Formation of supramolecular activation clusters on fresh *ex vivo* CD8$^+$ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells

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Upon productive interaction of CD4 T cells with antigen-presenting cells (APCs), receptors and intracellular proteins translocate and form spatially segregated supramolecular activation clusters (SMACs). It is not known whether SMACs are required for CD8 T cell activation. CD8 T cells, unlike CD4 T cells, can be activated by a single peptide-MHC molecule, or by purified monovalent recombinant peptide-MHC molecules. We studied, by three-dimensional digital microscopy, cell conjugates of fresh *ex vivo* CD8 T cells (obtained from OT-1 mice, which are transgenic for T cell antigen receptor reactive with the complex of H-2K^b and the ovalbumin octapeptide SIINFEKL) and peptide-pulsed APCs. Remarkably, even in T cell:APC conjugates that were formed in the presence of the lowest concentration of peptide that was sufficient to elicit T cell proliferation and IFN-γ production; the θ isoform of protein kinase C was clustered in a central SMAC, and lymphocyte function-associated antigen 1 and talin were clustered in the peripheral SMAC. Conjugation of T cells to APCs that were pulsed with concentrations of peptide smaller than that required to activate T cells was greatly reduced, and SMACs were not formed at all. APCs expressing mutant H-2K^b (Lys^{227}) molecules that do not bind CD8 were unable to form stable conjugates with these T cells, even at high peptide concentrations. Thus, although CD8 and CD4 T cells may display different sensitivity to the concentration and oligomerization of surface receptors, SMACs are formed and seem to be required functionally in both cell types. However, unlike CD4 T cells, which can form SMACs without CD4, CD8 T cells from OT-1 transgenic mice depend on their coreceptor, CD8, for the proper formation of SMACs .

During T cell activation, individual receptors form signaling complexes with intracellular proteins. Although the activation of the associated intracellular signaling molecules requires oligomerization of individual receptors, the efficient activation of T cells also depends on higher-order structural assemblies of multiple signaling complexes, termed supramolecular activation clusters [SMACs (1)] or the "immunological synapse" (2, 3). The central SMAC (c-SMAC) is identified by the clustering of the θ isoform of protein kinase C (PKCθ) (4). Surrounding the c-SMAC is a region referred to as the peripheral SMAC (p-SMAC) in which lymphocyte function-associated antigen-1 (LFA-1) and the cytoskeletal protein talin are clustered. No SMACs are formed in the absence of antigenic peptide or in the presence of antagonistic peptides (1). It has therefore been proposed that the formation of SMACs facilitates the efficient integration of the signals that lead to the activation of T cells by APCs (1, 5, 6).

Accompanying the commitment of a T cell to a functional T cell lineage are differences in the effector activities that are initiated on activation. Thus, at some stage after binding of the T cell antigen receptor (TCR) to MHC–peptide complexes, there is a divergence in the target genes that are activated in CD4 and CD8 T cells, and these genes presumably are regulated by different mechanisms. Both CD4 and CD8 molecules act as coreceptors and bind to the same MHC (class II or class I, respectively) molecule as the TCR (7–9). Although it is widely assumed that CD4 and CD8 perform similar roles during the recognition by T cells of MHC class II and class I molecules, respectively, there are also findings that suggest that there may be differences between these molecules. One such difference is the affinity of the coreceptor interaction with MHC molecules. In studies performed on the BIAcore, the CD8-MHC class I molecule affinity has been measured in the 10–40 μM range (10), whereas the affinity of CD4 for MHC class II molecules is below the limits of detection. In addition, in some BIAcore experiments, CD8 stabilized the TCR–MHC class I interaction; however, CD4 is unable to stabilize the TCR–MHC class II interaction in similar experiments (11). It has also been possible to cocrystallize CD8 bound to MHC class I molecules (12, 13), whereas no CD4–MHC class II cocrystals have been isolated.

The studies of molecular redistribution during T cell–APC interaction have been confined to CD4 T cells; similar SMAC formation on CD8 T cells has not been reported. Thus, SMAC formation may be a unique property of helper cells that is not shared by cytotoxic T lymphocytes. One possibility is that formation of SMACs may require a relatively high degree of TCR occupancy. Related to this possibility is the suggestion that the degree of TCR occupancy required for the activation of CD8 T cells is less than that required for the activation of CD4 T cells. Estimates of the number of MHC–peptide ligands required for the activation of CD4 T cells ranges from 200 to 300 molecules (14, 15). In contrast, estimates of the number of ligands required for the activation of CD8 T cells are as low as 10 molecules (16, 17). One study has even suggested that a single MHC–peptide molecule can activate CD8 T cells (18); and activation of CD8, but not CD4, T cells has been achieved with monovalent MHC–peptide ligands (19). In systems that use an artificial lipid membrane reconstituted with MHC–peptide complexes, a threshold density of 60 antigenic MHC–peptide molecules per square micrometer was required for formation of the immunological synapse on CD4 T cells (5). Thus, if such a high density of MHC–peptide complexes on APCs is required for SMAC formation on CD8 T cells, then such membrane redistribution events may not occur under the small concentrations of peptide that are sufficient to activate CD8 T cells.

