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Assessment of Specific T-cell Receptor Engagement Without Necessity for Identification of the
Relevant Antigen

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Harib Hassan Ezaldein
2016

Abstract

Individual cancers, even of the same cell type, express unique arrays of distinctive tumor antigens, requiring accurate laboratory measurement of induced immunity against them problematic.

Fluorescently tagged reagents (dextramers) that selectively engage clonal T-cell receptors (TCR) can cytofluorographically quantify both frequency and avidity antigen-specific T-cells, but cannot be synthesized without prior identification of the relevant antigen. Since clinically evident tumors may contain as many as 300 unique point mutations capable of generating a large number of uniquely antigenic proteins, and since procurement of such information for each cancer is currently unrealistic, it is presently only possible to assess responses to anti-cancer immunotherapy by clinical determination of estimation of the tumor burden capacity. Therefore, there is a need to develop methodology that can quantify the collective anti-tumor T-cell response, without prior identification of the full array of expressed tumor antigens. We have developed a practical high-resolution method to measure antigen-specific CD8 T-cell responses, via T-cell proton extrusion, an immediate result of selective TCR engagement by antigen presenting cells. The fluorescent emission characteristics of hydroxypyrene trisulfonate (HPTS) correlate with solution-phase proton concentrations, manifesting as increased emission signals. We exploit this TCR characteristic within the context of T-cell activation and show that stimulation with anti-CD3 immunoglobulin stimulates measurable TCR to release protons to a significantly higher degree than unengaged TCR ($p < 0.001$), both mouse and human systems. Specific mouse CD8 T-cell responses to an exogenous tumor antigen (the eight amino acid derivative SIINFEKL of transfected ovalbumin) and human CD8 T-cell responses to a melanoma-associate tissue antigen (MART-1) differed from control ($p < 0.001$). Human CD8 T-cell responses to MART-1 peptide, presented by dendritic antigen presenting cells (DC) could be similarly distinguished from that to control peptide (gp100), even when the frequency of MART-1 responsive CD8 T-cells was titrated down from 23 percent to 1% ($p < 0.001$), as measured in parallel by dextramers. When implemented in a

tumor-responsive animal model, treatment groups showed higher emission intensities compared to control groups at time points. These preliminary results confirm the practicality of real-time assessment of antigen-specific TCR engagement, by proton release, a methodology which may become applicable to T-cell responses to any collective group of antigens.

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Table of Contents

ABSTRACT	2
ACKNOWLEDGMENTS	4
BACKGROUND	7
<i>TRADITIONAL METHODS OF IMMUNOLOGICAL MONITORING:</i>	8
<i>BIOPHYSICS OF ACTIVATION: CALCIUM FLUX AND PROTON EXTRUSION</i>	9
<i>USE OF MODEL ANTIGENS</i>	10
<i>SUMMARY OF BACKGROUND AND SPECIFIC AIMS OF THIS STUDY</i>	13
MATERIALS, METHODS, AND APPROACH	14
<i>POLYCLONAL T-CELL STIMULATION MONITORING</i>	15
<i>APC:T-CELL COUPLET FORMATION AND IMAGING FLOW CYTOMETRY</i>	15
<i>ANTIGEN-SPECIFIC ASSESSMENTS</i>	17
<i>MART-I-REACTIVE CD8 EXPANSION</i>	18
<i>CORRELATION STUDIES WITH CALCIUM FLUX</i>	19
<i>MURINE TUMOR-RESPONSIVE MODEL AND IN VIVO FLUOROMETRIC ASSESSMENTS</i>	19
RESULTS	21
<i>DETECTABLE POLYCLONAL ACTIVATION WITH CELLULAR LIMITS</i>	21
<i>OBSERVABLE ANTIGEN-SPECIFIC ACTIVATION OF MURINE OTI CD8+ RESPONDERS.</i>	21
<i>CONJUGATE FORMATION AND DETECTABLE ACTIVATION OF HUMAN MART-I-SPECIFIC T-CELLS</i>	22
<i>DETECTABLE ANTIGEN-SPECIFIC INTERACTIONS WITH NAÏVE EXPANDED CD8 RESPONDERS</i>	22
<i>SIGNIFICANT DIFFERENCES IN EMISSION INTENSITY USING UNKNOWN ANTIGENS AS PROBES IN AN ANIMAL MODEL</i>	23
CONCLUSION	25
<i>DRAWBACKS OF THIS STUDY</i>	28
FUTURE DIRECTIONS	29

<i>METABOLIC PHENOTYPING AS INSIGHT INTO ACTIVATED CELL POPULATIONS</i>	29
<i>IMPROVING DETECTABILITY VIA TARGETED AGENT DELIVERY</i>	31
<i>A LABEL-FREE APPROACH TO ANTIGEN-SPECIFIC RESPONSES VIA NANOSCALE SEMICONDUCTORS</i>	32
FIGURES	35
FUNDING SOURCES	43
REFERENCES	44

Background

The role of immune cells in the development or regression of cancer has been a subject of investigation for decades and the importance of the innate and adaptive immune systems in tumor regression have provided the rationale for modern immunotherapy and cancer vaccines. The introduction of checkpoint inhibitors, along with newer immunotherapeutic targets, has ushered in a new era in cancer treatment strategy. Despite the promising circumstances, our ability to predict or assess whether a patient is capable of responding to such newer agents remains inconsistent. Significant focus is needed on developing consistent and standardized methods to evaluate CD8 T-cell responses to administered therapies, and such efforts are not limited to the field of cancer therapy.^{1,2} The principle of developing surrogate endpoints based on immunological readouts for every patient can have tremendous advantages in navigating treatment strategies, especially if such information can be obtained prior to a clinically observed outcome.

At the time of this study, there exist no more than 15 publications devoted to the subject of “immunological monitoring” within the context of cancer since 1978. Studying the elements of an immune response within different oncological frameworks therefore deserves more attention as the majority of efforts thus far have focused on predicting immunological rejection within the field of organ transplants. As cancer incidence rises, the utility of such data becomes increasingly relevant as newer therapies become available and costs for developing improved strategies continue to escalate.³

Current approaches to assessing T-cell activation have remained rather consistent, with little innovation, over the past few decades. The traditional approaches largely rely on measuring cell proliferation, cytotoxicity or cytokine release. However, the probes used in such studies involve a small group of model antigens that are “tumor-associated” for the relevant cancer and have been isolated and well studied over time. The domineering fallback of such a dependency lies precisely in the disregard

of the more immense cohort of antigens that are unknown or undefined and are arguably more relevant to the immune surveillance profiles of each patient. The uniqueness of each patient's tumor and immune response therefore has been consistently assessed using artificial probes or reductionist assumptions that have failed to prove consistency or clinical utility over time. Though readouts such as cytokine-mediated amplification or cytotoxicity are theoretically valid approaches to determine the reactivity of a patient's CD8 T-cells, newer approaches need to be developed that look towards the use of undefined sets of antigens to determine the state of a patient's treatment response. Our aim is thus to develop an approach that seeks to reconcile the need to identify causative antigens with an assay system that is capable of measuring early signs of immunological activation in CD8 T-cells that can unfailingly be identified in every patient.

Traditional Methods of Immunological Monitoring:

Enzyme-linked Immunospot (ELISPOT), cytokine flow cytometry, and tetramer or dextramer positivity are among the most widely available and utilized approaches. Though ELISPOT is the most frequently used method of detecting the number of reactive CD8 T-cells in patient samples, it was not shown to correlate effectively with the other aforementioned techniques in a recent cancer vaccination trial.⁴ This limited concordance suggests that the frequency of circulation of reactive populations of cells may not be adequate enough for detection and that perhaps the vaccinations themselves could have failed to elicit robust responses as well.

A longstanding immunological monitoring trial that spanned twelve academic centers, from five European countries, concluded that our capability of assaying antigen specificity is extremely variable and made recommendations for standardizing protocols.⁵ A cited reason for the inter-center variability included the differing yield of assayable T-cells at each institution, along with inconsistent levels of background noise with each readout strategy. Specifically, the study encountered difficulties with

baseline spot numbers of ELISPOT testing and was unable to correlate to distinct variables or outcomes of the study.

Intracellular cytokine analysis with flow cytometry and structural probes such as MHC tetramers or dextramers are also various methods charged with the universal challenge of developing a consistent assessment or readout from inconsistent starting material, such as the variable yield of CD8 T-cells seen in different patients. Both approaches require standardized protocols and quality control assessments prior to use, which can dictate the interpretation of results. For example, even factors such as staining on ice compared to room temperature affect the avidity of a tetramer-TCR interaction as the ensuing results can be influenced by background interactions. This has tremendous consequences for inter-institutional variability and consistency in the acquired results. Tetramer or dextramer analysis also has a crucial dependence on identifying the three-dimensional structure of immunodominant epitopes for TCR engagement, which is an insurmountable logistical challenge considering the innumerable tumor associated antigens seen in cancer patients.

Biophysics of Activation: Calcium flux and Proton Extrusion

Calcium flux, which represents the intracellular signaling cascade in the T-cell, is a well studied and characterized as a marker of immune activation.⁶ Calcium ions are released from intracellular stores during activation, mainly for purposes of secondary cellular signaling, and such stores may additionally induce an influx of exogenous calcium through selective channels on the cell membrane.⁷ These signals are essential for both short-term and longer-term processes such as cell motility, which can halt and can promote a more stable immunological synapse, or differentiation into effector or memory phenotype. It has been observed that even calcium ion flux can have variable patterns and manifestations depending on the biophysical interactions that occur in the vicinity of the TCR. Under fully activating conditions for example, the kinetics of the calcium ion changes resembles a sharp increase followed by a smooth plateau. Even a small disturbance of the full signal can result in a delay or loss of cellular activation or

proliferation and hence indicates the importance of sustained calcium signaling with respect to cellular metabolic processes. The importance of a sustained calcium signal was shown to enable transcription and its resulting processes, including downstream protein synthesis.⁸

Prior to activation, T-cells have low rates of cellular metabolic activity, though once activated, undergo antigen-specific differentiation, optimize cytotoxic processes, and engage in paracrine signaling via cytokine secretion. Downstream to the biophysical changes that occur such as calcium or proton signals, other signaling pathways can be activated such as the MAP kinase pathway via RAS and RAF. Costimulatory proteins such as the cytokine receptors or B7 can also contribute to T-cell activation.^{9,10} Such activities require significant biosynthetic efforts and hence rely on energy utilization and cellular respiration. Extracellular acidification or proton flux is related to the catabolic pathways that seek to generate ATP at the onset of cellular activation.^{11,12} Extracellular acidification is largely recognized as a byproduct of lactic acid production within a cell and a profound increase can be indicative of intracellular changes associated with ATP utilization.¹³ Such measurements can enable the identification of bioenergetic states associated with environmental-induced alterations in cellular physiology and even empower drug discovery efforts.

