Answers to reviewers

Monocyte HLA-DR expression in septic shock patients:
Insights from a 20-year real-world cohort of 1023 Cases

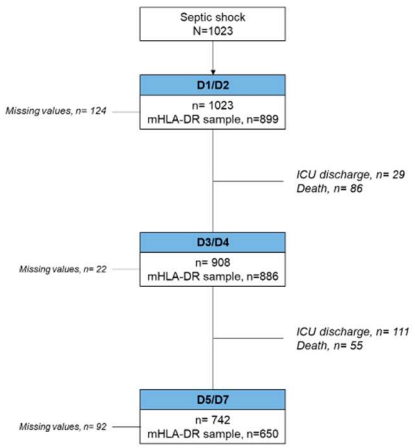
Reviewer #1:

With great interest I read the work of professor Monneret et al., who build upon previous works regarding the association between HLA-DR expression on monocytes (mHLA-DR) and adverse outcomes, being ICU-acquired infection and mortality. Before this study, it has already been demonstrated by other research groups that low mHLA-DR expression, which is thought to be reduced by sepsis, is indicative of immunosuppression and increases the risk secondary infections. In this unique, large real-world cohort that recruited patients over a 20-year long period, they confirm the association between mHLA-DR and ICU-acquired infections, but also with mortality. Their results provide unique insight in the changes in mHLA-DR expression over time, which allows them to demonstrate that mainly patients with a low mHLA-DR expression at d5-7 and those that fail to increase mHLA-DR levels are at increased risk of ICU-acquired infections and mortality.

We thank the reviewer for his/her kind comments and interest in our manuscript. We hope that we have adequately addressed his/her suggestions and comments in the following point-by-point response and in the revised version of the manuscript.

1. Is there a risk of bias due to survivorship or ICU-discharge (due to recovery) at the later time-points? The ICU LOS is 9 [4-17] in the whole cohort, so at least some patients seem to be discharged at d5-7. Was mHLA-DR also measured in the patients after discharge of the ICU? Please include the sample size per time-point and discuss whether the discharge from the ICU of recovered patients may have affected the results.

The number of patients with mHLA-DR samples were 899 at D1/D2, 886 at D3/D4 and 650, at D5/D7. This is reported in Figure 1D legend. To clarify this point, we now provide a flow chart detailing blood samples and mHLA-DR measurements performed during follow-up, taking into account early deaths and ICU discharges before the end of the first week (see below and novel Figure S2).



Unfortunately, mHLA-DR was not / could not be measured after ICU discharge. The referee is correct that, by nature, the results observed at D5/D7 are obtained in a specific subgroup of patients, as they exclude patients who died early (before D5/D7, and who would likely have had very low mHLA-DR values) and those discharged rapidly from the ICU due to clinical improvement (who would likely have had higher mHLA-DR values). Nevertheless, new Figure S7 (see also response to Reviewer #2 commentbelow), which includes patients with complete sampling at the 3 time points, shows that the trajectories observed in this subgroup of patients remain similar in comparison with trajectories obtained in the whole cohort (See Figure 4C). In addition, when comparing the main clinical characteristics at admission between patients with mHLA-DR values at D5/7 and those not sampled at this time-point due to survivorship or ICU-discharge, we did not observe any difference (see table below). This suggests that there is a limited bias when considering patients with available samples at D5/D7.

	Total cohort N=1023	Patients having D5/7 mHLA-DR value N=650	Patients without D5/7 sample N=373	*p-value*
Age (y), median [Q1-Q3]	68 [58-77]	68 [58-77]	70 [59-79]	*0.088*
SAPS II, median [Q1-Q3]	58 [47-72]	58 [48-71]	57 [45-75]	*0.538*
SOFA score, median [Q1-Q3]	10 [8-12]	10 [8-12]	10 [7-12]	*0.130*

Finally, it is also noteworthy that the Fine and Gray competing risk model (as described in the Methods section) accounts for death and ICU discharge as competing events when predicting the risk of ICU-acquired infections. This further reduces the risk of bias.

Importantly, the clinical course of the patients was not known to the immunologists in the laboratory when analysing mHLA-DR. Conversely, the mHLA-DR results were not available to the clinicians when taking care of the septic shock patients. This point has been clarified in the revised manuscript (Materials and Methods section, page S3.)

2. The authors conclude: "As such, it is widely regarded as a reliable marker of sepsis-induced immunosuppression [21-24]. The present data clearly demonstrate that, from the third day after septic shock onset, immune dysfunction becomes an independent factor associated with both mortality and the development of secondary infections." Yet, given that some patients are discharged from the ICU before day 5-7, the mean lower mHLA-DR levels at day 5-7 may also be due to case selection. Please include a paired sub-analysis of cases, of which mHLA-DR expression at multiple time-points is available, to identify whether temporal effects of sepsis on mHLA-DR expression. Figure S3 clearly demonstrates different expression levels at baseline associated with different trajectories of mHLA-DR expression over time. These results suggest, however, that mHLA-DR is not reduced by sepsis, but already low in expression in some patients upon diagnosis followed by a gradual increase over time, particularly in those patients with higher expression at day 1-2. Should the view that mHLA-DR expression is reduced by sepsis be revised?

We thank the reviewer for this interesting comment, which we have indeed illustrated through Figure S7 (former Figure S4). The alluvial plot represents the change of immune status from D1 to D7 for patients with all the 3 samples (n=541). This sub-analysis confirms the results of the main alluvial plot (Figure 4C).

