

Coreceptor affinity for MHC defines peptide specificity requirements for TCR interaction with coagonist peptide–MHC

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Recent work has demonstrated that nonstimulatory endogenous peptides can enhance T cell recognition of antigen, but MHCI- and MHCII-restricted systems have generated very different results. MHCII-restricted TCRs need to interact with the nonstimulatory peptide–MHC (pMHC), showing peptide specificity for activation enhancers or coagonists. In contrast, the MHCI-restricted cells studied to date show no such peptide specificity for coagonists, suggesting that CD8 binding to noncognate MHCI is more important. Here we show how this dichotomy can be resolved by varying CD8 and TCR binding to agonist and coagonists coupled with computer simulations, and we identify two distinct mechanisms by which CD8 influences the peptide specificity of coagonism. Mechanism 1 identifies the requirement of CD8 binding to noncognate ligand and suggests a direct relationship between the magnitude of coagonism and CD8 affinity for coagonist pMHCI. Mechanism 2 describes how the affinity of CD8 for agonist pMHCI changes the requirement for specific coagonist peptides. MHCs that bind CD8 strongly were tolerant of all or most peptides as coagonists, but weaker CD8-binding MHCs required stronger TCR binding to coagonist, limiting the potential coagonist peptides. These findings in MHCI systems also explain peptide-specific coagonism in MHCII-restricted cells, as CD4–MHCII interaction is generally weaker than CD8–MHCI.

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Abbreviations used: CHO, Chinese hamster ovary; pMHC, peptide–MHC.

The vast majority of the peptides presented by MHC molecules are derived from self-proteins and do not activate mature T cells. Antigen recognition and T cell activation must thus be

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tuned to allow for recognition of the small minority of disease-associated peptide–MHC (pMHC) “needles in the haystack” of nonstimulatory endogenous pMHC (Davis et al., 2007; Gascoigne, 2008; Gascoigne et al., 2010). Several experiments have shown that T cell activation by small amounts of antigen is enhanced by the presence of endogenous peptides (Irvine et al., 2002; Yachi et al., 2005). Although this activation enhancement or coagonist phenomenon has been reported for both MHC class I

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(MHCI)-restricted T cells and thymocytes (Yachi et al., 2005, 2007; Anikeeva et al., 2006; Juang et al., 2010) and for MHCII-restricted T cells (Irvine et al., 2002; Li et al., 2004; Krogsgaard et al., 2005), the relative importance of TCR recognition of the endogenous pMHC appears to be very different for CD4 and CD8 T cells (Davis et al., 2007; Gascoigne, 2008; Gascoigne et al., 2010).

The number of potential coagonist peptides for a given CD4 T cell are very limited (Krogsgaard et al., 2005; Ebert et al., 2009; Lo et al., 2009), whereas coagonism for CD8 T cells or thymocytes occurs with a wide range of different non-stimulatory peptides (Yachi et al., 2005, 2007; Juang et al., 2010). This evidence thus suggests that MHCII-restricted TCRs discriminate between endogenous peptides, whereas MHCI-restricted TCRs do not. However, recent data indicate that nonstimulatory pMHCI ligands show a very weak but possibly biologically significant interaction with TCR (Juang et al., 2010). This suggested that TCRs might play a role in coagonism in MHCI-restricted cells but that its specificity is only evident for very weakly stimulatory TCR ligands such as those involved in positive selection.

The CD8 coreceptor's interaction with nonstimulatory MHCI has been suggested to be important for coagonism in MHCI-restricted cells (Yachi et al., 2005; Gascoigne, 2008; Gascoigne et al., 2010). Nonstimulatory pMHC alone can recruit CD8 to the T cell-APC interface (Yachi et al., 2005; Rybakina et al., 2011). Also, coagonist pMHCs became antagonists in CD8-negative cells (Stone et al., 2011). These results, along with the lack of peptide specificity for coagonists, suggest that non-cognate CD8 coreceptor binding to nonstimulatory pMHC is the dominant mechanism of activation enhancement for MHCI-restricted T cells. In addition, CD8 affinity for the MHC presenting the antigenic peptide (agonist) plays a direct role in signaling through the TCR, where increasing the affinity of CD8 can increase ligand potency and even bypass peptide specificity requirements altogether (Laugel et al., 2007; Wooldridge et al., 2007, 2010). Because there is a range of affinities for CD8 binding to different MHCI molecules (Cole et al., 2012), the relative requirements for CD8, or for TCR interaction with the nonstimulatory ligand, might be expected to vary with the strength of CD8-MHC binding. Interestingly, the two mouse MHCI-restricted TCR models that have been analyzed in coagonism experiments (OT-I [Yachi et al., 2005, 2007; Juang et al., 2010] and 2C [Stone et al., 2011]) recognize H-2K^b or L^d, which show relatively high-affinity CD8 binding (Cole et al., 2012).

A stochastic, computational model has been used to investigate the role of coreceptors in TCR triggering, and results suggest that CD8 plays a dual role of stabilizing the TCR-pMHC interaction and of delivering the CD8-associated kinase Lck to the TCR to initiate signaling, with the latter effect being the more important (Artyomov et al., 2010). This model explicitly combined two key features, membrane-protein mobility and protein-protein interactions (Lis et al., 2009), which allowed incorporation of many biophysical measurements for

MHC, TCR, and coreceptor interactions. Here, an extension of this model allows us to describe coagonism enabled by self-peptides, taking into account the distinct activation states of Lck (Nika et al., 2010; Stirnweiss et al., 2013).

Because of the glaring discrepancies in the requirements for TCR discrimination between coagonist peptides in MHCI- and MHCII-restricted systems, there is a need for a unifying concept to explain activation enhancement for both T cell lineages. In this paper, we used H-2K^b and H-2D^b single chain (sc)-pMHCs (Yu et al., 2002; Choudhuri et al., 2005; Palmowski et al., 2009), which allowed us to dissect the distinct contributions of CD8 affinity and of TCR affinity for both antigenic and nonstimulatory pMHCs. Using H-2K^b- and H-2D^b-restricted TCRs and stochastic computer simulations of the kinetics of T cell activation, we describe two distinct mechanisms by which CD8 affinity for pMHC can influence the requirements for coagonists. Mechanism 1 describes CD8 binding to nonstimulatory pMHC as an absolute requirement for coagonism and shows that higher-affinity CD8-pMHC interactions can mitigate peptide specificity requirements for coagonists and increase the magnitude of enhancement. Mechanism 2 describes how the affinity of CD8 for agonist pMHC influences the requirements for TCR interaction with coagonist pMHC. A relatively simple kinetic model of T cell activation is sufficient to account for all coagonist phenomena, thus unifying disparate observations from CD4 and CD8 T cells.

RESULTS

Development of inducible, antigenic, sc-MHC Chinese hamster ovary (CHO) cell expression systems

sc-pMHCI molecules are produced by recombinant constructs in which the MHC-binding peptide is linked to the N terminus of β_2 -microglobulin, which in turn is linked to the N terminus of the MHCI heavy chain (Yu et al., 2002; Choudhuri et al., 2005; Palmowski et al., 2009). When expressed on the cell surface, these sc-MHCI constructs are very stable and can act as potent agonists for T cells. sc-K^bOVA (OVA peptide 257–264, SIINFEKL, on H-2K^b, recognized by OT-I TCR; Hogquist et al., 1994) and sc-D^bNP68 (influenza A/NT/60/68 nucleoprotein 366–374, ASNENMDAM, on H-2D^b, recognized by F5 TCR; Townsend et al., 1986; Mamalaki et al., 1992) in doxycycline-inducible vectors were transfected into CHO Trex cells (expressing tetracycline repressor). Doxycycline strongly up-regulated cell surface expression of sc-MHCI, allowing the CHO cells to stimulate antigen-specific T cell activation, as measured by CD69 up-regulation or TCR endocytosis (Fig. 1). In the repressed state (absence of doxycycline), “leaky” expression of sc-K^bOVA or sc-D^bNP68 presented enough agonist to the CD8 OT-I or F5 T cells to induce some CD69 up-regulation but not enough to induce significant TCR down-regulation (Fig. 1, B and D). Expression of sc-K^bOVA in the repressed state was virtually undetectable by flow cytometry with a K^bOVA-specific antibody (Fig. 1 A; and not depicted; Porgador et al., 1997),

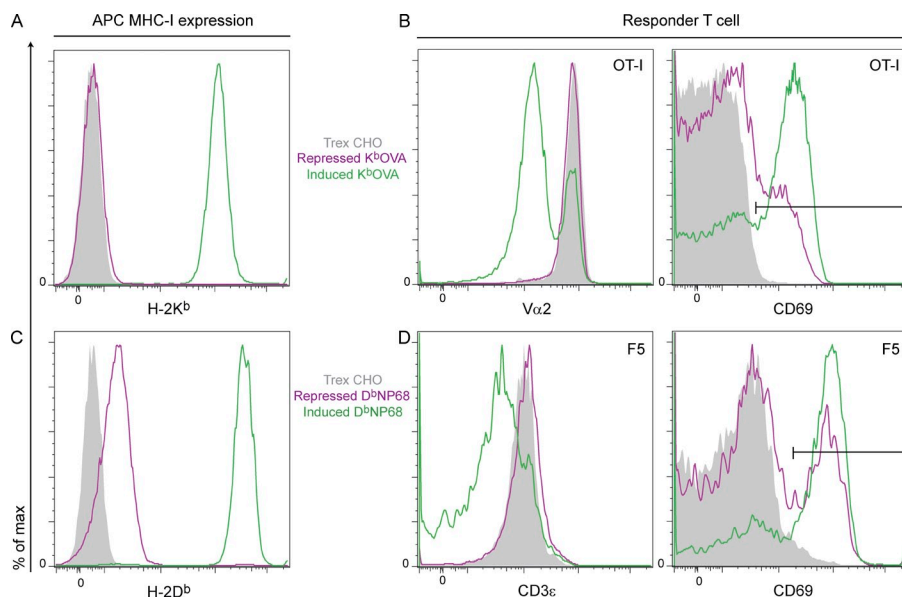


Figure 1. Expression of inducible sc-MHCI agonist molecules K^bOVA and D^bNP68 in CHO cells and activation of corresponding OT-I and F5 CD8 T cells. (A) Trex CHO cells expressing dox-inducible sc-MHC were stained with anti-K^b and analyzed by flow cytometry. Gray shading: untransfected Trex CHO cells. Purple: repressed (no dox) sc-K^bOVA. Green: induced (+dox) sc-K^bOVA. (B) OT-I T cells were incubated with the indicated CHO cell APCs for 3 h, and TCR endocytosis (anti-Vα2; left) and CD69 expression (right) were assessed by flow cytometry. The horizontal black line indicates the CD69^{hi} gate. The color coding is the same as in A. (C and D) As in A and B except CHO cells expressing dox-inducible sc-D^bNP68 were stained with anti-D^b or exposed to F5 responder T cells. TCR staining was by anti-CD3ε. Data are representative of three independent experiments.

whereas repressed sc-D^bNP68 was detectable above background (Fig. 1 C).