Rabinowitz and colleagues compared acid release, calcium flux, and T-cell proliferation responses to a variety of different stimulating ligands.¹⁴ Their study noted that significantly greater concentrations of antagonistic ligands or antibodies were needed to block the early events associated with T-cell activation such as proton release or calcium flux. They were the first to propose that a hierarchy exists within T-cell signaling in which various stages of activation correlate to the degree of a ligand's affinity and T-cell receptor engagement.

Use of model antigens

Classical approaches to predicting clinical responses have relied on delayed-type hypersensitivity approaches to help provide some measure of CD4 or CD8 reactivity.¹⁵⁻¹⁷ As mentioned

previously there is a reliance on T-cell defined antigens, which have shown to be poor correlates of post-therapy outcomes. Such efforts therefore have a poor track record in developing surrogate measures of the overall cellular immune response to therapy due to low precision, sensitivity, and relevance to the library of unclassified antigens encountered by a host's immune system. Tyrosinase, MART-1, and gp100 are widely expressed in melanoma, for instance, but are also proteins associated with normal tissues. Other melanoma probes include MAGE1, GAGE, and NY-ESO-1, all of which are also expressed in normal or neoplastic testicular and placental tissue. Therefore, the model antigens available to us are theoretically incapable of outputting tumor-specific trends for every patient. Some of the most commonly cited disadvantages to such approaches include the requirement to know and identify a peptide epitope and restricting allele, both of which require a significant level of technical expertise (i.e. protein folding conditions) to drive the construction of specific peptide:MHC multimers, which demonstrates how the evaluation of single or few epitopes may not provide a complete picture of the total immune response to the gamut of tumor antigens.¹⁸

Although MART-1 positivity is sometimes reported to correlate to tumor regression, Saleh and colleagues indicated that such an observation was restricted to tumor antigen-loss circumstances.¹⁹ It was nevertheless acknowledged that immune responses to this specific antigen may be insufficient to affect survival in patients with progressively-advanced disease. This is intuitive in the sense that such patients may have a complex and diverse repertoire of tumor-associated antigens and therefore trying to correlate a single, epitope-specific CD8 cell expansion to clinical tumor regression may not be a consistent strategy of deciphering a patient's immune response. In fact, it was shown that even though a modified gp100 peptide vaccination could correlate with the expansion of a peptide-specific CD8 population expansion, not all of these expanded cells were shown to be functionally responsive with respect to cytokine secretion and there was a lack of tumor regression in the trialed patients.²⁰

The dynamic antigen landscape of a progressing tumor could also account for the differences seen in such studies. Spontaneous immunoselection of dominant epitopes remains a subject of relevance, as many studies have shown that multiple metastatic nodules in the same cancer patient may show a progressive loss of tumor-associated antigen:MHC complexes with time.²¹⁻²³ It is thus crucial to avoid discounting the observation that tumor heterogeneity, especially in advanced cancer patients, can result in a diverse T-cell antigenic repertoire and how the limited pool of known antigens cannot logistically reflect the complexity of this larger antigen burden seen in such patients. Our current approaches to tumor vaccine therapy as well are simply too selective of a strategy to prevent tumor growth and this is largely due to an assumption that every tumor cell shares a common antigen and does not account for tumor heterogeneity and capacity for epitope selection with time.²⁴

Already, there is truly no consensus on evaluating unknown or undefined Ag and research efforts have recently been directed towards biophysical phenomena associated with early activation signals such as intracellular receptor domain phosphorylation or calcium flux, among others, since they can be assessed qualitatively and limit the need for specific biomarkers. Surface plasmon resonance, quartz crystal microbalances, cantilever-based microarrays, and field effect transistors are among a wide range of technologies with accelerating scientific development over the past decade with viable alternatives to measure biomolecules associated with various cellular outputs resulting from cellular phenomena such as an immune activation.²⁵⁻²⁸ Interestingly, the number of published journal articles including the terms “biosensor” or “lab on a chip” have enjoyed an exponential increase over the years, representing a keen interest in point-of-care diagnostics and its applicability to immunomonitoring.²⁹ The majority of such methods rely on amplification of biochemical signals arising from exceedingly small numbers of cellular subsets, such as activated CD8-APC pairs in our study, which would otherwise be largely undetectable.

Thus, it is more than evident that our strategies of monitoring responses need to be directed towards partially or undefined antigens, such as assessing cellular responses to tumor-cell lysates, modified tumor cells, or even DC-tumor cell fusions. The large variety of antigens may differentially induce adaptive immune responses, including both the humoral and cytotoxic domains, to help decipher a patient's response. It was previously proposed that a limited number of antigens in a tumor cell-lysate, or other composite antigen preparations, should at least be characterized in order to monitor specific components, though we believe the relevance of identifying such antigenic compartments remains debatable and subject to change with each assessment.³⁰⁻³²

Summary of Background and Specific Aims of this Study

Much scientific insight has been gained since the 1980s in understanding T-cell activation via the T-cell antigen receptor complex (TCR) and its downstream signaling effects.³³⁻³⁵ We now understand that activation depends on the careful interplay of early signaling cascades via phosphorylation, intra- and extracellular ion shifts, cytoskeletal reorganization, and eventual gene transcription of activation cytokines, such as IL-2.³⁶⁻³⁹ Despite our sophisticated mechanistic understanding, there is no reliable method of assessing the induction or augmentation of a patient-specific, tumor-directed T-cell response.³¹ Current laboratory efforts seek to assess T-cell activation by means of biochemical changes such as cytokine secretion, cell surface markers, clonal expansion, tetramer- or dextramer-positivity, and even apoptosis readouts. However, the scope of clinically relevant, identified cancer epitopes, utilized as probes in such assessments, remains narrow since such readouts are largely confined in potential on two fronts: the limited range of employable antigens (Ag) and the unique and dynamic nature of tumor-associated antigens within each patient.^{30,40,41} In malignant melanoma for example, antigens such as MART-1 that are common to even normal tissues are used in crude assessments to essentially guess the level of CD8 anti-tumor responses. The clinical utility and translational potential of our established antigen libraries are astounding imperfect, representing both a

need for increased standardization efforts and the exploration of newer strategies to keep pace with the clone-specific responses of each patient.⁴⁰⁻⁴²

Our study seeks to introduce a novel readout on the totality of an immune response to antigens that need not be defined, a potential turning-point in the current strategies used to monitor patient responses to therapies ranging in context from cancer, to autoimmunity, to vaccine therapy. The target of our study is to develop novel approaches to clinically applicable immune-monitoring by eliminating the reliance on a limited number of shared antigens to paradoxically address the complex and ever-adapting repertoire of immunogenic epitopes encountered by a host's immune system. Our strategy seeks to rapidly and collectively assesses CD8 T-cell reactions to sets of Ag that have been internalized and processed by autologous antigen-presenting cells (APC), with direct translational relevance to assessing the sets of patient-specific antigenic responses, regardless of their actual identities. We believe this approach is extremely useful in monitoring real-time T-cell activation following introduction to antigenic stimuli.⁴³⁻⁴⁷

Materials, Methods, and Approach

Our study seeks to demonstrate a system that characterizes T-cell activation from detectable extracellular proton flux via spectrophotometry using a pH-sensitive fluorescent dye, within polyclonal and antigen-specific contexts. It has been shown in previous literature that 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) is a photo-activated multivalent molecule whose fluorescent emission spectrum varies sensitively with pH changes.⁴⁸⁻⁵⁰ The compound is nontoxic and not perturbic to cells due to its inability to cross the membrane lipid bilayer, and its point emissions, which are reported ratiometrically, correlate well to the proton concentration of a solution in several studies.⁵¹⁻⁵⁶ We anticipate that this method, which represents an indirect method of proton “capture”, will serve as a conceptual validation of an alternative approach to assessing T-cell reactivity and support the development of downstream

strategies for monitoring T-cell responses directed towards undetermined populations of antigens.

Our approach is one of the simplest and most cost-effective methodologies yet proposed in assessing T-cell activation, and maintains a unique level of novelty and innovation, and promises to bring us closer to earlier and more effective assessments of patient treatment responses to help navigate clinical decisions.

Polyclonal T-cell stimulation monitoring

Spleens were obtained from a 8-week-old female C57/BL6 mice (Taconic Biosciences, NY, USA) and splenocytes were isolated through grinding and centrifugation of filtered splenic tissue in RPMI medium supplemented with antibiotics and 10% fetal bovine serum. Contaminating red cells were eliminated via treatment with ACK lysis buffer, followed by gradient centrifugation for purification using a Lympholyte-M protocol (Cedarlane Labs, Canada). Cells were counted, from which CD8 T-cells were purified using a negative selection EasySep protocol (Stem Cell Technologies, Canada). Cells were stimulated via 1 μ M anti-CD3e mouse antibody (eBiosciences, CA, USA) and plated into 96-well plates preloaded with 0.001M (in normal saline) of photoacid (8-hydroxypyrene-1,3,6-trisulfonic acid; Sigma-Aldrich, MO, USA) at indicated cellular concentrations. Spot emission assessments were taken at 2-minute time intervals for 30 minutes at excitation and emission wavelengths of 405nm/445nm and 520nm respectively using a Spectramax Photometer and SoftMax Pro Software (Molecular Devices, CA, USA). The point emissions are reported as mean ratiometric data corrected for background noise as determined from non-photoacid containing wells.

APC:T-cell Couplet Formation and Imaging Flow Cytometry

An Amnis ImageStream^XMark II fitted with 4 lasers (405nm, 488nm, 642nm, and 785nm) was used for imaging 60x magnification. A minimum of 1000 images were collected following mixing and centrifugation of the Ag-presenting cells (APC) and T-cells at a 2:1 ratio for 60 seconds at 4°C

(Eppendorf, NY, USA). Gating strategy is similar to a previous study, with a focus on FITC, BV421 double-positive populations.⁵⁷ Couplets were chosen due to good quality and resolution.

