Comprehensive Peripheral Blood Immunoprofiling Reveals Five Immunotypes With Immunotherapy Response Characteristics in Patients With Cancer

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Graphical abstract

**Highlights**

- Development of a machine learning-based clinical immunoprofiling platform
- Depiction of immune states by multiparameter flow cytometry and bulk RNA-seq using peripheral blood
- Identification and validation of five immunotypes conserved across diverse diagnoses
- Potential clinical utility in stratifying treatment responses via a simple blood test

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**In brief**

Dyikanov et al. developed a machine learning platform that uses a simple blood test to reveal cellular compositions reflective of a person's immune system. These compositions, delineated into five conserved immunotypes, can reflect a person's disease status or how someone responds to specific treatments, thus underscoring their potential clinical utility for cancer patients.
Comprehensive peripheral blood immunoprofiling reveals five immunotypes with immunotherapy response characteristics in patients with cancer

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SUMMARY

The lack of comprehensive diagnostics and consensus analytical models for evaluating the status of a patient’s immune system has hindered a wider adoption of immunoprofiling for treatment monitoring and response prediction in cancer patients. To address this unmet need, we developed an immunoprofiling platform that uses multiparameter flow cytometry to characterize immune cell heterogeneity in the peripheral blood of healthy donors and patients with advanced cancers. Using unsupervised clustering, we identified five immunotypes with unique distributions of different cell types and gene expression profiles. An independent analysis of 17,800 open-source transcriptomes with the same approach corroborated these findings. Continuous immunotype-based signature scores were developed to correlate systemic immunity with patient responses to different cancer treatments, including immunotherapy, prognostically and predictively. Our approach and findings illustrate the potential utility of a simple blood test as a flexible tool for stratifying cancer patients into therapy response groups based on systemic immunoprofiling.

INTRODUCTION

Human populations are genetically and developmentally diverse, with their immune systems shaped by unique immunological challenges from aging, microbial exposure, metabolic changes, and chronic diseases such as cancer. The composition of each immune system determines how an individual responds to different immunological stimuli, including those from anti-cancer therapies.

Increasingly, immunotherapies have become an essential part of treatment plans that typically also include aggressive chemotherapy and radiation, for patients with advanced solid tumors. These treatments affect the patient’s immune system and can interfere with its capacity to respond to subsequent therapy lines and combinations. Although immunotherapies such as immune checkpoint blockade (ICB) have been clinically successful over the last decade, response rates are still largely unpredictable in most patients, and serious immune-related adverse events often emerge. Unfortunately, analysis of gold-standard molecular biomarkers such as PD-L1, microsatellite instability (MSI), DNA mismatch repair alterations (dMMR), and tumor mutational burden (TMB) in tumors have only marginally improved response rates in some cancers, and do not fully reflect the complexity of a patient’s functional immunity.

Recently, the characterization of the tumor microenvironment (TME), including analyses of tumor-infiltrating T cells, functional genomic signatures from RNA expression, and the distribution of inflammatory and immunosuppressive cells within different...
tissue microdomains, have improved the positive predictive value for ICB response.8–10 While direct characterization of immune responses in tumor tissues is clinically useful, limitations to tissue-only approaches remain, including tumor heterogeneity, sampling bias, sample acquisition, and longitudinal monitoring. Additionally, these methods fail to assess a patient’s systemic immune status, which is intricately linked to tumor development, prognosis, and treatment response.11,12

Growing evidence suggests that analysis of peripheral blood leukocytes can inform immunotherapy selection in patients with solid tumors.13 Findings from a pan-cancer study have shown that high levels of naive B cells in peripheral blood associate with improved survival after ICB treatment.14 High levels of dendritic cells correlate with favorable responses to ICB in patients with non-small cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC).15,16 NSCLC patients responding to anti-PD-1 treatment exhibit proliferation of PD-1+ CD8+ T cells in the peripheral blood after four weeks of treatment,17 while patients with dysfunctional CD4+ T cells in the periphery fail to respond to PD-1 pathway inhibition.18 As such, this emerging consensus suggests that effective ICB treatment requires the early activation of tumor-specific T cells in the periphery. Ideally, these T cells then differentiate into exhaustion-resistant subtypes competent to infiltrate into the TME to perform their antitumor function.19

Currently, comprehensive analysis of systemic immunity is not routine when evaluating patients with cancer. Moreover, there are no consensus methods for profiling a patient’s immune system. Most approaches that focus on individual cell populations are subject to technical and natural variation, often leading to conflicting results in similar cohorts.20 On the other hand, unbiased yet resource-intensive approaches like single-cell RNA sequencing (RNA-seq) pose significant challenges in sample/data handling and bioinformatics analysis because of their low throughput, high coefficients of variation, and considerable cost per sample.21 This technique, while optimal for hypothesis generation and discovery in certain settings, is currently impractical for prospective validation and eventual clinical deployment.

Recently, systems immunology has emerged to address critical gaps in the study of human immunology by applying multidimensional machine-learning (ML) techniques to complex clinical datasets.22 Importantly, these approaches have used peripheral blood immunophenotyping data to develop predictive models for patient responses to different immunological stimuli, including vaccination.23 However, the translation of systems immunology to a wider breadth of clinical questions, including the evaluation of immunotherapy responses in patients with cancer, have been bottlenecked by a lack of standardized approaches that can be deployed to support large biomarker validation trials.

Overall, the aim of this study is to understand if a core group of immune response modules can be identified in different patient populations, quantified with a standardized platform and flexible analytical framework, and utilized to stratify patients with different advanced cancers into therapy-specific response groups. To accomplish these aims, we established a clinical immunoprofiling assay by pairing multiparameter flow cytometry with an automated high-throughput cytometry analysis platform. This platform was then used to evaluate immunological variation in cancer patients and identify associations between this variation and clinically significant responses to different cancer treatments, including immunotherapy.

RESULTS

Cell-typing analysis of peripheral blood using flow cytometry to develop a robust clinical immunoprofiling platform

A comprehensive immunoprofiling assay was developed using conventional flow cytometry of red blood cell (RBC)-depleted peripheral blood samples to evaluate the systemic immune characteristics of cancer patients. An end-to-end process was established with integrated wet- and dry-lab systems for sample processing, data acquisition, population identification, markup, and quantification, all supported by multiple layers of specimen and data quality control (Figure 1A).

Ten overlapping antibody panels were designed (nine cell type-specific panels and one general lineage-connecting backbone panel, Figure 1B; Table S1) to ensure broad coverage of immune cell subpopulations across different lineages. Each cell type-specific panel consisted of well-characterized cell-surface marker combinations for the following major immune cell populations: natural killer (NK) cells, dendritic cells, monocytes, CD4+ T cells, CD8+ T cells, non-conventional T cells, and B cells (Figure 1B). Multicolor flow cytometry was performed on isolated white blood cells (WBCs) from the peripheral blood, and these antibody panels were utilized to quantitate all CD45+ cells in the samples (Figures 1A and 1B; Table S2). We manually combined and aligned the flow cytometry data using our antibody panels for exhaustive subpopulation cell typing within the major cell populations (Figures S1A–S1I; Table S2). Together, 650 cell types and activation states were identified based on their marker expression profiles (Figure 1B; Table S2).

The manual cell-typing analysis thus defined an extensive cell-type hierarchy that was used to establish an automated cytometry analysis platform. Specifically, it was used to train ML gradient boosting event-type models to automatically and reproducibly identify immune cell subsets from each panel (Figures 1A and S2A; Table S3). These celltyping models accurately detected various immune cell populations and activation states from peripheral blood by corroborating the manual supervised gating analysis in validation tests (F1-scores: 0.74–0.95, P4-metrics: 0.84–0.97, for the 10 panels, Table S3; Figure S2B). This platform was then used to analyze blood samples from healthy donors and cancer patients to determine if differences in immune cell composition between these two groups could reveal additional findings of diagnostic or prognostic significance.

Preliminary comparisons were performed between healthy donors and cancer patients with the blood samples from our training set. These comparisons revealed striking differences in the relative frequencies of monocytes as well as naive, central memory, and terminally differentiated CD4+ and CD8+ T cells (Figure 1C). Consistent with published reports,24–27 this preliminary analysis supported further exploration of differences in immune profiles from healthy donors and patients with advanced cancer. Thus, we assembled a large cohort to examine if immune cell heterogeneity in patients with cancer differed significantly from healthy donors.
Figure 1. Development of the immunoprofiling pipeline

(A) Workflow of immunoprofiling pipeline development: white blood cells (WBCs) were isolated from peripheral blood samples collected from healthy donors and cancer patients. A hematology analyzer was used to measure complete blood count. WBCs were stained with custom antibody panels in 96-well plates. WBCs were processed by multiparameter flow cytometry. Manually labeled flow cytometry data were used to train machine learning models to automatically identify cell populations. Finally, the healthy/cancer and immunotype classifications were implemented.

(B) Schematic heatmap of cytometry panels showing a portion of the markers used (x axis) and examples of identified populations (y axis). The heatmap shows normalized signal intensities of cell-surface markers that define specific cell types, generated by combining readouts from the different antibody panels (e.g., general, NK cells, and dendritic cells). Representative tSNEs of immune cell families identified with this strategy are shown on the right.

(C) Differences in immune status, based on cell populations, between a representative healthy donor and a cancer patient, visualized with polar plots. Each dot represents 0.5% of all fractions of peripheral blood mononuclear cells (PBMCs) for a total of 200 fractions shown. See also Figures S1, S2, and Tables S1, S2, and S3.
Figure 2. Training and performance of our healthy/cancer classifier

(A) Cohort description with disease breakdown and clinical annotation. The cohort was first categorized into different clinical groups, and then split into training and validation subsets for the construction and evaluation of the healthy/cancer classifier, respectively.

(B) UMAP-2: Healthy donors vs. Cancer patients. UMAP-1: Age, Diagnosis, Treatment.

(C) Differentially represented populations.

(D) Comparison of Differentially Represented Populations, by Age and Diagnosis.

(E) True labels vs. Healthy/cancer classifier predictions.

