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Two new neutrophil subsets define a discriminating sepsis signature

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21 **Authorship contributions:**

22 AMK, BGC, AB and CC designed the study. AMK and NG performed experimental work. BGC, KG, CdR
23 and HV provided clinical samples, pathological diagnosis and patient clinical data. AMK compiled patient
24 data. AMK and AC run samples in the mass cytometer. AMK, SMB, SG and JMI performed data analysis.
25 AMK, JMI and CC developed the figures, and wrote the manuscript. CC provided financial support. All
26 authors contributed in reviewing the manuscript.

27 **Running head:** Sepsis neutrophil signature for patients' diagnosis

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29 **Text word count:** 4344

30 **At a Glance Commentary:**

31 There is an unmet need for specific and rapid diagnostic tests for sepsis, which would discriminate sepsis
32 patients from patients with aseptic inflammation. This work represents the first comprehensive evaluation of
33 whole blood circulating immune cells in septic patients using CyTOF high-dimensional technology coupled
34 with computational analysis. It allowed the identification of two novel sepsis-specific neutrophil subsets:
35 CD10-CD64+PD-L1+ and CD10-CD64+CD16low/-CD123+ immature neutrophils. This early sepsis immune
36 cell signature was validated computationally and biologically in an independent cohort and could be used for
37 sepsis diagnosis.

38 **Abstract:**

39 **Rationale:** Sepsis is the leading cause of death in adult intensive care units. At present, sepsis diagnosis relies
40 on non-specific clinical features. It could transform clinical care to have immune cell biomarkers that could
41 predict sepsis diagnosis and guide treatment. For decades, neutrophil phenotypes have been studied in sepsis,
42 but a diagnostic cell subset has yet to be identified.

43 **Objectives:** To identify an early specific immune signature of sepsis severity that does not overlap with other
44 inflammatory biomarkers, and that distinguishes patients with sepsis from those with non-infectious
45 inflammatory syndrome.

46 **Methods:** Mass cytometry combined with computational high-dimensional data analysis were used to measure
47 42 markers on whole blood immune cells from sepsis patients and controls, and automatically and
48 comprehensively characterize circulating immune cells, which enables identification of novel, disease-specific
49 cellular signatures.

50 **Measurements and Main Results:** Unsupervised analysis of high-dimensional mass cytometry data
51 characterized previously unappreciated heterogeneity within the CD64⁺ immature neutrophils and revealed
52 two new subsets distinguished by CD123 and PD-L1 expression. These immature neutrophils exhibited
53 diminished activation and phagocytosis functions. The proportion of CD123-expressing neutrophils correlated
54 with clinical severity.

55 **Conclusions:** This study showed that these two new neutrophil subsets were specific to sepsis and detectable
56 by routine flow cytometry using seven markers. The demonstration here that a simple blood test distinguishes
57 sepsis from other inflammatory conditions represents a key biological milestone that can be immediately
58 translated into improvements in patient care.

59 **Abstract word count:** 232

60 **Key words:** Sepsis, neutrophils, diagnosis, PD-L1, CD123

61 **Introduction**

62 Sepsis is the leading cause of death in the intensive care unit (ICU) (1-3). Diagnosis of patients relies on
63 clinical data rather than a robust biomarker that distinguishes sepsis from sterile inflammation and predict
64 its clinical outcome and prognosis can be evaluated by several scores including Simplified Acute
65 Physiology Score II (SAPS II) and Sequential Organ Failure Assessment (SOFA) Score. SOFA and
66 SAPS-II are indicators of severity, show poor performance regarding sepsis diagnostic and were
67 consistently shown to be non-specific of sepsis (1, 4-7). It is estimated that the survival rate decreases by
68 roughly 10% every hour that appropriate antimicrobial medication is delayed, emphasizing the urgent
69 need for early diagnosis techniques (8, 9). A comprehensive systems immunology approach using mass
70 cytometry is well-suited to characterize the diversity of disease-specific cellular states (10). Neutrophils
71 are a primary immune cellular barrier against pathogens, but they may be a double-edged sword in sepsis
72 having a role in both inflammation and immunosuppression (11-15). We hypothesized that phenotype of
73 circulating neutrophils might provide crucial early insight into immune features that drive sepsis and
74 distinguish this disease from non-infectious inflammatory syndrome.

75 For the systems immunology approach here, it was critical to track features that had been identified as
76 important in sepsis biology, but which individually had not the resolving power to specifically distinguish
77 sepsis. Neutrophils expressing the high-affinity immunoglobulin-Fc receptor I (CD64) were described in
78 numerous clinical studies over the last two decades (16). CD64 is normally expressed on monocytes, but
79 its expression on circulating neutrophils could be due to its upregulation during inflammation (17), or to
80 released immature granulocytes from the bone marrow, especially when it is associated with decreased
81 expression of neutral endopeptidase (CD10) and low-affinity immuno-globulin-Fc fragment III (CD16)
82 (13, 14, 18) (Supp.Tab.1) (19). Previous studies identified also the interleukin (IL)-3 as an orchestrator of
83 emergency myelopoiesis during sepsis and showed its association with hospital mortality (20, 21). In

84 parallel, programmed death ligand-1 (PD-L1) expressed on monocytes was also described as a mortality-
85 predictor in sepsis patients (22, 23).

86 A systems-level view is likely needed to identify cellular features that specifically distinguish sepsis
87 infection-induced immune phenotypes from those triggered by aseptic inflammatory signals. To identify
88 such early sepsis-specific cellular biomarkers, we developed a multi-parametric immune profiling strategy
89 (Fig.1). Cytometry by Time-Of-Flight (CyTOF) instrument was used to measure 42 markers on whole
90 blood immune cells from sepsis patients and controls (Fig.1A) (24). A computational analysis approach
91 was used to comprehensively characterize circulating immune cells and identify disease-specific cellular
92 signatures (25, 26). This approach consisted in a “discovery strategy” (Fig.1B) and a computational
93 “validation strategy” (Fig.1C) based on two complementary set of algorithms. We identified two
94 unreported early and sepsis-specific neutrophil subsets. A conventional “expert driven strategy” using a
95 limited set of markers confirmed that these two sepsis-specific neutrophil subsets were associated with
96 sepsis (Fig.1D). This result was confirmed using an independent cohort of patients and conventional flow
97 cytometry (Fig.1E).

98

99 **Methods**

100 *Study design*

101 This observational study was approved by the Comité de Protection des Personne Paris VII ethic
102 committee (CPP IDF VII A000142-53). Two cohorts were used in this study (Supp.Tab.2). Seventeen
103 sepsis (S) patients and twelve patients undergoing cardiac surgery considered as non-infected
104 inflammatory controls (NIC) were included in the discovery cohort of the study (Supp.Tab.2). The
105 validation cohort was composed of twenty-four sepsis patients and eighteen non-infected patients with

106 confoundable symptoms of sepsis (NIP) (Supp.Tab.2). Blood samples were drawn in heparin-coated
107 tubes, collected at the first- and seventh-day post admission of antibiotic treated sepsis patients or post-
108 surgery for NIC patients of the discovery cohort, and at the first-day post admission of the validation
109 cohort patients. In addition, blood samples of eleven age and gender matched healthy donors (HD)
110 were obtained from the French blood donation center. Five bone marrow (BM) biopsies from
111 orthopedic surgery patients were also included in this study.

112 *Mass cytometry analysis*

113 Whole blood samples were stained using a 42-dimensional mass cytometry panel (Supp.Tab.3). A
114 multi-step staining protocol was set up and is detailed in the supplementary methods section. Once the
115 collection of samples was completed, stained cells were thawed then measured on a CyTOF Helios
116 instrument. Acquired data were normalized with a MATLAB-based software (27) and analyzed using
117 the Cytobank platform (28).

118 *Computational data analysis*

119 To identify immune subsets and visualize all cells in a 2D map where position represents local phenotypic
120 similarity, we used two different dimensionality reduction tools depending on the strategy: the viSNE
121 implementation of t-SNE (29) and the UMAP (30). Cells were also grouped in phenotypically
122 homogenous clusters using either SPADE (31) or FlowSOM (32, 33). To phenotypically characterize
123 these clusters, Marker Enrichment Modeling (MEM) (34, 35) was used. The analysis process of each
124 strategy is detailed in the supplementary methods section.

125 *Flow cytometry validation panel*

126 To validate the sepsis-specific neutrophils signature a seven markers panel (Supp.Tab.4) was designed
127 for conventional florescent flow cytometry. The sepsis samples were analyzed in a blind cytometry
128 testing, along with the non-infected patients. The staining protocol is detailed in the supplementary
129 methods section.

130 *Activation and phagocytosis assay*

131 To address neutrophils activation and phagocytic capacities we used pHrodo-labeled BioParticles and
132 coated with *Staphylococcus aureus* (*S. aureus*) or Zymosan antigens (Invitrogen). The staining protocol
133 is detailed in the supplementary methods section.

