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 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 and II-restricted systems, the exact mechanisms by which coagonist peptides can enhance T cell recognition of antigen are not well understood.

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 coagonist pMHCI, 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.
which show relatively high-affinity CD8 binding (Cole et al., 2005; Artyomov 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 pMHC ligands show a weak but possibly biologically significant interaction with TCR (Juang et al., 2010). This suggested that TCRs might play a role in coagons in MHCII-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 MHC II has been suggested to be important for coagons in MHCII-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; Rybakin et al., 2011). Also, coagons pMHCs became antagonists in CD8-negative cells (Stone et al., 2011). These results, along with the lack of peptide specificity for coagons, suggest that non-cognate CD8 coreceptor binding to nonstimulatory pMHC is the dominant mechanism of activation enhancement for MHCII-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 MHCII 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 MHCII-restricted TCR models that have been analyzed in coagons experiments (OT-I [Yachi et al., 2005, 2007; Juang et al., 2010] and 2C [Stone et al., 2011]) recognize H-2Kb or Ld, 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 coagons 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 coagons peptides in MHCII- 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-2Kb and H-2Dd 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-2Kb- and H-2Dd-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 coagons. Mechanism 1 describes CD8 binding to nonstimulatory pMHC as an absolute requirement for coagons and shows that higher-affinity CD8–pMHC interactions can mitigate peptide specificity requirements for coagons and increase the magnitude of enhancement. Mechanism 2 describes how the affinity of CD8 for agonist pMHC influences the requirements for TCR interaction with coagons pMHC. A relatively simple kinetic model of T cell activation is sufficient to account for all coagons 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-pMHCII molecules are produced by recombinant constructs in which the MHC-binding peptide is linked to the N terminus of β2-microglobulin, which in turn is linked to the N terminus of the MHC heavy chain (Yu et al., 2002; Choudhuri et al., 2005; Palmowski et al., 2009). When expressed on the cell surface, these sc-MHCII constructs are very stable and can act as potent agonists for T cells. sc-KbOVA (OVA peptide 257–264, SIINFEKL, on H-2Kb, recognized by OT-I TCR; Högquist et al., 1994) and sc-DNP68 (influenza A/NT/60/68 nucleoprotein 366–374, ASNENMDAM, on H-2Dd, 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-MHCII, 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-KbOVA or sc-DNP68 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-KbOVA in the repressed state was virtually undetectable by flow cytometry with a KbOVA-specific antibody (Fig. 1 A; and not depicted; Porgador et al., 1997),
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-KbVSV and sc-DbUTY domains. When super-transfected into inducible KbOVA-expressing cells (Fig. 3A), we found that the sc-KbVSV CD8-binding mutant (sc-KbVSV-CD8m) did not provide coagonism to OT-I T cells (Fig. 3B) or preselection thymocytes (not depicted). Similarly, sc-DbUTY-CD8m expressed with repressed sc-DbNP68 did not provide coagonism for F5 cells (Fig. 3, C and D). Unexpectedly, the sc-DbUTY-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-2Kb–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 MHCI molecules unless exogenous peptide is provided (Ljunggren et al., 1990; Yachi et al., 2005, 2007). When H-2Kb–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...
We applied this formalism to a description of coagomism, incorporating separate on and off rates for TCR binding to agonist versus nonstimulatory pMHCI. The OT-1 TCR has approximately twofold higher affinity for agonist pMHCI than F5 (K_d of 5.9–6.5 µM for OT-I [Alam et al., 1996, 1999] versus 11 µM for F5 [Willcox et al., 1999]). The kinetics describing these equilibrium constants are markedly different. The on and off rates for OT-1 TCR–binding K_OVA 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 DNP68 based on the off rate and equilibrium affinity reported in the literature (k_{off} = 0.8/s, K_d = 11 µM, k_{on} = 72,727/M/s, calculated; Willcox et al., 1999). Therefore, we varied the TCR kinetics for agonist pMHCI 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 KbOVA 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^d nonstimulatory pMHCI, and the YFP fluorescence was used here to report on sc-D^dUTY 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.
coagonism on CD8 binding to the nonstimulatory pMHCI, and Fig. 3F showing the lack of F5 coagonist peptide specificity for peptides bound to the high CD8-binding affinity MHCI protein H-2K<sup>B</sup>.

