# Cellular and molecular mechanisms of IMMunE dysfunction and Recovery from SEpsis-related critical illness in adults: An observational cohort study (IMMERSE) protocol paper

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Abstract

Sepsis is a common illness. Immune responses are considered major drivers of sepsis illness and outcomes. However, there are no proven immunomodulator therapies in sepsis. We hypothesised that in-depth characterisation of sepsis-specific immune trajectory may inform immunomodulation in sepsis-related critical illness. We describe the protocol of the IMMERSE study to address this hypothesis. We include critically ill sepsis patients without documented immune comorbidity and age–sex matched cardiac surgical patients as controls. We plan to perform an in-depth biological characterisation of innate and adaptive immune systems, platelet function, humoral components and transcriptional determinants of the immune system responses in sepsis. This will be done at pre-specified time points during their critical illness to generate an illness trajectory. The sample size for each biological assessment is different and is described in detail. In summary, the overall aim of the IMMERSE study is to increase the granularity of longitudinal immunology model of sepsis to inform future immunomodulation trials.

Keywords

Sepsis, lymphocyte, immunology, transcriptomics

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## Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and includes septic shock as a more severe subset.1,2 Sepsis is a major global issue with an extrapolated incidence of 48.9 million cases worldwide and 11 mil-lion sepsis-related deaths, representing 19.7% of all deaths in 2017.3 Hospital mortality exceeds 20% for sepsis-related critical illness.4,5 Furthermore, patients who recover from sepsis (sepsis survivors) are at greater risk of rehospitalisation and death compared to both the age- and sex-matched general population and hospitalised patients without sepsis.6–8 Improving outcomes and enhancing survivorship are clinical priorities.

Immune responses are central to sepsis pathophysiology9 and are considered major determinants of outcome. Despite, or perhaps because of, a plethora of immune changes,9 there are no immune therapies confirmed to improve outcomes in large prospective trials. Improved patient selection and optimal timing of intervention could change this scenario.10,11 To achieve this in immunomodulation trials, we need to understand how immune abnormalities change over time (referred to as the longitudinal immunological model in this article), the major determinants of this change and the major immune abnormalities with a modifiable attributable risk.12 We hypothesised that this could be achieved by concurrently studying changes in immune cell phenotypes, functionality and their transcriptional determinants. Similar issues apply for coagulation abnormalities.13 Herein, we describe the protocol of the IMMERSE study to address these hypotheses.

## Rationale for the study and evidence gap addressed by the study

Sepsis-related immune responses involve both humoral and leukocyte components of the innate and adaptive immune systems.14 Previous studies have assessed the immune response in sepsis by characterising the pan-leukocyte transcriptome, with microarray as the analytic platform.15–18 The currently accepted sepsis immune trajectory model is a consensus model with limited data-driven evidence. Excessive inflammation and immunosuppression, which occur simultaneously in most patients, change over time. However, published studies are mostly single time point assessments from patients who are likely at various stages of immune activation or sup-pression. Averaging phenotypic profiles across patients at various stages of inflammation and immunosuppression may obscure critical disease pathways. Thus, there is a pressing need for standardised lymphocyte immunophenotyping in sepsis as described in the human immunology project,19 and, crucially, at multiple time points. Similarly, in terms of days from critical care admission, we do not know when the dominant immune signal in sepsis-related critical illness changes

from inflammation to immuno-suppression, how best to identify this change and therefore when to shift from immunosuppressive therapy towards possible immune stimulation. IMMERSE aims to increase the granularity of this longitudinal immunology model of sepsis to inform immunomodulation trial design.11

In most adult sepsis cohort studies, the study popu-lation has a mean age of around 60 years, and often have comorbidities or treatments such as malignancy and chemotherapy that can affect normal immuno-logical responses.15 This prompted us to consider the issue of control populations when designing our cohort study.20 We plan to use age- and sex-matched controls with blood sampling taken before and after uncomplicated cardiac surgery. The key rationale for our choice of control population is that their hospital admission diagnosis is unrelated to our exposure of interest (i.e. sepsis). Additional advantages include feasibility, comparable information quality, receipt of treatment in the same health care setting and the ability to perform paired sampling both before and after a sterile surgical insult at fixed time points to understand early immune changes.
There are numerous immunological interventions with known effects that may be of value.14 However, ex vivo assessment of the reversibility of these sepsis-related immunological abnormalities is seldom performed. Furthermore, in early phase clinical trials of these interventions,21,22 assessment of the biological pharmacodynamic effects of intervention is often limited, compared to pharmacokinetic and safety aspects. Thus, there is a need for systematic assessment of the ex vivo biological effect of putative interventions. The IMMERSE study addresses this question by concurrently evaluating biological reversibility, using the ex vivo experimental set-up as described previously,23 alongside immune cell phenotypes and molecular abnormalities.

