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Single-Cell Analyses Reveal Two Defects in Peptide-Specific Activation of Naive T Cells from Aged Mice

Gonzalo G. Garcia* and Richard A. Miller‡‡

Confocal fluorescent microscopy was used to study redistribution of membrane-associated proteins in naïve T cells from young and old mice from a transgenic stock whose T cells express a TCR specific for pigeon cytochrome C. About 50% of the T cells from young mice that formed conjugates with peptide-pulsed APC were found to form complexes, at the site of binding to the APC, containing CD3ε, linker for activation of T cells (LAT), and Zap-70 in a central area and c-Cbl, p95

vav

, Grb-2, PLCγ, Fyn, and Lck distributed more uniformly across the interface area. Two-color staining showed that those cells that were able to relocalize c-Cbl, LAT, CD3ε, or PLCγ typically relocalized all four of these components of the activation complex. About 75% of conjugates that rearranged LAT, c-Cbl, or PLCγ also exhibited cytoplasmic NF-AT migration to the T cell nucleus. Aging had two effects. First, it led to a diminution of ~2-fold in the proportion of T cell/APC conjugates that could relocalize any of the nine tested proteins to the immune synapse. Second, aging diminished by ~2-fold the frequency of cytoplasmic NF-AT migration among cells that could generate immune synapses containing LAT, c-Cbl, or PLCγ. Thus naïve CD4 T cells from old mice exhibit at least two separable defects in the earliest stages of activation induced by peptide/MHC complexes. The Journal of Immunology, 2001, 166: 3151–3157.

Activation of T lymphocytes by APC involves a complex series of interactions at the area of cell-to-cell contact. The three-dimensional organization of this TCR-APC interaction was described by Monk et al. using deconvolution imaging to study fixed T cell/APC conjugates (1, 2), and later by others using digital time-lapse microscopy to study live T cells (3–5). These studies have led to models in which the area of T cell/APC contact, the supramolecular activation cluster (SMAC) or “immune synapse,” is divided into a central area (c-SMAC) containing the TCR and associated proteins bound to it, and an immediately concentric peripheral area (p-SMAC) containing other key components including LFA-1 and cytosskeletal proteins (2). Engagement of the TCR by agonist peptides or anti-receptor Abs induces a reorganization of the T cell’s cytoskeleton (6) and accumulation of specific adaptor protein and enzymes (for review see Ref. 7). Localization of the tyrosine kinase Zap-70 within these complexes leads to the phosphorylation of adaptor proteins linker for activation of T cells (LAT), Slp-76, and p95

vav

(8, 9). These proteins in turn bring to the TCR complex other adaptor molecules, such as Grb-2 (10), and enzymes such as PLCγ and c-Cbl (11, 12) that together trigger later stages of the signal transduction cascade. It has recently been proposed that glycolipid-enriched microdomains, known as rafts or glycolipid-enriched membranes (GEMS), may play an important role in the translocation of enzymes and adaptor proteins to the area of TCR-APC contact (13, 14). In particular, it has been proposed that the raft domains help to concentrate the constitutively palmitoylated LAT to the c-SMACs, while excluding other molecules with negative regulatory roles, such as CD45 (15).

Aging leads to a decline in T cell response to new and previously encountered Ags (16). Several laboratories have shown that T cells from healthy old humans and mice exhibit a multitude of defects at early stages of the T cell signaling pathways, including changes in serine/threonine and tyrosine phosphorylation (17–23), development of calcium signals (24), activation of the Raf-1/mitogen-activated protein/extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways (25–26) and translocation of cytoplasmic NF-AT (NF-ATc) to the nucleus (27). Most of these alterations are demonstrable within the first 15 min of TCR engagement, and it is not yet clear which of the defects are primary, and which are merely secondary consequences of earlier abnormalities. One of the earliest detectable age-related defects is the decline with age in the phosphorylation of LAT by Zap-70 (28). In vitro kinase assays showed no effects of aging on Zap-70 protein kinase activity in CD4 T cells from young and old mice stimulated by anti-CD3/anti-CD4 cross-linking (29), suggesting that defective LAT phosphorylation might result from altered accessibility, i.e., differential compartmentalization of Zap-70 and its substrates. Indeed, confocal microscopic studies using anti-CD3 hybridoma cells as polyclonal APC analogs showed that the majority of CD4 T cells from old mice could not efficiently relocate protein kinase C (PKC)θ, LAT, and p95

