AACR ANNUAL MEETING 2022 New Orleans
American Association for Cancer Research

Poster #630

Dynamic imaging of T cell surveillance in live tumor fragments using camelid nanobodies

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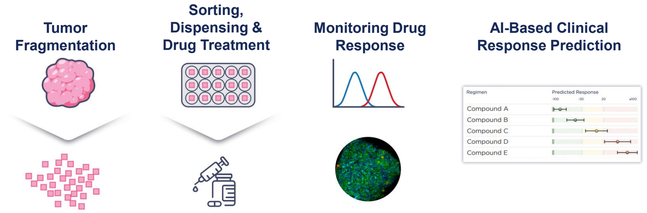
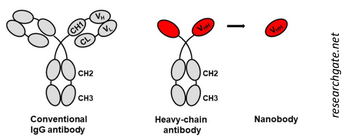
INTRODUCTION

Immunotherapies (ITx) have revolutionized the oncology landscape. However, predicting patient responses to ITx is difficult based solely on static correlates such as TILs (tumor infiltrating lymphocytes) localization and molecular signatures. Anti-tumor immune response depends on motile surveillance by TILs which recognize antigenic determinants and engage target cells in serial stop-and-go interactions that result in cell killing. However, the hostile tumor microenvironment (TME) can cause TIL dysfunction and lack of cytotoxicity which is manifested as either suppressed or aimless TIL motility.

- To determine which ITx works best for a given patient, we have developed a diagnostic platform using live tumor fragments (LTF) that preserve the TME and its immune cells.
- Antibody-based labeling of live tissues is hampered by slow diffusion and the potential for function-altering cross-linking.
- To overcome these limitations, we have used small camelid-derived monovalent antibodies (nanobodies) to monitor TIL motility in LTFs.



The Elephas Living Tumor Fragment (LTF) diagnostics Platform. Further description can be found in Posters #2779 and #2472



150 kDa

15 kDa

Nanobodies

- Antigen-binding fragments of heavy chain antibodies, typically from camelids or sharks
- Also termed VHH antibodies
- Single chain structure → structural stability
- Small size → rapid tissue perfusion, small spatial hindrance
- Monovalent binding → potentially low biological interference owing to lack of cross-linking activity.

- Human tumor excisions were cut into 300 x 300 x 200 µm LTFs, sorted into glass bottom multi-well plates and cultured.
- LTFs were incubated for 15 min – 24 h with an anti-hCD8a camelid VHH nanobody covalently labeled with AF594 and/or an anti-hCD4 camelid VHH nanobody covalently labeled with AF647 (Creative-Biolabs)
- 3-D imaging of LTFs was performed by multiphoton microscopy or spinning disk confocal microscopy.
- 3-D cell motility tracking was performed using Imaris software.

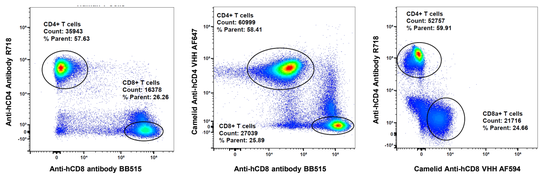


Figure 1. For specificity validation, human peripheral blood mononuclear cells were stained either with anti-hCD4 VHH-AF694 and anti-hCD8a whole antibody, or anti-hCD8a VHH-AF594 and anti-hCD4 whole antibody, and the staining patterns were compared by flow cytometry to that by anti-hCD4 and anti-hCD8a whole antibodies. We observed similar cell percentages in presumed CD4 and CD8 gates and minimal non-specific binding.

HUMAN CD4 AND CD8 CELL STAINING IN LTFs USING CAMELID NANOBODIES

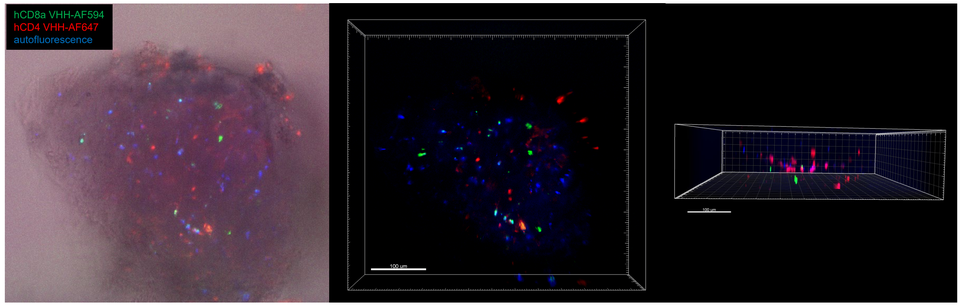


Figure 2. Anti-hCD8a camelid VHH-AF594 (green) and anti-hCD4 camelid VHH-AF647 (red) were added to human LTFs and imaged by spinning disk confocal microscopy without washing. The blue color indicates short wavelength tissue autofluorescence. The left image shows fluorescence and transmitted light overlay to visualize fragment shape. The center and right images show 3-D fluorescence. 20x objective, 24 x 5 µm z-stack MIP. Scale bars: 100 µm. We observed the expected mutually exclusive staining of lymphocyte-like cells, confirming the effectiveness of these VHH reagents for T cell staining in human LTFs. We also observed some additional co-staining of larger and autofluorescent cells, consistent with phagocytic uptake.

