A MICROFLUIDICS-BASED APPROACH FOR ISOLATION OF ANTIGEN-SPECIFIC CD8+ T CELLS

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A MICROFLUIDICS-BASED APPROACH FOR ISOLATION OF ANTIGEN-SPECIFIC CD8+ T CELLS

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A MICROFLUIDICS-BASED APPROACH FOR ISOLATION OF
ANTIGEN-SPECIFIC CD8⁺ T CELLS

A
THESIS

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for the Degree of
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by
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A MICROFLUIDICS-BASED APPROACH FOR ISOLATION OF ANTIGEN-SPECIFIC CD8+ T CELLS

Cancer is a global epidemic: there are predicted to be 200 million new cases this year alone. Almost a quarter of all cancer-related deaths are caused by lung cancer, for which 5-year survival rates are just above 20%. 85% of lung cancer diagnoses are classified as non-small cell lung cancer (NSCLC) for which 5-year survival rates in metastatic disease are less than 10%. Early detection and targeted therapies have improved prognoses, yet relapse is still common among patients.

Immunotherapies that leverage tumor-specific CD8+ cytotoxic T cells have shown great promise for the treatment of NSCLC. However, although highly promising, the success of these therapies has largely been hampered by the challenge of isolating tumor-specific CD8+ T cells from a bulk tumor-infiltrating lymphocyte (TIL) population, which are exceedingly rare. Current methods rely on the identification of tumor-specific antigens, which remains flawed. Current methods to isolate tumor-specific CD8+ T cells heavily rely on inaccurate antigen prediction models which require laborious functional validation in lab and make large-scale application infeasible.

The objective of this study is to develop a method which rapidly and specifically isolates antigen-specific CD8+ T cells that bypasses the need for prior antigen identification. In this work, I optimized a novel microfluidics method, “ATTACH” (Assessment of T cells Tethered to Antigen Class I and II Histocompatibility) within the model Ovalbumin (OVA) antigen system. Using a de facto pool of peptide-loaded MHC class I molecules on the surface of target cells, ATTACH enriches for antigen-specific CD8+ T cells based on binding avidity to cognate antigens. Here, I demonstrate that ATTACH specifically enriched for OVA-specific OT-I CD8+ T cells from both bulk splenocyte and heterogeneous CD8+ T cell populations. Importantly, enriched antigen-specific CD8+ T cell populations exhibited significantly greater antigen-specific effector function than both the bulk input and eluted non-specific CD8+ population.
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CHAPTER 1: INTRODUCTION

1.1 Innate versus adaptive immunity

The immune system is a collection of various cell types and components within our body which collectively protect against pathogens, infection, and autoimmune reactions [1]. The immune system is divided into two branches which contain distinct protective mechanisms [2]. The innate immune system is the first to respond to pathogens and does not retain immunological memory [2]. Innate immune responses are generally considered to be “non-specific” since they are mediated by conserved, germ-line encoded receptors expressed on the surface of immune cells such as macrophages, monocytes, neutrophils, and dendritic cells (DCs), which eliminate threats by phagocytosis, activation of the complement system, and release of pro-inflammatory cytokines such as TNF-α, IL-1, and type I interferons (IFNs) [1,3]. If the innate immune system is not able to clear a threat, an adaptive immune response is mobilized, which is largely mediated by T and B lymphocytes.

B cells mainly act as mediators to bridge the adaptive and innate immune responses and respond to circulating pathogens. Antigen-mediated activation through the B cell receptor (BCR) results in the production of antibodies which bind to and mediate clearance of circulating pathogens through direct neutralization, complement activation, or recruitment of T lymphocytes and other immune cells [4-5].

T cells mediate immune responses through specific recognition of peptides, termed “antigens,” presented by major histocompatibility complex (MHC) molecules on the surface of cells [6]. T cell antigen recognition occurs through the T cell receptor (TCR), a heterodimeric complex containing an immunoglobulin-like extracellular variable domain which recognizes peptides presented on (MHC) molecules on the surface of target cells [7]. Somatic V(D)J TCR rearrangement during T cell development results in the creation of a remarkably expansive TCR repertoire which contains up to $10^{15}$ different TCRs conferring unique antigen specificities [8]. Due to allelic exclusion during
development, each T cell expresses a TCR which recognizes a limited number of antigens [9,10] TCRs are known to have a range of affinities for cognate antigens but, regardless, antigen- specific TCRs bind to cognate antigens with higher affinity that non-specific antigens [11-13].

Two general classes of conventional (i.e. MHC-restricted) T cells exist based on the expression of the CD4 or CD8 co-receptor, which play a major role in stabilizing T cell and peptide-MHC (pMHC) interactions and facilitating T cell activation [14]. CD4+ T cells, expressing the CD4 co-receptor, are also called “helper” T cells because of their assistive role to activate the innate immune response as well as adaptive response through B and CD8+ T cell activation [15-16]. CD4+ T cells also play a major regulatory role in controlling the inflammatory response and dampening autoimmune reactions [16]. CD8+ T cells, also termed “cytotoxic” T cells, respond to intracellular pathogens and directly kill infected cells via cytotoxic mediators such perforin and granzyme B [15, 17]. CD4+ and CD8+ T cells also diverge in their MHC-restriction: CD4+ T cells recognize antigens presented by MHC class II molecules, while CD8+ T cells recognize antigens presented on MHC class I molecules (FIGURE 1A-B) [18-19].
Figure 1: CD8+ T cell antigen recognition. (A) Antigen recognition is mediated by the T cell receptor (TCR) binding to antigenic peptides presented by major histocompatibility complex (MHC) class I molecules. The CD8 co-receptor stabilizes TCR/pMHC interactions. (B) Antigen-induced CD8+ T cell activation results in the production inflammatory mediators, such as interferon-gamma (IFNγ) and (TNFα), and cytotoxic molecules, such as perforin (PFN) and granzyme B (GzmB).

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1.2 Major Histocompatibility Complex (MHC) molecules and their role in CD8+ T cell activation

Two classes of MHC molecules exist and while they both share the common role of presenting antigenic peptides for T cell recognition, structural differences create a divergence in the types of peptides presented by each. In humans, MHC class I and class II genes are in coded by the human leukocyte antigen (HLA) complex [20]. HLA-A, -B, and -C genes encode for MHC Class I molecules, while HLA-DR, -DP, and -DQ genes encode for MHC Class II molecules [20-21]. In mice, MHC class I and II genes are encoded by the H2 complex with “classical” MHC class I molecules encoded by the H2-K and H2-D genes, and MHC class II molecules encoded by the H2-A
and $H2-E$ genes [22-23]. Polymorphic variations enable MHC molecules to present peptides from, and mediate immune responses against, a wide range of pathogens [24].

MHC Class I molecules are expressed on the surface of all nucleated cells and generally present cytosolic peptides mostly derived from defective ribosomal translation products or viral proteins, for example [25]. Conserved tyrosine residues at either end of the peptide binding groove of MHC Class I molecules results in a “closed” binding groove which limits presentation to peptides of 8-13 amino acids in length [25]. Intracellular proteins destined for MHC Class I presentation are first degraded by cytosolic and further trimmed by ERAAP in the endoplasmic reticulum (ER) to appropriate lengths for loading onto MHC Class I molecules [25]. Chaperone proteins translocate degraded cytosolic proteins into the ER where they are loaded onto MHC Class I molecules which are subsequently trafficked to the cell surface [25]. In addition to endogenous antigens, some professional antigen presenting cells (APCs) can present exogenous antigens on MHC Class I in a pathway known as “cross presentation” which is important for immune surveillance, self-tolerance and priming of naïve CD8$^+$ T cells [27-28]. Continuous internalization and recycling MHC class I molecules via endocytic pathways helps mediate MHC class I loading and cross presentation of endogenous antigens [28-29].

Conversely, MHC Class II molecules are uniquely expressed on the surface of APCs, such as dendritic cells, B cells, macrophages, and thymic epithelial cells, and present exogenous proteins from the surrounding environment ranging from 10-34 amino acids in length [26,30]. APCs continuously surveillence the surrounding environment and internalize exogenous proteins through endocytic pathways which are then degraded into peptide fragments by endosomal proteases [31-32]. MHC Class II molecules recycled from the cell surface or newly-synthesized in the endoplasmic reticulum traffic to endosomal compartments containing internalized peptides which are subsequently loaded onto MHC Class II molecules and trafficked to the cell surface with the help of accessory proteins [31].
1.3 Antigen-specific CD8+ T cell activation

Antigen-induced CD8+ T cell cytotoxic function begins with DC-mediated priming of naïve antigen-inexperienced CD8+ T cells [32]. This process requires 3 sequential signals for naïve CD8+ T cell activation: antigen recognition, costimulatory signals, and cytokine stimulation [32]. APCs present exogenous antigens from tissues on MHC class I molecules which are recognized by cognate TCRs on effector CD8+ T cells [32]. TCR/pMHC binding triggers formation of the immunological synapese which drives T cell activation, proliferation, and differentiation into effector memory subsets [33-35]. Engagement of co-stimulatory molecules on the surface of CD8+ T cells, such as CD27, CD28, or 4-1BB, by ligands on activated APCs is necessary for perpetuation of CD8+ T cell activation [32,36]. Conversely, engagement of inhibitory molecules which are upregulated after initial T cell activation, such as programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), halt activation and serve as important regulators against autoimmune reactions [32-33]. In addition to APCs, tumor cells are able upregulate expression of inhibitory ligands, such as programmed death ligand-1 (PD-L1), and block CD8+ T cell activation within the tumor microenvironment [32, 37]. These inhibitory receptors and ligands are common targets for immune checkpoint blockade (ICB) therapy utilizing antagonistic monoclonal antibodies that block engagement of inhibitory molecules on CD8+ T cells [38]. The third signal required for CD8+ T cell activation comes from cytokine stimulation from activated APCs and/or autocrine stimulation from activation-induced interleukin-2 (IL-2) production, and expression of the high affinity IL2Rα receptor, completes CD8+ T cell activation and results in effector cell differentiation, rapid clonal expansion, and release of cytotoxic mediators [39]. Because of their powerful cytotoxic function, and specificity for target cells, CD8+ T cells offer great potential for anti-tumor therapies.
1.4 Non-small cell lung cancer

Cancer is a global epidemic; in 2022 alone, there are predicted to be almost 200 million new diagnoses [40-41]. Although early detection and prevention methods have decreased incidence, lung cancer still leads in the number of cancer-related deaths in both males and females and accounts for almost a quarter of all cancer-related deaths [41]. Over 230,000 Americans are diagnosed with lung cancer annually for which 5-year survival rates are only 22% [41].

Histologically, lung cancer is broadly divided into two categories: small cell (SCLC) and non-small cell lung cancer (NSCLC). 85% of lung cancer diagnoses are classified as NSCLC which includes several subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [42-43]. Unfortunately, over half of patients present with late stage metastatic disease at the time of diagnosis, for which 5-year survival rates are only 8% [40,44]. Due to its high prevalence and mortality rates, there is an urgent and significant need to develop novel therapies, and advance current ones, for the treatment of NSCLC.

1.5 Treatment of NSCLC

Current standard of care treatment depends on the stage at diagnosis. Historically, surgical resection has been considered the standard of care for early stage NSCLC, while radiation and/or adjuvant cisplatin-based chemotherapy have been indicated for the treatment of metastatic disease which elicit suboptimal response rates [45]. In patients with targetable mutations in pro-tumorigenic pathways, the addition of biological agents, such as epidermal growth factor receptor (EGFR) and Vascular Endothelial Growth Factor (VEGF) receptor inhibitors, have demonstrated superior response rates with less toxicity than chemotherapy alone [46]. Treatment with EGFR-targeted tyrosine kinase inhibitors (TKI), for example, has elicited impressive response rates of 58-85% in EGFR mutation-positive NSCLC tumors [47]. However, most patients develop acquired resistance to EGFR-TKI treatment and have disease reoccurrence after just 1-2 years [48-49].
Immunotherapies, which directly modulate the immune system and/or directly use its components to mount an anti-tumor response, have shown promising advancements in the treatment of NSCLC [50]. Immune checkpoint inhibitors, such as anti-CTLA4 and anti-PD-1/PD-L1, have been approved for the treatment of NSCLC both as monotherapies and combination treatments [51]. The synergistic effects observed in combination treatments of ICB and chemotherapy and/or radiation therapy have improved overall response rates (ORR) and progression-free survival (PFS) in NSCLC [52-53]. However, only a small subset of patients achieve a partial or complete response to ICB due to factors such as oncogenic driver mutations, low tumor mutational burden (TMB), low numbers of tumor-infiltrating lymphocytes (TIL), and an immunosuppressive tumor microenvironment (TME) [54-57]. Even when used in combination with first-line chemotherapy treatments, disease progression still occurs within 12 months in many patients [58-59]. Adoptive T-cell transfer (ACT) of ex vivo expanded autologous TIL has been predicted to help prime CD8+ T cell responses and overcome some of the barriers to ICB response [60] (FIGURE 2).

