Published in final edited form as:
J Immunol Methods. 2013 June 28; 392(0): 1–11. doi:10.1016/j.jim.2013.02.018.

Engineering improved T cell receptors using an alanine-scan guided T cell display selection system

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Abstract

T cell receptors (TCRs) on T cells recognize peptide-major histocompatibility complex (pMHC) molecules on the surface of antigen presenting cells and this interaction determines the T cell immune response. Due to negative selection, naturally occurring TCRs bind self (tumor) peptides with low affinity and have a much higher affinity for foreign antigens. This complicates isolation of naturally occurring, high affinity TCRs that mediate more effective tumor rejection for therapeutic purposes. An attractive approach to resolve this issue is to engineer high affinity TCRs *in vitro* using phage, yeast or mammalian TCR display systems. A caveat of these systems is that they rely on a large library by random mutagenesis due to the lack of knowledge regarding the specific interactions between the TCR and pMHC. We have focused on the mammalian retroviral display system because it uniquely allows for direct comparison of TCR-pMHC-binding properties with T-cell activation outcomes. Through an alanine-scanning approach, we are able to quickly map the key amino acid residues directly involved in TCR-pMHC interactions thereby significantly reducing the library size. Using this method, we demonstrate that for a self-antigen-specific human TCR (R6C12) the key residues for pMHC binding are located in the CDR3β region. This information was used as a basis for designing an efficacious TCR CDR3α library that

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AUTHOR CONTRIBUTIONS

K.M., and M.K., designed the study; M.K. supervised the project; K.M. conducted most of the experiments and K.M. and M.K. analyzed most of the data and wrote the manuscript; K.M. and S.Z. produced soluble proteins and performed SPR measurements. K.M., S.Z. and C.Y. generated DNA constructs and performed alanine scanning screening on cell surface. K.M.S and K.H worked on optimizing protein production of the wild-type TCR. LAJ isolated and cloned the R6C12 TCR as per ref Morgan, R.A., et al., J Immunol, 2003. 171(6): p. 3287-95.

allowed for selection of TCRs with higher avidity than the wild-type as evaluated through binding and activation experiments. This is a direct approach to target specific TCR residues in TCR library design to efficiently engineer high avidity TCRs that may potentially be used to enhance adoptive immunotherapy treatments.

Keywords

T cell receptor; T cell clones; Mammalian TCR display system; CDR3 alanine scanning mutagenesis; Combinatorial library design

1. Introduction

In nature, cytotoxic T cells are responsible for identifying and killing cells that display foreign or aberrant antigens and thus they have long been recognized for their potential in the immunotherapy of cancer (Rosenberg, Yang et al. 2004; Gattinoni, Powell et al. 2006; Johnson, Heemskerk et al. 2006; Johnson, Morgan et al. 2009; Stone, Chervin et al. 2009). The cytotoxic reaction is mediated through interactions between the T cell receptor (TCR) and co-receptor CD8 with peptide-major histocompatibility complex (MHC) presented on antigen-presenting cells (APCs). This interaction is followed by signaling through the TCR:CD3 signaling complex, which results in T-cell activation and the initiation of cellular immune responses (Germain and Stefanova 1999; Krogsgaard and Davis 2005).

The kinetics of the CD8/TCR/pMHC interaction is one of the parameters governing T-cell activation (Germain and Stefanova 1999; Krogsgaard and Davis 2005; Stone, Chervin et al. 2009); the initial interaction is a prerequisite for all downstream signaling cascades and T-cell activation (Kane, Lin et al. 2000; Smith-Garvin, Koretzky et al. 2009). Consequently, the biophysical properties of this complex are of great interest in terms of predicting T-cell activation outcomes. There is substantial evidence that describes the correlation between T-cell activation outcomes and the strength of the interaction between the TCR and pMHC (Davis, Boniface et al. 1998; Rudolph, Stanfield et al. 2006). Affinity and off-rate are widely accepted as the major kinetic parameters that determine how TCR-pMHC interaction contribute to efficient selection in the thymus as well as peripheral cell signaling and activation (McKeithan 1995; Valitutti, Muller et al. 1995; Rabinowitz, Beeson et al. 1996; Schodin, Tsomides et al. 1996; Goldrath and Bevan 1999; Lanzavecchia, Lezzi et al. 1999; Stone, Chervin et al. 2009).

During T cell development in the thymus, a theoretically large TCR repertoire is generated (up to ~10^{14} different TCRs) (Davis and Bjorkman 1988), although recent, deep sequencing analysis would indicate that the actual number is more likely around 10^{11} different TCRs (Robins, Srivastava et al. 2010). From this repertoire TCRs that bind to self peptide MHC (pMHC) complexes with intermediate affinity are positively selected (Goldrath and Bevan 1999) whereas TCRs with high affinity are deleted during negative selection (Sprent and Kishimoto 1998; Stockinger 1999) and the number of unique TCRβ CDR3 sequences expressed in peripheral blood T cells of a healthy adult is estimated to be ~3×10^6 (Robins, Campregher et al. 2009; Warren, Freeman et al. 2011). The absence of strong self pMHC interactions in the periphery serve to prevent autoimmunity although it poses a problem for efficient tumor recognition as most tumor antigens are self-derived and simply over-expressed on tumor tissues (Kawakami, Eliyahu et al. 1994; Boon, Coulie et al. 1997; Gattinoni, Powell et al. 2006). Due to high therapeutic demand to enhance tumor recognition many different strategies have been developed with the purpose of isolating tumor-specific, high affinity TCRs. *In vitro* isolation of high affinity TCRs has most commonly been done utilizing phage (Li, Moysey et al. 2005), or yeast (Holler, Holman et al. 2000; Weber,

Donermeyer et al. 2005) display systems and single or dual amino acid substitutions (Robbins, Li et al. 2008); recently the mammalian T cell display system has emerged as a promising alternative strategy facilitating specific selection of functional high affinity TCRs (Kessels, van Den Boom et al. 2000; Richman and Kranz 2007; Chervin, Aggen et al. 2008). Two recent reports have described mammalian display methods of engineering a combinatorial library of TCR mutants on the surface of TCR-negative T cells (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008) (reviewed in (Richman and Kranz 2007)). This strategy allows the TCR to be expressed on the T cell surface in complex with CD3 signaling subunits. However, as recently reported, one drawback of the mammalian cell surface display is the limited potential for combinatorial library diversity (Richman and Kranz 2007).

