FACULDADE DE MEDICINA
UNIVERSIDADE D
COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

MARIANA NUNES GONÇALVES AFONSO AMARAL

Immunoparalysis in critically ill children

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE PEDIATRIA

Trabalho realizado sob a orientação de:
DOUTORA ANDREA SOFIA DA SILVA DIAS
PROFESSORA DOUTORA GUIOMAR GONÇALVES DE OLIVEIRA

ABRIL/2021

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IMMUNOPARALYSIS IN CRITICALLY ILL CHILDREN

Mariana Nunes Gonçalves Afonso Amaral[1]; Andrea Sofia da Silva Dias[2,3], MD;
Guiomar Gonçalves de Oliveira[3,4], MD, PhD

1- Faculdade de Medicina da Universidade de Coimbra
2- Serviço de Cuidados Intensivos Pediátricos, Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra
3- Clínica Universitária de Pediatria, Faculdade de Medicina, Universidade de Coimbra
4- Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica, Hospital Pediátrico de Coimbra, Centro Hospitalar e Universitário de Coimbra

Mariana Nunes Gonçalves Afonso Amaral
Rua do Miradouro, nº54, r/c B, 3045-092 Coimbra
marianaamaral97@gmail.com

ABBREVIATIONS

PICU: paediatric intensive care unit

HLA-DR: human leukocyte antigen DR

MFI: mean fluorescence intensity

LPS: lipopolysaccharide

IFNγ: interferon-gamma

TNF-α: tumour necrosis factor alpha

IL-6: interleukin-6

PELOD-2: Paediatric Logistic Organ Dysfunction-2

IQR: interquartile range

AUC: area under the curve

SIRS: systemic inflammatory response syndrome

CARS: compensatory anti-inflammatory response syndrome

MODS: multiple organ dysfunction syndrome

PIM3: Paediatric Index of Mortality 3

IMV: invasive mechanical ventilation

VIS: vasoactive inotropic score

VFD: ventilator-free days

PB: peripheral blood

EDTA: ethylenediaminetetraacetic acid

HMR: high mortality risk

LMR: low mortality risk

ROC: receiver operating characteristic

PIMS-TS: paediatric multisystem inflammatory syndrome temporally associated with COVID-19

GI: gastro-intestinal

PAD: post admission day

BAL: bronchoalveolar lavage

BC: blood culture

UC: urine culture

MR: multiresistant

CPE: carbapenemase-producing *Enterobacteriaceae*

PCT: procalcitonin

CPR: C-reactive protein

WBC: white blood cells

GM-CSF: granulocyte-macrophage colony-stimulating factor

ABSTRACT

Introduction: Immunoparalysis is associated with poorer outcomes in the paediatric intensive care unit (PICU) setting. Downregulation of human leukocyte antigen (HLA)-DR and reduced cytokine production have been used to characterize it. We aimed to determine the group of patients with higher chances of immunoparalysis and correlate this status with increased risks of nosocomial infection and adverse clinical parameters.

Methods: We conducted an exploratory study including PICU patients with multiple organ dysfunction, over a period of six months. Monocyte HLA-DR expression (determined by the mean fluorescence intensity – MFI) and the frequency of monocytes producing intracellular cytokines (TNF-α and IL-6) after in vitro activation with LPS and IFNγ were measured by flow-cytometry at three distinct time points (T1=1-2 days; T2=3-5 days; T3=6-8 days) following PICU admission. Using the Paediatric Logistic Organ Dysfunction (PELOD)-2 score to assess initial disease severity, we established the optimal cut-off values of the evaluated parameters to identify the subset of patients with a higher probability of suffering from immunoparalysis. A comparative analysis based on demographic and clinical parameters was performed between them.

Results: Fifteen patients, 60.0% males, with a median age of 4.1 years were included. Considering the presence of two criteria in T1 (classical monocytes MFI for HLA-DR ≤1758.5, AUC 0.775; and frequency of monocytes producing IL6 ≤68.5%, AUC 0.905) or two criteria in T3 (classical monocytes MFI of HLA-DR ≤2587.5, AUC 0.675; and frequency of monocytes producing TNF-α ≤93.5%, AUC 0.833), a variable to define immunoparalysis was obtained (100% sensitivity, 81.5% specificity). Forty per cent of patients were assigned to the immunoparalysis group. In the immunoparalysis group, a higher frequency of nosocomial infection (p=0.011), a higher median vasoactive inotropic score (p=0.014) and a higher median length of hospital stay (p=0.036) was observed compared to the no immunoparalysis group. In the subgroup with the diagnosis of sepsis/septic shock (n=5), patients showed higher percentages of non-classical monocytes (p=0.004). No mortality was recorded.

Discussion: A reduction in classical monocytes HLA-DR expression, combined with lower frequencies of monocytes producing TNF-α and IL-6 at both early and later stages of critical illness appears to be a good marker of immunoparalysis and is associated with worse outcomes. On the other hand, increased frequency of non-classical monocytes in patients with sepsis/septic shock is suggestive of a better prognosis.

Conclusion: Immunoparalysis seems to be defined by low levels of monocytes HLA-DR expression and low frequencies of monocytes producing cytokines during the first week of critical illness and these findings relate to an increased risk of nosocomial infection and deleterious outcomes.

KEY WORDS

Immunoparalysis; Nosocomial Infection; MODS; Monocyte; Paediatric Intensive Care Unit

INTRODUCTION

Children are often exposed to a variety of inflammatory challenges. Some can result in critical illness and pose a real threat to their lives, requiring paediatric intensive care unit (PICU) support. In the acute phase of critical illness, the immune system plays a major role in the successful containment of these challenges.

The typical initial response of the host's immune system to critical illness consists of a proinflammatory surge that manifests clinically as an overwhelming systemic inflammatory response syndrome (SIRS).[1,2] This syndrome is mainly characterized by the systemic release of proinflammatory mediators that manifest as changes in vital signs and analytic parameters.[3] The degree of the proinflammatory stimulus has long been associated with increased risks for poor outcomes, with higher levels of proinflammatory cytokines being linked to multiple organ failure and overall mortality.[4]

In order to maintain homeostasis, a compensatory anti-inflammatory response syndrome (CARS) develops and seeks to limit the damage of the early dominant inflammatory phase. Thus, CARS serves as a counter-regulatory mechanism and often occurs concurrently with the proinflammatory insult.[1,5] If severe and persistent, however, the CARS response becomes pathological. In this setting, it represents an important form of acquired immune deficiency and is termed immunoparalysis.[6]

Strong evidence from adult and paediatric populations suggests that immunoparalysis itself is a predictor of morbidity and mortality,[7] as it results in ineffective clearance of a primary infection and renders the patient susceptible towards secondary infections and persistent organ failure.[2,8] Therefore, a proper balance between the often competing pro- and anti-inflammatory responses is a determining factor in patients' fate. Nonetheless, substantial critical illness-induced changes in both innate (e.g. monocyte) and adaptive (e.g. lymphocyte) immune systems can be observed in a majority of PICU patients.

