Aus der Klinik für Anästhesiologie
der Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. Bernhard Zwissler

Immunological effects of intravenous selenium administration in severe sepsis

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Anne Guo
aus Guangzhou

2021

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:
Prof. Dr. Alexander Choukér

Mitberichterstatter:
Priv.-Doz. Dr. med. Michael Dolch
apl. Prof. Dr. Wolfgang Hugo Hartl

Mitbetreuung durch den promovierten Mitarbeiter:
Prof. Dr. Matthias Feuerecker

Dekan: Prof. Dr. med. dent. Reinhard Hickel

Tag der mündlichen Prüfung:
24.06.2021

Table of Contents

1. Introduction

1.1. Sepsis

The condition has long been deemed to be difficult in its diagnosis and treatment. Even dating over 2000 years back, sepsis has already been described by historians and philosophers alike. In the present day, a definition for sepsis is still a constantly evolving work in progress [1], hotly debated in order to facilitate physicians in identifying and treating this critical illness. While many advances have been made in modern medicine, the mechanisms of disease and definitive treatment options remain elusive.

Earliest understandings of sepsis can be summarized as blood poisoning, the body's response to an infection with massive release of cytokines. This knowledge is still reflected in the present day definitions where inflammation is a hallmark of the disease and laboratory values signal the body's readiness in mounting an attack. The progression of the disease eventually leads to organ failures and death. There is no simple diagnostic tool for sepsis. Other than treating the infection, most therapy options are largely supportive. The prevalence of sepsis has gone up in the developed world and its mortality rate rivals that of myocardial infarction [2].

Despite advances in current medical therapies, the prognosis remains poor. Sepsis is associated with a mortality rate at around 10 – 20 %, this increases dramatically with severe sepsis (20 – 50 %) and septic shock (40 – 80 %) [3]. These extremely ill patients with severe sepsis and septic shock are the focus in this thesis. Efforts are continuously being made to improve the outcomes for this patient population. Despite being a significant challenge, this mysterious and fascinating illness is of great interest in the field of critical care medicine, a reflection of the complexities of the human immune system.

1.2. Selenium and oxidative stress

Critically ill patients, such as those suffering from severe sepsis, have considerable oxidative stress [4], a major promoter of systemic inflammation and organ failure through the production of excessive free radicals or a depletion of antioxidation defense mechanisms. Exogenous antioxidant supplementation has long been practiced by physicians when

treating critical illnesses, which has shown some positive effects especially for those at high risk of death [5]. Selenium, among other vitamins and trace elements, is one such well known antioxidant.

The body's battalion of antioxidant defense mechanisms include superoxide dismutase, catalase and glutathione peroxidase, which require trace elements for a functional enzyme to mop up free radical species in protecting cells from oxidative stress [6]. An inflammatory state also causes a loss of the intestinal mucosal integrity, which impairs absorption of essential nutrients in combatting oxidative stress [7]. The serum concentrations of trace elements suffer a significant decrease during sepsis and severe illness, and remain low for quite a period of time [8]. This trace element deficiency in sepsis patients may simply be a reflection of malnutrition but whether supplementation reduces mortality is unclear. European ICUs tend to be more liberal and North Americans more restrictive in administering trace elements to their patients [9].

1.3. Immune function during sepsis

Canadian physician Sir William Osler was a pioneer in modern medicine and made the observation that patient with sepsis did not die from the disease itself, but rather, from the own body's mounted immune response to infection. This is a double edged sword where a suitably mounted immune response is essential in getting rid of an infection, but on the other hand, too much inflammation causes irreparable damage to the host [10].

As discoveries are being made about the complex disease evolution in sepsis, the systemic inflammatory response syndrome (SIRS) remains a hallmark of the diagnosis. This entails the innate immune system being overactivated, where a pro-inflammatory cascade ensues, triggering cytokine, chemokine, complement and mediator release, as the body's defense against infection [11]. More recently in the literature, the opposite phenomenon of SIRS has been increasingly described – an inhibition of the immune system known as immune paralysis or compensatory anti-inflammatory response syndrome (CARS) [12, 13]. In fact, at the onset of severe sepsis, the immune system appears to be in a state of anergy which poses a significant susceptibility to secondary infections in these already critically ill individuals [14]. Lymphocyte apoptosis and a shift from the TH1-dominated to the TH2-

dominated state contribute to this immune paralysis. The interplay between hypoinflammation and hyperinflammation in the pathogenesis of sepsis is poorly understood. In fact, a third theory suggests that both these mechanisms are concurrently at play, known as the mixed anti-inflammatory response syndrome (MARS) [15].

There are many unknowns in the disease process. Whether mortality is attributed to the uncontrolled pro-inflammatory response or rather the later immunosuppressive state makes therapy decisions extremely difficult. The immune response can be desirable or detrimental at different stages of sepsis. Depending on the initial infection focus, different arms of the innate and adaptive immune system can be recruited, including a complex network of mediators and regulatory mechanisms. These small signaling molecules that are released by lymphocytes are called cytokines. They are implicated in all aspects of the cascade resulting in inflammation and are released by specific cell types as well as short lived. Therefore, cytokine levels can often provide an accurate snapshot of the degree of immune system activation.

1.4. Selenium as possible immune booster

Selenium is a vital nutrient with immunological, antioxidant and anti-inflammatory properties, which is also the cornerstone of the antioxidation defense mechanism [16]. Selenoenzymes play an important role in oxidation-reduction signaling, free radical scavenging and immune system responses [17]. There have been many systemic reviews and meta-analyses which demonstrated positive mortality benefits of selenium in sepsis patients, especially at higher intravenous dosages [18, 19, 20, 21, 22, 23]. Nevertheless, the most recent and largest randomized controlled prospective trial on selenium monotherapy in severe sepsis (SISPCT) did not find improved survival as compared to the placebo group [24].

Despite many selenium supplementation studies in sepsis patients having examined mortality as an endpoint, our aim was to elucidate the effects of selenium on immune function [25]. With the help of a conventional immune cytokine assay using whole blood samples from sepsis patients, we can uncover and monitor the immune function at multiple time points during the disease course.

1.5. Scope of Study

Building on the results of previous trials which have largely yielded no mortality benefits from sodium selenite in severe sepsis patients in the intensive care setting, it is our intention to further elucidate possible immunological effects of this medication. To this end, we centered our study based on three key questions. The first being whether selenium administration alters the immune capabilities in this critically ill patient population with known severe immune dysregulation. Secondly, we hope to uncover potential differences in the various immune pathways through examining key cytokine responses using a diverse selection of stimuli. The third question is whether certain tendencies towards hypo or hyperinflammatory states can be associated with early disease progression, using time points over a three-week course.

2. Materials and methods

2.1. Trial Design

2.1.1. SISPCT Study

The patient population at our hospital was recruited under the larger randomized clinical trial “Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis” (SISPCT https://clinicaltrials.gov/ct2/show/NCT00832039), where 33 German hospitals participated in. Primary and secondary outcomes were analyzed in this double blinded study to examine the effects of high dose sodium selenite infusions and procalcitonin guided antimicrobial therapy on the mortality and morbidities of sepsis patients in an intensive care setting. The enrolment period was from November 2009 until June 2013 with a 90-day follow-up period. The primary end point was mortality at 28 days and secondary outcomes included 90-day all-cause mortality, secondary infections, days without intervention, cost of antimicrobial and days without antibiotic use.

2.1.1.1. Inclusion and exclusion criteria

For enrolment in the SISPCT study, patients were stringently evaluated based on detailed predetermined guidelines which qualify them for either severe sepsis or septic shock. The sepsis diagnosis can be made with a laboratory proven infection focus or a high degree of clinical suspicion in combination with at least two of the SIRS criteria, as listed in Table 1 below. Sepsis definitions have since undergone two rounds of updates internationally and the criteria discussed in the SISPCT study were based on the most up to date guidelines at the time of study design. SIRS with an infection focus in combination with acute organ failure was defined as severe sepsis. Sepsis in addition to arterial hypotension or need for vasopressor use after aggressive and sufficient fluid resuscitation was defined as septic shock.

Table 1. Criteria for sepsis and related conditions.	
Condition	**Definition**
Systemic inflammatory response syndrome (SIRS)	At least two of the following conditions present: • Hypothermia (< 36°C) or hyperthermia (> 38°C) • Tachycardia (> 90 bpm) • Tachypnea (> 20/min) or $PaCO_2$ < 33 mmHg or mechanical ventilation • Leukopenia (WBC < 4000 cells/$mm^{3)}$ or leukocytosis (> 12000 cells/mm^3) or > 10% immature cells
Sepsis	SIRS and confirmed or presumed infection.
Severe sepsis	Sepsis with organ dysfunction.
Septic shock	Severe sepsis with refractory hypotension.
Multiple organ dysfunction syndrome (MODS)	Evidence of more than two organ systems failing.
Adapted from the dissertation of Lars Sudhoff [26] and the 1992 ACCP/SCCM Consensus Conference definitions [27].	

Mechanical ventilation (i.e. Intubated patients) overrides the criteria of an increased respiratory rate or decreased $PaCO_2$. To qualify for severe sepsis or septic shock, patients need to additionally demonstrate at least one dysfunctional organ system on top of sepsis. The specific criteria are listed below in Table 2 and were made by the admitting medical team. In order to qualify for the study, patients must be enrolled within 24 hours of their severe sepsis or septic shock diagnosis.

Exclusion criteria of the study included pregnancy, lactation period, selenium intoxication, use of antibiotics for other chronic causes, discontinuation of therapy, terminal or palliative diagnosis, severe immune compromise (CD4+ counts < 200/mm^3 or neutrophils < 500/mm^3), medication induced immune compromise (i.e. after organ transplantion), clinical trial involvement in the past month, earlier participation in SISPCT or personal relations to the principal investigator.

Table 2. Severe sepsis and septic shock.		
Clinical diagnosis		**Criteria**
Severe Sepsis	Acute encephalopathy	Pathologic alteration of global mental status in the absence of structural disease or substance use.
	Thrombocytopenia	Thrombocytes ≤ 100,000/µl *or* decrease in thrombocytes > 30% within 24 hours with no acute blood loss.
	Renal insufficiency	Urine output ≤ 0.5 ml/kg/h with adequate fluid infusion for over an hour *or* serum creatinine increase ≥ 2 above the reference range.
	Metabolic acidosis	Base deficit > 5 mEq/l *or* serum lactate ≥ 1.5 above the reference range.
	Arterial hypoxemia	Arterial oxygen partial pressure < 10 kPa (75 mmHg) on room air *or* Horowitz index ≤ 33 kPa (250 mmHg) with supplemental oxygen not due to pre-existing cardiac or pulmonary conditions.
	Arterial hypotension	Systolic blood pressure ≤ 90 mmHg *or* mean arterial pressure ≤ 70 mmHg for > 1 hour despite adequate fluid resuscitation without other causes for circulatory shock.
Septic shock		Diagnosis of SIRS *and* proven or presumed infectious origin *and* systolic blood pressure ≤ 90 mmHg or mean arterial pressure ≤ 70 mmHg for > 2 hours despite adequate fluid resuscitation and necessitates vasopressor use.
To be enrolled in the SISPCT trial, patients had to have severe sepsis or septic shock as well as meet the aforementioned inclusion criteria. Adapted from the dissertation of Lars Sudhoff [26].		

2.1.1.2. Informed consent

Written informed consent for the SISPCT study was obtained from 76 patients enrolled at Munich University Hospital. The study protocol was approved by the University of Jena Research Ethics Committee with local amendments (Eudra-CT-Nr. 2007-004333-42). In the case where the patient's ability was impaired and no medical proxy had been identified, a positive declaration from a certified physician not involved in the patient's treatment was obtained. This can be overturned once the patient was again capable or a substitute decision

maker was available. In case of refusal, no further data collection or follow up was performed.

2.1.1.3. SISPCT protocols

The two solutions, placebo or sodium selenite, were administered immediately following enrolment for the entire stay in the intensive care unit. The randomization process was coordinated centrally in a double blinded fashion. Initially, a bolus was administered over 20 minutes through the central line consisting of either 1000 µg of selenium, sodium selenite pentahydrate (selenase® T pro injection, biosyn Arzneimittel GmbH, Fellbach, Germany) in aqueous 0.9 % sodium chloride solution (50 ml total volume) or the 50ml aqueous 0.9% sodium chloride solution. The same concentration was then given slowly at 1000 µg of selenium over a 24-hour period or alternatively, the sodium chloride placebo solution. The continuous infusion lasted until discharge from ICU or for maximally 21 days.

All other treatment decisions were made at the discretion of the medical team based on individual circumstances such as parenteral nutrition, antimicrobial or corticosteroid therapy. Patient clinical and laboratory parameters were recorded throughout the duration of the ICU stay, to a maximum of 21 days. Additional follow ups were conducted at days 28 and 90. Patient blood sample collection took place on days 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, and 21. Study sample collections and interventions were carried out in addition to the routine care of the patients.

The non-selenium part of the SISPCT study involved independently assigning patients randomly to either the procalcitonin guided antimicrobial therapy group or the control group. The details of the PCT algorithm, where plasma procalcitonin levels directed antimicrobial therapy, can be found in the original study. For the purposes of the present immune function study discussed here, procalcitonin measurements were independent of the selenium administration protocol and will not be further elaborated upon.

2.1.2. Immune function assays

As an extension of the SISPCT study, we recruited a total of 76 patients at our center from June 2011 till February 2013 to participate in the immune function study. The randomization process was identical to that in the larger trial and additional blood samples were collected for cytokine stimulation assays. In our cohort, the centrally coordinated randomization led to 40 patients receiving sodium selenite and 36 receiving placebo.

2.2. Cytokine responses

2.2.1. Patient blood collection

Blood samples were collected from an arterial catheter in most cases, if that was not available, blood was drawn from the central venous catheter. Ex vivo whole blood samples for the cytokine assays were taken on days 0, 4, 7, 14, and 21. Routine bloodwork carried out on the ICU by the treatment team was carefully documented and also made available anonymously for the study. In each case, 9ml of patient blood was drawn into a lithium-heparinized tube (S-Monovette® 9 ml, Lithium-Heparin, 92x16 mm, Sarstedt AG & Co., Nümbrecht, Germany) and taken for further processing.

2.2.2. Whole blood stimulation

From the whole blood in lithium-heparinized tubes, 400 µl was transferred under aseptic conditions into tubes containing 400 µl of DMEM (Dulbecco´s Modified Eagle's Medium Nutrient Mixture F-12 HAM, Sigma-Aldrich, Steinheim, Germany) and the various immune stimulating agents. As a control group, the basal cytokine release response was measured without any addition of inciting antigens. The total volume in each tube was 800 µl. Pokeweed mitogen (PWM) (Sigma-Aldrich, Steinheim, Germany) strongly activates B and T cell division in a receptor independent manner. Lipopolysaccharide (LPS) (E.coli serotype 025:B6 Sigma-Aldrich, St. Louis, MO, USA) is an integral part of the outer gram-negative bacterial membrane and induces in animals a profound immune cascade. Phorbol myristate acetate and Ionomycin (PMA-I) (Sigma-Aldrich, St. Louis, MO, USA) stimulates cytokine production in immune cells through the protein kinase C (PKC) signaling pathway. A

CD3/CD28 mixture (Becton Dickinson, Franklin Lakes, NJ, USA) consisted of T cell receptor ligands which bind to major histocompatibility complexes on accessory cells. Additionally, influenza (1% Influvac, Solvay, Hannover, Germany), bacterial (1% Boostrix, GlaxoSmithKline, Munich, Germany), fungal (Candida lysate, Allergopharma, Reinbeck, Germany), and viral (CMV, EBV mixture, ABI, Columbia, SC, USA) stimulants were also used in our study as inciting agents. [25]

2.2.3. Assay analysis

Incubation of samples took place for 48 hours at body temperature (37°C) and the supernatant was subsequently frozen at minus 80°C for future analysis. For the processing at a later time point, samples were thawed and 200 µl of the supernatant mixture was withdrawn. The number of cytokines in the solution were analyzed using Luminex xMAP® technology (Bioplex®) and commercially sold reagents made by BioRad-Laboratories Inc. (Hercules, California, USA) in accordance with manufacturer instructions. The readouts were processed with software from Bioplex and had a sensitivity threshold of 2pg/ml for each analysis. [25]

2.3. Statistics

2.3.1. Data collection

Clinical and laboratory data were recorded initially without identifying information using SPSS (IBM Crop., New York, USA). Day 0 was defined as the timepoint of inclusion into the study until 7 o'clock the following day and blood samples were taken as soon as possible following enrolment. Disease scores were also calculated and recorded as a reflection of patient illness severity including the Simplified Acute Physiology Score SAPS II and Acute Physiology And Chronic Health Evaluation APACHE II at Day 0. Cytokine data were included in the database upon completion of the assays. For concentrations under the assay sensitivity threshold, the detection limit was used to enable statistic calculations.

2.3.2. Data analysis challenges

The overall goal to "investigate whether the evolution of the marker values over time was different in the two treatment groups, selenium and placebo" [25], presents a significant statistical challenge due to missing data points. Given the nature of our patient population and the experimental settings, there was significant attrition in the 21 days as patients pass away or got transferred to the normal inpatient ward. There was also "potentially strong correlation of the measurements within patients, there were individuals with very high average values and individuals with very low average values" [25]. We had a sample size of 76 at day 0, this number dropped significantly by the end of the study at day 21, to only 17 patients. Those patients who passed away represented a population that was likely more severely diseased and those patients who were well enough to leave the ICU likely represented milder disease severities. "It is also important to note that these missing values in this cohort were not completely at random. Moreover, the measurements of each individual were usually noticeably correlated over time (i.e. with progressions in a more similar range to each other on average than measurements from another person). Given this correlation, standard linear regression could not be used to model this data. Selecting an appropriate statistical approach to accurately model the evolution of the datapoints over time while taking all these aforementioned considerations into account required advanced statistical tools." [25]

2.3.3. Generalized least squares (GLS) models

In consultation with colleagues from the biostatistics department at our university, we decided to examine the "treatment effect on immune function over time by fitting generalized least squares (GLS) models with an unstructured correlation matrix." [25] This approach provided the benefit of accounting for a certain degree of correlation between the residuals in the regression to avoid a potentially misleading inference. This method also avoided having to imputate or exputate missing values, which would cause significant skewing given our relatively small sample size. The statistical software R was used. "The R function 'gls' from the R package 'nmle' was applied to each log transformed marker successively with treatment and time (coded as factors) as well as their interaction as covariates. An assumption was made that the probability a missing value was determined by

the last observed values." [25] This is realistic in our situation, for example, when a patient was recovering from sepsis, the laboratory values would tend to progressively trend relatively to previous values. This means we could yield a valid inference without imputation of the missing values. Additionally, correlations between measurements of each patient were accounted for.

"The log transformation log(1+x) was used to better approximate normality as there were values close to zero. For each marker, the global null hypothesis of no interaction between treatment and time was tested using a likelihood-ratio test as implemented in the R function 'anova'. This analysis was repeated for 42 different combinations of inciting antigens and measured cytokines. Holm's procedure was used to adjust for multiple testing. All statistical analyses were conducted with R (version 3.3.1)." [25]

3. Results

3.1. Study population

3.1.1. Overall patient characteristics

As a part of the larger clinical trial SISPCT, we recruited a total of 76 patients to be included in the immune function substudy at our intensive care units. Seven of which were either lost to follow up or retracted consent by the end of the 90-day study period. Six patients (8%) had a diagnosis of severe sepsis and 70 patients (92%) had septic shock at enrolment. 41 patients (54%) were admitted through the medicine service (non-surgical) and 35 patients (46%) were admitted due to a primarily surgical intervention. Of the surgical patients, 27 (36%) underwent unplanned operations and 8 (11%) were scheduled (elective and non-elective) operations.

Most common admission criteria included pneumonia (53%), intra-abdominal infections (19%) and urosepsis (7%). Approximately half of the patients had a microbiologically proven infection, from these 54% were gram-negative bacteria in origin, 38% gram-positive, and 8% viral. The vast majority of which (96%) had received antibiotics at study begin and half had been started on hydrocortisone therapy (routinely used in septic shock patients who are hemodynamically fragile).

In terms of patient outcomes, 26 (35%) required renal replacement therapy at some point during the ICU stay. The average number of days spent on the ICU was 11. The mortality rate of this subgroup while undergoing critical care was relatively low at 12% (8 patients) and the 90-day mortality rate was 20% (14 patients).

3.1.2. Comparing the selenium and placebo groups

With a grand total of 76 patients included at our hospital, 40 received infusions of sodium selenite and 36 sodium chloride solutions through the SISPCT study randomization (Table 3). The attrition rate over the 21-day period was comparable in both groups. The selenium arm began with 40 patients and dropped to 33 (at day 4), 24 (at day 7), 16 (at day 14), and 8 (at

day 21) respectively. In the placebo arm, the 36 patients at enrolment went down to 28 (at day 4), 26 (at day 7), 16 (at day 14) and 9 (at day 21) respectively. Age, gender, weight, and height comparisons are listed also in the table below.

Table 3.

Comparison of patient characteristics in the placebo and selenium groups.

	Placebo group	Selenium group
Patients at day 0/4/7/14/21	36/28/26/16/9	40/33/24/16/8
Age	61.3 ± 16.0	60.5 ± 17.4
Sex (m/f)	18/18	23/17
Weight (kg)	83.1 ± 19.8	84.5 ± 27.8
Height (cm)	172.1 ± 5.2	170.5 ± 10.2
GCS	6.4 ± 5.2	6.6 ± 5.2
APACHE II*	27.1 ± 7.6	27.7 ± 9.2
SAPS II	65.4 ± 15.6	66.0 ± 17.0
MOD	8.6 ± 3.4	8.2 ± 3.1
SOFA	12.4 ± 3.8	12.6 ± 3.8
MAP max. (mmHg)	94.8 ± 16.4	97.8 ± 17.6
MAP min. (mmHg)	62.9 ± 14.5	63.0 ± 12.1
HR max. (bpm)	127.2 ± 32.6	121.5 ± 22.3
HR min. (bpm)	87.6 ± 21.9	83.2 ± 26.0
Lactate max. (mmol/L)	3.8 ± 2.3	4.6 ± 5.1
CRP (mg/L)	18.4 ± 12.4	22.8 ± 16.2
Antibiotics prior to admission (y/n)	33/2	39/1
Hydrocortisone (y/n)	24/12	20/20

Continuous variables are summarized as mean ± SD. GCS = Glasgow coma scale, APACHE = acute physiology and chronic health evaluation, SAPS = simplified acute physiology score, MOD = multiple organ dysfunction, SOFA = sepsis-related organ failure assessment. m = male, f = female, y = yes, n = no. MAP = mean arterial pressure during entire ICU stay, HR = heart rate. *As patients were sedated, the APACHE II scores were also calculated assuming a GCS of 15: placebo group 18.5 ± 6.9, selenium group 18.8 ± 6.6.

Taken from Table 1 in Guo et al. [25] Use with copyright permission from Elsevier.

The selenium and placebo groups had comparably ill patients with similar simplified acute physiology score (SAPS II), acute physiology and chronic health evaluation (APACHE II), multiple organ dysfunction score (MODS) and sepsis-related organ failure assessment (SOFA) score. The mean arterial blood pressure and heart rate are physiological parameters that reflect disease severity in sepsis and whether adequate fluid resuscitation and/or pressor support has been initiated. C-reactive protein is a commonly used inflammatory

marker with huge variations in the critically ill. Serum lactate is a marker for tissue hypoperfusion and hypoxia due to anaerobic metabolism.

3.2. GLS analyses

Overall the GLS analyses yielded no statistically significant immune function difference between selenium and placebo groups over time after adjustment for multiple testing (42 combinations of inciting antigen and measured cytokine were tested). Because the assumption of constant variance was not satisfied in our data collection, we used the generalized least squares approach to account for inequalities of variance. The entirety of these results is tabulated in Section 9 and the corresponding visual representation of the data are included in the following results section. The x-axis demonstrates time progression (days 0, 4, 7, 14, and 21) for the two treatment groups, placebo and selenium. Logarithmically transformed longitudinal results (log (cytokine readout +1)) with the units picogram/mL are displayed along the y-axis. Despite the extremely widespread values and outliers, no individual datapoint was removed from the analysis to more closely model reality. Therefore, there are additional datapoints to be seen aside from the traditional box plots to better represent the spread of the data.

Figure 1. Sample GLS analysis from R.

```
> summary(model.IFNg.cmv)
Generalized least squares fit by REML
  Model: IFNg_levels ~ time.points * Groups
  Data: m.IFNg.cmv
       AIC      BIC    logLik
  506.9637 578.1342 -232.4818

Correlation Structure: General
 Formula: ~1 | ID
 Parameter estimate(s):
 Correlation:
  1      2      3      4
2  0.291
3  0.059  0.181
4 -0.042  0.313  0.294
5  0.034 -0.142  0.105  0.289

Coefficients:
                                     Value Std.Error   t-value p-value
(Intercept)                      0.7575372 0.1125340  6.731626  0.0000
time.pointsday_4                 0.0560129 0.1458279  0.384103  0.7013
time.pointsday_7                 0.2069734 0.1734083  1.193562  0.2339
time.pointsday_14                0.0204859 0.2044295  0.100210  0.9203
time.pointsday_21                0.3999618 0.2331150  1.715728  0.0876
GroupsSelenium                  -0.1070510 0.1551175 -0.690129  0.4908
time.pointsday_4:GroupsSelenium -0.3699261 0.2000811 -1.848881  0.0658
time.pointsday_7:GroupsSelenium -0.0239032 0.2428158 -0.098442  0.9217
time.pointsday_14:GroupsSelenium -0.0400189 0.2894122 -0.138276  0.8901
time.pointsday_21:GroupsSelenium -0.7423987 0.3649280 -2.034370  0.0431
```

After running the generalized least squares fit code in R using the datasets, regression coefficients were generated with accompanying p-values (a representative dataset is presented in Figure 1). The intercept, placebo group at day 0, was the comparison point to all later timepoints as well as the selenium group. These results were then tabulated and presented in excel tables including the p-values as well as box plots for all 42 combinations of reagents and cytokines. Representative tables are included below in the results section, the complete collection can be found in Section 9.

