

Costimulation and endogenous MHC ligands contribute to T cell recognition

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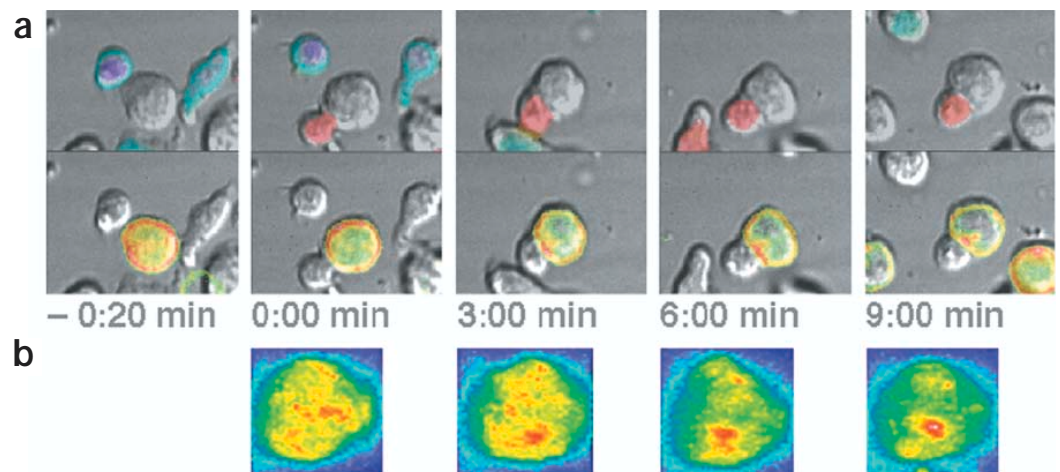
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To initiate an immune response, key receptor-ligand pairs must cluster in “immune synapses” at the T cell–antigen-presenting cell (APC) interface. We visualized the accumulation of a major histocompatibility complex (MHC) class II molecule, I-E^k, at a T cell–B cell interface and found it was dependent on both antigen recognition and costimulation. This suggests that costimulation-driven active transport of T cell surface molecules helps to drive immunological synapse formation. Although only agonist peptide–MHC class II (agonist pMHC class II) complexes can initiate T cell activation, endogenous pMHC class II complexes also appeared to accumulate. To test this directly, we labeled a “null” pMHC class II complex and found that, although it lacked major TCR contact residues, it could be driven into the synapse in a TCR-dependant manner. Thus, low-affinity ligands can contribute to synapse formation and T cell signaling.

T cells are normally activated through direct contact with an antigen-presenting cell (APCs). During this process a number of the key receptor-ligand complexes accumulate in discrete geometric patterns at the T cell–APC interface^{1–4}. In particular, intercellular adhesion molecule 1–lymphocyte function–associated antigen 1 (ICAM-1–LFA-1) tends to accumulate at the periphery of the interface, whereas complexes between T cell receptor (TCR) and peptide–major histocompatibility complex (pMHC) congregate at the center. This complex has been referred to as the

“supramolecular activation cluster” (SMAC)¹ or the “immunological synapse”² and it has been suggested that it potentiates TCR signaling⁵. As the pMHC on the APC plays a key role in this process, we decided to study the dynamics of these complexes on live cells using three-dimensional (3D) fluorescence video microscopy. In particular, we asked whether the clustering of MHC–TCR at the T cell–APC interface is mediated by a passive diffusion-driven interaction—as has been proposed^{6,7}, and which is referred to as mutual capping—or is instead the result of an active process

Figure 1. I-E^k accumulates at the T cell–APC interface. (a) Accumulation of I-E^k at the interface between an A20.I-E^k APC and a 5C.C7 T cell. Each panel consists of identical bright field images. The top images are overlaid with a transparent rainbow color-scale of the ratio of the fluorescence emissions of Fura-2 (which is a calcium-sensitive dye) when excited at 340 nm and 380 nm. This ratio correlated with the intracellular calcium concentration. Blue indicates low resting calcium; red indicates high resting calcium. The bottom images are overlaid with a transparent color-scale of I-E^k–GFP fluorescence. The I-E^k–GFP fluorescence was recorded in a stack of 21 z-planes per timepoint. The middle plane of the stack is shown overlaid with a rainbow color-scale. Blue indicates low, yellow indicates medium and red to white indicates high I-E^k–GFP concentrations. For clarity blue has been removed. Time relative to T cell activation is shown. This figure consists of individual frames taken from Web Movie 1. (b) A 3D reconstruction of the I-E^k–GFP intensity of an A20.I-E^k cell from a. With respect to its orientation in a, the 3D reconstruction has been rotated around the y-axis, so that the T cell–APC interface points up from the page. Time relative to T cell activation is shown. This figure consists of individual frames taken from Web Movie 2.