134 *Statistical information*

135 Numerical data are given as median and inter-quartile range (25th - 75th percentile) with the exception
136 of Fig.7 data that are given as mean±SD. Nonparametric two-tailed Mann-Whitney test with a
137 significance threshold of alpha ($\alpha=0.05$) was used to compare cellular abundances of cell subsets
138 between two groups of patients and MFI ratios. Nonparametric two-tailed Wilcoxon signed-rank test
139 with a significance threshold of alpha ($\alpha=0.05$) was used to compare cellular abundances of cell subsets
140 from patients at day-1 and day-7. Relationship between two data sets was assessed using Spearman's
141 rank correlation coefficient (r) and test with a significance threshold of alpha ($\alpha=0.05$), and linear
142 regression line was drawn on the corresponding plot. Statistical tests were performed using GraphPad7
143 software (GraphPad Software, San Diego, CA), as well as receiver operator characteristic (ROC)
144 analyses.

145

146 **Results**

147 *Mass cytometry and computational analysis revealed a sepsis-specific neutrophil signature*

148 We designed a longitudinal observational study with 40 individuals to explore the evolution of circulating
149 immune cell phenotypes of S patients (n=17), NIC patients (n=12) at day 1 and 7 (Supp.Tab.2) and HD
150 (n=11) in addition to BM biopsies (n=5) (Fig.1A). Whole blood immunostaining was performed with a
151 42-parameter mass cytometry panel designed to give a comprehensive evaluation of circulating leukocytes
152 (Fig.1A, Supp.Tab.3). We identified circulating immune cell populations. Using viSNE tool, neutrophils
153 were gated, and other circulating immune cells were independently analyzed.

154 The neutrophils were analyzed with a “discovery strategy” using viSNE and SPADE tools (Fig.1B).
155 viSNE is an unsupervised algorithm that reduces feature dimensions and allows cells visualization in a
156 two-dimensional map. SPADE is an unsupervised algorithm aiming to group cells into nodes that could
157 be displayed on the viSNE map. This strategy allowed to define an imprint for each sample group
158 (Fig.2A). On the resulting map, neutrophils of S and NIC day-1 patients and neutrophils of HD were
159 arranged in three different areas (Fig.2A). These S neutrophils were clustered in specific nodes that were
160 absent from NIC and HD (Supp.Fig.1, 2). Some of these S specific nodes were shared with BM,
161 suggesting the occurrence of myelocytosis for S patients (Supp.Fig.1, 2). Most cells from day-7 samples
162 were phenotypically similar to samples from HD (Fig.2A, Supp.Fig.1, 2). CD16, CD10 and CD64
163 markers split neutrophils signature into two positive and negative subpopulations for each marker
164 (Supp.Fig.1). To characterize all the nodes, their abundance in each sample and their average expression
165 of each marker were extracted and used to generate two heatmaps (Supp.Fig.3, 4). Hierarchical clustering
166 was used to arrange rows (nodes) and columns (samples) of the frequency heatmap (Supp.Fig.3) and
167 columns (markers) of the phenotype heatmap (Supp.Fig.4). In this unsupervised three arms analysis
168 (nodes, samples and markers), the resulting dendrograms led to the identification of 3 main samples
169 clusters (columns) as shown in Fig2B (Supp.Fig.3 before tree cut). Most of the samples were clustered

170 according to patient groups. S day-1 (pink) and BM (orange) samples were clustered together. S day-7
171 samples were split in two sample clusters, with half of them clustering with HD samples (Fig2B,
172 Supp.Fig.3) suggesting the acquisition of a “healthy” neutrophil phenotype profile (Fig2A). In addition,
173 this unsupervised strategy allowed the precise delimitation of four groups of cell nodes (Fig.2D): ⁽¹⁾HD-
174 abundant nodes representing neutrophils with CD16^{high}CD10^{med}CD64⁻ phenotype, ⁽²⁾ NIC and S day-7
175 common nodes harboring CD16⁺CD10^{med}CD64⁻ phenotype, ⁽³⁾ day-1 NIC and S common nodes defined
176 as CD16^{low}CD10⁻CD64^{low}, and ⁽⁴⁾ S day-1 and BM nodes with CD10⁻CD64⁺ phenotype. Node group ⁽⁴⁾
177 represents cells that are highly abundant in sepsis samples at day-1 when compared to other patient groups
178 (Supp.Fig.5A). The statistical analyses of these nodes are presented in Supp.Fig.5B. Among the nodes
179 that statistically discriminate S and NIC at day-1 (Supp.Fig.5B), a specific phenotypic characteristic was
180 observed: three nodes expressed CD123 and four other nodes expressed PD-L1 (Supp.Fig.5A, Fig.2C,
181 D). On the basis of phenotypic homogeneity meta-clusters were generated to group nodes that share
182 similar expression of these two markers and represent two neutrophil subsets specific to S at day-1 and
183 observed to be lacking in NIC neutrophils (Fig.2E). The first subset (in red) was composed of CD10⁻
184 CD64⁺CD16⁺PD-L1⁺ neutrophils (S median proportion: 18.08 (6.69-48.33) %, NIC median proportion:
185 0.81 (0.53-3.01) %, $p=0.0002$) and the second one (in blue) identified as CD10⁻CD64⁺CD16^{low}CD123⁺
186 immature neutrophils (S median proportion: 10.06 (1.12-39.35) %, NIC median proportion: 0.04 (0.02-
187 0.42) %, $p<0.0001$) (Fig.2E). We also recapitulated previously described results (13, 14, 18) regarding
188 the sepsis related increase of circulating immature CD10⁻CD64⁺ neutrophils when compared to NIC at
189 day-1 (S median proportion: 11.03 (1.41-40.39) %, NIC median proportion: 0.62 (0.12-1.46) %, $p=0.001$)
190 and we confirmed their phenotypic similarities with a third of BM neutrophils (BM median proportion:
191 37.39 (17.90-46.48) %) (Fig.2E). Also, we noticed that all HD specific-nodes were absent in S patient
192 day-1 samples (Fig.2B, D).

193 With this strategy, two novel neutrophil subsets were identified, including CD123⁺ cells (red) and PD-
194 L1⁺ cells (blue), and the absence of HD neutrophil phenotypes at an early stage of sepsis.

195 *A computational validation strategy confirmed sepsis day-1 specific neutrophil subsets*

196 To test whether the previously identified neutrophil subsets were sepsis-specific and robust, an
197 independent unsupervised data analysis strategy was applied on the same data files used in the discovery
198 strategy (Fig.1B, C). This “validation strategy” was based on UMAP and FlowSOM algorithms. UMAP
199 is an unsupervised dimensional reduction algorithm (Supp.Fig.6A) and FlowSOM is an unsupervised
200 clustering algorithm. This strategy allowed the identification of 50 neutrophil clusters and the complete
201 linkage hierarchical clustering of their relative cell abundance arranged again the samples according to
202 patient groups (Supp.Fig.6B). Two main cell cluster groups (pink gates) appeared to be more abundant
203 in sepsis samples (Supp.Fig.6B, C) and almost all HD associated-clusters (purple gate) were absent in
204 sepsis patient day-1 samples.

205 To phenotypically characterize the pink gate clusters, MEM phenotype annotation tool was used. The
206 MEM label of each cluster is an objective description of what makes that subset distinct from all the other
207 clusters. Among these clusters, three cell meta-clusters were identified, one with CD10⁻CD64⁺ immature
208 cells (pink clusters), and two meta-clusters phenotypically identical to the “discovery strategy” sepsis-
209 specific neutrophils nodes (Supp.Fig.6D, Fig.3A). Red clusters contained CD10⁻CD64⁺PD-L1⁺
210 neutrophils with a median cell proportion of 5.50 (1.15-38.03) % for S day-1 samples and 0.09 (0.02-
211 0.33) % for NIC day-1 samples ($p<0.0001$) (Fig.3B). Blue clusters gathered CD10⁻CD64⁺CD16^{low/-}
212 CD123⁺ immature neutrophils with median cell proportions of 2.43 (0.98-6.32) % and 0.04 (0.03-0.28)
213 % for S day-1 and NIC day-1 samples respectively ($p=0.0006$) (Fig.3B). We also visually noted that red
214 clusters (PD-L1⁺ cells) and blue clusters (CD123⁺ cells) from the “validation strategy” are co-localized

215 with red nodes (PD-L1⁺ cells) and blue nodes (CD123⁺ cells), respectively, from the “discovery strategy”,
216 when back mapped onto the t-SNE1-2/t-SNE2-2 axes (Fig.3C).

217 *Expert gating strategy based on a limited set of markers validated the sepsis day-1 neutrophil signature*
218 *that correlates with SAPSII and SOFA scores*

219 After cell subsets were identified by automatic and high-dimensional analysis strategies, we determined
220 whether the identified neutrophil signature could be found using conventional analysis applicable by
221 experts. The use of such gating strategy would make it easier to transpose it to clinical use.

