Monocyte and lymphocyte surface molecules in severe sepsis and non-septic critically ill Patients

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The aim of the present study was to investigate whether expression of monocyte and lymphocyte surface molecules differs between patients with severe sepsis and non-septic patients treated in the intensive care unit (ICU). The expression of monocyte CD14, CD40, CD80 and HLA-DR, and lymphocyte CD69 were analyzed using quantitative flow cytometry on three consecutive days in 27 patients with severe sepsis and in 15 non-septic patients. Receiver operating characteristic analyses were performed and each corresponding area under the curve (AUC) was determined. The results showed that the expression levels of CD40 on monocytes and CD69 on CD4+ T cells and on natural killer (NK) cells were highest in patients with severe sepsis (p < 0.05). Monocyte CD40 and NK cell CD69 expression levels were higher in patients with severe sepsis and positive blood culture compared with those with negative blood culture (p < 0.05).

The highest values of AUC for severe sepsis detection were 0.836 for CD40, 0.872 for CD69 on NK cells, and 0.795 for CD69 on CD4+ T cells. These findings suggest that monocyte CD40 and CD69 on NK cells and CD4+ T cells could prove useful for new approaches in the identification of severe sepsis in the ICU.

Key words: Severe sepsis; monocytes; lymphocytes; flow cytometry.

The results were partly presented at ICI 2013 in Milan and at ESICM annual meeting 2013 in Paris.

Distinguishing sepsis from non-septic causes of systemic inflammatory response syndrome (SIRS) still remains a challenge in clinical practice (1). Recently, it has been shown that one of eight patients had SIRS-negative sepsis (2). Therefore, the SIRS criteria do not exclude sepsis nor exclude the possibility of bacterial infection and the need for initiation of antimicrobials. Phenotyping of the cells of the immune system may serve as a tool for distinguishing infection from other causes of systemic inflammation more accurately. Moreover, in the future the immune status of a patient with sepsis needs to be evaluated when selecting immunomodulatory therapy (3). Some leukocyte surface markers, like neutrophil CD64, have already been demonstrated as a potential aid in sepsis diagnostics (4, 5). Monocytes and NK cells are activated to eliminate invading pathogens, and there are potentially useful cellular activation markers of lymphocytes and monocytes that have not been investigated, especially in severe sepsis. These include monocyte CD14, CD40, CD80 and HLA-DR, and CD69 on different lymphocytes (6–10).

Increased expression of CD40 on monocytes has been reported in sepsis (7, 9), but there is need for studies confirming this finding in patients with severe sepsis in the intensive care unit (ICU). There is...
also some evidence that monocyte CD80 (B7-1) expression is higher in sepsis (7). There are contradictory reports concerning monocyte CD14 expression in sepsis (6, 11). Human leukocyte antigen (HLA)-DR, part of the major histocompatibility complex (MHC) II, is expressed on both monocytes and lymphocytes, and decreased expression of HLA-DR on monocytes resulting from immune dysfunction has been reported in patients with different types of sepsis (10, 12). Expression of CD69 on T cells has been reported to be higher in sepsis compared with non-septic critically ill patients (8). Expression of CD69 on NK cells has been proposed as a marker of neonatal infection, but there is lack of studies on its expression in adult patients with sepsis (13).

The literature concerning these surface molecules as markers of infection in critically ill patients is limited. In this study, we explored the applicability of monocyte CD14, CD40, CD80 and HLA-DR, and lymphocyte CD69 expression (differently on CD4+ and CD8+ T cells, B cells, and NK cells) in distinguishing severe sepsis from non-septic conditions in patients requiring intensive care treatment. The aim was to investigate whether there are differences in the expression of monocyte and lymphocyte surface molecules between severe sepsis and non-septic intensive care patients.

MATERIALS AND METHODS

Study subjects

The three patient groups of this prospective study have been described in more detail elsewhere (5). Briefly, our study population consisted of (i) critically ill patients with severe sepsis (including septic shock) treated in the mixed surgical and medical ICU of Oulu University Hospital, Oulu, Finland; (ii) ICU patients without SIRS at the beginning of the ICU treatment (non-SIRS ICU, representing heterogeneous group of ICU patients without sepsis); and (iii) electively operated off-pump coronary artery bypass (OPCAB) surgery patients (representing inflammation caused by surgical trauma without sepsis). Ten healthy volunteers, seven of whom were male, with median age of 48 years (27–57) served as controls. The material was collected between May 2009 and March 2012. The local ethics committee approved the study protocol (The Regional Ethics Committee of the Northern Ostrobothnia Hospital District, protocol number: 54/2008). Written informed consent was obtained from all patient groups during the study period; in severe sepsis and non-SIRS ICU patients on day 0, and in OPCAB patients on the second postoperative day. Routine demographics and acute physiology and chronic health evaluation (APACHE) II (15) and sequential organ failure assessment (SOFA) (16) scores were collected.

