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THE Reanimation Low Immune Status Markers (REALISM) PROJECT: BROAD CHARACTERIZATION AND FOLLOW-UP OF INJURY-INDUCED IMMUNOSUPRESSION IN Intensive Care Unit (ICU) CRITICALLY-ILL PATIENTS

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ABSTRACT

Introduction. The host response to septic shock is dynamic and complex. A sepsis-induced immunosuppression phase has recently been acknowledged and linked to bad outcomes and increased healthcare costs. Moreover, a marked suppression of the immune response has also been partially described in patients hospitalized in ICU for severe trauma or burns. It has been hypothesized that immune monitoring could enable identification of patients who might most benefit from novel, adjunctive immune stimulating therapies. However, there is currently neither a clear definition for such injury-induced immunosuppression nor a stratification biomarker compatible with clinical constraints.

Methods and Analysis. We set up a prospective, longitudinal single-center clinical study to determine the incidence, severity and persistency of innate and adaptive immune alterations in ICU patients. We optimized a workflow to describe and follow the immuno-inflammatory status of 550 patients (septic shock, severe trauma/burn and major surgery) during the first 2 months after their initial injury. On each time point, two immune functional tests will be performed to determine whole blood TNF-alpha production in response to *ex-vivo* Lipopolysaccharide stimulation and the T lymphocyte proliferation in response to Phytohaemagglutinin. In addition, a complete immunophenotyping using flow cytometry including monocyte HLA-DR expression and lymphocyte subsets will be obtained. New markers (i.e. levels of expression of host mRNA and viral reactivation) will be also evaluated. Reference intervals will be determined from a cohort of 150 age-matched healthy volunteers**.** This clinical study will provide, for the first time, data describing the immune status of severe ICU patients over time.

Ethics and dissemination. Ethical approval has been obtained from the Institutional Review Board (#69HCL15_0379) and the French National Security agency for drugs and health related products. Results will be disseminated through presentations at scientific meetings and publications in peer-reviewed journals.

Trial registration. Clinicaltrials.gov Registration number: NCT02638779.

KEYWORDS: Injury-induced immunosuppression; Intensive Care Unit ; Innate immunity; Adaptive immunity; Biomarkers; Health Care Associated infections.

Strengths and limitations of this study:

- REALISM will be the first study to provide a comprehensive body of data on the immune status in a large cohort of ICU patients.
- REALISM will allow to precisely describe the occurrence and depth of injury-induced immunosuppression in septic, trauma, burns and surgical patients. This project will also provide long term assessment (D60) of the immune status in ICU patients, which has never been done before.
- New biomarkers of the immune status will be assessed in comparison to standardized tools and immune functional assays.
- The role of host genomics, microbiota, as well as checkpoint inhibitors expression will not be assessed in this study.
- Whether such biomarkers would permit to stratify patients for immunomodulatory treatments should be addressed in future studies.

INTRODUCTION

Sepsis is a major health problem and the main etiology for ICU admissions [1,2]. Its incidence is increasing over the years due to several factors, including a better awareness and an aging population [3]. Hospital admissions for sepsis have thus overtaken those for stroke and myocardial infarction [4]. Despite advances on its management, mortality of sepsis has remained stable over the last 20 years, reaching 30-40% in case of septic shock, the most severe form, and it is the leading cause of death in ICU.

Sepsis is a severe infection, defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" [5]. Besides circulatory and metabolic abnormalities, the multifaceted host response to the invading pathogen is amplified by comorbid conditions [6,7]. It is now acknowledged that the pro-inflammatory response, which can lead to organ failure, comes with a compensatory anti-inflammatory response. Recovery occurs when inflammation resolves quickly. However, in numerous patients, the anti-inflammatory response lingers on and leads to an immunosuppression state, associated with secondary infections, and increased morbidity and mortality [8]. This sepsis-induced immunosuppression could explain the failure of several previous clinical trials and support new innovative trials testing immune adjuvant drugs in septic shock [9].

Therefore, several studies and case reports now support the rational of boosting the immune system, in order to avoid the occurrence of health-care associated infection and therefore reduce the associated morbidity [10,11]. However, to avoid reproducing the errors from the past, such innovative treatments should be administered only to those individuals identified as immunosuppressed [11]. Some studies have already demonstrated that the concept of biomarker-guided therapeutic stratification can lead to clinical improvements [12].

A marked immunosuppression has been partially described in other patients admitted to the ICU for severe trauma/burns and other major surgeries [12–15]. In these "sterile" injuries, signs of injury-induced immune alterations have also been associated with increased susceptibility to secondary infections and mortality.

Given the complexity and heterogeneity of ICU patients, it is unlikely that any single biomarker will be sufficient to describe and diagnose injury-induced immunosuppression. On the contrary, a panel of validated biomarkers may bring enough information to accomplish such complex endeavor.

Rationale of the study

From a clinical perspective, no specific clinical signs or symptoms are associated with a state of altered immune response to allow prospective identification of at risk patients. Further, the outcomes of sustained immunosuppression are best defined by clinical relevant endpoints such as the occurrence of opportunistic and secondary infections. However, waiting for such a health-care associated infection to occur does not facilitate implementation of preventive strategies. Thus, diagnosis will rely on biomarkers.

From a biological perspective, sepsis-induced immunosuppression may be best identified by immune *functional* assays (such as cytokine release or lymphocytes proliferation after *ex-vivo* stimulation), or cell count parameters (such as, number of lymphocytes or level of expression of mHLA-DR) but both approaches present drawbacks. Indeed, such functional assays are not suitable to stratify patients in a prospective interventional clinical trial due to (1) the long time to results (up to 5 days for lymphocytes proliferation), and (2) poor reproducibility due to standardization issues and cumbersome technique. Due to such complexity, these reference tests are rarely performed in clinical studies evaluating biomarkers associated with deleterious outcomes in ICU.

On the other hand, HLA-DR expression on monocytes is currently the best biomarker available for such a routine use [17], and is being employed for patient stratification in a large multicenter interventional trial assessing the administration of GM-CSF in patients with septic shock [18]. However, its measurement requires flow cytometry analysis within 4hours of blood sampling which may not be available in all centers, making interlaboratory standardization challenging.

As a consequence of the previously discussed challenges, numerous biomarkers proposed to monitor injury-induced immune alterations have yet to be compared to these *reference* assays.

Hypothesis

Although several studies have shown an association between markers related to the immune system (e.g. HLA-DR) and the occurrence of healthcare-associated infections in septic patients [14,15,19], we still do not have a clear and operational definition of the immune deficiency that occurs in severely injured ICU patients. Precise description of injury-induced immunosuppression incidence and its characteristics are lacking. In the REALISM (REAnimation Low Immune Status Markers) project, we propose to broadly assess immune parameters over time and to correlate these findings with clinical epidemiologic data and outcomes in order to identify and define immunosuppression in ICU patients both in terms of magnitude and time duration.

To this aim, we have established two standardized functional immune assays (whole blood TNFα release after ex-vivo stimulation with LPS (Lipopolysaccharides) [20] and lymphocyte proliferation in response to *ex-vivo* stimulation with PHA (Phytohaemagglutinin) [21]. ***We propose to define the status of immunosuppression on the basis of an abnormal result (values outside the reference intervals) obtained in at least one of the two "reference" test.***

The REALISM project aims to provide a validated operational definition of injury-induced immunosuppression predicting clinically relevant outcomes. This will facilitate development of new tools and biomarkers with the goal of introducing diagnosis of immune suppression into routine clinical practice and allow patient stratification for the evaluation of new individual immunotherapies. It may also enable the identification of new targets and the development of new innovative therapeutics to treat ICU patients and prevent opportunistic infections in the future

Primary Aim

The primary objective of the study is to determine the incidence of injury-induced immunosuppression in ICU patients, during the first two months after injury.

Secondary Aims

The secondary objectives of the study are:

- To describe the occurrence of immunosuppression, its depth and impact on innate and adaptive immune responses, and its evolution during the first 2 months after injury.
- To assess the strength of the proposed definition, in particular by evaluating its association with secondary infections and mortality.
- To assess the accuracy of new biomarkers and immune functional assays to diagnose immunosuppression.

METHODS AND ANALYSIS

REALISM is a prospective longitudinal, single-center observational study, conducted in the anesthesiology and intensive care department at the Edouard Herriot hospital (University hospital, Lyon France, capacity of approximately 1,000 beds).

Study population

REALISM will include healthy volunteers (n=150) and patients at risk of injury-induced immunosuppression: (1) septic shock patients (n=160); (2) severe trauma patients (n=180); (3) severe burns patients (n=30); and (4) patients admitted to the ICU after major surgery (n=180).

Septic shock inclusion criteria follow the current definition [5] and require a state of shock defined by vasopressors administration and plasma lactate level above 2 mmol/L (18 mg/dL). An infection must be suspected, and microbiological sampling should have been performed, along with the administration of antimicrobials. Only primary septic shock will be considered (vasopressors should have been started within the first 48hours after ICU admission) [5].

