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Temporal changes in erythroid progenitors in critically ill patients: a prospective cohort study

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Data sharing statement: Requests for data should be made to the corresponding author and will be considered on an individual basis by the study group. Each request requires a research proposal including a clear research question and proposed analysis plan. Requests will be considered on an individual basis and are subject to review and relevant approvals.

Patients being cared for in an intensive care unit (ICU) display the hallmarks of anaemia of inflammation, characterised by hypoferremia, impaired erythropoiesis and decreased erythrocyte survival (1,2). Such defects in erythropoiesis and iron homeostasis associate with worse longer-term consequences for health and quality of life (3). Recent advances in transcriptional, phenotypic and functional single-cell sorting analyses enable identification and isolation of Haematopoietic Stem and Progenitor cells (HSPCs) from peripheral blood obviating the need for invasive bone marrow sampling (4,5). HSPCs differentiate into erythroid cells in a step-wise fashion through the intermediate multi-progenitor stages (MPP) and sequentially into the common myeloid progenitor (CMP), which gives rise to the bipotent megakaryocyte-erythroid progenitor (MEP). These in turn produce lineage-committed erythroid cells (Burst-Forming-Units-Erythroid (BFU-Es) and Colony-Forming-Units-Erythroid (CFU-Es), derived from Early Erythroid Progenitors (EEPs) and Late Erythroid Progenitors (LEPs)), which undergo the final stages of terminal erythroid differentiation to produce erythrocytes *(Online Supplementary Figure S1A).*

We aimed to determine the feasibility of assessing peripheral blood-derived subpopulations of erythroid progenitors from ICU patients during acute illness and recovery using advanced multi-colour flow cytometry, cell sorting and clonogenic assays.

We conducted a prospective observational cohort study at Oxford University Hospitals NHS Foundation Trust, Oxford, UK (approved by NHS South Central – Oxford C (18/SC/0545) and extended by Central University (R49648/RE002) Research Ethics Committees), enrolling adult patients (N=30, ≥16 years old) expected to require >72 hours of ICU care between March 19, 2019 and February 20, 2020. Inclusion and exclusion criteria are shown in *Online Supplementary Table* S1. Peripheral blood samples (40-50 mL) were collected within 72 hours of ICU admission (D0) and 28 (± 7) days post-enrolment (D28); samples from healthy controls were collected every 4-6 weeks. $CD34^+$ HSPCs were isolated from peripheral blood using Ficoll-Histopaque (Sigma) and Human $CD34^+$ microbead kit (Miltenyi Biotec) prior to cryopreservation. After thawing, $CD34^+$ HSPCs were stained with 12 fluorophore-conjugated monoclonal antibodies (*Online Supplementary Table S2*) (6) and frequencies of specific progenitor subsets were determined by flow cytometry (*Online Supplementary Figure S1B*). For assessment of functional capacity, single MEPs were sorted into 96-well plates containing Methocult and grown for 14 days prior to imaging or flow cytometric analysis using myeloid- and erythroid-specific cell surface markers (*Online Supplementary Figure S1C and Table S2*).

The primary outcome was successful isolation of $>1x10^5$ $CD34^+$ HSPCs from peripheral blood – a minimum number to enable analysis of sub-compartments giving rise to erythroid lineages (7). Secondary outcomes included assessment of BFU-E numbers, which reflect erythroid output, and clinical/biological factors associating with impaired erythropoiesis.

Baseline clinical characteristics and laboratory data at enrolment for all patients are displayed in **Table 1**. Mean (SD) APACHEII score was 21.1 (7.1) suggestive of high illness severity. Participants displayed the hallmarks of the anaemia of inflammation as evidenced by high CRP and ferritin concentrations, coupled with low Hb, transferrin saturation and serum iron concentrations (**Table 1)**. All but two participants were receiving invasive mechanical ventilation at D0. Clinical characteristics of participants from whom paired D28 samples were obtained (n=17) were similar to those of all

participants (*Online Supplementary Table S1*)**.** Four patients remained in hospital at D28. Patients did not receive supplementary iron or erythropoietin therapy during the study.