vav

 (Vav) to the immune synapse (28, 30–31), suggesting that at least some of the age-associated defects in the early stages of signal transduction could be the result of defects in the formation of immune synapses at the site of T cell/APC interaction. However, one weakness of this initial study was its reliance on anti-CD3 stimulation. Interactions between the 2C11 anti-CD3ε and the TCR are of much higher affinity, with a much slower off-rate, than the typical interaction between peptide-bearing MHC
molecules and the TCR (32, 33). Furthermore, the original studies used responder cells that contained both naive and memory T cells; because aging leads to major increases in memory T cell numbers at the expense of naive T cells (for review see Ref. 16), it seemed possible that differences in synapse formation between naive and memory CD4 might have contributed to the decline in age with the proportion of cells able to relocalize LAT, Vav, and PKCθ to the T cell/APC interface.

To help resolve these issues, we have now used fluorescence confocal microscopy to study the responses of T cells from young and old transgenic mice bearing TCR specific for a peptide fragment of pigeon cytochrome c (PCC), as presented by the CH12 B cell line. The results suggest two age-dependent changes in the early activation process, one that interferes with recruiting of a variety of proteins to the immune synapse, and another, postsynaptic defect that prevents NF-AT translocation even in T cells with apparently normal synapse composition.

Materials and Methods

Animals and cell culture

Breeding pairs of the AND line of TCR-transgenic mice, whose T cells respond to PCC, were a generous gift from Dr. Susan Swain (Trudeau Institute, Saranac Lake, NY). Transgene-positive mice were aged in a specific-pathogen-free colony at the University of Michigan (Ann Arbor, MI) and given free access to food and water. Sentinel animals from this colony were examined quarterly for serological evidence of viral infection; all such tests were negative during the course of these studies. Transgenic mice that were found to have splenomegaly or macroscopically visible tumors at the time of sacrifice were not used for experiments. Mice used were at 6–8 (young) and 18–20 (old) mo of age. The CH12 B cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% FCS and 2 mM l-glutamine at 37°C and 10% CO2.

Abs and reagents

Rabbit polyclonals anti-PLCγ, Vav, c-Cbl, and Grb-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the rabbit anti-Zap70, Lck, Fyn, and LAT from Upstate Biotechnology (Lake Placid, NY) and the anti-CD3ε from Dako (Carpinteria, CA). The mAb anti-c-Cbl was purchased from Transduction Laboratories (Lexington, KY), and the mAb to NF-ATc1 was purchased from Santa Cruz Biotechnology. Single-color detection of proteins for confocal microscopy analysis was performed using rabbit polyclonal Abs and goat anti-rabbit Ig coupled to FITC (Jackson ImmunoResearch), then the rabbit polyclonal and a goat anti-rabbit coupled to Alexa-594 (Molecular Probes, Eugene, OR).

Peptides were synthesized in the Protein Core Facilities of the University of Michigan. The agonist peptide sequence represents aa 88–103 of PCC (ANERADLIALYKQATK). The nonagonist peptide had the same sequence, with the exception of a single amino acid substitution (K to N) at position 99 (marked in bold, (34)).

Cell preparation

CD4+ T cells were obtained from transgenic mice using the negative selection methods described in (28). Flow-cytometry analysis of a typical preparation showed it to be 90–95% positive for both CD3 and CD4. For each experiment, CH12 cells in log-phase were pulsed with 20 μM agonist or nonagonist peptide for 2 h in fresh medium at 37°C.