Patients with severe trauma, defined by an injury severity score (ISS, Baker *et al*., 1974) > 15 [22], will be included in the study. As we hypothesized that the depth of immunosuppression might be related to severity, we will limit the group of patients between ISS = [15-25] to 90 patients to ensure at least 50% of the cohort includes patients with an ISS > 25. Severe burn patients will be selected for inclusion based on a total burn surface area over 30%.

Surgical patients will be screened according to the planned surgical procedure. This study will include patients undergoing: 1) eso-gastrectomy; (2) Bricker's bladder resection (total bladder resection with reconstruction from small bowel); (3) cephalic pancreaticoduodenectomy (Whipple's procedure); (4) abdominal aortic aneurism surgery by laparotomy.

Exclusion criteria are mainly related to factors that might impact the immune status and bias the results such as: severe neutropenia (neutrophil count <0.5 G/L), administration of immunosuppressive therapy, corticosteroids (IV if oral administration), use of therapeutic antibodies (such as anti-TNF alpha), onco-hematological disease (e.g. lymphoma, leukemia) under treatment or treated within 5 years before inclusion, end of chemotherapy within the 6 months prior to inclusion date. Patients with congenital/hereditary or acquired immune deficiency (for example severe combined immunodeficiency, HIV or AIDS, at any stage), and patients that have received extra-corporeal circulation in the month preceding inclusion will be excluded as well.

Considering the possible influence of gender bias on measured parameters, we will recruit healthy donors from both genders, following the age and gender distribution of the French population.

Complete lists of the inclusion and exclusion criteria for patients and healthy volunteers are presented in Table1 and Table 2 respectively.

Table 1. Inclusion and exclusion criteria for patients
Inclusion criteria
Male or female aged over 18 years
Patient or next of kin having been informed of the conditions of the study and having signed the informed consent form
Patient hospitalized for:
Septic shock, defined by:
Infection site suspected, and microbiological analysis sampling carried out
Vasopressor therapy needed to elevate MAP (Mean Arterial Pressure) ≥ 65 mm Hg and lactate > 2 mmol/L (18 mg/dL) despite adequate fluid resuscitation [26]
Noradrenalin > 0.20 µg/kg/min for at least 2 hours
Noradrenalin started within 48 hours after ICU admission
Serious trauma, defined by:
Patient admitted directly to the recruiting ICU
Injury Severity Score Baker *et al*., 1974 > 15 [22]
Severe burns, defined by:
Total burned surface area > 30%
Major surgery, defined by:

Surgery set for one of the following indications: (i) eso-gastrectomy; (ii) Bricker's bladder resection (total bladder resection with reconstruction from small bowel); (iii) cephalic pancreaticoduodenectomy (Whipple's procedure); (iv) abdominal aortic aneurism surgery by laparotomy. Categories i to iii concern management of solid tumors, while category iv concerns non-cancerous pathologies
Induction of anesthesia before 11 am (to permit same day processing of all samples)
Exclusion criteria
Patient with severe neutropenia (neutrophil count <0.5 G/L)
Patients receiving immunosuppressive therapy
Corticosteroids (IV or Per os).
Use of therapeutic antibodies
Onco-hematological disease (ex. Lymphoma, leukemia…) under treatment, or treated within 5 years before inclusion
End of chemotherapy within the 6 months prior to inclusion date
Patient with innate or acquired immune deficiency (for example severe combined immunodeficiency, HIV or AIDS, any stage)
Patients with a "do not resuscitate order" or a "withdraw of care" decision, at time of inclusion
Patient whose anticipated duration of hospitalization in the ICU is estimated at less than 48 hours
Participation in any interventional study
Extra-corporeal circulation in the month preceding inclusion in the case of cardiac surgery
Pregnant or breastfeeding women
Patient with no social security insurance, with restricted liberty or under legal protection

Table 2: Inclusion and exclusion criteria for healthy volunteers

Inclusion criteria
Male or female aged over 18 years
Normal clinical examination
Signed informed consent form
Person with social security insurance
Exclusion criteria
Person with an infectious syndrome during the last 90 days
Extreme physical stress within the last week
Person having received within the last 90 days, a treatment based on:
Antivirals
Antibiotics
Antiparasitics
Antifungals
Person having received within the last 15 days, a treatment based on non-steroidal anti-inflammatory drugs (NSAIDs)
Person having received within the last 24 months, a treatment based on:

Immunosuppressive therapy
Corticosteroids (IV or Per os)
Therapeutic antibodies
Chemotherapy
History of :
Innate or acquired immune deficiency
Hematological disease
Solid tumor
Severe chronic disease
Surgery or hospitalization within the last 2 years
Pregnancy within the last year
Participation to a phase I clinical assay during the last year
Participation to a phase I clinical assay during the last year
Pregnant or breastfeeding women
Person with restricted liberty or under legal protection

Sampling schedule

Samples and clinical data will be collected 3 to 4 times within the first week (early time-points) with the aim to evaluate the modulation of the immune status early after injury. Samples will be collected at day 1 (the morning following injury), at day 2 (for the severe trauma group) and at day 3/4 and day 5/7 (Table 3). Samples will also be collected before surgery, at day 0, as surgical patients are the only group for which sampling can be performed before injury. Additional samples will be collected during late time points to evaluate the recovery of the immune status, at day 14 (between day 13 to18), day 28 (between day 26 to 36) and day 60 (between day 52 to 68), depending on patient availability and technical constraints (Figure 1).

Definition of Immunosuppression

The REALISM project will monitor the immune function of the patients and healthy volunteers using two standardized immune functional tests: one reference test to evaluate the innate immune response (whole blood production of TNF-alpha in response to ex vivo stimulation by LPS) and a second reference test for the adaptive immune response (the lymphocyte proliferation in response to ex vivo T cell stimulation with PHA). Immunosuppression will be defined in comparison to the values as

obtained in a group of healthy volunteers for the two reference tests using the following methodology. First, reference intervals (RI) will be derived from the independent set of healthy volunteers. Second, immunosuppression will be defined in a patient when an abnormal result (value outside the reference intervals) is obtained in at least one of the two "reference" tests over at least two consecutive time points

Definition of Secondary infection

During the ICU stay, patients will be screened daily for exposure to invasive devices (intubation, indwelling urinary catheter, and central venous line) and occurrence of secondary infection. Information referent to infections will be collected, reviewed and validated by a dedicated adjudication committee, composed of 3 physicians not involved in the recruitment of the patients with confirmation of secondary infection made according to the definitions used by the European center for disease prevention and control [23] and the Infectious Diseases Society of America.

Immune functional assays

Innate immune response: TNF-α release after LPS whole blood stimulation

Innate immune response will be evaluated by measuring the production of TNF-α in response to *ex vivo* stimulation of whole blood by LPS [20]. The stimulation will be performed through the use of standardized TruCulture® tubes from MYRIAD RBM (MYRIAD RBM; Austin, USA) (the concentration, quality and activity of the LPS is guaranteed by the manufacturer MYRIAD RBM) [20]. The tubes contain the medium alone (Null) or the medium with LPS 100 ng/ml (LPS from *E.coli* O55:B5) (LPS-R; Null-R; MYRIAD RBM). The blood samples will be collected on heparin and transported to the laboratory where 1 ml of heparinized blood will be transferred to each TruCulture® tube and incubated for 24 hours at 37°C. Following incubation, the supernatant will be collected and stored at -80°C until batch quantification of TNF-α by ELISA (BE55001; BL International-Tecan; Männedorf, Switzerland).

Adaptive immune response: T lymphocyte proliferation after ex vivo PBMCs mitogenic stimulation

Adaptive immune response will be assessed by measuring T lymphocyte proliferation in response to *ex vivo* stimulation with a mitogen [21]. Briefly, Peripheral Blood Mononuclear Cells (PBMC) isolated by Ficoll density gradient centrifugation (U-04; Eurobio; Les Ulis, France) will be stimulated with PHA (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) in T cells (gated as CD3+) using flow cytometry [21].

Cellular Immunophenotyping

Phenotypic immune cells characterization and cell counting will be completed by flow cytometry. We will count the number of B-lymphocytes, CD4+ and CD8+ T-lymphocytes, NK cells, regulatory T-lymphocytes, mature and immature polymorphonuclear cells, as previously published [24,25]. In addition, the number of HLA-DR molecules per monocyte will be determined using the BD quantibrite standardized method (HLA-DR:340827; QuantiBRITE:340495; Becton Dickenson; New Jersey, USA) [26]. It is well known that the flow cytometry is highly sensitive to variation between labs and instruments; therefore a validation with the routine hospital immunology lab was performed to guarantee that all the protocols reproducible and standardized. All procedures generated results with less than 20% of variation when compared to reference protocols.

