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Microfluidic Platform to Selectively Enrich Rare Tumor-specific T-cells from Patient-derived Peripheral Blood

By

PREM SANTHOSH SUBRAMANIAN

THESIS

Submitted in partial satisfaction of the requirements for the degree of

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DAVIS

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TABLE OF CONTENTS

1. ABSTRACT	1
2. INTRODUCTION	2
3. RESULTS	3
3.1. Device Design and Validation	3
3.2. Phenotypic Validation of MART-1 specific T-cells and MART-1 pMHC expressing SKMEL5 Melanoma Cell Line.	4
3.2.1. C6 Jurkat cells express the TCR specific for MART-1 peptide:	4
3.2.2. SKMEL5 melanoma cell line expresses MART-1 pMHC complexes on its surface.	6
3.3. Devices coated with Biotinylated MART-1 pMHC selectively capture C6 Jurkat Cells.	6
3.4. The capture of C6 remained specific at physiological concentrations of MART-1 pMHC on a multi-shear microfluidic platform.....	7
3.5. Capture efficiency of C6 using native p-MHCs isolated from MEL624, SKMEL5 melanoma cell lines	8
3.5.1. Pilot Coating strategy using anti-HLA class I antibody to immobilize pMHC complexes on device channels.	8
3.5.2. Co-immunoprecipitation-based total pMHC isolation from tumor cell lines to improve C6 capture sensitivity in the device.	9
3.6. Captured C6 cells show a distinct Calcium influx associated with increased Fluo-4 AM fluorescence intensity	11
4. MATERIALS AND METHODS	12
4.1. Device fabrication, surface coating and perfusion.....	12
4.2. Jurkat Cell Culture and Staining	13
4.3. Flow cytometry analysis of C6 Jurkat and Melanoma Cells	13
4.4. Melanoma Cell Culture and Whole cell protein isolation	13
4.5. Isolation of pMHCs and Surface Coating.....	14
4.6. Loading Fluo-4 and Fura-RED in Jurkat Cells for analysis of Calcium influx.....	14
5. DISCUSSION	15
6. CONCLUSION	16
7. REFERENCES.....	17

1. Abstract

Adoptive cell therapy (ACT) therapies, including CAR-T and tumor-infiltrating lymphocyte (TIL) treatments have achieved remarkable clinical success in hematological malignancies. However, their efficacy in solid tumors remains limited by the rarity of naturally occurring tumor-specific T cells and the highly heterogenous tumor antigen profile. Current strategies rely on prior knowledge of specific antigen targets, a rather difficult requirement in personalized oncology. This thesis presents a microfluidic platform that utilizes shear force as a biophysical discriminator to selectively capture the rare tumor-specific T-cells in an antigen-agonistic manner. Using an eight-channel device designed to generate a defined range of shear, MART-1 specific T-cells are shown to be selectively captured from mixed populations on surfaces with immobilized native HLA class I peptide-major histocompatibility complexes (pMHC) isolated using immunoaffinity chromatography from melanoma cell lines. Our results demonstrate that capture sensitivity is robust even at low, physiologically relevant pMHC concentrations and is predominantly driven by the stability of agonistic TCR-pMHC interactions under a dynamic flow. Furthermore, ratiometric Ca^{2+} analysis confirms that the captured T-cells remain functionally active and elicit robust signals upon adhesion. This research establishes a framework for isolating rare, therapeutically active T-cells without needing prior antigen profiling, offering a potential paradigm shift in the manufacture of personalized immunotherapies for heterogenous tumors.

2. Introduction

Adoptive cell transfer therapy (ACT) has emerged as a paradigm shift in oncology, involving the isolation of immune-active cells from a patient, ex vivo expansion of these cells, and subsequent reinfusion to eliminate tumor cells¹. This approach was developed following the discovery of interleukin-2 (IL-2), which enabled a bulk expansion of T-cells while preserving their effective functionality. Within this field, the Chimeric Antigen Receptor (CAR) T-cell therapy, which involves genetic modification to incorporate co-stimulatory molecules into the isolated T-cells to enhance their persistence and proliferation². Modern generation CAR-T therapy has advanced to the level where they use an extracellular single-chain variable fragment (scFv) to recognize the surface antigens on tumors directly³⁻⁵. Upon successful binding, the T-cell forms an immunological synapse, releasing cytotoxic enzymes perforin and granzyme, or via the Fas-FasL apoptotic pathway to eliminate the tumor cell⁶. CAR-T therapy went on to achieve a well distinguished clinical success in hematological malignancies, achieving complete remission rates between 70-94% in patients with B-cell acute lymphoblastic leukemia^{1,7}. Another milestone was reached in 2024 when the first autologous tumor-infiltrating lymphocyte (TIL) therapy received FDA approval for treating advanced melanoma^{6,8}.

Despite the groundbreaking success, ACT still faces significant limitations that hinders its efficacy, a prominent constraint being the rarity of naturally occurring tumor-specific T-cells within the patient's peripheral blood or in the TIL population. Obtaining a sufficient quality of healthy and functional T-cells for genetic modification is a major challenge, particularly in patients who have received chemotherapy or radiation². Another important limitation is the intense and time-consuming manufacturing process, that can take until four weeks for production, which is a significant amount of time for patients with rapidly advancing cancers. Furthermore, this personalized manufacturing model can cost anywhere between \$370,000 and \$530,000 per single treatment in the United States³.

Most current strategies to enrich tumor-specific T-cells begin with the prior knowledge of one or more target antigens, an example being CAR-T cells to be engineered to recognize defined tumor-associated antigens such as CD19, BCMA, or NY-ESO-1. However, many solid tumors contain highly individualized neoantigen profiles, and even within the same patient, only 20% of predicted neoantigens are shared between the primary and metastatic lesions, indicating high spatial and temporal heterogeneity^{2,9,10}. Hence, therapies that only target a limited set of known antigens may fail to attain clinically relevant targets in individual patients⁷. Hence, there has been a progressive interest in antigen-agonistic strategies that select tumor-specific T-cells directly based on native TCR-pMHC interactions without requiring prior specifications of the tumor antigens^{7,11}. The work presented in this thesis builds on fundamental biophysical concepts of TCR-pMHC recognition. T-cells that bind to its agonistic pMHCs are observed to sustain strong bond lifetimes, enabling high functionality and stable immunological synapse that antagonistic pMHCs T-cells scan antigen-presenting cells under the physiological shear induced by blood flow and constant cell movement, implying the need for high-avidity interaction to elicit a response.^{1,7}

Recent work also indicates T-cells do not solely rely on TCR-pMHC affinity and dwell time, but also actively apply piconewton-scale forces to better discriminate the antigen. This mechanically productive engagement in the range of 12-19 pN across individual TCR-pMHC bonds precedes calcium flux and further mechanotransduction that correlates with T-cell

activation. When the TCR-pMHC forces are mechanically capped below this range, T-cells were found to still bind the antigen, but failed to display the activation signals, thereby unable to discriminate between strong and weak agonists. Conversely, conditions that permit higher force transmission between TCR-pMHC selectively amplified responses to agonistic ligands¹⁴⁻¹⁶. Microfluidics offers a great advantage for implementing shear as a discriminator for selective capture of rare cells as they offer precise control over important channel characteristics such as geometry, shear profile during flow and surface area to volume ratio¹². Though microfluidics has been used for high-throughput screening applications and on-chip simulation, their potential in antigen-agonistic T-cell selection remains uncommon.