Both H-2K^b and H-2D^b nonstimulatory pMHCs can be coagonists

CHO cells expressing inducible sc-K^bOVA or sc-D^bNP68 were super-transfected with nonstimulatory sc-MHCI constructs, enabling coexpression of agonist and nonstimulatory pMHC on the same cell (Fig. 2). This allowed us to probe coagonism by nonstimulatory peptides presented on H-2K^b and H-2D^b, with both H-2K^b- and H-2D^b-restricted TCRs. K^bVSV (RGYVYQGL) and D^bUTY (WMHHNMDLI) are nonstimulatory pMHCs for both OT-I and F5 T cells. We found that sc-K^bVSV provided strong coagonism for both OT-I and F5 T cells, measured as CD69 up-regulation or IL2 secretion (Fig. 2, B–D and F–H, respectively), or by phosphorylation of the NF-κB precursor p-p105 (not depicted). sc-D^bUTY also provided activation enhancement for both TCR transgenic T cells, but this was much weaker than the sc-K^bVSV effect and did not show statistically significant coagonism of IL2 secretion. Nonstimulatory sc-D^bNP68 also gave weak coagonism for OT-I T cells (not depicted). Analogous activation results were obtained with preselection OT-I thymocytes stimulated with K^bOVA-expressing CHO cells (not depicted). These data demonstrate that both H-2K^b and H-2D^b nonstimulatory pMHC molecules were capable of being coagonists for TCRs restricted by the same or another MHC, but H-2K^b provided much stronger coagonism than H-2D^b.

CD8 binding to nonstimulatory pMHC is directly related to the magnitude of coagonism

The lack of peptide specificity in coagonism in MHC-I-restricted T cells could be the result of noncognate CD8 interaction with nonstimulatory MHC-I molecules (Yachi et al., 2005;

Anikeeva et al., 2006; Gascoigne, 2008). To test this hypothesis, we generated D227K–E229K mutations, which are known to abrogate CD8 binding (Connolly et al., 1988, 1990; Potter et al., 1989), in the sc-K^bVSV and sc-D^bUTY α3 domains. When super-transfected into inducible K^bOVA-expressing cells (Fig. 3 A), we found that the sc-K^bVSV CD8-binding mutant (sc-K^bVSV-CD8m) did not provide coagonism to OT-I T cells (Fig. 3 B) or preselection thymocytes (not depicted). Similarly, sc-D^bUTY-CD8m expressed with repressed sc-D^bNP68 did not provide coagonism for F5 cells (Fig. 3, C and D). Unexpectedly, the sc-D^bUTY-CD8m had a mild and statistically significant inhibitory effect on F5 activation. These results revealed that the intact CD8-binding site is absolutely required for coagonism in both OT-I and F5 systems.

We also tested the ability of known H-2K^b-binding peptides (Santori et al., 2002; Yachi et al., 2005) to act as coagonists for F5 T cells. We used RMA-S cells as APCs, as these cells express very few MHC-I molecules unless exogenous peptide is provided (Ljunggren et al., 1990; Yachi et al., 2005, 2007). When H-2K^b-binding peptides Slc2a3 (VNTIFTVV), Nmt1 (AAYSFYNV), OVA, Stat3 (ATLVFHNL), and VSV were loaded on RMA-S cells pretreated with trace NP68 agonist peptide, we found that all peptides acted as strong coagonists for F5 CD8⁺ T cells (Fig. 3, E and F).

A computational model of T cell activation describes coagonism

Various computational and conceptual studies have dealt with the principles behind coagonism (Li et al., 2004; Wylie et al., 2007; Feinerman et al., 2008), but there has not yet been a treatment that simultaneously incorporates the diffusion, binding, and reactions of proteins in space and time with many available biophysical measurements. However, such kinetic models have been used to describe the different dependencies of T cell activation on CD4 and CD8 coreceptors

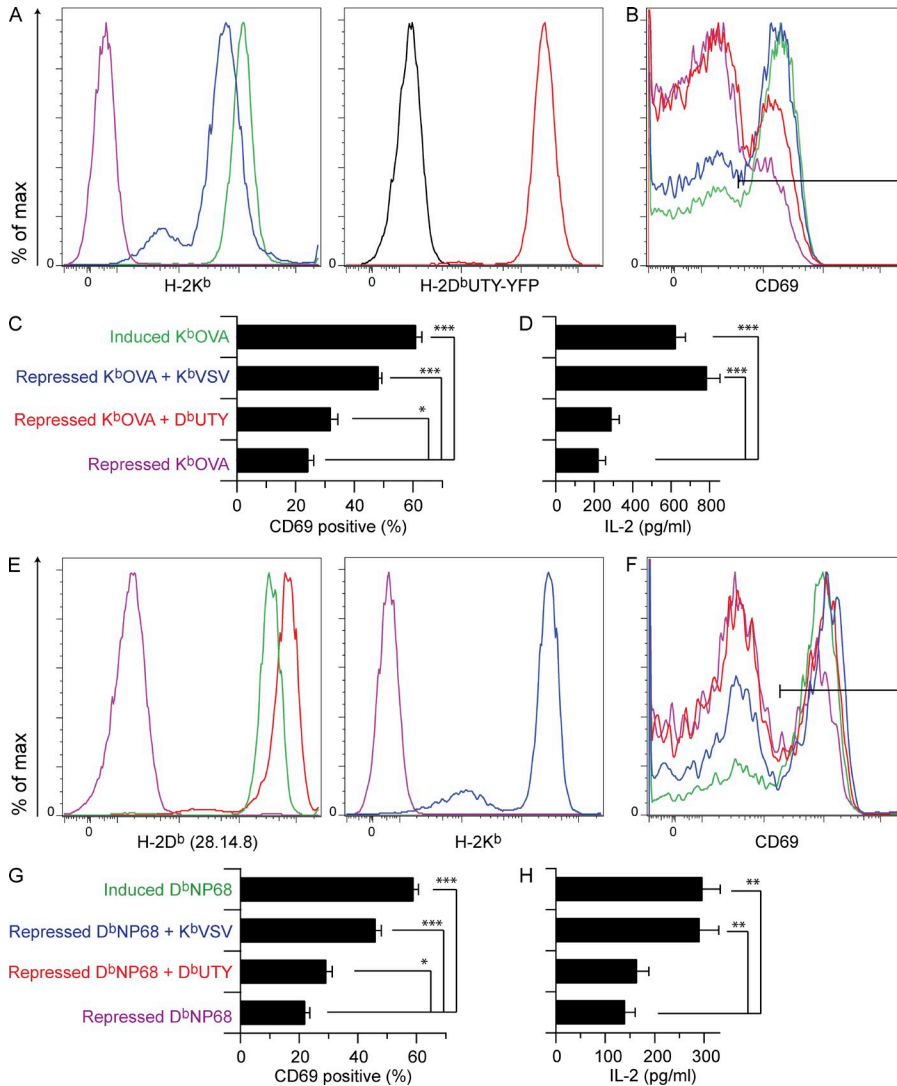


Figure 2. Coagonism of OT-I and F5 CD8 T cells by both sc-H-2K^b and sc-H-2D^b nonstimulatory sc-MHCI. Inducible sc-K^bOVA or sc-D^bNP68 CHO cells were super-transfected with constitutive sc-K^bVSV or sc-D^bUTY and used as APCs to assess coagonism of OT-I and F5 T cells. CHO cell APCs were stained for MHCI expression and in parallel exposed to responder T cells whose activation status was measured either by flow cytometry or IL2 ELISA. Inducible sc-K^bOVA APCs and OT-I responder T cells are described in the top panels, whereas inducible sc-D^bNP68 APC and F5 responder T cells are described in the bottom panels. (A and E) Analysis of K^b or D^b expression on CHO cell APCs. Color coding is as shown in the y-axis labels of the bar graphs. In A, sc-D^bUTY-YFP fusion construct was used to transfected cells expressing repressed K^bOVA, and the YFP fluorescence was used here to report on sc-D^bUTY expression relative to untransfected CHO cells (black). In the remaining panels, sc-MHCI expression was analyzed by antibody staining. (B and F) OT-I (B) and F5 (F) CD8 T cell expression of CD69 after incubation with the corresponding CHO APCs from A or E, respectively. Horizontal black lines indicate the CD69^{hi} gate. (C and G) Bar graphs represent mean ± SEM values of CD69^{hi} cells for OT-I (C) and F5 (G). (D and H) IL2 production after 8 h of exposure to CHO cell APCs as indicated for OT-I (D) and F5 (H). Statistical significance (*, P < 0.05; **, P < 0.01; ***, P < 0.001) results are from ANOVA analysis with Dunnett's post-test, referencing the repressed agonist sample as the standard. For the OT-I CD69 bar graph in C, n = 8 (individual mice) after removing outliers as defined by values >1.25 times the interquartile distance. For D, n = 4; G, n = 6 (individual mice); H, n = 6. Each data panel is representative of three independent experiments except C and G, which report the combined results from three independent experiments.

