T cell receptor (TCR) clustering in the immunological synapse integrates TCR and costimulatory signaling in selected T cells

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During T cell activation, T cell receptors (TCR) cluster at the center of the T cell/antigen-presenting cell interface forming a key component of the immunological synapse. The function of this TCR clustering is still unresolved. A comprehensive search for such a function yielded a very limited and specific result. A micrometer-scale receptor clustering integrated the TCR and CD28 signals required for IL-2 secretion in primary 5C.C7 T cells, a low-affinity/avidity TCR system. 5C.C7 TCR signaling itself was not affected. In addition, central TCR accumulation was not required for any T cell effector function tested in three other TCR transgenic models. Central TCR accumulation thus had a specific role in signaling integration in low-affinity T cells.

T cells are activated when the T cell receptor (TCR) engages cognate agonist peptide/MHC complexes expressed on the surface of the antigen-presenting cell (APC) (1). Complete T cell activation also requires the engagement of costimulatory receptors, in particular CD28 and LFA-1, by their APC ligands B7 and intercellular adhesion molecule-1 (ICAM-1) (2). T cell activation provides a striking example of localized signaling, the accumulation of TCR/MHC complexes at the center of the T cell/APC interface ("central TCR accumulation"), an event readily visualized by fluorescence microscopy (3–5). In response to strong T cell stimuli, 2,000–3,000 TCR and MHC molecules accumulate at the center of the interface (4, 6). Lower concentrations of agonist peptide can still elicit clearly detectable clustering (6, 7) with an estimated few hundred TCRs. Central TCR accumulation is a critical feature of the "mature immunological synapse," the segregation of multiple receptors, signaling, and adaptor proteins into micrometer-sized central and peripheral supramolecular activation clusters (8). Yet the function of central TCR accumulation has remained elusive and controversial (9). A major obstacle is the lack of an experimental tool to distinguish TCR signaling from its localization. To circumvent interference with TCR signaling, we have related central TCR accumulation to critical T cell signaling and effector functions over a wide range of physiological activation conditions. We used primary T cell/APC interactions from four different class II- and class I-restricted TCR transgenic mouse models. The comprehensive nature of our approach revealed a surprisingly limited and specific role for central TCR accumulation. The integration of costimulatory with TCR signaling required micrometer-scale receptor clustering only in the low-affinity/avidity 5C.C7 T cells. TCR signaling itself was not affected. In three additional TCR transgenic systems, central TCR accumulation was not required for any T cell signaling or effector functions.

Experimental Procedures

Cells and Reagents. *In vitro*-primed primary T cells from 5C.C7 (10), DO11.10 (11), HY (12), and P14 (13) TCR transgenic mice were generated as described in ref. 6. I-E^k-GFP-transfected A20 B cell lymphoma cells (6), A20 cells, or EL4 thymoma cells (for both HY and P14 T cells) were used as APCs. In the case of 5C.C7 T cells, agonist peptide concentrations were adjusted by dilutions into null peptide (6). In all other studies, the peptide was diluted in water. Costimulation blockade with antibodies against ICAM-1 or B7-1/B7-2 was as described in ref. 14. Use of Jasplakinolide and a membrane-permeable version of the Wiscott–Aldrich syndrome protein C-terminal domain has been described in ref. 15. The antibodies used were as follows: anti-IL-2 (JES6-5H4; Becton Dickinson), anti-IFN-γ (XMG1.2; Becton Dickinson), anti-phospho LAT Y191 and anti-phospho Akt S473 (Cell Signaling Technology, Beverly, MA), anti-CTLA-4 (UC10-4F10-11) (Becton Dickinson), anti-Vα2, anti-Vβ8 (P14 TCR) (Becton Dickinson), anti-Vα11 (Becton Dickinson), anti-Vβ3 (5C.C7 TCR) (Becton Dickinson), anti-DO11.10 (Becton Dickinson), anti-TCRβ (H27-597) (Becton Dickinson), and anti-HY (eBiosciences, San Diego).