The second part of the comment refers to the relevant hypothesis that mHLA-DR could already be low before sepsis in patients with underlying immunosuppression. However, by nature of the study, we had no access to blood samples collected prior to ICU admission. It was therefore impossible to determine the patient's immune status before the onset of sepsis. Moreover, and importantly, underlying/pre-existent immunosuppression was an exclusion criterion, as indicated in the Materials and Methods section. Although it is impossible to determine precisely the onset of an infection (as opposed to others injuries such as trauma, burn or surgery), we can hypothesize that differences at baseline (i.e. D1/2) may relate to differences in delays after infection onset in patients. Some patients may still be in the very early stage of the disease, whereas others may have already been infected for several days when sampled. Lastly, as shown in Figure 1D, at the time of ICU discharge, most patients had recovered normal HLA-DR values, suggesting they were likely at similar levels before septic shock. Thus, based on observation from the present study, we believe that we cannot revise the view that mHLA-DR expression is reduced by sepsis.

That said, we agree that underlying immunosuppression is becoming an increasingly important topic, given the growing number of patients who are already immunocompromised prior to ICU admission, regardless of the cause (e.g., cancer, CAR-T therapy, transplantation, biotherapies). The potential value of evaluating mHLA-DR in these specific patients warrants further investigation in future dedicated studies. We highlighted this point in the limitations section in the revised manuscript and added related references (page 9).

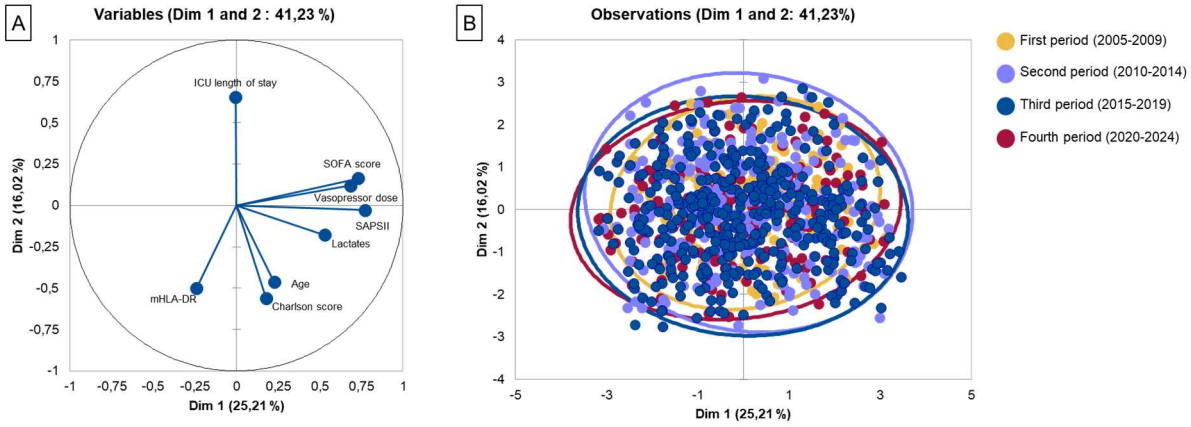
3. The study recruited patients over a large period of time, which allowed the researchers to collect data of an impressive number of patients. It is, however, how effective inclusion was, whether patients were missed, and why patients may not have been included. I would expect the hospital in Lyon to admit more than ±50 cases with sepsis to the ICU per year, but I might be wrong. The authors describe the cohort as "quasi-unselected cohort" (discussion). Please include a Consort diagram, if these data are available, to demonstrate the number of patients screened, included, and excluded, and specify the reasons, to facilitate understanding of the generalizability and risk of selection bias.

We agree with the referee's estimate of annual septic shock cases. However, our study focused on patients hospitalized in the ICUs of only 2 hospitals (Hôpital E. Herriot and Centre Hospitalier Lyon-Sud) within Hospices Civils de Lyon, not all ICUs from Lyon University hospitals. This explains the apparently low number of annual cases. We apologize for this lack of clarity. Futhermore, we missed patients with underlying immunosuppression (as explained above). This has been specified in the revised version of the Manuscript (Page S2). As IMMUNOSEPSIS is a real-world observational cohort (conducted outside the framework of a strict RCT), we did not maintain a complete and detailed

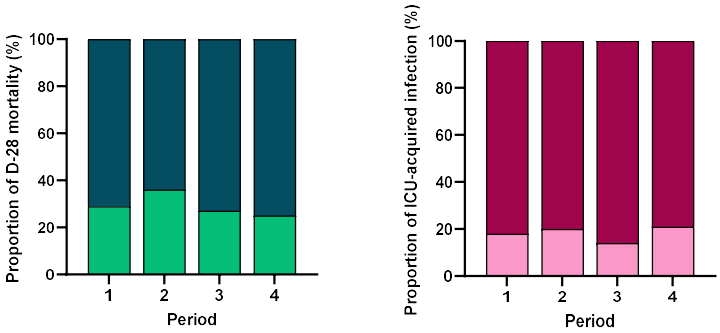
screening log over the 20-year study period. Consequently, the information required to produce a CONSORT diagram is unavailable. This limitation has been added to the revised manuscript (page 9). Nevertheless, we have provided a flow chart of patients included in the observational cohort (Figure S2) to facilitate understanding of the different samplings.

4. Over the course of 20 years, advances in ICU care led to a reduction in sepsis-related mortality. Similarly, changes in flow cytometry may have affected the measurements. The fact that mHLA-DR remains a robust predictor of adverse outcomes, despite changes in clinical practices over time, is a strong point of the study. Although the presented PCA based on clinical characteristics and mHLA-DR expression suggests no relevant changes over time (Fig 1b), this method reduces data dimensionality and potential relevant changes in mHLA-DR levels or the study endpoints (a.o., ICU acquired infections, mortality) may go unnoticed, which may affect the association between mHLA-DR and the study endpoints. The authors should consider including data on mHLA-DR levels and the incidence of endpoints over different time windows. They should consider performing a sensitivity analysis using a recent time window to demonstrate that their associations remain valid and not subject to developments in flow cytometry and ICU care over the last decade.

