

Activation associated accelerated apoptosis of memory B cells in critically ill patients with sepsis

Manu Shankar-Hari^{1,2,3*} PhD, David Fear²PhD, Paul Lavender²PhD, Tracey Mare³BSc,

Richard Beale^{2,3} MBBS, Chad Swanson⁵PhD, Mervyn Singer⁶MD, Jo Spencer¹*PhD

¹Peter Gorer Department of Immunobiology, Programme of Infection and Immunity, King's College London, London, UK

²Division of Asthma, Allergy and Lung Biology, King's College London, London, UK

³Department of Intensive Care Medicine, Guy's and St Thomas' NHS Foundation Trust, London, UK

⁴MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London, Guy's Hospital, London, UK

⁵Department of Infectious Diseases, Programme of Infection and Immunity, King's College London, London, UK

⁶Bloomsbury Institute of Intensive Care Medicine, University College London, London, UK

Institutions: This work was performed at the Program of Infection and Immunity at Kings College London, UK and the Critical Care Units at Guy's and St Thomas' Hospitals NHS Foundation Trust, London, UK

Financial support: Supported in part by the ICU research funds at Guy's and St Thomas' NHS Foundation Trust and by the NIHR Biomedical Research Centre at Kings Health Partners, London, UK

Address requests for reprints to:

Manu Shankar Hari, MSc (Epi), MD, FRCA, FFICM, PhD

ICU Support Offices, First floor, East Wing, St Thomas'

Hospital, London, SE1 7EH, United Kingdom.

E-mail: manu.shankar-hari@kcl.ac.uk

Corresponding author(s)

Dr Manu Shankar Hari

1st Floor, East Wing, St Thomas' Hospital, SE17EH

Tel: +44 (0)20 7188 3044; Fax: +44 (0)20 7188 2284

Email: manu.shankar-hari@kcl.ac.uk

Professor Jo Spencer

Head of Department of Immunobiology

Peter Gorer Department of Immunobiology, Programme of Infection and Immunity, 2nd Floor,
Borough Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT

Tel: + 44 (0)20 7188 8420

Email: Jo.spencer@kcl.ac.uk

Authors COI: The authors declare no competing financial interests.

Abstract

OBJECTIVE: Sepsis is life-threatening organ dysfunction due to dysregulated host responses to infection. Current knowledge of human B cells alterations in sepsis is sparse. We tested the hypothesis that B cell loss in sepsis is non-random and investigated the mechanisms of B cell depletion.

DESIGN: Prospective cohort study

SETTING: Critical care units

PATIENTS: Adult sepsis patients without any documented immune comorbidity.

INTERVENTIONS: None

MEASUREMENTS and MAIN RESULTS: B cell subsets were quantified by flow cytometry, annexin V status identified apoptotic cells and phosphorylation of intracellular kinases identified activation status of B cell subsets. B cell specific survival ligand concentrations were measured. Gene expression in purified B cells was measured by microarray. Differences in messenger RNA abundance between sepsis and healthy controls were assessed.

Lymphopenia seen in 74.2% of patients on admission day, was associated with reduction in absolute B cell counts (median (IQR) 0.133 (0.093 – 0.277) 10^9 cells/L) and selective depletion of memory B cells in presence of normal B cell survival ligand concentrations.

Greater apoptotic depletion of class-switched and IgM memory cells was associated with phosphorylation of extracellular signal-regulated kinases, implying externally driven lymphocyte stress and activation-associated cell death. This inference is supported by gene expression profiles highlighting mitochondrial dysfunction and cell death pathways, with enriched intrinsic and extrinsic pathway apoptosis genes.

CONCLUSIONS: Depletion of the memory B cell compartment contributes to the immunosuppression induced by sepsis, leaving survivors vulnerable to subsequent infections. Therapies targeted at reversing this immune memory depletion should be actively investigated.

Key words: Sepsis, B cells, Apoptosis, Immunosuppression, Immune memory

INTRODUCTION

Sepsis is life-threatening organ dysfunction caused by dysregulated host responses to infection(1). These dysregulated immune responses consist of concomitant pro-inflammatory and anti-inflammatory responses involving innate and adaptive immune systems(2-4). Sepsis-related immunosuppression describes a plethora of abnormalities also affecting both innate and adaptive immunity(2-4). Such immunoparesis predisposes septic patients to renewed bouts of infection (bacterial or fungal) and reactivation of dormant viruses(2, 4, 5). Immune abnormalities potentially persist in survivors well beyond their hospital discharge(6), and likely contribute to infection-related readmissions(7-11), worsening co-morbidities and worse long-term outcomes(12).

