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Active Surveillance Characterizes Human Intratumoral T Cell Exhaustion

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**Abstract:**

Intratumoral T cells that might otherwise control tumors are often identified in an ‘exhausted’ state, defined by specific epigenetic modifications and upregulation of genes such as CD38, CTLA-4 and PD-1. While the term might imply inactivity, there has been little study of this state at the phenotypic level in tumors to understand the extent of their incapacitation. Starting with the observation that T cells move more quickly through mouse tumors as residence time increases and they progress towards exhaustion, we elaborated a non-stimulatory live-biopsy method for real-time study of T cell behaviors within individual patient tumors. Using two-photon microscopy, we studied native CD8 T cells interacting with APCs and cancer cells in different micro-niches of human tumors, finding that T cell speed was variable by region and by patient and was inversely correlated with local tumor density. Across a range of tumor types, we found a strong relationship between CD8 T cell motility and exhausted T cell state that corresponds to observations made in mouse models where exhausted T cells move faster. Our study demonstrates T cell dynamic states in individual human tumors and supports the existence of an active program in ‘exhausted’ T cells that extends beyond incapacitating them.
**Introduction**

Tumors contain inflammatory infiltrates that might detect and eliminate tumor cells, and yet immune tolerance occurs, and cancer evasion persists. T cells isolated from the tumor microenvironment (TME) often exhibit an exhausted phenotype that is characterized by a unique epigenetic landscape and increased inhibitory receptor expression that leads to the reduced proliferative capability and tumor killing (1). Given that T cells are the targets for most current cancer immunotherapies, understanding how this tolerant state manifests in situ is important. Multiplexed flow cytometry and single-cell transcriptomics have been applied to isolated intratumoral T cells to investigate their function (2), however the spatial information is lacking. In contrast, fixed imaging methods reveal important links between protein phenotypes and the spatial organizations of immune infiltrates (3), but lack an understanding of dynamic behaviors and the plasticity of cellular ensembles.

Intravital imaging by two-photon laser microscopy—using near-infrared excitation to provide improved depth penetration with reduced phototoxicity over longer observation periods—is especially suited for in situ imaging of immune dynamics in intact tumors (4). We and others have established methods to study how immune cells behave in mouse tumors, often using genetically encoded fluorophores (5, 6). In one such setting, we found evidence that infiltrated T cells increase their motility following an initial arrest phase (7). The behaviors of T cells
in human tumors—which likely vary in myriad ways relative to mouse models — remains largely unstudied.

In one pioneering study using slices of human biopsies, exogenous T cells labeled with fluorescent dyes that were non-native to tumors were imaged (8). There, these newly introduced cells migrated actively in loose collagen areas whereas poorly in dense matrix areas. However, no systemic approach has yet been developed to study how tumor native T cells migrate nor how that links to their immune function in human tumors. Therefore, we developed and validated a ‘live biopsy’ method for direct imaging of immune function in human tumor specimens. We standardized the approach and applied it to tumors from multiple indications to measure immune cell behavior indicated by cell motility and interaction among antigen presenting cells (APCs), T cells and tumor cells. The results show both heterogeneity within human cancers with respect to exhaustion but also that the program of exhaustion defines a dynamic state resembling surveillance.
Results and Discussion

Develop Live Biopsy Imaging

To characterize the in situ phenotype of intratumoral high-affinity murine T cell, we generated a cell line from a well-described breast cancer mouse model (mammary tumor virus-polyoma middle T (MMTV-PyMT)) modified to express mCherry and OVA (PyMT-ChOVA) (5) and injected it into the fat pad of mice with the adoptive transfer of OT-I cells at two different time points during tumor progression (Fig. 1A). T cells resident in tumors for 14 days are characterized by upregulation of inhibitory molecules associated with exhaustion, including PD-1 and CD38, the latter albeit only in a fraction of cells (Fig. 1B) (9). A master transcriptional factor, often associated with exhausted T cells, Tox (10) is also upregulated when compared to effector T cells that just arrived (d4) in these tumors. In contrast, the prolonged resident T cells produced less IFNγ (Fig. 1B) with attenuated granzyme B and Nur77 expression as showed in a previous study (7), strongly suggesting the loss of effector functions and onset of exhaustion in these T cells. We found that similarly-generated (d14) T cells fail to arrest in the TME and were dynamic compared to d4 effector T cells (Fig. 1C). Specifically, 72.5% of d14 T cells moved at speed exceeding the mean of the d4 T cells and 44.6% were fast-moving (speed > Mean + 1 standard deviation (SD)).

