Gene signature of circulating platelet-bound neutrophils is associated with poor prognosis in cancer patients

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Abstract
Beyond their critical role in hemostasis, platelets physically interact with neutrophils to form neutrophil-platelet aggregates (NPAs), enhancing neutrophil effector functions during inflammation. NPAs may also promote disease worsening in various inflammatory diseases. However, characterization of NPAs in cancer remains totally unexplored. Using ImageStreamX (ISX) imaging flow cytometer, we were not only able to detect CD15+ CD14− CD36+ ITGA2B+ NPAs in both healthy donors’ (HDs) and cancer patients’ bloods, but we also showed that NPAs result from the binding of platelets preferentially to low-density neutrophils (LDNs) as opposed to normal-density neutrophils (NDNs). By reanalyzing two independent public scRNAseq data of whole blood leukocytes from cancer patients and HDs, we could identify a subset of neutrophils with high platelet gene expression that may correspond to NPAs. Moreover, we showed that cancer patients’ derived NPAs possessed a distinct molecular signature compared to the other neutrophil subsets, independently of platelet genes. Gene ontology (GO) term enrichment analysis of this NPAs-associated neutrophil transcriptomic signature revealed a significant enrichment of neutrophil degranulation, chemotaxis and trans-endothelial migration GO terms. Lastly, using The Cancer Genome Atlas (TCGA), we could show by multivariate Cox

Abbreviations: ANC, absolute neutrophil count; CPM, count per million; GO, gene ontology; HDs, healthy donors; HNC, head and neck cancer; HR, hazard ratio; ISX, ImageStreamX; LDNs, low-density neutrophils; LIHC, hepatocellular carcinoma cancer patients; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; NDNs, normal-density neutrophils; NETs, neutrophil extracellular traps; NLR, neutrophils-to-lymphocytes ratio; NPAs, neutrophil-platelet aggregates; NSCLC, nonsmall-cell lung cancer; OS, overall survival; PAAD, pancreatic adenocarcinoma cancer patients; PCA, principal component analysis; PCs, principal components; PFI, progression-free interval; RBC, red blood cells; RT, room temperature; scRNAseq, single cell RNA sequencing; ssGSEA, single sample Gene Set Enrichment Analysis; TANs, tumor-associated neutrophils; TCGA, The Cancer Genome Atlas; UMAP, uniform manifold approximation and projection; UPS, undifferentiated pleomorphic sarcoma.

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1 | BACKGROUND

Although, some recent evidences suggested that tumor-associated neutrophils (TANs) could display antitumor properties,1-3 a large body of evidence demonstrated that TANs could be protumoral and promote metastasis.4-6 Protumor TANs can assist metastasis by blunting antitumor T-cell responses,4 and stimulating proliferation and invasiveness of metastatic tumor cells.5,6 Accumulating evidence in mice and humans showed that blood neutrophils may also contribute to cancer progression. Numerous retrospective analysis across multiple different cancer types showed that an elevation in the absolute count of circulating neutrophils together with a decrease in the absolute count of circulating lymphocytes, both accounting for a high neutrophils-to-lymphocytes ratio (NLR) on blood, was associated with a worse prognosis in patients.7-10 More recently, studies have shown that a subset of circulating neutrophils, named low-density neutrophils (LDN) expanded in cancer patient’s blood and was associated with worse prognosis.11,13,15 Nevertheless, LDN remains a heterogeneous population of cells including mature and immature neutrophils.13,14 A subset of circulating LDN expressing the LOX-1 scavenger receptor was reported to display higher T-cell suppressive functions as compared to LOX-1− LDN.12 LOX-1+ T-cell suppressive neutrophils were recently reported to infiltrate lung tumors and were associated with a worse patient survival.15 Besides studies in humans, recent evidence in mice demonstrated that the preprogramming of neutrophils in the periphery was required for acquisition of their tumor-promoting functions once at tumor site16,17 supporting the idea that tumor-promoting neutrophils derived from a distinct subset of neutrophils preexisting in the periphery.

Beyond their critical role in hemostasis, platelets can bind to circulating leukocytes forming leukocyte-platelet aggregates, enhancing their effector functions. For instance, platelets have been recently shown to interact with peripheral blood monocytes to activate antigen cross-presentation.18 In sepsis, in contrast to free neutrophils, NPAs (neutrophil-platelet aggregates) produce higher amount of neutrophil extracellular traps (NETs)19-21 and display increased phagocytic functions.22-24 Furthermore, NPAs display enhanced trans-endothelial migration capacity25,26 therefore promoting neutrophil infiltration to site of injury and fueling pathological inflammation in various diseases.21,27-29 However, to our knowledge, the characterization of NPAs in cancer patients remains unexplored.

In our study, we showed that neutrophils from NPAs represent a unique subset of activated neutrophils in cancer patients by combining ImageStreamX (ISX) imaging flow cytometry and analysis of public single cell transcriptomic data of human circulating neutrophils. In particular, we showed that LDN as opposed to NDN were preferentially involved in NPA formation. Finally, we could generate a specific gene signature of neutrophils in NPAs that we found associated with a worse prognosis in pancreatic adenocarcinoma patients and liver hepatocellular carcinoma patients.

2 | MATERIALS AND METHODS

2.1 | Human cohorts

Whole blood from a cohort of six metastatic NSCLC patients (Table S1) at the Léon Bérard Hospital (France) was collected in...
EDTA-coated tubes (BB-0033-00050, CRB Centre Léon Bérard, Lyon, France). As an age and sex-matched control cohort, whole blood from healthy donors (HD) was collected via the Etablissement Français du Sang (Table S1).