Abbreviations: TCR, T cell antigen receptor; APC, antigen-presenting cell; SMAC, supramolecular activation cluster; c-SMAC, central SMAC; p-SMAC, peripheral SMAC; PKCθ, θ isoform of protein kinase C; LFA-1, lymphocyte function-associated antigen 1.

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Based in part on these potential differences in the function of CD4 and CD8, the notion that CD8 T cells will form SMACs in a manner similar to that of CD4 T cells cannot be inferred by studies of CD4 cells alone and must be tested experimentally. In this study we have examined the ability of freshly isolated CD8 T cells from a TCR transgenic mouse reactive with the complex of H-2K^b and the peptide SIINFEKL to form SMACs.

Materials and Methods

Mice. OT-1 TCR transgenic mice, which carry the TCR genes (Vα2, Vβ5.1) isolated from a T cell clone reactive with an H-2K^b–ovalbumin octapeptide (SIINFEKL) (20), were obtained from Frank Carbone (Monash University, Melbourne). Pooled lymph node cells were harvested from mice at 6–8 weeks of age. At this point, more than 95% of CD3$^+$ cells express Vβ5 and Vα2 and bind H-2K^b–SIINFEKL tetrameric complexes.

Measurement of T Cell Responses *in Vitro*. Purified lymph node cells were obtained from OT-1 mice by passage through StemSep columns (StemCell Technologies, Vancouver) according to the manufacturer's instructions. The purity of the population was greater than 99.5% as identified by CD3 staining and flow cytometry. The T cells were cultured in 96-well flat-bottom plates in the presence of various amounts of the SIINFEKL peptide. The APCs were M12 cells transfected with either the wild-type or mutant (Lys^{227}) H-2K^b gene. The M12 cells were irradiated with 20,000 rads before the addition of the purified T cells. The cultures comprised 5×10^4 T cells and 1×10^4 APCs in a total volume of 200 μl. To assay IFN-γ production, 5×10^5 purified lymph node T cells were cultured with 1×10^5 APCs in the presence of 0.1 pM to 1.0 μM SIINFEKL overnight in a volume of 200 μl. The presence of IFN-γ in the supernatant was detected by using a capture ELISA incorporating XMG1.2 (rat anti-mouse IFN-γ) as the capture reagent and biotinylated R4GA2 (rat anti-mouse IFN-γ) and horseradish peroxidase-conjugated streptavidin as the detecting system. The amount of IFN-γ was quantified by comparison with a standard curve of purified recombinant mouse IFN-γ.

The proliferation of 5×10^4 lymph node cells (in triplicate wells) in the presence of 0.1 pM to 1.0 μM SIINFEKL was determined after an overnight pulse with [^{3}H]thymidine on day 3 of culture. The cells were harvested and the incorporation of ^{3}H into DNA was measured.

Comparison of the Binding of SIINFEKL to Wild-Type and Mutant H-2K^b. The ability of SIINFEKL to bind to the mutant (Lys^{227}) and wild-type forms of H-2K^b was determined by using the 25D1.16 antibody, which recognizes the H-2K^b–SIINFEKL complex (21). M12.C3 (H-2^d) cells transfected with either the wild-type or mutant H-2K^b gene, were incubated with 50 nM SIINFEKL for 60 min at 37°C and then stained with the 25D1.16 antibody and FITC-conjugated goat anti-mouse IgG.

Formation and Staining of T Cell:APC Conjugates. *Ex vivo* OT-1 T cells were mixed at a 1:1 ratio with antigen-pulsed APCs. The APCs were M12 cells transfected with either the wild-type or mutant H-2K^b gene. The APCs were suspended at 1×10^6 per ml and pulsed with SIINFEKL for 60 min at 37°C. Unbound peptide was removed by washing the cells three times with DMEM/FBS. The peptide-pulsed APCs and the OT-1 T cells were brought to a final concentration of 3×10^6 per ml, and equal volumes (100 μl) of each cell suspension were mixed together. After a brief centrifugation (20 s) at room temperature in a microcentrifuge, the cells were incubated at 37°C for 3–6 min and then adhered to poly-D-lysine-coated glass coverslips for a further 4 min. Next, the cells were fixed with fresh 3% paraformaldehyde for 14 min and then stained with monoclonal antibodies specific for CD3 (hamster anti-mouse CD3; F-500), or LFA-1 (rat anti-mouse LFA-1; I21/7). To visualize intracellular proteins, the fixed and surface-labeled cells were treated with Triton X-100 (0.2% in PBS) for 5 min and then stained with antibodies to talin (raised in guinea pig) or PKCθ (raised in rabbits, Santa Cruz Biotechnology). Binding of the primary antibodies was monitored by the addition of fluorochrome-conjugated anti-IgG species-specific antibodies.