(F) Training and validation ROC curves.
Stratifying healthy donors and cancer patients using differences in immune cell composition between these two groups
Peripheral blood from 408 healthy donors and 442 cancer patients, aged 16 to 98 years (total n = 850, internal cohort), were collected. This cohort comprised 84 different solid tumor diagnoses within seven major therapy groups (Figure 2A; Table S4). Complete blood count (CBC) revealed significant differences in the absolute numbers of RBCs, platelets, neutrophils, and lymphocytes between healthy donors and cancer patients, while the absolute number of monocytes were similar, in agreement with published reports

This immunoprofiling platform was then used to analyze the peripheral immune cell distribution from each individual within this cohort. The frequencies of up to 650 cell types and activation states were measured for each sample, and Uniform Manifold Approximation and Projection (UMAP) was used to plot these features in two dimensions. This approach allowed us to evaluate the influence of different variables on immune cell heterogeneity in this cohort, such as the presence or absence of disease, patient age, solid tumor type, and administered therapies (Figure 2B). Patients with similar diagnoses did not form distinguishable clusters, neither did patients with similar therapy lines. Conversely, healthy donors and cancer patients formed separate clusters. Patients in different age groups tended to cluster as well, consistent with healthy donors being generally younger than patients with solid tumors (Figure 2B). Our findings suggest that differences in immune profiles between individuals in this cohort could be explained by the presence or absence of cancer, regardless of tumor or treatment types.

To explore the features contributing to the variance in this cohort, we used the Max-Relevance and Min-Redundancy (MRMR) algorithm with stepwise leave-one-out cross-validation to identify cell populations that were the most significantly different between healthy donors and cancer patients (Figure 2C; Table S5). Interestingly, CX3CR1þ CD8⁺ TEMRA and monocytes were significantly overrepresented in patients with cancer, whereas populations of naive CD4⁺ and CD8⁺ T cells as well as naive and memory B cells were underrepresented in healthy donors (Figures 2C and 2D; Table S5). These observations suggest that different cell types are enriched in patients with cancer, while others are enriched in healthy individuals. While some populations differed between old and young healthy donors, these differences were much starker between healthy donors and cancer patients (Figure 2C), suggesting disease state is a more prominent driver of variation in this cohort than patient age. We then selected 20 cell types and used their frequencies to train a TabPFN-based classifier model on a set of 503 samples from our internal cohort (Figure S3B). UMAPs were generated from these selected features to show the label assignments predicted by our classifier model to correspond to the true labels (Figure 2E).

This classifier was assessed using leave-one-out cross validation on the training dataset and shown to perform well in classifying healthy donors and cancer patients (area under the curve for receiver operating characteristics [ROC-AUC] = 0.91). Noteworthily, our binary classifier model trained on the features of the 20 previously selected cell populations outperformed a more basic model trained on populations that could be identified from a commonly used clinical cytometry panel (BD Multitest 6-color TBNK kit) combined with major populations from standard CBC: basophils, eosinophils, neutrophils, monocytes, NK cells, natural killer T (NKT) cells, B cells, CD4⁺ T cells, and CD8⁺ T cells (ROC-AUC = 0.81, Figure 2F, left). Similarly, this classifier performed better in selecting healthy donors and cancer patients on a validation subset of 347 patient samples from the internal cohort, compared to the TBNK/CBC panel (ROC-AUC = 0.84 and 0.77, respectively, Figure 2F, right). Overall, these results demonstrate that immune profiles can be used to stratify patients with distinct clinical characteristics with high specificity.

Classification of immune cell composition in peripheral blood into five distinct immunotypes
Having established differences between healthy donors and cancer patients, we aimed to understand if this analysis could be expanded to identify immune profiles with putative functional significance. Specifically, unsupervised spectral clustering was applied to the normalized frequencies of 34 cell types on samples from our internal cohort obtained by flow cytometry (Table S6). Initially, 30 cell types congruent with populations that could be identified by cellular deconvolution of bulk RNA-seq with Kassandra were selected. To that, four cell populations consistently identified as immunotherapy response biomarkers were incorporated: TIGIT⁺ PD-1⁺ CD8⁺ T cells, V62⁺ γδ-T cells, CD39⁺ regulatory T cells (Tregs), and HLA-DR⁺ monocytes.

Five immunotypes (G1–G5) were identified using this approach, with the separation into five clusters being associated with the highest median number of significantly different cell populations between clusters (Table S6, STAR methods). G1 exhibited high frequencies of naive CD4⁺ T cells, naive CD8⁺
Figure 3. Flow cytometry-based clustering of immunotypes and analytical validation with matched RNA-seq
(A) Unsupervised spectral clustering analysis was applied to normalized flow cytometry percentages to reveal five distinct immunotypes based on the distribution of selected cell populations. Samples were also categorized based on patient diagnosis (healthy donors or cancer patients).
T cells, and naive B cells. G2 showed greater percentages of differentiated CD4+ central and transitional memory T cells, as well as CD39+ Tregs. G3 showed increased frequencies of mature NK cells as well as CD8+ transitional memory and PD-1+ TIGIT+ CD8+ T cells. G4 was enriched with NKT cells as well as terminally differentiated effector memory CD45RA+ (TEMRA) and CD45RA- (TEM) of both CD4+ and CD8+ T cells. Finally, G5 was enriched with classical monocytes, HLA-DRlow monocytes, and neutrophils, and contained smaller frequencies of lymphocytes (Figure 3A). Consistent with our UMAP analysis of our internal cohort (Figure 2B), the presence or absence of a cancer diagnosis (healthy or cancer) and patient age were unevenly distributed among the immunotypes (Figures 3A and 3B). Importantly, immunotypes G4 and G5, enriched with terminally differentiated CD8+ T cells and classical monocytes, respectively, contained very few healthy donors. Conversely, immunotype G1 with the highest percentage of naive T and B lymphocytes contained the largest proportion of healthy donors (Figure 3A). The most frequently represented diagnoses in our internal cohort contained similar ranges of immunotypes, suggesting that cancer type was not a major driver of immunotype distribution (Figure 3C).

Bulk mRNA-seq libraries from 797 samples analyzed by flow cytometry from our internal cohort were prepared and sequenced (Table S4). Cell deconvolution of bulk RNA-seq by Kassandra was then used to match cell populations with those identified by flow cytometry. Cell population frequencies derived from RNA-seq and flow cytometry were concordant (Figures 3D and S4), thus confirming our flow cytometry findings. These RNA-seq data were then used to perform differential gene expression analysis on the immunotypes of our internal cohort. Here, a large number of genes uniquely up- or downregulated for each immunotype compared to the sum of all other immunotypes were identified (Figure S5A). Building upon this analysis, we selected the 200 most differentially expressed genes from each cluster and performed a gene set enrichment analysis (GSEA) using curated functional gene signatures for immunologically relevant pathways from MsigDB.29 G1 and G2 were shown to be enriched in signatures for transcriptional regulation involving TCF, LEF, and CTNNB1 as well as TCR- and WNT/beta catenin-mediated signaling. G4 was found to be enriched in genes associated with cytotoxic effector T cell responses, and G5 to be enriched in genes involved in signaling pathways associated with innate immune sensing and myeloid cells (Figures 3E and S5B; Table S6).

Of note, individual transcripts of cytokine and chemokine signaling-related genes displayed distinct expression patterns among our defined immunotypes. For instance, FLT3LG and CCR7, which are expressed by naive and central memory CD4+ and CD8+ T cells, were the most highly expressed in G1. Their expression decreased progressively through to G4 and were poor in G5. G4, enriched with terminally differentiated CD4+ and CD8+ T cells, showed the highest levels of CCL4 and TGFB. CXCL16 and IL1R1 transcript levels were greatest in G5, consistent with their expression by monocytes and neutrophils (Figure 3F). Collectively, these results demonstrate the presence of immunotype-specific gene expression patterns even at the level of individual transcripts.

**Analysis of T cell receptor repertoires and transcriptional signatures of T cell memory to show immunotype association with the characteristics of immunotherapy responsiveness**

Adaptive immunity generally and therefore patient responses to ICB are largely dependent on the clonal expansion and differentiation of tumor antigen-specific T cells.40 To better understand the adaptive response potential of our different immunotypes, we evaluated T cell repertoire composition using bulk RNA-seq data from our internal cohort,41 as T cell receptor (TCR) repertoire clonality and diversity have been characterized as biomarkers for ICB response.42 Our analysis showed the coverage of CDR3 sequences from TCRβ-chains to be consistent across the cohort and to reflect the overall frequency of T cells in each sample (Figure 4A, top). Dominant clones occupying more than 10% of total CDR3 in each patient, while infrequent in the cohort overall, were enriched in the G4-chronic immunotype (Figure 4A, bottom; Figures S6A and S6B).

The under- or over-representation of certain human leukocyte antigens (HLAs) between immunotypes could potentially explain differences in TCR repertoire composition. Therefore, the allele distributions of HLA-A, -B, and -C in our internal cohort were analyzed (Figure 4B, HLA-B). HLA distribution among the different immunotypes was heterogeneous (Figure 4B). Interestingly, of all the HLA types measured in this cohort, only the frequency of patients with the HLA-B07:02 allele were significantly lower in the G4-chronic immunotype compared to all other groups (Figure 4B). This analysis demonstrates that HLA skewing is not likely to have caused the differences in TCR repertoire distribution among different immunotypes.

TCRβ clonality index of individuals in the G4-chronic immunotype was approximately three times that of individuals in the other immunotypes (Figure 4C, left). Additionally, there was no association between TCRβ clonality and the number of patients with any specific HLA-B allele (Figure S6A). Conversely, immunotypes G1-naive, G2-primed, and G3-progressive had significantly higher TCRβ diversity (as indicated by Chao1) than immunotypes G4-chronic and G5-suppressive, decreasing from G1 to G3 (Figure 4C, right). This is consistent with the observed
A

TCR landscape

B

HLA-B allele distribution

C

TCRβ clonality

D

PD-1<sup>+</sup> CD8<sup>+</sup> T cells

E

TCF7

F

PDCD1

G

(legend on next page)
frequencies of naive, central, and transitional memory T cells in these immunotypes. Similar differences were present among immunotypes in TCRx clonality and diversity (Figures S6C and S6D). Additional analysis of B cell receptor (BCR) repertoire diversity between immunotypes revealed trends similar to those for TCR, with the G1-naive immunotype having the highest diversity index for BCR heavy and light chains that were significantly greater than that for immunotypes G3-progressive, G4-chronic, and G5-suppressive (Figure S6E). These immunotype-based differences in TCR repertoire suggest a putative connection to transcriptional programs that control T cell differentiation and clonal expansion.