Antigen-specific Assessments

Utilizing the well-established murine OT-1 system, naïve 8-week-old female OT1/RAG2^(-/-) mice (specific for a chicken ovalbumin peptide fragment 257-264 presented by the MHC class I molecule H2-Kb) and age-, sex-matched C57/BL6 (Taconic Biosciences, NY, USA) were sacrificed, underwent splenic harvest, and CD8 negative isolation protocols as described above. Bone marrow-derived dendritic cells (BMDC) were isolated from age and sex-matched C57/BL6 via an established protocol using mouse-GMCSF (eBiosciences, CA, USA) and were incubated with either SIINFEKL (OVA⁽²⁵⁷⁻²⁶⁴⁾) or control EIINFEKL (eBiosciences, CA, USA) at 1 μ M for 4 hours on day 7. OT1-derived CD8 T-cells were incubated at 37°C and combined with day 7 BMDCs at a concentration of 1E6 cells/mL and 2E6 cells/mL (1CD8:2APC), respectively. Titration experiments utilized identical configurations of peptide-pulsing doses and OT1 responder cell dilutions with control C57/BL6 CD8s, and vice-versa. The combined DC/T-cell suspension was spun (Eppendorf 5810R centrifuge, 1 minute, 4°C, 1650rpm) and lightly resuspended to maintain APC-CD8 interactions. The spatial orientation of conjugated APCs and CD8s was confirmed from an Amnis® imaging flow cytometer (Figure 2, EMD Millipore, MA, USA). The centrifuged solution was aliquotted into black Costar 96-well plates (Fisher Scientific MA, USA) into a final concentration of 0.001M HPTS (in normal saline) and subjected to spectrophotometric analysis as mentioned above.

For parallel experiments with human cells, we utilized Day-7 blood monocyte-derived dendritic cells (DCs) from HLA-A2 normal donors, which were either loaded overnight at 1 μ M concentration with a MART-1-derived 26-mer “long” peptide (MART-1₁₅₋₄₀ peptide KGHGHSYTTAEELAGIGILTVILGVL, “MART-1 LP”) which requires intracellular processing to expose the immunodominant MART-1²⁶⁻³⁵ peptide ELAGIGILTV, or with the 26-35 peptide itself for 4-6 hours prior to assessment, dubbed “short” peptide (MART-1 SP). A second melanoma-associated, HLA-A2-restricted peptide derived from the gp100 protein (gp100¹⁵⁴⁻¹⁶² KTWGQYWQV) acted as a

negative control. Responder cells included either the human MART-1-specific TCR transgenic CD8⁺ clone DMF5 (recognizing MART-127-35, a kind gift of John Wunderlich, NIH/NCI) which were defrosted and restimulated with IL-2 one day prior to use, or naturally-occurring MART-1²⁶⁻³⁵-specific T-cells expanded from normal donors as described below. Titration experiments utilized identical configurations of peptide-pulsing doses and naïve CD8 responder cell dilutions from the same donor.

MART-1-reactive CD8 Expansion

Blood was collected from HLA-A2 restricted donors and subjected to Ficoll gradient purification and washing, according to a previously-established protocol to yield peripheral blood mononuclear cells (PBMCs) which could be passed over a microfluidic chamber to convert blood monocytes to monocyte-derived dendritic cells by “plate passage”.⁵⁸⁻⁶⁰ PBMCs were divided into appropriate separate groups from which either APC populations or CD8⁺ responder cells could be derived as previously described. For plate passage, 25x10⁶ PBMCs in autologous plasma were pre-incubated on a flow chamber plate for 1 hour. The cells were then removed from the chamber, which was then connected to a syringe pump and the cells were passed through the chamber under flow conditions. Plate-passed PBMCs were combined with MART-1 long-peptide (1 μ M) and responder CD8 cells at a ratio of 1 PBMC:1 CD8 under an assumption that the former consists of a 10% monocyte population. These cells were then co-cultured in 12-well plates and were fed IL-2 (12.5 u/ml final) and IL-7 (5 ng/ml final) on day 3 and every 3 days until day 9, when they were harvested and stained for MART-1 specificity utilizing dextramer staining. For dextramer-based characterization, cells were resuspended in 50ul FACS staining buffer and 10ul of each dextramer (Immudex, Denmark, Sweden) added to the tube. Cells were mixed and incubated at room temperature for 10 minutes in the dark. CD3 and CD8 antibodies were then added and the cells incubated at 4 degrees for an additional 20 min. Cells were washed and spun and resuspended in FACS buffer containing 7-AAD for live/dead discrimination and analyzed on a Stratadigm 13L flow cytometer (Stratadigm, San Jose, CA). For titration experiments additional naïve

donor CD8 cells were isolated from the identical donor on the same day. In the event that dextramer positivity indicated that initial expansion was inadequate, a reboosting procedure was implemented, similar to a previously established protocol.⁶¹ Cells were boosted with gamma-irradiated and peptide loaded PBMC populations and supplemented with IL-2 and IL-7 as described above and reassessed at day 9 post-boost for clonality and expansion levels via dextramer-positivity.

Correlation studies with Calcium Flux

Intracellular calcium was measured as described previously.⁶² Negatively-purified T-lymphocytes were incubated with Indo-1-AM (Life Technologies, MA, USA) at 1 μ M dissolved in 20% Pluronic F-127 and dimethylsulfoxide for 45 min at 37 °C with limited light exposure and washed subsequently prior to murine CD8-PerCP-Cy5.5 staining (eBiosciences, CA, USA). Day 7 murine BMDCs, which were pulsed with antigen peptides (either SIINFEKL or EIINFEKL) for 4 hours at 37°C, were stained simultaneously with CD11c-FITC and subsequently washed. Human correlates were stained with CD11c-PerCP-Cy5.5 and CD8-FITC. Cells were placed on ice prior to flow cytometry (LSRII, Becton Dickinson NJ, USA) and warmed to room temperature via water bath immediately prior to mixing at 2:1(APC:CD8) concentrations. The cell mixture containing BMDCs and OT-1 CD8+ T-cells were centrifuged and lightly suspended prior to sample loading into the flow cytometer. Relative intracellular calcium concentrations were determined by the ratio of violet:blue (420:510 nm) wavelengths. Data is shown as a median ratiometric emission for Indo-1 for the time interval following conjugate formation. Raw data were analyzed with FlowJo software via median kinetic signal monitoring (Treestar OR, USA).

Murine Tumor-Responsive Model and In Vivo Fluorometric Assessments

Tumor-bearing C57/BL6 mice underwent six, semi-weekly “murine extracorporeal photopheresis” (m-ECP) treatment sessions, occurring on Day 10, 13, 16, 19, 22, and 25 following tumor inoculation with 10⁵ YUMM melanoma cells into the right flank. For each treatment session,

PBMC were collected from whole blood via retroorbital venous capillary draws and subjected to gradient centrifugation for purification using a Lympholyte-M protocol (Cedarlane Labs, Canada). YUMM cells were trypsinized (Trypsin/EDTA, 0.25%) and harvested from cell culture, washed, and subjected to 200nM 8-methoxypsoralen (UVADEX®) for 20 minutes, followed by 4J of UVA irradiation. Purified PBMC in autologous plasma were pre-incubated on a flow-chamber plate for 1 hour with PUVA-treated YUMM cells. The cells were then removed from the chamber, which was then connected to a syringe pump. This mixture of irradiated tumor cells and PBMCs was passed over a microfluidic chamber to convert blood monocytes to monocyte-derived dendritic cells by “plate passage”.⁵⁸⁻⁶⁰ The flow conditions were uniform during the plate passage step (0.49 mL/min) and washed afterwards at higher flow rates (1.49 mL/hr) with fetal bovine serum. Plate-passed cells were collected, washed, and spun and resuspended in PBS, before being reintroduced systemically via retroorbital injection. Tumor volumetric measurements were performed using precisely-calibrated calipers prior to each treatment.

Days 16, 22, and 25 involved in vivo fluorometric assessments during which a mouse was sacrificed from both groups (control and “m-ECP”), each undergoing CD8 negative selection purification via splenic and lymph node harvests as described earlier. YUMM tumor cell lysate was obtained by pelleting 20×10^6 cells in PBS ($2-4 \times 10^6$ cells/mL), and subjecting this pellet to four freeze-thaw cycles by alternating liquid nitrogen and 37°C water bath treatments. Cells were sonicated for 15 seconds at 38% amplitude (Teledyne Tekmar, Ohio, USA) to rupture cell membranes. Cells were spun at 12,000g for 20 minutes at 4°C to remove cellular debris. Supernatants were collected and stored at -20°C, before being administered to day 7 murine BMDCs, isolated using the aforementioned protocol, for 24 hours. Lysate-pulsed BMDCs were then combined with purified CD8 T-cells of the control- and treatment-group mice via centrifugation, and emission patterns were collected every 2 min for 60 min using the protocol described previously for antigen-specific experiments.

Results

Detectable Polyclonal Activation with Cellular Limits

Polyclonally activated CD8 T-cells demonstrated a sustained and consistent higher emission intensity than unstimulated cells. This is depicted graphically in Figure 1. This activated pattern of emission extends in sensitivity from $\sim 10^4$ cells down to ~ 10 cells per well ($p < 0.001$) with detection limits seen in 10^5 to 10^6 cells per well ($p > 0.05$). A somewhat noticeable decrease in activated intensity is also noted extending from 10^2 to 10^4 cells. Figure 1 depicts these results by indicating mean emission intensities with plotted standard error bars.

Observable Antigen-Specific Activation of murine OT1 CD8+ Responders.

Following DC/CD8 co-culture and centrifugation-driven dimer formation (Figure 2), CD8 stimulation via antigen-specific and antigen non-specific peptide-pulsed dendritic cells (BMDCs) was evaluated. At 1 μ M peptide loading, SIINFEKL-presenting DCs are capable of eliciting higher fluorescence signals than control EIINFEKL-presenting cells when interacting with specific OT1 CD8+ responder cells (Figure 3a). This observation was significant for all OT1 cell dilutions (diluted in C57/BL6 negatively-purified CD8s) tested, including 10% and 1% values. Upon pulsing with 10-fold less peptide (0.1 μ M), similar results were observed (Figure 3b).