Slide preparation and confocal microscopy

A total of 6 × 105 TCR-transgenic CD4+ T cells (resuspended at 4 × 106 cells/ml in RPMI 1640 plus 1% FCS) was combined with 3 × 105 CH12 cells (resuspended at 2 × 106 cells/ml) to achieve a 2:1 ratio, respectively. Cell mixtures were incubated at 37°C for 20 min and then gently resuspended and spread onto prewarmed poly(λ)-lysine-coated slides (Sigma, St. Louis, MO). Slides were incubated for another 20 min at 37°C, fixed with 3.7% formaldehyde, 1 mM MgCl2 in PBS (pH 8.5) for 10 min and washed three times for 5 min each with PBS. Slides were permeabilized with 0.2% Triton X-100 in PBS for 10 min at 4°C, washed with PBS and blocked overnight with 1% BSA/PBS. For single-color experiments, the slides were stained with the appropriate primary rabbit Abs diluted in blocking solution at 1 μg/ml for 1 h at 4°C, washed three times with PBS and then stained with goat anti-rabbit-FITC at 10 μg/ml in blocking solution for 1 h at 4°C. The slides were washed four times with PBS, mounted using SlowFade Light antifade reagents (Molecular Probes) and sealed with nail polish. All slides were coded for blind analysis, and then stored at 4°C protected from light. For two-color protocols, the slides were initially stained with the appropriate mAb diluted in blocking solution at 1 μg/ml for 1 h at 4°C, washed, and incubated with goat anti-mouse F(ab’)2-FITC at 10 μg/ml in blocking solution for 1 h at 4°C. The second stain was then applied using the method given for single stains, but using goat anti-rabbit reagent coupled to Alexa-594 as the second Ab.

Single- and two-color analyses were performed at ×100 magnification on a Nikon Diaphot microscope (Nikon, Melville, NY) equipped with a Bio-Rad MRC 600 confocal laser imaging system (Bio-Rad, Hercules, CA). Randomly selected T cell/APC conjugates were analyzed only if the following criteria were first met: 1) tightly formed cell-to-cell contact, 2) T cells in contact with only one APC, and 3) both cells in the same z-axis plane. At least 100 accepted conjugates on each slide were analyzed and scored as either positive or negative for translocation of the protein to the APC interface or for accumulation of NF-ATc in the nucleus. All slides were coded and scored blind, i.e., without knowledge of the age of the T cell donor. Two slides were examined for each sample (n > 200 total conjugates), and the mean of the two values was used for further statistical analysis.

Statistical analyses

Data in the text and tables represent the means ± SD of three mice from each age group, tested as one young and old pair in each set of experiments. Statistical significance was assessed using the paired Student’s t test at p = 0.05.

Results

Peptide-specific formation of immune synapses by naïve CD4+ transgenic T cells

Studies by Monks et al. (1, 2) have shown that T cells interacting with APCs form highly organized protein clusters at the site of cell contact. The TCR becomes concentrated in the c-SMAC of the interface, with other signaling proteins associated to this cluster or surrounding it. In our own investigations we made use of the CH12 B cell line, which expresses the MHC-II in the context of I-Ek and I-Ae, and high levels of ICAM-1, B7.1, and B7.2 (data not shown). As our source of T cells we used the AND line of PCC-specific transgenic mice, in which the majority of CD4+ T cells remain naïve throughout life, and express the Vα11 and Vβ3 specific for PCC (35). In preliminary studies using proliferation as endpoint (I. Dozmorov and R.A. Miller, unpublished observations), we confirmed previously published data showing that the agonist peptide sequence ANERADLIALYKQATK, at 2–20 μM, triggers strong proliferation of the transgenic T cells, and that a peptide in which N is substituted for K (shown in boldface) provides a nonstimulatory control.