This thesis proposes a microfluidic platform that utilizes hydrodynamic shear to discriminate tumor-specific T-cells in an antigen-agonistic manner. Native HLA class I pMHC complexes are isolated using immunoaffinity purification from melanoma as well as control tumor cell lines and are immobilized on the microfluidic channels to simulate physiological pMHC densities and heterogeneity. C6 Jurkat cells expressing a TCR specific for MART-1, along with Wild-type Jurkat cells are perfused through the channels at defined shear rates to quantitatively evaluate antigen-specific capture. This quantitative assessment is defined using the term ‘Capture Sensitivity’ which is the ratio of the C6 cell count adhered and the total amount of C6 cells perfused through the channel. Capture Sensitivity is assessed as a function of shear stress, to select the most optimal shear window for maximum C6 sensitivity. The downstream activation of the adhered C6 cells is also assessed using the ratiometric Ca^{2+} analysis. Integrating microfluidics, pMHC biochemistry and qualitative functional readouts, this research aims to establish an antigen-agonistic platform for enriching rare tumoricidal T-cells from patient derived peripheral blood samples, that can address some key limitations in the application of ACT to heterogeneous malignancies.

3. Results

3.1. Device Design and Validation

The eight-channel microfluidic device used in this study was specifically designed to generate a defined range of shear stresses within a single platform. With a common inlet and outlet, each channel consisted of equalized upstream micro-channels to ensure constant volumetric flow, though the width increases from channel 1 to channel 8. Quantitative pressure analysis using COMSOL verified that the shear profile is distinct across the channels, and since the device is hydraulically balanced, the magnitude of shear can be shifted upward or downward by simply adjusting the inlet flow rate. Furthermore, perfusion of cells through the device at a concentration of 0.5 million cells/mL for 30 minutes showed that the % influx of cells entering each channel did not have any significant difference. The hydrodynamic validation demonstrates the tunable and robust nature of the platform that is to serve as the experimental base for the biological capture and activation assays that were conducted subsequently.

The rationale of designing the multi-shear eight-channel device lies to address the challenge of detecting tumoricidal T-cells within the PBMC population, on channels coated with tumor-derived native pMHC complexes. In a physiological setting, the expected density of the relevant tumor-derived pMHCs is low, in addition to a low abundance of the cognate T-cell clones. Hence, it is essential for the platform to be functionally capable of discriminating these rare T-cells, despite weak antigen density and large background of non-specific cells. Since T-cells form

stronger and more stable bonds for agonistic pMHCs, they are likely to resist detachment at higher shears, in contrast to non-specific T-cells that engage in low-affinity interactions that easily detach upon increasing shear. Channels operating at lower shears provide high enough sensitivity for the rare T-cell capture while channels at higher shears only retain the stronger, agonistic pMHC - T-cell interactions.

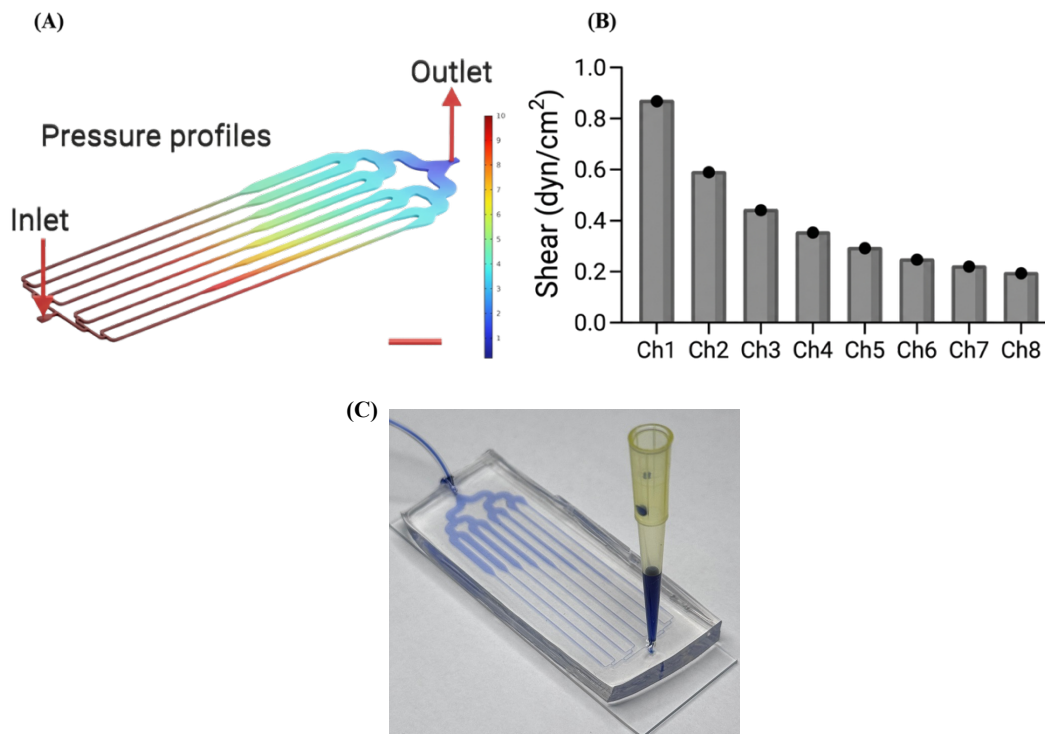


Figure 1. Device geometry and perfusion profile: (A) COMSOL pressure map across the eight channels (inlet on the left, outlet on the right; warmer colors indicate higher pressure). Upstream micro-resistors (narrow segments) were designed to equalize resistance, ensuring constant volumetric flow to each channel despite the increase in width across the channels (from Ch1 to Ch8). Additionally, the length of the channels was adjusted so that the total surface area across each channel is equal. Scale bar: 5 mm. (B) Shear stress across the channels decrease monotonically from Ch1 to Ch8, generating eight distinct shear conditions within a single experiment. (C) Representative photograph of the device.

3.2. Phenotypic Validation of MART-1 specific T-cells and MART-1 pMHC expressing SKMEL5 Melanoma Cell Line.

3.2.1. C6 Jurkat cells express the TCR specific for MART-1 peptide:

To validate the T-cell recognition of MART-1 peptide-containing MHC (MART-1 pMHC), we used the Clone 6 (C6) Jurkat cell line, which was engineered to express a TCR specific for the MART-1 peptide (insert sequence and details) presented on the MHC complex. The wild-type (WT) Jurkat cells lack this MART-1 pMHC recognizing TCR and hence served as a control population. To confirm that our C6 Jurkat cells express the MART-1 pMHC, we ran a simple flow cytometry by staining both C6 and WT cells with biotinylated MART-1 pMHC, followed by a secondary stain of Fluorescently tagged Neutravidin. The gated population of live cells showed that the C6 cells were positive for the MART-1 pMHC, whereas the WT population showed background level signal. This confirmed that our C6 Jurkat cells expressed the TCR specific to the MART-1 pMHC, and this specificity formed the base of all experiments covered in this article.