Greater than $1x10^5$ $CD34^+$ HSPCs were successfully extracted from 44/47 (93.6%) blood samples from study participants (mean (SD) cell count of 3.75 (2.83) $x10^5$) (**Table 2)**. Changes in progenitor frequencies between study timepoints are displayed in **Figure 1.** ICU patients had a higher proportion of haematopoietic stem cells (HSCs) at D0 compared to controls, albeit not reaching statistical significance; HSC proportions returned to baseline levels by D28 **(Figure 1A)**. Lymphoid-primed Multipotent Progenitor (LMPP) frequencies were significantly lower than controls at both timepoints and Granulocyte-Macrophage Progenitors (GMPs) were significantly reduced at D0; Common Myeloid Progenitors (CMPs) were increased at D0 compared with controls, while Multipotent Progenitor (MPP) and MEP frequencies were not different (**Figure 1A-F)**. These data suggest that critical illness may be associated with early changes in haematopoietic progenitor compartments. However, since proportions of MEPs were unaltered, reductions in frequencies of these erythroid progenitors may not be the primary cause of anaemia in critical illness.

Therefore, we assessed whether the functional potential of the MEPs was influenced by ICU admission. We sorted individual MEPs from controls (n=7) and 17 pair-matched ICU participants at D0 and D28 into methylcellulose and cultured them for 14 days; plating efficiency was marginally lower from D0 MEPs, but D28 plating efficiency was not different to controls **(Figure 1G)**. While numbers of GM colonies remained equivalent (**Figure 1H**), we observed reduced numbers of erythroid BFU-E colonies from ICU patients at both timepoints compared to controls (**Figure 1I)**. Although not statistically significant (p=0.0726), the number of BFU-E colonies was higher at D28 compared with enrolment in ICU patients. We used immunophenotyping (*Online Supplementary Figure S1)* as an orthogonal method to show that there was a reduction in the number of EEPs (**Figure 1J**) - the cells that give rise to BFU-Es (4) – consistent with a reduction in erythroid output in ITU patients at day 0. However, LEPs that give rise to CFU-Es were unaffected (**Figure 1K).** We found weak evidence of correlation between BFU-E forming potential of the MEPs in colony forming assays and immunophenotypically defined EEP frequency (**Figure 1L)**. In an exploratory analysis, we found no effect of an admission diagnosis of sepsis/septic shock on plating efficiency compared with patients without sepsis, although those with sepsis showed increased BFU-Es at D28 *(Online Supplementary Figure S1D).* Overall, this suggests that reduced erythroid output in ICU patients may relate to qualitative defects in MEPs.

The key findings of this study are: (i) $CD34^+$ HSPCs can be isolated in sufficient numbers from peripheral blood of ICU patients for evaluation of bone marrow activity; (ii) critical illness associates with early changes in progenitor compartments that may reflect reduced proliferation and lineage skewing, and qualitative defects may underly reduced erythroid output; (iii) there is weak evidence of recovery of BFU-E colonies at D28 compared with enrolment in ICU patients towards levels observed in controls. To our knowledge, the extent and combination of alterations in haematopoiesis we describe over the course of acute illness and recovery have not been reported previously in ICU patients.

In health, HSPCs undergo stepwise differentiation becoming progressively more restricted in lineage potential, ultimately producing unipotent or bipotent progenitors (8). Murine studies indicate that

inflammation affects bone marrow reprogramming, promoting myelopoiesis and lymphopoiesis at the expense of erythropoiesis (9). Direct bone marrow sampling from critically ill patients has previously revealed evidence of iron dysregulation, erythroid progenitor growth suppression, erythropoietin (EPO) dysfunction and excessive apoptosis (10-12). In one study, patients with septic shock showed reduced BFU-Es when compared with those without sepsis, similar to our exploratory analysis (10). Interestingly, growth capacity of BFU-Es in serum from patients with septic shock could be restored *in vitro* by culture by recombinant erythropoietin (rHuEPO) supplementation (10). In another study, bone marrow functional capacity assessed by BFU-E and CFU-E colony formation was significantly reduced, despite a marked increase in erythroid and myeloid progenitor numbers in peripheral blood (12). Progenitor egress from the bone marrow to the periphery could deplete the bone marrow of hematopoietic precursors, reducing bone marrow erythropoietic potential.