Imaging. As discussed in detail in *Supporting Text*, which is published as supporting information on the PNAS web site, TCR/MHC accumulation was determined by using GFP fusion proteins with I-E^k (for 5C.C7 T cells) (6), H2-D^b as generated in strict analogy (for P14 T cells), the TCR ζ-chain (for DO11.10 and HY T cells) (7), and the double Src homology 2 (SH2) domain of the kinase ZAP-70 (for P14 and HY T cells). The latter was generated by linking amino acids 1–276 of human ZAP-70 C-terminally through a 7-aa linker to EGFP. The location of ligand-engaged CD28 was determined with a B7-2/GFP fusion protein, as discussed in *Supporting Text*. Use of actin-GFP and tubulin-GFP has been described in refs. 15 and 16. TCR ζ-GFP, ZAP-70 double SH2-GFP, actin-GFP, and tubulin-GFP were introduced into primary T cells by retroviral transduction with a Moloney murine leukemia virus-derived system as described in ref. 15. The microscopy system, image acquisition, and image analysis have been described in detail in ref. 15. Briefly, primary T cells and APCs loaded with peptide were allowed to interact at 37°C on the microscope stage. Every 20 seconds, a differential interference contrast bright field image and GFP images were acquired (spaced 1 μm in z covering the entire cell). For the analysis of MHC/TCR accumulation, actin accumulation, and reorientation of the microtubule organizing center (MTOC), three-dimensional reconstructions of productive cell couples were generated and classified according to strict analysis criteria (refs. 6 and 15 and *Supporting Text*). For actin-GFP analysis, interface accumulation with fluorescence intensity of 40% more than the cellular background was considered as long it constituted the highest intensity within the cell (strong and partial phenotypes as defined in ref. 16).

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Abbreviations: APC, antigen-presenting cell; ICAM-1, intercellular adhesion molecule-1; LAT, linker of activated T cell; MCC, moth cytochrome c; MTOC, microtubule organizing center; SH2, Src homology 2; TCR, T cell receptor.

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Effector Functions. IL-2 and IFN-γ secretion were determined by intracellular cytokine staining of primary T cell/APC couples after 4 and 16 h of T cell/APC interactions according to standard procedures in ref. 17. Target cell killing was determined in 1-h chromium-release assays (16). TCR down-regulation was determined by staining for surface TCR expression levels after 1.5 h of T cell/APC contact according to standard procedures in ref. 18. Linker of activated T cell (LAT) and Akt phosphorylation was determined in cell extracts from T cell/APC couples that interacted for 5 min (LAT and Akt) or 30 min (Akt) by Western blotting with standard procedures. In control experiments, APCs were fixed with glutaraldehyde to assure that APC signaling did not contribute to observed changes in LAT and Akt phosphorylation (data not shown). TCR ζ-phosphorylation was assayed in ZAP-70 immunoprecipitates from T cell/APC couples that interacted for 5 min (19). Data were quantified as described in *Supporting Text* and in the legend to Table 1, which is published as supporting information on the PNAS web site.

Results

Central TCR Accumulation Is Specifically Linked to IL-2 Secretion in 5C.C7 T Cells. T cells from 5C.C7 TCR transgenic mice (10) have been used extensively to study the immunological synapse. The 5C.C7 TCR recognizes a peptide from moth cytochrome *c* (MCC) (amino acids 82–103) presented by I-E^k. To identify T cell signaling and effector functions that are consistently linked to central TCR accumulation, we performed agonist peptide dose–response experiments. The dose–responses of central TCR accumulation, induction of LAT phosphorylation as a readout of TCR signaling (20), TCR downmodulation as a readout of TCR engagement (18), and IL-2 production (Fig. 5, which is published as supporting information on the PNAS web site) were superimposable (Fig. 1*A*). These results suggested a direct role for TCR clustering in the regulation of 5C.C7 T cell activation. To further address this link, we used two approaches to block TCR clustering while maintaining TCR engagement. First, we slowed T cell actin dynamics by using either the inhibitor of actin depolymerization, Jasplakinolide, or a membrane-permeable version of the effector domain of the Wiskott–Aldrich Syndrome protein, the C-terminal domain (15). Second, we used antibodies to block CD28/LFA-1-mediated costimulation (6, 14). TCR down-regulation and LAT phosphorylation were unaffected with these inhibitors. In contrast, IL-2 secretion was reduced in parallel with the loss of central TCR accumulation (Fig. 1*A*). IL-2 secretion thus was consistently related ($R^2 = 0.75$) to central TCR accumulation, whereas LAT phosphorylation and TCR down-regulation were not ($R^2 < 0.3$) (Fig. 6, which is published as supporting information on the PNAS web site). These results imply that IL-2 production in 5C.C7 T cells selectively depended on central TCR accumulation. Interestingly, TCR signaling itself was not, as further addressed below.

