INTRODUCTION

Sepsis is defined as the uncontrolled response of the host immune system to infection leading to subsequent organ dysfunction; it is a life-threatening syndrome with increasing incidence worldwide. Sepsis can escalate to septic shock, representing a clinical condition of a persisting low blood pressure with vasopressor requirement and volume resuscitation to maintain a sufficient mean blood pressure with current high levels of blood lactate. In-hospital mortality ranges from 10% for sepsis, to 39% for septic shock. Although sepsis is a multifactorial disorder, regaining control of the inflammatory response seems to be crucial to maintain homoeostasis but despite decades of effort, mortality remains high. Clinical management of sepsis relies on early recognition of the condition, followed by identification and control of the source of sepsis, with simultaneous administration of antimicrobials and organ support to give patients time to recover. Early identification of patients with sepsis is based on evaluation of clinical symptoms and laboratory parameters. This article describes a novel approach to the early identification of patients with sepsis that may benefit from tailored therapy.
characteristics and laboratory markers equating to a change in the sequential organ failure assessment (SOFA) score of 2 points, or more. Many predictors of poor clinical outcome—such as high SOFA score, refractory course of shock, persistent hyperlactatemia, and hypothermia—have been described. However, no single, standardized predictive marker currently exists to define the group of patients with the worst prognosis.

Monocytes carry the long-term burden of sepsis. These cells are activated by pattern recognition receptors (PRRs) and sepsis-associated hypoxia; the hypoxia-inducible factor (HIF-1α) has been identified as having a key role in the functional reprogramming of these cells. Monocytes can be categorized into three major subpopulations: classical (CD14+ CD16−), intermediate (CD14+ CD16+), and non-classical (CD14− CD16+) monocytes. Fingerle et al. reported that CD16+ cells can represent up to 50% of all monocytes during sepsis. This finding led to studies of functional changes in monocytes during sepsis, although correlations between monocyte counts and sepsis severity have only been reported in the past 2 years.

In the search for diagnostic markers of sepsis progression, promising results have been provided by phenotypic changes in monocytes and the presence of monocyte-produced molecules in plasma. However, studies on the role of monocytes during sepsis have not generally considered the complexity of monocyte biology. The individual mechanistic roles of the three distinct monocyte subsets in sepsis progression remain to be investigated. Moreover, monocyte role in prediction of short-term survival in patients with sepsis has been studied.

Sepsis affects most of the immune system functions, profound changes indeed impact leucocyte subsets including CD4+ T cells. CD4+ T cells are important for regulation and orchestration of immune response and their impairment leads to loss of functional immune cells and subsequent immunosuppression; characteristic feature very often induced during sepsis.

In this study, we address whether the differences in monocyte subsets observed in patients with septic shock at the time of admission correlate with the severity of septic shock progression, including cytokine expression, changes in SOFA score, and early mortality. The diagnosis within the early stages of septic shock is an important factor in patient prognosis.

2 | MATERIAL AND METHODS

2.1 | Cohort design

In this prospective, observational cohort study, adult patients with early septic shock who were admitted to the intensive care unit (ICU) at St. Anne’s University Hospital in Brno, Czech Republic were consecutively enrolled. Patients with chronic immunosuppression and those who had received antibiotic therapy for more than 2 days were excluded. All patients were treated with tailored therapy according to current guidelines. The cohort was divided for study purposes into two groups: those who survived longer than five days after admission and those who died within five days (early deceased). Written informed consents were obtained from all enrolled patients and all procedures and protocols were approved by the institutional ethic committee (4G/2018).

2.2 | Blood sample isolation and preparation

Blood samples were obtained from patients within 12 hours of admission to the ICU and were processed within 2 hours of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by gradient centrifugation using Lymphoprep® (Alere Technologies AS; Oslo, Norway) (density 1.077 g/mL) following the manufacturer’s recommendations. Serum was collected in vials facilitating coagulation, centrifuged and immediately frozen and stored at −80°C.

