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Utilising mass cytometry with CD45 barcoding and standardised leucocyte phenotyping for immune trajectory assessment in critically ill patients

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Editor—Critically ill patients have rapidly changing longitudinal immune responses (*immune trajectory*) to infection and tissue injury. If we can delineate this immune trajectory, then we can time treatments that stimulate or depress the immune system (*immunomodulation*) to mirror the dominant immune signal at distinct stages of critical illness.

These rapidly changing longitudinal immune responses generate two major inferential challenges. First, the signal for illness-specific alterations must be delineated from the noise of inter-experimental variation within a study. Second, lack of standardisation of immune cell phenotyping assays between studies makes it difficult to discern whether observed differences between studies represent signal or noise.[1] These challenges need addressing in sepsis, as innate and adaptive immune systems are profoundly altered, immune phenotyping is not standardised, and rapidly changing sepsis-specific immune trajectory remains poorly characterised, for optimal immunomodulation.[1,2]

To address the challenge of inter-sample and inter-experiment variability and to enable simultaneous leucocyte phenotyping in longitudinal samples from the same patient, we developed a multiplexing approach using the stably expressed pan-leucocyte cluster of differentiation 45 (CD45) antigen as the target (referred to as barcoding) and cytometry by time of flight (CyTOF) as the phenotyping method.[3] To address the non-standardised phenotyping challenge, we used markers proposed in the standardised human immunophenotyping panel[4] and included selected immune state markers that are treatment targets such as checkpoint molecules and the human leucocyte antigen DR isotype (HLA-DR).[5] This report summarises the development of CD45 barcoding, assessment of major cell populations with a standardised human immunophenotyping panel,[4] and comparison of individual to multiplexed samples.

For all experiments, cryopreserved peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. For CD45 barcoding, commercially available CD45 89Y (Fluidigm Corp., South San Francisco, CA, USA) and in-house conjugated metal isotopes (115In, 159Tb, 209Bi) to the CD45 antibody (BioLegend®, BioLegend Inc., San Diego, CA, USA) were used. PBMCs were thawed in a 37°C water bath, washed in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 5% penicillin–streptomycin, resuspended in 1 ml, and counted. Then 3×10^6 cells from five donors were transferred to 5-ml polystyrene tubes for barcoding. Barcoded samples were washed once in phosphate-buffered saline (PBS), resuspended in 98 μl PBS, stained (1:100) with a unique combination of CD45 antibodies for 20 min, washed twice in 4 ml PBS, and combined into a single barcoded sample. The CD45 barcoding rationale, purpose, and novelty are summarised in Figure 1a.

For standardised leucocyte phenotyping, individual samples with 3×10^6 cells with combined barcoded samples were