To further test this, we expanded our GSEA analysis to evaluate the immunotypes using annotated gene signatures centered on gene expression patterns connected with T cell differentiation state, repertoire diversity, and PD-1 expression by T cells. First, an enrichment pattern for a general T cell differentiation signature (GSE14699, Figure 4D, right) was observed, containing genes differentially expressed between naive and activated CD8+ T cells that resembled the immune repertoire distribution pattern seen for TCRβ repertoire diversity (Figure 4C, right). In addition, individual gene expression levels of transcription factors TF-7, LEF1, and ID3 that are prominently expressed by naive and self-renewing central memory T cells and control the development of T cell memory were the highest in immunotypes G1-naive and G2-primed (Figure 4E, top), consistent with the T cell differentiation signature scores (Figure 4D, right). The G4-chronic immunotype had the highest enrichment score for the PD-1high CD8+ T cell signature (GSE26495, Figure 4D, left), which was concordant with our analysis on TCRβ clonality (Figure 4C, left) and the frequency of terminally differentiated T cells in this immunotype. Similarly, TBX21, EOMES, and TOX, encoding transcription factors that are critical regulators of effector T cell differentiation and exhaustion, showed the highest expression in the G4-chronic immunotype (Figure 4E, bottom). To examine the potential association between immune repertoire and immunotherapy response, we then analyzed 70 cancer patients with different diagnoses from our internal cohort who were on-treatment with a PD-1 pathway inhibitor. While no differences in the expression of PDCD1 (PD-1) or CD274 (PD-L1) were seen between immunotypes (Figure 4F), G1-naive, G2-primed, and G4-chronic showed an enrichment in gene signatures associated with PD-1 signaling and response to PD-1 inhibition (Figure 4G). Taken together, these results further support the association of immunotypes G1–G5 with immunotherapy treatment responses.

**Analysis of open-source peripheral blood RNA-seq data to reveal disease associations with immunotype distribution**

Having shown that these immunotype classifications are consistent with known states of immunological responsiveness, we examined if this approach could be expanded to other datasets. Initially, we attempted to assemble a cohort of healthy donors and cancer patients similar to our internal cohort. Here, peripheral blood transcriptomes from 17,800 individuals from open-source GEO and ArrayExpress databases were collected (Table S7). However, these datasets contained very few cancer patients (n = 160), with diagnoses and therapies that did not overlap with our internal cohort. As an alternative, samples from healthy donors and patients with 90 different diagnoses of immunological significance, including infectious, inflammatory, and autoimmune diseases, were used to evaluate the association between our immunotype framework and responses to well-characterized immunological challenges. First, the frequencies of immune cell populations were derived from these bulk transcriptomes using Kassandra. De novo unsupervised spectral clustering was then applied to understand the distribution of these immune subsets in this larger, more diverse dataset (Table S7). Encouragingly, we found these 17,800 transcriptomes to form five clusters with cell type distributions strikingly similar to those in our internal cohort (Figure 5A), thus allowing us to independently verify the existence of these immunotype clusters in open-source bulk RNA-seq datasets. Additionally, 3D principal component analysis (PCA) projection of these samples revealed a similar continuum of immunotypes to our internal cohort, and a direct comparison of healthy donors from both datasets showed no significant differences in immunotype distribution (Figures 5B, S7A, and S7B).

Next, each dataset was subgrouped based on disease pathogenesis (Figure 5C; Table S7). For instance, samples from patients with persistent Mycobacterium tuberculosis or Leishmania spp. infections were assigned to the “pathological pathogens” group, and samples from patients with influenza or coronavirus to the “respiratory viruses” group. Consistent with the analysis of our internal cohort (Figure 3A), healthy donors were most...
frequently assigned to the G1-naive and G2-primed immunotypes (Figure 5C). Compared to healthy donors, only patients from the “autoimmune diseases” group that contained a large number of pediatric cases were significantly clustered in G1-naive. While the G2-primed group contained the largest number of samples across the cohort overall, this immunotype was only significantly enriched among patients with phagosomal pathogens compared to healthy donors. The G3-progressive immunotype was significantly enriched among patients with viral infections (respiratory, enteric, and HIV) and phagosomal pathogens. Patients with chronic viral and extracellular bacterial infections were also assigned more frequently to the G4-chronic immunotype compared with healthy donors; G4-chronic was also the most common immunotype classification for patients with HIV. Interestingly, all disease groups contained significantly more patients with the G5-suppressive immunotype, particularly conditions associated with high levels of systemic inflammation such as bacterial sepsis where nearly 80% of the patients in this group were assigned to this immunotype (Figure 5C). Our analysis showed that beyond the observations from our internal cohort with healthy donors and cancer patients, these five immunotypes could be identified in a diverse external dataset and independently confirmed with cellular deconvolution of bulk RNA-seq only, further supporting the validity and broad applicability of our framework.

Development of immunotype signature scoring for the identification of cancer therapy response correlates

Considernig that frequencies of different immune cell populations in the peripheral blood of patients are normally distributed, clustering of patients using these data resulted in significant overlap between clusters. Consequently, classifying patients with intermediate or transitional immunotype characteristics was challenging, as visualized in a UMAP projection of our internal cohort (Figure 5D), suggesting that these immunotypes represent a continuum rather than discontinuous groups with clean separation between them. This is more precisely visualized with a pseudotime projection based on flow cytometry data from our internal cohort (Figure 5E, left). Patients with immunotypes G1-naive and G2-primed clustered most frequently at the origin of the plot and bifurcated into two branches terminating in nodes where G4-chronic and G5-suppressive were the most abundant. G3-progressive was located predominantly between clusters G1-naive and G5-suppressive, and to a lesser extent between clusters G2-primed and G4-chronic (Figure 5E), indicating potential transitional relationships between them. To more accurately quantify the dynamic nature of these immunotypes for therapy response evaluation, we used five different immunotype-specific linear regression models to transform each immunotype into a continuous score (Table S8). Importantly, this can be derived from individual patient samples independently from their putative immunotype assignment. These immunotype signature scores (ISSs) reflect the degree of similarity of each patient sample to the sample with the most ideal, or highest individual immunotype score within the cohort, between 0 and 10.

ISSs were then plotted onto five different pseudotime plots to illustrate the distribution of these scores throughout the internal cohort (Figure 5E, right). Essentially, these ISSs serve as a metric that quantifies the magnitude of a particular immunotype of an individual patient within a cohort; the higher the score, the closer that sample is to the ideal characteristics of that immunotype in that cohort. The maximum immunotype signature score (MIS) compares all five ISS values from a patient’s blood sample, and subsequently uses the largest ISS value to assign that patient to an immunotype. Immunotype assignment using the patients’ MISs showed high concordance with the initial spectral clustering-based immunotype assignment of our internal cohort (Figure 5F; Table S8). Therefore, the quantification of ISSs (Table S8) and determination of patients’ MISs are suitable analytical tools for evaluating dynamic, systemic immune responses to treatment and can be applied either individually or in a complimentary fashion even across small cohorts with adequate statistical power for correlative assessment.

Immunotype signature scores correlated with patient responses to cancer treatment

Understanding the potential utility of our immunotype framework as a tool to assess patient outcomes requires cohorts with homogeneouse diagnoses and treatments, detailed clinical data, and peripheral blood analysis where the frequencies of different immune cells could be quantified. Our internal cohort contained very few patients (n = 10) with available clinical response data and was heterogeneous with respect to diagnosis and treatment. Therefore, public datasets with specific treatments and diagnoses were used to first explore the utility of our immunotype signature framework as a prognostic tool to stratify cancer patients by response.

Figure 5. Immuno profiling of transcriptomes from healthy donors, cancer patients, and patients with other immune conditions in the open-source cohort

(A) Unsupervised spectral clustering analysis was applied to normalized cell percentages obtained from an open-source collection of 17,800 RNA-seq whole blood samples processed by Kassandra deconvolution. Only 1,000 randomly selected samples are shown to reduce figure complexity.

(B) A three-dimensional PCA representation of the open-source blood RNA-seq datasets processed by Kassandra deconvolution and then classified in immunotypes. Only 5,000 randomly selected samples are shown to reduce figure complexity.

(C) Bar plots showing distribution of the five immunotypes within each disease group (left). The statistical significance of the distribution for each disease group compared with healthy samples is shown on the bubble plot (right). Color gradient corresponds to log p values (Fisher’s exact test with Bonferroni correction). The circle size is proportional to the fraction value within the disease group.

(D) Immunotype distribution after UMAP analysis of the internal cohort’s cytometry data on selected cell populations used in clustering (left). Pseudotime analysis of flow cytometry data from our internal cohort based on the putative developmental trajectories of the different cell populations enriched in each immunotype (right).

(E) Distribution of signature scores for each immunotype mapped on the pseudotime trajectory in (D).

(F) Sankey-plot depicting the concordance between immunotype assignment by clusterization and by maximum immunotype signature scores (MIS) for the internal cohort. See also Figure S7, Tables S7, and S8.
Chemotherapy has profound effects on the composition of the immune system. As a proof of concept, we examined if these changes could be reflected in alterations in immunotype signatures identified in a cohort of breast carcinoma patients treated with neoadjuvant chemotherapy (NAC) prior to surgical resection (GSE201085, Figure 6A). Cellular deconvolution with Kassandra of bulk RNA-seq of patients’ peripheral blood mononuclear cells (PBMCs) collected 30 days after treatment initiation was used to calculate ISSs (Table S8), and patients were stratified into immunotypes using MIS. Interestingly, patients with a pathological complete response (pCR) to NAC were more frequently assigned to immunotype G5 than those with residual disease (RD) (Figure 6B). Patients with pCR had a significantly higher G5-suppressive signature score ($p = 0.01$) and a significantly lower score for the G1-naive signature ($p = 0.03$) than patients with RD (Figure 6C). Binary response stratification ROC-AUCs for G5-suppressive and G1-naive were 0.79 and 0.25, respectively, suggesting that our immunotype signatures are sensitive to systemic changes associated with response. G3-progressive and G4-chronic trended in the same direction as G5-suppressive while G2-primed trended toward G1-naive, indicating a broader shift in immune composition prognostic of effective responses to chemotherapy (Figure 6D).

Next, a cohort of patients with PDAC from a randomized phase II clinical trial (PRINCE) was evaluated. These patients were treated with a combination of chemotherapy and anti-PD-1 (nivolumab; A1) or anti-CD40L (sotigalimab; B2) immunotherapy, or all three (C2). PBMCs collected 30 days after treatment (CD21) were analyzed with high-parameter CyTOF (Figure 6E) to further demonstrate the utility of ISSs in stratifying cancer patients with different diagnoses and treatment lines. Cell populations derived from CyTOF data were used to calculate ISS. Each patient was assigned to an immunotype group using MIS, and then the combined cohort was evaluated for progression-free and overall survival (PFS and OS). While no significant association between immunotype and PFS was observed (Figure 6F, left), patients assigned to the G3-progressive immunotype had significantly longer OS ($p = 0.004$) than patients assigned to other immunotypes (Figure 6F, right). Patients were then stratified by the median survival time for this trial into short (<347 days) and long OS (≥347 days). Among patients with long OS, a greater proportion of them had a G3-progressive MIS compared with the MIS for the remaining immunotypes ($p = 0.04$, Figure 6G). These patients also had significantly higher G3-progressive ISSs overall ($p = 0.0009$, Figure 6H, left). A binary classifier using G3-progressive ISS was able to differentiate between patients with long OS and short OS in this cohort with high specificity (ROC-AUC = 0.74, Figure 6I, left).

