Unsupervised clustering identifies a CD161⁺ MAIT-like T cell population associated with anti–PD-1 response and immune-related toxicity in stage II-IV melanoma

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Background

Gating has been the standard approach to cytometry analysis for decades, but populations are defined by pre-specified, biaxial marker combinations, so cell types outside the gating hierarchy are missed regardless of panel size. On a 43-marker panel, possible marker combinations reach into the billions, and no gating scheme can exhaustively explore them.

Unsupervised methods overcome this limit by exploring the immune landscape. Algorithms such as FlowSOM for clustering and Uniform Manifold Approximation and Projection (UMAP) for visualization identify populations directly from the data, independent of prior assumptions about which markers define which cells.

Applied to 70 PBMC samples from 29 stage II-IV melanoma patients treated with anti-PD-1 therapy, our unsupervised clustering pipeline revealed a novel population associated with response and immune-related adverse events (IRAEs) that conventional gating did not capture.

Methods

We analyzed a 29-patient, 70 PBMC specimen melanoma dataset from the Huntsman Cancer Institute using a 43-marker mass cytometry panel.

Gating
Manual gating of FCS files was performed using CellEngine (CellCarta, Montreal, Canada) following the gating strategy found on app.teiko.bio/projects/HCI001/overview.

FlowSOM
Cells were clustered using FlowSOM. Self-organizing map (SOM) models were trained across grid sizes from 8×8 to 18×18. Each grid was evaluated using quantization error and topographic error. Both metrics were normalized to a 0–1 scale and summed, and the grid size that minimized the combined score was selected.

Following SOM training, hierarchical consensus metaclustering was applied to group SOM nodes into final clusters. Eleven k values (k=25 to k=75) were evaluated by expert review of cluster marker expression profiles on a QC dashboard. The k value with the most similar marker expression profile was selected.

Naming and Quality Control
Cluster names were assigned by evaluating marker expression profiles. The naming algorithm assigned biologically relevant labels (e.g., "CD8+ T Memory" vs. "CD8+ T Naive" based on CD27 and CD45RA), refined by marker-specific distinctions (e.g., CD11B). A cluster versus marker heatmap and per-cluster UMAP expression plots were generated for validation.

Statistical Analysis
Cluster frequencies were calculated as a percentage of the non-granulocyte population. Between-group comparisons of cluster frequencies (responder vs. non-responder) were performed using the Mann-Whitney U-test. Comparisons were performed across all timepoints pooled and within each idealized study timepoint.

Quantitative Metrics Identify Optimal SOM Grid Size

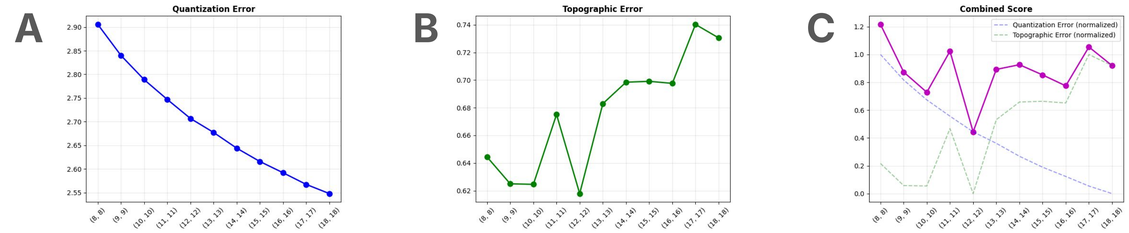


Figure 1. 12×12 SOM grid selected by normalized, combined scoring of QE and TE across grid sizes 8×8 to 18×18.
(A) Quantization error (QE), the mean Euclidean distance between each cell and its assigned SOM node, decreases steadily with larger grids. Lower values indicate each node more accurately represents its assigned cells. (B) Topographic error (TE), the proportion of cells whose two closest SOM nodes are not adjacent on the grid, remains low through 12×12 but increases at larger grids, indicating loss of map structure. (C) QE and TE are each normalized to a 0–1 scale across all grid sizes tested, then summed to produce a combined score. The 12×12 grid minimizes this score, representing the best balance of low quantization error and low topographic error. Dashed lines show individual normalized components.

