A Computational Framework for Deriving Mechanistic Hypotheses about Immune Interactions in the Tumor Microenvironment from Multiplex Immunohistochemistry Images

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Abstract

The complex network of interactions in the tumor microenvironment (TME) has yet to be fully elucidated. Spatial proteomic techniques like multiplex immunohistochemistry (mIHC) have made it possible for researchers to investigate numerous protein biomarkers simultaneously. While mIHC has been used to investigate tumor infiltration and some intercellular interactions on a spatial basis, the field has yet to establish a means of harnessing the power of observing and comparing the spatial interactions between all available markers for analysis simultaneously. To accomplish this, we propose a framework for analysis that incorporates a custom algorithm quantifying cell neighbors and machine learning techniques to elucidate the most important intercellular relationships investigated by immunologists and clinical researchers. To develop this framework, we used samples of tumor biopsies from a cohort of small cell lung cancer (NSCLC) patients. We developed neighborhood profiles surrounding each defined cell as features for analysis. Through orthogonalized Partial Least Squared Discriminant Analysis (OPLSDA), we connected the neighborhood profile of cells with their phenotype with high accuracy. Features with high Variable Importance in Projection ("VIP") scores highlighted intercellular relationships which have significantly strong associations to a given phenotype, implicating a relationship to be explored further by detailed pathway analysis. In a case study focusing on helper T cells, we found strong associations between interferon-gamma (IFNγ) expression and colocalization with activated T lymphocytes and Natural Killer (NK) cells. Additionally, we have established a similar pipeline that uses the cumulative neighbor scores between many cell types within each tissue region to separate different tumors based on broad clinical features. In an example distinguishing tumors by pathologic grade, VIP scores highlighted interactions between MHC-I expressing tumor cells and T lymphocytes. These methods have demonstrated to be useful in preliminary investigations of mechanism of action by immune cells in the TME and predictive power of tumor profiling. In the future, this is intended to be used towards implementing precision medicine techniques into immunotherapy treatment.

Keywords: multiplex immunohistochemistry, immunotherapy, spatial analysis, cancer

Introduction

Immunotherapy

The American Association of Cancer Research has highlighted the most promising areas of cancer research in 2023 to be immunotherapy and precision medicine [1,2]. The pioneering immunotherapy development, immune checkpoint inhibitors (ICIs), have been effective across several different cancer types. Many patients treated with ICIs have shown remarkable and lasting responses post-therapy [3]. Immunotherapy not only has promise of extended survival, but also frequently circumvents many of the side effects caused by current treatment plans. Modern pillars of cancer treatment include radiation, chemotherapy, and surgical resection, which carry long-term adverse effects towards patients, significantly decreasing their quality of life. The advancement of immunotherapy techniques will not only improve the ability for patients to recover but will also persuade more patients and physicians to choose immunotherapy, allowing those affected to experience a better quality of life during treatment[2]. Since its first approval in 2011, immunotherapy has accumulated numerous FDA approvals, including advancements like ICI, CAR-T cell, and adoptive T-cell therapy. The most prominent ICI therapies function by blocking receptors in immune cells and tumor cells that engage in immunosuppression, which reignites the immune response to tumors that have accumulated immunosuppressive effects[3].

While immunotherapies have been created to target specific ligand-receptor axes, the complete modulation of the tumor microenvironment is complex and has yet to be elucidated, making it difficult to target specific ways to improve the treatments or propose improved combination therapies[4]. In developing our pipeline (Aim 1), we looked to characterize this comprehensive modulation using cell-state level investigations.

Additionally, response to immunotherapy drugs is variable among patients, and while there are associations with tumor features and therapy response, improved capacity to predict responders are still needed to better identify candidates for specific treatments[5]. The most widely used immunotherapy, anti-PD-1 therapy (pembrolizumab) is used in the treatment of numerous types of cancer. It is most successful in malignant melanoma with a response rate at 53% [6]. For other soft tissue carcinomas treated with pembrolizumab, response rates vary between 20-40%. Predicting which patients will respond or how their tumors will evade particular immunotherapy treatments has proven to be difficult.[4]. Improvement remains a priority in choosing and optimizing treatment plans for patients undergoing immunotherapy. Towards this aim, we have developed a cumulative tumor-level analytical pipeline that looks to characterize tumors based on progression and future response to treatment.

The Tumor Microenvironment

Exploring the intricacies of the tumor microenvironment (TME) and its interface with the immune system is of particular interest when assessing and enhancing immunotherapy effectiveness. The TME represents the dynamic cellular environment surrounding a tumor, comprising a diverse array of

malignant and immune cells that engage in complex interactions shaping tumor progression and patient outcomes [7]. Recent advancements in imaging and sequencing technologies have unveiled the heterogeneous nature of TME, revealing substantial variability in cellular composition and spatial organization among patients. These findings underscore the importance of understanding the TME's role in cancer progression and treatment response, offering valuable insights for optimizing immunotherapeutic strategies.

Spatial Analysis within the TME

Complex interactions occur between malignant and immune cells within the TME. These interactions affect tumor progression and warrant further investigation. For example, uninhibited immune cells interact with tumor cells by attacking malignant cells through cytokine secretion or cascades resulting in killing by engulfment by nearby macrophages. Often, malignant cells also secrete cytokines and signaling molecules which reprogram and polarize immune cells to have an overall immunosuppressive effect. Immune cells in the stroma and infiltrating the tumor also interact with one another by activating cytotoxic effector cells, polarizing other immune cells, and promoting overall local inflammation [7].