HUMAN T CELL MOTILITY TRACKING IN LTFs USING CAMELID NANOBODIES

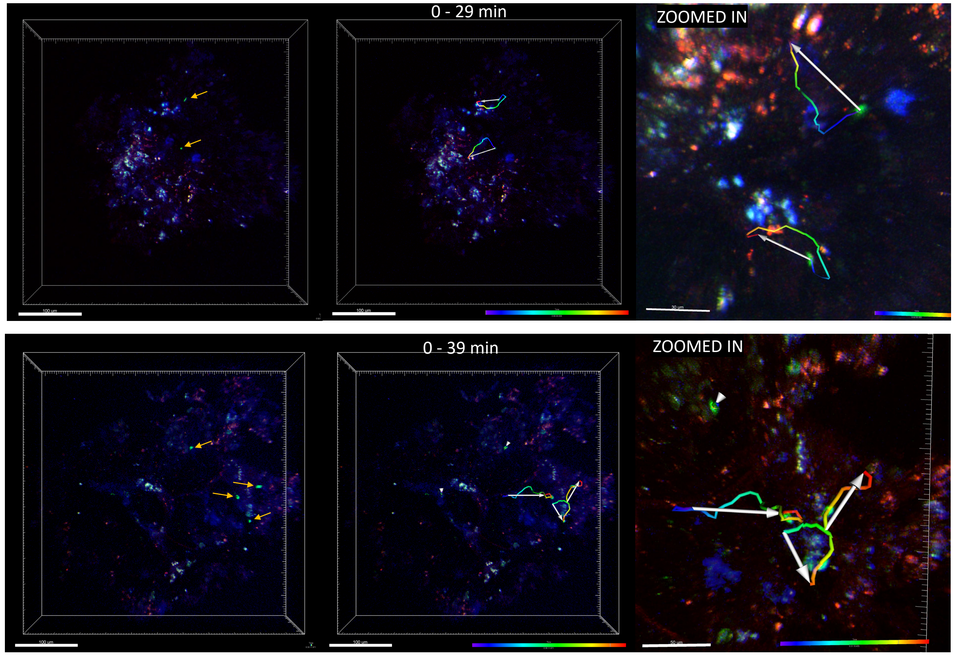


Figure 3. 3-D time-lapse multiphoton microscopy of human live tumor fragments stained with anti-hCD8a camelid VHH-AF594 (green). LTFs were cultured for 24 h followed by imaging for 29 or 39 min. The blue color indicates second harmonic generation (collagen) and short wavelength tissue autofluorescence. The red color indicates long wavelength fluorescence. The rainbow lines indicate cell tracks color-coded for time. The orange arrows point to CD8-stained cells. The white arrows indicate cell track displacements. 25x NA 1.1 objective, dz =5 µm z-stack MIP. We observed the expected staining of lymphocyte-like cells and larger cells. The smaller cells were 12 µm in diameter, cell surface-stained, and lacking autofluorescence, consistent with T cells. The larger cells were elongated, ramified, intracellularly stained, and distinctly autofluorescent, consistent with macrophages. 3-D motility tracking revealed characteristic translational motility of the smaller cells at up to ~10 µm/min along collagenous structures. In contrast, larger cells exhibited only slow motility.

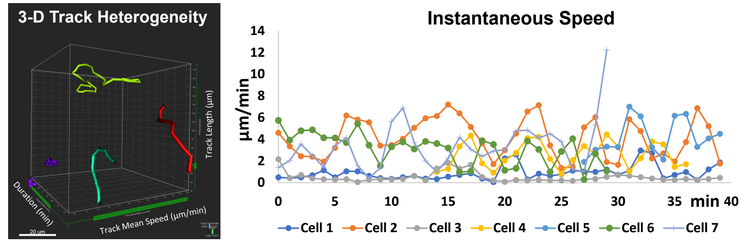


Figure 4. 3-D motility dynamics of anti-hCD8a camelid VHH-AF594 **stained cells in human LTF**. Left: 3-D representation of T cell tracks in one LTF from Fig. 3, bottom, showing movements in all directions and heterogeneity of track mean speed, length and duration. Right: Instantaneous velocity is defined as distance traveled during imaging time lapse (~60 s). The motile cells migrate in a stop and go pattern. This behavior is consistent with robust immune surveillance, similar to the T cell dynamics reported in *in vivo* systems.

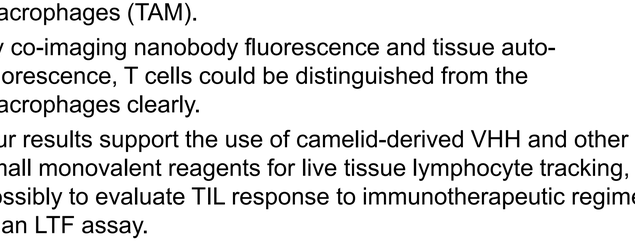


Figure 5. Motility analysis of human CD8 T cells in two LTFs. Each point represents a single cell. Confinement index is defined as the ratio of track displacement to track length. Low values indicate motility confinement, and high values (0.7 – 1) indicate directionality.

SUMMARY

- Individual T cells and CD4 vs. CD8 lineage identity can be tracked in living human tumor fragments using small, fluorescence-labeled VHH nanobody fragments.
- Sustained T cell motility suggests that camelid VHH reagent did not grossly interfere with T cell migratory behavior, but further examination is needed to understand if T cell function is altered.
- Camelid VHH nanobodies also labeled larger immotile cells that were autofluorescent, consistent with tumor-associated macrophages (TAM).
- By co-imaging nanobody fluorescence and tissue auto-fluorescence, T cells could be distinguished from the macrophages clearly.
- Our results support the use of camelid-derived VHH and other small monovalent reagents for live tissue lymphocyte tracking, possibly to evaluate TIL response to immunotherapeutic regimens in an LTF assay.