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Brief Report

Blood immune profiles reveal a CXCR3/CCR5 axis of dysregulation in early sepsis

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Abstract

We report on a pilot study exploring whether blood immune signatures can reveal early specific indicator profiles for patients meeting sepsis criteria upon hospital admission. We analyzed samples of sepsis-suspected patients ($n = 20$) and age-spanning healthy controls ($n = 12$) using flow cytometry–based assays. We measured inflammatory markers from plasma fractions and immunophenotyped freshly isolated unfixed peripheral blood mononucleated cells for leukocyte subset representation and expression of activation markers, including chemokine receptors. We found that besides IL-6 and sCD14, CXCR3 ligands (CXCL9 and CXCL10) separated sepsis-suspected patients from healthy controls. The abundance of $CD4^+$ T cells was significantly reduced in patients, while they displayed substantial losses of CCR5-expressing monocytes and CXCR3/CCR5 double-positive T cells. Post hoc subgrouping of patients according to their sepsis diagnosis on discharge identified CXCR3/CCR5 double expression on T cells as a separating characteristic for confirmed cases. This work suggests a potential novel axis of dysregulation affecting CXCR3 and CCR5 in early sepsis.

Keywords: blood biomarkers, chemokines and receptors, monocytes, sepsis, T cells

1. Introduction

Sepsis is a life-threatening complication of bacterial infections, accounting for 20% of deaths worldwide in 2017.[1] Early administration of antibiotics is the main treatment; acting within an hour of patient admission, it lowers the risk of complications and death.[2] The clinical recognition of sepsis is challenging; many meeting sepsis criteria upon admission are not assigned a discharge diagnosis of sepsis.[3,4]

Since bloodstream infections leading to sepsis are rarely associated with high bacteremia[5] but do alter inflammatory blood biomarkers,[6] the emphasis has been on defining biomarkers to rapidly predict sepsis.[3] The dynamics of sepsis means that identifying changes at a single point in time presents a challenge,[3] but profiling changes at the onset of clinical signs and symptoms may help.[7] Dominant cellular changes correlating with biomarker profiles from blood may expose specific signatures that can discriminate sepsis from other systemic inflammation.

Cytokines and chemokines (CCs) and their receptors are regulators of inflammation, affecting leukocyte recruitment, activation, and function, but also account for imbalances in the inflammatory network leading to sepsis.[8,9] A number of studies, including ours, have evidenced a direct effect of $Gram^-$ or $Gram^+$ bacteria cell wall components lipopolysaccharide or lipoteichoic acid on CC production, receptor expression, and cell activation.[10,11] Despite this, sepsis-induced leukocyte phenotypic changes remain poorly understood.

We performed a pilot study exploring differences in blood immune signatures for sepsis-suspected patients upon hospital admission compared to healthy controls by analyzing profiles of plasma biomarkers and relative representation of different leukocyte subsets with expression of activation markers and chemokine receptors. We report overlapping but distinct blood signatures with alterations in CCR5 expression and the CXCL9-10/CXCR3 axis as potential early indicators of sepsis.

2. Material and methods

2.1 Study cohorts and sample preparation

The study was sponsored by York & Scarborough Teaching Hospitals NHS Foundation Trust (Supplementary material). Clinical recruitment of patients at the emergency department (ED) of York Hospital with moderate to high risk of sepsis ($n = 20$) followed the National Institute of Clinical Excellence Clinical Guideline 51.[12] Age-matched healthy donors ($n = 12$) were recruited from university volunteers (reported good general health and no treatment for conditions that would impact their immunity) and consenting elderly participants attending York Hospital (elective orthopedic surgery).

Whole venous blood samples (9 mL) were collected in S-Monovette K3 EDTA tubes (Sarstedt) and stored at 4 °C before processing as previously described.[13] Plasma fractions were cryopreserved, while leukocytes were used immediately for live-cell immunophenotyping.

2.2 Measurement of biomarkers in human plasma fractions

Multiplex cytometry bead-based assay panels (Supplementary material) were used to measure plasma analytes. Data were acquired on a CytoFLEX (Beckman Coulter) and analyzed using

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LEGENDplex data analysis software to calculate concentrations within the bounds of intra-assay generated standard curves (sensitivity range 0.2 to 3.8 pg/mL). Serum-released CD14 (sCD14) was assessed with a CD14 human ELISA kit (Invitrogen, Thermo Fisher scientific; assay sensitivity 6 pg/mL).

2.3 Immunophenotyping of live, freshly isolated leukocytes

Leukocytes (5 to 6×10^6 cells) resuspended in ice-cold FACS buffer (FB: phosphate-buffered saline, 1% fetal calf serum, 0.05% sodium azide) were treated for 20 min in 50 µL FB with 20 µg/mL human IgG to saturate F_C receptors before adding 50 µL of either Blood Cell Panel (BCP) or T Cell Activation Panel (TCAP) antibody cocktail mix (Supplementary Fig. 1). After washes, samples were treated with Live/DEAD Fixable Near IR cell viability dye (Thermo Fisher Scientific UK, Winsford, UK). For each donor, controls included unstained and live/dead stain-only cells. Fluorescence Minus One experimental control cells were generated from healthy donors. Samples were fixed overnight, 4 °C in 250 µL 1% formaldehyde in FB before data acquisition. Results were preanalyzed using CytExpert Software (Beckman Coulter), and detailed analysis was performed with FCS Express Software (Dotmatics). Results are expressed as the percentage of cells positive for the indicated cell surface markers within the specified cell population(s).