To determine if this signature could be tied to a particular treatment arm, we analyzed the different therapy groups by OS. The Chemo/Nivo A1 group had significantly higher G3-progressive scores between patients with short and long OS ($p = 0.0006$) and an ROC-AUC of 0.94. Patients with long OS also had significantly lower G2-primed ISSs with an ROC-AUC of 0.19 (Figures 6H and 6I right), indicative of a broader systemic shift associated with response. This effect was not observed in either the Chemo/Sotiga B2 or Chemo/Nivo/Sotiga C2 groups (Figures 6H and 6I middle). Our findings are consistent with those of Padrón et al., where only patients from the Chemo/Nivo A1 group met the primary survival endpoints of the PRINCE trial. Collectively, this analysis supports our previous observations in NAC-treated breast cancer that ISSs have prognostic value in post-treatment response monitoring in an independent cohort.

**Application of immunotype signatures for the evaluation of PBMC samples from HNSCC patients treated with frontline immunotherapy**

Having shown our immunotype signature framework to differentiate responses between cancer patients with different diagnoses treated with chemotherapy and combination chemo/immunotherapy, we evaluated the utility of this platform in stratifying cancer patients treated with frontline immunotherapy by objective responses from PBMCs collected and analyzed at pre- and on-treatment time points. Two cohorts of patients with advanced head and neck squamous cell carcinoma (HNSCC) treated with checkpoint inhibitors targeting the PD-1 pathway were selected. Both trials included a second arm where an additional drug was combined with the checkpoint inhibitor. In the first trial, all 32 HNSCC patients (HNSCC-Durva cohort) were treated with PD-L1 inhibitor durvalumab; among these, 25 were also treated with anti-hyperglycemic agent metformin (25/32) (Table S9). Based on an overall objective pathological assessment of primary tumors and involved lymph nodes, 56.25% (18/32) of patients had responded to treatment. There was no statistical difference in response rates between patients treated with durvalumab plus metformin and durvalumab alone (Figure 7A), suggesting that therapy response in this trial was driven primarily by durvalumab. Pre- and post-treatment PBMCs from this trial were processed by bulk RNA-seq and cell percentages were identified using Kassandra. Signatures for immunotypes G1–G5 were calculated and patients were then classified into different immunotype groups. While there was no significant skewing of immunotype distribution based on response, more responders tended to be assigned the G4-chronic immunotype than non-responders (Figure 7B). A comparison of ISSs across the entire cohort showed significantly higher G3-progressive scores for responders at the on-treatment time point ($p = 0.04$), with a trend for increased G4-chronic ISSs and response classification ROC-AUCs of 0.74 and 0.68, respectively (Figure 7C).

Next, we explored if patient responses could be stratified based on the immunotype signature analysis of baseline samples collected before treatment. While immunotype classification with MIS was not statistically significantly different between responders and non-responders (Figure 7D), G4-chronic ISSs were significantly greater in responders ($p = 0.03$). Although not statistically significant, G3-progressive ISSs were also greater in responders, with ROC-AUCs of 0.73 and 0.66, respectively (Figure 7E). This corresponded with multiple CD8+ T cell populations that could be measured by Kassandra deconvolution being differentially represented among responders (Figure 7F). We then benchmarked the positive predictive value of our G4-chronic signature to the measurement of PD-L1 expression in the tumor by RNA-seq from patients in this cohort where next-generation sequencing (NGS) data were available. Our analysis showed the G4-chronic signature from this cohort to considerably outperform tissue PD-L1 expression in stratifying response at baseline (Figure 7G).
Figure 6. Immunoprofiling of clinical cohorts and analysis of treatment response

(A) Graphical description of the breast cancer cohort (n = 33 patients) with neoadjuvant chemotherapy (NAC), blood draw, and surgical resection time points.

(B) Bar graphs for distribution of pathological complete responders (pCR) versus non-responders with residual disease (RD) within immunotype clusters (Fisher’s exact test on a 5x2 table for count data, testing the relationship between immunotypes and treatment response) for on-treatment samples (left). Bar graphs for distribution of immunotype clusters between pCR and RD for on-treatment samples (right).

(C) Boxplots showing G1 and G5 signature score distribution between RD and pCR for on-treatment samples (Mann-Whitney U test).

(D) ROC-AUC for each of the five signature scores for pCR versus RD in the breast cancer cohort.

(E) Graphical description of the PDAC cohort (n = 71 patients) with combination treatments and the blood draw time point at 4 weeks after the start of the trial, noted as the C2D1 time point or day 30 (D30). The treatment types consisted of sotigalimab (sotiga) and/or nivolimab (nivo) combined with chemotherapy (CT), indicated as A1, B2, and C2.

(F) Kaplan-Meier plot (multiple log rank test) showing progression free survival (PFS, left) and overall survival (OS, right) distribution for PDAC patients assigned by immunotypes.

(G) Bar graphs for distribution of short overall survival (sOS) versus long overall survival (LOS) within immunotypes (Fisher’s exact test on a 5x2 table for count data, testing the relationship between immunotypes and treatment response) for blood samples at C2D1 time point (left). Bar graphs for distribution of immunotype clusters between sOS versus LOS for blood samples at C2D1 time point (right).

(H) Boxplots comparing the distribution of G3 signature scores between patients with sOS or LOS, for all that had received either A1, B2, or C2 treatment combinations, for patients with either B2 or C2 treatments, and patients with A1, as indicated, based on blood samples drawn at time point C2D1. The distribution of G2 signature score between patients with sOS and LOS was also compared (the median OS cutoff = 347 days, Mann-Whitney U test).

(I) ROC-AUC based on each signature score for all PDAC patients, patients with A1, and patients with B2 or C2, to show the performance of each signature score in classifying SOS and LOS patients. Boxplots in C and H indicate the median and the 25th and 75th percentiles, and whiskers extend to the lowest and highest value within 1.5x interquartile range. See also Table S8.
Figure 7. Establishing ICB response predictors for HNSCC cohorts based on immunotype signature scores (ISSs)

(A) Depiction of the HNSCC-Durva cohort (n = 32 patients) with treatment and blood draw timepoints. Bar plot shows the percentage of responders (R) and non-responders (NR) from the total cohort of patients treated with durvalumab alone or durvalumab plus metformin.

(D) Pre-treatment

(E) Pre-treatment

(F) ROC-AUC (pre-treatment: R vs NR)

(G) HNNSC predictive biomarker comparison

(legend continued on next page)
Our comprehensive immunoprofiling platform was then tested with a clinical cohort of 35 HNSCC patients (HNSCC-Nivo cohort) treated with first-line nivolumab alone or nivolumab in combination with indoleamine 2,3-dioxygenase-1 inhibitor BMS-986205 (IDOi) (Figure 7H, Tables S8, and S9). Similar to the HNSCC-Durva cohort, patients in different arms had similar response rates (Figure 7H). Cell population frequencies were obtained from flow cytometry and RNA-seq analysis of cryopreserved PBMCs collected at both on- and pre-treatment time points, and patients were classified into immunotype groups with corresponding MIS. Interestingly, responders were assigned most frequently to the G2-primed immunotype ($p = 0.004$) (Figure 7I). G2-primed scores were significantly higher in responders and could prognostically discriminate responders from non-responders with an accuracy of $76\%$ (Figure 7J). Analysis of pre-treatment samples revealed a similar pattern of G2-primed assignment, with all patients assigned to G2-primed responding to nivolumab (Figure 7K). Pre-treatment G2-primed scores were significantly higher in responders ($p = 0.02$) with an ROC-AUC of 0.74 (Figure 7L). In this context, the G2-primed signature demonstrated potential utility as both a prognostic and predictive biomarker for the treatment of advanced HNSCC with nivolumab. This was further supported by differential population analysis of baseline PBMCs showing ten significantly increased cell populations in the peripheral blood of responders, nine of which belong to the CD4+ T cell lineage (Figure 7M). Additionally, the accuracy of G2-primed scores in response stratification from this cohort was greater than that of multiple established and published biomarkers for HNSCC patients treated with anti-PD-1 ICB, including TMB, tumor PD-L1 expression by RNA-seq, and CD4+ T cells and PD-1+ CD8+ T cells from the peripheral blood52,53 (Figure 7N). Interestingly, approximately half of the patients in both HNSCC cohorts had the same MIS at both pre- and on-treatment time points, while the rest of patients transitioned between adjacent immunotypes during ICB therapy (Figures S8A and S8B). Of note, 6/8 responder patients with G2-primed MIS in the HNSCC-Nivo remained in G2-primed throughout treatment, while the remaining 2 patients transitioned to the adjacent G3-progressive MIS. Of the 11 patients that transitioned to G4-chronic or G5-suppressive MIS, 9 did not respond to PD-1 (Figure S8A). Conversely, in the HNSCC-Durva cohort, 7/8 patients that transitioned to G4-chronic or G5-suppressive MIS responded to anti-PD-L1 (Figure S8B). These observations are consistent with the association of different favorable systemic immune responses to specific immunotherapy drugs.

**Immunotype signatures improved response stratification in HNSCC based on therapy type and human papilloma virus infection status**

Human papillomavirus (HPV) is a major etiologic agent of HNSCC, and patient responses to PD-1 and PD-L1 blockade are associated with HPV status.54 Having shown the potential of ISSs to stratify HNSCC patients prior to treatment initiation with durvalumab or nivolumab, we then explored the utility of immunotype stratification in HPV+ and HPV− HNSCC patients from the two cohorts described in Figure 7. Segregation of patients from the HNSCC-Nivo cohort into HPV+ and HPV− groups revealed no significant differences among the G2-primed signature scores in HPV+ responders to nivolumab (Figure 8A). Interestingly, for anti-PD-1-treated HPV− patients in this cohort, the G2-primed scores were significantly higher ($p = 0.01$) in responders, with the G2-primed signature ROC-AUC at 0.86 in HPV− patients, versus 0.58 in HPV+ patients and 0.74 in the overall cohort (Figure 7L, 8B). HPV+ and HPV− patients from the HNSCC-Durva group were then analyzed separately. While not statistically significant, HPV+ responding patients had higher...
G4-chronic signature scores than non-responders with an ROC-AUC of 0.75 (Figure 8C). This trend was also seen in HPV+/C0 patients with an ROC-AUC of 0.75 (Figure 8D), similar to our analysis of the G4-chronic immunotype in the overall cohort which was statistically significant with an ROC-AUC of 0.73 (p = 0.04) (Figure 7E). On the other hand, the G3-progressive signature scores for HPV+/C0 patients in this cohort were significantly higher in responders (p = 0.03) with an ROC-AUC of 0.89 versus 0.58 in HPV+ patients (Figures 8C and 8D). Taken together, our findings indicate that immunotype signatures G2-primed and G3-progressive have potential predictive utility for HPV+/C0 HNSCC patients treated with nivolumab and durvalumab, respectively, while the immunotype signature G4-chronic can potentially stratify responders to anti-PD-L1 therapy independent of HPV status.

