Author's response to reviews

Title: Cytokine responses to Staphylococcus aureus bloodstream infection differ between patient cohorts that have different clinical courses of infection.

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We would like to thank the editor for the opportunity to submit a revised manuscript and the reviewers for their careful consideration of this work. We have used this opportunity to reconsider the data in light of all comments, and we believe that the changes made will improve the impact of this work. Below is a point by point response to each reviewer’s comments and the changes we have made to the manuscript. The revised manuscript has been uploaded and the changes to the text referred to below are also shown in a supplemental file which is also uploaded (McNicholasBMCInectDisREBUTTAL.doc). The changes have been approved by all authors.

We look forward to your reply.

Yours sincerely

Deirdre Fitzgerald Hughes, PhD

Reviewer 1 comments

Major Comment 1

The definition of complicated/uncomplicated is not valid and it selects for endocarditis in this setting. The conclusions can therefore not be extended to severe S. aureus septicaemia.

Authors response to comment 1

Definitions of complicated/uncomplicated in the literature are varied. Our definitions of complicated and uncomplicated clinical courses were defined specifically for this study but are similar to those previously defined in the literature, for example by Fowler et al [1] where cases that fulfill all of the following criteria: catheter-associated infection and removal of the catheter, negative result of follow-up blood culture, defervescence within 72 h, normal findings on transesophageal echocardiogram, no prosthetic material in the joints or intravascular space, and no symptoms suggestive of metastatic infection, are all defined as uncomplicated infection. In another study, Fowler defined complicated infection as the presence of either (1) attributable mortality, (2)
complicated infection present at the time of the initial hospitalization, (3) embolic stroke, or (4) recurrent infection within the 12-week follow-up period. We will clarify our definitions in the text. We believe that the definition is valid. While it is true that endocarditis is over-represented in our sample, we did not want to restrict our study to endocarditis alone.

Changes to text for comment 1

- Lines 85–89

An uncomplicated course of infection was defined specifically for this study as follows; negative follow-up blood culture, subsidence of fever within 72 h, normal findings on transesophageal echocardiogram and no symptoms suggestive of metastatic infection. A complicated course of infection was defined specifically for this study as; positive follow up blood culture, despite at least three days of appropriate antibiotics (e.g. flucloxacillin for methicillin-susceptible S. aureus (MSSA), vancomycin for methicillin-resistant S. aureus (MRSA)), disseminated infection such as osteomyelitis, or IE. Nosocomial, healthcare-associated and community-acquired infection were defined as described by Friedman et al. [2]

Reviewers comment 2

Besides in this setting, renal patients constitute a high proportion, The cytokine response of this patient group should be regarded as related to the renal disease at first, although they might have reactivating S. aureus

Authors response to comment 2

Both reviewers have concerns with the conclusions drawn based on the stratification of renal and non-renal patients. As a major revision is requested, we have removed this stratification and we have changed the focus of the study to concentrate on the cytokine response, as it relates to the clinical course of infection. See also response to reviewer 2, comments 2 and 3.

Changes to text, comment 2

- Line 65–69. The aims of the study are now stated as; identify cytokines or chemokines that are important in the immune response to SABSI, determine the levels of these molecules in sequential plasma samples taken over the course of SABSI and investigate these cytokine responses in SABSI with a complicated clinical course versus an uncomplicated course and those with MRSA infection compared to those with MSSA infection.

- Previous Figure 3 has been removed

- All data relating to comparison of renal with non-renal removed

Reviewers comment 3

Considering the small patient subgroups, the conclusion should be drawn carefully, this reduces the impact of the study.
Authors response to comment 3

The authors agree that this is a relatively small study and have therefore been careful not to over-interpret the data. Indeed, the study sample is relatively small, making the subgroups smaller still. However, the data are representative of patients with SABSI in two hospitals. Notwithstanding the study size, we believe that this study is important. The literature in this area largely concentrates on the bacterial contribution to varied courses of infection and most acknowledge that this contribution is unclear (except in cases such as CA-MRSA). Most would speculate that the bacterial contribution is but one factor in the pathogenesis of SABSI yet investigation of the immune response is sparse and often speculative [3, 4]. This study describes an in-vivo prospective study that not only investigates elements of the immune response in sequential samples in SABSI, but also shows that some of these responses are altered in those with a complicated course of infection.