2.4 Quantification, data integration, and statistical methods

Data were analyzed with GraphPad Prism version 10 software (GraphPad Software) using Mann–Whitney or analysis of variance (ANOVA) with multiple-comparison posttests where appropriate. Boxplots show minimum to maximum value of whiskers plus all points. A measure of group separation achieved by individual variables was defined by:

$$separation = \frac{beween\ group\ variance}{within\ group\ variance}. \quad (1)$$

Multivariate analyses were performed in the R programming environment.[14] Principal component analysis (PCA) was carried out using the function "prcomp" in base R. Partial least squares regression (PLSR) was performed using R package "pls" with the response variable encoded as 1.0 and 2.0 for healthy and sepsis-suspected (sepsis), respectively, and the class corresponding to the closest integer to the output response assigned. Random forest classification was performed using the R package "randomForest." Due to the potential for overfitting in supervised analyses, we used leave-one-out (L-O-O) cross-validation with the class for each observation predicted in turn from a model built without the data for that observation.

3. Results and discussion

3.1 Participants' characteristics and blood samples

Our study included 20 sepsis-suspected patients (mean age 70 yr, 45% female) and 12 healthy controls (mean age 51 yr, 58% female; Supplementary Fig. 2). Initial hospital white blood cell (WBC) counts for many patients were above the standard reference range,[15] but for both groups, the number of peripheral blood mononucleated cells (PBMCs) recovered after isolation was generally within the accepted yield range[16] (Supplementary Fig. 2), suggesting that the dominant increase in WBC for sepsis-suspected patients relates to neutrophilia.[17]

3.2 Plasma CC profiling

We performed multiplex plasma analyte analysis for CCs linked to sepsis.[9] IL-6, CXCL9, and CXCL10 showed statistical differences between healthy and sepsis-suspected samples (Fig. 1A), being elevated in patients. IL-5 and CXCL5 showed an inverse trend with reduced levels compared to healthy controls. Soluble CD14 (sCD14) enzyme-linked immunosorbent assay showed increased levels in sepsis-suspected patients (Fig. 1B), as previously shown.[18] PCA of the CC and sCD14 data indicated some grouping, with healthy patients clustering separately (Fig. 2A). Reanalysis using a simpler model with only IL-6, CXCL9, CXCL10, CXCL5, IL-10, CCL11, and sCD14 showed a similar level of discrimination—the variables with the greatest separation measure according to equation (1) (Fig. 2B; measure >0.1). This unsupervised analysis was complemented by PLSR analysis on the full CC data with sCD14 (Fig. 2C(i)). A correct classification rate of 93.75% in L-O-O confirmed the difference in profiles with all sepsis-suspected cases correctly classified and just 2 healthy controls incorrectly classified (Fig. 2C(ii)). Variable importance in projection (VIP) scores showed IL-6, CXCL9, CXCL10, and CXCL5 to be the most important variables (Fig. 2C(iii)).

Spearman correlation analysis was performed to determine any specific relationships between variables that could explain the dominance of IL-6, CXCL9, and CXCL10, but no clusters of pairwise correlations connected the discriminatory variables (Supplementary Fig. 3), suggesting that multiple unrelated immune events lead to plasma signatures. The profiles and pairwise correlations from our study were distinct from our previous results for COVID-19 ED-admitted patients, and early sepsis was not marked by a cytokine storm.[19]

3.3 Leukocyte changes in sepsis-suspected patients

We assessed the representation of innate and adaptive immune cell subpopulations from the blood samples using the isolated PBMCs. Sepsis-suspected patients showed significant accumulation of granulocytes (low density) and monocytes coinciding with a reduced frequency of lymphocytes (Fig. 3A), all known predictors of sepsis.[20] Expansion of atypical large monocytes reported in COVID-19[21] was not observed in our sepsis cohort. Multicolor panels of antibody (Supplementary Fig. 1) were used to immunophenotype freshly isolated unfixed cells to allow reliable detection of chemokine receptors known to be affected by fixation.[22,23] The BCP exposed a significant reduction of CCR5- and CCR7-positive PBMCs in the sepsis-suspected group compared to healthy controls (Fig. 3B). When gating on BCP subsets (Supplementary Fig. 4B)—namely, the monocytic CD14, T-cell receptor CD3, and B-cell CD19 markers—we confirmed a reduced frequency of T and B lymphocytes in sepsis-suspected patients (Fig. 3C). Chemokine receptors and TLR2 expression within each subset revealed significant changes for sepsis-suspected samples, including a fall in $CCR5^+$ and $CCR7^+$ monocytes, a small increase for $TLR2^+$ T cells, and inversely altered expression of CCR2 and CCR7 on B cells (Fig. 3D). Combining healthy controls and sepsis-suspected patients shows a strong anticorrelation between the frequency of CCR2- and CCR7-positive B cells (Fig. 3E), possibly due to CCR2 being found on immature B cells and downregulated with maturation, while CCR7 is a marker of naive and mature B cells.[24,25] The switch in expression of the 2 receptors may reflect an early alteration of the B-cell compartment, as previously reported with sepsis.[26] We used CD14 and CD16 expression to gate separately on classical, intermediate, and nonclassical monocytes (Supplementary Fig. 5). The loss of CCR5 expression affected all monocyte subpopulations