2.2 Preparation of whole blood, NDNs and LDNs-enriched fractions for multispectral imaging flow cytometry (ImageStreamX, ISX)

Blood from NSCLC patients or HD was withdrawn in EDTA-coated vacutainers 1 hour prior to the processing. Two tubes of 3 mL of blood were centrifuged at 120g (no brake) for 15 minutes at room temperature (RT) to separate platelet-rich plasma from leukocyte-enriched red pellet. Platelet-rich plasma was discarded for both tubes. One out of the two tubes was used for whole blood staining while the other served for the separation of LDNs from NDNs. For such separation, leukocyte-enriched red pellet was resuspended in 6 mL of PBS1X (5 mM EDTA) and then layered on the top of 3 mL of Ficoll (CMSSMSL01-01, EUROBIO), before being centrifuged at 600g for 20 minutes at RT (acceleration 5 and brake 1). LDNs-enriched PBMC ring and NDNs-enriched red pellet were washed with PBS1X, and pellets were resuspended in BD Pharm Lyse (SSS899) according to manufacturer's protocol to eliminate red blood cells. In parallel, whole blood leukocyte-enriched red pellet was also resuspended in BD Pharm Lyse for the same purpose. After incubation, red blood cells (RBC)-lysed leukocytes were washed twice with PBS1X (5 mM EDTA) were resuspended at a final concentration of 20.10^6 cells/ml prior to be stained. Samples were acquired on ImageStreamX (ISX) no more than 5 days following cell staining and fixation.

Two million cells per sample were stained for 30 minutes in the dark at 4°C in 100 μL staining buffer (PBS1X, 2% SVF, 5 mM EDTA) with antibodies directed against CD15 (561 584, Clone HI98), CD14 (562 335, MφP9), CD41a (559 777, HIP8) (all from BD), and CD36 (130-110-739, REA760) and CD62P (130-105-714, REA389) from Miltenyi Biotec. Cells were washed twice with PBS1X (5 mM EDTA, 2% fetal bovine serum [FBS]) before being fixed in 2% formaldehyde solution (252 549, Sigma-Aldrich). After incubation at 4°C, cells were washed twice with PBS1X (5 mM EDTA, 2% FBS) and resuspended in a final volume of 180 μL PBS1X (5 mM EDTA, 2% FBS).

NPAs were imaged with ImageStreamX (ISX) imaging flow cytometer (Aminis Corporation-Luminex, Seattle, WA) using 405, 488, 561, and 642 lasers and the 40X objective. Brightfield provided morphological and structural details of the cell. At least 300 000 RBC-lysed blood cells from NSCLC patients or HDs separately, excluding debris and free-platelet with low area, were collected for each sample. Since flow-speed could influence the proportion of NPAs, 30 RBC-lysed blood cells were acquired with a flow-speed between 500 to 1000 events/second for each sample. Data were analyzed using IDEAS image analysis software (Aminis Corporation, Seattle, WA). A specific gating strategy, with multiple filtering steps was set up to identify noncoincidental viable NPAs (Figure S6). Such strategy aimed first at retaining only focused cells based on Gradient RMS values for each event. Remaining debris and free platelets with low area were discarded. Dead cells positive for Zombie NIR (Biolegend) viability marker were then eliminated. Neutrophils were selected as cells with a high expression of CD15 and a low expression of CD14 markers. Low area event on CD15 object mask were eliminated to exclude CD15−debris or CD15+ insufficient quality staining for an accurate estimation of true NPAs. Platelet-related events were then selected by gating on events positive for both ITGA2B and CD36 cell-surface markers. A second filter was applied to narrow down the selection of platelet-related events by only considering the ones with high ITGA2B intensity on ITGA2B-related component with the largest area to remove residual platelet debris. To only retain noncoincidental NPAs, two distinct masks delimiting neutrophil-related CD15 stained-area and platelet-related ITGA2B stained-area were created. Noncoincidental NPAs were events for which neutrophil and platelet-associated masks were overlapping with at least one pixel, and confirmed by image examination of random samples. Only the most-focused platelet-related events were considered by selecting events with high gradient RMS of ITGA2B marker on platelet area. Frequencies of NPAs were calculated on at least 100 noncoincidental NPAs for each NSCLC patients and HDs.

2.3 May-Grünwald-Giemsa staining

A blood smear from a drop of NSCLC patients' whole blood was performed on a glass slide. Blood smear was dried up for 10/15 minutes at RT. May-Grünwald staining was layered on dried blood smear using Hema-Tek Stain Pak Kits designed for the Bayer Hema-Tek 2000 Slide Stainer. Pumps deliver fresh reagents (Stain, Buffer and Rinse solution) in precise volume. Slide was then taken out and completely dried at RT before being covered glasses and liquid mounting media using Tissue-Tek Coverslipping Film. Slides are immediately available for being digitized on Pannoramic scan II slide scanner by 3DHistech (x40, Z Stack, Multilayer Mode 5*0.2um) and being visualized using case viewer software.