Changes to text, comment 3

- While we have acknowledged the study limitation, we clarify that the size is one such limitation in Line 245 ‘providing a relatively small study size;’
- Lines 259-260 ‘While acknowledging that further studies on larger patient groups are required, these cytokines may be considered as potential prognostic markers....’

Reviewers Comment 4

Impact of resistance to different antibiotics is not evaluated with regard to treatment success. This may impair the response to treatment and it has therefore consequences for the stratification in ‘complicated/uncomplicated’

Authors response to comment 4.

The manuscript deals with the patient cytokine response as it contributes to clinical course of infection. We acknowledge that other factors such as antibiotic resistance are also important. However, in this study, we did not investigate the clinical isolate phenotypic features, such as antibiotic resistance. We have analysed the cytokine responses to MRSA infection compared to MSSA in patients with SABSI. Of the four cytokines studied, only GRO-γ was significantly increased in response to MRSA SABSI. However, only 4 patients with complicated infection had an MRSA and the numbers are therefore too low to make correlations between methicillin resistance and a complicated course in terms of the cytokine response.

However, we will include data on MRSA infection as it correlates with clinical course.

Changes to text, comment 4

- Lines 133-135 ‘Of the 15 patients with MRSA infection, four had a complicated clinical course and an increased risk of complications was not identified in those with MRSA Vs MSSA (4/15, 26 % Vs, 11/50 (22%), RR1.6);’
- Lines 170-175 Cytokine responses SABSI caused by MSSA Vs MRSA
The mean levels of GRO-γ were significantly greater in patient with SABSI in cases where an MRSA was the source of infection (p≤ 0.05) but this increase was only evident when comparing the samples taken on the day of diagnosis. The mean level of the other three cytokines investigated, were not significantly altered in patients with SABSI caused by MRSA Vs MSSA (data not shown).

- Figure 4 shows GRO-gamma levels in patients with MSSA or MRSA over the course of infection

- Lines 233-243 ‘We did not find evidence that GRO-γ levels were altered in patients with a complicated course of infection. However, our data suggest that this cytokine may play a role in the response to MRSA infection specifically. The cause and clinical implications of this finding are unclear. Bacterial components, such as peptidoglycan and LTA that stimulate GROγ production via IL-1 and TNF-α [5] are common to MRSA and MSSA. Furthermore, the chemoattractant properties of GROγ are equally important in MRSA and MSSA bloodstream infection. It has been shown that mecA augments the expression of virulence genes such as fnb [6] and increased expression of β-toxin and phenol soluble modulin has been shown in community-acquired MRSA [7]. It is therefore possible that differential host immune responses such as increased GROγ production may result indirectly from increased expression of virulence genes in MRSA.’

Reviewer 1, minor comment 1

In Figures 1-3 the amount of cytokines is related to mg protein. The reason for this should be explained

Authors response, minor comment 1

The cytokine concentrations were normalized to account for differences in plasma protein concentrations that may differ between SABSI patients especially given that many were undergoing haemodialysis.

Changes to text, minor comment 1

- Lines 117-120 ‘cytokine levels were normalised to plasma protein concentration to account for variability in blood processing and biological variations in plasma protein concentrations between patients.’

Reviewer 1, minor comment 2

Linguistic revision of the manuscript would be valuable

Author’s response

We have re-examined the manuscript and remain happy with the linguistic standard.

Reviewer 2 comments
Reviewers comment 1

The authors state in introduction and discussion that renal patient rapidly recover from S. aureus bacteraemia and rarely develop complications, whilst this may be true, this should be supported by data, especially since other studies have found S. aureus bacteraemia in renal patients is as severe as in non-renal.

Author’s response

Our suggestion that renal patients generally have a better clinical course is based on anecdotal evidence and over 20 years of clinical experience in this hospital. However, we accept that this is not evidence-based and may be specific to our hospital which includes the National Centre for Renal Transplantation. For this patient cohort we do not know their previous S. aureus status and subclinical infection may also be present. These factors would be important to support our interpretation of the data. As the other reviewer also had concerns in this area, we have removed this stratification and the interpretation of responses in renal vs non-renal patients.