The development of a markedly impaired immune function has been frequently described in the aftermath of multiple diagnostic groups, including sepsis,[9,10] multiple organ dysfunction syndrome (MODS),[11,12] trauma,[13,14] critical viral infections,[15] acute pancreatitis,[16] cardiac arrest,[17] and following cardiopulmonary bypass[18,19] and transplantation[20] in children.

From a clinical perspective, there are no specific signs or symptoms associated with a pathological state of immunosuppression.[5] Furthermore, it is not a static condition, and both magnitude and nature of the underlying immune defects differ considerably within the same individual over time.[21] Consequently, in order to prospectively identify patients at high risk for

adverse outcomes, assessment of the patient's immune status is essential and requires immune monitoring strategies.

Monocytes are key drivers of the acute immune response. These cells have the ability to display antigens on their surfaces within class II major histocompatibility complex (MHC) molecules, such as human leukocyte antigen (HLA)-DR, and subsequently activate the adaptive arm of the immune system. Circulating monocytes, which normally express HLA-DR very strongly on their surface, internalize their HLA-DR molecules as part of the CARS response.[2]

To date, flow-cytometric quantification of expression of the monocyte HLA-DR (mHLA-DR) on the cell surface has been used as standardized biomarker of the innate immune function, with persistently low levels being proposed as a reflection of monocyte unresponsiveness. A considerable body of literature has shown that a mHLA-DR expression level of < 30% is independently associated with both an increased risk of developing nosocomial infection and a higher mortality.[12,22,23] A recent study conducted in septic children reported that failure to increase mHLA-DR expression by at least 1,000 molecules per cell over the first week after onset of sepsis was associated with mortality, suggesting that change in expression over time may be more significant than absolute thresholds.[9]

In a state of health, proinflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 should be produced robustly when monocytes are stimulated ex vivo with lipopolysaccharide (LPS). Indeed, whole blood ex-vivo LPS-induced TNF-α production capacity is another biomarker of monocyte function frequently used in critical illness. A marked reduction in the TNF-α response has also been associated with the development of secondary infection, prolonged organ dysfunction and death.[10,12]

Early identification of the state of immunoparalysis is particularly relevant, since it is becoming increasingly clear that the attenuated immune function can be reversed. Furthermore, reconstitution of immunocompetence is now a promising therapeutic goal, as several immunostimulatory strategies are emerging.[24]

In the current study, we attempted to determine the group of patients at major risk of immunoparalysis among critically ill children admitted to a paediatric intensive care unit (PICU), based on cut-off determination of the evaluated immune function parameters. Secondarily, we aimed to establish the association between the state of immunoparalysis and an increased risk of nosocomial infection, and its correlation with clinical parameters associated with deleterious outcomes in this population.

METHODS

Study design and population

We conducted an exploratory study with prospective data collection of eligible patients admitted to the PICU over a period of six months, from August 1st 2020 to January 31st 2021.

The PICU at Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra (CHUC, EPE) is an academic medical-surgical ICU, with nearly 400 annual admissions. Median Paediatric Index of Mortality 3 (PIM3) was 5.6%, with a mortality rate of 5.9% and a nosocomial infection rate of 3.4%.

The study population consisted of patients older than 28 days and younger than 18 years of age with a diagnosis of sepsis, multiple organ dysfunction syndrome, acute pancreatitis, or after multiple trauma, transplantation and cardiopulmonary bypass. Considering the investigation's focus on the evolution of biomarkers' time course, all patients included had at least two blood sample collections, at the predefined timepoints.

A written informed consent was obtained from the legal guardians of all patients who fulfilled eligibility criteria. The study was approved by the Ethics Committee of Centro Hospitalar e Universitário de Coimbra (CHUC-124-20).

Data collection

During patient follow-up, demographic and clinical data were abstracted, and a restricted access hospital electronic database was used for the purpose. The data collection comprised age, gender, comorbidities, length of PICU and hospital stay, diagnosis on admission, laboratory results (complete blood cell count and biochemical markers), vital signs, infection status (timing and type of infection), need for invasive mechanical ventilation (IMV), use of vasoactive drugs, destination after discharge and outcome at 28 days.

Nosocomial infection was defined according to Centers for Disease Control (CDC) criteria[25] as a new bacterial or fungal infection occurring at least 48 hours after PICU admission.

Two clinical scores were recorded: initial severity was assessed by the PIM3[26] at PICU admission; the Paediatric Logistic Organ Dysfunction-2 (PELOD-2)[27] was calculated at PICU admission and for each study day.

The vasoactive inotropic score (VIS), calculated using the combined doses of inotropic and vasopressor infusions, was used as a surrogate measure of cardiovascular support.[28]

Ventilator-free days (VFD) were calculated according to the following rules: zero was attributed to surviving patients ventilated for ≥ 28 days; 28 was considered in patients free of ventilation for ≥ 28 days; non-surviving patients were assigned 0 or a score corresponding to the number of days free from ventilation between admission and death (if the period of unassisted breathing lasted ≥48 consecutive hours); 28 minus the total days of ventilation was calculated in the remainder; a ventilator day was defined as any amount of invasive mechanical ventilation on a given calendar day.[29]

Peripheral blood (PB) samples were collected from residual blood after completion of routine follow-up performed in the PICU. An initial day 1 to 2 (T1) blood sample was collected after PICU admission and upon informed consent. Additional blood samples were collected on days 3 to 5 (T2) and 6 to 8 (T3).

Phenotypic characterization of peripheral blood cells by flow cytometry

For the identification, quantification and phenotypic characterization of peripheral blood (PB) monocyte subpopulations, 100 µL of PB, collected in ethylenediaminetetraacetic acid (EDTA), were stained with the monoclonal antibody (mAb) panel described in **Annex I**, **Table 1** (tube 1), using a lyse and wash procedure. After the mAbs incubated for 10 min in the dark at room temperature (RT), 2 ml of FACSLysing solution (Becton Dickinson Biosciences (BD), San Jose, CA, USA) were added and after 10 min of incubation in the dark at RT, the samples were concentrated and washed in 1 ml of phosphate buffered saline (PBS, Gibco, Life Technologies, Paisley,UK). The cell pellet was resuspended in 500 µl of PBS and immediately acquired in a FACSCanto™ II flow cytometer (BD).

Cytokine production assessment by flow cytometry

To study cytokine production by monocytes, we used PB collected in heparin. PB cells were stimulated in vitro with LPS and interferon-gamma (IFNγ). To this end, LPS (100 ng/mL, Sigma-Aldrich, St. Louis, MO), IFNγ (100 U/ml, Promega, Madison), and Brefeldin-A (10 µg/mL, Sigma-Aldrich) were added to 500 µL of PB sample diluted 1:1 (v/v) in RPMI 1640 complete culture medium (Invitrogen, Life Technologies, Carlsbad, CA). All samples were then incubated in a 5% CO_2 humid atmosphere at 37ºC, for 6 hours. For all samples, a tube was included without stimulating agents in order to evaluate the basal production of TNF-α.