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Table 1. Peptide quantity and affinity determine the pattern of the I-E^k accumulation

T cell ^a	Peptide regime	Accumulation type					n ^c
		None ^b (no. 1)	Unstable (no. 2)	Diffuse (no. 3)	Diffuse→concentrated (no. 4)	Concentrated (no. 5)	
5C.C7	MCC ^d (10 μM)	9%	2%	26%	18%	45%	65
5C.C7	1:10 MCC:null ^e (10 μM)	14%	23%	32%	27%	5%	22
5C.C7	1:100 MCC:null (10 μM)	24%	58%	19%	0%	0%	21
2B4	1:100 MCC:null (10 μM)	19%	43%	29%	5%	5%	21
2B4	MCC (0.1 μM)	23%	64%	14%	0%	0%	22
5C.C7	MCC ^{T102S} (10 μM)	13%	22%	53%	6%	6%	32

^aTransgenic mouse model. ^bThe predominant phenotype is shown in bold. ^cNumber of cell couples analyzed, derived from at least three independent experiments. ^dMCC denotes MCC(82–103). ^e Null denotes MCC^{D93E,K99T,T102A}.

that is dependant on the T cell cytoskeleton^{8,9} and controlled by the costimulatory receptors CD28 and LFA-1^{10–15}. These receptors are central regulators of T cell activation efficiency and play a role in autoimmune disease, cancer and transplant rejection. The mechanism by which they augment the efficiency of T cell activation is still unclear, however^{8,9,16,17}. We show here that robust MHC class II clustering is costimulation-dependent, CD4-independent and appears to be driven by TCR movement on the T cell. These findings support previous suggestions that a major component of the costimulatory effect derives from the increased density of TCR-CD3 at the interface^{8,9}.

MHC on the surface of an APC displays a wide variety of peptides. Even during an immune response only a small fraction of these display a given antigenic peptide. Only these antigenic pMHC complexes have a high enough affinity to enable the TCR to induce T cell activation. However, it has long been suspected that endogenous pMHC complexes with an unmeasurably low affinity for the TCR play a key role in T cell physiology. They contribute to thymic selection^{18–20} and are implicated in the survival of mature T cells in the periphery^{21–26}. Studying MHC-TCR accumulation under conditions where agonist pMHC complexes were limiting, we still found a substantial accumulation of MHC class II at the T cell interface. It was far more than could be accounted for by the amount of agonist pMHC, which suggested that endogenous pMHC class II complexes could contribute to synapse formation. We tested this hypothesis directly by showing that a peptide that lacks most major T cell contact residues can nonetheless accumulate in T cell-induced synapses in the presence of agonist pMHC and enhance T cell activation. These data indicate that endogenous peptides that have no *in vitro* biological activity alone can accumulate and function in critical signaling structures in a TCR-dependent manner.

Results

Accumulation of MHC class II

We used 3D video microscopy^{3,4} to visualize the distribution of the murine MHC class II molecule I-E^k during T cell–APC interactions. We used live, *in vitro*-primed, primary T cells from either 5C.C7 or 2B4 αβ TCR-transgenic mice^{27–29} with a B cell lymphoma APC that expressed an I-E^k-green fluorescent protein (GFP) fusion protein at the physiological density of 245 molecules/μm² (see Methods). The 5C.C7 and 2B4 TCRs both recognize I-E^k that presents the moth cytochrome c peptide from amino acids (aa) 88 through 103 (full agonist MCC) and its derivatives.

After the initiation of T cell activation—as judged by a steep rise in the intracellular calcium concentration and the concomitant formation of a tight T cell–APC interface—we observed that I-E^k accumulated at the interface in less than 1 min (Fig. 1 and see Web Movies 1 and 2 on the supplementary information page of *Nature Immunology* on line). Two distinct types of accumulation occurred, which we refer to as “diffuse” and

“concentrated”⁴. The diffuse form of I-E^k accumulation covered the whole T cell–APC interface, whereas the concentrated form covered only 10–25% of the interface area. Both had a fluorescence intensity of between 40% and 100% greater than the fluorescence intensity of the parts of the APC surface that were not in contact with the T cell. These patterns were dynamic and changed from one phenotype to the other within a few minutes. I-E^k accumulation was confirmed by antibody staining, which showed that native I-E^k, but not the Fcγ receptor (CD16-CD32), accumulated in synapses formed between 5C.C7 T cells and B cell lymphoma APCs that expressed I-E^k endogenously (data not shown). Further evidence corroborates that MHC class II–GFP behaves in a similar manner to endogenous MHC class II is provided by a study showing that a COOH-terminal fusion of GFP to a MHC class II β-chain similar to the one used here displays undisturbed intracellular trafficking³⁰.

