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Combined dysfunctions of immune cells predict nosocomial infection in critically ill patients

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Background

Nosocomial infection occurs commonly in intensive care units (ICU). Although critical illness is associated with immune activation, the prevalence of nosocomial infections suggests concomitant immune suppression.

This study examined the temporal occurrence of immune dysfunction across three immune cell types, and their relationship with the development of nosocomial infection.

Methods

A prospective observational cohort study was undertaken in a teaching hospital general ICU. Critically ill patients were recruited and underwent serial examination of immune status, namely percentage regulatory T-cells (Tregs), monocyte deactivation (by HLA-DR expression) and neutrophil dysfunction (by CD88 expression). The occurrence of nosocomial infection was determined using pre-defined, objective criteria.

Results

Ninety-six patients were recruited, of whom 95 had data available for analysis. Relative to healthy controls, percentage Tregs were elevated 6-10 days after admission, whilst monocyte HLA-DR and neutrophil CD88 showed broader depression across time points measured. Thirty-three patients (35%) developed nosocomial infection, and patients developing nosocomial infection showed significantly greater immune dysfunction by the measures employed. Tregs and neutrophil dysfunction remained significantly predictive of infection in a Cox hazards model correcting for time effects and clinical confounders (HR 2.4 (95%

CI 1.1-5.4) and 6.9 (95% CI 1.6-30) respectively, p=0.001). Cumulative immune dysfunction resulted in a progressive risk of infection, rising from no cases in patients with no dysfunction to 75% of patients with dysfunction of all three cell types (p=0.0004).

Conclusions

Dysfunctions of T-cells, monocytes and neutrophils predict acquisition of nosocomial infection, and combine additively to stratify risk of nosocomial infection in the critically ill.

Key Words: Blood - neutrophils, Blood - lymphocytes, Complications - infections, INTENSIVE CARE

Many diseases that can precipitate the need for exogenous organ support and admission to intensive care are characterized by a profound systemic inflammatory response[1], with associated immune cell activation [2] and immune system-mediated organ damage[3]. However it is now increasingly apparent that this over-exuberant inflammation is accompanied by an equally vigorous counter-regulatory anti-inflammatory response [4].

The anti-inflammatory response to the systemic inflammatory response syndrome (SIRS) manifests across a range of cellular actions and functions, involving both the innate and adaptive arms of the immune system [4] Defects have been noted in neutrophils [5-8], monocytes [9], T lymphocytes [10] as well as B lymphocytes and splenic dendritic cells [11,12].

The recent identification of elevated levels of regulatory helper-T cells (Tregs) in sepsis [13] is in keeping with the supposition that much of the immunosuppression arises from the over-activation of counter-regulatory mechanisms. In human and experimental sepsis, Tregs impair the proliferative response of lymphocytes [14].

The demands of organ support require the disruption of physical and physiological barriers through the placement of devices such as endo-tracheal tubes. It is thought that the combination of immune vulnerability and such routes of microbial colonization are responsible for the high rates of nosocomial infection seen in critically ill patients [15]. These secondary infections typically

occur in 25-35% of those admitted to intensive care units (ICUs) [15], a rate that approaches that seen in neutropaenia [16]. These infections are associated with increased length of stay [17], morbidity [18] and mortality [19], and therefore are of considerable concern to patients and clinicians. Although it seems plausible that the immune defects found in critical illness are associated with the acquisition of nosocomial infection, there is little published evidence for this, and what data there are concentrates on single types of immune cell. Furthermore the temporal relationship between immune dysfunction and nosocomial infection is not always clear [6], limiting any inferences regarding causality.

This study aimed to characterise the temporal patterns of three measures of immune dysfunction, sampling both the innate and adaptive arms of the immune system, and to derive potential new biomarkers of susceptibility to nosocomial infection. The cell types and measures of dysfunction chosen were; the level of Tregs as a percentage of all CD4+ lymphocytes[14], monocyte deactivation assayed by monocyte HLA-DR expression [9] and C5a-mediated neutrophil dysfunction assayed by surface CD88 expression [6, 8].

Methods

Reagents

Fluorescein isothiocyanate (FITC)-conjugated murine anti-human CD4, allophycocyanin (APC)-conjugated murine anti-human CD25, and phycoerythrin (PE)-conjugated murine anti-human FOXP3 antibodies were obtained from eBioscience (San Diego, CA, USA). Red cell lysis buffer, fixation/permeabilization solution and flow staining buffer were obtained from eBioscience. Alexa Fluor™ 647-conjugated murine anti-human CD88 antibodies were obtained from AbD Serotec (Abingdon, UK), and QuantiBRITE monocyte HLA-DR assay was obtained from Becton Dickson Biosciences (Oxford, UK). Tri-colour (TC)-conjugated murine anti-human CD16 and CD62L, FITC-conjugated murine anti-human CD11b and CD14, and PE-conjugated murine anti-human CD3 and CD64 were obtained from Invitrogen (Paisley, UK).

Volunteers, Patients and Setting

Healthy volunteers were recruited from University of Edinburgh staff, to act as a reference group for the cellular markers examined.

The clinical study took place in an 18-bed teaching hospital medical-surgical ICU. Critically ill patients, defined as those admitted to ICU and requiring support of one or more organ systems (invasive ventilation; requirement for vasopressors and/or inotropes; or haemofiltration) and predicted to require such support for 48 hours or more, were screened for recruitment. Exclusion criteria were: age <16; pregnancy; known human immunodeficiency virus (HIV) infection; known

in-born errors of neutrophil metabolism; hematological malignancy; use of immunosuppressive drugs other than corticosteroids; and those thought unlikely to survive for more than 24 hours. Patients were also excluded if they were involved in another study that involved blood sampling, or if they had suspected H1N1 influenza. Informed consent was obtained directly from patients where possible, otherwise informed consent was obtained from the next of kin. Clinical data were collected regarding potential risk factors for nosocomial infection [15], these data included 'shock', defined by requirement for noradrenaline, adrenaline and/or dobutamine infusion. EDTA anti-coagulated blood was collected at study enrolment (within 48 hours of ICU admission), then at study day 2, days 3-4 and days 6-10 unless a study end-point was achieved. Study end-points were - ICU-acquired infection (see Supplementary Section for definition); death without ICU-acquired infection; or discharge from ICU without ICU-acquired infection.

Details of flow cytometric protocols and analysis for determination of immune dysfunction are included in the supplemental section.

Infections

Diagnostic criteria were pre-defined for the major ICU-acquired infections, namely ventilator-associated pneumonia (VAP), blood stream infection (BSI), vascular catheter-related infection (CRI), urinary tract infection (UTI) and surgical site/soft tissue infections, based on those from the HELICS programme[21] (see supplemental section for details). Data on infections were recorded by the study nurses (JA and CM), who were blinded to the immune phenotype. Day of

infection was defined as the day on which positive microbial culture was obtained from the patient.

Where infection was strongly clinically suspected but did not fulfil HELICS criteria (for instance when cultures were taken whilst on antibiotics and/or cultures were negative/equivocal), an expert panel (IFL, AWH, DGS, TSW and AJS), blinded to the immune phenotype, reviewed patients' data and the presence or absence of infection was adjudicated. In the absence of positive cultures the day of infection was defined as the day of clinical deterioration. The adjudication outcome could be 'confirmed', 'probable' or 'unlikely' infection. Details of diagnostic criteria and expert panel adjudication procedures are set out in the Supplementary Section.

Statistical analysis

Analysis was conducted using Prism (Graphpad Software, La Jolla, CA, USA) and PASW Statistics Version 18 (IBM Corp, Armonk, NY, USA).

Contingency tables were analyzed by Fisher's exact test (for 2x2) and chi-squared (for >2x2). Continuous data that were not normally distributed were log-transformed to normality to permit parametric analysis, with one way or two way ANOVA used as appropriate. A Cox hazards model was constructed to examine the effects of immune dysfunctions and other clinical variables on acquisition of infection over time. Variables for inclusion in the final Cox model

were selected by step-wise conditional entry with a threshold of P≤0.05. P≤0.05 was considered statistically significant.

Ethical approval

Written informed consent was obtained from the patient, or where incapacitated from their nearest relative. The study was approved by the Scotland A Research Ethics committee (study number 09/MRE00/19). Healthy volunteers provided written informed consent, and their involvement was approved by Lothian Research Ethics committee (study number 08/S1103/38).

