).

Pro-inflammatory and anti-inflammatory imbalance three days after admission

Reactive C Protein (RCP) showed higher levels at day +3 after admission (161.83±133.42 mg/ml) than basal time point (71.90±76.71 mg/ml) in the total of included patients (Fig 2 a). In contrast, lymphocyte count, the $_{M}$HLA-DR rate, the Mean Fluorescence Intensity (MFI) of $_{M}$HLA-DR, HLA-DR index and Sepsis Index (SI) did not show along the follow-up.

As shown in Figure 2 the dynamics of PCR (Fig 2 b), Sepsis Index (Fig 2 c) and $_{M}$HLA-DR expression (Fig 2 d) over time differed between groups. Septic patients showed increased levels of PCR (septic patients: 182.9±132.9 mg/ml; non-septic patients: 93.46±117.6 mg/ml, p=0.030) (Fig 2 b) and Sepsis Index (septic patients: 0.19±0.19; non-septic patients: 0.08±0.08, p=0.010) (Fig 2 c) at day +3 after admission. In contrast, a decreased $_{M}$HLA-DR rate was found (septic patients: 81.7±16.22%; non-septic patients: 88.53±12.13%, p=0.040)

(Fig 2 d). $_{M}$HLA-DR rate was slowly recovered before 6 days, while Sepsis Index remained higher in septic patients up to day +9. There were no differences in lymphocyte count, MFI of $_{M}$HLA-DR or HLA-DR Index between groups during the follow-up (Supplementary table 1).

A decrease in HLA-DR expression and an elevation of Sepsis Index enhance the probability to develop sepsis in critical patients

HLA-DR monocyte expression was evaluated in ICU admitted patients without infection and it was monitored during 15 days after admission. Considering the time of the diagnosis of sepsis in the analysis, the $_{M}$HLA-DR expression rate on monocytes in septic patients demonstrated to differ significantly over time before the sepsis diagnosis (p=0.001). As shown in Figure 3 a decrease in the means values of the expression of HLA-DR molecules in the surface of monocytes, measured as MFI HLA-DR on monocytes was found in septic patients and it was recovered after infection. At the mixed model-interaction test, the septic patients showed to differ significantly over time and remained as a significant factor after multivariate adjustments for gender, age and mechanic ventilation (p<0.019). On the other hand, the percentage of monocytes HLA-DR^{+} showed a trend to differ in septic patients over time (p=0.09).

Patients who presented high Sepsis Index showed a higher risk to develop sepsis (OR: 2.71, P<0.002). Moreover, statistical differences were found in the Sepsis Index means over time in septic patients *vs.* non-septic patients (p= 0.005).

$_{M}$HLA-DR expression rate present higher specificity and less sensibility than Sepsis Index and CRP levels to classify septic patients

In ROC curve analysis, CRP levels were significant predictors of the develop sepsis, followed by $_{M}$HLA-DR expression rate and MFI of $_{M}$HLA-DR, while lymphocyte count (p=0.286) and Sepsis Index (p=0.05) were not significant predictors. AUCs was highest for CRP levels (AUC 0.765, p<0.001), followed by $_{M}$HLA-DR expression rate (AUC 0.666; $p < 0.001$) and MFI of $_{M}$HLA-DR (AUC 0.654; <0.001). Boxplots of CRP levels and $_{M}$HLA-DR expression rate in those who developed sepsis and non-septic are displayed in Figure 4. To predict the development of sepsis, optimal cut-offs were CRP levels >106.90 mg/mL (74.19% sensitivity, 69.49 specificity), $_{M}$HLA-DR expression rate <72.80% (45.31% sensitivity, 89.47% specificity) and MFI of $_{M}$HLA-DR <1882 (73.53% sensitivity, 53.76% specificity).

DISCUSSION

The aim of this study was to find biomarkers of immunoparalysis to predict which patients have an increased risk of developing sepsis at the UCI. The candidate biomarkers evaluated were (i) HLA-DR expression rate on monocytes ($_{M}$HLA-DR), (ii) Mean fluorescence intensity of HLA-DR on monocytes, (iii) HLA-DR Index, and (iv) Sepsis Index (SI). Our results showed that a pro-inflammatory/anti-inflammatory imbalance - before infection - produces an increased risk of developing sepsis in critical neurologic patients and this risk is increased in patients who remain hospitalized longer.

The initial injury which leads to their admission at ICU could be triggering an imbalance between the processes of inflammation and immunosuppression. These processes involve the activation of several intracellular pathways resulting in the production of pro-inflammatory cytokines. In parallel, a Compensatory Anti-inflammatory Response Syndrome (CARS) is activated as a temporal protective effect during the first hours after the injury. If this immunosuppressive state would be maintained over time, it could produce immune-paralysis, predisposing the patient to infectious complications and the development of sepsis (7).

The decrease in the $_{M}$HLA-DR on circulating monocytes has been mostly accepted as a reliable marker of immune-paralysis in septic patients (20, 21). This molecule reflects the loss of ability of monocytes to present antigens and, consequently, to activate lymphocytes. Different authors have studied the association between expression levels of the $_{M}$HLA-DR and prediction of sepsis (10, 12, 19). However, the results obtained in those studies were not conclusive (12, 21-22). Differences in the study design such as monitoring time-points, as well as lack of standardization of flow cytometry protocols might be, in part, responsible of the discordant results. To avoid this variability due to the technical procedure, we used a standardized flow cytometry methodology which can be easily transferred to other centers.