(Artyomov et al., 2010). We applied this formalism to a description of coagonism, incorporating separate on and off rates for TCR binding to agonist versus nonstimulatory pMHC. The OT-I TCR has approximately twofold higher affinity for agonist pMHC than F5 (K_d of 5.9–6.5 μ M for OT-I [Alam et al., 1996, 1999] versus 11 μ M for F5 [Willcox et al., 1999]). The kinetics describing these equilibrium constants are markedly different. The on and off rates for OT-I TCR–binding K^bOVA are well characterized (3,720/M/s and 0.022/s, respectively; Alam et al., 1999). We calculated the on rate for the F5 TCR for D^bNP68 based on the off rate and equilibrium affinity reported in the literature (k_{off} = 0.8/s, K_d = 11 μ M, k_{on} = 72,727/M/s, calculated; Willcox et al., 1999). Therefore, we varied the TCR kinetics for agonist pMHC in the kinetic

model to be consistent with these binding data. The TCR on rate for nonstimulatory peptides for both OT-I and F5 simulations was set to the OT-I value for K^bOVA so that we could directly compare nonstimulatory peptides with equal affinity and kinetics. We developed this model (see [Supplemental text](#)) that describes signal enhancement caused by the coexistence of agonist and nonstimulatory peptides on the APC surface (Fig. 3 G). This model also describes increased TCR signaling in response to nonstimulatory ligands with higher CD8-binding affinities in both F5 and OT-I model systems (Fig. 3 H). This computational result is consistent with the following data: Fig. 2 showing a greater magnitude of enhancement by H-2K^b compared with H-2D^b nonstimulatory pMHC, Fig. 3 (B and D) showing the absolute dependence of

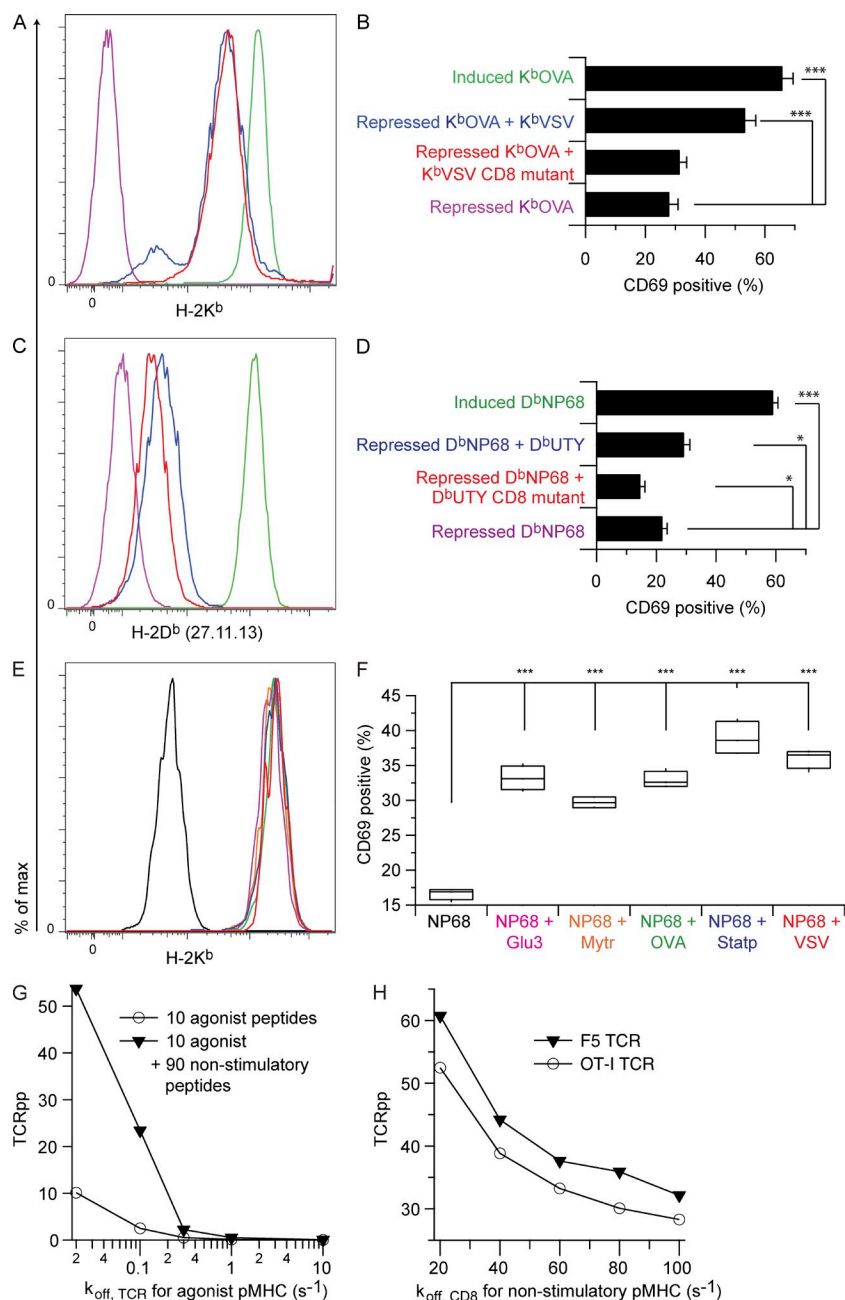


Figure 3. The effect of CD8 affinity for coagonist pMHC on coagonism. Inducible sc-K^bOVA or sc-D^bNP68 CHO cells were super-transfected with constitutive sc-K^bVSV CD8-binding mutant (sc-K^bVSV-CD8m) or sc-D^bUTY CD8-binding mutant (sc-D^bUTY-CD8m), respectively, and used as APCs to assess coagonism of OT-I and F5 T cells. CHO cell APCs were stained for MHCI expression and in parallel exposed to responder T cells whose activation status was measured by flow cytometry. (A) Anti-K^b staining of CHO cells expressing sc-MHCI. Color coding as shown in B. (B) Percentage of CD69^{hi} OT-I T cells (from $n = 10$ individual mice) after 3-h exposure to the CHO APCs in A. (C) Anti-D^b staining of CHO cells. Color coding as shown in D. (D) Percentage of CD69^{hi} F5 T cells ($n = 6$ individual mice) after 3-h exposure to the CHO APCs in C. RMA-S cell were also used as APCs to examine coagonism of F5 T cells. (E) Anti-K^b staining of RMA-S APCs loaded with combinations of NP68 agonist peptide and various H-2K^b-binding peptides: purple, Slc2a3; orange, Nmt1; green, OVA; blue, Stat3; and red, VSV. (F) Percentage of CD8⁺ F5 cells expressing CD69^{hi} after 3.5-h incubation with the RMA-S cells in E ($n = 4$). A kinetic model of T cell activation reproduces the effect of coagonism and describes a direct relationship between CD8 affinity for coagonist pMHC and TCR phosphorylation. (G) The kinetic model discriminates based on antigen quality and exhibits enhanced TCR phosphorylation (TCRpp) in the presence of non-stimulatory peptides. Results are shown for 10 agonist peptides alone and for 10 agonist plus 90 nonstimulatory peptides using OT-I kinetic parameters. (H) Results of computer simulations of the kinetic model describing coagonism as a function of CD8 affinity for nonstimulatory pMHC. Results are shown for F5 and OT-I kinetic parameters. Statistical significance (*, $P < 0.05$; ***, $P < 0.001$) results are from ANOVA analysis with Dunnett's post-test, referencing the repressed agonist sample (B and D) or the sample with agonist alone (F) as the standard. Data in A, C, E, and F are representative of three independent experiments. Data in B and D are the combined results of three independent experiments. Error bars represent SEM.

coagonism on CD8 binding to the nonstimulatory pMHC, and Fig. 3 F showing the lack of F5 coagonist peptide specificity for peptides bound to the high CD8-binding affinity MHCI protein H-2K^b.

Minimal contribution of OT-I TCR recognition of nonstimulatory pMHC to coagonism

Previous studies showed no peptide dependence for coagonism by nonstimulatory pMHC, with both OT-I preselection thymocytes and peripheral T cells (Yachi et al., 2005, 2007), nor was there any discrimination between endogenous nonstimulatory peptides in signaling for negative selection by covalent pMHC dimers containing one K^b-OVA with one

nonstimulatory K^b ligand (Juang et al., 2010). To probe the role of TCR interaction with the nonstimulatory ligands in coagonism using the sc-MHCI system, we used several strategies. Residues in the $\alpha 1\alpha 2$ -helices of MHCI comprise the main recognition structures for TCR CDR1 and CDR2 (Sun et al., 1995; Sim et al., 1998; Garcia et al., 2009). The E166K mutation disrupts recognition of K^bVSV by VSV-specific CTL (Sun et al., 1995) and of K^bOVA by OT-I CTL (not depicted). Second, we used a chimera between the $\alpha 1\alpha 2$ domains of the MHCI^b molecule H-2TL (which cannot bind or present peptide to TCRs; Devine et al., 2002; Weber et al., 2002; Liu et al., 2003) and the $\alpha 3$ from H-2K^b, enabling normal CD8 binding (Attinger et al., 2005). Both K^bVSV with the

E166K TCR-binding site mutation (sc-K^bVSV-TCRm) and the TL/K^b chimera provided significant coagonism for recognition of sc-K^bOVA (Fig. 4, A and B), indicating that the TCR interaction with nonstimulatory pMHC was at most a minor determinant of coagonism for OT-I T cells. We also noted that recruitment of CD8αβ to the immunological synapse (Yachi et al., 2005; Rybakin et al., 2011) was unperturbed by the sc-K^bVSV-TCRm mutation (not depicted).