3.2.1. Basal

The stimulation assays in this group did not have any antigens to provoke the immune system and the cytokine responses were therefore a reflection of the unprovoked release found in patient blood samples. They were, as compared to assays with added antigens, logarithmically less. The values were concentrated between the log0 and log1 interval. There was also no discernable trend, either an increase or a decrease, over the 21-day period and no significant difference between the placebo and selenium groups.

Table 4. No antigen addition immune assays ("basal") with GLS model readouts from IL2, TNF and IFN over time (day 0 to day 21).

Log IL2 - Basal				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,138	0,0005	-0,069	0,2015
Day 4	-0,009	0,8013	-0,015	0,7697
Day 7	0,119	0,0419	0,02	0,8027
Day 14	-0,057	0,1303	0,002	0,9743
Day 21	-0,033	0,4673	0,035	0,6031

Log IL2 - Basal				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,138	0,0005	0,069	0,2015
Day 4	0,129	0,8013	0,054	0,7697
Day 7	0,257	0,0419	0,089	0,8027
Day 14	0,081	0,1303	0,071	0,9743
Day 21	0,105	0,4673	0,104	0,6031

Log TNFa - Basal				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,321	0	-0,076	0,4265
Day 4	0,05	0,6563	-0,071	0,6489
Day 7	0,144	0,1312	0,076	0,5694
Day 14	-0,008	0,9433	-0,072	0,6255
Day 21	0,093	0,4685	-0,024	0,9025

Log TNFa - Basal				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,321	0	0,245	0,4265
Day 4	0,371	0,6563	0,174	0,6489
Day 7	0,465	0,1312	0,321	0,5694
Day 14	0,313	0,9433	0,173	0,6255
Day 21	0,414	0,4685	0,221	0,9025

Log IFNg - Basal				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,599	0	-0,096	0,4487
Day 4	-0,033	0,7924	-0,151	0,3796
Day 7	0,118	0,4033	0,079	0,6899
Day 14	-0,008	0,9601	-0,121	0,5795
Day 21	-0,382	0,0029	0,189	0,3427

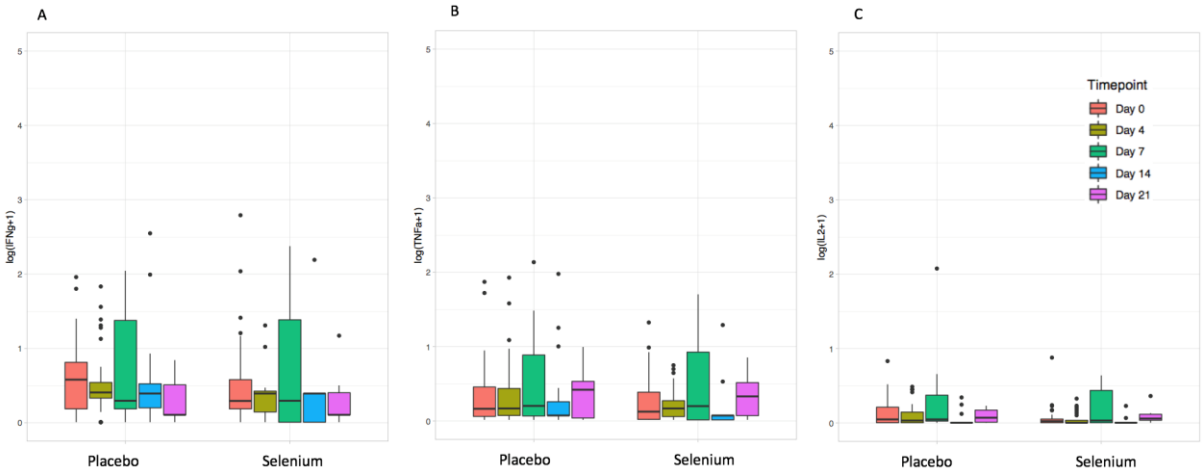
Log IFNg - Basal				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,599	0	0,503	0,4487
Day 4	0,566	0,7924	0,352	0,3796
Day 7	0,717	0,4033	0,582	0,6899
Day 14	0,591	0,9601	0,382	0,5795
Day 21	0,217	0,0029	0,692	0,3427

The analysis of variance (ANOVA) showed consistently large P-values, which suggests that the observed differences between treatment groups were likely due to random chance. There was no compelling evidence to reject the null hypothesis given the overwhelmingly non-significant P-values. The coefficients in the model represented an estimate of the change in the response variable for a one-unit change in a predictor variable. In our case, there were a number of predictor variables (treatment groups, time points, interaction of treatment and time point). All these predictors were treated as categorical variables in the model, so a one-unit change can be seen as whether or not a condition was present or absent. Given that the coefficients had large P-values, no significant relationship between individual predictors and the response can be concluded. This was seen in all test groups and the above table is a representative example for illustration. The placebo group Day 0 P-

values were almost always approaching zero, because this coefficient represented the intercept in the model. This makes sense intuitively because the average cytokine response on Day 0 in the placebo group was significantly different from zero (the intercept).

The cytokines examined without the addition of any inciting stimulating agent and their assay results are graphically displayed in Figure 2, which included interferon gamma (IFN), tumor necrosis factor alpha (TNF), and interleukin-2 (IL-2).

Figure 2. Logarithmic cytokine release profiles using no stimuli ("basal"); A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

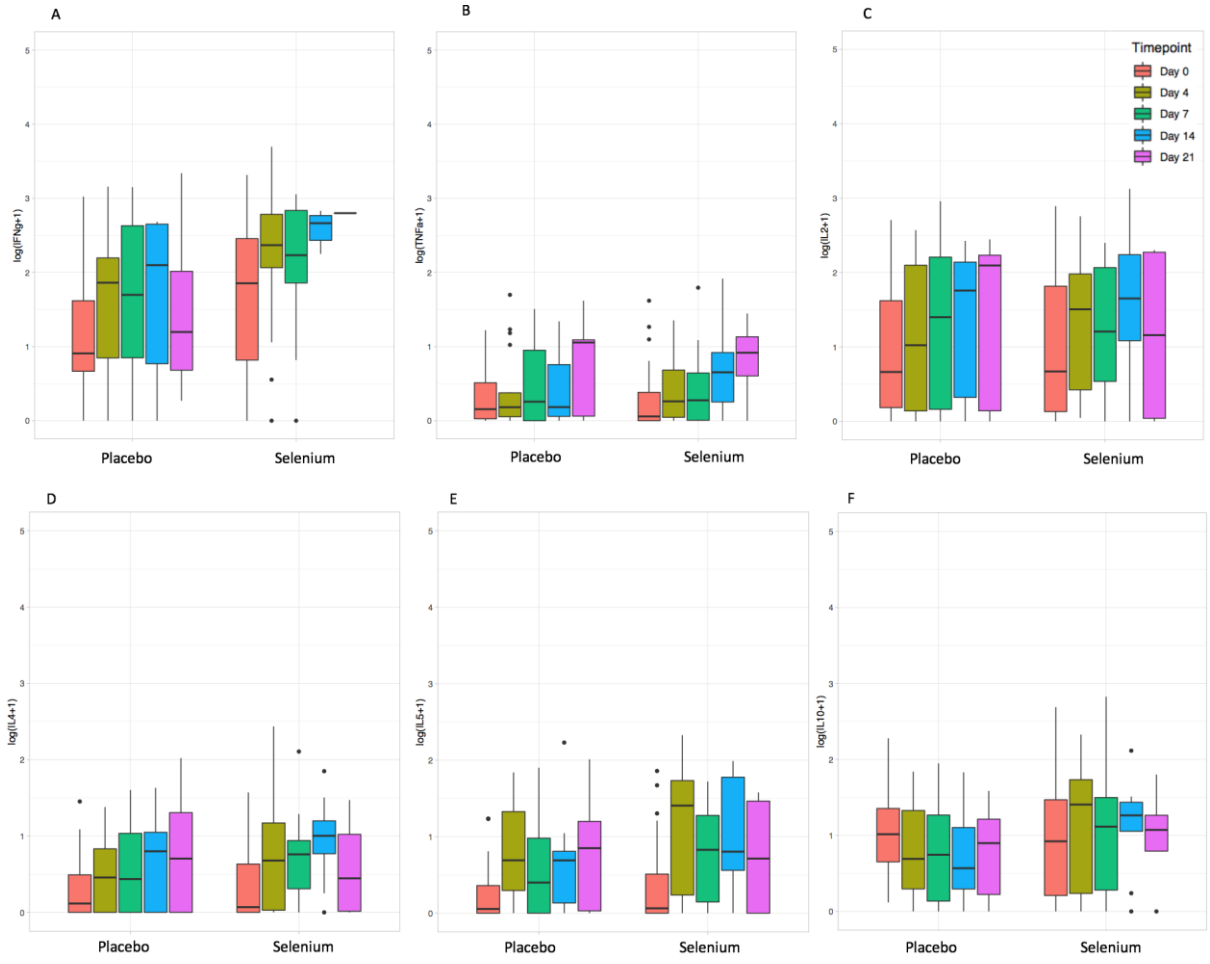


3.2.2. CD3 / CD28

The T-lymphocyte specific stimulators CD3 and CD28 partially mimic stimulation by antigen-presenting cells in the body [28]. CD3 is a part of the T-cell receptor complex and CD28 co-stimulation is necessary for activation. Without appropriate co-stimulation, the T-lymphocytes would go down the anergic response pathway. This combination of antigens provides a reflection of the body's acquired immune response.

CD3 / CD28 proved to be a powerful stimulant in this case, demonstrating an active acquired immunity in sepsis patients. Many cytokines were investigated, none of which showed a statistically significant difference with respect to time or to the treatment groups. The cytokine release profiles from IL-2, IL-4, IL-5, IL-10, IFN, and TNF can be seen in Figure 3.

Figure 3. Logarithmic cytokine release profiles using CD3 and CD28 as stimuli; A) interferon B) tumor necrosis factor C) interleukin-2 D) interleukin-4 E) interleukin-5 and F) interleukin-10, over time (day 0 to day 21).

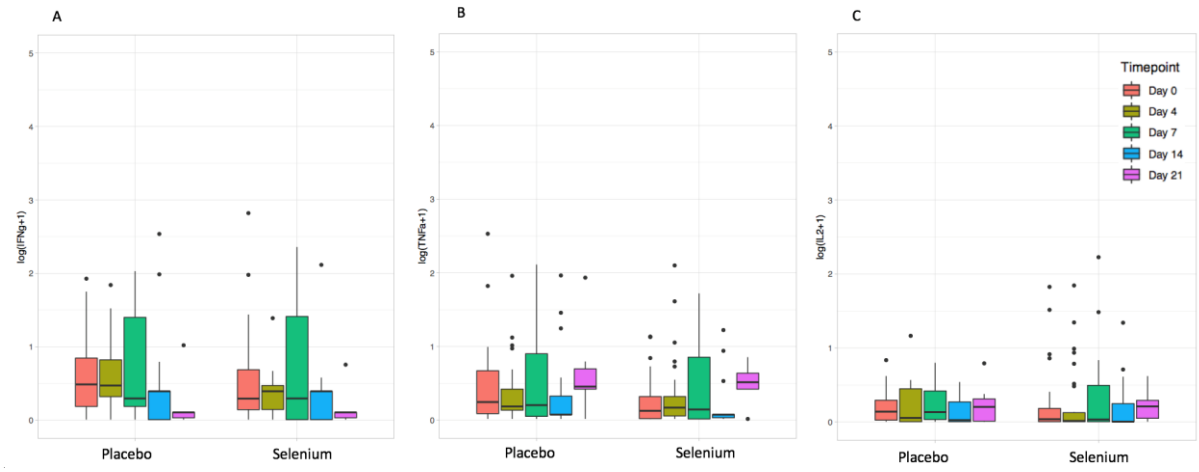


3.2.3. Aspergillus

Aspergillus is a fungus which can clinically cause severe disease, primarily through pulmonary infections. From an immune perspective, the innate immune response is largely involved given that in healthy individuals, cytokines drive the release and recruitment of neutrophils in order to clear this pathogen. It is therefore of particular significance in immunocompromised or severely ill patients [29]. The adaptive immune response also plays a role upon the exposure to airborne aspergillus species since fungal species are naturally found in the environment.

This set of stimulation assays showed a somewhat dampened cytokine release profile with many outliers and a huge spread but no significant differences between the placebo and selenium groups. There were also no notable trends over time. The logarithmic plots from the cytokines IFN, TNF, and IL-2 are shown in Figure 4 below.

Figure 4. Logarithmic cytokine release profiles using Aspergillus as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

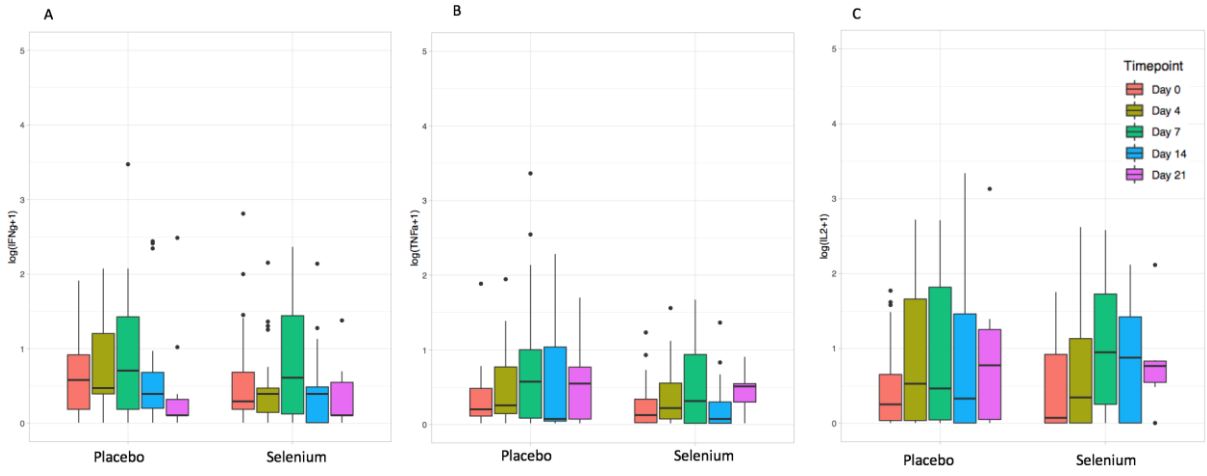


3.2.4. Bacteria

The human innate immune system has evolved for hundreds of millions of years under the selective pressure of bacterial peptides, allowing the modern eukaryote to possess highly complex immune mechanisms. Yet, they are still built on common molecular strategies through the recognition of conserved microbial peptides by a wide range of pattern recognition receptors (PRRs) to signal the first line defenses [30, 31, 32]. These diverse PRRs simultaneously activate the innate and acquired immune responses by detecting pathogen-associated (PAMPs) or danger-associated molecular patterns (DAMPs) in triggering the cytokine cascade.

Bacterial components did yield a good response in the cytokine assays, especially interleukin-2. The response demonstrated a very slight tendency of increase between days 0 and 7 followed by a slight decrease from days 7 to 21. There was no detectable difference between the placebo and selenium treatment groups. In Figure 5, the logarithmic data from IFN, TNF, and IL-2 are displayed as box plots respectively.

Figure 5. Logarithmic cytokine release profiles using bacteria as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

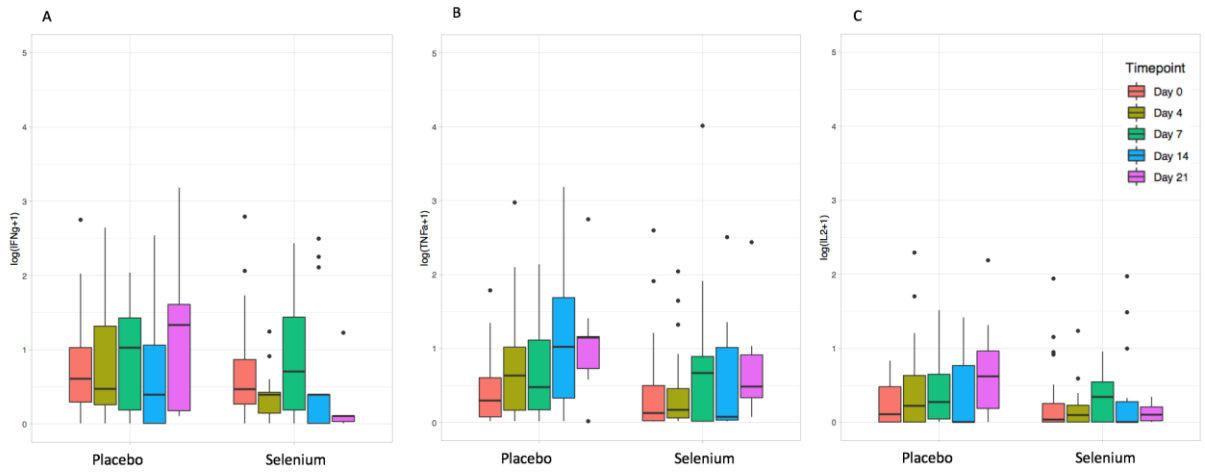


3.2.5. Cytomegaly virus

The human cytomegaly virus is an ancient herpes virus that has co-evolved with the immune system for a long time and rarely causes life-threatening symptoms in healthy individuals [33]. The host antiviral mechanisms implicate both the innate and adaptive compartments. Virus-infected monocytes differentiate into macrophages and are presented by the innate immune system to the pattern recognition receptors. The adaptive immune response to CMV is amongst the strongest in humans and actively engages the humoral and cellular responses [34].

The cytokine release assays with CMV showed a moderate response with some extremely high-valued outliers. In the placebo group, there was a trend for increased cytokine release over time, but this was not observed in the selenium group. Statistically significant differences were not detected amongst the treatment arms. The immune release assays measuring IFN, TNF, and IL-2 are shown below in Figure 6.

Figure 6. Logarithmic cytokine release profiles using bacteria as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

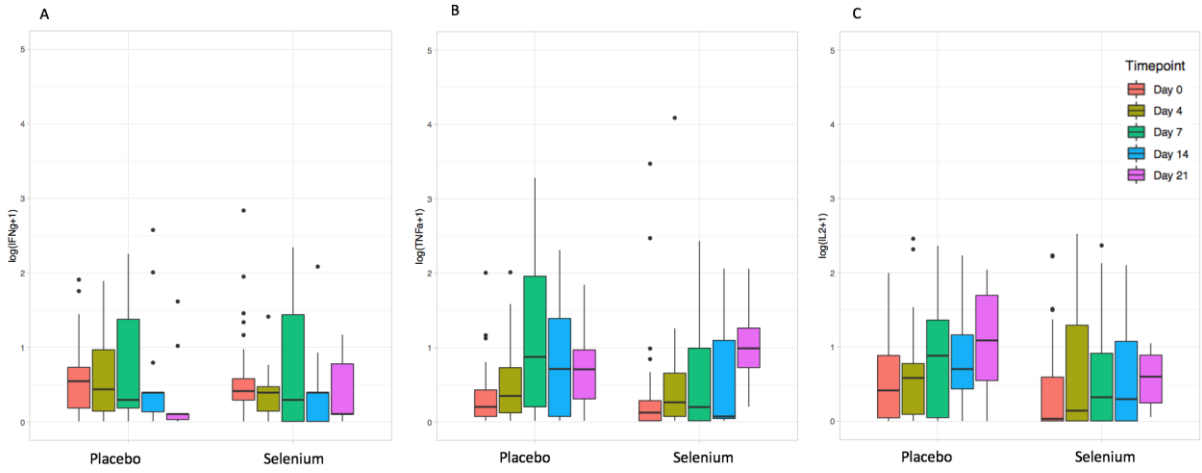


3.2.6. Fungal components

The ubiquitous exposure to fungi results in elaborate mechanisms to neutralize the pathogen for immune sufficient individuals. These responses engage both the innate and adaptive immune systems, producing an inflammatory response via the activation of nuclear factor kappa B and cytokines [35]. Binding to danger-associated molecular patterns DAMPs promote the activation of pathways that release substances due to tissue and cell damage, causing clinically significant disease in the immune compromised.

The cytokine stimulation assays showed a moderate response. The selenium group had a slightly decreasing trend over time whereas the placebo group did not. There was no difference statistically between the selenium and placebo arms. The logarithmically transformed data can be seen graphically below in Figure 7 with readouts from IFN, TNF, and IL-2.

Figure 7. Logarithmic cytokine release profiles using fungus as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

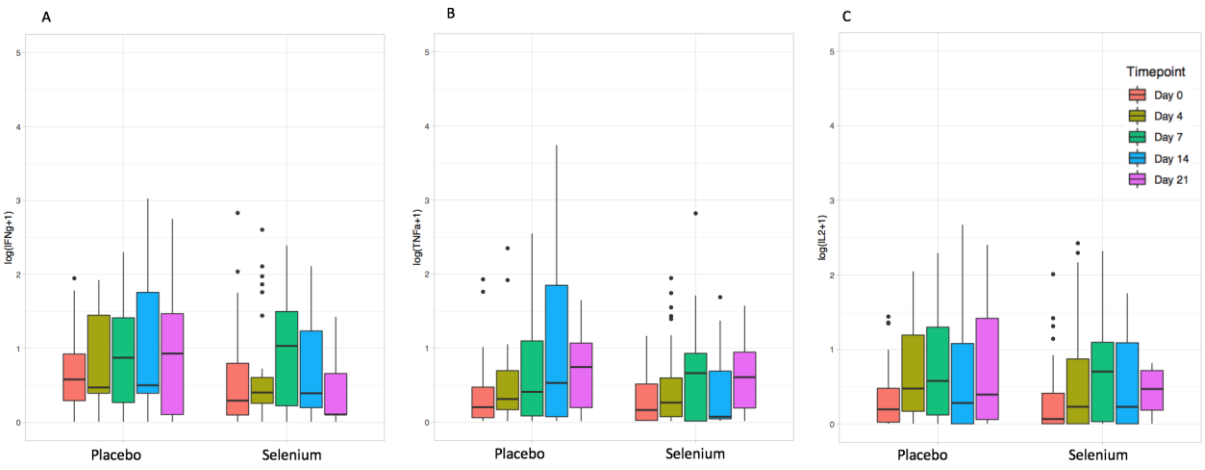


3.2.7. Influenza

Influenza viruses belong to a family of enveloped viruses which cause significant burden of respiratory diseases in the world. While the innate immune system rapidly responds to the initial infection, it then initiates the humoral immune system in producing antibodies and the cell-mediated immune system to activate helper and cytotoxic T-lymphocytes [36].

The influenza antigen elicited a moderate cytokine response in the whole blood ex vivo assay. There appeared to be an increased cytokine release over time in sepsis patients in the placebo group and an ever so slight decrease during the same period in the selenium group. We did not observe any differences statistically between the placebo and selenium groups. In Figure 8 below, the cytokine measurements using influenza as the inciting stimulus are shown with IFN, TNF, and IL-2 respectively.

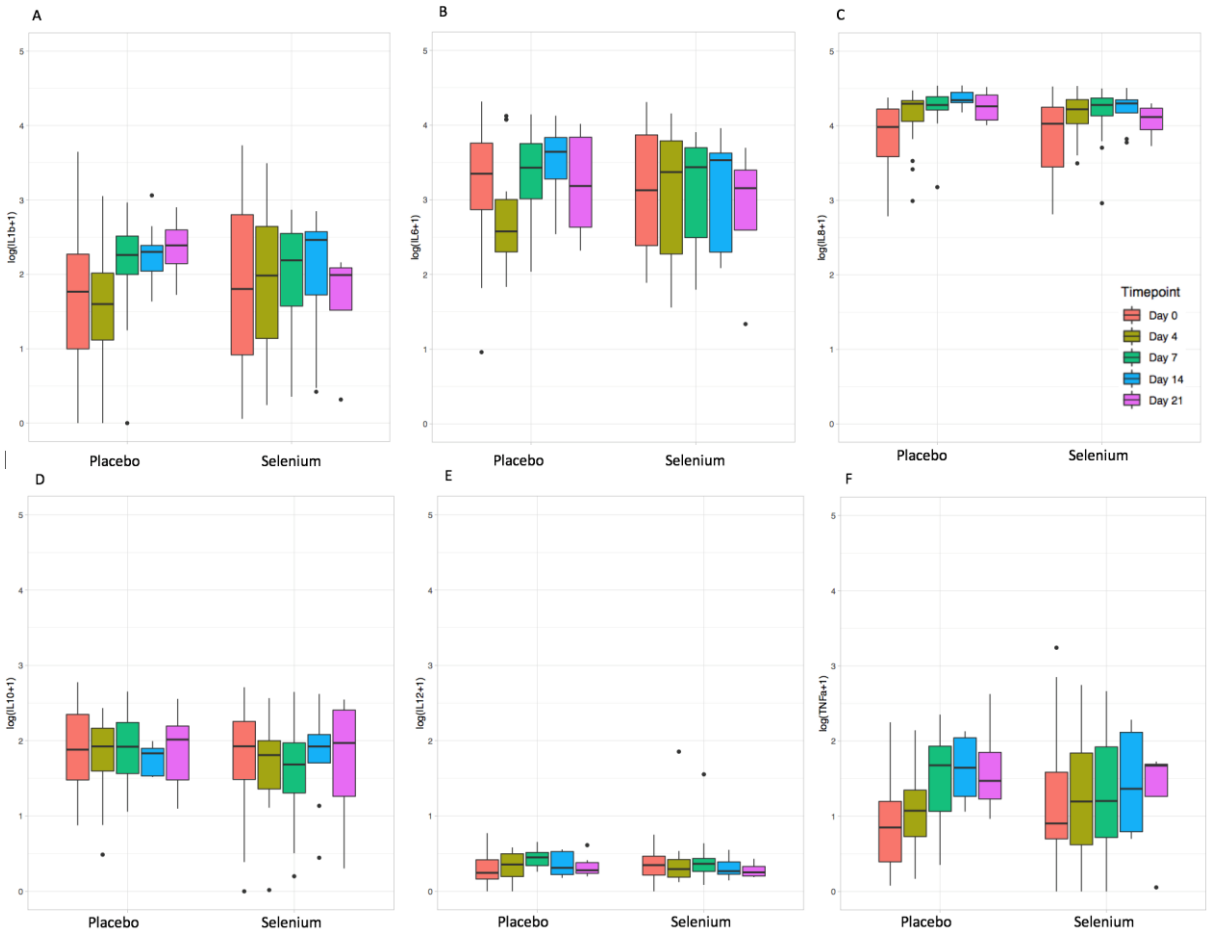
Figure 8. Logarithmic cytokine release profiles using Influenza virus as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).