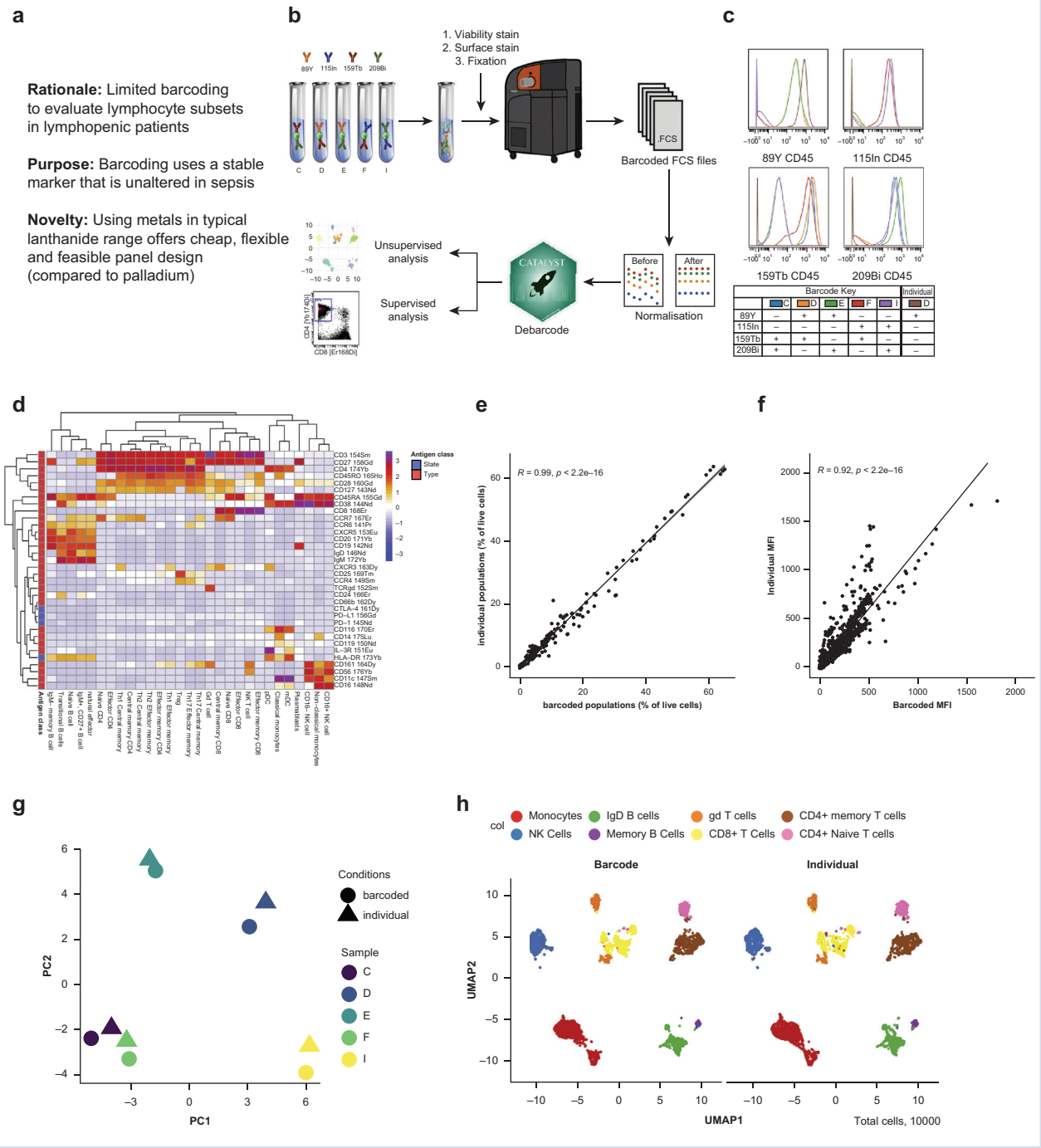

Fig 1. (a) CD45 barcoding rationale, purpose and novelty. (b) Five individual peripheral blood mononuclear cell (PBMC) donor samples (C, D, E, F, and I) were stained with a unique combination of CD45 conjugated antibodies (89Y, 115In, 159Tb, 209Bi). PBMCs were selected as input material as they are enriched for cells that are typically depleted in sepsis. Barcoded samples are combined into one tube for simultaneous downstream processing, minimising sample loss throughout the assay. The combined sample is stained for viability, surface markers and fixed, before being acquired on a Helios mass cytometer. Flow cytometry standard (FCS) files are normalised to EQTM bead standards, debarcoded in Catalyst package in R, and undergo supervised and unsupervised analysis. (c) Histograms depict debarcoded samples and an individually stained sample (D) with staining intensity of each metal conjugated CD45 antibody. Positive and negative peaks of histograms reflect the barcode key that defines each sample facilitating debarcoding. (d) Using 34 phenotypic surface markers (including four state and 30 type markers), we identify 29 manually gated leucocyte populations. (e) Proportions of live cells. (f) Median fluorescent intensity (MFI) measured on leucocyte populations highly correlated. (g) Principle component analysis (PCA) of the 29 leucocyte proportions identifies samples clustering together on donor characteristics as opposed to staining condition (barcoded or individual). (h) Using the unsupervised clustering algorithm FlowSOM, eight major populations were identified and projected onto uniform manifold approximation and projection (UMAP) demonstrating similar results.