222 A bi-parametric gating strategy on a limited set of markers allowed the identification of neutrophils
223 expressing CD123 and PD-L1 (Fig.4A). When CD123⁺ and PD-L1⁺ sepsis-specific neutrophils were
224 mapped back onto both t-SNE1-2/t-SNE2-2 axes and UMAP1/UMAP2 axes, they located in the same
225 regions as the cells identified by the two previous computational strategies meaning that they share the
226 same phenotype (Fig.4A). This expert gating strategy applied on the current dataset, allowed the selection
227 of PD-L1 expressing neutrophils that were significantly more abundant in blood of S day-1 patients (9.25
228 (3.61-36.97) %) when compared to NIC day-1 patients (0.12 (0.07- 0.60) %, p<0.0001) or HD (0.01 (0.00-
229 0.03) %, p<0.001) (Fig.4B). Similarly, expert gating allowed the selection of S-specific neutrophils (2.47
230 (0.44-17.42) %) that were consistent with CD123⁺ red subsets cells phenotype and that were almost absent
231 from NIC (0.04 (0.07-0.87) %, p<0.0001) or HD (0.04 (0.02-0.10) %, p<0.0001) (Fig.4B).

232 Although the proportion of CD10-CD64+CD16-CD123⁺ neutrophils could distinguish S and NIC
233 samples at day 1, we observed a large variability between patients. Interestingly, we noticed that patients
234 with the highest CD123⁺ neutrophil subset proportion (> 20%) tended to be more severe (requirement for
235 mechanical ventilation and catecholamine support). Later correlation with severity scores confirmed this
236 observation. The proportion of CD123⁺ sepsis-specific, assessed by the simple gating strategy on mass

237 cytometry data, positively correlated with Simplified Acute Physiology Score II (SAPS II) (Spearman
238 $r=0.62$, $p=0.0192$) and Sequential Organ Failure Assessment (SOFA) score (Spearman $r=0.55$, $p=0.0437$)
239 (Fig.4C). However, the proportion of CD123⁺ neutrophil was not influenced by sepsis endotype. The
240 proportions of PD-L1 neutrophil subset did not correlate with severity scores nor sepsis endotypes. ROC
241 analysis of these CD123⁺ neutrophils abundance was carried out to determine the optimal threshold
242 separating sepsis patients from non-infected patients. A cut-off point of 0.38% of the CD123⁺ neutrophil
243 subset abundance was able to identify sepsis patients with a specificity of 91.67% and sensitivity of
244 81.25% and display an area under the ROC curve (AUROC) of 0.91 (Fig.4D). When combining the
245 abundance of the CD123⁺ and PD-L1⁺ neutrophil subsets, the cut-off point changed to 0.93% and lowered
246 both the sensitivity, to 75%, and the specificity, to 83.33%. (Fig.4E). Whereas a clinical SOFA score >2
247 was discriminating with a good sensitivity (94.12%) but with a poor specificity (45.45%) and a worst
248 AUROC of 0.79 (Fig.4F). In addition, the AUROC of SAPS-II score was also lower (AUROC=0.82) with
249 a sensitivity of 88.24% and a poor specificity of 45.45% (Fig.4G).

250 Thus, a simple gating strategy assessing only 7 key markers identified successfully CD123⁺ and PD-L1⁺
251 sepsis-specific neutrophils and indicated that CD123⁺ neutrophils may be a marker of sepsis severity with
252 a better discriminating efficiency when compared to clinical scores.

253 *Mass cytometry and unsupervised analysis identified classical sepsis immune hallmarks*

254 Using two complementary computational strategies, we identified a sepsis-specific signature on the
255 neutrophil cells. We asked whether a signature in the non-neutrophil cells could reinforce the CD123⁺ and
256 PD-L1⁺ neutrophil subsets as sepsis biomarker candidates. The non-neutrophils circulating immune cells
257 were computationally analyzed using t-SNE and SPADE algorithms. A heatmap was generated to
258 characterize nodes phenotype and to delimitate the main circulating non-neutrophil immune cell

259 populations, according to complete linkage hierarchical clustering (Supp.Fig.7A). These populations
260 were then color coded and backgated on the t-SNE map (Supp.Fig.7B). Classical hallmarks of sepsis were
261 identified, including lymphopenia, monocytopenia and a persistent lower level of monocytes HLA-DR in
262 S patients when compared to HD group ($p<0.0001$, $p=0.0426$ and $p<0.0001$ respectively, Fig.5A). In
263 parallel, we observed an elevated number of circulating neutrophils ($p=0.0039$), and consistent with that,
264 a higher neutrophil to lymphocyte ratio ($p<0.0001$) in S vs. HD (Fig.5B). These trends were not exclusive
265 to S, but were also observed in NIC group when compared to HD group ($p=0.0003$, $p<0.0001$, $p=0.0034$,
266 $p<0.0001$, for lymphocytes and neutrophils counts, monocytes HLA-DR expression level and
267 neutrophils/lymphocytes ratio, respectively). No significant difference was observed between S and NIC
268 group at day-1 within these main immune cell populations (Supp.Tab.2).

269 To identify an early sepsis-specific signature within these immune populations, we compared the
270 abundance of the identified cell nodes of these immune populations between HD, NIC and S samples at
271 day-1. The abundance of 22 nodes was found selectively regulated in S at day-1 when compared to both
272 NIC and HD and 25 nodes differentiated S only from NIC at day-1 (Supp.Fig.7C, D). It included notably
273 15 nodes identifying classical monocytes with high expression of HLD-DR, 3 nodes of CD4⁺ T
274 lymphocytes and CD8⁺ T lymphocytes expressing CCR2 and CCR6, all were highly reduced in S patients,
275 one node of B lymphocytes with a low expression of B cells pan markers (HLD-DR, CXCR5, CD19 and
276 CCR6) and one node identified monocyte-derived DC (Fig.5C). Among the nodes that were massively
277 reduced in both S and NIC sample, 15 nodes out of 55 represent Basophils and Eosinophils subsets
278 (Fig.5D); the others being scattered among other cell populations.

279 Taken globally, the analysis of circulating non-neutrophil cells with a computational strategy allowed us
280 to resume sepsis hallmarks and identify the differences of several circulating immune subsets abundance.

281 *CD123⁺ and PD-L1⁺ sepsis-specific neutrophils are detectable by conventional cytometry and*
282 *discriminate infected and non-infected patients*

283 We identified two neutrophil subsets using 40 individuals and 42-marker mass cytometer and
284 computational analysis. These subsets might be detectable by conventional cytometry approach that is
285 used in routine in the clinic. To evaluate the efficiency and specificity of CD123⁺ and PD-L1⁺ neutrophil
286 subsets to discriminate sepsis patients from non-infected ones, we set up a fluorescent 7-marker flow
287 cytometry panel (Supp.Tab.4). We monitored an independent validation cohort composed of non-infected
288 patients (n=18) and sepsis patients (n=24).

289 With the overlay of full minus-two (FMT) stained control and the full panel stained tubes of three
290 representative patients of several expression levels of CD10, CD123 and PDL1, we appreciated the
291 increase of CD123⁺ and PD-L1⁺ sepsis-specific neutrophil subsets with the decrease of CD10 expression
292 by neutrophils (CD14⁻CRTH2⁻CD15⁺ cells) (Fig.6A). ROC analysis was performed using CD123⁺ and
293 PD-L1⁺ neutrophil subsets abundances, measured by conventional flow cytometry on an independent
294 validation cohort of sepsis and non-infected patients. A cut-off point of 0.35% of the CD123⁺ neutrophil
295 subset abundance was able to rule out sepsis patients with a specificity of 94.44% and sensitivity of 87.5%
296 and an AUROC of 0.95 (Fig.6B). When combining the abundance of the CD123⁺ and PD-L1⁺ neutrophil
297 subsets, the cut-off point changed to 0.60% with no effect on the sensitivity nor on the specificity (Fig.6C).
298 Whereas, a clinical SOFA score >2 was discriminating with a good sensitivity (91.30%) but with a poor
299 specificity (18.18%) and a worst AUROC of 0.61 (Fig.6D). In addition, the AUROC of SAPS-II score
300 was also lower (AUROC=0.69) with a sensitivity of 91.67% and a poor specificity of 25.00% (Fig.6E).
301 These results indicated that conventional flow cytometry recapitulates the results obtained by mass
302 cytometry and confirmed that the identified neutrophil subsets could be a marker of sepsis severity with

303 a better efficiency than clinical scores and reliably quantified by routinely performed clinical flow
304 cytometric profiling

305 In addition, we evaluated if the CD123+ neutrophil subset was only abundant in patients with the highest
306 severity scores. We used the data generated in both the discovery (Fig.4) and the validation cohorts (Fig.6)
307 and divided the cohorts by quartile of severity according to SOFA and SAPS II scores (Supp.Fig.8A, B).
308 While sepsis and non-infected patients overlap greatly their severity scores, the proportion of CD123+
309 neutrophils subset distinguishes efficiently sepsis and control groups in both discovery (Supp.Fig.8A) and
310 validation cohorts (Supp.Fig.8B).