Changes to text, comment 1

- See reviewer 1 comment 2

Reviewer’s comment 2

Patients with renal disease were included in the study because they may suffer repeated S. aureus bacteraemia. It is true that this cohort is particularly at risk but there is no evidence provided that the patient included in this study have suffered from previous episodes of bacteraemia.

Author’s response, comment 2

This is dealt with in the response to the previous comment of this reviewer

Reviewer’s comment 3

It is not clear which cytokines were screened for using the cytokine array and why these were chosen, or why a 1.4 fold change was chosen as the cut off, this needs to be explained.

Author’s response comment 3

We agree that as written, it is not clear how and why the cytokines were chosen. A commercially available pro-inflammatory panel of 42 cytokines and chemokines antibodies was used and pooled plasma from patients was hybridised to the cytokine antibody membranes which were then processed to measure and compare signal intensities between patient groups. This approach was used to guide the choice of which cytokines to investigate in all patients, i.e. which cytokines are present at different levels in those with complicated vs uncomplicated courses of infection. Because the cytokine response in SABSI is relatively uncharacterised in-vivo, particularly in complicated Vs uncomplicated infection, this approach was sensible. Regarding the interpretation of what constitutes a significant change (i.e. our use of 1.4 fold change), similar approaches have been used in gene expression studies when comparing groups and the use of pooled samples and ratio thresholds of at least 1.4 between groups have been used in other studies (eg [8] [9] [10]).
The methods and results sections have been changed to better explain the cytokine antibody array screening approach to identifying cytokines for further study.

**Preliminary identification of cytokines in plasma from patients with SABSI using a panel of pro-inflammatory cytokines**

A commercially available antibody based cytokine/chemokine microarray (RayBio® Human Cytokine Antibody Array 3 (RayBiotech Inc., GA, USA)) was used to identify cytokines that were differentially expressed in pooled plasma (taken on the day of diagnosis) from three patients with uncomplicated BSI and three with complicated BSI. Pooled plasma was used for the preliminary screen only, to minimise resources required. The panel consists of antibodies against 42 pro-inflammatory cytokines and chemokines and the manufacturer’s instructions were followed.

Cytokine signal intensities were captured and quantified by the chemiluminescence imaging system G:BOX Chemi XL (Syngene UK, Cambridge, UK). A change in cytokine levels in pooled plasma, at a ratio threshold of at least 1.4 fold between groups was considered to be potentially relevant and the results from this preliminary screen influenced the decision on which cytokines to investigate in individual patients over the course of their infection.

**Identification of cytokines for further investigation in complicated or uncomplicated SABSI**

A panel of 42 cytokine antibodies were probed by hybridization of pooled plasma taken on the day of diagnosis, from three patients with complicated SABSI and three with uncomplicated SABSI. The position and name of each of the 42 cytokine antibodies on the array is shown on the cytokine antibody array map (Figure 1A). An image of the resulting hybridisation patterns for representative pooled samples from each group is shown in Figure 1B and cytokines that showed ≥ 1.4 fold differences in levels between groups are highlighted. These cytokines; IL-6, GROγ and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) were identified for further study. Leptin levels were marginally lower in pooled plasma from patients with complicated SABSI compared to uncomplicated) but this cytokine was further studied because its levels were increased > 2-fold in pooled plasma from three patients with MRSA compared to MSSA BSI (Figure 1C).

A new figure has been introduced (now labelled Figure 1) to replace table 2. In this figure, the names of all 42 cytokines used in preliminary screening are shown along with images of the membranes hybridised with pooled plasma and the cytokines of further interest are highlighted.

**Reviewer’s comment 4**

*It is unclear why cytokine concentrations were determined per mg protein rather than per ml.*

**Author’s response to comment 4**

The cytokine concentrations were normalized to account for differences in plasma protein concentrations that may differ between SABSI patients especially given that many were undergoing haemodialysis.
Changes to text, comment 4

- Lines 117-120: "cytokine levels were normalised to plasma protein concentration to account for variability in blood processing and biological variations in plasma protein concentrations between patients many of whom were undergoing haemodialysis."