Plausible interventions to treat anaemia in ICU patients include red blood cell (RBC) transfusion, rHuEPO and/or intravenous iron (13). Intravenous iron would increase iron availability for erythroid development, but it is unclear how efficiently or directly this would support BFU-E numbers. The addition of rHuEPO may accelerate erythropoietic recovery. BFU-Es express high levels of erythropoietin receptor (EpoR), and EPO signalling supports proliferation, survival and differentiation. Similarly, MEPs also express EpoR and respond to exogenous EPO (14). Our data show that evaluating the response to such therapeutic options during recovery from critical illness by measuring the erythropoietic profile from peripheral blood is feasible. This in turn would allow for assessment of biomarkers of therapeutic response, aiding tailoring of therapies to the patients most likely to respond.

Limitations of our study include the small sample size, data from a single institution, lack of complete follow-up for all participants, and lack of direct comparison with bone marrow samples from the same patients. Although bone marrow sampling is the reference standard, this poses practical and ethical challenges. While it is well accepted that non-mobilised peripheral blood contributes to blood production in stress and disease, the true clinical potential of peripheral blood HSPCs compared to those from bone marrow remains unclear (15). Additionally, changes in concentrations of hepcidin, associated inflammatory cytokines (e.g. IL-6) and apoptotic markers were not measured in our study.

Through assessment of the $CD34^+$ HSPC compartment from peripheral blood of ICU patients, we conclude that critical illness is associated with progenitor alterations including reduced proliferation and lineage skewing. These changes appear within the first 72 hours and can persist for 3-4 weeks. Patients recovering from critical illness will demonstrate the hallmarks of anaemia of inflammation but demonstrate functional and immunophenotypic recovery of BFU-Es. Further research is needed to determine whether the changes observed are modified by treatments for anaemia.

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Table 1. Baseline characteristics of study cohort (n = 30)

Characteristic	Value
Age (years), median (IQR)	55 (46 - 60)
Sex, n (%)	
Male / Female	18 (60) / 12 (40)
BMI (kg/m^2), median (IQR)	26.1 (23.1 - 33.0)
Charlson Comorbidity Index, n (%)	
0	10 (33.3)
1	7 (23.3)
>2	13 (43.4)
APACHE II score, mean (SD)	21.2 (7.1)
Admission diagnoses, n (%)	
Elective operation	2 (6.7)
Emergency operation	13 (43.3)
Medical	15 (50)
No. with septic shock, n (%)	12 (40)
Organ support requirements, n (%)	
Advanced respiratory support	28 (93.3)
Advanced cardiovascular support	12 (40)
Advanced renal support	5 (16.7)
PaO_2 / FiO_2 ratio (mmHg)	159.5 (128 – 233)
Baseline laboratory parameters (Normal range), median (IQR)	
Haemoglobin (120 - 170), g/l	90 (76 – 103)
White cell count (4.0 – 11.0), x 10^9/l	13.9 (11.4 – 17.58)
Neutrophil count (1.5 – 10.0), x 10^9/l	11.5 (9.5 – 15.1)
Platelet count (150 – 400), x 10^9/l	219 (128 – 296)
Reticulocyte A (0.04 – 0.12), x 10^{12}/l	0.04 (0.02 – 0.06)
Reticulocyte % (0.5 – 2.5)	1.45 (1.05 – 1.95)
Creatinine (49.0 – 104), umol/l	87 (64 – 152)
Albumin (32 – 50), g/l	19 (16 – 22)
CRP (0 – 5), mg/l	226.3 (144 – 364.4)
Ferritin (10 – 300), mcg/l	642.3 (393.2 – 2057.5)
Iron (11 – 31), mmol/l	3.65 (2.6 – 5.55)
Tsat (16 – 50), %	15 (9.5 – 25)
Transferrin (1.8 – 3.6), g/l	0.99 (1.1 – 1.4)
EPO (5.0 – 25.0), IU/l	44.6 (22.2 – 65.4)
B_{12} (180 – 900), ng/l	820 (556 – 1895)
Folate (3.0 – 20.0), mcg/l	4.35 (3 – 5.4)
Vitamin D (50 – 250), nmol/l	13 (20.5 – 35)
ICU mortality, n (%)	2 (6.7%)
ICU length of stay (days), median (IQR)	11.5 (7 – 18)
Time between ICU admission and 1st sample (days), median (IQR)	3 (3 – 6)
Time between 1st sample and follow-up sample (days), median (IQR)	26 (20 – 68)

APACHEII, Acute Physiology and Chronic Health Evaluation; BMI, Body mass index; CRP, C-reactive protein; EPO, erythropoietin; ICU, Intensive care unit; IQR, interquartile range; SD, standard deviation. Data were analysed on STATA 14 (College Station, Texas, USA). None of the included patients received a red blood transfusion prior to enrolment.