**Minimal contribution of OT-I TCR recognition of nonstimulatory pMHCI to coagonism**

Previous studies showed no peptide dependence for coagonism by nonstimulatory pMHCI, 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<sup>B</sup>-OVA with one nonstimulatory K<sup>B</sup> 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 α<sub>1</sub>α<sub>2</sub>-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 KbVSV by VSV-specific CTL (Sun et al., 1995) and of KbOVA by OT-I CTL (not depicted). Second, we used a chimera between the α<sub>1</sub>α<sub>2</sub> domains of the MHCI<sup>B</sup> 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 α<sub>3</sub> from H-2K<sup>B</sup>, enabling normal CD8 binding (Attinger et al., 2005). Both K<sup>B</sup>VSV with the
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 (SSSYSYSSL), which should lack any TCR contact residues (Hogquist et al., 1994). Each of these peptides acted as a coagonist in an H-2Kb expression-dependent manner (Fig. 4 D), indicating that for the OT-1 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-KbOVA-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-KbOVA (Fig. 5 B and not depicted). Activation of OT-I T cells was rescued by sc-KbVSV but not by E166K TCR-binding site mutation (sc-KbVSV-TCRm) and the TL/Kb chimera provided significant coagonism for recognition of sc-KbOVA (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-KbVSV-TCRm mutation (not depicted).

When we plotted CD69 expression data for OT-I T cells versus MHCI expression on CHO cells for repressed and induced sc-KbOVA, or repressed sc-KbOVA plus sc-KbVSV, we found a linear relationship (Fig. 4 C). Repressed sc-KbOVA plus sc-KbVSV-TCRm revealed a minor defect in coagonism when TCR binding to nonstimulatory pMHC was attenuated. In contrast, the repressed sc-KbOVA plus the sc-KbVSV-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 Kβ but to lack TCR interaction residues, abrogated coagonism (Juang et al., 2010).

Figure 4. Minor contribution of OT-1 TCR binding to nonstimulatory pMHC in coagonism. Inducible sc-KbOVA CHO cells were super-transfected with constitutive sc-KbVSV TCR-binding mutant (sc-KbVSV-TCRm) or H-2TL-Kb (TL/Kb) and used as APCs to assess coagonism of OT-I 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) CHO cell staining for either H-2Kb 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 CD69hi OT-I T cells (n = 10 mice) after 3-h exposure to the CHO APCs in A. (C) Percentage of CD69hi OT-I T cells plotted as a function of the corresponding CHO APC H-2Kb expression, as indicated (n = 10 mice for CD69 data and n = 3 for H-2Kb expression on CHO cell APCs). A correction factor of 2.8 is applied to the H-2Kb staining of the repressed KbOVA plus KbVSV-CD8m to account for attenuated anti-Kb 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 CD69hi OT-I T cells plotted as a function of Kb expression of RMA-S cells loaded with increasing concentrations of nonstimulatory peptides R4, VSV, and SSSYSYSSL, as indicated (n = 2 for both CD69 and H-2Kb 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-KbVSV-TCRm (Fig. 5 B). This was in stark contrast to earlier results with repressed wild-type sc-KbOVA, in which sc-KbVSV-TCRm was a strong coagonist (Fig. 4, B and C).

To further probe this discrepancy in coagonism by sc-KbVSV-TCRm, we took advantage of the fact that CD8 has lower affinity for H-2D\(\beta\) than for H-2K\(\beta\) (Moody et al., 2001b; Huang et al., 2007). The CD8-binding affinity is a property of the MHCI \(\alpha\)3 domain, as a chimera of D\(\beta\)\(\alpha\)1\(\alpha\)2-Kb\(\alpha\)3 binds CD8 similarly to the native H-2K\(\beta\) molecule (Moody et al., 2001b). We therefore constructed and subsequently expressed inducible sc-KbOVA-D\(\alpha\)\(\alpha\)3 in CHO cells and super-transfected with sc-KbVSV or sc-KbVSV-TCRm. The altered \(\alpha\)3 domain epitope present in sc-KbOVA-D\(\alpha\)\(\alpha\)3 showed attenuated binding to the anti–H-2K\(\beta\) antibody, visible in the different anti-H-2K\(\beta\) staining of the induced sc-KbOVA-D\(\alpha\)\(\alpha\)3 versus wild-type sc-KbOVA (Fig. 5 C). The amount of H-2K\(\beta\)

![Figure 5. Modulation of KbOVA CD8-binding affinity and associated changes in coagonism by KbVSV-TCRm.](image)