Flow cytometry

Expression of CD14, CD40, CD69, CD80, and HLA-DR was measured using quantitative flow cytometry and whole blood method. For flow cytometry, the blood samples were collected using pre-cooled, siliconized vacuum tubes containing acid citrate dextrose (Venoject, Terumo, Leuven, Belgium) as an anticoagulant. The samples were stained and analyzed within 6 h of sampling keeping them at +4 °C to avoid the effects of temperature and time (5, 17). Flow cytometric analyses were available only during office hours.

The following anti-human antibodies from BD Biosciences (San Jose, CA, USA) were used for cell labeling at the concentration suggested by the manufacturer: CD3-peridinin chlorophyll protein (PerCP) (clone SK7), CD4-fluorescein isothiocyanate (FITC) (clone SK3), CD8-allophycocyanin (APC) (clone SK1), CD14-PerCP (clone MφP9), CD14-FITC (clone MφP9), CD19-APC (clone SJ25C1), CD40-FITC (clone 5C3), CD56-FITC (clone NCAM 16.2), CD69-phycocerythrin (PE) (clone L78), CD80-PE (clone L307.4), HLA-DR-PE (clone G46-6), Simultest IgG1/IgG2a-PE (clone X39/X40), and IgG1k-PE (clone X40).

FACSCalibur™ flow cytometer and CellQuest software (BD Biosciences), with regular equipment calibrations using CaliBRITE beads (BD Biosciences), were used for analysis. The forward-scatter characteristics, side-scatter characteristics, and specific monoclonal antibodies (CD14 for monocytes; CD3, CD4, and CD8 for T-lymphocytes; CD19 for B-lymphocytes; and CD3 and CD56 for NK cells) were used for cell identification.

Quantum FITC molecules of equivalent soluble fluorochrome (MESF) and R-phycocerythrin (R-PE) MESF microspheres (Bangs Laboratories, Inc., Fishers, IN, USA) were used for interassay standardization and fluorescence quantitation according to the manufacturer’s instructions. A standard curve was generated using QuickCal software v. 2.3 (Bangs Laboratories, Inc.) by plotting each bead population’s median fluorescence intensity.
against its assigned MESF value, and the standard curve was then used to convert the median fluorescence intensities of different cell populations in patient samples into MESF values. The method has been described and illustrated earlier (5). Fig. 1 shows an example of gating strategy.

In some samples, CD69 expression was below the lowest standard in the MESF calibration curve, and the MESF value was obtained by assuming that the MESF calibration curve is linear also below the lowest standard. Similarly, in some samples, CD14 expression was higher than the highest standard in the MESF calibration curve, and the MESF value was obtained by assuming that the calibration curve continues linearly above the highest standard.

Data analysis

For statistical analysis, SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp.) software was used. The results are expressed as medians with the 25th–75th percentiles. Kruskall-Wallis and Fisher tests were used to compare the demographics. Friedmann’s test was used to compare the surface molecule differences within the groups in repeated measurements, and Kruskall-Wallis and Mann-Whitney tests were used to compare the groups. To further investigate the expression in groups, OPCAB patients and non-SIRS ICU patients were combined into one group of non-septic ICU patients, and receiver operating characteristic (ROC) analyses were made and each related area under the curve (AUC) was determined. To investigate the diagnostic performance for severe sepsis, the D0, D1, and D2 values of the surface molecules in severe sepsis patients were compared to the peak values of the molecules in non-septic patients (non-SIRS ICU and OPCAB patients combined). Sensitivities and specificities were calculated according to Youden’s index (the point where sum of sensitivity and specificity reaches its maximum value). Two-tailed p-values were reported and statistical significance was determined as a p-value of <0.05.

RESULTS

Patients

Twenty-seven patients with severe sepsis and 15 non-septic ICU patients (seven OPCAB patients and eight non-SIRS ICU patients) were included in the study. Patient demographics are shown in Table 1 and in detail in our previous study report (5). The three patient groups did not differ statistically with regard to age and gender, while patients with severe sepsis had higher ICU scores (APACHE II and SOFA) and mortality and longer

![Fig. 1. An example of flow cytometry representing monocyte CD40 expression in a patient with severe sepsis. (A) The monocyte population was identified based on its side-scatter characteristics and CD14 positivity, and (B and C) FITC-conjugated CD40 antibody was used to define median fluorescence intensity (MFI) of CD40 expression, which was further converted into molecules of equivalent soluble fluorochrome (MESF) value.](image_url)

