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

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

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3 **Combined dysfunctions of immune cells predict nosocomial infection in**
4 **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%

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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: Neutrophils, monocytes, regulatory T-lymphocytes, critical illness, nosocomial infection

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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].

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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].

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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).

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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

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3 in-born errors of neutrophil metabolism; hematological malignancy; use of
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5 immunosuppressive drugs other than corticosteroids; and those thought
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7 unlikely to survive for more than 24 hours. Patients were also excluded if they
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9 were involved in another study that involved blood sampling, or if they had
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11 suspected H1N1 influenza. Informed consent was obtained directly from
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13 patients where possible, otherwise informed consent was obtained from the next
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15 of kin. Clinical data were collected regarding potential risk factors for
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17 nosocomial infection [15], these data included 'shock', defined by requirement for
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19 noradrenaline, adrenaline and/or dobutamine infusion. EDTA anti-coagulated
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21 blood was collected at study enrolment (within 48 hours of ICU admission), then
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23 at study day 2, days 3-4 and days 6-10 unless a study end-point was achieved.
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25 Study end-points were - ICU-acquired infection (see Supplementary Section for
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27 definition); death without ICU-acquired infection; or discharge from ICU without
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29 ICU-acquired infection.
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36 *Flow cytometry and Immune dysfunction*
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38 Flow cytometry was conducted on whole blood/EDTA samples. Neutrophils,
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40 monocytes and lymphocytes were identified by their size and granularity
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42 (forward and side scatter respectively) characteristics with confirmation that
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44 these populations were predominantly $CD16^{hi}$ (neutrophils), $CD14^{hi}$(monocytes)
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46 and $CD3_{pos}$ (lymphocytes).
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48 Tregs were identified by CD4, CD25, FOXP3 positivity [20], using the
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50 manufacturer's instructions for staining and permeabilisation, and expressed as
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52 a percentage of total CD4 (T_{helper}) lymphocytes.
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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).

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.

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Analysis plan

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 [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 [22] 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.

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

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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 $P \leq 0.05$. $P \leq 0.05$ 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].

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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 BAL 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.

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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 in supplemental section). By contrast lymphocyte and monocyte numbers didn't differ between the groups, although both showed significant rises over time (Figure S2C and D).

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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, although the difference only became significant between days 6-10 (Figure S3A supplemental section). 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).

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Monocyte deactivation

The expression of monocyte surface HLA-DR at various time points after study entry, in contrast to the Tregs, were suppressed relative to healthy volunteers at every time point from study enrolment onwards (Figure S3B supplemental section). 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, at all time points examined except on the first sample (Supplemental Figure S3C). 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 previous work [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 specific to CD88/C5a exposure and not reflective of generic activation.

Examining the cut-off for dysfunction by relationship to infection

At the interim analysis [8], the optimal cut-points were examined using ROC curves and Youden's index [22]; 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

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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 [23], 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.

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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

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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

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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].

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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 [24]. 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

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patients, given the risk of bladder colonisation with catheterisation [25]. 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

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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 [26], or even protective effects in animal models[27]. 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 [23] 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 [28]. 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.

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Although the finding of monocyte deactivation, and low HLA-DR expression, predicting nosocomial infection is not a new one [29, 30] the finding is not universally consistent [31]. The role of excessive complement activation in the pathogenesis of sepsis and non-septic critical illness is increasingly recognised [32,33]. Animal models of sepsis and trauma, and patient studies, have demonstrated C5a-mediated neutrophil dysfunction [6-8, 34].

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,

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but may indeed be synergistic. There is evidence of neutrophil subsets suppressing lymphocyte functions[35] and regulatory T-cells inhibiting neutrophil functions[36]. 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 [37-43] – there is relatively low concordance between studies, which often yielded different combinations of factors [37, 41-43]. 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 [40,42], with the added confounder that those acquiring nosocomial infection tend to stay in ICU longer [41]. 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[42]. This could be a genuine effect resulting from hetrologous blood's

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immunostimulatory effects[44], 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.

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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.

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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.

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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.

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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.