Table 2. Summary statistics of number of $CD34^+$ cells extracted from all samples.

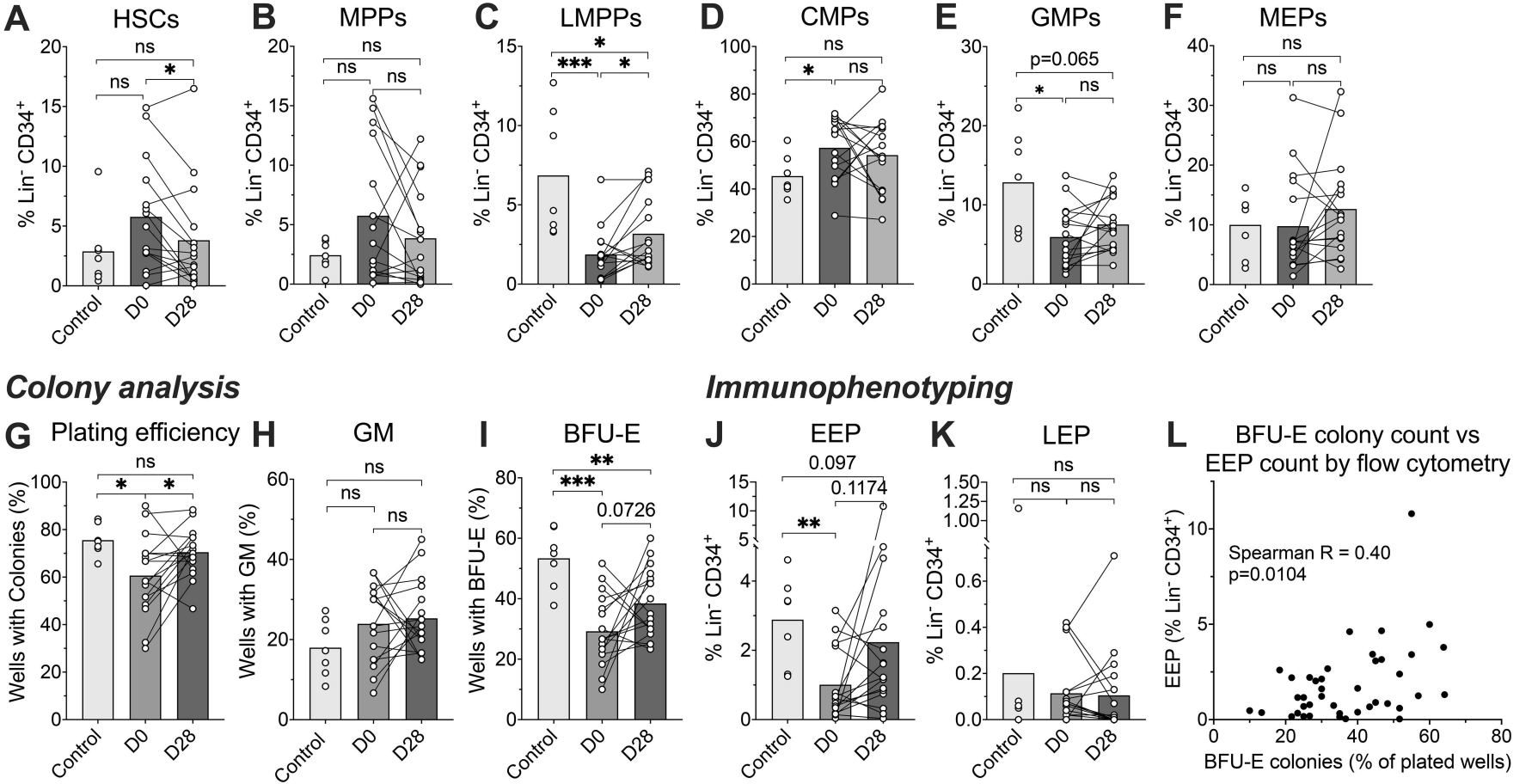
$CD34^+$ cell count ($x10^5$)	**Participants (n=48)**	**Healthy controls (n=7)**
Mean (SD)	3.75 (2.83)	6.10 (5.6)
SEM	0.41	2.12
Geometric mean (SD)	2.88 (2.11)	4.25 (2.51)
95% CI	2.32 – 3.58	1.8 – 9.99
Minimum	0.5	1.31
Maximum	15.0	17.00
Range	14.5	15.69