We thank the reviewer for this relevant comment and we fully agree with it. Accordingly, rather than categorizing patients by consecutive number of patients over time (approximately 255 as presented in the manuscript), we repeated the PCA analysis by defining 5-year temporal periods (n = 4) using the same variables. The results are identical and show no drift over time. Results are presented below.



In addition, at the reviewer's request, we illustrate the incidence of endpoints over different time windows (four 5-year periods, identical to those used for the PCA above). Day-28 mortality shows a tendency to decrease after 2015 (green histogram), in agreement with data reported in the literature. Regarding ICU-acquired infections (red histogram), we observed relative stability (19% before 2015 and 17% after 2015). Overall, there was no substantial drift likely to have significantly affected the results.



Those results have been added to supplementary figures (new figure S1).

Regarding the flow cytometry analyses, we continuously monitored the stability of results over time in line with methodological developments, and each step of this process has been published. The protocol is accredited by regulatory agencies in both the USA and France. We established an internal quality control program (based on stabilized blood) to ensure long-term reproducibility within our laboratory, and we also implemented an inter-laboratory quality control program to ensure reproducibility between laboratories. Based on these precautions, we are confident that our results are analytically robust over the years. Only a selection of these publications was cited in the manuscript, as we believe that the full methodological details of mHLA-DR flow cytometric measurements fall outside the scope of an ICU-focused journal. The complete list of references is provided below:

Multicenter inter-laboratory quality control of monocyte HLA-DR expression by flow cytometry.
Gossez M, Bonnet B, Boussaid I, Chapuis N, Cointe S, Cravat M, De Carvalho Bittencourt M, Dignat-George F, Evrard B, Jeannet R, Jourdi G, Lozano C, Paul S, Siguret V, Waeckel L, Monneret G.
Cytometry B Clin Cytom. 2025 Jan;108(1):95-97.

Monocyte HLA-DR expression as an enrollment biomarker in sepsis clinical trials: Evaluation of two sampling tubes and definition of respective clinical thresholds.
Haem Rahimi M, Conti F, Llitjos JF, Fleurie A, Cerro V, Venet F, Lukaszewicz AC, Monneret G.
Cytometry B Clin Cytom. 2023 Nov;104(6):468-470.

Accréditation de la mesure de l'expression des molécules HLA-DR à la surface des monocytes (mHLA-DR) par cytométrie en flux.
Frugier A, Malcus C, Baume M, Poitevin-Later F, Lukaszewicz AC, Venet F, Monneret G, Gossez M.
Ann Biol Clin (Paris). 2022 Mar 1;80(2):190-198.

Bicentric evaluation of stabilizing sampling tubes for assessment of monocyte HLA-DR expression in clinical samples.
Hamada S, Jeannet R, Gossez M, Cour M, Argaud L, Francois B, Daix T, Venet F, Monneret G.
Cytometry B Clin Cytom. 2022 Sep;102(5):384-389.

Inter-laboratory assessment of flow cytometric monocyte HLA-DR expression in clinical samples.
Demaret J, Walencik A, Jacob MC, Timsit JF, Venet F, Lepape A, Monneret G.
Cytometry B Clin Cytom. 2013 Jan-Feb;84(1):59-62.

Assessment of monocytic HLA-DR expression in ICU patients: analytical issues for multicentric flow cytometry studies.
Monneret G, Venet F, Meisel C, Schefold JC.
Crit Care. 2010;14(4):432.

Monitoring temporary immunodepression by flow cytometric measurement of monocytic HLA-DR expression: a multicenter standardized study.
Döcke WD, Höflich C, Davis KA, Röttgers K, Meisel C, Kiefer P, Weber SU, Hedwig-Geissing M, Kreuzfelder E, Tschentscher P, Nebe T, Engel A, Monneret G, Spittler A, Schmolke K, Reinke P, Volk HD, Kunz D.
Clin Chem. 2005 Dec;51(12):2341-7.

[Preliminary results in standardization of flow cytometry protocols for monocytic HLA-DR measurement and their application in the follow up of septic shock].
Finck ME, Elmenkouri N, Debard AL, Bohé J, Lepape A, Bienvenu J, Monneret G.
Ann Biol Clin (Paris). 2003 Jul-Aug;61(4):441-8.

Analytical requirements for measuring monocytic human lymphocyte antigen DR by flow cytometry: application to the monitoring of patients with septic shock.
Monneret G, Elmenkouri N, Bohe J, Debard AL, Gutowski MC, Bienvenu J, Lepape A.
Clin Chem. 2002 Sep;48(9):1589-92.

5. Site of infection: a relatively large number of patients (±20%) have another site of infection other than abdominal, urinary, or pulmonary. Please add some information on the (most common) sites of infection in this group.

Among the 214 patients classified with others site of infection, most common were soft tissue infection (n=63), intravascular catheter-related infection/infective endocarditis (n=38) and osteoarticular (n=20).

This information is provided in the legend of new supplementary figure 3.