**Figure 2: Overview of adoptive T-cell transfer (ACT).** *Tumor-infiltrating lymphocytes (TIL) are isolated from resected tumor tissue and expanded ex vivo with high doses of growth factors and cytokines such as IL-2 and IL-7.* Figure created with Biorender.com.
ACT was first reported over 50 years ago and has demonstrated robust response rates, especially in ICB-resistant tumors [61-64]. Specifically, in the metastatic melanoma setting, ACT has demonstrated significant response rates of 50% [61]. In this type of therapy, TILs are isolated from solid tumors and expanded to large numbers \textit{ex vivo} with high doses of IL-2 and IL-7 [65-66]. Expanding TIL outside of the immunosuppressive tumor microenvironment has been shown to relieve inhibition and promote the re-infusion of a non-exhausted effector memory CD8$^+$ T cell population capable of inducing tumor regression and durable remission [58, 65-66]. In fact, a recent phase I clinical trial in anti-PD-(L)1 treatment-naïve NSCLC tumors showed tumor regression in a small subset of advanced NSCLC patients treated with ACT [58]. However, responses varied across patients and only a small fraction of patients achieved a complete response [58]. Importantly, studies in ACT in metastatic melanoma have highlighted the importance of reinfusion of CD8$^+$ TIL which recognize \textit{neoantigen} peptides derived from somatic mutations which are exclusively expressed in tumor cells: patients who achieved complete tumor regression had higher numbers of neoantigen-specific CD8$^+$ TIL in their reinfusion product [67-71] [68] [69] [70]. While ACT has mediated tumor regression in NSCLC tumors, barriers such as low CD8$^+$ numbers and loss of tumor-specific clonotypes in the expanded TIL product have hindered its success in many patients, prompting the exploration into methods to identify and isolate neoantigen-specific CD8$^+$ TIL from solid tumors [58,65].

1.6 \textit{Strategies to isolate tumor-specific CD8$^+$ T cells and current challenges}

Most strategies commonly used to isolate neoantigen-specific CD8$^+$ cells use a combination of neoantigen-prediction models and functional validation in lab. Whole exome sequencing (WES) and RNA sequencing (RNA-seq) are commonly used to detect tumor-restricted somatic mutations which may give rise to neoantigen peptides [72-74]. However, intra-tumoral heterogeneity, low tumor ploidy, and insufficient read coverage makes identifying potential neoantigens with these methods difficult [75]. Furthermore, on their own, these methods fail to accurately predict epitope sequences
of neoantigen peptides presented on MHC molecules [72]. MHC prediction models are used in conjunction to predict antigen processing and presentation on MHC molecules; however, they require prior knowledge of MHC haplotype and have a success rate of only 1% in predicting immunogenic epitopes [76-77]. Moreover, prediction methods require functional validation of predicted neoantigens with synthesized peptides and pMHC multimers, which can be laborious and costly when screening hundreds to thousands of predicted neoantigens (FIGURE 3) [68,73].

![Antigen “A”-MHC Class I Tetramer](image)

**Figure 3: Isolation of antigen-specific CD8+ T cells with MHC multimers.** Engineered MHC Class I multimers, containing immobilized peptide-loaded MHC class I molecules, are commonly used to isolated antigen-specific CD8+ T cells. A fluorophore can be conjugated to multimers to visualize antigen-specific CD8+ T cell populations.

Markers such as PD-1 and 4-1BB have been speculated to identify activated T cells from a bulk TIL population, however, these methods fail to account for the intrinsic cross-reactivity of TCR specificity [78]. Recently, phenotypic-based identification of neoantigen-specific CD8+ T cells has demonstrated success in a limited number of patients; however, large-scale feasibility is not yet known [79]. There is a significant need to isolate tumor-specific CD8+ T cells from autologous tumors without the limitations of current isolation methods. **The goal of this study** was to develop a method that rapidly and specifically enriches for tumor-specific CD8+ T cells from a bulk population which bypasses antigen-identification methods. The Assessment of T cells Tethered to Antigen Class I/II Histocompatibility (ATTACH) assay uses a de facto pool of loaded MHC class I molecules on the surface of matched target cells and isolates autologous antigen-specific CD8+ T cells based on
binding avidity to cognate antigens (FIGURE 4A-B). **We hypothesize** that because of higher binding avidity to cognate antigens than non-specific T cells, antigen-specific CD8$^+$ T cells can be directly isolated from a bulk population by applying shear force. Herein, I describe the creation and optimization of a semi-automated ATTACH assay which significantly enriches for Ovalbumin$\text{257-264}$–specific CD8$^+$ T cells from a heterogeneous population.

**Figure 4: General overview of ATTACH.** By using a de novo pool of autologous peptide-loaded MHC class I molecules, ATTACH isolates antigen-specific CD8$^+$ T cells from a heterogeneous population based on binding avidity to cognate antigens. (A) A heterogeneous CD8$^+$ T cell population is added to matched target cells. In the context of the Ovalbumin (OVA) model antigen system, OVA-specific CD8$^+$ T cells bind tightly to OVA antigen on target cells. Force is applied which (B) removes any weak-binding, non-specific CD8$^+$ T cells while leaving OVA-specific CD8$^+$ T cells still bound to cognate antigen. Figure made with Biorender.com.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

Lewis Lung Carcinoma (LLC) cells were obtained from American Type Cell Collection (ATCC) (catalog ATCC-CRL-1642). 344SQ cells were kindly provided by the lab of Dr. John Heymach at The University of Texas MD Anderson Cancer Center. 344SQ cells transduced to endogenously express the H-2K\textsuperscript{b}-restricted OVA\textsubscript{257-264} SIINFEKL antigen (344SQ-SIINFEKL) were previously generated in lab using lentiviral transduction. LLC-SIINFEKL transduced cells were kindly provided by the lab of Tina Cascone at The University of Texas MD Anderson Cancer Center. Selection was maintained in transduced 344SQ-SIINFEKL and LLC-SIINFEKL cells by adding 0.05 mg/mL of Hygromycin B (Corning; #30-240-CR) to cell culture media. All cell lines were cultured at 37°C, 5% CO\textsubscript{2} in complete RPMI 1640 media (Corning 10040 CV) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin (referred to as “complete RPMI media” hereafter).

2.2 Mice

Female transgenic OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J; RRID:IMSR_JAX:003831), C57BL/6 mice (RRID:IMSR_JAX:000664), and pmel mice (B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J; RRID:IMSR_JAX:005023) were obtained from Jackson laboratories. CD8\textsuperscript{+} normal donor PMBCs expressing a TCR specific for the HLA-A03:01-restricted EGFR L858R epitope were previously isolated by single cell sorting. All murine T lymphocytes were cultured in complete RPMI media supplemented 50uM 2-mercaptoethanol, 1% Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS) (insulin 1.7uM, transferrin 68.8nM, and sodium selenite 39nM; Gibco), 0.3 ng/mL of recombinant mouse IL-2 (R&D systems #402-ML-020), and 0.5 ng/mL of recombinant mouse IL-7 (R & D systems #407-ML-005). Human T lymphocytes were cultured in LymphoONE T-Cell Expansion
Xeno-Free Medium (Takara Bio # WK552S) supplemented with 1000 U/mL of recombinant human IL-2 (STEMCELL Technologies #78036).

2.3 CD8+ T cell isolation and expansion

All animal protocols were approved by the MD Anderson Institutional Animal Care and Use Committee. Mice were 8-12 weeks at the time of sacrifice. Negative CD8+ selection was used to isolate CD8+ splenocytes via magnetic sorting under sterile conditions using the STEMCELL Technologies’ EasySep™ mouse CD8+ isolation kit (catalog #19853A) per manufacturer’s instructions. Briefly, isolated spleens were disrupted in complete RPMI media and cell suspensions were passed through a 40µm nylon mesh strainer (Fisher Scientific #22363547) to remove debris and aggregates. Strained cell suspensions were pelleted by centrifugation at 600xg for 6 min. All centrifugation steps were performed at room temperature. Red cell lysis buffer (Santa Cruz Biotechnology #sc-296258) was prepared at 1x concentration in sterile Millipore water and added to the pelleted cell suspension at a volume 10x that of the cell pellet. After incubating for 12 minutes at room temperature, complete RPMI media was added at 10x the volume of red cell lysis buffer to quench the lytic reaction. Cells were centrifuged at 600xg for 6 min and resuspended at ≤ 1 x 10^8 nucleated cells/mL in biotin, Ca^{2+}, and Mg^{2+} -free 1x phosphate buffered saline (PBS) supplemented with 2% FBS and 1mM EDTA (hereafter referred to as the “recommended media”). 50µL/mL of normal rat serum was added to samples before transferring them to 5mL polystyrene round-bottom tubes. 50µL/mL of EasySep™ mouse CD8+ isolation cocktail was added to samples and incubated at room temperature for 10 minutes. EasySep™ Streptavidin RapidSpheres™ were vortexed for 30 seconds and 125 µl/mL was added to samples. Samples were incubated at room temperature for 5 minutes, placed on an EasyEights™ magnet (STEMCELL Technologies #18103), topped off to 2.5mL with the recommended media, and incubated at room temperature for 5 minutes. The supernatant containing isolated CD8+ T cells was collected and counted using VitaStain™ AOPI
staining solution (Nexcelom Biosciences #CS2-0106) and Nexcelom Cellometer® K2 fluorescent viability cell counter.

Isolation of CD8⁺ T cells from non-PBMCs was performed by positive CD8⁺ selection using STEMCELL Technologies’ EasySep™ Mouse CD8α positive selection kit II (#18953) per manufacturer instructions. Briefly, cells were resuspended at ≤ 1 x 10⁸ nucleated cells/mL in the recommended media in 5mL polystyrene round bottom tubes. The included Fc receptor blocker was added to cells, 10µL/mL and allowed to incubate at room temperature while preparing the selection cocktail containing equal parts of component A and component B. The freshly-prepared selection cocktail was allowed to incubate for 5 minutes at room temperature. 50µL/mL of the selection cocktail was added to samples and incubated at room temperature for 3 minutes. EasySep™ Dextran RapidSpheres™ were vortexed for 30 seconds and 40µL/mL was added to samples. Samples were incubated with RapidSpheres™ for 3 minutes at room temperature. Tubes were topped to 2.5mL with the recommended media and placed on an EasyEights™ magnet and incubated for 10 minutes at room temperature. The supernatant was discarded, and samples were removed from the magnet and washed by adding 2.5mL of the recommended media and incubating on the magnet for 10 minutes. The supernatant was discarded, and samples were washed once more as described above. Isolated cells were resuspended in complete RPMI and counted using VitaStain™ AOPI staining solution and Nexcelom Cellometer® K2 fluorescent viability cell counter.

2.4 CD8⁺ T cell expansion

An expansion protocol was adapted from Lewis et al., Journal of Immunological Methods (2015) [106]. All cells were cultured at 37°C, 5% CO₂. Freshly-isolated CD8⁺ splenocytes were resuspended in complete RPMI media supplemented with 50uM 2-mercaptoethanol and 1% ITS and plated at 5x10⁶ cells/well in 6-well Nunc plates (Thermo Fisher Scientific) pre-coated with 0.5ug/mL anti-mouse CD3 (clone 2C11, Bio X Cell) and 5 ug/mL anti-mouse CD28 (clone 37.51, Bio X Cell). Cells were cultured
without cytokine stimulation for 24 hours. Recombinant mouse IL-7 (R & D systems #407-ML-005) and IL-2 (R&D systems #402-ML-020) were added to final concentrations of 0.5ng/mL and 0.1-0.4ng/mL, respectively. Cells were cultured for another 24 hours before subculturing cells at 1x10^6 cells/well in non-coated Nunc 6-well plates in freshly-made complete RPMI media supplemented with 50uM 2-mercaptoethanol, 1% ITS, 0.5ng/mL recombinant murine IL-7, and 0.1-0.4 ng/mL recombinant murine IL-2. Cells were subcultured every 24 hours for 8-10 days in freshly-made complete RPMI media containing all supplements and cytokines. Cells were frozen at 80-90% viability in FBS supplemented with 10% DMSO and stored in liquid nitrogen for later use.

