T cell activation enhancement by endogenous pMHC acts for both weak and strong agonists but varies with differentiation state

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T cells are extremely sensitive in their ability to find minute amounts of antigenic peptide in the midst of many endogenous peptides presented on an antigen–presenting cell. The role of endogenous peptides in the recognition of foreign peptide and hence in T cell activation has remained controversial for CD8+ T cell activation. We showed previously that in a CD8+ T cell hybridoma, nonstimulatory endogenous peptides enhance T cell sensitivity to antigen by increasing the coreceptor function of CD8. However, others were not able to detect such enhancement in naive and activated CD8+ T cells. Here, we show that endogenous peptides substantially enhance the ability of T cells to detect antigen, an effect measurable by up–regulation of activation or maturation markers and by increased effector function. This enhancement is most pronounced in thymocytes, moderate in naive T cells, and mild in effector T cells. The importance of endogenous peptides is inversely proportional to the agonist activity of the stimulatory peptide presented. Unlike for CD4+ T cells, the T cell receptor of CD8+ T cells does not distinguish between endogenous peptides for their ability to enhance antigen recognition.

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Abbreviations used: APL, altered peptide ligand; cSMAC, central supramolecular activation cluster; DP, double-positive; IS, immunological synapse(s); K^b, H-2K^b; MFI, mean fluorescent intensity; pMHC, peptide–MHC; VSV, vesicular stomatitis virus.

Antigen-specific T cell activation requires the interaction of TCRs with specific peptide–MHC (pMHC) complexes expressed on an APC. During their development, T cells are selected for weak or rare interactions with self–pMHC complexes presented in the thymus (1–3), and mature T cells require the presence of self-pMHC complexes in the periphery to survive (4–6). Recent data indicate that recognition of self-pMHC complexes enhances recognition of antigenic pMHC (7–11). Endogenous peptides enhance antigen reactivity of naive CD4+ T cells, such that interruption of T cell contact with self-pMHC ligands leads to a rapid decline in signaling and sensitivity to foreign antigens (7). An effect of endogenous peptides in contributing to CD4+ T cell activation has been noted at low antigen concentrations (8, 9). This led to a "pseudodimer" model of T cell activation, where CD4 acts as a bridge between two TCRs: one recognizing antigen and the other interacting with endogenous pMHC class II complexes (9, 12, 13).

Recently, we found that the presence of endogenous (or exogenous) nonstimulatory peptides enhances the formation of conjugates between APCs and CD8+ T cell hybridomas, and increases the antigen-induced interaction between CD8 and TCR (10). However, work with naive and antigen-experienced CD8+ T cells suggested a negligible effect for endogenous peptides on antigen recognition (14). In CD4+ T cells, only a subset of endogenous peptides aid in the recognition of antigen, suggesting that TCR recognizes endogenous pMHC (9). In contrast, our studies using CD8+ T hybridomas showed that each of the tested endogenous or exogenous nonstimulatory peptides was capable of this feat, and that CD8 became concentrated in the immunological synapse (IS) even without the influence of antigen, suggesting that the CD8–pMHC interaction is responsible for the enhancing effect of the endogenous pMHC (10). Recent data confirm that the CD8 interaction with nonstimulatory pMHC aids in recognition of antigenic pMHC (11). We proposed a "pre-concentration" model for the role of endogenous pMHC in aiding antigen recognition. In this view, CD8 plus associated Lck and

The online version of this article contains supplemental material.

pMHC are concentrated to the synapse as a result of the non-cognate CD8–pMHC interaction. Higher concentration of pMHC would enhance the ability of TCR to find its ligand, and the high concentration of CD8 (plus Lck) would enhance signal transduction (10).

These contrasting data suggest different roles for TCR in the recognition of endogenous pMHC by CD4$^+$ or CD8$^+$ T cells. To try to understand these controversial observations, we studied the response of different T cell populations to APCs presenting either an agonist peptide alone or in combination with different nonstimulatory peptides. We observed that nonstimulatory peptides were most effective in enhancing antigen recognition of less differentiated cells, such that their ability to enhance antigen recognition was most distinct in thymocytes, moderate in naive T cells, and mild in effector T cells. The recognition of weak agonists was more dependent on nonstimulatory peptides than was recognition of strong agonists. All of the tested nonstimulatory peptides were able to enhance T cell activation by weak or strong agonists, contrary to a prediction of the pseudodimer model (13) and suggesting that CD8$^+$ T cells do not require specific TCR recognition of the nonstimulatory pMHC to use them to enhance sensitivity of antigen recognition. Therefore, there seems to be an important difference between CD4$^+$ and CD8$^+$ T cells in their ability to take advantage of endogenous peptides.

RESULTS
Nonstimulatory peptide–induced enhancement of antigen recognition depends on the differentiation status of the T cell

We previously showed that nonstimulatory peptides enhanced antigen recognition by CD8$^+$ OT-I T cell hybridomas when presented together with an agonist peptide (10). To extend these findings to T cells proper, we analyzed up-regulation of the activation marker CD69 on thymocytes, naive T cells, and effector CD8$^+$ T cells. To investigate pre-selection CD4$^+$CD8$^+$ double-positive (DP) thymocytes, cells were isolated from the thymi of OT-I TCR transgenic *Tap1* KO mice, where development is arrested before positive selection (15). Naive T cells were harvested from the lymph nodes of OT-I transgenic mice, and activated T cells were obtained from a short-term antigen-stimulated culture of ex vivo OT-I T cells. OT-I T cells specifically recognize an OVA-derived peptide presented in the context of H-2K^b (K^b). A series of peptides that do not stimulate OT-I T cells in the context of K^b has been characterized (15–17).

To obtain APCs in which the only difference is the presence or absence of nonstimulatory peptides, we used *Tap2*-deficient RMA-S cells. These lack the ability to load most endogenous peptides onto their MHC class I molecules, resulting in a cell surface level of MHC class I of ∼5% of the *Tap2*$^+$ parental RMA cell line (18–20). The addition of exogenous peptides at 29°C stabilizes pMHC complexes on the cell surface, which therefore present almost exclusively the particular peptide(s) added (20). To compare T cell responses to RMA-S cells expressing different levels of K^b-OVA in the

absence or presence of different nonstimulatory peptides, we used the mAb 25-D1.16 that specifically binds to OVA, but not to the nonstimulatory peptides, in complex with K^b (21). Different peptides have different abilities to stabilize pMHC. Therefore, we titrated the different nonstimulatory peptides such that they would give equal K^b levels (∼25,000 molecules per cell). We titrated OVA such that in the presence of a nonstimulatory peptide the total MHC density was kept at a constant high level, whereas in the absence of any nonstimulatory peptide the total MHC density depended on the OVA-K^b amount, and therefore was lower than in the presence of a nonstimulatory peptide (except at the highest concentrations of OVA) (10). The data from the T cell stimulation assays were plotted as a function of 25-D1.16 (anti–K^b-OVA) staining intensity as measured by flow cytometry. Any differences between the groups in the OVA-K^b dose response curves were therefore a result of the presence of the nonstimulatory peptide or increased MHC density per se.

