Immune Cell Gene Signatures for Profiling the Microenvironment of Solid Tumors

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Abstract

The immune composition of the tumor microenvironment regulates processes including angiogenesis, metastasis, and the response to drugs or immunotherapy. To facilitate the characterization of the immune component of tumors from transcriptomics data, a number of immune cell transcriptome signatures have been reported that are made up of lists of marker genes indicative of the presence of a given immune cell population. The majority of these gene signatures have been defined through analysis of isolated blood cells. However, blood cells do not reflect the differentiation or activation state of similar cells within tissues, including tumors, and consequently markers derived from blood cells do not necessarily transfer well to tissues. To address this issue, we generated a set of immune gene signatures derived directly from tissue transcriptomics data using a network-based deconvolution approach. We define markers for seven immune cell types, collectively named ImSig, and demonstrate how these markers can be used for the quantitative estimation of the immune cell content of tumor and nontumor tissue samples. The utility of ImSig is demonstrated through the stratification of melanoma patients into subgroups of prognostic significance and the identification of immune cells with the use of single-cell RNA-sequencing data derived from tumors. Use of ImSig is facilitated by an R package (imsig). Cancer Immunol Res; 6(11); 1388–400. ©2018 AACR.

Introduction

Modulating the activity of the immune component of the tumor microenvironment holds potential in the treatment of cancer. Checkpoint inhibitors, particularly anti-PD1 and CTLA4, have advanced therapeutic options in the past decade producing benefit for some patients (1). However, multiple factors within the tumor microenvironment, including the immune infiltrate prior to treatment (2), influence the response to immunotherapy. IHC and flow cytometry are often used to study the immune status of tumors. However, the former analyses are limited to small areas of tissue and a few markers, and the latter requires tissue disaggregation, which may not always be practical. To overcome these limitations, computational methods have been developed to estimate the immune content of blood and tissue samples from transcriptomic data (3). Two approaches can be used to infer the relative proportion of cell types from transcriptomic data: (i) fitting reference gene-expression profiles from sorted cells to the data in question (4–7) and (ii) following cell type–specific genes to indicate the presence of certain cell populations (8–11). Both approaches rely on sets of gene markers (gene signatures); however, in the first case, the gene signature is not necessarily cell type–specific, and supervised learning algorithms are needed to distinguish between cell types.

A number of computational frameworks leveraging these approaches have been described to estimate the contribution of different immune cell types to the tissue transcriptome (5, 10–14). Across these studies, the range of immune cell types that each method detects varies. For instance, collectively, the published studies report gene signatures for 22 different T-cell subtypes, but with many “marker genes” expressed by nonimmune cell types and others used interchangeably to define different T-cell subtypes. Another shortfall is that these signatures are based on gene-expression data gathered from primary blood-derived cells generally collected from healthy donors. When the expression profiles of the same immune cell either from blood (peripheral blood mononuclear cell) or from tissue can differ (15), the predictive value of signatures is compromised (16).

Genes that contribute to a common biological process or define a given cell type are frequently coregulated and coexpressed, giving rise to expression modules (17, 18). We have previously validated gene correlation network (GCN) analysis of gene-expression data sets from human (including human cancers), mouse, pig, and sheep, as a means to define such expression modules (19–21). Here, we have analyzed human tissue transcriptomic data to identify coexpressed marker genes representing seven immune cell types and three cellular pathways present in data from many tissues. We have named this set of signatures ImSig. We demonstrate the advantages of ImSig over other reported signatures derived from the...
comparison of isolated blood cells and characterize ImSig’s utility for analyzing the immune microenvironment of tumors.