Biobanking

This study will provide the opportunity to establish four different types of biobanks to preserve the material collected, enabling exploration of innovative biomarkers: (1) Truculture® plasma biobank from whole blood stimulated with LPS, SEB *(Staphylococcus aureus* enterotoxin-B) or not stimulated, to study cytokines release. (2) EDTA plasma biobank to study viral reactivation markers and soluble host biomarkers. (3) Heparin plasma biobank for metabolomics/proteomics soluble host

biomarkers studies; (4) RNA biobank to study new transcriptomic host biomarkers (RNA will be extracted from whole blood collected in PAXgene® tubes).

Innovative immune functional assays and exploration of new biomarkers

Regarding the immune functional tests, other stimulants (e.g. SEB) and read-outs (e.g. Interleukin 2, Interferon gamma) will be tested using the TruCulture® tubes and commercial or home-made assays. Finally, a metabolomics and proteomics study will be performed using frozen (heparin) plasma. Biomarkers potentially associated to immune deficiency will be identified by LC-MS on high resolution mass spectrometry and 1H-NMR, after polar and non-polar samples extraction.

Sample size and data analysis plan

Population sizing

The number of healthy volunteers required to determine the reference intervals for the two immune reference tests was defined according to the methodology recommended by the Clinical and Laboratory Standards Institute (CLSI) C28-A3 guidelines [27]. The minimal number of subjects recommended being 120, after exclusion of aberrant values (confidence interval of 90%), we decided to include 150 healthy volunteers to take into account exclusions related to technical reasons, aberrant values or consent withdrawal.

For this reference population, the age range of healthy volunteers group has been carefully calculated to include the expected age range and gender distribution from ICU patients in France (Table 3).

Table 3. Age and gender distribution for the reference group

Age range	Male	F
[19-30[	14	14
[30-50[	25	25
[50-65[	18	19

[65-100[	15	20
Total	72	78

The main objective being descriptive, the computation of the sample size was based on secondary objectives, especially for 1) the analysis of the occurrence of immunosuppression, its depth and impact on innate and adaptive immune responses (Cohen d is 0.55) and 2) the correlation between new biomarkers and immune functional assays to diagnose immunosuppression (r>0.4). A Student t-test was used to approximate the number of patients needed and a minimum of 150 patients per group was required for a standardized Cohen's d effect = 0.55, if we get the recommended number of healthy volunteers of 120. It was therefore decided to include 160 septic shock patients, 180 severe trauma patients and 180 patients with a major surgery, to overcome secondary exclusions for technical causes or consent withdrawal. The severe burn patients group is an ancillary group that was arbitrary fixed at 30 subjects in order to collect data with the intent to inform a dedicated study on this population in the future.

Statistical analysis.

First, the percentage of patients meeting the definition of injury-induced-immunosuppression will be computed in each patients group to answer the main objective. Second, the occurrence of immunosuppression will be further described. The proportion of patients with at least one abnormal test will be computed for both immune reference tests and each patients group. The correlation between the 2 reference tests will be established from a Spearman correlation test. A mixed model will be constructed to describe the extent of the changes in the innate and adaptive measures over time, taking groups and time points into account. Third, a comparison of each biomarker or new functional tests with the two reference tests will be performed using a Spearman correlation test. For correlated biomarkers or functional tests, the performance for prediction of secondary infection will be estimated from a ROC curve. A Fine & Gray predictive model will be constructed [28] for the biomarkers harboring the best areas under curve, taking into account the competing risk of mortality.

Finally, multiple imputations will be taken into consideration in the case of a relevant amount of missing values.

ETHICS AND DISSEMINATION

Ethics approval

The protocol, information documents and consent forms received approval by the local Institutional Review Board (Comité de Protection des Personnes Sud-Est II, Bron, France) and the French National Security agency for drugs and health related products (Approval code: 69HCL15_0379, 30th of November of 2015). An amendment has been filled to extend sampling time points over the first week and add the metabolomics and proteomics study. This amendment has been approved on the 22nd of July of 2016 (protocol version 3). This study complies with the Declaration of Helsinki, principles of Good Clinical Practice and the French personal data protection act.

Informed consent

The free and informed consent of each patient and healthy volunteer will be obtained following a complete and faithful information, in comprehensive words, of the objectives, the proceedings and the constrains of the study, the right to refuse the enrollment or the possibility to withdraw at any time, when he/she is in capacity to understand. The patient (or next of kin) will also be informed of: (1) the existence of processing system for data concerning them; (2) Their right to access and rectify these data (accessible through the physician of their choice); (3) The possibility of the use of remaining biological material and associated data stored following the end of the study and their possible transfer to another academic or private party. This information is part of the written notice and the inform consent.

If the patient is not in capacity to understand and/or express his/her consent, the informed consent will be obtained from a next of kin. In the event that only the inform consent of a third party has been sought at the time of inclusion, the patients should be informed as soon as possible of their participation in this study and be asked to give their own consent to continue the study.

If the next of kin is not present and not available by phone, the patient may be included in emergency situation. The investigator will be required to record all steps for calling the next of kin in the medical record (Contact attempts with date, time and phone number ...) and justify patient inclusion in medical emergencies in accordance with French legislation. The written consent of the next of kin and the patient should be obtained as soon as the person is available and as soon as the patient's clinical condition allows. The consent form contains the possibility to refuse the storage of samples after the end of the study.

Safety of participants

This study includes no serious foreseeable risk to the health of the persons involved. The only potential risk is related to blood sample collection (maximum 192 ml collected over all time points – 2 months). However, this aspect of nursing is part of daily practice. Blood samples will be taken under the same conditions of safety as currently used for common diagnostic tests.

Study management

The study is managed by BIOASTER, and a dedicated team composed from members of all the consortium partners. The promoter of the study is the Hospices Civils de Lyon. The principal investigator is Dr Thomas Rimmelé.

Data management

Clinical data

For each patient, an electronic case report form including socio-demographic, clinical and para-clinical information will be completed by clinical research assistants (Table 4). A description of the hospital stay, the documentation on the type of injury (surgery, burn, trauma or septic shock) and the severity as defined by the ASA classification, SOFA score [29] and SAPSII score [30]. In addition, we will collect routine lab results about the CMV, HSV1 serology and Complete Blood Count (CBC).

Moreover, we will document if there is any specific treatments administered to the patient, such as antibiotics, exposure to invasive medical devices and secondary infections. All data will be transferred to a TranSMART [31] database following curation for data exploration and analysis.

Table 4 Clinical and biological data collection planning.

						ICU	Hospital	**J14**	J28	**J60**	
	D0 [a]	**D1 (2[f])**	**D2** [c]	**D3/4**	**D5/7**	Release	release	D13/18	D26/36	D52/68	**D90**
Inclusion/exclusion criteria		x [b]									
Consent form		x [b]									
Demography		x [b]									
Weight		x [b]									
Size		x [b]									
Description of hospital stay		x [b]									
IGS II score		x [b]									
McCabe score		x [b]									
CHARLSON score		x [b]									
Documentation of the septic shock, surgery, burn or trauma		x [b]									
SOFA score	x	x	x	x	X						
Treatments against infections	Steadily							x	x	x	x
Therapeutic management	Steadily										
Exposition to medical devices	Steadily										
Surveillance of health-care associated infections	Steadily							x[d]	x [d]	x [d]	x [d]
Concomitant events	Steadily							x	x	x	x
Vital status						X	x	x	x	x	x
Life quality (EQ5D)											x
Biology											
PAXgene® tube sampling	x	x	x	x	X			x	x	x	
EDTA tubes sampling	x	x	x	x	X			x	x	x	
Heparin tubes sampling	x	x	x	x	X			x	x	x	
Hematology	x	x	x	x	X			x	x	x	
Lactate	x[e]	x[e]	x[e]	x[e]	x[e]						
pH	x[e]	x[e]	x[e]	x[e]	x[e]						
Liver results (ASAT, ALAT, PAL)	x[e]	x[e]	x[e]	x[e]	x[e]						

Procalcitonin	x[e]	x[e]	x[e]	x[e]	x[e]			x[e]	x[e]	x[e]	x[e]
Serology (CMV, HSV1)		X[b]									
[a] Only for patients of the surgery group											
[b] Evaluation on day 0 for patients of the surgery group (not repeated on day 1)											
[c] Only for patients of the trauma group											
[d] only if related to a new hospitalization											
[e] if available											
[f] for the septic shock and burn patients: The enrollment at D2 will be accepted if D1 is not available											

Duration of the study

The study is planned to run for thirty months, starting December 2015. The expected end date for recruitment is June 2018. Some biomarkers will be quantified by batch analysis, at the end of the study. Primary data analysis is expected to be completed with subsequent dissemination of results by December 2018

Acknowledgement

Sponsorship

The project is funded by a consortium: bioMérieux, SANOFI, GlaxoSmithKline, Ecole Supérieure de Physique Chimie Industrielles de la ville the Paris – PSL Research University, the University Hospital Hospices Civils de Lyon and the microbiology technological institute BIOASTER. The project is financially supported in part by public funding through BIOASTER and Hospices Civils de Lyon. The project will be audited annually by the French National Research Agency ("Investissement d'Avenir" program; grant n°ANR-10-AIRT-03).