Each cultured sample was aliquoted (200 µL) into 2 tubes and stained with CD11c, CD33, CD14, HLA-DR and CD45. Cells were incubated for 15 min in the dark at RT, and then washed. For intracellular staining, Fix&Perm (GAS002, Life Technologies, Frederick) reagent was used in parallel with the mAbs, and according to the manufacturer's instructions, to stain cytokines at cytoplasmic level, as described in **Annex I**, **Table 1** (tube 2 and 3). After washing, cells were resuspended in 500 µl of PBS and immediately acquired in a FACSCanto™ II flow cytometer (BD).

Immunophenotypic identification of monocyte subpopulations

The gating strategy used to identify PB monocyte subpopulations was previously described by Laranjeira et al.,[30] and is illustrated in the two upper panels of **Annex I**, **Figure 1A**. In short, classical monocytes were identified based on their high expression of CD14 in the absence of CD16, together with high expression of CD33; intermediate monocytes also express high levels of CD14, but display an increasing expression of CD16, and a slight decrease in the expression of CD33 compared to classical monocytes. In turn, non-classical monocytes are positive for CD16 and display a decreasing expression of CD14; they also present the highest expression of CD45 among the three monocyte subpopulations, along with the lowest expression of CD33.

Evaluation of the HLA-DR and cytokines' expression by flow cytometry

We used flow cytometry to assess the expression of the MHC Class II molecule HLA-DR in monocytes, as a whole and within each individual monocyte subpopulation. HLA-DR expression was evaluated by the mean fluorescence intensity (MFI), which corresponds to a measure of the relative amount of protein.

Concerning TNF-α and IL-6, we evaluated the percentage of monocytes producing these cytokines, together with the expression levels (measured as MFI) in the whole monocyte population, and in classical and non-classical monocytes.

Data analysis was performed in Infinicyt™ 1.7 software (Cytognos SL, Salamanca, Spain).

Flow-cytometric analysis was conducted at Unidade de Gestão Operacional de Citometria, CHUC, EPE.

Statistical analysis

Demographic and clinical variables were analysed. For qualitative variables, such as female to male ratio and frequency of infection, Fisher's exact test was applied; the Mann-Whitney U test was used to compare quantitative variables.

Categorical variables are described as frequencies and percentages, and numerical variables as medians and interquartile ranges, for variables with non-normal distribution.

All reported *p* values were two-tailed and statistically significant differences were considered when $p < 0.05$. Data was analysed using SPSS® (Statistical Package for the Social Sciences) 26.0 software.

An initial PELOD-2 score cut-off value was established and patients were assigned to two groups, according to their organ dysfunction severity: "low mortality risk" – LMR (PELOD-2 score < 10) and "high mortality risk" – HMR (PELOD-2 score ≥ 10). It was hypothesized that the state of immunoparalysis would be more frequent amongst patients with higher organ dysfunction score.

The area under the curve (AUC) from a receiver operating characteristic (ROC) curve was analysed to determine the ability of the PELOD-2 score to predict organ dysfunction severity. The Youden index was used to calculate optimal cut-off values (i.e., maximized sensitivity and specificity) for HLA-DR expression, intracellular TNF-α and IL-6 production by classical monocytes at each study time point (T1, T2 and T3).

To define the immunoparalysis state, we established that two criteria at T1 or two criteria at T3 were required and we obtained a sensitivity of 100% and a specificity of 81.8% for this variable.

As a result, we found a subset of patients with increased chances of immunoparalysis – low HLA-DR expression and low IL-6 production at T1 or low HLA-DR expression and low TNF-α production at T3, by classical monocytes – and divided our sample into two groups: "Immunoparalysis" and "No Immunoparalysis".

A comparative analysis based on demographic and clinical parameters was performed between them.

RESULTS

Fifteen patients were included in this preliminary exploratory study. Demographic and clinical characteristics of the sample are summarized in **Table 1**. The median age was 4.1 years [IQR 1.25-13.75] and 60.0% were males. The most frequent diagnosis on admission was sepsis/septic shock (33.3%). The median PIM3 score was 4.53 [IQR 1.3-6.7] and median PELOD-2 score at admission was 9 [IQR 8-10]. About two-thirds of the patients (60.0%) had significant associated comorbidities. Median PICU and total length of hospital stay were six and 35 days, correspondingly. Vasoactive drugs were needed in the majority (86.7%) of patients and 60.0% of them underwent invasive mechanical ventilation (IMV). Six patients (40.0%) developed nosocomial infection. There was no mortality within our sample.

Table 1: Demographic and clinical data of patients included in the study

	Median or n	IQR or %
Age at admission (years)	4.1	[1.3-6.7]
Male gender	9	60
Admission diagnosis group		
Sepsis/Septic shock	5	33.3
Trauma	4	26.7
Post-transplantation	3	20
Post cardiopulmonary bypass	1	6.7
Sudden cardiac arrest	1	6.7
PIMS-TS	1	6.7
Comorbidities		
Active cancer disease	4	26.7
Syndrome / Malformation	3	20
Chronic GI disease	1	6.7
Chronic endocrine disease	1	6.7
No comorbidities	6	40
PIM3 score	4.63	[1.3-6.7]
PELOD-2 score	9	[8-10]
IMV	9	60
VFD (days)	26	[22-28]
Vasoactive drugs	13	86.7
VIS	11	[8.39-22.11]
Length of treatment with vasoactive drugs (days)	2	[1-4.5]
Length of PICU stay (days)	6	[3.5-13.5]
Length of hospital stay (days)	35	[20.5-52.5]
Nosocomial infection	6	40

IQR interquartile range, *PIMS-TS* paediatric multisystem inflammatory syndrome temporally associated with COVID-19; *GI* gastro-intestinal; *PIM3* paediatric index of mortality-3; *PELOD-2* paediatric logistic organ dysfunction-2; *IMV* invasive mechanical ventilation; *VFD* ventilator-free days; *VIS* vasoactive inotropic score; *PICU* paediatric intensive care unit.

Individual patient characteristics and infectious outcome of the 15 children enrolled are depicted in **Table 2**.