Image Analysis. Cell conjugates were selected for imaging based only on their appearance as cell pairs in Nomarski optics. Each three-dimensional image contained about 60 serial optical sections, 0.1 μm apart, for each label. The data were captured, subjected to constrained interactive deconvolution, and analyzed by using the SlideBook software (Intelligent Imaging Innovations, Denver). The T cell–APC contact region and the T cell were defined by manual delineation. The mean fluorescence intensity within these defined regions was used to calculate the degree of enrichment of a particular stain. An area outside of the cells was used to obtain background mean fluorescence intensities, which were subtracted from the mean fluorescence values in the defined regions. The fold enrichment of a fluorochrome in a particular region was calculated according to the formula: fluorescence intensity in the region (c-SMAC or p-SMAC) divided by the fluorescence intensity in the T cell.

To demonstrate the enrichment of molecules at the contact area, or the activation-induced changes (as imparted by the term SMAC), the images shown in Figs. 2 and 3 were obtained after the incorporation of lower limits of fluorescence for each fluorochrome. These lower limits were determined from the average values of regions in the T cell and APC outside the contact area and were less than 25% of the fluorescence values in the contact area. In Fig. 3 the lower limit of CD3 staining was set to show the staining of this marker on the T cell.

Results

OT-1 T Cells Form Conjugates with Peptide-Pulsed APCs at Doses of Peptide Similar to Those Required to Elicit T Cell Proliferation and Cytokine Production. The primary goal of these studies was to determine whether CD8 T cells, like CD4 T cells, form SMACs on interaction with peptide-pulsed APCs. An important consideration in these studies is whether the dose of peptide required for conjugate and SMAC formation is similar to the physiological range under which these T cells respond *in vitro*. We therefore compared the concentration of SIINFEKL peptide required for the formation of conjugates between OT-1 lymph node T cells and APCs with the peptide concentrations required for proliferation and IFN-γ production. For these experiments we used OT-1 lymph node T cells purified to >99.5% by passage through StemSep columns and M12 cells transfected with H-2K^b as APCs. As shown in Fig. 1*A*, OT-1 lymph node T cells readily proliferate in culture in the presence of SIINFEKL peptide. Proliferation of OT-1 T cells is readily observed with peptide concentrations of 1 pM and greater, but not in the presence of either 300 or 100 fM doses of SIINFEKL. At 1 pM the proliferative response was ≈25% of the maximal response. The concentration of peptide required to induce proliferation is certainly much less than the concentration required (low nanomolar) for the negative selection of thymocytes in fetal thymic organ culture (20). We also examined the requirement for CD8 binding for proliferation of OT-1 cells by using M12 cells that expressed a mutant H-2K^b molecule that is incapable of functional interaction with CD8 (9). The mutant H-2K^b molecule did not stimulate the proliferation of OT-1 T cells (Fig. 1*A*, triangles) even in the presence of micromolar concentrations of antigenic peptide, indicating that this response was strongly CD8-dependent. The amount of SIINFEKL required to elicit production of IFN-γ was in the same range (1 pM and greater) as that required for proliferation (Fig. 1*B*). Indeed, at 1 pM, the