Costimulation Enhances Receptor Clustering and IL-2 Secretion Preferentially in 5C.C7 T Cells. To investigate whether central TCR accumulation was associated with IL-2 secretion in other T cells, we analyzed primary T cells from DO11.10 TCR transgenic mice (11). The DO11.10 TCR recognizes an ovalbumin peptide 324–340 presented by I-A^d. In an agonist peptide dose–response, central TCR accumulation occurred only at 10 μM agonist peptide (Fig. 2*A*). In contrast, IL-2 production, LAT phosphorylation, TCR down-regulation, and naïve T cell proliferation occurred at peptide doses well below 10 μM (10 nM to 10 μM), severalfold less than that necessary for central TCR accumulation (Fig. 1*B*). Thus, central TCR accumulation was not associated with IL-2 production in all T cells. In addition, not a single DO11.10 signaling or effector function tested depended on central TCR accumulation, as further addressed below.

The disparate results between the D011.10 and the 5C.C7 T cells raised the question as to what distinguishes these two T cells. Most notably, I-E^k tetramer dissociation experiments showed that the avidity of the 5C.C7 TCR is at the very low end of the physiological range (21). The dissociation constant of the 5C.C7/MCC/I-E^k complex of 50 μM is high (22). In contrast to this difference, TCR surface expression as assayed by anti-TCR-β staining was the same. Transgenic TCR expression was >95% of total TCR for both. The percentage of T cells secreting IL-2 and the amounts of IL-2 produced were the same at 10 μM