2.4 Bioinformatic analysis of public scRNAseq data of human whole blood leukocytes

2.4.1 ScRNAseq data of NSCLC patients' whole blood leukocytes (GSE127465)

ScRNAseq data of six NSCLC patients' whole blood leukocytes were downloaded from GEO as normalized count per million (CPM) gene expression matrix. Briefly, preprocessing of scRNAseq data performed by the authors including the removal of: (i) dead cells identified by a mitochondrial gene content >20% among total gene transcribed; (ii) cells with poor quality transcriptome, as being cells with less than 300 counts; (iii) doublet cells performed with Scrublet. We next performed analysis of CPM gene expression matrix from six NSCLC patients' whole blood leukocytes by using Seurat R package (version 3.1.1). We first removed genes that were not expressed in at least three cells and filtered-out cells with less than 200 different
transcribed-genes (Seurat default parameters). Matrix was log-transformed and scaled prior to principal component analysis (PCA) with ScaleData Seurat function. PCA was performed on the 2000 most-variable genes, using RunPCA function. We used JackStraw function to determine the statistical significance of PCA scores that led us to retain the first nine principal components (PCs). We next define the number of clusters by using FindNeighbors and FindClusters functions that take into account the first nine PCAs and a resolution of 0.66, leading to 11 clusters. We ran the uniform manifold approximation and projection (UMAP) dimensional reduction technique, using RunUMAP function, and then display 2D UMAP projections. We used the authors major cell type annotations based on the validated leukocyte gene signature matrix (LM22).

2.4.2 | ScRNAseq data of healthy donors' whole blood leukocytes (GSE145230)

ScRNAseq data of three human males' and four human females' whole blood leukocytes were downloaded from GEO as normalized count per million (CPM) gene expression matrix. Preprocessing of scRNAseq data was performed identically as described above for GSE127465, except that doublet cells were removed based on UMI number and % of mitochondrial gene expression. We next define the number of clusters by using FindNeighbors and FindClusters functions that take into account the first 19 PCAs and a resolution of 0.6, leading to 22 clusters. Cells were annotated by combining Clustifyr (version 1.0.0) (using reference matrices from Seurat CBMC, Zilionis Blood datasets, and MCP-counter genes list with SingleR (version 1.2.4) annotation tools (Human Primary Cell Atlas used as reference).

2.4.3 | Differential gene expression analysis

From Seurat object generated from GSE127465 CPM gene expression matrix, differential gene expression analysis between two distinct clusters was performed using FindAllMarkers function from Seurat R package. Only genes detected in at least 25% of cells and with a positive average log₂ fold change of 0.25 were retained, but no cutoff value for the adjusted P-value was used. Union function from dplyr R package (version 1.0.2) was used to get the union of upregulated genes (Figure 4B; Table S4) from multiple differential gene expression analysis. Setdiff function was used to get the exclusive list of upregulated genes of a given cluster by opposition to another one.

2.4.4 | Single sample Gene Set Enrichment Analysis (ssGSEA) on bulk transcriptomic data

ssGSEA from GSVA R package (version 1.32.0) was performed on public microarray data of in vitro stimulated and unstimulated neutrophils (GSE15139 and GSE49757). For both data sets, control probes, and probes with ambiguous gene symbol were removed. For probes matching to identical genes, mean expression value per gene was calculated on probes matching to that gene to retain only one gene expression value per gene. SsGSEA of the Raghavachari platelet signature and the “in house 11 genes” neutrophil signature (Table S2) was performed on each replicate of each group using GSVA R package. All ssGSEA scores were bar-plotted with ggplot2 R package (version 3.2.1).

2.4.5 | Single sample Gene Set Enrichment Analysis (ssGSEA) on scRNAseq data

ssGSEA from R escape package (version 0.99.9) was used for calculating an enrichment score for Raghavachari platelet signature and “in house 11 genes” neutrophils signature (Table S2) of all individual cells. Only cells with at least one count per gene present in Raghavachari or “in house 11 genes” signatures were considered for calculating the enrichment score. Resulting ssGSEA scores obtained for each cluster was plotted using ggviolin function from ggpubr R package (version 0.4.0).

2.4.6 | Venn diagrams

Venn diagrams were generated by using the Venn and compute. Venn functions from the Vennerable package (version 3.1.0.9000).

2.4.7 | MCP counter on scRNAseq data

Normalized CPM expression matrix for all cells was generated from Seurat object. MCP counter scores (mean expression of all genes for a given signature [ln (log₂ TPM +1)] of our “in house 11 genes” neutrophil signature (Table S2) and the “16_gene_NPA_signature” (Table S6) were calculated for each individual cells using MCPcounter.estimate function from MCPcounter package (version 1.2.0). Comparison of mean MCP score between clusters was performed with the Stat_compare_means function from the ggpubr package (version 0.4.0) choosing nonparametric Wilcoxon rank-sum (Mann-Whitney) statistical test. Results were presented through violin plots that were generated with ggplot2 R package (version 3.3.2).

2.5 | Survival analysis

TCGA pan-cancer RSEM normalized log₂ transformed gene expression data were downloaded from UCSC Xena Browser and log₂ + 1 transformed. Updated clinical data with survival endpoints were retrieved from TCGA-CDR paper (Cell 2018, https://doi.org/10.1016/j.cell.2018.02.052).

Immune signatures (including the “NPA_12”) were calculated as the mean expression of each individual gene. Patients were then stratified into three groups based on each signature’s tercile values.

Survival analysis was performed using the survminer and survival R packages. For each cancer type, survival curves were estimated with
Kaplan-Meier and compared to the log-rank test. Multivariate analyses considering age and tumor stage as cofounding factors were then performed using a cox model with \( P \)-value correction using the Benjamini-Hochberg method.