Materials and Methods

Derivation of ImSig

Eight publicly available expression data sets derived from human tissue were extracted from the Gene-Expression Omnibus (GEO) database (ref. 22; GSE11318, GSE50614, GSE75214, GSE38832, GSE23705, GSE43833, GSE58812, and GSE65904). Prerequisites were that the unprocessed data files were available, the data set included a variety of normal and diseased samples, represented a variety of array platforms, and contained >20 samples (median size, 114 samples). The data sets were chosen to include the variety of immune cell types and differentiation states. Data sets were subjected to standard processing (i.e., conversion of raw platform-specific files into expression matrix and normalization) with the help of R packages such as “ oligo” (23) and “ limma” (24) for Affymetrix and Illumina data, respectively. The signal intensities were normalized using the robust multiarray average. The expression values for genes with multiple probes were reduced to one probe per gene by choosing the probe with maximum intensity across samples.

The resultant expression matrix was loaded into the network analysis tool Graphia Professional (Kajeka Ltd.), previously known as BioLayout Express3D (25, 26). Within the tool, a Pearson correlation matrix was generated, i.e., an all versus all comparison of expression profiles with genes exhibiting a similar expression pattern across the samples scoring highly (with a maximum correlation value equal to 1). A GCN was then generated using a correlation threshold value so as to include approximately 10,000 genes in the analysis for each data set. In the context of a GCN, nodes represent genes/transcripts and edges, correlations above the threshold. The optimal correlation threshold is data set–specific, as generally smaller data sets exhibit a higher overall correlation and all threshold values used also minimized chance associations. The GCN for each data set was then clustered using the Markov clustering (MCL) algorithm (27), an algorithm analyzes a graph’s structure to define gene clusters of nodes, in this case coexpression modules. Clusters were manually annotated based on domain knowledge with the help of Gene Ontology (GO) and Reactome pathway enrichment analyses (28, 29). Gene modules representing immune cell types and biological processes were identified for each of the eight data sets. The genes within the modules were consolidated into a list of genes for seven immune cell types and three biological processes. In order to identify the core set of genes that represents each cell type or process, these genes were further refined using eight independent validation data sets (GSE9891, GSE14580, GSE38832, GSE14951, GSE15773, GSE7305, GSE22619, and GSE52171) by the following procedure: Robust signatures were identified by excluding genes that were poorly coexpressed using an unbiased approach. Each data set was loaded into Graphia (r values were again selected so as to include approximately 10,000 genes in the analysis) and clustered using the MCL algorithm. To model the contribution of noise by random genes within signatures, 0 to 100% of genes within every MCL cluster were replaced with random genes (using the R function “sample”) in a stepwise manner, in 2% increments. For each of these replacements, the resultant median correlation of every cluster was noted. The combined data points were fitted to a sigmoidal curve using the nonlinear least squares method. On the basis of this model, we estimated the number of genes that might contribute to noise within the signatures and should be filtered out. To facilitate such estimation, the R package “ investr” was used. For example, based on the median correlation of signature genes, if the model suggested 30% of genes represented noise, then 30% of genes exhibiting the poorest median correlation were discarded. This process was repeated for each signature across the eight validation data sets. The set of genes that survived the filtration process were defined as ImSig. Our approach sought to identify the genes most correlated across data sets to arrive at the final list of genes for the individual ImSig signatures. TopGo was used to identify the five most enriched GO Biological Process (GO_BP) terms associated with each gene set (28), and P values were generated using the Fisher exact test.

Comparison of ImSig with other published signatures

Seven published immune signatures were taken from the literature (5, 8, 10–14). To visualize the concordance between the immune genes defined by the different studies, a chord diagram was built using the circlize package (30) in R. We used only genes reported as markers of immune cells, and signatures of nonimmune cells such as fibroblast or endothelial cells were omitted from this analysis. Due to the great variety of T cell–subtype signatures reported, these were further explored to identify how genes were used to define the different subtypes. Genes that were present in two or more studies and ascribed to a T cell or one of its subtypes were identified. Using these genes, a graph was constructed using Cytoscape (31) and visualized with a circular layout. The size of nodes representing individual signatures was adjusted according to the number of connections each signature had with others. A Jaccard similarity index was also calculated between all signatures. The LM22 signature (5) did not provide an absolute signature, that is, the same genes may represent multiple cell types and only a subset of genes that were unique to cell types were used for our analysis. For visualization of the results, genes pertaining to cell subsets [regulatory T cell (Treg) and Th1] were pooled to represent the parent population (T cells) and the Jaccard similarity index was recalculated.