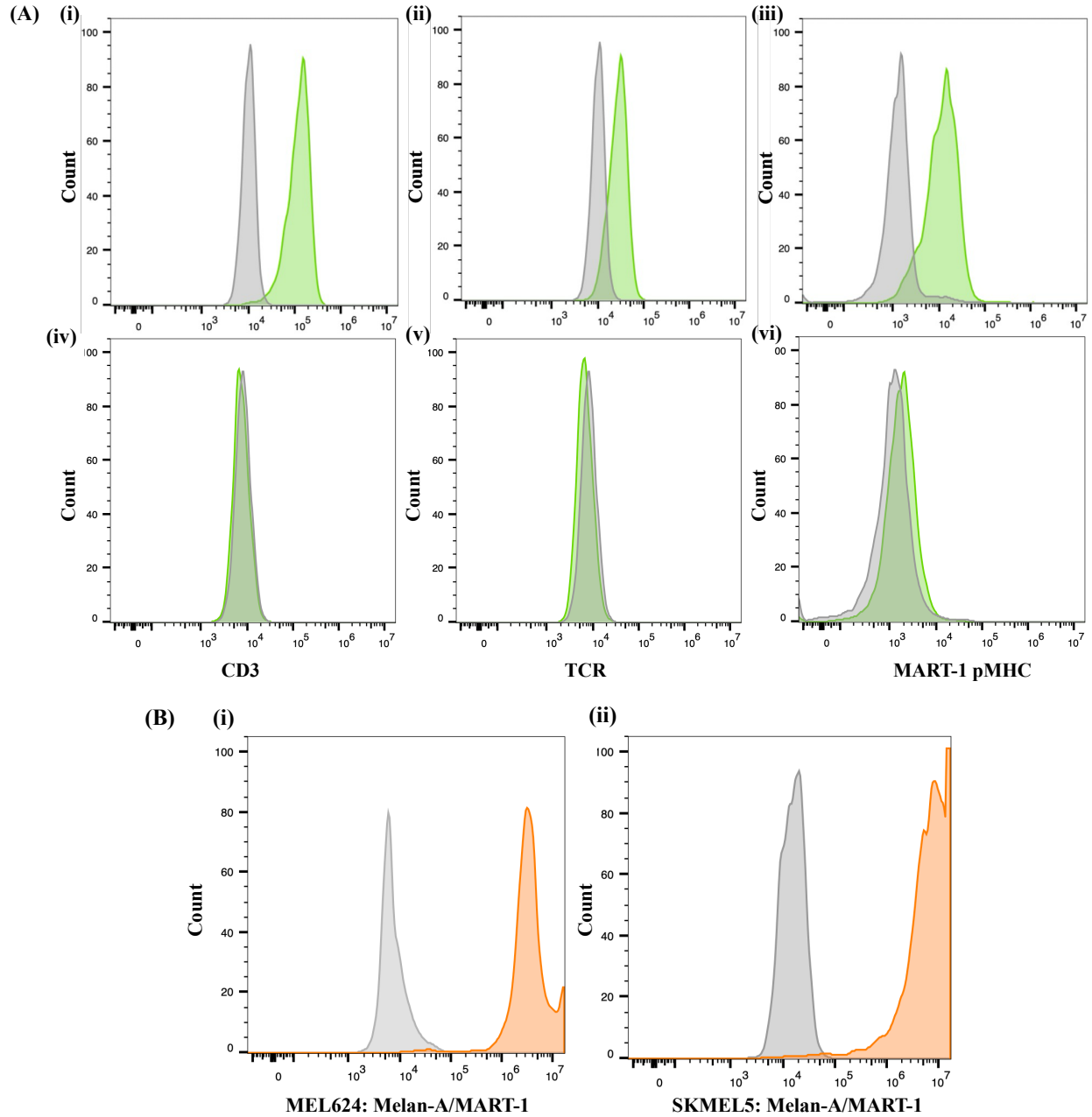


Figure 2. Flow cytometry validation of MART-1-specificity in C6 Jurkat cells and MART-1/Melan-A expression in SKMEL5. (A) C6 Jurkat cells: (i),(ii),(iii) and WT Jurkat cells: (iv),(v),(vi) were stained with anti-CD3, anti-TCR and MART-1 pMHC tetramer respectively. Histograms show tetramer fluorescence intensity for live cells, with unstained C6 and WT as the baseline intensity. C6 cells exhibit a clearly separated peak confirming the recognition of MART-1 pMHC, while WT signal remained close to the background. (B) MEL624 (i) and SKMEL5 (ii) melanoma cells were stained with anti-MART-1/Melan-A antibodies (pre-conjugated BD Alexa Fluor 647). Both cell lines produce noticeable rightward shifts in fluorescence intensities relative to the control, confirming MART-1/Melan-A pMHC expression.

3.2.2. SKMEL5 melanoma cell line expresses MART-1 pMHC complexes on its surface

To confirm the SKMEL5 cell line as a MART-1 positive target, the surface or intracellular expression of MART-1/Melan-A was assessed by flow cytometry. Using the Donkey anti-mouse IgG (H+L) Alexa Fluor 647 and pre-conjugated mouse IgG isotype as negative controls, the cells were stained with three independent MART-1/Melan-A specific antibodies. The TCR-like anti-MART-1 antibody displayed a distinct population positive for MART-1, indicating the recognition of the MART-1 pMHC complexes on SKMEL5. We yielded similar results using the Melan-A antibody A103 (mouse IgG1 k, Santa Cruz) and the pre-conjugated Anti-MART-1/Melan-A A103 antibody from BD. When compared to the isotype control and the unstained SKMEL5 samples, all the three different Anti-MART-1/Melan-A antibodies showed a distinct, well-separated positive peak, demonstrating that the SKMEL5 cell line expresses detectable levels of the target antigen for MART-1 pMHC-specific T-cell capture in a microfluidic device.

3.3. Devices coated with Biotinylated MART-1 pMHC selectively capture C6 Jurkat Cells.

To carry out a simple demonstration of the proof-of-concept, we qualitatively examined the capture specificity of C6 Jurkat Cells on single-channel devices coated with Biotinylated MART-1 pMHC. A 1:1 mixture of C6 and WT Jurkat cells were perfused at a defined shear rate, and time-lapse fluorescence recordings were acquired from a section of the device along the length of the perfusion. The cells that remained adherent were counted and classified as C6 or WT based on the cell counts with Cell Tracker Green signal (C6) and Cell Tracker Deep Red signal (WT). C6 cells had a higher frequency of adhesion on the channel, and remained adherent for the duration of the perfusion, leading to a noticeable accumulation along the coated surface. WT cells in the same field of view rarely established sustained adhesion, without any obvious enrichment over the C6 cells. Although this experiment was assessed qualitatively, it provides direct visual evidence that MART-1 pMHC-coated channels support the stable capture of C6 Jurkat cells from a mixed population under shear. This experiment serves as a proof-of-concept that pMHCs displaying tumor-associated peptides can be effectively used as a strategy to selectively capture T-cells bearing the agonistic TCR.

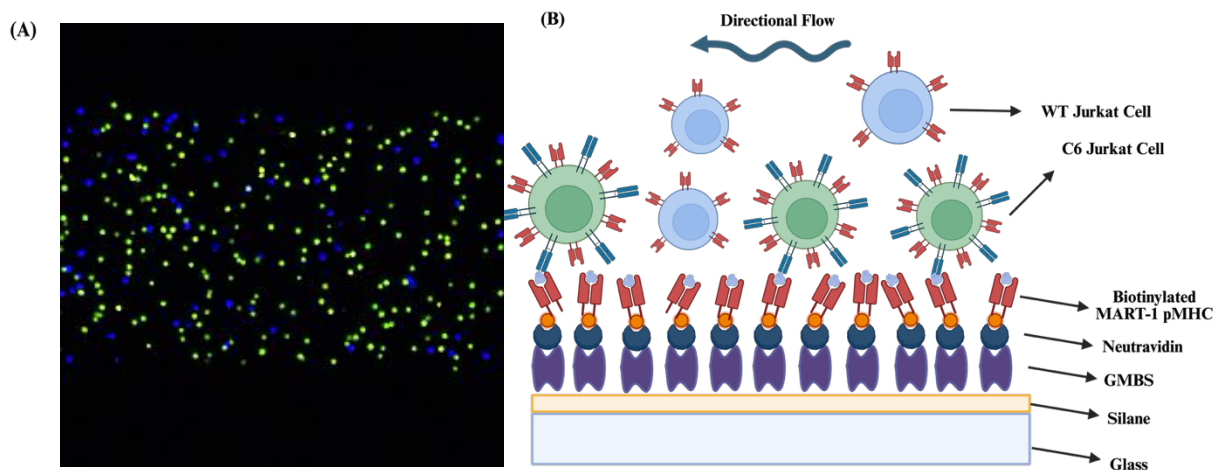


Figure 3. Proof-of-concept display of C6 Jurkat capture using MART-1 pMHC specificity. (A) Time-lapse fluorescence sequence from a single-channel device coated with biotinylated MART-1 pMHC and perfused with a 1:1 mixture of C6 (green) and WT (deep red) under a defined shear rate. The differential staining of C6 and WT cells with Cell Tracker Green and Cell Tracker Deep Red allows visual discrimination of the two populations during perfusion. C6 cells evidently appeared to adhere more to the surface, forming stable bonds whereas WT cells rarely sustained any interaction with the surface along the field of view. **(B)** Schematic representation of the experimental configuration, illustrating the coating technique used, and the specific interaction of C6 cells with the MART-1 pMHC on the channel surface.

