



Monitoring the dynamics of T cell clonal diversity using recombinant peptide:MHC technology

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The capacity to probe antigen specific T cells within the polyclonal repertoire has been revolutionized by the advent of recombinant peptide:MHC (pMHC) technology. Monomers and multimers of pMHC molecules can enrich for and identify antigen specific T cells to elucidate the contributions of T cell frequency, localization, and T cell receptor (TCR) affinity during immune responses. Two-dimensional (2D) measurements of TCR-pMHC interactions are at the forefront of this field because the biological topography is replicated such that TCR and pMHC are membrane anchored on opposing cells, allowing for biologically pertinent measures of TCR antigen specificity and diversity. 2D measurements of TCR-pMHC kinetics have also demonstrated increased fidelity compared to three-dimensional surface plasmon resonance data and are capable of detecting T cell affinities that are below the detection level of most pMHC multimers. Importantly, 2D techniques provide a platform to evaluate T cell affinity and antigen specificity against multiple protein epitopes within the polyclonal repertoire directly *ex vivo* from sites of ongoing immune responses. This review will discuss how antigen specific pMHC molecules, with a focus on 2D technologies, can be used as effective tools to evaluate the range of TCR affinities that comprise an immune response and more importantly how the breadth of affinities determine functional outcome against a given exposure to antigen.

Keywords: kinetics, 2D assays, T cell activation, recombinant pMHC, T cell affinity

DETECTION OF ANTIGEN SPECIFIC T CELLS

The ability to mount an effective immune response is essential to the survival of a living organism. Adaptive immunity in particular allows vertebrates a defense mechanism against countless pathogens. Antigen receptors on lymphocyte surfaces allow for recognition of a specific target, leading to activation and subsequent expansion of effector cells. This process is heavily dependent on affinity and on/off rate binding kinetics of the receptor for antigen. Though it is generally accepted that the highest affinity and thus most fit lymphocytes are selectively expanded (1, 2), the exact affinities of lymphocytes needed for an optimal immune response are still unknown.

During the course of a B cell response, somatic hypermutation in the germinal center allows for editing of the B cell receptor (BCR) to improve the affinity of the responding cells. This process involves the introduction of random mutations in the antigen binding site that can result in enhanced recognition of the target antigen. B cell affinity maturation allows higher affinity cells to outcompete less fit, lower affinity clones. While T cells also selectively expand responders based on specificity for antigen, T cells do not undergo receptor editing to improve the specificity of the response. Of interest, some reports have shown that mature T cells can re-express V(D)J recombination machinery and facilitate rearrangement of the T cell receptor (TCR) (3, 4). As the concept of TCR editing in the periphery may require further investigation, this review will assume that the TCR is fixed once the T cell has matured and entered the periphery. From the predetermined TCR

repertoire, mature T cells are still able to generate diverse antigen specific polyclonal responses. This leads to the questions of what affinity range defines an optimal T cell response and what technology is best suited to evaluate this aspect of T cell diversity.

One way to detect diversity of the TCR repertoire is through the analysis of antigen driven changes in V β chain usage and complimentary determining region (CDR3) sequences during the course of an immune response. The $\alpha\beta$ chains of the conventional TCR are encoded by V, D, J genes. Recombination of these gene segments concomitant with nucleotide insertions and imprecise joining events yields highly diverse T cell receptors. The CDR3 region, formed from the joining of the $\alpha\beta$ TCR chains, directly contacts the antigen in the binding groove and thus reflects the antigen specificity of the clone (5). Studies show that during the course of an immune response, certain V β chains are preferentially expanded to create a unique signature of antigen specificity and clonal dominance of an immune response (6–8). Spectratyping or immunoscope analysis is a technique in which the sequence length of the CDR3 is derived from the DNA of bulk clonal populations typically identified by V β usage (9). CDR3 sequence length has been used to subset and characterize T cell clonal populations for specific antigens (10, 11). Tracking CDR3 lengths and V β profiles can also provide insight in monitoring disease progression and for diagnostic purposes (12–15). Though repertoire analyses via these methods have revealed useful information, they lack the fine resolution to assess the diversity of a T cell clonal response. For example, these methods are primarily done on bulk cellular

populations resulting in conclusions based on a population average rather than on individual clones. More recent inquiries have shown this critical limitation fails to identify the paired TCR α and β chains responsible for the antigen recognition (16) and as a result, attempts to modify the techniques for single-clone analysis are being pursued (17). Future research combining single-cell analyses of TCR $\alpha\beta$ chain usage along with functional readouts and kinetic measurements will greatly enhance our knowledge of the T cells that comprise the polyclonal repertoire.