**DISCUSSION**

The environmental and genetic heterogeneity of human populations, coupled with the complex and ongoing nature of diverse immunological challenges, complicates the formation of clinically useful consensus models for analyzing human immune function in the context of a complex disease like cancer. This undertaking is further confounded by the lack of standardization among methods for measuring immune function, disagreement regarding the markers necessary and sufficient for accurate immunophenotyping, inconsistent methods for sample collection and specimen processing, and the prominence of reductionist approaches that focus only on a narrow range of molecules and cell types. Here, we attempted to address these bottlenecks by developing a platform that combines a comprehensive immunophenotyping assay based on flow cytometry and bulk RNA-seq with a ML-based analysis pipeline. This platform was then used to establish a robust analytical framework for describing systemic immune variation in individual patients that can be applied to diverse data and sample types to uncover peripheral blood-based response biomarkers either retrospectively or prospectively.

The application of this immunoprofiling approach to HNSCC, breast cancer, and PDAC patient cohorts illustrated the utility of this framework by stratifying responders and non-responders to different treatments. Moreover, this approach also revealed different immunotypes to be associated with responses to different therapies, highlighting how response-associated immune profiles can differ with regards to both disease pathogenesis and the engagement of a specific therapy target. For example, while inhibition of the PD-1 pathway by blocking either the receptor (PD-1) or its ligand (PD-L1) can lead to the reversal of immunosuppression,\textsuperscript{55} these molecules are expressed by different cell types both in the tumor and in the periphery and
display different efficacies depending on the drug formulation and cancer type.\(^6\) In this context, it is not surprising that different immunotype signatures correlated with responses to durvalumab and nivolumab in both HPV+ and HPV– HNSCC patients.

Collectively, these descriptions of systemic immunity suggest that immunotypes G1–G5 identified herein may represent peripheral immune meta-signatures conserved across different patient populations. In other words, individual biomarkers, such as immune cell population frequencies, cellular differentiation states, expression of individual genes and gene signatures, and TCR and BCR repertoire composition that have been evaluated independently in numerous response correlative studies,\(^5\) can also exhibit immunotype-specific distribution patterns. For example, evaluating TCR diversity in a cancer patient cohort where patients with G1-naive and G4-chronic immunotypes are mixed may yield unreliable conclusions. These types of discrepancies may explain, in part, why many blood-based biomarker approaches have not moved beyond the discovery stage in predicting treatment responses in patients with cancer.

Current standard practices only evaluate conditions in a patient’s tumor and the TME for predicting clinical responses to immunotherapy.\(^7,\)\(^56\) These conventions disregard an emerging consensus indicating that the recruitment of immune cells, particularly T cells, with anti-tumor potential from the periphery into the TME is critical for effective immunotherapy response.\(^57\) This consensus is supported by studies demonstrating a reduction in the efficacy of checkpoint inhibition in preclinical models where CXCR3-dependent T cell trafficking from the periphery is inhibited, and in patients with poor expression of chemokines CXCL9 and CXCL10.\(^58,\)\(^59\) Moreover, T cell recruitment, while necessary, may not be sufficient for immunotherapy responses. Therefore, T cell profiles of responders may also include a diverse TCR repertoire,\(^60\) memory subsets with high levels of TCF7 expression,\(^61\) and low levels of terminal differentiation and/or exhaustion.\(^62\) Conversely, the recruitment of immunosuppressive regulatory T cells and myeloid-derived suppressor cells (MDSCs) from the peripheral blood have been linked to immunotherapy failure,\(^63\)\(^65\) highlighting the importance of comprehensive approaches when evaluating therapy responses in cancer patients. It is our hope that this work and other complementary approaches will reshape this paradigm.

The immunoprofiling platform and immunotype framework described here can open additional avenues for translational research in immuno-oncology, by offering a simplified approach for multi-omics integration of systemic immunity with the TME. To date, only a few published studies have evaluated both peripheral blood and tumor tissue.\(^50,\)\(^66\)\(^68\) To the best of our knowledge, these studies did not use a similar systems immunology approach. Moreover, specimen procurement, sample processing, data generation, and analysis are often inconsistent.\(^66,\)\(^70\) Additionally, prospective longitudinal collection of peripheral blood cells is often deprioritized in favor of tissue and plasma-based liquid biopsies in oncology trials.\(^71,\)\(^72\) Therefore, additional experiments similar to those presented in our study will require a level of standardization that is not routine in clinical immuno-oncology research. Our immunotype signatures represent a diagnostic framework that attempts to translate the fine details of the human immune system into a practical bird’s eye view of human immunity. With additional validation, these findings could be applied to the rational selection of patients that may benefit from new treatment combinations and to the monitoring of effects of immune-conditioning interventions in the adjuvant or neoadjuvant setting.\(^73\) Additional studies are warranted to demonstrate the ability of this approach to identify (1) immunotype signatures related to the evolution of immune-related adverse events or immunotoxicity; (2) the development of resistance against a particular drug or treatment; and (3) the relative risk of developing a serious infection. Fundamentally, our systems analysis of patient immunotypes provides a springboard to a scenario where the routine evaluation of a cancer patient’s systemic immunity using a simple blood test may be used to predict and monitor responses to different treatments in a clinical setting.

**Limitations of the study**

Our initial analysis of healthy donors and cancer patients primarily involved advanced cancer patients with extensive treatment profiles. Consequently, very few patients in our internal cohort were treatment-naive or had early disease. Therefore, our selection of features differentiating these healthy donors and cancer patients would likely be insensitive to prior treatment history, which is known to influence subsequent therapy response.\(^74,\)\(^75\) RNA-seq or imaging-based surveys of the TME of our internal cohort were not included, also due to the heterogeneity of this cohort and lack of synchronization between tissue biopsy and blood draw, both of which may confound correlative analysis. Follow-up studies will require matched peripheral blood and tumor tissue from more homogenous cohorts of cancer patients.

Finally, a major limitation of this approach is the limited availability of standardized data and sample sets as well as routine specimen collection for this type of analysis that also includes sufficiently detailed clinical data. For this study, even though we scoured open-source databases and nearly every oncology publication from the past seven years, we only found very few studies that met these criteria and have included two in this manuscript. Similarly, attempts to engage collaborators that possessed specimen collections compatible with our platform were equally challenging. Subsequent clinical validation of this immunotype framework would require a large number of longitudinal blood samples from multiple oncology trials that adhere to a minimum set of requirements for specimen collection and storage. To the best of our knowledge, this level of standardization is uncommon.

**STAR METHODS**

**METHOD DETAILS**

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## STAR METHODS

### KEY RESOURCES TABLE

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**Biological samples**

- Whole blood from healthy donors, collected in K2-EDTA vacutainers (Purple top)
  - Source: Research Blood Components
  - Item#: 016-018
- Whole blood from healthy donors, collected in K2-EDTA vacutainers (Purple top)
  - Source: STEMCELL Technologies
  - Cat#:70508.1
- Whole blood from healthy donors, collected in K2-EDTA vacutainers (Purple top)
  - Source: Discovery Life Sciences
  - Order#:57762-A
- Whole blood from cancer patients, collected in K2-EDTA vacutainers (Purple top)
  - Source: BostonGene Clinical Program
- cryoPBMC, HNSCC Nivo cohort
  - Source: Adam Luginbuhl (TJU)
- cryoPBMC, HNSCC Durva cohort
  - Source: Joseph Curry (TJU)

**Chemicals, peptides, and recombinant proteins**

- 10x RBC lysis buffer
eBioscience
  - Cat# 00-4300-54
- Brilliant stain buffer, cat#:566385
BD Biosciences
  - RRID: AB_2869761
- ViaStain™ AOPI Staining Solution
Nexcelom Bioscience
  - Cat#: CSZ-0106
- Ghost Dye Violet 510 Viability Dye
Tonbo Biosciences
  - Cat#13-0870-T100
- CytoFix/CytoPERM
BD Biosciences
  - Cat#:554722
- Phosphate Buffered Saline (1X)
ThermoFisher Scientific
  - Cat#20021-027
- Ficoll-Paque™ PLUS
Cytiva
  - Cat#17144003

**Critical commercial assays**

- Maxwell® RSC simplyRNA Cells Kit
Promega
  - Cat#AS1390
- Illumina Poly(A) Capture
Illumina
  - Cat#20040894
- Illumina cDNA Synthesis
Illumina
  - Cat#20040896
- IDT for Illumina DNA/RNA UD Indexes
Illumina
  - Cat#20026121
- Illumina RNA Prep Ligation
Illumina
  - Cat#20040898

**Deposited data**

- Internal cohort RNA-seq data
This paper
  - EGA: EGAS50000000286
- Internal cohort FC data
This paper
  - ImmPort: SDY2583

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests should be directed to and will be fulfilled by the lead contact, Michael F. Goldberg (michael.goldberg@bostongene.com).