When we plotted CD69 expression data for OT-I T cells versus MHC expression on CHO cells for repressed and induced sc-K^bOVA, or repressed sc-K^bOVA plus sc-K^bVSV, we found a linear relationship (Fig. 4 C). Repressed sc-K^bOVA plus sc-K^bVSV-TCRm revealed a minor defect in coagonism when TCR binding to nonstimulatory pMHC was attenuated. In contrast, the repressed sc-K^bOVA plus the sc-K^bVSV-CD8m showed no coagonism.

A recent study showed that the OT-I TCR has a very weak but biologically relevant interaction with endogenous nonstimulatory ligands. Removal of this TCR interaction, for example with a peptide designed to bind K^b but to lack TCR interaction residues, abrogated coagonism (Juang et al., 2010).

To probe CD8 T cell coagonism with a similar strategy, RMA-S cells were first loaded with a very small amount of OVA peptide followed by loading with a titration of peptides including the OVA-derived antagonist/positively selecting peptide R4 (SIIRFEKL), the nonstimulatory peptide VSV (RGYVYQGL), and the engineered peptide poly-serine (SSYSYSSL), which should lack any TCR contact residues (Hogquist et al., 1994). Each of these peptides acted as a coagonist in an H-2K^b expression-dependent manner (Fig. 4 D), indicating that for the OT-I TCR, any discrimination based on the quality of the nonstimulatory peptide is at best very small.

Reduction in CD8-binding affinity for agonist pMHC enforces requirement for intact TCR interaction with nonstimulatory pMHC

To test the impact of CD8 affinity for agonist pMHC on coagonism, we tested sc-K^bOVA-CD8m (Fig. 5, A and B). As expected from previous work (Connolly et al., 1988, 1990; Potter et al., 1989), this mutation minimized direct activation of OT-I T cells by sc-K^bOVA (Fig. 5 B and not depicted). Activation of OT-I T cells was rescued by sc-K^bVSV but not by

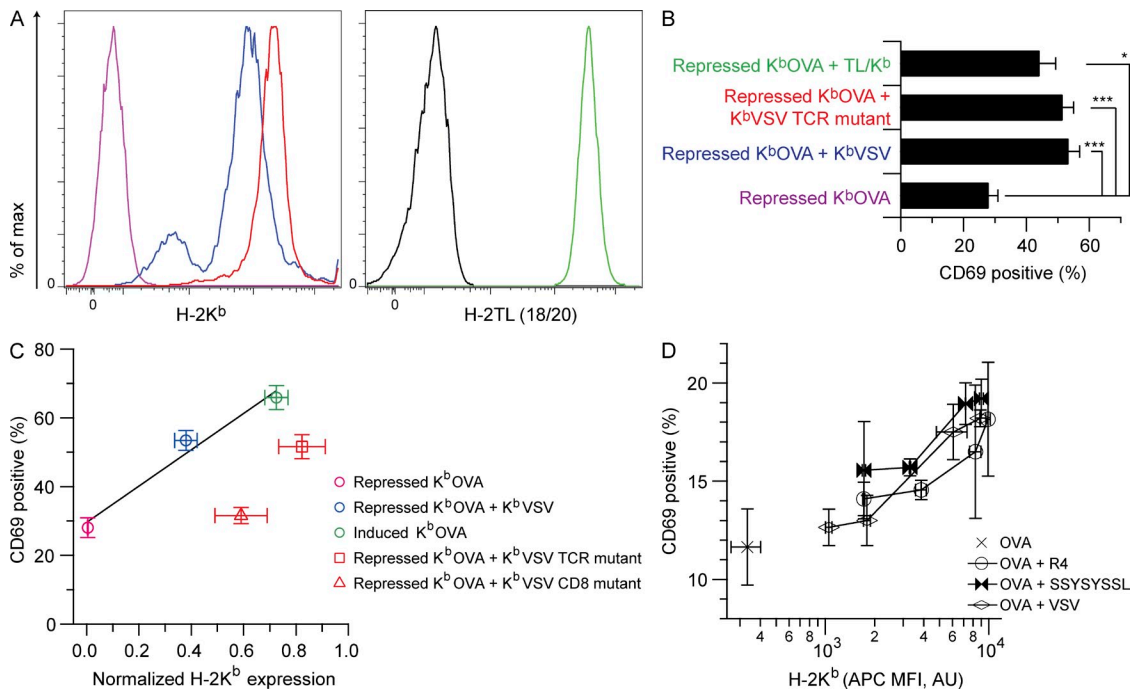


Figure 4. Minor contribution of OT-I TCR binding to nonstimulatory pMHC in coagonism. Inducible sc-K^bOVA CHO cells were super-transfected with constitutive sc-K^bVSV TCR-binding mutant (sc-K^bVSV-TCRm) or H-2TL-K^bα3 (TL/K^b) and used as APCs to assess coagonism of OT-I T cells. CHO cell APCs were stained for MHC expression and in parallel exposed to responder T cells whose activation status was measured by flow cytometry. (A) CHO cell staining for either H-2K^b or H-2TL expression as indicated. Black indicates TL-negative staining control (Trex CHO cells). Other colors are as indicated in B. (B) Percentage of CD69^{hi} OT-I T cells (*n* = 10 mice) after 3-h exposure to the CHO APCs in A. (C) Percentage of CD69^{hi} OT-I T cells plotted as a function of the corresponding CHO APC H-2K^b expression, as indicated (*n* = 10 mice for CD69 data and *n* = 3 for H-2K^b expression on CHO cell APCs). A correction factor of 2.8 is applied to the H-2K^b staining of the repressed K^bOVA plus K^bVSV-CD8m to account for attenuated anti-K^b staining resulting from the CD8-binding site mutation (not depicted). RMA-S cell APCs were also used to test coagonism of OT-I T cells. (D) Percentage of CD69^{hi} OT-I T cells plotted as a function of K^b expression of RMA-S cells loaded with increasing concentrations of nonstimulatory peptides R4, VSV, and SSYSYSSL, as indicated (*n* = 2 for both CD69 and H-2K^b expression). Statistical significance (*, *P* < 0.05; ***, *P* < 0.001) results are from ANOVA analysis with Dunnett's post-test, referencing the repressed agonist sample as the standard. Data in A are representative of three independent experiments, and data in D are representative of two independent experiments. Data in B and C are the combined results of three independent experiments. Error bars represent SEM (B and C) or SD (D).

sc-K^bVSV-TCRm (Fig. 5 B). This was in stark contrast to earlier results with repressed wild-type sc-K^bOVA, in which sc-K^bVSV-TCRm was a strong coagonist (Fig. 4, B and C).

To further probe this discrepancy in coagonism by sc-K^bVSV-TCRm, we took advantage of the fact that CD8 has lower affinity for H-2D^b than for H-2K^b (Moody et al., 2001b; Huang et al., 2007). The CD8-binding affinity is a property of the MHC I $\alpha 3$ domain, as a chimera of D^b $\alpha 1\alpha 2$ -K^b $\alpha 3$

binds CD8 similarly to the native H-2K^b molecule (Moody et al., 2001b). We therefore constructed and subsequently expressed inducible sc-K^bOVA-D^b $\alpha 3$ in CHO cells and super-transfected with sc-K^bVSV or sc-K^bVSV-TCRm. The altered $\alpha 3$ domain epitope present in sc-K^bOVA-D^b $\alpha 3$ showed attenuated binding to the anti-H-2K^b antibody, visible in the different anti-H-2K^b staining of the induced sc-K^bOVA-D^b $\alpha 3$ versus wild-type sc-K^bOVA (Fig. 5 C). The amount of H-2K^b