Data relating to C5a-mediated dysfunction in the first 60 patients described here have been published elsewhere in a paper delineating the mechanisms of C5a-dependent impairment of neutrophil phagocytosis and its clinical relevance [8].

Results

Recruitment

Ninety-six patients were recruited. Blood samples were missing for one patient and so 95 entered the final analysis. 42 (44%) patients were admitted with sepsis, 9 of whom had acquired their infection in hospital prior to ICU admission. Details of sites and organisms involved are shown in Tables S1 and S2 in the supplemental section.

Infections

Thirty-three patients (35%) developed nosocomial infection whilst in ICU (26 confirmed infections and 7 probable). Details of the sites of infections are shown in Table 1, infecting organisms are shown in Table 2. The median length of stay before developing infection was 6 days (IQR 5-7 days). In total 20 patients underwent adjudication panel review, 5 were ruled 'confirmed', 7 'probable' and 8 'unlikely'. Further details of adjudications can be found in the supplemental section. Amongst the patients admitted with sepsis, who subsequently developed ICU-acquired infection, the organisms differed in every case and in all but one case the site of the new infection was different (the exception being a patient admitted with Bronchoalveolar lavage PCR-positive Varicella pneumonitis who subsequently developed Gram negative bacterial VAP).

22 (23%) of patients died during their ICU admission, 11 (50%) were judged to have died of a septic insult, including 5 with an admission diagnosis of sepsis and 6 who developed secondary sepsis from an ICU-acquired infection.

Details of total and differential white cell counts at each time point are shown in the supplemental section (figure S2).

Regulatory T-cells

The percentage of Tregs at the various time points after study entry, compared to healthy donors Treg levels, were higher than those seen in healthy donors ($p=0.018$ by ANOVA, data not shown). When divided into patients developing nosocomial infection in the ICU and those not, Tregs were significantly higher amongst patients developing infection ($p=0.012$) (Figure 1A).

Monocyte deactivation

The expression of monocyte surface HLA-DR was significantly suppressed relative to healthy volunteers ($P<0.0001$ by ANOVA data not shown). Patients developing nosocomial infections had significantly lower levels of monocyte HLA-DR than those who did not ($p=0.018$) (Figure 1B).

Complement mediated neutrophil dysfunction

Significantly depressed levels of neutrophil CD88 were found in patients, relative to healthy donors ($P<0.001$ by ANOVA, data not shown). Again patients who subsequently developed nosocomial infection displayed lower levels of CD88, implying greater levels of C5a exposure ($p=0.001$) (Figure 1C). In keeping with our previous work showing the specific association between CD88 expression and neutrophil dysfunction [6,8], no association was found between nosocomial

infection and other markers of neutrophil activation (CD11b, CD64 and L-selectin, data not shown), suggesting that the effect seen is not reflective of generic activation. This is in contrast to other work suggesting a diagnostic role for CD11b and CD64 for infection[22] , however we examined the ability of markers to predict future infection rather than diagnose it after it had occurred.

Examining the cut-off for dysfunction by relationship to infection

At the planned interim analysis [8], the optimal cut-points were examined using ROC curves and Youden's index [23]; this revealed an optimal cut-point of 9.8% for Tregs, 10,000 molecules per cell for HLA-DR and a geometric mean fluorescence of 246 for CD88.

Thirty-seven patients had Treg levels >9.8% prior to achieving a study endpoint (i.e. ICU-acquired infection, death without infection or discharge without infection), the majority of whom (26, 70%) had normal levels on study admission. The remaining 58 patients had levels below 9.8%, although in 8 (14%) of them levels had started >9.8% and fallen prior to study endpoint. As noted above, in patients acquiring infection in ICU, data were censored for two days prior to infection for purposes of classifying Treg status. This changeability in Treg status led us to analyse this as a time-dependent variable in the Cox hazard analysis (see below). Of note many patients showed evidence of increased CD25 positive lymphocytes, a known marker of lymphocyte activation [24], reinforcing the need for FOXP3 staining in addition to cell surface markers of regulatory status [20].

62 patients had monocyte dysfunction whilst 69 had neutrophil dysfunction. In contrast to the Tregs, patients were far less likely to change from 'dysfunction' to 'no dysfunction' (or vice versa) with respect to monocyte HLA-DR and neutrophil CD88 expression. 75% of patients remained in the same monocyte group as their admission sample, whilst 13% progressed from 'no dysfunction' to 'dysfunction' and 14% went in the opposite direction. Of the minority who changed groups, all but 2 were in their eventual group by day 3 post-admission. Regarding neutrophil groups 84% of patients remained in the same group as their admission sample, whilst 13% progressed from 'no dysfunction' to 'dysfunction' and 3% went in the opposite direction. Of the minority who changed groups, all but 3 were in their eventual group by day 3 post-admission.

Effect of dysfunction on acquisition of infection

Those patients whose Tregs were above 9.8% had an increased risk of acquiring nosocomial infection, with a relative risk increase of 2.4 (95%CI 1.3-4.2, p=0.002 by Fisher's exact test). Monocyte deactivation, at the cut-off of 10, 000, was associated with a significantly increased risk of infection; relative risk of 3 (95%CI 1.3-6.9, p=0.0035 by Fisher's exact test). C5a-mediated neutrophil dysfunction was also associated with an increased relative risk of 4.7, (95% CI 1.2-18.3, P=0.007 by Fisher's exact test). In sensitivity analyses all three measures retained their significant values when 'probable' infections were excluded, as well as when urinary tract infections were excluded (data not shown).

Using these same cut-offs to examine other outcomes (all cause mortality and death from sepsis), none of the measures showed significant association with all-cause death (Tregs p=0.79, monocyte deactivation p=0.42 and neutrophil dysfunction p=0.2, all analyses by Fisher's exact test). When examining death from sepsis, only neutrophil dysfunction was significantly associated (p=0.03 by Fisher's exact test, Tregs p=0.09 and monocyte deactivation p=0.32).

Evaluation of the impact of other clinical variables on acquisition of infection

As an exploratory analysis data concerning demographic and clinical factors previously associated with nosocomial infection [15] were entered into a Cox hazards model, in a conditional stepwise approach using a threshold of $p \leq 0.05$ (Table 3). Elevated Tregs were treated as a time-dependent co-variate as this measure showed considerable variability over time. In this model both Tregs and neutrophil dysfunction retained their significant association (Table 4), however monocyte deactivation became non-significant (p=0.29) and was excluded from the final model. The only clinical predictor found to be significant was blood transfusion, which was associated with a lower risk of nosocomial infection. The overall model and hazard ratio estimates for the predictor variables are shown in table 4.

An alternative method of variable selection for the Cox model, using univariate regression resulted in Tregs and neutrophil dysfunction remaining significant at p=0.05 but again monocyte deactivation lost significance (Tables S3 and 4, supplemental section).

Finally the effect of cumulative occurrence of immune dysfunction was analyzed. Patients were analyzed by whether they had 0,1,2 or 3 dysfunctions (Table 5). This demonstrated an incremental risk of nosocomial infection with accumulating immune dysfunctions, ranging from none in those patients without immune dysfunctions to 75% of those who had all three (P=0.0004 by Chi^2 test for trend). Cumulative immune dysfunction did not predict all cause mortality (p=0.25 by Chi^2 test for trend), but was highly significant when looking at deaths from sepsis (p=0.0072 Chi^2 test for trend).

Effect of infection on dysfunction

To examine whether the development of ICU-acquired infection altered the measures of dysfunction, samples from time points before and after ICU-acquired infection were examined (Figure 2A-C) and demonstrated no significant change across the time intervals examined. Analysis of the measures of dysfunction in the first samples taken from patients with and without infection on admission revealed no significant difference between these two groups (% Tregs p=0.41 HLA-DR p=0.49 CD88 p=0.73, by t-test on log transformed data).

Discussion

This study demonstrates the temporal course of three measures of immune dysfunction amongst critically ill patients, illustrating that immune dysfunction is not an 'all or nothing' response and can affect different cell types at different

times. Although the magnitude of immune dysfunction appears to worsen as the critical illness progresses, in many cases patients demonstrate dysfunction early on (i.e. within 48 hours of ICU admission). Interestingly this study does not support the recent suggestion that immune dysfunction is restricted to patients with sepsis [12], as we found evidence of dysfunction amongst patients with both sterile and infective causes of critical illness.

