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

Patients being cared for in an Intensive Care Unit (ICU) display the hallmarks of anemia of inflammation, characterized 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 hematopoietic stem and progenitor cells (HSPC) from peripheral blood obviating the need for invasive bone marrow sampling.[4,5] HSPC 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-E] and colony forming units-erythroid [CFU-E], derived from early erythroid progenitors [EEP] and late erythroid progenitors [LEP]), 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-color 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 (Day [D]0) and 28 (±7) days post enrollment (D28); samples from healthy controls were collected every 4-6 weeks. $CD34^+$ HSPC were isolated from peripheral blood using Ficoll-Histopaque (Sigma) and Human $CD34^+$ MicroBead Kit (Miltenyi Biotec) prior to cryopreservation. After thawing, $CD34^+$ HSPC 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 MEP 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 Table S2* and *Online Supplementary Figure S1C*).

The primary outcome was successful isolation of >$1x10^5$ $CD34^+$ HSPC from peripheral blood, a minimum number to enable analysis of sub-compartments giving rise to erythroid lineage.[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 enrollment for all patients are displayed in Table 1. Mean APACHE II score was 21.1 (Standard Deviation [SD]: 7.1), suggestive of high illness severity. Participants displayed the hallmarks of the anemia of inflammation as evidenced by high C-reactive protein (CRP) and ferritin concentrations, coupled with low hemoglobin (Hb), transferrin saturation and serum iron concentrations (Table 1). All but 2 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 $1x10^5$ $CD34^+$ HSPC were successfully extracted from 44/47 (93.6%) blood samples from study participants (mean cell count: $3.75x10^5$ [SD: $2.83x10^5$]) (Table 2). Changes in progenitor frequencies between study timepoints are shown in Figure 1. ICU patients had a higher proportion of hematopoietic stem cells (HSC) 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 (GMP) were significantly reduced at D0; common myeloid progenitors (CMP) were increased at D0 compared with controls, while there was no difference between multipotent progenitor (MPP) and MEP frequencies (Figure 1A-F). These data suggest that critical illness may be associated with early changes in hematopoietic progenitor compartments. However, since proportions of MEP were unaltered, reductions in frequencies of these erythroid progenitors may not be the primary cause of anemia in critical illness.

Therefore, we assessed whether the functional potential of the MEP was influenced by ICU admission. We sorted individual MEP 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 MEP, but D28 plating efficiency did not differ from 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

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

Characteristic	Value	Normal range
Age in 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 1 >2	 10 (33.3) 7 (23.3) 13 (43.4)	-
APACHE II score, mean (SD)	21.2 (7.1)	-
Admission diagnosis, N (%) Elective operation Emergency operation Medical	 2 (6.7) 13 (43.3) 15 (50)	-
Septic shock, N (%)	12 (40)	-
Organ support requirements, N (%) Advanced respiratory support Advanced cardiovascular support Advanced renal support	 28 (93.3) 12 (40) 5 (16.7)	-
PaO_2 / FiO_2 ratio, mmHg (range)	159.5 (128-233)	-
Baseline laboratory parameters, median (IQR) Hemoglobin, g/dL White cell count, x10^9/L Neutrophil count, x10^9/L Platelet count, x10^9/L Reticulocyte A, x10^{12}/L Reticulocyte % Creatinine, umol/L Albumin, g/L CRP, mg/L Ferritin, mcg/L Iron, mmol/L Tsat, % Transferrin, g/L EPO, IU/L B12, ng/L Folate, mcg/L Vitamin D, nmol/L	 9 (7.6-10.3) 13.9 (11.4-17.58) 11.5 (9.5-15.1) 219 (128-296) 0.04 (0.02-0.06) 1.45 (1.05-1.95) 87 (64-152) 19 (16-22) 226.3 (144-364.4) 642.3 (393.2-2057.5) 3.65 (2.6-5.55) 15 (9.5-25) 0.99 (1.1-1.4) 44.6 (22.2-65.4) 820 (556-1895) 4.35 (3-5.4) 13 (20.5-35)	 12-17 4.0-11.0 1.5-10.0 150-400 0.04-0.12 0.5-2.5 49.0-104 32-50 0-5 10-300 11-31 16-50 1.8-3.6 5.0-25.0 180-900 3.0-20.0 50-250
ICU mortality, N (%)	2 (6.7%)	-
ICU length of stay in days, median (IQR)	11.5 (7-18)	-
Time between ICU admission and 1st sample in days, median (IQR)	3 (3-6)	-
Time between 1st and follow-up samples in days, median (IQR)	26 (20-68)	-

APACHEII: Acute Physiology and Chronic Health Evaluation; BMI: body mass index; CRP: C-reactive protein; EPO: erythropoietin; FiO_2 : fraction of inspired oxygen; ICU: Intensive Care Unit; IQR: interquartile range; N: number; PaO_2 : partial pressure of oxygen; Reticulocyte A: absolute reticulocyte count; SD: Standard Deviation. Data were analyzed on STATA 14 (College Station, TX, USA). None of the included patients received a red blood cell transfusion prior to enrollment.