Observing intercellular relationships within a relatively short radius (between 20-50 μm) reveals the potential for significant intercellular interactions through interactions of cell surface molecules[8]. While interactions occurring by way of paracrine signaling may occur over longer distances, they are still limited by the ability of small molecules to travel and maintain their effect against diffusion. It is postulated that paracrine signaling is largely limited between 200-250 μm[9]. When considering the colocalization required for many relevant intercellular interactions, spatial analysis becomes relevant to observe and reveals a milieu of potential and cumulative simultaneous interactions occurring.

Multiplex Imaging of the TME

Recent developments in high-dimensional, multiplex imaging have allowed for simultaneous phenotyping of cells within a large region of the tumor which preserve the spatial architecture with high resolution. Spatial proteomic and transcriptomic methods have been developed in allowing for high dimensional characterization across different levels. Spatial proteomic imaging methods such as multiplexed ion beam imaging (MIBI), imaging mass cytometry (IMC), multiplex immunohistochemistry (mIHC) have been developed in the last few decades and have revolutionized the capacity to visualize and analyze the tumor microenvironment on a protein level[10]. mIHC and other methods have become prevalent for their ability to visualize proteins on tumors, which for the analysis of multiple protein biomarkers simultaneously with single-cell resolution. It also allows for the visualization of spatial relationships between identifiable markers and has made it possible to quantify the frequency of spatial relationships between specified cell types[8,11].

Spatial analysis has gained popularity as a research method and is able to provide evidence for the mechanisms behind immune modulation and additionally associate potential intercellular interactions in the TME with prognosis and response to treatment. Methods of multiplex proteomic imaging and spatial analytical methods and theories have been employed to investigate mechanism of anti-tumor immunity in the tumor microenvironment and to find association and to predict prognosis and response to therapeutics. For example, previous studies have looked to synthesize spatial localization data about cytotoxic T cells with phenotypic and transcriptomic data in glioblastoma to successfully investigate signaling pathways within cytotoxic T cells[12]. Additionally, often in settings where other quantifications of tumor and immune cells within the tumor microenvironment are unable to show significant differences among responders and other tumor features, even simple spatial analysis does hold associative capacity. For example, the spatial relationship between PD-1/PD-L1 markers has been investigated using mIHC methods and spatial analytics and the strength of this relationships was found to be related to improved response to immunotherapy treatments in metastatic melanoma patients[13]. Most interestingly, a recent study published evaluating the ability of popular methods for analyzing the tumor microenvironment concluded that mIHC data was most capable of predicting response to immunotherapy treatment, especially when involving spatially informed analysis, supporting its use in investigations of this type[14]. Beyond a single spatial relationship, evaluating a milieu of spatial relationships has offered even more prognostic information and insight into treatment response. Recent groups have measured the strength and frequency of spatial relationship between various immune cell markers and used multiple relevant measurements to construct risk scores for patients with pancreatic ductal adenocarcinoma (PDAC). These risk scores had a higher capacity to distinguish groups based on their prognosis using spatial information from the TME[15]. The prior literature leads us to believe that our pipeline is the next logical step in the spatial analysis of the TME.

Precision Medicine

The efforts made associating biomarker information with response look to integrate a precision medicine approach into immunotherapy treatment. Precision medicine is a new approach to oncology which introduces a biomarker driven approach to cancer treatment. It has become well established in chemotherapy treatment were genetic tests guide use of therapies for treatment[16]. Response to treatment is often variable due to interpatient heterogeneity. In precision medicine, patients are stratified based on molecular features like gene expression data, tumor mutational burden, and proteomic data. Currently, investigations in immunotherapy seek to understand why patients resist response to immunotherapy, and what biomarker characteristics separate responders to treatment from non-responders[17].

Specific Aims

We propose to use similar concepts to investigate using multiplex analysis and a multitude of spatial relationships to evaluate the tumor-immune interface to gather predictive power to distinguish tumors and patients more comprehensively. While multivariate and machine learning analyses have been incorporated to understand and predict response in other analyses, they have not yet been sufficiently integrated alongside spatial analysis, a feature with powerful predictive capabilities. We have made analyses which are comprehensive, unbiased, and available for use by immunologists and clinicians in the form of easy-to-use tools.

We have designed a user-friendly pipeline that will use a custom cell neighbor finding algorithm to determine the spatial distribution of cells. The spatial analysis was then followed by machine learning techniques, namely orthogonalized partial least squares discriminant analysis (OPLSDA), to identify the neighborhood profiles of cells and their modulation based on specific conditions such as location, the presence of a specific marker, or colocalization with another relevant cell type to provide evidence to investigate a particular intercellular relationship (Aim 1).

We have designed a second, analogous pipeline using the cumulative neighborhood scores of all cell types in each region of a tumor biopsy as an input to OPLSDA analysis. We use subsequent cross validation to identify differences in neighborhood scores between different tumors, stratifying by features like low-grade and high-grade tumors and responders versus non-responders (Aim 2).

Results

Pipeline

The computational framework as outlined in Figure 1a uses object data from Vectra HALO analysis as input into a Python-based pipeline. It then spatially analyzes each cell and appends the neighborhood profiles as features for analysis into univariate analysis and machine learning analysis. The machine learning analysis used is an OPLSDA model. Interpretable outputs include accuracy metrics of the machine learning analysis and VIP scores ranking each feature for importance of separation from the OPLSDA model.

Spatial Analysis

Following quality control checks for cells and marker intensity, cells of interest were defined by a biologically informed immunohistochemistry marker combination. Subsequently, an in-house custom algorithm was used to construct neighborhood profiles. For each cell designated as the "center cell", a Euclidean distance was calculated between the center cell and all other cells within the focal region of interest. A user-specified radius was chosen, and each cell falling inside the specified radius was defined as a "target cell". A radius was chosen based on biological understanding and by evaluating the relationship between a center cell and a target cell across a range of radii. Each target cell defined by immune markers was used to construct a neighborhood profile which is illustrated in Figure 1b. A neighborhood profile was then generated for each center cell, and these neighborhood profiles were used as sets of features for analysis, which each sample being a cell or tumor region.