3.2.8. Lipopolysaccharide

Lipopolysaccharides (LPS) are large thermostable molecules with water and fat solubility embedded in the outer membrane of gram-negative bacteria. They help stabilize the membrane and generally elicit a strong immune response in humans. Their compositions are highly variable, which lead to a huge variation in their immunogenicity. LPS, also known as endotoxins, have been implicated in various disease processes such as endotoxemia, auto-immune illnesses, cancer and obesity [37]. Lipopolysaccharides induced a very high cytokine count in the whole blood assays and a consistently strong response. There did not appear to have a time-axis dependent trend and no statistically significant difference was observed between the two groups. Six cytokine full blood assays stimulated with lipopolysaccharide are shown in Figure 9 with IL-1b, IL-6, IL-8, IL-10, IL-12, and TNF.

Figure 9. Logarithmic cytokine release profiles using lipopolysaccharide as stimuli; A) interleukin-1b, B) interleukin-6, C) interleukin-8, D) interleukin-10, E) interleukin-12, and F) tumor necrosis factor; over time (day 0 to day 21).

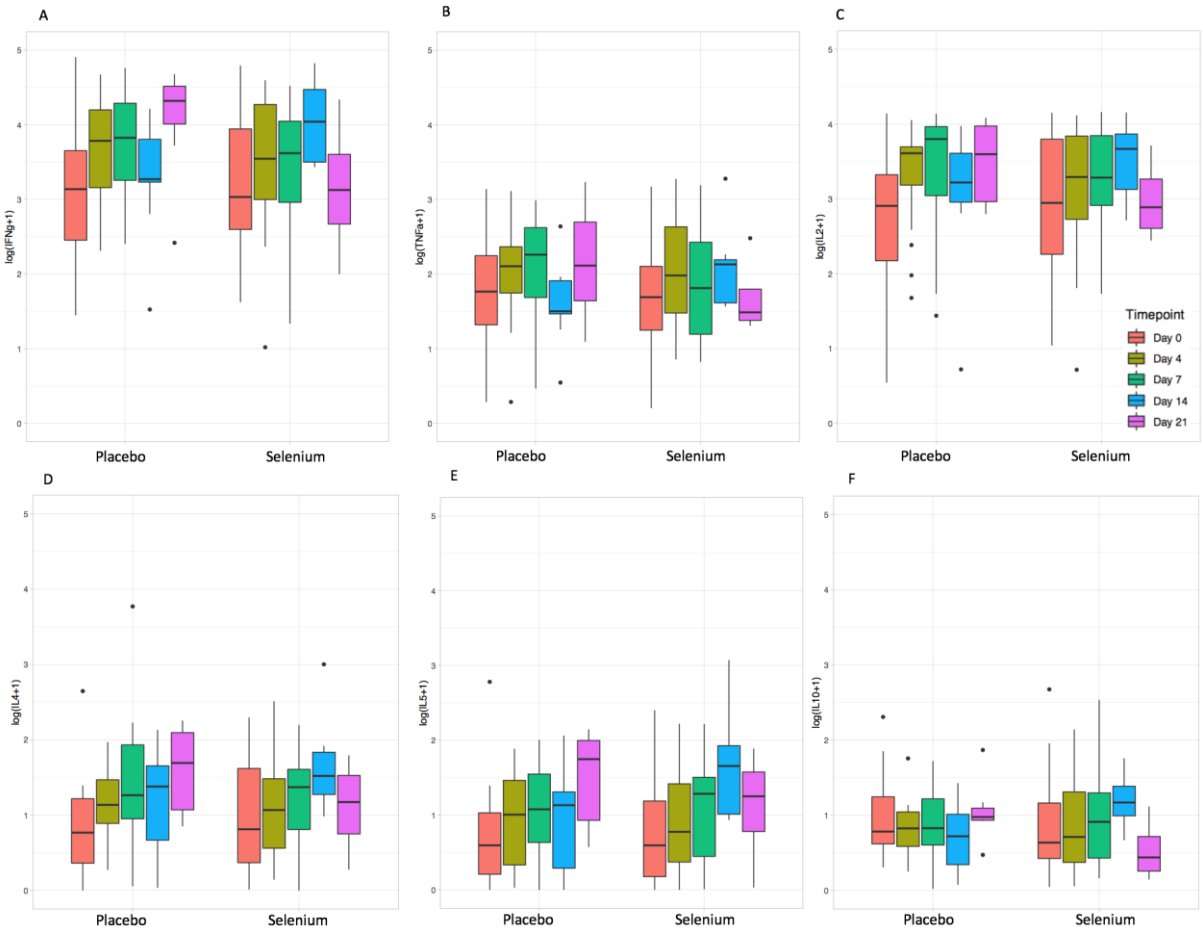


3.2.9. Phorbol myristate acetate and ionomycin

Phorbol myristate acetate (PMA) is a potent tumor promotor often used in research contexts to activate the protein kinase C signal transduction pathway in studying carcinogenesis [38]. Used in combination with ionomycin, it can stimulate T-lymphocyte activation, proliferation, and cytokine production.

Its strong immunogenicity is reflected in the assays with very robust cytokine release profiles. No particular trends were observed with respect to the time-axis. We found no difference statistically in the cytokine release profiles during the 21-day period between the selenium and placebo groups. The cytokine release assays are plotted logarithmically in Figure 10 with IFN, TNF, IL-2, IL-4, IL-5, and IL-10.

Figure 10. Logarithmic cytokine release profiles using lipopolysaccharide as stimuli; A) interferon, B) tumor necrosis factor, C) interleukin-2, D) interleukin-4, E) interleukin-5, and F) interleukin-10, over time (day 0 to day 21).

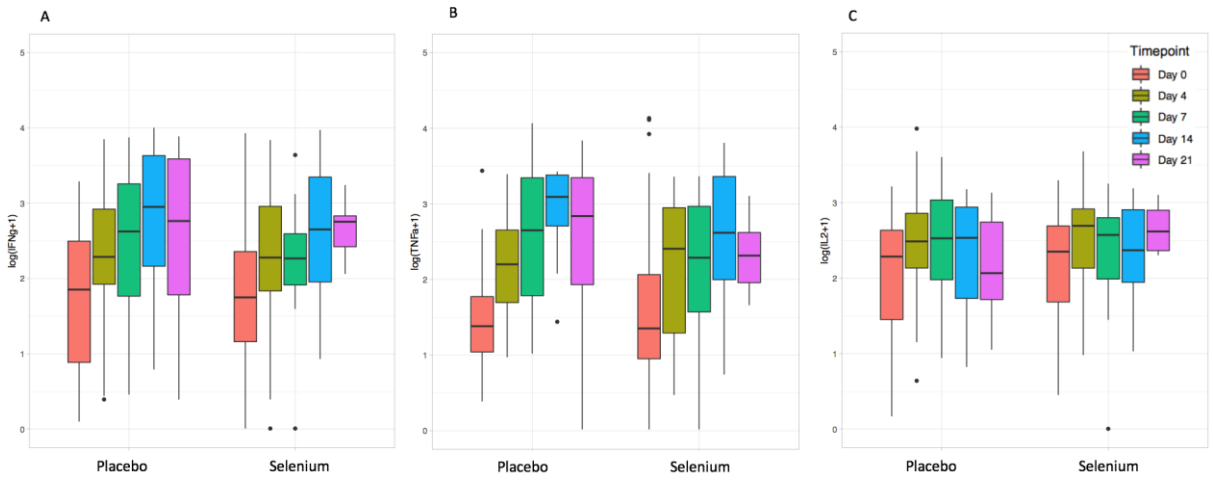


3.2.10. Pokeweed mitogen

Pokeweed mitogen is a glycoprotein that strongly activates lymphocytes and stimulates the proliferation of B-cells, T-cells and plasma cells [39]. The small peptide triggers mitogenesis by uninhibiting checkpoint proteins in the cell cycle.

An impressive immune response was seen in the whole blood assays with high cytokine readouts. The levels remained relatively unchanged over the entire 21-day period and there was again no significant difference between the place and selenium groups in the GLS analysis. The measurements collected from the cytokine assays using IFN, TNF, and IL-2 are shown in Figure 11.

Figure 11. Logarithmic cytokine release profiles using pokeweed mitogen as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).

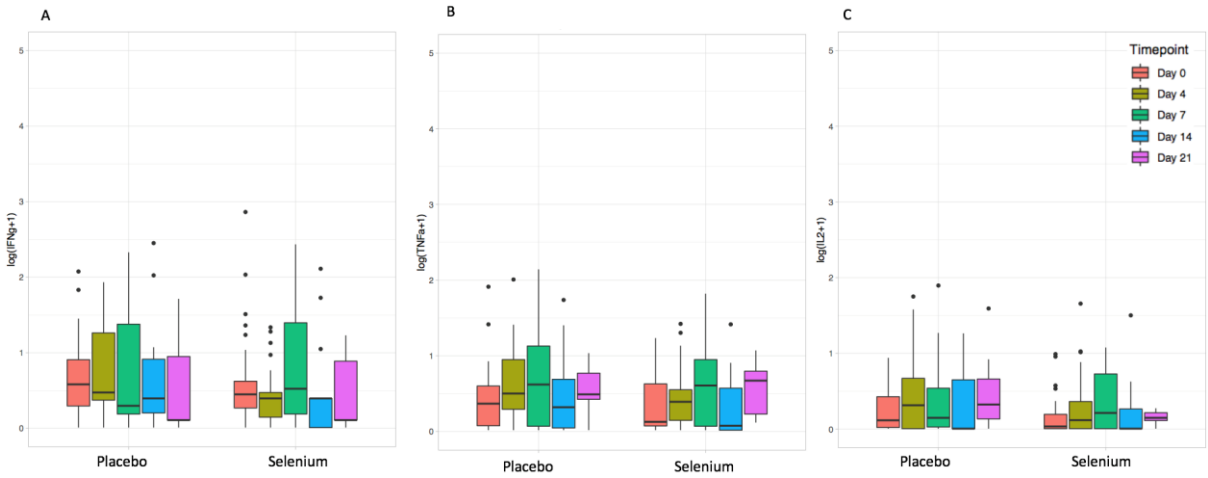


3.2.11. Virus

Viruses are highly adaptable and they have evolved a truly complex relationship with the human immune system. One of the first line defences include natural killer and cytotoxic cells mediated by antigen presenting cells [40]. Interferons that are released by the immune system also directly interfere with, as the name suggests, a virus' ability to replicate. Antibodies from B-lymphocytes facilitate agglutination and phagocytosis of infected cells, as a mechanism to rid the body of the pathogen.

The immune assays demonstrated a mild to moderate response to viral antigens using blood samples of sepsis patients. No particular trend with respect to time can be noted and no statistical difference was seen between the treatment groups. The logarithmically transformed assay data are shown below in Figure 12 with the cytokines IFN, TNF, and IL-2.

Figure 12. Logarithmic cytokine release profiles using viruses as stimuli; A) interferon B) tumor necrosis factor and C) interleukin-2, over time (day 0 to day 21).



3.3. Statistical considerations

3.3.1. Quantile-quantile plots

To visually assess the normal distribution of the 42 investigated cytokine stimulation assays, we created individual quantile-quantile (Q-Q) plots. We ran the GLS statistical analysis based on the assumption that the dependent variable was normally distributed. These Q-Q plots were able to confirm that our data more or less conform to a normal distribution, reaffirming the validity of our model. Below are two samples of Q-Q plots created using the statistical software R for the data from LPS and IL-12 as well as PWM and TNFa (Figure 13). All 42 Q-Q plots had such a roughly straight line and reassured us of a normal distribution.

LPS and IL-12 **PWM and TNF**

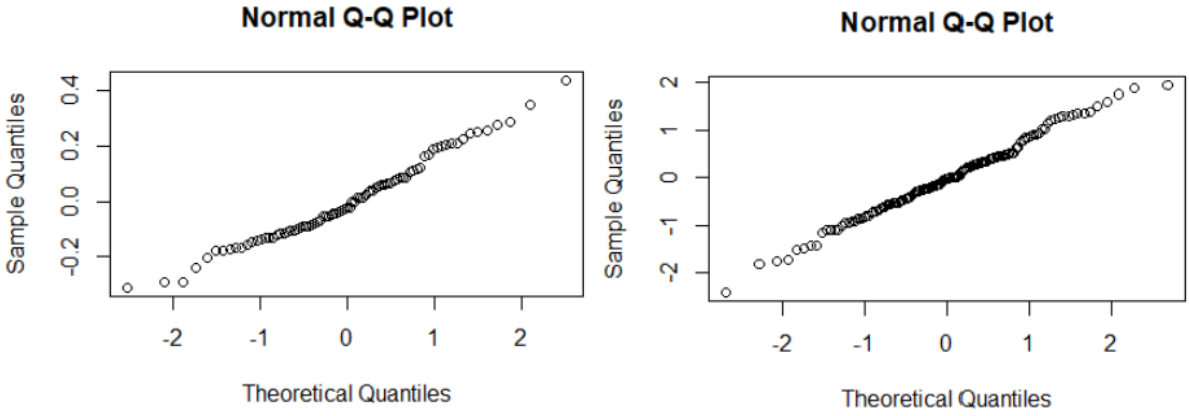


Figure 13. Representative Q-Q plots in evaluating normal distribution.

3.3.2. Holm method for multiple testing

Statistical inferences are often made based on a predetermined acceptable level of probability, such as 0.05. Which is why significance levels are of great importance in testing a null hypothesis in the sciences, to accept or reject. There are, however, many situations such as this particular immune function study, where a large number of hypotheses were being tested simultaneously, in our case, 42. This contributes to a statistical challenge known as multiple comparisons, where the probability of incorrectly rejecting the null hypothesis dramatically increases. When 100 hypothesis are tested with a significance level of 0.05, around 5 will be significant due to multiple comparisons [41]. This false positive rate may be even higher (and more complex), when the hypotheses are correlated, like in this case.

When all the whole blood assays with different combinations of inciting antigens and cytokines were run, we tested in effect 42 null hypotheses simultaneously where there was no difference in cytokine release over the 21-day period between the placebo and selenium groups. Indeed, we had a few statistically significant tests but once adjusted for the multiple comparisons, these effects were no longer. This demonstrated that between the placebo and selenium groups, we cannot discern a difference with respect to cytokine release profiles over time. In other words, there is no evidence of effect.

The method we chose to account for multiple testing was Holm's method, which is based on the Bonferroni method, where family-wise error rates (FWER) or type I errors are accounted for. Holm's method "iteratively accepts and rejects hypotheses" by progressively adapting the threshold values [42], making it more powerful than Bonferroni's method. After adjusting our results for multiple comparisons using Holm's procedure, the null hypotheses could not be rejected anymore.

3.3.3. Day 14 subgroup analysis

In order to determine whether the severely ill sepsis patients benefited from selenium supplementation, we analyzed the subgroup of patients who stayed longer than 2 weeks in the ICU. The rationale behind this is that the extremely critically ill patients would be particularly immune compromised. However, there was no significant difference to be found between the selenium and placebo groups. This further reinforces the immune neutral effects of selenium in this particular setting.

4. Discussions

4.1. The null hypothesis

Building upon over two decades of past studies on selenium administration in an intensive care setting, the most recent systematic review and meta-analysis demonstrated no mortality benefits [43]. The cited studies were all conducted on ICU patients and consisted of 4044 patients in aggregate worldwide. Selenium was administered intravenously either as monotherapy or in combination with other antioxidative nutrients. Ultimately, there were no discernable effects provided through selenium supplementation on mortality, length of ICU stay, days on the ventilator, renal function, or infection rates. The majority of studies focused exclusively on septic patients, while some were a mixed population. There is no mistaking the importance of this highly lethal disease process, as clinicians and scientists are to this day searching for better therapies to improve patient outcomes.

What these 21 randomized controlled trials did not investigate specifically was selenium's effects on immunity, we sought to scrutinize exactly this immune response in our cohort of septic patients. Despite its reputation as an immune booster, patients with severe sepsis or septic shock receiving intravenous sodium selenite did not have an altered cytokine release profile over a 21-day observation period at the study site. Our immune function assays further supported the clinical findings of no mortality benefit and lend evidence to the explanation, that selenium does not noticeably strengthen immune capabilities during sepsis.

4.2. Monitoring of immune modulation

The pathophysiology of sepsis is until today not completely understood. The immune system mounts an overwhelming pro-inflammatory response in the beginning and often shifts to an immunosuppressive phase, where deaths occur due to secondary infections [44]. We were curious whether selenium would have an impact on the immune response and chose to monitor cytokine release during the disease process through a simple yet comprehensive assay with proven sensitivity [45].

A panel of cytokines were selected and their concentrations measured upon stimulation as a reflection of the overall immune function. Interferon gamma and tumor necrosis factor are known to transfer signals from T cells to infected cells in enhancing the killing mechanisms. Based on the assay results, IFN and TNF levels were not significantly altered with selenium supplementation, or in other words, the cytotoxic mechanisms were not boosted. The various interleukins (IL-1b, -2, -4, -5, -8, -10, and -12) all play important and specific roles in immune modulation and regulation. Therefore, a detection of significant trends in the release of particular cytokines could have revealed in which arm of the immune response selenium played a role. Such an effect was ultimately not found in any combination of antigens and cytokines after statistical adjustment. This could likely be due to the severe nature of the disease, the immune system is so intensely compromised that the therapy requires multiple measures. Additionally, whether the patients were facing a hyper- or hypoinflammatory state at the time of selenium administration is unclear. Given the delicate interplay between fighting off an infection and causing too much damage in the process, it is perhaps unlikely to expect a simple fix to a complex problem.

4.3. Immunity and selenium

While there have been many clinical trials conducted on the efficacy of selenium supplementation in the critically ill, not very much evidence is present on how the trace element directly affects the immune system. There are, however, a handful of studies which have shown that selenium promotes the proliferation of activated T cells and natural killer cell activity, as well as enhances cytotoxic lymphocyte mediated capabilities in targeting cancer cells [46, 47]. In mice models, differentiation of CD4+ T cells favored the Th1 pathway, which leads to an increased cell-mediated response important in fighting intracellular bacteria and viruses, through dietary selenium supplementation [48]. Many of these studies were performed in elderly or cancer patients, both of which have a compromised immune system. Simple supplementation with 100µg selenium daily over a 6 months period resulted in a stronger proliferative response to antigen challenge in an elderly study population [49]. Most of the positive immune effects of selenium can be attributed to the insertion of the element into selenoproteins, 25 of which have been identified in humans and they are found in a wide variety of tissues [50].

The immune function in severe sepsis patients did not improve as a result of increased selenium, for which, there are many explanations. The dosage may not have been sufficient. While many clinical trials have been run, there is no laboratory model of sepsis and a paucity of experimental studies on selenium therapy, which could reveal dosing regimens that are not necessarily easy to test directly on patients. Timing could also play a role. While sodium selenite was administered at sepsis onset, this is a very loose definition based on the time of diagnosis. Perhaps some patients have been suffering from an infection without SIRS for a long time, that an immune boost would have been required at an earlier point in time. In addition, half of our study population received hydrocortisone, a standard therapy to prevent cardiovascular collapse. This immune suppressive medication could have mitigated the immune boosting effects of selenium in our particular group of patients.

4.4. Selenium and infections

Selenium deficiency has been associated with less favorable outcomes in HIV infected individuals [51] and tuberculosis patients [52]. Low levels of serum selenium in patients with Acquired Immune Deficiency Syndrome have been associated with decreased survival, decreased CD4+ Cell count and high viral load. The results hold true even after adjusting for antiretroviral regimen adherence and hepatitis C co-infection [53]. Similarly, the severity of pulmonary tuberculosis was positively correlated with low selenium levels in serum [54]. Supplementation with selenium and vitamin E have been shown to improve the antioxidant capacity in TB patients, although effects on the immune system were not elucidated [55].

There is definitive evidence for the utility of selenium supplementation in various viral and bacterial infections [56, 57]. It is interesting to note that the most compelling data currently available on selenium's immune enhancing properties are studies related to HIV, a severely immune compromised population. T cells are extremely sensitive to oxidative stress and the potential benefits from selenoenzymes most likely reside in their ability to regulate redox reactions [58]. There was no particular boost in immune function found in our series of critically ill sepsis patients given sodium selenite intravenously, despite having tested the individual arms of immunity. This could be, on the one hand, due to the acute nature of the disease, where the often sudden onset of sepsis is in stark contrast to patients who are living

with chronic illnesses such as AIDS or TB. On the other hand, this could be a reflection of the complex, poorly understood progression of sepsis, where the immune system goes through phases of hyper- and hypoinflammation.

4.5. Antioxidation and Dosing

Sepsis can often be characterized also by increased reactive oxygen species, low endogenous antioxidative capacities, as well as reduced selenium stores. Selenoproteins are located in the endoplasmic reticulum and help protect cells from stress-induced apoptosis. Glutathione peroxidases are a large family of antioxidant enzymes, where selenium plays an essential role in their function. There have therefore been many trials evaluating outcomes in sepsis patients with selenium supplementation based on this premise, but the results are definitely mixed.

One large meta-analysis found that supplementing with much higher dosages than what is recommended could potentially decrease mortality [18]. Most trials used a dosage of 1000µg and after one study with particularly high dosages nevertheless demonstrated no improvement of vasopressor use, length of ICU stay or mortality [59], protocols have generally turned to a bolus at the start, as a way of topping up the depleted selenoenzyme pool, followed by continuous infusion. Our study used such a high dose regime, which was well tolerated by patients. Most decisively, many questions remain unanswered regarding the mechanisms of disease in sepsis, perhaps oxidative stress ultimately plays a much more minor role than expected and the emphasis lies in the immune system.

4.6. Mechanisms of action

We intended at the outset to uncover possible affected immune pathways which could benefit from selenium administration during severe sepsis. Despite a wide range of tested cytokines and comprehensive antigen challenges, selenium did not appear to boost immune function in a discernable fashion. Studies which shed light on mechanistic details of selenium's impact on immunity are rather far and few between because of the vast number of cell functions selenium has an influence on.

A mouse model of T cell-specific knockout of all selenoproteins has demonstrated a reduction in the number of mature T lymphocyte production [60]. In further mice models, dietary selenium intake has been shown to mediate immune response through the interferon-γ and interleukin-6 pathways [61]. A recent study involving selenoprotein F knockout mice concluded its importance in regulating immunoglobulin levels in the endoplasmic reticulum [62]. Immune function studies with a positive effect through selenium supplementation have largely been shown in the elderly population or individuals with proven selenium deficits. In contrast, our patient population suffers from a typically acute illness and selenium was administered parenterally, rather than as dietary supplementation over prolonged periods of time. Since the baseline selenium reserves in our patients are not known and the age ranges from the young to the old, direct comparisons are difficult. That being said, the neutral effect on immunity of intravenous sodium selenite observed here is still most likely attributed to sepsis being a complex immunological disease process.

In patients with acute respiratory distress syndrome (ARDS), selenium supplementation has shown to modulate the interleukin-6 and interleukin-1β inflammatory responses by boosting the antioxidation capacities in the lungs [63]. Although the respiratory mechanisms were improved, there was no effect on overall survival or length of ICU stay. Similarly, without a better understanding of disease mechanisms during sepsis, a more refined evaluation of how selenium affects the immune system can be difficult.

4.7. Characteristics of the study population

Our anesthesia intensive care unit admits a wide range of patients, from trauma surgery to the chronically ill. This inadvertently complicates the range of pre-existing conditions and immune capabilities at sepsis onset, making it more challenging to elucidate the benefits of selenium than in a homogeneous population. There have been a number of previous discussions on whether the best route of administration is parenteral or enteral. Given that critically ill patients in the ICU are often intubated, our study also differs from selenium studies where the element was taken orally as nutritional supplementation.

It is interesting to note that European soils appear to be more depleted of selenium than North American soil [64], which was reflected as a relative deficiency of selenium in European studies done on healthy individuals [65]. Despite this finding, a large international antioxidant supplementation study performed by the Canadian Critical Care Trials Group, also did not find a therapeutic benefit [4]. In addition, irrespective of the overall lower selenium levels in Europeans, sepsis patients have a definite deficit given the inflammatory nature of the disease.

4.8. Strengths and weaknesses

This double blinded, randomized prospective clinical trial (SISPCT) has a robust design and high rate of adherence to protocol. This is also a very specifically defined patient population meeting stringent sepsis criteria in an intensive care setting and the efficacy of high dose intravenous selenium was compared to placebo. The characteristics of the selenium and control groups are comparable and blood samples were collected over a three-week period. The whole blood assays gave high fidelity cytokine readouts after antigen challenge, an effective and proven method to yield a snapshot of immune function. To our best knowledge, ours is the first of its kind to examine immune function of severe sepsis patients receiving high dose selenium therapy. To have clinical data over a three-week period in an intensive care setting also provides information on the intervention's effectiveness over time. To preserve the integrity of the individual datapoints without excluding outliers or extrapolating, we utilized the generalized least squares model to effectively compensate for the high rate of attrition over time. This complex statistical tool makes our conclusions much more reliable than conventional methods.

One obvious limitation of our study is the cohort size, starting at 76 patients, this unfortunately drops significantly by the end of the 3 weeks given the nature of the patient population. Ex vivo blood samples were collected as soon as possible upon enrolment and at the subsequent time points, but the laboratory processing was not immediate due to staffing constraints. This limited the choice of immune assays available, rendering more cell-specific tests or higher resolution of results difficult. Furthermore, the quantity of patient blood available also restricted the possible analyses, for example, western blots and cell

separation techniques were not feasible. Such experiments could have shed more light on mechanisms of action and altered signaling pathways. The heterogeneity of the group is fairly large, with a range of pathogens as well as non-microbiologically proven infections. Patients were admitted from both medical and surgical services, with varying pre-existing conditions that could be better subcategorized given a larger sample size. All statistical models become more powerful with lower attrition rates, to have patients followed up further outside of the ICU would yield a more complete data set.