This study demonstrates, for the first time, an association between elevated Tregs and the acquisition of nosocomial infection. The association with infection is also extended to diminished neutrophil CD88 expression, and both compare well to the more established marker, reduced monocyte HLA-DR [9].

This study involved assessment of several distinct mechanisms of immune dysfunction, and allowed for dynamic changes in immune cell function rather than relying on a single time point. Immunophenotyping in critical illness is a relatively new field, and determining what constitutes 'immune dysfunction' and how this could be determined by quantifying cell surface markers remains uncertain. This study, whilst exploratory in nature, has derived two potential new markers for susceptibility to nosocomial infection alongside a more established marker.

It is interesting that the cut-offs determined by examining patients with and without nosocomial infection were close to those suggested by more mechanistic work. A cut-off of 10% of CD4 cells was suggested as an indicator of inappropriately elevated Tregs in a recent study [14], very close to our value of

9.8%. Similarly the cut-off for CD88 of 246 arbitrary fluorescence units is close to the value of 250 which in our hands corresponded to 50% of neutrophils capable of efficient phagocytosis of zymosan in patients [8]. The cut off described here is also close to the value we observed when healthy volunteer neutrophils were exposed to 10nM C5a [8] which is well within the concentration range seen in severe sepsis [25]. Our cut-off for HLA-DR was higher than that described in previous studies [9] although the previous measure was looking at risk of death rather than development of nosocomial infection.

The infections had to meet rigorous, reproducible criteria and any cases not meeting these criteria underwent review by experts blinded to the immune cell data. Urinary tract infections (UTI) can be difficult to discern in critically ill patients, given the risk of bladder colonisation with catheterisation [26]. However those patients acquiring UTI had no clinical or microbiological evidence of infection from other sites and all exceeded the rigorous microbiological criteria set before the study (i.e. all UTIs were single organisms grown at high concentrations of $>10^6$ CFU ml^{-1}). Encouragingly, excluding UTIs or infections only judged 'probable' did not significantly reduce the predictive ability of our immune markers. These findings will, however, require further confirmation in an independent validation data set.

As with any observational study we cannot be certain that the observed associations are causative, however we took steps to minimize the risk of picking up epiphenomenal changes associated with infection. Indeed, analysis of the measures before and after the acquisition of infection did not support a simple,

epiphenomenal relationship. Furthermore, it is biologically plausible that immune dysfunction is causally linked to the acquisition of nosocomial infection. Although we cannot rule out the effect of residual confounding from unmeasured variables, we have accounted for many of the risk factors previously mooted for nosocomial infection [15].

Several studies have demonstrated elevated levels of Tregs in patients with sepsis [13, 14], and recent animal and *in vitro* work has shown Tregs mediating impaired T-cell proliferative responses in this disease [14]. The current study extends the findings of elevated Tregs to critically ill patients without sepsis, suggesting that their elevation is part of a stereotyped response to systemic inflammation rather than a specific response to severe infection. Although the association between elevated Tregs and adverse outcomes is consistent with some previous work [14], this finding is by no means universal with other studies showing no effect [27], or even protective effects in animal models[28]. It is also interesting to note the generally increased expression of CD25 noted on CD4 cells from critically ill patients, reflecting the nature of CD25 as a marker of lymphocyte activation [24] and emphasising the need for additional measures such as FOXP3 for identifying Tregs in this patient population. The field of regulatory T-cell identification is developing rapidly, and additional measures beyond those used in this study may further improve the predictive ability and add new information regarding expression of sub-sets of regulatory T-cells in critical illness [29]. Indeed use of CD127 negativity [14] as a marker would eliminate a step of the current process and produce a more rapidly available result, however these developments would require validation in a further study.

Although the finding of monocyte deactivation, and low HLA-DR expression, predicting nosocomial infection is not a new one [30, 31] the finding is not universally consistent [32]. The role of excessive complement activation in the pathogenesis of sepsis and non-septic critical illness is increasingly recognised [33,34]. Animal models of sepsis and trauma, and patient studies, have demonstrated C5a-mediated neutrophil dysfunction [6-8, 33-35].

To our knowledge this is the first study to examine several different cellular markers of immune dysfunction simultaneously at multiple time points, and to demonstrate a cumulative effect when it comes to predicting nosocomial infection. It remains a distinct possibility that the effects are not simply additive, but may indeed be synergistic. There is evidence of neutrophil subsets suppressing lymphocyte functions[36] and regulatory T-cells inhibiting neutrophil functions[37]. The current study was not designed to answer such questions, however investigation of potential interactions is the subject of ongoing studies.

It is interesting to speculate why our study did not show significant effects of many of the demographic and clinical factors that have been previously linked to nosocomial infection [15]. It is important to note that although there are a variety of acknowledged risk factors - including severity of illness, intubation, total parenteral nutrition and tracheostomy [38-44] – there is relatively low concordance between studies, which often yielded different combinations of factors [38, 42-44]. Furthermore many studies do not adequately account for the relationship between interventions such as tracheostomy, or total parenteral nutrition and

length of stay (i.e. duration of risk exposure), in that the longer a patient remains in ICU the more likely they are to receive one of these interventions [41,43], with the added confounder that those acquiring nosocomial infection tend to stay in ICU longer [42]. Many epidemiological studies of infection in ICU include all-comers, whereas in this study we deliberately recruited a group who were thought to be at high risk. All our patients had some form of invasive device in place, be it an endotracheal tube, central venous catheter or haemofiltration line, to facilitate the organ support that was an entry criterion. With a median APACHE II of 22 (IQR 18-28), these patients were a sicker subset of all ITU patients admitted to our unit. The apparent 'protective' effect of blood transfusion was an unexpected finding, as previous studies have suggested it as a risk factor for infection[43]. This could be a genuine effect resulting from hetrologous blood's immunostimulatory effects[45], or it may relate to failure to correct for unmeasured confounders.

Conclusions

This study has added new knowledge regarding the timing and magnitude of immune dysfunction occurring in critically ill adults, and related these findings to an important clinical outcome, namely the development of nosocomial infection. We have shown that these effects are not restricted to patients with sepsis but occur in those with sterile insults as well. We have also demonstrated

the utility of blood cell-based markers of immune dysfunction and thus set the scene for future validation and intervention trials.

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Declaration of interest

Conway Morris, Walsh and Simpson are co-applicants on a grant with Becton Dickinson Biosciences to fund development of flow cytometry-based markers of immune function. The application was submitted after completion of the work described here.

All other authors declare that they have no conflicts of interest related to this work.

Statement of authorship

ACM designed the study, obtained funding and performed the research and analysis, wrote the manuscript and approves the final version. NA designed the study, obtained funding, analyzed the data, revised the manuscript and approves the final version. MB performed the research, revised the manuscript and approves the final version. TS Wilkinson generated pilot data for the project and obtained funding, revised the manuscript and approves the final version. DFM modified the design of the study, revised the manuscript and approves the final version. JA recruited patients, collected data and approves the final manuscript version. CM recruited patients, collected data and approves the final manuscript version. LCB performed the research, revised the manuscript and approves the final version. KD performed the research, revised the manuscript and approves the final version. ROJ performed the research, revised the manuscript and approves the final version. CH obtained the funding, revised the manuscript and approves the final version. AWH designed the infection definitions, participated as an expert adjudication panel member, revised the manuscript and approves the final version. DGS designed the infection definitions, participated as an

expert adjudication panel member, revised the manuscript and approves the final version. IFL designed the infection definitions, participated as an expert adjudication panel member, revised the manuscript and approves the final version. DD designed the study, revised the manuscript and approves the final version. AGR designed the study, advised on technical aspects of experimental methods, revised the manuscript and approves the final version. TS Walsh designed the study, obtained funding, supervised the project, designed the infection definitions, participated as an expert adjudication panel member, revised the manuscript and approves the final version. AJS designed the study, obtained funding, designed the infection definitions, participated as an expert adjudication panel member, supervised the project, wrote the manuscript and approves the final version.

References

[1] Adibconquy M, Cavaillon J. Stress molecules in sepsis and systemic inflammatory response syndrome. *FEBS Lett* 2007;581:3723-3733.