**Materials availability**
This study did not generate new unique reagents.

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<td>PDAC (Prince)</td>
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<td>Padrón et al., 2022&lt;sup&gt;90&lt;/sup&gt;</td>
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<td>Breast cancer</td>
<td>Axelrod et al., 2022&lt;sup&gt;99&lt;/sup&gt;</td>
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**Software and algorithms**

| Kassandra | Zaitsev et al. 2022<sup>9</sup> | https://github.com/BostonGene/Kassandra |
| Cell-typing machine learning models | This paper |  |
| Scikit-learn v1.1.2 | Pedregosa et al. 2011<sup>119</sup> | https://github.com/scikit-learn/scikit-learn |
| Pandas v1.1.4 | McKinney, 2011<sup>78</sup> | https://github.com/pandas-dev/pandas |
| matplotlib | Hunter, 2007<sup>106</sup> | https://github.com/matplotlib/matplotlib |
| seaborn |  | https://seaborn.pydata.org/ |
| ggplot2 v3.4.4 | Wickham, 2016<sup>116</sup> | https://ggplot2.tidyverse.org/ |
| ggalluvial v0.12.5 | Brunson, 2023<sup>116</sup> Brunson, 2023<sup>117</sup> | https://corybrunson.github.io/ggalluvial/ |
| scipy | – | https://github.com/scipy/scipy |
| FlowKit v0.5.0 | White et al., 2021<sup>90</sup> | https://github.com/malcommac/FlowKit |
| FlowSom v0.1.1 | Van Gassen et al. 2015<sup>82</sup> | https://pypi.org/project/FlowSom/ |
| LightGBM v3.3.2 | Ke et al. 2017<sup>106</sup> | https://github.com/microsoft/LightGBM |
| FastQC v0.11.9 | https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ |  |
| FastQ Screen v0.11.1 | https://github.com/s-andrews/FastQC |  |
| MultiQC v1.14 | Ewels et al. 2016<sup>18</sup> | https://github.com/ewels/MultiQC |
| OptiType v1.0 | Szolek et al., 2014<sup>99</sup> | https://github.com/FRED-2/OptiType |
| MRMR v0.2.5 | Ding and Peng, 2005<sup>101</sup>; Zhao et al., 2019<sup>102</sup> | https://github.com/smazzanti/mrmm |
| TabPFN v0.1.8 | Hollmann et al. 2022<sup>29</sup> | https://github.com/automl/TabPFN |
| Monocle | Trapnell et al., 2014<sup>113</sup> |  |
| edgeR | Chen et al., 2023<sup>110</sup> |  |
| Strelka 2.9 | Kim et al. 2018<sup>115</sup> | https://github.com/Illumina/strelka |
| uMAP 0.5.3 | McInnes et al., 2020<sup>104</sup> | https://github.com/lmcinnes/umap |
| tSNE v0.6.2 | Maaten and Hinton, 2008<sup>84,120</sup> | https://pypi.org/project/openTSNE/ |
| Python 3.10 | – | https://www.python.org/ |
| R v4.3.3 | – | https://www.r-project.org/ |

**Other**

| BD FACSCelesta Cell Analyzer | BD Biosciences | Part #: 660345 |
| NovaSeq 6000 | Illumina | Ref. #: 20012850 |
| Maxwell® RSC Instrument | Promega | S/N:20000112 |
| DxH 500 Hematology Analyzer | Beckman Coulter | S/N:BD040017 |
| Cellometer K2 Fluorescent Cell Counter | Nexcelom Bioscience | S/N:6381 |
Article

Data and code availability
- Raw flow cytometry data are available at ImmPort under study accession SDY2583. Sequenced raw RNA-seq data of the internal cohort (376 healthy donors and 421 cancer patients) is deposited in the EGA (European Genome-Phenome Archive): EGASS0000000286. The accession IDs of open-source datasets analyzed in this study are included in Table S7.
- Software and algorithms used in this study are indicated in the key resources table. We had previously deposited the code for the Kassandra algorithm: https://github.com/BostonGene/Kassandra (Zaitsev et al. 2022).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

STUDY PARTICIPANT DETAILS

Blood samples
The peripheral blood of 408 healthy donors was obtained from Research Blood Components (Watertown, MA), STEMCELL Technologies (Vancouver, BC, Canada), and Discovery Life Sciences (Huntsville, AL). The peripheral blood of 442 cancer patients included in the internal cohort were processed in BostonGene laboratory. Each patient provided informed consent. The use of clinical samples was conducted in accordance with the Declaration of Helsinki and has been granted exemption from ethics approval by the Biomedical Research Alliance of New York (BRANY) Institutional Review Board (BRANY study #22-12-938-853). Additional information, including the age and sex of participants, is included in Table S4.

Internal cohort description
Peripheral blood samples of cancer patients were collected at multiple medical centers across the United States and delivered to BostonGene Laboratory. Blood of healthy donors were purchased from multiple collection centers throughout the United States; Research Blood Components (Watertown, MA), STEMCELL Technologies (Vancouver, BC, Canada), and Discovery Life Sciences (Huntsville, AL). All patients provided written consent per IRB-approved protocols. After excluding samples with insufficient quality (late receipt post-collection, low blood volume/cell count, viability lower than 75%), we analyzed 850 samples by flow cytometry (Table S4). The median age in our cohort was 47 years for healthy donors and 61.5 years for cancer patients. We included patients with sarcomas and carcinomas: pancreatic cancer \( n = 37 \), breast neoplasm \( n = 65 \), non-small cell lung carcinoma \( n = 32 \), colorectal neoplasm \( n = 41 \), melanoma \( n = 19 \), and prostate cancer \( n = 18 \). Therapeutic information was available for 417 patients (417/442, 94.3%). A total of 211 patients (211/417, 50.6%) underwent previous treatments within a year of blood draw, including chemotherapy, radiotherapy, immune checkpoint inhibitor (ICI), or other types of systemic therapy. A total of 234 patients (234/417, 56.1%) were on ongoing therapy during material collection, while 44 patients (44/417, 10.55%) had no evidence of therapy administration after cancer diagnosis. Additionally, we analyzed 797 patient-matched RNA samples from both healthy and cancer blood donors. We used this diverse cohort for our multi-scale analysis of the relationship between cancer and peripheral blood immunity.

Head and neck squamous cell carcinoma cohorts
To further investigate the implications of the newly discovered immunotypes on cancer immunotherapy, we applied our analytical framework to two cohorts of patients with head and neck squamous cell carcinoma (HNSCC-Nivo and HNSCC-Durva) (Table S9). The HNSCC Nivo cohort was part of a prospective phase II trial (NCT03854032) conducted at Thomas Jefferson University Hospital. During this trial, patients received anti-PD-1 monoclonal antibody nivolumab or nivolumab plus a specific IDO inhibitor (BMS986205). The HNSCC Durva cohort was part of a pilot phase I trial (NCT03618654) conducted at Thomas Jefferson University Hospital. Here, patients received anti-PDL1 monoclonal antibody durvalumab or durvalumab plus metformin. For the HNSCC-Nivo cohort, pre- and post-treatment cryopreserved WBCs were thawed and subject to a multicolor flow cytometry staining. Samples from the HNSCC-Durva cohort were subject to polyA RNA-seq described below due to insufficient number of cells for flow cytometry. Treatment response was determined by assessing overall pathological treatment response as previously described by Luginbuhl et al.76

METHOD DETAILS

Flow cytometry method development
Commercially available monoclonal antibodies against well-described lineage and activation-associated antigens were selected to identify a broad cross section of cellular phenotypes and cellular activation states from WBCs in the peripheral blood. Individual antibodies were titrated to give the optimal signal-to-noise ratio relative to each panel, using assay-specific instrument settings. This assay was then analytically tested for sensitivity and precision prior to wider implementation in this study. The lower limit of quantitation of this assay was measured at 0.01% of total live WBCs, intra-assay repeatability was 5.4% of CV on fresh whole blood, and inter-assay reproducibility was 7.36% of CV on cryopreserved PBMC.

Blood sample processing and white blood cell isolation
Upon receipt, all fresh peripheral blood samples underwent a CBC using the DxH 500 Hematology Analyzer (Beckman Coulter, Brea, CA). Samples received in K2-EDTA tubes within 24 h of collection at RT underwent RBC lysis with 3 mL whole blood to isolate WBCs.
Cryopreserved white blood cell thawing

WBC samples of both HNSCC cohorts, that were cryopreserved in the vapor phase of a remotely monitored liquid nitrogen freezer, were thawed at 37°C and transferred to an empty 15 mL tube. Thawing medium (20% NBCS in 500 mL RPMI 1640 media + 10 mM HEPES + 2% Penicillin/Streptomycin + 2% MEM non-essential amino acids + 1% GlutaMAX), in a 15 mL aliquot was pre-warmed to 37°C and supplemented with 75 μL DNase I (20 mg/mL) and 75 μL Glutathione (200 mM), and was slowly pipetted into the tube, gently layering the media over the sample. After 3–4 mL of layering, more thawing medium was slowly pipetted directly into the sample and the tube was simultaneously swirled until the sample was homogeneous. Finally, the sample was topped off with more thawing medium to a final volume of 15 mL. WBC samples from the HNSCC-Nivo cohort were then centrifuged at 300×g for 8 min and washed with thawing media at 300×g for 8 min before staining. WBC samples from the HNSCC-Durva cohort were subject to RNA extraction and sequencing.

Cell staining for flow cytometry

Isolated WBCs were centrifuged at 300×g for 5 min, and then resuspended and blocked with Blocking Buffer (IMDM + 10% NBCS + DNase I (20 mg/mL) + 2% (v/v) Human TrueStain FCx + 2% (v/v) Monocyte Blocker + 0.5% (v/v) Unlabeled Normal Mouse IgG) for 10 min at RT. After blocking, each sample was aliquoted into 10 unique wells on a 96-well plate, and centrifuged at 300×g for 3 min to remove supernatant. Each well was stained with 50 μL Ghost Dye Violet 510 Viability Dye in PBS (0.25% (v/v), Tonbo) at RT for 10 min. After staining with the viability dye, 200 μL of Sorter Buffer was added to each well, and the samples were centrifuged at 300×g for 3 min with the supernatant removed subsequently. Samples were stained with 10 custom flow cytometry antibody panels (Table S1) for 20 min at RT. The 10 custom antibody panels included the general lineage backbone panel with bridging antibodies to connect the remaining nine immune cell type-specific panels together. Each panel has a different combination of antibodies to detect different major immune cell populations and cell type subsets. Once stained, 200 μL of Sorter Buffer was added to each well, and centrifuged at 300×g for 3 min followed by supernatant removal. Cells were then fixed in a 1% paraformaldehyde solution (Cytofix/Cytoperm, BD Biosciences) overnight at 4°C. After fixation, 200 μL of Sorter Buffer was added to each well, centrifuged at 300×g for 3 min, and resuspended in Acquisition Buffer (PBS + 0.5% (w/v) BSA + 0.75% (w/v) Glycine + 5 mM EDTA + 0.05% (v/v) Tween 20 + 1% (v/v) sodium azide).

Flow cytometry data generation

Stained and fixed cells were acquired on the BD FACSCelesta Flow Cytometer using FACSDiva software. Assay-specific application settings were established and linked to cytometer set-up and tracking (CS&T) runs to ensure consistency with each acquisition. Instrument settings were calibrated daily using BD FACSCelesta and checked using CS&T Research Beads (BD Biosciences). Compensation matrices were generated through the FACSDiva software by calculating spectral overlap from single-stained controls. Single-stained controls were prepared in-house using Ultracomp eBeads Compensation Beads (Thermofisher) with unique antibodies for each channel titrated individually for optimal performance.