Comparative analysis of gene signatures in the context of a tissue data set

The median correlation of the signature genes from the same seven published immune signatures (5, 8, 10–14) was calculated within the context of a trachoma data set (GSE20436; ref. 32). The transcriptomics data set was generated from swabs taken from the eyes of children with symptoms of trachoma or controls and contained samples from three patient subgroups: 20 controls with normal conjunctivas; 20 individuals with clinical signs of trachoma but that tested negative for the bacteria C. trachomatis (these patients may have been in the resolution stage), and 20 individuals with symptomatic disease. This data set was chosen due to the immune cell infiltration associated with this disease. The presence of all immune cell populations was confirmed by ImSig. To facilitate comparison with ImSig, genes pertaining to cell subsets were pooled to represent the parent cell population. In addition, median correlations of nonpooled signatures (i.e., marker sets representing subpopulations of cells) were also analyzed.
To assess the ability of ImSig to define known clinical differences between patient subgroups and to illustrate the exploratory power of network-based analysis, we used the trachoma data set described above. In order to estimate the relative abundance of immune cells across patient groups, the average expression of the ImSig signature genes was computed. A two-tailed, unequal variance t test was conducted between groups to obtain P values. To explore the immune environment and extrapolate immune cell subsets, a GCN (r > 0.7) was visualized in Graphia. By visual inspection of the network graph, immunologically relevant genes (subtype/differentiation-specific) were identified in the vicinity of the ImSig modules, and their average expression profile across patient groups was plotted.

To validate ImSig in the context of tumor-derived samples, transcriptomic data from single-cell suspensions from lymph nodes of four patients with metastatic melanoma were analyzed (GSE93722). Here, the relative proportion of immune cells, CD4+ T cells, CD8+ T cells, B cells, natural killer (NK) cells, had been measured with flow cytometry. To facilitate direct comparison, proportions of CD4+ and CD8+ T cells were summed to estimate total T-cell content. The average expression of ImSig genes was calculated to determine the relative abundance of immune cells in each patient. Predicted and observed abundances were normalized between 0 and 1 to facilitate comparisons. This analysis also served to validate the applicability of ImSig to RNA-seq data.

Pan-cancer analysis of tumor data (TCGA)

Purified (level 3 data: the calculated expression signal of a gene per sample) transcriptomic data from 12 cancers were downloaded from The Cancer Genome Atlas (TCGA) database. For each cancer type, the patients were ordered based on the average expression of the individual ImSig signatures and split into two groups based on the median expression value of the signature genes. In cases such as brain lower grade glioma (LGG), kidney renal clear cell carcinoma (KIRC), and uterine corpus endometrial carcinoma (UCEC), B-cell signature genes were not coexpressed, indicating the absence or low abundance of these cells, and so were not included in the survival analysis. A univariate Cox-proportional hazard ratio (HR) analysis was performed for each cancer type, the patients were ordered based on the median expression value of the signature genes, and a score of >0.75 (upper quartile) were set as defined prediction, that is, a T-cell score of >0.75 in a T cell with a P value of <0.05 was judged as a correct prediction.

R implementation and availability of ImSig

We implemented ImSig as an R package called “imsig.” Users should call the “imsig” function, which takes a normalized gene-expression matrix made up of HUGO symbols in rows and samples in columns as its first argument, and a correlation threshold (r) as its second argument. Users can also generate a network graphic of ImSig genes and perform survival analysis using the package. A tutorial is available at https://github.com/ajitjohnson/imsig.

This package is available at CRAN (https://cran.r-project.org/web/packages/imsig/).