4.9. Future directions

The benefits of selenium administration in boosting immunity have been explored with mixed results, its utility partially demonstrated in select pathogens. With our study, we obtained a first glimpse into the immune function in patients who are critically ill with severe sepsis and observed no beneficial effects of selenium. Given the relative low cost of selenium and the high mortality rate of severe sepsis, this is a therapy option worth pursuing.

There have been many clinical studies on outcomes but experimental studies using sepsis models are lacking. This would be important in shedding light on mechanistic details of how exactly the large number of selenoproteins influence lymphocyte function. It would contribute to optimizing dosing without reaching toxicity. The research that has been carried out in selenium supplementation related to HIV would be relevant, in that both diseases embody an immunocompromised state. Glutathione peroxidase and specific antioxidant selenoenzymes have been implicated as key players in lymphocyte proliferation, their functions and levels in severe sepsis patients would offer further useful insights.

Future trials could have more targeted patient profiles, where the elderly or only non-surgical ICUs with severe sepsis are examined. With a large enough sample size, perhaps pathogens can be stratified, where selenium supplementation would be helpful only with certain types of infections. Last but not least, therapy options can be better devised when there is a thorough understanding of the illness at hand. Therefore, it is essential to make progress in understanding the immune function during sepsis at its different stages in order to treat it.

5. Summary

Background

Sepsis is a complex disease process with a high mortality rate and characterized by a breakdown of immune system function that is not yet completely understood. Selenium is a trace element important in enzymes which protects against oxidative stress, an immunological process seen in septic patients. While selenium has been administered to critically ill patients for decades, the outcomes according to the literature have been mixed in terms of clinical benefits. In this longitudinal study using whole blood samples from septic patients, we performed immune function assays to uncover whether selenium supplementation alters cytokine release profiles.

Methods

Our patient cohort consists of data collected at the University of Munich anesthesiological ICUs as part of the randomized, double blinded multicenter clinical trial SISPCT (registered with www.clinicaltrials.gov NCT00832039). Blood samples were collected upon sepsis onset (day 0) and subsequently at days 4, 7, 14, and 21. They were then incubated with a wide range of stimulating antigens such as bacteria or viruses and the supernatants were retrieved for cytokine measurements. A representative panel of cytokines were selected to reflect the function of different immune pathways. The statistical analysis utilized a generalized least squares model using the software R to compensate for missing values over time due to patient attrition.

Results

76 severe sepsis patients were enrolled at our center, 40 of which were randomized to receive selenium and 36 placebo. No statistically significant difference was seen in the immune response assay readouts between the two groups at any time point over the 21-day study period. There was, however, initial dampening of cytokine release at sepsis onset seen in both groups which recovered to different degrees over time.

Conclusion

High dose intravenous sodium selenite administration over a three-week period did not improve cytokine release in ex vivo stimulated sepsis patient blood samples. While the immune system is impacted by the availability of selenoproteins, there was no discernable benefit associated with selenium supplementation in those critically ill with sepsis. This further reinforces the complexity of immune responses that occur during sepsis, which include hyper as well as hypoinflammatory phases. The decision to administer sodium selenite in such a setting should therefore be considered carefully by the physician team, taking into account the possible side effects such as nausea and vomiting, fatigue, coagulopathies, as well as kidney and liver impairments.

6. Summary in German

Hintergrund

Sepsis ist ein komplexes Krankheitsbild mit einer hohen Mortalitätsrate, das von einem noch nicht ganz verstandenen Zusammenbruch des Immunsystems geprägt ist. Selen ist ein Spurenelement mit großer Bedeutung für Enzyme, die gegen oxidativen Stress schützen, einen in Sepsis-Patienten beobachteten immunologischen Prozess. Zwar ist Selen schon jahrzehntelang kritisch kranken Patienten verabreicht worden, die Ergebnisse hinsichtlich des klinischen Nutzens sind der Literatur nach jedoch gemischt gewesen. In dieser Längsschnittuntersuchung von Blutproben von Sepsis-Patienten haben wir Immunfunktions-Assays durchgeführt, um aufzuklären, ob die Gabe von Selen Zytokinausschüttungs-Profile verändert.

Methodik

Unsere Patientenkohorte besteht aus Daten, die an den anästhesiologischen Intensivstationen der Universität München im Rahmen der randomisierten, doppelblinden, multizentrischen klinischen Studie SISPCT erhoben worden sind (registriert bei www.clinicaltrials.gov, NCT00832039). Blutproben wurden beim Ausbruch von Sepsis genommen (Tag 0) und im Anschluss an den Tagen 4, 7, 14 und 21. Sie wurden dann mit einer Reihe stimulierender Antigene wie beispielsweise Bakterien oder Viren inkubiert, und die Überstände wurden für Zytokinmessungen gewonnen. Ein repräsentatives Panel von Zytokinen wurde ausgewählt, um die Funktion verschiedener Stränge des Immunsystems zu abzubilden. Die statistische Analyse basiert auf einem Modell der kleinsten Quadrate (Generalized Least Squares Model) mit Hilfe der Software R, um über die Zeit fehlende Werte wegen abnehmender Patientenzahl zu kompensieren.

Ergebnisse

76 Patienten mit schwerer Sepsis waren in unserem Zentrum registriert, von denen randomisiert 40 für die Gabe von Selen und 36 für die Gabe von Placebo ausgewählt wurden. Es wurde zu keinem Zeitpunkt über die 21 Tage der Studie ein statistisch signifikanter Unterschied in den Ergebnissen der Immunantwort-Assays zwischen den zwei

Gruppen beobachtet. Es wurde allerdings in beiden Gruppen einer anfänglichen Dämpfung der Zytokinausschüttung beim Ausbruch von Sepsis beobachtet, der sich mit der Zeit in unterschiedlichem Ausmaß erholte.

Fazit

Hochdosierte intravenöse Gabe von Natriumselenit über einen Zeitraum von drei Wochen verbesserte die Zytokinausschüttung in ex vivo stimulierten Blutproben von Sepsis-Patienten nicht. Obwohl das Immunsystem von der Verfügbarkeit von Selenoproteinen beeinflusst wird, war mit der Gabe von Selen in kritisch kranken Sepsis-Patienten kein erkennbarer Nutzen verbunden. Das unterstreicht die Komplexität der bei Sepsis auftretenden Immunantworten, die sowohl hyper- als auch hypoinflammatorische Phasen umfassen. Die Entscheidung zur Gabe von Natriumselenit in solch einer Situation sollte von dem Team der behandelnden Ärzte daher gründlich abgewogen werden, auch unter Berücksichtigung der möglichen Nebenwirkungen wie Übelkeit und Erbrechen, Müdigkeit, Gerinnungsstörungen, sowie Beeinträchtigungen der Nieren- und Leberfunktion.

7. References

[1] Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)*. JAMA, 2016. **315**(8): p.801-810.

[2] Angus, D.C., et al., *Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.* Crit Care Med, 2001. **29**(7): p.1303-1310.

[3] Martin, G.S., *Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes.* Expert Rev Anti Infect Ther, 2012. **10**(6): p.701-706.

[4] Heyland, D., et al., Canadian Critical Care Trials Group, *A randomized trial of glutamine and antioxidants in critically ill patients.* N Engl J Med, 2013. **368**(16): p.1489-1497.

[5] Manzanares, W., et al., *Antioxidant micronutrients in the critically ill: a systematic review and meta-analysis.* Crit Care, 2012. **16**(2): R66.

[6] Lee, Y.H., et al., *Serum Concentrations of Trace Elements Zinc, Copper, Selenium, and Manganese in Critically Ill Patients.* Biol Trace Elem Res, 2019. **188**(2): p.316-325.

[7] Rech, M., et al., *Heavy metal in the intensive care unit: a review of current literature on trace element supplementation in critically ill patients.* Nutr Clin Pract, 2014. **29**(1): p.78-89.

[8] McClave, S.A., et al., Society of Critical Care Medicine, American Society for Parenteral and Enteral Nutrition, *Guidelines for the Provision and Assessment of Nutrition Support Therapy in the Adult Critically Ill Patient: Society of Critical Care Medicine (SCCM) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.).* J Parenter Enteral Nutr, 2016. **40**(2): p.159-211.

[9] Singer, P., et al., ESPEN, *ESPEN Guidelines on Parenteral Nutrition: intensive care*. Clin Nutr, 2009. **28**(4): p.387-400.

[10] Hotchkiss, R.S., Karl, I.E., *The pathophysiology and treatment of sepsis.* N Engl J Med, 2003. **348**(2): p.138-150.

[11] Bone, R.C., *The sepsis syndrome. Definition and general approach to management.* Clin Chest Med, 1996. **17**(2): p.175-181.

[12] Rittirsch, D., Flierl, M.A., Ward, P.A., *Harmful molecular mechanisms in sepsis.* Nat Rev Immunol, 2008. **8**(10): p.776-787.

[13] Wiersinga, W.J., *Current insights in sepsis: from pathogenesis to new treatment targets.* Curr Opin Crit Care, 2011. **17**(5): p.480-486.

[14] Feuerecker, M., et al., *Early immune anergy towards recall antigens and mitogens in patients at onset of septic shock.* Sci Rep, 2018. **8**(1): 1754.

[15] Osuchowski, M.F., et al., *Sepsis chronically in MARS: systemic cytokine responses are always mixed regardless of the outcome, magnitude, or phase of sepsis.* J Immunol, 2012. **189**(9): p.4648-4856.

[16] Hardy, G., Hardy, I., Manzanares, W., *Selenium supplementation in the critically ill.* Nutr Clin Pract, 2012. **27**(1): p.21-33.

[17] Roman, M., Jitaru, P., Barbante, C., *Selenium biochemistry and its role for human health.* Metallomics, 2014. **6**(1): p.25-54.

[18] Alhazzani, W., et al., *The effect of selenium therapy on mortality in patients with sepsis syndrome: a systematic review and meta-analysis of randomized controlled trials.* Crit Care Med, 2013. **41**(6): p.1555-1564.

[19] Huang, T.S., et al., *Effect of parenteral selenium supplementation in critically ill patients: a systematic review and meta-analysis*. PLoS One, 2013. **8**(1): e54431.

[20] Landucci, F., et al., *Selenium supplementation in critically ill patients: a systematic review and meta-analysis.* J Crit Care, 2014. **29**(1): p.150-156.

[21] Kong, Z., et al., *Selenium supplementation for sepsis: a meta-analysis of randomized controlled trials.* Am J Emerg Med, 2013. **31**(8): p.1170-1175.

[22] Allingstrup, M., Afshari, A., *Selenium supplementation for critically ill adults*. Cochrane Database Syst Rev, 2015. **2015**(7):CD003703.

[23] Woth, G., et al., *The effect of Na-selenite treatment on the oxidative stress-antioxidants balance of multiple organ failure.* J Crit Care, 2014. **29**(5): 883e7-11.

[24] Bloos, F., et al., for SepNet Critical Care Trials Group, *Effect of Sodium Selenite Administration and Procalcitonin-Guided Therapy on Mortality in Patients With Severe Sepsis or Septic Shock: A Randomized Clinical Trial.* JAMA Intern Med, 2016. **176**(9): p.1266-1276.

[25] Guo, A., et al., *Immune function testing in sepsis patients receiving sodium selenite.* J Crit Care, 2019. **52**: p.208-212.

[26] Sudhoff, L., *Initial anergy of immune response in patients with severe sepsis and septic shock: implications for future treatment*. Dissertation, LMU München: Medizinische Fakultät, 2019.

[27] Bone, R.C., et al., The ACCP/SCCM Consensus Conference Committee, American College of Chest Physicians/Society of Critical Care Medicine, *Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis.* Chest, 1992. **101**(6): p.1644-1655.

[28] Kalamasz, D., et al., *Optimization of Human T-cell Expansion Ex Vivo Using Magnetic Beads Conjugated With anti-CD3 and Anti-CD28 Antibodies.* J Immunother, 2004. **27**(5): p.405-418.

[29] Park, S.J., Mehrad, B., *Innate Immunity to Aspergillus Species.* Clin Microbiol Rev, 2009. **22**(4): p.535–551.

[30] Janeway Jr., C.A., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. **54**(1): p.1–13.

[31] Kenny, B., et al., *Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells.* Cell, 1997. **91**(4): p.511–520.

[32] Kim, D.W., et al., *The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes.* Proc Natl Acad Sci USA, 2005. **102**(39): p.14046–14051.

[33] La Rosa, C., Diamond, D.J., *The Immune Response to Human CMV.* Future Virol, 2012. **7**(3): p.279-293.

[34] van de Berg, P.J., et al., *Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature.* J Infect Dis, 2010. **202**(5): p.690–699.

[35] Portnoy, J.M., Williams, P.B., Barnes, C.S., *Innate Immune Responses to Fungal Allergens.* Curr Allergy Asthma Rep, 2016. **16**(9):62.

[36] Bahadoran, A., et al., *Immune Responses to Influenza Virus and Its Correlation to Age and Inherited Factors.* Front Microbiol, 2016. **7**:1841.

[37] Moran, A.P., Prendergast, M.M., Appelmelk, B.J., *Molecular Mimicry of Host Structures by Bacterial Lipopolysaccharides and Its Contribution to Disease.* FEMS Immunol Med Microbiol, 1996. **16** (2): p.105-115.

[38] Goel, G., et al., *Phorbol Esters: Structure, Biological Activity, and Toxicity in Animals.* Int J of Toxicol, 2007. **26**(4): p.279-288.

[39] Yokoyama, K., Terao, T., Osawa, T., *Isolation and characterization of membrane receptors for pokeweed mitogens from mouse lymphocytes.* Biochem J, 1977. **165**(3): p.431–437.

[40] Mocikat, R., et al., *Natural killer cells activated by MHC Class I (low) targets prime dendritic cells to induce protective CD8 T Cell Responses.* Immunity, 2003. **19**(4): p.561-569.

[41] Chen, S.Y., Feng, Y., Zi, X.L., *A general introduction to adjustment for multiple comparisons.* J Thorac Dis, 2017. **9**(6): p.1725-1729.

[42] Holm, M., *A simple sequentially rejective multiple test procedure*. Scand J Statist, 1979. **6**: p.65-70.

[43] Manzanares, W., et al., *High-dose intravenous selenium does not improve clinical outcomes in the critically ill: a systematic review and meta-analysis*. Crit Care, 2016. **20**(1): 356.

[44] Boomer, J.S., et al., *Immunosuppression in Patients Who Die of Sepsis and Multiple Organ Failure*. JAMA, 2011. **306**(23): p.2594–2605.

[45] Feuerecker, M., et al., *A corticoid-sensitive cytokine release assay for monitoring stress-mediated immune modulation*. Clin Exp Immunol, 2013. **172**(2): p.290–299.

[46] Wood, S.M., et al., *β-Carotene and selenium supplementation enhances immune response in aged humans*. Integ Med, 2000. **2**(2): p.85-92.

[47] Broome, C.S., et al., *An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status*. Am J Clin Nutr, 2004. **80**(1): p.154-162.

[48] Hoffmann, F.W., et al., *Dietary selenium modulates activation and differentiation of CD4+ T cells in mice through a mechanism involving cellular free thiols*. J Nutr, 2010. **140**(6): p.1155-1161.

[49] Rayman, M.P., *The importance of selenium to human health*. Lancet, 2000. **356**(9225): p.233-241.

[50] Reeves, M.A., Hoffmann, P.R., *The human selenoproteome: Recent insights into functions and regulation*. Cell Mol Life Sci, 2009. **66**(15): p.2457-2478.

[51] Drain, P.K., et al., *Low serum albumin and the acute phase response predict low serum selenium in HIV-1 infected women*. BMC Infect Disn 2006. **6**:85.

[52] Grobler, L., et al., *Nutritional supplements for people being treated for active tuberculosis.* Cochrane Database Syst Rev, 2016. **2016**(6): CD006086.

[53] Hurwitz, B.E., et al., *Suppression of human immunodeficiency virus type 1 viral load with selenium supplementation: a randomized controlled trial.* Arch Intern Med, 2007. **167**(2): p.148-154.

[54] Eick, F., et al., *Food intake of selenium and Sulphur amino acids in tuberculosis patients and healthy adults in Malawai.* Int J Tuberc Lung Dis, 2009. **13**(10): p.1313-1315.

[55] Seyedrezazadeh, E., et al., *Effect of vitamin E and selenium supplementation on oxidative stress status in pulmonary tuberculosis patients.* Respirology, 2008. **13**(2): p.294-298.

[56] Carlson, B.A., et al., *Role of selenium-containing proteins in T-cell and macrophage function.* Proc Nutr Soc, 2010. **69**(3): p.300-310.

[57] Aribi, M., et al., *Macrophage bactericidal activities against Staphylococcus aureus are enhanced in vivo by selenium supplementation in a dose-dependent manner*. PLoS One, 2015. **10**(9): e0135515.

[58] Avery, J.C., Hoffmann, P.R., *Selenium, selenoproteins, and immunity*. Nutrients, 2018. **10**(9): p.1203-1223.

[59] Forceville, X., et al., *Effects of high doses of selenium, as sodium selenite, in septic shock: a placebo-controlled, randomized, double-blind, phase II study.* Crit Care, 2007. **11**(4): R73.

[60] Shrimali, R.K., et al., *Selenoproteins mediate T cell immunity through an antioxidant mechanism*. J Biol Chem, 2008. **283**(29): p.20181-20185.

[61] Tsuji, P.A., et al., *Dietary Selenium Levels Affect Selenoprotein Expression and Support the Interferon-γ and IL-6 Immune Response Pathways in Mice.* Nutrients, 2015. **7**(8): p.6529-6549.

[62] Yim, S.H., et al., *Role of Selenof as a gatekeeper of secreted disulfide-rich glycoproteins*. Cell Rep, 2018. **23**(5): p.1387-1398.

[63] Mahmoodpoor, A., et al., *The Effect of Intravenous Selenium on Oxidative Stress in Critically Ill Patients with Acute Respiratory Distress Syndrome*. Immunol Invest, 2019. **48**(2): p.147-159.

[64] Johnson, C.C., Fordyce, F.M., Rayman, M.P., *Symposium on 'Geographical and geological influences on nutrition': factors controlling the distribution of selenium in the environment and their impact on health and nutrition*. Proc Nutr Soc, 2010. **69**(1): p.119-132.

[65] Rayman, M.P., *Selenium and human health*. Lancet, 2012. **379**(9822): p.1256-1268.

8. Acknowledgements

Above and foremost, I would like to thank my supervisor Alexander Choukér, for his scientific guidance, positivity and mentorship. I am very grateful for the opportunity to work with him and his team on this project. Everyone who was involved in the SISPCT study, especially Matthias Feuerecker, Josef Briegel, Lorenz Frey, Ines Kaufmann, Brian Crucian (from NASA), Lars Sudhoff, and Judith-Irina Buchheim, thank you for your assistance and contributions.

Anne-Laure Boulesteix and Jyotsna Srinath from the Institute for Medical Information Processing, Biometry and Epidemiology (IBE, LMU München) provided invaluable discussions and help with the complex statistical analyses central to this project. Many thanks to Jana Stoilova for her expertise in helping me navigate R.

Last but not least, thank you to Prof. Zwißler and all the staff and patients who were involved in this study from the department of anesthesia intensive care units. It is my hope that we continue to gain new insights in order to provide the best possible care for sepsis patients.

This study was supported by funds from the German Federal Ministry of Economics and Energy (BMWi/DLR grant 50WB0719/WB0919 and WB1622).

9. GLS Modelling Tables

The following are tabulated raw outputs generated by the statistical model ‘generalized least squares’ using the software R using the immune assay data and were referred to in the results section. Shown in the tables are regression coefficients generated with accompanying p-values. Day 0 of the placebo group was used as the intercept and comparison to all later time points as well as the selenium group.

Aspergillus with readouts from IL2, IFN and TNF.

Log IL2 - Aspergillus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,194	0,0026	0,014	0,8736
Day 4	0,022	0,8284	0,005	0,9693
Day 7	0,051	0,5707	0,123	0,3187
Day 14	-0,055	0,5988	0,054	0,7114
Day 21	0,07	0,2306	0,011	0,8943

Log IL2 - Aspergillus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,194	0,0026	0,208	0,8736
Day 4	0,216	0,8284	0,213	0,9693
Day 7	0,245	0,5707	0,331	0,3187
Day 14	0,139	0,5988	0,262	0,7114
Day 21	0,264	0,2306	0,219	0,8943

Log IFNg - Aspergillus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,571	0	-0,033	0,7969
Day 4	0,019	0,882	-0,225	0,2044
Day 7	0,149	0,316	0,06	0,7722
Day 14	-0,027	0,8711	-0,135	0,5642
Day 21	-0,0366	0,0537	-0,167	0,5484

Log IFNg - Aspergillus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,571	0	0,538	0,7969
Day 4	0,59	0,882	0,313	0,2044
Day 7	0,72	0,316	0,598	0,7722
Day 14	0,544	0,8711	0,403	0,5642
Day 21	0,5344	0,0537	0,371	0,5484

Log TNFa - Aspergillus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,448	0	-0,191	0,0908
Day 4	-0,058	0,6117	0,131	0,401
Day 7	-0,003	0,9783	0,231	0,197
Day 14	-0,069	0,6322	0,037	0,8534
Day 21	0,155	0,3544	0,072	0,7847

Log TNFa - Aspergillus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,448	0	0,257	0,0908
Day 4	0,39	0,6117	0,388	0,401
Day 7	0,445	0,9783	0,488	0,197
Day 14	0,379	0,6322	0,294	0,8534
Day 21	0,603	0,3544	0,329	0,7847

Bacteria with readouts from IL2, IFN and TNF.

Log IL2 - Bacteria				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,467	0,0005	-0,027	0,8827
Day 4	0,403	0,0073	-0,03	0,882
Day 7	0,489	0,0025	0,148	0,5106
Day 14	0,501	0,0127	0,024	0,9317
Day 21	0,672	0	0,158	0,5004

Log IL2 - Bacteria				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,467	0,0005	0,44	0,8827
Day 4	0,87	0,0073	0,41	0,882
Day 7	0,956	0,0025	0,588	0,5106
Day 14	0,968	0,0127	0,464	0,9317
Day 21	1,139	0	0,598	0,5004

Log IFNg - Bacteria				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,67	0	-0,117	0,4142
Day 4	0,122	0,3794	-0,212	0,265
Day 7	0,242	0,1345	0,058	0,796
Day 14	0,069	0,7034	-0,077	0,7619
Day 21	-0,228	0,2091	-0,01	0,9713

Log IFNg - Bacteria				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,67	0	0,553	0,4142
Day 4	0,792	0,3794	0,341	0,265
Day 7	0,912	0,1345	0,611	0,796
Day 14	0,739	0,7034	0,476	0,7619
Day 21	0,442	0,2091	0,543	0,9713

Log TNFa - Bacteria				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,328	0,0002	-0,087	0,4654
Day 4	0,172	0,1262	-0,037	0,8099
Day 7	0,446	0,0011	-0,165	0,3841
Day 14	0,26	0,0546	-0,206	0,2776
Day 21	0,358	0,0416	-0,13	0,6071

Log TNFa - Bacteria				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,328	0,0002	0,241	0,4654
Day 4	0,5	0,1262	0,204	0,8099
Day 7	0,774	0,0011	0,076	0,3841
Day 14	0,588	0,0546	0,035	0,2776
Day 21	0,686	0,0416	0,111	0,6071

Cytomegaly virus with readouts from IL2, IFN and TNF.

Log IL2 - CMV				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,267	0,0004	-0,054	0,5952
Day 4	0,196	0,0243	-0,241	0,0434
Day 7	0,196	0,0116	-0,048	0,6559
Day 14	0,163	0,1659	0,011	0,9487
Day 21	0,428	0,0042	-0,393	0,0946

Log IL2 - CMV				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,267	0,0004	0,213	0,5952
Day 4	0,463	0,0243	-0,028	0,0434
Day 7	0,463	0,0116	0,165	0,6559
Day 14	0,43	0,1659	0,224	0,9487
Day 21	0,695	0,0042	-0,18	0,0946

Log IFNg - CMV				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,756	0	-0,107	0,4908
Day 4	0,056	0,7013	-0,37	0,0658
Day 7	0,207	0,2339	-0,024	0,9217
Day 14	0,02	0,9203	-0,04	0,8901
Day 21	0,4	0,0876	-0,742	0,0431

Log IFNg - CMV				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,756	0	0,649	0,4908
Day 4	0,812	0,7013	0,279	0,0658
Day 7	0,963	0,2339	0,625	0,9217
Day 14	0,776	0,9203	0,609	0,8901
Day 21	1,156	0,0876	-0,093	0,0431

Log TNFa - CMV				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,412	0,0003	-0,027	0,8609
Day 4	0,353	0,0546	-0,367	0,1452
Day 7	0,336	0,0581	0,017	0,946
Day 14	0,752	0,0001	-0,56	0,044
Day 21	0,764	0	-0,252	0,3705

Log TNFa - CMV				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,412	0,0003	0,385	0,8609
Day 4	0,765	0,0546	0,018	0,1452
Day 7	0,748	0,0581	0,402	0,946
Day 14	1,164	0,0001	-0,175	0,044
Day 21	1,176	0	0,133	0,3705

Fungal components with readouts from IL2, IFN and TNF.