Table 2. Patients characteristics and infectious outcome

Subject	Age[1]	Admission diagnosis group	Immunoparalysis group	Nosocomial infection	Cultures and date (PAD)[2]
1	9.5	Post-transplantation	No	No	-
2	16.7	Sudden cardiac arrest	**Yes**	**Yes**	BAL, *Enterobacter aerogenes*, PAD #8
3	1.4	**Sepsis/Septic shock**	No	No	-
4	0.3	**Sepsis/Septic shock**	**Yes**	**Yes**	BC, *Staphylococcus lugdunensis*, PAD #19
5	2.5	**Sepsis/Septic shock**	No	No	-
6	10	Post-transplantation	**Yes**	**Yes**	UC, *MR Escherichia coli*, PAD #11
7	12.25	Trauma	**Yes**	**Yes**	BAL, *Staphylococcus aureus*, PAD #4
8	3.5	**Sepsis/Septic shock**	No	No	-
9	4.1	Trauma	No	No	-
10	17.2	Trauma	**Yes**	No	-
11	13.8	**Sepsis/Septic shock**	No	No	-
12	0.4	Post-transplantation	**Yes**	**Yes**	BC, CPE *Klebsiella pneumoniae*, PAD #10
13	1.25	PIMS-TS	No	No	-
14	1.2	Post cardiopulmonary bypass	No	No	-
15	17	Trauma	No	**Yes**	BAL, *Candida albicans*, PAD #4

[1]Age in years; [2]*PAD* post admission day number; *BAL* bronchoalveolar lavage; BC blood culture; UC urine culture; *MR* multiresistant; *CPE* carbapenemase-producing *Enterobacteriaceae; PIMS-TS* paediatric multisystem inflammatory syndrome temporally associated with COVID-19.

Of the 15 patients included, 13 and 14 patients had blood samples to analyse on study days 3 to 5 (T2) and 6 to 8 (T3), respectively.

According to the initially established PELOD-2 score cut-off of 10, the HMR group was composed by 26.6% (n = 4) of patients (median PELOD-2 score of 10, [IQR 10-10.5]), while the LMR group consisted of 73.3% (n = 11) of patients (median PELOD-2 score of 8, [IQR 7.5-9]).

After analysis of the ROC curves **(Figure 1)**, we found that immunoparalysis was better predicted for study period T1 when classical monocytes HLA-DR expression was ≤ 1758.5 (MFI) (optimal sensitivity of 75.0% and specificity of 80.0%) with an AUC of 0.775 (p = 0.120); and the percentage of classical monocytes producing IL-6 was ≤ 68.5% (sensitivity of 100.0% and specificity of 86.0%), with an AUC of 0.905 (p = 0.053).

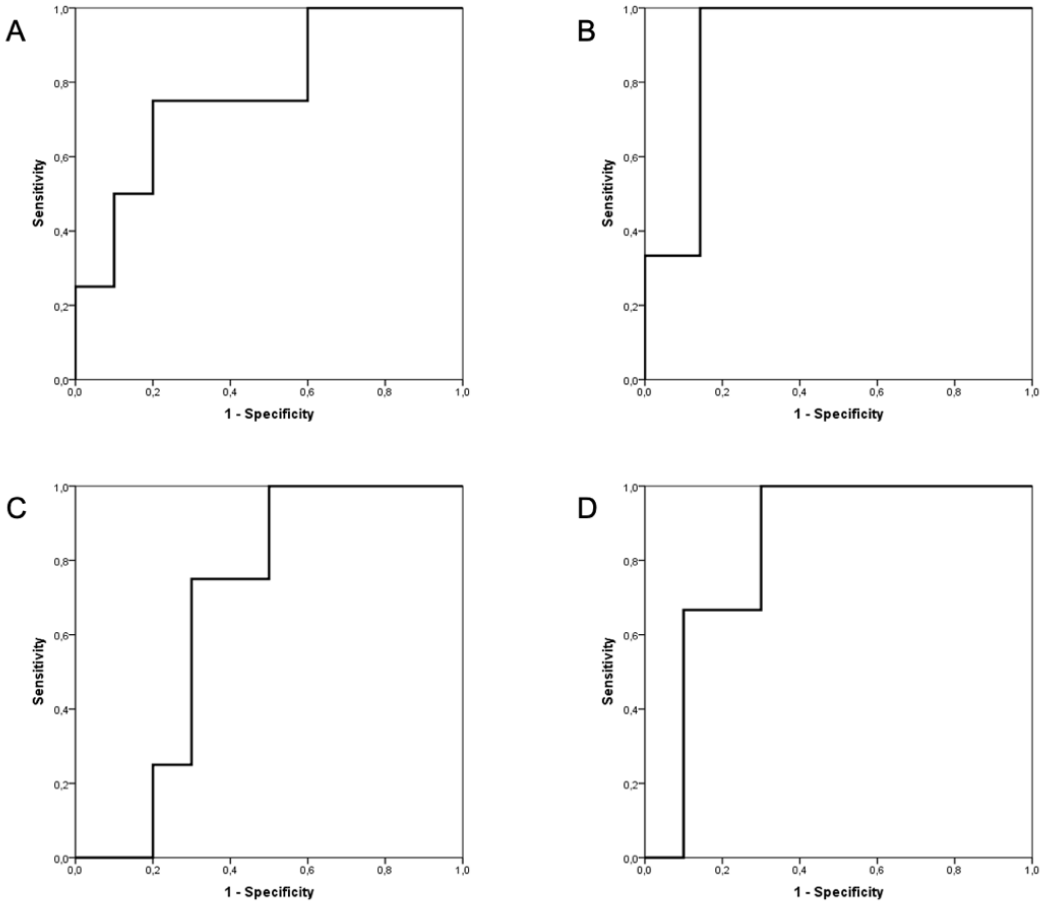
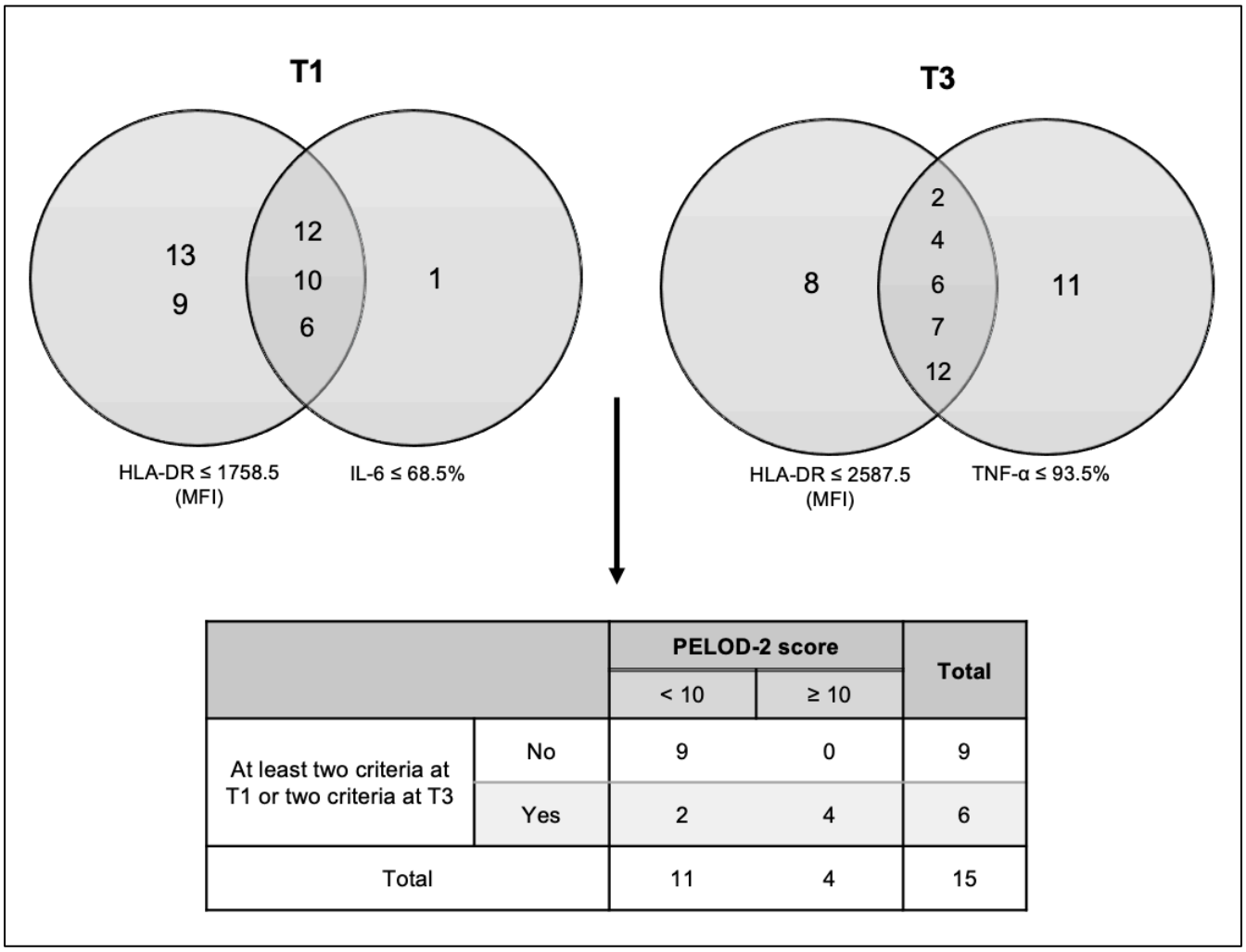