Both loss and decreased functionality of lymphocytes contribute to this immunosuppressed state(2, 3, 13). Secondary lymphoid organs taken from septic patients have lower cellular density compared to healthy controls, with loss of antigen-presenting cells and T cell exhaustion that may impair B cell maturation(3, 13, 14). Mouse models of sepsis identify early B cell activation by type-1 interferon yet despite depletion of the naïve mantle zone B cell compartment, peripheral B cell maturation progresses unhindered(15). B cells have both antibody-dependent and independent functions in such sepsis models. For example, impaired cytokine production and reduced survival in recombination-activating gene deficient ($RAG1^{-/-}$) septic mice could be replicated in wild-type models after depletion of B cells using anti-CD20 antibodies. Survival rates increased in these $RAG1^{-/-}$ mice when treated with either serum or with B cells from wild-type mice(15). In other models using chimeric and knockout mice, a novel B cell effector subset was identified that secretes granulocyte-monocyte colony stimulating factor, reduces illness severity by altering cytokine profiles, enhances pathogen clearance and improves sepsis outcomes(16).

Current knowledge of human B cells in sepsis are sparse, discordant, and also at variance with findings reported from animal models. Descriptions of sepsis related abnormalities in the B cell compartment are limited to inconsistent reports of changes in the absolute counts, in activation status(17-20), and immunoglobulin levels(21). Optimal

management of patients with sepsis requires a clear understanding of the potential for manipulation of the humoral response and how the B cell compartment could support healthy life after recovery. We tested the hypothesis that B cell loss in sepsis is non-random and investigated the mechanisms of B cell depletion.

MATERIAL AND METHODS

Study design and setting

This prospective observational cohort study was performed in the general medical-surgical critical care units of a university hospital. Ethics committee approval was obtained prior to start of recruitment (10/H0807/81 and 12/LO/0326; Camberwell St Giles Committee, England). Clinical management of patients were at the discretion of the attending physicians. Informed consent was obtained from patients or, where they lacked competency, from Personal Legal Representatives. Retrospective consent was then sought from patients after they regained mental competency.

Study population

Consecutive patients with sepsis (previously severe sepsis/septic shock(22)) within the first 12 hours following ICU admission between 05/2011 and 01/2015 were included. Sepsis was defined as evidence of two or more systemic inflammatory response syndrome (SIRS) criteria, with proven or suspected infection and at least one organ system dysfunction (cardiovascular, respiratory, renal, haematological or metabolic)(22). Exclusion criteria included patients < 18 years, those with congenital hypo-gammaglobulinemia; known protein-losing enteropathies; nephrotic syndrome; neoplastic or proliferative haematological diseases; those having received IVIg therapy in the last 3 months; those receiving high-dose corticosteroid therapy; on-going blood loss (defined by blood transfusion requirement > 2 units/24 hour period); retroviral disease and having immune dysfunction as defined by Acute Physiology and Chronic Health Evaluation (APACHE) II score co-morbidities(23).

Clinical data and blood sampling

Baseline demographic data, daily laboratory results, daily worst physiological variables, the APACHE II score(23) and the Sepsis-related Organ Failure Assessment (SOFA) scores(24) were obtained from the clinical information system (CareVue™, Philips and Electronic patient records). Fresh blood samples for all experiments were collected daily

from admission day until day-7 following ICU admission, if sooner on the day of demise or discharge (defined as final measurement (DF)).

Flowcytometry

For isolating peripheral blood mononuclear cells (PBMCs) and for whole blood flow cytometry, EDTA anti-coagulated blood was collected. All anti-human fluorochromes were BD unless otherwise specified. For all experiments, Amcyan (L34957; Invitrogen) was used to identify alive cells. For each target molecule, the fluorochrome and clone used are reported in parenthesis. To estimate the absolute B cell counts in sepsis patients, whole blood (50 μ L) and beads were labelled for 20 minutes at room temperature using CD19 (PE; LT19), CD45 (PeCY7; H130) and CD69 (FITC; FN50) antibody combinations and the corresponding isotype controls and estimated using flow-cytometry on Canto-A flowcytometer (BD) using Diva Software Version 6.1 following RBC lysis. Admission day and DF absolute B-cell counts are reported.

For all other experiments, PBMCs were isolated within 4 hours of blood sampling using Ficoll density centrifugation and stored in liquid nitrogen as viable cells for future analysis. To identify B cell subsets, we used: anti-CD19 (PerCP Cy5.5; HB19); anti-CD24 (PeCYTM7; ML5); anti-CD27 (APC; MT271), anti-CD38 (PE; HIT2; eBioscience); anti-IgM (V450; G20-127); anti-IgA (FITC; IS11-8E10; Miltenyi); anti-IgG (APCH7; G18-145); Annexin-V (PeCYTM7; eBioscience); anti-CD27 (FITC; O323; eBioscience); anti-p-ERK (PE; RateK1); anti-p-BTK (ALexafluor674; 24a/BTK); anti-p-SYK (ALexafluor488; human syk peptide); anti-p-AKT (APC; eBioscience). Staining for phosphorylation status of intracellular kinases was performed using the BD cytofix/Cytoperm kit and phosphoflow buffers as per manufacturer guidelines. Gating during analysis of FCS files were achieved using isotype controls, fluorescence minus-one controls and/or single stain controls. Admission day B-cell subset profiles are reported.

Enzyme linked immunosorbent assay (ELISA)

Quantitative detection of human B cell-activating factor of the tumour necrosis factor family (BAFF) and a proliferation inducing ligand (APRIL) in admission day serum in sepsis

patients and age-matched healthy controls were done in triplicate using commercial ELISA kits from eBioscience.

