

P3.140: Dynamic Visualization of T-Cell Exhaustion in Lung Cancer Immunotherapy Using a LAG-3-Targeted Nanobody-Based Immunotracer

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INTRODUCTION

Immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have become an important component of systemic therapy for non-small cell lung cancer (NSCLC)[1]. However, primary and acquired resistance continue to limit durable clinical benefit. Moreover, immunotherapy can dynamically remodel the tumor immune microenvironment (TIME), leading to changes in immune-cell infiltration and inhibitory checkpoint expression[2]. Therefore, identifying patients likely to benefit from immunotherapy and monitoring treatment-associated immune changes remain important goals in precision immunology.

Lymphocyte activation gene 3 (LAG-3) is an inhibitory immune checkpoint frequently expressed on activated and dysfunctional tumor-infiltrating lymphocytes. LAG-3 and PD-1 exert partially nonredundant inhibitory effects on T-cell function, providing a biological rationale for combined blockade[3,4]. The clinical efficacy of relatlimab plus nivolumab has further established LAG-3 as a relevant therapeutic target[5]. However, LAG-3 expression is spatially and temporally heterogeneous and may change during treatment, whereas conventional biopsy provides information from only a limited region and time point[6].

Radionuclide molecular imaging enables noninvasive, whole-body, and repeatable assessment of immune checkpoint expression[7]. LAG-3-targeted PET and SPECT tracers based on antibodies, peptides, and nanobodies have demonstrated the feasibility of visualizing LAG-3 expression and its heterogeneity in both preclinical models and patients[8-10]. Among these platforms, nanobodies are particularly attractive because of their small size, rapid tissue penetration, and fast blood clearance, allowing high-contrast imaging within a relatively short time window.

In this study, we screened high-affinity nanobodies targeting human LAG-3, humanized the lead candidate, and developed ^{99m}Tc- and ⁶⁸Ga-based LAG-3-targeted SPECT/PET tracers. We further evaluated their targeting specificity and investigated whether LAG-3 nanobody imaging could distinguish tumors with different immune microenvironments and dynamically monitor LAG-3-associated changes following anti-PD-1 treatment.

METHODS

Nanobody screening and humanization. A Bactrian camel was immunized with recombinant human LAG-3 extracellular domain, followed by construction and four rounds of panning of a VHH phage-display library. Candidate nanobodies were screened by ELISA and flow cytometry, and seven representative clones with distinct CDR3 sequences were expressed and purified. The lead nanobody was subsequently humanized by framework substitution while retaining the three CDRs.

Radiolabeling and stability. Nanobodies were reduced with TCEP and purified before radiolabeling. Reduced nanobodies were labeled with ^{99m}Tc using a GH-mediated labeling system. For ⁶⁸Ga labeling, reduced nanobodies were conjugated with MAL-NTA followed by ⁶⁸Ga chelation. Radiochemical purity was evaluated by iTLC/HPLC, and stability was assessed in PBS and 10% FBS. Radioactive SDS-PAGE was used to verify the association of ^{99m}Tc with the nanobody.

In vitro characterization. Human LAG-3 overexpressing A549 cells (LAG-3⁺-A549) and parental A549 cells were used as positive and negative models, respectively. LAG-3 expression was confirmed by Western blotting. Cellular uptake, blocking assays, and saturation binding experiments were performed to evaluate target specificity and binding affinity (K_d).

Animal models and molecular imaging. LAG-3⁺-A549 and LAG-3⁻-A549 xenografts were established in nude mice for SPECT/CT and PET/CT imaging. ^{99m}Tc-LAG-3-Nb imaging was performed from 0.5–8 h after injection, whereas ⁶⁸Ga-NOTA-LAG-3-Nb PET/CT was performed from 0.5–4 h. Blocking studies were conducted using excess unlabeled nanobody. Tumor uptake and tumor-to-muscle/blood ratios were quantified by ROI analysis.