### 2.5 Tetramer staining

All tetramers were provided by the Baylor College of Medicine MHC Tetramer Production core. Unless otherwise noted, all tetramer staining steps were performed in 100µL total volume, on ice, and obscured from light. Briefly, CD8+ cells were washed in FACS buffer (1x PBS + 1% bovine serum albumin (BSA) + 0.05% NaNO3). 0.5µg/µL of tetramer was added to cells and incubated for 30 minutes. A non-specific tetramer with the same fluorophore was used as a negative control. For tetramer staining incorporating a CD8 blocking antibody, prior to tetramer staining, CD8+ T cells were resuspended in FACS buffer and labeled with 5–10 µg/mL of an anti-mouse CD8-PE antibody (clone YTS156.7.7, Bio-Rad #MCA2805PE) for 30 minutes at 37°C, 5% CO2. IgG PE was used as a negative control. Cells were washed with FACS buffer and stained with tetramer as described above. For tetramer stains incorporating dasatinib treatment, a protocol was adapted from Lissina et al., J Immunol Methods (2009)[90]. Prior to tetramer staining, T cells were resuspended in FACS buffer in 5mL round bottom tubes and treated with a final concentration 50nM of dasatinib for 1 hour at 37°C, 5% CO2. Tetramer was immediately added to cells without washing and incubated for 30 minutes. Cells were washed with FACS buffer and immediately analyzed by flow cytometry.
2.6 H-2Kb-SIINFEKL surface staining

All surface staining was carried out in 100 µL total volume, on ice, and obscured from light, unless otherwise noted. To measure H-2Kb presentation of the SIINFEKL antigen (hereafter referred to as “SIINFEKL expression”) on target cells, adherent cells were first detached with 2.21 mM EDTA + 0.25% Trypsin (Corning) and washed in FACS buffer. To reduce non-specific binding of IgG molecules to Fc receptors, cells were blocked with 5µg/mL of an Fc blocking antibody (purified anti-mouse CD16/32; clone 93; Biolegend #101335) for 10 minutes on ice. An APC anti-H-2Kb bound to SIINFEKL antibody (clone 25-D1.16, Biolegend, #141606) was added to target cells at 0.2mg/mL and incubated on ice for 30 minutes. Non-transduced target cells served as a negative control. To measure the effect of dasatinib treatment on H-2Kb-SIINFEKL expression in transduced target cells, detached LLC-SIINFEKL cells were washed in FACS staining buffer in 5mL round bottom tubes and treated with 50nM of dasatinib for 1 hour at 37°C, 5% CO2. H-2Kb-SIINFEKL surface staining was carried out immediately following incubation as described above without washing cells. Samples were acquired by the University of Texas MD Anderson Flow Cytometry & Cellular Imaging Facility, which is supported in part by the National Institutes of Health through M. D Anderson's Cancer Center Support Grants CA016672 and P30CA16672. Samples were collected on BD FACSCanto™ and Beckman Coulter Gallios and Gallios 561 cytometers. Acquired data were analyzed using FlowJo™ v10.8.0 software (BD Biosciences), gating on live single cells.

2.7 CD8 and CD4 surface staining

All surface staining was carried out in 100 µL total volume, on ice, and obscured from light, unless otherwise noted. All CD8 and CD4 surface staining protocols used either anti-mouse CD4-FITC (clone RM4-5; Biolegend #100509), anti-mouse CD8-FITC (clone 53-6.7; BD Pharmigen #553030), or anti-mouse CD8-PE (clone 100708; Biolegend #100708) antibody. Cells were resuspended in FACS buffer and blocked with 5µg/mL of an Fc blocking antibody (purified anti-mouse CD16/32) for 10
minutes. Anti-CD4 and/or CD8 antibodies were added to samples at final dilutions of 1:100-1:1000 and incubated for 30 minutes. Cells were washed following staining and resuspended in FACS staining buffer for flow cytometric analysis. Just before flow cytometric analysis, 7AAD viability staining solution (Biolegend #420404) or DAPI was added to cells. Samples were acquired by the University of Texas MD Anderson Flow Cytometry & Cellular Imaging Facility, which is supported in part by the National Institutes of Health through M.D. Anderson Cancer Center’s Support Grants CA016672 and P30CA16672. Samples were collected on BD FACSCanto™ and Beckman Coulter Gallios and Gallios 561 cytometers. Acquired data were analyzed using FlowJo™ v10.8.0 software (BD Biosciences), gating on live single cells.

2.8 Intracellular staining

All intracellular staining was performed under sterile conditions, unless otherwise noted. Optimized working concentrations of fluorescent dyes were previously determined by titration experiments.

**CMAC BLUE:** CellTracker™ blue CMAC dye (Invitrogen, catalog #C2110) was dissolved in DMSO at a stock concentration of 10mM and stored in aliquots at -30°C. Aliquots were used immediately after thawing. Unused portions of stock aliquots were placed back at -30°C for future use, however, stock aliquots were not used more than two times (i.e. two freeze-thaw cycles). LLC and LLC-SIINFEKL cells were stained separately. Adherent LLC and LLC-SIINFEKL cells were detached with pre-warmed 2.21 mM EDTA + 0.25% Trypsin and resuspended to ≤ 5.33 x 10^5 cells/mL in 1x PBS and stained with 6µM of CMAC blue. Cells were incubated for 30 minutes at 37°C, 5% CO₂, obscured from light. Following incubation, cells were washed two times in complete RPMI media and immediately used in ATTACH.

**CFSE:** CFSE lymphocyte staining protocol was adapted from Boston University’s flow cytometry core facility. Briefly, CFSE dye (Invitrogen, catalog #65-0850-85) was dissolved in DMSO
at a stock concentration of 10mM and stored in aliquots at -30℃. Aliquots were used immediately after thawing. Unused portions of stock aliquots were placed back at -30℃ for future use, however, stock aliquots were not used more than two times (i.e. two freeze-thaw cycles). 100µM working solutions were made immediately prior to use in 1 x PBS. CD8+ T cells were freshly thawed prior to staining. Cells were resuspended to ≤ 3 x 10^5 cells/mL in 1x PBS + 0.1% FBS and stained with 0.7µM of CFSE for 8 minutes at room temperature, obscured from light. An equal volume of 100% FBS was added to quench staining before transferring cells to a 37℃ water bath and incubating for 10 minutes. After staining, cells were washed with 1 x PBS + 2% FBS and resuspended to 1x10^6 cells/mL in complete RPMI media for immediate use in ATTACH.

**CellTracker Deep Red:** CellTracker™ Deep Red dye (Invitrogen, catalog #C34565) was dissolved in DMSO at a stock concentration of 10mM and stored in aliquots at -30℃. Aliquots were used immediately after thawing. Unused portions of stock aliquots were placed back at -30℃ for future use, however, stock aliquots were not used more than two times (i.e. two freeze-thaw cycles). Lymphocytes were resuspended to 1 x 10^5 cells/mL in 1 x PBS. Cells were stained with 500nM of CellTracker Deep Red for 30 minutes at 37℃, 5% CO₂, washed, and resuspended in complete RPMI media for immediate use in ATTACH.

**MitoTracker Deep Red:** MitoTracker™ Deep Red dye (Invitrogen, catalog # M22426) was dissolved in DMSO at a stock concentration of 1mM and stored in aliquots at -30℃. Aliquots were used immediately after thawing. Unused portions of stock aliquots were placed back at -30℃ for future use, however, stock aliquots were not used more than two times (i.e. two freeze-thaw cycles). Lymphocytes were resuspended to ≤ 1 x 10^6 cells/mL in 1x PBS and stained with 250nM of MitoTracker Deep red. Cells were incubated for 30 minutes at 37℃, 5% CO₂, washed, and resuspended in complete RPMI media for immediate use in ATTACH.
2.9 Co-cultures for IFN-γ ELISA

All co-cultures were performed in duplicates in tissue culture-treated 96 well round (U) bottom plates (Thermo Scientific 163320) in 200μL total volume per well with 2:1-5:1 effector: target ratios. Incubations were performed at 37°C, 5% CO₂, overnight. Pre-warmed complete RPMI media was used in all wash steps and co-culture assays unless otherwise noted.

**CFSE co-cultures:** Freshly-thawed, expanded CD8⁺ lymphocytes were stained with 0.7 μM of CFSE as described above. After staining, cells were washed two times with 1 x PBS + 2% FBS and resuspended to 1x10⁶ cells/mL in complete RPMI media for immediate use in co-culture assays.

**CD8-blocking antibody:** Expanded OT-I CD8⁺ and LLC-SIINFEKL cells were plated in a tissue culture-treated 96 well plate in complete RPMI media at a 5:1 ratio, 2 x 10⁵ cells/well. LLC cells served as a negative control. An anti-mouse CD8-PE antibody (clone YTS156.7.7) was added directly to wells containing OT-I CD8⁺ and LLC-SIINFEKL cells at final concentrations of 0.5 μg/mL, 2.5μg/mL, and 5μg/mL and allowed to incubate for the duration of the co-culture. Following overnight incubation, plates were centrifuged at 1,000xg and the supernatant was collected before immediately proceeding with an IFN-γ ELISA.

**MHC blocking antibodies:** For MHC blocking assays, three anti-MHC antibodies were tested: a pan anti-mouse MHC Class I antibody (anti-mouse-H-2, clone M1/42.3.9.8, InvivoMAb #BE0077), an anti-H-2b antibody (clone B8-24-3, Leinco Technologies #H8300), and an anti-mouse H-2Kb-bound to SIINFEKL antibody (clone 25-D1.16, Biolegend, #141606).

For assays using the pan-anti-mouse MHC Class I antibody (clone M1/42.3.9.8), a protocol was adopted from Herz et al., Journal of Experimental Medicine (2015) [96]. Briefly, prior to co-cultures, LLC-SIINFEKL cells were treated with 10μg/mL, 100μg/mL, and 500μg/mL of the antibody and incubated for 30 minutes at 37°C, 5%CO₂ in complete RPMI media. Cells were washed...
and plated with expanded OT-I CD8+ T cells at a 5:1 effector: target ratio, 2 – 2.4 x 10^5 total cells/well. To measure the effect of the antibody in the co-culture itself, the highest concentration of antibody used in the pre-treated groups (500µg/mL) was added to wells containing untreated LLC-SIINFEKL target cells and expanded OT-I CD8+ effector cells and allowed to remain for the duration of the co-culture. Expanded OT-I CD8+ T cells were cultured with LLC cells as a negative control and untreated LLC-SIINFEKL cells as a positive control. Following overnight incubation, the supernatant was collected before immediately proceeding with an IFN-γ ELISA.

For functional assays using the anti-H-2b antibody (clone B8-24-3), a protocol was adapted from Gubin et al., Nature (2014) [93]. Briefly, 0.4 x 10^5 LLC-SIINFEKL cells/well were plated in a 96 well plate and treated with 25µg/mL and 250µg/mL of the antibody for 1 hour at 37°C, 5% CO₂. After incubation, 1.6 x 10^5 expanded OT-I CD8+ effector cells (4:1 effector: target) were directly added each well without removing the MHC block from target cells. Expanded OT-I CD8+ T cells were cultured with LLC cells as a negative control and untreated LLC-SIINFEKL as a positive control. Plates were incubated overnight at 37°C, 5% CO₂. Following incubation, the supernatant was collected before immediately proceeding with an IFN-γ ELISA.

For functional assays using the anti-H-2Kb-bound to SIINFEKL antibody, a protocol was adapted from Shore et al., J. Mol. Bio (2009) [105]. A 5:1 effector: target cell ratio was used. LLC-SIINFEKL cells were plated in a tissue culture-treated Nunc 96 well plate, 0.24 x 10^5 cells/ well in complete RPMI media. LLC cells served as a negative control. Expanded OT-I CD8+ effector cells were added to wells, 2.4 x 10^5/ well in complete RPMI media. Concentrations of 0.5µg/mL, 2.5µg/mL, and 5µg/mL of the anti-H-2Kb-SIINFEKL antibody were added to wells. Untreated cells served as a positive control. Plates were incubated overnight at 37°C, 5% CO₂. Following incubation, the supernatant was collected and immediately used in an IFN-γ ELISA.
Dasatinib co-cultures: The protocol for dasatinib treatment was adopted from Lissina et al., J Immunol Methods (2009) and Weichsel et al., Clin Can Res (2008) [90, 91]. Dasatinib (BMS-354825) (Selleck Chemicals #S1021) was dissolved in DMSO at a stock concentration of 100µM and stored in aliquots at -30°C. Aliquots were used immediately upon thawing. Working solutions were made immediately prior to use by diluting 100mM stock aliquots in complete RPMI media. Expanded OT-I CD8+ cells were treated with 50nM, 100nM, and 200nM of dasatinib in complete RPMI media for 1 hour at 37°C, 5% CO₂. Cells were washed in complete RPMI media and plated in a tissue culture-treated 96 well plate at a 4:1 effector:target ratio in complete RPMI media, 2 x 10⁵ total cells/well. All concentrations of dasatinib (i.e. 50nM, 100nM, or 200nM) were also added directly to the wells containing pre-treated effector cells and remained in culture for the entirety of incubation. Cells were incubated overnight at 37°C, 5% CO₂. The following day, the supernatant was collected before immediately proceeding with an IFN-γ ELISA.