3 | RESULTS

3.1 | Identification of NPAs in peripheral blood of cancer patients and healthy donors (HDs)

NPAs from NSCLC patients and HDs' whole blood were identified as CD15\(^ + \)CD14\(^ - \) neutrophils that were double positive for platelet glycoprotein 4 (CD36) and integrin alpha-IIb (ITGA2B, also known as CD41a) by using ImageStreamX (ISX) imaging flow cytometer (Figure 1A,B). Moreover, we found CD36\(^ + \) and ITGA2B\(^ + \) platelets from NPAs also expressed P-selectin (CD62P) (Figure 1B), known to influence neutrophil-platelet binding.\(^{30,38} \) We could also easily identify NPAs in unprocessed NSCLC patients' whole blood smear through May-Grunwald-Giemsa staining (Figure 1C). Because the frequency of NPAs was shown to be increased in the blood of patients with various pathologies as compared to HDs,\(^{38-40} \) we next quantified by ISX the frequency of NPAs among neutrophils between NSCLC patients and HDs with the same gating strategy. Although the frequency of NPAs between NSCLC patients (mean frequency = 1.48%) and HDs (mean frequency = 1.16%), was roughly the same, with no statistically significant difference (\( P \) value = .64), one NSCLC patient displayed more than a 3-fold increase of NPA proportion (mean frequency = 4.59%) compared to the mean frequency of NPA in NSCLC patients and HDs (Figure 1D). Interestingly, among all six NSCLC patients analyzed, this

**FIGURE 1** Identification of NPAs in both NSCLC patients and healthy donors' peripheral blood. (A) Gating strategy on ISX to identify CD36\(^ + \) ITGA2B\(^ + \) NPAs from CD15\(^ + \) CD14\(^ - \) neutrophils in NSCLC patients' peripheral whole blood (one representative data of \( n = 6 \) NSCLC patients' blood). (B) Visualization of CD15\(^ + \) CD14\(^ - \) CD36\(^ + \) ITGA2B\(^ + \) CD62P\(^ + \) NPAs by ISX in NSCLC patients and HDs' peripheral blood. Images were taken with X40 objective. CD15: Neutrophil marker; CD14: Monocyte marker; ITGA2B, CD36, CD62P: Platelet markers. (C) May-Grunwald-Giemsa coloration on NSCLC patient's whole blood smear. X100 magnification. Dark arrows show the presence of platelets aggregated to neutrophil (NPAs). Neutrophil is evidenced by segmented nucleus in dark purple. Free neutrophil refers to neutrophil free of platelets. (D) Box plot representing the frequency of noncoincidental CD15\(^ + \) CD14\(^ - \) CD36\(^ + \) ITGA2B\(^ + \) NPAs (among all neutrophils) in whole blood of NSCLC patients (\( n = 6 \)) and HDs (\( n = 10 \)), determined by ISX. Difference in terms of frequency of NPAs between NSCLC patients and HDs was assessed by the Wilcoxon rank-sum (Mann-Whitney) statistical test [Color figure can be viewed at wileyonlinelibrary.com]
NPAs define a subset of LDNs not present in NDNs. (A) Box plot representing the absolute number of LDNs (among PBMCs) per ml of whole blood in NSCLC patients (n = 8) and HDs (n = 5), determined by flow cytometry. Difference in absolute number of LDNs between NSCLC patients and HDs was assessed by the Wilcoxon rank-sum (Mann-Whitney) statistical test. (B) Box plot representing ssGSEA enrichment scores of the Raghavachari platelet signature (Table S2) across LDNs and patients’ matched NDNs from four HNC (blue dots) and two NSCLC (red dots) cancer patients (GSE79404). Differential enrichment of platelet signature between LDN and NDN groups was assessed by the Wilcoxon rank-sum (Mann-Whitney) statistical test. *P-value was displayed on graphs, **P ≤ .01. (C) Schematic representation of Ficoll density gradient centrifugation of human whole blood showing the separation of the low-density fraction (containing PBMCs) from the normal-density fraction (containing mostly granulocytes). Both fractions from NSCLC patients’ blood were analyzed by ISX, to identify CD15^+ CD14^- CD36^+ ITGA2B^- LDNs (in the low-density fraction) and CD15^- CD14^- CD36^- ITGA2B^+ NDNs (in the normal-density fraction). From either LDNs or NDNs gates, all CD36^+ ITGA2B^- events were gated as “NPAs.” (D) Visualization by ISX in NSCLC patients of noncoincidental CD36^+ ITGA2B^- CD62P^+ NPAs from LDN gate in the PBMC fraction and NDN gate in the granulocyte fraction. Images were taken with X40 objective. CD15: Neutrophil marker; CD14: Monocyte marker; ITGA2B, CD36, CD62P: Platelet markers. (E) Dot plot reflecting the frequencies of noncoincidental CD15^- CD14^- CD36^- ITGA2B^- NPAs among NDNs and LDNs determined by ISX, across n = 4 NSCLC patients [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3 Legend on next page.
patient with high percentage of NPAs also displayed by far the highest NLR value (34.56 as opposed to 7.21 for the others), mostly explained by a higher absolute neutrophil count (Table S1), and the highest tumor burden (7 vs 2 metastatic sites in the rest of the cohort) (Table S1).

3.2 | Platelets preferentially bind to low-density neutrophils (LDNs), rather than normal-density neutrophils (NDNs) in cancer patients’ blood

Given the well-known detrimental role of LDNs in cancer,11-14,41,42 we next analyzed a potential link between LDNs and NPAs. LDNs are known to be enriched in cancer patients’ blood in comparison with HDs.11,14,41,42 We could confirm such observations as we found a significant increase ($P < .0062$) in the absolute number of LDNs per ml of blood in NSCLC patients as compared to HDs (Figure 2A).