311 *Immature sepsis neutrophils exhibit an impaired microbial specific activation and phagocytosis*

312 To address sepsis-associated neutrophils activation and phagocytic capacities, whole blood of each tested
313 individual was incubated with *Staphylococcus aureus* (*S. aureus*) or Zymosan coated bio-particles labelled
314 with pHrodo, a pH-sensitive fluorochrome (36), in order to identify immature neutrophils bio-particles
315 uptake capacity and activation.

316 All immature circulating neutrophils (CD64+CD10⁻) were able to phagocytose *Staphylococcus aureus* (*S.*
317 *aureus*) beads independently from their group (HD, S-D1, BM). However, S day-1 neutrophils
318 phagocytosis of Zymosan Beads (Mean±SD=28.12±8.39%) was not as effective as that of HD
319 (Mean±SD=50.43±13.04, $p=0.02$) (Fig.7A). This sepsis-associated decrease of phagocytosis goes with
320 the proportion increase of both CD123+ and PD-L1+ immature neutrophil subsets in the blood of the
321 tested sepsis patients when compared to HD (Fig.7B). t-SNE visualization of PC and NC neutrophils of
322 both *S. aureus* (Fig.7C, D) and Zymosan (Fig.7E, F) bead stimulations highlighted the lower expression
323 level of CD11b marker by S day-1 neutrophils when compared to HD and the default of activation of
324 these cells after microbial beads activation. In fact, S neutrophils exhibited a lower ratio of CD11b and

325 CD66b MFI between PC and NC after activation, when compared to healthy donors after *S. aureus*
326 (Fig.7D) or Zymosan (Fig.7F) stimulations. The impaired phagocytic capacity of sepsis-patients'
327 immature neutrophils compared to HD neutrophils was confirmed by the measurement of phagocytosed
328 beads MFI ratios between PC and NC. This ratio was three times lower for S day-1 *S. aureus* response
329 (Fig.7D) and 30% lower for S day-1 Zymosan response (Fig.7F). These data allowed the identification
330 of an impaired capacity of immature sepsis neutrophils to form efficient phagolysosomes after bio-
331 particles stimulation and a default of activation when compared to HD.

332

333 **Discussion**

334 Whole blood mass cytometry and computational analysis identified classical hallmarks of sepsis, and
335 revealed two novel neutrophil subsets that distinguish early sepsis from aseptic inflammatory
336 syndromes. Two novel neutrophil subsets were identified, CD10⁻CD64⁺PD-L1⁺ neutrophils and CD10⁻
337 CD64⁺CD16^{low/-}CD123⁺ immature neutrophils that could be used for early identification of sepsis
338 patients. CD123⁺ and PDL1⁺ neutrophil subsets could help improving sepsis diagnosis and guide sepsis
339 treatment monitoring.

340 The results of this study recapitulated previous original findings and meta-analysis studies regarding the
341 sepsis-related increase of circulating immature CD10⁻CD64⁺ neutrophils (13, 14, 18, 37). Despite all these
342 large efforts, the CD64 detection-based tools are not yet standardized for sepsis diagnosis, because of the
343 heterogeneity of sepsis syndrome and inter-individual variability of CD64 basal level among sepsis
344 patients.

345 The CD10⁻CD64⁺CD16^{low/-}CD123⁺ population is most consistent with immature neutrophils. The
346 frequency of this population among total neutrophils positively correlates with both SAPS II and SOFA

347 severity scores, and need to be confirmed in a larger collection. The neutrophils expression of CD123 was
348 not described before during sepsis. In a previous study of Weber *et al.*, using a mouse model of abdominal
349 sepsis, the cytokine IL-3 was reported to potentiate inflammation in sepsis by inducing myelopoiesis of
350 neutrophils and IL-3 deficiency protects mice against sepsis (20). Moreover, the authors described an
351 association between high plasma IL-3 levels and high mortality. This result was also obtained in a recent
352 prospective cohort study, where higher levels of IL-3 were shown to be independently associated with
353 hospital mortality in septic patients (21). All these results identify IL-3 and its receptor CD123 as an
354 orchestrator of emergency myelopoiesis, and reveals a new target for the diagnosis and treatment of sepsis.

355 To our knowledge, the expression of PD-L1 by neutrophil during sepsis was not reported before. It was
356 defined on monocytes, macrophages and endothelial cells (38) but not granulocytes. Monocyte PD-L1
357 expression was described as an independent predictor of 28-day mortality in patients with septic shock
358 (22, 23). Peripheral blood transcriptomic analysis done by Uhle *et al.*, revealed the expression of PD-L1-
359 gene among the top 44 immune-related genes differentially expressed between patients with sepsis and
360 healthy donors (15). In parallel, mice in which the PD-1/PD-L1 interaction was inhibited show improved
361 survival to sepsis (39). Our results bring up a new target for the immune checkpoint therapies.

362 Controversial results were previously described regarding functional aspects of neutrophils during sepsis.
363 On one hand, Demaret *et al.*, described conserved phagocytosis and activation capacities of sepsis
364 neutrophils characterized as CD10^{dim}CD16^{dim} immature cells, after whole blood IL8, fMLP or FITC-
365 labeled *Escherichia coli* stimulation cells (40). On the other hand, Drifte *et al.*, by comparing mature and
366 immature neutrophils functions found that the latter were less efficient in phagocytosis and killing.
367 Accordingly, we observed an impaired capacity of cells to form efficient phagolysosomes after bio-
368 particles stimulation and a default of activation when compared to HD.

369 The immunosuppressive function was also attributed to G-MDSC neutrophils subset during sepsis (13-
370 15, 18). But, to date, human G-MDSC definition lacks consensual phenotypic characterization.
371 Published results on G-MDSC in cancer were obtained according to various phenotypes. Condamine
372 *et al.* described them as Lectin-type oxidized LDL receptor-1 (LOX1) expressing cells (41). Using
373 flow cytometry, we measured the expression of LOX-1 in sepsis patients (data not shown). No LOX-
374 1 co-staining was observed with neither CD123⁺ nor PD-L1⁺ subsets. More investigation is needed to
375 characterize if CD123⁺ neutrophils and PD-L1⁺ subset belong to G-MDSC.

376 Further research should be conducted to identify appropriate clinical actions for each identified
377 neutrophil subset and their evolution over time course and in different cohorts of patients
378 (undifferentiated shock patients, immunosuppressed patients, different types of infections, durability
379 of neutrophil population after antibiotics), to understand whether altered neutrophil production is
380 responsible for increased sepsis risk, and to determine how these subsets can be therapeutically
381 targeted.

382 In this study we show that the use of the identified neutrophil subsets gives complementary information
383 to severity scores such as SOFA and SAPS II and are specific of sepsis. In the discovery cohort, in
384 which stringent selection was applied for sepsis and non-infected control patient inclusion, few
385 differences were observed between AUROC of CD123⁺ neutrophils, SOFA and SAPS II scores (Fig.
386 4D, F, G). In contrast, the validation cohort, where blind analysis was performed, SOFA and SAPS II
387 lose their discrimination power (Fig. 6D, E) and CD123⁺ neutrophils biomarker remain highly specific
388 and sensitive for sepsis patient identification.

389 In addition, the diagnosis of sepsis was evoked for a significant proportion of patients (6/18) in the
390 ICU control group of the validation cohort, a third of them received antibiotics due to their clinical

391 characteristics but the diagnosis of sepsis was finally dropped out. They ended to be non-infected and
392 undistinguishable from other inflamed and non-infected controls (Supp.Tab.5). Of note, the CD123+
393 neutrophils proportion of these patients was <0.3%, below the cutoff value identified in our ROC
394 analysis. The use of this biomarker candidate would have avoided this unnecessary administration of
395 antibiotics. Especially that flow cytometry is a widely available technique in the clinic, with reasonable
396 costs and results can be rapidly obtained.

397 The use of a whole blood flow cytometry test to diagnose sepsis could change the fate of patient's care.
398 The clinician would have a rapid and specific result, obtained before microbiological cultures results,
399 that could guide their therapeutic decision.

400 In parallel, future studies should now be undertaken to validate the use of these new neutrophil subsets
401 in clinic by routine flow cytometry as an early biomarker predictive of sepsis. Larger cohorts that better
402 represent not only sepsis patients but also the diversity of aseptic inflammatory syndromes need to be
403 evaluated.

404 Delay to sepsis diagnosis has been shown to decrease survival and increase hospital costs, a better
405 diagnosis will definitely help to improve patient's care, avoid unnecessary treatments and reduce
406 hospital length of stay.