3.4. The capture of C6 remained specific at physiological concentrations of MART-1 pMHC on a multi-shear microfluidic platform

Following the proof-of-concept experiment, our next objective was to evaluate the C6 capture on channels coated with lower concentrations of MART-1 pMHC, closer or even lower than physiological range of tumor-associated pMHCs seen in patients (may need more info or reference on this). Devices were prepared with the coating concentrations of 1 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$ of MART-1 pMHC. A steady perfusion of a 1:1 mixture of C6: WT Jurkat cells was followed by a wash; the device was imaged and the cells that remained adherent were counted and classified as C6 or WT based on the cell counts with Cell Tracker Green signal (C6) and Cell Tracker Deep Red signal (WT). As the coating concentration of MART-1 pMHC decreased, there was a decline in the captured C6 cells, as expected from reduced antigen availability. However, the fraction of C6 cells captured remained significantly higher.

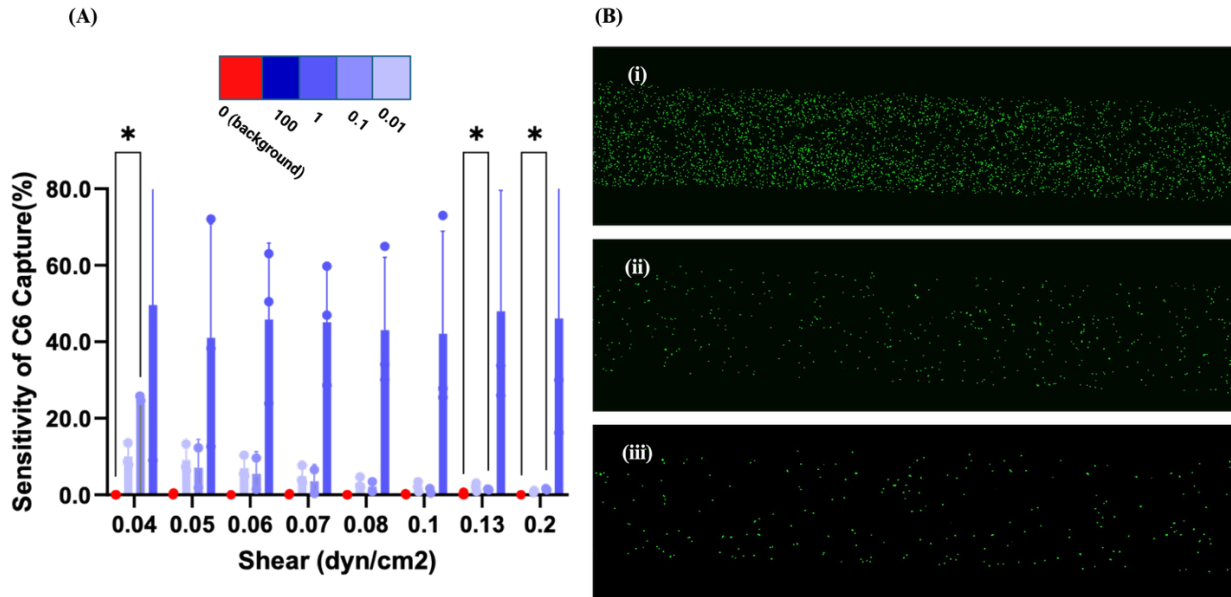


Figure 4. Sensitivity of C6 Jurkat Cell capture in varying densities of MART-1 pMHC spiked in Biotin-BSA coating. (A) Normalized sensitivity of C6 capture (%) as a function of shear stress for devices coated with 1%, 0.1% and 0.01% of MART-1 pMHC in 100 $\mu\text{g/mL}$ of Biotin-BSA. Each blue point represents an individual device, and the red points indicate the background C6 capture seen in devices with 100% Biotin-BSA. Even at lower (physiologically relevant) concentrations of MART-1 pMHC, the sensitivity of C6 capture is robust over the range of shears, whereas Biotin-BSA coatings show negligible C6 sensitivity. (B) Representative fluorescent images of Specific T-cell capture in devices coated with (i) 1% MART-1 pMHC; (ii) 0.1% MART-1 pMHC; and (iii) 0.01% MART-1 pMHC. Sensitivity of C6 Jurkat cells is preserved even when MART-1 pMHC is diluted into an excess of inert Biotin-BSA.

Even at concentrations as low as 0.1 $\mu\text{g/mL}$, we were able to achieve a capture sensitivity of 30% in the channel with the lowest shear. Overall, our microfluidic platform retains C6 specificity, allowing the quantifiable capture of MART-1 pMHC-reactive Jurkat cells even at a substantially weak coating concentration. The observed gradient in C6 capture depending on the MART-1: Biotin-BSA ratio further validates that our microfluidic platform can discriminate rare tumor-specific T-cells even in the presence of a large non-specific background. The sensitivity of capture scales with the density of cognate MART-1 pMHC and is hence predominantly driven by the specific TCR-MART-1 pMHC interactions. Alongside the proof-of-concept experiment, we also establish a reliable functional sensitivity to enrich tumor-specific T-cells from a mixed population.

3.5. Capture efficiency of C6 using native p-MHCs isolated from MEL624, SKMEL5 melanoma cell lines

3.5.1. Pilot Coating strategy using anti-HLA class I antibody to immobilize pMHC complexes on device channels.

With the established validation that the device can selectively capture C6 Jurkat Cells at varying levels of its cognate MART-1 pMHC even in the presence of a background, we next asked whether the device can detect antigen-specific T cells under a more physiologically relevant system. Alongside SKMEL5, a second melanoma cell line MEL624 was also selected, based on previous scientific evidence of MART-1 pMHC expression at steady levels. Prior to adopting co-immunoprecipitation for isolating tumor-derived pMHCs, we evaluated an alternate strategy where we functionalized the devices with a biotinylated anti-HLA class I antibody. The channels were then exposed to whole cell lysates from MEL624 and SKMEL5 melanoma cell lines. In this configuration, the antibody was intended to immobilize native pMHC complexes directly from the whole cell lysate, to generate a heterogenous surface display of tumor-derived pMHCs. Additionally, lysates from the breast cancer cell line MDA-MB-231, that do not express MART-1, served as a negative control for antigen specificity. Qualitative analysis of time-lapse recordings as well as end point images indicated a high non-specific adhesion of both C6 as well as WT Jurkat cells across all conditions. These observations suggest that residual anti-HLA binding sites, or other proteins within the lysate generated a highly non-specific adhesion pattern, thereby counterintuitive to discriminating specific TCR-pMHC interactions. Hence, we transitioned to the biochemical isolation of total pMHC complexes from the tumor cell lines, followed by immobilization of these purified complexes on the device surface, that substantially improved the specificity of C6 capture, as in the subsequent sections.