2.3 | Immunophenotyping of samples

To obtain the phenotype of monocytes, PBMCs were labelled using lineage antibodies (CD66b-biotin [RRID:AB_2566608] (BioLegend, Inc, San Diego, CA, USA), anti-CD3-biotin (RRID:AB_466323), anti-CD19-biotin (RRID:AB_466388), anti-CD20-biotin (RRID:AB_657690), anti-CD56-biotin (RRID:AB_10596499), anti-CD235α-biotin (RRID:AB_494036), followed by streptavidin eFluor450 (RRID:AB_10359737)), anti-CD45-PerCP-eFluor710 (RRID:AB_11041112), anti-CD16-APC (RRID:AB_2016663), anti-CD86-PE (RRID:AB_10732345) anti-HLA-DR-FITC (RRID:AB_2572542) (Affymetrix, Inc, Santa Clara, CA, USA), and anti-CD14-BV510 (RRID:AB_2833091) (Sony Biotechnology, Inc, San Jose, CA, USA). A LIVE/DEAD fixable near-IR dead cell stain kit (Thermo Fisher Scientific, Inc, Waltham, MA USA) was used to exclude dead cells from analysis. Monocyte subsets were classified as classical (CD14+ CD16−), intermediate (CD14+ CD16+), and non-classical (CD14− CD16+).

The T cell immunophenotype was determined by labelling with anti-CD3-APC-Cy7 (RRID:AB_2563410), anti-CD8-FITC (RRID:AB_314124), (BioLegend, Inc), and anti-CD4-BV510 (RRID:AB_2744424) (BD Biosciences, Franklin Lakes, NJ, USA). CD3+ T cells were used to distinguish between helper (T_h) CD4+ and cytotoxic (T_c) CD8+ T cells. Sample acquisition was performed using FACSCanto® (BD Biosciences) and data were analysed using FlowJo® software (FlowJo, LLC Ltd, Ashland, OR, USA).

2.4 | Cytokine detection

Preparation and measurement of samples were performed using a LEGENDplex™ kit (BioLegend, Inc) according to the manufacturer’s guidelines, with overnight incubation of twice-diluted serum samples with beads specific for following proteins: monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, IL-8, IL-10, IL-18, and IL-33.
Acquisition of samples was done on FACS Canto (BD Biosciences) and data were analysed using LEGENDplex™ software.

2.5 | Statistical analysis

Prism® (GraphPad Software, LLC, La Jolla, CA, USA) software was used for statistical analysis. Data were tested for normal distribution and statistical tests were applied as appropriate. Error bars are represented by SD. Statistical tests used are specified in the figure legends. The level of statistical significance was determined: *(P < 0.05), **(P < 0.01), and ****(P < 0.001). Correlation analysis was performed using Spearman’s rank correlation coefficient and visualization was done in R studio by corrplot package. AUC of ROC curves were calculated using Prism® software.

3 | RESULTS

3.1 | Demographic and clinical characteristics of patients

We enrolled a total of 41 patients (all Caucasians), 33 of whom survived longer than five days after admission. The characteristics of the cohort as a whole and by group are shown in Table 1, Figure S1. The mean age of patients was 71.3 years (range 49-89 years) and mean SOFA score of enrolled patients was 11.46 indicating septic shock. The most frequent comorbidities include ischaemic heart disease, obesity, ischaemic disease of the lower limbs, obesity and diabetes mellitus. The most common cause of septic shock in the cohort, as a whole was pneumonia (n = 17), where all patients survived past day five. Among those who died before day five, the most common origin of septic shock was infection of the urinary tract. The most frequently detected microbial agents were Staphylococcus Aureus, E Coli, Enterococcus faecalis and Enterobacter Cloacae. In 13 patients the primary aetiological agent was not identified.