407

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412

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422

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568

569 **Figure Legends:**

570 **Fig.1. Study design.** (A) Blood samples from sepsis patients (S) (n=17) or non-infected post-
571 cardiothoracic surgery patients (NIC) (n=12) were enrolled in the discovery cohort of the study, in
572 addition, blood samples were obtained from healthy donors (HD) (n=11) and bone marrows biopsies
573 from orthopedic surgery patients (BM) (n=5). Immunostainings targeting 42 parameters were
574 performed and analyzed by mass cytometry. A computational “discovery strategy” was used to
575 identify sepsis-specific subsets (B), a “computational validation” analysis was used to check whether
576 the identified sepsis-specific subsets are strategy-dependant (C), and with an additional “expert driven
577 validation” we defined a small set of markers to gate on the sepsis-specific neutrophil subsets (D). A
578 second independent validation cohort, with sepsis patients (S) (n=24) and noninfected patients (NIP)
579 (n=18), was used for the “biological validation” of these sepsis-specific neutrophil subsets by
580 conventional flow cytometry (E).

581 **Fig.2. Identification of sepsis day 1-specific neutrophils with a discovery analysis strategy.** (A) t-SNE
582 analysis was performed on neutrophils from all samples with cells being organized along t-SNE-1-2

583 and t-SNE-2-2 according to per-cell expression of CD11b, CD66b, CD16, CD10, CD64 and CD123,
584 PD-L1. Cell density for the concatenated file of each group is shown, on a black to yellow heat scale,
585 for each group time-point. **(B)** A heat map shows samples clustering (columns) according to nodes
586 cell proportion log₂-transformed and centered around the mean proportion of all samples' nodes
587 (rows). Samples and mean-centered log₂-transformed nodes cell proportion were arranged according
588 to complete linkage hierarchical clustering. Heat intensity (from blue to yellow) reflects the mean-
589 centered log₂-transformed cell proportion of each sample's node. **(C)** A heatmap shows
590 characterization of cell nodes identified by SPADE (columns) according to mean expression of 7
591 markers (rows). Markers were arranged according to complete linkage hierarchical clustering and
592 nodes were pre-ordered according to **(B)** heat map nodes order. Heat intensity (from blue to red)
593 reflects the mean expression of each marker for each node. **(D)** Four groups of nodes were back-
594 viewed on t-SNE1-2 / t-SNE2-2 map. **(E)** cells abundance of each meta-cluster subset (CD10-
595 CD64+CD16+PD-L1+ cell subset in red, CD10-CD64+CD16lowCD123+ cell subset in blue and
596 CD10-CD64+ cell subset in green) was presented as cell proportion among total neutrophils of each
597 group samples. Statistics: Nonparametric two-tailed Mann-Whitney test was used to compare
598 differences in cellular abundance of cell subsets between NIC-D1 and S-D1 (see the Methods
599 section). Sample sizes: HD=11, BM=5, NIC=12 and S=17.

600 **Fig.3. Validation of sepsis day-1-specific neutrophil subsets by a second computational strategy.** As
601 a first step, UMAP analysis was performed on all samples neutrophils and cells were organized along
602 UMAP-1 and UMAP-2 axes according to per-cell expression of CD11b, CD66b, CD16, CD10, CD64
603 and CD123, PD-L1. As a second step, FlowSOM clustering was done to separate neutrophils subsets
604 into 50 clusters. MEM was then used to quantify the enriched features of the 50 clusters. Protein
605 enrichment was reported on a +10 to -10 scale, where +10 indicates that protein's expression was

606 especially enriched and -10 indicated that the protein's expression was excluded from those cells,
607 relative to the other neutrophils clusters. (A) Among these clusters, two meta-clusters were identified
608 as phenotypically identical to the strategy-1 sepsis-specific neutrophils: clusters 18 and 19 (in red)
609 composed of CD10-CD64+PD-L1+ neutrophils and clusters 6 and 7 (in blue) composed of CD10-
610 CD64+ CD16lowCD123+ neutrophils. (B) Cells abundance of each meta-cluster subset (CD10-
611 CD64+CD16+PD-L1+ cell subset in red and CD10-CD64+CD16lowCD123+ cell subset in blue) was
612 presented as cell proportion among total neutrophils of each group samples. Statistics: Nonparametric
613 two-tailed Mann-Whitney test was used to compare differences in cellular abundance of cell subsets
614 between NIC-D1 and S-D1 (see the Methods section). Sample sizes: HD=11, BM=5, NIC=12 and
615 S=17. (C) each meta-cluster cells (red and blue) was back-viewed on both UMAP-1 / UMAP-2 map,
616 and t-SNE1-2 / t-SNE2-2 map.

617 **Fig.4. Sepsis day 1-specific neutrophil subsets validated by expert gating correlate with severity**
618 **scores.** Expert gating strategy with 7 markers set (A) allowed the selection of CD10-CD64+PD-L1+
619 cell subset (in red) and CD10-CD64+CD16lowCD123+ cell subset (in blue), back-viewed on both
620 discovery (t-SNE1-2 / t-SNE2-2) and validation (UMAP-1 / UMAP-2) maps. The two neutrophil
621 subsets are significantly more abundant in sepsis patients (S) blood collected at day-1 post-admission
622 to ICU when compared to day-1 or day-7 non-infected post-cardiothoracic surgery patients (NIC) or
623 Healthy donors (HD) (B). Correlation between the log10 transformed frequency of CD10-CD64+PD-
624 L1+ neutrophils subset (in red) or CD10-CD64+CD16^{low}CD123+ neutrophils subset (in blue) and
625 SAPS II score (green squares) or SOFA score (purple squares) are shown in (C). ROC curve obtained
626 using only CD123+ neutrophil subset is shown in (D) and the one using CD123+PD-L1+ neutrophil
627 subsets is shown in (E) and with the SOFA and SAPS II clinical scores are shown in (F) and (G)
628 respectively. Statistics: Nonparametric two-tailed Mann-Whitney test was used to compare cellular

629 abundances of cell subsets between S-D1 and NIC-D1, NIC-D7 or HD. Nonparametric two-tailed
630 Wilcoxon signed-rank test was used to compare cellular abundances between the two matched groups
631 S-D1 and S-D7. Linear regression lines and Spearman's rank correlation were used to assess
632 relationship between neutrophil subsets frequency and severity scores (see the Methods section).
633 Spearman r and two-tailed p value are presented. * $p < 0.05$. Sample sizes: HD=11, BM=5, NIC=12
634 and S=17.

635 **Fig.5. Non-neutrophil cells analysis resume sepsis immune hallmarks.** (A) Lymphocytes and
636 monocytes numbers and intensity of HLA-DR expression on monocytes (mHLA-DR) were obtained
637 from non-neutrophils computational analysis and presented for each group. (B) Neutrophils numbers
638 were obtained previously from the computational separation of neutrophils from non-neutrophil cells
639 and used to calculate Neutrophils/Lymphocytes ratio. Cell number of the main immune subsets that
640 were differentially abundant in S group from HD and NIC were presented in (C) and the ones that
641 were differentially abundant in S group from only HD were presented in (D).

642 **Fig.6. Sepsis-specific neutrophils are detectable by conventional cytometry and discriminate**
643 **infected from non-infected patients.** The gating strategy applied on fluorescent flow cytometry data
644 of three sepsis patients from the validation cohort is showed in (A). The overlay of full minus-two
645 (FMT) stained control and the full panel (FP) stained tubes of each representative patient, showed the
646 increase of sepsis-specific neutrophil subsets with the decrease of CD10 expression by neutrophils
647 (CD14-CRTH2-CD15⁺ cells). The ROC curves were obtained using only CD123⁺ neutrophil subset
648 (B), the two CD123⁺ and PD-L1⁺ neutrophil subsets (C) or using the SOFA (D) and SAPS II (E)
649 clinical scores.

650 **Fig.7. Staphylococcus aureus and Zymosan specific activation and phagocytosis are impaired in**
651 **immature sepsis neutrophils.** To address sepsis immature (CD64+CD10-) neutrophils phagocytic
652 capacities, 100µL of blood were incubated with 20µL or 40µL of beads coated with Staphylococcus
653 aureus or Zymosan, respectively, coated-particles and coupled with pH acidification-sensitive
654 fluorochrome. After 1h incubation at 37°C (PC: positive control) or 4°C (NC: negative control) cells
655 were stained and analyzed by flow cytometry. (A) represents gating strategy of CD15+CD14-CD3-
656 CD19- neutrophils from healthy donors (HD), sepsis day-1 samples (S-D1) and bone marrow samples
657 (BM). Cells were separated in 2 gates based on CD10 expression and phagocytosis marker intensity
658 (Staphylococcus aureus or Zymosan) and cells from PC (red dots) were overlaid on NC cells (blue
659 dots). The proportion of total phagocytic neutrophils were presented for the three groups. t-SNE
660 analysis organized cells along t-SNE axes according to per-cell expression of 5 proteins and
661 phagocytosis fluorescence. Cell expression of CD11b after Staphylococcus aureus (B) or Zymosan
662 (C) stimulations, for one representative individual of HD and S-D1 stimulated at +4°C (NC) and
663 +37°C (PC) is shown on a heat scale. The ratio between PC and NC CD66b CD11b and particles
664 MFI, of each individual after Staphylococcus aureus (D) or Zymosan (E) stimulations, in each group
665 were plotted in histograms. CD10- cells have less phagocytic capacity whatever it is appreciated by
666 MFI or proportion. Stimulated CD10- cells exhibit a lower level of expression of CD11b and CD66b.
667 Statistics: Nonparametric two-tailed Mann-Whitney test was used to compare differences in cellular
668 abundance of cell subsets and MFI ratios (see the Methods section). Sample sizes: HD=4, S-D1=6
669 and BM=3.