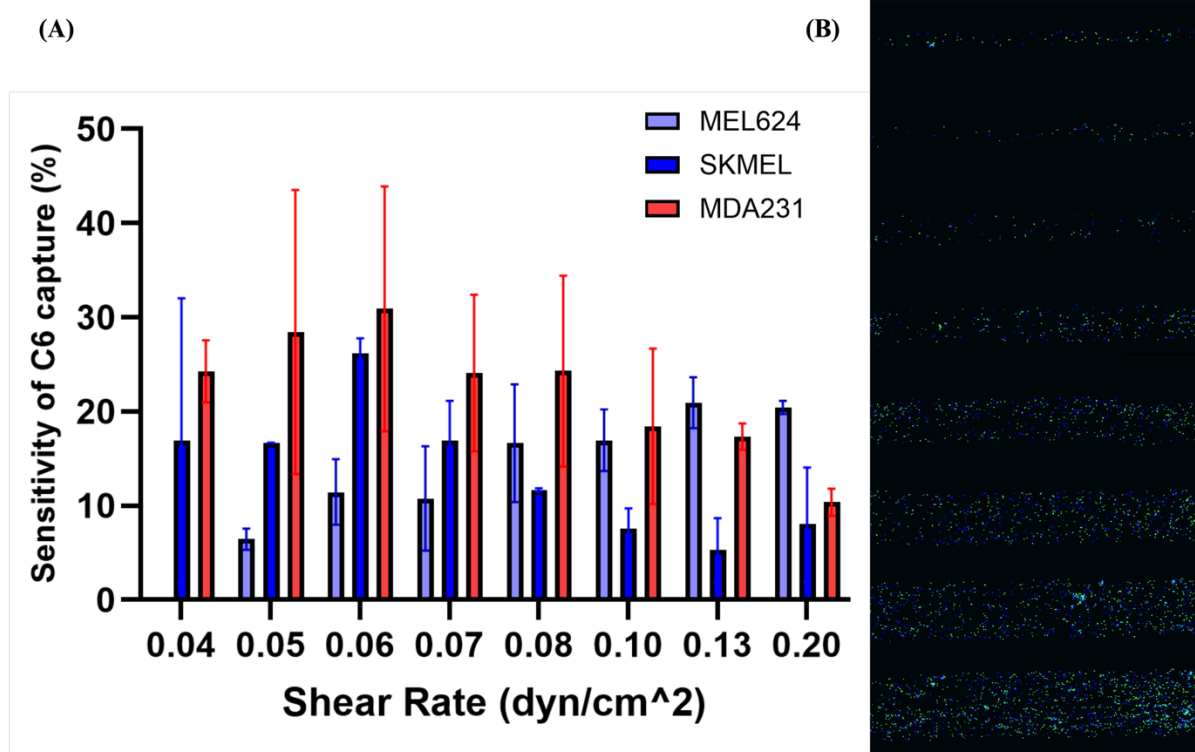


Figure 5. Anti-HLA class I- based immobilization of tumor-derived pMHCs from whole cell lysates yields high non-specific adhesion. (A) Normalized sensitivity of C6 capture (%) as a function of shear rate for devices coated with biotinylated anti-HLA class I antibody following exposure to whole cell lysates from MART-1 positive melanoma cells SKMEL5 and MEL624 or MART-1 negative breast cancer cell line MDA-MB-231. C6 capture remained comparable or higher in the MDA-MB-231 whole cell lysate-coated device, indicating non-specific adhesion of C6 cells. Furthermore, a high capture of WT Jurkat cells was also seen across all shears, following a 1:1 perfusion of C6 and WT Jurkat cells, consistent with high non-specific binding. **(B)** Representative fluorescence image showing all channels of a device coated exposed to MDA-MB-231 whole cell lysate. The data suggests residual anti-HLA binding sites or other adsorbed lysate proteins that led to a broadly adhesive surface that disrupts specific TCR- pMHC interactions, requiring an alternate coating strategy.

3.5.2. Co-immunoprecipitation-based total pMHC isolation from tumor cell lines to improve C6 capture sensitivity in the device.

The total HLA-1 pMHC complexes from both MEL624 and MDA-MB-231 cell lines were extracted using immunoaffinity chromatography. The isolated pMHC complexes were then biotinylated and used to surface coat the devices. To serve as a non-pMHC background/control, devices were coated with Biotin-BSA. A 1:1 mixture of C6: WT Jurkat cell suspensions were perfused through the devices coated with all the conditions that were described and the sensitivity of C6 Capture was plotted relative to the background from Biotin-BSA at each corresponding shear. Having established that our device can demonstrate the capture of C6 selectively at diluted levels of its cognate MART-1 pMHC, we moved on to assess whether this sensitivity can extend to native pMHCs that are isolated from MEL624 and SKMEL5 melanoma cell lines.

Since the entire pool of pMHCs were extracted in bulk using immunoaffinity chromatography, the expected fraction of the MART-1 pMHCs among the total pMHCs extracted and coated on the device is presumably small. Comparing the sensitivity of C6 capture normalized to Biotin-BSA background, we observed that the MEL624-derived pMHCs produced a modest, but consistent sensitivity values in the range of 8-12% across different shears, peaking around 0.049 dyn/cm² (CH7). SKMEL5-derived pMHCs also followed a similar trend but showed a slightly enhanced sensitivity range of 8-23% of C6 capture peaking at 0.056 dyn/cm². The sensitivity of C6 capture were generally lower and more variable, suggesting that MART-1 pMHC densities were low, and the exact quantity of these pMHCs in both the melanoma cell lines remained unknown. Taken together, the data from native pMHC fit into the sensitivity trend established in the proof-of-concept experiments that used recombinant MART-1 pMHCs diluted with ns-pMHCs. The devices coated with high MART-1 pMHC concentrations served as the higher end of the performance range in terms of C6 sensitivity.

As the MART-1 pMHC is progressively diluted, the total capture declines, yet the C6 sensitivity remains clearly detectable even at very low MART-1 pMHC coating concentrations at lower shears. In this context, the conditions with MEL624 and SKMEL-derived pMHCs behave as physiologically relevant analogues of the low-fraction MART-1 pMHC conditions in the earlier experiments. Moreover, the consistent observation that sensitivity is highest at lower shear highlights the importance of a gentler perfusion rate when attempting to capture rare tumor-specific T cells engaging with extremely sparse pMHC targets in a physiologically relevant setup. Overall, the results from the native pMHC perfusion experiments show that our microfluidic platform can maintain sensitivity at biologically plausible scenarios, reinforcing its suitability for use in rare tumoricidal T-cell selection in patient-derived tumor profiles with unknown pMHC heterogeneity.