Log IL2 - Fungi				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,524	0	-0,129	0,3903
Day 4	0,179	0,138	0,063	0,7023
Day 7	0,273	0,0713	-0,041	0,8438
Day 14	0,326	0,0511	-0,08	0,7351
Day 21	0,62	0	-0,226	0,261

Log IL2 - Fungi				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,524	0	0,395	0,3903
Day 4	0,703	0,138	0,458	0,7023
Day 7	0,797	0,0713	0,354	0,8438
Day 14	0,85	0,0511	0,315	0,7351
Day 21	1,144	0	0,169	0,261

Log IFNg - Fungi				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,578	0	-0,016	0,8981
Day 4	0,046	0,7417	-0,24	0,2149
Day 7	0,153	0,3029	0,037	0,8591
Day 14	-0,027	0,8656	-0,135	0,5527
Day 21	-0,229	0,1794	-0,002	0,9937

Log IFNg - Fungi				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,578	0	0,562	0,8981
Day 4	0,624	0,7417	0,322	0,2149
Day 7	0,731	0,3029	0,599	0,8591
Day 14	0,551	0,8656	0,427	0,5527
Day 21	0,349	0,1794	0,56	0,9937

Log TNFa - Fungi				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,35	0,0027	-0,025	0,8763
Day 4	0,233	0,0201	-0,116	0,4003
Day 7	0,703	0,0001	-0,43	0,0834
Day 14	0,457	0,0266	-0,214	0,4561
Day 21	0,866	0,0001	-0,19	0,5226

Log TNFa - Fungi				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,35	0,0027	0,325	0,8763
Day 4	0,583	0,0201	0,209	0,4003
Day 7	1,053	0,0001	-0,105	0,0834
Day 14	0,807	0,0266	0,111	0,4561
Day 21	1,216	0,0001	0,135	0,5226

Influenza with readouts from IL2, IFN and TNF.

Log IL2 - Influenza				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,348	0,0014	-0,038	0,8001
Day 4	0,386	0,0066	-0,102	0,5977
Day 7	0,443	0	-0,013	0,9301
Day 14	0,47	0,0038	-0,114	0,6132
Day 21	0,497	0,0137	0,075	0,7974

Log IL2 - Influenza				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,348	0,0014	0,31	0,8001
Day 4	0,734	0,0066	0,208	0,5977
Day 7	0,791	0	0,297	0,9301
Day 14	0,818	0,0038	0,196	0,6132
Day 21	0,845	0,0137	0,385	0,7974

Log IFNg - Influenza				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,658	0	-0,068	0,6711
Day 4	0,179	0,2797	-0,123	0,5885
Day 7	0,23	0,1724	0,15	0,5229
Day 14	0,398	0,033	-0,19	0,4688
Day 21	0,389	0,0835	-0,394	0,2428

Log IFNg - Influenza				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,658	0	0,59	0,6711
Day 4	0,837	0,2797	0,467	0,5885
Day 7	0,888	0,1724	0,74	0,5229
Day 14	1,056	0,033	0,4	0,4688
Day 21	1,047	0,0835	0,196	0,2428

Log TNFa - Influenza				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,363	0,0004	-0,052	0,7088
Day 4	0,141	0,3858	0,045	0,8396
Day 7	0,323	0,0456	0,05	0,8234
Day 14	0,639	0,0003	-0,509	0,0363
Day 21	0,634	0,0019	-0,247	0,406

Log TNFa - Influenza				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,363	0,0004	0,311	0,7088
Day 4	0,504	0,3858	0,356	0,8396
Day 7	0,686	0,0456	0,361	0,8234
Day 14	1,002	0,0003	-0,198	0,0363
Day 21	0,997	0,0019	0,064	0,406

Lipopolysaccharide with readouts from IL1b, IL6, IL8, IL10, IL12 and TNF.

Log IL1b - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,622	0	0,161	0,5112
Day 4	-0,052	0,7319	0,127	0,5436
Day 7	0,53	0,0074	-0,253	0,3577
Day 14	0,485	0,0838	-0,178	0,6521
Day 21	1,071	0,0042	-1,751	0,0011

Log IL1b - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,622	0	1,783	0,5112
Day 4	1,57	0,7319	1,91	0,5436
Day 7	2,152	0,0074	1,53	0,3577
Day 14	2,107	0,0838	1,605	0,6521
Day 21	2,693	0,0042	0,032	0,0011

Log IL6 - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	3,206	0	-0,057	0,778
Day 4	-0,45	0,0118	0,306	0,2085
Day 7	0,135	0,4785	-0,106	0,694
Day 14	0,161	0,5347	-0,091	0,8025
Day 21	-0,062	0,8337	-0,569	0,2251

Log IL6 - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	3,206	0	3,149	0,778
Day 4	2,756	0,0118	3,455	0,2085
Day 7	3,341	0,4785	3,043	0,694
Day 14	3,367	0,5347	3,058	0,8025
Day 21	3,144	0,8337	2,58	0,2251

Log IL8 - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	3,851	0	0,021	0,8469
Day 4	0,235	0,0027	0,074	0,4836
Day 7	0,386	0,0003	-0,083	0,5717
Day 14	0,358	0,0007	-0,034	0,8131
Day 21	0,643	0,0001	-0,452	0,0494

Log IL8 - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	3,851	0	3,872	0,8469
Day 4	4,086	0,0027	3,946	0,4836
Day 7	4,237	0,0003	3,789	0,5717
Day 14	4,209	0,0007	3,838	0,8131
Day 21	4,494	0,0001	3,42	0,0494

Log IL10 - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,891	0	-0,112	0,4803
Day 4	-0,105	0,4125	0,079	0,6523
Day 7	-0,003	0,9817	-0,189	0,3371
Day 14	-0,069	0,7002	0,202	0,4194
Day 21	0,149	0,5535	-0,56	0,1452

Log IL10 - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,891	0	1,779	0,4803
Day 4	1,786	0,4125	1,858	0,6523
Day 7	1,888	0,9817	1,59	0,3371
Day 14	1,822	0,7002	1,981	0,4194
Day 21	2,04	0,5535	1,219	0,1452

Log IL12 - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,293	0	0,044	0,4448
Day 4	0,029	0,6025	0,006	0,9394
Day 7	0,119	0,0242	-0,041	0,5704
Day 14	0,109	0,1572	-0,178	0,1027
Day 21	0,034	0,6151	-0,018	0,8519

Log IL12 - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,293	0	0,337	0,4448
Day 4	0,322	0,6025	0,343	0,9394
Day 7	0,412	0,0242	0,296	0,5704
Day 14	0,402	0,1572	0,159	0,1027
Day 21	0,327	0,6151	0,319	0,8519

Log TNFa - LPS				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,878	0	0,314	0,0983
Day 4	0,172	0,1943	-0,0141	0,4377
Day 7	0,711	0	-0,56	0,0106
Day 14	0,762	0,0018	-0,47	0,1641
Day 21	0,863	0,0046	-1,083	0,0193

Log TNFa - LPS				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,878	0	1,192	0,0983
Day 4	1,05	0,1943	1,1779	0,4377
Day 7	1,589	0	0,632	0,0106
Day 14	1,64	0,0018	0,722	0,1641
Day 21	1,741	0,0046	0,109	0,0193

CD3/CD28 with readouts from IL2, IL4, IL5, IL10, and TNF.

Log IL2 - CD3/CD28				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,89	0	0,072	0,7681
Day 4	0,266	0,0893	0,137	0,5213
Day 7	0,342	0,064	-0,03	0,9051
Day 14	0,442	0,0192	0,028	0,9144
Day 21	0,601	0,0024	-0,011	0,9698

Log IL2 - CD3/CD28				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,89	0	0,962	0,7681
Day 4	1,156	0,0893	1,099	0,5213
Day 7	1,232	0,064	0,932	0,9051
Day 14	1,332	0,0192	0,99	0,9144
Day 21	1,491	0,0024	0,951	0,9698

Log IL4 - CD3/CD28				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,287	0,0081	0,105	0,4775
Day 4	0,207	0,0616	0,152	0,3151
Day 7	0,228	0,1079	0,144	0,4669
Day 14	0,288	0,0452	0,199	0,3217
Day 21	0,573	0,0007	-0,218	0,3923

Log IL4 - CD3/CD28				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,287	0,0081	0,392	0,4775
Day 4	0,494	0,0616	0,544	0,3151
Day 7	0,515	0,1079	0,536	0,4669
Day 14	0,575	0,0452	0,591	0,3217
Day 21	0,86	0,0007	0,174	0,3923

Log IL5 - CD3/CD28				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,251	0,0326	0,111	0,4913
Day 4	0,563	0,001	0,22	0,3389
Day 7	0,284	0,613	0,104	0,6258
Day 14	0,329	0,0271	0,185	0,3728
Day 21	0,692	0,0001	0,02	0,9403

Log IL5 - CD3/CD28				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,251	0,0326	0,362	0,4913
Day 4	0,814	0,001	0,582	0,3389
Day 7	0,535	0,613	0,466	0,6258
Day 14	0,58	0,0271	0,547	0,3728
Day 21	0,943	0,0001	0,382	0,9403

Log IL10 - CD3/CD28				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,031	0	-0,116	0,515
Day 4	-0,223	0,1285	0,462	0,022
Day 7	-0,315	0,0412	0,395	0,0661
Day 14	-0,322	0,0564	0,396	0,0916
Day 21	-0,13	0,5131	0,382	0,2112

Log IL10 - CD3/CD28				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,031	0	0,915	0,515
Day 4	0,808	0,1285	1,377	0,022
Day 7	0,716	0,0412	1,31	0,0661
Day 14	0,709	0,0564	1,311	0,0916
Day 21	0,901	0,5131	1,297	0,2112

Log TNFa - CD3/CD28				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,315	0,0009	-0,018	0,8895
Day 4	0,06	0,4869	0,09	0,4487
Day 7	0,177	0,1452	-0,023	0,8922
Day 14	0,214	0,0735	0,215	0,2015
Day 21	0,527	0,0025	0,121	0,6611

Log TNFa - CD3/CD28				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,315	0,0009	0,297	0,8895
Day 4	0,375	0,4869	0,387	0,4487
Day 7	0,492	0,1452	0,274	0,8922
Day 14	0,529	0,0735	0,512	0,2015
Day 21	0,842	0,0025	0,418	0,6611

Phorbol myristate acetate and ionomycin with readouts from IL2, IL4, IL5, IL10, IFN and TNF.

Log IL2 - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	2,71	0	0,141	0,5474
Day 4	0,542	0,012	-0,207	0,4796
Day 7	0,646	0,0097	-0,339	0,3301
Day 14	0,052	0,8192	0,625	0,0551
Day 21	0,502	0,0276	-0,32	0,3838

Log IL2 - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	2,71	0	2,851	0,5474
Day 4	3,252	0,012	2,644	0,4796
Day 7	3,356	0,0097	2,512	0,3301
Day 14	2,762	0,8192	3,476	0,0551
Day 21	3,212	0,0276	2,531	0,3838

Log IL4 - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,81	0	0,145	0,4057
Day 4	0,268	0,0207	-0,066	0,6749
Day 7	0,631	0,0004	-0,422	0,0887
Day 14	0,367	0,0354	0,288	0,2373
Day 21	0,793	0	-0,403	0,1274

Log IL4 - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,81	0	0,955	0,4057
Day 4	1,078	0,0207	0,889	0,6749
Day 7	1,441	0,0004	0,533	0,0887
Day 14	1,177	0,0354	1,243	0,2373
Day 21	1,603	0	0,552	0,1274

Log IL5 - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,653	0	0,106	0,5464
Day 4	0,238	0,0424	-0,019	0,9044
Day 7	0,445	0,0036	-0,205	0,3366
Day 14	0,313	0,07	0,494	0,0421
Day 21	0,805	0	-0,242	0,4289

Log IL5 - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,653	0	0,759	0,5464
Day 4	0,891	0,0424	0,74	0,9044
Day 7	1,098	0,0036	0,554	0,3366
Day 14	0,966	0,07	1,253	0,0421
Day 21	1,458	0	0,517	0,4289

Log IL10 - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,925	0	-0,072	0,6058
Day 4	-0,11	0,298	0,145	0,3143
Day 7	-0,008	0,9535	0,073	0,7102
Day 14	-0,169	0,3704	0,047	0,0768
Day 21	0,305	0,1149	-0,387	0,1836

Log IL10 - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,925	0	0,853	0,6058
Day 4	0,815	0,298	0,998	0,3143
Day 7	0,917	0,9535	0,926	0,7102
Day 14	0,756	0,3704	0,9	0,0768
Day 21	1,23	0,1149	0,466	0,1836

Log IFNg - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	3,042	0	0,177	0,421
Day 4	0,605	0,0039	-0,316	0,2664
Day 7	0,677	0,0042	-0,532	0,1076
Day 14	0,061	0,7919	0,854	0,0102
Day 21	1,309	0	-0,851	0,0567

Log IFNg - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	3,042	0	3,219	0,421
Day 4	3,647	0,0039	2,903	0,2664
Day 7	3,719	0,0042	2,687	0,1076
Day 14	3,103	0,7919	4,073	0,0102
Day 21	4,351	0	2,368	0,0567

Log TNFa - PMA-I				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,727	0	-0,006	0,9766
Day 4	0,264	0,1356	0,046	0,8488
Day 7	0,378	0,0684	-0,265	0,362
Day 14	-0,238	0,2051	0,703	0,0082
Day 21	0,498	0,0634	-0,406	0,3384

Log TNFa - PMA-I				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,727	0	1,721	0,9766
Day 4	1,991	0,1356	1,767	0,8488
Day 7	2,105	0,0684	1,456	0,362
Day 14	1,489	0,2051	2,424	0,0082
Day 21	2,225	0,0634	1,315	0,3384

Pokeweed mitogen with readouts from IL2, IFN and TNF.

Log IL2 - PWM				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	2,048	0	0,075	0,6521
Day 4	0,351	0,0137	0,065	0,7362
Day 7	0,338	0,0539	-0,06	0,8036
Day 14	0,299	0,01489	-0,036	0,9011
Day 21	-0,016	0,9432	0,7	0,0407

Log IL2 - PWM				
	Placebo	P-Value	Selenium	P-Value
Day 0	2,048	0	2,123	0,6521
Day 4	2,399	0,0137	2,188	0,7362
Day 7	2,386	0,0539	2,063	0,8036
Day 14	2,347	0,01489	2,087	0,9011
Day 21	2,032	0,9432	2,823	0,0407

Log IFNg - PWM				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,736	0	0,12	0,5841
Day 4	0,526	0,0067	-0,099	0,7081
Day 7	0,756	0,0024	-0,324	0,3457
Day 14	1,145	0	-0,227	0,539
Day 21	0,965	0,0012	0,433	0,3315

Log IFNg - PWM				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,736	0	1,856	0,5841
Day 4	2,262	0,0067	1,757	0,7081
Day 7	2,492	0,0024	1,532	0,3457
Day 14	2,881	0	1,629	0,539
Day 21	2,701	0,0012	2,289	0,3315

Log TNFa - PWM				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	1,426	0	0,222	0,2664
Day 4	0,745	0	-0,215	0,3176
Day 7	1,139	0	-0,595	0,0359
Day 14	1,544	0	-0,529	0,1147
Day 21	0,945	0,0003	0,142	0,7228

Log TNFa - PWM				
	Placebo	P-Value	Selenium	P-Value
Day 0	1,426	0	1,648	0,2664
Day 4	2,171	0	1,433	0,3176
Day 7	2,565	0	1,053	0,0359
Day 14	2,97	0	1,119	0,1147
Day 21	2,371	0,0003	1,79	0,7228

Viral antigens with readouts from IL2, IFN and TNF.

Log IL2 - Virus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,244	0,0002	-0,095	0,2923
Day 4	0,235	0,0124	-0,111	0,3871
Day 7	0,185	0,0324	0,037	0,7612
Day 14	0,162	0,0804	-0,056	0,6675
Day 21	0,234	0,0096	-0,139	0,302

Log IL2 - Virus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,244	0,0002	0,149	0,2923
Day 4	0,479	0,0124	0,038	0,3871
Day 7	0,429	0,0324	0,186	0,7612
Day 14	0,406	0,0804	0,093	0,6675
Day 21	0,478	0,0096	0,01	0,302

Log IFNg - Virus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,684	0	-0,105	0,4473
Day 4	0,096	0,5169	-0,268	0,1896
Day 7	0,115	0,466	0,132	0,5517
Day 14	0,002	0,9895	-0,019	0,9394
Day 21	-0,174	0,3432	0,043	0,8799

Log IFNg - Virus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,684	0	0,579	0,4473
Day 4	0,78	0,5169	0,311	0,1896
Day 7	0,799	0,466	0,711	0,5517
Day 14	0,686	0,9895	0,56	0,9394
Day 21	0,51	0,3432	0,622	0,8799

Log TNFa - Virus				
	Placebo (ΔInt)	P-Value	Selenium(ΔInt)	P-Value
Day 0	0,412	0	-0,066	0,5358
Day 4	0,233	0,0211	-0,143	0,301
Day 7	0,278	0,0141	0,011	0,9433
Day 14	0,109	0,3593	-0,076	0,6473
Day 21	0,067	0,472	0,216	0,1352

Log TNFa - Virus				
	Placebo	P-Value	Selenium	P-Value
Day 0	0,412	0	0,346	0,5358
Day 4	0,645	0,0211	0,203	0,301
Day 7	0,69	0,0141	0,357	0,9433
Day 14	0,521	0,3593	0,27	0,6473
Day 21	0,479	0,472	0,562	0,1352

10. Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

„Immunological effects of intravenous selenium administration in severe sepsis”

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Darmstadt, 28.06.2021 Anne Guo

Journal of Critical Care 52 (2019) 208–212

Contents lists available at ScienceDirect

Journal of Critical Care

journal homepage: www.journals.elsevier.com/journal-of-critical-care

Immune function testing in sepsis patients receiving sodium selenite

Anne Guo [a], Jyotsna Srinath [b], Matthias Feuerecker [a], Brian Crucian [c], Josef Briegel [a], Anne-Laure Boulesteix [b], Ines Kaufmann [d], Alexander Choukèr [a,*]

[a] Department of Anaesthesiology, University Hospital, LMU Munich, Munich, Germany
[b] Institute for Medical Information Processing, Biometry and Epidemiology, LMU Munich, Munich, Germany
[c] Johnson Space Center (JSC), NASA, 1601 NASA Parkway, Houston, TX 77058, USA
[d] Department of Anaesthesiology, Munich-Neuperlach Hospital, Munich, Germany

ARTICLE INFO

Keywords:
Selenium
Sepsis
Immune function
Cytokines

ABSTRACT

Purpose: We examined in a longitudinal study the role of sodium selenite in sepsis patients in strengthening the immune performance in whole blood samples using immune functional assays.
Materials and methods: This was a sub-study from a randomized, double blinded multicenter clinical trial (SISPCT) registered with www.clinicaltrials.gov (NCT00832039) and with data collected at our center. Full blood samples were incubated with various recall antigens and the supernatants were measured for their cytokine concentrations as markers for immune response. Data from days 0, 4, 7, 14, and 21 (from sepsis onset) were analyzed using a generalized least squares model in R to appropriately take the longitudinal structure and the missing values into account.
Results: From the 76 patients enrolled in the study at our center, 40 were randomized to selenium therapy and 36 to placebo. The analyses of immune response assay data showed no statistical difference between the selenium and placebo groups at each of the time points. There was however an overall dampening of cytokine release, which tended to recover over time in both groups.
Conclusion: Selenium has long been an adjuvant therapy in treating sepsis. Recently, it was proven to not have beneficial effects on the mortality outcome. Using data from our center in this sub-cohort study, we identified no relative improvement in cytokine release of stimulated blood immune cells ex vivo from patients with selenium therapy over a three-week period. This offers a potential explanation for the lack of beneficial effects of selenium in sepsis patients.

1. Introduction

Severe sepsis and septic shock are a leading cause of mortality in critical care. Selenium is a trace element that is often given to these patients to improve clinical outcomes. In septic patients, selenium levels are often already decreased [1] and are not reconstituted through volume resuscitation or transfusions. Being critically ill also increases the demand for selenium in order to neutralize radicals due to oxidative stress in the body [2]. It all appears logical that administration of this trace element may be an important part of therapy in sepsis.

The first comprehensive study which supports the use of selenium in sepsis showed a reduction in mortality [3]. The discussion quickly became heated as many research teams were unable to reproduce the positive results. The current Surviving Sepsis Campaign guidelines from 2016 state that "evidence for the use of intravenous selenium to provide a pharmacologic effect through an antioxidant defense is not convincing" [4]. There was no significant impact on mortality or secondary outcomes such as length of stay or development of nosocomial pneumonia. Indeed, early parenteral [5,6] administration of selenium has not proven to be effective in altering clinical outcomes despite selenoproteins' known role in upregulation of anti-inflammatory pathways [7,8]. While we now know selenium does not impact mortality, this study takes a step further in uncovering if and how selenium affects elementary functions of the immune system. We have applied an established cytokine release assay to run short time cultures with diluted whole blood as a proxy for the assessment and monitoring of the immune performance in a homeostatic environment with no cell separations and included proteins and complement [9–11] over the course of 3 weeks of the disease. To answer the question of whether intravenous selenium improves immune function in severe sepsis or septic shock, we analyzed blood samples from patients with immune

* Corresponding author at: Laboratory of Translational Research "Stress and Immunity", Department of Anaesthesiology, University Hospital, LMU Munich, Marchioninistraße 15, 81377 Munich, Germany.
E-mail address: achouker@med.uni-muenchen.de (A. Choukèr).

https://doi.org/10.1016/j.jcrc.2019.05.001
0883-9441/

stimulation assays over the course of 21 days using a generalized least squares model.

2. Methods

2.1. Clinical study design

A prospective, longitudinal study at the anaesthesia ICU, Munich University Hospital was conducted as part of the multicenter "Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis" (SISPCT) [5] cohort [NCT00832039]. 76 Patients were recruited at our hospital following ethical approval by the University of Jena Research Ethics Committee with local amendments [Eudra-CT-Nr. 2007-004333-42] and written informed consent was obtained from the medical proxy. As part of the study protocol, patients were randomized to receive intravenous sodium selenite (1 mg loading dose followed by continuous infusion of 1 mg daily until discharge) or placebo 24 h within sepsis onset.

To be included, patients had to meet the criteria either for severe sepsis or septic shock at the time of admission to the ICU and were enrolled within 24 h into the study. This entailed meeting two or more of the SIRS criteria of tachycardia (>90 bpm), tachypnea (>20 bpm or mechanical ventilation), body temperature above 38 °C or below 36 °C, and white blood cell count over 12,000/mm^3 or below 4000/mm^3. There must be a clinical suspicion for or microbiologically proven infection. In addition, one or more of acute encephalopathy, thrombocytopenia, renal dysfunction, metabolic acidosis, arterial hypoxemia, and arterial hypotension must be present. Patients were excluded if they were pregnant, breastfeeding, otherwise severely immune compromised, requiring long-term antimicrobial therapy, or if they had experienced selenium intoxication or were allocated as participants in another trial.

The administration of study solutions, either placebo or sodium selenite, was started as soon as possible until discharge from the ICU. All other medical management decisions, such as antimicrobial or corticosteroid therapy and enteral or parenteral nutrition, took place independently at the discretion of the ICU physicians. Clinical data were collected throughout the entire ICU stay. Blood samples for running the study relevant ex vivo immune assays were drawn on days 0, 4, 7, 14, and 21 for our mono-center sub-study to assess immune function.

2.2. Immune response assays

Blood samples were drawn into 9 ml lithium heparinized tubes (Sarstedt AG & Co., Nümbrecht, Germany) through either arterial or central venous catheters. 400 µl of patient blood was transferred to tubes with equal volume of Dulbecco modified eagles medium (Sigma-Aldrich, Steinheim, Germany) along with the different stimulating agents including pokeweed mitogen (PWM) (Sigma-Aldrich, Steinheim, Germany) and a CD3 / CD28 (Becton Dickinson, Franklin Lakes, NJ, USA) mixture. Pokeweed mitogen is a strong immune activator that induces T and B cell mitosis in a non-receptor specific fashion. CD3 and CD28 are T cell receptor ligands that stimulate T cell activation via binding to antigen presenting cells. Lipopolysaccharide (LPS) (*E.coli* serotype 025:B6 Sigma-Aldrich, St. Louis, MO, USA) is a component of the outer membrane of gram-negative bacteria and elicits a strong immune response in animal cells. Additional stimulating antigens used include bacterial (1% Boostrix, GlaxoSmithKline, Munich, Germany), fungal (Candida lysate, Allergopharma, Reinbeck, Germany) components, or Phorbol myristate acetate and Ionomycin (PMA-I) (Sigma-Aldrich, St. Louis, MO, USA) to activate protein kinase C (PKC) signaling pathways and stimulate immune cell cytokine production.

These mixtures were incubated for 48 h at 37 °C and immediately frozen at −80 °C in Eppendorf tubes. The frozen supernatants were then processed in a blinded fashion after thawing. The concentrations of the cytokines IL-1b, IL-2, IL-6, TNF and IFN-γ were analyzed using Luminex xMAP technology with commercially available reagents from BioRad-Laboratories Inc. (Hercules, California, USA). The readouts were processed using software provided by Bioplex. Further cytokines that were measured include IL-4, IL-5, IL-8, IL-10, and IL-12.

2.3. Statistical analysis

The main objective of the statistical analyses was to investigate whether the evolution of the marker values over time was different in the two treatment groups (selenium and placebo). Important challenges in the analysis of longitudinal data were missing data points (with patients who passed away or became healthy enough to leave the ICU, there was a substantial attrition over the three week period) and the potentially strong correlation of the measurements within patients (there were patients with high average values and patients with low average values). With the starting number at $n = 76$, complete case analysis including only the 17 patients with follow-up until day 21 would be skewing the results toward the remaining, likely sicker patients. It is indeed important to note that the missing values in this case were not missing completely at random. On the one hand, the patients who have left the ICU in comparison to those who remained were likely less severely diseased. On the other hand, patients whose values were missing because of death were likely more severely diseased. Moreover, the measurements of a patient were usually noticeably correlated (i.e. more similar to each other on average than measurements from different patients). Because of this correlation, standard linear regression could not be used. The selection of a statistical approach modelling the evolution of the markers over time while taking these issues into account required advanced statistical expertise.