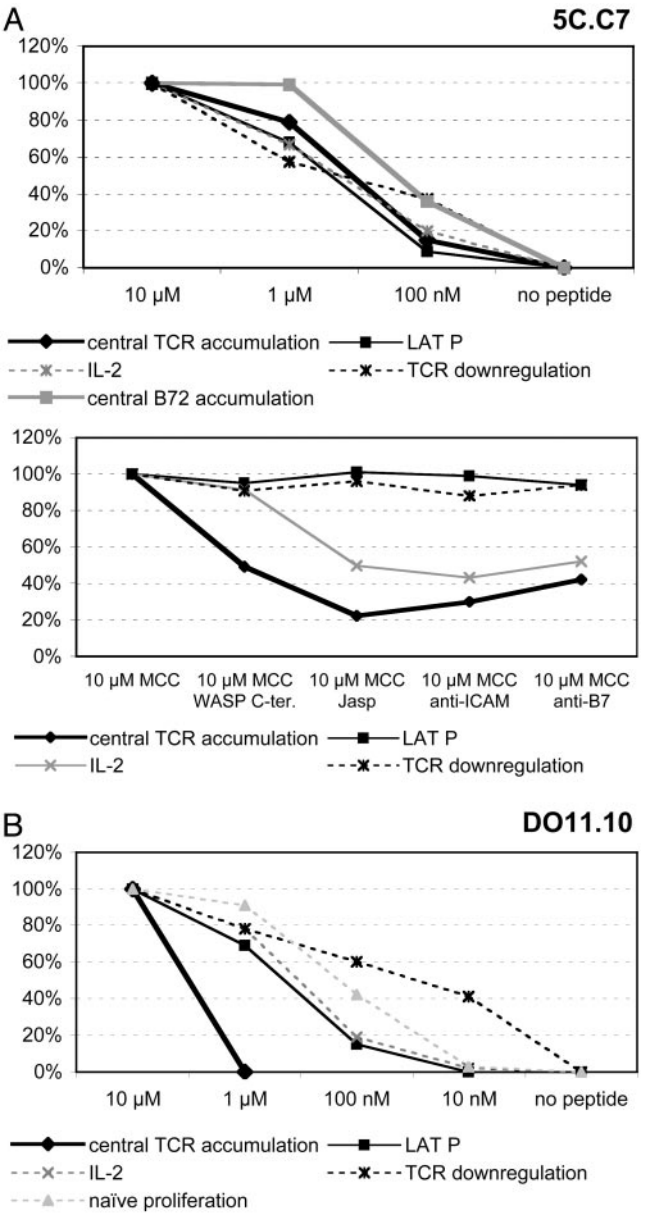

Fig. 1. Central TCR accumulation associates with IL-2 secretion in 5C.C7 T cells. (*A*) Central TCR and B7-2 accumulation and effector functions in 5C.C7/I-E^k-GFP transfected A20 B cell lymphoma APC interactions under the indicated conditions are shown as normalized data. Values for 10 μM MCC are set to 100%, and values for no agonist peptide are set to 0%. All other values are scaled accordingly. Raw data are either published (TCR accumulation) (6) or given in Fig. 7 (B7 accumulation) or Table 1 (all other data). WASP C-ter, 200 nM of the tat Wiscott–Aldrich syndrome protein C-terminal domain; Jasp, 0.5 μM Jasplakinolide; anti-ICAM and anti-B7′, 10 μg/ml blocking antibodies against ICAM-1 and B7-1/B7-2, respectively. (*B*) Normalized data for primary DO11.10/A20 APC interactions are shown similarly. Raw data are given in Table 1 and Fig. 2.

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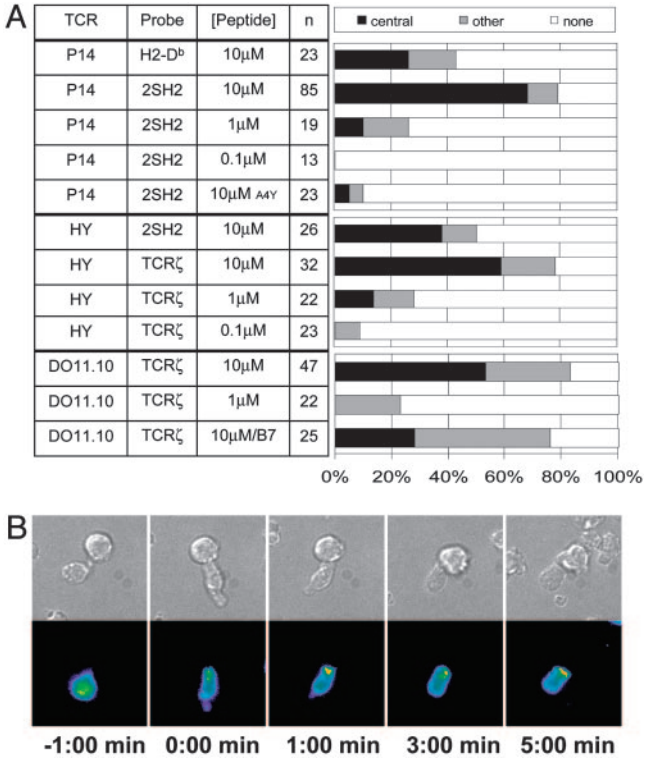
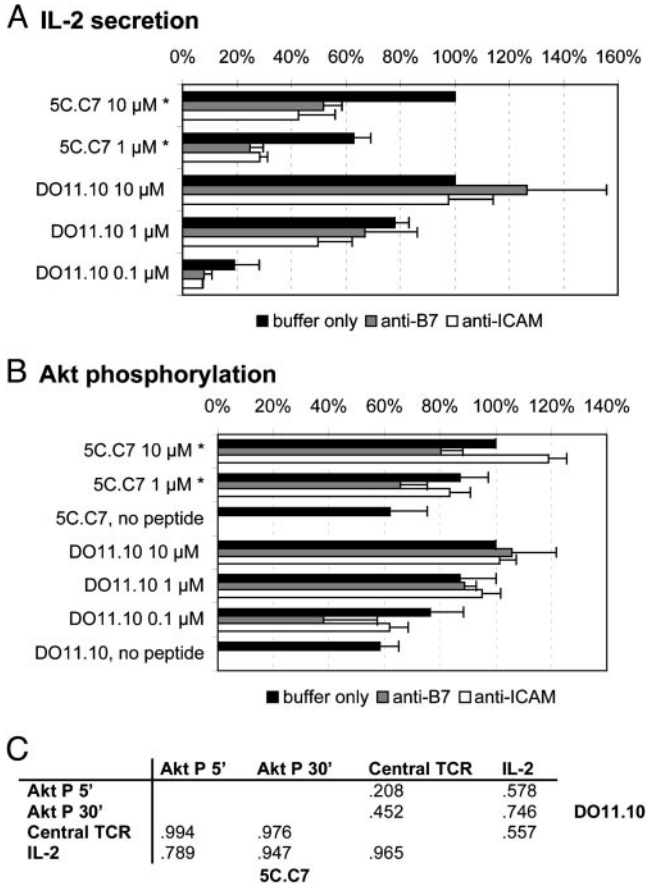


