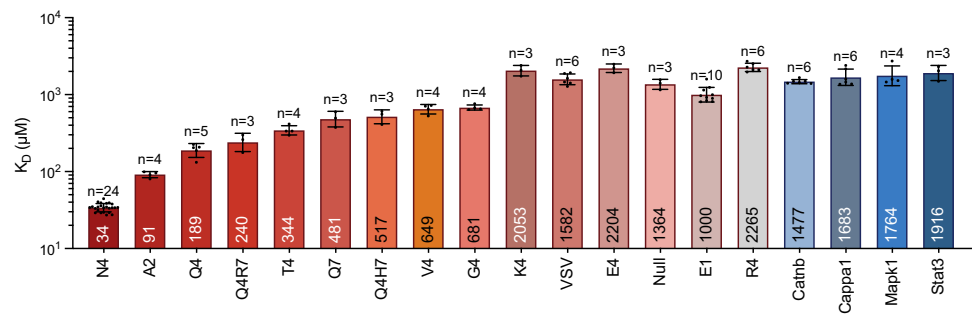
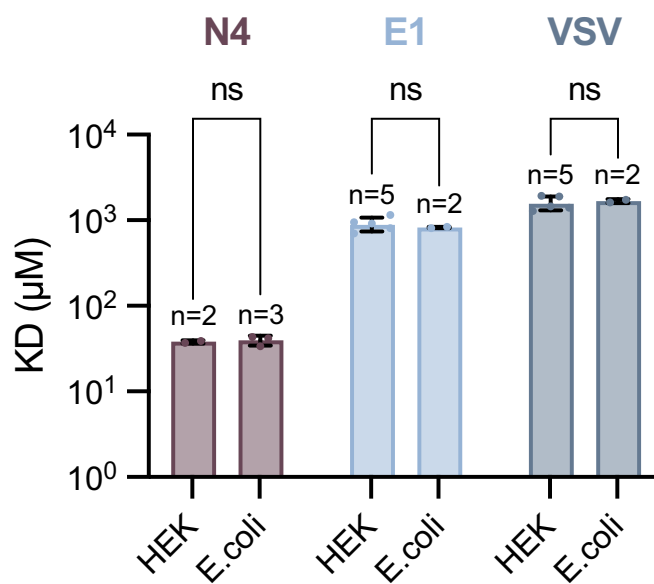