We sought to extend this result to human tumors and in doing so shed light on the variety of T cell behaviors within the more diverse domain of human tumors. For
this, we needed to label and image human cells in biopsy in a way that did not alter
their biology (Fig. 1D). We first validated imaging strategies for mouse tumor
biopsies, whereby we could compare the data obtained from sliced tumors against
data taken from intravital imaging of the same tumor (Fig. 1D). To maintain intact
endogenous T cell behaviors, we found an optimum for storage/transport using
pre-warmed and oxygenated media and times post-surgery of less than 5 hours,
and for imaging using a heated perfusion chamber with a constant flow of oxygen-
saturated media. Under these conditions, T cell motility rates were well maintained
in the tumor slices of PyMT-ChOVA, B78 melanoma and MC38 colon carcinoma,
being indistinguishable from those observed using intravital imaging (Fig. 1E and
Supplemental Fig. 1A).

Since transgenic fluorescent markers are not experimentally tractable in human
samples, we next screened and identified a panel of non-stimulatory antibodies
that label human T cells to aid visualization and do not trigger well-established
signaling pathways downstream of their ligands (Fig. 1D). For example, we
selected a single clone that bound to a TCRβ (Vβ13.1) (Fig. 1F) and an anti-CD8α
antibody (Supplemental Fig. 1B) that did not induce a detectable calcium influx in
Jurkat cells or in human peripheral blood mononuclear cells when cross linked
(Supplemental Fig. 1C), as compared to the majority of monoclonal antibodies
against TCR-associated proteins (e.g. CD3, TCR, etc.) which did so. Where
possible, Quantum dot (Qdot) conjugates of these clones were used, due to
exceptional brightness and photostability (11). As a functional test of this panel in situ we examined human tonsil slices to visualize CD8 T cells and CD14+ myeloid cells (Supplemental Fig. 2A and video 1) and found that motility of human T cells fell in the range 1-10μm/min (Supplemental Fig. 2B), consistent with mouse lymphocyte motility in lymph nodes (12). We observed diverse CD8 T cell behaviors in a micro-region of the tonsil, including some with a random walk and others arrested on myeloid cells (Supplemental Fig. 2C), that might be attributed to the presence of antigens in these inflamed tonsils.

Application of this method to tumors, including head and neck (HNSC), colorectal (CRC), gynecological (GYN), kidney (KID) and hepatic (HEP) cancers (Supplemental Fig. 3), showed well-maintained immune infiltrates, stroma, and tumor structure. An example of a HNSC tumor highlighted diverse levels of immune infiltrations and T cell speed among five different regions of interest (ROIs), chosen at random during acquisition (Supplemental Fig. 4A, B). Neither the density of CD8 T cell or that of HLA-DR+ myeloid cells nor the contact time between them in these five ROIs were well-correlated with T cell speed (Supplemental Fig. 4C-F).

**CD8 T cell motility was inversely correlated with local tumor density**

To extend our analysis to additional tumors and seek out other tissue parameters that might impact motility, we imaged a colorectal tumor, where CD8 T cell
behaviors in three ROIs with various abundance of EpCAM$^+$ tumor cells were quantified (Fig. 2A, B, and supplemental video 2). T cell tracks demonstrated significant regional differences (Fig. 2C, D), while were not correlated with the density of either T cells or myeloid cells, nor the contact time between these two cell types (Supplemental Fig. 5A-C). However, tumor cell density was inversely correlated with T cell motility across the three ROIs (Fig. 2E, F). This relationship repeated in another colorectal cancer (Supplemental Fig. 5D-G) and the HNSC shown in Supplemental Figure 4 (Fig. 2F).