Here we have addressed this limitation by employing an alanine mutagenesis screen to evaluate the individual contribution of the CDR3 alpha and beta regions to TCR-pMHC binding before TCR library design. This method allows targeting of key amino acids in the TCR CDR3 regions important for the pMHC-TCR interaction identified through alanine scanning mutagenesis. Moreover, in contrast to previous methods (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008) after the final round of selection we conducted additional analysis on selected T cell clones on functional potency (such as cytokine production) in addition to TCR binding potency for pMHC. This strategy allowed the selection of T cell clones not only with increased binding avidity but also increased functionality. This additional component in our selection strategy is important, as recent data have shown that increased TCR-pMHC binding affinity does not always translate into increased functional activities (Dai, Huseby et al. 2008; Adams, Narayanan et al. 2011). Using the TCR display mammalian system we were able to generate both specific and non-specific (cross-reactive) T cell clones expressing mutated TCRs with a range of half-lives, affinities and activation potencies. Our data demonstrate that T-cell activation correlates with both TCR binding avidity and off-rate to pMHC. Importantly, this correlation is only applicable to the TCRs that specifically recognize pMHC, suggesting other mechanisms underlying cross-reactivity and non-specific T cell signaling. In conclusion, this work provides the basis for a novel, systematic method of efficient TCR display selection and characterization processes that provide a powerful strategy to understand biophysical parameters of TCR-binding and relation to function in a physiological setting.

3. Results

3.1. Ala scanning mutagenesis analysis of R6C12 TCR indicates that the critical contacting residues are located in the CDR3β region of the TCR

Knowledge of the specific residues important for the interaction between the TCR and pMHC could facilitate the design of optimal libraries for the selection of high-affinity TCRs. CDR1 and CDR2 regions of the TCR have been shown to mainly contact the MHC (sometimes CDR1 and CDR2 also contact the peptide) while the CDR3 region mainly contacts the peptide and thus mediates the peptide-specificity of T cell recognition (Rudolph, Stanfield et al. 2006). For this reason CDR3 α and β are two regions where mutations may increase TCR affinity without compromising specificity (Kessels, van Den Boom et al. 2000). In order to determine the energetically critical amino acid residue(s) at the TCR-pMHC binding interface, an alanine scanning screening system was applied for R6C12, a HLA-A2 restricted and gp100:209-217-specific TCR isolated from vaccinated melanoma patients (Dudley, Wunderlich et al. 2001; Morgan, Dudley et al. 2003; Zhong, Malecek et al. 2013). Each residue in the CDR3 α and β region of the TCR was mutated to alanine using site-directed mutagenesis (Narimatsu, Yoshioka et al. 2011) and expressed on the 58 α-/β-hybridoma cell line with human CD8 (hCD8 58−/− cells (Letourneur and Malissen 1989)) (Fig. 1). Since, the functional activity of R6C12 TCR is CD8 dependent

(Zhong, Malecek et al. 2013) we co-expressed the WT TCR or the alanine mutants in the presence of hCD8 to display TCR in its native environment and conformation in which the TCR is normally on the T-cell surface. CD8 enhances the sensitivity of TCR-signaling (Purbhoo, Boulter et al. 2001; Laugel, Cole et al. 2011) and binding (Holler and Kranz 2003; Laugel, Cole et al. 2011), therefore including CD8 in this system also facilitates measurement of mutated clones with low activity. For these clones CD8 is known to have a greater impact on the functional activity and tetramer binding measurements compared to high affinity TCRs (Purbhoo, Boulter et al. 2001; Choi, Chen et al. 2003; Zhong, Malecek et al. 2013). Finally, including hCD8 in the original screening of TCRs would allow us to advance the use of these TCRs for more clinical setting by addressing the need to screen for cross-reactivity in the presence of hCD8 on human T cells (Overwijk, Theoret et al. 2003; Gattinoni, Powell et al. 2006; Morgan, Dudley et al. 2006).

Single amino acid alanine substitutions in CDR3β diminished affinity for tetrameric pMHC binding when compared to both WT and alanine substitutions introduced in CDR3α (Fig. 1A). Accordingly, CDR3β alanine substitutions significantly decreased cellular cytokine production when stimulated with APCs *in vitro* (Fig. 1B). These results suggest that the CDR3β residues are critically important for maintaining specific contacts with the pMHC and eliciting proper T cell signaling responses. In contrast, alanine substitutions in CDR3α had minor effects on tetrameric pMHC binding (Fig 1A) and cytokine production (Fig. 1B). Together, these results suggest an opportunity to improve TCR binding to pMHC by introducing specific mutations that would strengthen binding of the CDR3 region as this chain is more tolerant to amino acid substitutions, likely because it does not significantly contribute to binding. However, owing to the numerous and dynamic interactions that occur on the cell surfaces during T cell signaling, e.g. TCR clustering, force sensitivity, molecular flexibility or steric effects that may be profoundly influenced by active cellular processes (Stone, Chervin et al. 2009; Huang, Zarnitsyna et al. 2010; Huppa, Axmann et al. 2010) it is important to first ensure that the observed differences in the TCR-pMHC binding interactions are entirely attributable to alanine mutations on the protein level.

3.2. Soluble TCR-gp209-2M -HLA-A2 interactions show a similar trend as cell surface derived binding parameters

To confirm that the observed differences in binding affinity and cellular signaling were the direct result of the engineered alanine substitutions alone and not attributable to intrinsic properties of the T cell, binding affinities were measured using soluble proteins. The wild-type (WT) TCR (R6C12) and four alanine substituted TCRs - represented by two CDR3 β chain (P96 and G97) and two CDR3 α chain (Q95 and K96) mutations - were cloned, expressed, refolded *in vitro* and subsequently purified by ion-exchange and size exclusion chromatography (Fig. S1A) (Huppa, Axmann et al. 2010; Newell, Ely et al. 2011). To increase the solubility and protein yield, the variable domains of the TCR α and β chains were fused to the human constant domain (Newell, Ely et al. 2011). To measure the binding affinity of alanine substituted TCRs to gp209-2M/HLA-A*0201 complexes surface plasmon resonance (SPR) was performed using a BIAcore X100 (Fig. S1B). In order to verify the proper refolding of the WT (Fig. S1C) and Ala-mutated (Fig. S1D) heterodimer TCRs, the binding to an epitope-specific antibody (anti-human Vβ8) was analyzed. Binding to this antibody is only observed when the TCR is properly folded (Choi, Kotzin et al. 1991). An antibody specific for an irrelevant human Vβ chain (V 17) was used (Fig S1C) as a negative control to ensure that the anti-human Vβ8 antibody was specific for the properly refolded R6C12 WT TCR. Binding of human Vβ8 antibody was then analyzed for binding to selected mutant TCRs, which exhibited specific binding at several antibody titrations (Fig S1D), indicating correct *in vitro* refolding. The CDR3α mutants, Q95 and K96, showed a modest three to five-fold reduction in binding affinity to gp209-2M/HLA-A2*0201 complexes (26

and 48 μM, respectively) when compared to the WT TCR binding affinity (9.0 μM) (Zhong, Malecek et al. 2013). In contrast, the two CDR3β mutants, P96 and G97, showed no binding (Fig 2). These results confirm that the diminished cellular functions and tetramer binding for the CDR3β mutants are due to reduced TCR affinity rather than other cellular contributions. Both, the soluble binding affinity and the cell surface results reinforce the importance of an intact CDR3β sequence for specific binding and proper cell signaling and strongly suggest the prospect of tuning specificity through CDR3α.