Univariate Analysis

Features such as neighborhood profiles at contact signaling distances and paracrine signaling distances, were incorporated for each cell, enriching subsequent univariate analyses and machine learning assessments. Univariate analysis facilitated the comparison of individual neighborhood profiles among distinct cell populations. Center cell populations were separated based on marker expression, activation status, spatial localization within specified microenvironments, or other distinctive attributes. Pairwise comparisons between designated target cells are depicted in Figure 2b. Statistical evaluations of neighborhood profiles employed a non-parametric Mann-Whitney U test, with Bonferroni false discovery rate correction applied to control for $p < 0.05$.

Multivariate Discriminate Analysis and Sampling Techniques

While univariate comparisons were effective at drawing statistically significant associations between phenotype and tumor environment with elements of the neighborhood profile, we were drawn to observe the ability of supervised machine learning analysis to separate the two populations. To this end, we employed a model of OPLSDA that has been implemented in the Dolatshahi Lab using Python based methods.

We incorporated methods of model evaluation into the pipelines to evaluate the reliability of each OPLSDA model and quantified the results. A confusion matrix was displayed and analyzed for each test set within each fold of the cross validation and was summed and displayed along with other OPLSDA figures. From these results, accuracy, precision, F1 scores were calculated and used to assess model fit which can be seen in Figure 3c and Figure 5c.

Under-sampling

In instances of considerable data imbalance, we undertook supplementary analysis using under-sampling methodologies. Specifically, for cell-level examinations, a reduction in the sample size of the predominant central cell population was applied prior to conducting PLS-DA analysis. Across various case studies, although accuracy metrics exhibited a decline, permutation testing, precision, and F1 scores demonstrated enhancement, suggesting an improved model fit to the dataset. The under-sampling process was iteratively executed,

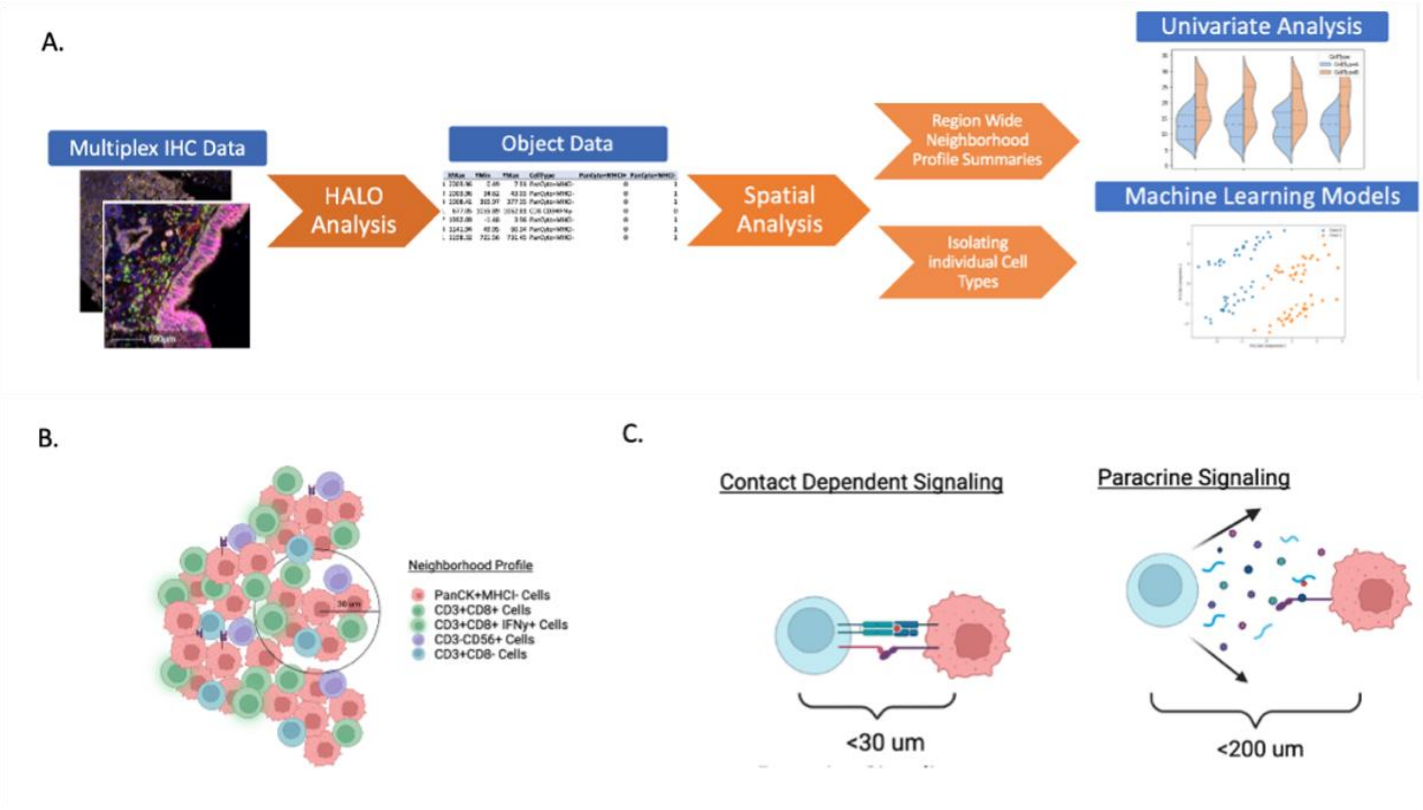

Fig. 1. (A) A flow chart describing the computational framework with spatial analysis followed by simultaneous univariate and machine learning analysis. **(B)** Neighborhood profiles were calculated using object data that was output from Halo analysis. Scores were constructed by counting the cells of each immune marker designation within a user-specified radius. **(C)** Contact-dependent signaling was explored at a radius of 30 µm while paracrine signaling was explored at a distance of 200 um.

and a distribution showcasing accuracy value was provided to evaluate the efficacy of under-sampling, as depicted in Figures 3d and 5d.