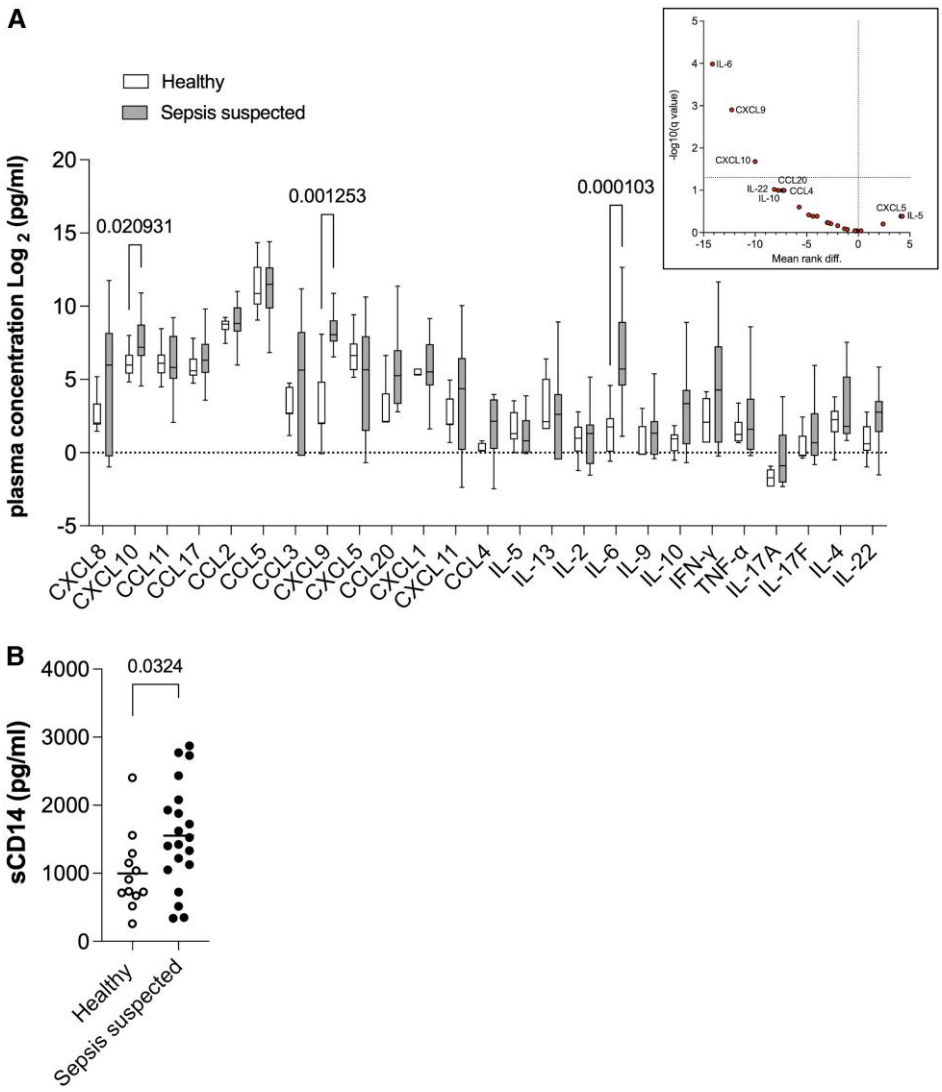


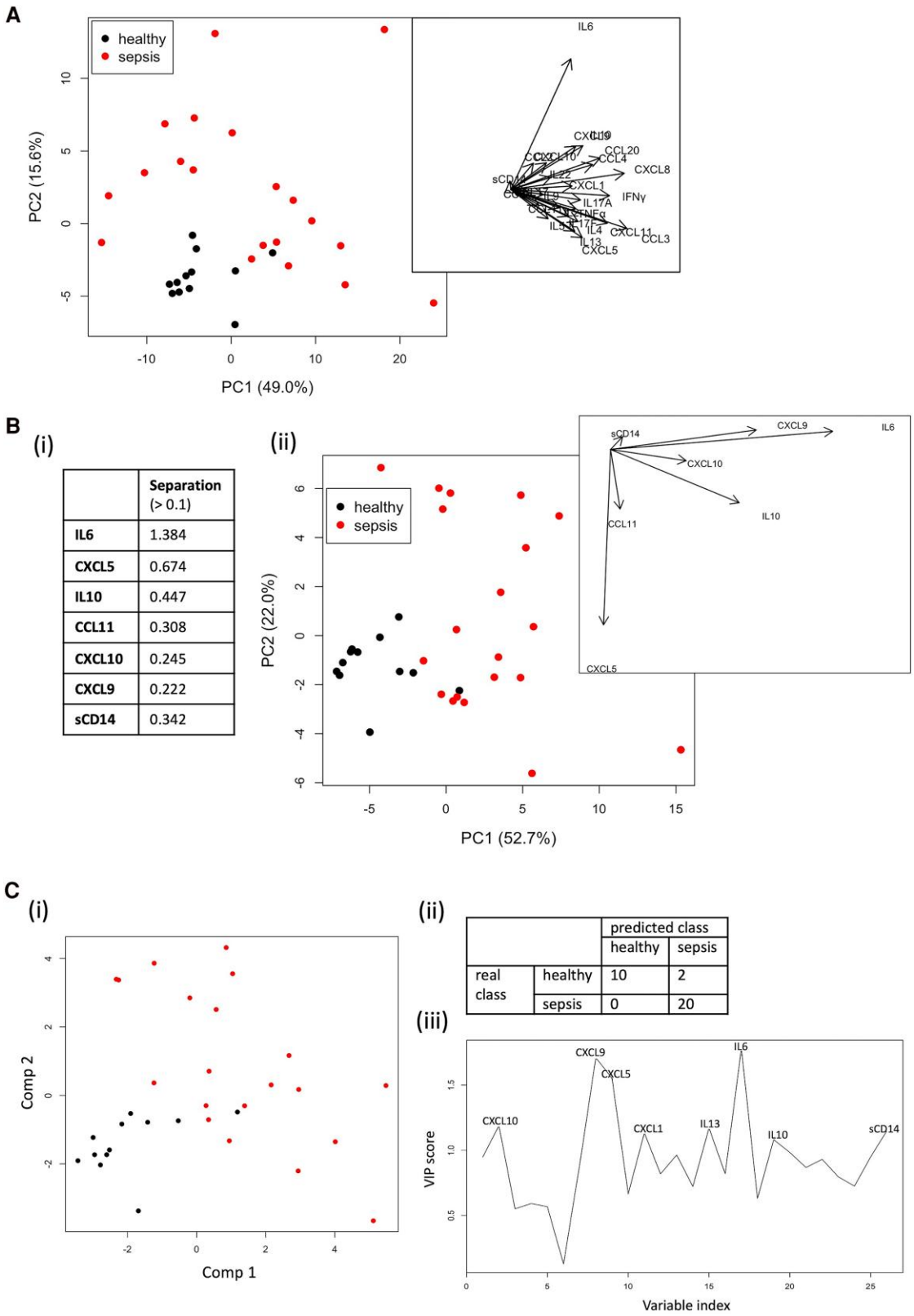
Fig. 1. Circulating CC signatures for healthy volunteers compared to sepsis-suspected patients. (A) Box-and-whisker plot of measured cytokines and chemokines from blood-isolated plasma samples with statistical significance from Mann–Whitney tests using Benjamini, Krieger, and Yekutiel adjustment for multiple comparisons. The volcano plot reports the mean rank differences, either increased or decreased, for all markers between the healthy and sepsis-suspected groups. (B) Scatterplot of sCD14 blood concentration measured by enzyme-linked immunosorbent assay, with statistical significance ($P = 0.0324$) between healthy and sepsis-suspected groups defined using a Mann–Whitney test.