Consent for publication

Not applicable

Competing interests

AP, JT, VM are employees of bioMérieux, an in-vitro diagnostic company. PC, LK and DG are employees of Sanofi-Aventis R&D, Sanofi-Pasteur SA, and GlaxoSmithKline, three pharmaceutical companies.

Data sharing statement

Results will be disseminated through presentations at scientific meetings and publications in peer-reviewed journals. New markers and immune functional tests will be evaluated for the diagnostic immune deficiency and may be patentable.

Contributorship statement:

All authors (MLR, FV, TR, VM, PC, LQ, DG, AG, AP, JT, GM) fulfils ICMJE guidelines and (1) provided substantial contributions to conception, design and cquisition of data, (2) drafted and revised critically the manuscript and, (3) approved the final version of the manuscript.

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REFERENCES

1 Jawad I, Lukšić I, Rafnsson SB. Assessing available information on the burden of sepsis: global estimates of incidence, prevalence and mortality. *J Glob Health* 2012;2:4.

2 Vincent J-L, Marshall JC, Ñamendys-Silva SA, *et al.* Assessment of the worldwide burden of critical illness: the Intensive Care Over Nations (ICON) audit. *Lancet Respir Med* 2014;2:380–6.

3 Walkey AJ, Lagu T, Lindenauer PK. Trends in sepsis and infection sources in the United States. A population-based study. *Ann Am Thorac Soc* 2015;12:216–20.

4 Seymour CW, Rea TD, Kahn JM, *et al.* Severe Sepsis in Pre-Hospital Emergency Care: Analysis of Incidence, Care, and Outcome. *Am J Respir Crit Care Med* 2012;186:1264–71.

5 Singer M, Deutschman CS, Seymour CW, *et al.* The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016;315:801. doi:10.1001/jama.2016.0287

6 Angus DC, van der Poll T. Severe Sepsis and Septic Shock. *N Engl J Med* 2013;369:840–51.

7 Cohen J, Vincent J-L, Adhikari NK, *et al.* Sepsis: a roadmap for future research. *Lancet Infect Dis* 2015;15:581–614.

8 Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013;13:862–74.

9 Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *Lancet Infect Dis* 2013;13:260–8.

10 Delsing CE, Gresnigt MS, Leentjens J, *et al.* Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series. *BMC Infect Dis* 2014;14:166.

11 Venet F, Lukaszewicz A-C, Payen D, *et al.* Monitoring the immune response in sepsis: a rational approach to administration of immunoadjuvant therapies. *Curr Opin Immunol* 2013;25:477–83.

12 Meisel C, Schefold JC, Pschowski R, *et al.* Granulocyte–Macrophage Colony-stimulating Factor to Reverse Sepsis-associated Immunosuppression: A Double-Blind, Randomized, Placebo-controlled Multicenter Trial. *Am J Respir Crit Care Med* 2009;180:640–8.

13 Gentile LF, Cuenca AG, Efron PA, *et al.* Persistent inflammation and immunosuppression: A common syndrome and new horizon for surgical intensive care. *J Trauma Acute Care Surg* 2012;72:1491–501.

14 Angele MK, Chaudry IH. Surgical trauma and immunosuppression: pathophysiology and potential immunomodulatory approaches. *Langenbecks Arch Surg* 2005;390:333–41.

15 Kimura F, Shimizu H, Yoshidome H, *et al.* Immunosuppression following surgical and traumatic injury. *Surg Today* 2010;40:793–808.

16 Timmermans K, Kox M, Vaneker M, *et al.* Plasma levels of danger-associated molecular patterns are associated with immune suppression in trauma patients. *Intensive Care Med* 2016;42:551–61.

17 Gossez M, Malcus C, Demaret J, *et al.* Evaluation of a Novel Automated Volumetric Flow Cytometer for Absolute CD41 T Lymphocyte Quantitation. Cytometry B Clin. Cytom. 2016. 10.1002/cyto.b.21360

18 http://www.clinicaltrial.gov/.

19 van Vught LA, Klein Klouwenberg PMC, Spitoni C, *et al.* Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis. *JAMA* 2016;315:1469-79.

20 Duffy D, Rouilly V, Libri V, *et al.* Functional Analysis via Standardized Whole-Blood Stimulation Systems Defines the Boundaries of a Healthy Immune Response to Complex Stimuli. *Immunity* 2014;40:436–50.

21 Poujol F, Monneret G, Friggeri A, *et al.* Flow cytometric evaluation of lymphocyte transformation test based on 5-ethynyl-2'deoxyuridine incorporation as a clinical alternative to tritiated thymidine uptake measurement. *J Immunol Methods* 2014;415:71–9.

22 Baker S, O'Neill B, Haddon W, *et al.* The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. J Trauma. 1974;14:187–96.

23 European Centre for Disease Prevention and Control. Point prevalence survey of healthcareassociated infections and antimicrobial use in European acute care hospitals. ECDC. 2012;ISBN 978–92 – 9193–366 – 2.

24 Demaret J, Venet F, Friggeri A, *et al.* Marked alterations of neutrophil functions during sepsis-induced immunosuppression. *J Leukoc Biol* 2015;98:1081–90.

25 Venet F, Chung C-S, Kherouf H, *et al.* Increased circulating regulatory T cells (CD4+CD25+CD127−) contribute to lymphocyte anergy in septic shock patients. *Intensive Care Med* 2009;35:678–86.

26 Docke W-D. Monitoring Temporary Immunodepression by Flow Cytometric Measurement of Monocytic HLA-DR Expression: A Multicenter Standardized Study. *Clin Chem* 2005;51:2341–7.

27 Horowitz, Gary L, Altaie, Sousan, Boyd, James C, *et al.* CLSI C28-A3 Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory Approved Guideline. 2008.

28 Fine JP, Gray RJ. A Proportional Hazards Model for the Subdistribution of a Competing Risk. *J Am Stat Assoc* 1999;94:496.

29 Vincent JL, Moreno R, Takala J, *et al.* The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996;22:707–10.

30 Le Gall JR, Lemeshow S, Saulnier F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA J Am Med Assoc* 1993;270:2957–63.

31 TranSMART [http://transmartfoundation.org/].

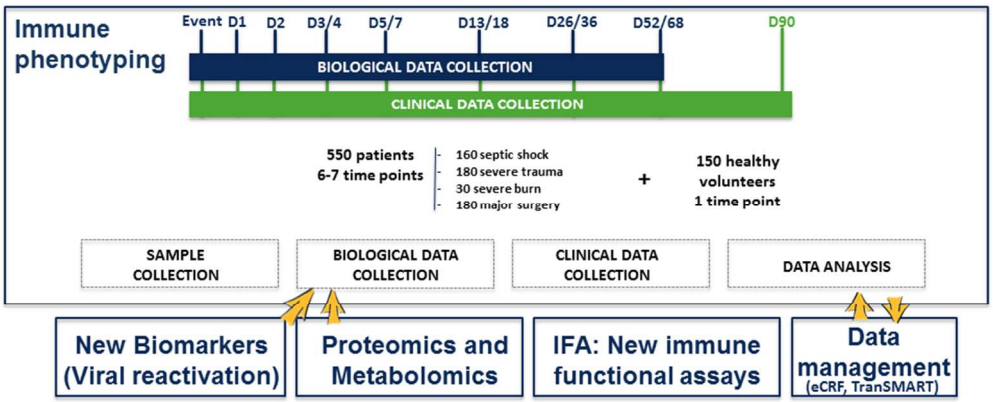

Figure 1

254x101mm (96 x 96 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Yes
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Yes
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	Yes
Funding	4	Sources and types of financial, material, and other support	Yes
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Yes
	5b	Name and contact information for the trial sponsor	Yes
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Yes
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Yes
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Yes
	6b	Explanation for choice of comparators	Yes
Objectives	7	Specific objectives or hypotheses	Yes

Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Yes
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Yes
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Yes
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	NA
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Yes
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Yes
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Yes
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Yes
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	No
Methods: Assignment of interventions (for controlled trials)			

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Yes
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Yes
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Yes
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Yes
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Yes

	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Yes
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Yes
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	Yes
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Yes
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	Yes
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Yes
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	Yes
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Ye
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Yes
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Yes

Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Yes
	31b	Authorship eligibility guidelines and any intended use of professional writers	Yes
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Yes
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Yes

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

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

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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

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ABSTRACT

Introduction. The host response to septic shock is dynamic and complex. A sepsis-induced immunosuppression phase has recently been acknowledged and linked to bad outcomes and increased healthcare costs. Moreover, a marked suppression of the immune response has also been partially described in patients hospitalized in ICU for severe trauma or burns. It has been hypothesized that immune monitoring could enable identification of patients who might most benefit from novel, adjunctive immune stimulating therapies. However, there is currently neither a clear definition for such injury-induced immunosuppression nor a stratification biomarker compatible with clinical constraints.

