PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	THE Reanimation Low Immune Status Markers (REALISM) PROJECT: A PROTOCOL FOR BROAD CHARACTERIZATION AND FOLLOW-UP OF INJURY-INDUCED IMMUNOSUPRESSION IN Intensive Care Unit (ICU) CRITICALLY-ILL PATIENTS
AUTHORS	ROL, Mary-Luz; Venet, Fabienne; RIMMELE, Thomas; MOUCADEL, Virginie; CORTEZ, Pierre; QUEMENEUR, Laurence; GARDINER, David; GRIFFITHS, Andrew; PACHOT, Alexandre; Monneret, G; Textoris, Julien

VERSION 1 - REVIEW

REVIEWER	William Carson University of Michigan - USA
REVIEW RETURNED	26-Jan-2017

GENERAL COMMENTS	In this report, Rol et al. describe a protocol for the identification of biomarkers of immunosuppression in patients who have recovered from septic shock, severe trauma, severe burns or major surgery requiring admittance to intensive care. The authors plan to collect peripheral blood from large cohorts of these patient populations using well-defined criteria for inclusion and exclusion, along with reference samples from healthy volunteers. These peripheral blood samples will then be analyzed for cytokine production in response to stimulation with lipopolysaccharide, along with proliferative responses of CD3+ T cells in response to phytohemagglutinin. The healthy patient sample results will be used to generate reference intervals for each assay, and patients will be categorized with immunosuppressive phenotypes if their results fall repeatedly outside the healthy reference interval. This proposed study would provide important diagnostic insights into the development and persistence of immunosuppressive phenotypes in patients that have recovered from severe inflammatory responses. It is expected that by the conclusion of the study period, the authors will have generated data sets identifying clear parameters for the measurement of post-septic immunosuppression in human patients, as well as described identifiable differences in peripheral immune cell responses in septic patients vs. other severe inflammatory responses (trauma, burn or major surgery). ELABORATIONS ON REVIEW CHECKLIST: 4. Are the methods described sufficiently to allow the study to be repeated? The authors should include more information regarding their planned

	evaluation of peripheral blood T cell proliferative responses. The dose of PHA should be provided, the timepoints for EdU treatment of cells should be indicated (along with EdU concentrations and information on the supplier used), and the method for flow cytometric analysis of EdU incorporation should be explained (% of proliferating cells, for example). It would also be helpful to identify the fluorescent conjugates to be used for both the EdU incorporation and the surface staining of CD3. 12. Are the study limitations discussed adequately? In the "strengths and limitations" bullet point section of the manuscript, the authors should address the relative short-term aspect of their proposed study. The persistence of post-septic immunosuppression is hypothesized to be long lasting, primarily based on studies in experimental animal models. However, there is a paucity of experimental data in human patients linking cellular immune phenotypes to observed long-term immunosuppression in these patients (years after the resolution of inflammation). The two-month timepoint proposed by the authors is presumably well past the resolution of inflammation in their patient populations; however, it does not directly address the persistence of immunosuppressive phenotypes for months/years after the septic event. This is an important limitation of this study, and if the authors are successful in identifying biomarkers of immunosuppression at 60 days, these data sets will serve as strong supports for more expansive timepoints in future studies. MINOR COMMENTS 1) Rationale of the study/Hypothesis: The authors argue that proliferation assays are not suitable for prospective interventional clinical trials due to "…the[ir] long time to results (up to 5 days for lymphocytes proliferation)…" (Page 6). Despite this critique, the authors plan to use PHA-stimulated T cell proliferation as one of their major diagnostic parameters. The authors should provide some rationale for why their approach to T cell proliferation studies overcome the issues mentioned in the "rationale" section. For example, will their proliferation assay be completed in a shorter amount of time? Does their approach lead to improved standardization across samples? Etc. 2) Celluar Immunophenotyping: The authors should directly indicate in the text the cell surface markers they plan to utilize to identify each lymphocyte subset. A brief explanation of their proposed gating strategy would also be helpful. 3) Innovative immune functional assays and exploration of new biomarkers: The reference to "home-made assays" is vague. The authors should explain the nature of these assays, specifically the targets to be analyzed (e.g. cytokine production), and a general identification of the experimental methodology (e.g. ELISA, Luminex, qPCR, etc.).