RNA isolation

Isolated WBCs for RNA sequencing were centrifuged at 300×g for 5 min with a maximum of 10⁶ cells per vial. The supernatant was removed, and the cells were resuspended in cold Homogenization Buffer (2% 1-Thioglycerol, Promega). Samples were then frozen at −80°C until extraction. RNA extraction was performed from frozen samples with Maxwell RSC simplyRNA Cells Kit (Promega) using the benchtop automated Maxwell RSC Instrument (Promega).

Library preparation and sequencing of samples

Libraries were prepared with Illumina TruSeq Stranded mRNA Library Prep (Poly-A mRNA; stranded). Libraries were sequenced on NovaSeq 6000 as Paired-End Reads (2×150) with targeted coverage of 50 mln reads.

Flow cytometry data processing

Flow cytometry data went through several quality control steps to ensure the consistency and overall high quality of the input data. All the selected patient samples contained no less than 10⁷ cells in one panel. We also excluded files with poor compensation or occasional PMT failure. Flow cytometry data were exported in fcs 3.0 file format and analyzed as Pandas DataFrames (v 1.1.4, 77,78 https://pandas.pydata.org/) with compensation matrices applied using FlowKit (v. 0.5.0) for data processing and analysis. The values of all fluorochrome-marker channels were divided by a coefficient of 190 with the following inverse hyperbolic sine: arcsinh x = ln(x + √(x² + 1)) transformation. Forward scatter and side scatter values (FCS-A/H/W and SSC-A/H/W) were divided by 10⁵ to meet the order of data transformed with arcsinh.
Manual analysis of flow cytometry data

A framework was constructed for the precise manual analysis of cell populations combining classical gating within 2D scatterplots and clustering steps. Each panel was analyzed separately in accordance with its own specific strategy. Every strategy consists of several consecutive steps as follows.

1. Events were clustered using FlowSOM (v0.1.1, \cite{1,2} https://pypi.org/project/FlowSom/). Data were visualized with the tSNE algorithm (openTSNE, v 0.6.2, \cite{3,4} https://pypi.org/project/openTSNE/) and colored both by clustering and by marker intensities, thus enabling the visualization of the combination of marker intensities on specific clusters. Each cluster was manually matched with a cell population based on the combination of marker intensities on this cluster.

2. Prior to clustering, the processing of cytometry data may include a noise transformation to adjust marker intensities in order to reduce the influence of noise on the clustering results. This step involved reducing the marker intensities to levels lower than a certain threshold. The noise threshold for a marker was defined manually based on a two-dimensional plot of the intensity of this marker versus intensity of another marker in the panel. The boundary between the noise and positive signal of the marker was chosen at the point of visually observed local minimum of the marker distribution. The equations below describe the intensity of a marker after noise transformation:

\[
I_{\text{after transform}} = \begin{cases} 
I_{\text{initial}}, & \text{if } I_{\text{initial}} \geq \text{border} \\
0, & \text{if } I_{\text{initial}} < \text{border} 
\end{cases}
\]

where \(I_{\text{initial}}\) is the initial intensity of the marker from the cytometry data file, \(\text{border}\) is the noise threshold for the intensity of the marker, and \(k\) is the coefficient of noise reduction. The coefficient of noise reduction was not a constant; rather, it linearly increased from 1 at the selected noise threshold to its maximum value (defined as 20) at the minimum intensity of the marker.

(1) Population selection by two-dimensional plots yielded pairwise projections of data distribution histograms and the distribution density of events represented by color (the same as done with classical gating process). The boundary between the positive and negative population was manually chosen at the visually observed local minimum point of the marker distribution. Kernel density estimate plots were used above the density plots to simplify the visualization of the local minimum of the distribution.

These manual data labeling steps yielded the cell population labels for every event in the fcs file.

Manual analysis of CyTOF data

CyTOF data from blood samples for Prince phase II trial underwent logarithmic transformation and were manually analyzed using the consecutive 2D plot gating strategy described in Figure S6 in Padrón et al. \cite{5} The list of populations and their marker composition are shown in Supplementary Table S2.

Cell-typing model training for flow cytometry analysis platform

Manually labeled samples were used to train the LightGBM decision tree boosting ML models (Figure S2A; Table S3, with default parameters \cite{6,7} https://lightgbm.readthedocs.io/en/latest/Parameters.html). These cell-typing models were trained to predict labels for each cytometry event. Approximately 200–300 labeled FCS samples were utilized for the models for each cytometry panel. Forward scatter, side scatter, and compensated fluorescence channel signal values were used as input, along with those normalized on maximum value and those normalized on different quantiles selected for each panel (Figure S2A; Table S3). A voting model was trained for each panel. The base model for voting was composed from two types of submodels, each represented by a LightGBM decision tree boosting classifier (LightGBM, v 3.3.2, \cite{8} https://github.com/microsoft/LightGBM). The first type predicted “top-level” populations such as leukocytes in the general panel and CD8+ T cells in the CD8+ T cells panel. The second type classified the target population into subtypes (Figure S2A). Model performance was checked using validation sample sets (~30 samples for each panel) that were not used in model training. Predictions were generated for each validation sample. These predictions were then compared to the manual labels of these samples based on the F1-score and p4-score metrics (see Table S3 for average F1-scores and P4-scores for each panel among all populations used in the article).

Quality control of predicted labels

All predicted labels, generated by cell-typing models, underwent a manual quality control procedure. The quality of predicted labels was assessed using a panel-specific set of two-dimensional plots with the intensity of one marker against the intensity of another marker. Key populations for the panel were plotted in distinct colors on these plots to check the accuracy of population selection and the accuracy of separation of the populations from one another. In cases of errors in the predicted label of the file, the gating of this population was corrected manually.
HNSCC-Nivo cohort data processing
All markers for all panels underwent thorough analysis of their performance after cryorecovery. A list of markers, including CD62L, CXCR3, CXCR5, CCR6, and CD161, showed significantly decreased intensity (decrease in median of 95th percentile intensity of more than 1.5 times with p-value less than 0.05) in comparison with internal cryopreserved PBMC and WBC cohorts. Thus all cytometry populations defined by those markers were excluded from further analysis. Due to the instability of the monocyte fraction in flow cytometry samples after cryorecovery (Pearson correlation less than 0.6 between different panels), monocyte fractions were counted using RNA-seq deconvolution data by Kassandra. Fractions of other cells were adjusted by multiplying by the ratio between their total fraction in cell deconvolution and cytometry.

Determination of cell percentages
To calculate the final population percentages from the labeled data, the results from different cytometry panels were combined via the general lineage backbone panel. The cell count values in corresponding populations from other panels were multiplied by normalization coefficients to match results from the general panel. The normalization coefficient was obtained by dividing the number of cells in the reference population in the linear panel by the number of cells in the reference population in the other panels (Monocytes for monocytes panel, T cells for CD4+ T cells panel, etc.). Table S2 contains the list of reference populations used to combine results from different panels in order to calculate cell percentages for subpopulations. After this procedure, the percentage of leukocytes for each cell population was calculated. The final percentages were obtained by multiplying the percentage of each cell type by the corresponding normalization coefficient calculated using the ratio of three major reference populations (monocytes, lymphocytes, and granulocytes) within the data generated using the hematology analyzer. As such, we used a dual-platform system to acquire concentrations of cell populations in blood. As for CyTOF data, after labeling, the final results were calculated as percentages of cell populations out of total PBMC fraction.

Bulk RNA-seq quality metrics and data processing
The quality of raw FASTQ files was analyzed using FastQC (version 0.11.9), FastQ Screen (0.11.1), and MultiQC (version 1.14, https://github.com/ewels/MultiQC/releases). The reference genomes used for the creation of BWA aligner indices (for FastQ Screen) included information from Homo sapiens (GRCh38), Mus musculus, Danio rerio, Drosophila melanogaster, Caenorhabditis elegans, Saccharomyces cerevisiae, Arabidopsis thaliana, Mycoplasma arginini, Escherichia virus phiX174, microbiome downloaded from NIH Human Microbiome Project website, adapters provided with FastQ v0.11.9, and UniVec (NCBI). All open-source blood RNA-seq type datasets also went through the same quality metric procedure.

Bulk RNA-seq fastq files were processed by Kallisto (version 0.46). The Kallisto index file was downloaded from the Xena project. Bulk RNA-seq processing was performed as previously described in Zaitsev et al. (2022). Calculated expression results were presented in the TPM (transcripts per million) normalization method. All open-source blood RNA-seq datasets obtained from GEO or ArrayExpress were processed the same way as internal RNA-seq data.

Cell deconvolution with Kassandra
Kassandra is a cell deconvolution algorithm used for the digital reconstruction of the cellular composition of samples from gene expression data. Kassandra algorithm is a decision tree ML technique trained on artificial mixes made from a broad collection of 9,414 tissue and blood sorted cell RNA-seq samples. Here, we used Kassandra to estimate cell type fractions in the paired bulk RNA-seq data of our internal cohort samples and the open-source transcriptomes.

Open-source RNA NGS data
More than 20,000 bulk whole blood RNA-seq samples across several diagnoses were gathered from the GEO and ArrayExpress databases (Table S7). The raw RNA-seq datasets were combined, homogeneously annotated, and bioinformatically recalculated for comparable measurements of transcript expression within each cell type to reduce batch effects. Purified RNA-seq of blood samples from healthy donors and patients with over 90 different diagnoses were grouped based on common diagnostic features (Table S7). After extensive quality control, cell population percentages were extracted from each dataset using Kassandra. Samples with neutrophil percentages below 20% were labeled as PBMC and removed from WBC collection. After these QC steps, 17,800 samples were left for subsequent analysis.

RNA-seq data from peripheral blood of the triple negative breast cancer patient cohort were obtained from open-source dataset GSE201085. Only samples from patients that underwent NAC were selected for the analysis.

T cell receptor/B cell receptor analysis
Extraction of data for TCR or BCR clonotypes from raw FASTQ files was executed with MiXCR (version 3.0.12) with default parameters for bulk RNA-seq extraction. For each clonotype, subsequent fractions were recalculated by chain summarized numbers. Additional clonotype groups that corresponded to similar (differed by 1 amino acid) CDR3 sequence in clonotypes were identified for BCR. The diversity of TCR and BCR was estimated by calculating the Chao1 index:

\[
\text{Chao1} = N + \frac{N(N-1)}{2N_2}
\]
where \( N \) is the number of observed clonotypes, \( N_1 \) is the number of singletons and \( N_2 \) is the number of doubletons. The clonality index\(^95\) was estimated as:

\[
\text{Clonality} = 1 + \sum_{i=1}^{N} p_i \log_2 p_i.
\]

where \( N \) is the number of observed clonotypes and \( p_i \) is the frequency of clonotypes. The estimation was performed using a down-sampled repertoire of 100 clonotypes in order to exclude coverage bias in 10 replicates. An average value was used for final analysis.