Table 1. Demographic details of the patients

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Severe sepsis (n = 27)</th>
<th>Non-SIRS ICU (n = 8)</th>
<th>OPCAB (n = 7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>65.9 (58.1–74.5)</td>
<td>56.4 (54.1–71.9)</td>
<td>67.7 (63.4–74.7)</td>
<td>0.411</td>
</tr>
<tr>
<td>Male %</td>
<td>56</td>
<td>63</td>
<td>71</td>
<td>0.818</td>
</tr>
<tr>
<td>Comorbidities¹ (%)</td>
<td>14 (52)</td>
<td>3 (38)</td>
<td>7 (100)</td>
<td>0.051</td>
</tr>
<tr>
<td>APACHE II</td>
<td>21 (15–24)</td>
<td>16 (11–21)</td>
<td>14 (11–15)</td>
<td>0.018</td>
</tr>
<tr>
<td>SOFA, Peak</td>
<td>9 (7–12)</td>
<td>3 (1–5)</td>
<td>6 (5–6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ICU treatment, days</td>
<td>5.7 (2.4–8.7)</td>
<td>1.4 (0.94–2.6)</td>
<td>0.96 (0.91–1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28-day mortality (%)</td>
<td>6 (22)</td>
<td>0</td>
<td>0</td>
<td>0.215</td>
</tr>
</tbody>
</table>

APACHE II, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment.

¹At least one of the following comorbidities: coronary artery disease, diabetes, asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, chronic renal disease.
stay at the ICU and in the hospital. The median time to blood sampling from the beginning of the first detected organ failure in severe sepsis at ICU was 19 h (11–30), with delay of more than 24 h in nine cases. Of the 27 patients with severe sepsis, 25 (93%) had septic shock receiving vasopressors, and sepsis cortisone was used in 23 septic shock patients (92%). The primary foci of the infection were bacterial pneumonia (n = 8); urine tract infection (n = 3); intra-abdominal infection (n = 8); and bone, joint, skin or connective tissue infection (n = 8). Eleven patients with severe sepsis (41%) had positive blood culture (one gram-positive, nine-gram-negative, one anaerobe).

**Monocyte surface markers**

Monocyte CD40 expression was highest in patients with severe sepsis compared with OPCAB and non-SIRS ICU patients and healthy controls (p < 0.001) (Fig. 2). The peak value of the severe sepsis patients was observed on D0 (p < 0.001) (Fig. 2). Monocyte CD40 expression increased after OPCAB surgery (p = 0.008) (Fig. 2).

Monocyte CD80 expression was higher in patients with severe sepsis than in non-SIRS ICU patients and healthy controls, but the expression did not separate severe sepsis and OPCAB patients (Fig. 2). Monocyte CD14 expression was lower in patients with severe sepsis than in non-septic ICU patients and healthy controls, but the expression did not separate severe sepsis and OPCAB patients (Fig. 2). The highest CD14 expression was observed in non-SIRS ICU patients. Monocyte HLA-DR expression was higher in healthy controls than in severe sepsis patients, postoperative OPCAB patients, and non-SIRS ICU patients (p < 0.001) (Fig. 2). In OPCAB patients, the HLA-DR expression decreased after surgery (p = 0.001) (Fig. 2).

**Lymphocyte CD69**

The expression of CD69 on NK cells was highest in patients with severe sepsis compared with the other groups (p < 0.001, Fig. 2). The expression of CD69 on CD4+ T cells was highest in patients with severe sepsis compared with the other groups (p = 0.002) (Fig. 2). The expression of CD69 on CD8+ T cells and B cells was low, and there were no significant differences between the groups in its expression (data not shown).

**ROC analyses**

The diagnostic utility of the surface molecules was further assessed in ROC analyses in which severe sepsis patients were compared with non-septic ICU patients (OPCAB and non-SIRS ICU groups combined). In the ROC analyses, the best AUC for severe sepsis diagnosis was with NK cell CD69 at D1 (0.872) with a sensitivity of 92% and specificity of 80%. However, 100% sensitivity with 69% specificity was achieved with monocyte CD40 with an AUC of 0.836 at admission, while in CD4+ T cell CD69, the best AUC was 0.795 with 68% sensitivity and 87% specificity at D1 (Fig. 3).

**Expression in severe sepsis patients with positive blood culture**

The highest AUC, 0.766 (0.578–0.955), was on D1 for monocyte CD40, and 0.770 (0.593–0.947) on D0 for NK cell CD69 for discriminating blood culture positivity (p = 0.025 and p = 0.019, respectively).

**DISCUSSION**

In this prospective 3-day observational study, the expression levels of CD69 on CD4+ T cells and NK cells and CD40 on monocytes were higher in patients with severe sepsis than in non-septic patients. According to ROC-analyses, these lymphocyte and monocyte surface molecules may be advantageous for sepsis diagnostics in ICU population.