REVIEWER	Naeem Patil MD, PhD Vanderbilt University Medical Center, USA
REVIEW RETURNED	31-Jan-2017

GENERAL COMMENTS	The manuscript describes a study protocol relating to a highly significant clinical problem of sepsis. Numerous clinical trials have

	failed to identify an appropriate therapy for sepsis. Immunosuppression has been shown to play a critical role during sepsis, rendering the host susceptible to myriad of secondary infections and impaired ability to clear existing infections. Therefore, this study is very timely, as there is a need to identify biomarkers that could be standardized across all critically ill patients, in order to accurately classify patients as immunosuppressed. The manuscript is very well written, easy to follow and represents an extensive study. Results from this study could serve as a good reference for future studies in this area and it is an excellent study. Comments: 1. Could the authors also measure differential leucocyte count from the whole blood of the patients? This is relatively easy standardized test and widely performed across hospitals and will provide will additional information with respect to circulating immune cell numbers. 2. Lymphocyte proliferation assay as described by authors will take 72 hours to complete. Therefore, the future clinical applicability of this test is questionable. Would it have been more appropriate to analyze other T cell functional assay such as interferon-gamma production upon ex vivo stimulation (for example with PMA/Ionomycin or anti-CD3/CD28), which could be accomplished relatively fast. 3. Please mention the amount of blood that will be collected from pateints and volunteers for this study. 4. For the TruCulture tubes, authors mention that the tubes contain a medium. Please add details about this medium. 5. On page 13, authors mention that following stimulation in TruCulture tubes, supernatant will be collected? Will this be plasma alone? Or will it be mixed with the medium? Please add more details regarding the supernatant collection procedure. 6. For the cell proliferation assay, it would be more informative to include CD4+ and CD8+ surface markers along with CD3+ for flow cytometry analysis.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: William Carson

Dear Dr Carson,

Thank you very much for this positive analysis of our study protocol and for your comments, they helped us to improve our manuscript. Please find below the answers to your comments.

The authors should include more information regarding their planned evaluation of peripheral blood T cell proliferative responses. The dose of PHA should be provided, the timepoints for EdU treatment of cells should be indicated (along with EdU concentrations and information on the supplier used), and the method for flow cytometric analysis of EdU incorporation should be explained (% of proliferating cells, for example). It would also be helpful to identify the fluorescent conjugates to be used for both the EdU incorporation and the surface staining of CD3.

As suggested by the reviewer, we added more details concerning the T-cell proliferation assay to the revised version of the manuscript (page 14, lines 250-257) (the protocol is also fully described in the reference 21, from our group). More precisely:

- the concentration of PHA used for the reference test is 4µg/mL.
- the EdU treatment is performed at 72h of proliferation
- the supplier is Life technologies, Carlsbad, California, USA
- the gating strategy and method of measurement is now explained (percentage of EdU positive cells among CD3 gated (positive) cells).
- The anti-CD3 antibody is linked to APC, while EdU is linked with AF488.

“Briefly, Peripheral Blood Mononuclear Cells (PBMC) isolated by Ficoll density gradient centrifugation (U-04; Eurobio; Les Ulis, France) will be stimulated with PHA at 4µg/mL (HA16; Remel; Lenexa, USA), at 37°C for 72 hours. Following incubation, the cells will be harvested and cell’s proliferation will be determined by the incorporation of EdU (5-ethynyl-2'-deoxyuridine, 10µM for 2h) in T cells using the commercial kit Click-It EdU AF488 flow kit (C10420, Life Technologies, Carlsbad, California, USA). Cell proliferation is measured as the percentage of EdU positive T cells (gated as CD3+ cells using a CD3-APC staining) using flow cytometry [21].”