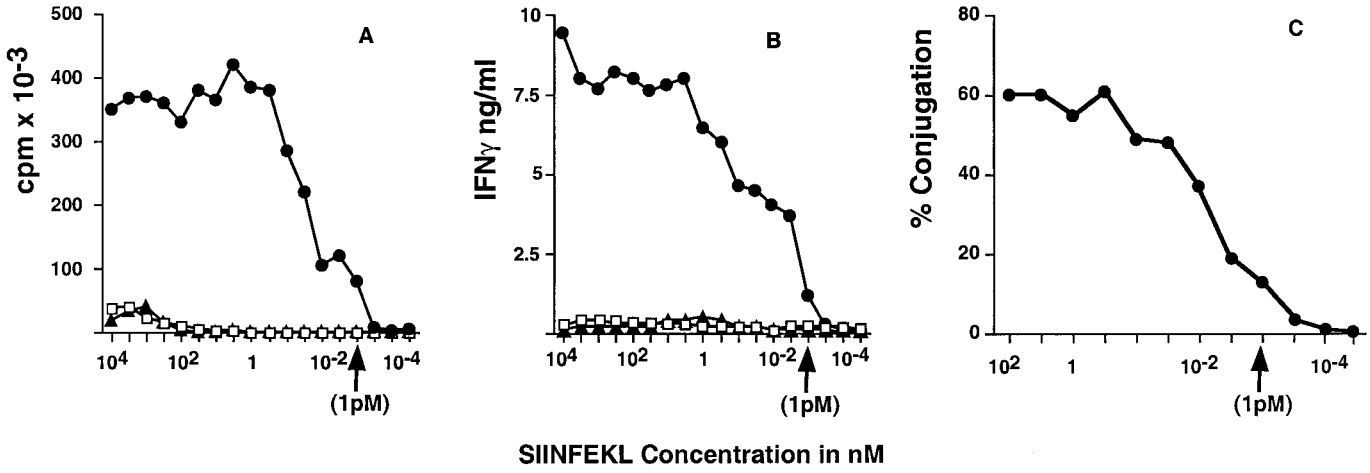

Fig. 1. Proliferation (*A*), cytokine (IFN-γ) production (*B*), and conjugate formation (*C*) by OT-1 lymph node T cells in the presence of various doses of SIINFEKL. (*A*) Cultures were established between OT-1 lymph node T cells separated on StemSep columns and irradiated M12 cells that expressed either wild-type (●) or mutant (▲) H-2K^b. Cultures that were not given any APCs are represented by □. Cultures were established in triplicate wells and pulsed overnight with [^{3}H]thymidine on day 3. In this experiment and other replicate experiments, proliferation of OT-1 T cells was detectable in the presence of 1 or 3 pM SIINFEKL, but not at concentrations (300 or 100 fM) below 1 pM. (*B*) Cultures were established as described for *A*. After overnight culture, the supernatants were harvested and assayed for the presence of IFN-γ by using an ELISA. As observed for the proliferative response in *A*, IFN-γ production could be detected in SIINFEKL concentrations of 1 pM and greater, but was not observed at concentrations in the femtomolar range. The amount of IFN-γ was calculated on the basis of the comparison with a standard curve obtained by using a titration of purified recombinant mouse IFN-γ. (*C*) Conjugates were formed between OT-1 lymph node T cells and M12.K^b (wild-type) APCs that had been pulsed with the indicated dose of SIINFEKL. The conjugates were formed for 7 min before fixation and staining. The percentage of CD3$^+$ cells that formed a conjugate with a peptide-pulsed APC was determined by counting 200 CD3$^+$ cells in the fluorescence microscope. The presence of an APC in the conjugate was identified with use of transmitted light and/or autofluorescence. The extent of conjugate formation at 1 pM (13%) was approximately one-fifth of the maximal response at the higher peptide concentrations. At concentrations of peptide below 1 pM, no appreciable conjugate formation occurred. Experiments in which either nontransfected M12 cells or M12 cells transfected with the mutant H-2K^b gene were incubated with OT-1 T cells always had less than 5% of conjugated T cells, irrespective of the dose of peptide.

production of IFN-γ was ≈10% of the maximal response. We also examined the requirement for CD8 binding for the production of IFN-γ by OT-1 cells with M12 cells that expressed the mutant H-2K^b molecule. The mutant H-2K^b molecule did not stimulate the production of IFN-γ by OT-1 T cells (Fig. 1*B*, triangles) even in the presence of micromolar concentrations of antigenic peptide. The addition of antibody to CD8 (YTS-169) completely ablated the production of IFN-γ by the OT-1 cells across the complete range of peptide concentrations (data not shown). Thus, responsiveness of the OT-1 T cells *in vitro*, as measured by either proliferation or cytokine production, was considered to be CD8-dependent.

To analyze conjugate formation between OT-1 T cells and APCs in the presence of different concentrations of SIINFEKL, APCs were pulsed with the peptide for 1 h and the unbound peptide was removed by washing the cells. T cell:APC conjugates were formed for 7 min before fixation and staining. The percentage of CD3$^+$ cells that formed a conjugate with a peptide-pulsed APC was determined. As shown in Fig. 1*C*, approximately half (range of multiple experiments, 31–61%) of the CD3$^+$ T cells formed a conjugate with the peptide-pulsed APCs at concentrations of peptide down to 10 pM. In the absence of peptide, or in the presence of 100 fM SIINFEKL, less than 4% (in general, 0–3%) of the T cells bound to the APCs (Fig. 1*C*). (In other experiments we noted that T cell clones maintained *in vitro*, unlike the fresh *ex vivo* T cells, show higher frequencies of conjugate formation in the absence of antigenic peptide.) In the experiment shown in Fig. 1*C*, the frequency of T cells that formed conjugates in the presence of 3 and 1 pM peptide was 19% and 13%, respectively. Therefore, the extent of conjugate formation at 1 pM was ≈20% of the maximal response, demonstrating that conjugate formation between CD8 T cells and peptide-pulsed APCs occurs at the doses of antigen that are similar to those required to elicit other responses in these T cells.

c-SMAC and p-SMAC Are Formed on OT-1 T Cells Interacting with Peptide-Pulsed APCs. As mentioned above, the c-SMAC on CD4$^+$ T cells is identified by the presence of PKCθ, whereas the p-SMAC is identified by the presence of LFA-1 and talin. We examined the distribution of these molecules in conjugates between OT-1 lymph node T cells and peptide-pulsed M12.K^b cells by using three-dimensional fluorescence microscopy. During the interaction of OT-1 T cells with APCs, there are definite c-SMAC and p-SMAC zones, defined by the clustering of the cytoplasmic proteins PKCθ and talin, respectively. This clustering is shown in Fig. 2 in which PKCθ (stained red with Cy3) is located primarily in a central region that is surrounded by talin (stained green with FITC). Similarly, in Fig. 3 *A–D*, the segregation of LFA-1 (green) from PKCθ (red) and the colocalization of LFA-1 (stained green) with talin (stained red; Fig. 3 *E–H*) in the p-SMAC are shown. The images presented in Fig. 3 *A–H* are characteristic of conjugates formed in the presence of 5–100 pM SIINFEKL.