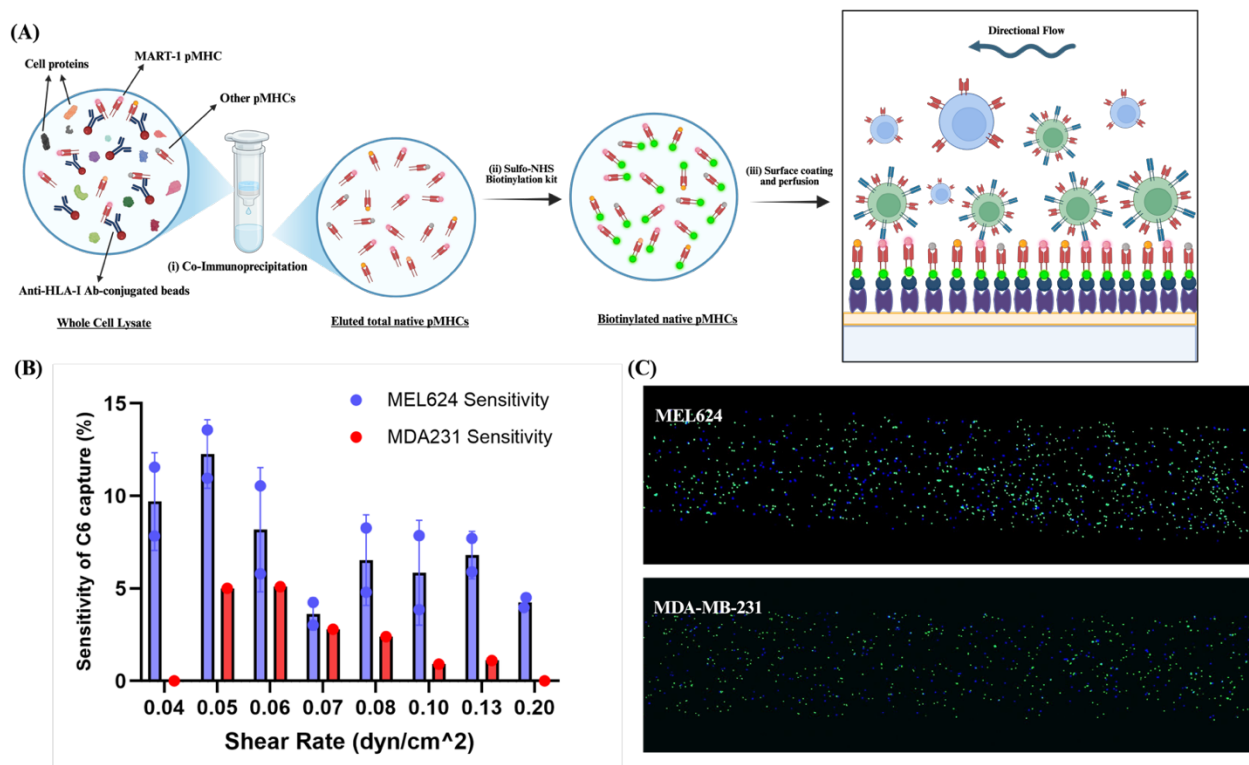


Figure 6. Direct immobilization of Co-immunoprecipitation-based isolation of total pMHCs from tumor cell lines yielded significantly improved C6 capture sensitivities. (A) Schematic representation of the co-immunoprecipitation (Co-IP) to isolate total pMHCs from MART-1 positive MEL624 and MART-1 negative MDA-MB-231 tumor cell lines. Whole cell lysates from both cell lines were incubated with anti-HLA class I antibody-coated agarose resin beads and then eluted in affinity chromatography spin columns to recover total pool of native pMHCs, which were subsequently biotinylated and immobilized on the Neutravidin-coated devices. (B) Sensitivity of C6 Capture (%) normalized to Biotin-BSA background. MEL624 pMHC-coated devices showed modest but reproducible C6 capture, yielding a sensitivity of upto 12% in channel 7 (0.05 dyn/cm²). Sensitivity from the MDA-MB-231 pMHC-coated devices were significantly lower, consistent with no expression of MART-1 pMHC. (C) Representative fluorescence images of channels from MEL624 pMHC (top) and MDA-MB-231 pMHC coated devices (bottom). Perfusion of 1:1 mixture of C6 and WT Jurkat cells show a detectable enrichment of C6 cells in the MEL624 devices, relative to the MDA-MB-231 devices. The adhesion of WT cells, though still present, is noticeably lower in both devices. These results suggest that the devices retain C6 sensitivity at biological densities of MART-1 pMHC densities, highlighting the need for a better blocking strategy to further reduce non-specific cell binding.

3.6.Captured C6 cells show a distinct Calcium influx associated with increased Fluo-4 AM fluorescence intensity

The interaction of T-cells with their agonistic antigen elicits a distinct influx of Calcium, making it one of the standard markers of T-cell activation. To determine whether the pMHC-mediated C6 capture on our microfluidic platform is associated with functional activation, we examined the intracellular Ca²⁺ dynamics on devices coated with MART-1 pMHC, alongside Biotin-BSA coated devices as the control. C6 cells were loaded with Fluo-4 AM and Fura-Red AM, the two standard dyes that are sensitive to change in intracellular Calcium levels. Cells that were captured on the channel surface within the field of imaging were monitored over 220 seconds using confocal microscopy to quantify the fluorescence intensity of Fluo-4 and Fura-Red over time on a single-cell basis. C6 cells that stably bound to MART-1 pMHC-coated regions showed a pronounced Fluo-4 and reduced Fura-Red fluorescence following adhesion.

In the majority of the responding C6 cells, there was a rise in Ca²⁺ signal within several minutes of binding, reaching a defined peak, followed by a plateau. C6 cells perfused on devices coated with Biotin-BSA did not indicate any level of Ca²⁺ dynamics, though they adhered to the surface. The mean change in fluorescence observed in C6 cells over a MART-1 pMHC surface remarkably exceeded that of the Biotin-BSA surface, where the cells remained under threshold. The ratio of the fluorescence intensities (Fluo-4/Fura-Red) are plotted relative to time, with the baseline fluorescence in the pre-activation frames subtracted. This mean change in fluorescence increased noticeably in the C6 cells bound to the MART-1 pMHC-coated surfaces, while remaining at baseline levels in the Biotin-BSA coated devices. These findings are consistent with robust T-cell activation, demonstrating the functional responsiveness of the captured T-cells to its cognate pMHCs.

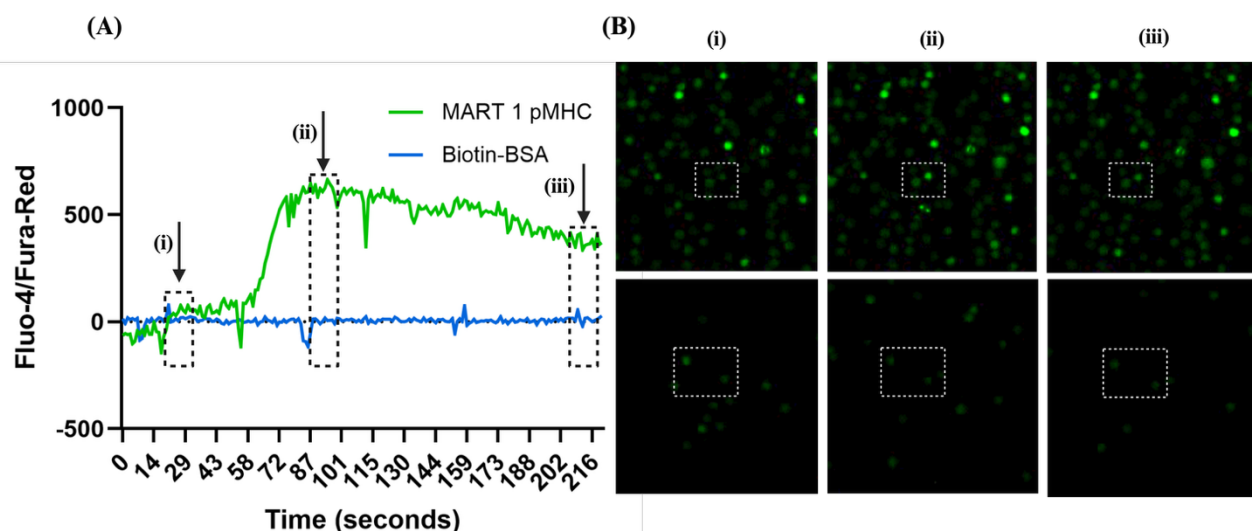


Figure 7. Ratiometric Ca^{2+} analysis of C6 Jurkat cells captured on devices coated with MART-1 pMHC. (A). Time course of Fluo-4/Fura-Red fluorescence intensity for C6 Jurkat cells that adhered to MART-1 pMHC-coated (green) or Biotin-BSA-coated (blue) devices. Cells were loaded with Fluo-4 AM and Fura-Red AM and fluorescence profiles were monitored on a single-cell basis by confocal microscopy over 220 seconds; ratios were normalized with the background fluorescence intensity in the initial frame. C6 cells that stably bound to MART-1 pMHC displayed a rapid spike in Fluo-4/Fura-Red ratio within minutes of binding, followed by a sustained plateau, which indicates robust Ca^{2+} influx pertaining to activation. (B) Representative confocal images of C6 cells captured on Biotinylated MART-1-coated (top) and Biotin-BSA-coated (bottom) channels at the time points indicated: (i) pre-activation baseline; (ii) peak Ca^{2+} response; and (iii) late plateau phase. Regions of interests (marked by dashed boxes) highlight single cells that were observed in the specific time frame of acquisition. C6 cells captured on Biotin-BSA, though adhered, did not elicit any influx of Ca^{2+} throughout the frame of the time-lapse. This demonstrates that the functional activation of T-cells is highly specific to the interaction between the specific TCR and its cognate pMHC complex. Non-specific interactions do not activate the T-cells though they may transiently bind to the antigen.