In CD4$^+$ T cells only certain nonstimulatory peptides have been shown to enhance antigen recognition (9), whereas our experiments showed that each of the nonstimulatory K^b-binding peptides tested enhanced recognition as defined by T cell–APC couple formation, TCR endocytosis, and the induced TCR-CD8 interaction (10). Therefore, it was of interest to validate our earlier findings in ex vivo CD8$^+$ T cell populations, and for other markers of T cell activation. We therefore used a nonstimulatory peptide derived from vesicular stomatitis virus (VSV) and a series of endogenous nonstimulatory peptides (17) assaying for CD69 up-regulation, an early marker for thymocyte or T cell activation through the TCR (22). Simultaneous presentation of nonstimulatory peptides with agonist peptide was able to enhance CD69 up-regulation. After a 5-h stimulation, this was most clear in pre-selection DP thymocytes (Fig. 1 and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20062610/DC1). For naive CD8$^+$ T cells, the percentage of CD69$^+$ cells did not change greatly, although the amount of CD69 on the cell surface was considerably increased by the nonstimulatory peptides (Fig. 1 H). The effect of nonstimulatory peptides was more evident at earlier time points (see next section). CTLs already expressed some CD69, and neither the percentage of CD69$^+$ cells nor the amount of CD69 was substantially changed by the presence of the nonstimulatory peptides after 5 h of stimulation (but see below). Similar data have been obtained with four more endogenous peptides (unpublished data). Thus, the effect of nonstimulatory peptides decreased during differentiation. A similar phenomenon was observed for TCR endocytosis (unpublished data). Therefore, the extent to which nonstimulatory peptides enhance antigen recognition was dependent on the differentiation status of the CD8$^+$ T cell.

Nonstimulatory peptides are most important in the early stages of antigen recognition

We have previously suggested that nonstimulatory peptides act by helping the TCR to find the antigenic pMHC by concentrating pMHC to the IS through interactions with CD8 (10).
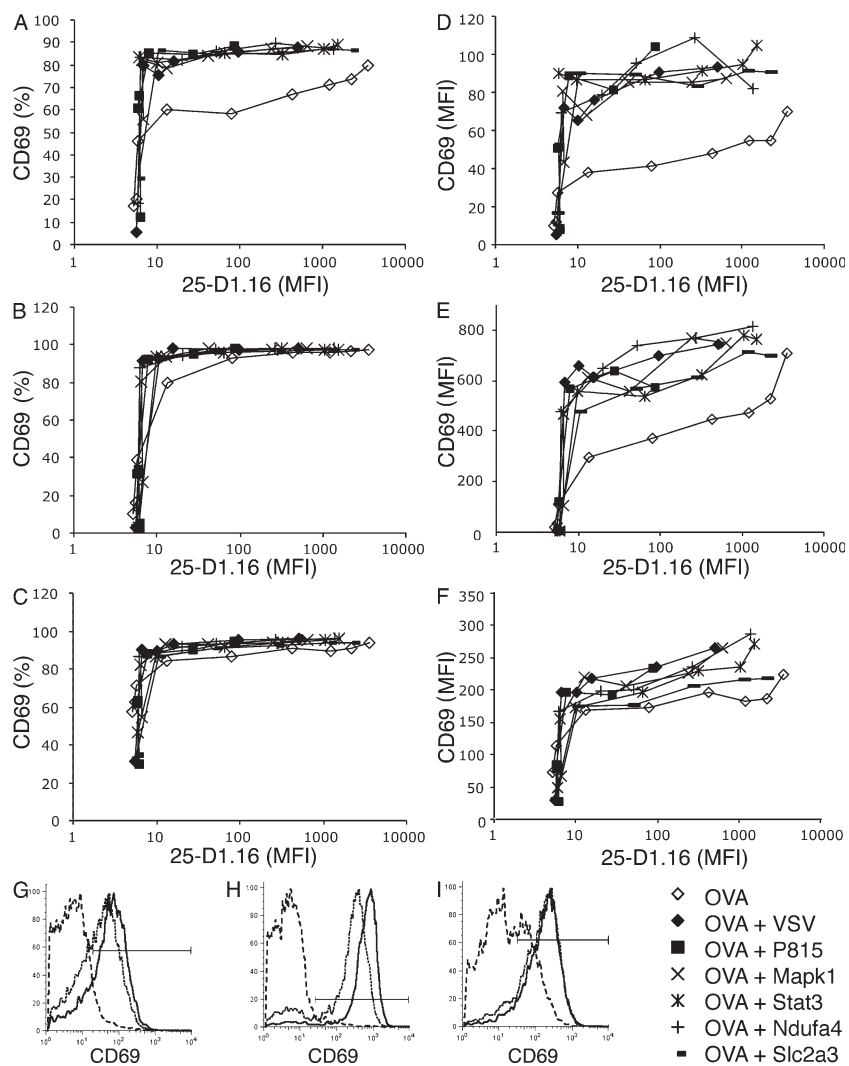
Figure 1. Nonstimulatory peptides increase antigen recognition by T cells at different differentiation states. RMA-S cells were loaded with various amounts of OVA alone or OVA plus nonstimulatory peptide. The RMA-S cells were allowed to interact with thymocytes (A, D, and G), naive T cells (B, E, and H), or effector CTLs (C, F, and I) for 5 h. The cells were stained with anti-CD8 and anti-CD69, and the level of CD69 was assessed by flow cytometry. The results are shown as percentage of CD69+ cells (A, B, and C) or as mean fluorescent intensity (MFI) of CD69 staining (D, E, and F) from CD8 gated cells at different OVA-K^b concentrations. For thymocytes, the overall population behaves uniformly, and the graphs for MFI and percent CD69^{hi} populations look similar. For naive cells, CD69 up-regulation is bimodal. However, in the presence of nonstimulatory peptide, the MFI of the CD69^{hi} cells is higher than in the absence of nonstimulatory peptides, and therefore the two groups display bigger differences when presented as MFI as opposed to percent CD69^{hi}. Example histograms (G, H, and I) are shown for each of the cell subsets. In the histograms, the dashed lines correspond to thymocytes or T cells incubated with RMA-S cells without added peptide, the dotted lines to cells incubated with RMA-S cells loaded with OVA alone, and the solid black lines to cells incubated with RMA-S cells loaded with OVA together with a large excess of the nonstimulatory VSV peptide. The example histograms have been chosen in such a way that the OVA-alone group displays more antigen than the group with the nonstimulatory peptide. (Fig. S1 shows an OVA-K^b–matched comparison.) The results are representative of at least three independent experiments.