### Table 1 Demographic and clinical characteristics of patients with septic shock

| Characteristic                     | Total | D5+ Survivors | Early deceased | P value |
|------------------------------------|-------|---------------|----------------|---------|
| Recruited patients                 | 41    | 33 (80.5%)    | 8 (19.5%)      | —       |
| Gender                             |       |               |                |         |
| Female                             | 17 (41.5%) | 14 (82.4%) | 3 (17.6%)     | —       |
| Male                               | 24 (58.5%) | 19 (79.2%) | 5 (20.8%)     | —       |
| Age, mean (range)                  | 71.3 (49-89) | 70.6 (49-89) | 74.1 (66-85) | .3850   |
| Comorbidities, mean                | 2.23 | 2.22          | 2.25           | .8253   |
| BMI, mean                          | 27.80 | 27.48         | 29.12          | .3699   |
| SOFA, mean                         | 11.46 | 10.82         | 14.13          | .0360   |
| CRP mg/l, mean                     | 224.32| 213.38        | 268.06         | .5388   |
| Lactate mmol/l, mean               | 2.15  | 1.72          | 3.90           | .0045   |
| Origin of septic shock             |       |               |                |         |
| Pneumonia                          | 17    | 17 (100%)     | 0 (0%)         | —       |
| Abdominal infection                | 7     | 5 (71.4%)     | 2 (28.6%)      | —       |
| Urosepsis                          | 6     | 3 (50%)       | 3 (50%)        | —       |
| Soft tissue infection              | 5     | 3 (60%)       | 2 (40%)        | —       |
| Mediastinitis                      | 3     | 3 (100%)      | 0 (0%)         | —       |
| Other                              | 3     | 1 (33.3%)     | 2 (66.7%)      | —       |

Abbreviations: BMI, body mass index; CRP, c-reactive protein; SOFA, sequential organ failure assessment.
cells in all monocyte subsets. Early deceased patients had significantly higher frequency of HLA-DR$_{\text{low,neg}}$ CD86$_{\text{low,neg}}$ cells in classical (P < 0.001) and intermediate (P < 0.05) monocyte subset in comparison to D5+ survivors. Furthermore, the correlation between monocyte activation assessed as surface expression of CD86 and HLA-DR and SOFA score was evaluated, revealing close to significant negative correlation (P = 0.069, P = 0.055 respectively; Figure 1G, H). The increased presence of HLA-DR$_{\text{low,neg}}$ CD86$_{\text{low,neg}}$ cells in classical monocyte subset revealed significant correlation with sepsis severity represented by SOFA score (P = 0.024) (Figure 1I).

### 3.3 Cytokine production and correlation between monocyte characteristics

Differences in cytokine production between survivors and early deceased patients were analysed. The production of MCP-1, IL-6, IL-8, IL-10, and IL-18 was significantly higher in early deceased patients than in those who survived to day 5 (Figure 2). Correlation analysis performed to determine the relationships between measured characteristics including T cells (Figure 3) are summarized in Figure 4A, values of correlations are presented in Figure S2. The reduced frequency of classical monocytes negatively correlated (P < 0.001) with the increased frequency of intermediate and non-classical monocytes. Reduced surface expression of activation markers—HLA-DR and CD86 showed positive correlation related to each other in classical, intermediate and non-classical monocytes. The decreased frequency of classical monocytes negatively correlated with the increased production of MCP-1 (P < 0.01), IL-6 (P < 0.01) and IL-8 (P < 0.05). On the contrary, a positive correlation was observed between the elevated frequency of intermediate monocytes and the serum level of different cytokines—MCP-1 (P < 0.001), IL-6 (P < 0.01), IL-8 (P < 0.05), IL-10 (P < 0.01) and IL-18 (P < 0.01). Significant negative correlations were also observed.
between the decreased CD86 expression levels in all monocyte subsets with increasing levels of majority of measured cytokines. Intermediate and classical monocyte subsets showed significant potential to predict risk of early death (AUC 0.894 for intermediate subset and AUC 0.767 for classical monocyte subset). Similarly to monocyte subsets, analysed cytokines may predict short-term survival (Figure 4B).

### 3.4 T cell immunophenotype and correlation with cytokines levels

T cells were characterized by the presence of CD3, and subsequently, positivity on CD4 (T\(_h\)) and CD8 (T\(_c\)) was determined. The gating strategy is shown in Figure 3A.