Results

Derivation of ImSig

Using a network-based approach, we identified a set of coexpressed gene modules associated with human tissue immune cell populations and frequently observed biological processes, from eight independent tissue transcriptomics data sets. An illustrative example of a GCN is shown in Fig. 1A. These initial
gene signatures were refined and validated by testing for coexpression of the genes associated with each signature across an additional eight independent data sets (Fig. 1B). The result was 569 marker genes representative of seven immune populations [B cells (37 genes), plasma cells (14 genes), monocytes (37 genes), macrophages (78 genes), neutrophils (47 genes), NK cells (20 genes), T cells (85 genes)] and three biological processes [Interferon response (66 genes), translation (86 genes), proliferation (99 genes)]. We named this set of genes collectively ImSig (Tables 1 and 2; Supplementary Table S1). The data-driven definition of each immune signature is internally validated by association of known markers with the specific gene signatures, e.g., CD3D and CD3E (T cells), CD19, CD22, and CD79 (B cells), CD14 (monocytes), CD68 and CD163 (macrophages), KIR family (NK cells) and immunoglobulin family members (plasma cells). Furthermore, GO enrichment analysis of the gene signatures and data from the published literature supported the association of markers with relevant cell types and processes. The top five significant enrichment terms for all signatures are listed in Supplementary Table S2 and the top significant term is given in Fig. 1C. Unlike other published immune signatures, our gene signatures do not distinguish immune cell subtypes, such as subpopulations of T cells or activation states of macrophages. We found no support for distinct modules of coexpressed markers describing T cell or macrophage subpopulations. Indeed, analysis of isolated human macrophages responding to different stimuli did not support the existence of distinct activation states of macrophages but rather indicated a continuum of states depending on the stimulus (38). Where present, “activation-specific” transcripts, such as receptors, cytokines, or transcription factors, tend to form part of the overall cell expression module. By inference, coexpression of a gene with a particular cell type–specific signature in a particular data set indicated that the gene is likely expressed by those cells or at least a subpopulation of them.

Comparison between ImSig and published immune signatures

The gene content of seven published immune signatures, all derived from comparisons of isolated blood cells (5, 8, 10–14), was compiled and compared. We excluded signatures for non-immune cell types, e.g., endothelial cells, fibroblasts etc. When ImSig was added to the list, the list contained a total of 3,658 genes (Supplementary Table S3). To compare these gene signatures, we calculated a Jaccard similarity index
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Figure 2.
Comparison of ImSig with other published signatures. A, Chord diagram showing the overlap between marker genes across studies. In most studies, a large proportion of genes were unique to the signatures defined by them. ImSig showed the best overlap (81%) with other studies. B, Network diagram showing the relationship between T-cell subtype–specific genes among six studies and ImSig. Only genes that were present in two or more studies are represented in this plot (98 genes representing 13.4% of all T-cell marker genes). Nodes are sized relative to the number of shared genes between one signature and others. ImSig included genes describing various subtypes and was the most conserved set among all studies compared. C, Heat map of the median correlation between genes from published signatures as assessed in the context of the trachoma data set (GSE20436). Where a cell-type signature was split into subsets, subset signatures were combined to represent the parent population. The median correlation values of signatures without combining them into parent population is available (Supplementary Table S5). D, Bar plots of the average expression of signature genes (estimated relative abundance) across the data set, each bar representing the average expression of signature genes in an individual patient sample. Samples are ordered according to T-cell content, low to high (left to right), and this order is maintained for other plots. ***, P = 0.01; ***, P = 0.001.
subsumed into one module, such as T cells. Their median correlation in the context of the trachoma data set is shown in Fig. 2C. A noncollated version of the results is provided in Supplementary Table S5. Regardless of whether data were aggregated by broad cell type, or by subtype, none of the blood-derived modules were strongly coexpressed across the set of trachoma patient samples. In contrast, all of the ImSig signatures displayed a high median correlation (coexpression) value. The gene signatures from ref. 8 performed next best. The bacterial infection that gives rise to the pathology of trachoma leads to recruitment of immune cells to the site of infection (32). In order to evaluate the ability of ImSig to estimate the relative abundance of immune cells, the average expression of each gene signature was used as a proxy for immune cell number in the trachoma data set. All immune cell populations increased in patient groups relative to controls, with greater increases seen in patients with an active infection (Fig. 2D).