When APCs are pulsed with a peptide concentration of 0.01 μ M, higher emission signals are observed in responder dilutions of 100% and 10%, but not seen in 1% populations (Figure 3c). At the lowest titration level of 0.001 μ M peptide, significant difference is only seen at the 100% OT1 responder level, but lost at the 10% and 1% levels. These data indicate that Ag-specific T-cell activation can be monitored effectively utilizing the pH sensitive dye at physiologically relevant peptide and APC to T-cell concentrations.

Conjugate Formation and Detectable Activation of Human MART-1-specific T-cells

Utilizing human DC loaded initially at 1 μ M of peptide, conjugate formation and T-cell stimulation assays were carried out with a MART-1-specific human CD8⁺ T-cell readout line to confirm antigen-specific APC-CD8 interactions. Figure 4a illustrates that significant differences were seen when plate-passed cells, a source of 10-12% dendritic APC, are incubated with either unprocessed (26-mer) and processed (10-mer) MART-1-derived peptides (LP and SP, respectively) versus control gp100 peptide, when these APCs are complexed with a 100% specific DMF5 responder cells. DMF5 is a MART-1-specific TCR transgenic CD8⁺ clone (recognizing MART-1²⁷⁻³⁵) which should respond in an Ag-specific manner following stimulation with DC either surface-labeled with the 10-mer MART-1 peptide or which had processed and presented the 26-mer “long” peptide. Significant differences are noted between emissions observed following conjugate formation between DC and T-cell (black bars) versus cells passively mixed in adjacent wells (grey bars). Dextramer staining (Figure 4b) confirmed a high percentage of dextramer positivity for the DMF-5 line utilized in these assays. Interestingly, MART-1 SP conjugation had the widest difference between conjugated cells than unconjugated passive interactions, perhaps as a result of the higher density of specific peptides presented on the cell surface following exogenous loading of pre-processed, form-fitting peptides. Nonspecific (gp100) peptide-loaded cells were unable to establish notable signal differences from baseline, even with DC/T-cell complexation.

Detectable Antigen-Specific Interactions with Naïve Expanded CD8 Responders

Since normal human donors have an unusually high frequency of naïve, MART-1-specific precursors in their blood, it is possible to derive Ag-specific T-cells directly from normal HLA-A2 donors which can act as fresh Ag-specific T-cells for stimulation assays.^{61,63} Separate expansion trials were accomplished utilizing normal donor blood as a source of naïve T-cells as described in Materials and Methods. Following 1-week exposure of CD8 T-cell populations to MART-1 peptide-loaded DC,

successful expansion of MART-1-specific T-cells was accomplished from all donors. MART-1 dextramer positivity of the T-cells was confirmed following expansions, which resulted in T-cell populations in the 0.9% to 21.1% range.

Staining for the most successful expansion, 21.1%, is shown in Figure 5a. Each of the three expanded T-cell populations was then utilized as responder T-cells in DC stimulation assays, which mirrored those, previously carried out on the DMF5 transgenic line experiments. In each trial as shown in Figure 5b, conjugated pairs showed notable increases in emission signal over control peptide-loaded DC, consistent with antigen-specificity. One of these expansions successfully generated a dextramer-positive population of 2.3% and the expanded T-cells were used or diluted to increasingly lower specific T-cell ratios (1.2%, 0.2%, and 0.02% respectively) with autologous, non-specific T-cells. At all specific T-cell ratios tested, T-cell activation and fluorescent emissions were higher in cells stimulated with specific MART-1 loaded DC compared to control gp100, with the exception of two trials (0.2% and 0.02% dextramer-positive groups). These observations indicate that specific T-cell populations at levels as low as 90 per 10,000 in peripheral blood could be amenable to this analysis. The lowest MART-1 naïve CD8 expansion group was also capable of yielding detectable differences in calcium signaling by means of intracellular calcium flux (Figure 7). To look at this data as a whole and determine whether a relationship exists between mean fluorescent differences and antigen specificity, a regression plot was developed (Figure 6). Mean emission differences between Ag-specific and –nonspecific differences for the MART-1 expansion assessments are summarized and demonstrate a linear correlation to dextramer values, with few notable statistical outliers, reasons for which are elaborated upon in the “Discussion” section below.

Significant Differences in Emission Intensity using Unknown Antigens as Probes in an Animal Model

Mice from control and treatment groups were analyzed at three separate time points of six total treatments. When subjected to APCs harboring tumor cell lysate antigens, CD8s from the treatment

group revealed higher emission signals compared to controls for all assessments (Figure 8). The largest difference was seen during the earlier half of scheduled treatments. There are notable fluctuations seen with the emission patterns of the control group, though this group's T-cells were consistently less "reactive" for all time points. It is not until the final treatment (Day 25) that a significant difference in tumor volume is clinically observed between the groups for the remainder of the experiment.

Conclusion

The ability to simply and accurately quantitate the upregulation or downregulation of T-cell based responses has vast implications for treatment of T-cell mediated diseases and vaccination-based targeting in cancer. Since there is no longer a need to follow only defined Ags, the use of dynamic antigen sources such as cell lysates or disease-associated whole proteins defined by personalized genomic sequencing allow potential treatment monitoring against a panoply of T-cell relevant Ags. This capability is especially relevant within the context of immunotherapy, as each patient engages to his or her own unique array of tumor-associated antigens presented via class 1 major histocompatibility complexes (MHCs) to clone-specific T-cell receptors. Our study provides evidence advocating for a shift in clinical readout strategies to assess the level of global TCR engagement in a host and a capability to sensitively discriminate between specific and nonspecific T-cell stimulation by APC. We established this with a rapid assay in murine and human antigen systems via spectrophotometry. Due to the reliance on an extracellular analyte, our strategy is capable of detecting signals deriving from rare subsets of cells, that are amplified extracellularly to increase detection capability down to levels of approximately 10 polyclonally activated cells, 0.001M peptide-pulsed APC:CD8 conjugates, and ~90 antigen-specific T-cells out of 10,000 unpurified donor PBMC within our MART-1 system. Although the latter sensitivity may be attributable to the higher number of stimulating APCs in the unpurified expansion samples, the translational capability of utilizing unpurified patient PBMC for routine TCR engagement assessments is conserved.

The underlying motivation of our fluorometric approach is to overcome the severe limitations that exist for monitoring Ag-specific T-cell responses in cancer and autoimmune therapy, particularly attempts to quantify the complexity of an immune response based on limited set of probes.⁶⁴⁻⁶⁷ As mentioned previously, current favored strategies rely heavily on tetramer or dextramer positivity and in vitro re-stimulations using APC pulsed with defined peptide epitopes in an attempt to quantify the

degree of a patient's immune reactivity and correlate to clinical outcomes.⁴² Such approaches to assess tumor immunity often rely on following responses only to established tumor antigens, many of which are shared with normal tissues (i.e. MART-1, gp100, prostate-specific antigen), and have failed repeatedly due to the dynamic expression of tumor-specific neo-antigens and response kinetics of an ensuing immune response. The complexity, cost and reality that many diseases do not possess a fully characterized library of antigens renders the "single-antigen, single-readout" a currently intractable challenge. The rate of tumor mutation heterogeneity renders these methodologies, which need to uncover MHC-restricted epitopes at nearly every phase of treatment, rather impractical across virtually every tumor type.⁶⁸⁻⁷¹ In addition, bona fide immune responses leading to tumor regressions often have not correlated with T-cell responses against the limited number of Ag followed in clinical trial, indicating that targeting and monitoring is neither practical or expected to yield satisfactory clinical outcomes.⁷²⁻⁷⁴ Yet another inconsistency in the field of immunological monitoring is determining what an appropriate threshold would be for a "positive response", as this is currently unknown and amounts to relative estimation.⁷⁵⁻⁷⁸

Here, we present an approach that provides a collective assessment of immune reactivity, qualitatively and quantitatively, that can provide prompt and accurate clinical information. The model antigen systems utilized in our study undergo the same internalization, processing, and presentation as do the distinct tumor antigens and are detectable at frequencies that rival clinical MHC dextramer values.⁷⁹ Moreover, at steady state, a single cell is capable of producing $\sim 10^8$ protons per second, a rate that can be raised between ~ 10 to 100%, depending on the method and degree of receptor stimulation.¹¹ By utilizing an approach that detects such extruded protons, our system may also possess a signal intensity that correlates to the presence of relevant polyclonal antigens, regardless of their identity. For these reasons, our proposed strategy has major ramifications for the field of immune-monitoring as it shifts the focus towards quantifying the total reactivity of clonal T-cell populations.

The utility is understood considering the numerous potential immunotherapeutic and cancer vaccine agents are currently being trialed, including immune checkpoint inhibitors and DC-based vaccination strategies. But with limited options of immune-monitoring tools for evaluation, oftentimes researchers are forced to use only gross assessments such as tumor size or symptom amelioration to define therapeutic benefit. Monotherapies such as extracorporeal photopheresis (ECP), used for both cutaneous T-cell lymphoma and autoimmune diseases such as graft-versus-host diseases, could benefit from such an analysis, as treatment-resistant cases exist could benefit from earlier shifts in treatment course, particularly since ECP has uniquely shown both immunogenic and tolerogenic effects.^{58,80-83}

Several existing technologies are capable of sensing proton differences in solution, including pH microelectrodes, pH radiolabeled and fluorescent probes, and silicon field effect transistors.⁸⁴⁻⁸⁸ Previous technologies looking at proton flux within the context of activated T-cells include light-addressable potentiometric sensors (LAPS) and chemically-modified field effect transistors (CHEM-FET), based on similar semiconductor-based technologies.^{11,45} Both showed tremendous sensitivity in detecting proton flux from cells following soluble stimulatory factors and have shown more promise than traditional assays.⁸⁹ Though Stern's one-dimensional CHEM-FETs showed tremendously sensitive detection capabilities (~200 cells within 10 seconds of a stimulus), issues commonly acknowledged with CHEM-FETs includes inter-device manufacturing variations potentially blunting sensitivity, temporal deterioration of device performance, inconsistent calibration efforts and of course overall expense.¹² Moreover, such technologies have yet to evaluate real-world cellular interactions vital to CD8 activation such as the use of a patient's autologous dendritic cells as activation agents, not only a crucial wing of modern cancer immunotherapy, but also a promise to monitor patient responses to immunomodulatory treatments using personalized antigens and responder cells.⁹⁰⁻⁹⁴ Our fluorometric approach therefore potentially offers a simple and low cost alternative with respect to many of these aforementioned

challenges as it enables the local and rapid detection of extracellular proton flux without a manufactured nanodevice, allowing monitoring of APC:CD8 interactions in a variety of clinical settings.