Case Study 1: Analysis of activated (IFNγ+) and inactivated (IFNγ-) cells shows significant differences in neighborhood profiles, suggesting variation in spatially dependent interactions in cells.

To achieve Aim 1, we fist conducted analyses to investigate the relationship between the activation status of helper T cells and their neighborhood profiles. Cells were used from mIHC-stained tumor sections from a cohort of NSCLC patients. Biopsies collected across tumor grades and histologic types were included for analysis. Cells expressing CD3 but not CD8 (CD3+CD8-) are recognized as helper T cells. Helper T cells recognize environmental antigens presented by professional antigen-presenting cells (APCs) and subsequently release cytokines and chemokines to coordinate the responses of cytotoxic T cells (CTLs) and other adaptive immune cells[18]. Given the pivotal role of helper T cells in immune responses, we postulated that activated helper T cells (CD3+CD8-IFNγ+) exhibit distinct behaviors and interactions with other cells compared to inactive helper T cells (CD3+CD8-IFNγ-). This is anticipated to result in altered colocalization patterns with other tumor and immune cells. To investigate this hypothesis, we assessed the neighborhood profiles of all helper T cells quantified in tumors within the NSCLC cohort at both contact-signaling distance (30 µm) and paracrine signaling distance (200 µm). Subsequently, we developed an orthogonal partial least squares discriminant analysis (OPLSDA) model using IFNγ expression as the classifier and helper T cell neighborhood profiles as features (see Figure 3). To mitigate the challenge of low activation fraction among helper T cells, we applied under-sampling techniques to the model, which enhanced the precision and reliability of our analysis.

We then applied the OPLSDA model and validation techniques to separate the two cell populations and evaluated the reliability of the model. Confusion matrices and re-under-sampling distributions were created towards this end. We found that the OPLSDA model was successful in separating the distinct phenotypic populations. The cross-validation accuracy taken of an under-sampled population of helper T cells was 89% with a promising precision metric of 92%. Among 100 iterations of under-sampling, accuracy remained impressive with limited variation and a standard deviation of 0.15 among accuracy results. VIP scores, delineating the primary contributors to group differentiation, prominently featured activated T lymphocytes, including CTLs and helper T cells, alongside activated natural

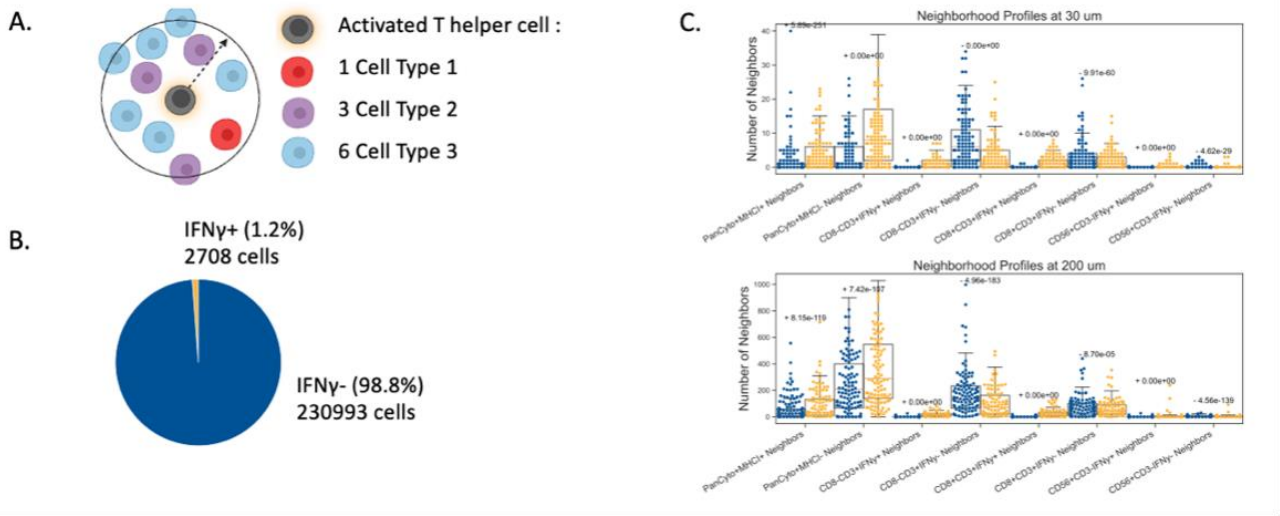

Fig. 2. Analysis of helper T cells shows activation status shows significant differences in neighborhood profiles, suggesting variation in spatially dependent interactions in cells. A. Schematic of spatial analysis neighborhood profile construction **B.** Proportion of cells within each population indicate need for under-sampling. **C.** Comparison of neighborhood profiles between activated helper T cells and inactivated helper T cells. Statistical

