Multiplexed Spatiotemporal Proteomics-Guided CAR-T Cell Engineering: A Modular Pipeline for Antigen Drift Compensation in Relapsed/Refractory Hematologic Malignancies

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Abstract. Antigen escape remains a principal mechanism of treatment failure following chimeric antigen receptor T-cell (CAR-T) therapy in relapsed/refractory hematologic malignancies. We present a modular, multiplexed spatiotemporal proteomic framework integrating mass cytometry (CyTOF), single-cell RNA sequencing (scRNA-seq), and CRISPR-Cas9-mediated tandem antigen-targeting to counteract clonal antigen drift in B-cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma (DLBCL). Utilizing a cohort of 84 post-CAR-T relapse specimens, our pipeline systematically mapped intratumorally heterogeneous surface antigen profiles at single-cell resolution, enabling iterative CAR construct recalibration. Dual-targeting CD19/CD22 constructs engineered via our protocol demonstrated a 61.3% reduction in antigen-loss escape variants in murine xenograft models. These findings establish a reproducible, clinically translatable methodology for adaptive CAR-T cell redesign informed by real-time proteomic surveillance.

Keywords: CAR-T cell therapy, antigen escape, spatiotemporal proteomics, single-cell RNA sequencing, CRISPR-Cas9 multiplexing, hematologic malignancies, CD19/CD22 dual targeting, clonal tumor heterogeneity, adoptive cell immunotherapy

Introduction

Chimeric antigen receptor T-cell (CAR-T) immunotherapy has fundamentally transformed the therapeutic landscape of relapsed and refractory hematologic malignancies over the past decade. Since the landmark approvals of tisagenlecleucel and axicabtagene ciloleucel, CD19-directed CAR-T products have achieved complete remission rates exceeding 70–80% in heavily pretreated B-cell acute lymphoblastic leukemia (B-ALL) cohorts. However, the durability of these responses remains critically undermined by a well-characterized immunoediting phenomenon: antigen escape, wherein tumor subclones harboring diminished or absent surface expression of the targeted antigen selectively expand under CAR-T-mediated selective pressure. As of 2024–2026, antigen escape accounts for approximately 30–50% of post-CAR-T relapses across CD19- and BCMA-directed constructs, representing one of the most formidable unresolved barriers in cellular immunotherapy. The mechanistic underpinnings of antigen drift are multifactorial and encompass transcriptional silencing of target loci, lineage switching from B-cell to myeloid or T-cell phenotypes, alternative splicing of surface receptor exons, and stochastic epigenetic remodeling within tumor microenvironmental niches. Conventional single-time-point immunophenotyping, while diagnostically informative, fails to capture the longitudinal, spatially heterogeneous proteomic dynamics that precede and accompany antigen loss. This diagnostic insufficiency precludes proactive therapeutic adaptation and perpetuates a reactive clinical paradigm that is ill-suited to the biological complexity of post-immunotherapy relapse. Recent advances in mass cytometry (CyTOF), spatially resolved transcriptomics, and single-cell multi-omics integration have opened new methodological avenues for resolving intratumorally heterogeneous antigen architectures at single-cell resolution. Concurrently, CRISPR-Cas9 genome editing platforms have matured sufficiently to enable scalable, multiplexed reconfiguration of CAR construct specificity, including the incorporation of tandem or bicistronic dual-targeting cassettes directed against antigenically redundant surface epitopes such as CD19, CD22, and CD10. The convergence of these technologies creates a tractable, albeit technically demanding, opportunity to implement an adaptive, proteomics-informed CAR-T redesign cycle that responds directly to evolving tumor antigen landscapes. Despite this conceptual promise, no standardized, end-to-end methodological pipeline currently exists that integrates spatiotemporal proteomic surveillance with iterative CAR construct engineering within a unified, clinically translatable framework. Existing efforts remain fragmented across discrete discovery and manufacturing phases, with insufficient cross-validation between ex vivo proteomic readouts and in vivo CAR-T functional outcomes. The present study addresses this gap by delineating a modular, multiplexed spatiotemporal proteomics-guided pipeline for adaptive CAR-T cell engineering. The manuscript is organized as follows: Section 2 details the patient cohort characteristics and specimen acquisition protocols; Section 3 describes the integrated CyTOF/scRNA-seq antigen profiling workflow; Section 4 presents the CRISPR-Cas9 tandem-targeting engineering strategy; Section 5 reports in vitro and xenograft functional validation data; and Section 6 discusses translational implications and prospective clinical integration pathways.

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