The detection of antigen specific T cells concomitant with the characterization of their functional responsiveness has been key to providing insights into the factors that promote pathogenic specific and protective immunity. Historically, the tracking of antigen specific T cells in a polyclonal environment has been performed with functional assays assessing proliferation, production of cytokines, cytotoxic mediators, and protein markers of cell activation. These indirect markers are important for characterizing T cell phenotype but may poorly represent the entire polyclonal repertoire because detection depends on antigen dose utilized in the assay as well as the efficiency of the assay itself. Stimulation with high dose, cognate antigen may negatively bias T cell detection toward a low affinity profile by eliminating the higher affinity clones though activation induced cell death (18), while low dose cognate antigen may selectively detect cells with higher affinity TCR. Therefore, a direct means for quantifying antigen specific T cells utilizing recombinant cognate or variant peptide:MHC (pMHC) molecules could provide more sensitive analytical tools for assessing the complexity of the entire responding T cell population (19).

The development of recombinant pMHC molecules for detection of a myriad of MHC class I and II epitopes from both foreign (bacteria, viruses, and parasites) and self proteins (tumors and targets of autoimmune attack) provide a method for specific assessment or targeting of the T cell repertoire. Multimers of pMHC, especially the biotin:streptavidin-based pMHC tetramer technology, provide accessible tools to determine the frequency of antigen specific T cells via flow cytometry (19) and to deplete antigen specific T cells *in vivo* (20, 21). Importantly, tetramers are useful for enumerating and enriching antigen specific T cells. The fluorophore attached to tetramers can allow for the “pull down” or enrichment of antigen specific cells from a polyclonal population for downstream applications such as determining precursory frequency of tetramer positive cells (22, 23). The efficiency of detection by multimers is due to the aggregation of TCR:antigen interactions that increase avidity and circumvent the short half life of interactions between TCR and pMHC (19, 24). MHC class I and II tetramers are the most commonly utilized multimer largely because monomers and dimers exhibited insufficient binding kinetics for TCR to facilitate detection by flow cytometry and were less stimulatory than tetramers (25). Advancements on multimer technology have been focused on increasing avidity through creation of progressively higher order oligomers, most notably the commercially available 5-armed pentamers (26–28) or 10-armed dextramers (29, 30). Despite the increased avidity provided by these reagents, multimers of higher order magnitude beyond pentamers provide, at most, modest increases in sensitivity of T cell detection (29, 30), possibly due to the physical constraints needed

for multiple simultaneous TCR–pMHC interactions (31). Even in the case of pMHC tetramers, it is unlikely that all four monomers bind simultaneously due to steric hindrances (25, 32).

The efficiency of pMHC molecules to detect antigen specific T cells is also dependent on peptide orientation within the MHC groove. Peptide–MHC anchor residues, which typically lie at positions 1, 4, 6, and 9 of the core peptide for MHC class II, are key to the stability of the peptide within the MHC. Variations in the amino acid residues that contact MHC, termed MHC variant peptides, can weaken or stabilize the interaction between TCR and pMHC (33–36). Though MHC variant peptides have been used to stabilize interactions with MHC to enhance T cell detection, these modifications could confound downstream analyses. For instance, these changes could modify the secondary structure, altering the TCR contact residues (37, 38) and may impact accurate kinetic and functional analysis. Furthermore the non-covalent interactions between peptide and MHC class II are of particular concern because the binding groove is open at both ends and can allow for the peptide to slide into different binding registers and influence TCR detection of the pMHC complex (39). For example, we and others identified three to four peptide registers in the well described OVA_{323–339} 17-mer peptide (40, 41) that have made uniform recombinant pMHC monomer production and especially the creation of tetramers somewhat difficult (41). One method to improve the tetramer production is through the use of a limited set of linker amino acids used to covalently attach peptide to the N-terminus of the MHC class II molecule (42). In addition, multiple binding registers can be limited by creating a disulfide bond or “lock” engineered via a cysteine residue on the peptide and on the MHC as reported for insulin B_{9–23}, OVA_{323–339}, and HA_{126–138} peptides (41, 43). Despite the effective use of recombinant pMHC and tetramers for the identification of antigen reactive T cells, their use as direct measures of TCR frequency and affinity during an immune response can be problematic.