To first test if NPAs could be a subset of LDNs, we then performed a single sample Gene Set Enrichment Analysis (ssGSEA) on publicly available transcriptomic data of bulk LDNs and bulk NDNs from NSCLC and Head and Neck Cancer (HNC) patients’ blood (GSE79404). We showed that the published platelet-specific gene signature “Raghavachari”43 (Table S2) was significantly (P-value = .0022) more enriched in LDNs than in NDNs (Figure 2B).

We next isolated LDNs and NDNs (Figure 2C). Using ISX, we could visualize CD15+ CD14- CD36+ ITGA2B+ CD62P+ NPAs from LDNs but not from NDNs (Figure 2C,D). NPAs represented around 2% of LDNs but were negligible in the NDN population (Figure 2C-E). Collectively, these results demonstrate that platelets are preferentially bound to LDNs rather than NDNs.

3.3 | Identification of NPAs in public scRNAseq datasets from whole blood leukocytes in both cancer patients and HDs

We next aimed at characterizing NPAs from cancer patients at the transcriptomic level. Indeed, given the small size of platelets, we hypothesized that NPAs could be isolated as a single cell (Figure 1B), just like free neutrophil, and therefore be present in scRNAseq data. For this purpose, we took advantage of a recently published scRNAseq dataset of NSCLC patients’ whole blood leukocytes (GSE127465).31 Therefore, for our analysis we used normalized counts tables and kept author’s major cell type annotations to identify blood cell populations. We could recluster all leukocytes (Figure 3A) and distinguished six clusters of neutrophils (named Neu 1, Neu 2, Neu 3, Neu 4, Neu 5 and Neu 6 (Figure 3B). Interestingly, we observed that Neu 5 cluster seemed to be distinct from the other neutrophil clusters, through its close proximity with the platelet cluster (Figure 3B). Using our own neutrophil-specific gene signature “in house 11 genes” (Table S2) generated by intersecting multiple published neutrophil gene signatures, we showed that the bulk of neutrophils had a MCP counter score (mean abundance score = 0.71) significantly greater to platelets, monocytes, B cells/pDC, T cells and NK cells (mean abundance score = 0.15, 0.13, 0.05, 0.05, 0.09, respectively) (Figure 3C), thus validating the specificity of this signature. Moreover, neutrophil-specific genes (CSF3R, CXCR2 and FcGR3B) were expressed at the highest level in all six clusters of neutrophils, including Neu 5 cluster (Figure 3E). We then evaluated the ssGSEA enrichment score of the published platelet-specific gene signature “Raghavachari” (Table S2) across all clusters identified. Platelet cluster was by far the most enriched cell type for the platelet-specific gene signature (mean ssGSEA score = 0.28) as compared to neutrophils, monocytes, B cells/pDC, T cells and NK cells (mean ssGSEA score = −0.32, −0.19, −0.30, 0.31, −0.30, respectively) (Figure 3D), validating the specificity of this signature. Looking at the gene level, PF4, PPBP and NRGN (Table S3) platelet genes were in the five most-differentially upregulated genes (Table S4) able to discriminate the Neu 5 cluster from the other neutrophils and immune cell clusters (Figure 3F). In conclusion, the Neu 5 cluster of platelet gene-expressing neutrophils seems to correspond to NPAs.

To validate that this Neu 5 cluster of platelet gene-expressing neutrophils containing NPAs was not exclusive to this particular scRNAseq dataset, we next investigated for the presence of platelet gene-expressing neutrophils in a public scRNAseq dataset of HDs’ whole blood leukocytes (GSE145230). By performing the same analysis as for the NSCLC patients scRNAseq dataset (GSE127465), we were able to identify a cluster of neutrophils (Neu 17) located in between the platelet cluster and the remaining neutrophil clusters (Figure S1A,B). The neutrophil-specific gene signature “in house 11 genes” (Table S2) was once again proven to be specific to
neutrophils (mean abundance score = 0.45) as compared to platelets, monocytes, B cells/pDC, T cells and NK cells (mean abundance score = 0.02, 0.06, 0.01, 0.01, 0.01, respectively) (Figure S1C). The same was true for the platelet-specific gene signature “Raghavachari” (Table S2) whose mean ssGSEA score (0.65) was significantly greater in platelet cluster than in neutrophils, monocytes, B cells/pDC, T cells and NK cells (mean ssGSEA score = 0.23, 0.38, 0.30, 0.29, 0.35, respectively) (Figure S1D). Consistent with what we previously found in the NSCLC patients’ scRNAseq dataset, a fraction of Neu 17 cells was expressing high level of neutrophil (Figure S1E) and platelet-specific genes (Figure S1F), suggesting that the identification of platelet gene-expressing neutrophils could be generalized to any humans’ whole blood scRNAseq datasets.

In order to rule out that upregulation of platelet genes in neutrophils is not a consequence of neutrophil activation, we next tested whether the enrichment of the platelet-specific genes signature “Raghavachari” (Table S2) was increased upon neutrophil activation. We used public transcriptomic data of in vitro activated neutrophils by either GM-CSF or plasma from septic patients and we show that the enrichment score of the platelet signature in neutrophils did not change significantly (Figure S1G).