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) [SEM]	3.75 (2.83) [0.41]	6.10 (5.6) [2.12]
Geometric mean (SD) [95% CI]	2.88 (2.11) [2.32-3.58]	4.25 (2.51) [1.8-9.99]
Minimum-Maximum (range)	0.5-15.0 (14.5)	1.31-17.0 (15.69)

CI: Confidence Interval; N: number; SEM: Standard Error of Mean; SD: Standard Deviation.

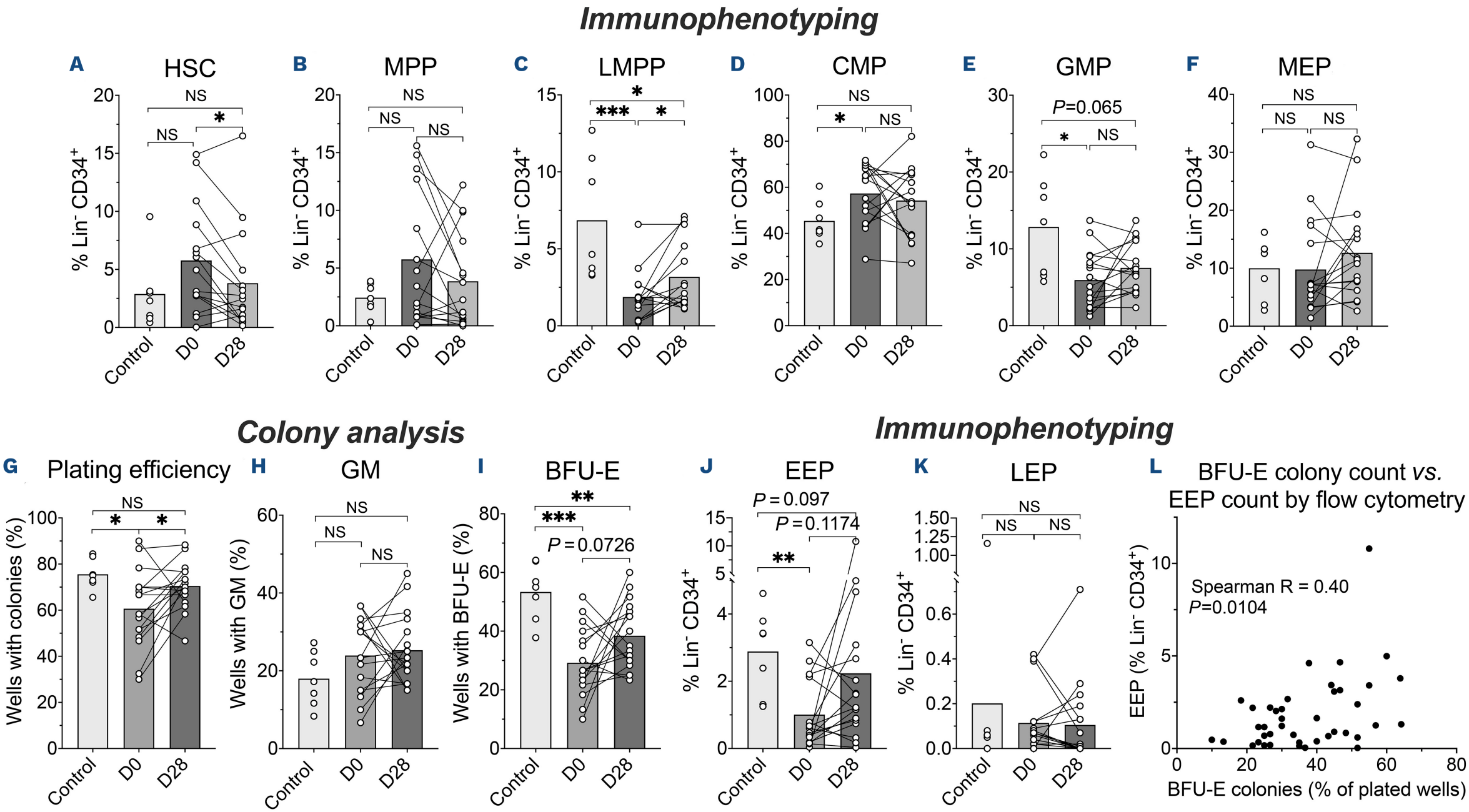

Figure 1. Characterization of hematopoietic progenitors from Intensive Care Unit (ICU) patients by flow cytometry, clonogenic assays, and flow cytometry assessment of megakaryocyte-erythroid progenitor (MEP) erythroid output, indicate changes in progenitor compartments at admission to ICU. (A-F) Individual immunophenotyping data showing the frequency of (A) hematopoietic stem cells (HSC), (B) multipotent progenitors (MPP), (C) lymphoid-primed multipotent progenitors (LMPP), (D) common myeloid progenitors (CMP), (E) granulocyte-macrophage progenitor (GMP), and (F) MEP 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 MEP showing (G) plating efficiency of MEP from controls (N=7) and participants (N=17 matched pairs). (H and I) Number of burst-forming unit-erythroid (BFU-E) and myeloid colony-forming unit-granulocyte macrophage (GM) colonies grown from MEP 14 days after single-cell sorting into methylcellulose from controls, or pair-matched participants at Day (D) 0 or D28. (J and K) Proportion of immunophenotypic early erythroid progenitors (EEP) that give rise to BFU-E, and immunophenotypic late erythroid progenitors (LEP) that give rise to colony forming units-erythroid (CFU-E) in controls and participants at D0 and D28. (L) Correlation of visually scored BFU-E with the numbers of EEP determined immunophenotypically using flow cytometry. 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: not significant ($P>0.05$); (L) Spearman rank correlation. Data were analyzed on STATA 14 (College Station, TX, USA) and Prism 9 and 10 (GraphPad software); statistical significance was defined as $P<0.05$.

timepoints compared to controls (Figure 1 I). Although not statistically significant (*P*=0.0726), the number of BFU-E colonies was higher at D28 compared with that at enrollment 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 EEP (Figure 1J) - the cells that give rise to BFU-E[4] - consistent with a reduction in erythroid output in critically ill patients at D0. However, LEP that give rise to CFU-E were unaffected (Figure 1K). We found weak evidence of correlation between BFU-E forming potential of the MEP in colony forming assays and immunophenotypically defined EEP frequency (Figure 1L). In an exploratory analysis, we found no effect of a diagnosis of sepsis / septic shock on admission on plating efficiency compared with patients without sepsis, although those with sepsis showed increased BFU-E at D28 (*Online Supplementary Figure S1D*). Overall, this suggests that reduced erythroid output in ICU patients may relate to qualitative defects in MEP.