We further examined the enrichment of these molecules in the T cell–APC contact area across a range of peptide concentrations. In all T cell:APC conjugates formed in the presence of SIINFEKL at a concentration of 1 pM or greater, both talin and LFA-1 were enriched in the contact area. At the 100 fM concentration of peptide the number of conjugates formed was indistinguishable from that formed between OT-1 T cells and APCs either in the absence of peptide or in the absence of H-2K^b (0–4%). In conjugates formed under such circumstances, there is generally no enrichment of either LFA-1 or talin, although a few conjugates may show slight incremental increases in the localization of these molecules in the contact area. The enrichment of PKCθ in the contact area was also apparent in most (≈90%) of the conjugates formed in the presence of 100 pM or greater. The number of conjugates with PKCθ enrichment declined at 10 and 1 pM to 70% and 50%, respectively. In contrast to the readily apparent enrichment of LFA-1 and talin,

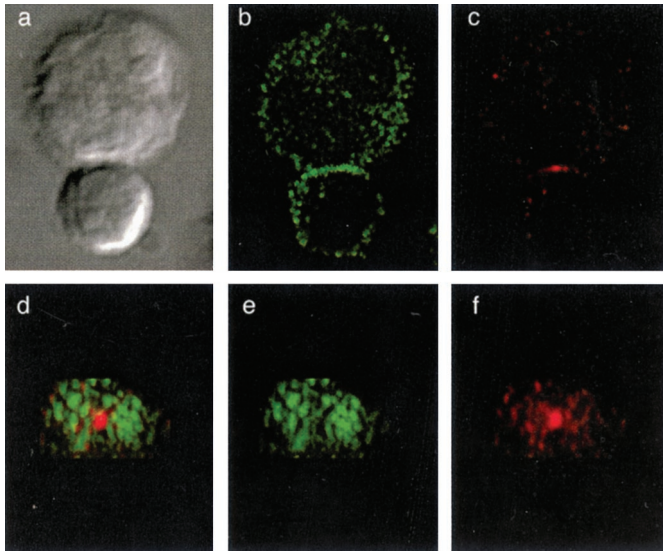

Fig. 2. Staining for talin (FITC in *b*, *d*, and *e*) and PKCθ (Cy3 in *c*, *d*, and *f*) in conjugates between OT-1 T cells and APCs pulsed with 100 pM SIINFEKL. This figure shows the views from the top (*a–c*) and the rendered image of the contact area rotated 90° (and magnified 150%) (*d–f*). PKCθ is in the c-SMAC region, whereas talin is excluded from this area and is confined to the p-SMAC region. The mean fluorescence intensity of talin staining in this image is 219 units in the p-SMAC region and 19 units in the remainder of the cell (11.5-fold enrichment). The mean fluorescence intensity of PKCθ staining is 1,106 units in the c-SMAC region and 6.7 units in the remainder of the cell (166.3-fold enrichment).