Methods and Analysis. We set up a prospective, longitudinal single-center clinical study to determine the incidence, severity and persistency of innate and adaptive immune alterations in ICU patients. We optimized a workflow to describe and follow the immuno-inflammatory status of 550 patients (septic shock, severe trauma/burn and major surgery) during the first 2 months after their initial injury. On each time point, two immune functional tests will be performed to determine whole blood TNF-alpha production in response to *ex-vivo* Lipopolysaccharide stimulation and the T lymphocyte proliferation in response to Phytohaemagglutinin. In addition, a complete immunophenotyping using flow cytometry including monocyte HLA-DR expression and lymphocyte subsets will be obtained. New markers (i.e. levels of expression of host mRNA and viral reactivation) will be also evaluated. Reference intervals will be determined from a cohort of 150 age-matched healthy volunteers**.** This clinical study will provide, for the first time, data describing the immune status of severe ICU patients over time.

Ethics and dissemination. Ethical approval has been obtained from the Institutional Review Board (#69HCL15_0379) and the French National Security agency for drugs and health related products. Results will be disseminated through presentations at scientific meetings and publications in peer-reviewed journals.

Trial registration. Clinicaltrials.gov Registration number: NCT02638779.

KEYWORDS: Injury-induced immunosuppression; Intensive Care Unit ; Innate immunity; Adaptive immunity; Biomarkers; Health Care Associated infections.

Strengths and limitations of this study:

- First prospective study to provide a broad immune status characterization in a large cohort of ICU patients.
- Mid-term assessment (D60) of the immune status in ICU patients, which has never been done before.
- Long term follow up will not addressed here and should be examined in future studies.
- New biomarkers of the immune status will be assessed in comparison to standardized tools and immune functional assays.
- Whether such biomarkers would permit to stratify patients for immunomodulatory treatments should be addressed in future studies.
- The role of host genomics, microbiota, as well as checkpoint inhibitors expression will not be assessed in this study.

INTRODUCTION

Sepsis is a major health problem and the main etiology for ICU admissions [1,2]. Its incidence is increasing over the years due to several factors, including a better awareness and an aging population [3]. Hospital admissions for sepsis have thus overtaken those for stroke and myocardial infarction [4]. Despite advances on its management, mortality of sepsis has remained stable over the last 20 years, reaching 30-40% in case of septic shock, the most severe form, and it is the leading cause of death in ICU.

Sepsis is a severe infection, defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" [5]. Besides circulatory and metabolic abnormalities, the multifaceted host response to the invading pathogen is amplified by comorbid conditions [6,7]. It is now acknowledged that the pro-inflammatory response, which can lead to organ failure, comes with a compensatory anti-inflammatory response. Recovery occurs when inflammation resolves quickly. However, in numerous patients, the anti-inflammatory response lingers on and leads to an immunosuppression state, associated with secondary infections, and increased morbidity and mortality [8]. This sepsis-induced immunosuppression could explain the failure of several previous clinical trials and support new innovative trials testing immune adjuvant drugs in septic shock [9].

Therefore, several studies and case reports now support the rational of boosting the immune system, in order to avoid the occurrence of health-care associated infection and therefore reduce the associated morbidity [10,11]. However, to avoid reproducing the errors from the past, such innovative treatments should be administered only to those individuals identified as immunosuppressed [11]. Some studies have already demonstrated that the concept of biomarker-guided therapeutic stratification can lead to clinical improvements [12].

A marked immunosuppression has been partially described in other patients admitted to the ICU for severe trauma/burns and other major surgeries [13–16]. In these "sterile" injuries, signs of injury-induced immune alterations have also been associated with increased susceptibility to secondary infections and mortality.

Given the complexity and heterogeneity of ICU patients, it is unlikely that any single biomarker will be sufficient to describe and diagnose injury-induced immunosuppression. On the contrary, a panel of validated biomarkers may bring enough information to accomplish such complex endeavor.

Rationale of the study

From a clinical perspective, no specific clinical signs or symptoms are associated with a state of altered immune response to allow prospective identification of at risk patients. Further, the outcomes of sustained immunosuppression are best defined by clinical relevant endpoints such as the occurrence of opportunistic and secondary infections. However, waiting for such a health-care associated infection to occur does not facilitate implementation of preventive strategies. Thus, diagnosis will rely on biomarkers.

From a biological perspective, sepsis-induced immunosuppression may be best identified by immune *functional* assays (such as cytokine release or lymphocytes proliferation after *ex-vivo* stimulation), or cell count parameters (such as, number of lymphocytes or level of expression of mHLA-DR) but both approaches present drawbacks. Indeed, such functional assays are not suitable to stratify patients in a prospective interventional clinical trial due to (1) the long time to results (up to 5 days for lymphocytes proliferation), and (2) poor reproducibility due to standardization issues and cumbersome technique. Due to such complexity, these reference tests are rarely performed in clinical studies evaluating biomarkers associated with deleterious outcomes in ICU.

On the other hand, HLA-DR expression on monocytes is currently the best biomarker available for such a routine use [17], and is being employed for patient stratification in a large multicenter interventional trial assessing the administration of GM-CSF in patients with septic shock [18]. However, its measurement requires flow cytometry analysis within 4 hours of blood sampling which may not be available in all centers, making interlaboratory standardization challenging.

As a consequence of the previously discussed challenges, numerous biomarkers proposed to monitor injury-induced immune alterations have yet to be compared to these *reference* assays.

Hypothesis

Although several studies have shown an association between markers related to the immune system (e.g. HLA-DR) and the occurrence of healthcare-associated infections in septic patients [14,15,19], we still do not have a clear and operational definition of the immune deficiency that occurs in severely injured ICU patients. Precise description of injury-induced immunosuppression incidence and its characteristics are lacking. In the REALISM (REAnimation Low Immune Status Markers) project, we propose to broadly assess immune parameters over time and to correlate these findings with clinical epidemiologic data and outcomes in order to identify and define immunosuppression in ICU patients both in terms of magnitude and time duration.

To this aim, we have established two standardized functional immune assays (whole blood TNFα release after ex-vivo stimulation with LPS (Lipopolysaccharides) [20] and lymphocyte proliferation in response to *ex-vivo* stimulation with PHA (Phytohaemagglutinin) [21]. ***We propose to define the status of immunosuppression on the basis of an abnormal result (values outside the reference intervals) obtained in at least one of the two "reference" test.***

The REALISM project aims to provide a validated operational definition of injury-induced immunosuppression predicting clinically relevant outcomes. This will facilitate development of new tools and biomarkers with the goal of introducing diagnosis of immune suppression into routine clinical practice and allow patient stratification for the evaluation of new individual immunotherapies. It may also enable the identification of new targets and the development of new innovative therapeutics to treat ICU patients and prevent opportunistic infections in the future

Primary Aim

The primary objective of the study is to determine the incidence of injury-induced immunosuppression in ICU patients, during the first two months after injury.

Secondary Aims

The secondary objectives of the study are:

- To describe the occurrence of immunosuppression, its depth and impact on innate and adaptive immune responses, and its evolution during the first 2 months after injury.
- To assess the strength of the proposed definition, in particular by evaluating its association with secondary infections and mortality.
- To assess the accuracy of new biomarkers and immune functional assays to diagnose immunosuppression.

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.

METHODS AND ANALYSIS

REALISM is a prospective longitudinal, single-center observational study, conducted in the anesthesiology and intensive care department at the Edouard Herriot hospital (University hospital, Lyon France, capacity of approximately 1,000 beds).

Study population

REALISM will include healthy volunteers (n=150) and patients at risk of injury-induced immunosuppression: (1) septic shock patients (n=160); (2) severe trauma patients (n=180); (3) severe burns patients (n=30); and (4) patients admitted to the ICU after major surgery (n=180).

Septic shock inclusion criteria follow the current definition [5] and require a state of shock defined by vasopressors administration and plasma lactate level above 2 mmol/L (18 mg/dL). An infection must be suspected, and microbiological sampling should have been performed, along with the administration of antimicrobials. Only primary septic shock will be considered (vasopressors should have been started within the first 48hours after ICU admission) [5].

Patients with severe trauma, defined by an injury severity score (ISS, Baker *et al*., 1974) > 15 [22], will be included in the study. As we hypothesized that the depth of immunosuppression might be related to severity, we will limit the group of patients between ISS = [15-25] to 90 patients to ensure at least 50% of the cohort includes patients with an ISS > 25. Severe burn patients will be selected for inclusion based on a total burn surface area over 30%.