Figure 1. Receiver operating characteristic (ROC) curves for prediction of organ dysfunction severity. **A.** ROC curve of HLA-DR expression as mean fluorescence intensity (MFI) by classical monocytes at T1. The area under the curve (AUC) was 0.775 (p = 0.120); **B.** ROC curve of IL-6 production by classical monocytes at T1 as percentage (%) of positive cells. AUC was 0.905 (p = 0.053); **C.** ROC curve of HLA-DR expression as MFI by classical monocytes at T3. AUC was 0.675 (p = 0.322); **D.** ROC curve of TNF-α production by classical monocytes as % of positive cells. AUC was 0.833 (p = 0.091).

Similarly, for study period T3, immunoparalysis thresholds were best predicted when HLA-DR expression on classical monocytes was ≤ 2587.5 (MFI) (75.0% sensitivity and 70.0% specificity), with an AUC of 0.675 (p = 0.322); and percentage of classical monocytes

producing TNF-α was ≤ 93.5% (100.0% sensitivity and 70.0% specificity), with an AUC of 0.833 (p = 0.091). By contrast, exploration of the ROC curves at study period T2 revealed no sufficient AUC to determine an adequate cut-off.

Based on the calculated cut-offs that compose our variable, 40.0% (n = 6) of patients were included in the immunoparalysis group, while 60.0% (n = 9) were included in the no immunoparalysis group (**Figure 2**).



		PELOD-2 score		Total
		< 10	≥ 10	
At least two criteria at T1 or two criteria at T3	No	9	0	9
	Yes	2	4	6
Total		11	4	15

Figure 2. Variable used to define the study groups (100.0% sensitivity and 81.8% specificity). Each number inside the circles represents a subject described in Table 2.

In **Table 3**, demographic and clinical comparisons between the two study groups are presented.

Table 3. Demographic and clinical data of study groups

	No Immunoparalysis (n=9)		Immunoparalysis (n=6)		
	Median or n	**IQR or %**	**Median or n**	**IQR or %**	***p* value**
Age at admission (months)	42	[16-139.5]	133.5	[4.75-202.25]	0.689**
Male gender	5	55.6	1	16.7%	0.287#
PIM3 score at admission	4.63	[1.23-6.58]	4.18	[1.27-34.5]	0.776**
PELOD-2 score at admission	8	[6.5-9]	10	[8.75-10.5]	**0.018****
Length of PICU stay (days)	5	[2-8.5]	14	[4.75-37]	0.113**
Length of hospital stay (days)	27	[14-38]	52.5	[40.5-109.25]	**0.036****
VFD	228	[24-28]	17.5	[0-27.25	0.145
VIS	10	[5.67-11]	22.11	[17.25-40.75]	**0.014****
Length of treatment with vasoactive drugs (days)	2	[0.5-4.5]	2	[1-6.25]	0.607**
Nosocomial infection	1	11.1	5	83.3	**0.011#**
PCT T1 (ng/mL)	5.13	[0.81-43.5]	3.49	[1.74-13.35]	0.864**
CPR T1 (mg/dL)	11.08	[9.77-16.44]	12.05	[8.67-14.5]	0.188**
WBC T1 (10^9 cell/L)	7.1	[0.2-11.5]	6.45	[2.45-16.37]	0.776**
Neutrophils T1 (10^9 cell/L)	5.07	[0.02-8.29]	5.04	[2.01-12.96]	0.529**
Monocytes T1 (10^9 cell/L)	0.27	[0.01-0.52]	0.3	[0.04-1.38]	0.529**
Lymphocytes T1 (10^9 cell/L)	0.47	[0.16-1.97]	1.01	[0.4-1.94]	0.529**
Classical monocytes (%)					
T1	94	[68-98]	85.5	[61.75-89]	0.181**
T3	81.5	[77.25-91.5]	92.6	[73.25-97.75]	0.181**
Intermediate monocytes (%)					
T1	5.86	[2.4-16-5]	11	[5.88-12.25]	0.755**
T3	10.06	[6.27-17.5]	5.92	[1.95-9.55]	0.181**
Non-classical monocytes (%)					
T1	1.38	[0.3-10]	2.66	[0.5-20.51]	1**
T3	4.53	[3.15-9]	1.52	[0.47-17.19]	0.108**

IQR inter-quartile range; *PIM3* paediatric index of mortality-3; *PELOD-2* paediatric logistic organ dysfunction-2; *PICU* paediatric intensive care unit; *VFD* ventilator-free days; *VIS* vasoactive inotropic score; *PCT* procalcitonin; *CPR* C-reactive protein; WBC white blood cells; **Mann-Whitney U test, #Fisher's exact test.

The immunoparalysis and no immunoparalysis groups did not vary significantly on age (p = 0.689) and gender (p = 0.287). PIM3 score at admission showed no significant differences between groups (p = 0.776).

Although length of PICU stay among immunoparalysis patients tended to be longer (median 14, [IQR 4.75-37]) than those with no immunoparalysis (5, [IQR 2-8.5]), the difference between groups did not reach statistical significance (p = 0.113). However, the immunoparalysis group had a significantly higher median total length of hospital stay (52.5 [IQR 40.5-109.25] *vs*. 27 [IQR 14-38], p=0.036).