When the I-E^k-GFP-transfected A20 cells (A20.I-E^k) were incubated in a high concentration (10 μM) of the full agonist peptide MCC(82–103), 60–70% of the T cell–APC complexes showed concentrated accumulation either by itself or, more rarely, against a background of diffuse accumulation (Table 1, no. 5). This often developed from an initial diffuse accumulation over several minutes (Table 1, no. 4). Based on staining of T cell–B cell couples¹ and studies that show T cells interact with lipid bilayers containing mobile I-E^k², the concentrated form was expected. Use of the partial agonist peptide MCC^{T102S}, in which Thr¹⁰² of MCC is mutated to Ser¹⁰²

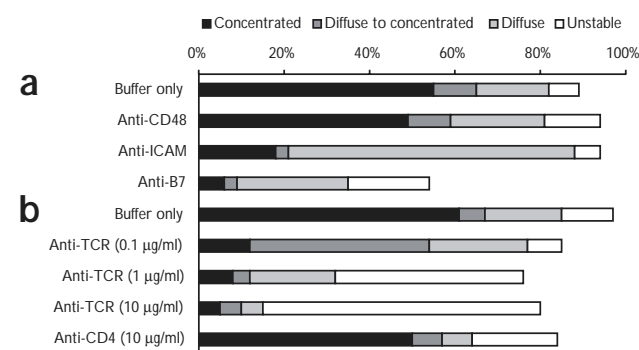


Figure 2. I-E^k accumulation is controlled by costimulation and mediated by TCR but not by CD4 binding. I-E^k-GFP transfected B cell lymphoma cells were loaded with 10 μM agonist peptide and used to activate 5C.C7 T cells in the presence of the indicated antibodies or antibody fragments. The percentage of T cell–APC couples that showed I-E^k accumulation in a specific pattern, as defined in Table 1, is shown. Between 20 and 42 cells were analyzed per condition. (a) Patterns of I-E^k accumulation in the presence of blocking antibodies (10 μg/ml) to different accessory ligands, showing its dependence on costimulation. (b) TCR or CD4 were blocked with mAb KJ25 (anti-TCR Fab) fragments or mAb GK1.5 (anti-CD4). Each experiment was done at least three times on at least two separate days.

Table 2. I-E^k accumulation substantially exceeds agonist pMHC class II at limiting peptide concentrations

Agonist dilution ^a	Activation ^b	I-E ^k loaded with agonist		I-E ^k accumulated ^e	Ratio of I-E ^k accumulated to amount of agonist
		Mean ^c	Biased ^d		
Undiluted	51±7.5% (n=7)	1.3±0.35%	1.3–2.75%	1.85±0.25% (n=12)	1.4±0.4
1:10	53±6% (n=4)	0.13%	0.13–0.275%	1.4±0.15% (n=10)	11
1:100	42±5.5% (n=8)	0.013%	0.0145–0.0275%	2.35±0.45% (n=10)	180

^aDilution of agonist peptide MCC(82–103); null peptide MCC^{D93E,K99TT102A}. ^bCalculated as the percentage of APCs in contact with a T cell that activate the T cell. The number of independent experiments is shown in parentheses. ^cMean determined, by RIA, for the maximal amount of agonist peptide and for the dilutions. ^dCalculated assuming that T cells are only activated by the part of the APC population that expresses the highest peptide densities (see Methods). ^eCalculated as a percentage of total I-E^k; the numbers of cell couples assessed are shown in parentheses (see Methods).

(refs. 31–33), resulted in little concentrated accumulation of I-E^k (Table 1). This was consistent with published data^{1–3} and supported the hypothesis that robust synapse formation correlates with efficient T cell activation.

Control of MHC class II accumulation

To determine whether APCs or T cells drive the accumulation of I-E^k, we paralyzed the actin cytoskeleton of either cell by pretreatment with cytochalasin D²⁹. Although pretreatment of the T cells disrupted most aspects of T cell recognition and activation^{29,34,35}, identical pretreatment of the A20 cells had no effect on the accumulation of I-E^k at the interface (data not shown). This indicated that the T cell drives the accumulation of I-E^k, as it does ICAM-1 accumulation²⁹; this was consistent with published data, which shows that T cells can form synapses on an artificial lipid bilayer². These results suggested that MHC accumulation is driven by the action of the CD4 coreceptor, the TCR or both (see below).