[2] Rittirsch D, Flierl M, Ward P. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol* 2008;8:776-787.

[3] Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;420:885-91.

[4] Wang T, Deng J. Molecular and cellular aspects of sepsis-induced immunosuppression. *J Mol Med* 2008;86:495-506.

[5] Ahmed NA, McGill S, Yee J, Hu F, Michel RP, Christou NV. Mechanisms for the diminished neutrophil exudation to secondary inflammatory sites in infected patients with a systemic inflammatory response. *Crit Care Med* 1999;27:2459-2468.

[6] Conway Morris A, Kefala K, Wilkinson TS, et al. C5a mediates peripheral blood neutrophil dysfunction in critically ill patients. *Am J Respir Crit Care Med* 2009;180:19-28.

[7] Huber-Lang M, Younkin EM, Sarma JV, et al. Complement-induced impairment of innate immunity during sepsis. *J Immunol* 2002;169:3223-31.

[8] Conway Morris A, Brittan M, Wilkinson TS, et al. C5a-mediated neutrophil dysfunction is RhoA-dependent and predicts infection in critically ill patients *Blood* 2011;117:5178-5188.

[9] Meisel C, Schefold JC, Pschowski R, et al. GM-CSF to Reverse Sepsis-associated Immunosuppression: A Double-blind Randomized Placebo-controlled Multicenter Trial. *Am J Respir Crit Care Med* 2009;180:640-8.

[10] 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;166:6952-6963.

[11] Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 2003;348:138-50.

[12] Boomer JS, To K, Chang KC, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA* 2011; 21:2594-605.

[13] Venet F, Chung CS, Monneret G, et al. Regulatory T cell populations in sepsis and trauma. *J Leukoc Biol* 2008;83:523-35.

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

[15] Vincent JL. Nosocomial infections in adult intensive-care units. *Lancet* 2003;361:2068-77.

[16] Prevention of bacterial infection in neutropenic patients with hematologic malignancies. A randomized, multicenter trial comparing norfloxacin with ciprofloxacin. The GIMEMA Infection Program. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto *Ann Intern Med* 1991;115:7-12.

[17] Chastre J, Fagon JY. Ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2002;165:867-903.

[18] Baker AM, Meredith JW, Haponik EF. Pneumonia in intubated trauma patients. Microbiology and outcomes *Am J Respir Crit Care Med* 1996;153:343-349.

[19] Vallés J, Pobo A, García-Esquirol O, Mariscal D, Real J, Fernández R. Excess ICU mortality attributable to ventilator-associated pneumonia: the role of early vs late onset. *Intensive Care Med* 2007;33:1363-8.

[20] Langier S, Sade K, Kivity S. Regulatory T cells: The suppressor arm of the immune system. *Autoimmunity Rev* 2010;10:112-5.

[21] Hospitals in Europe Link for Infection Control through Surveillance protocol available from http://helics.univ-lyon1.fr/home.htm accessed 09/06/2012.

[22] Davis BH. Improved diagnostic approaches to infection/sepsis detection. *Exp Rev Mol Diag*. 2005;5:193-207

[23] Youden WJ. Index for rating diagnostic tests. *Cancer* 1950;3:32-5.

[24] Urdal DL, March CJ, Gillis S, Larsen A, Dower SK. Purification and chemical characterization of the receptor for interleukin-2 from activated human T lymphocytes and from a human T cell lymphoma cell line. *Proc. Natl. Acad. Sci USA* 1984;81:6481-5.

[25] Solomkin JS, Jenkins MK, Nelson RD, et al. Neutrophil dysfunction in sepsis. II. Evidence for the role of complement activation products in cellular deactivation. *Surgery* 1981;90:319-27.

[26] Garibaldi RA, Burke JP, Dickman ML, Smith CB. Factors predisposing to bacteriuria during indwelling urethral catheterization. *N Engl J Med* 1974;291:215-9.

[27] Hein F, Massin F, Cravoisy-Popovic A, et al. The relationship between CD4+CD25+CD127- regulatory T cells and inflammatory response and outcome during shock states. *Crit Care*. 2010;14(1):R19. Epub 2010 Feb 15.

[28] Heuer JG, Zhang T, Zhao J, Ding C, Cramer M, et al. 2005 Adoptive transfer of in vitro-stimulated CD4+CD25+ regulatory T cells increases bacterial clearance

and improves survival in polymicrobial sepsis. *J Immunol* 2005; 174: 7141–7146.

[29] Miyara M, Yoshioka Y, Kitoh A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 2009;19;30:899-911.

[30] Landelle C, Lepape A, Voirin N, et al. Low monocyte human leukocyte antigen-DR is independently associated with nosocomial infections after septic shock. *Intensive Care Med* 2010;36:1859-66.

[31] Asadullah K, Woiciechowsky C, Döcke W, et al. Immunodepression following neurosurgical procedures. *Crit Care Med* 1995;23:1976-83.

[32] Lukaszewicz AC, Grienay M, Resche-Rigon M, et al. Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction. *Crit Care Med* 2009;37:2746-52.

[33] Ward PA. The dark side of C5a in sepsis. *Nat Rev Immunol* 2004;4:133-42.

[34] Amara U, Kalbitz M, Perl M, et al. Early expression changes of complement regulatory proteins and C5A receptor [CD88] on leukocytes after multiple injury in humans. *Shock* 2010;33:568-75.

[35] Unnewehr H, Rittirsch D, Sarma JV et al. Changes and Regulation of the C5a Receptor on Neutrophils during Septic Shock in Humans. J Immunol. 2013 Mar 11. [Epub ahead of print]

[36] Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1.
J Clin Invest 2012; 122:327–336.

[37] Lewkowicz P, Lewkowicz N, Sasiak A, Tchórzewski H. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. *J Immunol* 2006;177:7155-63.

[38] Pratikaki M, Platsouka E, Sotiropoulou C, et al. Risk factors for and influence of bloodstream infections on mortality: a 1-year prospective study in a Greek intensive-care unit. *Epidemiol Infect* 2008;137:727-735.

[39] Vincent JL, Bihari DJ, Suter PM, Bruining HA, et al. The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care [EPIC] Study. EPIC International Advisory Committee. *JAMA* 1995;274:639-44.

[40] Crowe M, Ispahani P, Humphreys H, Kelley T, Winter R. Bacteraemia in the adult intensive care unit of a teaching hospital in Nottingham, UK, 1985-1996. *Eur J Clin Microbiol Infect Dis* 1998;17:377-84.

[41] Sugerman HJ, Wolfe L, Pasquale MD, et al. Multicenter, randomized, prospective trial of early tracheostomy *J Trauma* 1997;43:741-747.

[42] Moro ML, Viganò E, Cozzi Lepri A. Risk factors for central venous catheter-related infections in surgical and intensive care units. The Central Venous Catheter-Related Infections Study Group. *Infect Control Hosp Epidemiol* 1994;15:253-64.

[43] Levine J, Kofke A, Cen L, et al. Red blood cell transfusion is associated with infection and extracerebral complications after subarachnoid hemorrhage. *Neurosurgery* 2010;66:312-8.

[44] Beyersmann J, Gastmeier P, Grundmann H, et al. Transmission-associated nosocomial infections: prolongation of intensive care unit stay and risk factor analysis using multistate models. *Am J Infect Control* 2008;36:98-103.

[45] Brunson ME, Ing R, Tchervenkov JI, Alexander JW. Variable infection risk following allogeneic blood transfusions. *J Surg Res* 1990;48:308-12.

Figure legends

Figure 1. Measures of immune dysfunction by subsequent infection status

Panel A: T-regs as a percentage of all CD4+ T_H cells. p=0.012 for difference between outcomes, p=0.025 for difference over time by two way ANOVA.

Panel B: Monocyte HLA-DR expression. p=0.018 for difference between outcomes, p=0.56 for difference over time by two way ANOVA.

Panel C: Neutrophil CD88 expression p=0.0034 for difference between outcomes, p=0.001 for difference over time by two way ANOVA.

Data shown as mean and 95% confidence intervals. Analyses performed on log-transformed data.

PE, phycoerythrin; geoMCF, geometric mean cell fluorescence.

Figure 2. Changes in measures of immune dysfunction before and after ICU-acquired infection (n=33).