Therefore, we wondered whether giving the TCR a longer time to find antigenic pMHC would render the effect of non-stimulatory peptides negligible. We also wondered whether the effect of endogenous peptides might be stronger at different time points after stimulation. We measured CD69 up-regulation at 2, 7, and 20 h in naive T cells (Fig. 2), CTLs (Fig. S2, A–C, available at http://www.jem.org/cgi/content/full/jem.20062610/DC1), and pre-selection DP thymocytes (Fig. S2, D–F). For the naive CD8$^+$ T cells, the effect of the nonstimula-

tory peptide was strongest at early time points, but by 20 h even minute amounts of OVA peptide were able to induce CD69 up-regulation so that any effect of nonstimulatory peptide was undetectable. The effect of nonstimulatory peptides faded faster in CTLs, being gone by 7 h. In thymocytes, there was an observable difference in response in the presence or absence of nonstimulatory peptides even at 24 h. This finding suggests that nonstimulatory pMHC complexes are most important in the early stages of recognition of agonist pMHC but that, given
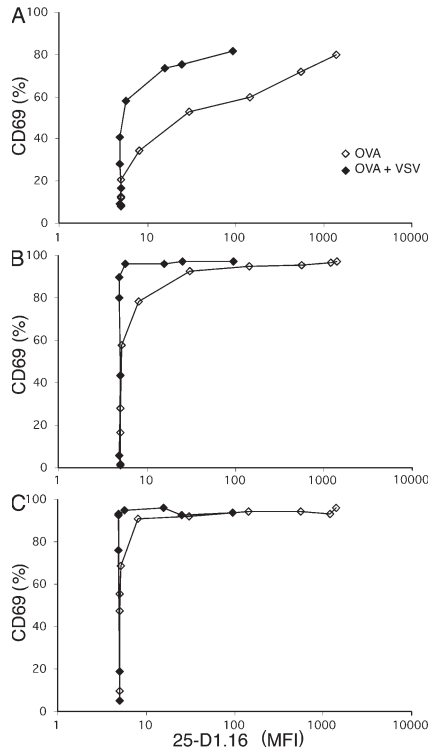
Figure 2. The effect of nonstimulatory peptides is most pro-nounced at early time points. RMA-S cells were loaded with various amounts of OVA alone or OVA plus VSV. The RMA-S cells were allowed to interact with naive T cells for 2 (A), 7 (B), or 20 h (C). The cells were stained with anti-CD8 and anti-CD69, and the level of CD69 was assessed by flow cytometry. The results are shown as percentage of CD69^{+} cells from CD8 gated cells at different OVA-K[b] concentrations. The results are representative of four independent experiments.

sufficient time, even very low numbers of antigenic pMHC (in the presence of the low level of endogenous pMHC expressed on RMA-S cells) can be enough to stimulate the T cell.

The effect of nonstimulatory pMHC on antigen recognition is more pronounced with weaker agonists

Given the strong effects of nonstimulatory peptides on recognition of the strong agonist ligand OVA, we wondered whether nonstimulatory peptides would affect recognition of weaker agonist altered peptide ligands (APLs). We found that the single amino acid variants of OVA, Q4 and T4, were recognized by 25-D1.16, and therefore we could use these peptides similarly to OVA. Q4 and T4 are both weaker agonists than OVA, with T4 being much weaker than Q4 (23). Indeed, T4 is at the border between positive and negative selection in the thymus, and as such is one of the weakest ligands that in physiological circumstances would be expected to give a functional response upon recognition by the OT-I TCR. Up-regulation of the activation markers CD25 and CD69 on naive OT-I T cells by Q4 and T4 was delayed and required a higher peptide concentration compared with OVA for stimulation (T4<Q4<OVA) (Fig. 3, A and B). When TCR down-regulation was used as

a direct indicator of TCR engagement, both Q4 and T4 were weaker ligands than OVA (Fig. 3 C). The presence of the non-stimulatory peptide VSV resulted in enhanced CD25 up-regulation by both Q4 and T4, whereas the effect on OVA-induced CD25 up-regulation was almost negligible (Fig. 3 A). Increased up-regulation of CD69 in the presence of nonstimulatory peptides was seen with both APLs; the enhancement was the most pronounced for the weakest ligand, T4 (Fig. 3 B). In addition, the presence of nonstimulatory peptide during recognition of OVA and both APLs enhanced TCR down-regulation on OT-I CTL, with the effect being small for OVA, stronger for Q4, and strongest for T4 (Fig. 3 C). Therefore, nonstimulatory peptides are more important in the recognition of weaker agonists.

Nonstimulatory peptides enhance antigen recognition equally well regardless of the agonist strength

A prediction of the pseudodimer model for T cell activation is that as the half-life of TCR for agonist decreases, a smaller subset of the available endogenous peptides are able to act as "co-agonists" (13). For CD4^{+} T cells, stronger binding agonists were able to synergize with a larger fraction of endogenous
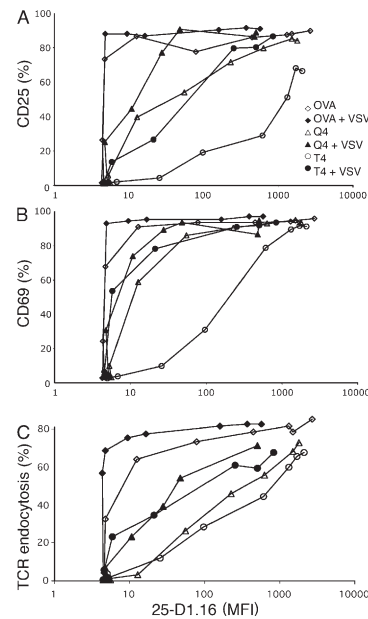