The treatment effect on immune function over time was assessed by fitting "generalized least squares (GLS) models" with an unstructured correlation matrix. This was done by applying the R function 'gls' from the R package 'nmle' to each log-transformed marker successively with treatment and time (coded as factors) as well as their interaction as covariates. Assuming the probability that a missing value was determined by the last observed values (a reasonably realistic assumption in the present case), this approach adequately coped with missing values and yielded a valid inference, so that imputation was not needed. It also adequately took the correlation between measurements from the same patient into account. The log-transformation $\log(1 + x)$ was performed to achieve approximate normality (1 was added to better cope with values close to 0). For each marker, the global null-hypothesis of no interaction between treatment and time (i.e. that the treatment has no effect on the changes in marker levels over time) was tested using a likelihood-ratio test as implemented in the R function 'anova'. This analysis was repeated for 42 different combinations of antigens and cytokines. Holm's procedure was used to adjust for multiple testing. All statistical analyses were conducted with R (version 3.3.1) by two data analysts (JS and ALB) independently for crosschecking.

3. Results

Among the 76 severe sepsis or septic shock patients enrolled, 40 were randomized to receive sodium selenite or 36 placebo (Table 1). Top admitting diagnoses were pneumonia, intra-abdominal infections and urosepsis. The subjects were comprised of surgical and to a smaller percentage, non-surgical patients. The vast majority have already received antimicrobial therapy upon admission and over half hydrocortisone therapy. Patient characteristics and disease severity were comparable in the two groups based on SAPS II, APACHE II and SOFA scores.

The GLS analyses showed no statistically significant immune enhancement with selenium versus placebo over time after adjustment for multiple testing. Quantile-quantile plots were generated for each investigated marker to visualize the distribution of the residuals as a model fit check. There were no substantial deviations from the normal distribution. Logarithmically transformed longitudinal results from

Table 1
Comparison of patient characteristics in the placebo and selenium groups.

	Placebo group	Selenium group
Patients at day 0/4/7/14/21	36/28/26/16/9	40/33/24/16/8
Age	61.3 ± 16.0	60.5 ± 17.4
Sex (m/f)	18/18	23/17
Weight (kg)	83.1 ± 19.8	84.5 ± 27.8
Height (cm)	172.1 ± 5.2	170.5 ± 10.2
GCS	6.4 ± 5.2	6.6 ± 5.2
APACHE II*	27.1 ± 7.6	27.7 ± 9.2
SAPS II	65.4 ± 15.6	66.0 ± 17.0
MOD	8.6 ± 3.4	8.2 ± 3.1
SOFA	12.4 ± 3.8	12.6 ± 3.8
MAP max. (mmHg)	94.8 ± 16.4	97.8 ± 17.6
MAP min. (mmHg)	62.9 ± 14.5	63.0 ± 12.1
HR max. (bpm)	127.2 ± 32.6	121.5 ± 22.3
HR min. (bpm)	87.6 ± 21.9	83.2 ± 26.0
Lactate max. (mmol/L)	3.8 ± 2.3	4.6 ± 5.1
CRP (mg/L)	18.4 ± 12.4	22.8 ± 16.2
Antibiotics prior to admission (y/n)	33/2	39/1
Hydrocortisone (y/n)	24/12	20/20

Continuous variables are summarized as mean ± SD. GCS = Glasgow coma scale, APACHE = acute physiology and chronic health evaluation, SAPS = simplified acute physiology score, MOD = multiple organ dysfunction, SOFA = sepsis-related organ failure assessment. m = male, f = female, y = yes, n = no. MAP = mean arterial pressure during entire ICU stay, HR = heart rate. *As patients were sedated, the APACHE II scores were also calculated assuming a GCS of 15: placebo group 18.5 ± 6.9, selenium group 18.8 ± 6.6.

two stimulating antigens pokeweed mitogen and CD3 / CD28 co-stimulation are shown (Fig. 1) with immune function assay markers IL-2, TNF and IFNγ. Pokeweed mitogen is a strong non-specific lymphocyte activator. Interleukin-2 levels in the supernatant increased moderately over time (1A). TNF and IFNγ levels were more pronouncedly dampened at sepsis onset and appeared to recover already at day 4 (1B and 1C). The T-lymphocyte specific stimulators CD3 / CD28 showed a similar progression as well, where the immune response was initially inhibited but bounced back throughout the 3-week period (2A to 2C). In the LPS stimulation assays, the immune function assays with the cytokines TNF (Fig. 1 3A), IL-6 (3B) and IL-1b (3C) also showed comparable readouts with no substantial difference between the selenium and the control groups.

Additional stimulation assays using either bacterial or fungal recall antigens or PMA-I were also unable to demonstrate an effect of selenium therapy on the characteristic cytokine release patterns. A subpopulation analysis of patients who stayed at least 2 weeks in ICU was conducted to isolate the severely ill sepsis patients, the rationale being that the immune system in these patients was particularly compromised and selenium levels are known to be low in the critically ill. Again, no significant difference was detected between selenium and placebo groups. Interleukin-1b, Interleukin-2, Interleukin-6, TNF and IFNγ were only selected cytokines whose release is representative of the general immune function. Additional immune markers including IL-4, IL-5, IL-8, IL-10, and IL-12 (data not shown) were also tested and analyzed demonstrating no difference between the test groups, further reinforcing the immune neutral effects of selenium on the panel of immune stimuli and read-out cytokines as tested in this study.

4. Discussion

Given selenium's reputation as an immune booster, it was a surprise that the early administration of sodium selenite did not alter cytokine release of ex vivo stimulated blood from sepsis patients over a three-week period. Our study furthered the clinical findings of no benefit in mortality with one possible mechanistic explanation, that administration of selenium does not per se strengthen immune capabilities in sepsis.

Analyses performed using data upon admission to the ICU have shown that, compared to healthy controls, cytokine release upon stimulation with all kinds of stimuli or antigens was severely dampened at the onset of sepsis [12]. This means the patients from the two groups, selenium and placebo, were all in an immune paralyzed state due to the nature of their illness before randomization occurred. As proper functioning of the human immune system is pivotal in fighting the disease process, we were able to examine for the first time whether selenium administration in sepsis patients alters the cytokine release from stimulated whole blood over time using a generalized least squares model.

Bacteremia accounts for only a portion of the sepsis patient population, many are indeed culture negative patients. Therefore, a spectrum of immune stimuli was used to test the bacterial and fungal re-call antigen response, specific lymphocyte response as well as the innate immune response. Pokeweed mitogen is a strong and non-specific activator of B and T lineage cells. CD3 and CD28 are T-cell specific adaptive immune activators, while LPS is a strong ligand and activator at the TLR-4 of innate immune cells. Bacterial and fungal recall antigens were also included in the panel but we did not detect a difference in any of these arms of the immune response with selenium therapy. This does further emphasize the severity of the disease and the full blown impact sepsis has on the immune system. It also suggests that selenium does not have a selective effect on either adaptive or innate immune cell subtypes in combatting against immune suppression. Even over a time course of 3 weeks, the insult to immune system functioning could not be improved with selenium.

The applied immune assay has been tried and proven to be effective [9] in providing an overall measure of the immune response using incubated ex vivo whole blood samples. The release of three cytokines, IL-2, TNF and IFNγ, which are important in innate and adaptive immunity were unaltered by selenium. Accordingly, selenium did not show an effect in ameliorating the activation of innate immune cells (monocytes, granulocytes) by LPS. Over the time course, release of TNF, IL-6 and IL1ß moderately increased but a further selenium dependent effect was not detected. This suggests a neutral effect of selenium on cell-mediated immune response.

While an immune booster like selenium is expected to enhance our defenses, sepsis remains a mysterious immunological process where a dynamic balance between pro- and anti-inflammatory activities exert their seemingly contradictory effects, making treatment possibilities difficult and limited. In a way, it is perhaps not as surprising that selenium does not have a discernable impact on cytokine release during the sepsis disease process because the immune system does not necessarily require a simple boost. In fact, a complex interplay between pro- and anti-inflammation is known to exist at various time points during sepsis. This intricate balance is unlikely to be improved with a silver bullet, such as selenium supplementation, but requires deeper understanding on its mechanisms of action on each specific branch of immunity.

On the other hand, it has been shown in in vitro endothelial cell models with conditions mimicking sepsis that cytokine levels were not drastically altered through selenium therapy but there was an observable effect on mitochondrial function [2]. Our results have extended these findings using real patient blood samples over a time axis, rather than simulated laboratory conditions. Perhaps its direct incorporation into selenoproteins makes high selenium reserves already necessary at the onset of sepsis, during the initial pro-inflammatory phase. In the time to follow and transition into the immunosuppressive phase of sepsis, the irreparable damage has already occurred, as one might speculate.

The debate also extends into dosing, whether bolus or continuous administration could have explained differences observed in past selenium studies. In a sheep peritonitis model where the two methods of administration were compared, the bolus injection group did have a better outcome than the continuous administration group [13]. Our study was a part of the largest multicenter trial to date where the protocol includes a one-time administration of bolus at study onset and continued selenium therapy throughout the ICU stay. Nevertheless, the positive effects as seen in animal models have not been observed in

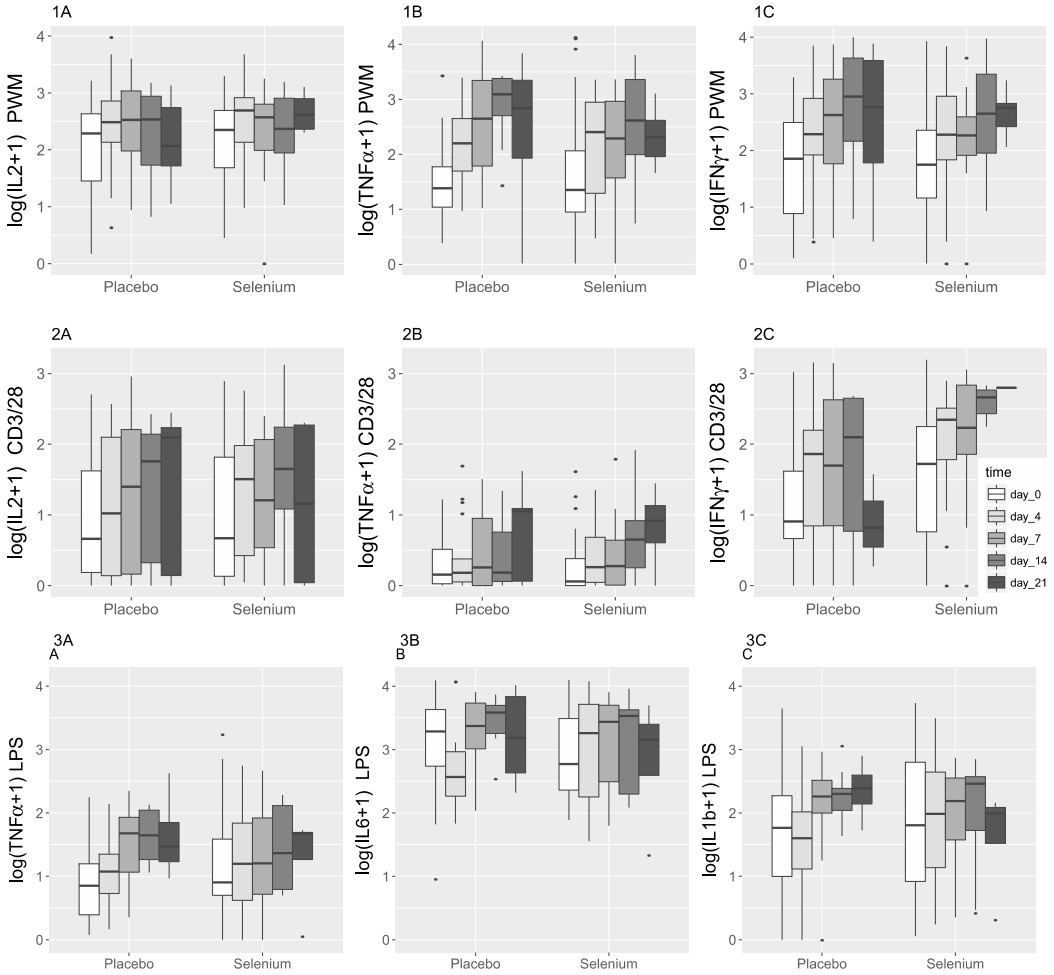


Fig. 1. Logarithmic values of stimulation assays using pokeweed mitogen (1) or CD 3/CD28 (2) co-stimulation as boxplots for the cytokines Interleukin-2 (A), TNF (B), and IFNγ (C). Logarithmic values of stimulation assays using lipopolysaccharide (3) stimulation as boxplots for the cytokines TNF (A), Interleukin-6 (B) and Interleukin-1b (C).

clinical studies. Indeed, a recent meta-analysis of 21 randomized controlled trials in ICU patients showed no mortality difference between intravenous selenium administration and placebo [14].

4.1. Strengths, weaknesses, approaches

The strength of this study lies in its randomized and double-blinded design, with rigorous criteria for patient recruitment and the high rate of adherence to protocol. The immune response assays in turn provided high fidelity readouts to cytokine levels. The study cohort size was limited to 76 and despite the high quality of the data, immune response assays generate a broad response range. Moreover, patients leave the ICU at various time points, making conventional statistical methods altogether unsuitable. The generalized least squares model we have utilized is an advanced statistical tool which preserves integrity of data points despite the attrition of sample size. This enabled the analysis over time, which demonstrated even with over 3 weeks of selenium supplementation, the immune system does not get a significant boost. The exact pharmacological mechanisms and other potential benefits of selenium in sepsis remain obscure. This is due to our experimental setup where patients were enrolled at any time of the day and the limited blood samples, which did not allow more cell-specific immune assays or analyses, including specific cell separation and/or higher resolution of the time points. Our patient population was already severely immune compromised at the start of our study, the immune anergy observed prevented us from examining any potential beneficial effects of selenium in lessening the global immune weakening.

5. Conclusion

With our study, we have gained the insight that selenium therapy did not improve cytokine release of ex vivo stimulated blood from sepsis

patients over time. The decision to administer sodium selenite should therefore be weighed even more carefully in the ICU given the possible side effects including nausea and vomiting, fatigue, irritability, coagulation problems as well as liver and kidney impairment. To add to existing studies showing no improvement in clinical outcomes, we now demonstrated that selenium does not discernably affect the investigated immune system function in sepsis.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This paper is a part of A. Guo's medical doctoral thesis. This investigation was supported by the German Federal Ministry of Economics and Energy (BMWi/DLR grant 50WB0719/WB0919 and WB1622). Funding to support NASA participation in immune functional assays was provided by the NASA Human Research Program, Human Health and Countermeasures Element. The authors express their special thanks to M. Hörl, S. Matzel, J. Göschl, L. Sudhoff, I. Kumpreij and C. Ladinig for the on-site support and analyses, respectively, in the anesthesiologic laboratory of the University of Munich. Also to S. Greven from the University of Munich for the input on statistical methods. Furthermore, the authors are very thankful for the continuous support of the anesthesiologic intensive care units, its staff under the guidance of Dr. Lorenz Frey, and the national SISPCT study organising SepNet Critical Care Trials Group.

References

[1] Lee YH, Bang ES, Lee JH, Lee JD, Kang DR, Hong J, et al. Serum concentrations of trace elements zinc, copper, selenium, and manganese in critically ill patients. Biol Trace Elem Res 2019 Apr;188(2):316–25 [Epub ahead of print].

[2] Mertens K, Lowes DA, Webster NR, Talib J, Hall L, Davies MJ, et al. Low zinc and selenium concentrations in sepsis are associated with oxidative damage and inflammation. Br J Anaesth 2015 Jun;114(6):990–9.

[3] Angstwurm MW, Engelmann L, Zimmermann T, Lehmann C, Spes CH, Abel P, et al. Selenium in Intensive Care (SIC): results of a prospective randomized, placebo-controlled, multiple-center study in patients with severe systemic inflammatory response syndrome, sepsis, and septic shock. Crit Care Med 2007 Jan;35(1):118–26.

[4] Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Crit Care Med 2017 Mar;45(3):486–552.

[5] Bloos F, Trips E, Nierhaus A, Briegel J, Heyland DK, Jaschinski U, et al. Effect of sodium selenite administration and procalcitonin-guided therapy on mortality in patients with severe sepsis or septic shock: A randomized clinical trial. JAMA Intern Med 2016 Sep 1;176(9):1266–76.

[6] Chelkeba L, Ahmadi A, Abdollahi M, Najafi A, Ghadimi MH, Mosaed R, et al. The effect of high-dose parenteral sodium selenite in critically ill patients following sepsis: A clinical and mechanistic study. Indian J Crit Care Med 2017 May;21(5):287–93.

[7] Lee Byung Cheon, Lee Sang-Goo, Choo Min-Kyung, Kim Ji Hyung, Lee Hae Min, Kim Sorah, et al. Selenoprotein MsrB1 promotes anti-inflammatory cytokine gene expression in macrophages and controls immune response *in vivo*. Gladyshev Sci Rep 2017;7:5119.

[8] Shrimali RK, Irons RD, Carlson BA, Sano Y, Gladyshev VN, Park JM, et al. Selenoproteins mediate T cell immunity through an antioxidant mechanism. J Biol Chem 2008 Jul 18;283(29):20181–5.

[9] Feuerecker M, Mayer W, Kaufmann I, Gruber M, Muckenthaler F, Yi B, et al. A corticoid-sensitive cytokine release assay for monitoring stress-mediated immune modulation. Choukèr A Clin Exp Immunol 2013 May;172(2):290–9.

[10] Feuerecker M, Crucian BE, Quintens R, Buchheim JI, Salam AP, Rybka A, et al. Immune sensitization during one year in the Antarctic high altitude Concordia Environment. Allergy 2019 Jan;74(1):64–77 [Epub ahead of print].

[11] Yi B, Rykova M, Jäger G, Feuerecker M, Hörl M, Matzel S, et al. Influences of large sets of environmental exposures on immune responses in healthy adult men. Sci Rep 2015 Aug 26;5:13367.

[12] Feuerecker M, Sudhoff L, Crucian B, Pagel JI, Sams C, Strewe C, et al. Early immune anergy towards recall antigens and mitogens in patients at onset of septic shock. Sci Rep 2018 Jan 29;8(1):1754.

[13] Wang Z, Forceville X, Van Antwerpen P, Piagnerelli M, Ahishakiye D, Macours P, et al. Shock 2009 Aug;32(2):140–6.

[14] Manzanares W, Lemieux M, Elke G, Langlois PL, Bloos F, Heyland DK. High-dose intravenous selenium does not improve clinical outcomes in the critically ill: a systematic review and meta-analysis. Crit Care 2016 Oct 28;20(1):356 [Review].

SCIENTIFIC REPORTS

OPEN

Early immune anergy towards recall antigens and mitogens in patients at onset of septic shock

Received: 31 July 2017
Accepted: 10 January 2018
Published online: 29 January 2018

M. Feuerecker[1], L. Sudhoff[1], B. Crucian[2], J.-I. Pagel[1], C. Sams[2], C. Strewe[1], A. Guo[1], G. Schelling[1], J. Briegel[1], I. Kaufmann[1,3] & A. Choukèr[1]

The pathology of sepsis is typically characterized by an infection and excessive initial inflammation including a cytokine storm, followed by a state of immune suppression or paralysis. This classical view of a two peak kinetic immune response is currently controversially discussed. This study was a sub-study of the randomized clinical Trial SISPCT registered with www.clinicaltrials.gov (NCT00832039, Registration date: 29/01/2009). Blood samples from 76 patients with severe sepsis and septic shock were incubated for 48 h at 37 °C *in vitro* with bacterial or fungal recall-antigens or specific mitogen antigens within 24 hours of sepsis onset. Recall-antigen stimulation led to a severe dampening of normal cytokine release. This immunologic anergy was similarly observed after mitogen stimulation. Moreover, patients under hydrocortisone therapy or with lowered arterial oxygen tension had further reductions in cytokine levels upon B- and T-cell mitogen stimulation. This investigation reveals an early onset of immunoparalysis during sepsis. This immune incompetence in mounting an adequate response to further infections includes previously sensitized pathogens, as seen with recall-antigens. Also, the immune-suppressive role of hydrocortisone and low PaO_2 is highlighted. Aside from early broad-spectrum antimicrobial therapy, our findings reinforce the need for maximal immunological support and protection against further infections at the onset of sepsis.

Sepsis and sepsis-associated disease states are not only an observation in modern medicine, there have long been many reports dealing with this particular condition in the past. Sir William Osler (1849–1919) observed that patients apparently died from the body's response to the infection rather than from the infection itself[1]. His observations of the immune system still hold true today, making sepsis a particularly dangerous and tenacious disease. At the end of the 20th century, incidence of sepsis has increased annually by 8.7% in the US, peaking in the year 2000 at 240.4 per 100,000 inhabitants. Although the in-hospital mortality decreased from 27.8% to 17.9%, the total number of deaths continued to rise due to the increase in incidence[2]. Despite all advances in modern medicine and antimicrobial therapy, sepsis and in particular septic shock are still the leading causes for death in critically ill patients in the United States[1,3]. Considering sepsis to cause a comparable number of annual deaths as acute myocardial infarction[4], further research leading to new therapeutic strategies is directly needed.

Besides systemic inflammatory response syndrome (SIRS), which still is the hallmark sign of sepsis, another contrary condition in the progression of sepsis is described in the literature. It is associated with an inhibition of the immune system, resulting in a lack of response to pathogens, and goes by different names, such as compensatory anti-inflammatory response syndrome (CARS) or immune paralysis[5–7]. This condition causes a severe susceptibility to secondary infection and might be responsible for a significant number of deaths in the later phases of sepsis. There are different hypotheses concerning this immunosuppressive state. It is assumed that a shift from a TH_1-dominated initial immune response resulting in excessive inflammation and, subsequently, SIRS, to a TH_2-dominated anti-inflammatory state might contribute to the development of CARS[3]. Different works state that extensive lymphocyte apoptosis during sepsis progression seems to be, at least in part, responsible for the genesis of CARS[8]. Recent findings suggest that hyperinflammation and hypoinflammation are two concurrently

[1]Laboratory of Translational Research "Stress and Immunity", Department of Anaesthesiology, University Hospital, LMU Munich, Marchioninistraße 15, 81377, Munich, Germany. [2]Johnson Space Center (JSC), NASA, 1601 NASA Parkway, Houston, Texas, 77058, USA. [3]Department of Anaesthesiology, Hospital Munich-Neuperlach, Oskar-Maria-Graf-Ring 51, 81737, Munich, Germany. M. Feuerecker and L. Sudhoff contributed equally to this work. Correspondence and requests for materials should be addressed to M.F. (email: matthias.feuerecker@med.uni-muenchen.de)

Patient and general variables	Patient Group	Healthy Volunteers Group	p
Age [yrs][a]	60.9 ± 16.6	55.5 ± 12.5	0.15[b]
Body weight [kg][a]	83.9 ± 24.2 (n = 75)	82.6 ± 19.0	0.22[c]
Height [cm][a]	171.3 ± 9.5 (n = 73)	175.0 ± 8.0	0.94[b]
Body Mass Index[a]	28.6 ± 8.4 (n = 73)	27.0 ± 6.2	0.57[b]
Sex (male/female)	41/35	8/3	0.24[d] 0.34[e]
Glasgow Coma Scale (GCS)[a]	6.5 ± 5.2 (n = 76)	15	
Acute Physiology And Chronic Health Evaluation II (APACHE II)[a]	27.4 ± 8.4 (n = 74)	Not assessed	n.a.
Simplified Acute Physiology Score (SAPS II)[a]	65.7 ± 16.2 (n = 74)	Not assessed	n.a.
Ventilation (none/non-invasive/invasive)	13/10/53	11/0/0	n.a.
Vasopressors (yes/no)	70/6	0/11	**$4.42e^{-10}$**[c]
Antimicrobial therapy (yes/no)	73/3	0/11	**$4.87e^{-11}$**[c]
Hydrocortisone (yes/no)	41/35	0/11	**<0.001**[c]

Table 1. Comparison of general patient and healthy volunteers data, including important Intensive Care Unit scores and therapy variables. [a]Values are mean ± SD; [b]Mann-Whitney U test, [c]t-test, [d]Chi Square test, [e]Fisher's exact test; n.a. = not applied.

developing processes in sepsis, terming it as mixed anti-inflammatory response syndrome (MARS)[6,9]. There are numerous further theories concerning the pathophysiology of immunosuppression in sepsis, including impaired leukocyte recruitment and decreased cell surface protein expression[10]. After all, the exact pathophysiology of sepsis and the accompanying hyperinflammatory and immunosuppressive states are still poorly understood.

To gain better insight into the early phase of septic shock we have recruited patients for the present clinical trial. The main focus was to evaluate the patients' initial immune function shortly after onset of severe sepsis or septic shock. This was carried out with a recall-antigen whole blood assay, along with specific innate and adaptive immune cell activation assays. We hypothesized a pre-existing global immunosuppression at this time point. Moreover, this report looks at the capacity of these assays to detect effects of additional early hydrocortisone (HC) treatment as well as the immunosuppressive consequences of hypoxia in this patient cohort.

Results

Study design and demographic data. The *immune function study* was conducted from June 2011 to February 2013 as part of the clinical trial SISPCT[11] (www.clinicaltrials.gov: NCT00832039, Registration date: 29/01/2009). In total, 76 patients with severe sepsis or septic shock were included. An age- and sex matched healthy volunteer (HV) control group was recruited between September 2012 and December 2012. No significant group differences could be determined based on age, sex, height, body weight and body mass index (BMI) (Table 1).

At the time of enrolment, 96.0% (n = 73) of patients received antimicrobial therapy. In 53% (n = 40) of patients, pneumonia was identified as the primary focus, followed by intra-abdominal foci (19%, n = 14). Pathogen detection was achieved in 51% (n = 39) of the cases. Therein 54% (n = 21) were shown to be gram-negative sepsis, 38% (n = 15) gram-positive and 8% (n = 3) viral. Increased "Simplified Acute Physiology Score II" (SAPS II) and "Acute Physiology and Chronic Health Evaluation II" (APACHE II) scores were seen (Table 1). About 95% (n = 70) of patients were treated with inotropic or vasopressor medications for an average of 14.2 ± 8.3 h (n = 70) at study enrolment. The majority of patients on multiple catecholamines (n = 40) received hydrocortisone within the first 24 h (n = 30). The 90-day mortality was 18.7% (n = 14) with a mean survival of 30.6 ± 33.4 days. Immediately after patient enrolment, blood was drawn for the study. In mean average this was 14.5 hours (SD 5.8 hours) after the first symptoms occurred.