To our knowledge, there are no other prospective studies comparing monocyte CD40 expression in severe sepsis patients with that in non-septic ICU controls. However, monocyte CD40 has been reported to be activated by the CD40 ligand (CD154) on T cells during antigen presentation or directly via bacterial stimulus (18). Previously, other reports have shown contradictory results, the variation being explained by various pathogens (e.g., gram-negative bacteria) or the clinical severity of the disease (7, 9, 19, 20). In our study, monocyte CD40 expression was higher in severe sepsis patients with positive blood culture typically yielding gram-negative bacteria. The expression of CD40 on monocytes slightly increased after OPCAB surgery but remained lower than the levels in severe sepsis, which was also a new finding.

Other investigators have shown that natural cytotoxicity receptors of NK cells (CD314) are significantly lower in septic ICU patients than non-septic ICU patients (21). We studied CD69 expression on NK cells, which is expressed at the very early phase of T-cell activation and promotes the release of proinflammatory cytokines (22, 23).

In our study, CD69 expression on NK cells was higher in severe sepsis patients compared with the
other groups and higher in those severe sepsis patients who had positive blood culture than in those with negative blood culture. Expression of CD69 on CD4+ T cells was also higher in patients with severe sepsis than in controls being in harmony with previously published studies comparing sepsis cases and healthy controls (24) or non-septic critically ill patients (8).

In contrast to an earlier study on major surgery, in our study major cardiac surgery had no effect on
CD69 expression on NK or CD4+ T cells. The difference may be explained by the fact that the earlier study, which reported increased CD69 expression on CD4+ T cells, was performed among patients who underwent oesophagectomy or pancreaticoduodenectomy due to malignancy (25). In our study malignancies were excluded and none of our OPCAB patient developed postoperative sepsis.

In our study, monocyte CD80 expression was significantly higher on D0 in patients with severe sepsis compared with healthy controls and non-SIRS group. A similar result has been reported earlier between sepsis and healthy controls (7). However, OPCAB patients had pre-operatively higher monocyte CD80 expression compared with healthy volunteers and non-SIRS patients. This is in accordance with previous evidence that CD80 is increased on monocytes in patients with coronary artery disease (26).

Our results are in harmony with earlier studies, in which HLA-DR expression has been shown to be decreased in severe sepsis (10, 12) and after surgery (27). Our results suggest that HLA-DR expression is not applicable in sepsis diagnostics.

It has been shown earlier that septic ICU patients have lower or equal monocyte CD14 expression than non-septic patients or healthy controls (6, 11, 21). In our series, CD14 expression did not have diagnostic value.
The strengths of our study included the strict pre-analytical methods to avoid in vitro activation of the leukocytes; these entailed, for example, the use of +4 °C temperature for sample collection and storage (17). Furthermore, during the analytical process, we quantitated the expression levels by using commercial calibration beads allowing standardization of the results by converting unstandardized median fluorescence intensity values into standardized MESF values (3, 4). Our study also involved a major surgical patient group allowing comparison with patients with sepsis. Since sepsis is not a rare complication in surgical patients, it is essential also to know to what extent surgery alone induces changes in cell surface markers.

This explorative study has some limitations. Our patient sample size was quite small and the study was a one-center study. Furthermore, it was not possible to validate our findings in a separate independent test cohort due to the challenges in flow cytometry methods. Nonetheless, we used quantitative flow cytometry to investigate the kinetics of these molecules in different ICU populations allowing comparisons between clearly selected patient groups. Our setting could overestimate the diagnostic performance of the molecules since severe sepsis patients had higher APACHE II and SOFA scores, and thus the differences in surface marker expression in the present study may be a reflection of disease severity. However, all our severe sepsis patients had microbiologically or clinically verified infections. It is clear that further investigation concerning CD69 on CD4+ T cells and NK cells and CD40 on monocytes with unselected patient groups is necessary to determine whether these leukocyte surface molecules could serve as biomarkers for sepsis diagnostics. Our septic patients were investigated a median of 19 h after severe sepsis. Our results on monocyte CD40 expression with decreasing values on consecutive days suggest that the time of sampling is relevant. It is known that both adrenergic drugs and hydrocortisone may modulate expression of lymphocyte subpopulations (28, 29). In the current study, 93% of the patients with severe sepsis received vasopressors, and sepsis cortisone was given to 92% of the septic shock patients. Therefore, the possible effects of vasopressors and sepsis cortisone on our results must be taken into consideration.

As a conclusion, our findings suggest that the expression of CD40 on monocytes and CD69 on CD4+ T cells and NK cells distinguish patients with severe sepsis from non-septic critically ill patients and healthy controls. Further studies are needed to confirm the clinical applicability of these findings.

CONFLICTS OF INTEREST

The authors declare that they do not have any conflicts of interest.

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