in the sepsis-suspected group (Fig. 3F), when only nonclassical monocytes showed a loss of CCR2 positivity, with no difference for the other markers (Supplementary Fig. 5C). Interestingly, CCR5$^+$ monocytes have recently been reported as crucial to control sepsis in a murine model.[27] PCA analysis on all BCP parameters showed some clustering of the healthy controls away from sepsis-suspected patients (Supplementary Fig. 6A), and L-O-O classification in PLSR achieved an 87.5% accuracy rate (Fig. 3G) due to parameters such as CD3, CD14/CCR5, CD19/CCR7, and CD19/CCR2 (Supplementary Fig. 6B). These findings suggest sepsis affects the innate and adaptive immune system even at an early stage.[17]

3.4 T-cell activation profiles in sepsis-suspected patients

Recent studies have indicated that T-cell dysregulation impairs the host response in sepsis, affecting effector CD4 and CD8, regulatory (Treg), and memory T cells.[28,29] We used a TCAP with gating strategies (Supplementary Figs. 4 and 7) to investigate sepsis changes and confirmed the significant loss of CCR5$^+$ PBMCs seen with the BCP (Supplementary Fig. 8A). Gating showed that CD8 cells comprised both CD8$^{+(high)}$, corresponding to classical CD8$^+$ T cells, and CD8$^{+(low)}$ cells, reported as a distinct subpopulation of activated CD8 effector cells in human blood[30] (Supplementary Fig. 4C). Comparing CD4$^+$ and CD8$^+$ lymphocyte populations in healthy controls and sepsis-suspected patients, we found a highly significant reduction in CD4$^+$ T cells associated with sepsis (Fig. 4A), as previously reported.[31] From the percentage of CD4$^+$ and CD8$^{+(high\ \&\ low)}$, we calculated the CD4/CD8 ratio, which reduces with age but is expected to be greater than 1 for healthy individuals.[32] The average value was significantly lower for the sepsis-suspected group, with many patients presenting a CD4/CD8 ratio <1 (Fig. 4B).

We assessed the percentage of cells expressing the chemokine receptors CXCR3 and CCR5 as markers of T-cell activation,[33,34] CD25 as a marker of activated T cells and Tregs,[35] CD45RO as a T-cell memory marker,[36] and TLR2 as a regulator of T-cell activation in response to infection.[37] No significant difference was seen for individual marker expression (Fig. 4C). However, combinations of markers showed a



	Separation (> 0.1)
IL6	1.384
CXCL5	0.674
IL10	0.447
CCL11	0.308
CXCL10	0.245
CXCL9	0.222
sCD14	0.342

		predicted class	
		healthy	sepsis
real class	healthy	10	2
	sepsis	0	20

Fig. 2. Integration of sCD14 plasma levels with CC profiles. (A) PCA scores plot for the first 2 components for cytokine and chemokine data sets with sCD14 with the biplot showing the loadings as vectors (black arrows) and scores by sample names in gray. (B) PCA restricted to variables with separation scores >0.1 (i), with score plot and biplot (ii) showing the loadings. Here, separation is defined by the between-group variance divided by the within-group variance (equation (1); see Material and methods). (C) PLSR score plot (i) for the first 2 latent variables obtained using scaled sCD14 and CC data. The confusion matrix (ii) shows the results obtained using leave-one-out cross-validation on scaled data. (iii) The VIP graph highlighting the most important variables in the data. The dotted line shows the threshold, VIP score = 1, above which the variables are labeled.