2.10 IFN-γ ELISA

Mouse IFN-γ ELISAs were performed using Biolegend’s ELISA MAX™ Deluxe Set Mouse IFN-γ kit (#430804) according to manufacturer’s instructions. When necessary, sample supernatants were diluted 1:10-1:100 in the included 1x assay diluent and placed in duplicates in the ELISA plate. Murine IFN-γ standards of 0-1,000 pg/mL were prepared in 1x assay diluent by serial dilution, using aliquots of the included standard reconstituted in 1x PBS. The plate was incubated at room temperature with shaking (500rpm with a 0.3cm circular orbit) and subsequently washed 4x with ELISA wash buffer. 1x Mouse IFN-γ ELISA MAX™ detection antibody was added to wells and plates were incubated for 1 hour with shaking at room temperature. Plates were washed 4x in ELISA wash buffer and 1x of the included Avidin-HRP solution was added to wells. Plates were incubated for 30 minutes at room temperature with shaking and washed 5x with ELISA wash buffer, making sure to let the wash buffer sit in wells for 30-45 seconds per wash to reduced background noise. The
included TMB substrate was added to wells and incubated at room temperature without shaking and in the dark for 5-20 minutes, or just before saturation was reached. An equal volume of 2N HCl was added to wells to stop the reaction and plates were immediately read at 450nm using an automated plate reader. Blank-corrected 450nm absorbance readings were plotted against standard concentrations to create a standard curve. IFN-γ concentrations of unknown samples were interpolated using the standard curve’s equation of the line and absorbance values.

2.11 Killing Assays:

Caspase-3 killing assay: Cleaved Caspase 3-killing assays were performed using expanded fractions collected in ATTACH v1 experiments and the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, catalog #C10740) per manufacturer’s instructions. Fractions were expanded for 14 days in 6 well plates with 0.6 ng/mL of recombinant mouse IL-7 and 0.4 ng/mL of recombinant mouse IL-2 as previously described. CD8+ lymphocytes were isolated from expanded fractions post-ATTACH using STEMCELL Technologies’ EasySep Mouse CD8a positive selection kit II as described previously. 1 mL of cell suspensions containing 0.1 x 10^5 of LLC-SIINFEKL target cells and 0.5 - 4 x 10^5 of freshly isolated CD8+ lymphocytes from fractions were added to 5mL polystyrene round bottom tubes fractions (effector: target ratios of 5:1, 10:1, 20:1, 30:1, and 40:1). The included CellEvent™ Caspase-3/7 green detection reagent was added to samples to a final concentration of 500nM. Samples were gently mixed and incubated in the dark for 4 hours at 37°C, 5% CO₂. Tubes containing CD8+ cells only were stained with only CellEvent™ Caspase-3/7 green detection reagent or SYTOX™ AADVanced™ dead cell stain for compensation controls. In Caspase-3/7 compensation tubes, apoptosis was induced by heating cells at 50°C for 10 minutes. A 1mM solution of the included SYTOX™ AADVanced™ dead cell stain was prepared in DMSO and added to samples during the final 5 minutes of incubation (final staining concentration of 1µM). Cells were immediately analyzed by flow cytometry without washing or fixing. Cells were gated on lymphocytes based on FSC vs SSC plots.
Chromium release assay: Antigen-specific killing was measured by a chromium release assay. Briefly, H1975-A3-GFP cells were resuspended to $1 \times 10^6$ cells in 1 mL of complete RPMI media. 100µL of Chromium-51 ($^{51}$Cr) was added to cells. Cells were incubated for 1 hour at 37°C, 5% CO₂ and washed twice with medium. Cells were resuspended to $2 \times 10^5$ cells/mL in LymphoONE T-Cell Expansion Xeno-Free Medium (Takara Bio # WK552S). 100 µL ($2 \times 10^4$) target cells were added to each well of a tissue culture-treated 96 well U bottom plate. CD8+ T cells were resuspended in LymphoONE T-Cell Expansion Xeno-Free Medium (without IL-2 supplementation) and added to wells at 40:1 and 20:1 effector: target ratios. Medium plus $^{51}$Cr-labeled target cells alone were used as a negative control (“minimum”). $^{51}$Cr-labeled target cells plus lysis buffer were used as a positive control (“maximum”). All samples were run in replicates of 4. Samples were incubated at 37°C, 5% CO₂ for 4 hours. 50µL of supernatant was collected from each well and transferred to a 96-well Luma plate (Fisher Scientific catalog #50-905-0456) which was placed under a laminar flow hood overnight to dry. The following day, the filter plate was read in a gamma counter machine. Percent of specific cell lysis was calculated by averaging all 4 replicates and using the equation: 

\[
\frac{\text{sample average} - \text{minimum average}}{\text{maximum average} - \text{minimum average}} \times 100.
\]

2.12 ATTACH v1 experimental setup

OVA$_{257-264}$ SIINFEKL peptide (Genescript) was reconstituted to 6µg/µL in DMSO and stored in aliquots at -80°C. LLC cells were seeded in tissue culture-treated vented T25 flasks and placed at 37°C, 5% CO₂ overnight to adhere. Cells were seeded at 80% confluency so that they would be at 95% confluency for ATTACH the following day. The day of ATTACH, adhered cells were pulsed with 10µg/mL of SIINFEKL peptide by adding stock aliquots directly to T25 flasks containing adhered cells + 3 mL complete RPMI media. Cells were incubated for 1.5 hours at 37°C, 5% CO₂. Meanwhile, expanded CD8+ T cells were thawed and resuspended to $1-2 \times 10^6$ cells/mL in complete RPMI media and placed in 6 well plates at 37°C, 5% CO₂ for 1 hour. Following rest, non-specific T cell populations were stained with 6µg/mL of MitoTracker Deep Red as described above and
resuspended in complete RPMI media. Media containing SIINFEKL peptide was removed from flasks and flasks were washed with 1 x PBS by gentle swirling. 1 X PBS was removed and replaced with complete RPMI media containing the input population (either bulk OT-I splenocytes or a 1:1 mixture of OT-I: B6 CD8+ T cells). Part of this “input” fraction was set aside at 37°C, 5% CO2 for downstream flow cytometric analysis. Flasks were spun down at 300 x g for 5 min to promote contact between T cell and target cell and immediately placed at 37°C, 5% CO2 and incubated for 90 minutes. After incubation, media in flasks was carefully removed by pipette and placed on ice in a labeled tube (“aspirate” fraction). Flasks were washed with 6mL of 1x PBS which was removed and placed on ice in labeled 15mL tubes (“wash” fraction). Finally, bound cells were detached with 0.25% Trypsin + 2.21 mM EDTA and transferred to labeled tubes (“detach” fraction). For downstream functional assays, fractions were expanded in vitro to sufficient numbers for downstream functional assays, no longer than 10 days.

2.13 ATTACH v2 experimental setup

Sterilized ibiTreat: #1.5 polymer coverslip µ-Slide I0.4 Luer tissue culture-treated channel slides (catalog #80176), luer lock female connectors (catalog #10825), elbow luer male connectors (catalog #10802), and 1.6mm silicone tubing (catalog #10842) were obtained directly from the manufacturer ibidi. All fluorescent staining and incubation steps were performed under sterile conditions, unless otherwise noted. All complete RPMI media was pre-warmed to 37°C prior to use unless otherwise noted. Live/dead cell counts were determined using VitaStain™ AOPI staining solution (Nexcelom Biosciences, catalog #CS2-0106) and Nexelom Cellometer® K2 fluorescent viability cell counter. A 1:4 ratio of effector: target cells was used (1x 10⁵ CD8+ T cells: 4 x 10⁵ target cells). SIINFEKL expression on LLC-SIINFEKL target cells was measured regularly to confirm antigen expression prior to running ATTACH. LLC and LLC-SIINFEKL target cells were seeded in separate channel slides, without antibiotic selection, in complete RPMI media. Channel reservoirs were topped off with complete RPMI media (LLC) or complete RPMI media with selection (LLC-SIINFEKL), capped
with included caps, and placed in 100mm x 20mm sterile culture dishes (Falcon™, catalog #353003). To prevent media evaporation from insufficient sealing by included caps, a damp Kim wipe was placed in each dish to maintain humidity. Slides were incubated at 37°C, 5% CO₂ overnight to allow target cells to adhere to channel bottoms. The following day, slides were flushed by pipetting 125 µL of complete RPMI through channels to remove dead and detached cells prior to loading T cells. After fluorescently staining CD8⁺ effector cells (described above), effector cells were loaded into channels by first removing media from channel slides containing adhered target cells. For experiments using single T cell populations (i.e. expanded OT-I CD8⁺ or B6 CD8⁺ splenocytes), 1 x 10⁵ of fluorescently-stained of CD8⁺ T cells were loaded separately into channels containing either LLC-SIINFEKL or LLC cells and incubated at 37°C, 5% CO₂ for 15 minutes, obscured from light. For experiments using a mixed input CD8⁺ T cell population, a 1:1 ratio of OT-I: B6 CD8⁺ cells was used, 1x 10⁵ total effector cells/channel. For experiments where T cells were loaded into empty slides, the same staining and incubation protocols were used, but target cells were not added to slides. Slides were run one at a time and loaded in succession every 7 minutes to ensure that no slide incubated longer than 15 minutes. T cell populations not yet loaded were kept at 37°C, 5% CO₂, obscured from light. After incubation, slides were removed from the incubator and immediately connected to an automated syringe pump for shear stress application.

2.14 ATTACH v2 shear stress application

Microscopy and shear stress application during ATTACH was done in non-sterile conditions at room temperature and atmospheric CO₂ and O₂ levels. Slides were connected to a Harvard Apparatus PHD ULTRA™ automated syringe pump with ibidi luer lock female connectors (catalog #10825), elbow luer male connectors (catalog #10802), and 1.6mm silicone tubing (catalog #10842). One end of the slide was connected to a 140mL syringe mounted on the automated pump system (Covidien Monoject™ 140mL piston syringe with luer-lock tip # 8881114030), while the other end drained into
Shear stress, defined as the force applied per area, to flow rate conversions were calculated per ibidi’s conversion calculation:

\[ \tau = \eta \cdot \gamma \]

Where \( \tau \) = shear stress, defined as the force acting on the cell (dyne/cm²)

\( \eta \) = dynamical velocity (dyne·s/cm²)

For cell culture media supplemented with 10% serum, \( \eta = \sim0.0072 \text{ dyne·s/cm²} \)

\( \gamma \) = shear rate, which characterizes the velocity profile of the perfused media (1/s).

For \( \mu \)-Slide I0.4 Luer channel slides, the shear rate (\( \gamma \)) at the wall of the channels is defined by:

\[ \gamma = 131.6 \cdot \phi \]

Where \( \phi \) = flow rate (mL/min)

So, for \( \mu \)-Slide I0.4 Luer channel slides, the final equation used to convert shear stress to flow rate is:

\[ \tau = \eta \cdot 131.6 \cdot \phi \]

\[ \tau = 0.0072 \cdot 131.6 \cdot \phi \]

\[ \tau/0.948 = \phi \]

Shear stress was applied to channels by infusion via 140mL syringes filled with pre-warmed complete RPMI media. For ramped “enrichment” experiments, applied shear stress increased linearly with time and ranged from 0.5-20 dyne/cm² (0.53- 21.1 mL/min) over the course of 12 minutes. For de-enrichment experiments where a low shear stress was constantly applied to wash off non-specific CD8+ T cells, a shear stress of 1 dyne/cm² (1.05 mL/min) was selected based on the point at which OT-I CD8+ became detached from negative control slides containing LLC cells (no antigen) during ramped shear experiments. For experiments using flow cytometry post-ATTACH for quantification,
cells were gated on live single cells. For experiments using fluorescent imaging, slides were visualized under Nikon A1RSi (Center for Advanced Microscopy, Department of Integrative Biology & Pharmacology at McGovern Medical School, UTHealth) and Nikon ECLIPSE Ti confocal microscopes using Nikon NIS- Elements AR 5.21.03. Fluorescent images were captured via time lapse, 1 image captured/second, within 1 region of interest (ROI) in the middle of the channel. The ROI was consistent across all slides within an experiment. After shear stress application, images were converted into TIFF files for downstream analysis and quantification with ImageJ Fiji software.

2.15 CD8+ T cell treatments in ATTACH v2

CD8 block: For ATTACH experiments incorporating the anti-mouse-CD8-PE antibody (clone YTS156.7.7) blocking antibody, CFSE-stained OT-I CD8+ T cells were resuspended in 1 x PBS + 0.1% FBS and stained with 0.5 and 5μg/mL of anti-mouse CD8-PE (clone YTS156.7.7) for 30 minutes at 37°C, 5% CO2. After incubation, cells were washed in complete RPMI media and immediately used in ATTACH.