Early deceased patients had significantly fewer CD4\(^+\) cells and significantly more CD8\(^+\) cells (\(P < 0.05\)) compared to patients who survived past day five (Figure 3B); the ratio of CD4\(^+\) and CD8\(^+\) populations was calculated (Figure 3D). No correlations between the frequency of CD4\(^+\) or CD8\(^+\) cells and the SOFA score were found (Figure 3D,E).

Correlation analysis was performed using individual parameters for immunophenotyping and cytokine production. The reduced frequency of CD4\(^+\) cells strongly negatively correlated with the increasing relative count of CD8\(^+\) cells (\(P < 0.001\)). The overall decreased frequency of CD4\(^+\) T cells was significantly associated with IL-10 production (\(P < 0.05\)) (Figure 4, Figure S2).

### 4 DISCUSSION

Using data from a cohort of 41 patients admitted to the ICU with septic shock, we investigated whether the frequency of different monocyte subsets and their activation status assessed within 12 hours of admission, is associated with a fulminant course of septic shock. In order to identify the immune-phenotype associated with fulminant course of septic shock, the studied cohort was retrospectively divided into two groups; one group contained patients with very short survival (within 5 days) and second was the group of patients, who survived this crucial 5-day period. As we mainly focused to analyse phenotype of the dynamically changing monocytes subsets, the 12 hours time-point of analysis provided an important advantage comparing to other reports focused to monocytes where authors used later sampling (1-5 days after admission to ICU).

The prompt risk stratification of patients with septic shock, is a key information for adjusting the care and later improvement of the therapeutics outcomes remain a key research focus. Many different experimental strategies, including single cell expression analysis, have been used to improve our understanding of the immune response in the early phase of sepsis and to identify new therapeutic targets.

Here we report shift in frequencies of classical and intermediate monocyte subset and strong negative correlation (\(P < 0.001\)) between these parameters. From this we speculate that the 'inflammatory' status of patients detected through frequency of intermediate monocytes reflects the septic shock severity, however the increased
frequency of intermediate monocytes does not correlate with increasing SOFA score. Mukherjee et al and Liepelt et al also reported the similar changes in frequency of monocyte subsets of septic patients. They showed frequency of intermediate and also non-classical monocytes, which was increased, whereas the frequency of classical monocyte subset was decreased in comparison to healthy controls.21,25 Unlike published studies21,25 comparing healthy volunteers, or critically ill non-septic patients with septic patients, we used a more appropriate way to assess markers of septic shock prognosis dividing the cohort of septic shock patients to survivors and early deceased patients. We showed that shift in monocyte subsets can serve as predictive marker of early survival first five days of septic shock. The increased variability in monocyte population was also used as predictive marker of sepsis severity.26 Greco et al showed that intensified variability in monocyte population was originated by increased presence of intermediate and non-classical monocytes.27

Interestingly, sepsis and septic shock affect not only monocyte subsets frequency, but also their activation status represented by surface expression of HLA-DR and CD86.28,29 We observed significantly decreased surface expression of CD86 on classical monocytes and non-significant decrease trend in intermediate and non-classical monocytes. The changes in CD86 expression were also showed by Washburn et al, who did RNAseq of monocytes from septic patients and observed decreased expression levels of CD86 gene.18 We also focused on HLA-DR surface expression levels. Our data showed a trend in decreased surface HLA-DR expression in early deceased patients in comparison to D5+ survivors. HLA-DR is well established predictive marker of sepsis seriousness,30,31 moreover, reduced amounts of HLA-DR result in functional impairment of antigen-driven immunity, which can lead to increased susceptibility to secondary infections,13,32-35 nevertheless, its use never reached routine diagnostics. According to the dynamics of HLA-DR and CD86 in sepsis, we determined the frequency of HLA-DRlow, neg CD86low, neg cells in all monocyte subsets and revealed a significantly increased frequency of HLA-DRlow, neg CD86low, neg in early deceased patients in comparison to patients, who survived day five. The population of HLA-DRlow, neg CD86low, neg cells across all monocyte subsets showed the correlations with various monocytic parameters and also with levels of produced cytokines. Furthermore, the increased frequency of HLA-DRlow, neg CD86low, neg showed significant correlation with severity of the septic shock.