Patients in the immunoparalysis group scored a significantly higher median VIS: 22.11 [IQR3.17.25-40.75] *versus* 10 [IQR 5.67-11] in the no immunoparalysis group (*p* = 0.014). In contrast, no differences were observed between cohorts regarding length of treatment with vasoactive drugs (p = 0.607) or VFD (p = 0.145).

Both procalcitonin (PCT) and C-reactive protein (CPR) values at T1 revealed no significant differences between groups (p = 0.864 and p = 0.188, respectively). In a similar manner, statistical significance was not obtained when comparing complete white blood cell (WBC) count between the two groups (p = non-significant, all).

Overall, five patients (83.3%) in the immunoparalysis group developed nosocomial infection *versus* one patient (11.1%) in the no immunoparalysis group: frequency of nosocomial infection was more than seven times higher among patients with immunoparalysis (p = 0.011).

HLA-DR expression on classical monocytes and intracellular cytokine production levels over time are shown in **Figure 3**, along with the PELOD-2 score. In the immunoparalysis group, HLA-DR expression on classical monocytes was persistently lower. In a similar fashion, the frequency of classical monocytes producing TNF-α tended to be lower among patients with immunoparalysis, specially at T3. Regarding intracellular IL-6 production, median levels were visibly reduced at T1 in the immunoparalysis group, while the difference between the two groups was not so evident at T3. Median PELOD-2 score was higher at all moments within Immunoparalysis group patients.

Concerning monocytes subpopulations, no statistically significant disparities were identified between the two groups (**Table 3**). However, in a subsequent evaluation, we found that in the subgroup of patients with the admission diagnosis of sepsis/septic shock (n=5), at some time point during the first week after PICU admission, non-classical monocytes frequency was higher than 20.0% (4/5 (80.0%) *vs.* 0/10 (0.0%), p = 0.004, Fisher's exact test). This increased percentage of non-classical monocytes is illustrated in **Annex I**, **Figure 1C.** Median PELOD-2 score was lower in this subgroup of patients when compared with the remainder subjects (7.5 [IQR 6.25-8.75] *vs*. 9 [IQR 8-10], p = 0.078, Mann-Whitney U test).

Among this subgroup, only one patient belonged to the immunoparalysis group and that same patient was the only one to develop nosocomial infection (**Table 2**).

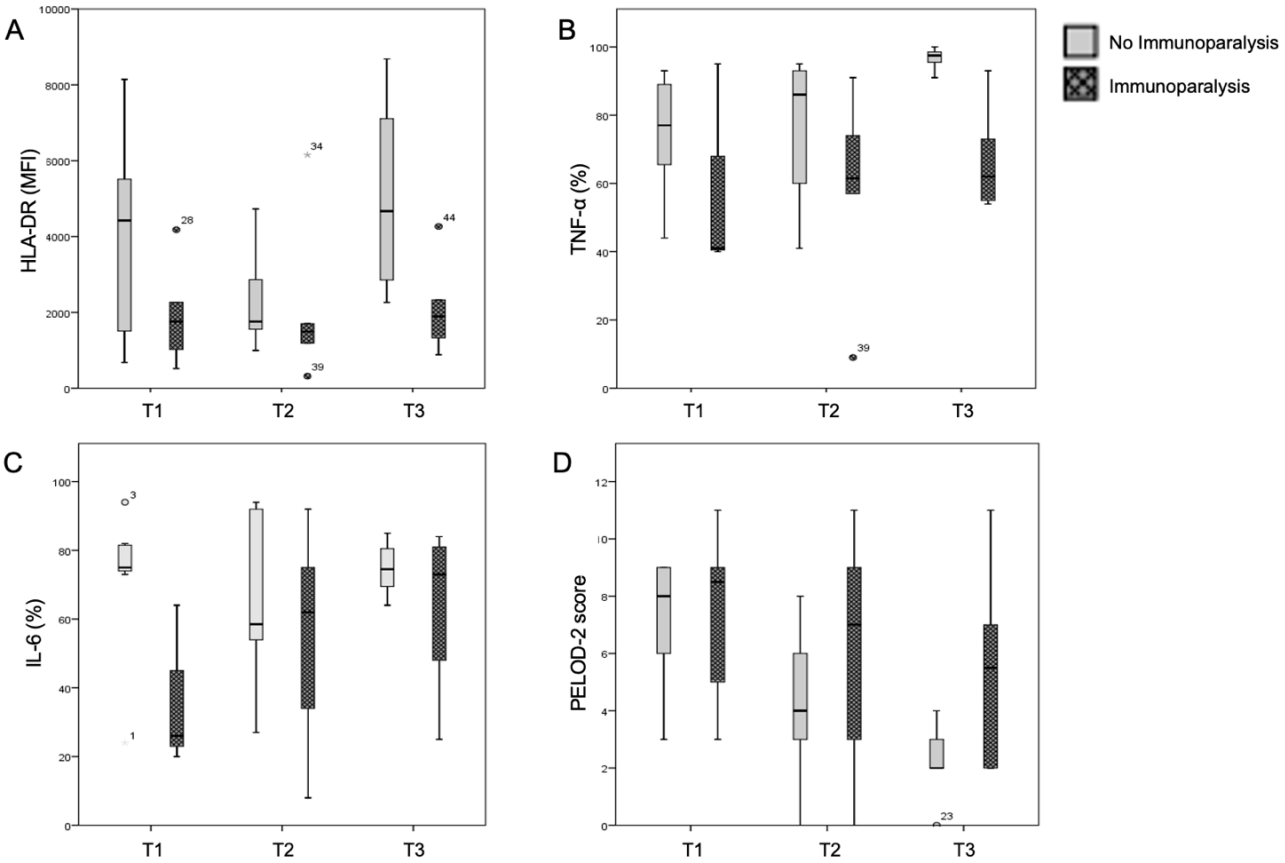


Figure 3. HLA-DR expression on classical monocytes, intracellular TNF-α and IL-6 production and PELOD-2 score in the two study groups at T1, T2 and T3. **A.** HLA-DR expression on classical monocytes, as mean fluorescence intensity (MFI); **B.** Percentage of classical monocytes producing TNF-α; **C.** Percentage of classical monocytes producing IL-6; **D.** PELOD-2 score; Data are presented in box plot analysis with the median lines, 25- and 75-percentile boxes, and 10- and 90-percentile error bars.

DISCUSSION

In the PICU setting, a wide range of diagnoses result in severe acute injury, involving activation of several cellular systems by the innate immune system and the overwhelming production of proinflammatory cytokines and other inflammatory mediators. While transient immunosuppression can be understood as counterbalancing an inflammatory overreaction, persistent immunoparalysis has been shown to increase susceptibility to secondary infection, which in turn can lead to multiple organ failure and death. Such consequences in the outcome of these patients have led to multiple intensive care unit studies over the years and biomarkers of innate immune suppression have been proposed.