CI, Confidence Interval; SEM, Standard error of the mean; SD, Standard deviation

Figure legends

Figure 1: Characterisation of haematopoietic progenitors from intensive care unit (ICU) patients by flow cytometry, clonogenic assays, and flow cytometry assessment of Megakaryocyte-Erythroid Progenitor (MEP) erythroid output, indicate changes in progenitor compartments at admission to ICU. (A-F) Individual immunophenotyping data showing the frequency of (A) Haematopoietic Stem Cells (HSCs), (B) Multipotent Progenitors (MPPs), (C) Lymphoid-primed Multipotent Progenitors (LMPPs) (D) Common Myeloid Progenitors (CMPs), (E) Granulocyte-Macrophage Progenitor (GMPs) and (F) MEPs as % of Lin^- $CD34^+$ in 17 pair-matched samples from ICU patients as compared to control samples (G-I) Colony forming capacity of single index-sorted MEPs showing (G) Plating efficiency of MEPs from controls (n=7) and participants (n=17 matched pairs); (H, I) number of Burst-Forming Unit- Erythroid (BFU-Es) and myeloid colony-forming unit-granulocyte macrophage (GM) colonies grown from MEPs 14 days after single-cell sorting into methylcellulose from controls, or pair-matched participants at D0 or D28; (J, K) Proportion of immunophenotypic early erythroid progenitors (EEPs) that give rise to BFU-Es, and immunophenotypic late erythroid progenitors (EEPs) that give rise to Colony Forming Units-Erythroid (CFU-E), in controls and participants at D0 and D28. (L) Correlation of visually scored BFU-Es from (2I) with the numbers of EEPs determined immunophenotypically using flow cytometry (from 2J). *Statistics*: (A-K) Mann-Whitney test for comparison with control and Wilcoxon matched-pairs test for comparison of pair matched samples; *p<0.05, **p<0.01, ***p<0.001, ns: p>0.05; (L) Spearman rank correlation. Data were analysed on STATA 14 (College Station, Texas, USA) and Prism 9 and 10 (GraphPad software); statistical significance was defined as $p < 0.05$.

Figure 1


Immunophenotyping
A HSCs
B MPPs
C LMPPs
D CMPs
E GMPs
F MEPs
% Lin- CD34+
Control
D0
D28
ns
*
***
p=0.065
Colony analysis
G Plating efficiency
H GM
I BFU-E
Wells with Colonies (%)
Wells with GM (%)
Wells with BFU-E (%)
**
0.0726
Immunophenotyping
J EEP
K LEP
0.097
0.1174
L BFU-E colony count vs EEP count by flow cytometry
Spearman R = 0.40
p=0.0104
EEP (% Lin- CD34+)
BFU-E colonies (% of plated wells)

Online Data Supplement

Supplementary Table S1

Table S1A - Detailed inclusion and exclusion criteria;

Inclusion criteria

- ICU patients (≥16 years old), both with capacity and without capacity (for medically induced reasons such as therapeutic sedation), who have required at least 72 hours of adult ICU care

Exclusion criteria

- Active haematological malignancy
- Chemotherapy or myelosuppressive treatment within last 30 days
- Documented or suspected HIV infection
- Received massive transfusion during index admission defined as 'replacement of >1 blood volume in 24 hours or >50% of blood volume in 4 hours'.
- Palliative care intent
- Participants who are lacking capacity for non-medically induced reasons e.g. dementia
- Death is imminent or likely during this admission
- Patients residing outside a reasonable geographic follow-up area (defined as within 30 miles of the John Radcliffe Hospital, Oxford, UK)

S1B - Characteristics of study cohort with paired samples (n=17)