Costimulatory signals through CD28 and LFA-1 can trigger the movement of the T cell cortical actin cytoskeleton toward, and the accumulation of membrane patches at, the T cell–APC interface^{8,9}. Therefore, we used the blocking antibodies 16-10A1 (anti-B7-1), GL1 (anti-B7-2), YN1 (anti-ICAM-1) or HM48-1 (anti-CD48)⁸ to assess whether this costimulation-controlled mechanism is involved in the accumulation of I-E^k. We found that neither blocking antibody disturbed the formation of a tight interface. However, both anti-B7 and anti-ICAM-1, but not anti-CD48, interfered with I-E^k accumulation (Fig. 2). In the presence of anti-B7, only 50% of the cell couples accumulated I-E^k; most of the accumulation was

transient or diffuse. Blocking ICAM-1 still allowed substantial clustering of I-E^k at the interface, but concentrated accumulation was inhibited. The costimulation dependence of MHC class II accumulation was consistent with published data, which showed that ICAM-1 and peptide–I-E^k in a lipid bilayer can trigger the clustering of MHC class II underneath an activated T cell². However, alone in a bilayer system, MHC class II cannot produce this clustering phenomenon, even though T cell activation can still occur, as judged by T cell adhesion to the bilayer and increases in the intracellular calcium concentration²⁹ (and C. Wülfing, C. Sumen and M. L. Dustin, unpublished data). Collectively, these results showed that costimulation is critical to the accumulation of I-E^k at the T cell–APC interface and probably other aspects of synapse formation.

Nonagonist pMHC accumulation

To study the role of agonist peptide concentration in MHC class II–TCR accumulation, we diluted the agonist peptide MCC(82–103) with a null variant (MCC^{D93E,K99TT102A}), which lacks two of the three TCR contact residues critical for T cell activation. Although it binds I-E^k as efficiently as the agonist peptide^{31,33}, the null peptide does not elicit a biological response in 5C.C7 and 2B4 T cells. We found that I-E^k accumulation still occurred at dilutions of 1:10 and even 1:100, the minimal concentration necessary to activate 5C.C7 T cells. However, the geometrical pattern of this accumulation was different. At a 1:10 dilution of agonist peptide with null peptide (Table 1, row 2) this concentrated pattern, if it formed at all, only formed slowly (Table 1, no. 4). At limiting agonist concentrations (Table 1, row

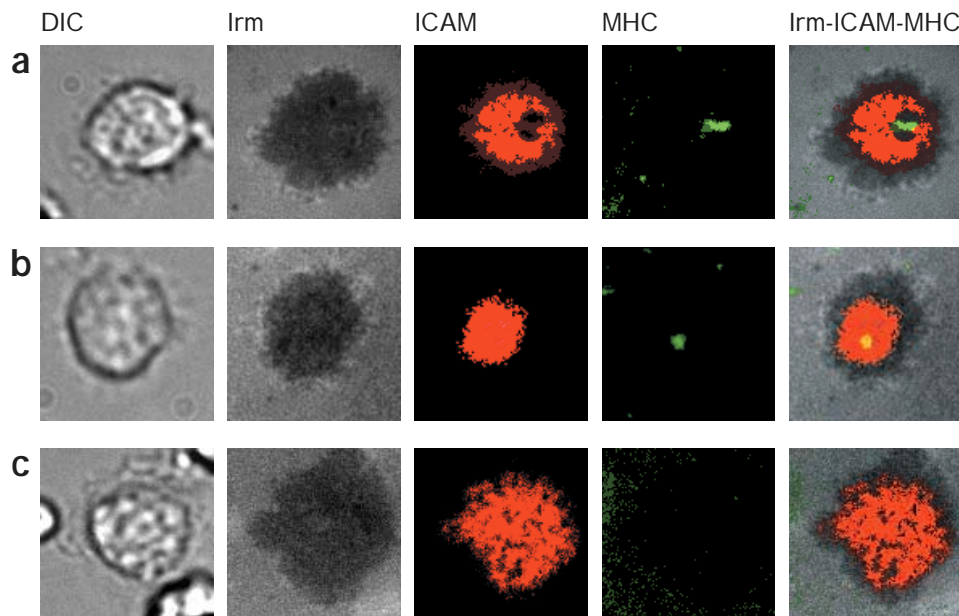


Figure 3. Null peptide–loaded I-E^k complexes accumulate at the interface. Supported lipid bilayers presenting 30/μm² of unlabeled activating gE^k–α32, 150/μm² of Cy5–ICAM-1 (red) and 200/μm² of OG-gE^k (30% peptide-loaded) to primed 5C.C7 T cells. Either (a,b) the null peptide MCC^{D93E,K99TT102A} or (c) the MCC^{K99E} variant was used to load gE^k. Irm, interference reflection microscopy (in which tight T cell–bilayer contacts appear dark). With respect to the ICAM accumulation in the T cell contacts with the supported lipid bilayers, we found that ICAM was excluded from the central MHC zones in 50% of the contacts (a). In contacts where ICAM exclusion was absent or incomplete, distinct MHC accumulation near the center was still present (b), although usually reduced in density. Accordingly, the MHC cluster density was 380 I-E^k molecules/μm² in a and 180 I-E^k molecules/μm² in b. Cells were representative of respective phenotypes from three independent experiments.