However, differences in measurement methodologies and values studied (mainly expressed as 'percentages of positive monocytes' or 'mean fluorescence intensity'), which are generally specific for a given laboratory, defy interpretation and comparison of these findings. As a consequence, to date there exists no defined criteria and identification of immunoparalysis remains challenging, especially in the paediatric population where research is even scarcer.

Therefore, in the present study we evaluated monocyte function over the first week after PICU admission for the purpose of determining which of the evaluated biomarkers better defined a state of immunoparalysis in our sample and at what time.

In order to select the group of patients with greater chances of suffering from innate immune suppression, our sample was divided into two groups according to their initial severity of organ dysfunction, using the PELOD-2 score. Our selection criteria was based on the emerging understanding that immunoparalysis commonly occurs in children with severe organ dysfunction.[10,12]

In fact, throughout the entire study period, median PELOD-2 score values remained consistently higher in the subgroup of patients with immunoparalysis, which supports the idea that this initial severity score is a good criterion to discriminate patients who develop persistent organ dysfunctions at early phases of critical illness, as it was previously demonstrated.[31] This is particularly relevant if we consider the importance of the early identification of these patients in the interest of achieving prompt and lasting restoration of immune function.

Diminished mHLA-DR expression has been proposed as the most reliable marker and is used in most of the studies about ICU-acquired immune suppression.[7] On the other hand, the largest body of evidence in critically ill children, spanning multiple diagnostic groups, uses measurement of the capacity of whole blood cells to produce the proinflammatory cytokine TNF-α when stimulated ex vivo.[6,12,13,19] Even so, the optimal timing of these measurements has been unclear. Instead of being limited to a single monocyte function assessment, we

aimed to strengthen this study's ability to identify the state of immunoparalysis in our sample by determining the functional response of the innate immune system through measurement of multiple markers.

From our results, a combination of diminished HLA-DR expression on classical monocytes and reduced frequencies of monocytes producing IL-6 after in vitro stimulation, one to two days after admission to the PICU (T1), appears to be a good marker of immunoparalysis. The rapid activation of proinflammatory cytokines has a central role in the host defence. Since IL-6 plays a key part in the systemic inflammatory response, it makes sense that, as our findings suggest, low production capacity of this cytokine is present in this group of patients with immunoparalysis.

Conversely, other studies in the field reported elevations in plasma levels of IL-6 responses occurring along with innate immune suppression.[12,13,15] However, it must be noted that IL-6 is one of the most complex cytokines, since it is produced by immune and non-immune cells across multiple organ systems.[4] Hence, measuring its plasmatic levels may be misleading. Instead, assessment of intracellular IL-6 production capacity by classical monocytes is more likely to be representative of the innate immune function and seems to provide early discrimination of these patients.

Measurement of HLA-DR expression on classical monocytes and intracellular TNF-α production at study days six to eight (T3) also showed good potential to identify immunoparalysis patients. Monocyte deactivation is well characterized by the reduction of HLA-DR expression. In addition, it appears to be accompanied by changes in proinflammatory cytokine levels,[6] as was the case in our study. This is most likely indicative that the immune system was unable to recover after the peak of the compensatory anti-inflammatory response.

Interestingly, most of the patients that compose the immunoparalysis group were selected by criteria applied for this period of time (T3). Thus, we could hypothesize that low levels of HLA-DR expression and intracellular cytokines production by monocytes at later stages of onset of critical illness are highly characteristic of an immunosuppressive state. This is consistent with previous adult and paediatric trials that report persistence of immunoparalysis beyond day three to be a strong predictor of poor prognosis.[9,14,23,32]

Regarding patients' characteristics, there were no differences in age or gender, meaning that, in theory, our study provided a homogenous cohort of patients. No differences were found in the PIM3 score at admission between groups, suggesting that this may not be a good initial risk assessment score to predict worse clinical outcomes associated with a state of immunoparalysis, as has also been reported before.[9]

According to our findings, acute phase markers, such as CRP and PCT, and WBC counts appear to have no value for the identification of patients with immunoparalysis. For this reason, beyond use of routine biomarkers and leukocyte quantification, direct assessment of immune function seems necessary to better characterize the dynamic of the immune systems' status.

As expected, the immunoparalysis group had a higher median VIS, indicating that illness was more severe in patients with seriously impaired immune responses, which is also consistent with immunoparalysis. From another perspective, catecholamines have an immunomodulation effect, which is also a possible rationale for this correlation found. Additionally, increased severity of disease has also been demonstrated in this investigation through significant association between patients from the immunoparalysis group and a longer length of hospital stay.

More importantly, the results obtained in this study reinforce the growing evidence[12,13,19,23] that immunoparalysis is associated with adverse clinical outcomes, demonstrated in this study by an increased frequency of nosocomial infection. In spite of the high mortality risk inherent in this group of patients, there were no deaths among our sample.

Our data showed no difference in the percentage of monocytes subpopulations between the two groups; however, in the sepsis/septic shock subgroup, our findings are in line with previously published reports that described increased numbers of non-classical monocytes among septic patients.[33,34] Non-classical monocytes are more inflammatory phenotype cells with properties for antigen presentation. This monocyte subtype exhibits inflammatory characteristics on activation and is the primary producer of TNF-α. Against this background, the increased percentage of non-classical monocytes in sepsis/septic shock patients is a feasible explanation for the fact that the majority of these patients belonged to the no immunoparalysis group and developed no nosocomial infection, achieving better outcomes.

Mechanisms of immunoparalysis in paediatric critical care remain poorly understood. Apart from patient-related factors, it is likely that many of the therapies employed in the PICU have overt or unintended immunomodulatory properties. Post-transplant patients and those with active cancer disease necessarily receive exogenous immune suppressants and antineoplastic agents that worsen immune compromise. Moreover, many routinely used medications have immunosuppressive effects, such as opiates, catecholamines, antibiotics and steroids.[2]

Several therapies have the potential to restore innate immune function in this setting, of which granulocyte-macrophage colony-stimulating factor (GM-CSF) has the largest body of evidence to date.[12,35,36] Interferon-gamma (IFNγ) treatment is another attractive path for the future, as it also demonstrated clinical benefit to improve immune host defence in the presence

of immunosuppression.[36,37] These immunostimulatory strategies highlight the need for an early stratification of patients driven by prospective immune function testing.

A number of potential limitations can be pointed out in this study. First, given the single-centre design of this study and its preliminary character, the reduced size of the sample restricted its robustness. Despite this, we were able to show strong associations between immune function and outcome. Second, different times between sample collection and analysis might affect expression of the evaluated biomarkers. Third, longitudinal data was missing from 13.3% and 6.7% of patients at T2 and T3, respectively. Thus, missing data not only reduces statistical power, but could introduce a selection bias. Fourth, we cannot account for genetic or acquired (according to underlying disease and received treatment) heterogeneity of immune response across subjects. Lastly, the heterogeneity of the participants' diagnosis at admission suggests that the underlying mechanisms contributing to immunoparalysis are likely to be varied within this group, which might imply the need for further studies with more homogenized samples.