6. How was the diagnosis of infection and sepsis made? The diagnosis may not always be clear upon ICU admission, the coded diagnosis in the (electronic) health record may be incorrect and as such, post-hoc adjudication by an expert panel is considered the most reliable way to confirm a diagnosis of sepsis, albeit very time consuming. The methods states that patients were included based on the presence of a microbiologically confirmed or suspected infection combined with Sepsis-2/3 criteria and further states: "presence or absence of infection acquired during ICU hospitalization) were collected blindly from results of biological analyses by clinicians". What was the qualification of these clinicians (e.g., residents, consultants/specialists?) and was it performed by one person or multiple persons per case? Were patients who did not have an infection according to retrospective chart review retained in the study or excluded?

We thank the reviewer for this pertinent comment regarding the diagnosis of infection. As the data were collected outside a strict RCT (IMMUNOSEPSIS is a real-world observational cohort), we did not have the benefit of a formal adjudication committee for establishing the diagnosis of infection, which would have required substantial additional resources. As specified in the Supplementary Methods section (page S2), infections were initially identified at the time of clinical suspicion by the attending physician (a fully trained intensivist, not a resident). Subsequently, all records of septic shock patients included in this report were retrospectively reviewed and confirmed by an external physician (hospital practitioner, Dr. T. Lafon, first co-author of the article). The diagnosis of infection was based on clinical or radiological findings, with or without microbiological confirmation. This validation was similar to a recent standardized protocol to define true infection status (Whitfield NN, Diagn Microbiol Infect Dis. 2024 Sep;110(1):116382). We added this information in supplementary methods section (page S2).

Using this method and chart review, we describe an epidemiological data consistent with others cohorts of septic shock, particularly in terms of distribution of infectious sites, proportion of microbiological identification and associated bacteremia (Timsit JF. Intensive Care Med. 2020 Feb;46(2):266-284; Tabah A, Intensive Care Med. 2023 Feb;49(2):178-190; Tabah A, Intensive Care Med. 2025 Jul 14. doi: 10.1007/s00134-025-08026-5).

7. The authors mention "Source controlled" in table 1. How was this defined?

Source control included drainage of abscess, abdominal surgery (perforation, peritonitis, cholecystitis, pyelonephritis associated with obstruction…), debriding infected necrotic tissue, removal infected device or control of a source of ongoing microbial contamination. As we use prognostic outcomes, we thought it was important to describe the proportion of patients that requires emergent source control, as recommended by the SSC.

We added this precision in Table 1 footnotes (page 13).

8. Diuresis (ml) (table 1): please add the time window ("per 24h").

We added this precision in the different tables.

9. The authors suggest, with some caution, that mHLA-DR can be used to enroll patients in clinical trials that test immunostimulants. "*Our data confirm the relevance of this threshold to identify patients at higher risk of unfavorable outcomes. However, we demonstrate that an initial mHLA-DR value < 8,000 AB/C (measured on D1/2) does not carry predictive value on its own. The clinical significance of mHLA-DR expression becomes clearer over time: the longer a patient remains in the ICU with persistently low mHLA-DR, the greater the risk of clinical deterioration. Beyond the theoretical concern of administering immunostimulants too early, which could potentially reboot a cytokine storm, our findings indicate that early mHLA-DR measurements alone are insufficient to reliably identify patients who will remain severely immunosuppressed".* Since reduced mHLA-DR expression levels beyond day 1-2 (i.e, day 3-4, 5-6) are associated with adverse outcomes, how do the authors propose to use the mHLA-DR expression levels for clinical trial enrichment? Ideally, such treatment should likely be initiated early in the course of the disease. Given the unmet medical need to develop targeted therapies for patients with sepsis, it would be interesting to learn more on how the authors would envision the use of mHLA-DR for clinical trial enrichment: at what time-point should it be measured, would it be a single time-point or should the change in mHLA-DR expression trigger inclusion? Do the authors envision mHLA-DR-directed therapy (if so, which?) or should mHLA-DR be considered a marker of immunosuppression and thereby any immunostimulant could potentially be effective? See also the review below, where mHLA-DR is described as a marker of immunosuppression and how it can support clinical trial enrichment, including some examples of studies.

* S Deinhardt-Emmer, BG Chousterman, JC Schefold, SB Flohé, T Skirecki, M Kox, MS Winkler, A Cossarizza, WJ Wiersinga, T van der Poll, MA Weigand, S Cajander, EJ Giamarellos-Bourboulis, G Lachmann, M Girardis, BP Scicluna, R Ferrer, D Payen, S Weis, A Torres, JF Bermejo-Martín, MF Osuchowski, I Rubio, HR Bouma. Sepsis in patients who are immunocompromised: diagnostic challenges and future therapies. Lancet Respir Med. 2025 May 20;S2213-2600(25)00124-9.

We thank the referee for this comment, which encompasses several aspects. A comprehensive discussion of all these points is likely beyond the scope of the present report, owing to space limitations (it could be the matter of a position paper); however, we may briefly address them here.

As stated in the manuscript, and based on our results, we consider that the initial decrease in mHLA-DR (i.e., measured within 48 h after onset of septic shock) may represent a physiological mechanism (observed in most patients) in response to the exacerbated inflammation. At this early stage, mHLA-DR does not allow us to predict which patients will fail to restore their immune function. Consequently, initiating early immunostimulation is challenging, as it may carry the risk of rekindling the cytokine storm. In line, a recent study using IFNg from the time of ICU admission was halted for safety reasons (reactivation of the inflammatory response) (Roquilly et al., Intensive Care Med. 2023 May;49(5):530-544.). For these reasons, we suggest that immunostimulation should be restricted to patients who remain in the ICU beyond day 3–4 and who present signs of non-recovering immune function. As shown in the manuscript, at this later stage, mHLA-DR can identify patients at increased risk of secondary infections and death. In this context, mHLA-DR becomes a valuable enrichment factor, as proposed by the referee. Notably, in agreement with our perspective, ongoing studies testing IFN-γ are enrolling patients several days after ICU admission, rather than at admission (IGNORANT #NCT05843786, PLATINIUM #NCT06774235).