MEASURING TCR AFFINITY FOR pMHC

A critical determinant for an antigenic response is the strength of signal derived through the TCR (44, 45). Although many factors contribute to the translation of signals into a biological response (i.e., costimulation (46), duration of signal (47, 48), etc.), affinity is a major parameter that establishes and controls the contribution of all additional factors in this response. Characterization of T cell response dynamics requires methods to obtain biophysical measures of affinity and kinetic on/off rates between TCR and recombinant pMHC. Many of the models describing T cell activation have been postulated based on kinetic-binding data from three-dimensional (3D) and two-dimensional (2D) binding assays.

Purified TCR and pMHC proteins can be used to study binding kinetics in 3D using techniques such as surface plasmon resonance (SPR). In this case, TCR and pMHC protein interactions occur in a fluid filled 3D space and affinity is measured in terms of the molar concentration needed to generate binding; TCR–pMHC affinity can range from 1 to 100 μ M while the half life of the interaction can range from 10 to 100 s (49–52). SPR analysis provided the biophysical basis for models correlating TCR binding kinetics and T cell triggering in order to explain the functional

differences seen between agonists, weak agonists, and antagonists (53–55). The most popular models are the kinetic proofreading and kinetic discrimination models, which ascribe optimal T cell responses to binding kinetics that allow sufficient time for TCR triggering (53, 54, 56, 57). Ligands that stimulate outside of this optimal time range, i.e., too long or too short, will not lead to a productive response according to these models. Despite the accuracy of these models in predicting agonist responses, several instances were identified where the biophysical measures did not relate to T cell activation state, particularly in response to weaker ligands (51, 57–62). These exceptions raised questions regarding the accuracy of 3D kinetic measurements derived from purified molecules to reflect the kinetics of proteins within the membrane environment. 3D assays are also limited in their ability to assess the full scope of a response due to the difficulty in purifying TCR from all participating antigen specific T cells. Therefore, alternative technologies are needed to probe the breadth of a polyclonal T cell response.

Analysis of receptor/ligand interactions using 2D technologies provides a physiologically relevant context in which to assay TCR affinity and the scope of polyclonal T cell responses because the TCR and pMHC are bound within cell membranes. Therefore these assays, namely the fluorescent based assays of FRAP and FRET, as well as the mechanical based micropipette techniques, biomembrane force probe and flow cell, can potentially better interrogate T cell kinetics with pMHC (63–69). The interactions between pMHC and TCR were found to occur more rapidly when analyzed in 2D rather than 3D, lending support to the serial triggering model where high affinity interactions generate fast off rates and rapid on-rates amenable for sampling multiple pMHC (69, 70). For the most part, one cannot readily convert the 2D area based measurements to 3D volume based affinities and on rates. A conversion of affinity from 2D FRET data to 3D measurements was suggested based on approximations of the contact area and intercellular volume between the T cell and surrogate APC bilayer (66). The approximations of contact area and intercellular volume are difficult to attain for T cells, which possibly explains why there is a discrepancy between the converted 3D K_d and SPR values for the MCC agonist and T102S weak agonist peptides. In contrast, the 2D and 3D half-life measurements are comparable because they are reported in units of time, yet in 2D, the time of interaction is more rapid than found in 3D analyses (66, 70).