In the “strengths and limitations” bullet point section of the manuscript, the authors should address the relative short-term aspect of their proposed study. The persistence of post-septic immunosuppression is hypothesized to be long lasting, primarily based on studies in experimental animal models. However, there is a paucity of experimental data in human patients linking cellular immune phenotypes to observed long-term immunosuppression in these patients (years after the resolution of inflammation). The two-month timepoint proposed by the authors is presumably well past the resolution of inflammation in their patient populations; however, it does not directly address the persistence of immunosuppressive phenotypes for months/years after the septic event. This is an important limitation of this study, and if the authors are successful in identifying biomarkers of immunosuppression at 60 days, these data sets will serve as strong supports for more expansive timepoints in future studies.

We agree with the reviewer that in the REALISM project, we will determine a “mid-term” (2 months) follow up, rather than a long term one. Our wording was derived from the complete lack of data in these patients in the literature. We plan to develop future projects to determine the immune status of patients in the longer term. This point has now been added to the “strengths and limitations” section (page 4, line 64).

• “Mid-term assessment (D60) of immune status of ICU patients. Long term follow up is not addressed here and should be examined in future studies.”

MINOR COMMENTS
1) Rationale of the study/Hypothesis: The authors argue that proliferation assays are not suitable for prospective interventional clinical trials due to “…the[ir] long time to results (up to 5 days for lymphocytes proliferation)…” (Page 6). Despite this critique, the authors plan to use PHA-stimulated T cell proliferation as one of their major diagnostic parameters. The authors should provide some rationale for why their approach to T cell proliferation studies overcome the issues mentioned in the “rationale” section. For example, will their proliferation assay be completed in a shorter amount of time? Does their approach lead to improved standardization across samples? Etc.
We thank both reviewers for raising this point. Obviously, our message was not clear on this point. We fully agree that T-cell proliferation is not suitable for immune status assessment in the routine management of ICU patients. We decided to use T-cell proliferation as a reference test for the adaptive immunity, due to its wide use in the characterization of immune deficiencies. However, one of the secondary objective of the REALISM project is indeed to identify new biomarkers or functional assays, that could replace such reference assay in the future, being more suited to the clinical management of ICU patients. In order to clarify this point to the readers, we have now added the

following sentence to the manuscript (page 8, line 165-168):

“These new biomarkers / immune functional assays could therefore replace assays such as the T-cell proliferation assay, the current protocol of which is not suited to the routine management of ICU patients. We therefore expect to provide data to validate simpler diagnostic tools to determine and follow the immune status in hospitalized patients.”

2) Cellular Immunophenotyping: The authors should directly indicate in the text the cell surface markers they plan to utilize to identify each lymphocyte subset. A brief explanation of their proposed gating strategy would also be helpful.

We have now completed the methods section with a detailed list of the cell surface markers used to determine each leukocyte subpopulation, (page 14, line 263-266):

“We will count the number of number of B-lymphocytes (CD45+, CD3-, CD19+), T-lymphocytes, CD4+ (CD45+, CD3+, CD8-, CD4+) and CD8+ (CD45+, CD3+, CD8+, CD4-), NK cells (CD45+, CD3-, CD56+), regulatory T-lymphocytes (gated on T CD4+, CD25high, CD127low), mature (CD10High, CD16High, CD14-, CRTH2-) and immature mature (CD10dim, CD16dim, CD14-, CRTH2-) polymorphonuclear cells, as previously published [24,25].”

3) Innovative immune functional assays and exploration of new biomarkers: The reference to “home-made assays” is vague. The authors should explain the nature of these assays, specifically the targets to be analyzed (e.g. cytokine production), and a general identification of the experimental methodology (e.g. ELISA, Luminex, qPCR, etc.).

We agree with the reviewer that the wording “home made assays” is vague and non-informative. However, as the precise list of readouts is not definitive, we have not decided yet which technology will be used (ELISA, SiMOA, luminex, etc …). In order to enhance our manuscript, we have now replaced the initial sentence with the following (page 15, lines 287-288):

“The cytokine production levels in the supernatants of the functional assays will be quantified using commercial IVD or RUO assays.”

We hope that we have addressed all your questions and that you will be satisfied with the modifications added to the revised manuscript.

Reviewer: 2
Reviewer Name: Naeem Patil MD, PhD

Dear Dr Patil,

Thank you very much for this positive analysis of our study protocol and your questions, they have helped us to clarify and improve our manuscript.