3), no concentrated accumulation was observed. The majority of the MHC class II accumulation was transient (Table 1, no. 2). When, instead of diluting the agonist peptide with the null peptide, we pulsed the A20 cells with smaller amounts of agonist peptide, similar results were obtained (Table 1). These data suggested that TCR–MHC class II accumulation in a concentrated form is not strictly required for T cell activation *in vitro* but, rather, is an indicator of its efficiency.

We quantified both the total amount of agonist peptide–I-E^k on the APC surface and the amount of I-E^k, irrespective of the peptide bound, that accumulated at the interface. We used this data to determine whether nonagonist pMHC class II can accumulate in addition to agonist pMHC at the T cell–APC interface. The amount of I-E^k that accumulated at the T cell–APC interface was independent of the ratio of agonist to nonagonist peptide (Table 2). At the highest agonist peptide dilution, the amount of accumulated I-E^k at the interface exceeded that of total agonist peptide–I-E^k by 180-fold (Table 2). Thus, I-E^k molecules that are not loaded with agonist peptides must also be able to accumulate at the T cell–APC interface, at least when agonist peptide is limiting.

TCR binding in MHC class II accumulation

As shown above and in artificial bilayer experiments², MHC class II accumulation is driven by the T cell. Thus, its movement must be dependent on a T cell molecule that is mobilized into the synapse; one candidate is CD4. To investigate this, we blocked the I-E^k–CD4 interaction with the monoclonal antibody (mAb) GK1.5 (Fig. 2). Although the frequency of T cell activation was reduced (data not shown), I-E^k accumulation was undisturbed. When we blocked CD4 at limiting concentrations of antigenic peptide, only a few T cell–APC couples formed, which prevented comprehensive analysis. But even under those conditions, MHC–TCR accumulation was observed consistently (data not shown). To corroborate the anti-CD4–blocking data, we also constructed an I-E^k mutant in which the two major CD4 contact residues, Glu¹³⁷ and Thr¹⁴⁰^{36,37} on the β2 domain, were mutated to alanine. Again, T cell activation was impaired, but I-E^k accumulation was not (data not shown). To test whether TCR engagement was required for I-E^k accumulation, we blocked TCR recognition with an anti-TCR Fab fragment (mAb KJ25, which binds to CDR2 on the V_β3 surface). Even relatively low concentrations of the anti-TCR Fab fragment had marked effects, delaying formation of the concentrated pattern of I-E^k accumulation at 0.1 μg/ml and virtually eliminating it at 1 μg/ml (Fig. 2). We concluded, therefore, that CD4 on the T cell surface generally cannot engage I-E^k with a high enough affinity to result in substantial binding and that the TCR is likely the principal mediator of MHC class II accumulation, although scenarios involving other molecules are possible.

Direct visualization of null pMHC accumulation

If the TCR is responsible for MHC class II accumulation, it should be sensitive to which peptide is bound to the MHC. Nonagonist pMHC complexes found in the synapse should, therefore, contain endogenous peptides

Table 3. Low affinity TCR–pMHC class II interactions can contribute to T cell activation

I-E ^k concentration ^a	Agonist I-E ^k ^b	Additional null-I-E ^k ^c	Activation ^d	<i>n</i> ^e	<i>P</i>	Activation I-E ^k mt only ^f
<0.45/μm ²	<0.45/μm ²	None	0%	5	0.04	0%
<45/μm ²	<0.45/μm ²	100×	8%			5%
>0.6/μm ²	>0.6/μm ²	None	7%	4	NS	9.5%
>60/μm ²	>0.6/μm ²	100×	5%			7.5%
<1.5/μm ²	<1.5/μm ²	None	3%	6	0.0003	0%
<45/μm ²	<1.5/μm ²	30×	20%			12%
>2/μm ²	>2/μm ²	None	16%	6	NS	20.5%
>60/μm ²	>2/μm ²	30×	15%			12.5%
<4.5/μm ²	<4.5/μm ²	None	9.5%	11	0.0001	11.5%
<45/μm ²	<4.5/μm ²	10×	27%			24%
>6/μm ²	>6/μm ²	None	31%	4	NS	32.5%
>60/μm ²	>6/μm ²	10×	37%			37.5%