We have focused on the mechanical 2D micropipette adhesion frequency assay as it provides a novel platform for evaluating T cell antigen specificity, frequency, and cross reactivity between epitopes within a polyclonal repertoire. Importantly, small numbers of T cells can be individually analyzed directly *ex vivo* from the blood and sites of ongoing immune responses. This assay allows for the visualization of TCR binding events with pMHC on opposing cells via a modified inverted microscope (Figure 1). The T cell and pMHC coated red blood cell are placed on opposing micropipettes and moved in and out of contact by means of a piezoelectric actuator for a defined contact and retraction cycle that will facilitate a binding event at equilibrium (71). A binding event is seen as distension of the RBC membrane on the video monitor as the cells are moved out of contact. The concentration of pMHC monomer coating on the RBC is optimized to yield an average binding frequency between 10 and 90% for several repeated

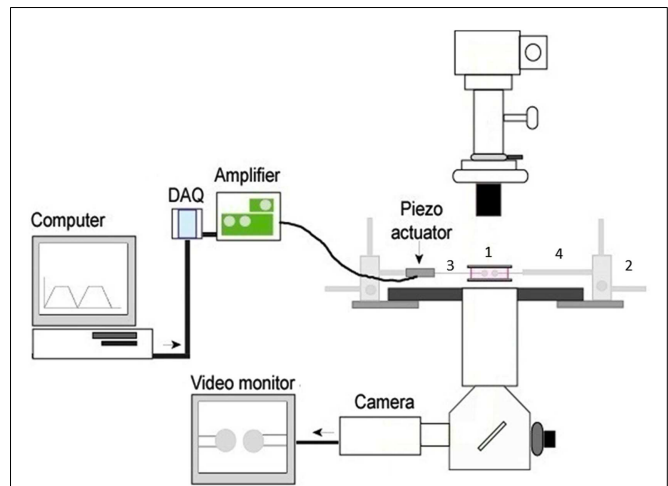


FIGURE 1 | The basic 2D micropipette adhesion frequency assay system.

The foundation for this system is an inverted microscope. The stage has been modified with a metal adapter (1) to rest a media filled chamber containing the T cells and pMHC coated RBCs above the 100× oil immersion objective lens. The stage is also fitted with course micromanipulators (2) allowing for the movement of the micropipettes (3) within the chamber. The micropipettes are attached to a hydraulic pressure system (not shown) by means of a micropipette holder (4) allowing for individual cells to be aspirated and held within the chamber. A piezoelectric actuator is attached to one micropipette holder such that it can be moved in and out of contact with the opposing cell. The DAQ, digital acquisition board, converts the cyclical digital signal from a computer program to an analog voltage signal that drives the piezoelectric actuator. Cells can be visualized on the video monitor and adhesion events can be subsequently recorded.

contacts (usually 50 contacts). The micropipette assay is sufficiently sensitive to measure the binding of a single receptor-ligand bond, a feature that cannot be achieved with pMHC multimer technology. The higher sensitivity of the micropipette assay is not based on increased valency but likely due to the 2D orientation of the pMHC and TCR incorporated within the cell membranes. This closely replicates the interaction as it would occur between two cells and allows for measurement of TCR:pMHC kinetic parameters in a biologically relevant context. The effective 2D binding affinity, with a detection range from high to low, 10^{-2} to $10^{-7} \mu\text{m}^4$, is a composite term that incorporates the affinity (K_a) and the contact area (A_c) between the T cell and surrogate APC. Derivation of the effective 2D binding affinity ($A_c K_a$) requires quantification of the receptor density (m_r), the ligand density (m_l), and the frequency of adhesion (P_a) between the cells as represented by $A_c K_a = -\ln(1 - P_a)/m_r m_l$ (71, 72). The adhesion frequency assay is the fundamental model of 2D mechanical assays where binding frequencies can be used to derive affinities and on/off rate kinetic readouts (73). The biomembrane force probe is a modified adhesion frequency assay that can allow for detection of individual bonds with increased sensitivity for detecting faster on/off rates and it can be modified to readout the effects of force on the bond between TCR and pMHC (73). Furthermore, the 2D micropipette system can be altered to permit visualization of functional fluorescent readouts such as calcium signaling, which has already been integrated into the FRET based 2D assays (66). The capacity of

multiple readouts and increased sensitivity with the 2D mechanical tools is evidence for the power/significance of these techniques.