3.3. Selection of high affinity T cells from the CDR3α library

In order to generate TCRs with unique specificities, based on the finding that CDR3 would be a good strategic target for enhancing the TCR-pMHC affinity, CDR3α mutated libraries were designed and displayed on 58−/− mammalian cells expressing human CD8 (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008). A seven codon degenerate amino acid library in CDR3α of R6C12 TCR was created by overlapping PCR (Chervin, Aggen et al. 2008). To stabilize surface expression of human TCRs on hybridoma cells, constant regions of human TCRs were replaced by mouse constant regions ensuring pairing with the mouse CD3 expressed on hybridoma cells (Cohen, Zhao et al. 2006). The human-mouse hybrid TCR chains were linked by a self-cleavable 2A peptide for optimal TCR gene expression (Szymczak and Vignali 2005) and subcloned into a retroviral vector pMXs (Kessels, van Den Boom et al. 2000; Cohen, Zhao et al. 2006; Zhong, Malecek et al. 2010). This retroviral expression system was used to introduce recombinant TCR α and β chains into a TCR-negative T cell hybridoma (58−/− TCR^{-}, $hCD8^{+}$, $CD3^{+}$) that has previously been used to display mutagenized TCR libraries (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008) as well as the R6C12 WT TCR (Zhong, Malecek et al. 2010). The library was enriched for tetramer binding, where one percent of the cells were determined to be tetramer positive (Fig. 3A) before single cell sorting into 96-well plates. Based on E. coli transformation a library size of 3.1×10^4 clones was estimated (Chervin, Aggen et al. 2008). 574 individual T cell clones were isolated and their activation properties were determined by the level IL-2 cytokine production after stimulation with gp209-2M-pulsed T2 A2-K^b cells (Fig. 3B). A significant proportion of the analyzed T cell clones (67.48%) were less potent than the WT (<WT). The remaining T cell clones (32.54%) can be subdivided into categories as clones that exhibited IL-2 production upon stimulation at 1-2, 2-4, and 4-10 times higher cytokine levels than the WT clone. Nine representative T cell clones (three from each category) exhibiting higher IL-2 production than WT were selected for further characterization. This selection strategy allowed us to focus on mutated T cell clones that were enriched for increased binding avidity as well as increased T cell functionality. Our initial analysis of individual TCR sequences show that we obtained multiple mutated TCR sequences from each individual clone. This is, however, a commonly observed phenomenon when using hybridoma technology and has been observed previously both when generating antibodies and TCRs using this technology (Kubota, Ishikawa et al. 1981; Brioen, Sijens et al. 1982; Chervin, Aggen et al. 2008). For future clinical applications, subcloning and isolation of the TCR and/or TCRs, which is/are responsible for the observed T cell activation phenotype is required. Alternatively, for clinical applications total RNA for a pool of TCR genes could be isolated and expressed on human T cells (Zhao, Zheng et al. 2006; Mitchell, Karikari et al. 2008).

3.4. Characterization of the specificity of individual T cell clones identifies pMHC-specific as well as cross-reactive clones

The selected T cell clones produced greater IL-2 cytokine levels at several peptide concentrations when compared to the WT (Fig 4A). For most of the T cell clones, IL-2 production decreased when peptide concentration was decreased. However, two of the nine T cell clones (clone 1 and 2) exhibited elevated levels of cytokine production irrespective of

peptide concentration. Surprisingly, these clones produced high levels of cytokine even in the absence of the cognate antigen (gp209-2M) or in the presence of an irrelevant peptide, Mart-1(27L) (Fig 4B). However, APC stimulation was needed for IL-2 production as the two clones (clone 1 and 2) displayed six times reduced levels of activation without APC stimulation (Fig 4B), which was probably due to some weak levels of baseline T-cell activation. This was not due to the differences in TCR or CD8 expression as clone 1 and 2 did not have significantly higher TCR expression (Fig S2A) and only clone 2 had higher CD8 expression (less than 2-fold) (Fig S2B). Because clone 3 also exhibited equal CD8 expression to clone 2 but had different T cell potency these differences are likely not attributable to variations in TCR or CD8 expression level. Rather, these clones are cross-reactive because they are activated in the presence of APCs regardless of the peptide sequence presented. Overall, these results indicate that we can generate both specific and cross-reactive clones with improved potencies when compared to WT. The cross-reactive clones appeared to be less frequent than specific clones (2/9 = 22%), however they comprise an important class of mutated clones that in the future can be used to study the biophysical and structural properties of cross-reactive TCRs in order to extend previous studies on this class of TCRs (Holler, Chlewicki et al. 2003; Zhao, Bennett et al. 2007).

3.5. Cytokine production, avidity and half-life correlate for specific T cell clones

In order to better understand the TCR-pMHC binding properties underlying T-cell activation and specificity, we compared the binding parameters with the biological activities of the panel of the nine mutated T cell clones. Gp209-2M-HLA-A2 tetramer binding assays were used to measure both half-life ($t_{1/2}$) and avidity for each TCR, as previously described (Altman, Moss et al. 1996; Savage, Boniface et al. 1999). We found that the mutated TCRs exhibited a narrow window of measured dissociation half-life values, which were one to two-fold greater than the WT dissociation half-life (WT, $t_{1/2}$ = 21.58 min vs $t_{1/2}$ ranging from19.38 to 32.57 min for the mutated clones) (Fig. 5A & B, upper panels), consistent with previous observations (Gascoigne, Zal et al. 2001). Conversely, the avidities, measured as mean fluorescence intensity, were up to ten times greater than that of the WT (WT, MFI = 4.25 vs MFI ranging from 8.77 to 41.9 for mutated clones) (Fig. 5A & B, lower panels). We then plotted cytokine production for the T cell clones versus their corresponding half-life or T cell avidity for pMHC, but only observed weak correlations between the individual parameters (R^2 = 0.20477 and 0.19363, respectively) (Fig 5A). However, when excluding the two cross-reactive T cell clones (clone 1 & 2) we observed a much stronger correlation between cytokine production and half-life or avidity (R^2 = 0.61792 and 0.65956, respectively, Fig 5B). A good correlation was also observed between avidity and half life of these clones (excluding cross-reactive clones) (Fig 5C) indicating that their on-rates are similar. This suggests that a stronger correlation exists between the avidity or half-life measured for the engineered TCRs and their T-cell activation outcome (Stone, Chervin et al. 2009). Taken together, these results support the notion that it is possible to improve T cell function by generating TCRs with longer half-life and higher avidity.