Finally, we observed that while most CD8 T cells moved freely when they were not in contact with tumor cells, they occasionally alternatively engaged with myeloid cells and tumor cells to form stable conjugates for at least 90 mins (Fig. 2G and supplemental video 2). Thus, CD8 T cell motility differed among subregions of a tumor, which was potentially caused by heterogeneous local tumor density.

**CD8 T cell motility was correlated with immune states in human tumors**

We next sought to broaden this approach toward defining the rate of T cell motility across tumors and across site of origin. In total, 17 fresh tumors of five indications were split for flow cytometry (Supplemental Fig. 6A) and live biopsy imaging (Fig. 3A). Though T cell speed was not a binary variable, we classified those with substantial motility across the tumor (motile) from those with very limited T cell motility (Immotile) using a cutoff if the mean was $<$1 μm/min (Fig. 3B), to investigate
if the immune component and cell state were linked to fast versus slow T cell speed. Although these groups had different ischemia time (Supplemental Fig. 6B), we found no statistical support that these were different between motility groups. Average CD8 T cell density, the percentage of CD45+ live cells, CD4 to CD8 T cell ratio, and the abundance of regulatory T cells (Supplemental Fig. 6C-F) were also quantified for these samples, all showing no relationship to motility.

We then turned to markers of exhaustion, guided by the mouse data (Fig 1), as a hypothesis. We examined the frequency of CD8 T cells expressing the exhaustion markers CD38 and CTLA-4, finding that these were upregulated in the motile group when compared to their expression in the immotile group (Fig. 3C-F). PD-1 was also mildly upregulated in the motile group. (Fig. 3G, H).

We also sought to determine whether motility and exhaustion had other correlates and found that Ki67 expression in CD8 T cells followed the same trend (Fig. 3I, J). Though Ki67 is often used as a marker for proliferative capability, it also marks a population of recently generated terminal exhausted T cells that lose the ability to respond to additional stimulation (13). From the tumor biopsies that we imaged, Ki67^{hi} CD8 T cells showed significantly higher expression levels of both PD-1 and CD38 than Ki67^{low} CD8 T cells from the same tumors (Supplemental Fig. 6G).
To further examine the link between T cell exhaustion and motility, we performed RNA-sequencing of T cells sorted from a cohort of CRC patients, one of the most abundant indications analyzed by live biopsy, and grouped the samples based on CD38 upregulation as a representative marker for exhausted T cells. *ENTPD1*, *LAG3*, *HAVCR2*, and *TOX2* were upregulated, supporting that these cells are preferentially exhausted whereas *TNF* was downregulated, indicating a reduced effector phenotype (Fig. 4A). Referencing gene ontology of the differentially expressed genes also showed that protein localization to microtubule and regulation of cell migration related pathways were both enriched in CD38 high group (Fig. 4B). These included genes that are associated with cytoskeletal rearrangement and cell polarization, *PDLIM4*, *AFAP1L2*, and *PLK4*, microtubule motor protein-encoding genes, *KIF20A*, *MYO7A* and *MYO7B*, and a Rho-GTPase associated protein *CAV-1* (Fig. 4A). We then plotted the fold change for these promotility genes that revealed in our data by comparing ‘exhausted’ versus ‘non-exhausted’ cells based on the RNA-sequencing data of four recent publications on exhaustion and found they were all upregulated in exhausted T cells (Fig. 4C) in human lung cancer or melanoma (14-17). Together, this supports that ‘exhausted’ cells, though defective for sensitivity and ability to mount cytokine responses, are nevertheless upregulated for genes associated with tissue surveillance.

To directly compare markers of exhaustion with cell motility on a cell-by-cell basis, we monitored the T cell motility and the expression of exhaustion markers.
simultaneously in PyMT tumor slices (Fig. 4D). We found that long resident T cells expressed higher PD-1 when compared to the recent arrivals (Fig. 4E) and moved faster with a longer track (Fig. 4F). When quantifying these parameters on individual cells from the same tumor volumes, d14 T cells exhibited higher PD-1 expression and speed, while d4 T cells expressed less PD-1 and were slower, with a positive overall correlation between T cell motility and exhaustion (Fig. 4G).