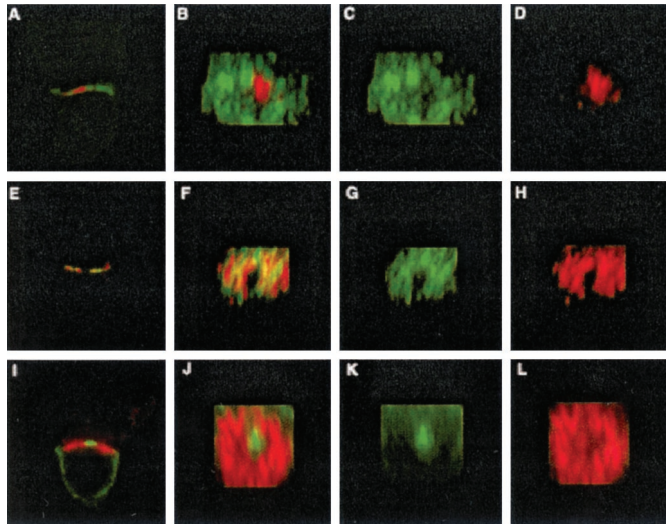

Fig. 3. Staining for PKCθ, talin, LFA-1, and CD3 in conjugates between OT-1 T cells and APCs pulsed with either 100 pM (*A–H*) or 10 nM (*I–L*) SIINFEKL. On the left-hand side of the figure (*A*, *E*, and *I*), a single plane showing both fluorochromes is presented. The remainder of the figure shows the rendered images of the contact area rotated 90° (and magnified 150%). (*A–D*) Staining for LFA-1 (FITC) and PKCθ (Cy3). The mean fluorescence intensity of LFA-1 staining in this image is 2,705 units in the p-SMAC region and 453 units in the remainder of the cell (6-fold enrichment). The mean fluorescence intensity of PKCθ staining is 21,897 units in the c-SMAC region and 491 units in the remainder of the cell (44.6-fold enrichment). (*E–H*) LFA-1 (FITC) and talin (Cy3) staining. The mean fluorescence intensity of talin staining in this image is 820 units in the p-SMAC region and 174 units in the remainder of the cell (4.7-fold enrichment). The mean fluorescence intensity of LFA-1 staining is 437 units in the p-SMAC region and 103 units in the remainder of the cell (4.2-fold enrichment). (*I–L*) CD3 (FITC) and talin (Cy3) staining. The mean fluorescence intensity of talin staining in this image is 1,393 units in the p-SMAC region and 136 units in the remainder of the cell (10.2-fold enrichment). The mean fluorescence intensity of CD3 staining is 881 units in the c-SMAC region and 199 units in the remainder of the cell (4.4-fold enrichment). *A–D* demonstrate that, although PKCθ is present in the c-SMAC region, LFA-1 does not colocalize with PKCθ and is confined to the p-SMAC region. *E–H* demonstrate that neither LFA-1 nor talin is present in the c-SMAC region, and they are confined to the p-SMAC region. *I–L* demonstrate that at these relatively high concentrations of peptide (10 nM), there is enrichment of CD3 in the c-SMAC region. This enrichment of CD3 into the c-SMAC region is not observed in the presence of SIINFEKL in the picomolar range.

and to a lesser extent PKCθ, observed in the contact area at peptide doses of 1 pM or greater, we did not observe significant enrichment of CD3 in the presence of 1–100 pM SIINFEKL. Although it is likely that very few CD3/TCR complexes were clustered in a c-SMAC, it is technically impossible to detect such a small amount of clustering in the presence of a much larger number of unengaged TCRs. Accordingly, enrichment of CD3 in the contact area was readily observed in the presence of 1, 10, and 100 nM SIINFEKL (in 9%, 48%, and 51% of conjugates, respectively), as shown in Fig. 3 *I–L*. Thus, on interaction of $CD8^+$ T cells with peptide-pulsed APCs, enrichment of LFA-1, talin, and PKCθ in the contact area, and the formation of SMACs was consistently observed at doses of antigenic peptide that were similar to the dose required for T cell responsiveness. In contrast, visualizing the enrichment of the CD3/TCR molecular complex in the contact area required higher doses of peptide.

Binding of the CD8 Coreceptor to H-2K^b Is Required for Conjugate Formation of OT-1 T Cells with Peptide-Pulsed APCs. To investigate the contribution of CD8 coreceptor function to conjugation and SMAC formation, we used APCs that express mutant H-2K^b molecules that are incapable of binding CD8. Mutation from an acidic to a basic residue at position 227 of murine MHC class I molecules ablates their ability to bind CD8 (9, 22). We have provided evidence that this mutation does not affect the binding of peptides to H-2K^b (23). For the SIINFEKL epitope, an antibody, 25D1.16, that recognizes this peptide when bound to H-2K^b has been produced (21). We used this antibody to confirm that the mutation at residue 227 did not affect the binding of the SIINFEKL peptide. As shown in Fig. 4, the 25D1.16 antibody readily bound to M12 cells expressing either the wild-type or Lys^{227} mutant H-2K^b molecule, after a 60-min pulse with 100 nM SIINFEKL peptide. The relative increase in the binding of 25D1.16 to wild-type and mutant H-2K^b was similar with doses of peptide ranging from 10 nM to 100 μM (Fig. 4). Thus the mutation at residue 227 does not alter the binding of the SIINFEKL peptide. When OT-1 T cells were mixed with peptide-pulsed M12 cells that express the mutant form of H-2K^b no proliferation or production of IFN-γ occurred (Fig. 1). In addition, when these cells were used as APCs, there was no detectable increase in conjugate formation with OT-1 T cells above background (0–5%) levels. Even in the presence of concentrations of SIINFEKL up to 10 μM (7 orders of magnitude greater than the amount required for conjugate formation with cells expressing wild-type H-2K^b), there was still no significant increase above background levels in conjugate formation, The inability of OT-1 T cells to form conjugates with cells expressing the mutant H-2K^b molecule cannot be attributed to a reduction in H-2K^b expression on these cells, because both transfectants (wild-type or mutant H-2K^b) expressed identical amounts of H-2K^b (data not shown) and H-2K^b SIINFEKL (Fig. 4).