Regarding time points, a kinetic assessment of at least 2 samples from day 3-4 would indeed be ideal, but even a single measurement from day 3-4 (< 8,000 AB/C) provides valuable information as shown in the present work. To date, while the use of immunostimulation in the ICU is still in its infancy, we should avoid being dogmatic and we should only propose recommendations. Each center may determine its own number of time point for clinical decision-making according to its local resources and capabilities.

The Deinhardt-Emmer et al. article has been added to the manuscript (new ref #25, page 7).

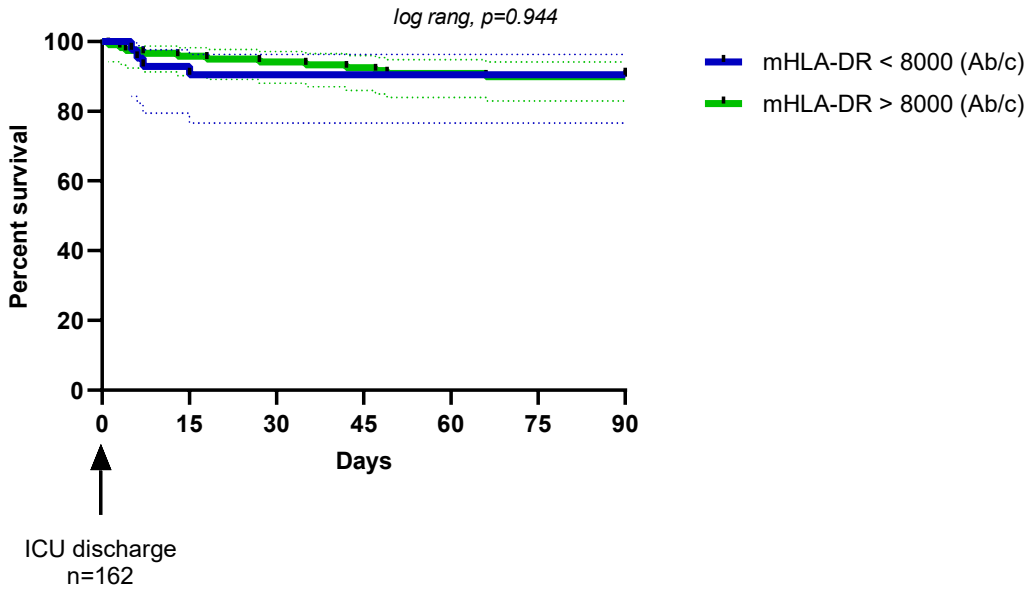
10. The authors demonstrate an association between mHLA-DR and adverse outcomes, including survival up to 90 days. How many of the deaths occurred in hospital, and how many after discharge? As low mHLA-DR expression is an indicator of immunosuppression, one may assume that late mortality was due to recurrent sepsis: is this known for this cohort? Can (or should) low mHLA-DR expression be used to identify patients at risk for recurrent sepsis or long-term mortality, which is not uncommon for patients who have had severe sepsis?

In the whole cohort, among the 395 deceased patients, 376 (95%) died in hospital, with a median delay of 10 days [3–25] after ICU admission; 311 of these deaths occurred in the ICU (83 % of hospital deaths). The remaining 19 patients (5% of deceased patients) died after hospital discharge.

Data on recurrent sepsis are not available in our study. However, several previous studies have reported that approximately 25% of patients who survive sepsis are readmitted to the hospital within 30 days of discharge due to a new infectious episode. We remain convinced that mHLA-DR measurement may help identify patients at risk of recurrence, either at the time of hospital discharge or at an additional follow-up point, by detecting persistent immunosuppression. Nevertheless, this hypothesis cannot be confirmed with the present study design and would require dedicated prospective investigations.

To further address this point indirectly, while mHLA-DR may help identify patients at persistent risk of infection, our results show no association between mHLA-DR measured at ICU discharge and

90-day mortality (although immunosuppression is likely a contributing comorbidity). As specified in the Results section (Page 5), at ICU discharge, mHLA-DR values (measured in 162 patients) were not associated with 90-day survival, mainly because 95% of deaths occurred during the index hospital stay. We generated a Kaplan–Meier curve using the 8,000 AB/C threshold to illustrate this finding (data not shown in the manuscript).



11. "In a substantial cohort of 1023 patients with septic shock, we demonstrated that low monocyte HLADR (mHLA-DR) expression is a robust and consistent biomarker associated with mortality and the risk of developing ICU-acquired infections." I do not agree with this statement, as the authors did not demonstrate an association between mHLA-DR at day 1/2 and mortality, and neither between D1/2 or D3/4 and ICU-acquired infections (Fig 3). The statement needs to be nuanced.

We agree and we rephrased the sentence accordingly (page 6).

12. The finding of low mHLA-DR in patients with septic shock and its association with adverse outcomes is not new, as also described by the authors already in the introduction - the novelty seems to be in the real-world setting of this study, which is highly valuable to translate previous findings into clinical applications. To aid readers in understanding what was known before this study and what this study adds, it would be helpful if the authors could include a box to mention this.
Further, the study by Schefold et al. also measures mHLA-DR expression in septic shock: this study should be cited and discussed to understand which knowledge is confirmed and what novelty is generated in the current work.