Panel A: Tregs as a percentage of all CD4+ve lymphocytes, p=0.28 by ANOVA.

Panel B: Monocyte HLA-DR expression, p=0.56 by ANOVA.

Panel C: Neutrophil CD88 expression, p=0.95 by ANOVA.

All data shown as mean and 95% CI, all analyses performed on log-transformed data. Hatched line indicates the 'cut-off' for immune dysfunction.

PE, phycoerythrin; geoMCF, geometric mean cell fluorescence.

Confirmed infections	
	Pneumonia-10 (7 VAP)
	Blood stream infections-4
	Catheter-related blood stream infections-3
	Urinary tract infections-5
	Surgical site/soft tissue infections-4
Probable infections	
	Pneumonia-4 (all VAP)
	Intra-abdominal infection-3

Table 1. Site of infections acquired in ICU.

Infection category	Organism	Frequency
Confirmed	*Staphylococcus aureus*	3
	Coagulase negative *Staphylococci*	1
	Streptococcus pneumoniae	1
	Other *Streptococci*	1
	Enterococcus faecalis	2
	Burkholderia cepacia	1
	Citrobacter braakii	1
	Coliform –no further specification	1
	Enterobacter cloacae	3
	Escherichia coli	5
	Klebsiella pneumoniae	2
	Haemophilus influenzae	1
	Pseudomonas aeruginosa	3
	Anaerobes	1
	Candida albicans	4
	Herpes simplex	1
Probable	*Staphylococcus aureus*	1
	Culture negative	4
	No samples taken as care withdrawn	2
Unlikely	*Staphylococcus aureus*	2
	Coagulase negative *Staphylococci*	2
	Streptococcus pneumoniae	1
	Acinetobacter baumannii	1
	Haemophilus influenzae	1
	Klebsiella pneumoniae	1
	Candida albicans	1
	Culture negative	2

Table 2. Culture results from patients with confirmed, suspected and unlikely infections.

More than one organism was isolated from some patients.

	Variable	Infection	No infection	P value for Cox hazards analysis
Pre-morbid factors	Number of co-morbid conditions by Charlson index, median (IQR)	3 (1.5-4)	3 (1-5)	0.08
	% with chronic lung disease	19%	9%	0.62
	% with diabetes mellitus	18%	19%	0.36
	Age, median (range)	60 (20-88)	59 (16-85)	0.34
	% male	61%	63%	0.06
Admission factors	Admission SOFA score, median (IQR)	9 (6.5-11)	8 (4-10)	0.11
	Admission APACHE II Score, median (IQR)	21 (18-26)	23.5(18.5-29)	0.26
	% fulfilling criteria for shock on admission	76%	56%	0.28
	% admitted following surgery	30%	23%	0.33
	% admitted following trauma	1%	5%	0.79
Illness course/interventions prior to endpoint achievement	Peak SOFA score, median (IQR)	9 (8-12.5)	8 (5-11)	0.29
	% intubated	94%	88%	0.22
	% with central venous catheter	90%	88%	0.19
	% receiving renal replacement therapy	42%	33%	0.23
	% with tracheostomy	8%	6%	0.30
	% with urinary catheter	92%	97%	0.30
	% receiving H_2	100%	98%	0.70

	antagonist/proton pump inhibitor			
	% receiving corticosteroids	30%	23%	0.74
	% receiving a blood transfusion	52%	44%	0.002*
	% receiving total parenteral nutrition	27%	18%	0.92
Immune profiling	% with elevated Tregs	60%	29%	0.026*
	% with low CD88	94%	62%	0.009*
	% with low HLA-DR	85%	55%	0.29

Table 3. Demographic and clinical factors amongst those with and without nosocomial infection.

Right hand column indicates p value for hazard ratios determined during stepwise conditional evaluation, variables marked * entered the final model (see table 4 below).

Variable	P value	**Hazard Ratio (95% CI)**
Overall model	0.001	NA
*Elevated Tregs	0.026	2.4 (1.1-5.4)
Neutrophil dysfunction	0.009	6.9 (1.6-30)
Blood transfusion	0.002	0.3 (0.1-0.6)

Table 4. Cox model for occurrence of nosocomial infection.

*****Elevated Treg cells were expressed as a time-dependent co-variate.

NA = not applicable.

Number of dysfunctions [a]	N=	% acquiring nosocomial infection (95% CI)
0	11	0 (0-0%)
1	21	10% (0-22%)
2	43	37% (23-52%)
3	20	75% (56-94%)

Table 5. Relationship between burden of immune dysfunction and acquisition of nosocomial infection.

([a]i.e. neutrophil dysfunction (as indicated by low CD88), monocyte deactivation (as indicated by low HLA-DR) and elevated regulatory T-cells). P=0.0004 by Chi squared test for trend.

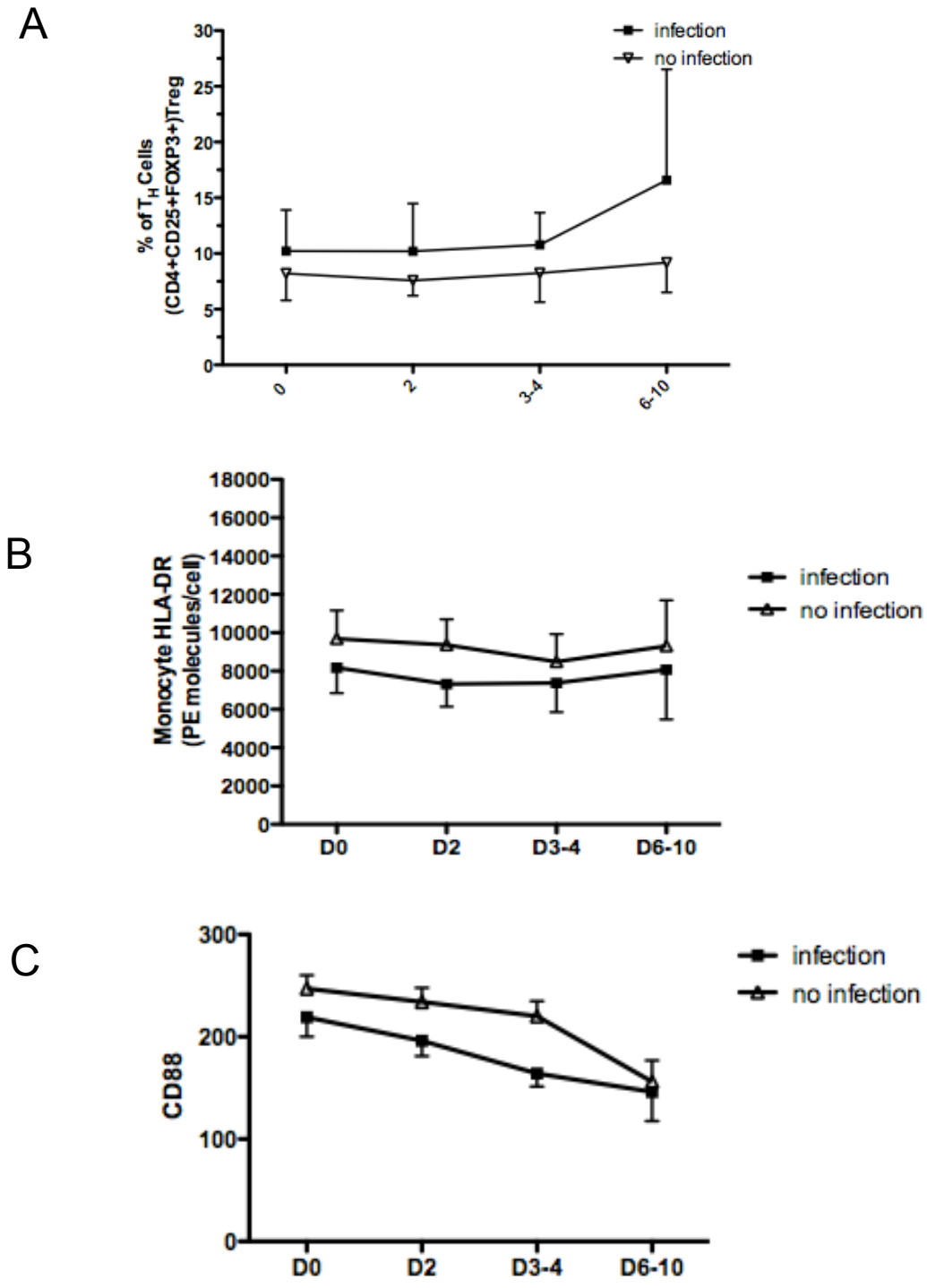

Figure 1: Measures of immune dysfunction by subsequent infection status
X axis indicates time after study enrolment (Day 0)
Panel A: T-regs as a percentage of all CD4+ T_H cells. p=0.012 for difference between outcomes, p=0.025 for difference over time by two way ANOVA.
Panel B: Monocyte HLA-DR expression. p=0.018 for difference between outcomes, p=0.56 for difference over time by two way ANOVA
Panel C: Neutrophil CD88 expression p=0.0034 for difference between outcomes, p=0.001 for difference over time by two way ANOVA
Data shown as mean and 95% confidence intervals. Analyses performed on log-transformed data.