Microarray

CD19+ B cells from sepsis and healthy controls were isolated to a purity of >99% using EasySep® Positive Selection kit. Purity was confirmed with flow cytometry. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was converted to cDNA using the Ovation® Pico WTA Systems V2 and labelled using the Encore® BiotinIL Module (Nugen). Labelled cDNA was hybridized onto Infinium Illumina HT12v4 arrays and data collected on an iSCAN array scanner (Illumina). Following scanning, basic QC statistics were completed, displaying hybridisation controls, background signal and mean gene intensity of all genes and those of housekeeping genes. Array results were compiled using Genome Studio (illumina) following quantile normalisation. Differences in messenger RNA abundance between the sepsis and healthy controls were assessed using Partek Genomic Suite (Partek Inc.). The similarities and differences in the cell specific gene expression pattern in health and in sepsis were assessed using heat maps and the top canonical pathways identified using Ingenuity Pathway Analysis (IPA) (Qiagen). Apoptosis genes and pathways were also studied. Manufacturer's instructions were followed in all steps.

Healthy controls

For all comparisons of sepsis patients reported with ELISA assays, flowcytometry and microarrays, we measured healthy controls who were consented prior to sampling as per the Infectious Diseases Biobank at King's College London protocol (25). Only for Figure 1b, which tests whether the B-cell counts in sepsis on admission day and DF are lower than healthy subjects, we used the published reference adult population median of 0.233×10^9 cells/L (26). Aside from mean fluorescent intensity (MFI) comparisons by apoptosis status shown in Figure-4, all other comparisons are between healthy and sepsis.

Statistics

Continuous data is presented as mean and standard deviation (SD) when normally distributed and as median and interquartile range (IQR) when not. Frequency and percentages are presented for categorical data. Statistical tests included unpaired t-test or paired t-tests the corresponding non-parametric equivalents where appropriate accounting for

unequal variances. All analyses were performed using Prism6. Reported p values are two sided with p values <0.05 representing a statistically significant result.

RESULTS

Study cohort

Mean (SD) age was 64.8 (13.7) years and 62.4% were male. The respiratory tract was the most common infection site (64.4%), followed by intra-abdominal (15.0%), wound, bone and soft tissue (11.0%), and urinary tract (6.9%). The mean (SD) APACHE II score was 19.5 (5.7) and the median (IQR) SOFA Score was 7 (6, 9). Acute hospital mortality was 26.7% (eTable1; eFigure1).

Sepsis patients have a high prevalence of lymphopenia, low absolute B cell counts and normal B cell specific survival factor concentrations.

Lymphopenia was defined as an absolute lymphocyte count $<1.2 \times 10^9$ cells/L. Persistent and more severe lymphopenia was observed in non-survivors compared to survivors over the first seven days of ICU stay ($p < 0.001$ on days 2, 3, 4 and 5; comparing absolute lymphocyte counts; Figure1a). On admission day, the median (IQR) B cell count in sepsis patients was 0.133 (0.093 – 0.277) 10^9 cells/L, significantly lower than population median ($p = 0.01$) and 58.3% of sepsis patients had counts less than population median of 0.233×10^9 cells/L (26). As there was no statistically significant differences in B-cell counts between admission day and DF ($p = 0.23$), subsequent experiments presented in this paper were done using admission day samples (Figure1b). There were no differences between patients with sepsis and healthy controls in the serum levels of B cell specific survival factors (BAFF - Figure1c; APRIL - Figure1d).

Sepsis associated B cell depletion is most severe in memory B cell subsets

B cell subsets were quantified by flow cytometry as the proportion of live CD19 cells in PBMCs. The gating strategy is shown in eFigure2. No difference was seen between sepsis patients and healthy controls in the frequency of transitional B cell subsets ($p = 0.70$ for T1; $p = 0.21$ for T2; Figure2a) and the proportion of naïve B cells (Figure2b). In marked contrast, the proportions of plasmablasts, IgM memory B cells and class-switched memory B cells

were all significantly lower ($p=0.002$ for plasmablasts; $p=0.0002$ for IgM memory; $p=0.001$ for IgA memory cells and $p=0.013$ for IgG memory) (Figure2c-f).

B cell apoptosis in sepsis is highest in memory B cell subsets that have the highest relative ERK phosphorylation

B cell apoptosis was assessed by flow cytometry as the proportion of Annexin-V status in live-dead stain negative (live) CD19 cells. In each sample, each B cell subset was used as its own denominator to estimate the proportion of apoptotic cells within. The gating strategy is shown in eFigure3. B cell apoptosis was highest in the memory subsets (Figure3a), which were also the B cell subsets with greatest proportional depletion (Figures2d-f).