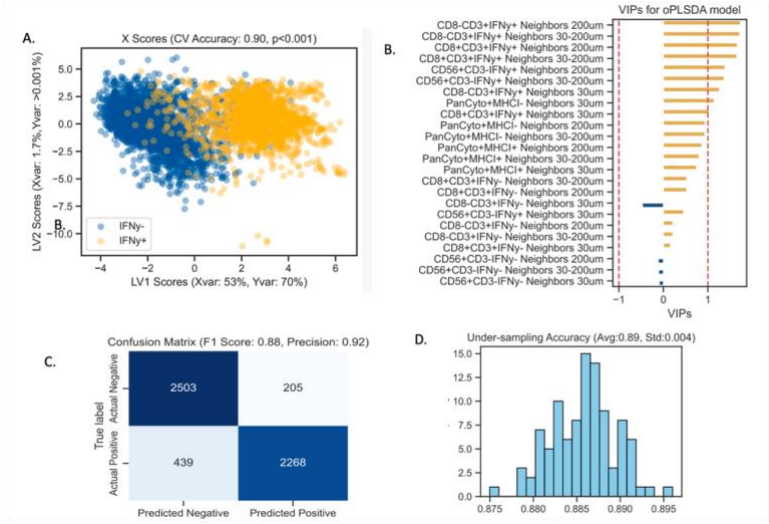

Fig. 3. OPLSDA provides a reliable model to distinguish helper T cells based on their activation status (A) The cohort of T helper cells is separated using the latent variables calculated within OPLSDA analysis. **(B)** The magnitude of importance of each feature in the first two latent variables is displayed in the Variable Importance in Projection (VIP) scores plot. **(C)** The confusion matrix shows the results applying the model's prediction summed over the 5-fold cross validation. **(D)** A distribution of model accuracy across 100 iterations of under-sampling shows limited variation and strong model reliability.

killer (NK) cells (CD56+CD3-) at paracrine signaling distances. These findings facilitated the formulation of the following mechanistic hypotheses regarding the interactions among these central and target cells.

Upon evaluating VIP scores greater than 1, we found that in NSCLC patients, intercellular colocalization involving IFNγ as a signaling mediator occurs between T lymphocytes and NK cells at paracrine distances. Other previous immune models provide evidence of a positive feedback loop, wherein IFNγ secreted by helper T cells stimulates other immune cells, including NK cells and CTLs. This occurs within proximal regions of the TME, leading to activation via autocrine and paracrine signaling pathways. This cascade subsequently induces activation and IFNγ expression, a phenomenon supported by murine models exploring immune cell activation[19,20]. Further elucidation of these interactions may be achieved through molecular pathway analysis and in vitro studies in NSCLC cohorts, offering insights into the mechanism behind this co-localization. Spatial analysis has identified several notable intercellular interactions warranting further investigation, thereby providing valuable evidence for the exploration of these relationships.

Case Study 2: OPLSDA provides a reliable model to distinguish helper T cells based on their colocalization with CTLs

Intercellular interactions in the TME are essential for a coordinated immune response, enabling communication between different immune cells to regulate their activities and mount an effective defense against malignant cells. We reasoned that close spatial relationships offer insight into possible interactions which are related to other consequential colocalizations and behaviors of the cells. T cells play a crucial role in the immune response by activating cytotoxic T lymphocytes (CTLs). They do this by recognizing antigens presented by APCs and releasing cytokines that stimulate the proliferation and differentiation of CTLs. This collaboration is essential for the effective elimination of malignant, highlighting the fundamental relationship between helper T cells and CTLs in adaptive immunity[18].

After we noticed the significant colocalization between helper T cells and CTLs in the first case study shown in Figure 3 and in the broader literature, we looked to examine its effect of its presence on the behavior of helper T cells. Our goal was to investigate the hypothesis by assessing the correlation between

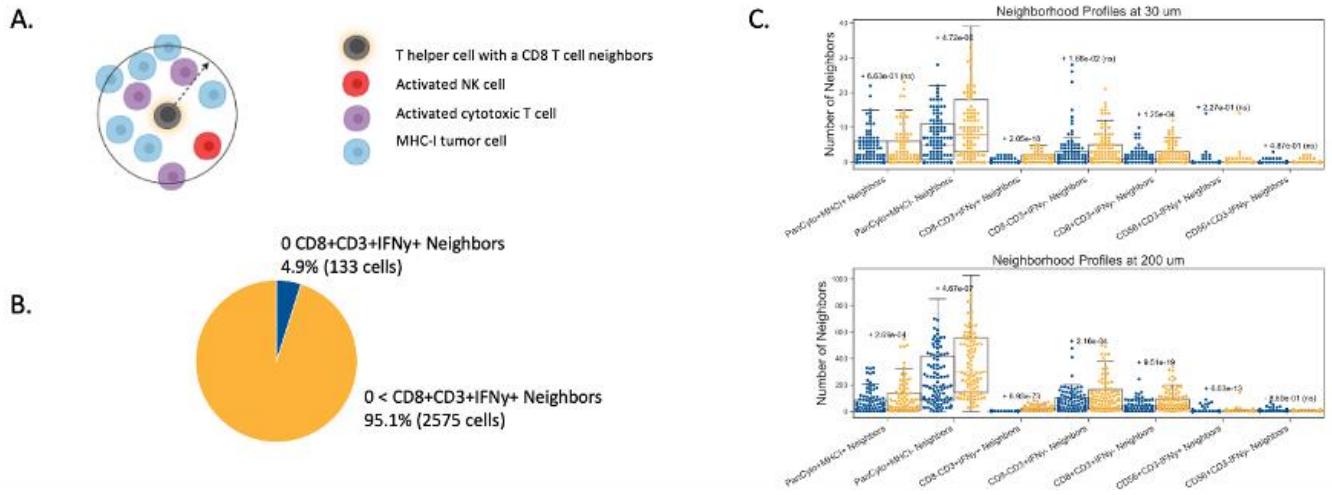

Fig. 4. Analysis of helper T cells shows with and without CTL neighbors shows significant differences in neighborhood profiles, suggesting variation in spatially dependent interactions in cells. (A) Schematic of the spatial analysis neighborhood profile. **(B)** Proportion of cells with and without cytotoxic T cell neighbors. **(C)** Comparison of neighborhood profiles between the two populations of cells based on neighborhood status. Statistical significance is calculated using Mann-Whitney U test with significance accounted for using Bonferroni correction.