Benefits from the fluorometric proton flux immunoassay itself include its simplicity and versatility, which enable a considerably more rapid readout than current assaying protocols, and allows for the combination of readouts from numerous techniques to evaluate whether clinical correlations can be predicted more robustly and with increased accuracy. Further studies will naturally be crucial to evaluate whether clinical correlations can be drawn from fluorometric proton flux data, particularly since evaluation guidelines are unclear in regard to using sets of undefined antigens, such as tumor cell lysates.^{31,42,95} Unknown antigens, which are dynamic in frequency and vary between patients and even within a patient's tumor itself, are the key to providing insight to the competency of any immunomodulatory treatment. The complexity of tumor associated antigens are undeniable and matched by the inner workings and sensing capabilities of an immune response, neither of which can adequately assayed or understood using the sparse repertoire of known cancer epitopes. The significance of our study lies in the validation of an early-stage, alternative strategy and establishing the potential for downstream advances in immunological monitoring that will undeniably translate into more effective treatment response prediction models, tailoring of clinical decision-making, and increased "personalization" as we approach the unique case of every patient.

Drawbacks of this Study

By no means is our approach the solely appropriate one for discriminating overall TCR engagement. Our methods are a rudimentary strategy that seeks to provide conceptual evidence for an approach that would yield clinically meaningful data in a variety of immunogenic and immunotolerant settings. While it is established to correlate with T-cell activation, extracellular acidification ultimately represents a single facet of a complex set of reactionary biochemical changes. With respect to the

materials and protocols, HPTS may also not be the best indicator of extracellular $[H^+]$ as other photoacidic fluorescent agents exist.^{96,97} Additionally, there are other agents that can help determine the analyte levels resulting from other cellular processes apart from cellular acidification. Calcium flux, which is investigated in this study, can be studied with a fluorescent microscopic or plate-reader approach, the latter allowing for increasingly high-throughput studies.⁹⁸ A study was successful at using functionalized glass slides to assess short-term cytokine secretion (IFN- γ , IL-2, TNF- α) and cytolytic activity in a high-throughput fashion using fluorescent imaging of the cell populations.

Our experiments fell short of displaying consistent emission intensity baselines, which may be a product of inconsistent manual solution titration. The various baseline levels were hedged by the comparison of the emission changes associated with an activated population from a control, which we assumed and proved would be related to the reactivity, or dextramer-positivity, of a T-cell group. The inconsistent background in our experiment renders modeling the kinetics of proton extrusion rather difficult and is certainly a potential area of improvement.

Future Directions

Metabolic Phenotyping as Insight into Activated Cell Populations

Our presented system, though far from completion, lays the framework for a collective effort to discount the need for identifying immunodominant epitopes for each patient's tumor. Though rudimentary, several elements of our approach can be improved upon and standardized for a method to collectively assessing a subject's T-cell reactivity to presented antigens. A variety of different procedures can exist when focusing on biophysical phenomena associated with cellular activation. The energy-consuming nature of such a process entails a concatenation of measurable chemical changes that occur both inside and outside of the activated cell. In addition to the extracellular proton flux or intracellular calcium flux investigated in this study, approaches can also seek to characterize metabolic events such as oxygen consumption rates (OCR) or carbon dioxide generation from such events. OCR

can be readily measured and is shown to be higher in CD8 cells than CD4s upon stimulation via a preference for aerobic glycolysis.⁹⁹ Looking at ratios that relate oxygen consumption or carbon dioxide generation can even indicate which catabolic pathways such metabolically reprogrammed cells preferentially utilize. Theoretically, recording the basal oxygen consumption of a cell population and assessing for differences following antigen stimulation, or the admixture of responder cells, could shed light on the overall metabolic profile of intracellular events that follow TCR engagement.

Along similar lines, assessment of the byproducts of cellular respiration or other aerobic metabolic processes within the activated lymphocyte can be another wing of reassurance with which to approach the issue. Studying these key tenets of the metabolic reprogramming that occurs to provide the materials for biosynthetic processes will shed light on discriminated an engaged group of cells from a nonreactive counterpart. As activated populations are more oxidative, they tend to be associated with greater levels of reactive oxygen species and elevated production of glycolytic compounds such as lactate. Such processes are necessary for cellular survival, growth, and proliferation, with the existence of data indicating that stimulation can also increase the observable median cell size in T-cells with time. The signaling pathways that result in the distinct metabolic signatures associated with lymphocytic activation can be explored using probes aimed at assessing compounds seen along the entire spectrum of activated T-cells. This allows us to study differences in the reprogramming that occurs in activated populations and directs our attention to downstream effects of an antigen-specific interaction, rather than focusing on the molecular structure or identity of that stimulus. Our strategy therefore hedges against the uncertainty associated with the unique immunogenic stimuli in each patient by reporting instead on the downstream features of an activated receptor that can be observed independent of a tumor's antigen expression profile.

Improving Detectability via Targeted Agent Delivery

The TCR:MHC interactions that occur at the immunological synapse can dictate the fate of an effector cells. The synaptic complex consists of a variety of costimulatory receptors and adhesion proteins, on both the T-cell and APC, that can be targeted for an increasingly localized detection of extruded electrolytes. It is established that targeted agent delivery is more effective for eliciting a certain cellular response than systemic delivery. The impressive effects of targeted delivery can be understood from mathematical modeling of the localized concentration gradients that occur at the surface of a responder cell. The Laplace Diffusion equation can shed light on the paracrine effects of a APC on its target effector by showing that the concentration of a secreted protein or molecule will decrease inversely with radial distance, with the highest concentration noted at its surface.¹⁰⁰ In a paracrine modeling system, in which a cell is secreting protein factors to another, we can observe that the gradient on the targeted cell surface is exponentially greater than the one found in solution. Thus, when an APC and T-cell interact at the TCR:MHC complex, it is extremely important for the two cells to do so as closely as possible in order to increase the release and absorptive capacities of both cells.

Along these lines, a similar assumption can be made for extruded cellular analytes deriving from a stimulating immunological synaptic interaction. For our study, the protons extruded by membrane-bound channels and pumps provide a conceptual framework for enabling detection as close as possible to the T-cell surface as possible via nanoscale vehicles. Liposomes are well studied platforms with ideal chemical properties for the encapsulation of HPTS, a strongly hydrophilic substance that would otherwise be relatively more difficult to load into the hydrophobic core of other alternatives.¹⁰¹ In addition, liposomes also have a surface modifiable lipid bilayer, to which targeting ligands can be conjugated and help with the navigation of such particles to specific targets.¹⁰² The formulation enables encapsulation and sustained release of the loaded agent over time through a two-step approach in which the internal polymer matrix is synthesized and entrapped within a liposomal shell structure that can have

targeting elements conjugated to its surface. We hypothesize that targeting such elements to adhesion molecules that participate peripherally in the TCR engagement process may provide a more sensitive reading of the acidification or proton gradient emanating from the surface of an activated T-cell.

Our design for a murine prototype for such an endeavor would involve the conjugation of anti-LFA1 (anti-leukocyte functional antigen-1, or anti-CD11a) antibodies to the surface of the liposomes with the intent of targeting the T-cell receptor that interacts with the ICAM-1 (Intercellular adhesion molecule-1) ligand on the surface of the dendritic cell. The formation of an immunological synapse relies heavily on the dynamic characteristics of the secondary receptors and adhesion factors associated with APC:T-cell crosstalk. Targeting this particular area with the nanoscale agents will allow for localized delivery of our fluorometric indicator and a heightened sensitivity to the T-cell surface, where the kinetics of proton release are highest and therefore the concentration gradient will be most detectable. One obvious advantage is that such an approach will heighten the sensitivity of distinguishing activated T-cells within a heterogeneous population of immune cells. Rather than relying on the global acidification of a soluble medium, this approach allows us to report on the microenvironment associated with activated lymphocytes during and after the process of antigen-specific priming. Another advantage of this approach would be in the ability to render time-lapsed assessments for populations of cells to look for temporal trends that may indicate rapid rises in emission patterns that could correlate to the stimulation of a certain cell populations. The nontoxic nature of the approach could allow for longer and more comprehensive assessments of a patient's immune cells.

A Label-free Approach to Antigen-Specific Responses via Nanoscale Semiconductors

An important hurdle to overcome from our various approaches entails the variability of baseline fluorescent characteristics seen with every assessment. Though a variety of factors may be associated with spectrophotometric approaches, such as the standard error associated with manual titration of HPTS in solution, our approach hedged against such inconsistencies by standardizing conditions for

each assessment and seeking the most ideal controls for each assessment. An incentive for transitioning our approach using a device-based approach is attractive due to the standardized conditions associated with a fabricated device that may be utilized several times for assessments from a particular patient, rendering the baselines of comparison increasingly standardized and aiding with the discrimination of significant changes in assessed reactivity or TCR engagement over the course of treatment. One-dimensional materials, such as nanowires, can be implemented for many applications such as the aforementioned “lab-on-a-chip” purposes. The unique electrical and surface-to-volume ratios of such devices, render their clinical integration increasingly exciting.⁴⁴ The top-down approach of selective etching yields a high standard of manufacturing to the molecular-scale that can output consistent clinically useful data.¹⁰³ Additionally, such technology has advanced tremendously in the past decade with more advanced techniques for chemical functionalization that allow utility to extend beyond simple analyte measurements, but also can help with the biophysical elements of cellular activation such as adhesion, morphology, and differentiation.¹⁰⁴

A newer, optoelectronic approach was investigated aimed at rapidly detecting solution pH in minute fluid volumes from a semiconducting nanodevice which showed a capability to measure the absolute concentration of molecular analytes in real-time. However, this particular design was uniquely deviant for the ability to calibrate each semiconductor using fluorescent, reducing inter-device variability, and showing unprecedented success in monitoring continuous cellular metabolic processes. In an experiment, Peretz-Soraka and colleagues were able to show real-time detection of pH changes resulting from the metabolic activity of Jurkat cells upon the administration of glucose. This could have tremendous consequences on our ability to identify metabolic signatures associated with antigen-specific activation with time and enables such studies to occur at the picomolar level.¹² Interestingly, the photoactive molecular species and chief sensing element of this highly-sensitive nanoscale semiconductor is actually a derivatized form of HPTS conjugated to the surface of the device. Although

the derivatized form possesses different functional groups, the study nevertheless points to the heightened sensitivity of spectroscopic agents as effective reporters for clinically relevant cellular processes. The capability to sensitively detect such information is extremely useful in the realm of immunological monitoring as it provides functional and molecular correlates, helping clinicians navigate the complexity of a patient's immune response to decipher risks for tumor progression, organ rejection, sepsis, or other life-threatening conditions prior to encountering clinically observable symptoms.