Tumor immune microenvironment and immunotherapy models. LLC and TC-1 tumors were established in hLAG-3 transgenic mice to compare LAG-3 imaging across different immune microenvironments. For immunotherapy studies, LLC-bearing mice received anti-PD-1 antibody or PBS every 3 days for three doses. ^{99m}Tc-LAG-3-h221 SPECT/CT was performed before treatment and on day 9. Tumors were subsequently analyzed by immunohistochemistry and flow cytometry for CD3, CD4, CD8, LAG-3.

Biodistribution and pharmacokinetics. Major organs and tumors were collected after imaging for γ -counting, and radioactivity was expressed as %ID/g. Blood pharmacokinetics of ^{99m}Tc-LAG-3-h221 were evaluated in BALB/c mice and fitted using a two-phase decay model.

Molecular dynamics analysis. All-atom molecular dynamics simulations were performed using GROMACS with the AMBER force field for 100 ns to compare nanobody systems before and after C-terminal modification. RMSD, RMSF, radius of gyration, molecular surface area, and short-range electrostatic/van der Waals interactions were analyzed to evaluate structural dynamics and radionuclide-associated interactions.

Statistical analysis. Data are presented as mean $\pm$ SD. Two-group comparisons were performed using Student's t-test, and multiple-group comparisons using one-way ANOVA. $P < 0.05$ was considered statistically significant.

DISCUSSION AND CONCLUSION

• KEY DISCUSSION POINTS

- This study developed a ^{99m}Tc-labeled humanized LAG-3 nanobody, h221, for rapid and specific imaging of LAG-3-positive tumors. During candidate screening, LAG-3-221 showed the most favorable overall imaging performance. After humanization, h221 retained favorable targeting specificity. In contrast, ⁶⁸Ga-NOTA-h221 showed lower tumor uptake and prominent renal and bladder accumulation, suggesting that chelator incorporation and labeling chemistry may alter tracer conformation and pharmacokinetics.
- In hLAG-3 transgenic mice, TC-1 tumors showed higher tracer uptake and LAG-3 expression than LLC tumors, indicating that h221 can distinguish LAG-3-associated differences across distinct tumor immune microenvironments. Following anti-PD-1 treatment, increased tumor uptake of ^{99m}Tc-LAG-3-h221 was accompanied by a higher proportion of LAG-3-positive T cells, supporting its potential for monitoring treatment-associated immune changes. In addition, h221 showed no obvious competition with relatlimab or the flow-cytometry antibody, suggesting recognition of a distinct or non-overlapping epitope.
- We also established a simplified ^{99m}Tc-labeling strategy using a C-terminal specific peptide sequence. Molecular dynamics and DFT analyses suggested that engineered nanobody may facilitate Tc association by modulating the local electrostatic and conformational environment, although the exact coordination mode remains to be clarified.
- This study established a LAG-3 nanobody-based radionuclide molecular imaging platform and developed both ^{99m}Tc- and ⁶⁸Ga-labeled tracers for noninvasive assessment of LAG-3 expression within the tumor immune microenvironment. Through candidate screening and subsequent humanization, we identified ^{99m}Tc-LAG-3-h221 as a lead tracer with favorable targeting properties. This probe was able to distinguish differences in LAG-3 expression across tumor models with distinct immunogenic profiles and to detect tumor immune microenvironment changes associated with anti-PD-1 treatment.
- In parallel, we developed a simplified ^{99m}Tc-labeling strategy based on a genetically encoded C-terminal specific peptide sequence. Our findings further suggest that radionuclide selection and labeling architecture may influence nanobody conformational dynamics and in vivo biodistribution, providing a rationale for future optimization of nanobody-based radiotracers. Collectively, this work provides a foundation for the development of LAG-3-targeted immunoimaging agents and optimization of nanobody radiolabeling strategies, while supporting the potential of LAG-3 molecular imaging as a noninvasive tool for patient stratification and assessment of immunotherapy-associated changes.

• CONCLUSION

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Artificial Intelligence (AI) Use Disclaimer

We used ChatGPT for language polishing.

RESULTS