Dasatinib: CFSE-stained OT-I CD8+ T cells were resuspended to 1x 10^6 cells/mL in complete RPMI media. Dasatinib was added to cells at a final concentration of 50nM. Cells were incubated at 37°C, 5% CO2 for at least 30 minutes. Cells were directly taken from incubation and loaded into channel slides (i.e. without washing off dasatinib) before proceeding with shear stress application.

2.16 Statistical analysis

All statistical analyses were performed using GraphPad Prism v9 (GraphPad Software). Ordinary one and two-way ANOVA tests (with multiple comparisons) and paired or unpaired t-tests were performed when appropriate. Quantification of fluorescent images captured during live imaging was performed using ImageJ Fiji software.
CHAPTER 3: RESULTS

3.1 ATTACH v1 enriches for OT-I CD8+ from a bulk splenocyte population

We previously developed a version of ATTACH (“ATTACH v1”) which was performed manually in T25 flasks using the H-2Kb-restricted SIINFEKL antigen. LLC cells pulsed with SIINFEKL peptide were used as target cells. Non-pulsed LLC cells served as a negative control. Previous titration experiments in the lab optimized the concentration of SIINFEKL peptide used for pulsing based on its saturation point. Non-expanded bulk OT-I splenocytes were added to flasks containing LLC and LLC cells pulsed with the SIINFEKL peptide. To encourage CD8+ T cell/target cell contact, an additional centrifugation step was performed prior to incubation. Flasks were incubated for 90 minutes at 37°C, 5% CO2. After incubation, “aspirate” fractions were collected by removing media from flasks. 1x PBS was used to gently wash flasks and collected as the “wash” fraction. Bound splenocytes and target cells were detached and collected as the “detach” fraction. CD4 and CD8 surface staining and flow cytometric analysis was used to measure relative proportions of T cell populations across fractions. Results showed a significant enrichment of CD8+ splenocytes from the bulk population in the final fraction of LLC-OVA flasks, which was 4x greater than that of LLC flasks (p = 0.002) (FIGURE 5A-B). Furthermore, OT-I CD8+ enrichment was most significant in the final “detach” fraction versus earlier fractions, confirming differential binding avidities between antigen-specific CD8+ cells and non-CD8+ cells (FIGURE 5C). Importantly, centrifugation increased enrichment of CD8+ T cells nearly 3-fold, highlighting the importance of close contact between CD8+ cells and target cells for optimal enrichment (FIGURE 5D).
Figure 5: ATTACH v1 enriches for CD8\(^+\) T cells from a bulk splenocyte population. Bulk OT-I splenocytes were added to flasks containing LLC and LLC-OVA target cells. (A) Relative proportions of CD4\(^+\) and CD8\(^+\) T cell populations in fractions were analyzed by flow cytometry. Red text indicates the ratio of CD8\(^+\)/CD4\(^+\) T cells. (B) Median percentages OT-I CD8\(^+\) cells in final fractions of LLC-OVA (blue) and LLC flasks (white). (C) Graph tracking CD8:CD4 ratios in the bulk splenocyte input population and collected fractions from LLC (gray) and LLC-OVA (blue) flasks (normalized to input). (D) The addition of a centrifugation step (pink) increased OT-I CD8\(^+\) retention nearly 3-fold in LLC-OVA slides.
3.2 **ATTACH v1 enriches for antigen-specific CD8\(^+\) T cells from a heterogeneous CD8\(^+\) population**

After confirming antigen-dependent CD8\(^+\) T cell enrichment from a bulk splenocyte population, a heterogeneous CD8\(^+\) population containing OVA-specific OT-I and non-specific B6 CD8\(^+\) T cells was used in ATTACH to further elucidate its specificity. CD8\(^+\) cells were isolated from OT-I and C57BL/6 splenocytes by magnetic sorting and expanded *in vitro* with IL-2 and IL-7. OVA specificity of expanded OT-I CD8\(^+\) T cells, and non-OVA specificity of expanded B6 CD8\(^+\) cells, was confirmed via tetramer staining and IFN-\(\gamma\) ELISA (**FIGURE 6A-C**).
Figure 6: Tetramer staining of expanded OT-I and B6 CD8\(^+\) T cell populations. Representative flow cytometry plots of expanded (A) OT-I and (B) C57BL/6 ("B6") CD8\(^+\) cells stained with an H-2K\(^b\)-SIINFEKL-APC tetramer. An irrelevant tetramer, H-2D\(^b\)-E7-APC, was used as a negative control. (C) OVA\(_{257-264}\) specificity of OT-I (red bar) and non-OVA\(_{257-264}\) specificity of B6 (blue bar) expanded CD8\(^+\) cells was confirmed functionally by IFN-\(\gamma\) ELISA.

To differentiate between CD8\(^+\) populations in post-ATTACH cytometric analysis, B6 CD8\(^+\) cells were stained with MitoTracker APC mitochondrial dye prior to ATTACH. OT-I and MitoTracker APC-stained B6 CD8\(^+\) cells were combined at a 1:1 ratio and added to flasks containing SIINFEKL-pulsed LLC and non-pulsed LLC cells. A portion of the combined T cell input population was set aside for later use in downstream flow cytometric analysis. Flasks were centrifuged to promote T cell/target cell contact and immediately placed at 37°C, 5% CO\(_2\) for 90 minutes. After incubation, “aspirate”, “wash”, and “detach” fractions were collected as described above. All fractions, including the input, were stained with an anti-CD8-FITC antibody to separate CD8\(^+\) cells during downstream flow cytometric analysis. Results showed nearly a 4-fold enrichment of OT-I CD8\(^+\) in the presence of
OVA antigen when compared to both the heterogeneous input population and final fraction of OVA-negative flasks (FIGURE 7A-C). Enriched antigen-specific populations also exhibited greater cytotoxic capacity than eluted non-specific populations when stimulated with OVA antigen. IFN-γ production of enriched CD8+ populations in the “detach” fractions of LLC-SIINFEKL flasks was increased 12-fold from that of the heterogeneous input population and was 5x greater than that of populations enriched in the absence of OVA antigen (FIGURE 7D) (p = 0.0312) (p = 0.0220). Moreover, killing ability of OVA-enriched CD8+ cells was almost 3-fold greater than cells isolated in the absence of antigen, as measured by expression of cleaved caspase 3/7 on apoptotic target cells (FIGURE 7E).
Figure 7: OT-I CD8+ cells are enriched from a heterogeneous CD8+ population in ATTACH v1 and retain antigen-specific effector functions. A 1:1 mixture of OT-I and APC-stained B6 CD8+ cells were added to LLC and LLC-OVA flasks (“input”). (A) Gating strategy (B) CD8+ populations in the input and final “detach” fractions of LLC and LLC-OVA slides were analyzed by flow cytometry. (C) OT-I CD8+ were enriched nearly 4-fold in LLC-OVA flasks (blue) when compared to both the heterogeneous input population (gray) and “detach” fraction of LLC flasks (white). (D) When stimulated with OVA antigen, IFN-γ production in CD8+ cells enriched in the final “detach” fraction of LLC-OVA slides (blue) is significantly higher than the heterogenous “input” population (gray) and “detach” fraction of LLC flasks (red) (E) OVA-specific killing is increased 3-fold in CD8+ cells enriched from LLC-OVA slides (blue) when compared to the heterogenous input population (gray) and LLC flasks (red).
3.3 Optimization of ATTACH v2: microfluidic isolation of antigen-specific CD8+ T cells

After ATTACH v1 confirmed the feasibility of isolating antigen-specific CD8+ T cells from a mixed population, we sought to optimize a semiautomated version (ATTACH v2) to overcome barriers to widespread application of ATTACH v1 in a clinical setting, namely the number of required target and effector cells required and the risk of human error in performing a manual version of the assay. In ATTACH v2 experiments, laminar flow was used to apply shear stress to TCR/pMHC interactions, as has been used in other studies exploring TCR/pMHC binding kinetics [80-81]. 0.4mm high Ibidi µ-Slide I0.4 Luer tissue culture-treated channel slides, which have been used to study an array of cellular responses under laminar flow, were used in place of T25 flasks for ATTACH v2 experiments [82-84]. Shear stress applied was via infusion using Harvard Apparatus’ PHD Ultra™ syringe pump. A general experimental overview is shown in FIGURE 8.

**Figure 8: General overview of ATTACH v2 experimental setup.** Schematic showing general ATTACH v2 experimental setup and dimensions of µ-Slide I0.4 Luer channel slides from (A) side and (B) top views. Circles represent wells on either side of the channel. (C) Tumor cells (orange) and a mixture of antigen-specific (red) and non-specific (blue) CD8+ cells are added to channel slides. (D) An automated pump system applies shear stress to the channel via infusion of media which detaches weak-binding non-specific CD8+ cells (blue) and leaves strong-binding antigen-specific cells (red) still bound to cognate antigens.
Due to the small, 100 µL volume of channel slides and possibility of inaccurate peptide pulsing, and to more faithfully replicate a cell whose antigens are expressed endogenously rather than pulsed, target cells which were transduced to endogenously express SIINFEKL antigen on MHC Class I were considered for ATTACH v2 assays. Two NSCLC murine model cell lines (LLC and 344SQ) were transduced to express the SIINFEKL antigen. Flow cytometric analysis of SIINFEKL expression on H-2K\(^b\) of transduced LLC-SIINFEKL and 344SQ-SIINFEKL showed that SIINFEKL expression was almost 2x higher on LLC-SIINFEKL which were subsequently chosen for use in ATTACH v2 (87.4% vs 53.7%, \(p=0.0160\)) (FIGURE 9A-C). Importantly, H-2K\(^b\)-SIINFEKL expression on transduced LLC-SIINFEKL cells was not significantly different than that SIINFEKL-pulsed cells \((p=0.1504)\) (FIGURE 10A-C).

**Figure 9:** H-2K\(^b\)-SIINFEKL surface expression on LLC-SIINFEKL and 344SQ-SIINFEKL cells.

Representative flow plots measuring H-2K\(^b\)-SIINFEKL expression on transduced (A) LLC-SIINFEKL and (B) 344SQ-SIINFEKL cells. Non-transduced cells were used as a negative control. (C)
Quantification of flow cytometry results showing H-2K^b-SIINFEKL surface expression in transduced 344SQ-SIINFEKL (light gray) and LLC-SIINFEKL (dark gray) cells.

Figure 10: H-2K^b-SIINFEKL surface expression in transduced LLC-SIINFEKL cells and LLC cells pulsed with SIINFEKL peptide. (A) Gating strategy for flow cytometric analysis of H-2K^b-SIINFEKL expression on (B) transduced LLC-SIINFEKL cells and (C) LLC cells pulsed with SIINFEKL peptide. (D) Quantification of flow cytometry results showing average H-2K^b-SIINFEKL surface expression in SIINFEKL-pulsed (white) and transduced LLC-SIINFEKL (checkered) cells.

Shear stress ranges for ramped experiments were selected based on previous studies in steady state shear stress applied to epithelial cells and T lymphocyte binding kinetics under shear stress [85-87]. LLC-SIINFEKL target cells were seeded at 100% confluency in channel slides and allowed to
adhere overnight at 37°C, 5% CO₂. Brightfield microscopy confirmed uniform seeding of target cells across channel slides (FIGURE 11).

Figure 11: Representative brightfield images of LLC (left) and LLC-OVA (right) target cells seeded at 100% confluency in µ-Slide I°.4 Luer channel slides.

3.4 ATTACH v2 “ramp shear” enriches for OT-I CD8+ T cells from a heterogenous CD8+ population

Expanded C57BL/6 (B6) and OT-I CD8+ T cells were mixed at a 1:1 ratio and loaded into channel slides. A portion of the mixed B6/OT-I CD8+ T cell population was set aside for downstream analysis. Shear stress values ranging from 0.5-20 dyne/cm² were applied to the channel via infusion in a “ramped” fashion (i.e. shear stress increasing linearly with time) over the course of 12.5 minutes (FIGURE 12A-B). We predicted that non-specific CD8+ cells would wash off at low shear stress values, while tightly-bound antigen-specific CD8+ cells would remain bound throughout higher shear stress. Fractions were collected at specified time points correlating with applied shear stress of 2.84, 8.3, 16.1, and 20 dyne/cm², as calculated using the equation of the ramped line, y = 1.56x + 0.5 (FIGURE 12B).
Figure 12: Ramped shear pattern of shear stress application. (A) Graphical illustration of a “ramp” shear pattern where applied shear stress increases linearly to time. (B) Shear stress values of 0.5-20 dyne/cm² were applied to channel slides in a “ramped” pattern over the course of 12.5 minutes. Fractions were collected at 1, 5, 10, and 12.5 minutes which correlated to shear stress values of 1, 3.92, 7.8, and 20 dyne/cm², respectively.