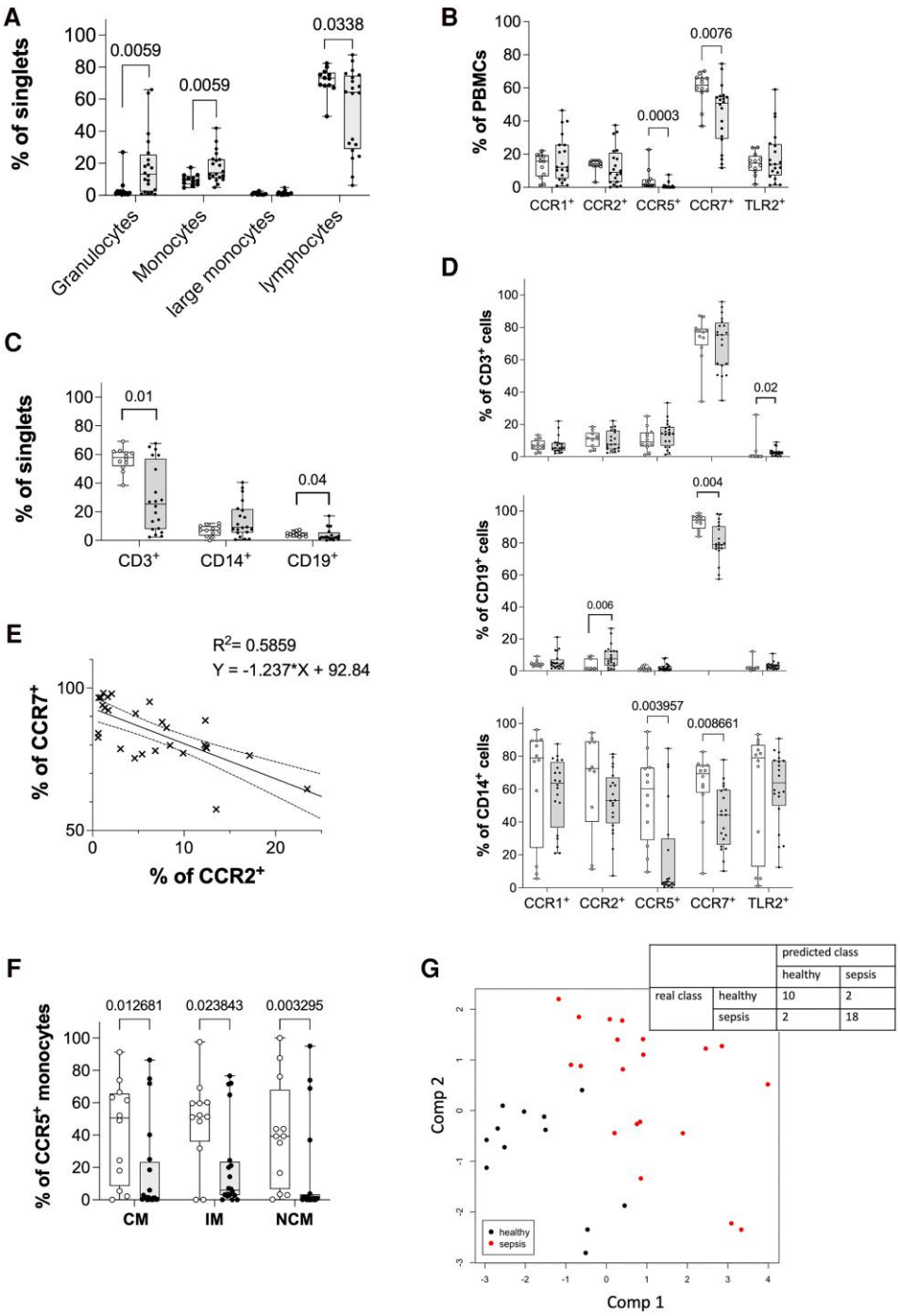


Fig. 3. PBMC profiles and expression of cell surface markers detected with the BCP. (A) Representation of leukocyte subpopulations identified by flow cytometry based on forward and side-scatter profiles, comparing healthy volunteers to sepsis-suspected patients. Results are expressed as the percentage of total single cells (singlets) recorded by the cytometer using the mean value from $n = 5$ samples run for each individual. (B) Frequency of cells expressing CCR1, 2, 5, or 7 and TLR2 within PBMCs. (C) Frequency of T cells, monocytes, and B lymphocytes among single cells recorded by the cytometer (singlets) based on CD3, CD14, and CD19 expression, respectively. (D) Expression of CCR1, 2, 5, or 7 and TLR2 on $CD3^+$, $CD14^+$, and $CD19^+$ cells; insert shows scatterplot for $TLR2^+$ distribution on $CD3^+$ cells with Mann–Whitney test. (E) Negative correlation between CCR7 and CCR2 expression on $CD19^+$ B lymphocytes (Spearman correlation $r = -0.6776$). The linear regression equation and goodness-of-fit coefficient (R^2) are shown, $P < 0.00001$. (F) Distribution of $CCR5^+$ monocytes across the classical monocyte (CM), intermediate monocyte (IM), and nonclassical monocyte (NCM) subpopulations, as defined in Supplementary Fig. 5A. For all boxplots shown with all points, statistical significance was determined using Mann–Whitney tests with adjustment for multiple comparisons (adjusted P values) using Benjamini, Krieger, and Yekutiel secondary tests. (G) The partial least squares regression scores plot for the first 2 latent variables obtained using the BCP variables show clear separation between the healthy and sepsis-suspected groups. Accuracies shown in the table were obtained using a leave-one-out cross-validation on scaled data.

significant loss of CXCR3/CCR5 dual-expressing T cells in sepsis-suspected patients (Fig. 4D). CXCR3/CCR5 dual expression has been reported for Th1-associated T effector/memory cells linked with inflammatory reactions.[38,39] The reduced frequency of dual expression affected $CD4^+$ and $CD8^+$ cells independently of which markers were coexpressed (Fig. 4D and E), and within each subset, only cells with dual CCR5/CXCR3 expression were affected, as shown with $CD45RO^+$ T memory cells (Fig. 4F). The sepsis-suspected group