**Figure 4** NPA gene signature is enriched for neutrophil degranulation, chemotaxis and trans-endothelial migration GO terms, as opposed to the other neutrophil subsets. (A) Strategy to make the Neu 5 population more homogeneous (public scRNAseq data from NSCLC patients’ whole blood leukocytes—GSE127465). Left panel represents the two-dimensional-UMAP projection plot of cells from Neu 5 cluster (comprising 437 cells), based on the UMAP parameters used to discriminate the major subsets of leukocytes. Middle panel shows the selection of Neu 5 cells based on the coexpressed high level of the three platelet specific genes (PPBP, PF4 and NRGN) above 1 log2 gene expression (CPM). Right panel represents the two-dimensional-UMAP projection plot of Neu 5 cells coexpressing PPBP, PF4 and NRGN genes (comprising 48 cells annotated Neu 5 PPBPhigh PF4high and NRGNhigh). Cells were plotted based on the UMAP parameters used to discriminate the major subsets of leukocytes. (B) Differential gene expression approach to get the union of upregulated genes in Neu 5 PPBPhigh PF4high and NRGNhigh (annotated NPA cluster) as opposed to each of the remaining neutrophil clusters (Neu 1, Neu 2, Neu 3, Neu 4 and Neu 6). The statistical test used in the differential gene expression analysis was the Wilcoxon rank-sum (Mann-Whitney) test. (C) Venn diagram representing mutually and exclusive upregulated genes between the NPA cluster (union of upregulated genes in NPA cluster compared to each of the remaining neutrophil clusters—see Table S4) and the platelet cluster (Table S3). (D) Top 10 most-enriched GO biological processes, cellular components and KEGG gene sets calculated based on the 120 specific upregulated genes in NPA compared to platelet cluster (Table S5). [Color figure can be viewed at wileyonlinelibrary.com]
not significantly differ upon activation as compared to controls (Figure S2). The expression of platelet genes in Neu 5 (GSE127465) or Neu 17 (GSE145230) clusters seems therefore not to result from neutrophil activation. Collectively, all these results strongly suggest that Neu 5 and or Neu 17 neutrophil cluster displaying a high expression of platelet genes most likely contained NPAs.

3.4 Gene signature of neutrophils from NPAs reveals enhanced degranulation, chemotaxis and trans-endothelial migration capacities

To go deeper into the characterization of NPAs from NSCLC patients’ whole blood leukocytes at the mRNA level, we focused our analysis on NSCLC patients’ scRNAseq dataset (GSE127465). We noticed that only a fraction of cells of the Neu 5 cluster expressed platelet specific genes (PF4, PPBP and NRGN) (Figure 3F). When we reprojected cells for each individual neutrophil cluster, keeping the same UMAP parameters used to discriminate each individual subset of leukocytes, we could show that the Neu 5 cluster was more heterogeneous than the other neutrophil clusters based on visual cell dispersion (Figures 4A and S3). We selected the 48 cells coexpressing the three platelet specific genes (PF4, PPBP and NRGN) at a level above 1 log2 CPM (Figure 4A) (named “Neu 5 PPBP\text{high} PF4\text{high} NRGN\text{high}”) out of the 437 of the whole Neu 5 cluster. 48 cells out of the 8873 neutrophils analyzed represents 0.54%, which is consistent with the median frequency of NPAs among all neutrophils (0.85%) found in our cohort of NSCLC patients (Figure 1D and Table S1).

To next determine if neutrophils from the NPA cluster were distinct from any other subset of neutrophils, we first performed a differential gene expression analysis of the NPA cluster (Neu 5 PPBP\text{high} PF4\text{high} NRGN\text{high}) in comparison to each individual neutrophil clusters (Figure 4B). We then took the union of upregulated genes in NPA cluster from each pairwise comparison, therefore yielding 191 genes. We then removed the platelet genes (Figure 4C) and identified 120 nonplatelet genes discriminating NPAs-derived neutrophils from any other subset of neutrophils.

We next performed a pathway analysis and found a significant enrichment of neutrophil degranulation/chemotaxis-related gene ontology (GO) biological process, secretory granules-related GO cellular component and trans-endothelial migration-related KEGG gene set (Figure 4D). Collectively, these data demonstrate that NPAs-derived neutrophils display enhanced degranulation, chemotaxis and trans-endothelial migration functionalities.

3.5 Specific NPAs-associated neutrophil signature is associated with a worse prognosis in pancreatic adenocarcinoma and liver hepatocellular carcinoma patients

Next we addressed the prognostic significance of neutrophils from NPAs across the different tumor types available in The Cancer Genome Atlas (TCGA). The first step was to generate a specific NPAs-associated neutrophil signature excluding any other genes expressed in the other nonneutrophil blood immune cell type and platelets (Figure 5A) yielding 16 nonplatelet NPAs-associated neutrophils specific genes (Figure 5B). Using scRNAseq data from human NSCLC tumor samples (discovery dataset) matching previously-used patients’ whole bloods, we then eliminated RBP1, IDH2, HELLPAR and CCNI that were highly expressed by multiple other tumor-associated cell types (Figure S4), thus yielding a final 12-genes NPAs-associated neutrophil signature (Figure 5B). We next validated the specificity of this new NPAs-associated neutrophil signature in the discovery dataset (scRNAseq dataset from NSCLC tumors) by showing that cells coexpressing at least 3 out of the 12-genes NPAs-associated neutrophil signature were significantly more enriched in tumor-associated neutrophils than any other major cell types present in the tumor microenvironment (Figure 5C).