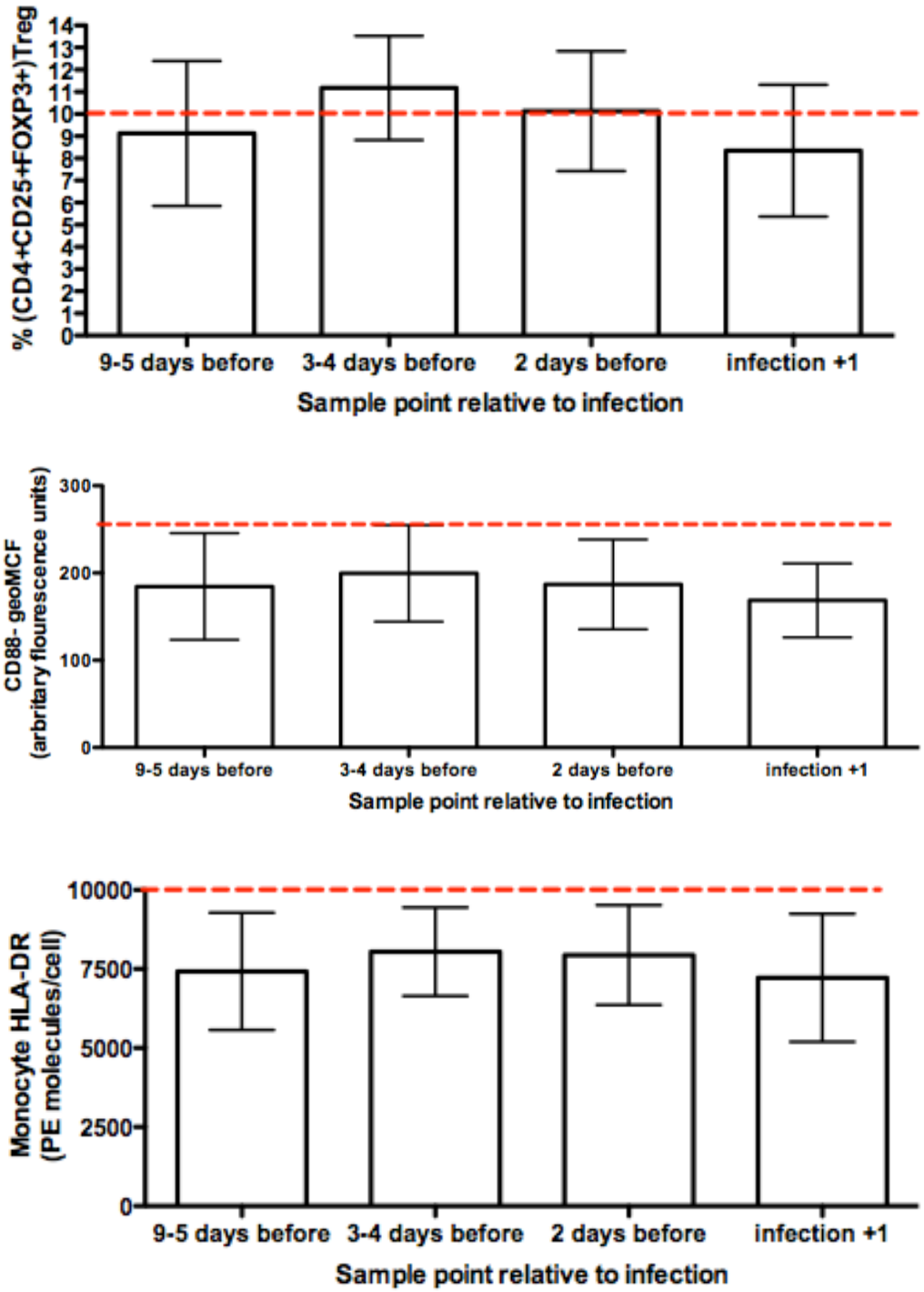

Figure 2:Changes in measures of immune dysfunction before and after ICU-acquired infection (n=33 patients).

Panel A: Tregs as a percentage of all CD4+ve lymphocytes, p=0.28 by ANOVA

Panel B: Monocyte HLA-DR expression, p=0.56 by ANOVA

Panel C: Neutrophil CD88 expression, p=0.95 by ANOVA

All data shown as mean and 95% CI, all analyses performed on log-transformed data. Hatched line indicates the 'cut-off' for immune dysfunction.

Supplemental methods – definition of infection

These criteria were based on those provided by HELICS (main text reference 21).

Any new infection occurring after 48 hours of ICU admission was deemed 'ICU-acquired'. For consistency infections arising within 48 hours of ICU discharge were deemed 'ICU-acquired'.

Infections were defined prior to the start of the study as follows, based on the HELICS criteria.

*a) Ventilator-associated pneumonia***:** Requires radiographic, clinical and microbiological criteria to be met:

i. Radiological criteria.
CXR or CT scan showing new infiltrates, or worsening infiltrates without evidence of pulmonary oedema, and either pyrexia of >38^{o}C or white cell count >12 x10^{9} L^{-1} or <4x10^{9} L^{-1}.

These must be combined with one or more clinical criteria.

ii. Clinical criteria.

- Worsening oxygenation – any increase in FiO_2 to maintain PaO_2 target, or an increase in PEEP, frequency or tidal volume, proning or paralysis to facilitate ventilation.

- Relevant clinical chest findings – auscultatory finding of crepitations, crackles or decreased air entry.

- Increased/changed sputum – any increase in volume, presence of muco-purulent or muco-purulent-bloody sputum.

iii. Microbiological criteria.

The above radiological and clinical criteria must be combined with positive quantitative BAL culture of $>10^4$CFU ml^{-1} or positive pleural fluid or pulmonary/pleural abscess culture.

Where the diagnosis of VAP has been suggested by mini-BAL, endotracheal aspirate or where growth is below the 10^4 CFU ml^{-1} threshold or without any positive microbiology, adjudication is required.

Hospital-acquired pneumonia (HAP), i.e. nosocomial pneumonia in non-mechanically ventilated patients, requires the same fulfilment of criteria as VAP except that sputum cultures with heavy growth of a single organism constitute a confirmed infection.

b) Catheter-associated infections

Positive culture (semi-quantitative >15CFU) from an indwelling vascular line combined with either

- Local inflammation and pus (catheter-related infection (CRI)) or
- Improvement of inflammatory markers within 48 hours of removal (CRI) or

- Culture of the same organism from a peripheral blood culture (catheter-related blood stream infection (CRBSI)).

c) Blood stream infection

One positive culture of a typical pathogen, coupled with evidence of systemic inflammation (WCC >12 $x10^9$ L^{-1} or <4$x10^9$ L^{-1}, temperature ≥ 38^oC).

d) Urinary tract infection

Growth of 2 or fewer organisms at ≥ 10^5 CFU ml^{-1} combined with evidence of systemic inflammation (WCC >12 $x10^9$ L^{-1} or <4$x10^9$ L^{-1} , temp >38^oC or shock without another identifiable cause).

e) Soft-tissue or surgical site infection

Evidence of pus/inflammation at site of presumed infection combined with a positive culture.

Suspected infections which did not meet these criteria were referred to the consensus panel for adjudication. The panel was constituted from a pool of five experienced doctors, 3 intensivists (DGS, AWH,TSWalsh), 1respiratory physician (AJS) and 1 microbiologist (IFL), all of whom had at least 12 years post-qualification experience.