In conclusion, with a limited cohort of critically ill children, we were able to find a strong relationship between the state of immunoparalysis, defined by diminished mHLA-DR expression and lower frequencies of monocytes producing inflammatory cytokines during the first week after PICU admission, and severe multiorgan dysfunction, evidenced by a greater necessity for vasoactive drugs and longer length of hospital stay. Our study provided evidence that this group of patients had an increased risk of nosocomial infection. Nevertheless, we reported no mortality in this sample, either during PICU stay or total hospital stay. In addition, our data showed that increased frequencies of non-classical monocytes seem to determine a better prognosis.

Mainly, this study suggested possible thresholds for immunoparalysis, which is predictive of the development of nosocomial infection, in the first week following onset of critical illness in the paediatric population, allowing stratification of these patients into higher and lower risk groups. Ultimately, these results should motivate further investigation in subsequent larger cohorts. In light of the future possible immunological interventions, a greater understanding of the mechanisms underlying immunoparalysis, along with the development and implementation of prospective, standardized, immune-monitoring regimens capable of identifying patients at risk are needed to further improve outcomes in critically ill children.

ACKNOWLEDGMENTS

I would like to express my deep gratitude to Professor Artur Paiva, Doctor Paula Laranjeira and Doctor Sandra Silva from Unidade de Gestão Operacional de Citometria, Centro Hospitalar e Universitário de Coimbra, EPE without whom I would not have been able to conduct this research.

Special thanks should be given to Doctor Andrea Dias for sharing her insight and knowledge of the subject and for her professional guidance, encouragement and patience throughout this research.

Assistance provided by Doctor Margarida Marques from Serviço de Tecnologias e Sistemas de Informação, Centro Hospitalar e Universitário de Coimbra, EPE in the statistical data analysis was greatly appreciated.

My grateful thanks are also extended to Professor Guiomar Oliveira, for her valuable and constructive recommendations on this research.

Finally, I thank Doctor Filipa Neves for her support and encouragement at every stage of this project.

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ANNEX I

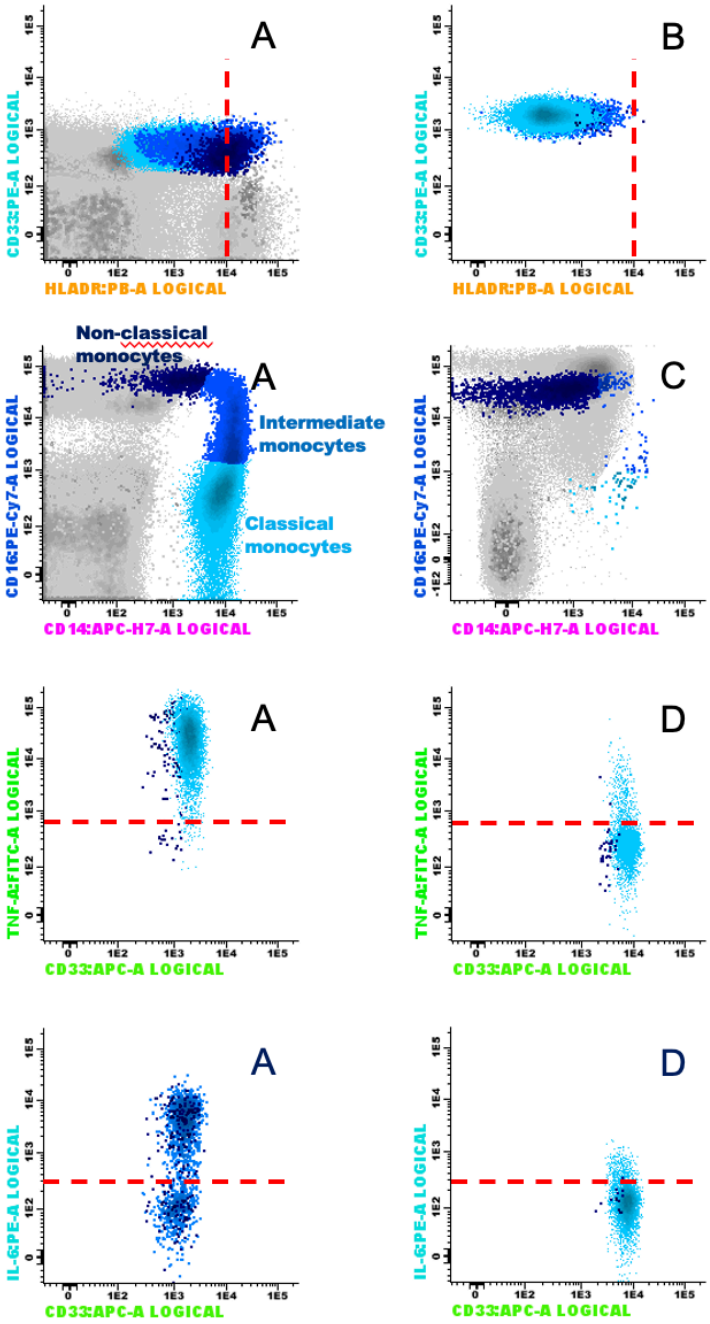

Figure 1. Dot plots histograms representing **(A)** a patient displaying normal phenotypic and functional features, concerning HLA-DR expression, distribution of peripheral blood monocyte subpopulations (classical, intermediate and non-classical monocytes), and TNF-α and IL-6 production by monocytes, upon LPS + IFNγ stimulation; **(B)** a patient whom monocytes display a decreased expression of HLA-DR; **(C)** a patient with an increased percentage of non-classical monocytes; and **(D)** a patient with a marked decreased expression of TNF-α and IL-6 by monocytes, after LPS + IFNγ stimulation. Light blue events represent classical monocytes, blue events represent intermediate monocytes, dark blue events correspond to non-classical monocytes, and the remaining peripheral blood cells are represented in grey.

Table 1. Panel of mAb reagents (with clones and commercial source) used for immune cells' phenotypic characterization

Tube	FITC	PE	PercP-Cy5.5	PE-Cy7	APC	APC-H7	PB	PO
1	CD11b	CD33	CD11c	CD16	CD300e	CD14	HLA-DR	CD45
2		IL-6	CD11c		CD33	CD14	HLA-DR	CD45
3	TNFα		CD11c		CD33	CD14	HLA-DR	CD45

mAb, monoclonal antibody; *PB*, pacific blue; *PO*, pacific orange; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP-Cy5.5*, peridinin chlorophyll protein-cyanine *5.5; PEC-y7*, phycoerythrin-cyanine 7; *APC*, allophycocyanin; *APC-H7*, allophycocyanin-hilite 7. Commercial sources: BD Pharmingen (San Diego, CA, USA); Beckman Coulter (Miami, FL, USA); BD (Becton Dickinson Biosciences, San Jose, CA, USA).