This table should be read in pair-wise rows. For example, the first row described T cell activation by a bilayer that had been generated with 1/100th of the amount of I-E^k than the bilayer in the second row (I-E^k concentration: <0.45/μm² versus <45/μm²). The bilayer in the first row was loaded with agonist peptide only while the bilayer in the second row was loaded with a 1:100 dilution of agonist:null peptide. This generated to the same total amount of agonist peptide (agonist-I-E^k: <0.45/μm² for both) but different amounts of additional null peptide–I-E^k (additional null peptide–I-E^k: none versus 100×). The second row of each couple therefore shows the effect of additional null peptide on T cell activation. ^aThe concentration <45/μm² is 35±9/μm²; >60/μm² is 67±9/μm². All other values are dilutions as explained above. ^bAssumes 100× peptide-loading efficiency. ^cThese are shown as multiples of the agonist peptide concentration. ^dActivation values are expressed as % T cells that showed an increase in the intracellular calcium concentration. ^eFor each pair-wise comparison from *n* independent experiments the statistical significance, *P*, was calculated. NS, not significant. ^fNo differences were seen between the wild-type I-E^k or the E137A T140A double mutant (I-E^kmt) that showed defective CD4 interaction. Therefore, the data was pooled in all columns but this one. Here, the subsets of the results obtained with the E137A T140A mutant are shown separately.

that are similar to the agonist peptide, but not endogenous peptides that interfere with TCR binding. To directly visualize the accumulation of nonagonist peptide–I-E^k and to test this hypothesis, we used a lipid bilayer system that incorporates two different types of MHC. The first was a glycosyl-phosphatidyl inositol (GPI)-linked I-E^k molecule (gE^k-α32), which presented wild-type MCC(88–103) covalently linked to the I-E^k α-chain so that it could not exchange with other MHC molecules (L.C. Wu and M. M. Davis, unpublished data). These unlabeled, laterally mobile, activating complexes were seeded at low densities (<45 molecules/μm²). The second type of MHC incorporated into the bilayer was I-E^k linked to GPI and labeled with Oregon green (OG-gE^k), which can be loaded with peptide and visualized by video fluorescence microscopy. GPI-linked ICAM-1 labeled with Cy5 was also included to provide costimulation and to assess overall synapse formation².

We found that MHC class II loaded with the null peptide accumulated to an average density of 350 molecules/μm², at a frequency comparable to that of the agonist peptide alone (Fig. 3a,b). To test whether a peptide variant—which likely interfered with TCR recognition, behaved in the same way—we used MCC^{K99E}, in which lysine in the main CDR3-interacting position is charge-reversed to glutamate but MHC binding is the same as in wild-type MCC³⁸. When this peptide was loaded onto I-E^k, the T cells were no longer able to cluster the pMHC to the contact area at densities of >30 molecules/μm², which is well below the threshold for forming an immunological synapse² (Fig. 3c). Thus, by specifically visualizing null pMHC class II complexes in supported lipid bilayers that acted as APC substitutes, we provided direct evidence that such complexes can accumulate in a T cell contact zone after T cell activation, as long as they do not interfere with TCR–MHC binding.

Nonagonist pMHC and T cell activation

We next determined whether nonagonist pMHC could also influence T cell signaling. We activated 5C.C7 T cells with supported lipid bilayers that contained a small amount of agonist peptide–I-E^k either alone or in

combination with null peptide-I-E^k. At a low MHC density of <45 molecules/ μm^2 , bilayers that contained null peptide-I-E^k + agonist peptide-I-E^k, as opposed to bilayers that contained only agonist peptide-I-E^k, were significantly more potent T cell activators (Table 3). This was determined as an increase in the number of T cells that showed increased intracellular calcium concentrations. These results suggested nonagonist pMHC class II could play an important role in T cell activation. Using bilayers with a MHC density of >60 molecules/ μm^2 , we no longer observed this effect (Table 3). Our experimental MHC class II densities were within the physiological range of MHC class II densities on APCs, such as macrophages, with inducible MHC class II expression^{39,40}. This suggested that the amplification of T cell activation by low-affinity pMHC class II complexes is a physiologically relevant mechanism with which to enhance T cell activation at lower MHC class II densities. It has been hypothesized that the amplification of cellular signaling by low-affinity receptor-ligand interactions or “background noise” is important⁴¹, particularly at limiting activation conditions. Our data showed signal amplification occurred at lower MHC class II densities only, which was consistent with this idea. In summary, by systematically varying MHC class II and peptide concentrations we have provided direct evidence for signal amplification by “background noise” in T cell recognition.

Discussion

We have shown here that the accumulation of I-E^k at the immunological synapse is T cell-driven and requires costimulation. Thus, at least this part of synapse formation is an active process that is not due to the passive accumulation of receptors and ligands, as has been proposed^{6,7}, or cytoskeletal reorganization by the B cell. An active process driven by TCR-CD3 and other molecules that migrate to the center of the interface is also required to explain the rapidity with which synapse formation occurs^{2,4,29}. The resulting increase in TCR-CD3 density will likely potentiate signaling and thus could make up a large part of the costimulatory effect, as has been suggested^{8,9}. It remains to be determined how the immunological synapse segregates TCR to the center and LFA-1 to the edge of the interface, although a number of models have been proposed^{6,42} (and N. J. Burroughs and C. Wülfing, unpublished data). Dendritic cells—as opposed to the B lymphoma cells that we used here as APCs—actively contribute to the formation of the immunological synapse⁴³.