Following shear stress application, bound lymphocytes and target cells were detached and collected. All fractions, including the input and detached fractions, were stained with a SIINFEKL-APC tetramer to measure relative proportions of B6 and OT-I CD8⁺ populations. Although not statistically significant, results clearly showed nearly a 2-fold enrichment of OT-I CD8⁺ and an equivalent de-enrichment of B6 CD8⁺ in the final fraction (FIGURE 13A-D, p = 0.0728).
Figure 13: ATTACH v2 “ramped shear” enriches for OT-I CD8+ T cells from a heterogenous CD8+ population

(A) Representative gating strategy for ATTACH v2 “ramped shear” experiments. (B) A 1:2 mixture of OT-1: B6 CD8+ was added to LLC-SIINFEKL channels. OVA-specific and non-specific CD8+ populations in the input and collected fractions were visualized post-ATTACH by (C) flow cytometry using an H-2Kb-SIINFEKL tetramer (representative flow plots). (D) OT-I CD8+ are enriched (green), and B6 CD8+ de-enriched (red), 2-fold from a heterogenous CD8+ population in ATTACH v2 ramped shear experiments (note: relative proportions of CD8+ populations were normalized to baseline).
3.5 Ramp shear results in large amounts of cell death

Fractions collected during ramp shear contained many dead cells, perhaps due to the large amount of shear stress applied to cells, which we worried would be a barrier in downstream therapeutic applications utilizing ATTACH-enriched tumor-specific CD8⁺ T cells (FIGURE 14A-B). The need for higher variability, as well as more flexibility in other antigen settings, prompted exploration into a “de-enrichment” method of shear stress application whereby a low amount of shear stress was applied at a constant rate to wash off any weakly-bound non-specific T cells and leave more tightly-bound antigen-specific CD8⁺ T cells still bound to target cells (FIGURE 15A-B). The feasibility of enriching for antigen-specific CD8+ cells via a de-enrichment strategy was also supported by results in ramped shear experiments where the most significant enrichment of antigen-specific OT-I CD8⁺ occurred at low shear stress values within 1-3.92 dyne/cm² (FIGURE 13D).

Figure 14: Ramped shear results in large amounts of cell debris and death. (A) Representative FSC vs SSC flow cytometry plots of collected fractions depicting a large amount of cell debris which (B) increases with applied shear stress.
Figure 15: General overview of “de-enrichment” strategy in ATTACH v2. (A) Ramped shear separates antigen-specific and non-specific CD8$^+$ cells at distinct shear values. (B) De-enrichment strategy, whereby a low amount of shear stress is applied to wash off any weak-binding non-specific CD8$^+$ cells (red line), can enrich for a wider range of antigen-specific TCR/pMHC avidities (black dotted line) than ramp shear.

3.6 Optimizing “de-enrichment” strategy in ATTACH v2

To more easily distinguish between effector cell populations, intracellular staining of CD8$^+$ populations was used instead of tetramer staining. Additionally, fluorescent imaging during ATTACH was used in place of flow cytometric analysis post-ATTACH to analyze cell populations in real time. Target cells were stained with CMAC blue, B6 CD8$^+$ with CellTracker™ Deep Red, and OT-I CD8$^+$ with CFSE. To confirm that CFSE staining did not affect OT-I TCR/pMHC binding, CFSE-stained OT-I CD8$^+$ cells were labeled with H-2Kb-SIINFEKL-PE tetramer and analyzed by flow cytometry. Results showed comparable levels of OVA-tetramer$^+$ cells in unstained and FSE-stained OT-I CD8$^+$ (78.8% vs 83.7%, respectively, FIGURE 16A-D). Functionally, we observed a slight decrease (18%) in IFN-γ production of CFSE-stained cells versus untreated cells (p=0.0065, FIGURE 16E).
Importantly, OT-I CD8+ CFSE staining did not increase non-specific activation in wells containing LLC cells ($p=0.0745$, Figure 16E).

Figure 16: CFSE staining does not impact TCR/pMHC binding but slightly reduces IFN-γ release in OT-I CD8+ cells. (A) Representative gating strategy. (B) Unstained and (C) CFSE-stained OT-I CD8+ cells were labeled with an H-2Kb-SIINFEKL tetramer to confirm CFSE staining did not inhibit TCR/pMHC binding. An irrelevant H-2Db-E7 tetramer was used as a negative control. (D) Quantification of flow cytometry results showing the percent of tetramer+ cells in unstained (white) and CFSE-stained (green) OT-I CD8+ cells. (E) IFN-γ production as measured by ELISA in unstained (white bar) and CFSE-stained (light green bar) OT-I CD8+ T cells when co-cultured with LLC-SIINFEKL cells.
3.7 OVA-specific CD8$^+$ T cells are retained during “de-enrichment” method of shear stress application

To optimize de-enrichment experiments, single OT-I CD8$^+$ effector cell populations were used at first instead of a combined population of antigen-specific and non-specific effector cells as in previous ATTACH experiments. CFSE-stained expanded OT-I CD8$^+$ T cells were loaded into channels containing CMAC blue-stained LLC and LLC-SIINFEKL cells. Since slides were only able to be visualized one at a time under live imaging, slides were loaded with effector cells one at a time in succession so that the incubation time was consistent. Slides were incubated for 15 minutes at 37°C, 5% CO$_2$ and immediately connected to a Harvard Apparatus PHD Ultra™ syringe pump which was programmed to apply a constant rate of 1 dyne/cm$^2$ of shear stress via infusion for 5 minutes. During shear stress application, a time lapse series of fluorescent images were captured 1 image/second within a single ROI of 2.54 pixels in the center of the channel, which was kept consistent across slides (FIGURE 17A-B). Quantification of CD8$^+$ population proportions pre- and post-ATTACH confirmed antigen-dependent enrichment of OT-I CD8$^+$ during “de-enrichment” experiments: 5x more OT-I CD8$^+$ cells were retained in LLC-OVA slides than LLC slides (23.25% vs 4.37% of input, respectively) (FIGURE 17, $p=0.0003$). However, although tetramer staining confirmed OVA- specificity of expanded OT-I CD8$^+$ cells, LLC-OVA slides retained a quarter of loaded OT-I CD8$^+$ cells which prompted investigation into mechanisms contributing to the loss of antigen-specific CD8$^+$ T cells.
3.8 Optimizing retention of antigen-specific CD8+ T cells during “de-enrichment”

TCR internalization upon TCR/pMHC binding has been reported and the magnitude of downregulation is said to be proportional to TCR/pMHC avidity [88-89]. We wondered whether loss of antigen-specific CD8+ cells in the presence of antigen was perhaps due to TCR down-regulation upon pMHC binding and the resulting destabilization of TCR/pMHC interactions. In the context of multimer staining, pre-treating CD8+ T cells with dasatinib, a tyrosine kinase inhibitor, has been shown to improve TCR/pMHC-multimer binding by preventing TCR signaling thereby stabilizing TCRs at the cell surface which could bind to multimers [90-91]. If the loss of antigen-specific CD8+ T cells was indeed due to TCR downregulation, we predicted the dasatinib treatment prior to ATTACH would increase the amount of OT-I CD8+ retained in the presence of antigen by stabilizing TCRs at the cell surface and increasing the number of potential T cell/pMHC interactions.
To identify any detrimental impact on surface MHC presentation, target cells were pre-treated with dasatinib and analyzed for SIINFEKL presentation on H-2K\textsuperscript{b} molecules by flow cytometry. Results showed no difference in H-2K\textsuperscript{b}-SIINFEKL expression between treated and untreated LLC-SIINFEKL cells, confirming that dasatinib treatment did not hinder presentation of the SIINFEKL antigen (FIGURE 18A-C). Impairment of TCR signaling in dasatinib-treated OT-I CD8\textsuperscript{+} was next confirmed functionally by IFN-\(\gamma\) ELISA. Expanded OT-I CD8\textsuperscript{+} T cells were treated with 50nM, 100nM, and 200nM of dasatinib and added to wells containing LLC-SIINFEKL cells. As the reported half-life of dasatinib is only a few hours, all concentrations of dasatinib were also added directly to the wells containing pre-treated CD8\textsuperscript{+} cells and remained in culture for the entirety of incubation [91]. When compared to the untreated group, results showed significant reductions in IFN-\(\gamma\) production across all treatment groups, confirming inhibition of TCR signaling and antigen-induced T cell activation (\(p< 0.05\), FIGURE 18D). To confirm that dasatinib treatment did not inhibit TCR/pMHC binding, dasatinib-treated OT-I CD8\textsuperscript{+} T cells were stained with an H-2K\textsuperscript{b}-SIINFEKL-APC tetramer. No substantial difference in OVA-tetramer\textsuperscript{+} cells was observed between treated and untreated groups (81.8% vs 80.5%, respectively), confirming that dasatinib treatment does not interfere with TCR/pMHC interactions (FIGURE 18E-G).
Figure 18: Dasatinib treatment inhibits TCR signaling but does not disrupt TCR/pMHC binding in OT-I CD8+ cells. (A) Representative gating strategy for measuring H-2Kb-SIINFEKL expression on (B) LLC and LLC-OVA untreated and dasatinib-treated cells. (C) Quantification of flow cytometry results showing H-2Kb-SIINFEKL expression in untreated (white) and Dasatinib-treated (green) LLC and LLC-SIINFEKL cells. (D) IFN-γ production in dasatinib-treated OT-I CD8+ and untreated cells (green). (E) Representative gating strategy for tetramer staining. (F) Dasatinib-treated OT-I CD8+ cells were labeled with an H-2Kb-SIINFEKL tetramer to confirm that dasatinib treatment did not impair TCR/pMHC binding. An irrelevant H-2Dk-E7 tetramer was used as a negative control. (G) Quantification of flow cytometry results showing the percent of tetramer+ cells in untreated (white) and dasatinib-treated (green) OT-I CD8+ cells.

3.9 Dasatinib treatment does not increase retention of antigen-specific CD8+ T cells during ATTACH

Dasatinib treatment was incorporated into ATTACH by treating CFSE-stained OT-I CD8+ effector cells with 50nM dasatinib immediately prior to ATTACH. Untreated and dasatinib-treated OT-I CD8+ were loaded into slides containing CMAC blue-stained LLC and LLC-SIINFEKL cells. Slides were incubated for 15 minutes at 37°C, 5% CO2 and immediately placed under 1 dyne/cm² of shear stress for 5 minutes. Results showed that dasatinib treatment did not increase retention of OT-I CD8+ T cells in either the presence or absence of antigen when compared to untreated slides (p = 0.4151, LLC-SIINFEKL; p = 0.8115, LLC; FIGURE 19A-B).
3.10 Elucidating the specificity of ATTACH: inhibiting MHC-dependent TCR/pMHC interactions

To confirm that enrichment of effector cells in the presence of antigen was truly antigen-specific, we explored methods to block non-specific MHC-dependent binding.

Three different methods were explored to block TCR/pMHC interactions: pan-MHC blocking antibodies, a SIINFEKL-specific MHC antibody, and a CD8 blocking antibody. Mouse IFN-γ ELISAs, and tetramer staining when appropriate, were used to evaluate the ability of antibodies to inhibit TCR/pMHC interactions and antigen-induced T cell activation.

3.11 Two pan-MHC antibodies fail to inhibit SIINFEKL-specific TCR/pMHC interactions

Two pan-MHC blocking antibodies were tested. First, a pan anti-mouse-H-2 antibody (clone M1/42.3.9.8, InvivoMAb) previously reported to block ex vivo MHC-dependent interactions was tested [92]. LLC-SIINFEKL cells were treated with 10µg/mL, 100µg/mL, and 500µg/mL of the antibody and plated in a 96 well plate with expanded OT-I CD8⁺ cells. The highest concentration of

Figure 19: Dasatinib treatment does not increase retention of OT-I CD8⁺ T cells ATTACH v2.

Median values (as a percent of input) of untreated (solid) and Dasatinib-treated (checkered) OT-I CD8⁺ retained in (A) LLC-OVA and (B) LLC slides.
antibody (500µg/mL) was added directly to wells containing untreated LLC-SIINFEKL target cells and OT-I CD8\(^+\) cells and allowed to remain for the overnight co-culture. Analysis of collected supernatant by IFN-\(\gamma\) ELISA revealed no difference in IFN-\(\gamma\) production among any of the groups treated with the pan-MHC blocking antibody when compared to untreated cells \((p > 0.05)\) (FIGURE 20A).