670 **Figures:**

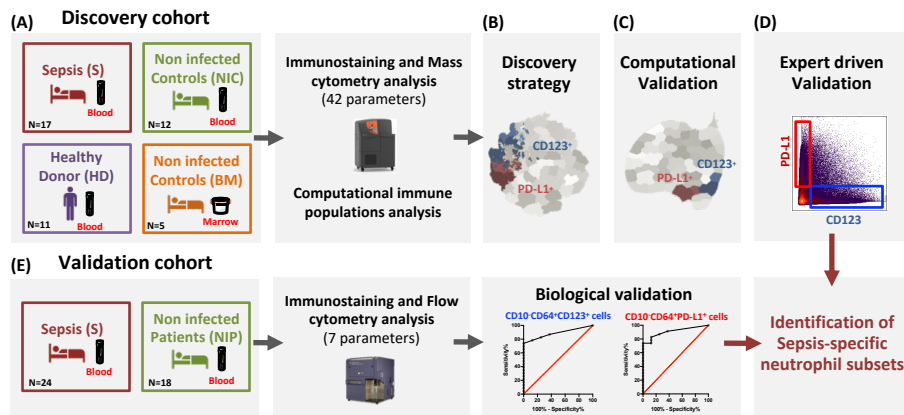


Figure 1. Study design

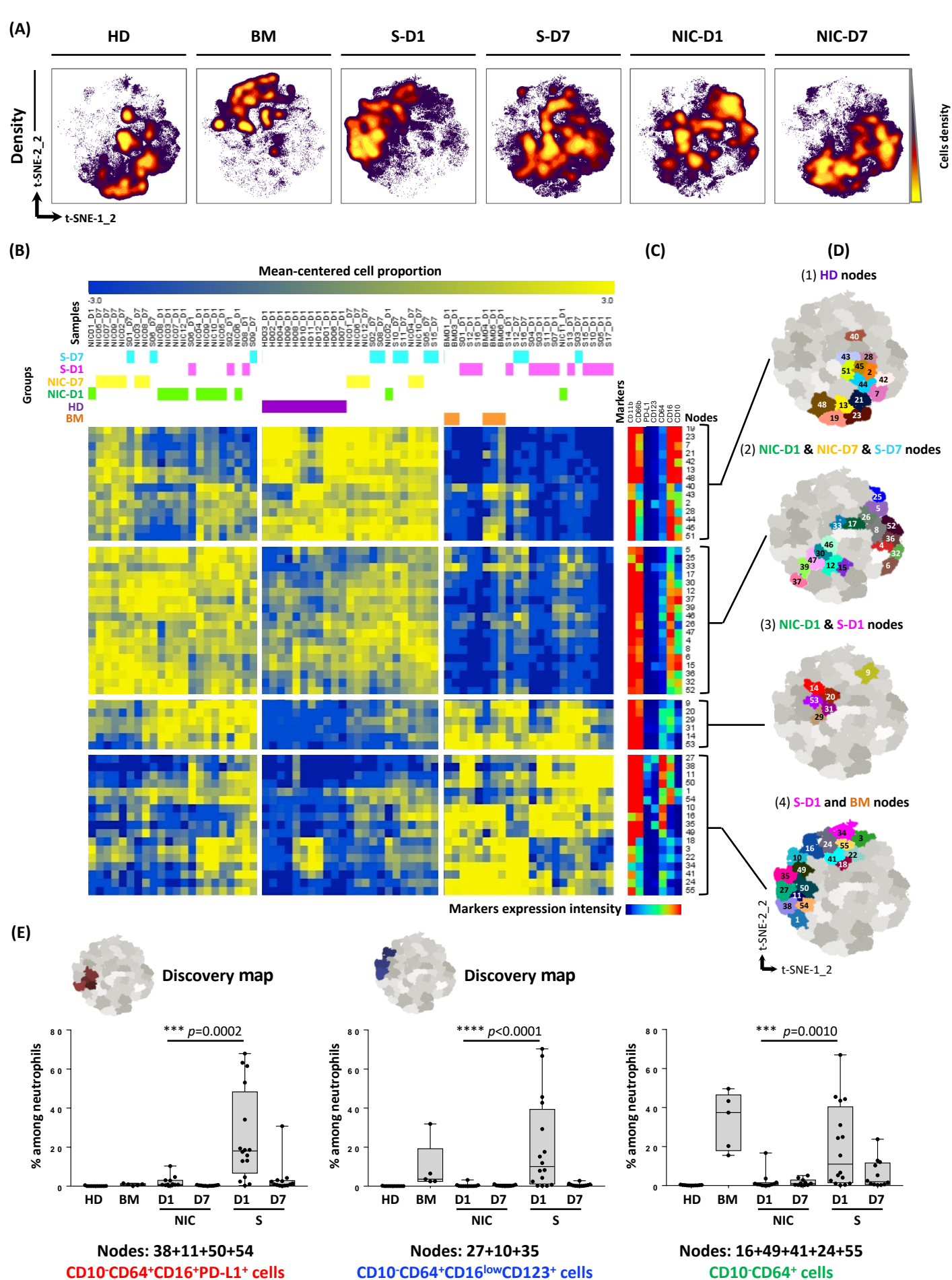


Figure 2. Identification of sepsis day 1-specific neutrophils with a discovery analysis strategy

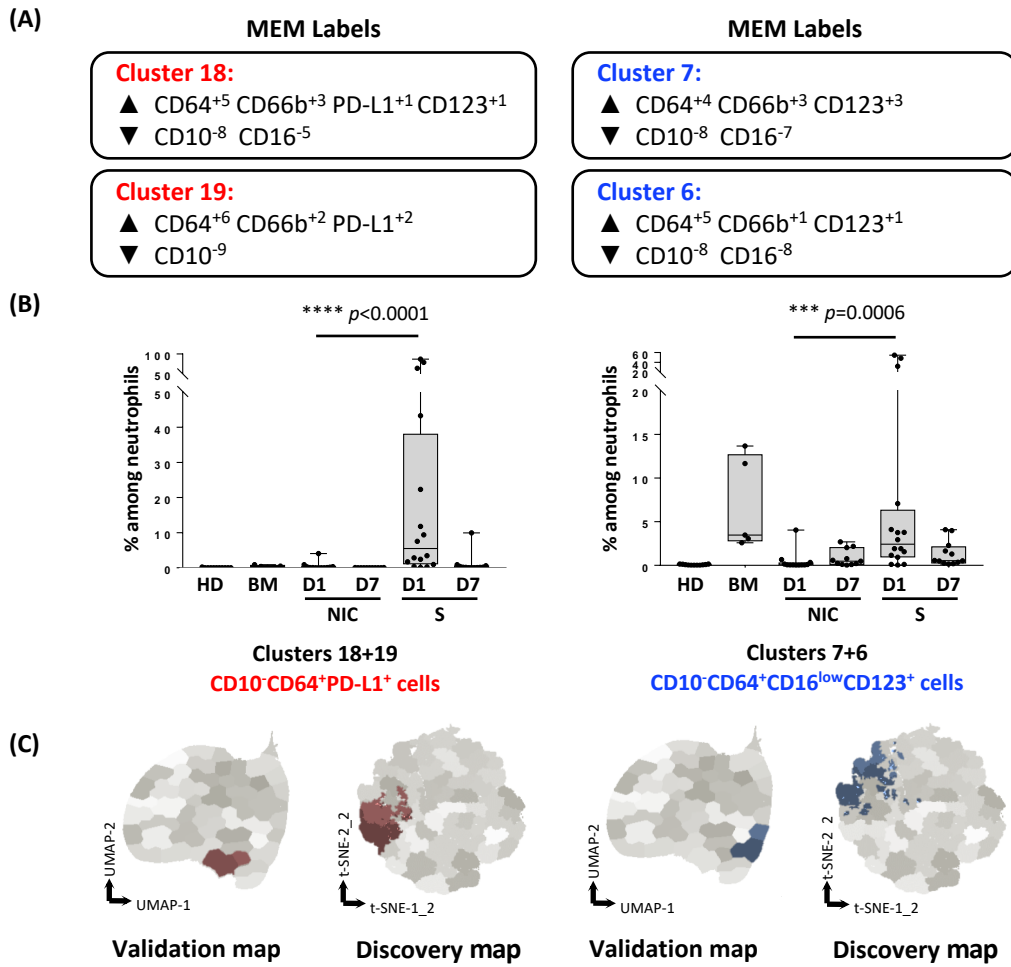


Figure 3. Validation of sepsis day-1-specific neutrophil subsets by a second computational strategy