4. Materials and Methods

4.1. Device fabrication, surface coating and perfusion

All the microfluidic devices used in this article have eight channels, with a common inlet (source) and outlet (sink). The channels vary in dimension, while keeping the area of each channel equal. The purpose of this design is to create a range of shear forces that each have its effect on the sensitivity and the capture efficiency of the device. Studying a range of shears will also enable the selection of the best channel width that achieves the most optimal capture of tumor-specific T-cells from the perfused cell suspension. The microfluidic geometry was etched into a silicon wafer master mold via SU-8 photolithography. Polydimethylsiloxane (PDMS; Sigma-Aldrich, Cat: 504823) was prepared by mixing with Sylgard 184 Silicone Elastomer Base and Curing Agent in a 10:1 ratio. The prepared PDMS mixture was cast onto the mold and allowed to polymerize in heat (60 °C) for a minimum of 3 hours. The solidified PDMS with the design engraved is peeled out of the mold gently, and plasma bonded to a clean glass coverslip. The bonded devices were cured in heat (120 °C) for 24 hours. The surface coating of the devices are optimized to utilize the

very well-known NeutrAvidin-Biotin bond to attach the tumor antigen-containing MHC complexes onto the channels. The cured devices were coated with NeutrAvidin at a concentration of 100 µg/mL, over the base coatings of 3.65% Silane and 1 mM GMBS. The NeutrAvidin-coated devices were stored at 4 °C (can be stored up to 2 weeks) until the day of the experiment.

On the day of experiment, the devices were equilibrated to 37 °C in the incubator prior to perfusion. The tips at the sink were carefully removed and connected to 5 mL syringes filled with 1 mL of 0.2% BSA in DPBS (without ions; perfusion buffer) via an appropriate length of tubing. The tips at the source were filled with 200 µL of perfusion buffer and gently perfused at 360 µL/hr for 5 minutes. After washing, the volume of buffer in the source was replaced with the appropriate cell suspensions based on the experimental design. For all experiments, the perfusion was set at 360 µL/hr for 10 minutes, followed by a washing phase with the perfusion buffer for 15 minutes. After the washing phase, the total device was imaged using the confocal microscopy and analyzed using ImageJ (Fiji).

4.2. Jurkat Cell Culture and Staining

The human T-lymphocyte cell line, Jurkat was cultured in RPMI media supplemented with HI-FBS and PenStrep. A genetically modified clone of the Jurkat cell line (named Clone 6 or C6) was generated via lentivirus infection, to express the TCR specific to MART-1 peptide that is displayed on the MHC complexes in the melanoma cell lines. For the perfusion experiments, the wild-type (unmodified) Jurkat cells and C6 Jurkat cells, were stained with Cell Tracker Deep Red (insert product) and Cell Tracker Green (insert product) respectively and then mixed at a ratio of 1:1 at a final concentration of 0.5 million cells per mL of RPMI media.

4.3. Flow cytometry analysis of C6 Jurkat and Melanoma Cells

C6 and WT cells were collected in two microcentrifuge tubes each containing about 200,000 cells. The tubes were centrifuged to yield the pellet and then resuspended in 200 µL of 0.2% BSA in DPBS (without ions). The tubes were centrifuged and the supernatant was discarded. This washing step was repeated once again. One vial each of the C6 and WT samples were labelled with 200 µL of MART-1 pMHC tetra-APC at the concentration of 10 µg/mL, and the other vials were left unstained. The samples were incubated in ice for 30 minutes. The samples were then washed twice with DPBS (without ions) and resuspended in fresh DPBS. The expression of MART-1 specific TCR was analyzed using flow cytometry by the qualitative detection of APC signal at 647 nm.

SKMEL5 cells were collected in microcentrifuge tubes containing about 200,000 cells each. The tubes were centrifuged and washed twice with 0.2% BSA in DPBS (without ions) as mentioned above. Intracellular staining was performed and analyzed via flow cytometry using Cyto-Fast™ Fix/Perm kit according to the manufacturer's instructions. Three MART-1/Melan-A antibodies were used: Anti-MART-1 TCR like antibody (Creative Biolabs), Melan-A antibody clone A103 (Santacruz) and pre-conjugated mouse anti-human MART-1/Melan-A Alexa Fluor 647 antibody.

4.4. Melanoma Cell Culture and Whole cell protein isolation

Two melanoma cell lines, SKMEL5 and MEL624 were cultured in DMEM media supplemented with Fetal Bovine Serum and PenStrep. The melanoma cell lines chosen to have been reported to have stable expression of MART-1 peptide-expressing MHC complexes. Each of the cell lines were cultured in 4 T175 flasks and maintained until confluency. The cells were then

lifted with Versene (DPBS -/- + 2 mM EDTA) per flask, with gentle rocking. Once cells detached, the Versene was neutralized using fresh cold DPBS, and collected on Ice. The cell suspensions were then centrifuged to collect the pellet. After discarding the supernatant, the pellet from each cell line were combined and reconstituted in a 1% w/v Digitonin Lysis Buffer, prepared with Tris-HCL, EDTA, NaCl and Halt's protease inhibitor. The mixture was incubated on ice for 40 minutes with gentle rocking, after which was centrifuged at 16,000 - 20,000g for 20 minutes. The clear supernatant was carefully collected in a new tube to acquire the Whole Cell Lysate from each cell line. The protein in the whole cell lysates of both the MEL624 and SKMEL5 cell lines was quantified using the Micro BCA Protein Assay kit (Thermofisher Scientific: 23235). The samples were stored in -80 until further use.

4.5. Isolation of pMHCs and Surface Coating

pMHC complexes from MEL624 and SKMEL5 cell lines were isolated using the Pierce Co-Immunoprecipitation kit. To preserve the peptide-MHC bond in the samples, the Gentle Ag/Ab buffer (pH 6.6) was used in the final elution step of the Co-IP. The collected eluates were now adjusted to a pH of 7.2-7.3 using 1M NaOH. Using the EZ-link Sulfo-NHS Biotin Labelling kit, the eluates containing pMHCs were tagged with Biotin, and excess biotin was removed using the Zeba dye and Biotin Removal Columns. All the steps were performed in ice to reduce the risk of peptide dissociating from the MHC complexes. The biotinylated pMHCs from the melanoma cell lines were stored at 4 C. To coat the microfluidic devices, 250 uL of the biotinylated pMHCs were added to the source side using a gel loading pipette tip and incubated at 4 C for one hour to allow capillary forces to evenly coat the channels with the pMHCs. Once the fluid levels equilibrate on the source and the sink, the fluid from the sink is collected using a gel loading pipette tip and added back into the source followed by incubation at 4 C for one hour. The recirculation and incubation steps were repeated twice to ensure maximum saturation of all the NeutrAvidin sites present in each channel. The completed devices, now coated with biotinylated pMHCs from each melanoma cell line was stored in 4 C for not more than 2 days.