* Schefold JC. Measurement of monocytic HLA-DR (mHLA-DR) expression in patients with severe sepsis and septic shock: assessment of immune organ failure. Intensive Care Med. 2010;36(11):1810-2;

As we were not certain that a boxed figure would fit the original paper format, we have reformulated our conclusion (page 9) to better highlight the novelties and confirmations that the present report brings to the field. The editorial mentioned by the referee (Schefold, Intensive Care Med. 2010;36(11):1810–1812) has been added to the manuscript (new ref #25) in the part addressing organ failures.

13. The authors mention "Beyond the simplicity and robustness of the protocol, access to flow cytometry is not universally available", and propose the use of point-of-care flow-cytometers and whole-blood transcriptome analysis. However, these technologies may also not be available, specifically not in low/middle income countries, where the mortality rate of sepsis is even higher than in high income countries. Would it be possible to develop affordable methods to allow mHLA-DR analysis in low/middle-income countries as well?

The referee is correct regarding the situation in low- and middle-income countries. While point-of-care cytometers are already available for CD4+ T lymphocyte counts in studies including HIV-infected patients, it is conceivable that POC flow cytometers capable of measuring mHLA-DR could be developed. That said, to stimulate diagnostic companies to invest in such device and its broader implementation, it is beforehand essential to demonstrate the clinical impact in sepsis of immunoadjuvant treatments tailored based on mHLA-DR measurement in a well-designed randomized controlled trials. .

One alternative could be to define a surrogate marker for mHLA-DR using simple, routinely available biological markers, such as leukocyte values extracted from a complete blood count. To our knowledge, such approach has not yet been explored. This would require demonstrating that such markers identify the same population as that defined by mHLA-DR < 8000 AB/C. Dedicated studies are therefore necessary to confirm this and to define clinically relevant thresholds.

Reviewer #2:

This is the largest study published to the present day evidencing the ability of HLA-DR expression in monocytes to predict mortality and infections in patients with septic shock, developed by the pioneer group in this issue.

Mayor strength of this study are:

- Large sample size
- Use of an standardized the flow cytometry protocol quite reproducible between different flow cytometry devices and centers
- Use of a previously derived cut-off (8,000 AB/C) which the study confirms as useful to predict the outcomes of mortality and secondary infections.
- The work confirms the predictive ability of HLA-DR for these outcomes

The results confirm that it is necessary to monitor HLA-DR values to get an adequate picture of those patients who will deteriorate, instead of using an isolated measurement at day 1. Translational implications are multiple: monitoring HLA-DR could help to avoid overuse of immunosuppresses in patients showing low expression levels. Initiating immune stimulatory therapies too early in the disease course may be deleterious. Identifying the most immunosuppressed patients could help to properly allocate resources to prevent bad outcomes. At the light of the results, immunological parameters should be integrated into severity scores such as SOFA.

We thank the reviewer for these nice remarks.

I have nonetheless some questions:

1. In the appendix you mention that immunosuppressed patients are excluded. These patients are those showing the highest risk of poor outcomes in sepsis, mostly those with haematological malignancies. How do you think HLA-DR profiling could perform in these patients to predict mortality and secondary infections? Please comment in the discussion

We agree that underlying immunosuppression is becoming an increasingly important topic, given the growing number of patients who are already immunocompromised prior to ICU admission, regardless of the cause (e.g., cancer, haematological malignancies, CAR-T therapy, transplantation, biotherapies). Although we hypothesize that mHLA-DR would be also helpful in these circumstances, the potential value of mHLA-DR in these specific subgroups of patients warrants further investigation in future studies. This point has been added to the limitations section within the discussion (page 9).

2. Could you provide AUCs for mortality and secondary infections?

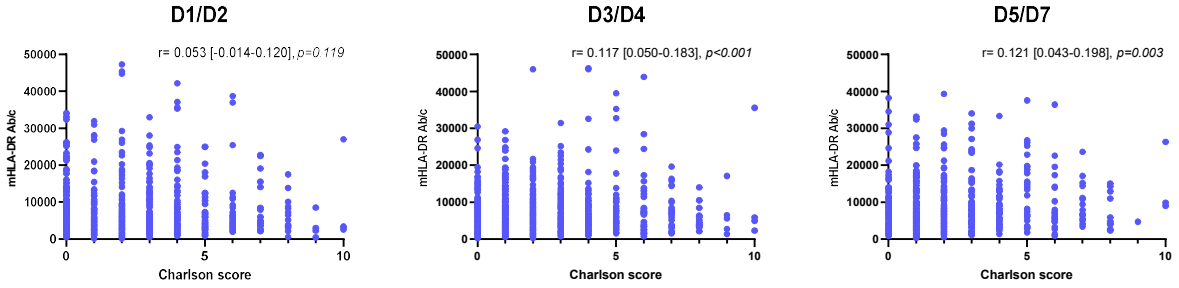
AUC with ROC curve were generated at each time point for the 2 outcomes. We added these results in new Supplementary figure S3.

3. Could the results help to identify patients deserving longer antibiotic courses or wider initial empiric antibiotic therapies? Have you evaluated this? Are you considering further studies addressing the potential use of HLA-DR for antibiotic stewardship? Would you recommend increased surveillance of secondary infections to start earlier with the antimicrobial therapy in these patients?

We thank the reviewer for raising this important point. We did not evaluate the relationship between mHLA-DR evolution and various aspects of antibiotic treatment (such as duration or efficacy), although such analysis could yield highly valuable insights. We believe this should be addressed in a well-designed protocol focused specifically on antibiotic use, which was not the case in our real-world study. To our knowledge, a study currently underway in the UK investigates this topic and includes mHLA-DR as one of the parameters for diagnosing immunosuppression (Role of immunosuppression in an antibiotic stewardship intervention and its association with clinical outcomes and antibiotic use: protocol for an observational study (RISC-sepsis). Scott J, Trevi L, McNeil H, Ewen T, Mawson P, McDonald D, Filby A, Lall R, Booth K, Boschman G, Melkebeek V, Perkins G, McMullan R, McAuley DF, McCullagh IJ, Walsh T, Rostron A, Shankar-Hari M, Dark P, Simpson AJ, Conway Morris A, Hellyer TP.BMJ Open. 2022 Dec 9;12(12):e068321).Results from this study are eagerly awaited.