The second pan-MHC blocking tested was an anti-H-2\(^b\) antibody (clone B8-24-3) previously reported to block \textit{in vitro} TCR/pMHC interactions [93]. Blocking ability of the anti-H-2\(^b\) antibody was again measured by IFN-\(\gamma\) ELISA. LLC-SIINFEKL cells were plated in a 96 well plate and treated with 25µg/mL and 250µg/mL for 1 hour before adding OT-I CD8\(^+\) cells. Following overnight incubation, supernatant was collected and immediately analyzed by IFN-\(\gamma\) ELISA. Results showed no inhibition of IFN-\(\gamma\) production in either of the treated groups when compared to untreated cells (25 µg/mL \(p = 0.0544\); 250µg/mL \(p = 0.3588\)) (FIGURE 20B).
Figure 20: Two pan-MHC blocking antibodies fail to inhibit SIINFEKL-specific OT-I/pMHC interactions. IFN-γ results of co-cultures incorporating pan-MHC blocking antibodies reported to block MHC-dependent CD8+ activation. (A) LLC-SIINFEKL cells were treated with 10 (light purple), 100 (orange), and 500 (light blue) µg/mL of a pan-H-2 blocking antibody prior to adding OT-I CD8+ cells. The highest concentration of antibody (500 µg/mL) was also added directly to wells containing untreated target cells (dark blue). Untreated cells served as a positive control (pink). (B) 25 (light blue) and 250 (dark blue) µg/mL of an anti-H-2b antibody were directly added to wells containing LLC-SIINFEKL and OT-I CD8+ cells. Untreated cells served as a positive control (orange).
3.12 A SIINFEKL-specific anti-MHC antibody hinders antigen-dependent OT-I CD8+ T cell activation

We questioned whether the pan-MHC blocking antibodies failed to block TCR/pMHC interactions due to incomplete hinderance of the SIINFEKL epitope and decided to test an antibody specific for SIINFEKL-bound H-2Kb complexes (clone 25-D1.16). Blocking ability of the anti-H-2Kb-SIINFEKL-APC antibody was measured by IFN-γ. Concentrations of 0.5 µg/mL, 2.5 µg/mL, and 5 µg/mL of the anti-H-2Kb-SIINFEKL antibody were added to wells containing LLC-SIINFEKL and OT-I CD8+ cells. Plates were incubated overnight and the supernatant was used in an IFN-γ ELISA. When compared to the untreated control, results showed impairment of IFN-γ production in a dose dependent manner; however, impairment was incomplete (56%) and only statistically-significant in the group treated with 5 µg/mL of antibody (p=0.0413, FIGURE 21A). Since a saturation point of the antibody did not seem to be reached, we repeated the co-culture with increased antibody concentrations to see if greater blocking would be observed. 5 µg/mL, 25 µg/mL, and 50 µg/mL of the anti-H-2Kb-SIINFEKL antibody were added to wells containing LLC-SIINFEKL and OT-I CD8+ cells and incubated overnight. The supernatant was collected and immediately analyzed by IFN-γ ELISA. Results showed significant and dose-dependent impairment, ranging from 29-84%, of IFN-γ production across all treatment groups when compared to the untreated control (p=0.0033, p=0.0016, p=0.0084) (FIGURE 21B). Even though significant blocking of antigen-dependent CD8+ T cell activation was observed with the anti-H-2Kb-SIINFEKL antibody, we wanted a blocking method which would block close to 100% of SIINFEKL-specific TCR/pMHC- dependent interactions. Due to this, and fear that blocking across a 2D surface and in a small channel volume could lead to ineffective blocking, direct blocking of CD8+ T cells, instead of target cells, was further explored.
Figure 21: SIINFEKL-specific TCR/pMHC interactions are significantly inhibited by an anti-H-2Kb-SIINFEKL antibody. IFN-γ results of co-cultures incorporating various concentrations of an anti-H-2Kb-SIINFEKL antibody (A) 0.5 (light green), 2.5 (dark green), and 5.0 (purple) µg/mL of an anti-H-2Kb-SIINFEKL antibody were directly added to wells containing LLC-SIINFEKL and OT-I CD8+ cells. Untreated cells served as a positive control (yellow). (B) 5 (purple), 25 (light blue), and 50 (dark blue) µg/mL of antibody were added directly to wells containing LLC-SIINFEKL and OT-I CD8+ cells. Untreated cells served as a positive control (yellow).

3.13 A CD8 blocking antibody (clone YTS156.7.7) inhibits nearly 100% of SIINFEKL-specific TCR/pMHC interactions

Blocking the CD8 co-receptor has been reported to destabilize, and therefore inhibit, TCR/pMHC interactions [94]. An anti-mouse-CD8-PE antibody (clone YTS156.7.7) reported to specifically block OT-I/SIINFEKL-MHC interactions was tested for its ability to block these interactions [105]. OT-I CD8+ and LLC-SIINFEKL cells were plated in a 96 well plate and 0.5-5 µg/mL of the CD8 blocking antibody was added directly to co-culture wells and incubated. The supernatant was collected and analyzed in an IFN-γ ELISA. Results showed significant reduction, ranging from 92-96%, in IFN-γ
production across all concentrations of antibody when compared to the untreated control ($p=0.0211$, $p=0.0193$, $p=0.0193$; FIGURE 22A).

After confirming the ability of the CD8 blocking antibody to functionally block TCR/pMHC interactions, treated OT-I CD8$^+$ effector cells were stained with tetramer to confirm the antibody’s ability to block TCR/pMHC binding, as would be required in ATTACH experiments. Expanded OT-I CD8$^+$ T cells were treated with 0.5 $\mu$g/mL, 5$\mu$g/mL and 10$\mu$g/mL of CD8 blocking antibody and stained with H-2K$^b$-SIINFEKL-tetramer-APC. Flow cytometric analysis showed nearly complete blocking of OT-I/SIINFEKL-MHC tetramer binding in both treated groups when compared to the untreated control (98%, $p=0.0003$; FIGURE 22B-C). Importantly, no difference was seen between groups treated with 5$\mu$g/mL and 10$\mu$g/mL of the antibody which served as an indication that a saturation point was reached ($p=0.7645$).
**Figure 22: A CD8 blocking antibody inhibits nearly 100% of SIINFEKL-specific TCR/pMHC interactions.** (A) IFN-γ results of a co-culture incorporating various concentrations of an anti-CD8-PE blocking antibody. Final concentrations of 0.5 (light red), 2.5 (medium red), and 5.0 (dark red) µg/mL were added directly to co-culture wells containing OT-I CD8⁺ and LLC-SIINFEKL cells. Untreated cells served as a positive control (blue). (B) Gating strategy and controls used to set negative gates. (C) OT-I CD8⁺ cells treated with the CD8 blocking antibody were stained with an H-
2Kb-SIINFEKL tetramer to confirm inhibition of TCR/pMHC binding. (D) Quantification of flow cytometry results showing the percent of tetramer⁺ cells in untreated OT-I CD8⁺ cells (white) and those treated with 5µg/mL (light red) and 10µg/mL (dark red) of the CD8 blocking antibody.

To test the antibody’s blocking ability against other epitopes, expanded pmel CD8⁺ T cells, specific for the H-2Dᵇ-restricted pmel-17 GP100₂₅₋₃₃ (GP100) epitope, were treated with the CD8 blocking antibody stained with an H-2Dᵇ-GP100₂₅₋₃₃ tetramer. No difference in the amount of tetramer⁺ cells was seen between untreated and treated groups (77.8% and 72%, respectively; FIGURE 23A). After confirming SIINFEKL-specific blocking of TCR/pMHC interactions, the CD8 blocking antibody was incorporated into ATTACH to identify any non-OVA-specific TCR/pMHC sources of background binding.

Figure 23: CD8 blocking antibody does not inhibit GP100₂₅₋₃₃ specific TCR/pMHC binding (A)

Gating strategy and controls used to set negative gates. (B) pmel CD8⁺ cells were treated with 5µg/mL of the CD8 blocking antibody and stained with an H-2Dᵇ-GP100₂₅₋₃₃ tetramer to measure the
antibody’s ability to block non-SIINFEKL TCR/pMHC interactions. (C) Quantification of flow cytometry results.

3.14 Treatment with anti-CD8 clone YTS156.7.7 decreases OT-I CD8\(^+\) T cell retention both in the presence and absence of OVA antigen in ATTACH

To optimize the amount of CD8 blocking antibody to use in ATTACH, CFSE-stained OT-I CD8\(^+\) cells were treated with two concentrations of the CD8 blocking (0.5 µg/mL and 5 µg/mL) and loaded into channel slides containing CMAC-blue stained LLC and LLC-SIINFEKL cells. Slides were incubated for 15 minutes and immediately placed under 1 dyne/cm\(^2\) of shear stress for 5 minutes. CD8-blocked CD8\(^+\) T cells were identified via co-localization of GFP and PE. Both concentrations of the blocking antibody showed a similar 2-fold (2.6 and 2.2) reduction in the percentage of OT-I CD8\(^+\) cells retained in treated LLC-OVA slides when compared to the untreated control (for 0.5 µg/mL \(p=0.0753\); FIGURE 24A-B). However, when comparing duplicates within the 0.5µg/mL- treated group, the degree of blocking between LLC-SIINFEKL varied: some slides achieved complete blocking while others showed minimal blocking (FIGURE 24C). No significant difference in OT-I retention was seen between LLC slides treated with the antibody versus untreated slides (\(p=0.5351\); FIGURE 24D-E).
**Figure 24:** Treatment with anti-CD8 (clone YTS156.7.7) blocking antibody decreases OT-I CD8⁺ retention in both the presence and absence of antigen in ATTACH. Treatment with (A) 0.5 µg/mL and (B) 5 µg/mL of a CD8 blocking antibody reduces OT-I CD8⁺ retention in LLC-SIINFEKL slides when compared to untreated slides (white). (C) Variability in OT-I CD8⁺ retention among groups treated with 0.5 µg/mL of the antibody was observed. Treatment with (D) 0.5 µg/mL and (E) 5 µg/mL reduces OT-I CD8⁺ retention in LLC slides when compared to untreated slides (white).
3.15 Elucidating the specificity of ATTACH: identifying MHC-independent TCR/pMHC interactions

Since LLC cells do not grow in a monolayer, even when 100% confluent, we wondered whether a source of MHC-independent background binding could be from CD8+ cells binding to the slide itself in between target cells. CFSE-stained OT-I CD8+ effector cells were loaded into empty channel slides without any target cells and incubated as usual for 15 minutes at 37°C, 5% CO2. A constant shear stress of 1 dyne/cm² was applied to slides for 5 minutes. Fluorescent imaging results revealed that an average of 6.05% of OT-I CD8+ remained bound to empty slides after shear stress application (FIGURE 25A-C). Importantly, this amount was not significantly different from the number of OT-I CD8+ cells retained in negative control LLC slides (5.75%), highlighting the possible contribution of MHC-independent binding to background retention of OT-I CD8+ (p = 0.5660) (FIGURE 25D).

**Figure 25: CD8+ T cells bind to channel slides in absence of target cells.** Representative images of CFSE-stained OT-I CD8+ cells in empty channel slides (A) pre-ATTACH and (B) post-ATTACH. (C) Median values of OT-I CD8+ retained (as a percent of input) in empty slides. (D) Median values of OT-I CD8+ T cells retained (as a percent of input) in LLC (solid) and empty (striped) slides.
3.16 De-enrichment strategy enriches for antigen-specific CD8⁺ T cells from a heterogeneous CD8⁺ T cell population

To further elucidate the specificity of CD8⁺ enrichment in ATTACH v2, and to recapitulate results from similar experiments in ATTACH v1, a heterogeneous CD8⁺ population of OT-I CD8⁺ and B6 CD8⁺ were used in de-enrichment experiments. To distinguish between the two populations under fluorescent imaging, CFSE-stained OT-I CD8⁺ and CellTracker Deep Red-stained B6 CD8⁺ cells were used. A 1:1 ratio of OT-I: B6 CD8⁺ was added to channels containing CMAC blue-stained LLC and LLC-SIINFEKL cells. Slides were incubated for 15 minutes at 37°C 5% CO₂ and immediately placed under 1 dyne/cm² of shear stress for 5 minutes. Fluorescent images pre- and post-ATTACH revealed a 2-fold enrichment of OT-I CD8⁺ T cells from the heterogeneous population in LLC-OVA slides (p=0.0260, FIGURE 26A-C), which was not observed in the absence of antigen (p=0.6461, FIGURE 26D-F). In fact, although not statistically-significant, a slight de-enrichment of B6 CD8⁺ T cells was observed in the presence of OVA antigen versus without antigen (p=0.7676, FIGURE 26B-C).
Figure 26: “De-enrichment” strategy enriches for OT-I CD8+ T cells from a heterogeneous CD8+ population. OT:1 and B6 CD8+ T cells were combined at a 1:1 ratio for use in ATTACH v2 de-enrichment experiments. A-C: Quantifying results from LLC-SIINFEKL slides. (A) Ratio of OT-I to B6 CD8+ T cells pre (checkered) and post (solid) ATTACH. (B) Relative proportions of OT-I (green) and B6 (red) CD8+ T cells at the beginning and end of ATTACH (normalized to input population). (C) The percentage of OT-I (green) and B6 (red) CD8+ T cells retained in LLC-SIINFEKL slides. D-F: Quantification of the same results as A-C in LLC slides.