To validate the applicability of ImSig on RNA-seq data and in the context of tumor biology, we computed the relative abundance of immune cells in four metastatic melanoma patients from whom samples were collected from lymph nodes. A fraction of the single-cell suspension was used to measure cell-type proportions by flow cytometry and the other fraction was used for RNA-seq analysis. We observed good agreement (r = 0.91, RMSE = 0.1, and P = 2.74E−05) between predictions of relative cell number made using ImSig and experimentally determined cell numbers (see also Supplementary Fig. S2). Thus, ImSig accurately predicted relative cell numbers for all cell types, as confirmed by the low root-mean-square error (RMSE).

Deconvolution of tissue data
In the context of GCN analyses, the ImSig signatures can be used to identify other context-specific genes expressed by immune populations. For example, the T cell and macrophage signatures were correlated with each other, consistent with an immune-mediated inflammatory process, and many immune-related genes were coexpressed with ImSig genes in the context of the trachoma data (Fig. 3A). The expression profile of genes such as IFNG, LAG3, CD44, FOX3, FOXP3, CD80, IL20, STAT4, and IL17A was correlated with T-cell signature genes, indicating that the T-cell population included Th17, Treg, and Th1 subtypes (Fig. 3B). Similarly, genes associated with the macrophage signature contained many M1 markers. Performing a network analysis such as this can also provide a broader perspective of the transcriptional signatures of other cell types present in clinical samples. When the data set is examined as a whole, many GCN clusters can be assigned to other cell populations or processes (41).

Satisfied with the performance of ImSig in the context of tissue transcriptomics data, we explored its utility in the analysis of transcriptomics data derived from cancer.

Analysis of immune infiltrates in cancer
Our previous analysis of the cancer transcriptome showed that expression signatures of immune cells can be extracted from large cancer transcriptomic data sets, but we did not at that time correlate gene-expression signatures with patient outcomes (20). To test the use of ImSig in the study of the tumor microenvironment, the 12 largest TCGA cancer data sets were examined and HRs were computed between high and low-immune cell infiltrate groups (Fig. 4A). Although the survival analysis was not adjusted for potentially confounding variables (such as tumor stage, grade, age, or treatment), the findings were consistent with the literature. In melanoma (SKCM), we reaffirmed the known association between tumor-infiltrating lymphocytes (TIL) and a good

Figure 3.
Coexpression of other immune genes with ImSig core signatures. A, Correlation network of genes associated with the immune clusters during trachomatis infection. ImSig genes are colored according to the different immune cell types they represent, whereas the genes coclustering with the ImSig immune genes are shown as nodes without color and reduced in size. Highlighted with a greater node size and label are a few well-known immune-modulatory genes present in the immediate vicinity of the signature genes. B, Bar plots of the average expression intensity of a few well-known immune-modulatory genes across the three patient groups.
prognosis (42, 43). Breast cancer (BRCA) is not as immunogenic as melanoma, but several studies have associated TILs with a good prognosis as observed here (44). A negative association between TILs and prognosis was evident in LGG (45, 46) and lung squamous cell carcinoma (LUSC; refs. 47, 48) in accordance with previous literature. We did find prognostic value of the interferon response in LGG. We confirmed an association between the proliferation signature and a good prognosis in colorectal cancers (COAD; as shown in ref. 49) and also in LUSC. Analysis of individual proliferation-related genes in LUSC supported this observation (log2HR: G2E3, 0.66; MND1, 0.56; CHEK2, 0.53; RFC4, 0.51; CEP192, 0.48; CDKN3, 0.47; CENPA, 0.47; CCND2, 0.47; CDC7, 0.46; P < 0.05). One possible explanation for this counterintuitive observation is that the mitotic signal in these tissues originates from proliferating immune cells, not from cancer cells (50, 51).