4. Are low HLA-DR levels related to higher CHARLSON scores? Could you assess correlation between both factors?

Spearman correlation coefficients, calculated for each time point, are presented in the graphs below. Overall, we found no relevant correlation between mHLA-DR at any time point and the Charlson score.



5. Are low HLA-DR levels related to higher CRP or PCT levels?

Unfortunately, as these two biomarkers are not routinely monitored in septic shock patients in the ICUs from Hospices Civils de Lyon; they were not collected in the current study and we are unable to evaluate their correlation with mHLA-DR.

Reviewer #3:

The decrease expression of HLA-DR expression on monocytes from septic patients is a well-known phenomenon which reflects the status of immune suppression observed in these patients. Monneret et al. offer a survey of this biomarker on 1023 septic shock patients, and report that its measurement on day 1 does not correlate with mortality while it does on day 3/4 and 5/7. This biomarker does correlate with the occurrence of infection whenever the measurement was made. The authors point out that, as immuno-stimulant therapies enter clinical trials, effective stratification is critical to identify patients with the most profound immune dysfunction.

Main concern

1. It is unclear what are the novelties of this manuscript (except the number of included patients) as compared to the author's previous publication (Leijte et al. Critical Care 2020 ; 24:110). In fact, it is even unclear whether the 241 patients of their previous publication where included in the present one. In addition, there is a contradiction between both papers since in the previous one, there was no significant correlation between HLA-DR expression measured on day 1/2 and 3/4 and the occurrence of a secondary infection (fig. 2A, versus fig.2E in the present submitted paper). If more than 1000 patients are needed to observe an association between the low level of HLA-DR expression on day 1 to 4 and the occurrence of infection, the probability that it could be meaningful for ONE given patient is rather limited. But, this is a question the physician asks himself for each of his/her individual patient. So as a biomarker of immunosuppression for a personalized medicine to decide whether the patient should receive an immuno-stimulant treatment does not appear very helpful.

We apologize for the lack of clarity in describing the key points of our work. We acknowledge that we are not presenting novelties in the strictest sense, as numerous articles have already described the modulation of mHLA-DR expression in septic patients. However, based on real-world data, we believe that the main strength of the current study lies in firmly demonstrating the robustness of this marker in a cohort of more than 1,000 patients hsopitalized in ICU over a 20-year period. In this real-word cohort, mHLA-DR — measured using a standardized protocol with long-term quality control — remained associated with both mortality and the occurrence of infections when applying the usual threshold of 8,000 AB/C from day 3–4 onward. To our knowledge, such a study has never been reported in the literature, and in itself, this constitutes a novel contribution. We have reformulated our conclusion to better highlight the novelties and confirmations that the present report brings to the field.

From our perspective, the fact that mHLA-DR performs better in 1,023 patients than in 241 patients is not a contradiction between studies but rather illustrates the advantage of large cohorts with

increased statistical power. That said, we fully agree with this Referee that mHLA-DR is far from being a perfect marker (as reflected by the AUC values in ROC analysis). Nonetheless, as highlighted in many recent reviews (*Cajander, S., et al., Lancet Respir Med, 2024, Torres, L.K et al. Annu Rev Physiol, 2021, Pei, F., et al., Mil Med Res, 2022, Martin-Loeches, I., et al., EClinicalMedicine, 2025, Deinhardt-Emmer, S., et al., Lancet Respir Med, 2025*), mHLA-DR remains the most well-established biomarker in the field of sepsis-induced immunosuppression to date.

Thus, while awaiting any marker with better performances, our results show that using the proposed clinical threshold of 8,000 AB/C, currently employed in ongoing RCTs, mHLA-DR serves as a reliable and robust enrichment marker. This allows the selection of a subgroup of patients who are more likely to respond to immunostimulation, while excluding those who do not require this treatment. Even if this is not yet perfect, this represents a step toward abandoning the “one size fits all” approach that has prevailed for decades in sepsis immunomodulation. In that sense, we believe mHLA-DR is indeed a valuable tool for clinicians and for patients suffering from sepsis.

2. The second problem of this biomarker aimed to help to initiate the administration of immuno-stimulant treatment, is the fact that the authors have investigated septic shock patient. So, should the ICU doctor wait till his/her sepsis patient develop a shock to start considering his patient as significantly immunosuppressed for initiating an immuno-stimulant treatment? In fact, similar investigations with similar goals have also been regularly reported in ICU, or sepsis patients (Lekkou et al. Clin. Diagn. Lab. Immunol 2004 ; 11 : 161-167 ; Wu et al. Critical Care 2011, 15:R220, Drewry et al. Critical Care. 20, 334. (2016) ; de Roquetaillade et al. Ann. Intens. Care 2022 ; 12:39 ; Cui et al. Clin Biochem. 2025, 135: 110851). According to these papers, the status of severe immunosuppression could be defined without waiting the patient to develop a septic shock. Furthermore, in the present study, a significant proportion of patients died in the early time before developing any secondary infection (Fig.1C).