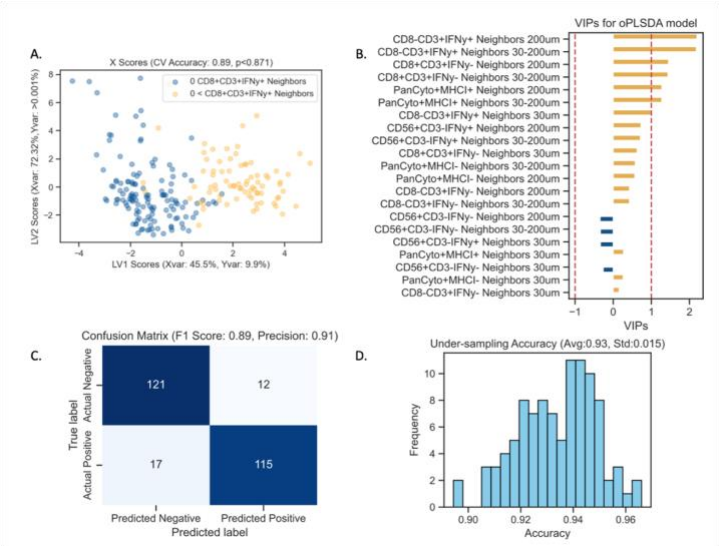

Fig. 5. OPLSDA provides a reliable model to distinguish helper T cells based on their colocalization with a cytotoxic T cell. (A) The cohort of T helper cells is separated using the latent variables calculated within OPLSDA analysis. **(B)** The magnitude of importance of each feature in the first two latent variables is displayed as the Variable Importance in Projection (VIP) scores plot**. (C)** The confusion matrix shows the results applying the model prediction summed over the 5-fold cross validation. **(D)** A distribution of model accuracy across iterations of under-sampling shows limited variation and reassuring model reliability.

helper T cells and CTLs and how it influences the behavior and spatial relationships of helper T cells. To accomplish this, we isolated activated helper T cells and categorized them based on the presence or absence of activated CTLs within a 200 µm radius. Subsequently, we analyzed the neighborhood profiles of these two populations and compared the spatial distribution patterns of the immune cells through univariate analysis (Figure 4).

We then constructed an OPLSDA model using the presence of a CTL neighbor as the model classifier and neighborhood profiles of all helper T cells as model features (Figure 5). Under-sampling techniques were applied to the model to address the low fraction of helper T cells without a CTL neighbor within 200 µm.

The pivotal features discerned in distinguishing this relationship, as elucidated by the VIP scores of the OPLSDA, were the presence of other activated T lymphocytes, encompassing both cytotoxic T cells (CTLs) and helper T cells, at paracrine signaling distances. We can again formulate mechanistic hypotheses regarding the interactions associated with this phenomenon.

It is plausible that helper T cells are colocalizing with one another to better recruit CTLs to their vicinity. Drawing from existing evidence in other tumor models, we postulate that helper T cells that colocalize with each other may generate additive signals, leading to an overall increased production of IFNγ, thereby enhancing the activation and recruitment of CTLs near the CD4 center cells[19]. Additionally, previous studies have demonstrated that helper T cells coordinate IFNγ production to recruit and bolster the activation of CTLs, which may elucidate the augmented presence of helper T cells surrounding this relationship[21].

Moreover, other noteworthy features include the presence of MHC-I-expressing tumor cells at paracrine signaling distances. The association between MHC-I and helper T cells has been extensively documented in other malignancies in existing literature, demonstrating that helper T cells utilize IFNγ as a signaling molecule to induce and amplify MHC-I expression in malignant cells, and subsequently facilitating the recognition of the malignant cells by CTLs[22]. We postulate that a similar phenomenon may be occurring within the NSCLC cohort in the vicinity of CTLs, warranting further investigation via spatial transcriptomic analysis and in vitro studies.

The mechanistic hypotheses generated from the case studies involving helper T cells were developed using findings

from machine learning analysis corroborated with information from literature exploring these relationships in other malignancies. This framework can be applied to broaden the understanding of mechanism across a range of other malignancies and diseases.

Case Study 3: OPLSDA model predicts tumor grade effectively using relationships between activated immune cells and tumor cells.

Given the pivotal role of intercellular interactions in shaping the tumor microenvironment (TME) and influencing patient outcomes, our study aims to investigate how patterns of cellular colocalization within the TME correlate with clinical characteristics. We began this attempt by examining whether distinct colocalization patterns could differentiate between high-grade and low-grade tumors within the NSCLC cohort, as tumor grade is a critical prognostic factor.

This analysis employed the framework that was developed to achieve Aim 2. For a comprehensive analysis, we performed spatial analysis for every cell type within the cohort. Then we summarized each region of interest (ROI) by taking summary statistics of each colocalization. To do this, we isolated each cell type of interest (for example, CD8+CD3+IFNγ+) and took an average of their entire neighborhood profile. We repeated this for every cell type of interest, and with 8 cell types, we created 64 features for evaluation per ROI. Then we separated ROIs based on whether the tumor they were collected from was designated as high-grade or low-grade tumors and used this as the designation for analysis.