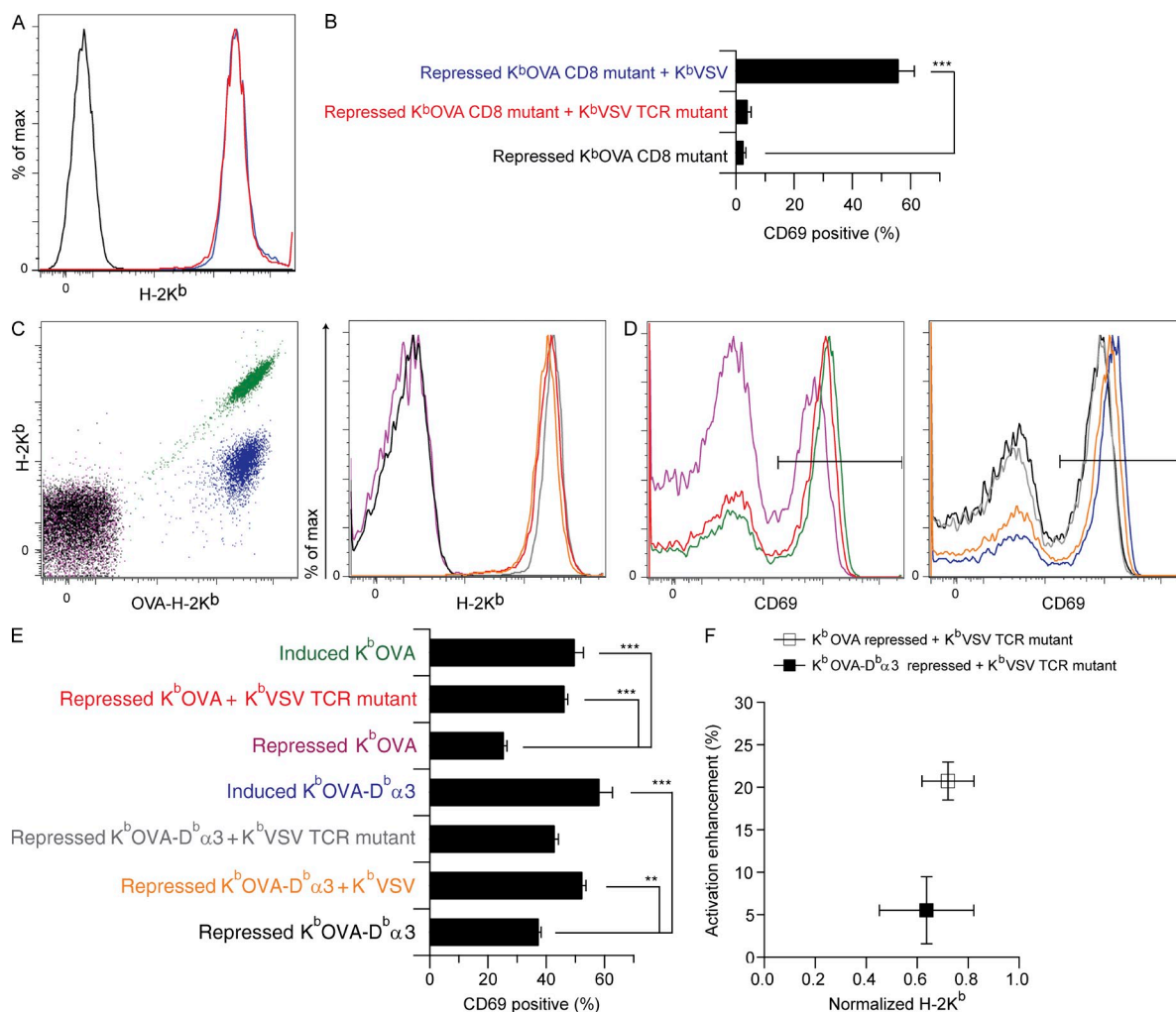


Figure 5. Modulation of K^bOVA CD8-binding affinity and associated changes in coagonism by K^bVSV-TCRm. Inducible sc-K^bOVA CD8-binding mutant (sc-K^bOVA-CD8m) CHO cells were super-transfected with constitutive sc-K^bVSV or sc-K^bVSV-TCRm and used as APCs to assess coagonism of OT-I T cells. CHO cell APCs were stained for MHC I expression and in parallel exposed to responder T cells whose activation status was measured by flow cytometry. (A) Anti-K^b stain of CHO cell APCs. Color coding is as shown in B. (B) Percentage of CD69^{hi} OT-I T cells ($n = 10$ mice) in response to the CHO cell APCs in A. Inducible domain-swapped sc-K^bOVA-D^b $\alpha 3$ CHO cells were also super-transfected with both sc-K^bVSV or sc-K^bVSV-TCRm and used as APCs to assess coagonism of OT-I T cells. (C) Repressed and induced K^bOVA (purple and green, respectively) and K^bOVA-D^b $\alpha 3$ (black and blue, respectively) constructs were analyzed as a function of both OVA-K^b and total H-2K^b staining, whereas repressed K^bOVA plus K^bVSV-TCRm (red), repressed K^bOVA-D^b $\alpha 3$ plus K^bVSV (orange), and repressed K^bOVA-D^b $\alpha 3$ plus K^bVSV-TCRm (gray) were analyzed with H-2K^b staining alone. (D) CD69 expression of CD8⁺ OT-I T cells responding to the APCs described in C, with color coding as in C. The horizontal black line indicates the CD69^{hi} gate. (E) Percentage of CD69^{hi} OT-I T cells ($n = 4$ mice) in response to the CHO cell APCs in C. (F) Calculated value of activation enhancement of OT-I T cells plotted as a function of anti-K^b staining of the associated CHO APCs. Repressed K^bOVA plus K^bVSV-TCRm and repressed K^bOVA-D^b $\alpha 3$ plus K^bVSV-TCRm are shown. Statistical significance (**, $P < 0.01$; ***, $P < 0.001$) results are from ANOVA analysis with Dunnett's post-test, referencing the repressed agonist sample as the standard. Data in A are representative of three independent experiments. Data in B are the combined results of three independent experiments. Data in C, D, and F are representative of two experiments, whereas the data in E are the combined results from two experiments. Error bars represent SEM (B and E) or SD (F).

expression in the nonstimulatory sc-K^bVSV and sc-K^bVSV-TCRm were comparable (Fig. 5 C). The CD69 expression profiles for OT-I CD8⁺ T cells (Fig. 5 D) and quantification of CD69^{hi} T cells (Fig. 5 E) clearly showed the lack of statistically significant coagonism for sc-K^bOVA-D^bα3 plus sc-K^bVSV-TCRm compared with either of the significant responses found for the sc-K^bOVA-D^bα3 plus wild-type sc-K^bVSV or wild-type sc-K^bOVA plus sc-K^bVSV-TCRm.

Both repressed and induced sc-K^bOVA-D^bα3-expressing CHO cells elicited systematically higher CD69 expression than the wild-type sc-K^bOVA in the responder OT-I T cells. This result was unexpected, but we examined several different clones of the inducible sc-K^bOVA-D^bα3 construct and each one behaved in a similar manner (not depicted). This phenomenon could be explained by the serial TCR engagement model (Valitutti, 2012), which proposes that a few agonist pMHC complexes can initiate signaling from many TCRs. Optimum induction of T cell activation occurs in response to agonist pMHC complexes that engage TCR with intermediate affinity, with half-lives sufficient to initiate productive TCR ITAM phosphorylation but short enough to allow an individual pMHC molecule to serially trigger several TCRs. We suggest that the attenuated CD8 affinity reduces the half-life of OT-I/CD8 engagement by K^bOVA-D^bα3, bringing it closer to the optimum values that allow more effective serial TCR engagement and T cell activation. Because of this unexpected result, and to explicitly show the altered behavior of sc-K^bVSV-TCRm coagonists for the recognition of sc-K^bOVA-D^bα3 versus recognition of wild-type sc-K^bOVA, we calculated the activation enhancement of OT-I T cells (percent CD69^{hi} in response to repressed agonist with sc-K^bVSV-TCRm minus percent CD69^{hi} in response to repressed agonist alone; Fig. 5 F). This enabled comparison of coagonism by sc-K^bVSV-TCRm with these two different agonists, revealing that the nonstimulatory ligand with reduced TCR interaction provided greater coagonism for recognition of sc-K^bOVA than for chimeric sc-K^bOVA-D^bα3. The results presented in Fig. 5 suggest that reduced CD8 affinity for antigenic pMHC I enforces an increased reliance on TCR interaction with nonstimulatory ligand to achieve coagonism.

Coagonist activity by H-2D^b for F5 T cells requires TCR discrimination of nonstimulatory pMHCs

The two antibodies used to stain sc-H-2D^b-transfected CHO cells in this study recognize different H-2D^b epitopes. The clone 27.11.13 recognizes a peptide-dependent conformational epitope, whereas the 28.14.8 clone recognizes the H-2D^b α3 domain independent of peptide (Fig. 6 A; Palmowski et al., 2009). We prepared sc-D^bGP33 and sc-D^bGP33-TCRm sc-MHC molecules, expressed them constitutively in CHO cells, and stained with both 27.11.13 and 28.14.8 anti-D^b antibodies. These results show that the 27.11.13 antibody staining was diminished by the TCR-binding site mutation on H-2D^b (Fig. 6 A). This offers strong evidence that the 27.11.13 antibody actually reports on the presence of amino acid residues that are important for TCR recognition, both in the peptide

sequence and MHC heavy chain. The sc-D^bUTY clones used in our assays stained strongly with 28.14.8 but relatively weakly with 27.11.13 (Fig. 6 A). Linear regression analysis of CD69^{hi} F5 T cells after activation with repressed sc-D^bNP68, repressed sc-D^bNP68 plus sc-D^bUTY, or induced sc-D^bNP68 revealed a very strong correlation between the percentage of CD69^{hi} T cells and 27.11.13 staining of the corresponding CHO cells (Fig. 6 B). No such correlation existed for these same CD69 data plotted against 28.14.8 staining, suggesting that coagonism for the F5 TCR was sensitive to the conformation of the α1α2 domains of the nonstimulatory pMHC and/or to the peptide presented. This result suggested that there are fundamental differences in coagonism/activation enhancement between the D^bNP68-F5 and K^bOVA-OT-I systems.

To probe the question of the specificity of coagonism of F5 T cells for nonstimulatory H-2D^b pMHC, we screened a small library of ~40 H-2D^b-binding peptides (Table S1) for H-2D^b binding and the ability to act as coagonists for F5 T cells when presented by RMA-S, identifying only two variant influenza peptides as coagonists (not depicted). Neither of these peptides (NP34, ASNENMETM; and NP371I, ASNENIDTM) stimulates F5 T cells (Townsend et al., 1986; Price et al., 2000), but NP34 can act as an antagonist (Williams et al., 1998) and possibly so can NP371I (Price et al., 2000). We therefore carefully tested these peptides for direct activation of F5 T cells. RMA-S cells were loaded with 100 μM each of NP34, NP371I, and PA224 (SSLEN-FRAYV, a D^b-binding peptide which did not act as coagonist in our screen) or with 10 nM NP68 agonist peptide. H-2D^b was stabilized on RMA-S cells by binding of NP34, NP371I, and PA224, but the H-2D^b loaded with 10 nM NP68 agonist was undetectable (Fig. 6 C). There was no significant difference in T cell activation between saline-treated RMA-S cells and those presenting high amounts of PA224, NP34, or NP371I on H-2D^b (Fig. 6 D). RMA-S cells treated with 10 nM NP68 peptide activated ~15% of F5 T cells. To test for coagonism by PA224, NP34, and NP371I, we first loaded RMA-S cells with 10 nM NP68 agonist, followed by 100 μM each of PA224, NP34, and NP371I, as previously described (Yachi et al., 2007). After peptide loading, these RMA-S cells expressed comparable amounts of H-2D^b (Fig. 6 E), and when used to stimulate F5 T cells, both NP34 and NP371I, but not PA224, showed strong coagonist activity (Fig. 6 F).