Phosphorylation of intracellular kinases such as ERK can be considered as surrogates for intracellular stress signalling(27, 28). In the patients with sepsis, the p-ERK MFI was significantly higher in IgM memory ($p=0.0002$) and CSR memory ($p=0.004$) subsets when compared to naive B-cells (Figure 3b; left panel). In contrast, this difference was not observed in B-cells from healthy subjects (Figure3b, right panel). In sepsis, annexin-V positive CD19 B cells had significantly higher p-ERK MFI compared to annexin-V negative cells ($p=0.002$; Figure3c); these differences were replicated in the B cell subsets (Figure3d-f). Reciprocal changes were observed in the CD22 MFI, another key BCR signalling modulator (29) (Figure4a-d), supporting the hypothesis that B cell death is a non-random event associated with an activated profile. No differences between apoptotic and non-apoptotic B cells were seen in phosphorylation of the more BCR proximal kinases, Bruton's tyrosine kinase (BTK) and spleen tyrosine kinase (SYK) (eFigure4) and in protein kinase B phosphorylation (p-AKT) in sepsis compared to health, though memory cells in general had more p-AKT than naïve B cells (Figure4e).

Gene expression profiles of purified B cells also supports activation-induced cell death in sepsis

Salient clinical characteristics of the six sepsis patients used in microarray experiment are shown in eTable2. Heat maps show clear clustering segregating the sepsis and healthy groups (Figure5a; eTable3). IPA identified significant alterations in canonical

pathways of oxidative phosphorylation ($p=0.0007$), cyclins and cell cycle regulation ($p=0.001$), mitochondrial dysfunction ($p=0.005$) and in the super pathways of serine and glycine biosynthesis ($p=0.005$). Using the B cell apoptosis regulator genes' status in sepsis compared to health are (eFigure6), IPA analysis highlighted two B cell apoptosis networks in sepsis involving both mitochondrial and death receptor pathways (eFigures7). ERK either influenced ($n=15$) or was influenced by ($n=14$) or had a bidirectional relationship with ($n=5$) 34 apoptosis regulator genes in this dataset (Figure5b), implying a potentially important role for ERK in activation-associated B cell death in sepsis. A simplified schematic overview of activation associated B cell death using the genes identified in the two apoptosis networks in eFigures7 is shown in Figure5c.

DISCUSSION

Changes in the relative proportions of B cell subsets in critically ill patients with sepsis are reported here for the first time. B cell depletion is an acute phenomenon as it was observed on admission day samples, with an exaggerated proportional loss of antigen-experienced B cell subsets, i.e. plasmablasts and memory cells. B cell apoptosis is greater in memory cells compared to naïve B cells, consistent with the finding that these B cell subsets are the most depleted. This suggests that B cell depletion is not a consequence of impaired bone marrow production. The observed cell death events occurred despite normal concentrations of BAFF and APRIL and involve well-characterised, activation-associated apoptosis pathways. In health, generation of high affinity antibody responses depends on T cell functionality since it requires B cell: T cell cognate interaction for the generation of germinal centre responses (30, 31). There are well-characterised abnormalities in T cell effector function in sepsis (3, 32-38) that could be considered to be an explanation for the lower frequencies of memory cells and plasmablasts we observe. However data presented here show that their depletion is related to their higher susceptibility to death rather than a failure to create them due to a lack of T cell help.

The B-cell receptor (BCR) complex is composed of a cell-surface immunoglobulin molecule, which is non-covalently bound to the antigen-nonspecific signalling protein dimer CD79a and CD79b. The BCRs have two main roles, antigen binding and transmitting signals

to regulate B cell fate decisions(29). BCR crosslinking activates SRC family kinases such as LYN, which phosphorylates the immune tyrosine based activation moieties (ITAM). Binding of SYK to these double phosphorylated ITAM moieties activates SYK, which then rapidly phosphorylates the scaffold protein B cell linker (BLNK). Phospholipase C-gamma 2 is activated secondary to BLNK and BTK interactions, which results in transcription factor activation and activation of downstream ERK1/2 kinases via phosphorylation (39). Of the three BCR signalling pathway kinases studied, only the p-ERK MFI was significantly higher in the more apoptotic IgM and CSR memory B cell subsets, when compared to naïve B cells from the same subjects, with no evidence for increased phosphorylation of the more BCR proximal kinases BTK and SYK. This implies that on admission day, B cells in patients with sepsis are not in the early stages of activation through their BCR. It would be highly unlikely that the memory populations with activated profiles would all have antigenic specificity for the causal infectious agent(40).

Phosphorylation of ERK can be associated with transition to an apoptotic state(41-43). Memory B cell subsets have different susceptibilities to apoptosis when compared to naïve B cells (44). Determinants of the four potential fates of activated B cells (division, death, differentiation, and class switch), are complex and only predictable with limited certainty(45). Data presented here suggest that cell death in sepsis is externally driven rather than through the BCR and ERK plays an important role in activation-associated B cell death in sepsis. The microarray analyses of the B cells identified mitochondrial dysfunction with alterations in ATP5J, ATP50, COX8A (cytochrome C oxidase subunit 8A), NADH dehydrogenase 1 sub complexes alpha-1 and beta-10 (NDUFA1, and NDUFB10) genes and the serine/glycine super pathways (which included the genes PHGDH and SHMT2)(46, 47). When taken together, these alterations in gene expression also support the concept of activation-associated B cell death in sepsis (Figure5b and 5c).