Consensus panel members were asked to come to an independent decision as to whether an infection was 'confirmed', 'probable' or 'unlikely'.

A 'confirmed' infection was where the panel member was convinced that infection was present and would definitely initiate antibiotic treatment and/or pursue source control. Positive microbial antigen detection (e.g. by Gram film or PCR), microbial cultures above an accepted threshold (i.e. $>10^4$ CFU ml^{-1} from BAL sample), microbial cultures from a normally sterile site or serology confirming a probable pathogen was obligatory, with other evidence of infection.

A 'probable' infection is where the panel member thought there was, on the balance of probabilities, an infection present and would consider antibiotic treatment and/or source control if the patient's clinical condition merited it. This category could include positive microbial cultures. An example would be culture of a classically non-pathological organism (e.g. single cultures of coagulase negative cocci or diphtheroids) associated with clinical evidence of infection/systemic inflammation.

An 'unlikely' infection is where the panel member thought there was a low probability of infection and would not consider antibiotic treatment and/or source control. Although positive microbial cultures could be included in this, this would be culture of a classically non-pathological organism (e.g. single cultures of coagulase negative cocci or diphtheroids) without evidence of systemic inflammation/infection or mixed growth of commensal organisms.

Systemic evidence of infection would require the presence of SIRS – specifically 2 or more of the following: heart rate>90 beats per minute, WCC>12 $x10^9$ L^{-1} or <4

$x10^9$ L^{-1}, respiratory rate >20 breaths per minute, or mechanical ventilation and temperature of >38°C or <36°C. Additional evidence to consider would include reports of large numbers of neutrophils on sample microscopy, and clinical examination findings of pus or inflamed tissue.

'Unlikely' infection combined with a positive microbial culture would constitute colonization.

The consensus panel was constituted by two members drawn from the pool, and asked to arrive at an independent opinion in one of the three categories above. Where there was agreement the verdict stood, where there was disagreement the panel members met to try and agree a consensus view. If this failed a third member was drawn from the pool and asked to make an opinion between the two options selected by the initial panel members.

Flow cytometry strategy

Flow cytometry was conducted on whole blood/EDTA samples. Neutrophils, monocytes and lymphocytes were identified by their size and granularity (forward and side scatter respectively) characteristics with confirmation that these populations were predominantly $CD16^{hi}$ (neutrophils), $CD14^{hi}$(monocytes) and $CD3_{pos}$ (lymphocytes).

Tregs were identified by CD4, CD25, FOXP3 positivity (main text reference 20), using the manufacturer's instructions for staining and permeabilisation, and expressed as a percentage of total CD4 (T_{helper}) lymphocytes.

The flow cytometer (FACSCalibur, BD Bioscience, Oxford, UK) was calibrated weekly using caliBRITE^{tm} beads (3 colour and APC, BD Biosciences), linearity and

sensitivity by 8 peak beads (Spherotech, Lake Forrest, IL, USA). QuantiBRITE^{tm} beads (BD Bioscience) were run for quantification of HLA-DR-PE expression (see supplemental section, Figure S1).

Analysis plan and immune dysfunction

To allow cellular analysis to be dichotomised into 'dysfunction' or 'no dysfunction', the cut-off points for percentage of Tregs, monocyte HLA-DR and CD88 were examined in a planned interim analysis at 60 patients (main text reference 8) . This analysis was performed by constructing receiver operator characteristic (ROC) curves comparing the sample most temporally related to infection (censored for two days prior) with samples from patients who did not develop infection, using Youden's method (main text reference 23) to determine the optimal cut-off.

Patients were categorized by the sample taken most proximally to an end-point (death, infection, or discharge without infection) although in the case of those acquiring infection dysfunction was censored for 2 days prior to the diagnosis of infection in order to reduce the risk that observed dysfunction levels might reflect the presence of new infection. Patients were also analyzed to determine whether their immune dysfunction status changed during admission.

Supplemental results

Detail regarding adjudication panel results

Of the non-confirmed adjudications, 6 of the 7 'probable' infections had either negative cultures whilst on antibiotics, with other strong clinical evidence of infection or did not have cultures taken as care was being withdrawn but with strong clinical evidence. The remaining 'probable' patient grew bacteria below the 10^4 colony forming units per ml cut off for quantitative culture of bronchoalveolar lavage fluid. Of the 'unlikely' infections, two had negative cultures with no potential focus of infection identified, 5 had positive cultures without any evidence of infection (and were classified as 'colonisation') and 1 had persistence of the same organism cultured on admission with a change in antibiotic sensitivities.

Leucocyte counts in patients

Patients had elevated total white cell counts on admission (mean 14.1 $x10^9\ L^{-1}$, 95% CI 12.4-15.9 $x10^9\ L^{-1}$, normal range (NR) 4.0-12.0 $x10^9\ L^{-1}$). Neutrophil counts were also elevated (mean 11.8 $x10^9\ L^{-1}$, 95% CI 10.2-13.4, NR 2-7.5 $x10^9\ L^{-1}$). Lymphocyte counts were suppressed (mean 1.1 $x10^9\ L^{-1}$, 95% CI 0.9-1.2 $x10^9\ L^{-1}$, NR 1.5-4 $x10^9\ L^{-1}$), whilst mean monocyte counts were within the normal range (mean 0.7 $x10^9\ L^{-1}$, 95% CI, 0.6-0.9 $x10^9\ L^{-1}$, 0.2-0.8 NR $x10^9\ L^{-1}$). When stratified by patients subsequently developing infection or not, total white cell count was significantly more elevated in patients developing infection, with this effect persisting when samples were censored for two days prior to infection. The increased white cell count was mostly due to neutrophilia (see Figure S2A and B). By contrast lymphocyte and monocyte numbers didn't differ

between the groups, although both showed significant rises over time (Figure S2C and D).

Data relating to patients who had evidence of infection prior to admission to ICU

The relevant site of infection is described in Table S1, and the range of organisms isolated in Table S2.

Alternative modelling for effects of immune dysfunctions and other clinical variables on acquisition of infection over time.

An alternative model to that described in Tables 3 and 4 of the main manuscript was employed. The alternative model used univariate regression analysis and entering variables with a p value of ≤0.1. Low CD88 expression and elevated Tregs retained significance in this model (Tables S3 and S4).

Calibration of flow cytometer

Example data from the QuantiBRITE beads from two calibration runs are shown in figure S1.

Changes in differential leucocyte count

Changes in total leucocyte and differential counts over time, dichotomised by infection outcome, are shown in figure S2.

Source of Sepsis	Number of patients	Number hospital-acquired
Pneumonia	17	6
Intra-abdominal collection	8	1
Pelvic collection	1	0
Urinary tract	4	1
Soft tissue infection	5	0
Central nervous system	2	0
Blood stream (no other source identified)	4	1
Endocarditis	1	0

Table S1. Source of sepsis present at admission to ICU.

Organism	N
Staphylococcus aureus	6
Group A Streptococcus	3
Haemophilus influenzae	3
Escherichia coli	9
Citrobacter braakii	1
Enterobacter aerogenes	1
Acinetobacter baumannii	1
Candida albicans	2
Enterovirus	1
Varicella zoster	1
Influenza virus A	1
Parainfluenza virus	1
Culture negative	15

Table S2. Organisms cultured from patients admitted to ICU with sepsis.

Some patients had more than one organism isolated.