4. Discussion

In this study, we report a strategy for generating mutated T cell clones with increased potency that combines an alanine scanning mutagenesis analysis with a mammalian TCR display. The mammalian TCR display system exploits the characteristic ability of T cells to assemble the TCR αβ heterodimer with the CD3 complex on the surface of transformed T cell hybridomas (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008), a complex that is not possible in other systems such as phage or yeast display (Holler, Holman et al. 2000; Li, Moysey et al. 2005; Weber, Donermeyer et al. 2005). An important stipulation for the mammalian TCR display system is the limited size of the library that can

be displayed (Chervin, Aggen et al. 2008). The mammalian TCR system typically displays 10^5 clones or less (Kessels, van Den Boom et al. 2000; Chervin, Aggen et al. 2008), a comparatively small library considering the estimated 10^5-10^7 clones or 10^7-10^{12} clones displayed by yeast (Shusta, Kieke et al. 1999; Holler, Holman et al. 2000) or phage (Li, Moysey et al. 2005; Dunn, Rizkallah et al. 2006), respectively. Here we propose that this caveat can be overcome through systematic evaluation of the CDR3 amino acids responsible for contacting the pMHC in order to generate more focused, smaller libraries. A central step in this approach is to determine those key amino acids in the CDR3α and β chains that contribute to TCR-pMHC binding. We found that an alanine mutation at any position in CDR3β chain is detrimental to binding and cell signaling, highlighting the critical importance of an intact beta chain for peptide recognition and providing an opportunity to increase T cell potency through mutations in the alpha chain. None of the alanine mutations in our study increased TCR affinity as was observed in a previous alanine scanning study (Manning, Schlueter et al. 1998; Robbins, Li et al. 2008; Narimatsu, Yoshioka et al. 2011), however, it is worth mentioning that most of the alanine mutagenesis of CDR3 residues in these studies showed a diminishing effect of both activity and binding. It could be possible that alanine mutagenesis in the CDR 1 and 2 regions would produce enhanced binding to the pMHC (Manning, Schlueter et al. 1998; Robbins, Li et al. 2008). The cytokine secretion and tetramer binding results obtained from the alanine scanning mutagenesis were used to establish the relative importance of each CDR3 chain; however the contribution of individual, key residues within these chains remained unclear (Fig. 1). Based on these results we decided to generate a seven-codon library representing the full-length CDR3α region. Although this strategy reduced the effective size of the generated library (3.1×10^4 clones out of 7×10^{20} theoretical possible combinations) and is a limitation of this system it still allowed us (Fig. 3 and 5) and others (Chervin, Aggen et al. 2008) to successfully isolate higher potency TCRs from less effective libraries. We expect the reason is probably because the mammalian expression system can dramatically reduce the possible library size by excluding TCRs that are not properly folded to be expressed on the cell surface.

Due to the library size limitations the length of CDR3 length for each individual TCR should be taken in consideration. Recently, we have successfully displayed TCR and selected more potent TCRs from libraries with longer CDR3s (nine codons CDR3 alpha sequence) (Fig. S3). However, to increase the efficiency and to narrow down the required size of the library, a prior alanine scanning step as described in this paper could be included with the purpose of identifying key residues for introduction of mutations. For shorter CDR3 regions we do not anticipate any issues in library size; however the library might be too small to provide enough mutations to screen for increased T-cell activity although recent studies from Kranz and colleagues displaying libraries of the 2C TCR with a CDR3 length of 5 codons where they generate high affinity mutants would argue against this issue (Holler, Holman et al. 2000).

Binding parameters obtained from SPR measurements on soluble, purified proteins confirmed the importance of an intact CDR3β as suggested by cell signaling assays. Such investigations of TCR and pMHC interactions on the soluble protein level are critical for optimal library design, in which selection is based on complex, dynamic interactions occurring on the cell surface.

The mammalian library display system generated a panel of both specific and cross-reactive variant TCRs and facilitated a direct investigation into the biophysical properties of related TCRs recognizing the same pMHC antigen. Additionally, the high level of sequence similarity (identical TCR sequences except for changes in the CDR3α region) affords the opportunity to dissect the contribution of individual amino acid in the TCR/pMHC interface

using experimental data and ultimately can inform future work aimed at increasing or decreasing TCR specificity for a given peptide.

Using this system, we analyzed the relationship between TCR avidity for pMHC on the cell surface and the corresponding T cell functional outcomes for a panel of nine TCRs. Previous studies in the area of relating biophysical binding parameters to functional outcomes are controversial (Krogsgaard, Prado et al. 2003; van der Merwe and Davis 2003; Krogsgaard and Davis 2005; Stone, Chervin et al. 2009; Corse, Gottschalk et al. 2011; Zhong, Malecek et al. 2013): Some studies indicate a positive correlation between TCR affinity and functional outcome, while others report the dissociation rate to be the key determinant for T-cell function (Gascoigne, Zal et al. 2001; Krogsgaard, Prado et al. 2003; Krogsgaard and Davis 2005; Stone, Chervin et al. 2009; Corse, Gottschalk et al. 2011). In our study, the panel of engineered specific TCRs demonstrated a correlation between T-cell activation and both functional avidity and half-life indicating that the TCRs displayed via this method fit the biophysical parameters of previously reported engineered TCRs (Stone, Chervin et al. 2009; Corse, Gottschalk et al. 2011). Discrepancies between previous studies have been reconciled by considering factors that also influence T cell sensitivity such as coreceptor expression (Holler and Kranz 2003; Laugel, van den Berg et al. 2007; Jiang, Huang et al. 2011), TCR oligomerization (Boniface, Rabinowitz et al. 1998; Minguet, Swamy et al. 2007), co-agonist peptides (Krogsgaard, Li et al. 2005), conformational changes (Krogsgaard, Prado et al. 2003), and confinement time (Aleksic, Dushek et al. 2010). Previous studies have compared different affinity complexes obtained by using the same TCR recognizing different pMHC complexes, restricted to different MHCs or by just comparing different TCRs altogether (Kersh, Kersh et al. 1998; Tian, Maile et al. 2007; Chervin, Stone et al. 2009; Persaud, Donermeyer et al. 2010). In the former cases variations of peptide stability in these experiments could lead to deviations from the correlations that might not be due TCR-pMHC binding but rather differences in peptide stability for the MHC binding (Kersh, Kersh et al. 1998; Tian, Maile et al. 2007; Chervin, Stone et al. 2009; Persaud, Donermeyer et al. 2010).