adjusted to 2×10^7 cells ml^{-1} in PBS, stained for viability in pre-warmed 5 μM cisplatin solution for 60 s, Fc receptors blocked (5 μl FC block [TruStain FcX BioLegend®; BioLegend] for 10 min) and incubated with an antibody cocktail containing 34 surface markers for 30 min, as per the Fluidigm staining protocol.[6] Samples were then fixed in 1.6% formaldehyde for 10 min, centrifuged, and incubated in 125 μM Intercalator-Ir fix and perm buffer (Fluidigm) at 4°C until acquisition the following day using a Helios mass cytometer (Fluidigm). Samples were washed once in CSB, twice in cell acquisition solution (CAS), adjusted to 5×10^5 per ml in CAS with 1:10 EQ™ four element calibration beads (Fluidigm), and data were acquired at 300 events s^{-1} (Fig. 1b).

For comparisons between individual and multiplexed samples, we normalised flow cytometry standard (FCS) files with EQ beads. Barcoded samples recorded in multiple recordings were concatenated and debarcoded using the Catalyst package.[7] All samples were manually gated (https://www.cytobank.org). Proportions of live cells and median fluorescent intensity (MFI) of all markers on all populations were exported as comma separated values (CSV) files for analyses. We compared proportions of cell populations, the MFI of all 34 markers between staining conditions, and conducted unsupervised analyses using principal component analyses (PCA). We also conducted unsupervised analysis of single cell data using self-organising map[8] (FlowSOM) projected onto uniform manifold approximation and projection (UMAP).[9]

The stain intensity of each CD45 metal isotope was compared on debarcoded and individual samples as a surrogate for its binding characteristics. Histogram peaks of in-house conjugated metal isotopes for CD45 were similar to commercially available CD45 89Y. There was an expected loss of 89Y signal in barcoded samples owing to binding competition between two antibodies. Clear positive and negative signals reflect the key for debarcoding (Fig. 1c).

The 34-surface marker panel consisted of 30 class and four state markers. This enabled identification of 29 major leucocyte populations, including lymphocyte, monocyte, natural killer, and dendritic cell subsets (Fig. 1d). These also included cellular subsets of therapeutic relevance in sepsis such as T-helper subsets (Th-1, Th-2, Th-17, regulatory T cells), expression of checkpoint molecules on T cells, and expression of HLA-DR on monocytes.

High correlation was observed between individually stained and barcoded multiplexed samples in proportions of 29 leucocyte subsets ($R=0.99$; $P<0.001$; Fig. 1e) and MFI of 34 markers ($R=0.92$, $P<0.001$; Fig. 1f). In the PCA, the barcoded and individual samples clustered together for each donor (Fig. 1g). Furthermore, barcoded and individually stained leucocyte population projections were similar with FlowSOM projected on UMAP (Fig. 1h).

Overall, CD45 barcoding facilitates longitudinal patient sample measurements to derive immune trajectories, while maintaining standardised immunophenotyping to identify lymphocyte subsets in sepsis patients in whom lymphopaenia is common, as described in our study protocol.[2] This formed the rationale for using PBMCs instead of whole blood for phenotyping and limited barcoding to maximise detection of lymphocyte subsets that are treatment targets (such as terminally exhausted T cells) in sepsis patients. Our use of lanthanide metals is a low-cost, flexible panel design method, whereas barcoding on CD45 potentially enables our methods to be used in whole-blood phenotyping and *ex vivo* stimulation experiments. Thus, our approach is a useful advance on a recent proof-of-concept report of mass cytometry in five sepsis patients.[10] Although variability between barcoded experiments has not been addressed, this could be facilitated by using the same technical control in each experiment for batch normalisation. Discriminant biomarkers for trajectory identified using our approach to standardised immunophenotyping can then be applied to near patient testing using flow cytometry.