For proof-of-concept experiments, the devices coated with NeutrAvidin were coated with Biotinylated MART-1 pMHCs (sequence) acquired from Tetramer facility, at varying concentrations (100 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL) prepared in a solution of 100 µg/mL Biotin-BSA. The surface served as a MART-1 pMHC coated platform "spiked" with Biotin-BSA, in order to saturate all the Neutravidin binding sites in the channels. As a negative control, devices coated only with 100 µg/mL Biotin-BSA were used.

4.6. Loading Fluo-4 and Fura-RED in Jurkat Cells for analysis of Calcium influx

3 million cells of both WT and C6 Jurkat cells were loaded stained according to the article (insert article) 4 Fluo-4 AM (insert product details), 10 µg/mL Fura-Red AM prepared in HBSS supplemented with 0.02% Pluronic F-127 at a concentration of 1 million cells/mL. After incubation in the dye solution for 30 minutes at 37 C, the cells were washed once with DPBS supplemented with HEPES at a concentration of 10 mM. The pellet was resuspended in fresh HEPES buffered DPBS at a concentration of 0.25 million cells/mL and incubated at room temperature for 45 minutes to allow sufficient cleaving of the AM esterases.

5. Discussion

The microfluidic platform used in this thesis supports the feasibility of using shear as a discriminator to enrich tumor-specific T cells by taking advantage of the TCR-agonistic pMHC interactions. Currently used platforms on a clinical level involve CAR-T and engineered TCRs, largely depending on prior knowledge of a small antigen pool, and require an expensive and resource-intensive manufacturing process⁹. In contrast, our approach requires no prior knowledge about the antigen pool expressed on the tumor, letting the biophysics of the specific TCR-cognate pMHC recognition and hydrodynamic shear select multiple tumor-reactive clones. The platform works based on two well-studied principles of T-cell recognition. First, TCRs form longer and higher-avidity interactions with its agonistic pMHC, enabling an immunological synapse and functional activation. Weaker non-specific interactions do not form sustained bonds and hence do not elicit any immunological response¹³. Second, T-cells scan antigen-presenting cells in vivo under the shear forces generated by blood and constant cell movement, implying that only sufficiently strong TCR-pMHC bonds survive to achieve a response in the dynamic microenvironment^{14–16}. Using controlled ranges of shear in an eight-channel microfluidic platform, the thesis mimics a physiological filter and shows that C6 Jurkat cells can be selectively retained on MART-1 pMHC coated surfaces, whereas WT Jurkat cells fail to form sustained adhesions. This is also consistent with prior microfluidic-based studies of leukocyte adhesion and T-cell interaction under flow, which have also shown that shear can be used to discriminate high-affinity interactions between receptor and its ligand pairs, which have better resistance to detachment than low-affinity interactions^{12,17–19}.

A point advance of this work is demonstrating that C6 specificity is preserved as MART-1 pMHC density is reduced towards physiologically relevant ranges and diluted into an excess of non-specific Biotin-BSA. Even at the lowest fractions of MART-1, the sensitivity of C6 capture clearly remained elevated over the WT across the range of shears, with maximal sensitivity observed at lower shear. This sensitivity at low ligand density addresses a major challenge in solid tumors where both cognate pMHCs and tumor-specific T cells often exist at low populations, where only 20% of predicted neoantigens may be shared even between the primary and metastatic lesions of the same patient. Other existing antigen-agonistic strategies have also targeted the capture of a rare functional T-cell repertoire but typically require large antigen panels and extensive tumor sequencing^{20–23}. The shear-based method here offers a much simpler, biophysical route to selectively enrich antigen-agonistic interactions.

To move towards a more physiological antigen presentation, the thesis tested whether the device could sustain C6 capture sensitivity when channels were functionalized with native pMHCs derived from tumor cell lines. An initial pilot approach that used biotinylated anti-HLA class I antibody to capture native pMHCs from MART-1 positive (MEL624 and SKMEL5 melanoma cell lines) and MART-1 negative (MDA-MB-231 breast cancer cell line) lysates yielded an extensively non-specific adhesion of both C6 and WT cells across all lysate conditions, with C6 capture sometimes higher on the MDA-MB-231 lysate-coated devices. These data suggest residual, unoccupied anti-HLA binding sites, or other lysate protein adsorption that may have generated a very adhesive surface that overwhelmed the subtle difference between agonistic and non-agonistic interactions. This negative result helped to improve the workflow to an immunoaffinity chromatography-based isolation of total pMHC complexes from MEL624 and MDA-MB-231, followed by biotinylation and immobilization in the channels. Perfusion through these devices showed a reproducible C6 capture with sensitivities ranging from 8-12% in the MEL624 pMHC-coated devices compared to the much lower and more variable sensitivity observed in MDA-MB-

231 pMHC-coated devices. Most importantly the sensitivity values acquired with native pMHCs aligned with the continuum established by the MART-1/biotin-BSA dilution experiments, where progressive MART-1 dilution reduced but not disrupt the sensitivity at lower shear.

Beyond mechanical retention, it is essential that the captured C6 cells were functionally competent. Ratiometric Ca^{2+} analysis showed a robust increase in the Fluo-4/Fura-Red ratio following adhesion, as seen in canonical T-cell activation via the TCR²¹. This result is also consistent with previous studies that show that sustained intracellular calcium flux along with effector cytokine production, marks the stimulation of the TCR by an agonistic pMHC²⁴⁻²⁶. Hence, the platform not only enriches high-avidity T-cells, but also preserves their functional activation, an essential prerequisite for subsequent expansion for therapeutic use in ACT.

The thesis however does carry certain limitations that should be acknowledged. First, the primary effector T-cell population was an engineering Jurkat T-cell line that expresses a TCR specific for MART-1 pMHC. These cells differ from primary effector cells in their activation thresholds, cytokine production and mechanobiological properties that may influence capture dynamics and optimal shear windows. Hence, the quantitative results of capture sensitivity reported here is a system-specific benchmark rather than having direct and clinical parameters. Extending this platform to primary T-cells from both patients and healthy donors will be an important step in evaluating the feasibility of clinical translation. Second, despite switching to total pMHC isolation for the surface coating, there was non-specific adhesion of WT cells. Even with good C6 capture sensitivity, the non-specific binding of WT cells may interfere with the later stages of the project that involve recovery and expansion. Third, the device was optimized for sensitivity, and important parameters such as cell recovery from the channels, post-capture viability and compatibility with rapid expansion workflows were not assessed.

Despite these limitations, the work presented in this thesis supports the conceptual advance that hydrodynamic shear can serve as a power biophysical filter to selectively enrich tumor-specific T cells based solely on TCR-pMHC interactions without requiring prior antigen profiling. This approach complements existing ACT strategies that rely on genetic engineering and sequencing and shows great potential in treating solid tumors with highly heterogenous and patient-specific antigen landscapes.

6. Conclusion

The thesis demonstrates that a shear-based microfluidic platform can selectively capture rare tumor-specific T cells using native TCR and cognate pMHC interactions under a controlled dynamic flow. A major strength of the platform is that the capture sensitivity depends on ligand density as well as shear stress, with the highest cell capture was generally observed at a lower shear. This supports that mechanobiological principle that agonistic TCR-pMHC interactions are likely to resist detachment when subjected to mechanical filtering in comparison to the weaker non-specific interactions. Notably, the captured C6 cells not only remain adhered, but also had sustained Ca^{2+} input upon binding to MART-1 pMHC, showing their functional responsiveness. While the thesis also revealed crucial limitations, the platform shows great potential in ACT applications with further optimization and validation using primary patient samples. The proof-of-concept displayed in the thesis could become a useful enrichment tool for adoptive cell therapy in heterogenous tumors.

7. References

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