	Variable	Infection	No infection	P value for univariate analysis
Pre-morbid factors	Number of co-morbid conditions by Charlson index, median (IQR)	3 (1.5-4)	3 (1-5)	0.44
	% with chronic lung disease	19%	9%	0.2
	% with diabetes mellitus	18%	19%	0.89
	Age, median (range)	60 (20-88)	59 (16-85)	0.88
	% male	61%	63%	0.82
Admission factors	Admission SOFA score, median (IQR)	9 (6.5-11)	8 (4-10)	0.17
	Admission APACHE II Score, median (IQR)	21 (18-26)	23.5(18.5-29)	0.5
	% fulfilling criteria for shock on admission	76%	56%	0.07
	% admitted following surgery	30%	23%	0.39
	% admitted following trauma	1%	5%	0.05
Illness course/interventions prior to endpoint achievement	Peak SOFA score, median (IQR)	9 (8-12.5)	8 (5-11)	0.18
	% intubated	94%	88%	0.88
	% with central venous catheter	90%	88%	0.70
	% receiving renal replacement therapy	42%	33%	0.37
	% with tracheostomy	8%	6%	0.7
	% with urinary catheter	92%	97%	0.35
	% receiving H_2 antagonist/proton pump inhibitor	100%	98%	0.14

	% receiving corticosteroids	30%	23%	0.58
	% receiving a blood transfusion	52%	44%	0.42
	% receiving total parenteral nutrition	27%	18%	0.26
	Length of stay prior to infection, median (IQR)	6(5-7)	5(3-8)	0.22
Immune profiling	% with elevated Tregs	60%	29%	0.002
	% with low CD88	94%	62%	0.001
	% with low HLA-DR	85%	55%	0.004

Table S3. Demographic and clinical factors amongst those with and without nosocomial infection.

Right hand column indicates p value for univariate analysis (regression for continuous/ordinal variables and phi coefficient for binary categorical variables).

Variable	P value	**Hazard Ratio (95% CI)**
Overall model	0.037	NA
Shock on admission	0.51	1.3 (0.6-3.2)
Admission with trauma	0.65	0.75 (0.2-2.6)
*Elevated Tregs	0.05	2.1 (1.0-4.2)
Neutrophil dysfunction	0.05	4.0 (1.0-19.1)
Monocyte deactivation	0.07	2.4 (0.92-6.5)

Table S4. Cox model for occurrence of nosocomial infection.

*****Elevated Treg cells were expressed as a time-dependent co-variate.

NA = not applicable.

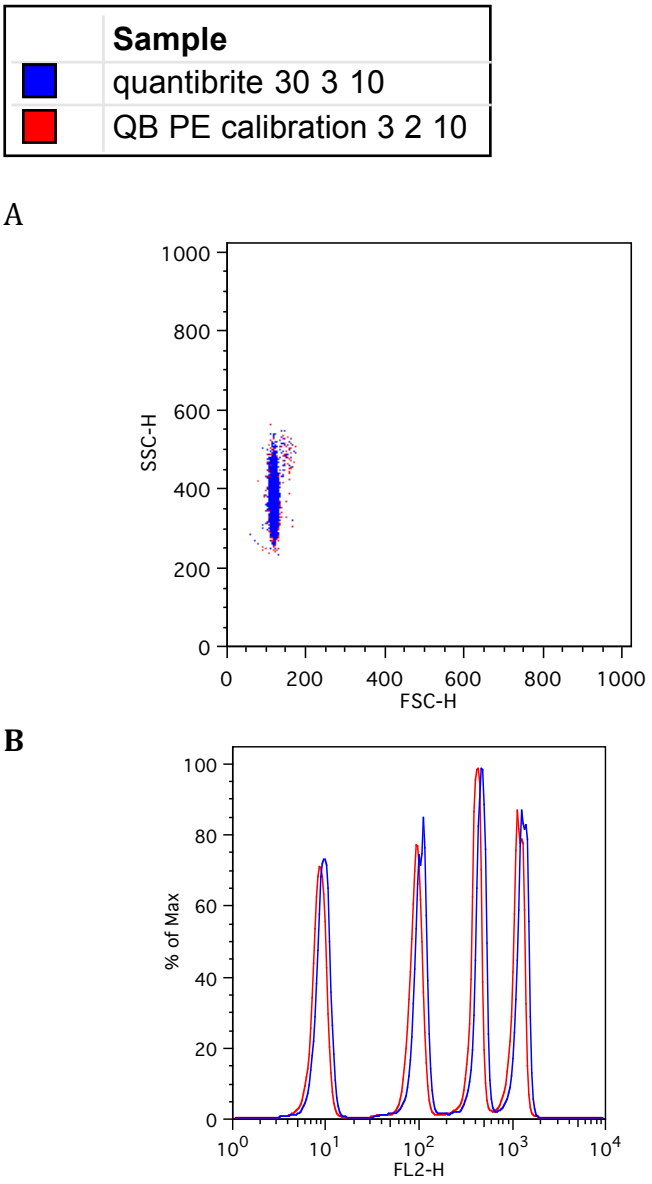

Figure S1 Example overlaid flow plots of QuantiBRITE beads, used to quantify HLA-DR-PE expression.

Panel A shows Forward (FSC) and Side (SSC) scatter, Panel B shows histograms.

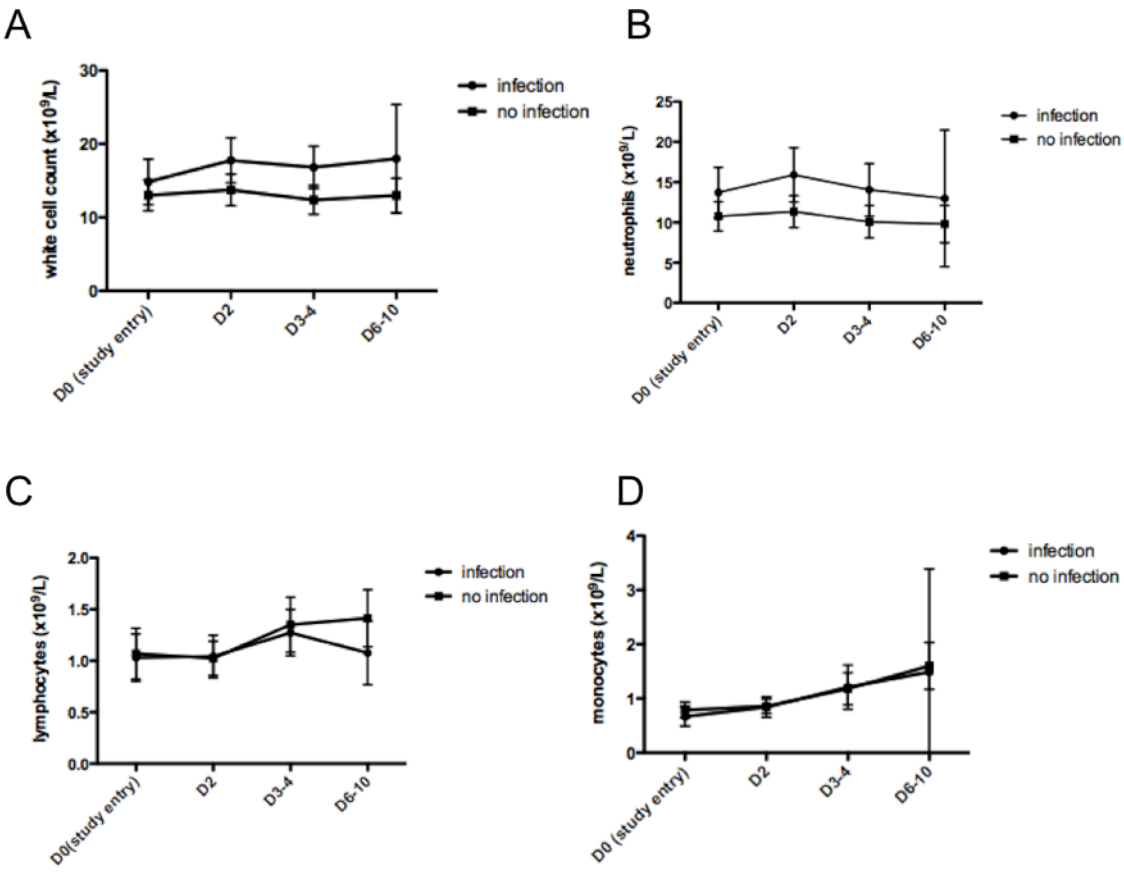

Figure S2: changes in total and differential white cell counts during study amongst patients developing and not developing ICU-acquired infection.
A: Total white cell count, p=0.0006 for difference between outcomes and p=0.43 for difference over time by two way ANOVA
B: Neutrophils, p 0.0014 for difference between outcomes and p=0.77 for difference over time by two way ANOVA
C: Lymphocytes, p=0.92 for difference between outcomes and p=0.0003 for difference over time by two way ANOVA
D: Monocytes, p=0.64 for difference between outcomes and p=0.0001 for difference over time by two way ANOVA
Data shown as mean and 95% confidence intervals, analyses performed on log-transformed data