Figures

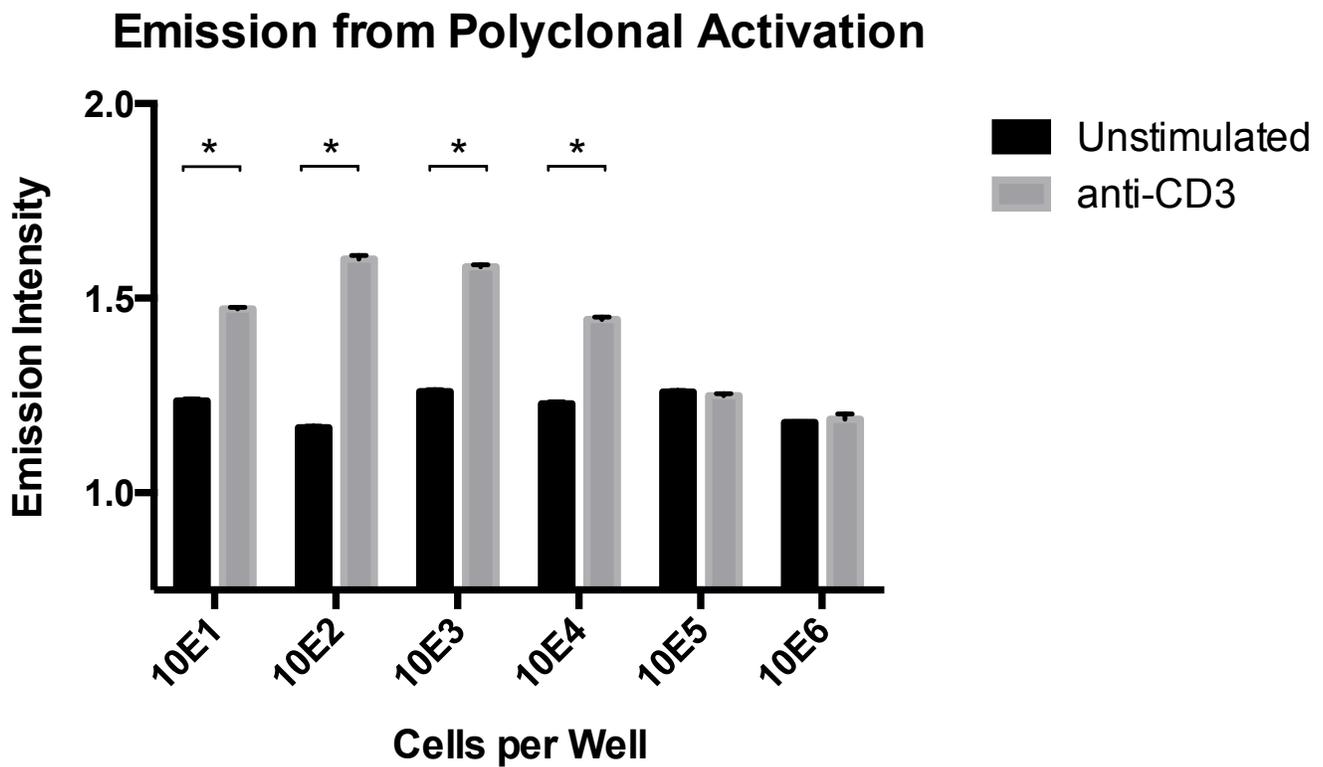


Figure 1. Polyclonal activation with cellular limits of assay detection and monitoring. Various numbers of cells (indicated) were plated in single wells and subjected to 1 μ M anti-CD3e mouse antibody for stimulation. Emission signals were collected for a 60-minute duration. Significant differences are marked with an asterisk ($p < 0.001$).

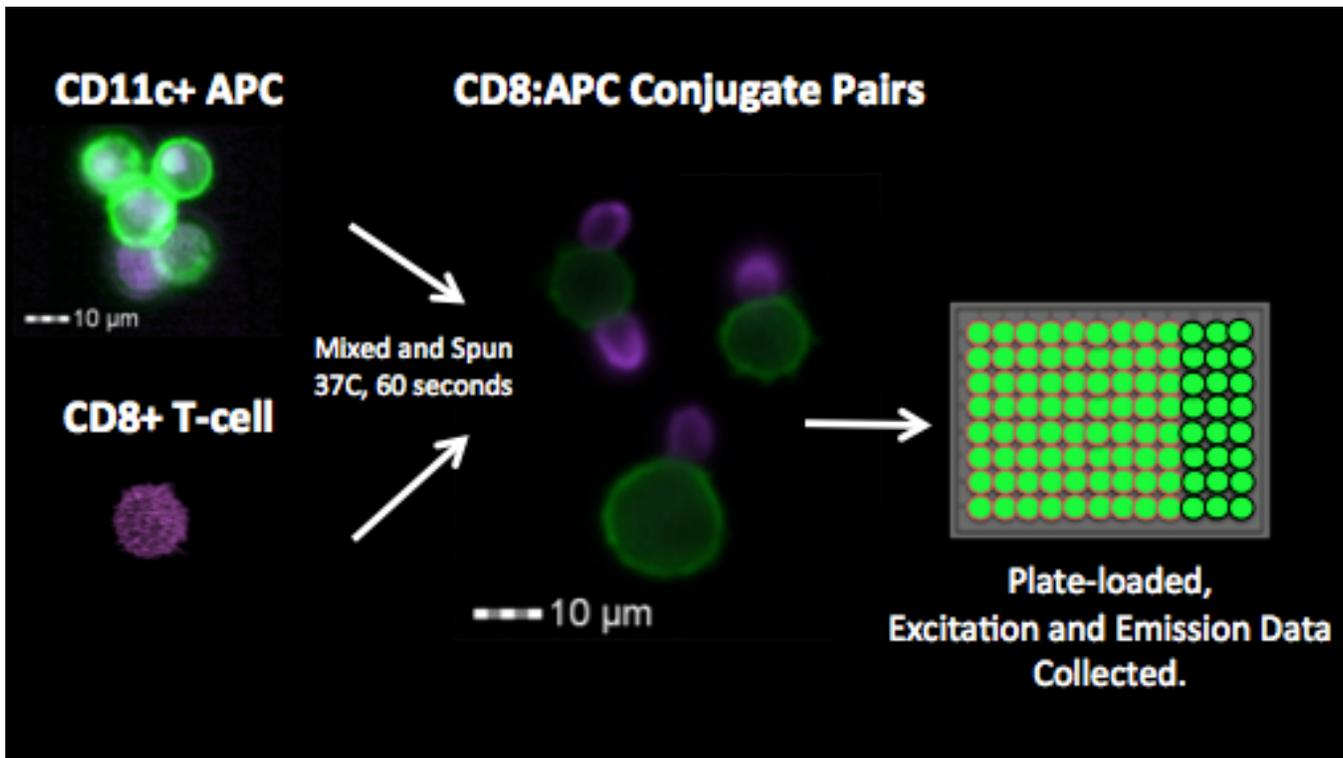


Figure 2. Imaging flow cytometer depictions of APC:T-cell couplets formed after centrifugation and light resuspension. Antigen-presenting-cells and T lymphocytes are labeled in CD11c-FIT and CD8-Brilliant Violet 421 antibodies, respectively. Ch02 corresponds to FITC-labeled APCs and Ch07 renders BV421-labeled CD8s. The three couplets shown are representative from a single experiment.