In sepsis patients, thrombocytopenia is associated with a more severe disease and an increased risk of death.24 In addition to their role in haemostasis and thrombosis, platelets are widely accepted as key com-ponents of the cellular immune system. Platelets con-tribute to the inflammatory response in both allergic25 and non-allergic inflammation26 and are critical to host defence in infectious disease.26,27 Although plate-lets have been heavily discussed in the context of sepsis,28 little work has been done to assess how their function may change through the trajectory of the disease, both in terms of haemostasis and immune (inflammatory) function. The IMMERSE study will help define how platelet function is altered during sepsis, its association with immune changes and the potential of highlighting novel therapeutic avenues.

Aims

First, to describe longitudinal changes in B and T cell counts and phenotypic changes within immune cell subsets19 during sepsis-related critical illness commencing at critical care admission, at one or more time points during their critical care stay and at critical care discharge. Second, to explain the observed phenotypic changes in B and T cell subsets at the

molecular level using functional readouts with cell culture and transcriptomic experiments. Third, to describe changes in platelet phenotype and function. Fourth, to contribute towards refining the longitudinal sepsis immunology model by integrating these orthogonal (statistically independent) multilevel data with repeated measurements.

## Methods and analysis

### Study design

IMMERSE is a prospective observational cohort study in adult critically ill sepsis patients, with age-and sex-matched non-sepsis controls.

### Setting

The study will recruit from multiple intensive care units, high dependency units and cardiac surgical wards at Guy's and St Thomas' NHS Foundation Trust, London, England. This is a tertiary referral hospital with 120 critical care beds, a mixed medical–surgical case mix and includes a severe respiratory failure centre. The basic science work will be conducted within the School of Immunology & Microbial Sciences and the Institute of Pharmaceutical Science at King's College London.

### Study cohorts and eligibility criteria

We plan to enrol two hospitalised cohorts (sepsis and cardiac surgery) and age–sex matched non-hospitalised participants to address our research aims.

Sepsis (including septic shock) will be defined as per the Sepsis-3 criteria.1 Inclusion criteria include patients >18 years of age, admitted with suspected or proven infection with an admission day Sepsis-related Organ Failure Assessment (SOFA) Score of 2 or higher, and provision of written consent by patient or by professional or personal consultee.

Elective uncomplicated surgical control participants will also be enrolled to explore how the immune system responds to the sterile insult of cardiac surgery. Inclusion criteria include patients >18 years of age, admitted for planned cardiac surgery and provision of written consent by patient or by professional or personal consultee.

Exclusion criteria for the sepsis and cardiac surgical cohorts are aimed at removing confounding effects of concomitant immune abnormalities in host immune responses to infection and injury. Such exclu-sions include any limitations of care order set, including 'do not attempt cardiopulmonary resuscitation' or not for readmission to critical care, and one or more of the following conditions associated with immuno-suppression as defined in the APACHE II score29: human immunodeficiency virus (HIV) diagnosed at any point prior to enrolment; leukaemia within the last 5 years; lymphoma (treated or eligible for treatment by radiotherapy or chemotherapy) within the last 5 years; multiple myeloma (treated or eligible for treatment by radiotherapy or chemotherapy within the last 5 years); malignancy

(treated or eligible for treatment by radiotherapy or chemotherapy) within the last 5 years; being treated with immuno-suppressive therapy including corticosteroids for 2 weeks or longer at a daily prednisolone (or equivalent) dose of 30 mg or higher; receipt of an organ (including bone marrow) transplant with ongoing immunosuppressive medication and immunosuppression due to another cause including congenital hypogammaglobulinaemia or other congenital immunodeficiency; nephrotic syndrome; known protein-losing enteropathies; or receipt of treatment with intravenous immunoglobulins in the preceding 3 months.

Clinical data

For all patients included in the cohort, we will collect and summarise demographic data, daily laboratory results for white blood counts, C-reactive protein, daily worst physiological and biochemical variables to derive the SOFA score, critical care length of stay, critical care mortality, hospital length of stay and hospital mortality.