There is ongoing work to generate higher affinity, antigen-specific TCRs directly from human patients, a time-consuming task yielding few TCRs with limited efficiency for adoptive immunotherapies (Rosenberg, Yang et al. 2004; Gattinoni, Powell et al. 2006; Johnson, Heemskerk et al. 2006; Stone, Chervin et al. 2009). We propose that systematic alanine scanning of the CDR3 regions that confer peptide specificity as a precursor to library display is of immense experimental benefit. Further, mammalian cell display is advantageous over both yeast and phage display as it facilitates both TCR affinity measurements and correlations to T-cell activation. Collectively this mammalian TCR display approach facilitates efficient and targeted TCR library generation against a specific antigen and can be applied to any TCR. Even though, the affinities obtained using this technique might not be in the pico-molar range like those obtained from phage or yeast display (Holler, Holman et al. 2000; Li, Moysey et al. 2005; Dunn, Rizkallah et al. 2006); however, our recent work (Zhong, Malecek et al. 2013) has demonstrated that this range might not be advantageous or physiologically relevant. As with monoclonal antibodies, it will take time to optimize the *in vitro* affinity maturation system but the potential benefits of TCR-based therapies (Rosenberg, Yang et al. 2004; Gattinoni, Powell et al. 2006; Johnson, Heemskerk et al. 2006; Robbins, Li et al. 2008; Johnson, Morgan et al. 2009; Stone, Chervin et al. 2009) make the challenges worth the effort.

2. Materials and Methods

2.1. Peptides, antibodies and pMHC tetramer production

gp209-2M peptide (IMDQVPFSV) and Mart 27L (ELAGIGILTV) were synthesized by Biosynthesis Inc. Allophycocyanin (APC) anti-mouse TCRβ constant (Clone H57-597) and phycoerythrin (PE) anti-mouse CD3ε (Clone 145-2C11) were from eBiosciences; PE anti-human TCR Vβ8 (clone JR2) was from Biolegend; For p209-2M/HLA-A2-K^b tetramer production, HLA-A2-K^b heavy chain with a biotinylation sequence at C-terminus (kindly provided from Dr. Cerundolo, John Radcliffe Hospital, UK) and human β2M were purified as inclusion bodies from *E.coli*. The complexes were refolded in vitro with gp209-2M peptide, as previously described (Krogsgaard, Prado et al. 2003; Juang, Ebert et al. 2010). The folded protein was concentrated and biotinylated with BirA Biotin Protein Ligase (Avidity) accordingly to manufacturer's instructions. Protein purification and tetramer production were performed as previously described (Laugel, van den Berg et al. 2007) by adding PE-labeled streptavidin (BD Pharmingen) to the biotinylated monomeric complexes in a 1 to 4 molar ratio.

2.2. Cell Lines and Cell Cultures

58 α-/β-hybridoma cell line (Letourneur and Malissen, 1989, kindly provided from Dr. Kranz, University of Illinois) was transfected with retroviral human CD8 α and β chains linked by a self-cleavable 2A peptide. T2 cells (a gift from Dr. L. Sherman, Scripps Research Institute) expressed a chimeric HLA-A2-H2-Kb (allows for mouse CD8 binding of mouse hybridoma cells) on T2 cells. Both these cell lines were cultured in RPMI media supplemented with 10% FBS, Glutamax™-I, Sodium pyruvate, Non-Essential Amino Acids and Penicillin-Streptomycin. Fetal bovine serum (FBS) was from Thermo Scientific HyClone and all other cell culture reagents were from Invitrogen. Human recombinant IL-2 (Aldesleukin Proleukin) was from Novartis.

2.3. DNA constructs and library construction

The R6C12 TCR construct (Dudley, Wunderlich et al. 2001; Morgan, Dudley et al. 2003) was cloned and expressed on hybridoma cells (Zhong, Malecek et al. 2010; Zhong, Malecek et al. 2013). Human/mouse chimeric TCR constructs were generated by PCR and a self-cleavable 2A peptide was introduced for optimal TCR gene expression as previously described (Szymczak and Vignali 2005; Cohen, Zhao et al. 2006) and sub-cloned into retroviral vector pMXs (Kitamura, Koshino et al. 2003) The seven amino acid degenerate codon library in CDR3α of the R6C12 TCR (LIQGAQK, positions 90-96) was created by amplifying the WT construct (Zhong, Malecek et al. 2013) with the degenerate primer (reverse 3′ to 5′ AGTCAGCCTGGTTCCTTGGCCAAATACCAGSNNSNNSNNSNNSNNSNNSNNTGAG GCAGCACAGATGTAGACGGCAGAGTCTCT).

2.4. Transduction of T cells, flow cytometry analysis and cell sorting

Retroviral transduction of hybridoma cells was performed as described (Zhong, Malecek et al. 2010; Kerkar, Sanchez-Perez et al. 2011). The transduced hybridoma cells were dual stained with PE HLA-A2-gp100-2M tetramer for 1 hour at 4°C and then for 30 min at 4°C with APC anti-TCRβ (Clone H57-597) constant antibodies. Cells were washed twice and then sorted at room temperature using MoFlo Cell Sorter (Beckman Coulter). A total number of 10^5 cells were sorted in the first round of selection and then expanded for five days. For single cell sorting, 10^6 cells were dual stained as above and single-cell sorted into each well of a 96-well plate. After clonal expansion cells were further characterized in binding and T cell functional assays.

2.5. Cytokine ELISA

1×10^5 APCs (T2/A2-K^b) loaded with different concentrations of gp209-2M peptide and were co-incubated with 1×10^5 T hybridoma cells for 16 hr at 37 °C, 5% CO^2. IL-2 production was quantified by a standard sandwich ELISA. All antibodies and cytokine standards were from Ebiosciences. Streptavidin-HRP was from BD Biosciences and tetramethylbenzidine (TMB) ELISA substrate was from Sigma.

2.6. Protein Production and Surface Plasmon Resonance (SPR) Measurements

For soluble TCR production, variable domains of TCR α and β chain genes were fused to the human constant of LC13 (Newell, Ely et al. 2011) (a generous gift from Mark M. Davis) and sub-cloned into pET30a vector (Novagen). A BirA biotinlyation sequence was engineered at the C-terminus of the TCRα chain for immobilization purposes. The TCR α and β chains were purified individually from inclusion bodies expressed in BL-21 CodonPlus cells (Stratagene) and solubilized in 8 M Urea. Soluble TCR was refolded *in vitro* by rapid dilution into 100 mM Tris pH 8 buffer, 400 mM L-Arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione and 2 mM EDTA (Newell, Ely et al. 2011). The refolded TCR was dialyzed four times in 10 mM Tris-HCl (pH8) followed by purification on a Hitrap DEAE column (GE Lifesciences) and biotinylated with BirA Biotin Protein Ligase (Avidity) accordingly to manufacturer's instructions. The protein was further purified by a HiLoad S200 gel filtration columns (GE Lifesciences).

For SPR measurements, biotinylated TCR or pMHC molecules were immobilized on a SA sensor chip (GE Lifesciences) at 300 to 500 RU in a Biacore X100 machine (GE Lifesciences) and soluble pMHC or TCR molecules were injected at various concentrations at 25 °C. The collected data were analyzed using Biacore X100 evaluation software as previously described (Krogsgaard, Prado et al. 2003).