Expanded View Figures

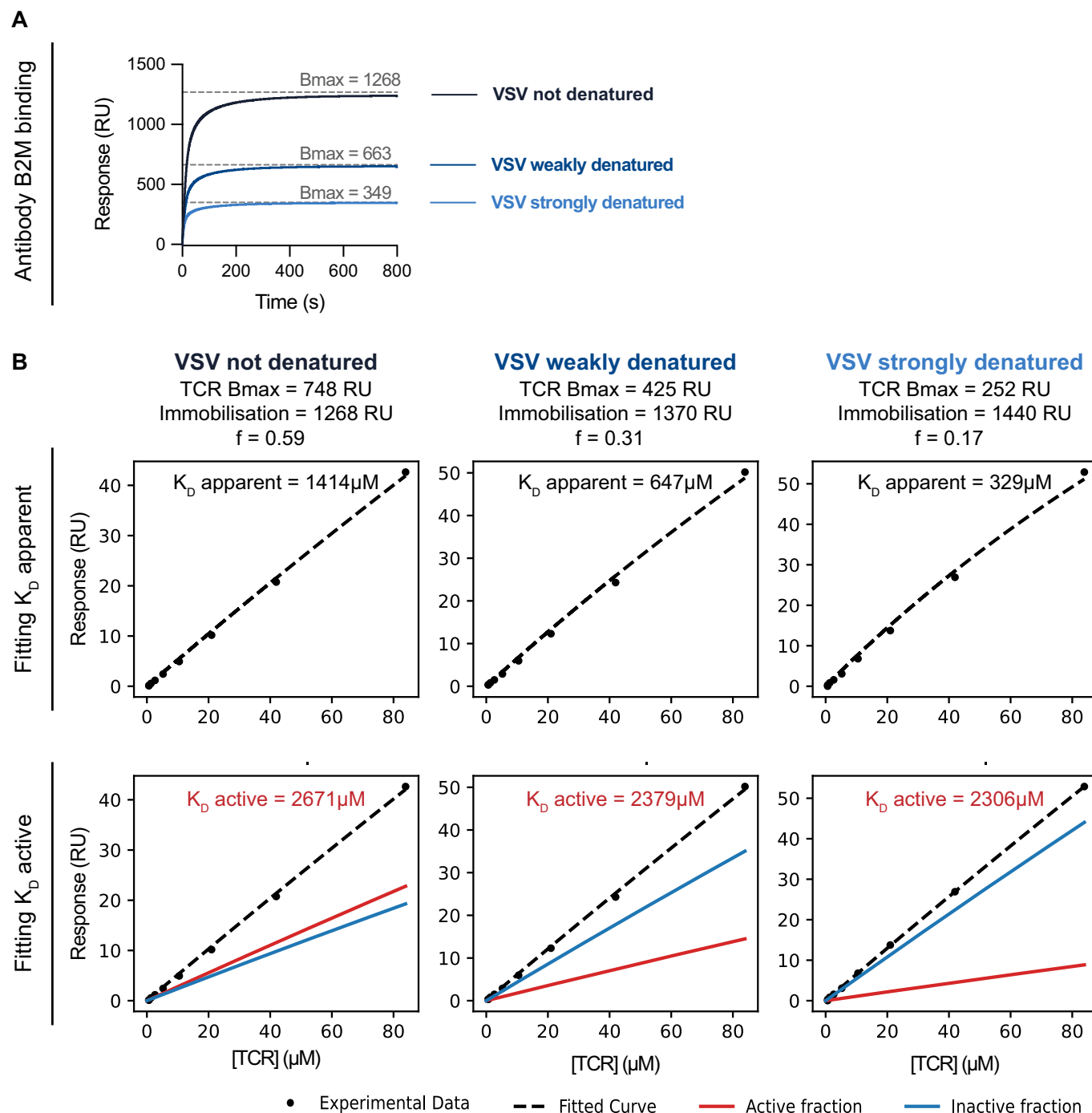


**Figure EV1. Apparent  $K_D$  values for OT-I specific peptides.**  
The  $K_D$  values were obtained by fitting the steady-state binding using a 1:1 model with  $B_{max}$  constrained to value obtained from the B2M or Y3 antibody binding using the standard curve (see Fig. 1 and "Methods"). Geometric mean is displayed within the bars, mean values, error and  $N$  are listed in Table 1.



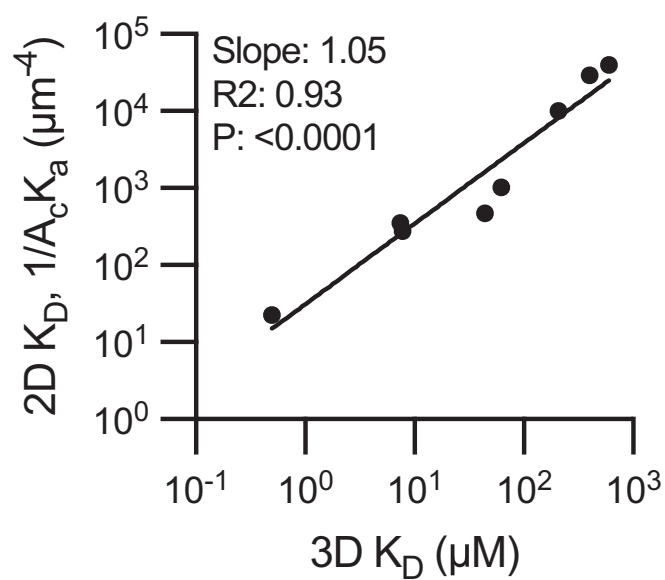
**Figure EV2. The OT-I TCR displays similar affinity to pMHC produced in *E. coli* and HEK293T cells.**

The *E. coli*-produced pMHC were produced in-house, whereas the HEK293T-produced pMHC were supplied by the NIH tetramer facility. Bars show mean values  $\pm$  SD. Statistical significance was determined using a two-way ANOVA test.