Because of the positive coagonist results with sc-D^bUTY-expressing CHO cells, we also tested the ability of the UTY peptide to support activation enhancement when presented on RMA-S cells. UTY peptide loaded at 100 μM did stabilize H-2D^b on RMA-S cells as measured by anti-D^b staining with antibody clone 28.14.8, but staining with the complementary anti-D^b antibody 27.11.13 was negative for UTY but positive for NP34 and PA224 (Fig. 6, G and H). The fraction of F5 CD8⁺ T cells that up-regulated CD69 expression showed that the UTY peptide did not support activation enhancement (Fig. 6 I).

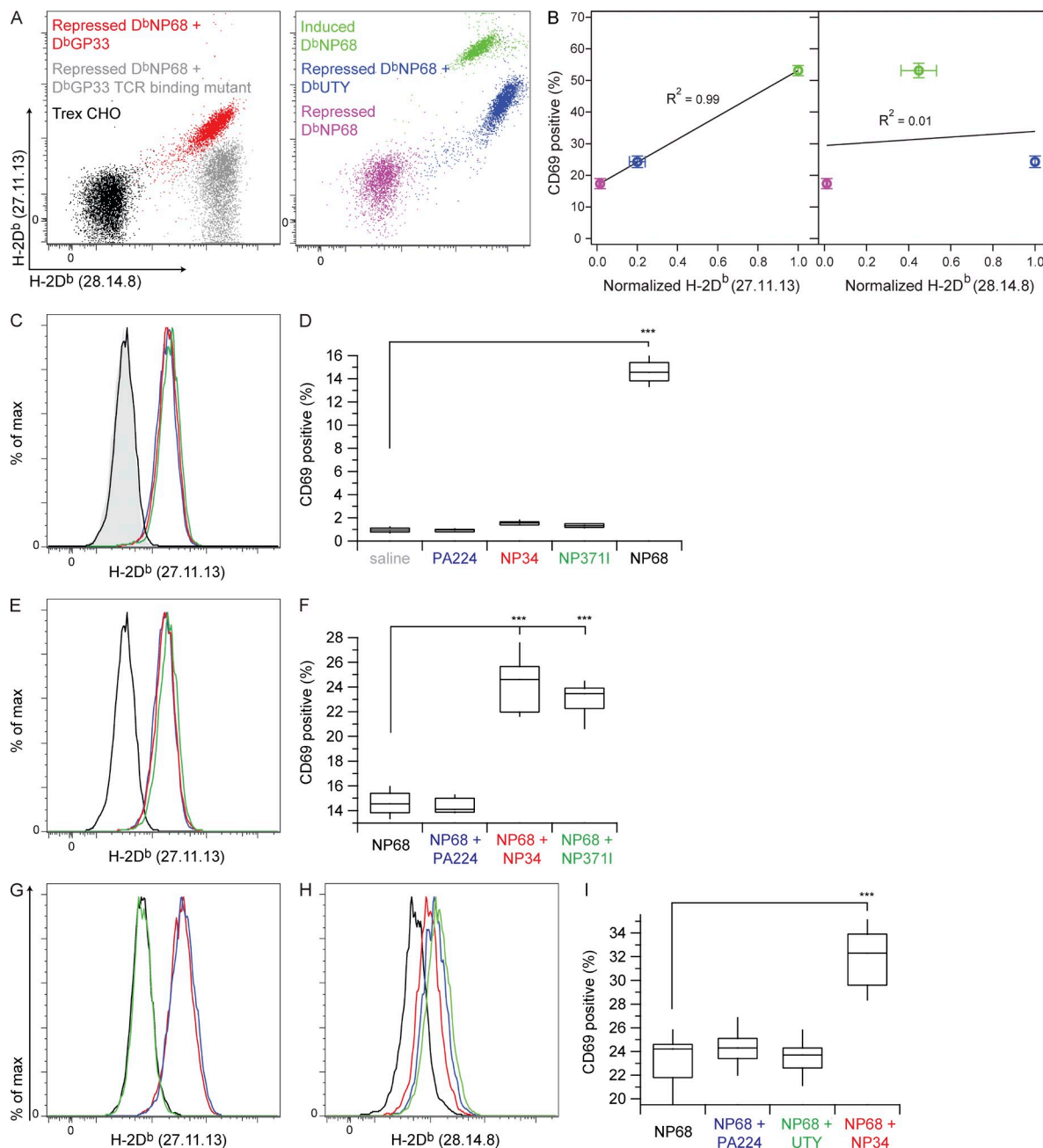


Figure 6. Contribution of F5 TCR recognition of nonstimulatory H-2D^b pMHC to coagonism. Inducible sc-D^bNP68 CHO cells were super-transfected with constitutive sc-D^bGP33, sc-D^bGP33-TCRm, or sc-D^bUTY and used either as APCs to assess coagonism of F5 T cells or as probes of anti-D^b antibody specificity. (A) Dual anti-D^b antibody staining (clones 28.14.8 and 27.11.13) of CHO cell APCs. (B) Percentage of CD69^{hi} F5 T cells plotted separately versus the 27.11.13 and 28.14.8 anti-D^b stains of corresponding CHO cell APCs in A. The black lines are linear fits to the data with the R² value for the fit reported on the graph ($n = 6$ mice). Error bars represent SEM. RMA-S cell APCs were also used to assess coagonism of F5 T cells by H-2D^b-binding peptides. (C) Anti-D^b staining of RMA-S APCs loaded with individual peptides. Gray shading shows RMA-S alone, and black shows RMA-S after 10 nM NP68 agonist addition. 100- μ M additions of peptides were as follows: blue, PA224; red, NP34; and green, NP3711. (D) Percentage of CD8⁺CD62L⁺CD44^{lo} F5 cells expressing CD69^{hi} after 3.5-h incubation with the RMA-S APCs in C. (E) Anti-D^b staining of RMA-S cells loaded with combinations of peptides to test coagonist activity, color coded as indicated in C. (F) Percentage of CD8⁺CD62L⁺CD44^{lo} F5 cells expressing CD69^{hi} after 3.5-h incubation with the RMA-S cells in E. (G and H) Anti-D^b antibody staining with clones 27.11.13 (G) and 28.14.8 (H) of RMA-S cell APCs loaded with trace NP68 agonist with or without coagonists. Black indicates NP68 alone. 100 μ M additions of peptides were as follows: blue, PA224; red, NP34; and green, UTY. (I) Percentage of CD8⁺ F5 cells expressing CD69^{hi} after 3.5-h incubation with the RMA-S cell APCs in G and H. Statistical significance (***) results are from ANOVA analysis with Dunnett's post-test, referencing the sample with saline (D) or agonist alone (F and I) as the standard. Data in A are representative of three independent experiments, and data in B are the combined results from three independent experiments and are the same data as presented in Figs. 2 G and 3 D. For D and F, $n = 5$; for I, $n = 15$. For RMA-S assays with NP34 and PA224, data are representative of six independent experiments, and for NP3711 and UTY, data are representative of two independent experiments.

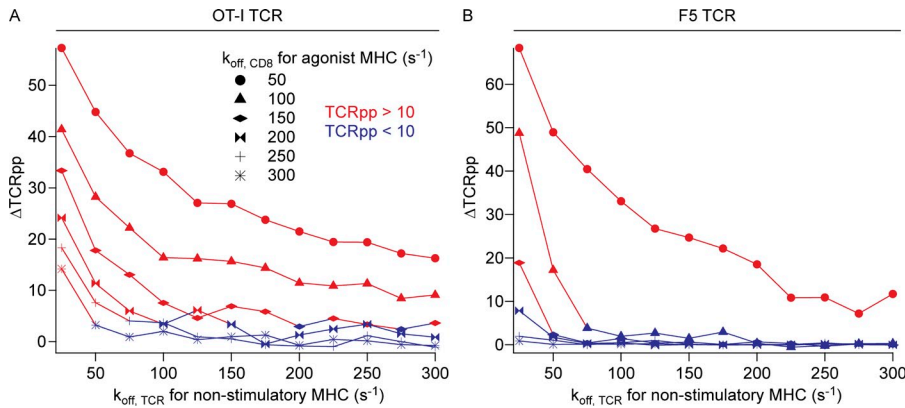


Figure 7. Results of computer simulations of the kinetic model describing coagonism as a function of both TCR affinity for nonstimulatory pMHC and CD8 affinity for agonist pMHC. (A) Data represent simulations for an OT-I-like TCR affinity (modeled as $k_{on,TCR}$ for agonist = 3,720/M/s and $k_{off,TCR}$ for agonist = 0.02/s) with 10 agonist and 90 nonstimulatory peptides. (B) Data represent simulations for an F5-like TCR affinity (twofold lower affinity than OT-I, modeled as $k_{on,TCR}$ for agonist = 72,727/M/s and $k_{off,TCR}$ for agonist = 0.8/s) with 20 agonist and 80 endogenous peptides. $\Delta TCRpp$ enhancement values were calculated from two different simulations for

each point on each curve. Symbols for different CD8 agonist MHC off rates are indicated, and the curves are color coded according to the TCRpp value of the agonist plus nonstimulatory peptide simulations, where TCRpp values above the activation threshold of 10 (Artyomov et al., 2010) are red and values below that threshold are blue.