3.17 Examining the specificity of ATTACH in a human antigen system

We next wanted to evaluate the feasibility of ATTACH in a human antigen system. To do so, CD8+ T cells recognizing the HLA-A03:01-restricted EGFR L858R epitope were isolated from healthy donor PBMCs via tetramer sorting and expanded ex vivo. (A) Tetrimer staining confirmed expansion of L858R-specific CD8+ T cells (FIGURE 27A). H1975 cells, which naturally harbor the L858R mutation, were transduced ex vivo to overexpress HLA-A03:01 (HLA-A3”) and used as target cells. Parental H1975 cells, which are homozygous for HLA-A01:01, were used as a negative control.

L858R-specificity of isolated CD8+ T cells was also confirmed by a chromium release killing assay (FIGURE 27B).
Figure 27: Confirming antigen-specificity in isolated L858R-specific CD8+ T cells. L858R-specific CD8+ T cells were isolated from healthy donor PBMCs by tetramer sorting and expanded ex vivo (A) Tetramer staining confirmed expansion of L858R-specific CD8+ T cells. (B) A chromium-51 release assay confirmed antigen-specific killing of isolated cells.

A single L858R-specific CD8+ population was used for optimization. Because the A03:01-transduced target cells contained a GFP tag, CellTracker Deep Red was used instead of CFSE to stain CD8+ T cells. A 1:1 ratio of effector: target cells was used in channels. CMAC blue stained H1975 and H1975-A3 cells were seeded in channels and brightfield microscopy was used to visualize confluency adhered (FIGURE 28A). HLA-A3 overexpression on transduced cells was confirmed by GFP expression. Deep red-stained L858R-specific CD8+ cells were added to channels containing H1975-HLA-A3 and H1975 parental cells. Since the optimal shear stress for L858R-specific CD8+ cells was unknown, a ramped shear pattern ranging from 0.1-10 dyne/cm² was applied over the course of 10 minutes. A significant number CD8+ were washed off negative control H1975 slides by 4.6 dyne/cm² (FIGURE 28B). CD8+ populations still bound at that point were used for enrichment
quantification. Although not statistically significant, 2x more CD8+ cells were retained in H1975-A3 slides when compared to negative control slides (FIGURE 28C-D). Importantly, this preliminary data highlights the feasibility of using ATTACH to isolate neoantigen-specific polyclonal CD8+ T cells in a human antigen setting.

**Figure 28: ATTACH ramp shear enriches for L858R-specific human CD8+ T cells.** (A) Brightfield microscopy images of channel slides seeded with parental H1975 (left) and H1975-A3 transduced (right) target cells. (B) CD8+ T cells in channel slides containing H1975 parental cells pre (left) versus post (right) ATTACH. White circles indicate retained CD8+ T cells. (C) CD8+ T cells in channel slides containing H1975 parental cells pre (left) versus post (right) ATTACH. White circles indicate retained CD8+ T cells. (D) Quantification of ATTACH results showing a slight enrichment for L858R-specific CD8+ T cells in the presence of antigen (green) versus negative control slides (white).
CHAPTER 4: DISCUSSION

Herein we have developed a method which enriches for OVA\textsubscript{257-264}-specific CD8\textsuperscript{+} T cells from bulk lymphocyte and heterogeneous CD8\textsuperscript{+} cell populations based on binding avidity to cognate antigens. The Assessment of T cells Tethered to Antigen Class I/II Histocompatibility (ATTACH) assay specifically enriched for OVA\textsubscript{257-264}-specific CD8\textsuperscript{+} cells 7-fold over CD4\textsuperscript{+} cells from a bulk OT-I splenocyte population in significantly less time than current isolation methods [76, 95]. Moreover, retention of OT-I CD8\textsuperscript{+} cells was 4x greater in the presence of OVA\textsubscript{257-264} antigen versus its absence, which confirmed antigen-specific CD8\textsuperscript{+} enrichment. Importantly, enriched CD8\textsuperscript{+} T cell populations were responsive to antigen re-stimulation and retained cytotoxic effector function as measured by IFN-\(\gamma\) production and killing ability. Greater feasibility of applying ATTACH in a clinical setting was achieved by optimizing a semi-automated version of ATTACH which required significantly less target and effector cells than ATTACH v1 and reduced inter-assay variability and risk of human error.

The small amount of OT-I CD8\textsuperscript{+} retention observed in the absence of antigen in ATTACH v1 and v2 experiments prompted investigation into MHC-dependent sources of non-specific background binding. In order to identify any non-specific MHC-dependent binding, we tested two pan-MHC blocking antibodies, both of which failed to functionally inhibit OT-I/SIINFEKL-MHC interactions. Previous studies employing the pan-H-2 antibody (clone M1/42.3.9.8) demonstrated its ability to block H-2D\textsuperscript{b}-restricted CD8\textsuperscript{+} activation, but none have reported on its ability to block H-2K\textsuperscript{b}-restricted epitopes such as the SIINFEKL antigen [96]. Our results suggest that while the antibody may recognize pan-H-2 molecules, it is unable to block the H-2K\textsuperscript{b}-restricted SIINFEKL epitope. However, this blocking antibody may still be used to block other H-2K\textsuperscript{b}-restricted antigens and/or H-2D\textsuperscript{b}-restricted antigens such as the gp100\textsubscript{25-33} epitope. A second anti-H-2\textsuperscript{b} blocking antibody (clone B8-24-3) reported block to H-2K\textsuperscript{b}-restricted T cell activation \textit{in vitro}, also failed to functionally inhibit SIINFEKL-specific TCR/pMHC interactions [93]. While this antibody has been reported to
recognize SIINFEKL-H-2Kb complexes, our findings suggest that it cannot block SIINFEKL-H-2Kb and TCR binding [97]. While it is possible that the pan-MHC antibodies failed to block antigen-induced T cell activation because of MHC recycling and complete internalization of antibody complexes, this seems unlikely given the fact that an anti-H-2Kb-SIINFEKL antibody effectively blocked OT-I activation at 1/50th of the concentration of the pan-MHC antibodies [29].

A CD8-blocking antibody reported to specifically inhibit SIINFEKL-specific TCR/pMHC interactions showed greater inhibition of TCR/SIINFEKL-MHC interactions than any of the MHC blocking antibodies tested. SIINFEKL-specific blocking of TCR/pMHC interactions was evident by a 92% reduction in IFN-γ production and nearly complete blocking of OT-I/SIINFEKL-MHC tetramer binding. However, when incorporating the CD8 block in ATTACH, treated OT-I CD8+ cells were still retained both in the presence and absence of antigen. While this blocking antibody failed to inhibit H-2Db-restricted gp10025-33 TCR/pMHC binding, further investigation is required into whether this antibody can inhibit other non-SIINFEKL H-2Kb-restricted TCR/pMHC interactions, especially low-affinity interactions which heavily rely on CD8 co-receptor stabilization for proper TCR/pMHC binding and antigen-induced CD8+ activation [98].

Although a low level of CD8+ background binding was observed in both manual and automated ATTACH assays, ATTACH clearly demonstrated the feasibility of isolating antigen-specific CD8+ T cells based on binding affinity to cognate antigens. In manual ATTACH assays, the most significant enrichment of OT-I CD8+ from LLC-OVA flasks was observed in the final “detach” fraction rather than the earlier “aspirate” and “wash” fractions. ATTACHv2 experiments corroborated this finding and demonstrated that antigen-specific CD8+ were enriched from heterogenous CD8+ population with high shear stress during “ramped” shear experiments, and weakly-bound non-specific CD8+ cells were de-enriched with low shear stress during “de-enrichment” experiments. Interestingly, background binding of OT-I CD8+ in the absence of antigen slides was not significantly different than that of B6 CD8+, which suggested an OVA-independent source of background binding. Indeed, further
investigation identified an MHC-independent source of background binding in which OT-I CD8+ cells remained bound to the slide itself in the absence of target cells. This seemed to be a likely source of background binding since LLC cells grow in a patchy pattern rather than a monolayer, which leaves regions of exposed slide between target cells for CD8+ cells to bind to. Our findings emphasize the importance of a monolayer of target cells for optimal CD8+ enrichment. Incorporating alternate strategies to adhere target cells to slides, such as mechanical adhesion with centripetal force, or using antibodies and/or biotin-streptavidin linkage to immobilize target cells instead of natural adhesion, could help overcome this. Alternative blocking methods are also being explored to reduce MHC-independent CD8+ T cell retention.

In ATTACH v2 experiments using a single OT-I CD8+ population, a quarter of the input CD8+ population was retained in LLC-OVA slides. This may have been due, in part, to overloading the slide with effector cells, which was done to increase the likelihood of effector and target cell contact. However, a more likely reason for the loss of antigen-specific effector cells might be the absence of a centrifugation step. Results from ATTACH v1 experiments confirmed that the addition of a centrifugation step prior to incubation encouraged effector/target cell contact and increased OT-I CD8+ enrichment almost 3-fold. Due to the risk of losing loaded effector cell populations from centripetal force through the wells on either side of the channel slides, ATTACH v2 experiments did not contain a centrifugation step prior to incubation. We predict that the addition of a centrifugation step in ATTACH v2 or encouraging target/effector cell contact in a different way, would increase OT-I CD8+ enrichment in ATTACH v2. Future studies include developing a prototype slide to maximize tumor/ T cell interactions.

Although CFSE staining of OT-I CD8+ T cells did not seem to affect TCR/pMHC binding, it decreased IFN-γ production when CFSE OTI-CD8+ T cells were cultured with LLC-SIINFEKL cells. It is possible that the CFSE staining is interfering with H-2Kb-SIINFEKL expression on target cells.
Future experiments will investigate whether this is negatively impacting OT-I CD8+ retention in ATTACH.

Antigen-induced TCR downregulation has been reported to destabilize TCR/pMHC binding [88,99]. Treating CD8+ cells with the tyrosine kinase inhibitor, dasatinib, has been reported to increase CD8+/pMHC multimer binding by preventing signaling through the TCR and inhibiting antigen-induced TCR downregulation [90]. We predicted that treating effector cells with dasatinib would increase retention by increasing the amount of TCRs within the immunological synapse that could bind to pMHC complexes. Although Dasatinib treatment did impair CD8+ T cell cytotoxic function, incorporation into ATTACH did not affect OT-I CD8+ retention which, with the current 15-minute incubation time, suggests that TCR downregulation may not be a challenge in ATTACH as it is in other isolation methods, namely, multimer-based isolation [90, 100].

A major hurdle of CD8+-mediated anti-tumor therapies is the challenge of identifying CD8+ T cells that selectively target tumor cells and not healthy cells [79]. The customizability of ATTACH provides a potential strategy to de-enrich for cross-reactive CD8+ clones from a bulk TIL population by first running ATTACH using normal lung tissue, for example, to de-enrich “bystander” CD8+ clonotypes that target normal tissue, and subsequently using the de-enriched product with matched tumor cells to enrich for tumor-specific clonotypes. Yet to be determined, however, is the sensitivity of ATTACH in enriching for rare CD8+ populations such as neoantigen-specific clonotypes which have been reported to comprise less than 1% of the CD8+ TIL population [79, 101-102]. We plan to investigate whether running ATTACH serially would enable the isolation of low-frequency CD8+ clonotypes.

In the human antigen setting, data from preliminary experiments suggested antigen-specific enrichment of L858R-specific CD8+ T cells; however, enrichment was not statistically significant. Due to ongoing optimization of experiment conditions, slides containing H1975 and H1975-A3 cells were only loaded to ~60% confluency which could have hampered enrichment. Previous tetramer
staining confirmed L858R-specificity in nearly 70% of expanded CD8⁺ T cells; however, tetramer staining was not repeated prior to running ATTACH to confirm that antigen-specificity was still maintained. Ongoing optimization experiments to more clearly define shear stress values, seeding densities, and incubation times for the L858R antigen system are underway.

In conclusion, we have herein demonstrated the feasibility of isolating antigen-specific CD8⁺ cells based on binding affinity in an OVA\textsubscript{257-264} using the ATTACH method. By using a pool of loaded MHC class I molecules on the surface of matched target cells, ATTACH rapidly enriches for OVA\textsubscript{257-264}-specific CD8⁺ cells in as little as 5 minutes and circumvents many of the challenges of current isolation methods such as antigen identification and expensive multimer production. Moreover, the customizability of shear stress application in ATTACH enables isolation of varying TCR/pMHC affinities, which, due to the intrinsic interpatient variability in TCR affinities, is especially important for the therapeutic capability and success of CD8⁺-based therapies [103, 104]. Future studies in alternate antigen and neoantigen systems will further define the specificity of ATTACH and highlight its feasibility for use in a variety of antigens.
REFERENCES


VITA

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