Surgical patients will be screened according to the planned surgical procedure. This study will include patients undergoing: 1) eso-gastrectomy; (2) Bricker's bladder resection (total bladder resection with reconstruction from small bowel); (3) cephalic pancreaticoduodenectomy (Whipple's procedure); (4) abdominal aortic aneurysm surgery by laparotomy.

Exclusion criteria are mainly related to factors that might impact the immune status and bias the results such as: severe neutropenia (neutrophil count <0.5 G/L), administration of immunosuppressive therapy, corticosteroids (IV if oral administration), use of therapeutic antibodies (such as anti-TNF alpha), onco-hematological disease (e.g. lymphoma, leukemia) under treatment or treated within 5 years before inclusion, end of chemotherapy within the 6 months prior to inclusion date. Patients with congenital/hereditary or acquired immune deficiency (for example severe combined immunodeficiency, HIV or AIDS, at any stage), and patients that have received extra-corporeal circulation in the month preceding inclusion will be excluded as well.

Considering the possible influence of gender bias on measured parameters, we will recruit healthy donors from both genders, following the age and gender distribution of the French population.

Complete lists of the inclusion and exclusion criteria for patients and healthy volunteers are presented in Table1 and Table 2 respectively.

Table 1. Inclusion and exclusion criteria for patients
Inclusion criteria
Male or female aged over 18 years
Patient or next of kin having been informed of the conditions of the study and having signed the informed consent form
Patient hospitalized for:
Septic shock, defined by:
Infection site suspected, and microbiological analysis sampling carried out
Vasopressor therapy needed to elevate MAP (Mean Arterial Pressure) ≥ 65 mm Hg and lactate > 2 mmol/L (18 mg/dL) despite adequate fluid resuscitation [26]
Noradrenaline > 0.20 µg/kg/min for at least 2 hours
Noradrenaline started within 48 hours after ICU admission
Serious trauma, defined by:
Patient admitted directly to the recruiting ICU
Injury Severity Score Baker *et al*., 1974 > 15 [22]
Severe burns, defined by:
Total burned surface area > 30%
Major surgery, defined by:

Surgery set for one of the following indications: (i) eso-gastrectomy; (ii) Bricker's bladder resection (total bladder resection with reconstruction from small bowel); (iii) cephalic pancreaticoduodenectomy (Whipple's procedure); (iv) abdominal aortic aneurysm surgery by laparotomy. Categories i to iii concern management of solid tumors, while category iv concerns non-cancerous pathologies
Induction of anesthesia before 11 am (to permit same day processing of all samples)
Exclusion criteria
Patient with severe neutropenia (neutrophil count <0.5 G/L)
Patients receiving immunosuppressive therapy
Corticosteroids (IV or Per os).
Use of therapeutic antibodies
Onco-hematological disease (ex. Lymphoma, leukemia…) under treatment, or treated within 5 years before inclusion
End of chemotherapy within the 6 months prior to inclusion date
Patient with innate or acquired immune deficiency (for example severe combined immunodeficiency, HIV or AIDS, any stage)
Patients with a "do not resuscitate order" or a "withdraw of care" decision, at time of inclusion
Patient whose anticipated duration of hospitalization in the ICU is estimated at less than 48 hours
Participation in any interventional study
Extra-corporeal circulation in the month preceding inclusion in the case of cardiac surgery
Pregnant or breastfeeding women
Patient with no social security insurance, with restricted liberty or under legal protection

Table 2: Inclusion and exclusion criteria for healthy volunteers

Inclusion criteria
Male or female aged over 18 years
Normal clinical examination
Signed informed consent form
Person with social security insurance
Exclusion criteria
Person with an infectious syndrome during the last 90 days
Extreme physical stress within the last week
Person having received within the last 90 days, a treatment based on:
Antivirals
Antibiotics
Antiparasitics
Antifungals
Person having received within the last 15 days, a treatment based on non-steroidal anti-inflammatory drugs (NSAIDs)
Person having received within the last 24 months, a treatment based on:

Immunosuppressive therapy
Corticosteroids (IV or Per os)
Therapeutic antibodies
Chemotherapy
History of :
Innate or acquired immune deficiency
Hematological disease
Solid tumor
Severe chronic disease
Surgery or hospitalization within the last 2 years
Pregnancy within the last year
Participation to a phase I clinical assay during the last year
Participation to a phase I clinical assay during the last year
Pregnant or breastfeeding women
Person with restricted liberty or under legal protection

Sampling schedule

Samples and clinical data will be collected 3 to 4 times within the first week (early time-points) with the aim to evaluate the modulation of the immune status early after injury. Samples will be collected at day 1 (the morning following injury), at day 2 (for the severe trauma group) and at day 3/4 and day 5/7 (Table 3). Samples will also be collected before surgery, at day 0, as surgical patients are the only group for which sampling can be performed before injury. Additional samples will be collected during late time points to evaluate the recovery of the immune status, at day 14 (between day 13 to18), day 28 (between day 26 to 36) and day 60 (between day 52 to 68), depending on patient availability and technical constraints (Figure 1). Total volume of sampling will be 30 mL at each time point.

Definition of Immunosuppression

The REALISM project will monitor the immune function of the patients and healthy volunteers using two standardized immune functional tests: one reference test to evaluate the innate immune response (whole blood production of TNF-alpha in response to ex vivo stimulation by LPS) and a second reference test for the adaptive immune response (the lymphocyte proliferation in response to ex vivo T cell stimulation with PHA). Immunosuppression will be defined in comparison to the values as

obtained in a group of healthy volunteers for the two reference tests using the following methodology. First, reference intervals (RI) will be derived from the independent set of healthy volunteers. Second, immunosuppression will be defined in a patient when an abnormal result (value outside the reference intervals) is obtained in at least one of the two "reference" tests over at least two consecutive time points

Definition of Secondary infection

During the ICU stay, patients will be screened daily for exposure to invasive devices (intubation, indwelling urinary catheter, and central venous line) and occurrence of secondary infection. Information referent to infections will be collected, reviewed and validated by a dedicated adjudication committee, composed of 3 physicians not involved in the recruitment of the patients with confirmation of secondary infection made according to the definitions used by the European center for disease prevention and control [23] and the Infectious Diseases Society of America.

Immune functional assays

Innate immune response: TNF-α release after LPS whole blood stimulation

Innate immune response will be evaluated by measuring the production of TNF-α in response to *ex vivo* stimulation of whole blood by LPS [20]. The stimulation will be performed through the use of standardized TruCulture® tubes from MYRIAD RBM (MYRIAD RBM; Austin, USA) (the concentration, quality and activity of the LPS is guaranteed by the manufacturer MYRIAD RBM) [20]. The tubes contain the medium alone (Null) or the medium with LPS 100 ng/ml (LPS from *E.coli* O55:B5) (LPS-R; Null-R; MYRIAD RBM). The blood samples will be collected on heparin and transported to the laboratory where 1 ml of heparinized blood will be transferred to each TruCulture® tube and incubated for 24 hours at 37°C. Following incubation, the supernatant (medium+plasma) will be collected using a separation valve (according to manufacturer instructions) and stored at -80°C

until batch quantification of TNF-α by ELISA (BE55001; BL International-Tecan; Männedorf, Switzerland).

Adaptive immune response: T lymphocyte proliferation after ex vivo PBMCs mitogenic stimulation

Adaptive immune response will be assessed by measuring T lymphocyte proliferation in response to *ex vivo* stimulation with a mitogen [21]. 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+ using a CD3-APC staining) using flow cytometry [21].

Cellular Immunophenotyping

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 cells characterization and cell counting will be completed by flow cytometry 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]. In addition, the number of HLA-DR molecules per monocyte will be determined using the BD quantibrite standardized method (HLA-DR:340827; QuantiBRITE:340495; Becton Dickenson; New Jersey, USA) [26]. It is well known that the flow cytometry is highly sensitive to variation between labs and instruments; therefore a

validation with the routine hospital immunology lab was performed to guarantee that all the protocols reproducible and standardized. All procedures generated results with less than 20% of variation when compared to reference protocols.

Biobanking

This study will provide the opportunity to establish four different types of biobanks to preserve the material collected, enabling exploration of innovative biomarkers: (1) Truculture® plasma biobank from whole blood stimulated with LPS, SEB *(Staphylococcus aureus* enterotoxin-B) or not stimulated, to study cytokines release. (2) EDTA plasma biobank to study viral reactivation markers and soluble host biomarkers. (3) Heparin plasma biobank for metabolomics/proteomics soluble host biomarkers studies; (4) RNA biobank to study new transcriptomic host biomarkers (RNA will be extracted from whole blood collected in PAXgene® tubes).