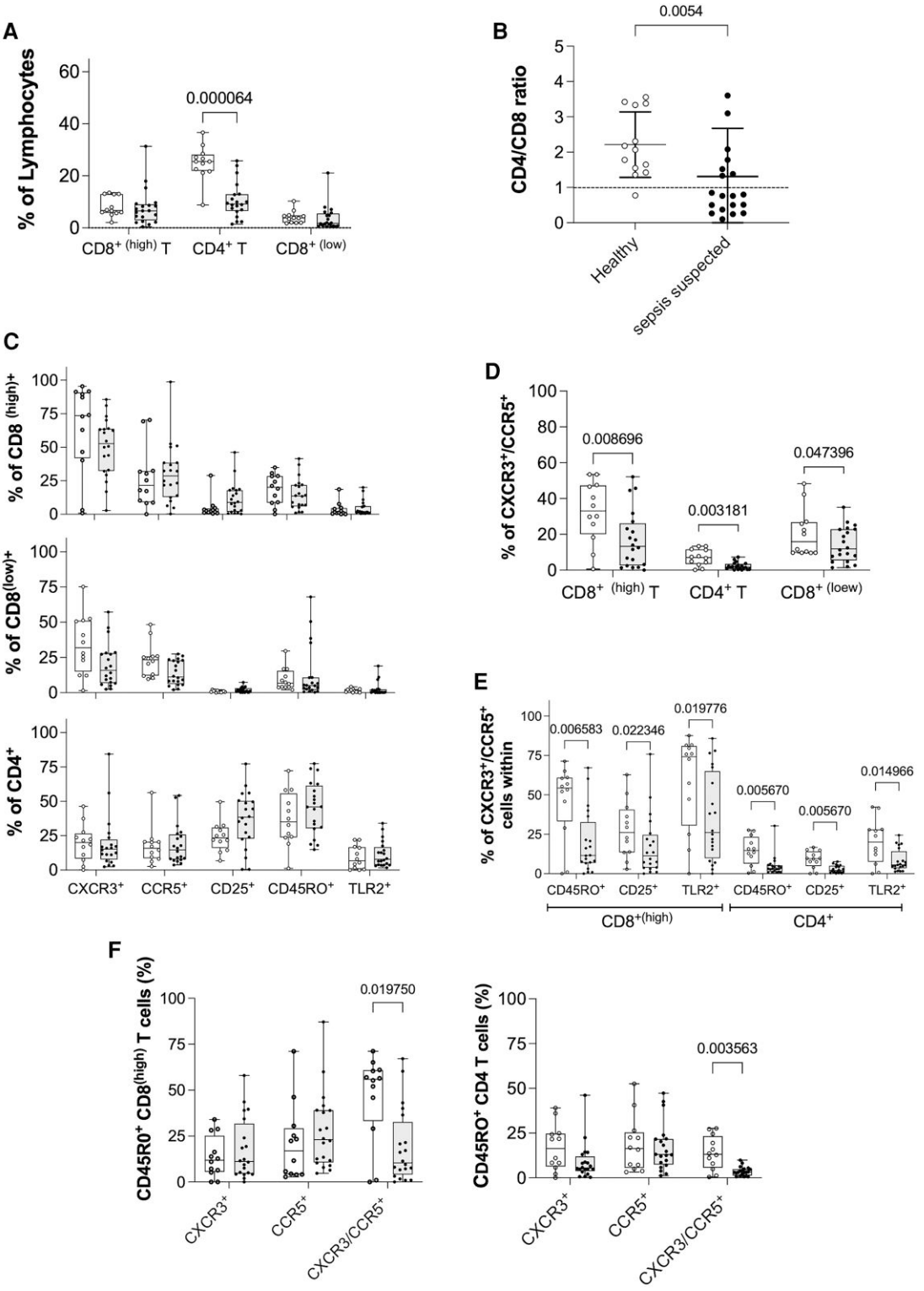


Fig. 4. Expression profiles of activation markers on T-cell subpopulations. All boxplots with all points comparing healthy and sepsis-suspected groups report on the percentage of cells positive for the indicated markers as measured with the defined TCAP. (A) Frequency of CD4 and CD8 cells among gated lymphocytes. (B) Scatterplot comparing CD4/CD8 cell ratios in each group (dotted line marks normal ratio >1.0). (C) Distribution of CXCR3, CCR5, CD25, CD45RO, and TLR2 expression on $CD4^+$ T, $CD8^{+(high)}$ T, and $CD8^{+(low)}$ cells. (D) Loss of CXCR3/CCR5 coexpression on $CD4^+$ T, $CD8^{+(high)}$ T, and $CD8^{+(low)}$ cells for sepsis-suspected patients. (E) With reduced frequency of $CXCR3/CCR5^+CD8^{+(high)}$ and $CD4^+$ T cells across the board. (F) A reduction not affecting single expressing CXCR3 or CCR5 memory T cells ($CD45RO^+$). Statistical significances were determined using Mann–Whitney tests with adjustment for multiple comparisons (adjusted *P* values) by Benjamini, Krieger, and Yekutiel secondary test.

also showed a reduced frequency of $CD4^+$ CD45RO/CD25-positive cells (Supplementary Fig. 8), a subset reported as type 1–like regulatory T cells,[40] but this was not linked to coexpression of CCR5/CXCR3. However, PCA analysis of all TCAP variables showed no separation of healthy controls from the sepsis-suspected group (Supplementary Fig. 9).