CDR3 specificity of TCR was analyzed using a comprehensive internal database consisting of data from VDJdb,\(^96\) McPAS,\(^97\) and TBAdb from PIRD,\(^98\) as well as data for identified CDR3 sequences in different experiments from manually annotated articles. HLA alleles of MHC I class were extracted by OptiType (v 1.0\(^99\)). These data were used to analyze allele distributions.

### Healthy/cancer classifier and ROC-AUC calculation

Robust cell populations that were differentially represented between the peripheral blood samples from healthy donors and patients with solid tumors were selected to train a ML-based classifier. The transformer-based TabPFN model\(^{29}\) (https://arxiv.org/abs/2207.01848), Python package version 0.1.8\(^{100}\) (https://github.com/automl/TabPFN) was used for the cancer/healthy classification of our samples. The most significant number of features (cell populations) distinguishing between healthy donors and cancer patients were identified using the MRMR algorithm (minimum-redundancy-maximum-relevance\(^{101,102}\), Python package version 0.2.5\(^{103}\) (https://github.com/smazzanti/mrmr) in a stepwise leave-one-out cross approach, by testing for numbers ranging from 10 to 100 features in increments of 10 (Figure S3B). This approach revealed 20 features that yielded the best model performance (highest ROC-AUC value) between these two groups (Figure 2C; Table S5). The UMAP approach\(^{104}\) (https://arxiv.org/abs/1802.03426), Python package 0.5.3\(^{105}\) (https://github.com/mcinnes/umap) was used to ensure data similarity between the training (\( n = 503 \)) and validation sets (\( n = 347 \) samples of the internal cohort). This process allowed us to visualize both sets and compare them to detect any discrepancies. The verification of data similarity allowed us to accurately assess the quality of the trained model using the features sampled from the training set for the validation set.

AUC values of ROC curves were calculated using the function “roc_curve” from the sklearn.metrics package. The predicted value was used as “scores” and healthy/cancer labels were taken as values “0” and “1”. The same was done for response and OS evaluation. “fpr” and “tpr” were used for visualization using the plot function from the matplotlib.pyplot package.\(^{106}\)

### Clusterization algorithm

Flow cytometry data were represented as cell percentages (from total number of WBCs for granulocyte populations and from total number of PBMCs for all other populations, Table S6). A total of 30 major cell populations lying on the same hierarchical tree level as in the deconvolution output by Kassandra were selected for cluster analysis with an additional four manually selected ICB-relevant cell populations based on previously published reports: TIGIT\(^{+}\) PD-1\(^{+}\) CD8\(^{+}\) T cells,\(^30\) Vi\(\delta\)2\(^{+}\) γ-δ T cells,\(^31\) CD39\(^{+}\) Tregs\(^32\), HLA-DR\(^{low}\) monocytes.\(^{14,33–35}\) Prior to clusterization, the data were rescaled just as for min-max normalization, but with the 2\(^{nd}\) and 98\(^{th}\) percentiles instead of minimum and maximum values, respectively. All values outside the 0–1 range were clipped to the closest value. The formula for normalization is the following:

\[
\text{scaled value } V_x = \frac{P_x - P_{q2}}{P_{q98} - P_{q2}}
\]

Unsupervised spectral clustering (scikit-learn version 1.1.2,\(^{107–109}\) https://scikit-learn.org/stable/modules/generated/sklearn.cluster.SpectralClustering.html) was applied to the normalized frequencies of the 34 selected cell types for clusterization of the flow cytometry data.

Kassandra cell deconvolution analysis was used to calculate percentages of an open-source RNA-seq collection of 17,800 samples. A total of 25 immune cell population percentages (Table S7) predicted by Kassandra were normalized to the PBMC fraction and selected as input features. Subsequently, the data were rescaled using min-max normalization and clipped in the same manner as described in the clustering method (see clusterization algorithm Method). The same unsupervised spectral clustering method, as described above, was applied for the clusterization.

### Cluster number evaluation

To determine the optimal number of clusters and cluster decomposition that would produce the most distinct immunotypes, we tested various numbers of clusters from 2 to 14 (Table S6). For each number of clusters in those decompositions, we calculated the number of statistically differentially distributed (\( p \)-value <0.05) cell populations (out of 34 selected). Mann-Whitney U test with Bonferroni correction was applied to obtain \( p \)-values for all comparisons. For each number of clusters, we calculated the median values of statistically differentially distributed cell populations between all these paired combinations. Median numbers of features that significantly distinguished each cluster pair for the decompositions for each tested number of clusters (from 2 to 14) are presented in Table S6. The median number of features was the highest for four and five clusters. Decomposition with five clusters was chosen because it covered greater diversity of the data and still produced significantly different cluster groups.
Differential gene expression and gene set enrichment analysis

Differential expression analysis was conducted using the edgeR tool\textsuperscript{110} (https://bioconductor.org/packages/release/bioc/html/edgeR.html). Heat shock genes and sex genes were excluded from the analysis.

GSEA analysis\textsuperscript{111} was performed on an unfiltered list of 200 genes, ranked in descending order of differential expression test statistics. The Compute Overlaps tool\textsuperscript{112} (https://www.gsea-msigdb.org/gsea/msigdb/help_annotations.jsp#overlap) was used to compare our gene sets with the H gene set (hallmark gene sets) and the CP gene set (canonical pathways) from the MSigDB collection. For each cluster, 20 gene sets from the collection that overlapped the most with our gene set were chosen. Out of this list, immunity-related signatures were selected for this analysis. Signature score values were calculated using ssGSEA, normalized by median scaling, and shown as a heatmap with the range clipped at \((-2, 2)\). The ssGSEA score of PD-1-related signatures for patients on PD-1 therapy were also calculated (Figure 4G). The full list of GSEA gene sets used is presented in Table S6.

Pseudotime analysis

Pseudotime analysis was performed using Monocle.\textsuperscript{113} Monocle is an unsupervised algorithm initially developed for use on single-cell RNA-seq data to analyze the cell fate decisions based on gene expression data. Here, it was used to analyze the connection between different blood samples using cell percentages obtained from flow cytometry data analysis.

Immunotype signature scores

Immunotype cluster-based signature score is a linear metric that separates all samples belonging to a given cluster from all other samples in a multidimensional space of percentages of cells. For each immunotype cluster, there was a separate signature score based on a linear regression model. Before the implementation of this approach, the cytometry data were rescaled for min-max normalization and clipped the same way as it was done for the clustering method (see clusterization algorithm method). Then, Elastic Net regression\textsuperscript{114} (https://scikit-learn.org/stable/modules/generated/sklearn.linear_model.ElasticNet.html) was used to identify coefficients that linearly transformed cell population percentages to a number from 0 to 1 (separating samples from the chosen cluster and other clusters). Features were normalized percentages of cell populations, where 1 was assigned to samples from the chosen cluster and 0 to samples from other clusters. Model parameters alpha and l1_ratio were selected by grid search. The score for grid search was cross-validated by AUC. Cross-validation was performed with StratifiedShuffleSplit(n_splits = 5, test_size = 0.3). After the model was trained, predictions for all cohort samples were considered in the calculation of 0.01 and 0.99 quantiles of cohort predictions for normalization (q01 and q99, respectively). The final score was calculated as:

\[
\begin{align*}
\mathbf{a} &= 9.9 + \frac{(\text{clip} - q01)}{(q99 - q01)} + 0.1 \\
\text{with all predictions being clipped at those quantile values. Therefore, the presented signature score always lies between 0.1 and 10.}
\end{align*}
\]

Deconvolution-based signature scores were trained on quantile-normalized deconvolution fractions of the internal cohort with output being assigned as ISSs calculated on flow cytometry data. Then, the same Elastic Net approach and final score calculation were applied.

These signature scores were calculated for the internal cohort and for all external clinical cohorts that were used to demonstrate clinical utility (HNSCC-Nivo, HNSCC-Durva, PDAC and breast cancer cohorts). The models used for each cohort were trained at the intersection of populations measured for the internal cohort with populations that could be measured for each external cohort.

Immunotype assignment for external datasets

For external datasets (HNSCC-Nivo, HNSCC-Durva, PDAC, breast cancer), cluster assignment was performed using the scores of the corresponding signatures. The signature values underwent a quantile normalization procedure using a subset of cancer patients from the internal cohort. Quantile normalization was performed by aligning the signature values to the 10\textsuperscript{th} and 90\textsuperscript{th} percentiles with the following formula:

\[
G_{\text{new}} = G_{\text{dataset}} \ast a + b
\]

\[
\begin{align*}
\mathbf{a} &= \frac{(q90_{\text{cohort}} - q10_{\text{cohort}})}{(q90_{\text{dataset}} - q10_{\text{dataset}})} \\
\mathbf{b} &= \frac{(q10_{\text{cohort}} * q90_{\text{dataset}} - q90_{\text{cohort}} * q10_{\text{dataset}})}{(q90_{\text{dataset}} - q10_{\text{dataset}})}
\end{align*}
\]

Cohort: values for the internal cohort of cancer patients; Dataset: values calculated within a particular external dataset. The cluster label was assigned according to the maximum value from the five immunotype signatures after normalization.
Assessment of immunotherapy biomarkers tumor mutational burden and PD-L1
The evaluation of PD-L1 was taken as the expression of CD274 in TPM as described in Zaitsev et al. (2022). Somatic mutation calling was performed using Strelka2 v2.9. TMB was calculated based on protein-affecting mutations as the number of somatic mutations divided by megabases called out within the coverage range. Tumor content was determined using variant allelic frequency of somatic mutations. Only samples with tumor content higher than 20% were included in TMB analysis (Figure 7N).

Assessment of blood biomarkers
To compare the potential prognostic value of our findings with that of known blood biomarkers, we used available cohorts for published cytometry-based biomarkers as the benchmark. Most published blood-based biomarkers specific to certain disease types, therapies, or blood draw timepoints for the chosen cohorts were observed using a hematology analyzer. From these biomarkers, we chose to use CD4+ T cells and PD-1+ CD8+ T cells with our panels to evaluate anti-PD-1 therapy response in the HNSCC cohort (Figure 7N).

QUANTIFICATION AND STATISTICAL ANALYSIS
Fisher’s Exact test on a 5 × 2 data were calculated with R. Other statistical analyses were performed using the scipy.stats module in Python 3. Sankey plots were created using ggalluvial and ggplot2 packages in R, while other graphs were plotted using custom implementation of matplotlib and seaborn libraries of Python 3. The workflow diagrams were drawn on the website www.draw.io.