Blood sampling and biological assessments

In the sepsis and cardiac surgical cohorts, we will collect, process and store blood samples for leukocyte phenotyping, whole blood transcriptomics, platelets for functional analyses (aggregation and chemotaxis), cellular and molecular analysis of antigen expression, receptor repertoire and serum and plasma for bio-marker assessments. In sepsis patients, these samples will be collected at four time points: within 24 h of critical care admission, on day 3 (±1 day), on day 5 (±1 day) and on critical care discharge. In the cardiac surgical cohort, these samples will be collected before and 24 h following cardiac surgery. In healthy co-trols, we will collect one sample only. To reduce technical variation between samples, we plan to multiplex leukocyte phenotyping, use the same technical control in every experiment and publish our experimental methods. Leukocyte phenotyping will be done using flow cytometry and mass cytometry (CyTOF) to gain insights into cellular phenotype and function.30–32 Strand-specific, poly(A)þ ribonucleic acid sequencing (RNA-seq) will be performed with a sequencing depth of 35–40 million 150 bp paired-end reads per sample to determine mRNA expression.

To understand the importance of platelets through-out the trajectory of sepsis, circulating platelet concentrations will be quantified and platelet activation markers (e.g. beta-thromboglobulin and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES)) will be measured by ELISA. Changes in haemostatic and inflammatory platelet function will be evaluated using in vitro functional assays of platelet aggregation and chemotaxis, respectively. Platelets will be stimulated using endogenous purinergic receptor agonists, and down-stream function assessed, as previously described.33 Platelet surface expression of purinergic receptors, P2Y1, P2Y12 and P2Y14 will be measured using flow cytometry and other molecular techniques.

Sample size

The overall study sample size of the sepsis cohort of 110 patients was determined using precision estimates for lymphocyte counts based on our previous study, where we observed a standard deviation of 1.07 cells x $10^9$/L34 and we wanted a precision of 0.2 cells x $10^9$/L for a Z value of 1.95 for 95% confidence interval. This will be used as a guide for recruiting patients. Each research question will address experiment-specific sample size requirements, as described.

For paired transcriptomics experiments, we used pilot microarray data in sepsis patients to inform sample size calculations. Among the 522 differentially expressed genes in T cells, there were 12 genes with more than a threefold increase and four genes with more than a threefold decrease in expression, com-pared with age- and sex-matched healthy controls. Among 182 differentially expressed genes in B cells, there were 26 genes with more than a twofold increase and nine genes with more than a twofold decrease in expression compared with age- and sex-matched healthy controls.34 Based on the above, we will sample a minimum of 18 sepsis patients and controls to perform paired analysis where required (such as with ex vivo stimulation experiments and longitudinal data), which will allow for the detection of candidates with a minimum of 50 reads that differ by a factor of 2, achieving 0.9 power with an alpha error of 0.05.35

For platelet studies, the coefficient of variation in platelet counts prior to cardiac surgery (n = 20 patients) and at admission day in sepsis (n ¼ 30 patients) were 32.3% and 58.4%, respectively. Therefore, we estimated a sample size of 36 sepsis patients with at least two repeated time-point measurements for platelet experiments, to achieve 0.8 power with an alpha error of 0.05, for functional differences.

Data analysis plan

The primary exposure is Sepsis-3 criteria defined sepsis. The primary outcome is sepsis-specific bio-logical traits to refine the longitudinal immunology model. Transcriptomic experiments will generate RNA-seq data which will be analysed to determine changes in RNA abundance at the gene level as well as the transcript isoform level using open source soft-ware (such as kallisto,36 DESeq2,37 and sleuth36) and our High-Performance Computing Cluster (Rosalind) within King's College London. Post-transcriptional steps of gene expression, such as alternative splicing and polyadenylation, will also be analysed.

Gene set enrichment analysis (GSEA) will be performed using Ingenuity Pathway Analysis. Whole blood flow cytometry assays will be used to measure absolute counts of CD4 þ and CD8þ T cells, B cells and all by memory and naıve phenotype. FlowJo will be used to manually gate populations of interest to determine cell count changes over time and sepsis-specific changes compared to sterile inflammation. For mass cytometry experiments, we will analyse complex cellular interactions in multiple immune populations. All time points from a

patient and a healthy technical control will be barcoded prior to staining and acquisition. Samples will be acquired in multiple FCS files on the Helios (Fluidigm). For analysis, FCS files will be normalised,38 concatenated and debarcoded.39 This process is advantageous as it reduces non-biological variation derived from experiment procedure and machine variability over time. To analyse immune cells of interest, manual gating will identify defined cell populations of interest. Exploratory analysis will be carried out using unsupervised clustering and dimensional reduction techniques.40 Populations identified will be used to understand sepsis trajectory and sepsis-specific alterations by comparing to sterile inflammation.