Our analysis revealed disparate colocalization patterns in high-grade tumors, suggesting that these patterns could serve as potential biomarkers for tumor aggressiveness and patient outcomes. VIP scores highlighted the paracrine distance relationship between CTLs and MHC-I expressing tumors as hallmarks of low-grade tumors. Low-grade tumors also showed a higher degree of infiltration by inactivated NK cells in the tumor regions. This indicates that appropriate recognition of MHC-I expressing cells by CTLs is occurring in low grade tumors and that appropriate tumor infiltration of NK cells is occurring simultaneously. Univariate analysis and Mann-Whitney U tests confirmed these trends. In high grade tumors, the relationship between helper T cells and malignant (PanCyto+) cells were strong regardless of MHC-I expression. We reasoned that the relationship between helper T cells is stronger in high-grade tumors and that helper T cells allow the immune assault in NSCLC patients to persist even in more aggressive tumors.

We interpreted these results to determine that lower grade tumors elicit an immune response characterized by NK cells and CTLs which we hypothesize is related to the normal expression of surface markers on malignant cells. On the other hand, we hypothesize that higher grade tumors, while also becoming more abnormally differentiated, undergo loss of MHC-I and other helpful surface markers, working towards immune evasion, eliciting a more robust response from helper T cells. This pipeline not only allowed us to use spatial architecture to predict

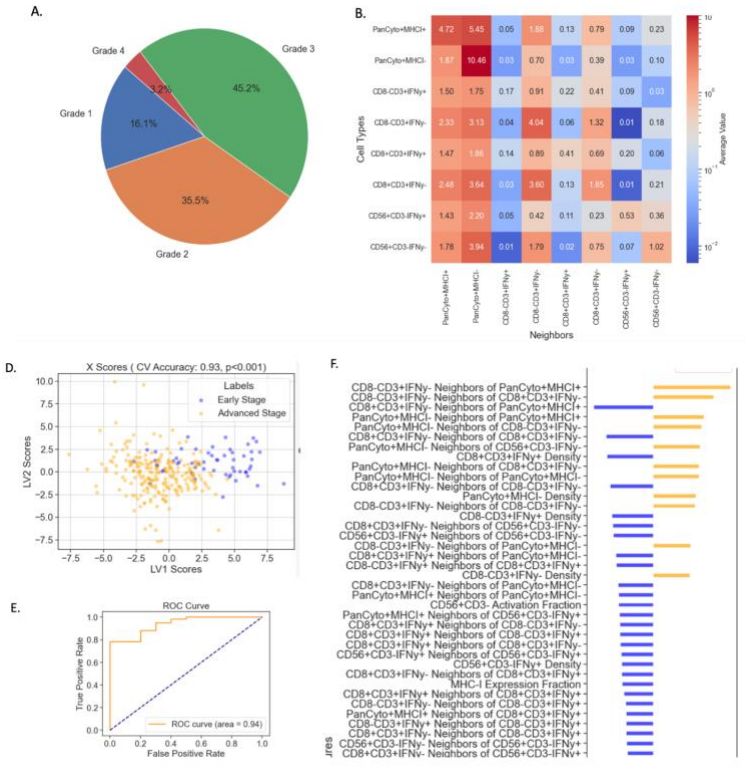

Fig. 6. OPLSDA model predicts tumor grade effectively using relationships between activated immune cells and tumor cells. All possible neighborhood relationships may be measured and summarized over an entire region of interest (ROI) in order to distinguish between tumors with different features, in this case clinical grade. **(A)** The distribution of tumor grades among all ROIs measured is shown. **(B)** The average number of cell neighbors surrounding each cell type (average neighborhood profile) across all ROIs is shown in heatmap form. Neighborhood relationships between all cell types in a given ROI were used as input to analysis. **(D)** The PLSDA model was able to distinguish ROI's based on their tumor grade. **(E)** ROC curve shows the models ability to balance true positive and false positive rates at all regions of interest. **. (F)** Relationships between T lymphocytes and MHC-I expressing tumor cells are most relevant in distinguishing low versus high grade tumors.

a clinical feature of the tumor but also allowed for us to form specific hypotheses about the TME modulation using VIP scores.

Discussion

Towards Aim 1, this analytical pipeline was successful in correlating cellular phenotypes and specific colocalizations with a broader neighborhood profile. The highlighted relationships in each test case were related to strong existing evidence in the literature of these relationships, highlighting their accuracy and applicability. A limitation of the case studies employed was the size of the marker panel which focused primarily on identifying immune cell and malignant cell types, along with their activation status. More novel and specific hypotheses could be generated from an improved marker panel.

Towards Aim 2, the cumulative analytical pipeline was successful in correlating tumor grade with a broader picture of the spatial architecture of the TME. The analysis highlighted spatial colocalizations which had known relationships to behaviors

related to tumor grade. These findings give us confidence that the panels will soon allow for broad investigations in a variety of malignancies which will speed the process from exploring the modulation of individual intercellular relationships. While this panel was successful, it is subject to the same limitations of a small biomarker panel and a limited patient population as in the cell-state level case studies. More comprehensive panels and larger patient populations could improve our confidence in the model.

Limitations and Future Direction

The next stage of pipeline development will be to apply it to existing tumor biopsies and train the model with the distinction being response to treatment. This has been tested and has shown positive preliminary results in studies performed on other malignancies and will likely allow the model to move forward into aims that are closely related to the needs of immunotherapy research.