In summary, we report a feasible barcoding approach for standardised immunophenotyping to derive immune trajectory in critically ill patients. Our method would highlight leucocyte immune trajectory in sepsis patients and inform development of near patient immunophenotyping tests to provide targeted and timely immunomodulation in sepsis.

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Declarations of interest

All authors declare that they have no conflicts of interest.

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Authorship patterns in contemporary anaesthesia literature: a cross-sectional study

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Editor—Recent studies have shown an increase in the number of authors of scientific publications in the medical field.[1–4] Several reasons have been proposed, including greater complexity of the scientific question requiring interdisciplinary collaboration, pressure to publish in academia for promotion purposes, and honorary or gift authorship. Gift authorship is defined as naming a person as an author who does not meet authorship criteria. The last reason is a cause for potential concern. To date, no study evaluating the number of authors in the anaesthesiology literature has been published.

We analysed articles published from 2008 to 2018 in the five anaesthesia journals with the highest 2018 impact factors (based on Thomson Reuters-Clarivate Analytics) as an extension of a previous study on gender authorship presented at the American Society of Anesthesiology annual meeting. Our objective was to determine the trend of number of authors and the factors associated with high author numbers over time in the anaesthesiology literature.

This study was prospectively registered with the International Prospective Register of Systematic Reviews (Registration number 151092).[5] The journals *Anesthesiology*, *British Journal of Anaesthesia* (*BJA*), *Anaesthesia*, *European Journal of Anaesthesiology* (*EJA*), and *Anesthesia and Analgesia* (*A&A*) were included (Fig. 1a). Original research articles, systematic reviews, and meta-analyses published in 2008, 2010, 2012, 2014, 2016, and 2018 were selected. The number and gender of authors, year of publication, country of origin, departmental affiliations, type of study, and source of funding were extracted for each article.

Continuous variables, normally distributed based on visual inspection and the Shapiro–Wilk normality test, were reported as mean and standard deviation (SD) and compared using the Mann–Whitney *U*-test. Categorical variables were reported as counts and percentages and compared using the χ^2 test. Two-sided significance testing was used and *P*-values <0.05 were considered significant.

A total of 4720 articles were included over the study period. Although the number of articles overall slightly decreased over time (1642–1506 to 1572), the number of co-authors per article increased significantly from a mean (SD) of 5.80 (SD 2.23) in 2012–2014 to 6.35 (SD 2.72) in 2014–2016 to 7.10 (SD 3.46) in 2016–2018, all *P*<0.001. In all five journals, there was a statistically significant increase in the number of authors per article over time (*P*<0.001). *Anesthesiology* had the highest mean number of authors at 7.28 (SD 3.23) and *Anaesthesia* had the lowest at 5.56 (SD 2.71) (Fig. 1a, Supplementary Table S1). The number of authors increased significantly over time in all considered subgroups, including basic research articles, clinical articles, retrospective and prospective studies, single and multiple institutions studies, different funding sources, and for all continents of origin (Fig. 1b–d).

We examined the number of authors over 10 years in the five anaesthesia journals with the highest impact factors and found a significant increase from a mean of 5.80 authors per manuscript in 2008–2010 to 7.10 in 2016–2018. This trend held true for each journal. The percentage of articles with more than eight authors, defined as the highest quartile of author number per article, more than doubled from 9.8% in 2008 to 25.9% in 2018. *Anesthesiology* had the highest mean number of authors and percentage of articles with more than eight authors (28.0%), while *Anaesthesia* had the lowest mean number and percentage of articles with more than eight authors (9.3%).

Our results are similar to results from previous studies on authorship patterns in general medicine. Studies found that the number of authors of manuscripts published in high impact medical journals increased by 53% from 1980 to 2000,[4] and by 23% from 1995 to 2005.[2] A significant increasing trend in