2.7. Tetramer decay and MFI determination

PE-labeled HLA-A2 tetramers bearing gp209-2M peptide were used for tetramer decay determination. All stains and washes were prepared using media consisting of 1x Phosphate Buffered Saline (PBS), 5% FBS, and 0.1% sodium azide. For staining, 2×10^6 cells were washed and stained with tetramer for 2 hrs at 4°C. After incubation, cells were washed 3 times and stained with anti-cTCRβ-APC mAb for 30 min. After 2 washes cells were re-suspended in 100 μL of media and 8 μL samples were taken before or after 11 μg of blocking antibody addition (HLA-A2 clone BB7.2, Genetech Inc). Cells were immediately treated in 4% paraformaldehyde (PFA). Aliquots were taken at various time points and treated the same way. The mean fluorescence intensity (MFI) was determined based on gating of TCR positive cells. For K_D determination the MFI was normalized by subtracting the initial time point and plotted on a logarithmic scale. The K_D was derived from the negative reciprocal of the slope of the line fit to Scatchard plots of bound tetramer/free tetramer versus bound tetramer (Savage, Boniface et al. 1999).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the NYU flow cytometry core for technical assistance. We thank Drs. Evan Newell and Mark M. Davis, Stanford University for protocols and advice for soluble TCR expression and purification. We thank GE healthcare for the use of the Biacore X100. We also thank Drs. Arianne Perez, Wenjuan Wang, Hila Novak and Ngozi Monu for critical discussions of the manuscript. This work was supported by the National Institute of Health grant 1U01CA137070 (to M.K.), American Cancer Society Research Scholar grant

RSG-09-070-01-LIB (to M.K), Cancer Research Investigator grant (to M.K.), Pew Scholar in Biomedical Sciences supported by the Pew Trust (to M.K.), NIGMS grant 5R01GM085586-04 (to M.K.) American Heart Association fellowship (to K.M.) and NIH Molecular Biophysics training grant (to K.M.)

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Highlights

- Alanine scanning of TCR CDR3 regions for optimal design of TCR display library
- Conduct analysis and selected T-cell clones based on functional T-cell potency
- Generate T cell clones with increased binding affinity and increased functionality
- Use mammalian display system to generate specific and cross-reactive T cell clones
- TCR binding avidity and off-rate determine functionality for specific T cell clones

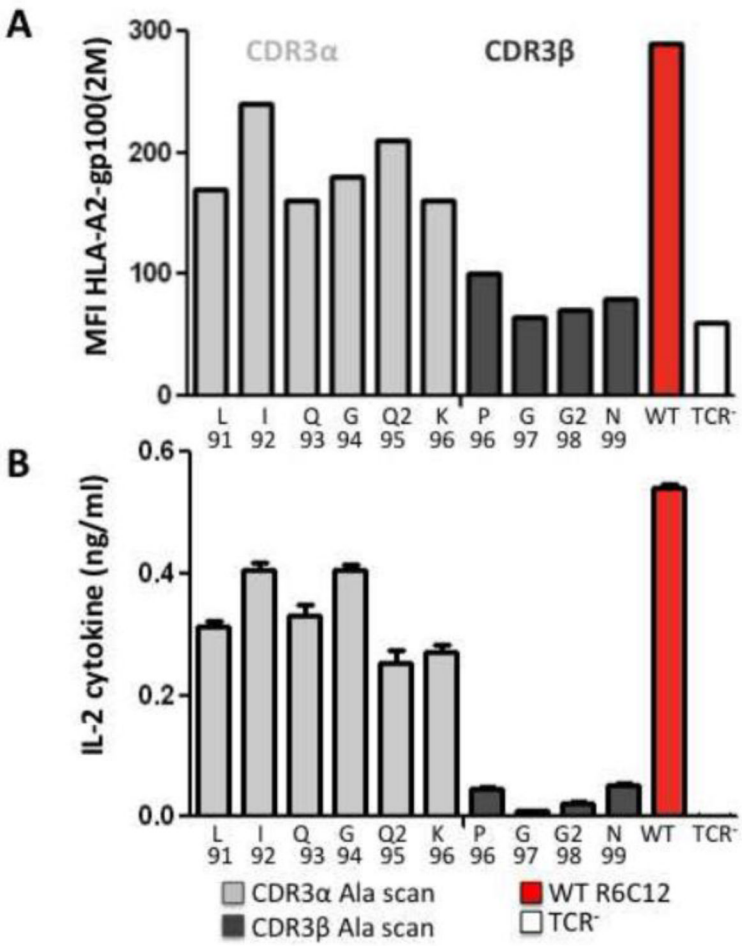

Figure 1.

Alanine scanning of R6C12 TCR indicates the CDR3β region to be more important for TCR-pMHC binding

We used an alanine scanning screening method (CDR3α - light gray and CDR3β - dark gray) to map key amino acid residues in the TCR-pMHC binding interface. A. Mean fluorescence intensities (MFI) of PE HLA-A2-p209-2M tetramer binding for the indicated clones was quantified via flow cytometry. TCRs were expressed on hCD8 58−/− hybridoma cells. B. IL-2 cytokine production for the indicated clones after 20h incubation with T2 cells expressing A2Kb loaded with 1μM gp209-2M peptide. IL-2 cytokine release into the supernatant was quantified via ELISA. Experiments repeated three times, and a representative experiment is shown.

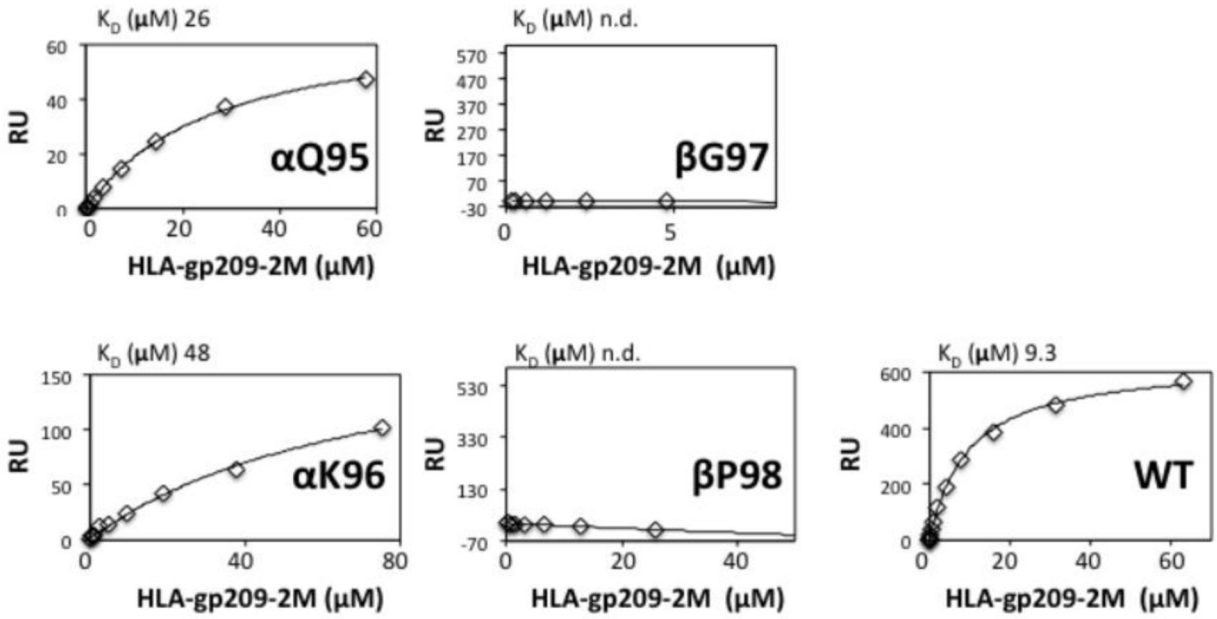

Figure 2.