Innovative immune functional assays and exploration of new biomarkers

Regarding the immune functional tests, other stimulants (e.g. SEB) and read-outs (e.g. Interleukin 2, Interferon gamma) will be tested using the TruCulture® tubes. The cytokine production levels in the supernatants of the functional assays will be quantified using commercial IVD or RUO assays. Finally, a metabolomics and proteomics study will be performed using frozen (heparin) plasma. Biomarkers potentially associated to immune deficiency will be identified by LC-MS on high resolution mass spectrometry and 1H-NMR, after polar and non-polar samples extraction.

Sample size and data analysis plan

Population sizing

The number of healthy volunteers required to determine the reference intervals for the two immune reference tests was defined according to the methodology recommended by the Clinical and Laboratory Standards Institute (CLSI) C28-A3 guidelines [27]. The minimal number of subjects

recommended being 120, after exclusion of aberrant values (confidence interval of 90%), we decided to include 150 healthy volunteers to take into account exclusions related to technical reasons, aberrant values or consent withdrawal.

For this reference population, the age range of healthy volunteers group has been carefully calculated to include the expected age range and gender distribution from ICU patients in France (Table 3).

Table 3. Age and gender distribution for the reference group

Age range	Male	F
[19-30[	14	14
[30-50[	25	25
[50-65[	18	19
[65-100[	15	20
Total	72	78

The main objective being descriptive, the computation of the sample size was based on secondary objectives, especially for 1) the analysis of the occurrence of immunosuppression, its depth and impact on innate and adaptive immune responses (Cohen d is 0.55) and 2) the correlation between new biomarkers and immune functional assays to diagnose immunosuppression ($r>0.4$). A Student t-test was used to approximate the number of patients needed and a minimum of 150 patients per group was required for a standardized Cohen's d effect = 0.55, if we get the recommended number of healthy volunteers of 120. It was therefore decided to include 160 septic shock patients, 180 severe trauma patients and 180 patients with a major surgery, to overcome secondary exclusions for technical causes or consent withdrawal. The severe burn patients group is an ancillary group that was arbitrary fixed at 30 subjects in order to collect data with the intent to inform a dedicated study on this population in the future.

Statistical analysis.

First, the percentage of patients meeting the definition of injury-induced-immunosuppression will be computed in each patients group to answer the main objective. Second, the occurrence of immunosuppression will be further described. The proportion of patients with at least one abnormal test will be computed for both immune reference tests and each patients group. The correlation between the 2 reference tests will be established from a Spearman correlation test. A mixed model

will be constructed to describe the extent of the changes in the innate and adaptive measures over time, taking groups and time points into account. Third, a comparison of each biomarker or new functional tests with the two reference tests will be performed using a Spearman correlation test. For correlated biomarkers or functional tests, the performance for prediction of secondary infection will be estimated from a ROC curve. A Fine & Gray predictive model will be constructed [28] for the biomarkers harboring the best areas under curve, taking into account the competing risk of mortality. Finally, multiple imputations will be taken into consideration in the case of a relevant amount of missing values.

ETHICS AND DISSEMINATION

Ethics approval

The protocol, information documents and consent forms received approval by the local Institutional Review Board (Comité de Protection des Personnes Sud-Est II, Bron, France) and the French National Security agency for drugs and health related products (Approval code: 69HCL15_0379, 30th of November of 2015). An amendment has been filled to extend sampling time points over the first week and add the metabolomics and proteomics study. This amendment has been approved on the 22nd of July of 2016 (protocol version 3). This study complies with the Declaration of Helsinki, principles of Good Clinical Practice and the French personal data protection act.

Informed consent

The free and informed consent of each patient and healthy volunteer will be obtained following a complete and faithful information, in comprehensive words, of the objectives, the proceedings and the constrains of the study, the right to refuse the enrollment or the possibility to withdraw at any time, when he/she is in capacity to understand. The patient (or next of kin) will also be informed of: (1) the existence of processing system for data concerning them; (2) Their right to access and rectify these data (accessible through the physician of their choice); (3) The possibility of the use of remaining biological material and associated data stored following the end of the study and their possible transfer to another academic or private party. This information is part of the written notice and the informed consent.

If the patient is not in capacity to understand and/or express his/her consent, the informed consent will be obtained from a next of kin. In the event that only the informed consent of a third party has been sought at the time of inclusion, the patients should be informed as soon as possible of their participation in this study and be asked to give their own consent to continue the study.

If the next of kin is not present and not available by phone, the patient may be included in emergency situation. The investigator will be required to record all steps for calling the next of kin in the medical record (Contact attempts with date, time and phone number ...) and justify patient inclusion in medical emergencies in accordance with French legislation. The written consent of the next of kin and the patient should be obtained as soon as the person is available and as soon as the patient's clinical condition allows. The consent form contains the possibility to refuse the storage of samples after the end of the study.

Safety of participants

This study includes no serious foreseeable risk to the health of the persons involved. The only potential risk is related to blood sample collection (maximum 192 ml collected over all time points – 2 months). However, this aspect of nursing is part of daily practice. Blood samples will be taken under the same conditions of safety as currently used for common diagnostic tests.

Study management

The study is managed by BIOASTER, and a dedicated team composed from members of all the consortium partners. The promoter of the study is the Hospices Civils de Lyon. The principal investigator is Dr Thomas Rimmelé.

Data management

Clinical data

For each patient, an electronic case report form including socio-demographic, clinical and para-clinical information will be completed by clinical research assistants (Table 4). A description of the hospital stay, the documentation on the type of injury (surgery, burn, trauma or septic shock) and the severity as defined by the ASA classification, SOFA score [29] and SAPSII score [30]. In addition, we will collect routine lab results about the CMV, HSV1 serology and Complete Blood Count (CBC).

Moreover, we will document if there is any specific treatments administered to the patient, such as antibiotics, exposure to invasive medical devices and secondary infections. All data will be transferred to a TranSMART [31] database following curation for data exploration and analysis.

Table 4 Clinical and biological data collection planning.

						ICU	Hospital	**J14**	J28	**J60**	
	D0 [a]	**D1 (2[f])**	**D2** [c]	**D3/4**	**D5/7**	Release	release	D13/18	D26/36	D52/68	**D90**
Inclusion/exclusion criteria		x [b]									
Consent form		x [b]									
Demography		x [b]									
Weight		x [b]									
Size		x [b]									
Description of hospital stay		x [b]									
IGS II score		x [b]									
McCabe score		x [b]									
CHARLSON score		x [b]									
Documentation of the septic shock, surgery, burn or trauma		x [b]									
SOFA score	x	x	x	x	X						
Treatments against infections	Steadily							x	x	x	x
Therapeutic management	Steadily										
Exposition to medical devices	Steadily										
Surveillance of health-care associated infections	Steadily							x[d]	x [d]	x [d]	x [d]
Concomitant events	Steadily							x	x	x	x
Vital status						X	x	x	x	x	x
Life quality (EQ5D)											x
Biology											
PAXgene® tube sampling	x	x	x	x	X			x	x	x	
EDTA tubes sampling	x	x	x	x	X			x	x	x	
Heparin tubes sampling	x	x	x	x	X			x	x	x	
Hematology	x	x	x	x	X			x	x	x	
Lactate	x[e]	x[e]	x[e]	x[e]	x[e]						
pH	x[e]	x[e]	x[e]	x[e]	x[e]						
Liver results (ASAT, ALAT, PAL)	x[e]	x[e]	x[e]	x[e]	x[e]						

Procalcitonin	x[e]	x[e]	x[e]	x[e]	x[e]			x[e]	x[e]	x[e]	x[e]
Serology (CMV, HSV1)		X[b]									
[a] Only for patients of the surgery group											
[b] Evaluation on day 0 for patients of the surgery group (not repeated on day 1)											
[c] Only for patients of the trauma group											
[d] only if related to a new hospitalization											
[e] if available											
[f] for the septic shock and burn patients: The enrollment at D2 will be accepted if D1 is not available											

Duration of the study

The study is planned to run for thirty months, starting December 2015. The expected end date for recruitment is June 2018. Some biomarkers will be quantified by batch analysis, at the end of the study. Primary data analysis is expected to be completed with subsequent dissemination of results by December 2018

Figure Legends

Figure 1: Schematic design of the REALISM project, illustrating the type of patients included in the study, the various time-points, and major planned analysis.

Acknowledgement

Sponsorship

The project is funded by a consortium: bioMérieux, SANOFI, GlaxoSmithKline, Ecole Supérieure de Physique Chimie Industrielles de la ville the Paris – PSL Research University, the University Hospital Hospices Civils de Lyon and the microbiology technological institute BIOASTER. The project is financially supported in part by public funding through BIOASTER and Hospices Civils de Lyon. The project will be audited annually by the French National Research Agency ("Investissement d'Avenir" program; grant n°ANR-10-AIRT-03).

Consent for publication

Not applicable

Competing interests

AP, JT, VM are employees of bioMérieux, an in-vitro diagnostic company. PC, LK and DG are employees of Sanofi-Aventis R&D, Sanofi-Pasteur SA, and GlaxoSmithKline, three pharmaceutical companies.