Fig. 1A shows a representative set of digital images of CD3ε localization in transgenic T cells conjugated to peptide-pulsed CH12 cells. The T cells in each image are smaller than the CH12 cells, and show strong CD3ε fluorescence signals. The image at left shows a conjugate between a T cell and CH12 bearing the nonagonist control peptide; in this as in other negative controls, tight conjugation was confirmed by Nomarski optics (not shown; but see Ref. 28 for examples). In the presence of the control peptide, CD3ε remains distributed evenly around the outside of the T cell, as expected, consistent with the lack of immune synapse formation. CH12 cells pulsed with agonist peptide generate two types of tight conjugates. Some conjugates, like the one shown in the middle of Fig. 1A, show no redistribution of the CD3ε-chain whereas others, such as the one shown at the right of Fig. 1A, exhibit highly concentrated ε-chain staining in the center of the area of cell-to-cell contact. These latter, positive responses closely resemble the images generated previously by other authors using
parallel methods (2, 5) and represent the c-SMAC area of the immune synapse.

Age-dependent decline in the proportion of naive CD4\(^+\) T cells that can form effective immune synapses after conjugation with peptide-pulsed APC

Fig. 1B shows the proportion of conjugates, from three young and three old mice, that showed strong CD3\(\varepsilon\) relocalization in responses to agonist and nonagonist peptide stimuli. As expected, the nonagonist peptide triggers CD3\(\varepsilon\) relocalization in only a small proportion (<5%) of conjugates from young or old donors. The agonist peptide triggered \(\varepsilon\)-chain redistribution in 48% of T cells from young mice, and in 20% of the cells from old mice; this difference is significant at \(p < 0.03\). These data suggest that aging diminishes the proportion of naive T cells that can form effective SMACs at the site of interaction with peptide-bearing APC.

The redistribution of multiple proteins to the immune synapse declines with age in naive CD4\(^+\) T cells

In some systems (1, 2, 28), the percentage of CD3\(\varepsilon\) molecules relocating to the c-SMAC is much smaller than the fraction of other TCR-dependent signaling proteins, such as PKC\(\theta\) and LAT, that move to the site of the synapse. Thus the defect in CD3\(\varepsilon\)-chain redistribution in T cells from aged transgenic mice does not exclude the possibility that the small numbers of TCR molecules at the site of APC interaction might still be able to complex with other signaling proteins and form functionally efficient immune synapses. To examine this problem we performed a series of experiments with nonagonist and agonist peptides, staining the conjugates for proteins known in other models to be associated with the TCR complex. We selected three groups of proteins for these analyses. The first group included PLC\(\gamma\) and c-Cbl, enzymes thought to play positive and negative roles, respectively, in the progress of the TCR signal transduction. The second group included the tyrosine kinases Lck, Fyn, and Zap-70, which are responsible for phosphorylation of both CD3 molecules and other proteins in the TCR signal transduction complex. The third group included LAT, Grb-2, and Vav, adapter molecules that help to recruit other elements of the transduction chain. Fig. 2 shows representative digital images from this series of experiments. The upper left panel of Fig. 2 shows a negative control, using nonagonist peptide stained for distribution of c-Cbl; similar negative controls using nonagonist peptide were obtained for all the Abs used (not shown). The other eight panels show examples of conjugates in which the indicated target molecules did indeed relocate to the synapse in response to agonist peptide-pulsed CH12 cells.

We noted consistent differences in the degree to which the tested molecules were or were not concentrated in the central area of the T cell/APC interface. Specifically, a high percentage of the conjugates positive for LAT and Zap-70 rearrangement showed fluorescence localized in a small area of cell-to-cell contact, a pattern resembling that noted for CD3\(\varepsilon\) (see Fig. 1); these proteins seem likely to be bound tightly to the TCR/CD3 complex within the c-SMAC. In contrast, distribution of c-Cbl, PLC\(\gamma\), Lck, Fyn, Grb2, and Vav was typically much more uniform along the area of membrane contact. These differences in distribution were not altered appreciably by age (not shown).