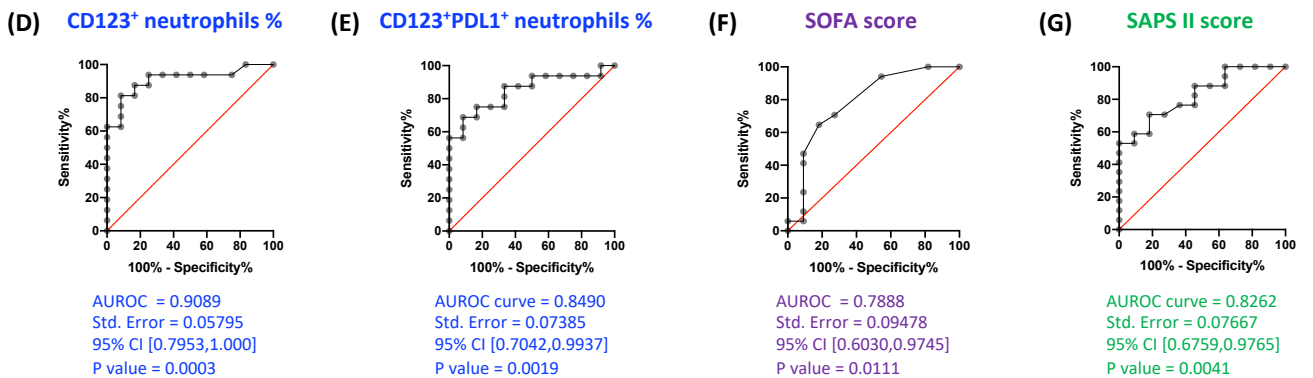
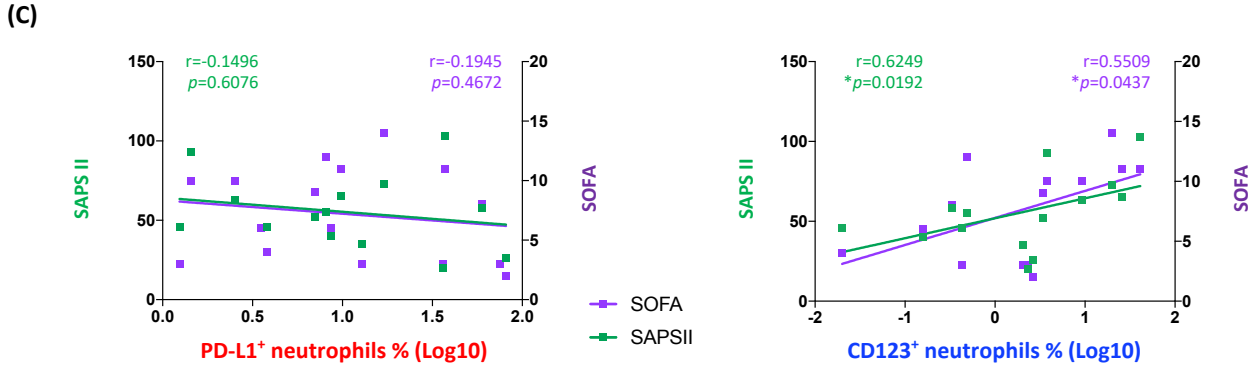
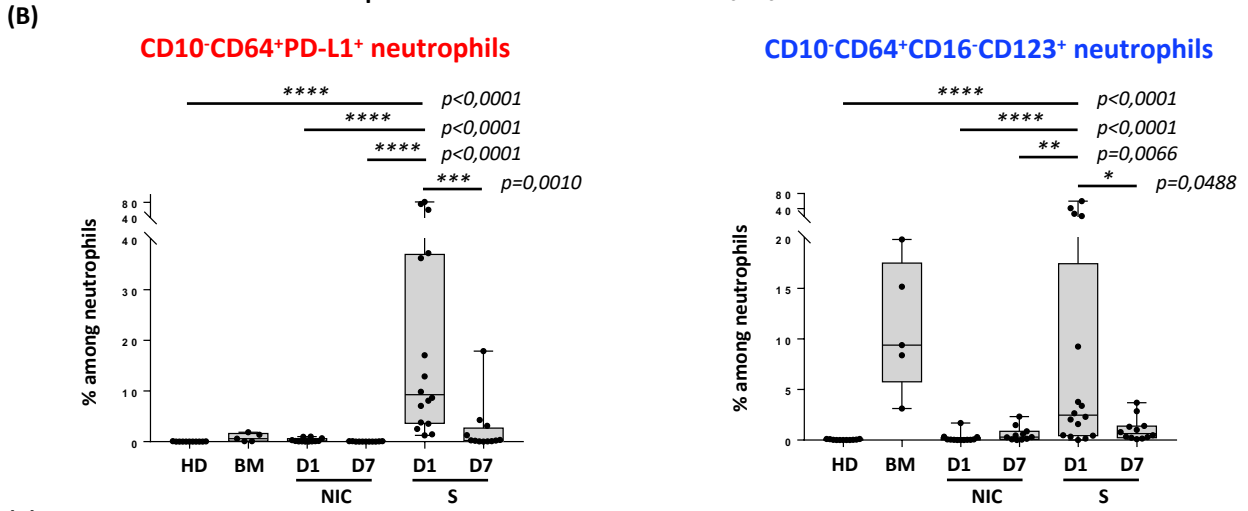
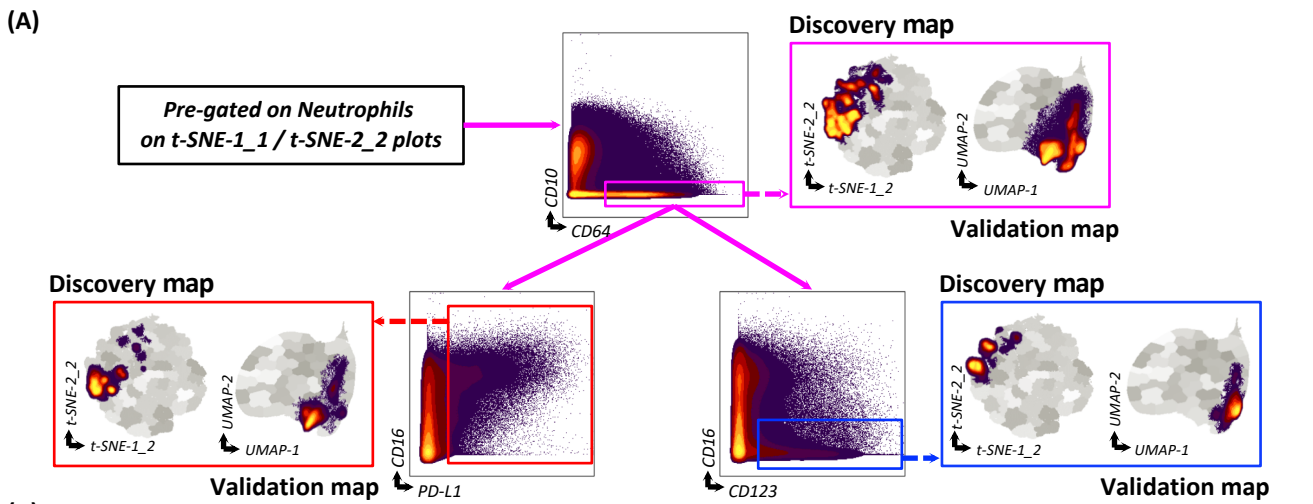


Figure 4. Validation of sepsis day 1-specific neutrophil subsets by expert gating