BREADTH OF AFFINITY IN THE POLYCLONAL REPERTOIRE

The affinity of TCRs for antigen can be discussed at both the single clone and population levels. A single TCR clone expands into multiple daughter cells that will possess a measurable but narrow range in affinity despite expressing an identical TCR. A polyclonal population of cells will possess a wider range or distribution of affinities comprised of all TCR clones activated to expand in response to any specific antigenic stimuli. Additionally, TCRs by their very nature are degenerate or crossreactive and can interact with many different peptide antigens. The estimated $\alpha:\beta$ TCR diversity is $\sim 10^{18}$, a seemingly large number that is significantly reduced to several hundred millions of T cells during thymocyte maturation (74, 75). Even with these reduced numbers, T cells still possess the ability to respond to most possible antigens. Therefore, the ability of a single TCR to recognize multiple antigens, albeit with varying degrees of affinity, is critical to increase the number of T cells that recognize each antigen.

The concept of TCR degeneracy is demonstrated by the capacity of monoclonal TCRs to recognize MHC variant peptides or altered peptide ligands, defined as epitopes with modified affinity for cognate TCR (76, 77). They also provide insight into the effective affinity range recognized by a single TCR. For instance, OT-1 CD8⁺ TCR transgenic T cells exhibit a V α 2/V β 5 rearranged TCR that recognizes the cognate SIINFEKL (OVA) peptide on H2-K^b with a high 2D effective affinity ($\sim 10^{-3} \mu\text{m}^4$). Modifying this peptide sequence changes the affinity of OT-1 TCR for pMHC generating a breadth of affinities (70) that can alter downstream functional outcomes to yield agonist (A2, 2D affinity of $\sim 10^{-4} \mu\text{m}^4$), weak agonist (G4 and E1, 2D affinity of $\sim 10^{-5} \mu\text{m}^4$), or antagonist (V-OVA and R4, 2D affinity of $\sim 10^{-6} \mu\text{m}^4$) responses (70). Overall the OT-1 TCR exhibits an approximate, 1000-fold range in affinities depending on the peptide being presented by MHC class I. Additional T cells will have to be analyzed to determine whether this breadth of the 2D affinity range is characteristic of all CD8⁺ or CD4⁺ T cells.

The identification that a single TCR can exhibit a broad range of affinities to different peptide antigens led to the study of the array of affinities found within a polyclonal CD4⁺ T cell response against one peptide antigen. The breadth of 2D effective affinities for a single antigen within a polyclonal population exhibited a Gaussian distribution possessing a defined mean and standard deviation. For example, CD4⁺ T cells primed with the LCMV (lymphocytic choriomeningitis virus) GP_{61–80} peptide epitope, showed between a 100- and 1000-fold range of affinities by the 2D micropipette assay with a mean of $4.21 \pm 1.48 \times 10^{-4} \mu\text{m}^4$ (78). A similar distribution and range with a lower mean affinity $1.63 \pm 0.48 \times 10^{-5} \mu\text{m}^4$ was also observed for the polyclonal response against the myelin oligodendrocyte glycoprotein self antigen MOG_{35–55} (78). As one would expect, the analysis of a single TCR does not replicate the affinity range found within a polyclonal population. It is currently unclear how well conclusions made based on monoclonal models informs on the polyclonal response to the same antigen (79). This is affirmed by comparing the 2D affinities between the monoclonal CD4⁺ SMARTA

T cell clone and the polyclonal CD4⁺ T cell population. Both populations are specific for the same GP_{66–77}: IA^b antigen, but the monoclonal SMARTA population only represents a fraction of the affinity breadth seen in the polyclonal response. In this case, the monoclonal cells have a mean affinity of $\sim 10^{-3} \mu\text{m}^4$ which is ~ 10 -fold higher than the mean polyclonal affinity of $\sim 10^{-4} \mu\text{m}^4$ [Figure 2A adapted from Ref. (48, 78)]. Although the SMARTA TCR is monoclonal, it is interesting that this TCR exhibits a range of affinities, albeit more narrow than the responding polyclonal T cells. An affinity range can even be detected