Fig. 2. Central TCR accumulation is rapid and requires high concentrations of agonist peptide. (*A*) The percent of T cell/APC couples with central TCR accumulation in at least one of the four time points analyzed (1′, 3′, 5′, and 7′–15′; central, black bars); any other accumulation phenotype (other, gray bars) or no accumulation (none, unfilled bars) are given for different TCR transgenic T cells as indicated in the first column. Fig. 9 provides a kinetic analysis of the same data. TCR accumulation was determined by using EL-4 target cell transfection with H2-D^b-GFP or T cell transduction with TCR-ζ/GFP or ZAP-70 double SH2-GFP (2SH2) as indicated in the second column at the given concentration of peptide (third column). The ZAP-70 double SH2 domain-GFP protein is further characterized in *Supporting Text* and Fig. 11, which is published as supporting information on the PNAS web site. Imaging probes are discussed in *Supporting Text*. Agonist peptides were used throughout with the exception of the A4Y partial P14 agonist and the addition of 10 μg/ml blocking B7-1/B7-2 antibodies (B7) to one set of DO11.10 samples. *n*, cells from at least three independent experiments were analyzed per condition. (*B*) An interaction of a P14 T cell that has been transduced with ZAP-70 double SH2–GFP (*Lower*) with an EL4 APC incubated with 10 μM p33 peptide (*Upper*) is shown at the indicated time points relative to tight cell couple formation (t = 0:00) in still images derived from Movie 1, which is published as supporting information on the PNAS web site. (*Upper*) Bright field images are displayed. (*Lower*) Matching projections of the three-dimensional ZAP-70 double SH2 domain–GFP fluorescence data in a false color scale (increasing from purple to red and white).



C

	Akt P 5'	Akt P 30'	Central TCR	IL-2	
Akt P 5'			.208	.578	
Akt P 30'			.452	.746	DO11.10
Central TCR	.994	.976		.557	
IL-2	.789	.947	.965		
		5C.C7			

Fig. 3. IL-2 secretion and Akt phosphorylation are particularly costimulation-dependent in 5C.C7 T cells. (*A*) The dependence of IL-2 secretion (Fig. 5) on costimulation in 5C.C7/I-E^k-GFP transfected A20 versus DO11.10/A20 interactions in the presence of the indicated concentrations of agonist peptide is shown. Blockade of ICAM-1 and B7–1/B7–2 with 10 μg/ml antibody is indicated. IL-2 secretion was normalized (the value for 10 μM agonist peptide = 100%). Two to five independent experiments were performed, and averages plus standard deviations are given. *, a significant ($P < 0.05$) difference between buffer only and anti-B7. (*B*) Akt S473 phosphorylation after 30 min of T cell/APC interactions (Fig. 8*B*) is shown similar to *A*. In *C*, correlation coefficients (R^2 values) of normalized Akt phosphorylation against central TCR accumulation and IL-2 data as determined by linear regression (similar to Fig. 6) are listed.