Immunosuppression is a clinically valuable term used to communicate the profound acute impairment of the immune system in sepsis. However, this persists beyond the acute illness in sepsis survivors, with a marked reduction in cellularity in the secondary lymphoid organs(3, 13)(48). Reversing this suppression will require a better understanding of

contributory factors during the acute phase, and factors that maintain this state thereafter. Current paradigms to reverse this sepsis related immunosuppression focus on interventions such as anti-interleukin-3, interleukin-7, anti programmed death ligand-1 antibody, and granulocyte-monocyte colony stimulating factor aimed at managing excess inflammation and/or acute functional impairment(49). There may be two fundamentally different facets to sepsis immunosuppression that should perhaps be considered independently - functional (immune dysfunction) and structural abnormalities (immune depletion). The latter is particularly relevant when considering infection-related re-admissions in sepsis survivors (7-11). In this study we identify that B cell depletion preferentially affects memory B cell subsets, which could be either due to impaired secondary lymphoid organ maturation or accelerated apoptosis in peripheral circulation or combinations thereof. Relative contributions of these different mechanisms on B-cell subsets need further studies. Restoring immune capability will therefore require replenishment of B cell numbers as well as boosting the memory cell pool to replenish lymphocyte cellularity in secondary lymphoid organs of sepsis survivors.

CONCLUSIONS

We report selective depletion of antigen-experienced B cells in sepsis. As sepsis survivors suffer from long-term susceptibility to infection, therapies targeted at reversing this immune depletion state should be actively investigated.

ACKNOWLEDGEMENTS

MSH, PL, JS and MS acknowledge the support of the UK NIHR Biomedical Research Centre schemes. MS is a recipient of UK NIHR Senior Investigator Fellowship 2009 - 2017.

CMS is funded by the U.K. Medical Research Council grant MR/M019756/1DF.

AUTHOR CONTRIBUTIONS

MSH conceived the clinical study and performed all the experiments, except data presented in Figure-1b (performed by TM). MSH did all the analyses presented Figures 1-5. MSH, PL and DF did the analysis for Figure-5. MSH, CS, DF and PL conceived the micro-array study plan. JS and MS supervised all the experiments. All authors contributed to the interpretation of data, critical revision of the manuscript and approved the final manuscript.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016;315(8):801-810.
2. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013;13(12):862-874.
3. Boomer JS, To K, Chang KC, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA* 2011;306(23):2594-2605.
4. van Vught LA, Klein Klouwenberg PM, Spitoni C, et al. Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis. *JAMA* 2016;315(14):1469-1479.
5. Limaye AP, Kirby KA, Rubenfeld GD, et al. Cytomegalovirus reactivation in critically ill immunocompetent patients. *JAMA* 2008;300(4):413-422.
6. Yende S, D'Angelo G, Kellum JA, et al. Inflammatory markers at hospital discharge predict subsequent mortality after pneumonia and sepsis. *Am J Respir Crit Care Med* 2008;177(11):1242-1247.
7. Sun A, Netzer G, Small DS, et al. Association Between Index Hospitalization and Hospital Readmission in Sepsis Survivors. *Crit Care Med* 2016;44(3):478-487.
8. Jones TK, Fuchs BD, Small DS, et al. Post-Acute Care Use and Hospital Readmission after Sepsis. *Ann Am Thorac Soc* 2015;12(6):904-913.
9. Donnelly JP, Hohmann SF, Wang HE. Unplanned Readmissions After Hospitalization for Severe Sepsis at Academic Medical Center-Affiliated Hospitals. *Crit Care Med* 2015;43(9):1916-1927.
10. Dharmarajan K, Hsieh AF, Lin Z, et al. Diagnoses and timing of 30-day readmissions after hospitalization for heart failure, acute myocardial infarction, or pneumonia. *JAMA* 2013;309(4):355-363.
11. Goodwin AJ, Rice DA, Simpson KN, et al. Frequency, cost, and risk factors of readmissions among severe sepsis survivors. *Crit Care Med* 2015;43(4):738-746.
12. Shankar-Hari M, Ambler M, Mahalingasivam V, et al. Evidence for a causal link between sepsis and long-term mortality: a systematic review of epidemiologic studies. *Crit Care* 2016;20(1):101.
13. Hotchkiss RS, Tinsley KW, Swanson PE, et al. Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans. *J Immunol* 2001;166(11):6952-6963.
14. Venet F, Lukaszewicz AC, Payen D, et al. Monitoring the immune response in sepsis: a rational approach to administration of immunoadjuvant therapies. *Curr Opin Immunol* 2013;25(4):477-483.
15. Kelly-Scumpia KM, Scumpia PO, Weinstein JS, et al. B cells enhance early innate immune responses during bacterial sepsis. *J Exp Med* 2011;208(8):1673-1682.
16. Rauch PJ, Chudnovskiy A, Robbins CS, et al. Innate response activator B cells protect against microbial sepsis. *Science* 2012;335(6068):597-601.