The key findings of this study are: (i) CD34$^+$ HSPC 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 enrollment in ICU patients towards levels observed in controls. To our knowledge, the extent and combination of alterations in hematopoiesis we describe over the course of acute illness and recovery have not been reported previously in ICU patients.

In health, HSPC 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-E when compared with those without sepsis, similar to our exploratory analysis.[10] Interestingly, growth capacity of BFU-E 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 anemia 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-E express high levels of erythropoietin receptor (EpoR), and EPO signaling supports proliferation, survival, and differentiation. Similarly, MEP 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-mobilized peripheral blood contributes to blood production in stress and disease, the true clinical potential of peripheral blood HSPC compared to those from bone marrow remains unclear.[15] In addition, 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 anemia of inflammation but will also demonstrate functional and immunophenotypic recovery of BFU-E. Further research is needed to determine whether the changes observed are modified by treatments for anemia.

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https://doi.org/10.3324/haematol.2024.285530

Received: March 23, 2024.
Accepted: September 26, 2024.
Early view: October 3, 2024.

Disclosures

AS and HD have received consultancy fees from Pharmacosmos UK outside of the submitted work. All of the other Authors have no conflicts of interest to disclose.

Contributions

AS, NBAR, CS, HD, PAR, SRM and SJS are responsible for study concept and design. CS, ID-H, AEA, TJ, NBAR and AS acquired and analyzed data. CS, ID-H, AEA, NBAR, AS, AP and HD interpreted data. CS, ID-H, AA, AP, NBAR and AS wrote the first draft of the manuscript. All authors critically revised the manuscript for important intellectual content.

Acknowledgments

We thank Katherine Wray and Kevin Clark for their assistance with the experiments. We also thank Professor Anindita Roy for constructive feedback and discussions that refined the manuscript. We are grateful to all the patients and their families, and healthy volunteers who participated in this study, without whom this research would not have been possible.

Funding

This study was supported by a National Institute for Health Research grant (NIHR-DRF-2017-10-094) (to AS). CS is supported by Action Medical Research (grant reference: GN2855). The flow cytometry facility is supported by the MRC TIDU, MRC MHU (MC_UU_12009), NIHR Oxford BRC, Kay Kendall Leukaemia Fund (KKL1057), John Fell Fund (131/030 and 101/517), the EPA fund (CF182 and CF170), and by the WIMM Strategic Alliance awards G0902418 and MC_UU_12025.

Data-sharing statement

Requests for data should be made to the corresponding author. 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.

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