Inducible sc-KbOVA CD8-binding mutant (sc-KbOVA-CD8m) CHO cells were super-transfected with constitutive sc-KbVSV or sc-KbVSV-TCRm and used as APCs to assess coagonism of OT-I 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\(\beta\) stain of CHO cell APCs. Color coding is as shown in B. (B) Percentage of CD69\(\text{hi}\) OT-I T cells (\(n = 10\) mice) in response to the CHO cell APCs in A. Inducible domain-swapped sc-KbOVA-D\(\alpha\)\(\alpha\)3 CHO cells were also super-transfected with both sc-KbVSV or sc-KbVSV-TCRm and used as APCs to assess coagonism of OT-I T cells. (C) Repressed and induced KbOVA (purple and green, respectively) and KbOVA-D\(\alpha\)\(\alpha\)3 (black and blue, respectively) constructs were analyzed as a function of both OVA-K\(\beta\) and total H-2K\(\beta\) staining, whereas repressed KbOVA plus KbVSV-TCRm [red], repressed KbOVA-D\(\alpha\)\(\alpha\)3 plus KbVSV [orange], and repressed KbOVA-D\(\alpha\)\(\alpha\)3 plus KbVSV-TCRm [gray] were analyzed with H-2K\(\beta\) 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\(\text{hi}\) gate. (E) Percentage of CD69\(\text{hi}\) 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\(\beta\) staining of the associated CHO APCs. Repressed KbOVA plus KbVSV-TCRm and repressed KbOVA-D\(\alpha\)\(\alpha\)3 plus KbVSV-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-KbOVA and sc-KbOVA-TCRm were comparable (Fig. 5 C). The CD69 expression profiles for OT-I CD8+ T cells (Fig. 5 D) and quantification of CD69+ T cells (Fig. 5 E) clearly showed the lack of statistically significant coagonsism for sc-KbOVA-Dα3 plus sc-KbOVA-TCRm compared with either of the significant responses found for the sc-KbOVA-Dα3 plus wild-type sc-KbOVA or wild-type sc-KbOVA plus sc-KbOVA-TCRm.

Both repressed and induced sc-KbOVA-Dα3—expressing CHO cells elicited systematically higher CD69 expression than the wild-type sc-KbOVA in the responder OT-I T cells. This result was unexpected, but we examined several different clones of the inducible sc-KbOVA-Dα3 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 KbOVA-Dα3, 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-KbOVA-TCRm coagonists for the recognition of sc-KbOVA-Dα3 versus recognition of wild-type sc-KbOVA, we calculated the activation enhancement of OT-I T cells (percent CD69+ in response to repressed agonist with sc-KbOVA-TCRm minus percent CD69+ in response to repressed agonist alone; Fig. 5 F). This enabled comparison of coagonsism by sc-KbOVA-TCRm with these two different agonists, revealing that the nonstimulatory ligand with reduced TCR interaction provided greater coagonsism for recognition of sc-KbOVA than for chimeric sc-KbOVA-Dα3. 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 coagonsism.

Coagonsist activity by H-2Db for F5 T cells requires TCR discrimination of nonstimulatory pMHCs

The two antibodies used to stain sc-H-2Db-transfected CHO cells in this study recognize different H-2Db epitopes. The clone 27.11.13 recognizes a peptide-dependent conformational epitope, whereas the 28.14.8 clone recognizes the H-2Db α3 domain independent of peptide (Fig. 6 A; Palmowski et al., 2009). We prepared sc-DbGP33 and sc-DbGP33-TCRm sc-MHC molecules, expressed them constitutively in CHO cells, and stained with both 27.11.13 and 28.14.8 anti-Db antibodies. These results show that the 27.11.13 antibody staining was diminished by the TCR-binding site mutation on H-2Db (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-DbUTY 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+ F5 T cells after activation with repressed sc-DbNP68, repressed sc-DbNP68 plus sc-DbUTY, or induced sc-DbNP68 revealed a very strong correlation between the percentage of CD69+ 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 coagonsism 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 coagonsism/activation enhancement between the DbNP68-F5 and KbOVA–OT-I systems.

To probe the question of the specificity of coagonsism of F5 T cells for nonstimulatory H-2Db pMHC, we screened a small library of ~40 H-2Db–binding peptides (Table S1) for H-2Db binding and the ability to act as coagonsists for F5 T cells when presented by RMA-S, identifying only two variant influenza peptides as coagonsists (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 (SSLKENFRAVVY, a Db-binding peptide which did not act as coagonsist in our screen) or with 10 nM NP68 agonist peptide. H-2Db was stabilized on RMA-S cells by binding of NP34, NP371I, and PA224, but the H-2Dk loaded with 10 nM NP68 agonist peptide 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-2Dk (Fig. 6 D). RMA-S cells treated with 10 nM NP68 peptide activated ~15% of F5 T cells. To test for coagonsism 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-2Dk (Fig. 6 E), and when used to stimulate F5 T cells, both NP34 and NP371I, but not PA224, showed strong coagonsist activity (Fig. 6 F).