17. Monserrat J, de Pablo R, Diaz-Martin D, et al. Early alterations of B cells in patients with septic shock. *Crit Care* 2013;17(3):R105.
18. Gogos C, Kotsaki A, Pelekanou A, et al. Early alterations of the innate and adaptive immune statuses in sepsis according to the type of underlying infection. *Crit Care* 2010;14(3):R96.
19. Venet F, Davin F, Guignant C, et al. Early assessment of leukocyte alterations at diagnosis of septic shock. *Shock* 2010;34(4):358-363.
20. Andaluz-Ojeda D, Iglesias V, Bobillo F, et al. Early natural killer cell counts in blood predict mortality in severe sepsis. *Crit Care* 2011;15(5):R243.
21. Shankar-Hari M, Culshaw N, Post B, et al. Endogenous IgG hypogammaglobulinaemia in critically ill adults with sepsis: systematic review and meta-analysis. *Intensive Care Med* 2015;41(8):1393-1401.
22. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 2003;31(4):1250-1256.
23. Knaus WA, Draper EA, Wagner DP, et al. APACHE II: a severity of disease classification system. *Crit Care Med* 1985;13(10):818-829.
24. Vincent JL, Moreno R, Takala J, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996;22(7):707-710.
25.

http://www.kcl.ac.uk/lsm/research/divisions/diuid/about/facilities/bio_bank/index.aspx. Accessed on 09/09/2016.
26. Valiathan R, Deeb K, Diamante M, et al. Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida. *Immunobiology* 2014;219(7):487-496.
27. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298(5600):1911-1912.
28. Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *FEBS J* 2010;277(1):2-21.
29. Niiron H, Clark EA. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol* 2002;2(12):945-956.
30. Gitlin AD, Shulman Z, Nussenzweig MC. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* 2014;509(7502):637-640.
31. Paus D, Phan TG, Chan TD, et al. Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J Exp Med* 2006;203(4):1081-1091.
32. Cabrera-Perez J, Condotta SA, James BR, et al. Alterations in antigen-specific naive CD4 T cell precursors after sepsis impairs their responsiveness to pathogen challenge. *J Immunol* 2015;194(4):1609-1620.

33. Cabrera-Perez J, Condotta SA, Badovinac VP, et al. Impact of sepsis on CD4 T cell immunity. *J Leukoc Biol* 2014;96(5):767-777.
34. Condotta SA, Rai D, James BR, et al. Sustained and incomplete recovery of naive CD8+ T cell precursors after sepsis contributes to impaired CD8+ T cell responses to infection. *J Immunol* 2013;190(5):1991-2000.
35. Guignant C, Lepape A, Huang X, et al. Programmed death-1 levels correlate with increased mortality, nosocomial infection and immune dysfunctions in septic shock patients. *Crit Care* 2011;15(2):R99.
36. Boomer JS, Shuherk-Shaffer J, Hotchkiss RS, et al. A prospective analysis of lymphocyte phenotype and function over the course of acute sepsis. *Crit Care* 2012;16(3):R112.
37. Kobayashi Y, Iwata A, Suzuki K, et al. B and T lymphocyte attenuator inhibits LPS-induced endotoxic shock by suppressing Toll-like receptor 4 signaling in innate immune cells. *Proc Natl Acad Sci U S A* 2013;110(13):5121-5126.
38. Shubin NJ, Monaghan SF, Heffernan DS, et al. B and T lymphocyte attenuator expression on CD4+ T-cells associates with sepsis and subsequent infections in ICU patients. *Crit Care* 2013;17(6):R276.
39. Xu Y, Xu L, Zhao M, et al. No receptor stands alone: IgG B-cell receptor intrinsic and extrinsic mechanisms contribute to antibody memory. *Cell Res* 2014;24(6):651-664.
40. Kurosaki T, Kometani K, Ise W. Memory B cells. *Nat Rev Immunol* 2015;15(3):149-159.
41. Adem J, Hamalainen A, Ropponen A, et al. ERK1/2 has an essential role in B cell receptor- and CD40-induced signaling in an in vitro model of germinal center B cell selection. *Mol Immunol* 2015;67(2 Pt B):240-247.
42. Mizuno T, Rothstein TL. B cell receptor (BCR) cross-talk: CD40 engagement enhances BCR-induced ERK activation. *J Immunol* 2005;174(6):3369-3376.
43. Yasuda T, Kometani K, Takahashi N, et al. ERKs induce expression of the transcriptional repressor Blimp-1 and subsequent plasma cell differentiation. *Sci Signal* 2011;4(169):ra25.
44. Klein U, Tu Y, Stolovitzky GA, et al. Transcriptional analysis of the B cell germinal center reaction. *Proc Natl Acad Sci U S A* 2003;100(5):2639-2644.
45. Duffy KR, Wellard CJ, Markham JF, et al. Activation-induced B cell fates are selected by intracellular stochastic competition. *Science* 2012;335(6066):338-341.
46. Chiarla C, Giovannini I, Siegel JH. High phosphoserine in sepsis: panel of clinical and plasma amino acid correlations. *Springerplus* 2014;3:279.
47. Desler C, Lykke A, Rasmussen LJ. The effect of mitochondrial dysfunction on cytosolic nucleotide metabolism. *J Nucleic Acids* 2010;2010.
48. Shankar-Hari M and Rubinfeld GR. Understanding Long-Term Outcomes following Sepsis: Implications and Challenges. DOI: 10.1007/s11908-016-0544-7. *Curr Infec Dis Reports*. 2016. In press