Figure 3. The effect of nonstimulatory peptides is increased with weaker agonists. RMA-S cells were loaded with various amounts of OVA, Q4 or T4 alone, or the same peptides plus VSV, respectively. The RMA-S cells were allowed to interact with naive T cells for 24 (A) or 5 h (B), or with effector cells for 3 h (C). The cells were stained with anti-CD8 in combination with anti-CD25 (A), anti-CD69 (B), or anti-Vα2 (C), and the level of these molecules was assessed by flow cytometry. The results are shown as percentage of CD25^{+} (A) and percentage of CD69^{+} (B) CD8 cells at different OVA, Q4, or T4-K[b] concentrations. The TCR down-regulation data (C) are shown as percentages of Vα2 expression on the surface of CD8 cells incubated with RMA-S cells presenting different amounts of OVA, Q4, or T4-K[b] compared with cells incubated with RMA-S cells in the absence of an exogenously added peptide. The results are representative of four independent experiments.

peptides compared with weaker binding agonists (9, 13). Therefore, we wondered whether the universal ability of nonstimulatory peptides to increase the sensitivity of CD8$^+$ T cell recognition in the presence of OVA-K^b (Fig. 1) was due to the comparatively high affinity interaction between OT-I TCR and OVA-K^b (24, 25). We tested the ability of several different nonstimulatory peptides to enhance antigen recognition of OVA (Fig. 4 A), the weak agonist Q4 (Fig. 4 B), the very weak agonist T4 (Fig. 4 C), and a very weak agonist/antagonist G4 (unpublished data). Similarly to OVA, the recognition of each APL was enhanced in the presence of all the tested nonstimulatory peptides. Therefore, unlike CD4$^+$ T cells, the ability of different nonstimulatory peptides to enhance antigen recognition in CD8$^+$ T cells is not dependent on the quality of the nonstimulatory peptide.
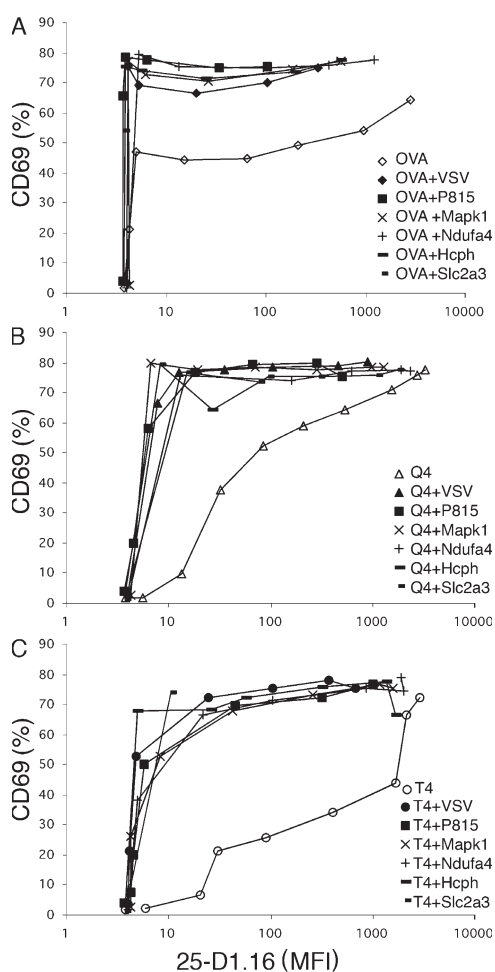

Figure 4. The ability of nonstimulatory peptides to enhance antigen recognition is independent of the agonist strength. RMA-S cells were loaded with various amounts of OVA (A), Q4 (B), or T4 (C) alone or together with a nonstimulatory peptide. The RMA-S cells were allowed to interact with DP thymocytes for 5 h. The cells were stained with anti-CD8 and anti-CD69, and the level of CD69 was assessed by flow cytometry. The results are shown as percentage of CD69$^+$ CD8 gated cells at different OVA, Q4, or T4-K^b concentrations. The results are representative of at least two independent experiments.

The effect of nonstimulatory peptides on antigen recognition depends on quantity but not quality of the nonstimulatory pMHC

To test whether different nonstimulatory peptides had a different threshold for antigen recognition enhancement, we titrated the nonstimulatory peptides. For these experiments, the RMA-S cells were first loaded with antigen, which was then carefully washed away. The cells were divided into different groups into which the nonstimulatory peptides were titrated, followed by incubation and washing. This way we obtained cells that after incubation with different amounts of nonstimulatory peptides expressed the same amount of antigenic peptide (Fig. S1 and unpublished data). T cell sensitivity to antigen depended on the amount of nonstimulatory pMHC. The different nonstimulatory peptides gave similar enhancement at similar cell surface densities of K^b (Fig. 5). Therefore, the ability of different nonstimulatory peptides to enhance antigen recognition in CD8$^+$ T cells depends on the quantity of nonstimulatory pMHC. Fig. 5 also indicates that there were $\sim$550 endogenous K^b-peptide molecules on the RMA-S cells. As this estimate derives from quantitative flow cytometry using fluorescent beads (see Materials and methods), we believe this to be a more accurate estimate than the usually quoted number: $\sim$5% of perhaps 100,000 molecules on a parental RMA cell, therefore $\sim$5,000 molecules. Comparison of the number of CD69$^+$ cells after stimulation with RMA-S without added peptide and with those loaded with OVA only indicates that the endogenous pMHC of the RMA-S cells did not have a measurable effect on CD69 up-regulation in this assay.