Kinetic features of wild-type and alanine mutated TCRs binding pMHC show similar trends as cell surface determined affinity binding

Steady-state surface plasmon resonance experiments determines the affinities of alanine substituted TCRs and wild-type TCR for gp209-2M/HLA-A2*0201. 500 RU of the indicated soluble biotin-labeled TCRs was immobilized to a streptavidin sensor chip. Each data point represents the value of recorded experimental RU (response units) when injecting increasing amounts of gp209-2M/HLA-A2*0201 and the corresponding buffer baseline surface value of the reference flow cell was subtracted. n.d. = not determined.

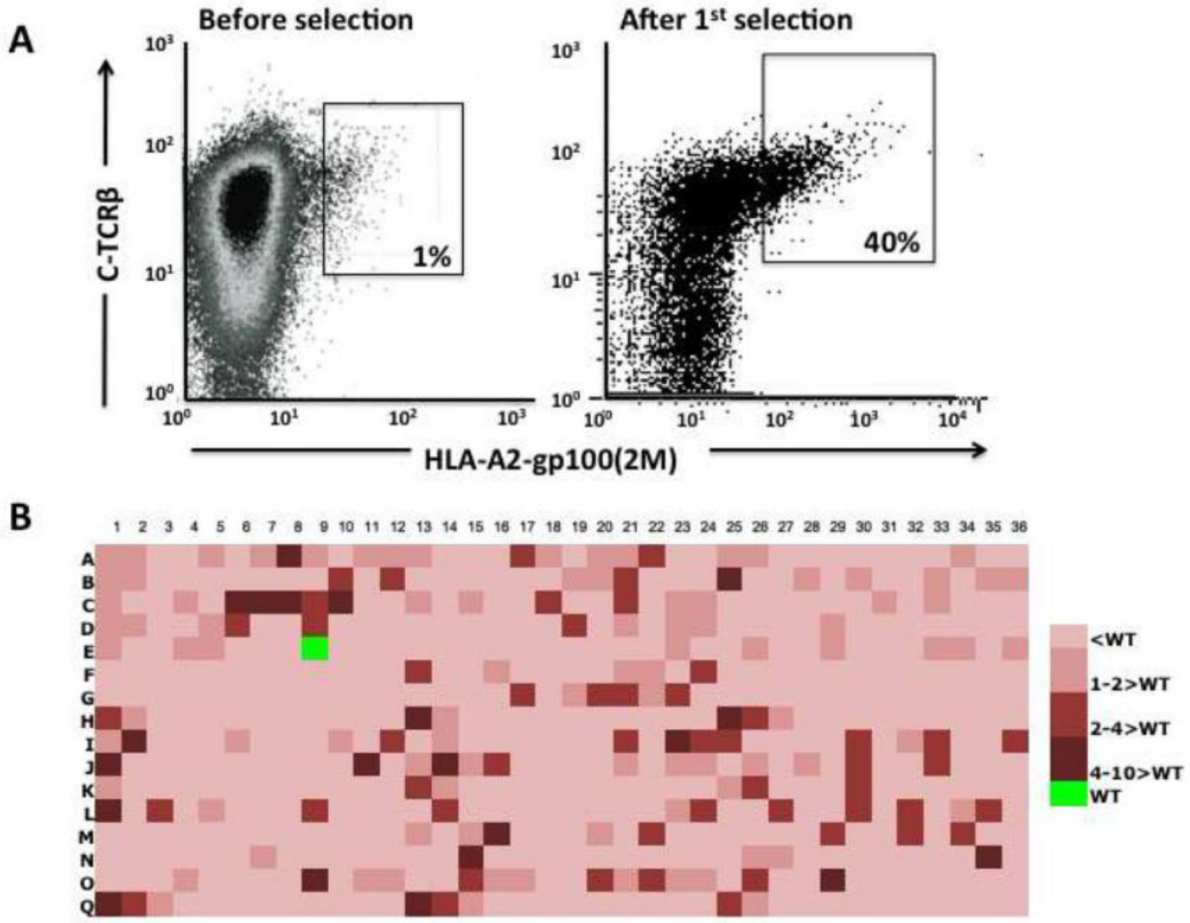

Figure 3.

Selection of T cell clones with enhanced T-cell activity from a R6C12 CDR3α library

A. Analysis of T cell hybridoma transduced with the R6C12-CDR3α library. hCD8+, 58−/− TCR-negative T cell hybridoma were transduced with retrovirus generated from the R6C12-CDR3α library and populations were dual-stained with anti-cTCR APC mAb (Clone H57-597) and HLA-A2-gp209-2M tetramer using a MoFlo sorter. Cells were sorted once. B. Single cell sorted selected T cell hybridomas expressing mutated TCRs were incubated with 10uM gp209-2M -loaded T2 A2Kb cells for 24 hr and their level of IL-2 cytokine production was evaluated. A subset of clones were compared to the WT (green) and placed in color coded categories characterized by making less cytokine than the WT, 1-2, 2-4, or 4-10 times more cytokine than the WT.