A series of three experiments, each using an old and a young mouse, was conducted to determine the effects of age on the proportion of CD4\(^+\) naïve T cells that could rearrange these proteins to the immune synapse. Table I summarizes these results. Nonagonist peptide controls were included for c-Cbl, Lck, and LAT, and showed that fewer than 10% of conjugates from young or old mice were able to induce protein redistribution in response to the peptide-pulsed CH12 cells.
control peptide, in good agreement with the CD3ε and proliferation data.

CH12 cells pulsed with agonist peptide induced relocalization of each of the eight tested molecules in a higher proportion of young T cells than of T cells from aged mice. For Lck, Zap-70, Fyn, LAT, Grb2, and Vav, the proportion of responding T cells in young mice ranged from 45 to 51%, and in old mice from 17 to 22%; each of these is significant by paired t test at p < 0.05, except for Fyn, which is marginal at p = 0.06. PLCγ rearrangement was noted in 62% of T cells from young mice (p < 0.05 compared with the 24% of T cells from old donors); the slightly higher results for PLCγ may represent a technical artifact, in that the fluorescent signal was very strong for this reaction. The effect of aging on c-Cbl relocalization is more difficult to assess, in part because the c-Cbl localization to the APC interface was noted in ~10% of conjugates generated using the nonagonist control peptide. The effect of age is somewhat smaller than for the other molecules examined, and is not statistically significant (p = 0.15). Taken together, the data suggest that aging can decrease the translocation of many TCR-associated proteins to the immune synapse, and are consistent with models in which altered protein relocation contributes to defects in LAT phosphorylation (28) and in later stages of activation in T cells from aged donors.

All-or-none redistribution of multiple proteins in the immune synapses of naive CD4 T cells

The close agreement between the proportions of T cells showing rearrangement of each protein (see Table I) suggests that most T cells either rearrange all the tested proteins, or none of them. However, single-color experiments cannot formally exclude the hypothesis that defects in the ability to relocalize specific coupling molecules might be differentially distributed among the cells of young or old mice. We tested this possibility by using a double-staining system, in which conjugates are examined using both anti-c-Cbl Ab and Abs to CD3ε, LAT, or PLCγ. The method uses a mouse anti-c-Cbl monoclonal and goat anti-mouse Fcy-FITC to avoid cross-reaction with the IgM expressed on the surface of CH12 cells. No significant background interference was seen using this secondary Ab alone (not shown). The second molecule (LAT, CD3ε, or PLCγ) was then detected using specific rabbit antisera followed by goat anti-rabbit coupled to Alexa-594. Fig. 3 shows typical pairs of digital images showing both fluorescent channels for individual conjugates. The examples chosen are ones in which both c-Cbl and the other molecule are rearranged in the same cell; these constitute the majority of cells examined (see below). The distribution of the proteins within the synapses is similar to that noted in single-color experiments (see Fig. 2), with CD3ε and LAT more centrally located than c-Cbl or PLCγ. Table II presents results of these two-color tests, from three mice of each age, showing the proportion of cells with each staining pattern as a percentage of all cells in which at least one protein was rearranged. Double-positive cells made up at least 83% of all stained cells for each case. Fewer than 15% of the positive cells stained for c-Cbl alone, and fewer than 5% stained positive for CD3ε, LAT, or PLCγ but not for c-Cbl. There were no statistically significant differences for any of these values between the young and old samples. These findings are consistent with our previous data looking at colocalization of LAT and PKCθ in individual CD4 cells from nontransgenic young mice stimulated by an anti-CD3 hybridoma cell line (28). The observations show that defects in the ability to relocalize effector proteins to the immune synapse are not randomly distributed among cells, but instead that the redistribution reaction may be “all-or-nothing” at least with respect to the specific proteins we have examined.