Nonstimulatory peptides enhance effector functions of CD8$^+$ T cells

Next, we wanted to find out whether nonstimulatory peptides are able to enhance effector functions, such as cytokine production, in CD8$^+$ T cells. This was a particularly important question because previous attempts by others had failed to show enhancement of effector functions of CD8$^+$ T cells (14). We measured IL-2 production of naive lymphocytes by intracellular cytokine staining after 5 h of incubation with peptide-loaded RMA-S cells. IL-2 expression was enhanced compared with OVA alone by the presence of a nonstimulatory peptide (Fig. 6 A). Similarly, IFN-γ production of activated CTLs was increased in the presence of a nonstimulatory peptide (Fig. 6 B and Fig. S4, which is available at http://www.jem.org/cgi/content/full/jem.20062610/DC1). IL-2 expression was also enhanced by the nonstimulatory peptides in these cells (Fig. S5). To determine whether nonstimulatory peptides enhance CTL killing of target cells as well, we assessed the influence of nonstimulatory peptides during CTL killing of OVA and T4-labeled target cells. An enhancing effect on cytotoxic activity (measured as survival of APCs after 17 h of coincubation with activated OT-I CTLs) was detectable for the weak agonist T4 but not for OVA (Fig. 7). Therefore, endogenous or exogenous nonstimulatory peptides can enhance cytokine expression and the cytotoxic activity of CTLs.
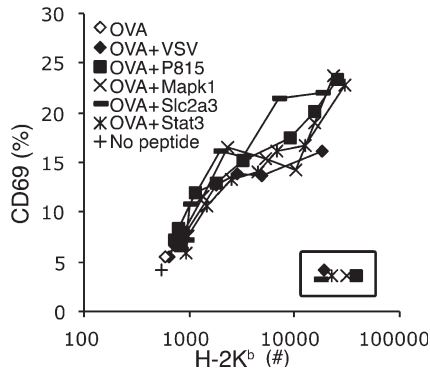
Figure 5. The effect of nonstimulatory peptides on antigen recognition depends on quantity but not quality of the nonstimulatory pMHC. RMA-S cells were loaded with OVA peptide, washed, and aliquoted to different groups. Nonstimulatory peptides were titrated on the antigen-loaded cells and, after incubation, washed away. The cells were allowed to interact with DP thymocytes for 2 h. The cells were stained with anti-CD8 and anti-CD69, and the level of CD69 was assessed by flow cytometry. The results are shown as percentage of CD69+ CD8 gated cells in the presence of different numbers of K^b molecules. The effect of the nonstimulatory peptides without any OVA is shown surrounded by a box, but using the same symbols as for those with added OVA. The results are representative of at least three independent experiments. Fig. S1 shows histograms of CD69 up-regulation by RMA-S cells matched for low expression of OVA-K^b in the presence or absence of excess nonstimulatory peptide-K^b.

In addition to CD69 and CD25, we measured the effect of nonstimulatory peptides on several other phenotypic markers, such as CD5, CD44 (on naive cells), and HSA, which were all
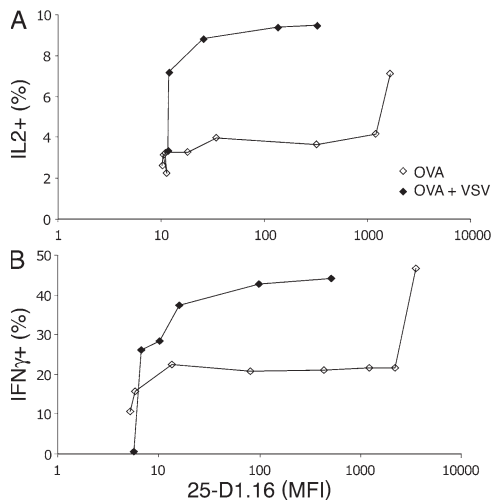

Figure 6. Nonstimulatory peptides enhance cytokine production. RMA-S cells were loaded with various amounts of OVA alone or together with VSV. The RMA-S cells were allowed to interact with naive T cells (A) or effector cells (B) for 5 h (A and B). The cells were first stained with anti-CD8, and then intracellularly with anti-IL-2 (A) or anti-IFN-γ (B), and the cytokine level was assessed by flow cytometry. The results are shown as percentage of cytokine+ cells at different OVA-K^b concentrations. The results are representative of eight independent experiments.

affected by the nonstimulatory peptides (unpublished data). In conclusion, nonstimulatory peptides enhance effector functions in CD8+ T cells.

Nonstimulatory peptides enhance formation and dissolution of T cell–APC conjugates

Next, we assessed whether the presence of nonstimulatory peptides and the resulting higher density of pMHC complexes on the APCs enhanced the formation of conjugates between T cells and APCs, and hence T cell activation by agonists. We incubated OT-I T cells with RMA-S cells, which had been loaded with OVA or T4, in the presence or absence of nonstimulatory peptides. At various time points cells were pipetted up and down to separate any weakly conjugated cells and fixed. Formation of conjugates between naive OT-I T cells and RMA-S cells was measured by flow cytometry. For the strong agonist OVA, initial conjugate formation was enhanced if the nonstimulatory peptide was also present (Fig. 8 A). This effect was seen for low amounts of OVA peptide and at early time points (up to 30 min of incubation; unpublished data). In the absence of added nonstimulatory peptides, the number of conjugates accumulated over time. However, this was not the case when nonstimulatory peptides were present. At later time points (>30 min), the number of conjugates for OVA plus nonstimulatory peptide was lower than for OVA alone (Fig. 8 B). For the weak ligand T4, nonstimulatory peptides increased the number of conjugates even at later time points (Fig. 8 B).

We conclude from these experiments that the co-presentation of nonstimulatory peptides favors not only the initial conjugate formation but, as observed for the strong agonist OVA, also shortens the interaction time of T cells and APCs required for T cell activation. As a result, the T cells dissociate faster.

DISCUSSION

In this work we have shown that nonstimulatory peptides enhance antigen recognition as measured by effector functions, such as cytokine production and CTL killing, by inducing
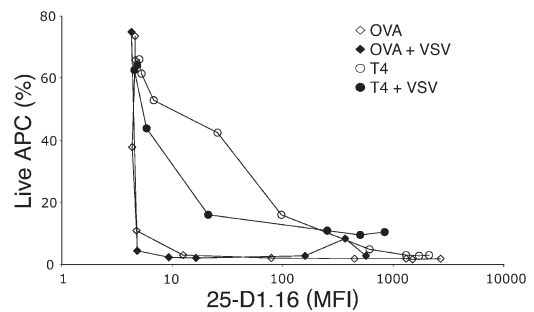

Figure 7. Nonstimulatory peptides enhance CTL killing of target cells. RMA-S cells were loaded with various amounts of OVA or T4 alone or together with VSV. The Cy5-labeled RMA-S cells were allowed to interact with effector cells for 17 h. Cell death was determined by death-associated changes in the forward- and side-scatter properties among the RMA-S cell population (Cy5+). Results are shown as percentage of live RMA-S cells for different OVA or T4-K^b concentrations and are representative of four independent experiments.
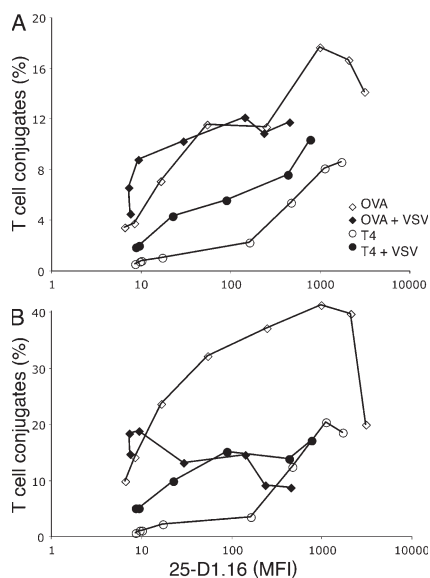
Figure 8. Nonstimulatory peptides affect duration of T–APC conjugates. RMA-S cells were labeled with Cy5 and loaded with various amounts of OVA or T4 alone or together with VSV. The RMA-S cells were allowed to interact with naive T cells for the indicated times. The cells were fixed and stained for CD8, and cell conjugate formation was assessed by flow cytometry based on simultaneous staining for CD8 and Cy5. Results are shown as percentage of cell conjugates among CD8 cells for different OVA or T4-K^b concentrations at 20- (A) and 60-min (B) time points. The data are representative of at least four independent experiments.