Thus, at least for OT-1 T cells, which seem to be "CD8-dependent," conjugate formation absolutely depends on the ability of CD8 to bind to the same MHC molecule (H-2K^b) as the

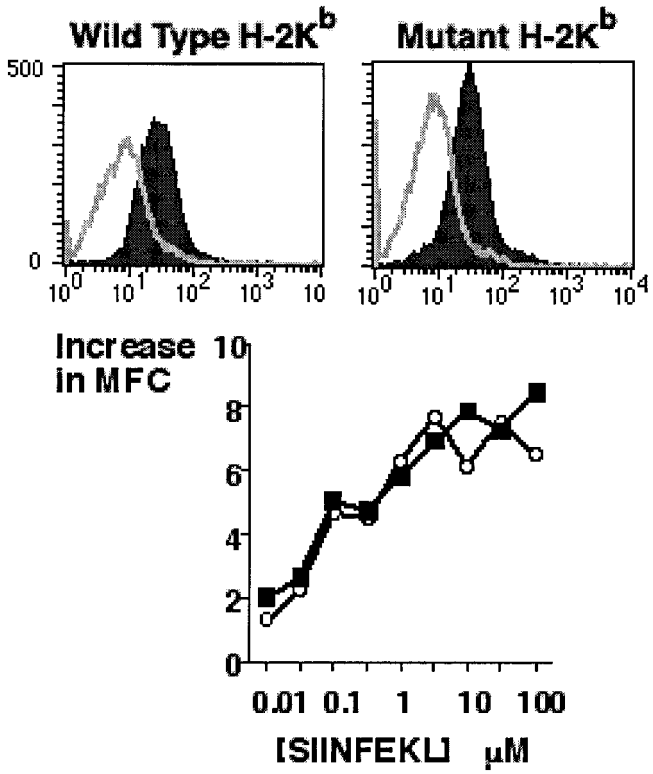

Fig. 4. Binding of the SIINFEKL peptide to wild-type (*Upper Left*) or mutant (*Upper Right*) H-2K^b. Cells expressing either form of the H-2K^b molecule were incubated with 100 nM SIINFEKL for 60 min at 37°C and then stained with the 25D1.16 antibody, which recognizes the H-2K^b–SIINFEKL complex (solid histogram). In both panels, the unfilled histogram represents the amount of binding of 25D1.16 to nonpulsed cells. For either cell type, binding of the 25D1.16 antibody required the addition of at least 10 nM SIINFEKL. At all doses of SIINFEKL greater than 10 nM, the shift in mean fluorescence channel (MFC) was approximately the same for both cells (*Lower*: ■, M12.K^b wild-type; ○, M12.K^b mutant, Lys^{227}) suggesting that the mutation at residue 227 does not affect the binding of SIINFEKL to H-2K^b.

TCR. Obviously, in the absence of T cell:APC conjugates, SMACs did not form on the OT-1 T cells.

Discussion

During the interaction of CD4$^+$ T cells with APCs, several cytoplasmic components, as well as receptors on the surface of the T cells, move to the area of contact with the APC. The redistribution of these molecules into distinct zones such as the c- and p-SMACs suggests that the activation of T cells does not arise from the stimulation of random individual T cell receptors. Instead, the formation of SMACs, or the ''immunological synapse'' (24), may facilitate the integration of signals from several receptors and may provide a mechanism to account for the ability of T cells to be activated under conditions of low TCR occupancy. It certainly has been suggested that the degree of TCR occupancy required for the activation of CD8 T cells is less than that required for the activation of CD4$^+$ T cells. The activation of most CD4$^+$ *ex vivo* T cells, for example, T cells from the IE^k-PCC-reactive TCR transgenic AND mouse (25), generally requires doses of peptide in the micromolar range. In contrast, CD8 T cells, such as the OT-1 T cells used in this study, are often able to recognize and respond to APCs in the presence of concentrations of peptide in the low picomolar range (Fig. 1). Although some studies have provided evidence for the redistribution of intracellular organelles, such as secretory granules (26, 27) and the microtubule organizing center (28–30), during the activation of cloned cytotoxic T lymphocyte, there has been no analysis of SMAC formation on CD8 T cells during interaction with APCs. It is quite plausible that SMAC formation required a high degree of TCR occupancy and thus was a unique property of CD4$^+$ helper cells that is not shared by CD8$^+$ cytotoxic T lymphocyte.

The c-SMAC formed in CD4 T cells can be visualized by several criteria. First, it is the area in the contact that is surrounded by the p-SMAC molecules (such as talin and LFA-1). Second, PKCθ is clustered in the c-SMAC. Third, the engaged TCR/CD3 complex is found in the c-SMAC. The results of this study demonstrate that OT-1 CD8$^+$ T cells show molecular redistribution similar to that observed on CD4$^+$ T cells. This finding further validates the significance of SMAC formation during T cell activation.