Data sharing statement

Results will be disseminated through presentations at scientific meetings and publications in peer-reviewed journals. New markers and immune functional tests will be evaluated for the diagnostic immune deficiency and may be patentable.

Contributorship statement:

All authors (MLR, FV, TR, VM, PC, LQ, DG, AG, AP, JT, GM) fulfils ICMJE guidelines and (1) provided substantial contributions to conception, design and acquisition of data, (2) drafted and revised critically the manuscript and, (3) approved the final version of the manuscript.

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REFERENCES

1 Jawad I, Lukšić I, Rafnsson SB. Assessing available information on the burden of sepsis: global estimates of incidence, prevalence and mortality. *J Glob Health* 2012;2:4.

2 Vincent J-L, Marshall JC, Ñamendys-Silva SA, *et al.* Assessment of the worldwide burden of critical illness: the Intensive Care Over Nations (ICON) audit. *Lancet Respir Med* 2014;2:380–6.

3 Walkey AJ, Lagu T, Lindenauer PK. Trends in sepsis and infection sources in the United States. A population-based study. *Ann Am Thorac Soc* 2015;12:216–20.

4 Seymour CW, Rea TD, Kahn JM, *et al.* Severe Sepsis in Pre-Hospital Emergency Care: Analysis of Incidence, Care, and Outcome. *Am J Respir Crit Care Med* 2012;186:1264–71.

5 Singer M, Deutschman CS, Seymour CW, *et al.* The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016;315:801. doi:10.1001/jama.2016.0287

6 Angus DC, van der Poll T. Severe Sepsis and Septic Shock. *N Engl J Med* 2013;369:840–51.

7 Cohen J, Vincent J-L, Adhikari NK, *et al.* Sepsis: a roadmap for future research. *Lancet Infect Dis* 2015;15:581–614.

8 Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013;13:862–74.

9 Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. *Lancet Infect Dis* 2013;13:260–8.

10 Delsing CE, Gresnigt MS, Leentjens J, *et al.* Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series. *BMC Infect Dis* 2014;14:166.

11 Venet F, Lukaszewicz A-C, Payen D, *et al.* Monitoring the immune response in sepsis: a rational approach to administration of immunoadjuvant therapies. *Curr Opin Immunol* 2013;25:477–83.

12 Meisel C, Schefold JC, Pschowski R, *et al.* Granulocyte–Macrophage Colony-stimulating Factor to Reverse Sepsis-associated Immunosuppression: A Double-Blind, Randomized, Placebo-controlled Multicenter Trial. *Am J Respir Crit Care Med* 2009;180:640–8.

13 Gentile LF, Cuenca AG, Efron PA, *et al.* Persistent inflammation and immunosuppression: A common syndrome and new horizon for surgical intensive care. *J Trauma Acute Care Surg* 2012;72:1491–501.

14 Angele MK, Chaudry IH. Surgical trauma and immunosuppression: pathophysiology and potential immunomodulatory approaches. *Langenbecks Arch Surg* 2005;390:333–41.

15 Kimura F, Shimizu H, Yoshidome H, *et al.* Immunosuppression following surgical and traumatic injury. *Surg Today* 2010;40:793–808.

16 Timmermans K, Kox M, Vaneker M, *et al.* Plasma levels of danger-associated molecular patterns are associated with immune suppression in trauma patients. *Intensive Care Med* 2016;42:551–61.

17 Gossez M, Malcus C, Demaret J, *et al.* Evaluation of a Novel Automated Volumetric Flow Cytometer for Absolute CD41 T Lymphocyte Quantitation. Cytometry B Clin. Cytom. 2016. 10.1002/cyto.b.21360

18 http://www.clinicaltrial.gov/.

19 van Vught LA, Klein Klouwenberg PMC, Spitoni C, *et al.* Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis. *JAMA* 2016;315:1469-79.

20 Duffy D, Rouilly V, Libri V, *et al.* Functional Analysis via Standardized Whole-Blood Stimulation Systems Defines the Boundaries of a Healthy Immune Response to Complex Stimuli. *Immunity* 2014;40:436–50.

21 Poujol F, Monneret G, Friggeri A, *et al.* Flow cytometric evaluation of lymphocyte transformation test based on 5-ethynyl-2'deoxyuridine incorporation as a clinical alternative to tritiated thymidine uptake measurement. *J Immunol Methods* 2014;415:71–9.

22 Baker S, O'Neill B, Haddon W, *et al.* The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. J Trauma. 1974;14:187–96.

23 European Centre for Disease Prevention and Control. Point prevalence survey of healthcareassociated infections and antimicrobial use in European acute care hospitals. ECDC. 2012;ISBN 978–92 – 9193–366 – 2.

24 Demaret J, Venet F, Friggeri A, *et al.* Marked alterations of neutrophil functions during sepsis-induced immunosuppression. *J Leukoc Biol* 2015;98:1081–90.

25 Venet F, Chung C-S, Kherouf H, *et al.* Increased circulating regulatory T cells (CD4+CD25+CD127−) contribute to lymphocyte anergy in septic shock patients. *Intensive Care Med* 2009;35:678–86.

26 Docke W-D. Monitoring Temporary Immunodepression by Flow Cytometric Measurement of Monocytic HLA-DR Expression: A Multicenter Standardized Study. *Clin Chem* 2005;51:2341–7.

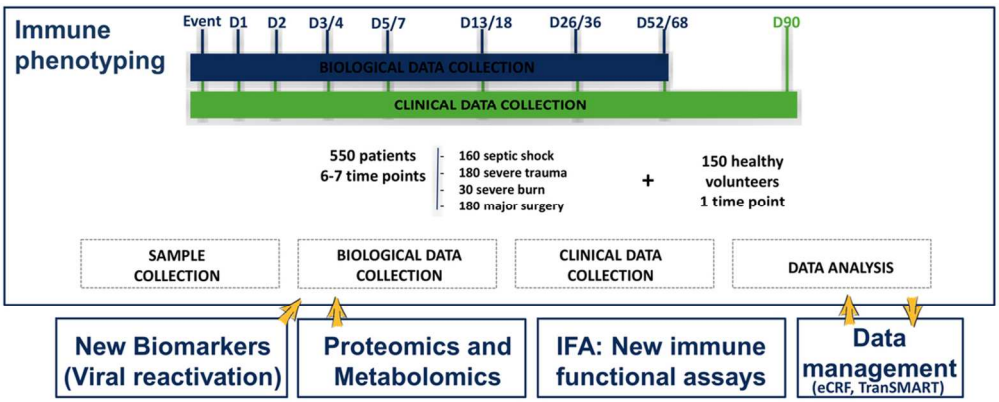
27 Horowitz, Gary L, Altaie, Sousan, Boyd, James C, *et al.* CLSI C28-A3 Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory Approved Guideline. 2008.

28 Fine JP, Gray RJ. A Proportional Hazards Model for the Subdistribution of a Competing Risk. *J Am Stat Assoc* 1999;94:496.

29 Vincent JL, Moreno R, Takala J, *et al.* The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996;22:707–10.

30 Le Gall JR, Lemeshow S, Saulnier F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA J Am Med Assoc* 1993;270:2957–63.

31 TranSMART [http://transmartfoundation.org/].



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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym (page 1)	Yes
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry (page 4)	Yes
	2b	All items from the World Health Organization Trial Registration Data Set (NA)	
Protocol version	3	Date and version identifier (page 18)	Yes
Funding	4	Sources and types of financial, material, and other support (page 21)	Yes
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors (page 22-23)	Yes
	5b	Name and contact information for the trial sponsor (page 21)	Yes
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities (page 22)	Yes
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee) (page 19)	Yes
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention (page 5 and 6)	Yes
	6b	Explanation for choice of comparators	NA
Objectives	7	Specific objectives or hypotheses (page 7-8)	Yes

Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory) (page 9)	Yes
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained (page 9)	Yes
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)(page 9-12)	Yes
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	NA
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	NA
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial (page 12)	Yes
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended (page 12-13)	Yes
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (page 12)(see Figure 1)	Yes
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations (page 15-16)	Yes
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	No
Methods: Assignment of interventions (for controlled trials)			

Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	NA
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	NA
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	NA
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	NA
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol (pages 4,15,16,19 and 20)	Yes
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol (page 19)	Yes
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol (page 16-17)	Yes
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses) (page 16)	Yes

	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation) (page 16)	Yes
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct(page 19)	Yes
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor (page 13)	Yes
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval (page 18)	Yes
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators) (page 18)	Yes
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32) (page 18)	Yes
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable(page 18)	Yes
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial (page 19)	Yes
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site(page 21)	Yes

Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators(page 4)	Yes
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions(page 22)	Yes
	31b	Authorship eligibility guidelines and any intended use of professional writers(page 22)	Yes
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	No
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates(page 18)	Yes
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable (page 14)	Yes

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.