phenotypic maturation of the T cells, such as up-regulation of CD25, CD69, CD44, CD5, and HSA expression, and by the number of TCRs stimulated, as measured by TCR down-modulation. With the strong agonist OVA we saw enhancement of T cell–APC conjugate formation at early time points when nonstimulatory pMHC was available, but at later time points there was a decrease in the number of conjugates compared with the cells in the absence of nonstimulatory peptide. These results indicate that nonstimulatory peptides do not just simply enhance adhesion between cells but that they allow the T cell activation program to proceed faster, leading to earlier dissociation of the conjugates in the presence of nonstimulatory peptides. This would allow a more robust immune response, as T cells would be able to change their polarization status for cytokine production, proliferation, differentiation, killing of the next target, and homing to target organs, for example. For the weak agonist T4, the conjugates were increased in the presence of a nonstimulatory peptide even at 60 min, reflecting the lower and slower capability of T4 to execute the full signaling program needed to terminate the cell contact.

In contrast to our work (10 and this work) and that of others (11), an earlier study did not support an enhancing role for self-pMHC class I complexes in CD8$^+$ T cell antigen recognition (14). This is particularly surprising in that Sporri and Reis e Sousa performed a similar experiment to us, comparing stimulation by RMA cells, which express endogenous peptides, versus RMA-S cells, which express relatively few endogenous

peptides ($\sim$550 K^b molecules in our experiments) (Fig. 5). RMA-S is a mutant subline of RMA, and has therefore been separated from RMA by many passages. RMA-S could therefore potentially have other differences to RMA than simply the lack of Tap2 protein. Our experiments used the same RMA-S cells to compare the effect of presence or absence of added nonstimulatory peptides on antigen recognition, and therefore the two groups were identical in all respects except for the presence of nonstimulatory pMHC. In addition, we compared RMA-S to RMA cells, and in contrast to Sporri, found that RMA induced a better response to antigen than did RMA-S (Fig. S3). RMA has a slightly higher K^b expression than the maximum expression that we reached on RMA-S with added peptides (unpublished data). Another difference between the studies is that Sporri and Reis e Sousa irradiated the APCs. However, this did not explain the differing results (unpublished data), which are most likely due to differing sensitivities of the assays used, with the relatively few endogenous peptides present in RMA-S cells sufficient to induce some responses, particularly that of CTL to strong agonist (Fig. 7 and reference 14).

We have shown that nonstimulatory peptides are most important in antigen recognition by less differentiated cells, such that their ability to enhance antigen recognition is most evident in thymocytes, moderate in naive T cells, and mild in effector T cells. The different time courses of nonstimulatory peptide contribution to antigen recognition also underline the developmental differences in the importance of nonstimulatory peptides in antigen recognition. This fits well with data showing that DP thymocytes respond better to pMHC than anti-CD3 mAb activation as compared with naive T cells (26). Evidence indicates that immature thymocytes are more sensitive to low affinity ligands than mature T cells, but that both respond well to high affinity ligands (26, 27). The sensitivity of immature thymocytes was recently correlated to miR-181a expression levels, which in part affects phosphatases and therefore affects the threshold of TCR signaling (28). Low affinity ligands are generally more CD8 dependent (29). This developmental dependence may be due to higher CD8-MHC avidity in thymocytes caused by differential glycosylation (30). In addition, immature thymocytes express 10-fold less TCR compared with mature T cells (31). Therefore, due to higher affinity and a higher ratio of CD8 to TCR in immature thymocytes, pMHC binding to CD8 is more prominent in thymocytes compared with mature T cells. Alternatively, cells at different stages of development may have different membrane compartmentalization of TCR and CD8 and their associated molecules such as Lck that might affect their ability to take advantage of nonstimulatory peptides during antigen recognition. The more mature cells are also more sensitive to minute amounts of peptides in the absence of added nonstimulatory peptides (Fig. 1), so they may simply not be as reliant on nonstimulatory pMHC because they have other means such as adhesion and costimulatory molecules to enhance their sensitivity to antigen, or they may be sensitive enough to make use of the relatively few endogenous pMHC complexes present in the RMA-S cells. In addition, activated T cells were shown to

have 20–50 times higher TCR avidity for pMHC complexes as compared with naive T cells, a finding that was linked to TCR reorganization and cholesterol content (32).

T cells have been shown to form organized structures called IS between T cells and APCs. Although the exact function of the IS remains elusive, it has been proposed to act in signal integration. In particular, modeling studies suggested that formation of central supramolecular activation cluster (cSMAC) enhances weak signals by concentrating TCR, pMHC, and Lck while dampening strong signals by enhancing TCR degradation (33). The organization of the IS differs depending on the state of T cell differentiation, and this may contribute to different abilities of nonstimulatory peptides to enhance antigen recognition at different states of differentiation. Recent data using total internal reflection fluorescence microscopy indicate that TCR signaling occurs in microclusters in the periphery of the synapse (34), suggesting that the main function of cSMAC is to down-regulate signaling. This is supported by a recent paper showing that the stimulatory potency of a peptide was reduced when cSMAC formation was increased (35). However, this may not be the case with weaker agonists or in physiological circumstances where antigen is limiting. Our finding that the ability of nonstimulatory peptides to enhance antigen recognition is more pronounced with weaker agonists, and at lower antigen concentrations, would support a role for nonstimulatory peptides in clustering key molecules for enhanced antigen recognition.