In CD4$^+$ T cells, conjugate formation and talin, but not PKCθ, clustering can be observed in the presence of suboptimal antigen concentrations (4). We examined whether molecular redistribution on CD8$^+$ T cells was influenced by the concentration of antigen. Specifically, we investigated whether SMAC formation required high peptide doses consistent with a requirement for high TCR occupancy. Peptide concentrations in the low picomolar range were sufficient to stimulate these T cells *in vitro* (Fig. 1). This amount of peptide was also sufficient to mediate conjugate formation between OT-1 T cells and APCs that express wild-type H-2K^b. Within this range of peptide concentrations, enrichment of talin and LFA-1 was readily apparent in virtually all conjugates. The proportion of conjugates showing redistribution of PKCθ was somewhat reduced in the range of 1–10 pM peptide; however, ≈50% of conjugates at 1 pM peptide showed PKCθ enrichment in the contact area. At suboptimal concentrations of antigen (100 fM), there were very few conjugates, and talin was clustered at the contact area in only a few of these conjugates. Thus, the establishment of distinct c-SMAC (identified by PKCθ) and p-SMAC (identified by talin and LFA-1) regions occurred under ''physiological'' conditions similar to those required to activate other events (proliferation, cytokine production) in OT-1 CD8$^+$ T cells. To detect enrichment of CD3 in the T cell–APC contact area, much higher concentrations of peptide were required. We attribute the failure to observe enrichment of CD3 at the lower concentrations of peptide (picomolar range) to reflect the extent of occupancy of the TCR. At low peptide concentrations the number of H-2K^b–SIINFEKL complexes is very low but is sufficient for T cell activation. This amount of H-2K^b–SIINFEKL complexes is insufficient to bind enough TCR/CD3 complexes so that an enrichment of CD3 molecules in the contact area could be visualized in the presence of a vast excess of unbound TCR complexes. Presumably, the ability of higher antigen concentrations to facilitate detectable CD3 enrichment reflects a higher degree of occupancy of the TCR/CD3 complexes by H-2K^b–SIINFEKL.

The essential role of the coreceptor CD8 was demonstrated in the *ex vivo* T cells, because target cells that express mutant H-2K^b, in which lysine is substituted for aspartic acid at residue 227, do not form conjugates with OT-1 T cells in the presence of SIINFEKL. This substitution ablates the interaction of H-2K^b with CD8 (9, 22) and is a major contact residue in cocrystals of CD8 and MHC class I molecules (12, 13). The ablation of the CD8–MHC class I interaction resulting from the mutation at residue 227 generally results in the inability of CD8-dependent T cells to recognize and respond to cells expressing the mutant molecule. The inability of OT-1 T cells to form conjugates in the absence of CD8 coreceptor function was not overcome by the addition of very high concentrations (high micromolar range) of the antigenic peptide SIINFEKL. Most functional interactions of CD8 T cells with APCs require that CD8 and the TCR bind the same MHC class I molecule. The coreceptor function of CD8

and CD4 in T cell activation most likely includes the activation of $p56^{lck}$ and the subsequent phosphorylation of CD3 and/or other signaling components (31) and the stabilization of the TCR–MHC interaction (10). It has also been suggested that subsequent to activation through the TCR, the binding of CD4 and CD8 to nonantigenic MHC class I molecules leads to an increase in the avidity of the T cell–APC interaction. Certainly with OT-1 T cells, it is clear that TCR binding in the absence of CD8 is insufficient to mediate conjugate formation and the initiation of molecular redistribution.

Investigations into the movements of CD4 during T cell–APC interaction have revealed that CD4 is clustered together with CD3ζ at the interface at the same time as the initial rise in intracellular Ca^{2+} level (2). At later times, CD4 moves to the periphery of the interface, suggesting that the role of CD4 is confined to the initial activation phase, during which it may serve to augment the TCR–MHC–peptide interaction; however, at later times CD4 is not required (2). In this scenario, the mechanism by which this interaction is terminated after the initial activation events is not apparent. Localization of the tyrosine phosphatase CD45 is also temporally regulated during interaction with APCs (32). Examining the movement of CD8 during the interaction of OT-1 T cells with peptide-pulsed APCs should allow us to determine whether it redistributes in a manner similar to that described for CD4.

Finally, the T cells used in this study were freshly isolated from the lymph nodes of the OT-1 transgenic mouse. The T cells were not exposed to antigen *in vivo* and were essentially "resting naïve" T cells before incubation with peptide-pulsed APCs. Analysis by flow cytometry revealed that less than 6% of the OT-1 T cells were $CD44^{hi}$ (data not shown); thus, only a minor fraction of T cells had been activated *in vivo*. Therefore, because the number of T cells that formed conjugates was significantly greater than the number of $CD44^{hi}$ T cells, naïve CD8 T cells are able form conjugates and SMACs with APCs in the absence of exogenous cytokines, chemokines, or other activation stimuli.

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