We have also shown that a null pMHC class II complex can accumulate at the interface of T cell–B cell couples and between T cells and ligands on a supported lipid bilayer. Because neither the B cell nor the bilayer provides an active MHC class II transport mechanism, this pMHC class II must have been brought into and held at the interface by binding interactions with the T cell. We have shown that the TCR, but not CD4, is the relevant MHC class II ligand. Two different mechanisms might explain how this occurs. One is that null peptide complexes can bind directly to the TCR, although the affinity must be low because it is undetectable in plasmon resonance studies³² (L. C. Wu, unpublished data). Another possibility is that the null pMHC complex associates “laterally” with agonist pMHC complexes. If the latter were true, it would not matter which null peptide was bound to the MHC, which would not explain why the MCC^{K99E} variant does not accumulate at a synapse. In addition, if only agonist pMHC binds the TCR at the T cell–APC interface, one would predict that this interaction would be rather insensitive to blocking of either TCR or MHC with antibodies at the highest agonist pMHC concentration, where ~100-fold more agonist pMHC is present than is minimally required. However, even relatively low concentrations of anti-TCR, as shown above, or anti-MHC (C. Wülfing and M. M. Davis, unpublished data) disturbed MHC class II accumulation at the T cell–APC interface. Both sets of data suggest

that at least part of the null pMHC class II accumulation is mediated by direct binding of the TCR. That such binding could occur in the absence of negative interaction (for example, MCC^{K99E}) is consistent with the finding that >50% of the binding energy in at least one TCR–pMHC complex can be attributed to direct TCR–MHC contacts⁴⁴.

We have therefore shown that a pMHC complex in which the peptide lacks two of the three major TCR determinants nevertheless directly interacts with the TCR at the T cell–APC interface and that this interaction can contribute to T cell signaling. We would therefore propose calling such a pMHC complex an “accessory ligand”. As TCR–MHC accumulation as a consequence of this low-affinity interaction can be seen in the presence and absence of an externally added defined null peptide, our data strongly suggests that such “accessory ligands” are an integral part of the endogenous peptide repertoire of each APC. Further support for key low-affinity pMHC–TCR interactions comes from data that showed antigen-specific T cells selected on low densities of MHC can be stimulated by the same MHC expressed at higher densities, regardless of the peptide bound⁴⁵.

Our results also have implications for the interpretation of another study² that found a marked correlation between TCR-ligand half-life and MHC density at the synapse. The simplest interpretation is that pMHC accumulation is a direct result of TCR binding. Our finding that a pMHC complex with no measurable half-life (<0.1 s) accumulated to a density equal to that of the most stable agonists suggests that this interpretation is untenable. Instead, we propose that limiting amounts of an agonist can dictate the rate of MHC accumulation, depending on its signaling strength, which would be dependant on TCR binding stability. This could, for example, be accomplished by influencing the velocity at which TCRs migrate to the interface after costimulation.

Methods

Cells, constructs, peptides and antibodies. 5C.C7 and 2B4 primary T cells were isolated and maintained as described³⁹. We fused GFP-bex1⁴⁶ to the COOH terminus of the β -chain of I-E^k with a 17-aa linker, as described for the ICAM-1–GFP fusion protein²⁹. Both I-E^k chains were then expressed from the plasmid pBJ1neo, as described⁴⁷. The E137A and T140A I-E^k double-mutant was expressed as a GFP fusion protein in an analogous manner. Similar mutations in I-E^k and I-A^b have major deficiencies in activating T cells^{48–51}. Peptides are derivatives of MCC(88–103). MCC(82–103) is functionally equivalent to MCC(88–103) and was used in its place in some of the experiments. Antibodies and antibody incubations were as described⁸. Anti-CD4 (mAb GK1.5) and the anti-V β 3 (mAb KJ25, both from Pharmingen, San Diego, CA) were preincubated with the cells for 30 min and were present during the experiment at concentrations of 0.1, 1 and 10 $\mu\text{g}/\text{ml}$.