In CTL assays, nonstimulatory peptide did not seem to play a major role when presented with OVA. However, the sensitivity of OVA-K^b staining by the 25-D1.16 antibody is not sufficient to identify the very small number of ligands that are sufficient to mediate killing by CTLs (12, 36), so it is possible that at such low levels of peptide nonstimulatory peptides are important. Alternatively, the relatively few endogenous pMHC complexes present on RMA-S may have been sufficient to help recognition of the strong agonist by CTLs, even though this was not evident in other assays. For the very weak agonist peptide T4, which requires higher amounts of peptide to induce a response, and therefore where minimally stimulatory concentrations of T4-K^b fall within the limits of 25-D1.16 resolution, we saw increased killing in the presence of nonstimulatory peptides.

All the nonstimulatory endogenous (or exogenous) peptides that we have tested were able to aid in antigen recognition by CD8$^+$ T cells, and their effect was more evident in recognition of weaker ligands. This is in contrast to CD4$^+$ T cells where only certain endogenous peptides acted to enhance recognition by agonist, and their ability to function was dependent on the strength of the agonist; stronger agonists were helped by a larger proportion of endogenous peptides than weaker agonists (9, 13). This suggests that there is a pronounced difference between CD8$^+$ and CD4$^+$ T cells in their ability to take advantage of endogenous nonstimulatory peptides. It has been suggested (for CD4$^+$ T cells) that two TCRs binding, respectively, to agonist and endogenous pMHC complexes, are bridged by the coreceptor CD4 to form a pseudodimer (9, 12). Only certain endogenous pMHC complexes were shown to synergize with agonist, suggesting that TCR binding to the endogenous pMHC is important (9). This does not seem to be the case for CD8$^+$ T cells, as all the peptides tested were able to aid antigen recognition. Therefore, as a mechanism of synergism by nonstimulatory peptides in antigen recognition by CD8$^+$ T cells, we proposed a pre-concentration model, in which noncognate interactions between CD8 and MHC concentrate CD8, its associated Lck, and pMHC complexes to the synapse, thus aiding the TCR to find its ligand and allowing easier access and a high concentration of CD8-Lck to enhance signal transduction (10). Pre-concentration could potentially change the quality of the signal received by T cells, as we found that recognition of APLs is translated to different CD8–TCR interaction kinetics, such that the interaction is induced faster for stronger ligands (37). Therefore, pre-concentration of CD8 could speed up the antigen-induced interaction between CD8 and TCR, leading to signaling characteristic of a stronger agonist than in the absence of endogenous peptides. This notion is supported by the finding that thymocytes respond better to low affinity ligands than do mature T cells (26), by our data showing that thymocytes are the most efficient at taking advantage of nonstimulatory peptides (Fig. 1), and that nonstimulatory peptides are most important in recognition of weaker ligands (Fig. 3).

The differing results obtained with CD4$^+$ and CD8$^+$ T cells may in part be due to differences in methods. In the CD8$^+$ T cell system we have used RMA-S cells to present the pMHC complexes, whereas in the CD4$^+$ T cell system Krogsgaard et al. (9) used soluble pMHC heterodimers. These soluble heterodimers are well defined in regards to their pMHC content, consisting of one agonist and one nonstimulatory peptide, allowing precise control of stimulation. RMA-S cells have the caveat that they do express a few percentages of endogenous pMHC complexes compared with physiological levels (18–20), and therefore in our RMA-S system we have several hundred endogenous pMHC molecules as a background. We have described above why these do not pose a problem for our major conclusions. Soluble pMHC heterodimers have their own caveats, for example, that the ratio of antigen to nonstimulatory peptide is 1:1. In a natural antigen-presenting environment the ratio of endogenous pMHC to antigenic pMHC is enormous. RMA-S is therefore more useful in approximating this situation, which is highly relevant to our proposed pre-concentration model. It is also possible that the lack of an APC surface could be important in experiments with oligomers, perhaps for correct alignment of the molecules relative to each other, whereas this would not be a problem for RMA-S studies. Clearly there are advantages and disadvantages to both methods, and it will be important to use different methods to truly elucidate all the layers of endogenous peptide participation in antigen recognition.

Another possibility is that the difference between CD4$^+$ and CD8$^+$ cells is that the higher affinity of CD8 rather than CD4 for noncognate pMHC (38) overcomes and obscures the requirement for the TCR interaction with endogenous

pMHC that is seen in the class II–restricted system. Thus, all of the endogenous pMHC complexes work in the class I system, even with weak agonists like Q4, T4, and G4. Indeed, Q4 and T4 have tetramer-binding avidities close to that of the noncognate CD8–MHC class I avidity (23). In addition to our data showing that the noncognate CD8–pMHC interaction concentrates these molecules at the IS and may therefore explain the role of any of the nonstimulatory pMHC complexes in aiding antigen recognition (10), recent data show a dominant effect of the noncognate CD8–pMHC interaction in enhancing TCR recognition (11), and that the CD8–pMHC interaction precedes the TCR–pMHC interaction (39). These data support the idea (pre-concentration model) that the noncognate interaction of CD8 with class I is responsible for the effect of the nonstimulatory peptides.

In summary, we have shown that nonstimulatory peptides universally lower the threshold for antigen recognition, an effect measurable at many different levels. This effect is more important for weaker agonists and is fine-tuned depending on the differentiation status of the T cell.

MATERIALS AND METHODS

Peptides and antibodies. Peptides OVA (SIINFEKL), VSV (RGYVYQGL), Q4 (SIIQFEKL), G4 (SIIGFEKL), and T4 (SIITFEKL) were generated at the Protein and Nucleic Acid Core Facility at The Scripps Research Institute and purified by HPLC. P815 (HIYEFPQL), Mapk1$_{(19–26)}$ (amino acid range in parentheses; VGPRYTNL), STAT3$_{(53–60)}$ (ATLVFHNL), Ndufa4$_{(61–68)}$ (VNVDYSKL), Slc2a3$_{(314–321)}$ (VNTIFTVV), and Hcph$_{(503–510)}$ (AQYKFIYV) were provided by S. Jameson (University of Minnesota, Minneapolis, MN). Antibodies used in this study were PE-conjugated Fab goat anti–mouse IgG (Protos Immunoresearch) and H2-K^b OVA-specific 25-D1.16 (provided by R. Germain, National Institutes of Health [NIH], Bethesda, MD). Anti-CD8β (clone H35-17.2), anti-K^b (clone AF6-88.5), anti-CD8α (53–6.7), anti-CD69 (H1.2F3), anti-CD25 (PC61), anti-Vα2(B20.1), and anti–IFN-γ (XMG1.2) were obtained from BD Biosciences. Anti-CD8 (CT-CD8α) from Caltag/Invitrogen was used for staining of fixed cell conjugates. Anti–IL-2 (JES6-5H4) was from eBioscience.