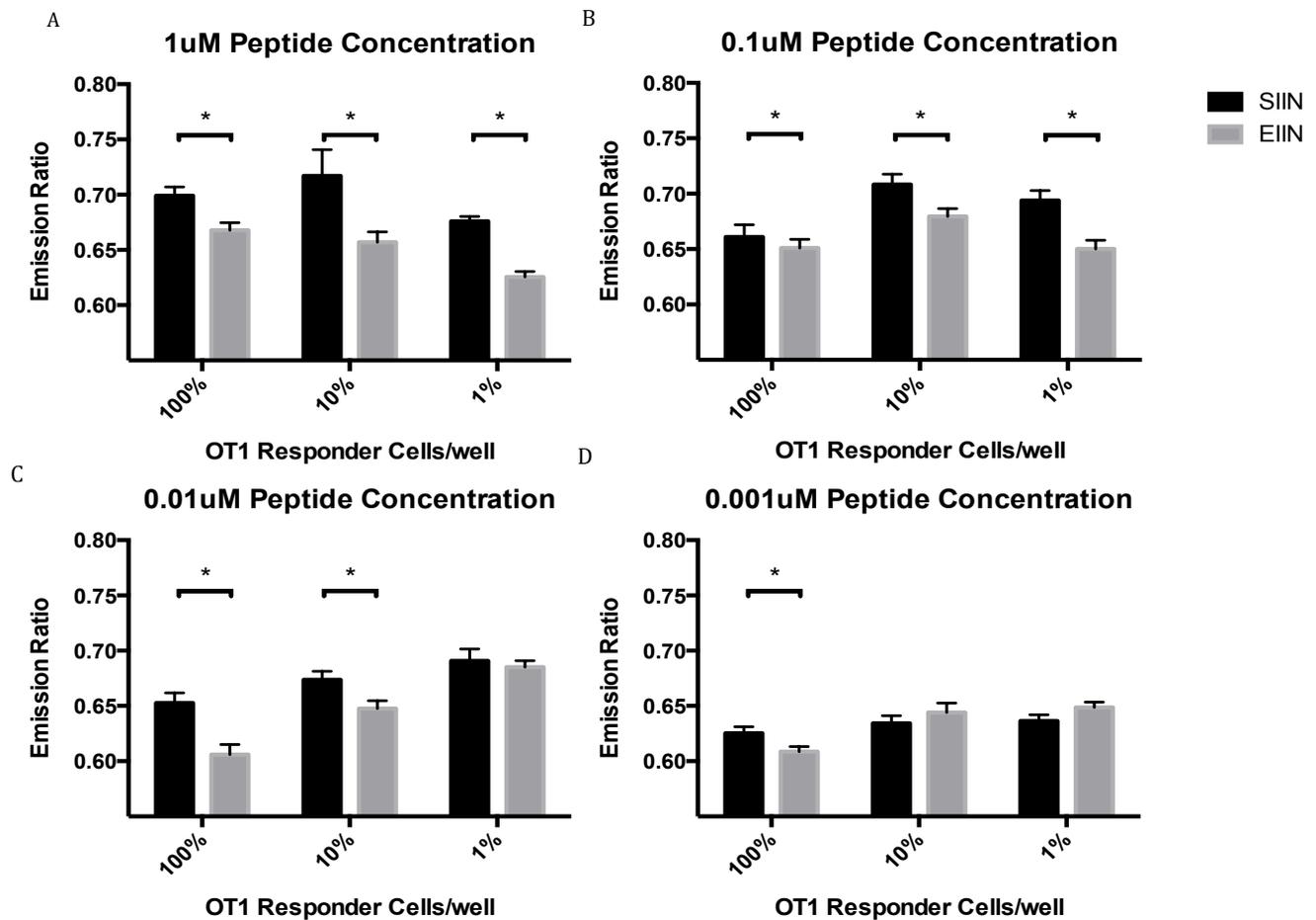


Figure 3. Fluorometric differences between Ag-specific and –nonspecific interactions in the OT-1 system. Such interactions occur following presentation by BMDCs via MHC class I H2-Kb within the context of OT1/RAG2(-/-) mice. A) At 1uM peptide pulsing concentrations, SIINFEKL-presenting DC:T-cell interactions following complexation emit at more significantly than EIINFEKL-based control groups extending from 100% to 1% OT1 Responder CD8 cells. B) When peptide pulsing concentration is reduced tenfold, this effect remains preserved throughout the OT1 responding cell titrations. C) At 0.01uM this significant differences are maintained from 100% until the 10%, but lost at a 1% OT1 responder level. D) The lowest titration showed a significant difference only at the 100% OT1 responder level, with a loss of the effect for lower percentages of OT1 responders. Significant differences are marked with an asterisk (p<0.001).

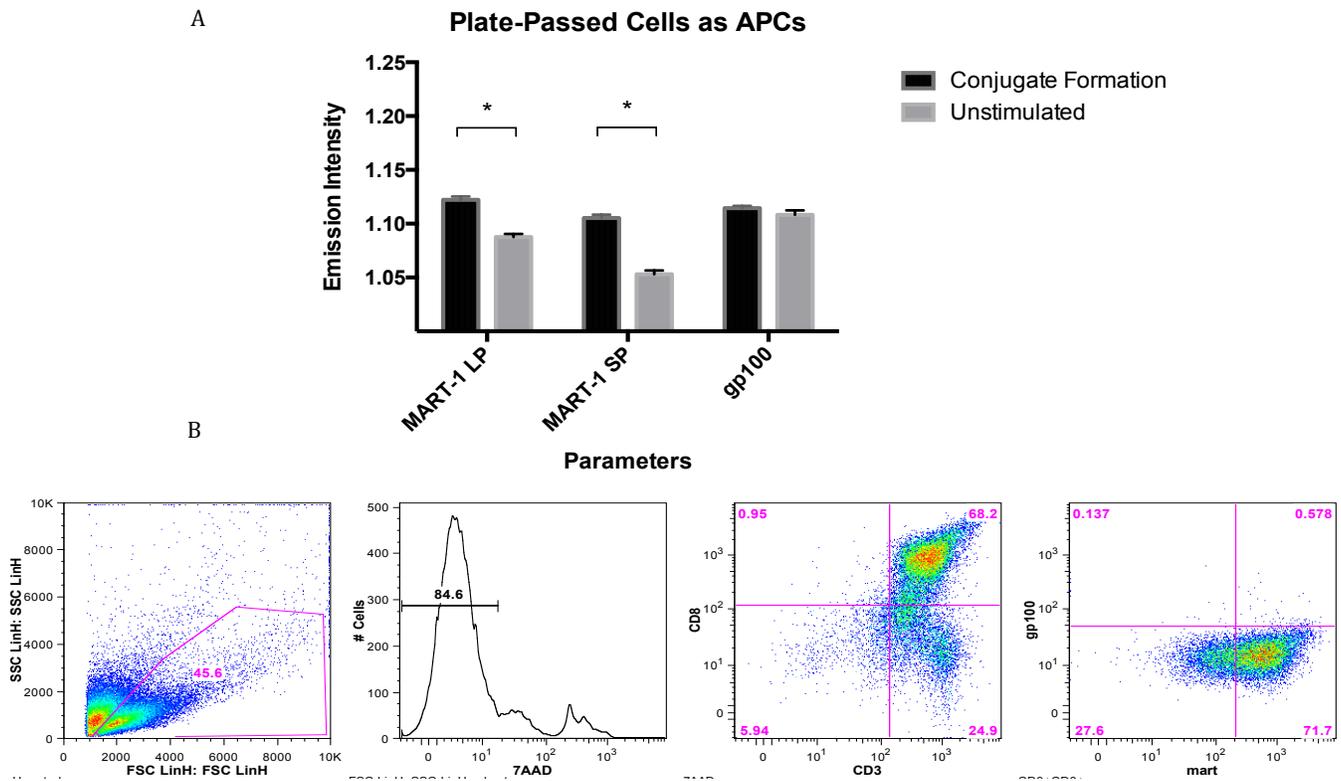


Figure 4. Antigen-Specific processing and presentation by “plate-passed” APC cells to a 100% specific MART-1 responder human T-cell line (DMF5). A) Plate-passed cells incubated with 1uM of unprocessed (MART-1 LP), processed peptide (MART-1 SP), or a form-fitting control peptide (gp100) showed significant differences upon complexation with DMF5, rather than passive mixing in adjacent wells. This held for all groups except the control gp100 group. B) DMF5 cells displayed a high percentage of dextramer positivity (71.7%) confirmed by flow cytometry, indicating antigen-specific reactivity.

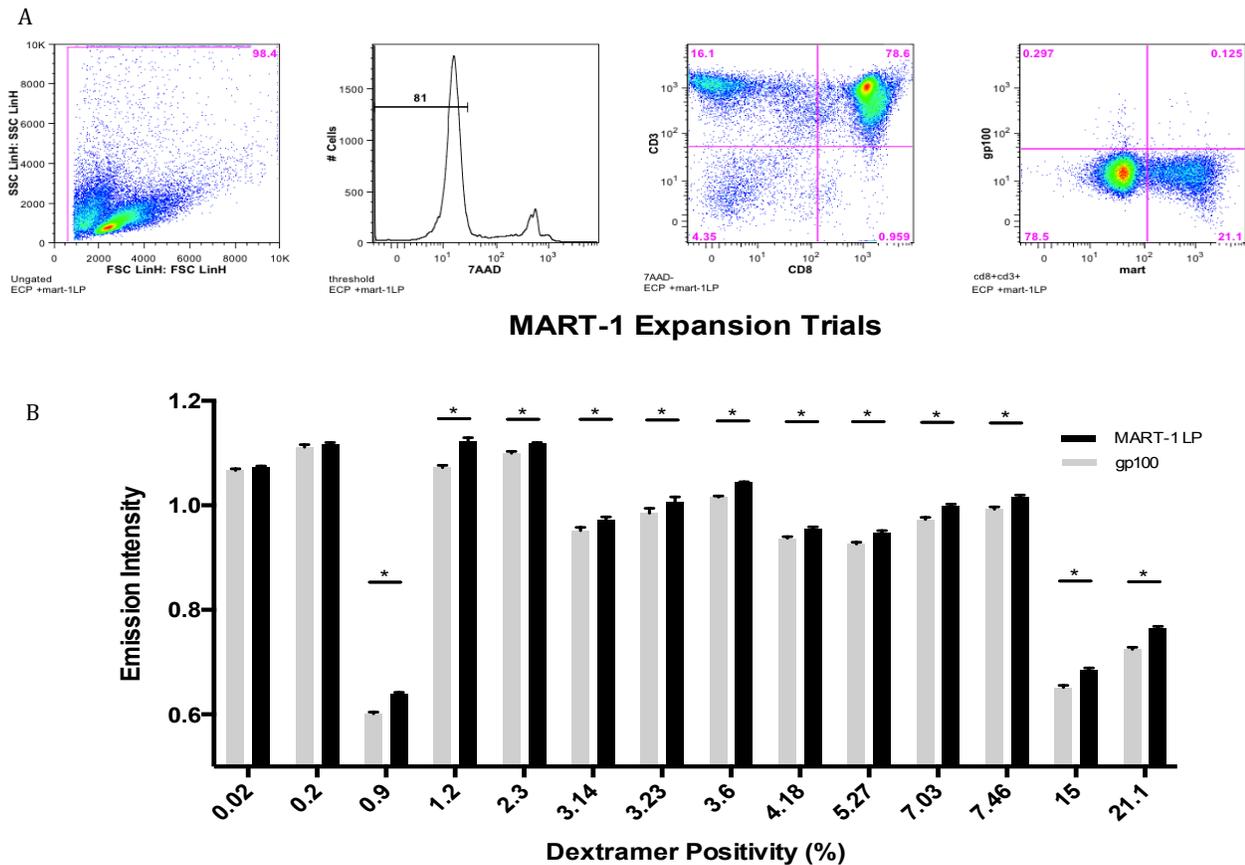


Figure 5. Flow cytometry gating strategy and summary of overall expansion photometric data. A) Cells are initially gated and isolated by 7-AAD negativity, indicating viability. Of the 7-AAD+ cells, CD3+CD8+ double-positives are selected and focus is placed on the MART-1+ cells. B) An amalgamation of successful MART-1 expansions utilizing plate-passed APCs is indicated, along with their emission intensity differences when complexed with cytokine-generated dendritic cells presenting either MART-1 “long” peptide or gp100 (form-fitting control) peptide. All pairs are significant with the exception of the 0.2% and 0.02% dextramer group.