49. Hotchkiss RS, Sherwood ER. Immunology. Getting sepsis therapy right. *Science* 2015;347(6227):1201-1202.

FIGURE LEGENDS

Figure 1 | Sepsis associated B cell depletion occurs with normal survival factor concentrations.

Figure 1a: Dot plot shows lymphopenia is common in both survivors (n=74) and non-survivors (n=27), with a hospital mortality of 26.3% (eTable-1). Lymphopenia was defined as absolute lymphocyte counts (ALC) less than 1.2×10^9 cells/L, which is marked by the shaded region under the reference line. Non-survivors have more severe lymphopenia than persists longer when compared to survivors ($p < 0.01$ on days 2, 3, 4, 5 and 6 using t-tests) and patients with persistent lymphopenia by day-4 had poor survival at 90 days (eFigure-1). Proportions of survivors versus non-survivors with lymphopenia by days of ICU stay are shown as italics in the x-axis and were not compared for statistical differences. The proportions of patients with severe lymphopenia ($ALC < 0.6 \times 10^9$ cells/L) was also more common in non-survivors (D1= 39.1% vs. 59.3% ($p=0.03$) and D2 = 25.7% vs. 59.3% ($p=0.007$); compared using χ^2 tests). **Figure 1b:** Dot plot of admission (D1) and final day (DF) absolute B cell count shows significantly lower counts in sepsis patients (n=24) on admission day when compared to population median values (median reference line= 0.233×10^9 cells/L). Absolute lymphocyte count on D1 and DF in sepsis patients were 0.133 (0.93 – 0.277) and 0.224 (0.12 – 0.525) $\times 10^9$ cells/L respectively. Shaded regions represent normal range in healthy adults. Comparisons made using one-sample t-test. The admission day and DF absolute B-cell counts were compared to assess whether the B-cell counts change significantly during the 7-day period. As there was no statistically significant differences in B-cell counts between D1 and DF ($p=0.23$), subsequent experiments presented in this paper were done using admission day samples. **Figure 1c:** Shows the BAFF concentrations; Shows the median (IQR) ng/mL BAFF concentrations (ICU admission day in sepsis (n=62) patients = 0.14 (0.09 – 0.20) versus health = 0.11 (0.07 – 0.25)); **Figure 1d:** Shows the APRIL concentrations (ICU admission day in sepsis (n=26) patients = 2.31 (1.29 – 8.26) versus health = 2.39 (2.35 – 5.65)) were similar (Mann-Whitney Test). D= days following ICU admission. Admission day=D1; DF represents either day-7 samples or

last day of ICU stay or death. BAFF and APRIL concentrations in sepsis compared to age-matched healthy controls measured using ELISA to test the hypothesis that low absolute B-cell counts in sepsis were not due to lack of B cell specific survival factors.

Figure 2 | In the context of absolute low B cell counts, the memory B cells and plasmablasts are proportionally more depleted in sepsis

The proportions of functional B cell subsets (naïve, transitional, plasmablasts and memory B cells) in sepsis were analysed using flowcytometry on PBMCs stained with CD19, CD24, CD38, CD27, IgG, IgA, IgM and amcyan. B cell subsets are reported as proportion of the live CD19 population. Proportions were compared between healthy samples (n=23) and sepsis patients (N=29) for statistically significant differences using Mann Whitney tests. Dot plots reporting proportions shows all data points alongside median and IQR as summary distribution. The gating strategy used to identify B cell subsets shown in eFigure2. **Figure 2a:** Shows the median (IQR) transitional (T1) B cells in healthy individuals and sepsis patients were 1.04% (.76% - 1.63%) and 1.38% (0.62% - 1.87%) respectively (p=0.70) and T2 B cells were 2.37% (1.48% - 3.06%) and 2.95% (1.69% - 4.72%) respectively (p=0.21). **Figure 2b:** Shows the median (IQR) naïve B cells as a proportion of alive CD19 population in health was 54.9% (45.1% - 66.6%) and in sepsis was 70.9% (56.8% - 74.7%); p=0.043). **Figure 2c-f:** Shows that sepsis patients have a significantly lower proportion of potentially antigen experienced cells (**Figure 2c:** plasmablasts (0.36% [0.14% - 0.80%] in sepsis compared to 1.05% [0.49 - 1.64%]; in health: p=0.002); **Figure 2d:** IgM memory (4.0% [2.3-9.5%] in sepsis compared to 9.1% [7.1%-8.2%] in health; p=0.0002); **Figure 2e:** IgA memory (2.54% [1.39% - 4.50%] compared to 5.33% [3.75% - 8.48%] in health; p=0.001) and **Figure 2f:** IgG memory (4.23% [2.38% - 6.47%] in sepsis compared to 6.63% [4.62% - 10.5%] in health; p=0.013).