Reviewer’s comment 5

It is unclear why IL-6, Gro-gamma and RANTES were selected for further study. Were these the only ones to show differences between study groups? If so fine, but this needs to be stated. It is also unclear why pooled plasma was used to examine leptin levels. A single sample could hugely influence the overall level using such an approach.

Author’s response to comment 5

As explained in response to comment 3, the cytokine antibody array was used as a preliminary screen to flag cytokines for further, more extensive and sensitive investigation by ELISA. We acknowledge that this approach, in which 3 samples were pooled, may not account for inter-patient differences in cytokine levels (which may be substantial) and the outcome is largely dependent on the plasma that is pooled for screening. However, in defence of this approach, the alternative, under budget constraints would have been more random, such as looking at the literature and choosing cytokines to investigate quite arbitrarily. This cytokine array screening approach used here could be compared to the more widely used global measurement of gene expression in a small number of samples, with follow up of the most interesting genes in a larger sample size and in that regard it was a valid preliminary investigation to identify cytokines that may be important in SABSI. Pooled plasma was used to identify cytokines from the cytokine antibody array, leptin was among the 4 that were investigated further because it was 2 fold increased in MRSA vs MSSA infections in the initial cytokine screen.

Text changes, comment 5

- The changes outlined in response to comment three also clarify the issues raised here.

- The new Figure 1, shows the leptin levels in each group (including MRSA vs MSSA) using the pooled plasma preliminary screen (cytokine antibody array) and its inclusion in further investigation based on these data, is clarified as follows;

  Line 149 ‘Leptin levels were marginally lower in pooled plasma from patients with complicated SABSI compared to uncomplicated) but this cytokine was further studied because its levels were increased > 2-fold in pooled plasma from three patients with MRSA compared to MSSA BSI (Figure 1C).’

Reviewer’s comment 6

The presentation of data as bar graphs with SEM is not particularly useful in assessing the inter-patient variability of data. It would be useful to see the full range of values, especially if such data are to be considered as prognostic markers
Author’s response, comment 6

We choose bar graphs as a clear way for the reader to appreciate the comparisons between groups. However we accept the importance of appreciating also the full-range of data. In the interests of clarity of presentation, where several sets of data are presented (e.g. complicated vs uncomplicated SABSI cytokine levels at three time points), we would argue that the bar chart is a better approach. However to see the full range of data, we have changed one of the figures to a scatter plot.

Changes to text

➢ Figure 2 is now a scatter plot.

Reviewer’s comment 7

Table 2, it is not clear what is being shown. The changes in the apparent size/intensity of dots looks greater than the fold differences described. How was this assay calibrated?

Author’s response, comment 7

This table has been replaced in response to an earlier comment and now the entire cytokine membrane is shown for each group. The intensity of each dot is normalised to the mean of 6 positive spots present on each membrane. Therefore the fold change in intensity may not be obvious from inspection of the signal directly.

Changes to text, comment 7

➢ Lines 105-110 Mean values for each cytokine were expressed relative to the mean positive value for each array membrane (mean of 6 positive control spots per membrane). A change in cytokine levels in pooled plasma, at a ratio threshold of at least 1.4 fold between groups was considered to be potentially relevant and the results from this preliminary screen influenced the decision on which cytokines to investigate in individual patients over the course of their infection.

➢ The figure 1 legend also explains the calibration ‘Fold change was estimated from cytokine signal intensity from the compared groups (uncomplicated/complicated , MSSA/MRSA) where signal intensity was normalised with respect to the signal intensity of six positive control spots on each membrane located at positions A1, A2, B1, B2, L7, L8.’

Reviewer’s comment 9

The authors state that they could not obtain baseline cytokine values from the study patients, which would certainly be a big undertaking. However, data from matched controls would be extremely useful in the interpretation of the data, especially since the author’s cite evidence that cytokine levels in renal patients can vary in the absence of infection.

Author’s response, comment 9

While matched control groups would have been desirable, it was not possible to identify a suitable control group for each comparison (e.g. should one choose, healthy controls, those without infection but with similar underlying conditions, similar groups but without infection) or indeed to resource
the study if several control groups were included. However, we did investigate cytokine levels in four healthy people. The range found in healthy people is now highlighted in figure 2.

Changes to text, comment 9

Mean levels found in four healthy controls included in Figure 2

References list