We performed a molecular subgrouping of melanoma based on ImSig, using the signature genes only to group patient samples. Unsupervised clustering based on the immune cell gene expression data was performed using the MCL algorithm. Here, every node is a patient, and the edges correspond to the correlation between them. C, Expression profile of ImSig-related genes within the various clusters/grouping as defined in B. Here, the y-axis is the average expression of the signature genes, and x-axis describes patient groupings as shown in B. D, Univariate Cox-proportional analysis between the patient groups as defined in B. 

Figure 4.
Application of ImSig to tumor data. A, Prognostic map of 12 cancer types based on immune cell content. The average expression of each ImSig signature was calculated for each sample/tumor type. Samples were then ordered according to each signature (low–high, black plot in each square), and the HR was calculated between the lowest and highest expressing samples. Blue represents a good prognosis with increased expression of the signature genes and red a poor prognosis. A, HR P < 0.05. BCLA-bladder urothelial carcinoma; BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; HNSC, head and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LGG, brain lower grade glioma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma. B, Sample–sample correlation graph of melanoma patient samples based on expression of ImSig genes in and clustered using MCL algorithm. Here, every node is a patient, and the edges correspond to the correlation between them. C, Expression profile of ImSig-related genes within the various clusters/grouping as defined in B. Here, the y-axis is the average expression of the signature genes, and x-axis describes patient groupings as shown in B. D, Univariate Cox-proportional analysis between the patient groups as defined in B.
profile revealed five groups of patient samples (Fig. 4B). Clinical features such as the tissue of origin and tumor type (metastatic or primary) did not affect the subtyping. Nearly half the patients were in cluster-1, characterized by little immune infiltrate (Fig. 4C). HR analysis between these low-immune (cluster 1) and high-immune infiltrate (clusters 2 and 3) tumors revealed a significant difference in patient survival (HR: 0.38, P = 3E−9). The median survival of patients in the group with high-immune infiltrate was 10 years greater than that of patients with low-immune infiltrate (Fig. 4D). Within the high-immune infiltration subgroup, cluster 2 appeared to have more B cells and plasma cells than cluster 3 (Fig. 4C), but overall survival (HR) was not significantly different between the two groups (Fig. 4D). Cluster-4 samples displayed higher expression of interferon response genes and also showed improved survival compared with the low-immune infiltrate group (Fig. 4D). Finally, patients in cluster 5 had a low-immune infiltrate, showed greater expression of keratin-related genes, and presented the worst survival rates (median survival = 2.34 years). Although patients in clusters 2, 3, and 4 did not differ in HR, they could differ in other ways, such as responses to treatment. Following an analogous analysis, we reproduced the five patient groupings on an independent validation data set (GSE65904), which showed a similar infiltration pattern (Supplementary Fig. S3A), survival analysis, and prognostic pattern (Supplementary Fig. S3B). High-immune and keratin subgroups have been identified and described in melanoma (52, 53) but these studies did not describe the type and variation in the immune infiltrate in melanomas. Our analysis reveals the nature of the immune landscape of these tumors and differences in their survival.

Use of ImSig in identifying immune cells in single-cell data

To extend these analyses and validate the ImSig signatures in the context of single-cell data, we examined single-cell data derived from melanoma (35). The immune component of the melanoma single-cell analysis included 515 B cells, 126 macrophages, 52 NK cells, and 2,069 T cells. Cell type-specific expression of ImSig markers was observed (P < 7E−15) as illustrated in Fig. 5A. For each patient, the estimated proportion of immune cells was compared with the true proportion. The estimated proportion was concordant with the measured number of cells (P < 0.05), with the poorest observed correlation being r = 0.97. Randomized permutation analysis with the same-sized gene sets produced no significant correlation (Fig. 5B). Figure 5C illustrates the concordance between the measured and estimated number of cells.