A prospective study in trauma patients without infection admitted to ICU showed that the $_{M}$HLA-DR expression was decreased in patients who developed sepsis during the follow-up (19). Similar results were found in our study – in our case in

patients with severe neurological injury - where the $_{M}$HLA-DR on day +3 of follow-up was lower in patients that developed sepsis at later time points. To our knowledge, no other longitudinal and predictive studies have been performed analyzing $_{M}$HLA-DR expression in ICU patients. These results together, support that the analysis of the $_{M}$HLA-DR expression is the best marker to monitor the immunocompetent status of the patients, and assess the susceptibility to the development of infections.

Regarding CRP levels, we observed higher values in patient who developed sepsis on day +3 of follow-up. At the same time point, Sepsis Index – which provides information between pro-inflammatory and anti-inflammatory balance – was increased, supporting that patients that develop sepsis suffered an imbalance due to an increase of inflammatory mediators and a decrease of HLA-DR molecules.

Early diagnosis of the septic process is important to establish an adequate therapeutic strategy, because it is associated to longer survival (23). Currently, the markers used in clinical practice to support the diagnosis of sepsis are CRP and procalcitonin (PCT), but they have limitations. While the CRP is highly sensitive, it lacks specificity for the diagnosis of sepsis; oppositely, PCT is more specific but lacks sensitivity. Therefore, there is a need to look for biomarkers able to provide a reliable diagnosis (24). In this context, the analysis of the evaluated biomarkers ($_{M}$HLA-DR expression rate and Sepsis Index), showed changes before the diagnosis of sepsis. In contrast, no differences were found in those patients that did not develop sepsis during follow-up. The ROC analysis

showed $_{M}$HLA-DR expression rate the biomarker with a highest specificity, and CRP the one with the highest sensitivity to predict sepsis.

Taking all the mentioned above into consideration, we propose a diagnostic algorithm that could be implemented in the monitoring of critical neurological patients admitted at the ICU. First, immune-monitoring the Sepsis Index, CRP and $_{M}$HLA-DR expression rate biomarkers at the admission and on day +3, to allow an early predictive stratification of susceptible sepsis patients. In this context, our preliminary results showed that the optimal cut-off values could be >106.90 mg/ml for CRP and <72.80% for $_{M}$HLA-DR expression rate. Patients who would fulfill both criteria should be classified as potentially susceptible to develop sepsis. These biomarkers should be used in addition to other laboratory tests, such as PCT, and evaluated in the patient's context to avoid misdiagnosis.

The present study has a number of limitations. First, as it is a single-center study. The findings need to be confirmed in a larger and independent cohort. Moreover, we have only analyzed a specific group of patients (patients with severe neurological injury). The applicability of these biomarkers should be tested in different pathological contexts as severe acute pancreatitis, trauma, burn or surgery.

We found a combination of biomarkers in peripheral blood able to stratify ICU patients with high risk to develop sepsis. These results have a potentially

important implication in the hospital care area, as the combination of parameters studied can facilitate the management of critical ill patients.

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BQ contributed to the development, performed the immunological monitoring, analyzed raw data and contributed to writing the article. OP collected clinical information about critical patients. EL performed the immunological monitoring. FA contributed to the study concept, development and writing of the article. EM designed the study and contributed to writing the paper. All the authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. **Gateing strategy analysis by flow cytometry of CD64 expression on neutrophils and HLA-DR expression on monocytes and lymphocytes.**

Figure 2. **Changes in measures of immune dysfunction by subsequent sepsis status.** (a) PCR levels of total patients included for difference over time ($p<0.05$). (b) PCR levels for differences between groups ($p<0.05$). (c) Sepsis Index values for differences between septic and non-septic patients ($p<0.05$). (d) Expression rate of $_{M}$HLA-DR on monocytes for differences between groups ($p<0.05$).

Figure 3. Differences in the mean fluorescence of $_{M}$HLA-DR on monocytes from septic and non-septic patients before infection. Dynamic values of mean fluorescence intensity of $_{M}$HLA-DR on monocytes from septic (red) and non-septic (blue) patients during 15 days of follow-up over time.

Figure 4. ROC curves for PCR levels (a), the expression rate of HLA-DR on monocytes (b) and MFI of $_{M}$HLA-DR (c) for the diagnosis of patients with higher risk to develop sepsis during their stay in ICU.

Characteristics	Total cohort n=77	Septic n=55	Non septic n=22	p-value
Female sex (n. of patients, (%))	26(33)	17(19)	9(7)	0.400
Age (years), (IQR)	54±16	54±16	56±16	0.520
Basal SOFA* score (IQR)	7±4	8±3	4±3	**<0.001**
APACHE **II score (IQR)	21±7	22±6	19±9	0.050
Median hospital days (IQR)	21±15	25±15	10±7	**<0.001**
Mechanic ventilation days (IQR)	14±13	17±13	4±8	**<0.001**

Comorbidities (n. of patients, (%))				
COPD***	6 (8)	4 (4)	2 (2)	0.790
Smoker	25 (32)	16 (18)	9 (7)	0.320
Alcoholims	15 (19)	9 (11)	6 (4)	0.270
Cardiopathy	8 (10)	6 (6)	2 (2)	0.810
Chronic kidney disease	7 (9)	5 (5)	2 (2)	1.000
Cirrhosis	2 (3)	2 (1)	0 (0)	0.360
Exitus (n.patients, (%))	14 (18)	11(10)	3(4)	0.510
Blood culture (n. of patients, (%))	36 (71)		NA	
Adequate antibiotic treatment (n. of patients, (%))	41 (79)		NA	
Shock septic (n. of patients, (%))	9 (12)	9 (16)	NA	

Table 1. Demographic and clinical characteristics of patients.

* *SOFA= Sepsis-related Organ Failure Assesment scale, ** APACHE=* Acute Physiology and Chronic Health Evaluation*, ***COPD= Chronic obstructive pulmonary disease*