Blood Samples. *Complete Blood Count (CBC).* Almost all patients had abnormal CBC results. 11 patients showed leukopenia and 39 patients had leukocytosis (Table 2).

Plasma Inflammation Markers. Except for one patient with a normal C-reactive protein (CRP) value and another with normal procalcitonin (PCT), all others had an elevated CRP, interleukin (IL) 6 and PCT (Table 2).

In vitro Recall Antigen and Mitogen Stimulation Assays. Determination of the initial immune function: Spontaneous cytokine release in the unstimulated (basal) assay showed significantly lower levels of IFN-γ and IL-2 in septic shock/ septic patients (SS). After stimulation with recall antigens, patients had significantly lower pro-inflammatory cytokine levels (interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis-factor (TNF)-α) compared to healthy volunteers (HV), irrespective of the type of antigen used (bacterial, fungal) (Fig. 1). Cell specific stimulation assays with pokeweed mitogen (PWM), lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA)/Ionomycin, and CD3/CD28 (Fig. 2) also revealed a significant reduction in pro-inflammatory cytokine

Septic patients	Standard Value	Mean ± SD	Range
Complete blood count (CBC)			
Leukocytes [G/l]	*4.5–10.5*	**14.6 ± 10.6 ↑**	0.8–46.8
Erythrocytes [T/l]	*4.2–5.1*	**3.5 ± 0.7 ↓**	2.3–5.5
Hemoglobin [g/dl]	*12.0–16.0*	**10.7 ± 2.3 ↓**	7.0–18.0
Hematocrit [%]	*36.0–46.0*	**31.3 ± 6.4 ↓**	20.9–50.7
Thrombocytes [G/l]	*150–400*	191.6 ± 97.6	32–571
Mean Corpuscular Volume (MCV) [fl]	*79.0–92.0*	89.8 ± 6.3	67.6–104.0
Mean Corpuscular Hemoglobin (MCH) [pg]	*26.5–32.5*	30.1 ± 2.4	21.8–35.6
Mean Corpuscular Hemoglobin Concentration (MCHC) [g/dl]	*32.0–36.0*	33.6 ± 1.5	29.8–36.7
Plasma inflammation markers			
CRP [mg/dl]	*≤0.5*	**20.7 ± 14.6 ↑**	0.3–66.5
Interleukin-6 [pg/ml]	*≤5.9*	**18989.6 ± 71652.2 ↑**	26–499,000
Procalcitonin [ng/ml]	*≤0.1*	**16.9 ± 25.5 ↑**	0.1–105.0

Table 2. Complete Blood Count (CBC) and Plasma inflammation marker. Deviation from standard values are marked bold. Data are mean ± SD; n = 51–68.

release (IL-2, IFN-γ, TNF-α) in comparison to HV. Differences between groups after LPS stimulation, which mimics bacterial endotoxins and assesses the innate immune response, were less pronounced but reached statistical significance for Interleukin 1β and TNF-α. Anti-inflammatory interleukins (IL-4, IL-5, IL-10) were significantly lower in sepsis patients and close to the lowest detection limit of the assays. Statistically significant group differences for IL-10 however were only observed after stimulation with soluble antibodies to both CD3 and CD28, given a low significance level ($p < 0.05$). No differences were observed between male vs. female patients (n.s.).

Disease severity classification systems and the stimulation assays: In order to correlate with disease severity, we used SAPS II and APACHE II. Correlation of SAPS II and APACHE II values with the 90-day mortality showed no statistically significant effect although a tendency towards a positive correlation was present (Supplemental Table 1 and Fig. 3).

An increase in disease severity, represented by SAPS II and APACHE II scores, was associated with an impaired TNF-α response both after bacterial antigen (Fig. 3A,B) as well as PWM stimulation (Fig. 3C,D).

Immune answers of septic patients with or without hydrocortisone: In patients with refractory septic shock, a daily dose of 200–300 mg of hydrocortisone is recommended[12]. In our patient collective, 43 patients (56.6%) received hydrocortisone (HC) during the first 24 h of admission to the intensive care unit (ICU). We compared this group to the patients who received no hydrocortisone within this time period with respect to assays cytokine release. Analysis of the two groups revealed no significant differences in the CBC, but the disease severity scores varied significantly (SAPS II: HC: 72.7 ± 14.9, n = 43; no HC: 55.9 ± 12.8, n = 31; APACHE II: HC: 31.0 ± 7.4, n = 43; no HC: 22.4 ± 7.1, n = 31). Patients on HC therapy had higher IL-6 levels compared to the other group (*HC:* 29854 ± 93424 pg/ml, n = 35; *low HC:* 4906 ± 16335 pg/ml, n = 27; Mean ± SD; Mann- Whitney- U test, $p = 0.058$). Patients receiving hydrocortisone showed a highly significant suppression of TNF-α in the PWM assay (Fig. 4A, $p < 0.001$) and IL-1β release in the LPS assay (Fig. 4B, $p < 0.01$). This significant difference was present although the stimulated cytokine responses in all septic patients were enormously reduced as compared to the healthy volunteers.

Hypoxemia and immune response: Using the normal range of PaO_2 when breathing room air (PaO_2 80–100 mmHg[13]), we classified the subgroups as "hypoxemic" patients ($PaO_2 < 80$ mmHg) or "normoxemic" patients (PaO_2 80–100 mmHg)[14]. Figure 5 shows correlations between PaO_2 and cytokine release in the assays stimulated with PWM and LPS, respectively. In both assays, supernatant cytokine concentrations (TNF-α, IL-1β) in the "normoxemic" group were higher than those in the hypoxemic group. APACHE II and SAPS II showed statistically similar scores irrespective of the PaO_2 status.

Receiver operating characteristics Initiation of renal replacement therapy: For receiver operating characteristic (ROC)-analyses using the endpoint "initiation of renal replacement therapy (RRT) during stay on ICU", the cytokine assay read-outs from PWM TNF-α and LPS IL-1β as well as disease severity classification systems SAPS II and APACHE II showed similar results, with high statistical significance (Fig. 6).

Discussion

Testing blood samples from septic patients with a new *in vitro* recall antigen whole blood assay[15] and immune-cell-specific and unspecific mitogenic stimulation assays revealed, that the human immune system is in a paralyzed state at the onset of sepsis. This blunted immune response from monocyte, T- and B-cell activation can account for the almost entirely extinct cytokine release upon bacterial and fungal recall antigen stimulation. These responses and the immunologic memory to such re-call antigens is one of the key adaptive immune competencies of the host when re-exposed to known pathogens. This old preserved mechanism seems entirely suppressed at the onset of sepsis and might be considered as one explanation for the long lasting immune compromised state and high morbidity.

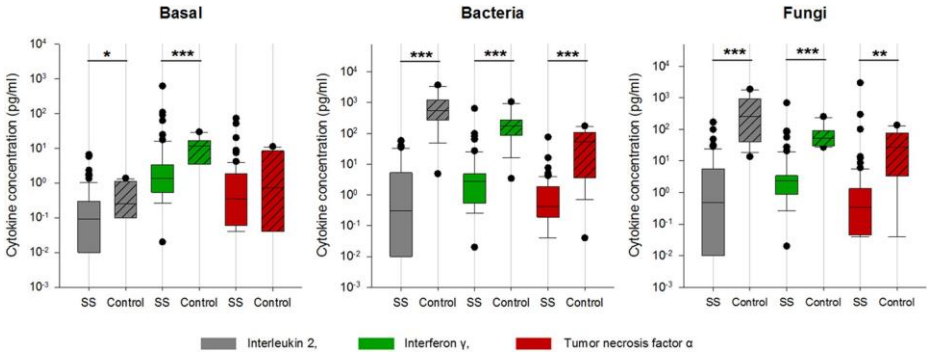


Figure 1. Patients versus control group in unstimulated assay and after stimulation with recall antigens. *SS:* severe sepsis/septic shock patients, *Basal:* unstimulated test assay, *Bacteria:* bacterial antigen mixture, *Fungi:* fungal antigen mixture. Blood samples were taken subsequently to study enrolment (SS) or at a time of subjective physical well-being (control group), respectively. In boxplots, boxes show the median and interquartile range (IQR), whiskers represent the 10th and 90th percentile. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. y-axis: logarithmic scale.

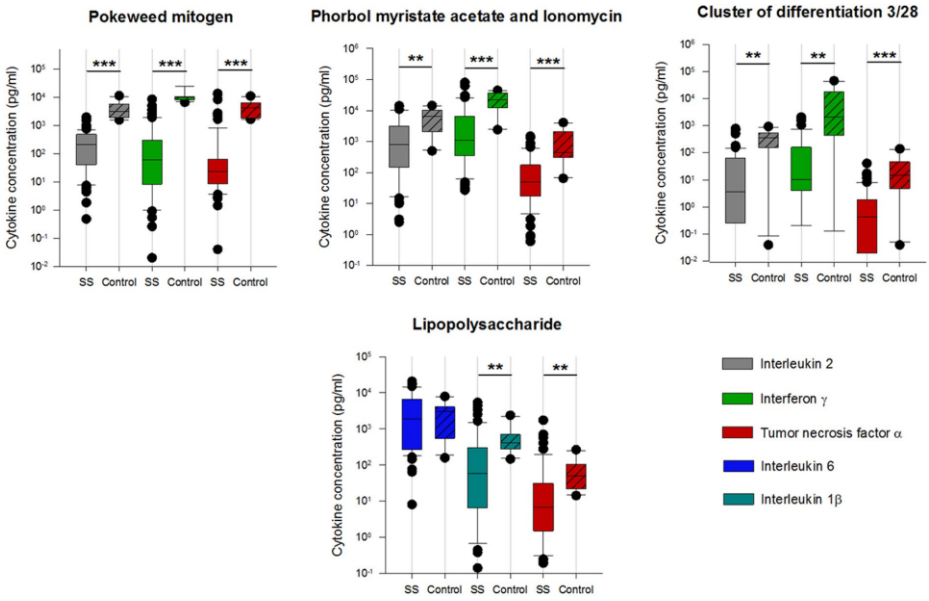


Figure 2. Patients versus control group after stimulation with PWM, PMA, CD3/28 and LPS. PWM: Pokeweed mitogen, LPS: Lipopolysaccharide, PMA-I: Phorbol myristate acetate and Ionomycin, CD3/28: Cluster of Differentiation 3/28, SS: severe sepsis/septic shock patients. Blood samples were taken subsequently to study enrolment (SS) or at a time of subjective physical well-being (control group), respectively. In boxplots, boxes show the median and interquartile range (IQR), whiskers represent the 10th and 90th percentile. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Y-axis: logarithmic scale.

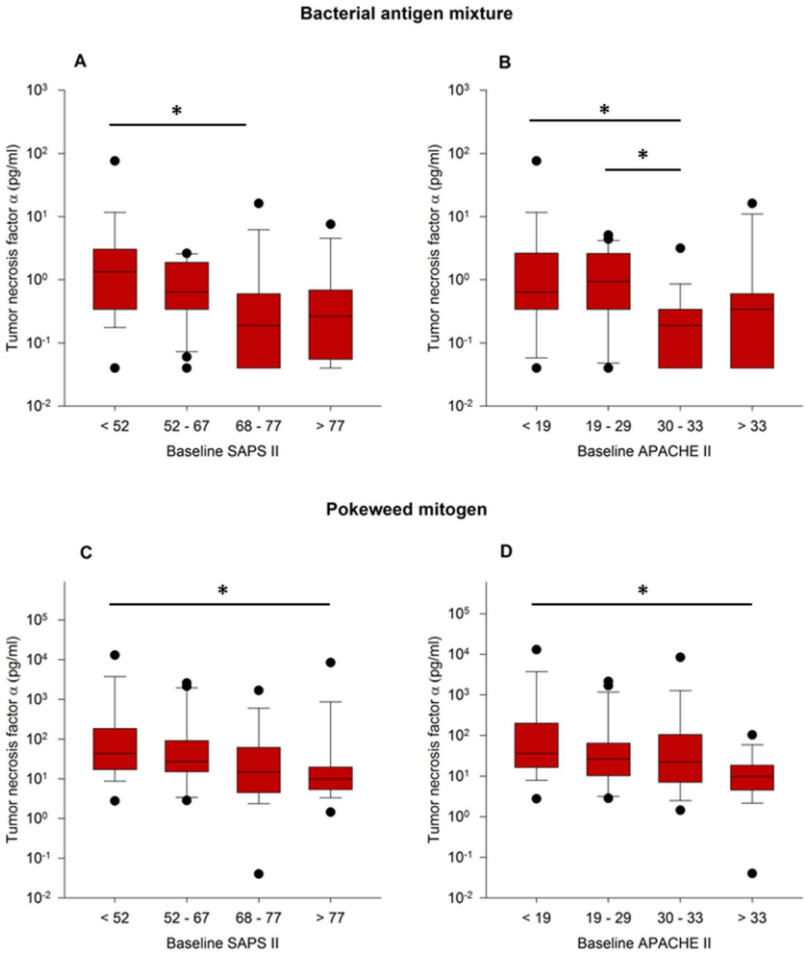


Figure 3. Relationship between disease severity and TNF-α release in Bacteria and PWM assay. TNF-α release from whole blood was correlated with quartile groups for disease severity as measured by SAPS II (**A**,**C**) and APACHE II (**B**,**D**) disease severity classification systems. Panels A and B show hereby stimulations with bacterial antigen mixtures, Panels C and D with Pokeweed mitogen assay. Statistically significant differences (One-way ANOVA on RANKS followed by Dunn's test) are indicated *$p < 0.05$.

These findings expand our understanding of the consequences of severe immune dysfunction, frequently described as "immunoparalysis"[16], by using this battery of *ex vivo* stimulation assays. Furthermore, this clinical trial demonstrates that patients who received stress doses of hydrocortisone (HC) had even more suppressed immune responses. Immune suppression in septic shock patients was further aggravated when the degree of oxygenation resulted in PaO_2 levels lower than 80 mmHg.

Early on in the development of sepsis, it is classically described that an initial excessive inflammatory state occurs, typically known as a cytokine storm[3]. The entire organism appears to be in an ultimate inflammatory state with pro-inflammatory processes adding up, resulting in tissue injury, organ failure and further inflammation. Pro- and anti-inflammation are two concurrently developing processes emerging early in these pathophysiologic processes[9,17,18], which are most commonly referred to as mixed antagonist response syndrome (MARS). In the literature, there is relatively little information on the exact kinetics of septic conditions and inflammatory responses. Tamayo *et al.*[17] described that pro- and anti-inflammatory responses are simultaneously regulated in the first onset of sepsis. Herein, plasma levels of IL-6 and IL-8 were up-regulated but also IL-10. Murine sepsis

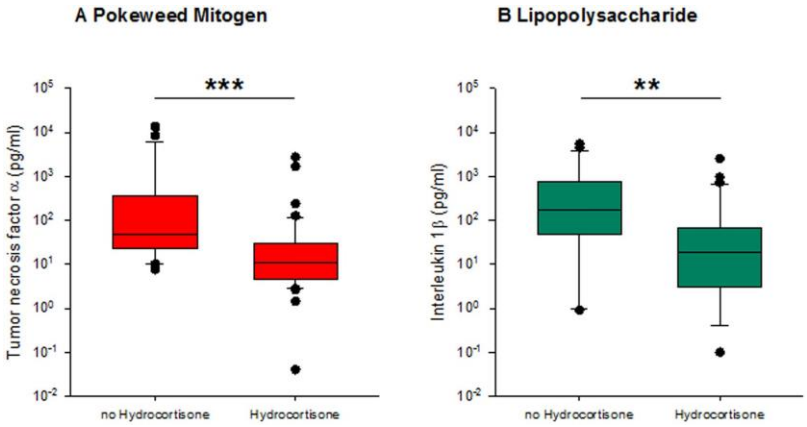


Figure 4. Relationship between hydrocortisone administration and cytokine release. Patients were allocated to two groups (Hydrocortisone (HC) or no HC) for comparison of stimulated cytokine release. (**A**) TNF-α in supernatants of whole blood stimulated with PWM. (**B**) IL-1β in supernatants of LPS stimulated whole blood. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated as follows: $^{**}p < 0.01$, $^{***}p < 0.001$.

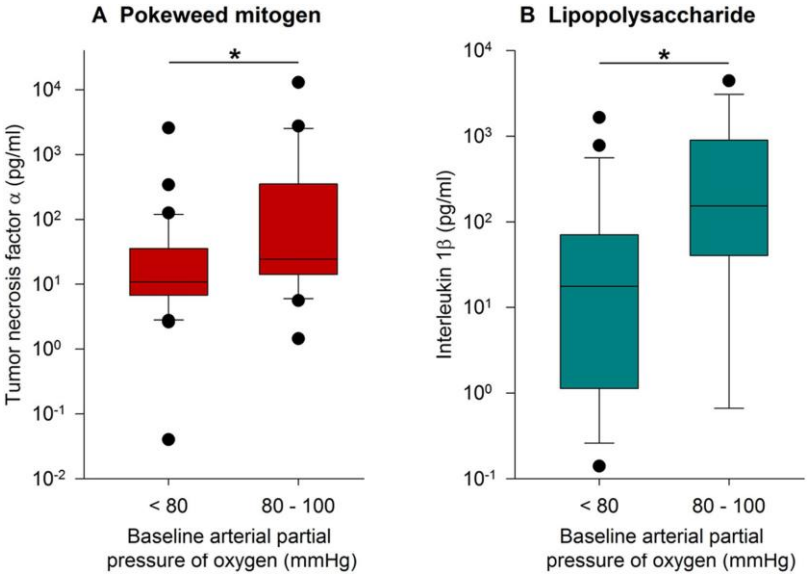


Figure 5. Relationship between PaO_2 and cytokine release. Patients were allocated to groups regarding their arterial partial pressure of oxygen (PaO_2) for comparison of stimulated cytokine release: hypoxemia ($PaO_2 < 80\,mmHg$) and normoxemia (PaO_2 *80–100 mmHg*). (**A**) TNF-α measured after stimulation with PWM. (**B**) IL-1β measured after stimulation with LPS. Statistically significant differences (Mann-Whitney Rank Sum Test) are indicated $^{*}p < 0.05$.

models based on cecal ligation and puncture (CLP) showed that the systemic inflammatory response represented by elevated levels of pro-inflammatory cytokines in the blood plasma began to emerge within 2–8 hours after the insult, depending on the individual cytokine[19]. Anti-inflammatory processes are assumed to begin within the first 24 hours in human sepsis[20].

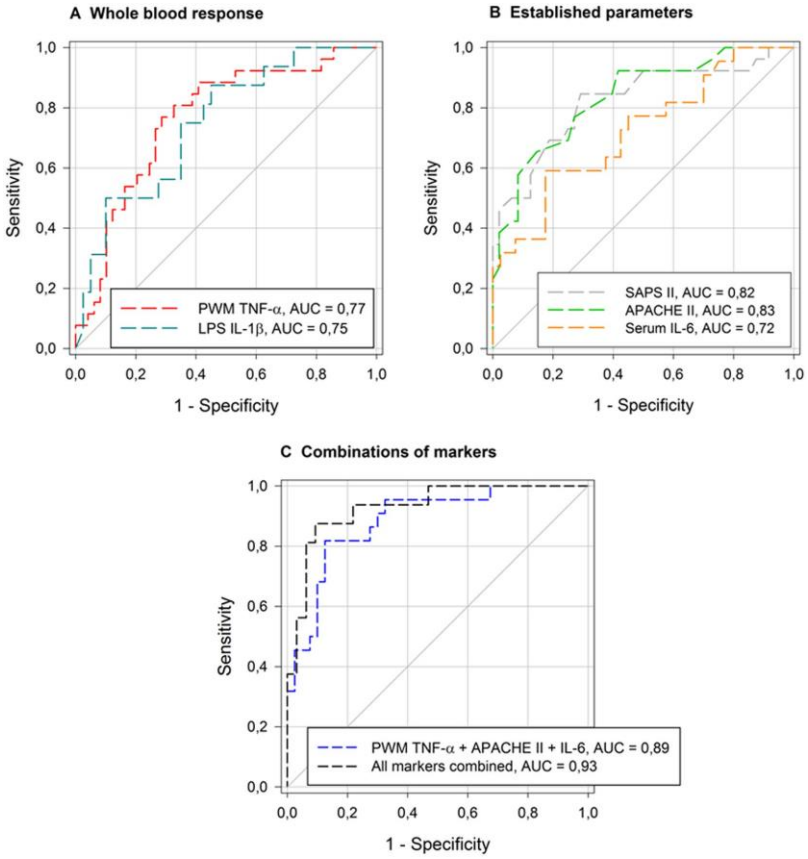


Figure 6. ROC curves for the endpoint"RRT during ICU stay" ROC-curves for (**A**) whole blood response, (**B**) established parameters used in sepsis and (**C**) different combinations of markers referring to (**A** and **B**) regarding the endpoint "initiation of RRT during ICU stay": *ROC:* Receiver operating characteristic curve, all markers combined: combination of PWM stimulated TNF-α release, LPS stimulated IL-1β release, SAPS II, APACHE II and serum IL-6.

Overall immune response in sepsis. The here observed ambivalent behavior of high IL-6 serum levels and corresponding low whole blood assay responses can be attributed to the finding that in subacute phases of infectious conditions, the resulting "cytokine storm" is not a physiologic reaction to pathogens, but rather a massive liberation of so-called damage-associated molecular patterns (DAMPs)[21]. The degree of tissue injury and thus the disease severity, as seen by a marked rise in pro-inflammatory markers such as CRP and IL-6, is negatively correlated with the whole blood response to immunologic stimulants. This results in a heightened susceptibility to secondary infections and thus late-phase mortality. The critical players include all immunologic cells and especially cells with leukocytic origin. Regardless of leukocyte differentiation, the cell count in itself already indicates an upset in the immune response. Unsurprisingly, our patients with moderate leukocytosis had the best immune response, whereas both leukopenia as well as severe leukocytosis were associated with increased immune dysfunction. This suggests that higher disease severity is related to the lowest whole blood assay responses.

Furthermore, when correlating assay response in the latter patient group with SAPS II and APACHE II, this relation between significantly impaired immune function with disease severity scores was demonstrated again. Both scores incorporate physiologic parameters which are typically deranged in cases of organ failure and tissue injury[22,23]. These results also substantiate the aforementioned relationship to inflammatory parameters, that our assays were able to help further quantifying disease severity from an immunologic standpoint.

Pan-immune cell paralysis?! The battery of assays display a lack of adequate immune responsiveness in almost all major leukocyte lineage cells, stimuli and antigens used. Recall antigen responses to bacterial toxoids and fungal antigens as well as to polyclonal B- and T-cell activation were highly suppressed and almost entirely blunted. Selective activation of T-cells via co-receptor CD3 and the co-stimulant CD28 was also strongly suppressed in septic patients. In light of the mechanisms of Ca^{++}-ionophore (Ionomcyin)/PMA action which leads to reduced T cell activation and cytokine synthesis, a reduction in the signaling capacity of the proteinkinase C (PKC) pathway can be anticipated. Moreover, activation of CD14/TLR4 from innate immune cells (monocytes and granulocytes[24]) through the endotoxin stimulus LPS led to a strong and significant reduction in the release of several key cytokines (IL-1β, TNF-α). This activation may be linked to the PKC pathway and its isoforms[25] as a key cellular target potentially explaining this cell-mediated immunologic anergy. The role of PKC isoforms in separated cell subsets could not be further elucidated using complementary assays due to the limited blood volumes available.

The highly suppressed cytokine responses to the strong mitogenic (PWM) and receptor specific stimuli (LPS) were still in a well detectable range which allowed for comparisons of either activation or further suppression in sub-cohorts of patients. PWM and LPS assay results were identified as suitable and included in further analyses for the role of HC, oxygenation (Figs 4 and 5) and other Receiver Operating Characteristic (ROC) findings (see supplemental Table 1 and supplemental Fig. 3).

Effects of hydrocortisone and oxygenation. A highly significant suppression of pro-inflammatory cytokine release was observed in patients receiving stress doses of HC. Considering the clinical indications as well as effects of HC therapy, it is in many ways a double-edged sword. The genomic effects of HC results in immunosuppression which impairs the whole blood assay response and further dampens the immune cells performance in raising a TH1 response. One must take into account that according to clinical guidelines, application of HC should be restricted to patients in refractory septic shock, where hemodynamic stability cannot be restored despite adequate fluid resuscitation and vasopressor use[12,26]. Within our patient population, this applies to patients in the most fulminant phase of septic shock and defines a high severity of the septic state. Therefore, the observed immune function differences between the two sub-groups (low and high HC) could be also due to patient selection as indicated in the administration of hydrocortisone in septic shock.

Hypoxemia can impact the immune response, as shown by our and other studies[27]. Hypoxia-induced anti-inflammatory mechanisms are mediated by the A2 adenosine receptor (A2AR)[28], HIF and other pathways[29,30]. The anti-inflammatory effects are enabled by various mechanisms such as the inhibition of oxidative burst and a reduction in platelet activation minimizing microvascular occlusion; especially through a reduction in pro-inflammatory cytokines being released (mainly TNF-α or IFN-γ) and an increased release of anti-inflammatory cytokines such as IL-10 or IL-4, suggesting a shift of the lymphocyte TH1/TH2-equilibrium towards the TH2-pathway[31,32]. In the here presented study, we observed a greater immune suppressive response in the hypoxemic patient population. The disease scores and elevated levels of serum lactate as a surrogate parameter for hypoxia were used to identify the state of hypoxia. The blood assays show a significant attenuation of the TH1-responses (IL-1β, TNF-α) when PaO_2 was below 80 mmHg as compared to normoxemic conditions. The TH2 responses were almost uniformly low and close to the detectable threshold of the respective cytokine assays (e.g. IL-10, PMA stimulation, $PaO_2 < 80$ mmHg: 11.4 ± 16.0 pg/ml), suggesting that a TH2 shift and the role of HC or oxygen play a more significant role than the dampening of TH1 responses.