Figure 3 | B cell apoptosis in sepsis was proportionally higher in memory B-lymphocyte subsets and associated with higher MFI for phosphorylated ERK1/2 without concomitant higher MFI for BTK and SYK

Figure 3a: Dot plots showing proportions of apoptosis (Annexin-V staining) in B cell subsets analysed using flow cytometry. To ascertain statistical differences between healthy samples (n=12) and sepsis (n=19), the proportions of Annexin-V positive cells within each subset were compared (gating shown in eFigure2). Sepsis samples had a significantly higher proportion apoptotic naïve B cells: (2.33% [1.70% - 4.03%] in sepsis compared to 1.25 [0.51% - 1.68%]; p=0.0002 in health); IgM memory (3.85% [2.50% – 7.04%] in sepsis compared to 1.81% [0.76% - 2.67%]; p=0.0002) and class switched memory B cells (8.97% (7.77%-11.57%) in sepsis compared to 2.76% (1.92% - 4.52%) in health; p=0.0007) (Figure-3a). The Class switched memory B cell subset showed the greatest extent of apoptosis, which also was the proportionally most deplete B cell subsets (refer Figure-2). **Figure 3b-e:** To ascertain differences in key regulatory and BCR signalling molecules, after cell surface with CD19, CD27, CD22, IgM and annexin-V, the cells were fixed and permabilised followed by intracellular staining for phosphokinases and AKT (gating shown in eFigure-3). Only paired tests were used to compare the differences within sepsis and within health by apoptosis status to account for experimental differences in MFI, importantly no statistical comparisons between sepsis versus health were conducted. Shows the significant differences observed between apoptosis and non-apoptosis B cell population for p-ERK status in sepsis patients (n=10) compared to health (n=5). There was significant differences between p-AKT, p-BTK and p-SYK (eFigure 4) status with these comparisons.

Figure 4 | B cell apoptosis in sepsis was associated with reciprocal lower MFI for CD22 without concomitant higher MFI for BTK and SYK

To ascertain differences in key regulatory and BCR signalling molecules, after cell surface with CD19, CD27, CD22, IgM and annexin-V, the cells were fixed and permabilised followed by intracellular staining for phosphokinases and AKT (gating shown in eFigure-3). Only paired tests were used to compare the differences within sepsis and within health by apoptosis status to account for experimental differences in MFI, importantly no statistical comparisons between sepsis versus health were

conducted. Figure 4a-d shows the significant differences observed between apoptosis and non-apoptosis B cell population for CD22 status in health samples (n=7) and sepsis (n=9). There was significant differences between p-AKT, p-BTK and p-SYK (eFigure 4) status with these comparisons.

Figure 5 | Microarray based gene expression supports activation associated B cell apoptosis

Figure 5a: Heat maps were used to assess the clustering of gene expression between B cells from healthy individuals and patients with sepsis. Sepsis patient characteristics are presented in eTable-2. RNA quality was assessed using RNA pico chips on Agilent Bioanalyser (N=6 sepsis samples with RIN values of 8.7; 8.5; 8.7; 8.9; 9.3; 9.3) and N=6 healthy samples (with RIN values of 9.9; 9.5; 9.1; 8.7; 9.5; and 9.1). Clear clustering within within and clear separation between sample groups, with 182 differentially expressed genes in sepsis with a ≥ 1.5 -fold change (57 down-regulated and 125 up-regulated genes in B cells from sepsis). The numbers of samples contributing to each of these clusters are represented underneath the top horizontal sample group bar. The fold changes are colour coded, with yellow representing up-regulated genes and blue representing down-regulated genes. Genes are presented in the y-axis. Each column represents data from a separate donor. For clarity, genes presented in the y-axis are tabulated (eTable-4). **Figure 5b:** Shows the relationship of ERK to the 93 apoptosis genes identified in the current dataset (shown in eFigure6). Descriptive assessment of the relationship between apoptosis genes and ERK expression was performed using IPA. A direct relationship between ERK and expression of two apoptosis regulators, DAPK1 and PEA15 was observed. Both had lower expression in sepsis compared to health. **Figure 5c:** Schematic digram highlighting the potential apoptosis regulator molecules in sepsis. We identified 93 apoptosis regulator genes and changes in these individual genes are shown in eFigure5. The top two apoptosis networks highlighted by lthe Ingenuity pathways anlysis involved intrinsic pathway with higher expression of CASP2, CASP6, pro-apoptotic BCL2 proteins (APAF1, HTRA2, HIP1 and BOK) (eFigure7)

and DR pathway higher expression of plasma membrane death receptor (DR) genes (FAS, TNFRSF25, TNFRSF10B, TNFRSF10A, DR4, DR5 and Trail-R), DR adaptor proteins (CRADD, PIDD1 and FADD), CFLAR, CASP8 and CASP10.