## Current study status

We started recruitment on 10 July 2019. To date, we have recruited 30 cardiac surgical patients, 32 sepsis patients and 7 healthy controls.

## Strengths and limitations of this study

We include non-sepsis controls with age and sex matching. We perform in-depth phenotyping of immune cell subsets at multiple time points during the sepsis illness, using multiplexing to reduce technical variation. Finally, there is concurrent evaluation of molecular mechanisms to explain phenotypic changes in immune cells. Our study has limitations. We are including all sepsis patients to increase generalisability but at the risk of increasing heterogeneity by not restricting by site of infection. The use of cardiac surgical controls could be confounded by their well-understood risk of endotoxaemia, which may lower the chances of identifying a sepsis-specific sig-nature. Our sample size for transcriptomic experiments is based on previous microarray data and may be insufficient.

## Ethics, regulations and governance

## Study management

The study is managed by the Department of Critical Care Medicine at Guy's and St Thomas' Hospital NHS Foundation Trust.

## Sponsorship

The study sponsors are Guy's and St Thomas' Hospital NHS Foundation Trust.

## Funding

This study is partly funded by the National Institute of Academic Anaesthesia – British Journal of Anaesthesia and Royal College Of Anaesthetists non-clinical PhD Studentship grant. The funders had no role in study design.

## Duration of the Study

The Study recruited its first patient on 10 July 2019. Recruitment is expected to take a maximum of 3 years and be completed by 7 February 2022.

## Ethical and regulatory approval

The study will be conducted according to the ethical principles outlined in the declaration of Helsinki. South Central Berkshire Research Ethics Committee has granted ethical approval 19/SC/0187. The study has been registered with the UK National Institute for Health Research (NIHR) Clinical Research Portfolio.

## Protocol compliance

We will conduct the study as per the protocol given a favourable opinion by the Research Ethics Committee (REC). Protocol changes will require REC favourable opinion prior to implementation. The study team will monitor protocol compliance as it is an observational cohort study. We will have a data dictionary for all variables collected in the study.

## Patient confidentiality

At the point of sample collection, all patient samples are assigned a unique study ID. The only link between patient identity and unique identifier will be held securely at the study site.

## Dissemination

Our four major aims will be addressed using multiple peer-reviewed publications, and we plan to provide the corresponding open access resource data with each publication.

## Summary

The IMMERSE study is a cohort study in adult critically ill patients with sepsis, with age- and sex-matched hospitalised patients acting as the clinical and biological comparator population. Our aims are to describe the longitudinal immune system changes at a cellular and molecular level with enough granularity to refine the sepsis illness model.

## Executive summary

Introduction: Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Despite the immune system being a key driver of sepsis pathophysiology, there are no proven immunomodulatory therapies for sepsis. This is explained partly by the illness heterogeneity and poorly characterised illness trajectory in sepsis patients. Therefore, we hypothesised that understanding immune heterogeneity and trajectory could inform patient selection for future clinical trials. We aim to describe sepsis-specific changes in leukocyte phenotype, function and transcriptional state, over the course of critical illness.

Methods and analysis: We plan to conduct a prospective, observational cohort study analysing blood samples from patients admitted to the critical care unit with sepsis defined

as per the Sepsis-3 criteria (sepsis cohort), and compare against age- and sex-matched cardiac patients undergoing elective cardiac surgery (cardiac cohort) and/or age- and sex-matched healthy volunteers, who have not been hospitalised in the previous 6 months (healthy cohort). We will use flow cytometry and mass cytometry to describe sepsis-specific changes in the leukocyte phenotype, cell culture and fluorospot, for functional characterisation, and next-generation sequencing for gene expression analysis, alongside clinical data.

Ethics and dissemination: This study will be con-ducted in accordance with principles outlined in the Declaration of Helsinki. Ethical approval has been obtained from the South-Central Berkshire research ethics committee. On conclusion of the study, results will be disseminated through peer-reviewed journals and conferences.

Checklist: We have included a STROBE Checklist for observational studies (see Supplementary Material). As a protocol paper, the results and discussion sections are not applicable.

Authors' contributions

MSH conceived the study. MF/KA/MSH wrote the first draft of the manuscript. All authors contributed to the crit-ical care vision of the manuscript. All authors read the final draft of the manuscript prior to publication.

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Declaration of conflicting interests

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Supplemental Material
The supplementary material for this article is available online.

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