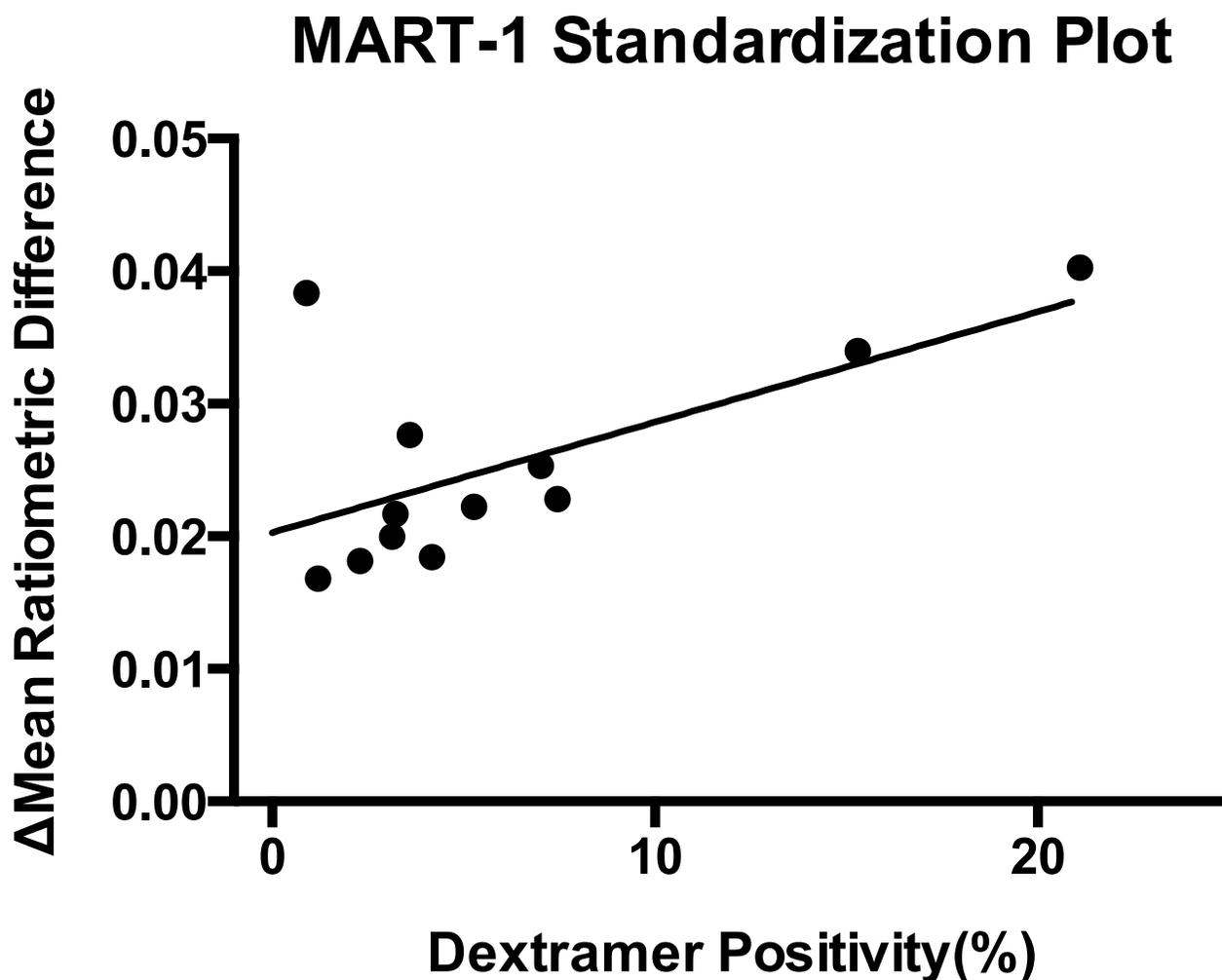


Figure 6. Regression plot for Mean Emission differences between significant Ag-specific and – nonspecific interactions in the MART-1 system. Values are standardized and based on dextramer positivity, as determined by flow cytometry. A positive, linear correlation (R-squared=0.89) was obtained with significance ($p=0.0015$) and minimal outlier values.

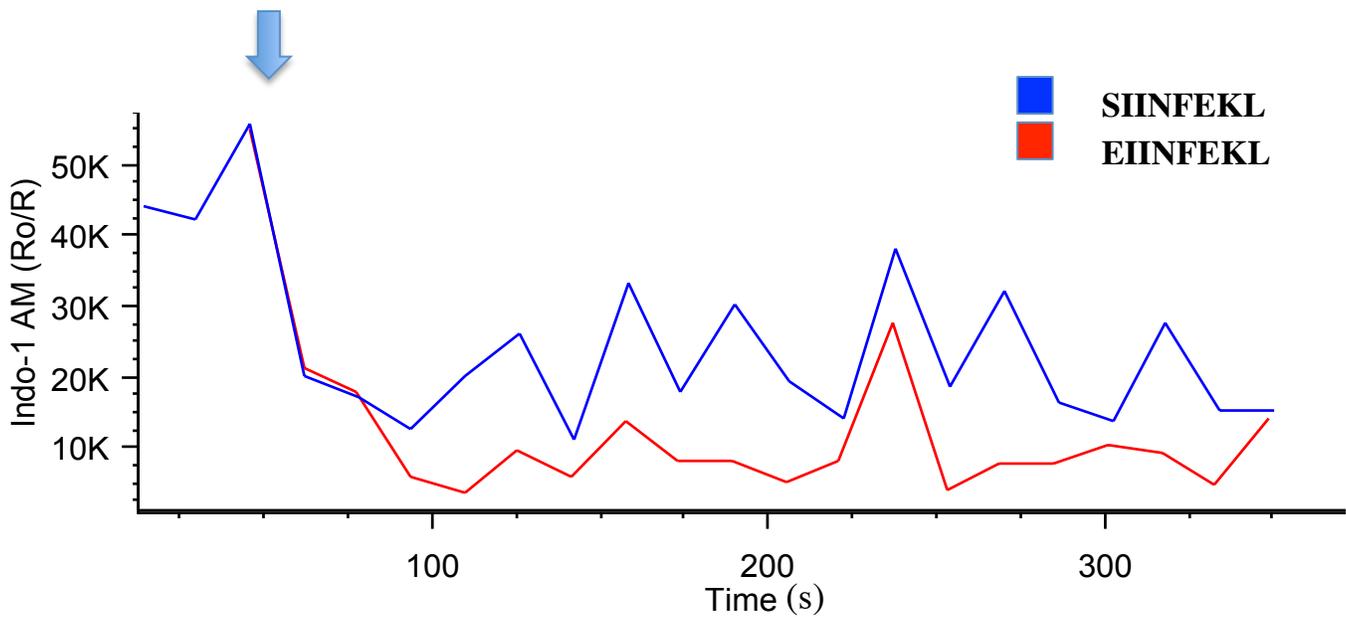


Figure 7. Calcium Flux Correlation for 0.9% MART-1 Dextramer Population. A qualitative, yet distinct signal is seen following conjugate formation, indicated with a blue arrow. SIINFEKL represents an antigen-specific interaction which displays a higher magnitude and frequency changes when compared to EIINFEKL, which is a much weaker, stimulatory interaction. Time in seconds.

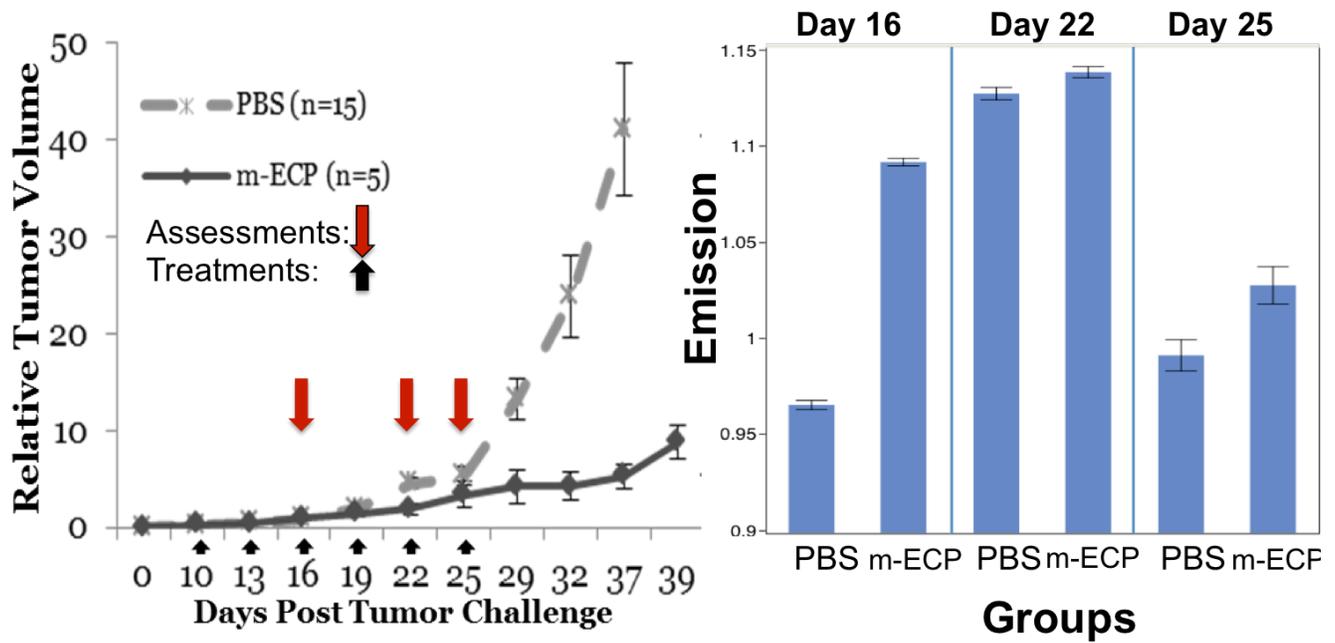


Figure 8. In vivo Fluorometric Assessments in an Animal Tumor-responsive Model. Our treatment group (m-ECP) showed significant differences in emission intensity, or T-cell reactivity, compared to our control group (PBS) prior to an observable clinical response. Treatment days are indicated with a black arrow and assessment time points are indicated in red arrows. Error bars indicate standard error measurements.

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