Characteristic	Value
Age (years)	58 (50 – 63)
Sex, n (%)	
Male	8 (47.0)
Female	9 (53.0)
BMI ($kg.m^{-2}$)	27.0 (23.5 – 35.9)
Functional Comorbidity Index, n (%)	
0	5 (29.5)
1	4 (23.5)
2	4 (23.5)
>3	4 (23.5)
APACHEII score	21.8 (6.1)
Admission diagnoses, n (%)	
Elective operation	0(0.0)
Emergency operation	9 (53.0)
Medical	8 (47.0)
No. with septic shock, n (%)	8 (47.0)
Organ support requirements, n (%)	
Advanced respiratory support	16 (94.1)
Advanced cardiovascular support	9 (53.0)
Advanced renal support	13 (76.4)
PaO_2 / FiO_2 ratio (mmHg)	160 (128 – 248)

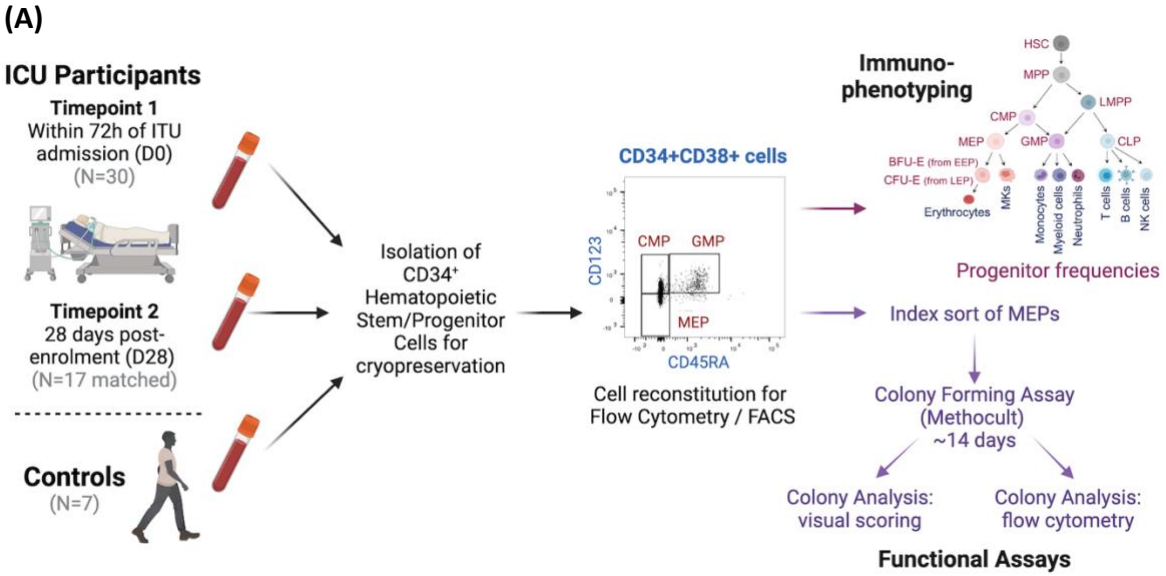
ICU length of stay, days	9 (6 – 17)

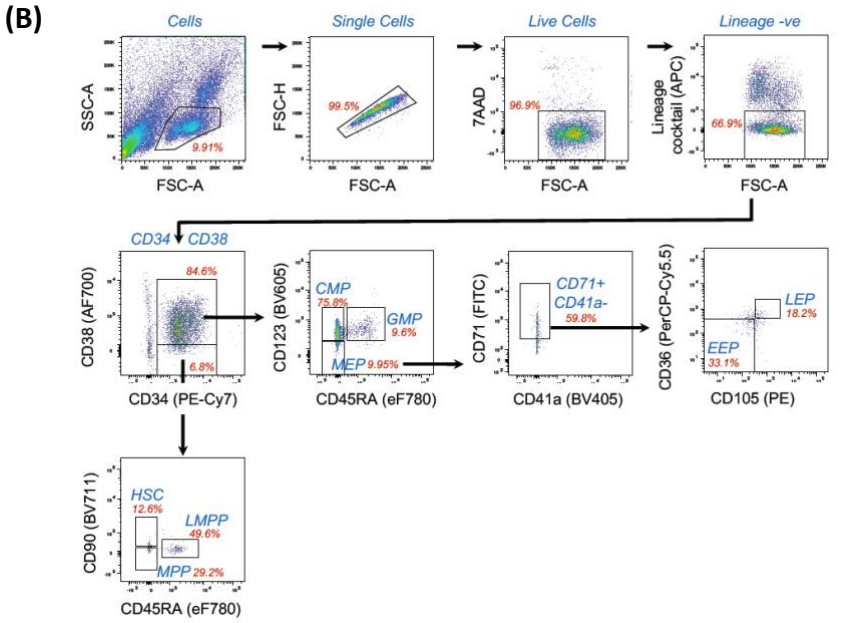
APACHEII, Acute Physiology and Chronic Health Evaluation; BMI, Body mass index; CRP, C-reactive protein; EPO, erythropoietin; ICU, Intensive care unit; IQR, interquartile range; SD, standard deviation