Computer simulations reveal the importance of TCR interaction with nonstimulatory pMHC when CD8 binding to agonist pMHC is weak

To provide a mechanistic context to the experimental findings described here, that sensitivity of coagonism to the nature of the nonstimulatory peptide originates in part from differences in CD8 affinity for agonist pMHC, we modified the computational model described above to introduce separate $k_{off,CD8}$ values for agonist and nonstimulatory pMHC species. This allowed us to systematically vary coreceptor binding affinity to agonist pMHC while keeping coreceptor binding to nonstimulatory pMHC constant. To test the different requirements of OT-I and F5 T cells for nonstimulatory pMHC in responses to low amounts of their specific agonist pMHCs, we performed simulations with agonist peptides only or with agonist plus nonstimulatory peptides. These simulations were performed over a range of high, intermediate, and low CD8 affinities for agonist pMHC ($k_{off,CD8}$ for agonist pMHC of 50–300/s; Fig. 7 and not depicted; Cole et al., 2012). Simulations were performed for relevant combinations of parameters while varying the quality of the nonstimulatory pMHC ($k_{off,TCR}$ for nonstimulatory pMHC ranging from 20/s to 300/s). The results are presented as the increase in TCR/CD3 phosphorylation $\Delta TCRpp$,

$$\Delta TCRpp = TCRpp_{agonist, nonstimulatory} - TCRpp_{agonist only},$$

and color coded according to the TCRpp value of the agonist plus nonstimulatory peptide simulations, where TCRpp values >10 are considered activating (TCRpp denotes the mean number of fully phosphorylated TCR; Artyomov et al., 2010). The results revealed that for TCR with an OT-I-like agonist affinity, coagonism showed little dependence on nonstimulatory pMHC quality for small values of $k_{off,CD8}$ for agonist pMHC (i.e., high CD8 affinity; Fig. 7 A). With intermediate or weak CD8 binding to agonist pMHC, however, there was stronger dependence on the quality of the nonstimulatory

pMHC. At these weak CD8 agonist pMHC-binding affinities, the lowest affinity nonstimulatory peptides provided little signal enhancement, and the absolute TCRpp value for these simulations fell below the activation threshold of $TCRpp = 10$. For the TCR with F5-like affinity for agonist pMHC (Fig. 7 B), there was a steeper dependence on nonstimulatory pMHC–TCR off rate at low $k_{off,CD8}$ (high CD8 affinity) for agonist pMHC. This indicates that the kinetics of the TCR–agonist pMHC interaction play a role in the requirement for specific coagonists. However, at the smallest values of $k_{off,CD8}$ for agonist pMHC, coagonism of F5 was still significant and the absolute values of TCRpp for the simulations of agonist plus nonstimulatory peptides remained above the threshold of 10. For intermediate $k_{off,CD8}$ for agonist pMHC, only the highest affinity nonstimulatory peptides gave any appreciable activation. At the highest values of $k_{off,CD8}$ for agonist pMHC, the nonstimulatory pMHC gave no coagonism for F5. These computational results provide a conceptual framework describing our experimental results, in which we found that CD8 affinity for agonist pMHC is a key factor for the dependence of coagonism on the TCR’s interaction with the nonstimulatory pMHC.

DISCUSSION

Recent work has demonstrated that nonstimulatory peptides bound to MHC can enhance T cell recognition (Krogsgaard et al., 2005; Yachi et al., 2005, 2007; Anikeeva et al., 2006; Davis et al., 2007; Gascoigne, 2008; Gascoigne et al., 2010; Juang et al., 2010). To investigate the parameters of the coagonist phenomenon for CD8 T cells, we developed a system where very low expression of sc-pMHC agonists for specific TCRs was coupled with constitutive expression of nonstimulatory sc-pMHC. Using OT-I and F5 TCRs, we tested combinations of H-2K^b and H-2D^b, as well as mutations in the cognate or nonstimulatory MHC, to identify the interactions important in coagonism. In addition, we used computer

simulations of T cell activation to verify our key conclusions and develop a conceptual model.

Our results describe two distinct mechanisms for which coreceptor binding to pMHC influences the phenomenon of coagonism. In the first and most obvious mechanism, we find that CD8 affinity for the nonstimulatory pMHC exerts several effects on coagonism. We found that both H-2D^b and H-2K^b molecules supported coagonism but that activation enhancement by nonstimulatory D^b ligands was less effective than K^b ligands for both (K^b restricted) OT-I and (D^b restricted) F5 T cells. This demonstrated that the phenomenon of coagonism is not restricted to a single MHC I molecule and suggested that coagonism correlated with the CD8 binding affinity for nonstimulatory MHC, as CD8 affinity for H-2K^b is stronger than for H-2D^b (Moody et al., 2001b; Huang et al., 2007; Cole et al., 2012). In addition, when coagonist peptides were presented to F5 on H-2K^b with RMA-S cell APC, we found that the higher CD8 affinity for the nonstimulatory K^b enabled coagonism to occur for all the peptides tested. To provide a conceptual framework within which to test our experimental results, we modified a kinetic model of T cell activation (Artyomov et al., 2010) to include nonstimulatory pMHC. In stochastic computer simulations, we systematically varied the affinity of CD8 for pMHC. The results showed that coagonism depends on the affinity of CD8 for nonstimulatory pMHC, rendering our model consistent with experimental findings showing that higher CD8 affinity for nonstimulatory H-2K^b gives stronger coagonism than does the lower CD8 affinity for H-2D^b.

The direct correlation between CD8 affinity for nonstimulatory pMHC and the magnitude of coagonism suggested that CD8 binding to nonstimulatory pMHC is a dominant factor in coagonism. By introducing CD8-binding site mutations (CD8m) in the $\alpha 3$ domains of sc-K^bVSV and sc-D^bUTY, we found that CD8 binding to nonstimulatory pMHC is an absolute requirement for coagonism. In the F5 system, the sc-D^bUTY-CD8m actually manifested a statistically significant inhibition of activation, which we interpret as competitive inhibition, similar to the finding that coagonist pMHCs become antagonists in the absence of CD8 (Stone et al., 2011).

The requirements for the interaction of TCR with the nonstimulatory pMHC for coagonism are complex. For OT-I T cells recognizing sc-K^bOVA, we detected a very mild coagonist defect using sc-K^bVSV-TCRm as nonstimulatory pMHC. This defect was only clear when we accounted quantitatively for T cell activation as a function of total MHC expression on the APCs. The minimal contribution of the affinity of OT-I TCR for nonstimulatory pMHC to coagonism was confirmed by two other findings. First, the TL/K^b chimera that cannot bind peptide significantly enhanced activation of OT-I T cells and, second, that a peptide designed to have no TCR interaction (poly-serine; Hogquist et al., 1994) was an effective coagonist, similar to other nonstimulatory peptide-K^b complexes.

This complete lack of specific coagonism for OT-I T cells is a convolution of the relatively strong CD8 binding to nonstimulatory H-2K^b pMHC, mechanism 1 described above,

and a second mechanism involving the affinity of CD8 for agonist pMHC, consistent with the experimental data described hereafter and described by the kinetic model used in our study. By testing a gradient of CD8 affinities for the agonist sc-K^bOVA, using sc-K^bOVA-D^b $\alpha 3$ chimera and CD8m constructs, we found that the requirement for TCR recognition of the nonstimulatory sc-K^bVSV became more stringent as the ability of CD8 to bind agonist sc-K^bOVA was reduced. sc-K^bVSV-TCRm gave slightly reduced coagonism compared with wild-type sc-K^bVSV for agonist sc-K^bOVA stimulation, but sc-K^bVSV-TCRm coagonism for sc-K^bOVA-D^b $\alpha 3$ agonist was significantly reduced. sc-K^bVSV-TCRm was unable to provide coagonism for sc-K^bOVA-CD8m. Thus, loss of CD8 binding to sc-K^bOVA introduced an absolute requirement for the OT-I TCR to bind to the nonstimulatory pMHC to drive activation, whereas weak CD8 binding to agonist revealed a partial requirement for TCR recognition of the nonstimulatory ligand.

Using the H-2D^b-restricted F5 TCR and correlations with two different anti-H-2D^b antibody-mediated measures of CHO H-2D^b expression, the associations that we uncovered suggested that coagonism of F5 cells by sc-D^bUTY nonstimulatory pMHC was dependent on the TCR interaction with nonstimulatory H-2D^b. This requirement for TCR discrimination of the nonstimulatory pMHC suggests that coagonism for F5 recognition of D^bNP68 should be more peptide specific than OT-I recognition of K^bOVA. To further probe the question of peptide-specific coagonism of F5 T cells by nonstimulatory peptides bound to H-2D^b, we used the RMA-S cell APC system. From a library of ~ 40 peptides, we found only two H-2D^b-binding peptides that acted as coagonists for F5 T cells. Both of these peptides were variant influenza peptides, each differing in only two amino acid residues from the NP68 agonist. These data show that coagonism for F5 T cells requires TCR binding to nonstimulatory pMHC, where only peptides closely related to the antigen worked as coagonists. In contrast, as described above, when nonstimulatory peptides are presented to F5 T cells on H-2K^b (along with agonist NP68 on H-2D^b), the peptide specificity of coagonism is lost because of the increased CD8 affinity of the coagonist H-2K^b pMHC (i.e., mechanism 1).

We tested the ability of the UTY peptide bound to RMA-S to act as a coagonist for F5 T cells. As shown in Fig. 6 I, the UTY peptide did not support activation enhancement of F5 T cells when presented on RMA-S cells. However, sc-D^bUTY expressed on CHO cells did give some degree of F5 coagonism. We can offer two potential explanations for this discrepancy. It is possible that coagonism by sc-D^bUTY results from the relatively high H-2D^b expression on CHO compared with RMA-S (Fig. 6, compare A and B with G and H, for example) or is an artifact supplied by the single-chain design, for example by the presence of the stabilizing linker between the peptide and $\beta 2m$. However, based on the 27.11.13 and 28.14.8 anti-D^b antibody correlation analysis of sc-UTY (Fig. 6, A and B), we concluded that an observable anti-D^b 27.11.13 antibody stain correlated with activation enhancement

by the UTY peptide (Fig. 6 B). Consistent with this requirement, we found that H-2D^b bound to UTY peptide on RMA-S cells did not stain with the 27.11.13 antibody but did stain with the 28.14.8 antibody (Fig. 6, G and H). Therefore, based on the lack of 27.11.13 staining of RMA-S cells loaded with UTY peptide, we would not predict UTY to act as a coagonist.