Age-dependent defect in nuclear translocation of NF-ATc after successful synapse formation in CD4 naive T cells

Formation of the immune synapse is followed within minutes by activation of several downstream pathways whose linkage to the TCR signal is not yet fully elucidated. Many of these early steps are diminished by aging, including activation of mitogen-activated protein kinases of the extracellular signal-related kinase (36) and JNK families (25, 26), and activation of RAF-1 (19), together with

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Table I. Single-color analysis of c-Cbl, PLCγ, Lck, Zap-70, Fyn, LAT, Grb-2, and Vav localization in naive CD4+ cells conjugated with CH12 cells bearing either nonagonist or agonist peptide

<table>
<thead>
<tr>
<th>Protein Stain</th>
<th>Nonagonist</th>
<th>Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Old</td>
<td>Young</td>
</tr>
<tr>
<td>c-Cbl</td>
<td>10 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>PLCγ</td>
<td>6 ± 9</td>
<td>24 ± 8*</td>
</tr>
<tr>
<td>Lck</td>
<td>7 ± 3</td>
<td>22 ± 2*</td>
</tr>
<tr>
<td>Zap-70</td>
<td>50 ± 11</td>
<td>19 ± 2*</td>
</tr>
<tr>
<td>Fyn</td>
<td>49 ± 13</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>LAT</td>
<td>4 ± 1</td>
<td>18 ± 1*</td>
</tr>
<tr>
<td>Grb-2</td>
<td>47 ± 12</td>
<td>17 ± 1*</td>
</tr>
<tr>
<td>Vav</td>
<td>45 ± 8</td>
<td>17 ± 3*</td>
</tr>
</tbody>
</table>

* Mean ± SD. ** Not determined.
* Value was statistically different (p < 0.05) from that for the same stain in the young mice.

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Table II. Two-color analyses of protein localization to immune synapse in CD4 naive, TCR-transgenic T cells from young and old mice in response to agonist peptide

<table>
<thead>
<tr>
<th>Protein Stain</th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Cbl/LAT</td>
<td>86 ± 5</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>c-Cbl/CD3ε</td>
<td>91 ± 3</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>c-Cbl/PLCγ</td>
<td>91 ± 6</td>
<td>9 ± 6</td>
</tr>
</tbody>
</table>

* Mean ± SD. +/+ Relocalization of both c-Cbl and the indicated protein (LAT, CD3ε-chain, or PLCγ). +/− Relocalization of c-Cbl only. −/+ Relocalization of LAT, CD3ε, or PLCγ, but not c-Cbl. There were no significant differences between age groups.
impaired activation of transcriptional factors such as NF-ATc (27, 28). To see whether defects in the assembly of protein complexes at the site of TCR/APC interaction might contribute to age-dependent alterations in these downstream events, we performed a series of experiments examining both NF-ATc translocation and relocalization of LAT, PLCγ, or c-Cbl in individual T cells. The proportion of LAT relocalization of NF-ATc alone as rare among aged T cells as in the majority (70%) of old T cells that show synaptic responses fail to proceed to NF-ATc migration. In contrast, not all naive CD4 T cells, freshly isolated from mouse spleens, are able to form synapses involving LAT, c-Cbl, and PLCγ typically proceed to NF-ATc migration. The majority (~70%) of old T cells that show synaptic responses fail to proceed to NF-ATc translocation, with rearrangement of NF-ATc alone as rare among aged T cells as in the ones from younger donors. The proportion of LAT+ cells that also score as NF-ATc-positive is significantly different (p = 0.03) between young and old mice, and the effects of age in the experiments using c-Cbl and PLCγ markers are also significant at p < 0.04.

Using this approach we then conducted a series of tests involving three young and three old mice. For each mouse and each staining combination we examined at least 100 conjugates that exhibited a positive response either for NF-ATc, for the synapse-localized protein, or for both. Table III summarizes these results. For naive CD4 cells from young mice, ~75% of the cells that scored positive for the synaptic protein also exhibited NF-ATc translocation (scored as +/+). Approximately 24% of the responding conjugates showed a relocalization of the synapse component but not translocation of NF-ATc (−/+), and fewer than 3% showed translocation of NF-ATc alone (+/−). The data show that young T cells that are able to form synapses involving LAT, c-Cbl, and PLCγ typically proceed to NF-ATc migration. In contrast, fewer than 32% of those CD4 naive T cells from old mice that relocalize LAT, c-Cbl, or PLCγ also exhibit NF-ATc migration to the nucleus. The majority (~70%) of old T cells that show synaptic responses fail to proceed to NF-ATc translocation, with rearrangement of NF-ATc alone as rare among aged T cells as in the ones from younger donors. The proportion of LAT+ cells that also score as NF-ATc-positive is significantly different (p = 0.03) between young and old mice, and the effects of age in the experiments using c-Cbl and PLCγ markers are also significant at p < 0.04.