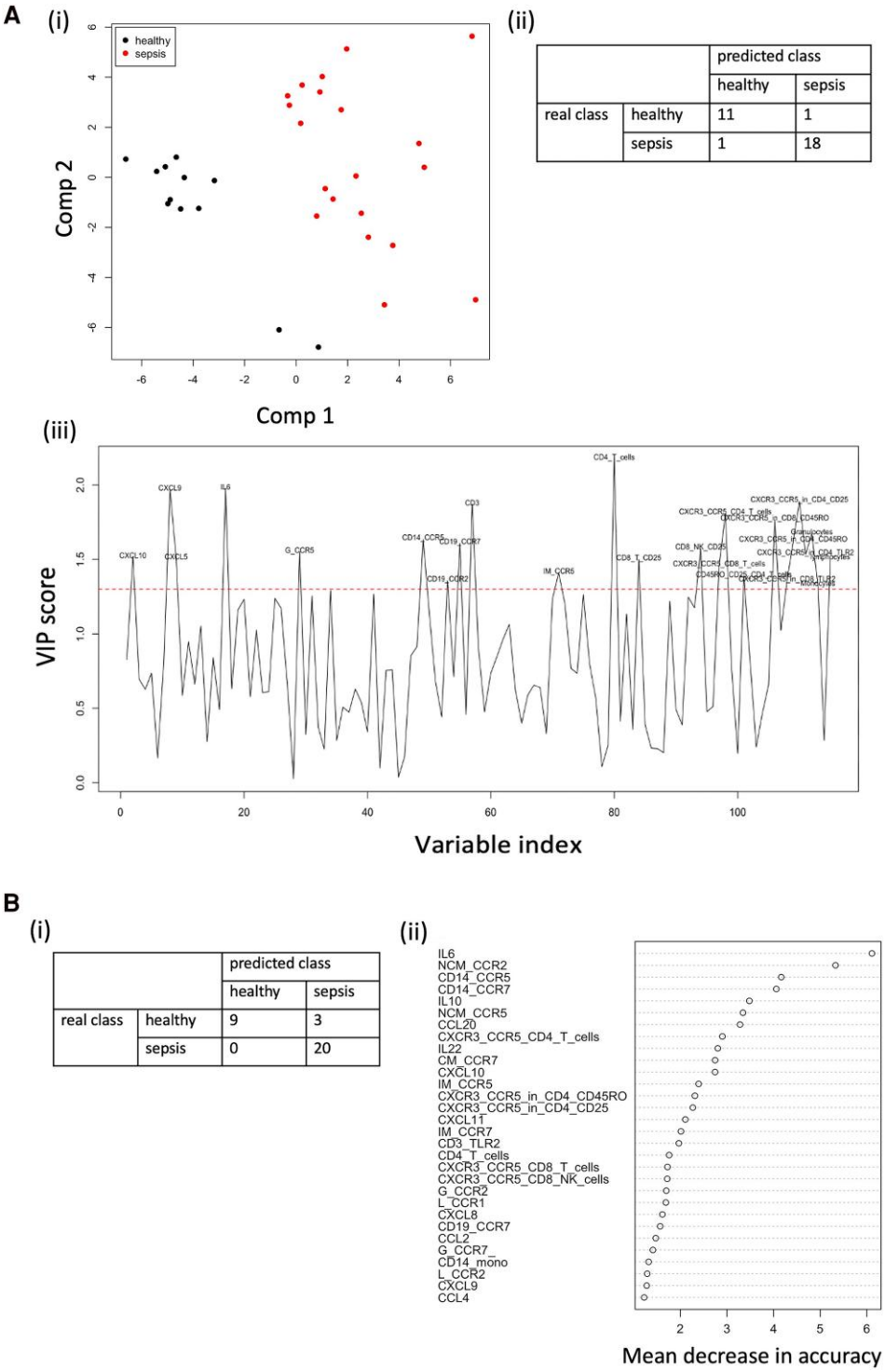


Fig. 5. Separation of healthy and sepsis-suspected profiles based on variables measured across all panels. (A) PLSR results from the model obtained with all variables. (i) The partial least squares (PLS) score plot generated from all data shows clear separation between healthy and sepsis-suspected (sepsis) profiles. (ii) The results from L-O-O cross-validation show 1 classification error for each group. (iii) VIP score graph highlighting the most important predictor variables in the PLS model. The dotted line shows the threshold, VIP score = 1.3, above which the variables are labeled. (B) (i) Confusion matrix showing the results from random forest classification using L-O-O cross-validation. (ii) The most discriminatory variables sorted by importance based on mean decrease in accuracy.

3.5 Integrated full data set analysis with clinically validated sepsis patients' status

To determine the parameters within our data set (Supplementary Table 2) that dominate the changes in the sepsis cohort, we used 2 supervised methods, PLSR (Fig. 5A) and random forest (Fig. 5B), that allow the most important variables to be identified. Both methods achieved discrimination between healthy controls and sepsis-suspected patients with correct classification rates of 93.8% (PLSR; Fig. 5A (ii)) and 90.6% (random forest; Fig. 5B (i)). Discriminatory variables were identified from VIP scores in PLSR (VIP > 1.3, Fig. 5A (iii))