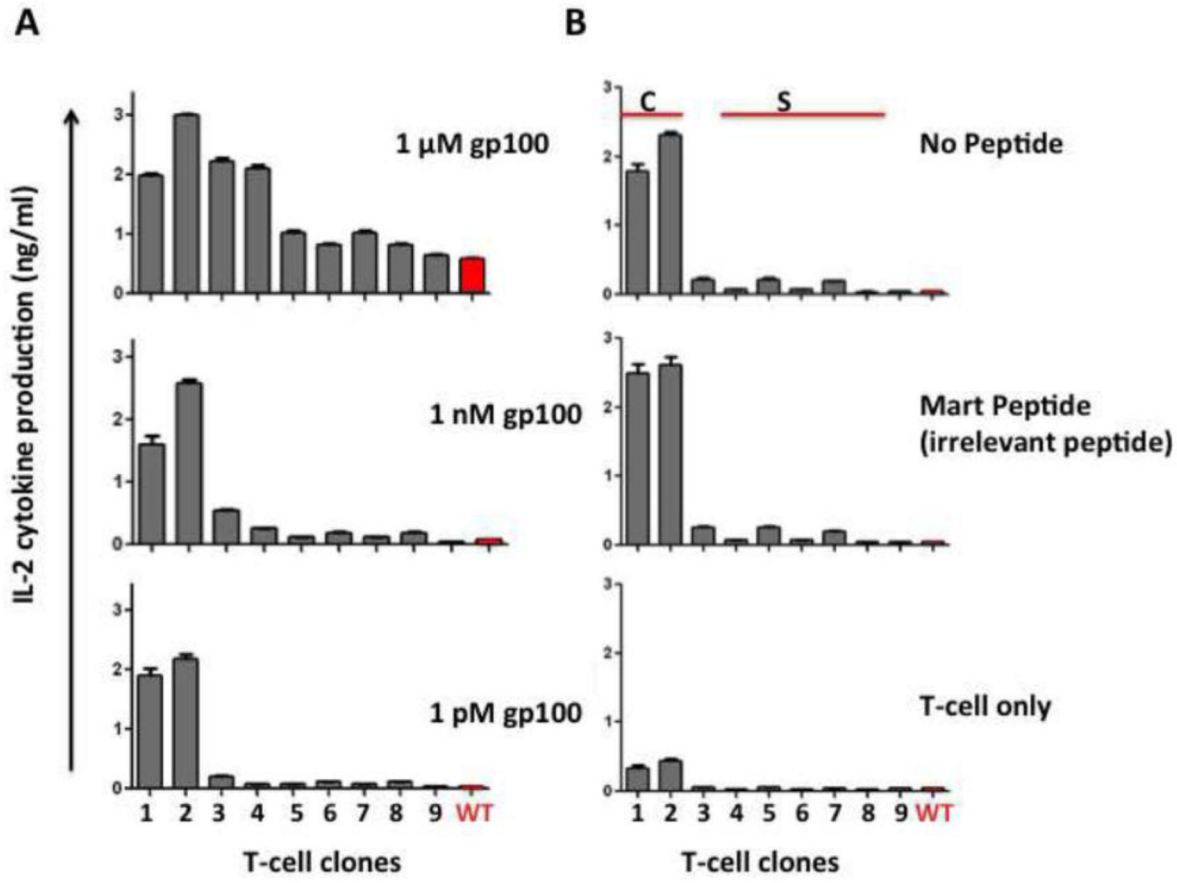

Figure 4.

Identification of specific and cross-reactive TCR clones

T hybridoma cell populations of clonally expanded CDR3α mutants were incubated for 24h with T2 A2Kb cells and A. varying indicated concentrations of gp209-2M, B. no peptide (top); irrelevant Mart 1 (27L) (10 μM) peptide (middle) or no peptide and no T2 A2Kb cells (bottom). Secreted IL-2 was quantified using IL-2 ELISA. Specific clones (S) and cross-reactive clones (C) are defined as those that do not and do respectively, secrete IL-2 in the absence of relevant peptide, respectively. Error bars represent standard deviation of triplicate repeats. Experiments were repeated twice.

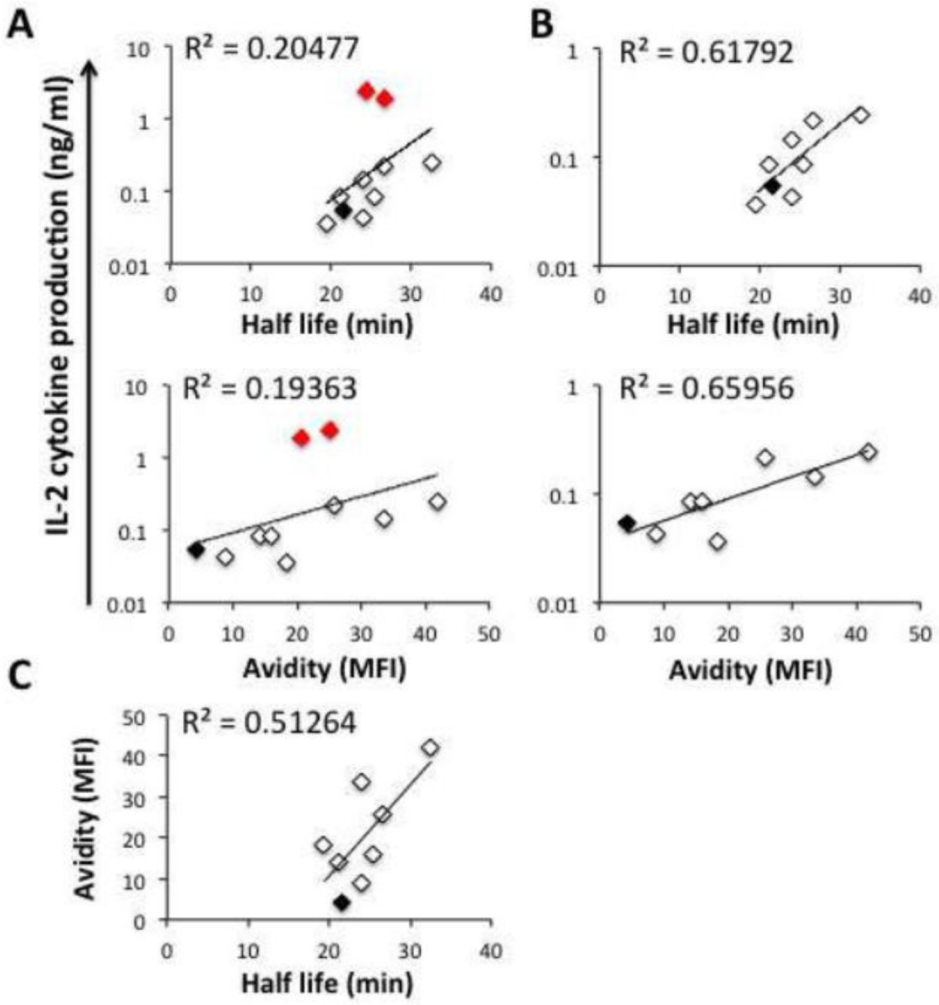

Figure 5.

Cytokine production correlates with half-life and avidity for specific clones

Half-life and binding avidity was determined via tetramer dissociation and binding assays to determine half life ($t_{1/2}$) and the avidity of binding, respectively, for the panel of representative specific (gray diamonds), cross-reactive (red diamonds), and WT TCR (black diamonds) expressed on hybridoma cells. IL-2 cytokine production was correlated to the experimentally determined dissociation half life ($t_{1/2}$) and tetramer avidity. Data was fitted using linear regression to derive R-squared values when including (A) or excluding (B) cross-reactive clones. The half-life value was calculated from the equation: $t_{1/2} = \ln 2/\text{slope}$. Tetramer avidity is determined as the mean fluorescence intensity (MFI) of gp209-2M-HLA-A2 tetramer staining. (C) Correlation between TCR avidity and half-life for CDR3α engineered and wild-type TCRs.