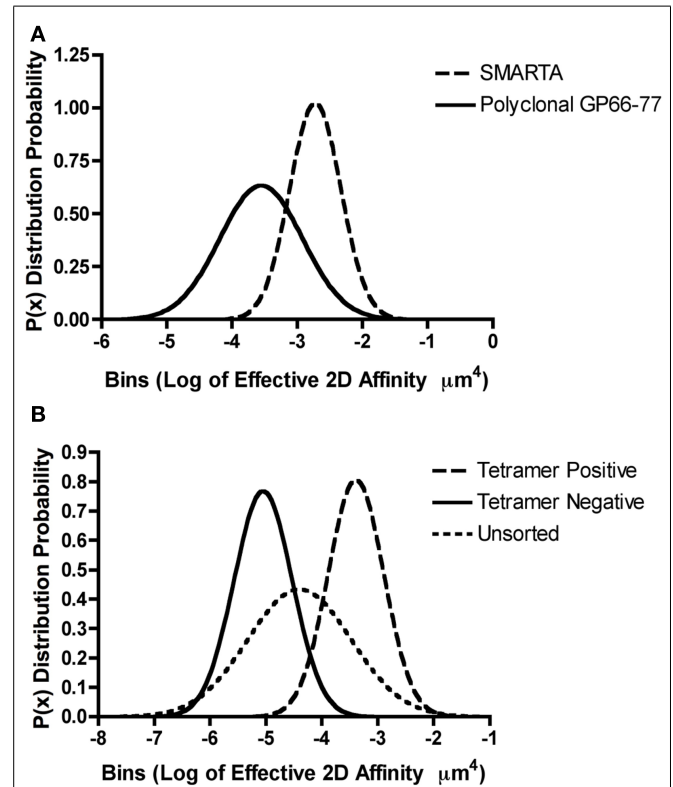


FIGURE 2 | Range of TCR affinities for an individual pMHC in a polyclonal repertoire. (A,B) Gaussian distributions were modeled for the described T cells by utilizing previously published effective 2D affinity means and standard deviations using the equation $P(x) = 1/(\sigma \times \sqrt{2\pi}) \times e^{-(X-\mu)^2/(2\sigma^2)}$ where $P(x)$ is the probability density function or distribution, σ is the standard deviation, X is the variate or bin interval, and μ is the mean log of the TCR affinities. **(A)** The monoclonal SMARTA T cells and the polyclonal GP_{61–80} population both recognize GP_{66–77}: IA^b. The 2D micropipette adhesion frequency assay was used to determine the mean effective 2D affinities and standard deviations as previously reported (48, 78). Gaussian distributions indicated that SMARTA T cells exhibit a higher log of affinity μm^4 (-2.7 ± 0.39) ~ 10 -fold higher than the polyclonal T cell populations (-3.5 ± 0.63), indicating that monoclonal population underrepresented the polyclonal affinity range. **(B)** The 2D micropipette adhesion frequency assay was used to ascertain the mean effective 2D affinities and standard deviations for the polyclonal GP_{61–80} repertoire (unsorted) and FACS sorted GP_{66–77}: IA^b tetramer positive and negative populations (78). Gaussian distributions indicated that both the tetramer positive (\sim peak at -3.0) and tetramer negative (\sim peak at -5.0) populations under represented the range of affinities exhibited by the polyclonal (unsorted) repertoire.

among TCRs expressed on a single T cell because FRET analyses with the 5C.C7 CD4⁺ TCR have shown a 250-fold 2D affinity range for MCC between microclusters of the same cell (66). Furthermore, monoclonal models are often thought to represent the highest affinity TCRs within a polyclonal response, which is not necessarily the case. Clones are often selected *in vitro* for optimal growth, effector function, and reagent availability for tracking the TCR *in vivo*. For example, we have found that the transgenic 2D2 TCR, widely used for the study of demyelinating autoimmune disease, is of low affinity for its antigenic ligand yet shows measurable reactivity through proliferation and cytokine production assays (48, 80).