Unsupervised Clustering Reveals CD161⁺ T Cell Subset

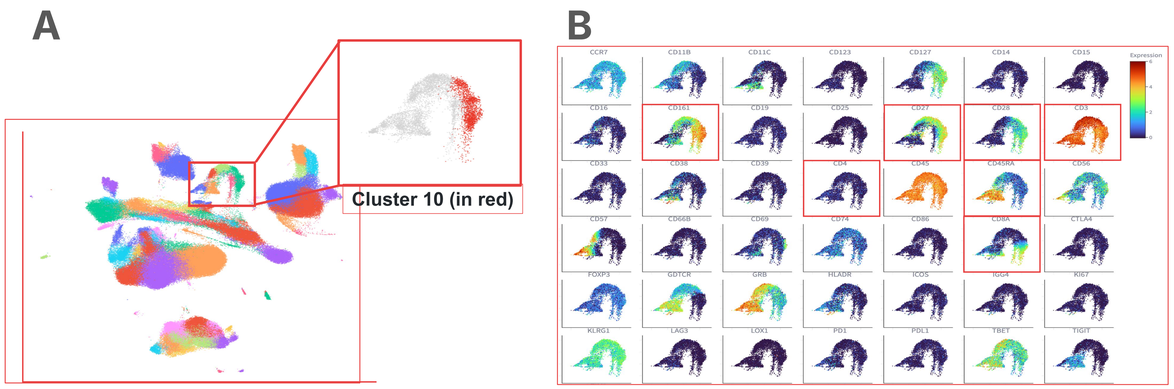


Figure 2. CD161⁺ T cell subset identified by unsupervised clustering that falls outside conventional gating hierarchies (n=29 patients, 70 PBMC samples, ~11M cells, 43-marker CyTOF panel).
(A) UMAP of all clustered cells colored by cluster assignment; Cluster 10 (red) localizes within the T cell region but does not correspond to any standard gated population. Zoomed view shows red Cluster 10 cells against all other gray cells. (B) Per-marker UMAP expression overlays colored by median channel value (MCV; blue = low, red = high). Red-boxed markers highlight the defining profile of Cluster 10: strong CD161, partial CD8, medium CD28, variable CD27, absent CD45RA, consistent with an innate-like or MAIT-like memory T cell phenotype not captured by standard CD4/CD8 gating.

CD161⁺ T Cell Frequencies Show a Significant Difference Between Responders and Nonresponders

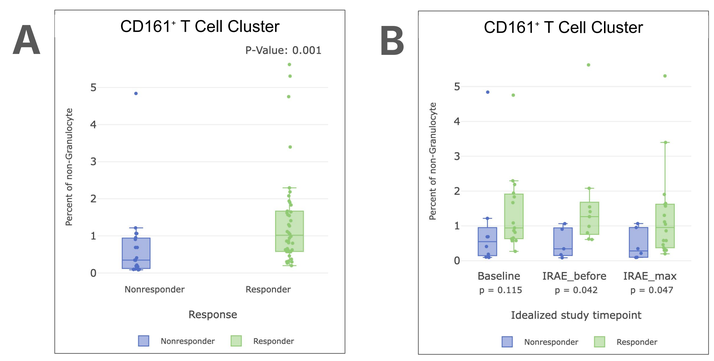


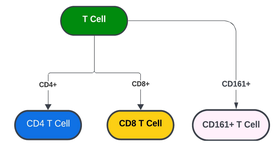
Figure 3. CD161⁺ T cell (Cluster 10) frequency is associated with response and immune-related adverse events in melanoma patients treated with anti-PD-1 therapy
(n=29 patients, frequency as percent of non-granulocytes).

(A) CD161⁺ T cell cluster frequency by response status across all timepoints (p = 0.001).

(B) CD161⁺ T cell cluster frequency by response status at each idealized study timepoint. Enrichment in responders reaches significance at IRAE_before (= 0.042) and IRpAE_max (p = 0.047) but not at baseline (p = 0.115).

Key Takeaways

Unsupervised clustering revealed a distinct CD161⁺ T cell population (Cluster 10) that was not captured by the conventional gating scheme. This subset showed heterogeneous CD8 expression, low CD45RA, and variable CD27, consistent with a memory-like phenotype, and expressed very high levels of CD161 compared to other T cells.



The frequency of this cluster was significantly higher in responders to anti-PD-1 therapy and was also elevated during on-treatment timepoints in patients who developed IRAEs. This enrichment suggests that CD161⁺ T cells may expand alongside therapeutic activation of the immune system, potentially contributing to both anti-tumor immunity and immune toxicity.

Published studies have linked CD161⁺ and MAIT-like T cells to Th17 cytokine programs, IL-17/IL-23-driven inflammation, and checkpoint inhibitor-associated toxicity. Our findings build on this by providing direct evidence from high-dimensional cytometry that a CD161-rich T cell compartment is associated with clinical outcomes in melanoma.

Together, these findings highlight the value of computational clustering approaches in uncovering rare, biologically meaningful immune subsets that may contribute to both therapeutic response and autoimmune toxicity in melanoma patients treated with PD-1 blockade.

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