In summary, we developed a two-photon microscopy-based imaging method of tumor slices from fresh biopsies taken from cancer patients that maintained immune dynamics and spatiotemporal information. Focusing on in situ endogenous T cell behavior in human cancer, our study provides a view of regional and patient-specific variations in cellular dynamics and further elaborates our understanding of a critical T cell state, namely ‘exhaustion’.

To the point of methods development, the biology observed using these imaging conditions and non-stimulatory antibodies highly resembles that observed in mouse lymph nodes and in situ (4, 5, 12), suggesting that we have broadly captured the durable biology of these tissues. Though the full-size immunoglobulins used in our methods were non-disturbing, using a monovalent Fab or nanobodies to eliminate Fc-region-mediated effects would be ideal. Although we optimized our slice imaging method, immune behavior was possibly
not perfectly recapitulated because vascular circulation, chemokine milieu in addition to enervation and lymphatic flow were lacking (18).

Regardless of different histological tumor type and their genetic variations, the combination of immune profiling by flow cytometry/RNA-Seq with live biopsy imaging enabled us to group tumors according to T cell motility and link this to the aggregated state of the T cell population. In our study, the aggregated abundance of human exhausted T cells marked with Ki67\textsuperscript{hi} CTLA-4\textsuperscript{hi} PD-1\textsuperscript{hi} CD38\textsuperscript{hi} correlates well with the observation of fast motility within the same tumor sample. While multiple factors could contribute to the progression of T cell motility with tumor-residence and during transitions towards exhaustion, including varied T cell priming in the lymph nodes and desensitization to the existence of tumor antigens (19), our data suggested that T cells exhibited the exhausted phenotype gradually with a wide spectrum of exhaustion markers expressed at different levels. Correspondingly, though T cell speed within a given human sample varied, the overall motility was positively correlated to exhausted phenotype. Given the observation of an absolute shift in motility pattern during the establishment of exhaustion in mouse models (Figure 1 and 4) we believe that this relationship is likely to be at least partially cell intrinsic. This is further suggested by the upregulation of motility genes in T cells taken from patients with an abundance of exhausted cells. Further analysis of single-cell sequencing data for exhausted T cells from patient samples, not currently available, may reinforce this as could in
vitro experiments taken from patients. However, we consider it likely that this faster motility rate is also a consequence of the combination of cell intrinsic and extrinsic features and may even represent the co-evolution of microenvironmental features along with the induction of exhaustion. With our live biopsy method established, testing if TCR/HLA interaction blockade could lead to the increase of motility or in contrast, if exogenous peptide or anti-PD-1 blockade could arrest exhausted T cells in tumors are possible. It will be intriguing to explore how other micro-regional factors, including cell types, soluble factors, local oxygen levels, protease, and metabolites, regulate immune cell behavior and T cell function, e.g. exhaustion. To understand all these factors together, significantly more studies will be required, notably spatial transcriptomics and/or proteomics.

Given that the composition and type of immune cells in the TME affect clinical outcomes (20), live biopsy developed in our study will provide a platform to test drugs, e.g. immune checkpoint inhibitors in tumors demonstrating these distinct T cell phenotypes. Following the disseminated use and elaboration of these methods, live biopsy studies may act as mini-patients, to predict the efficacy of the same therapy in patients.
Methods

Detailed experimental methods are included in the Supplemental Material.

The accession number for the RNA-sequencing data reported in this paper is GSE179975.

Study Approval. Patients in this study provided written and informed consent to tissue collection under a University of California, San Francisco (UCSF) institutional review board (IRB)-approved protocol (no. 13-12246). All mice were housed and bred at UCSF in accordance with the regulatory standards of the National Institutes of Health (NIH) and American Association of Laboratory Animal Care standards and that were consistent with the UCSF Institution of Animal Care and Use Committee (no. AN170208-01B).