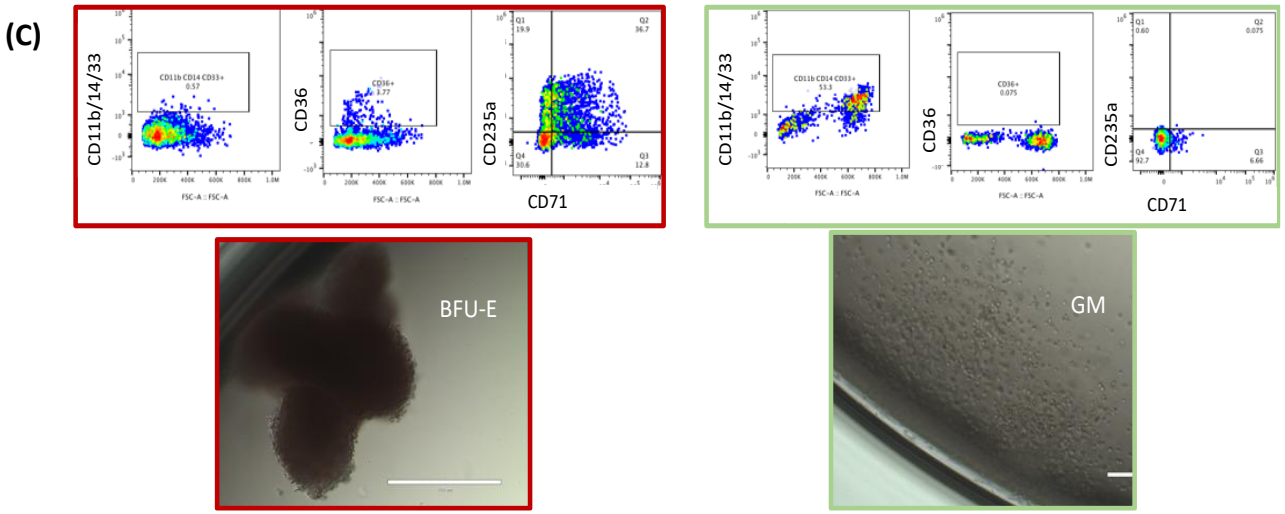
Supplementary Table S2: Details of antibodies used

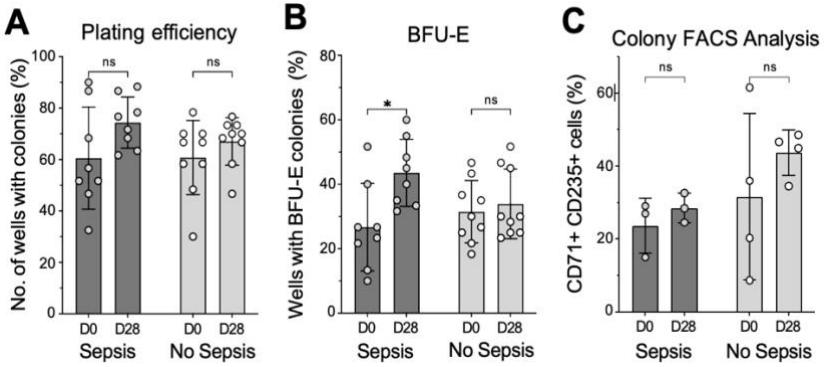
Anti-human antibodies used for analysis and sorting			
Antibody	Clone	Fluorochrome	Supplier
CD34	4H11	PE-Cy7	eBioscience
CD36	CB38	PerCP-Cy5.5	BD Biosciences
CD71	OKT9	FITC	eBioscience
Lineage Cocktail (CD2, CD3, CD14, CD16, CD19, CD56, CC235)	n/a	APC	eBioscience
CD105	43A3	PE	Biolegend
CD38	HIT2	AF700	eBioscience
CD123	7G3	BV605	BD Biosciences
CD235a	HIR2	PerCp-Cy5	Biolegend
CD45RA	Hl100	APC-eFluor780	eBioscience
CD90	5E10	BV711	BD Biosciences
CD41a	HIP8	AlexaFluor405	eBioscience
Viability dye	na	7AAD	Life Technologies
Antibodies used for FACS analysis of colonies			
CD235	HIR2	PE	Life Technologies
CD71	CY1G4	PE-Cy7	Biolegend
CD36	CB38	APC	BD Bioscience
CD11b	ICRF44	FITC	eBioscience
CD14	6ID3	FITC	Biolegend
CD33	P67.6	FITC	Biolegend
Viability dye	na	Hoechst	Invitrogen