We next evaluated the prognostic value of the 12-genes NPAs-associated neutrophil signature with a pan-cancer approach in the TCGA database (Figure 5B). For comparison purposes, we interrogated the prognostic value of conventional neutrophils by using our neutrophil signature “in house 11 genes” (Table S2) that we proved to be highly specific to neutrophils in two independent scRNAseq datasets (Figures 5C and S1C). By performing a multivariate Cox analysis, we did not find any association between the NPAs-associated neutrophil signature and the prognosis of either types of NSCLC cancer patients, lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) (Figure 5D). Nevertheless, we could find that the signature was statistically associated with a worse progression-free interval (PFI) (P-value <.001, Hazard Ratio [HR] = 2.9) and overall survival (OS) (P-value <.001, HR = 2.6) in pancreatic adenocarcinoma cancer patients (PAAD) (Figures 5D and S5A), as well as in liver hepatocellular carcinoma cancer patients (LIHC) (PFI: P-value = .063, HR = 1.45; OS: P-value = .004, HR = 2.1) (Figures 5D and S5B). The canonical neutrophil signature “in house 11 genes” could not recapitulate such significant prognostic values in PAAD and LIHC reinforcing the idea that NPAs-associated neutrophils is a unique population of neutrophils with different clinical outcomes (Figure 5D). Kaplan-Meier univariate analysis also showed the poorer prognostic values of the 12-genes NPAs-associated neutrophil signature in PAAD (Figure 5E) and LIHC cancer patients (Figure 5F). The canonical neutrophil signature “in house 11 genes” did not show any statistically significant association with prognosis in PAAD cancer patients (Figure 5E), nor in LIHC cancer patients (Figure 5F). This implies again that NPAs-associated neutrophils are distinct from canonical neutrophils. Interestingly, conventional neutrophil signature was recently reported to be associated with better clinical outcome in a subtype of sarcoma, known as undifferentiated pleomorphic sarcoma (UPS). We confirmed such finding as we found that a high expression of our neutrophil signature “in house 11 genes” correlated with a significant better PFI (P-value = .036), but not a significant greater OS (P-value = .24) in UPS patients (Figure 5G). Nevertheless, in such patients, the 12-genes NPAs-associated neutrophil signature did not show any statistically significant association with prognosis, ruling out the fact that good
FIGURE 5 Legend on next page.
prognosis neutrophils in UPS are NPAs. Collectively, our results therefore suggest that a subset of neutrophils, corresponding to NPAs-associated neutrophils, when present in tumors may be detrimental in PAAD and LIHC cancer patients.

4 | DISCUSSION

To our knowledge, our study is the first to report the identification and characterization of NPAs at the transcriptomic level, based on scRNAseq data from human whole blood leukocytes. Based on their transcriptomic profile, we found that neutrophils bound to NPAs displayed enhanced degranulation, chemotaxis and trans-endothelial migration features. Our results collectively showed that neutrophils from NPAs represent a distinct subset of neutrophils, related to LDNs. Lastly, using TCGA database, we showed that our specific NPAs-associated neutrophil signature was associated with a worse prognosis in PAAD and LIHC cancer patients. Hence, NPAs may hold clinical utility as novel noninvasive blood prognostic biomarker in cancer patients with solid tumors.

Using ISX, we were able to distinguish and quantify accurately NPAs in our cohort of NSCLC cancer patients and HDs. Although we did not find a significant difference in the percentage of NPAs between NSCLC cancer patients and HD, one NSCLC patient displayed more than a 3-fold increase of NPA proportion compared to the mean frequency of NPAs in NSCLC patients and HDs (Figure 1D). Interestingly, this patient also displayed the highest number of different metastatic sites (Table S1), as well as an extremely high NLR value of 34.56 (Table S1). This is consistent with the poorer clinical outcome of patients with elevated NLR values, including NSCLC patients. This high NLR value was mostly explained by a massive increase in the absolute neutrophil count (ANC), rather than a decrease in the absolute lymphocyte count. Mounting evidence have shown that high ANC alone, was also significantly associated with poorer prognosis in NSCLC cancer patients, including in response to immune checkpoint inhibitors (ICIs). It would be of great interest in future studies to determine if the percentage of circulating NPAs, in comparison to the other cost-effective and noninvasive NLR and ANC, is more accurately associated with response to immunotherapy.