Quantification I-E^k and agonist peptide-I-E^k on the APC surface. I-E^k on the surface of the A20.I-E^k cells and, as a control, on CH27 cells that expressed I-E^k endogenously was quantified with a radioimmunoassay (RIA) with the mAb 14.4.4, which recognizes I-E^k irrespective of the peptide bound. In brief, 5×10^5 cells were resuspended in 20 μl of assay buffer (PBS with 2% bovine serum albumin, 1 mM CaCl₂, 1 mM MgCl₂, 0.1% NaN₃) in the presence of 50 $\mu\text{g}/\text{ml}$ of anti-CD16 and anti-CD320 (Pharmingen). Radiolabeled mAb (Iodogen reagent, Pierce, Rockford, IL) (2 μg in 50 μl) was added to each well for 2 h at 4 °C. The extent of nonspecific binding (<5%) was assessed with identical wells pretreated for 1 h with 50-fold excess (100 μg) unconjugated mAb before the addition of radiolabeled mAb. For the molecule/ μm^2 extrapolations, average cell radius (r) was measured microscopically on hypotonically (in 10% PBS) swollen cells. The total experimental error of I-E^k surface densities, given in the results, was in all cases <15%. The peptide-loading efficiency of the A20.I-E^k cells was determined by comparing the surface density of total I-E^k (RIA with 14.4.4) with that of MCC-I-E^k (RIA with G35). Although 14.4.4 recognizes I-E^k irrespective of the peptide bound, G35 is specific for MCC⁵². In a representative experiment in which the total density of I-E^k was set to 100%, we found $5.8 \pm 1.0\%$ G35 binding after peptide pulsing with 10 μM MCC and $4.5 \pm 0.6\%$ in the absence of peptide. The 1.3% difference was the peptide-loading efficiency and was statistically significant ($P < 0.01$, Student's t -test). This value was consistent with published reports of peptide-loading efficiencies of MHC class II on mouse B cell lymphomas of <1%⁵³ and <3%⁵⁴. T cell activation might be skewed towards the part of the APC population with the highest amount of agonist pMHC class II on its surface. To calculate what the MCC-I-E^k surface density of such a population would be, we first determined by G35 fluorescence-activated cell sorting (FACS) that 99% of the APC population covered a 25-fold range of MCC-I-E^k surface densities. Assuming a normal distribution and that all APCs in our experiments (about 50 per experiment) were represented by 99% of the APC population, we calculated the range of MCC-I-E^k densities for the percentage of APCs that can activate T cells, assuming that only the APCs with the highest MCC-I-E^k density activate T cells (Table 2, column 4).

Microscopy and image analysis. The microscopy system used for 3D time-lapse imaging and the experiments in Table 3 was a Zeiss/Universal Imaging system (Thornwood, NY/Downingtown, PA)³⁴. The basic T cell–APC interaction on the microscope stage—including T cell and APC preparation, excitation wavelengths and the use of pharmacological agents—was as described^{8,29}. For each of the 15-min 3D experiments, every 20 s, one differential interference contrast (DIC) image, one Fura-2 ratio and 21 stacked I-E³-GFP z-planes (spaced 1 μm apart) were collected. Only cells that could be observed for at least 5 min after the formation of the tight T cell–APC interface were analyzed. For analysis, 3D reconstructions of the I-E³-GFP images were made at four maximally spread timepoints at and after T cell activation and the pattern of accumulation of MHC class II was classified as described in the Results. A rare concentrated accumulation on the background of a diffuse one was counted as “concentrated”. An “unstable” accumulation was one that could not be sustained at >140% of the background intensity during the 15-min experiment. “No accumulation” denoted an I-E³-GFP intensity at the interface at background amounts or <140% of background intensity. To quantify the amount of I-E³ accumulated at the interface, using the Metamorph analysis software (Universal Imaging, Downingtown, PA), we first measured the area of interface I-E³ accumulation and the diameter of the APC. Assuming spherical geometry, for the APC we calculated the size of the area of I-E³ accumulation as a percentage of the total APC surface area. Next, we measured the average I-E³-GFP intensity at the area of accumulation and the rest of the APC, we then calculated which percentage of the total I-E³-GFP had moved to the area of accumulation.

Supported lipid bilayers. Supported lipid bilayers were prepared and used for microscopy as described²⁹. Synapse formation on these bilayers was analyzed with microscopy as described². Cluster densities were analyzed and I-E³ incorporated on the supported lipid bilayer was quantified by RIA²⁴⁵. Egg phosphatidylcholine bilayers without incorporated I-E³ were used to assess nonspecific binding, which represented the lower limit of detection (~5 molecules/μm²). The construct gE³-α32 contained the MCC(91–103) sequence fused to the NH₂ terminus of the I-E³ α-chain through a 32-aa linker (L. C. Wu, unpublished data). The density of gE³-α32 was also determined by RIA, as described³⁵. We took into account that G35 (which mimics the TCR epitope) recognizes only 50% of all of gE³-α32, as determined by comparison with mAb 14.4.4 (L.C. Wu and C. Sumen, unpublished data). With this considered, gE³-α32 was able to stimulate T cell blasts to a similar extent as agonist-loaded gE³, as judged by standard proliferation assays. OG-gE³ was purified, labeled and peptide-loaded as described².

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