Supplementary Figure S1









Supplementary Figure

S1A Overview of the experimental strategy.

$CD34^+$ haematopoietic and stem progenitor cells (HSPCs) were extracted from controls (n=7) or study participants within 72 hours of admission to an intensive care unit (ICU) (D0 baseline samples) or 28 days later (D28) and cryopreserved. We had seven controls in total – all of whom were healthy volunteers (4 males, 3 females, age range 24-48), and were recruited from our research facility. Four of these volunteers (all male) provided blood samples every 4-6 weeks in parallel with batch analysis of study participants' samples. Peripheral blood was also collected from the remaining three volunteers (all female) at one timepoint. Cells were immuno-stained with a 12-colour fluorochrome panel. For frequency analysis, proportions of different cellular subsets were determined from flow cytometry plots with gates set using Fluorescence-minus-one controls and single stains. For functional analysis, Megakaryocyte-Erythroid Progenitors (MEPs) were index sorted into 96 well plates containing Methocult; colonies were allowed to grow for 14 days before being imaged and selected for FACS analysis to determine colony lineage. *Abbreviations*: HSC = Haematopoietic Stem Cell; MPP = Multipotent Progenitor; CMP = Common Myeloid Progenitor; LMPP = Lymphoid-primed Multipotent Progenitor; MEP = Megakaryocyte-Erythroid Progenitor; GMP = Granulocyte-Monocyte Progenitor ; CLP = Common Lymphoid Progenitor; BFU-E = Blast-forming Unit - Erythroid ; EEP = Early Erythroid Progenitor; CFU-E = Colony-forming Unit - Erythroid; LEP = Late Erythroid Progenitor; MKs = Megakaryocytes; NK = Natural Killer.

S1B FACS gating/sorting strategy.

$CD34^+$ HSPCs were immuno-stained with a 12 colour fluorochrome panel and gates were set using FMOs and single stains.

S1C Colony analysis by FACS.

Representative examples of a BFU-E and GM colony grown for 14 days in Methocult from single cell sorted MEPs (defined by flow cytometry as Lin^- $CD34^+$ $CD38^+$ $CD45RA^-$ $CD123^-$) (A) Flow cytometric analysis shows expression of the erythroid markers (CD71, CD36 and CD235) and myeloid markers (CD11b/CD14/ and CD33) (B) images of a BFU-E and GM colony.

S1D Patients admitted with sepsis showed a significant increase in the number of BFU-Es at D28 compared to baseline and this was not observed in non-sepsis patients. (A) Plating efficiency of single index sorted MEPs from all participants showing the effect of sepsis (n=8) vs non-sepsis (n=9). (B) The number of Burst Forming Units-Erythroid (BFU-Es) grown from MEPs 14 days after single-cell sorting into methylcellulose from pair-matched participants admitted with either sepsis (n=8) or non-sepsis (n=9). (C) Flow cytometric analysis of BFU-E colonies at day 14 showing the expression levels of the erythroid markers $CD71^+$ $CD235^+$ (data available from n=3 with sepsis and n=4 non-sepsis). Two-way repeated measures ANOVA; overall effect of timepoint in (A) $p=0.024$ and in (B) $p=0.035$; *$p<0.05$ Sidak's multiple comparison test.