**Discussion**

Reorganization of kinases, substrates, and coupling molecules at the site of T cell/APC interaction plays an important role in T cell activation (37). Using the B cell line CH12 as APC and TCR-transgenic mice as the source of naive CD4 T cells, we have studied the effect of aging on formation of the immune synapse. We noted that in this system CD3ε, Zap-70, and LAT relocalized to the central domain of the synapse, similar to the c-SMAC pattern previously documented in other systems (2). The distribution of PLCγ, Grb-2, Vav, c-Cbl, and Fyn resembles that seen for cytoskeletal proteins, such as talin, which localize to the p-SMAC of the synapse (2, 4).

However, not all naive CD4 T cells, freshly isolated from mouse spleens, are able to form synapses of this composition after conjugation to peptide-loaded APC. When the T cells are derived from young donors, only ~50% of those forming tight conjugates with APC translocate any of the listed proteins to the synapse (Table I). These values are consistent with those noted in previous studies of LAT and PKCθ translocation in T cells activated by exposure to

**FIGURE 4.** Two-color analysis of NF-ATc nuclear translocation and LAT, PLCγ, and c-Cbl localization in naive CD4 T cells conjugated to CH12 in the presence of agonist peptide

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**Table III.** Two-color analysis of NF-ATc nuclear translocation and LAT, PLCγ, and c-Cbl localization in naive CD4 T cells conjugated to CH12 in the presence of agonist peptide

<table>
<thead>
<tr>
<th>Protein Stain</th>
<th>Young</th>
<th>Old</th>
</tr>
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<tbody>
<tr>
<td>+/+</td>
<td>76 ± 12</td>
<td>74 ± 17</td>
</tr>
<tr>
<td>+/-</td>
<td>23 ± 14</td>
<td>24 ± 17</td>
</tr>
<tr>
<td>−/+</td>
<td>31 ± 5</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>−/−</td>
<td>1 ± 1</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

**Note:** Mean ± SD. +/++: Relocalization of both NF-ATc and the indicated protein (LAT, e-chain, or PLCγ). ++/−: Relocalization of NF-ATc only. −/+/−: Relocalization of the synaptic protein, but not of NF-ATc.
hybridoma cells bearing cell surface Ab to mouse CD3ε (28, 30, 31). In the current study, the proportion of T cells showing trans-
location of CD3ε was higher than in our previous work (28) and in
reports from other groups (2), perhaps because our current system
uses higher concentrations of agonist peptide (20 μM, instead of 2
μM). The distribution of Lck also deserves comment. In our stud-
ies we found Lck to be distributed throughout the area of APC-T
cell contact (presumably within the p-SPMAC). Consistent with our
findings, Krummel et al. (38) found that CD4/Lck complexes, ini-
tially present within the central area of the synapse, migrated
within minutes of synapse formation to more peripheral areas of
the contact zone.