While the ability to track antigen specific T cells within a polyclonal repertoire has been revolutionized by the use of pMHC tetramers recent investigations call into question the fidelity of these reagents to sufficiently capture all participating effectors in an immune response. Our laboratory and others have shown a discrepancy in the use of tetramers to determine the breadth of CD4⁺ T cell responses. Tetramers will not detect T cells where the affinity of the TCR for antigen is below the avidity threshold needed for binding. We have estimated this 2D effective affinity cutoff to be between 10^{-5} and 10^{-4} μm^4 for MHC class II restricted T cells; it is more difficult to define for MHC class I based tetramers as CD8 significantly contributes to the overall stability and binding while CD4 does not (48, 78, 81). Analysis of the tetramer positive subset of polyclonal GP₆₆₋₇₇ reactive T cells showed enrichment for higher affinity T cells with a mean 2D affinity of $\sim 10^{-3}$ μm^4 as compared to the affinity of the intact polyclonal T cell population of $\sim 10^{-4}$ or the tetramer negative T cells $\sim 10^{-5}$ μm^4 (78). Of interest, the tetramer positive and negative cells are a subset of the overall polyclonal affinity repertoire [Figure 2B, adapted from Ref. (78)]. For both the self and pathogen specific CD4⁺ T cell response, the percentage of tetramer reactivity was lower and did not correlate to the percentage of cytokine responders or the frequency of antigen specific T cells measured by the 2D micropipette adhesion frequency assay (78). Therefore, the sole use of tetramers to monitor the antigen specificity, frequency, magnitude, and affinity of a polyclonal repertoire in order to predict the overall composition of an immune response appears somewhat limited, missing the contribution of the lower affinity T cells.

Underestimating the contribution of low affinity T cells is a significant issue for models of autoimmune disease where negative selection likely enriches for a low affinity repertoire reactive against self antigen. Our studies with MOG₃₅₋₅₅ specific CD4⁺ T cells indicated that tetramer generally reacted with 7–10% of T cells within the target organ, while functional effector responses and 2D affinity analysis detected much higher levels of cells (78). In the 2D2 TCR transgenic model of EAE, the 2D2 T cells promoted spontaneous paralytic disease (4%) or spontaneous optic neuritis (35%) (80). This TCR has very low affinity for antigen (48) and does not interact with MOG-specific tetramers and therefore contrasts with data suggesting low affinity or low avidity T cells are less pathogenic. Furthermore, retrogenic derived monoclonal TCR models suggest that TCR of low avidity can support the development of spontaneous EAE in the absence of higher

avidity T cells (82). The challenge in studying the contributions of low affinity T cells has been the lack of reagents to do so. The 2D adhesion frequency assays gives us one such tool to characterize lower affinity T cells alongside the higher affinity contributors within a polyclonal population.

BENEFITS OF AN INCLUSIVE RESPONSE

To date, current models of T cell clonal expansion suggest that high affinity T cell clones are preferentially enriched over low affinity clones (83–85). As a result, many current T cell therapeutic initiatives seek to elicit or artificially create high affinity T cells to enhance pathogen specific and anti-tumor responses (86). However, recent investigations have shown that T cells manipulated to have supraphysiological affinity were unexpectedly less potent effectors than lower affinity counterparts due to triggering of inhibitory mechanisms (87, 88). It would therefore be plausible that an effective immune response may benefit from a balance of high, intermediate, and low affinity T cell responders.

Polyclonal TCR affinity composition can be shaped by TCR activation thresholds. For example, CD27 costimulation has been shown to support the emergence of lower affinity CD8⁺ T cells that mediate greater protection against reinfection with an influenza variant (89). Similarly, clones with low functional avidity have been shown to be important in the maintenance of an effective anti-tumor response (90). Although affinity was not the sole focus of this study, reduction of p56^{Lck} expression significantly decreased T cell sensitivity to activation which mimics a lower affinity response. These low affinity effectors were less susceptible to an exhausted phenotype and mediated better protection in subsequent rechallenge. Such investigations provide evidence for why low affinity clones may exist within the repertoire and how therapeutics to limit them may be shortsighted.