Because of the positive coagonsist results with sc-DbUTY—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-2Db on RMA-S cells as measured by anti-Dα3 staining with antibody clone 28.14.8, but staining with the complementary anti-Dβ 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\(^{b}\)NP68 CHO cells were super-transfected with constitutive sc-D\(^{\alpha}\)GP33, sc-D\(^{\alpha}\)GP33-TCR\(^{m}\), or sc-D\(^{\alpha}\)UTY 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 R\(^{2}\) 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-µM additions of peptides were as follows: blue, PA224; red, NP34; and green, NP371I. (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\(^{hi}\) 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 µM 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 (***, P < 0.001) 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 NP371I and UTY, data are representative of two independent experiments.
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 coagonsm to the nature of the nonstimulatory peptide originates in part from differences in CD8 affinity for agonist pMHCI, we modified the computational model described above to introduce separate koff, CD8 values for agonist and nonstimulatory pMHCI 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 pMHCI in responses to low amounts of their specific agonist pMHCIIs, 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 (koff, CD8 for agonist pMHCI 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 pMHCI (koff, TCR for nonstimulatory pMHCI constant). T o test the differences of agonist plus nonstimulatory peptides remained above the threshold of 10. For intermediate koff, CD8 for agonist pMHCI, the nonstimulatory pMHCI–TCR off rate at low koff, CD8 (high CD8 affinity) for agonist pMHCI. This indicates that the kinetics of the TCR–agonist pMHCI interaction play a role in the requirement for specific coagonsms. However, at the smallest values of koff, CD8 for agonist pMHCI, coagonsm of F5 was still significant and the absolute values of TCRpp for the simulations fell below the activation threshold of TCRpp = 10. For the TCR with F5-like affinity for agonist pMHCI (Fig. 7 B), there was a steeper dependence on nonstimulatory pMHCI–TCR off rate at low koff, CD8 (high CD8 affinity) for agonist pMHCI. This indicates that the kinetics of the TCR–agonist pMHCI interaction play a role in the requirement for specific coagonsms. However, at the smallest values of koff, CD8 for agonist pMHCI, coagonsm of F5 was still significant and the absolute values of TCRpp for the simulations fell below the activation threshold of TCRpp = 10. For intermediate koff, CD8 for agonist pMHCI, the nonstimulatory pMHCI gave no appreciable activation. At the highest values of koff, CD8 for agonist pMHCI, only the highest affinity nonstimulatory peptides gave any appreciable activation. At the highest values of koff, CD8 for agonist pMHCI, the nonstimulatory pMHCI gave no coagonsm for F5. These computational results provide a conceptual framework describing our experimental results, in which we found that CD8 affinity for agonist pMHCI is a key factor for the dependence of coagonsm on the TCR’s interaction with the nonstimulatory pMHCI.

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-pMHCI agonists for specific TCRs was coupled with constitutive expression of nonstimulatory sc-pMHCI. Using OT-I and F5 TCRs, we tested combinations of H-2Kk and H-2Dd, as well as mutations in the cognate or nonstimulatory MHC, to identify the interactions important in coagonsm. In addition, we used computer