Mice. C57BL/6J (B6) mice were bred and maintained at The Scripps Research Institute. OT-I mice bearing a transgenic TCR specific for OVA-K^b (Vα2, Vβ5) (16) and OT-I mice deficient for *Tap1* (OT-I *Tap1* KO) (15) were obtained from S. Jameson and K. Hogquist (University of Minnesota, Minneapolis, MN). All mice were maintained at The Scripps Research Institute, and all experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of The Scripps Research Institute.

APC preparation. *Tap2*-defective RMA-S cells that are defective in binding endogenous peptides to newly synthesized MHC class I molecules (18) were used as APCs. Stable expression of K^b or D^b can be achieved by adding synthetic peptides able to bind to K^b or D^b exogenously to the cell culture (19, 20). The RMA-S cells were maintained in RPMI medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 50 μM β-mercaptoethanol. RMA-S cells were stained with Cy5 20 h before experiments by incubating cells with 0.1 mg/ml of Cy5 monomeric succinimidyl ester (GE Healthcare) in RPMI at room temperature for 5 min, washing with RPMI, and quenching with 10% FCS in RPMI. Different peptides have a different ability to stabilize pMHC, and the different nonstimulatory peptides were titrated to give comparable cell surface pMHC quantity. The peptide amounts added to the culture were as follows: 80 μM VSV, 28 μM P815, 36 μM Mapk1, 9 μM Ndufa4, 15.8 μM Hcph, 19.5 μM Slc2a3, and 27 μM STAT3. The RMA-S cells were incubated at 29°C overnight, pulsed with peptides for 30 min at 29°C, incubated at 37°C for 3 h, and washed

once. For OVA, Q4 or T4-K^b quantitation RMA-S cells were stained with 25-D1.16 antibody together with PE-conjugated Fab goat anti–mouse IgG. The total pMHC was quantitated with PE-labeled K^b-specific antibody (AF6-88.5). The QuantiBRITE phycoerythrin fluorescence quantitation kit (Becton Dickinson) was used to calculate the number of molecules.

Preparation of T cells. Pre-positive selection thymocytes, CD4$^+$CD8$^+$ DP thymocytes, were isolated from OT-I Tap$^{-/-}$ mice. Naive OT-I T cells were prepared from pooled lymph nodes of 6–12-wk-old OT-I mice. Activated T cells were generated by antigen-specific stimulation of naive OT-I T cells. In brief, pooled OT-I lymph node cells (2.5 × 10^6 cells) were seeded in 24-well plates and stimulated with irradiated OVA-loaded C57BL/6J spleen cells (3 × 10^6 cells) in RPMI complete medium supplemented with rat Con A supernatant at a final concentration of 5% (vol/vol). Fresh medium containing 2% of rat Con A supernatant was added at days 2 and 4. T cell cultures were used as effector cells on day 6 after antigen stimulation.

T cell activation assays. T cells (2–3 × 10^5 cells in 50 μl) were incubated with peptide-pulsed RMA-S cells (10^5 cells in 20 μl) in round-bottom 96-well plates at 37°for the indicated times, and the T cell activation status was measured as described below.

Antibody staining and flow cytometric analysis. After incubation, cells were stained for CD8, CD69, and CD25 or Vα2 in FACS buffer (0.02% azide, 10% FCS in PBS) and washed, and the samples were run on a flow cytometer. For FACS analysis, T cells were gated according to the appropriate scatter profile and CD8 expression. To avoid unspecific APC background, RMA-S–T cell conjugates were excluded based on simultaneous staining for CD8 and Cy5 (Cy5-labeled RMA-S cells) and their forward- and side-scatter profile. The TCR down-regulation data are shown as a percentage of Vα2 expression on the surface of cells compared with cells incubated with RMA-S cells in the absence of an exogenously added peptide. For CD69 and CD25 up-regulation, the percentage of CD8$^+$ T cells expressing CD69^{high} and CD25^{high} population is presented. The gate defining CD69 or CD25^{low} versus CD69 or CD25^{high} was determined from corresponding samples in which T cells were incubated with nonpeptide-pulsed RMA-S cells.

Cell conjugate assay. For the conjugate assay, cells were pipetted up and down three times at the indicated time points to separate any weakly conjugated cells and fixed in 4% paraformaldehyde. Paraformaldehyde was inactivated by 10 mM Tris, pH 7.4, in PBS. Cells were washed in PBS and stained with anti–CD8 antibody. Cell conjugates were analyzed by flow cytometry based on simultaneous expression of CD8 (CD8 antibody staining) and Cy5 (Cy5-labeled RMA-S cell).

Cytotoxicity assay. To determine T cell–mediated cytotoxicity, T cells and RMA-S cells were incubated for 17 h. After incubation, cells were stained for CD8. Cell death was determined by death-associated changes in the forward- and side-scatter properties among the Cy5$^+$ (CD8$^-$) RMA-S cell population.

Intracellular cytokine staining. For intracellular IL-2/IFN-γ staining, T cells and RMA-S cells (Cy5-labeled) were incubated in the presence of 0.67 μg/ml Monensin (GolgiStop; BD Biosciences) to block the release of cytokines from the cells. After incubation, cells were stained for CD8 and then fixed with 4% paraformaldehyde. After inactivation of paraformaldehyde with 10 mM Tris, pH 7.4, in PBS, the cells were washed in FACS buffer and permeabilized in FACS buffer containing 0.2% saponin. Subsequent staining for IL-2 or IFN-γ and washing were performed in FACS buffer containing 0.2% saponin. Cells were analyzed by flow cytometry. IL-2$^+$ and IFN-γ$^+$ cells were determined among CD8$^+$ T cells, excluding APCs, by cell size and Cy5 staining.

Online supplemental material. Fig. S1 shows example histograms for K^b and K^b-OVA expression on RMA-S cells treated with OVA peptide with or without excess nonstimulatory VSV peptide, plus the up-regulation

of CD69 expression on naive OT-I T cells induced by these RMA-S cells. Fig. S2 shows the effect of nonstimulatory peptides on up-regulating CD69 on activated T cells or preselection DP thymocytes at different time points. Fig. S3 shows a comparison of CD69 up-regulation on naive OT-I T cells in response to RMA or RMA-S cells presenting antigen. Fig. S4 shows that nonstimulatory peptides enhance IFN-γ expression in effector T cells responding to antigen. Fig. S5 shows that nonstimulatory peptides enhance IL-2 expression in effector T cells in response to antigen. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062610/DC1.

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