1. Could the authors also measure differential leucocyte count from the whole blood of the patients? This is relatively easy standardized test and widely performed across hospitals and will provide will

additional information with respect to circulating immune cell numbers.

We agree with your suggestion that the report from the hematology lab will be a valuable source to compare our results from the flow cytometry cell counts. These data are indeed part of our data collection (see Table 4, section Biology, line 3). To increase clarity for the reader we completed our manuscript with the following sentence (page 14, lines 260-261):

“Complete blood cell count report from the hematology lab will be collected on each time point, this information will be compared to our cell counts results by flow cytometry. Beside, phenotypic immune […]”

2. Lymphocyte proliferation assay as described by authors will take 72 hours to complete. Therefore, the future clinical applicability of this test is questionable. Would it have been more appropriate to analyze other T cell functional assay such as interferon-gamma production upon ex vivo stimulation (for example with PMA/Ionomycin or anti-CD3/CD28), which could be accomplished relatively fast.

We thank both reviewers for raising this point. Obviously, our message was not clear on this point. We fully agree that T-cell proliferation is not suitable for immune status assessment in the routine management of ICU patients. We decided to use T-cell proliferation as a reference test for the adaptive immunity, due to its wide use in the characterization of immune deficiencies. However, one of the secondary objective of the REALISM project is indeed to identify new biomarkers or functional assays, that could replace such reference assay in the future, being more suited to the clinical management of ICU patients. In order to clarify this point to the readers, we have now added the following sentence to the manuscript (page 8, line 165-168):

“These new biomarkers / immune functional assays could therefore replace assays such as the T-cell proliferation assay, the current protocol of which is not suited to the routine management of ICU patients. We therefore expect to provide data to validate simpler diagnostic tools to determine and follow the immune status in hospitalized patients.”

3. Please mention the amount of blood that will be collected from patients and volunteers for this study.

As requested, a clarification on your question about the volume of blood sampled at each time point has been added in the methods section (page 12, line 213):
“Total volume of sampling will be 30 mL at each time point.”

4. For the TruCulture tubes, authors mention that the tubes contain a medium. Please add details about this medium.
We agree with the reviewer that this information could be useful to the reader. Unfortunately, the formulation of the TruCulture tubes medium is proprietary of MYRIAD. Therefore, we do not have access to its precise composition.

5. On page 13, authors mention that following stimulation in TruCulture tubes, supernatant will be collected? Will this be plasma alone? Or will it be mixed with the medium? Please add more details regarding the supernatant collection procedure.

More details about the TruCulture stimulation and supernatant recovery have been added in the

methods section as follow (page 13, lines 244-245):

“Following incubation, the supernatant (medium+plasma) will be collected using a separation valve (according to manufacturer instructions) and stored […]”.

6. For the cell proliferation assay, it would be more informative to include CD4+ and CD8+ surface markers along with CD3+ for flow cytometry analysis.

We agree with the reviewer that it might be interesting to analyze the proliferation of CD4 and CD8 T-cells subgroups. However, we thought in this project that focusing on T-cells was already an improvement (compared to tritiated thymidine assay for example). Moreover, a previous study published by our group (Venet et al, J Immunol 2012) has provided evidence that in septic shock patients the proliferation of CD4+ or CD8+ T cells are reduced in a similar manner. Nevertheless, your suggestion will be certainly taken into account for future projects to explore in more details the role of T cells proliferation in other types of patients, such as trauma, post-operatory or burn patients.

We hope that we have provided you enough clarification to your questions and that you agree with the modifications added in the revised manuscript

VERSION 2 – REVIEW

REVIEWER	William Carson University of Michigan Medical School, USA
REVIEW RETURNED	08-Mar-2017

GENERAL COMMENTS	The authors' revision has satisfactorily addressed all of the comments regarding the original manuscript.

REVIEWER	Naeem Patil Vanderbilt University Medical Center, USA
REVIEW RETURNED	23-Feb-2017

GENERAL COMMENTS	Authors have addressed this reviewers concerns.