Interestingly, it is well known that CD86, as a co-stimulatory molecule, has a key role in supporting the development of T cells, therefore we also analysed basic changes in T cells. Considering our search for dynamically changing parameters among monocytes we also assessed some basic information from T cells immune-phenotype, impaired T cells functions, exhaustion and apoptosis are hallmarks of sepsis.18 Although usually these changes are reported in later time-points of septic shock and also can persist after patient recovery.36 Our data demonstrate the differences in CD4+ and
The CD8⁺ frequency occurred in both observed patient groups with septic shock at the time of their admission to ICU. The differences in CD4⁺ and CD8⁺ cell frequency to reduced amount CD4⁺ cells, probably indicates higher susceptibility to fulminant septic shock. Xia et al reported the decreased frequency of CD4⁺ cells and also CD4⁺/CD8⁺ ratio inclined to increased abundance of CD8⁺ cells, is associated with higher risk of development the postoperative sepsis in HIV-infected patients. Hotchkiss et al also observed, the depletion of CD4⁺ T cells induced by sepsis in non-surviving patients. This phenomenon is highlighted by strong inverse correlation between the frequency of CD4⁺ and CD8⁺ T cells in our data. Furthermore, we also speculate, whether the significant changes in the surface levels of the key T cell co-stimulatory molecule—CD86 observed on monocytes can eventually affect the pool of T cells. Others reported that decreased surface localization of CD86 can be associated with exhaustion and increased apoptosis of T cells.

Sepsis and septic shock are associated with increased production of pro- and anti-inflammatory mediators, also known as cytokine storm. The promising results of earlier research on systemic markers of the inflammatory response, such as levels of cytokines originating from immune cells, have not been fully translated to clinical use eventually due to broad pleiotropic effects and diverse impact on immune cell populations in different timeframe of sepsis.

The changes in proportion from classical to intermediate and non-classical monocyte subsets, are also associated with increased levels of produced pro-inflammatory cytokines. We correlated changes in monocyte subsets frequency together with secreted cytokines and evaluated their possible crosstalk. The reduced frequency of CD8⁺ cells highly negatively correlate with increased frequency of CD8⁺ cells (P < 0.001). Visualization was done in R studio by corrplot package. * (P < 0.05), ** (P < 0.01), *** (P < 0.001). B, ROC curve analysis was performed to evaluate the potential to predict five-day survival, monocyte subsets showed significant predictive potential. AUC, area under curve; GMF, geometric mean of fluorescence; SOFA, sequential organ failure assessment.
Our findings clearly highlight the possible predictive roles of monocyte subsets and their activation markers in short-term septic shock survival. Furthermore, we suggest an important crosstalk of changes in monocytes with changes in T cell compartment, possibly explained through the cytokine levels changes or CD86 expression. However, validation of our data will be required as our study had several limitations including size (41 patients) recruited from a single centre; the average age of patients was 71.3 years, which limits how our findings can be generalized to other age groups.

Even though we analysed data from cohort with a very broad clinical sources of septic shock including different types of bacterial agents, our results demonstrate that monocyte subset frequency can serve as predictive marker of septic shock survival independent on stimuli, which induced the septic shock. Further analysis in a larger cohort will allow us to determine whether the observed differences in monocyte subsets can be used to predict patient outcome and be used to risk stratify patients and detect those with excessive inflammatory response who may profit from immunomodulatory therapy.

Altogether, we demonstrated that proportional changes in monocyte subsets and their correlation with increased cytokine production and decreased status of activation of these cells, can serve as predictive marker of septic shock progression (Figure 5). Future research should deeply focus on the dynamics of monocytes subsets in progression of septic shock. We have identified markers that have the potential to be used in prognostic tests; the sample may be taken and analysed at the time of admission, and parameters correlating with survival quantified within hours of sample collection. This approach may help to apply more tailored immunotherapy of patients in septic shock.
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