Figure 5.
Validation of ImSig using single-cell RNA-seq data from melanoma samples. A, The immune component of the melanoma single-cell data displayed as a correlation network, each node representing a cell from melanoma. Box plots display the average expression of cell type-specific ImSig genes in their respective cell types compared with the average expression of other ImSig genes. Process-specific ImSig signature genes (proliferation, interferon, and translation) were omitted in this analysis. B, Linear regression plots showing the concordance between the estimated and measured abundance of immune cells in 10 patients. For five patients (P1, P3, P5, P7, and P9), the regression line was also calculated using a random set of genes to highlight the specificity of ImSig genes. C, Stacked bar plots showing the concordance between measured and estimated proportions of immune cells. ***. P = 0.001.
The single-cell community depends on gene markers, gene signatures, and clustering algorithms to define cell types. Here, we show the utility of ImSig when used in association with classification algorithms, such as support vector machine (SVM), to predict cell types from single-cell RNA-seq data. To demonstrate the potential for automation, we used the SVM-based deconvolution tool Cibersort (5) with a reference profile generated with ImSig to predict immune cells within a single-cell data set from head and neck tumors (HNSCC; ref. 36). The immune component of the HNSCC data set contained 1,473 cells. Prediction using ImSig yielded a high degree of accuracy for B cells (88.4% correct prediction), macrophages (98.8%), and T cells (99.8%; Table 3). Only 63 immune cells remained unclassified (\( P < 0.05 \)). With respect to the other 4,087 cells, which consisted of myocytes, mast cells, malignant cells, fibroblast, dendritic cells, and endothelial cells, only 2.2% of cells were misclassified as macrophages, B cells, or T cells. In contrast, Cibersort’s (5) default blood-derived signature (LM22) showed an accuracy rate for B cells of 15.2%; macrophages, 0%; and T cells, 75.3%. However, the LM22 signature was not designed to deconvolute single-cell data, and its poor performance is likely a result of using a blood-derived signature and a reference gene matrix based on microarrays.

### Discussion

Cellular heterogeneity is a hallmark of cancer, in terms of both the tumors themselves and the normal cells that both support and control their growth. A wealth of transcriptomics data has been generated from cancer samples and a number of studies report approaches to deconvolute these data and to define the set of cell types present therein. However, we and others (16) found that immune signatures derived by comparing the expression profile of immune cells isolated from blood do not perform optimally when applied to tissue data.

The current work is based on the observation that genes associated with a specific cell population or biological process form highly connected cliques of nodes when large collections of transcriptomics data are subjected to network-based correlation analysis (18, 41). Although the main goal of this study was to define immune gene signatures for the deconvolution of cancer data, we have derived ImSig from a range of tissue pathologies and stages, and pathologies. Although heterogeneity among immune cell populations exists, few markers can identify this heterogeneity outside of the context of flow cytometry and IHC. For instance, tissue macrophages are sometimes named depending on their tissue of origin (microglia, Kupffer cells, etc.) or activation state (M1, M2, etc.) and in other cases are referred to as dendritic cells (53, 54). In the literature we have cited, signatures for 22 T-cell subsets are reported, and this does not include all T-cell subsets that are defined in the literature (55). In a given pathologic state, multiple cellular subtypes or populations whose biology is adapted to different niches are likely to

### Table 3. Identification of immune cells within single-cell data

<table>
<thead>
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<th>Cells</th>
<th>Correct prediction</th>
<th>Wrong prediction</th>
<th>Accuracy (%)</th>
<th>Error (%)</th>
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<td>100.0</td>
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**NOTE:** ImSig was used in conjunction with the SVM-based classifier Cibersort, to identify immune cells within the head and neck tumor (HNSCC) single-cell data. The table shows the accuracy of identification. Sixty-three immune cells were unassigned as their \( P \) value was greater than 0.05.
be present. We would argue that it is unrealistic to categorically identify their individual signatures from bulk tissue data, especially when the differences between them are more likely to be a spectrum than a series of absolute states (38). Even among different myeloid populations, i.e., monocytes, macrophages, and neutrophils, we have found few markers that are specific to one population or another. Markers that define the presence of these populations do so more by their coexpression than by absolute expression in the context of tissue.