Author Contributions

R.Y. performed all experiments unless specified. J.A., A.F. and A.E. assisted in imaging analysis. A.C. and G.R. participated in preparation and analysis of the flow cytometry of human samples. B.S processed and analyzed RNA-seq data. R.Y. and M.F.K designed the experiments, wrote and revised the manuscript.

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Running Title: Intratumoral Exhausted T cells are Active
References


Figure 1. Establishment of live biopsy imaging using non-perturbing antibodies. (A) Schematic diagram of the experimental design. (B) Measurement of exhaustion molecules and IFNγ in GFP or RFP OT-I transferred 4 (arrival) or 14 days (exhausted) before tumor harvest. N = 5. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, as determined by the Paired Student’s t-test. (C) Quantification of T cell motility in tumor slices. Mean and SD: 1.846 and 1.088 (d4); 3.637 and 2.639 (d14). Data is pooled from four ROIs in two slices, representative of two independent experiments. **** P<0.0001, as determined by the Student’s t-test. (D) Schematic diagram of live biopsy imaging strategy. (E) Color-coded tracks and quantification of mouse T cells from intravital and slice imaging of fat-pad injected PyMT-ChOVA tumor. Speed range: 0 to 0.15 μm/sec (red to white). Scale bar: 50 μm. (F) Calcium influx assay showed Jurkat cell activation. Star indicated the non-stimulatory T cell antibody.
Figure 2. Inverse correlation of CD8 T cell motility with tumor density in cancer biopsies. (A) A scan of a colorectal cancer slice. (B) Three ROIs of panel A. Scale bar: 50 μm. (C) Color-coded track displacement of CD8 T cells. Length range: 0.5 (red) to 25 μm (white). (D) The speed of top 10% fastest CD8 T cells was plotted with mean±SD. (E) Percentage of EpCAM$^+$ volume in total volume. (F) The speed mean of CD8 T cell from three tumor samples plotted respectively against the tumor cell density of the corresponding ROIs from the biopsy. (G) Snapshots of time-lapse video from ROI2 showing: 1) CD8 T cell movement (blue rectangle); blue arrow: a T cell moved to a CD14$^+$ cell; 2) T-APC-tumor cell conjugates remained through 90 mins (yellow circle). Also see supplemental Video 2.
Figure 3. Linkage of T cell motility and exhaustion phenotypes in human tumors. (A) Diagram of a fresh tumor split for live biopsy imaging and flow cytometry. (B) CD8 T cell speed across multiple tumor biopsies. Blue bars: Specimens with motile T cells; black/grey: the immotiles (speed mean less than 1 μm/min, indicated by the red dashed line). Each bar (min to max with line at mean) represents the CD8 T cell motility data pooled from ≥ three ROIs of one biopsy. (C-J) Flow cytometry of cells from the same tumor biopsies of panel B. Percentage data plotted as both bar graphs and together with normalized gMFI grouped into motile or immotile to compare. Each dot represented one biopsy. * P<0.05, ** P<0.01 as determined by the Student’s t-test. N =17.
Figure 4. Exhausted T cells exhibited motile features. (A) Volcano plots of differentially expressed genes between CD38\textsuperscript{hi} (top 33\textsuperscript{rd} percentile of all samples) and CD38\textsuperscript{low} (bottom 33\textsuperscript{rd} percentile) colorectal samples. Red dots are above the cut off for p value = 0.05. (B) Gene ontology analysis of the upregulated genes in the CD38\textsuperscript{hi} compared to CD38\textsuperscript{low} samples. (C) Fold change of promotility genes in exhausted versus non-exhausted T cells from RNA-sequencing of melanoma or lung cancer samples in four publications. (D) Schematic diagram of the experimental design for panel E-G. (E) Representative images showing PD-1 staining on RFP (d14) and GFP OT-I cells (d4) that resided in the same field. Scale bar = 30 μm. (F) Speed-based color-coded track displacement of GFP and RFP OT-Is. (E) PD-1 intensity on OT-I cells was plotted against the mean speed of each cell. Data was pooled from three different ROIs and was representative of two independent experiments. P and r\textsuperscript{2} values were obtained by linear regression model.