The one-color data summarized in Table I show that relatively
few naive CD4 T cells from old mice are able to generate synapses
that include any of the tested molecules: c-Cbl, PLCγ, Lck, Zap-
70, Fyn, LAT, Grb-2, and Vav. In each case the proportion of
responsive naive CD4 cells from aged donors is about half that of
cells from young donors. These results are similar to those seen in
our previous studies of unseparated CD4 cells from young and old
nontransgenic donors responding to anti-CD3 stimulation, as mon-
tored by translocation of LAT and Vav (28). The new data show
that aging affects T cell responses to peptide Ags in addition to
responses triggered by high affinity anti-receptor Abs, show that
the changes affect all eight of the tested components of the syn-
apse, and show that the aging effect is seen in naive T cells, and
thus not due simply to the accumulation of memory CD4 T cells in
old age. The two-color experiments of Table II show that cells,
from young or old donors, which exhibit defects in any one of the
four proteins tested (c-Cbl, LAT, PLCγ, and CD3ε) usually show
defects in all of them; in this sense the changes are “all or nothing”
at the single-cell level. We have not examined the effects of aging
on the time course of translocation of these proteins to the synapse
in transgenic T cells, and, therefore, it is possible that differences
in response to peptide-APC conjugates may be delayed, rather than
absent, in T cells from old donors, even though our previous studies
using an anti-CD3 hybridoma cell line as a polyclonal stimu-
lator found no evidence for an effect of aging on the time course of
synapse assembly in CD4 or CD8 cells (31). Although not all of
the eight molecules summarized in Table I have been examined in
two-color experiments, the excellent agreement among them in the
proportions of responsive cells in mice of either age are consistent
with the idea that they, too, show parallel responses, all on, or all
off, within individual cells. Although many of these proteins are
either constitutively associated with high viscosity raft domains in
the T cell membrane, or can become raft-associated by binding to
LAT (13, 14), our previous work (28) found no evidence for an age
effect on distribution of LAT, RAFT-constitutive GM-1, or raft-
excluded CD45 in resting or anti-CD3-activated T cells. Thus def-
ects in immune synapse formation probably cannot be attributed
with changes in the initial composition of the raft microdomains
(28). However, it is possible that further studies of raft-associated
proteins may provide clues to the mechanism of the alterations in
complex formation we have documented. It is also plausible that
age effects on formation of functional immune synapses might be
due to changes in cytoskeleton reorganization during responses to
TCR stimulation. In this context it is noteworthy that at least one
of the cytoskeletal proteins, talin, can apparently become recol-
lized to the immune synapse in T cells exposed to nonagonist pep-
tides (1, 2), whereas other proteins, such as PKCθ, migrate only in
response to agonist peptides. Biochemical and microscopic tech-
niques will help to sort out the ways in which aging might alter
association of signaling proteins with cytoskeletal proteins before
and during responses to agonist peptides.

Two-color experiments can also test the linkages between events
immediately tied to TCR stimulation, and those down-
stream events, such as migration of transcription factors and in-
duction of new gene expression, that are triggered by kinase-de-
pendent cascades. The data presented in Table III show that T cells
from aged mice have, over and above the problems in synapse
formation summarized in Table II, a diminished ability to trans-
locate NF-ATc to the nucleus. Among young CD4 T cells that
show LAT migration, for example, 77% proceed to NF-ATc mi-
gration, but this figure falls to 31% for cells from aged donors,
with very similar changes seen when c-Cbl or PLCγ is used as the
index of functional synapse formation. Thus there seem to be three
classes of CD4 T cells that can be discriminated in responses to
peptide-bearing APCs: 1) those that form synapses and induce NF-
ATc migration; 2) those that form synapses but do not undergo
NF-ATc migration; and 3) those that do neither, with the latter two
classes increasing as a function of age. T cells from aged donors
also show defects in activation of the Raf-1 and JNK-dependent
protein kinase pathways (19, 25), the latter of which depends upon
CD28-mediated signaling. It is possible that alterations in CD28/
JNK signals or in PLCγ-independent generation of calcium signals
(39) might contribute to derangements in the postsynaptic pro-
cesses required for NF-ATc translocation and induction of IL-2
gene expression. Further work should now be able to define addi-
tional biochemical and functional differences among these three
classes of cells, and may help to develop a more detailed picture of
the ways in which T cells discriminate between agonist and non-
agonist peptides, and of the changes that impair activation of T
cells in old mice.

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