We fully agree that the relevance of decreased mHLA-DR is not specific to septic shock. This marker can also be applied in contexts such as sepsis, trauma, surgery, pancreatitis, and cirrhosis to identify immunosuppressed patients. Accordingly, in ongoing RCTs testing immunostimulation (IGNORANT, PLATINIUM), septic shock is not an inclusion criterion.

The referee is correct in noting that some of our analyses excluded severely ill patients who died within the first hours or days without developing an infection. That said, this potential bias was accounted for by using a Fine and Gray competing risk model.

3. In terms of personalized medicine, the need to perform a survey over 7 days to rank the patients in three clusters, themselves associated with different frequencies of secondary infection and mortality is interesting, but probably of limited interest, regarding the need to wait one week to define in which cluster the patient belongs and before deciding whether an immuno-stimulant treatment would be beneficial.

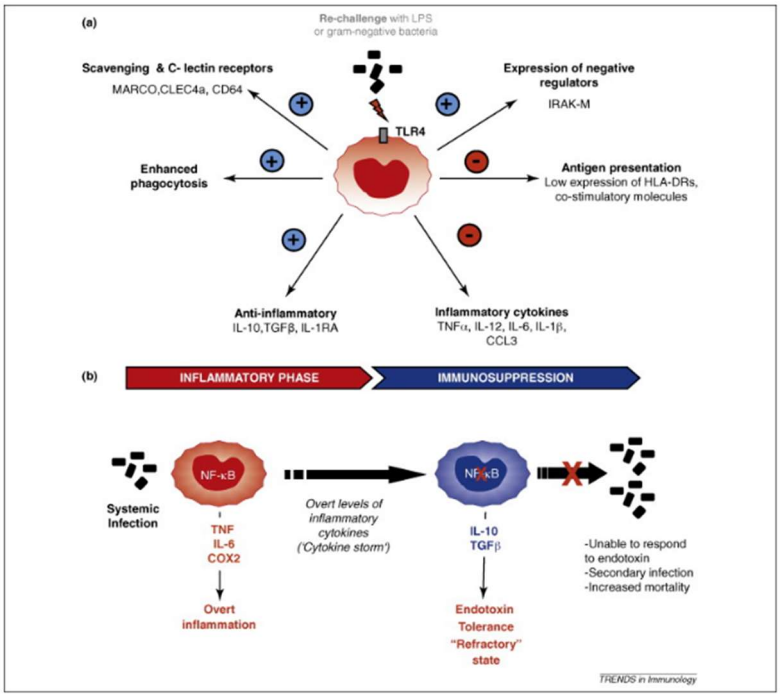
Again, we apologize for the lack of clarity in describing the key points of our work. Regarding time points, while a kinetic assessment would indeed be ideal, our results show that even a single measurement on day 3–4 or after (< 8,000 AB/C) provides valuable information. The cluster analysis is presented here to support the concept that it is the persistence of immunosuppression (or lack of recovery) that is associated with unfavorable outcomes and we fully agree that plotting a full evolution curve over the week is not mandatory to identify the most immunosuppressed patients. In agreement, ongoing RCTs (IGNORANT, PLATINUM) are based on a single mHLA-DR value rather than a kinetic profile.

Other comments

1. Some references are not illustrating the point raised by the authors : Ref. 15 deals with endotoxin tolerance while the authors address the impaired capacity of monocytes of septic shock patients to release inflammatory cytokines; Ref.13, 19 are global reviews of the authors far from the specific aspects mentioned by the authors referring to the inflammatory and immunosuppressive signals influencing the monocytes phenotypes. For example, the paper by Fumeaux et al. Am J Respir Crit Care Med. 2002 Dec 1;166(11):1475-82, which precisely addresses that point would be a far more appropriate reference.

Ref 19 has been deleted. Fumeaux's reference has been added (page 3).

We suggest to keep Biswas' reference as this review clearly indicates that endotoxin tolerance during sepsis is characterized by low mHLA-DR expression and loss of inflammatory cytokine production – see figure below from Biswas' reference.



2. The authors seem to have identified 100% of the infectious organisms. This is quite impressive. How was it possible?

As mentioned in Table 1, only 58% (n=598) of patients had an identification of infection organism and 34% with associated bacteremia. This proportion corresponds to previously published data, notably the study by Vincent JL et al. *(Vincent JL, Sakr Y, Singer M, Martin-Loeches I, Machado FR, Marshall JC, Finfer S, Pelosi P, Brazzi L, Aditianingsih D, Timsit JF, Du B, Wittebole X, Máca J, Kannan S, Gorordo-Delsol LA, De Waele JJ, Mehta Y, Bonten MJM, Khanna AK, Kollef M, Human M, Angus DC; EPIC III Investigators. Prevalence and Outcomes of Infection Among Patients in Intensive Care Units in 2017. JAMA. 2020 Apr 21;323(15):1478-1487).*

3. SOFA score, SAPS II, lactate, etc… are far more predictive of mortality than HLA-DR on day 1 (table 1), but are less predictive values in terms of secondary infection (table S2)? What correlation exists between these scores or markers and HLA-DR within the three clusters?

We agree that mHLA-DR measured at day 1 has no predictive value regarding mortality, as clearly stated in the manuscript. As requested, we calculated day-1 correlations (Spearman test) within the three clusters between mHLA-DR and SOFA, SAPS II scores, and lactate (all measured at admission; see results in the table below). No significant correlations were observed.

	r	p
Cluster 1		
SOFA	- 0.01	ns
Lactate	0.10	ns
SAPS II	-0.07	ns
Cluster 2		
SOFA	0.07	ns
Lactate	0.07	ns
SAPS II	0.02	ns
Cluster 3		
SOFA	0.12	ns
Lactate	0.04	ns
SAPS II	-0.06	ns