We suggest that many immune subtype markers are too poorly defined to reliably distinguish immune cell subsets in the context of transcriptomics data derived from tissue. However, network analysis can provide a comprehensive picture of the immune microenvironment. By examination of the genes that correlate with the core signature genes, even if those genes expression cannot be reliably assigned to one cell type or another, it is possible to capture the overall profile of the immune microenvironment of a tissue. It may, after all, be the sum of the individual parts that matter. How these findings are used to identify immune cell subtypes, we leave to the individual analyst.

After satisfying ourselves of the validity of ImSig and comparing it to other signatures for defining immune populations in tissue data, we used it to analyze transcriptomics data sets derived from 12 cancer types. In each case, the majority of signature genes were tightly coexpressed, apart from instances where we believe the target cell was not present or was in low abundance. When the samples for each tumor type were ranked according to their immune cell content (as defined by the average expression of the signature genes), we were able to demonstrate variation in the immune microenvironment between tumors and the association of specific immune cell populations with good or poor prognoses.

Despite an established association between the immune system and survival in melanoma (56), there has been little effort to subgroup patients based upon what immune cell types are present in their tumors. Previous studies have merely defined tumors as having a high- or low-immune-cell content (34, 57). We, therefore, explored the use of ImSig in the molecular subtyping of melanoma patients. The analysis demonstrated a greater heterogeneity in the immune infiltrate of melanoma than previously reported (52, 54). We distinguished tumors characterized by T cells and macrophages (cluster 3), interferon enrichment (cluster 4), or B-cell infiltration (cluster 2). Treating the immune infiltrate of tumors as an overall signature limits the potential to identify prognostically significant subgroups. In other cases, merging the immune infiltrate into one immune-subgroup might result in opposing survival differences cancelling each other out, for example, if T cells were associated with a good prognosis and macrophages a bad prognosis. Understanding the immune heterogeneity of tumors may be key to predicting responses to immunotherapy (58, 59).

The advent of single-cell transcriptomics and its application to understanding the microenvironment of cancer promises to facilitate the profiling of all the cells of a tumor as never before possible (60) and may eventually circumvent the need to deconvolute tissue data, as described here. The technology to perform these analyses is improving and may in the future answer many questions about immune cell heterogeneity. However, at present, the data available are limited and the droplet-based RNA-seq methods being widely used may not provide a sufficient depth of sequencing to go beyond the identification of cell type. Here, we demonstrate how ImSig was able to accurately define the type and relative abundance of immune cells in single-cell transcriptomics data derived from melanoma, as well as head and neck cancer. As the quantity and quality of single-cell cancer data sets improve and our understanding of the expression profile of these cells improves, markers that are able to differentiate between immune subtypes or activation states, specifically in the context of the tumor microenvironment, may emerge.

ImSig is directly derived from tissue data. Although its gene content is not entirely novel, we believe ImSig performs better than previously published immune signatures as a subtype-agnostic means to estimate the relative abundance of immune cells across tissue samples. We also demonstrate the ability of ImSig to facilitate identification of biomarkers when applied in the context of network coexpression analyses. We anticipate that ImSig will aid studies of immune cell variation in tumors, responses to therapy, and predictive biomarkers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A.J. Nirmal, A.H. Sims, T.C. Freeman
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.J. Nirmal, B.B. Shih, D.A. Hume, A.H. Sims, T.C. Freeman
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