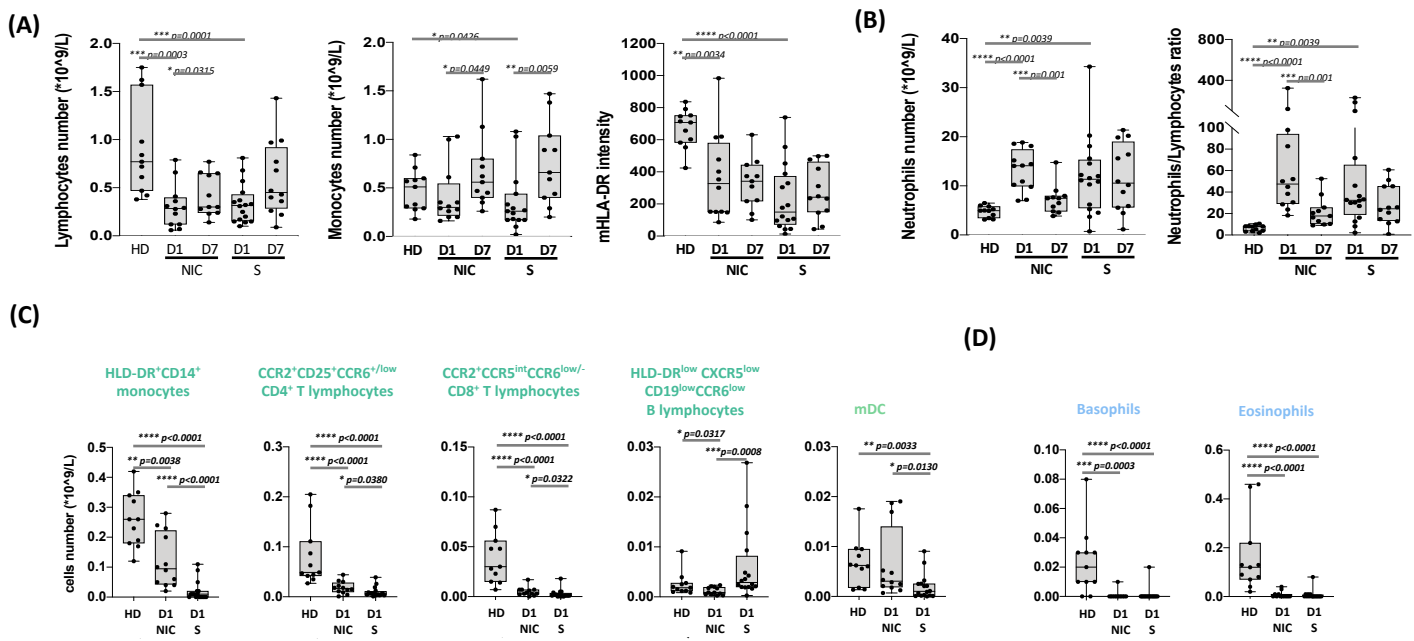
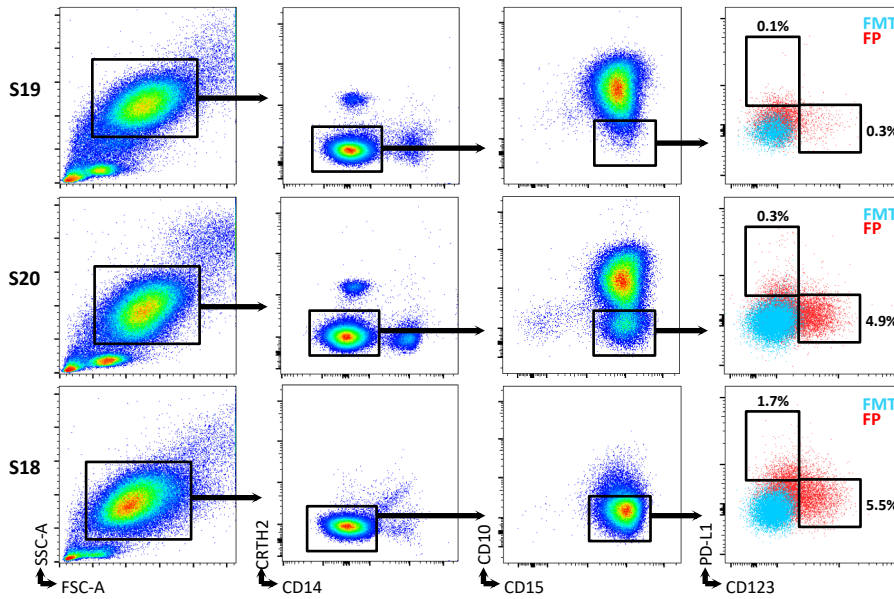
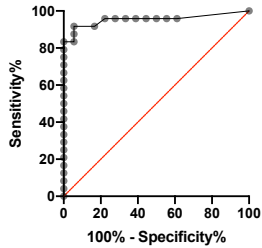


Figure 5. Non-neutrophil cells analysis resume sepsis immune hallmarks

(A)

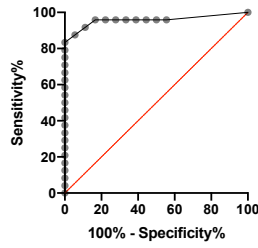


(B) CD123⁺ neutrophils %



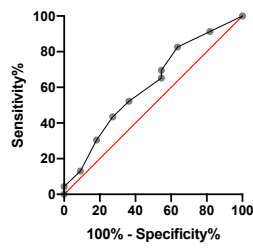
AUROC = 0.9572
Std. Error = 0.03387
95% CI [0.8908,1.000]
P value < 0.0001

(C) PD-L1⁺CD123⁺ neutrophils %



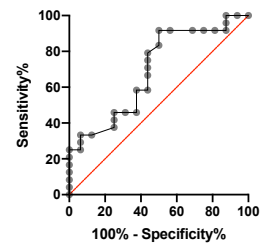
AUROC = 0.9537
Std. Error = 0.03527
95% CI [0.8846,1.000]
P value < 0.0001

(D) SOFA score



AUROC = 0.6126
Std. Error = 0.1052
95% CI [0.4065,0.8188]
P value = 0.2941

(E) SAPS II score



AUROC curve = 0.6940
Std. Error = 0.08660
95% CI [0.5243,0.8637]
P value = 0.0397

Figure 6. Sepsis-specific neutrophils are detectable by conventional cytometry and discriminate infected from non-infected patients

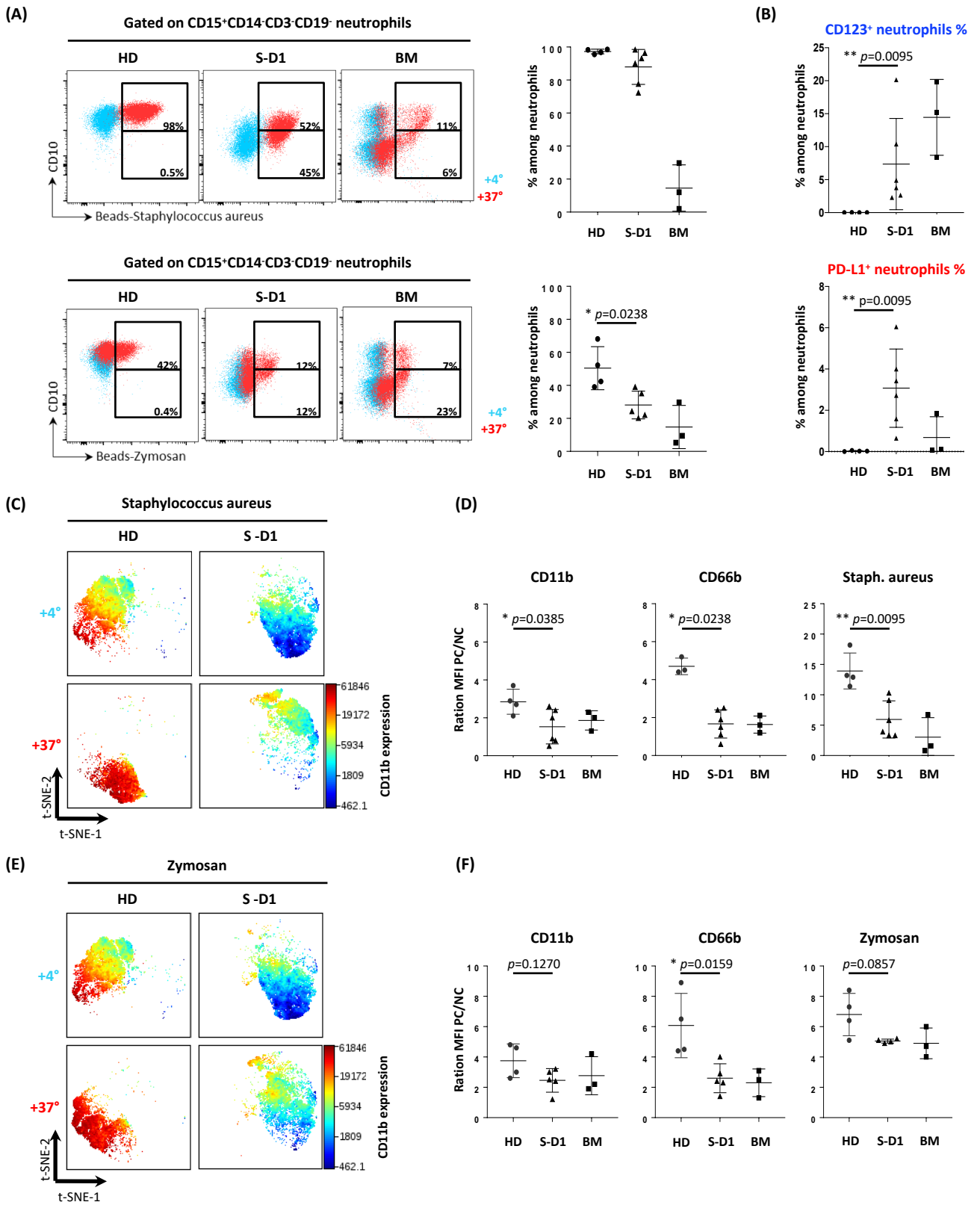


Figure 7. Staphylococcus aureus and Zymosan specific activation and phagocytosis are impaired in immature sepsis neutrophils