We propose that work in the future will focus on applying the same general techniques and model evaluations to other biomarker to elucidate other relationships that are occurring with the tumor microenvironment. Suggested biomarkers include those which include PD-1 and PD-L1, surface receptors which immunotherapies like pembrolizumab act on. Other possible biomarkers include markers for immune cell proliferation and exhaustion as well as markers for other types of immune cells such involved in the adaptive immune system such as memory T cells and regulatory T cells. This model should also be tested for applicability to evaluate tumors from other tissues, especially those with especially strong or weak responses to immunotherapy. Additionally, larger populations will be helpful to further validate tumor and patient-level analyses and will help researchers to better prove its generalizability and use among a wide range of cases.

This computational framework is flexible in that within each step of the framework, alternative tools and methods could be used to ask different questions and potentially gather increased predictive capacity. Other spatial analysis metrics may be substituted for the K-function based method to draw similar conclusions while evaluating a range of different radii. These include methods based in the G-function and L-function which are supported by existing tools such as R Spatstat, SPIAT, and Monkeybread[13,23,24]. To improve the predictive power of the model, use of other, non-linear machine learning models is suggested to potentially improve model performance. Artificial neural networks (ANN) is one of several promising techniques to accomplish this feat[25]. It must be advised that the conclusions drawn from the model will change based on the tools used and the current conclusions have been made based on the use of K-function based spatial analysis and OPLSDA models.

Future Framework Applications

One application of the proposed pipeline attempts to leverage the comprehensive nature of multivariate analysis to investigate intercellular interactions from a spatial perspective. This will help scientists to select specific relationships for further study, and to correlate spatial relationships with biomarker expression, providing evidence for mechanisms. These principles have been used by the Dolatshahi group to investigate intercellular interactions that occur in the tumor microenvironment which have the potential to reveal new potential molecular targets for therapies and diagnostics. The method also has the capacity to provide evidence for mechanistic explanations why therapies might fail in certain candidates. If the heterogenous weaknesses of anti-PD-1 therapy and ICI therapy can be revealed, candidates for new therapies and complementary combination therapies may be chosen in a more informed way, speeding up the process for development.

The pipeline may also be used to use the cumulative intercellular relationships to predict patients' response to treatment. This aims to contribute to the integration of precision medicine into immunotherapy. Precision medicine is broadly defined, but in cancer immunotherapy has historically referred to using molecular features of cells in the tumor microenvironment to better select therapy for treatment [17]. Concepts of precision medicine have already been applied to chemotherapy, where genetic testing is used to select the chemotherapy that is the most likely to work for each patient based on historical data. These methods have been very successful in improving overall treatment plans and disease-free survival. Precision medicine not only improves patients' chances at responding to treatment, but it also allows patients to forego chemotherapy treatments with harsh side effects that are unlikely to be of benefit to them, giving them potential for greater comfort and quality of life. This method aims to be applied to clinical biopsies in the development of precision medicine strategies in immunotherapy, to make more informed treatment plans than are currently available. More informed treatment plans also reduce the trial-and-error method that is commonly used in cancer treatment plans, allowing patients to receive effective treatments faster, and further improving survival and comfort [16].

The purpose of this project is to utilize spatial distribution methods and machine learning techniques to gain evidence for interactions between immune cells in the tumor microenvironment and to use the immune spatial milieu to predict which patients will be responsive to treatment. The synergy of bringing personalized medicine into immunotherapy treatment would bring together the benefits of both legs of advancement in medicine.

Our work will help to advance immunology and clinical research by presenting users with a framework to attempt unbiased, comprehensive analysis of the spatial associations in the tumor microenvironment. The single cell approach to the pipeline will help research scientists to leverage machine learning to find important spatial relationships in the tumor microenvironment and to plan subsequent experiments. The methods will also offer a framework that can be used by institutions with large biopsy cohorts to create models to help make treatment decisions in a clinical setting.

Materials and Methods

Multiplex immunohistochemistry imaging and geospatial analysis.

NSCLC patient tissues were procured from 49 patients among varying tumor grades (G1-G4) and two distinct histologic subtypes (adenocarcinoma and squamous cell carcinoma). 395 ROIs that displayed 3 mm^2 sections of tumor were used for analysis. Sections of formalin-fixed, tumors were stained with a 7-color panel and imaged using the Vectra system. Tissues were stained with antibodies against PanCK, MHC I, CD3, CD8, CD56, IFNg, and DAPI. Regions that were extremely sparse (>50 cells) were excluded from analysis. HALO analytical software was used to segment individual cells and assign stain positivity. Combinations of these markers were used to classify cellular phenotypes. In cell-state level case studies (Case Study 1 & 2), all cells of interest were incorporated for analysis regardless of tumor characteristics.

Cellular neighborhood analysis.

Custom cell-cell neighborhood scoring algorithm. Intercellular geospatial colocalizations in NSCLC tumors from the patient cohort were determined in Python using single cell 2-dimensional coordinates obtained from HALO. The Euclidian distance between each cell and every other cell on the slide was computed; nearest neighbors were defined as cells with a center-to-center Euclidian distance of less than the user-specified radius from the center cell (Figure 1). The nearest neighbors of each phenotype were enumerated to yield the neighborhood profile for every individual cell.

Machine learning analysis

Orthogonalized Partial Least Squares Discriminant Analyses (OPLSDA) two-component (latent variable) models were generated in Python using the PLSRegression function in the scikit-learn package. The scripts used for this purpose were developed in-house. Prior to input into OPLSDA, all data were log-transformed, centered, and scaled. In the case of PLSDA classification models, significance was calculated by comparing the constructed model's mean squared error against one thousand randomly permuted null models.

End Matter

Author Contributions and Notes

K.G., S.D., R.W and G.H. designed research, K.G. and G.H. performed research, K.G wrote software, K.G. analyzed data; and K.G. wrote the paper.

The authors declare no conflict of interest.

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