ΔTCRpp = TCRppagonist, nonstimulatory − TCRppagonist 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, coagonsm showed little dependence on nonstimulatory pMHCI quality for small values of koff, CD8 for agonist pMHCI (i.e., high CD8 affinity; Fig. 7 A). With intermediate or weak CD8 binding to agonist pMHCI, however, there was stronger dependence on the quality of the nonstimulatory pMHCI. At these weak CD8 agonist pMHCI–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 pMHCI (Fig. 7 B), there was a steeper dependence on nonstimulatory pMHCI–TCR off rate at low koff, CD8 (high CD8 affinity) for agonist pMHCI. This indicates that the kinetics of the TCR–agonist pMHCI interaction play a role in the requirement for specific coagonsms. However, at the smallest values of koff, CD8 for agonist pMHCI, coagonsm 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 koff, CD8 for agonist pMHCI, only the highest affinity nonstimulatory peptides gave any appreciable activation. At the highest values of koff, CD8 for agonist pMHCI, the nonstimulatory pMHCI gave no coagonsm for F5. These computational results provide a conceptual framework describing our experimental results, in which we found that CD8 affinity for agonist pMHCI is a key factor for the dependence of coagonsm on the TCR’s interaction with the nonstimulatory pMHCI.
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 MHCI 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 (CD8\(^m\)) in the \(\alpha_3\) domains of sc-K\(^b\)VSV and sc-D\(^b\)UTY, we found that CD8 binding to nonstimulatory pMHC is an absolute requirement for coagonism. In the F5 system, the sc-D\(^b\)UTY-CD8\(^m\) 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\(^b\)OVA, we detected a very mild coagonist defect using sc-K\(^b\)VSV-TCR\(^m\) 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\(^b\)OVA, using sc-K\(^b\)OVA-D\(\alpha_3\) chimera and CD8\(^m\) constructs, we found that the requirement for TCR recognition of the nonstimulatory sc-K\(^b\)VSV became more stringent as the ability of CD8 to bind agonist sc-K\(^b\)OVA was reduced. sc-K\(^b\)VSV-TCR\(^m\) gave slightly reduced coagonism compared with wild-type sc-K\(^b\)VSV for agonist sc-K\(^b\)OVA stimulation, but sc-K\(^b\)VSV-TCR\(^m\) coagonism for sc-K\(^b\)OVA-D\(\alpha_3\) agonist was significantly reduced. sc-K\(^b\)VSV-TCR\(^m\) was unable to provide coagonism for sc-K\(^b\)OVA-CD8\(^m\). Thus, loss of CD8 binding to sc-K\(^b\)OVA 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\(^b\)UTY 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\(^b\)NP68 should be more peptide specific than OT-I recognition of K\(^b\)OVA. 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 \(\sim\)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\(^b\)UTY 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\(^b\)UTY 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-2Db 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 nonstimulatory pMHC that may be nearby. The kinetic model validated in this study suggests that agonists presented by MHCII 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-2Db 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; Krosggaard 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 Dα-restricted TCR. In addition, a study of LCMV CTL responses showed that H-2Dβ–restricted CTL numbers were decreased in H-2Kb 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-2Dβ–restricted CTL by H-2Kβ–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 Dα-restricted CD8α 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 KbOVA and DαNP68 (Choudhuri et al., 2005; Palmowski et al., 2009) were cloned into the pcDNA5/TTO vector (hygromycin resistance; Invitrogen) for inducible expression of agonist. KbOVA (Yu et al., 2002) and the TL/Kε chimera (Attinger et al., 2005) were used in the pcDNA3.1 vector. Constitutive expression of DNP68, DPUTC, DPUTY–YFP fusion, and DPG3 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.

Antibodies. CD8α (53.6-7), CD3ε (145-2C11), Vα2 (B20.1), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), OVA-Kb (25.D1.16), H-2Kb (Af6-88.5), H-2Dβ (27.11.13), and H-2Dβ (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 Rag−1−/−, and F5 Rag−1−/− 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 (SIINFEKL), VSV (RGYVYQGL), UTY (WMHHNMDLI), and NP371I (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 (SSLENFRAYV), and several other H-2Dβ–binding peptides (Table S1) were provided by J. Sidney and A. Sette (La Jolla Institute for Allergy and Immunology) or D. Popkin (TSRI). Slc2a3 (VNTFTVV), Nmt1 (AAYSFYNV), PA224 (SSLENFRAYV), and several other H-2Dβ–binding peptides (Table S1) were provided by J. Sidney and A. Sette (La Jolla Institute for Allergy and Immunology) or D. Popkin (TSRI). Slc2a3 (VNTFTVV), Nmt1 (AAYSFYNV),
Hepes, pH 7.3) for 1–3 h at 37°C. Lymphocyte suspensions were diluted to

Lymphocytes were extracted from the TCR

T cell activation assays.

RMA-S MHC staining and one plate used for T cell activation.

resuspended in 100 µl of fresh Aim V media. The cells were then shifted to

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

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 sep-

ate MHC staining. CHO cells were scraped for MHC staining analysis.

Dosycycline 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 10⁶ 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 10⁶

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 10⁻⁹ 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 eRPMI (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

2–2.5 × 10⁶ cells/ml for OT-I cells and 1–1.5 × 10⁶ cells/ml for FS RAG-1⁻⁻ 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 cytome-

eters 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 imple-

mented 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 param-

eters, and a list of chemical species for the computer simulations of the ki-

netic model. Online supplemental material is available at http://www.jem.

org/cgi/content/full/jem.20122528/DC1.

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