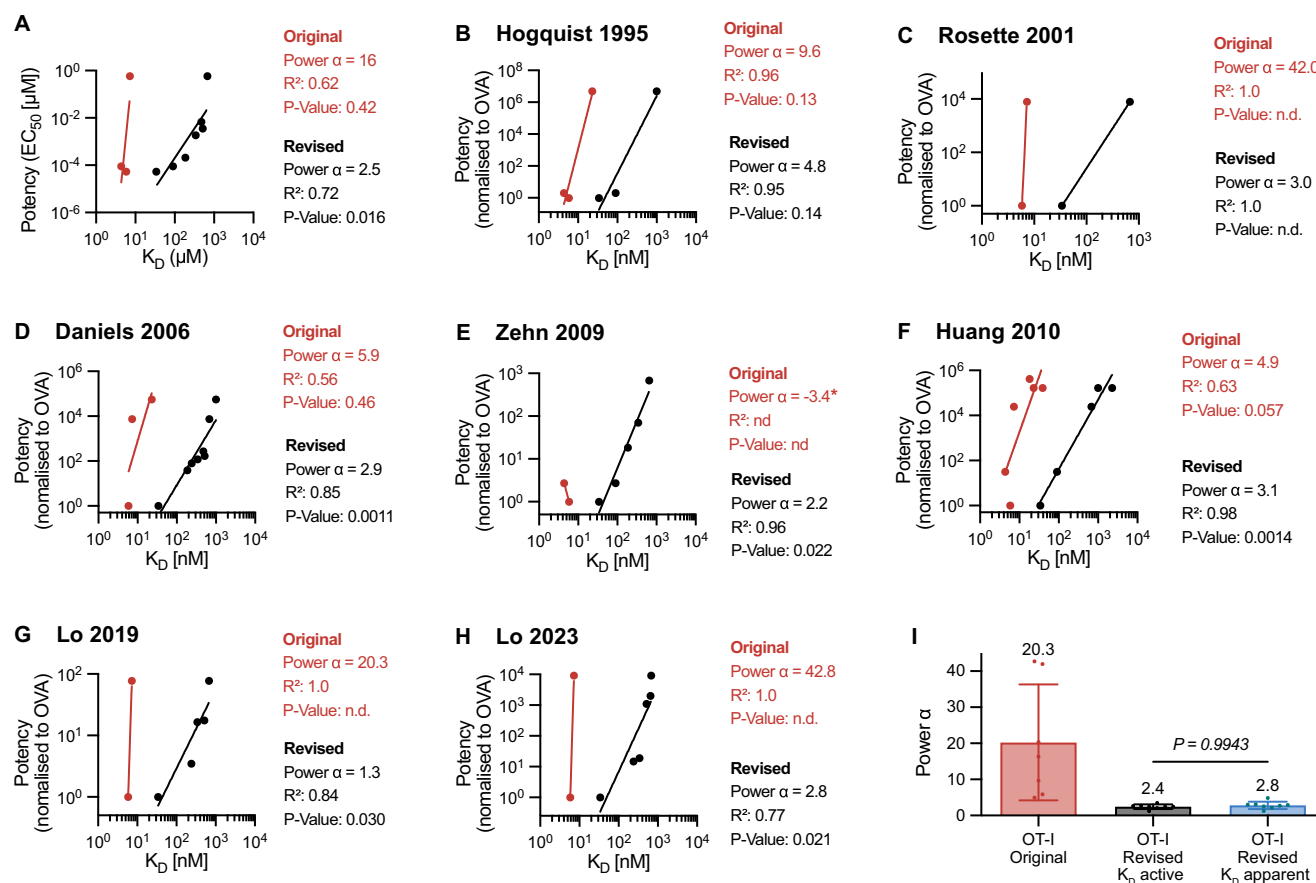
**Figure EV3. Calculating the active  $K_D$  provides similar results for OT-I binding to surfaces with large differences in the fraction of active pMHC.**

The VSV pMHC was immobilised at similar levels on 3 flow cells in SPR before inducing denaturing by a short (weakly denatured) or long (strongly denatured) injection of glycine solution (pH 1.7). (A) B2M antibody binding curves to the three VSV pMHC surfaces. The TCR  $B_{\max}$  was estimated using the standard curve in Fig. 1B. (B) Steady-state TCR binding response to the 3 surfaces (columns). To determine the apparent  $K_D$ , the data was fit with a 1:1 binding model with constrained  $B_{\max}$  to determine  $K_D$  apparent (top row). To determine the active  $K_D$ , the workflow in Fig. 2G-I was used, where the fraction of active pMHC was calculated from the ratio of TCR  $B_{\max}$  to pMHC immobilisation, and  $K_D$  inactive was fixed to 2180  $\mu\text{M}$ . While the apparent  $K_D$  displayed large differences, the active  $K_D$  produced consistent results across all VSV pMHC surfaces.



**Figure EV4. Quantitative comparison of 2D and 3D affinities for the 1E6 TCR.**

The log-transformed data was fitted with a linear regression. An  $F$  test was used to determine a  $P$  value for the null hypothesis that the slope is equal to zero. All data were taken from Cole et al (Cole et al, 2016).



**Figure EV5. Discriminatory power of OT-I TCR calculated with  $K_D$  apparent shows imperfect discrimination.**

Plots show peptide potency over original or apparent  $K_D$  values. (A) Potency data from Fig. 5A. Data is mean of  $N = 2$  independent experiments. (B–H) Published potency data from the indicated study over original or apparent  $K_D$  values. A power law (potency  $\sim (K_D)^\alpha$ ) is fit to the data to estimate the discriminatory power  $\alpha$ . A Pearson correlation is used to determine  $R^2$  and  $P$  values on log-transformed values. (I) The discriminatory power from (A–H) in comparison with discriminatory power calculated with active  $K_D$  values. The  $P$  value is determined using a t-test. Number of data points included in each category:  $N = 7$ ,  $N = 8$ ,  $N = 5$ ,  $N = 15$  for OT-I Original, OT-I revised, other mouse TCRs and other human TCRs, respectively. Bars show mean values (also indicated above the bar)  $\pm$  SD.