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	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

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	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

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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).

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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.

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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 immuno-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.

For Peer Review

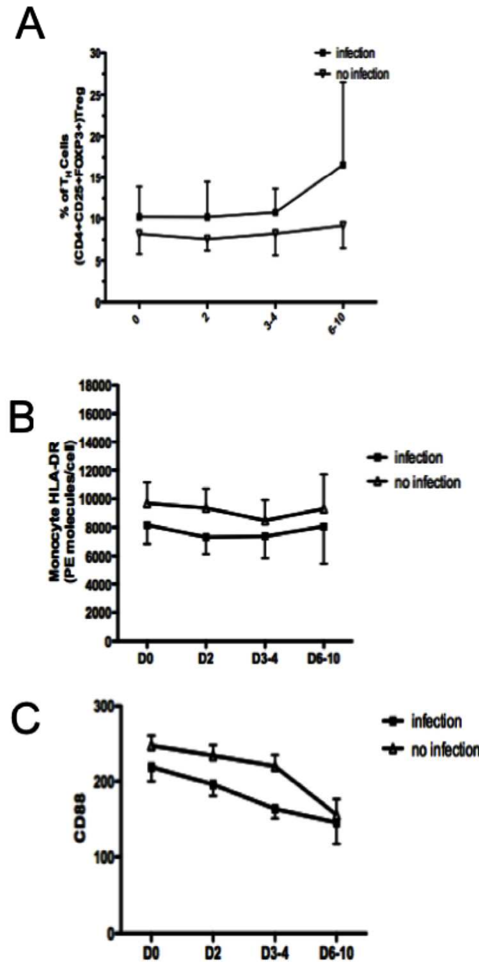

Figure 1. Measures of immune dysfunction by subsequent infection status
Panel A: T-regs as a percentage of all CD4+ TH cells. p=0.012 for difference between outcomes, p=0.025 for difference over time by two way ANOVA.
Panel B: Monocyte HLA-DR expression. p=0.018 for difference between outcomes, p=0.56 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.
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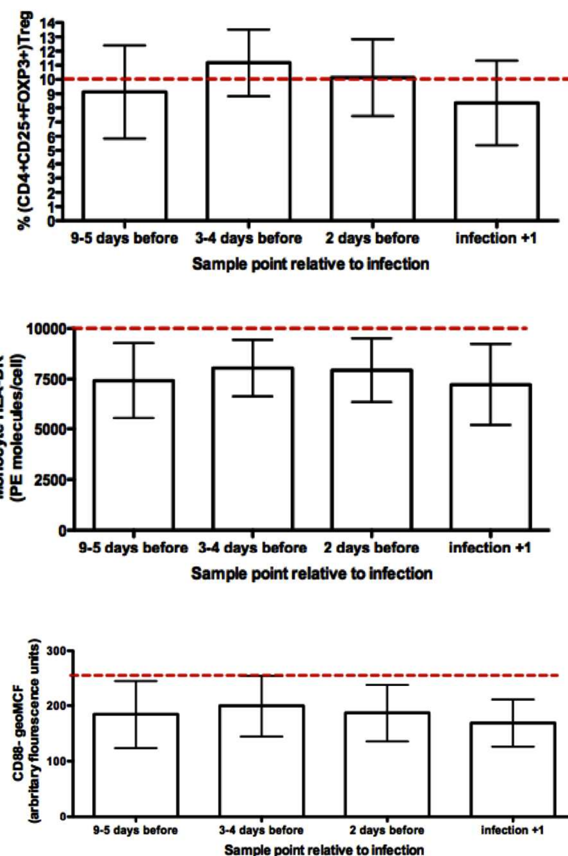

Figure 2. Changes in measures of immune dysfunction before and after ICU-acquired infection (n=33).
Panel A: Tregs as a percentage of all CD4+ve lymphocytes, p=0.28 by ANOVA.
Panel B: Monocyte HLA-DR expression, p=0.56 by ANOVA.
Panel C: Neutrophil CD88 expression, p=0.95 by ANOVA.
All data shown as 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.

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