Role of the immune response in the prediction of renal replacement therapy (RRT). Acute renal failure (ARF) is a common complication of severe sepsis or septic shock. It is not only an indicator of disease severity, but also an independent risk factor for death, occurring in as often as 41% severe sepsis and septic shock cases in a 2007 prevalence study carried out in German ICUs[33]. ARF and the initiation of renal replacement therapy are predictors of unfavorable disease progression and mortality. Sepsis survivors are at high risk for suffering from chronic kidney disease[34]. While considering the endpoint "initiation of renal replacement therapy", the immune dysfunction of RRT patients became apparent. ROC analyses using TNF-α and IL-1ß from the *in vitro* assays after stimulation with PWM and LPS can sensitively and specifically predict need for RRT, as good as the widely used SAPS II, APACHE II scores. Combining these immune functional parameters with Serum IL-6, SAPS II and APACHE II, one could generate a useful predictive value in the ROC for RRT with high specificity and sensitivity. The predictive value exceeds that of the conventional parameters in use today. These findings show that immune responses to strong immunologic stimuli (PWM, LPS) might be an additional immune functional tool which can help predicting the need for RRT when combined with established markers and scoring systems in sepsis patients. Parameters on its own, even when a strong positive correlation can be established, are not sufficiently powerful predictors and should be aggregated for better utility. This could be used clinically to identify patients at high risk for receiving RRT and to adjust treatment strategies early.

Consequences. *Hygiene, reverse isolation and quarantine.* Our data demonstrate that in the very first moments after septic shock onset, immune competence is severely compromised and adaptive immune system responses seem almost non-existent. Liu *et al.*[35] were recently able to show in 35,000 sepsis patients that with every hour of delay in antibiotic treatment within the recommended 6 hour window, mortality increases. Patients suffering from critical illness and associated immune suppression are also easy targets for secondary infections from viruses and opportunistic pathogens[36,37]. More direct anti-infective therapy does not seem effective when dealing with this issue in sepsis. As soon as one organism is successfully eliminated with antibiotics, antimycotic or virostatic agents, another one not covered by the first round of medications or by the immune system often leads to a superinfection[36], and many of these pathogens tend to be multidrug-resistant[37].

In order to minimize the risk of acquiring an opportunistic infection, patients benefit from strict compliance with infection prevention and control[38]. More efforts should be invested in creating better awareness for this issue amongst medical personal. Moreover, temporary reverse isolation and quarantine, typical in other immune suppressed patients i.e. organ transplantation post-op, could have beneficial effects. Although a recent review showed that noncompliance resulted in a paradoxical increase in adverse events[38].

Restriction of hydrocortisone administration. Administration of hydrocortisone in sepsis should be considered on a case by case basis, since HC can further weaken the immune response. The effects of both high-dose as well as low-dose corticosteroids were examined previously in septic patients, none of which showed conclusive improvement in survival[39,40]. There is evidence that an earlier reversal of shock may be accomplished by corticosteroid application[40,41]. As such, corticosteroid administration in current guidelines is restricted to cases of refractory septic shock that does not respond adequately to fluid resuscitation and vasopressor therapy[12,26]. Our data on the additional immunosuppressive effects is in line with current recommendations that the use of corticosteroids in sepsis should be limited to refractory cases.

Immune function monitoring and modulation. Severe immunosuppression at the onset of sepsis as seen in this trial and increasingly being discussed in recent literature suggest the potential role of immune modulatory therapies such as GM-CSF or IFN-γ[42]. One of the latest reviews on this topic concluded that in the absence of deleterious side effects from GM-CSF administration in sepsis patients, multiple clinical benefits such as rapid recovery from infection, reduced length of hospital stay and decreased need for mechanical ventilation were seen[43]. The immune cell expansion and other potential immune stimulatory therapies in sepsis are compelling concepts. With increasing evidence that patient deaths are a consequence of the immunosuppressive state, immune enhancing drugs could become the next milestone in sepsis therapy[16].

Conclusion

The stimuli used in these whole blood immune assays are based on the principles of re-call antigen responses, specific T and B cell answers or innate cell activation. Taken together it reflects the body's ability to mount an immune response against a broad spectrum of pathogens, showing anergy and immune-paralysis early on in sepsis. This suppressed state of the immune system requires immediate additional protection against opportunistic infections including an early anti-infective treatment, proper hygiene measures and reverse isolation or quarantine. The roles of HC therapy and PaO_2 values should also be considered. The broadly used early hydrocortisone application in septic shock should be carefully evaluated on a case by case basis. This study reveals that further research needs to be done to establish a profound marker and immune functional assay – directed therapy.

Limitations and Considerations

The focus of this investigation was the clinical evaluation of the immune competence upon further distinct immune stimulation in whole blood. Due to recruitment time point and the overall clinical study setup (including strict blood volume limitations) it was not possible to test for specific cell subsets, cell numbers or cell viability under all conditions. To overcome some concerns, *in vitro* experiments were carried out to test for the effects of the assay incubation on the apoptosis and necrosis of the cytokine producing cells. Here CD3, CD4, CD8 and NK cells as well as granulocytes revealed only a moderately increased immune cell apoptosis and necrosis after 48 h assay incubations (e.g. range of necrosis was ~1% in non-stimulated T-cells and up to 10% in PWM stimulated assays).

Moreover, data as presented on this clinical study were analyzed and presented as they were collected, and no extreme outlier exclusion or other statistical tools were applied in order to evaluate the strength and weaknesses of this immune assay approach test to function despite the huge inter-individual pattern. The authors are aware that patients with severe sepsis are a very heterogeneous group and preexisting co-morbidity impacts the longterm outcome as Mansur *et al.* described[44]. As our study focused primarily on the onset of sepsis, co-morbidities were not taken into calculations.

Furthermore, the here presented control group is small but data of the controls are in the margins of what other control experiments in healthy male and female have shown. This presented control group was run in the study period, with the same operators, assay procedures (incl. reagent's lot etc.) and subsequent analyses.

Samples from healthy volunteers were taken venously and from an arterial line in the septic patients which may have led to some bias. Any kind of inflammatory or systemic diseases were excluded in the healthy subjects and no relevant difference between arterial and venous blood shall be hence anticipated. Moreover, in the light of this knowledge and due to the very strict handling of ethical boards to minimize risks to volunteers, an arterial draw was not possible in the healthy subjects as it seemed hardly justifiable due to an increased risk of potential severe side effects such as malperfusion or necrosis. The blood draw from an arterial line was performed in septic patients to avoid any contamination or bias (dilution) as if blood would have been taken from central i.v. line, which is used otherwise for infusion therapy or drug administration or parenteral nutrition. Interestingly, several publications also addressed the question e.g. if proteins or functions of blood components change in arterial or venous blood. Kelly *et al.*[45] were able to show that in patients with COPD, biomarkers were comparable in arterial and venous blood samples. Some other reports[46,47] describe in severely sick patients some differences indicating some advantage to the draw of blood from the arterial line in inflammatory lung disease, but however still, these reports overall judge a similar value of venous to arterial blood. Fernández-Serrano *et al.*[46] report some preference to arterial blood when lung sick people are investigated. In the current study on sepsis the lung was often affected and one might anticipate-since we have drawn arterial blood-that the "better sample" was collected in the study population. Overall a bias in the immune parameters analyzed just because of the different sites of blood draw in healthy volunteers or septic patients, respectively, can be excluded to a large extent.

Methods

Study design. The present *immune function study* was part of a prospective, randomized, multicenter clinical trial named "Placebo Controlled Trial of Sodium Selenite and Procalcitonin Guided Antimicrobial Therapy in Severe Sepsis" (SISPCT, NCT00832039)[11,48].

Informed Consent. Ethical study approval for additional experiments performed at our center was obtained as a local amendment to the approved SISPCT study [Eudra-CT-Nr. 2007-004333-42] from the ethical board of the University of Jena.

After positive patient screening, written informed consent signed by the patient or by the legal representative had to be present for enrolment. In case of withdrawal, the patient was immediately excluded from the study and no further follow-up was performed.

All reported experiments and methods were approved by the ethical board of the University of Jena and were in accordance with the relevant guidelines and regulations. The respective experiment protocols were also approved by the ethical committee of the University of Jena.

Data recording. In addition to the acquisition of data on demographics, biometrics, past medical history and clinical and laboratory findings, the disease severity classification scores Simplified Acute Physiology Score II (SAPS II)[22] and Acute Physiology And Chronic Health Evaluation II (APACHE II)[23] were calculated. Furthermore, the Glasgow Coma Scale (GCS) was assessed. The control group was small but based on our previous experiences with other healthy volunteers it was in the margins of what other control experiments in healthy male and female have shown and it was hence considered a valid size, also since it was matched to the expected age and gender composition of the studied patients at the time of the study. In the volunteers, we have been also using the same lot of reagents/antigens and the exactly same methods and operators to try minimizing the risk of a methodologic bias. All recorded data and analyses were anonymized and stored in a database (SPSS® Statistics 21, IBM Corp., New York City, NY, USA).

Study specific blood Sample Collection. Immediately after patient enrolment, 9 ml blood was collected from an *in situ* arterial line in lithium-heparinized tubes (Sarstedt, Nümbrecht, Germany). In the control group, blood was collected by venipuncture of a cubital vein.

Blood Processing. *Complete Blood Count* (*CBC*): Erythrocyte, leukocyte and platelet count as well as hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were assessed upon admission as standard at the intensive care unit (ICU) (Institute of Laboratory Medicine, University of Munich, Germany).

Plasma Inflammation Markers. C-reactive protein (CRP), interleukin-6 and procalcitonin (PCT) were routinely assessed upon ICU admission and measured according to standard procedures (Institute of Laboratory Medicine, University of Munich, Germany).

In vitro Recall Antigen and Mitogen Stimulation Assays. Immediately following sample collection, 400 µl of whole blood were transferred into assay tubes prefilled with DMEM (Dulbecco´s Modified Eagle's Medium Nutrient Mixture F-12 HAM, Sigma-Aldrich, Steinheim, Germany) and the different stimulants, as previously described[15]. The *in vitro* recall antigen and mitogen stimulation assay tubes contained DMEM only or DMEM and either a bacterial recall antigen mixture containing Diphteria-, Tetanus- and Pertussis-toxoid or a fungal antigen mixture containing Candida-Lysate and Trichophyton-Lysate. Additionally, the following mitogens were used: 1) Pokeweed mitogen (PWM), a strong immune activator, induces mitosis in T and B lymphocytes in a non-receptor specific fashion[49,50]; 2) Phorbol-12-myristate 13-acetate (PMA/Ionomycin), an unspecific activator of Protein kinase C (PKC)[51] affecting multiple cell types[52] but is also reported as a pan-specific activator of B-cells[53] and mitogen for T lymphocytes[54]; 3) CD3/28 mixture, which activate T cells via the T cell receptor (CD3) and the cell receptor (CD28), CD28 provides, via binding to antigen presenting cell, costimulation for T cell activation[55]; 4) lipopolysaccharide (LPS), targets the innate pathways via CD14 cell surface receptors and Toll like receptor (TLR) 4 signaling cascades[56].

Incubation time was 48 h at 37 °C. The supernatant was subsequently transferred into Eppendorf tubes and immediately frozen at −80 °C for future cytokine analyses. Frozen supernatants were measured after thawing in a blinded fashion by Luminex xMAP® technology (Bioplex®) with commercially available reagents from BioRad-Laboratories Inc. (California, USA) according to the manufacturer´s guidelines. The concentrations of the pro- and anti-inflammatory cytokines were analyzed (pg/ml).

Statistical analyses. In order not to distort raw data, but to fully illustrate the variability of extreme responses and to investigate the effects under real clinical conditions, no outlier analysis was performed and all data in this study cohort was kept for analysis. After testing for normal distribution, data were analyzed either by Student's T-test, Mann-Whitney Rank Sum Test or One-way analysis of variance (ANOVA) on ranks followed by Dunn´s post-hoc test. Correlation analyses were performed using Pearson's correlation coefficient or Spearman rank-order correlation coefficient, dependent on presence of a normal distribution. All p-values were calculated in a two-sided manner and statistical significance was set at a p-value of 0.05.

For better comparability of non-normally distributed data, variable values were divided into specific groups (indicated in the individual charts) or four quartile groups, with the 25th, 50th and 75th percentile representing the cut-off values for group allocation.

The predictive value of the *in vitro* cytokine release of TNF-α, IFN-γ and IL-2 after PWM challenge in patients receiving extracorporeal *r*enal *r*eplacement *t*herapy (RRT, dialysis) while in the ICU was further assessed alone or in combination with other markers of disease severity under a receiver operating characteristic (ROC) curve. ROC curves were applied to obtain cut-off values for sensitivity and specificity for the respective cytokines as well as the need for dialysis.

Results are expressed as means ± SD in tables and as boxplots in graphs. Boxes show the median and interquartile range (IQR), whiskers represent the 10th and 90th percentile. Data are plotted and were statistically analyzed using IBM SPSS® Statistics 21 as well as SigmaPlot 11.0 (Systat Software Inc., San Jose, California, USA).

References

1. Martin, G. S. Sepsis, severe sepsis and septic shock: changes in incidence, pathogens and outcomes. *Expert Rev. Anti. Infect. Ther.* **10**, 701–706, https://doi.org/10.1586/eri.12.50 (2012).
2. Martin, G. S., Mannino, D. M., Eaton, S. & Moss, M. The epidemiology of sepsis in the United States from 1979 through 2000. *N. Engl. J. Med.* **348**, 1546–1554, https://doi.org/10.1056/NEJMoa022139 (2003).
3. Hotchkiss, R. S. & Karl, I. E. The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* **348**, 138–150, https://doi.org/10.1056/NEJMra021333 (2003).
4. Angus, D. C. *et al.* Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* **29**, 1303–1310 (2001).
5. Rittirsch, D., Flierl, M. A. & Ward, P. A. Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* **8**, 776–787, https://doi.org/10.1038/nri2402 (2008).
6. Wiersinga, W. J. Current insights in sepsis: from pathogenesis to new treatment targets. *Curr. Opin. Crit. Care* **17**, 480–486, https://doi.org/10.1097/MCC.0b013e32834a4aeb (2011).
7. Ertel, W. *et al.* Inhibition of the defense system stimulating interleukin-12 interferon-gamma pathway during critical Illness. *Blood* **89**, 1612–1620 (1997).
8. Hotchkiss, R. S. *et al.* Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction. *Crit. Care Med.* **27**, 1230–1251 (1999).
9. Osuchowski, M. F., Craciun, F., Weixelbaumer, K. M., Duffy, E. R. & Remick, D. G. Sepsis chronically in MARS: systemic cytokine responses are always mixed regardless of the outcome, magnitude, or phase of sepsis. *J. Immunol.* **189**, 4648–4656, https://doi.org/10.4049/jimmunol.1201806 (2012).
10. Huttunen, R. & Aittoniemi, J. New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. *J. Infect.* **63**, 407–419, https://doi.org/10.1016/j.jinf.2011.08.004 (2011).
11. Bloos, F. *et al.* Effect of Sodium Selenite Administration and Procalcitonin-Guided Therapy on Mortality in Patients With Severe Sepsis or Septic Shock: A Randomized Clinical Trial. *JAMA Intern. Med.* **176**, 1266–1276, https://doi.org/10.1001/jamainternmed.2016.2514 (2016).
12. Reinhart, K. *et al.* Prevention, diagnosis, therapy and follow-up care of sepsis: 1st revision of S-2k guidelines of the German Sepsis Society (Deutsche Sepsis-Gesellschaft e.V. (DSG)) and the German Interdisciplinary Association of Intensive Care andEmergency Medicine (Deutsche Interdisziplinare Vereinigung fur Intensiv- und Notfallmedizin (DIVI)). *Ger. Med. Sci.: GMS e-journal* 8, Doc14, https://doi.org/10.3205/000103 (2010).
13. Jacob, S. & Cory, F. In *Common Surgical Diseases* (eds J. A. Myers, K. W. Millikan, & T. J. Saclarides) Ch. 97, 391–394 (Springer New York, 2008).
14. respiratoryupdate. *Levels of Hypoxemia*, http://www.respiratoryupdate.com/members/Levels_of_Hypoxemia.cfm (2015).
15. Feuerecker, M. *et al.* A corticoid-sensitive cytokine release assay for monitoring stress-mediated immune modulation. *Clin. Exp. Immunol.* **172**, 290–299, https://doi.org/10.1111/cei.12049 (2013).
16. Leentjens, J., Kox, M., van der Hoeven, J. G., Netea, M. G. & Pickkers, P. Immunotherapy for the adjunctive treatment of sepsis: from immunosuppression to immunostimulation. *Time for a paradigm change? Am. J. Respir. Crit. Care Med.* **187**, 1287–1293, https://doi.org/10.1164/rccm.201301-0036CP (2013).
17. Tamayo, E. *et al.* Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. *Eur. Cytokine Netw.* **22**, 82–87, https://doi.org/10.1684/ecn.2011.0281 (2011).
18. Novotny, A. R. *et al.* Mixed antagonist response and sepsis severity-dependent dysbalance of pro- and anti-inflammatory responses at the onset of postoperative sepsis. *Immunobiology* **217**, 616–621, https://doi.org/10.1016/j.imbio.2011.10.019 (2012).
19. Remick, D. G., Newcomb, D. E., Bolgos, G. L. & Call, D. R. Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* **13**, 110–116 (2000).
20. Frazier, W. J. & Hall, M. W. Immunoparalysis and adverse outcomes from critical illness. *Pediatr. Clin. North Am.* **55**, 647–668, xi, https://doi.org/10.1016/j.pcl.2008.02.009 (2008).
21. Zhang, Q. *et al.* Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* **464**, 104–107, https://doi.org/10.1038/nature08780 (2010).
22. Le Gall, J. R., Lemeshow, S. & Saulnier, F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. *JAMA* **270**, 2957–2963 (1993).
23. Knaus, W. A., Draper, E. A., Wagner, D. P. & Zimmerman, J. E. APACHE II: a severity of disease classification system. *Crit. Care Med.* **13**, 818–829 (1985).
24. Haziot, A., Tsuberi, B. Z. & Goyert, S. M. Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide. *J. Immunol.* **150**, 5556–5565 (1993).
25. McGettrick, A. F. *et al.* Trif-related adapter molecule is phosphorylated by PKC{epsilon} during Toll-like receptor 4 signaling. *Proc. Natl. Acad. Sci. USA* **103**, 9196–9201, https://doi.org/10.1073/pnas.0600462103 (2006).
26. Dellinger, R. P. *et al.* Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit Care Med.* **41**, 580–637, https://doi.org/10.1097/CCM.0b013e31827e83af (2013).
27. Chouker, A. *et al.* Critical role of hypoxia and A2A adenosine receptors in liver tissue-protecting physiological anti-inflammatory pathway. *Mol. Med.* **14**, 116–123, https://doi.org/10.2119/2007-00075.Chouker (2008).
28. Kaufmann, I. *et al.* Effects of adenosine on functions of polymorphonuclear leukocytes from patients with septic shock. *Shock* **27**, 25–31, https://doi.org/10.1097/01.shk.0000238066.00074.9000024382-200701000-00005 (2007).
29. Thiel, M. *et al.* Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury. *PLoS Biol.* **3**, e174, 04-PLBI-RA-0506R3, https://doi.org/10.1371/journal.pbio.0030174 (2005).
30. Eltzschig, H. K. & Carmeliet, P. Hypoxia and inflammation. *N. Engl. J. Med.* **364**, 656–665, https://doi.org/10.1056/NEJMra0910283 (2011).
31. Nowak, M. *et al.* The A2aR adenosine receptor controls cytokine production in iNKT cells. *Eur. J. Immunol.* **40**, 682–687, https://doi.org/10.1002/eji.200939897 (2010).
32. Linden, J. Adenosine in tissue protection and tissue regeneration. *Mol. Pharmacol.* **67**, 1385–1387, 105.011783/10.1124/mol.105.011783 (2005).

33. Oppert, M. *et al.* Acute renal failure in patients with severe sepsis and septic shock–a significant independent risk factor for mortality: results from the German Prevalence Study. *Nephrol. Dial. Transplant.* **23**, 904–909, https://doi.org/10.1093/ndt/gfm610 (2008).
34. Anderberg, S. B., Luther, T. & Frithiof, R. Physiological aspects of Toll-like receptor 4 activation in sepsis-induced acute kidney injury. *Acta physiol.* **219**, 573–588, https://doi.org/10.1111/apha.12798 (2017).
35. Liu, V. X. *et al.* The Timing of Early Antibiotics and Hospital Mortality in Sepsis. *Am. J. Respir. Crit. Care Med.*, https://doi.org/10.1164/rccm.201609-1848OC (2017).
36. Hotchkiss, R. S., Coopersmith, C. M., McDunn, J. E. & Ferguson, T. A. The sepsis seesaw: tilting toward immunosuppression. *Nat. med.* **15**, 496–497, https://doi.org/10.1038/nm0509-496 (2009).
37. Opal, S. M. New perspectives on immunomodulatory therapy for bacteraemia and sepsis. *Int. J. Antimicrob. Agents* **36**(Suppl 2), S70–73, https://doi.org/10.1016/j.ijantimicag.2010.11.008 (2010).
38. Huang, G. K., Stewardson, A. J. & Grayson, M. L. Back to basics: hand hygiene and isolation. *Curr. Opin. Infect. Dis.* **27**, 379–389, https://doi.org/10.1097/QCO.0000000000000080 (2014).
39. Bone, R. C. *et al.* A controlled clinical trial of high-dose methylprednisolone in the treatment of severe sepsis and septic shock. *N. Engl. J. Med.* **317**, 653–658, https://doi.org/10.1056/NEJM198709103171101 (1987).
40. Annane, D. *et al.* Corticosteroids in the treatment of severe sepsis and septic shock in adults: a systematic review. *JAMA Intern. Med.* **301**, 2362–2375, https://doi.org/10.1001/jama.2009.815 (2009).
41. Annane, D. *et al.* Corticosteroids for treating sepsis. *Cochrane Database Syst. Rev.*, CD002243, https://doi.org/10.1002/14651858.CD002243.pub3 (2015).
42. Heming, N., Lamothe, L., Ambrosi, X. & Annane, D. Emerging drugs for the treatment of sepsis. *Expert Opin. Emerg. Drugs* **21**, 27–37, https://doi.org/10.1517/14728214.2016.1132700 (2016).
43. Mathias, B., Szpila, B. E., Moore, F. A., Efron, P. A. & Moldawer, L. L. A Review of GM-CSF Therapy in Sepsis. *Medicine* **94**, e2044, https://doi.org/10.1097/MD.0000000000002044 (2015).
44. Mansur, A. *et al.* Chronic kidney disease is associated with a higher 90-day mortality than other chronic medical conditions in patients with sepsis. *Sci. rep.* **5**, 10539, https://doi.org/10.1038/srep10539 (2015).
45. Kelly, E. *et al.* Comparison of arterial and venous blood biomarker levels in chronic obstructive pulmonary disease. *F1000Res* **2**, 114, https://doi.org/10.12688/f1000research.2-114.v1 (2013).
46. Fernandez-Serrano, S. *et al.* Molecular inflammatory responses measured in blood of patients with severe community-acquired pneumonia. *Clin Diagn Lab Immunol* **10**, 813–820 (2003).
47. Shah, B. *et al.* Comparison of platelet activity measurements by use of arterial and venous blood sampling. *Journal of thrombosis and haemostasis: JTH* **11**, 1922–1924, https://doi.org/10.1111/jth.12370 (2013).
48. Sepsis, K. http://clinicaltrials.gov/show/NCT00832039.
49. Suzuki, N. & Sakane, T. Mechanism of T cell-derived helper factor production upon stimulation with pokeweed mitogen in humans. *Clin. Exp. Immunol.* **71**, 343–349 (1988).
50. Waldmann, T. A. & Broder, S. Polyclonal B-cell activators in the study of the regulation of immunoglobulin synthesis in the human system. *Adv. Immunol.* **32**, 1–63 (1982).
51. Iliodromitis, E. K. *et al.* The PKC activator PMA preconditions rabbit heart in the presence of adenosine receptor blockade: is 5′-nucleotidase important? *J. Mol. Cell. Cardiol.* **30**, 2201–2211, https://doi.org/10.1006/jmcc.1998.0780 (1998).
52. Nishihira, J. & O'Flaherty, J. T. Phorbol myristate acetate receptors in human polymorphonuclear neutrophils. *J. Immunol.* **135**, 3439–3447 (1985).
53. Masilamani, M., Kassahn, D., Mikkat, S., Glocker, M. O. & Illges, H. B cell activation leads to shedding of complement receptor type II (CR2/CD21). *Eur. J. Immunol.* **33**, 2391–2397, https://doi.org/10.1002/eji.200323843 (2003).
54. Touraine, J. L. *et al.* Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. *J. Exp. Med.* **145**, 460–465 (1977).
55. Trickett, A. & Kwan, Y. L. T cell stimulation and expansion using anti-CD3/CD28 beads. *J. Immunol. Methods* **275**, 251–255 (2003).
56. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088 (1998).

Acknowledgements

This work is part of L. Sudhoff's doctoral thesis. This investigation was supported by the German Federal Ministry of Economics and Energy (BMWi/DLR grant 50WB0719/WB0919 and WB1622). The authors express their special thanks to M. Hörl, S. Matzel, J. Göschl, I. Kumpreij and C. Ladinig for the on-site support and analyses, respectively, in the anesthesiologic laboratory of the University of Munich. Furthermore, the authors are thankful for the support of the anesthesiologic intensive care units under the guidance of Dr. Lorenz Frey.

Author Contributions

M.F., L.S., G.S., I.K. and A.C. designed the work. M.F., L.S., I.K., A.C. collected the data. Data analysis and interpretation as well as drafting the article and critical revision for important intellectual content were performed by all authors (M. F., L.S., B.C., J.-I.P., Cl.S., C.S., A.G., G.S., J.B., I.K., A.C.). All authors gave final approval of the version to be submitted and any revised version.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-19976-w.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.