By performing a Ficoll density gradient, we showed that NPAs define a subset of LDNs but are absent in NDN fraction, illustrating that platelets preferentially bind to LDN in contrast to NDN (Figure 2C-E). Several studies have reported a link between LDNs and cancer progression. Many research groups indeed reported that LDNs displayed enhanced T-cell suppressive properties, expanded in cancer patients’ blood as opposed to healthy donors. Recent evidence have shown that high LDN expansion in cancer patients’ blood was associated with worse prognosis including NSCLC patients. Nevertheless, it remains to be known why platelets bind preferentially to LDNs. The lower density of neutrophil bound to platelet may be a consequence of platelet binding. Since NDN activation is sufficient to convert NDNs into LDNs, one can hypothesize that platelets interacting with NDN activate and convert them into LDNs. It is also likely that platelets may behave as floats changing NDNs’ density and retaining them at the surface of the Ficoll gradient, explaining their recovery within peripheral blood mononuclear cells. Although we did not document a significant increase of NPAs frequency in patient, likely as a consequence of the small size of the cohort, the fact that LDNs increase in cancer patients strongly suggests that the frequency of NPAs also increases in cancer patients.
Although unexpected, our study identified a yet unreported new subset of neutrophils in scRNAseq data with high platelet gene expression that most likely correspond to NPAs. We ruled out the possibility that platelet genes could be expressed by a subset of activated neutrophils as we did not find enrichment of platelet signature in stimulated neutrophils (Figure S2). Moreover, it is also unlikely that neutrophils expressing platelet genes correspond to a common progenitor of megakaryocyte (platelet-producing cell) and neutrophils, as the closest common progenitor between the two is the common myeloid progenitor (CMP), which gives rise to all myeloid cells. High expression of neutrophil-specific genes at this stage of differentiation is therefore unlikely to occur. Taken together, platelet genes are more likely to be expressed exogenously by neutrophils through aggregated-platelets. It is, however, plausible that platelets could have been isolated in the same droplet than neutrophils, without any interaction. The potential random contamination of platelets during single cell isolation of immune cells would have yielded cluster of cells expressing platelet-specific genes in all immune cell types. However, expression of platelet specific genes was mostly observed in neutrophils as opposed to other major immune cell types (Figure 3F). To note that some monocytes also displayed expression of platelet specific genes which is consistent with the fact that platelets may also aggregate with circulating monocytes. Interestingly, we found that the frequency of neutrophil coexpressing the three platelet specific genes (PF4, PBPB and NRGN) was very similar to the one we accurately determined by ISX in our cohort of NSCLC patients. This further supports that the cluster of neutrophils with high expression of platelet genes identified in Zilionis’ scRNAseq dataset (GSE127465) corresponds to NPAs.

In contrast to the other subsets of circulating neutrophils, we found that neutrophils from NPAs displayed enhanced degranulation, chemotaxis and trans-endothelial migration features, based on the specific transcriptomic signature of NPAs. This is consistent with previous reports showing that platelet binding to neutrophils boosted their potential to release granules, increased their capacity to trans-migrate through endothelial cells to exacerbate inflammation thereby promoting disease progression. One can speculate that if neutrophils from NPAs display higher transmigration potential, they would infiltrate tumors more efficiently and then fuel tumor growth and metastasis.

We found that NPA signature was highly detected in fewer tumor types in the TCGA data base and was strongly associated with a poorer prognosis in PAAD and LIHC cancer patients. This is consistent with the tumor-promoting role of TAN documented in pancreatic and liver cancer. Interestingly, we found that the canonical neutrophil signature “in house 11 genes” could not recapitulate such significant poorer prognostic values in PAAD and LIHC, reinforcing the idea that neutrophil from NPA would account for most of tumor-promoting role of TAN in such cancer types. Moreover, we provided evidence that NPAs-associated neutrophils more likely resemble LDNs (Figure 2) that are described to possess tumor-promoting functions, supporting the idea that NPAs-associated neutrophils once becoming TANs after tumor infiltration would fuel tumor growth. Beyond their potential role in cancer, recent studies have reported an increase in the percentage of circulating NPAs in COVID-19 patients in contrast to HDs. Interestingly, patients who had severe forms of COVID-19 displayed a significant higher level of NPAs in contrast to patients who had moderate forms. We showed that NPAs displayed enhanced degranulation/activation, based on their specific transcriptomic signature (Figure 4D). This is in accordance with a recent report showing in bulk granulocyte RNAseq data from COVID-19 patients an increase expression of granulocyte activation-associated factors in severe COVID-19 patients in contrast to mild ones. Whether NPAs may not just be a consequence but also a cause of COVID-19 severity remains to be investigated in future studies.

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CONFLICT OF INTEREST
All authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Conceptualization: Pacôme Lecot, Christophe Caux and Marie-Cécile Michallet; methodology: Pacôme Lecot, Maude Ardin, Sébastien Dussurgey, Manuela Pereira Abrantes, Vincent Alcazer, Lyvia Moudombi, Margaux Hubert, Hector Hernandez-Vargas, Alain Viairi, Christophe Caux and Marie-Cécile Michallet; formal analysis: Pacôme Lecot, Maude Ardin, Sébastien Dussurgey, Vincent Alcazer, Lyvia Moudombi and Margaux Hubert; investigation: Pacôme Lecot, Maude Ardin, Sébastien Dussurgey, Vincent Alcazer, Lyvia Moudombi and Margaux Hubert; writing—original draft: Pacôme Lecot; writing—review and editing: Maude Ardin, Sébastien Dussurgey, Vincent Alcazer, Lyvia Moudombi, Christophe Caux and Marie-Cécile Michallet; resources: Aurélie Swalduz, Christophe Caux and Marie-Cécile Michallet; visualization: Pacôme Lecot, Maude Ardin, Sébastien Dussurgey, Vincent Alcazer, Lyvia Moudombi, Margaux Hubert; supervision: Christophe Caux and Marie-Cécile Michallet; funding acquisition: Pacôme Lecot, Alain Viairi, Christophe Caux and Marie-Cécile Michallet. All authors read and approved the final manuscript. The work reported in the study has been performed by the authors, unless clearly specified in the text.
DATA AVAILABILITY STATEMENT
Data sources and handling of the publicly available datasets used in our study are described in the Materials and Methods and in Tables S1 to S6. Further details and other data that support the findings of our study are available from the corresponding author upon request.

ETHICS STATEMENT
Metastatic NSCLC patients (Table S1) included in the LIBIL clinical trial (NCT02511288) at the Léon Bérard Hospital as well as healthy donors from Etablissement Français du Sang provided informed consent regarding the use of their blood for research purpose. The LIBIL protocol was approved by the Comité de Protection des Personnes Ouest VI, ref: CPP Ouest 6-CPP 1023-HPS2.

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