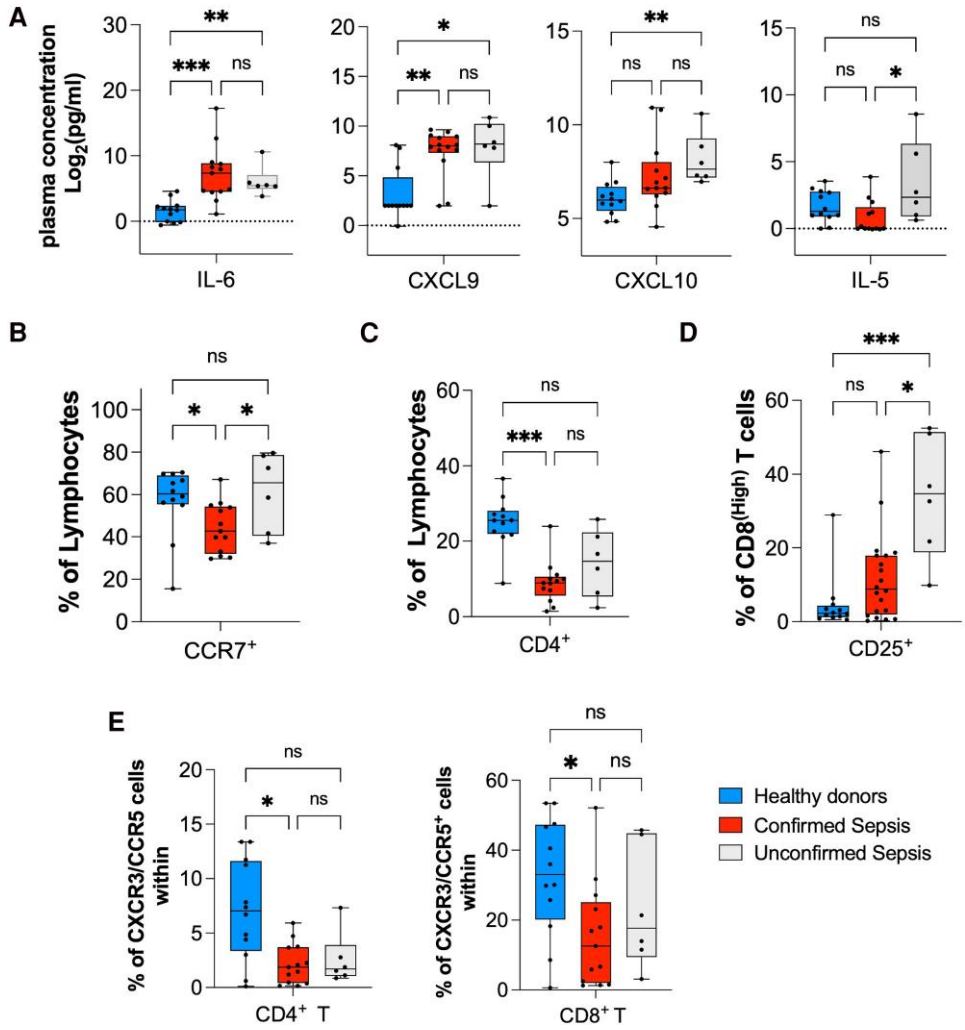


Fig. 6. Subgroup analysis by individual markers. Analysis of each marker individually identified a series of variables with significant differences between healthy donors, confirmed and unconfirmed sepsis subgroups. Cytokines and chemokines showing significant differences between at least 1 pair of subgroups (A), the percentage of $CCR7^+$ (B) or $CD4^+$ (C) lymphocytes, $CD25^+CD8^{+(high)}$ T cells (D), and CXCR3/CCR5 double-positive cells in $CD4^+$ and $CD8^{+(high)}$ subpopulations (E). Statistical significance was determined using ANOVA and Kruskal–Wallis multiple comparisons test (adjusted P values: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, ns = nonsignificant).

and mean decrease in accuracy in random forest classification (Fig. 5B (ii)). In both analyses, IL-6, CXCL9, CXCL10, $CCR5^+$ monocytes, $CD4^+$, and CXCR3/$CCR5^+$ T cells emerged as important parameters.

Clinical diagnoses at discharge were used for further analysis dividing patients into subgroups of confirmed ($n = 13$) or unconfirmed ($n = 6$) cases of sepsis for all but 1 patient, who withdrew in the later phase (see Supplementary Table 1). PCA analysis indicated some separation between the profiles of healthy controls and patients but could not distinguish the sepsis subgroups (Supplementary Fig. 10A). Although Spearman correlation analysis of the parameters identified as discriminatory by partial least squares analysis (Fig. 5B) showed distinct correlation profiles for each of our groups and subgroups, no unique sepsis biomarker-based signature was found (Supplementary Fig. 10B).

Considering individual variables, ANOVA tests revealed some significant differences between the confirmed and unconfirmed subgroups (Fig. 6). For CC data, we found an increase in IL-6 and CXCL9 for both subgroups in comparison to the healthy group, CXCL10 accumulation was only significant for the unconfirmed sepsis group, and IL-5 levels were significantly different between the confirmed and unconfirmed subgroups (Fig. 6A). In the BCP data set, confirmed sepsis patients exhibited a loss of $CCR7^+$ lymphocytes (Fig. 6B), while the TCAP data set showed that the $CD4^+$ T-cell–driven lymphocytopenia was specific for the sepsis-confirmed subgroup (Fig. 6C). There was a significant reduction in the frequency of activated $CD25^+CD8^{+(high)}$ T cells for confirmed cases vs unconfirmed sepsis and healthy controls (Fig. 6D). Interestingly, the severe reduction in CXCR3/CCR5-coexpressing $CD4^+$ and $CD8^{+(high)}$ T cells specifically affected confirmed sepsis patients (Fig. 6E). CXCR3/CCR5 T cells have been linked with infiltration of inflammatory sites and inflammatory reactions,[38,39] and the loss of these chemokine receptors can impair T-cell response to infection.[41] Here the loss of receptor coincided with accumulation of the CXCR3 ligands CXCL9/10 and not CXCL11, which interacts differently with CXCR3 with distinct biological activity.[42,43] CXCR3 activity impacts the development and function of both $CD4^+$ and $CD8^+$ T-cell compartments.[43,44] Mechanistically, it has been shown that CXCL9/10-mediated activation of CXCR3 leads to receptor degradation and loss of surface expression on activated T cells.[45] It can also trigger CCR5 cross-phosphorylation on CXCR3/CCR5 T cells blocking their migration,[46] suggesting a relationship between our observed increase of CXCL9/10 and loss of CXCR3/CCR5 T cells in sepsis-suspected patients. With these 2 chemokine receptors driving efficient Th1-type

adaptive immunity by influencing the positioning and balance of Tregs and T effector and memory cells during inflammation,[47] the question remains whether the collapse in blood T cells CXCR3/CCR5 we observed is part of the cause or a consequence of sepsis.

3.6 Summary and limitations

Our study confirms that early-stage sepsis changes in the innate and adaptive immune system are difficult to distinguish from nonsepsis conditions[3,48] but expose alterations in CCR5 and CXCR3 expression as novel sepsis indicators. We acknowledge this is a small-scale single-center study, and patient heterogeneity may affect some observations. Similar issues were raised in a recent study of 77 patients with sepsis that identified the frequency of T cells and CXCR3 expression on $CD4^+$ T cells as dominant parameters without investigating CCR5.[49] A larger multicenter follow-up study is required to validate our early observations and the potential of a CXCR3/CCR5 axis of dysregulation to recognize sepsis.

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Author contributions

N.S., N.T., and D.Y. designed the study. T.J., N.T., and D.Y. supervised the clinical side of the study, including collection and analysis of clinical data; R.C. oversaw the recruitment with informed consent of patients and healthy volunteers from the hospital and blood sample collection; G.F. was the study clinical manager. D.K. and N.S. performed the biological experiments, K.H. contributed to the flow cytometry analyses, and J.W. performed the statistical analyses. D.K., J.W., and N.S. were responsible for data interpretation and writing the manuscript.

Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

Conflict of interest statement. None declared.

Data availability

The data underlying this article not included in the supplementary document will be shared on reasonable request to the corresponding author.

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