The picture of mechanism 2 that emerges from the kinetic model is one in which activation of Lck upon TCR binding to agonist pMHC (Stirnweiss et al., 2013) is followed by this active Lck-CD8 complex finding other TCR-pMHC complexes in the vicinity on the cell membrane. This Lck can phosphorylate even short-lived complexes of TCR with non-stimulatory pMHC that may be nearby. The kinetic model validated in this study suggests that agonists presented by MHCI with high affinity for CD8 promote a higher likelihood (or effective concentration) of CD8 complexes with active Lck than do agonists presented by MHC molecules with low CD8 affinity. For coagonism to occur for weakly CD8-binding agonist pMHC, a longer half-life of the ternary TCR-nonstimulatory pMHC-CD8 complex is required because of the lower amount of active Lck. This increase in stability can be achieved with either a higher-affinity TCR-nonstimulatory pMHC interaction or a higher-affinity CD8-nonstimulatory pMHC interaction (i.e., mechanism 1).

Thus, the overarching principle that emerges from our study and from the literature is the relationship between coreceptor affinity for pMHC and the peptide specificity of T cell activation. CD8 affinity for agonist pMHC plays a direct role in signaling through the TCR, where increasing the affinity of CD8 increases ligand potency and the number of peptides recognized as agonists, and can even bypass peptide specificity requirements altogether (Laugel et al., 2007; Wooldridge et al., 2007, 2010). In addition to greater cross-reactivity, increased CD8 affinity will make a larger proportion of nonstimulatory ligands potential coagonists through both mechanisms described here. Collectively, these data therefore suggest that the highest peptide specificity of T cell activation is achieved with MHC molecules with the lowest affinities for coreceptor.

Our results provide a unifying view of coagonism as these conclusions are also applicable to CD4 T cells, in which CD4 affinity for MHCII is lower than CD8 affinities for MHCI (van der Merwe and Davis, 2003). The lower-affinity H-2D^b MHCI agonists behave more like the previously characterized CD4 T cell systems, where only a very restricted set of coagonist peptides can support enhancement. Previously proposed qualitative and mathematical models of coagonism studied in the context of CD4 T cells are closely related to the model we have studied here (Li et al., 2004; Krogsaard et al., 2005). These earlier calculations were modeled in the “well-mixed” limit and did not explicitly study the mechanism by which active Lck coreceptor created by agonist pMHC-TCR complexes “found” the vicinal endogenous pMHC-TCR complexes and interacted with them via the coreceptor. Here, among other extensions, we have explicitly included considerations

of protein mobility and modeled the relative motion of integral membrane proteins. The work presented here not only reveals a consistent set of requirements for CD8 and TCR-binding affinities to the disease-related agonist and the self-nonstimulatory pMHC complexes, but also allows us to formulate predictions about coagonism for systems in which it has not yet been systematically investigated and suggests explanations for some enigmatic reports in the literature. Mouse CD8 affinity for MHC is higher on developing thymocytes than on mature CD8⁺ T cells (Daniels et al., 2001; Moody et al., 2001a), and we predict that a larger set of peptides will be coagonists for thymocytes than for peripheral CD8⁺ T cells, especially for T cells bearing D^b-restricted TCR. In addition, a study of LCMV CTL responses showed that H-2D^b-restricted CTL numbers were decreased in H-2K^b knockout mice (Kotturi et al., 2008), a result which is likely caused by the action of mechanism 1 as described in this study, on both positive selection and coagonism of H-2D^b-restricted CTL by H-2K^b-bound coagonists. More importantly, human CD8 affinity for HLA is generally lower than that of mouse CD8 for H-2 MHCI molecules (Cole et al., 2012), so we predict that during human CD8 T cell activation, only a restricted set of nonstimulatory peptides will support activation enhancement, similar to mouse CD4⁺ and D^b-restricted CD8⁺ T cells. Moreover, several HLA alleles confer human disease susceptibility or protection, and the work presented here suggests that different coagonism potentials of distinct MHCI alleles could contribute to this association.

MATERIALS AND METHODS

Plasmids. Single-chain trimer MHC K^bOVA and D^bNP68 (Choudhuri et al., 2005; Palmowski et al., 2009) were cloned into the pcDNA5/TO vector (hygromycin resistance; Invitrogen) for inducible expression of agonist. K^bVSV (Yu et al., 2002) and the TL/K^b chimera (Attinger et al., 2005) were used in the pcDNA3.1 vector. Constitutive expression of D^bNP68, D^bUTY, D^bUTY-YFP fusion, and D^bGP33 was from the pKG4 vector backbone. D227K-E229K CD8-binding mutations (“CD8m”) and the E166K TCR-binding mutation (“TCRm”) were introduced by site-directed mutagenesis. The α 3 domain-swapped construct was made by overlap PCR. All constructs were prepared by Maxiprep (QIAGEN) before transfection.

Antibodies. CD8 α (53-6.7), CD3 ϵ (145-2C11), V α 2 (B20.1), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), OVA-K^b (25.D1.16), H-2K^b (AF6-88.5), H-2D^b (27.11.13), and H-2D^b (28.14.18) were obtained from BD, eBioscience, BioLegend, or Abcam. Anti-H-2TL (18/20) was a gift of C. Lena and H. Cheroutre (La Jolla Institute for Allergy and Immunology, La Jolla, CA).

Mice. OT-I, OT-I *Rag1*^{-/-}, and F5 *Rag1*^{-/-} mice, all on the B6 background (at least 10 backcross generations), were bred at the Scripps Research Institute (TSRI). F5 mice were provided by K. Walsh and M. Oldstone (TSRI). Protocols were approved by the Institutional Animal Care and Use Committee of TSRI.

Peptides. OVA (SIINFELK), VSV (RGYVYQGL), UTY (WMHHNMDLI), and NP3711 (ASNENIDTM) were obtained from Peptides International; R4 (SIIRFEKL), poly-serine (SSYSYSSL), and NP68 (ASNENMDAM) were obtained from the Scripps Peptide Core Facility; and NP34 (ASNENMETM), PA224 (SLENFRAYV), and several other H-2D^b-binding peptides (Table S1) were provided by J. Sidney and A. Sette (La Jolla Institute for Allergy and Immunology) or D. Popkin (TSRI). Slc2a3 (VNTFTVY), Nmt1 (AAYSFYNV),

and Stat3 (ATLVFHNL) were provided by S. Jameson (University of Minnesota, Minneapolis, MN).

CHO cell culture, transfection, and cloning. CHO cells expressing the tetracycline repressor (Trex; Invitrogen) under blasticidin selection (10 $\mu\text{g}/\text{ml}$) were grown in Ham's F12 media with 10% (vol/vol) FCS, 100 U/ml penicillin, and 10 mg/ml streptomycin. CHO cells were passaged with trypsin. Transfection of CHO cells was accomplished with FuGENE 6 (Roche) transfection reagent, and the cells were subsequently drug selected (0.3 mg/ml hygromycin and 0.8 mg/ml G418), cloned by limiting dilution, and screened for MHC transgene expression by FACS. To prepare for a T cell activation experiment, CHO cells were trypsinized, counted using a Beckman Coulter Z1 particle counter, and plated overnight at 20,000 cells per well of a 96-well plate for T cell activation or 200,000 cells per well of a 12-well plate for separate MHC staining. CHO cells were scraped for MHC staining analysis. Doxycycline was added to a final concentration of 50 ng/ml for induction of agonist expression.

RMA-S cell peptide loading. RMA-S cells were maintained in serum-free Aim V media (Invitrogen). The afternoon before an experiment, RMA-S cells near confluence were washed, diluted to 10^6 cells/ml in Aim V media, and plated at 28°C overnight. The next morning, dilute agonist peptide (100 pM OVA or 10 nM for NP68) was added to the cells and incubated for 30 min at 28°C. The cells were washed with Aim V media, counted, diluted to 10^6 cells/ml in Aim V media, and plated at 100,000 cells per well of replica 96-well plates. RMA-S cells incubated overnight at 28°C but without agonist peptide were plated for controls. Nonstimulatory peptides were added to the RMA-S cells over a final concentration range of 10^{-7} to 10^{-4} M, mixed well, and incubated for another 30 min at 28°C. The cells were then washed and resuspended in 100 μl of fresh Aim V media. The cells were then shifted to 37°C, where after 3 h they were ready for the assay. One plate was used for RMA-S MHC staining and one plate used for T cell activation.

T cell activation assays. Lymphocytes were extracted from the TCR transgenic mice and incubated as single-cell suspensions in cRPMI (RPMI medium supplemented with 10% [vol/vol] FCS, 100 U/ml penicillin, 10 mg/ml streptomycin, 292 mg/ml glutamine, 50 mM 2-mercaptoethanol, and 25 mM Hepes, pH 7.3) for 1–3 h at 37°C. Lymphocyte suspensions were diluted to $2\text{--}2.5 \times 10^6$ cells/ml for OT-I cells and $1\text{--}1.5 \times 10^6$ cells/ml for F5 *Rag1*^{-/-} cells. 100 μl of the lymphocyte suspension was added per well of the 96-well plate for CHO and RMA-S activation assays, mixed, allowed to interact at 37°C for the allotted time (typically 3–4 h), chilled on ice, and stained for FACS analysis of activation. For assays of IL-2 in the supernatant, plates were spun at the designated stopping point, and the supernatant was removed and frozen for later analysis by IL-2 ELISA (EMD Millipore).

Flow cytometry. All flow cytometry was conducted on BD LSR-II cytometers at the Scripps Research Flow Cytometry Core Facility. Flow cytometry data were analyzed in FlowJo (Tree Star) and exported where necessary.

Statistical analysis. Data analysis was performed in Excel (Microsoft) and Igor Pro (WaveMetrics). All indications of statistical significance stem from p-values derived from ANOVA analysis with Dunnett's post-test as implemented in Igor Pro.

Online supplemental material. Table S1 lists the library of peptides tested in RMA-S cell assays. The supplemental text includes SSC code, rate parameters, and a list of chemical species for the computer simulations of the kinetic model. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122528/DC1>.

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