The role for low affinity T cell populations can be obscured by the nature of the assay used to analyze the response. In a study examining the therapeutic efficacy of tumor vaccines, high affinity clones (as determined by SPR) responded optimally in *in vitro* assays, but intermediate affinity clones mediated the best anti-tumor responses *in vivo* (91). Similarly, a study evaluating optimal T cell responses to peptide in the 5C.C7 model (85, 92) showed that intermediate affinity clones mediated the most optimal *in vivo* responses while high affinity clones demonstrated the strongest response *in vitro*. Future studies may benefit from understanding the interplay of individual T cell affinity subsets in the overall efficacy of tumor and pathogen specific responses. These findings underscore the potential role for lower affinity effectors in an immune response and therefore they should be an important consideration in the design of therapeutic interventions.

Understanding how T cell affinity mediates protective immunity also has important implications for vaccine design because recent studies have shown the priming agent and the adjuvant can alter the CD4⁺ TCR affinity composition. In one study, vaccinations using either cytochrome C peptide or whole protein were compared (93). Though both vaccination regimens generated diverse clonal responses, peptide vaccines elicited high

affinity dominated responses while protein vaccines generated a repertoire inclusive of both low and high affinity responses. The maintenance of low affinity effectors was found, at least in part, to require CD27–CD70 signaling. Another study demonstrated the ability of vaccine adjuvants to affect the affinity composition of T cells generated in response to pigeon cytochrome C, PCC (94). All the adjuvants tested were effective in enhancing a PCC-specific T cell response, but alum, IFA, and CFA induced lower avidity responses while CpG and monophosphoryl lipid A generated higher avidity responses as determined by pMHC tetramer and CDR3 spectrotyping. This observation suggests that adjuvants could differentially influence recruitment into the polyclonal response. The effect was dependent on the dispersive ability of the adjuvant and activation of different TLRs that resulted in changes in CD4⁺ T cell recruitment and/or migration. It is worth noting that even adjuvant choice can affect the balance of low and high affinity clonotypes (94, 95) and should be furthered explored with 2D assays. The application of 2D based pMHC technologies to these questions will allow us to uniquely explore the breadth of TCR affinities and redefine our understanding of the dynamic interplay between TCR affinity subsets within the polyclonal repertoire.

CONCLUSION

The use of pMHC technology is at the forefront of monitoring antigen specific immune responses. We promote 2D mechanical based assays with purified pMHC for several reasons. First, they display increased sensitivity for detecting antigen specific T cells when compared to functional responses or pMHC tetramer based assays. Secondly, the polyclonal repertoire can be monitored without purification of individual TCRs because the analysis is carried out using intact T cells. Lastly, 2D assays provide a more accurate

representation of the relationship between T cell affinity and functional responsiveness. The findings to date have highlighted the presence of antigen specific CD4⁺ T cells exhibiting a range of affinities from low to high in both autoimmune and pathogen specific models. Low affinity, tetramer negative populations elicit effector functions and expand in response to antigen suggesting their capacity to contribute to adaptive immune responses. The idea that lower affinity T cells effectively compete within and contribute to the effector T cell repertoire at the very least modifies our view that high affinity T cell clones would dominate the lower affinity counterparts. Future work is needed to examine how affinity of the initial TCR:pMHC interaction contributes functionally to the initiation, maintenance, and/or resolution of a polyclonal immune response. In addition, we need sensitive techniques that allow for analysis of TCR crossreactivity and in the case of autoimmunity, epitope spread to new antigens. At this point in time, 2D based assays together with recombinant pMHC molecules are useful tools available to characterize individual T cell affinity contributions to the breadth of an immune response. Potential clinical outcomes for this research include the use of TCR affinity as a biomarker to monitor disease progression and to provide information for the development of high efficacy antigen specific therapies.

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