agonist peptide (data not shown). I-E^k-GFP-transfected A20 B cell lymphoma cells were used as APCs for both. These data suggest that an intrinsically weak 5C.C7 TCR signal alone might not be sufficient to drive effective IL-2 secretion, unless costimulatory molecules are integrated with the TCR signals. Micrometer-scale receptor clustering might mediate the integration of TCR and costimulatory signaling. In fact, IL-2 secretion depended more on costimulation in 5C.C7 than in DO11.10 T cells. Using antibodies to block B7 and ICAM-1, a procedure that does not significantly inhibit cell-couple formation (6, 14), IL-2 secretion in 5C.C7 T cells was significantly ($P < 0.05$) reduced ≥50% at all agonist peptide concentrations. In DO11.10 T cells, a reduction was found only at 0.1 μM agonist peptide (Fig. 3*A*). Interestingly, central TCR accumulation particularly depended on CD28 in 5C.C7 T cells. Blocking B7, only 13% of the T cell/APC couples exhibited central TCR accumulation, instead of 64% under control conditions (6), a reduction by 80%. In DO11.10 T cells, the reduction was moderately less (49%), where 24% of the cell couples instead of 47% under control conditions exhibited central TCR accumulation (Fig. 2*A*). Supporting a role of receptor clustering as a mediator of signal integration, efficient accumulation of CD28 at the center of the T cell/APC interface has been demonstrated in 5C.C7 T cells by using a CD28–GFP fusion protein (23). Clustered CD28 is likely engaged by its ligand B7 because we observed central accumulation of B7-2 in the interaction of 5C.C7 T cells and B7-2/GFP-transfected CH27 APCs (*Supporting Text* and Fig. 7, which is published as supporting information on the PNAS web site). In addition, central accumulation of TCR/MHC and CD28/B7 showed a comparable dependence on the concentration of the MCC agonist peptide (Fig. 1*A*). Coclustering of TCR and CD28 has been directly demonstrated by using 2B4 primary T cells with supported lipid bilayers as APC substitutes (24). In summary, in 5C.C7 T cells, IL-2 secretion and central TCR accumulation particularly depended on costimulation. In addition, ligand-

engaged TCR and CD28 both clustered at the center of the interface with a similar dependence on the agonist-peptide concentration. Together, these data suggest that micrometer-scale receptor clustering, including TCR and CD28, integrates signaling to IL-2 secretion.

Costimulatory Signaling Is Linked to Receptor Clustering Specifically in 5C.C7 T Cells. To further substantiate the differential link between CD28 engagement, micrometer-scale receptor clustering including central TCR accumulation, and IL-2 secretion, we analyzed CD28 signaling. The kinase Akt is a critical downstream effector of both TCR and CD28 (25, 26). Similar to IL-2 secretion, Akt activation, as assayed by S473 phosphorylation, particularly depended on CD28 engagement in 5C.C7 T cells. At all agonist peptide concentrations, blocking B7 significantly ($P < 0.05$) reduced 5C.C7 T cell Akt phosphorylation, whereas DO11.10 Akt phosphorylation was reduced only at 0.1 μM agonist peptide (Fig. 3*B* and Fig. 8, which is published as supporting information on the PNAS web site). Akt phosphorylation was highly correlated (R^2 values between 0.79 and 0.99) with IL-2 secretion and central TCR accumulation in 5C.C7 T cells but not in DO11.10 T cells (R^2 values between 0.21 and 0.75) (Fig. 3*C*). In 5C.C7 but not DO11.10 T cells, CD28 engagement was thus particularly required for Akt activity, central TCR accumulation, and IL-2 secretion. In addition, these signaling and effector functions were linked in a statistically significant fashion (Fig. 3*C*). The extensive and specific nature of these correlations suggests a causal relation. Specifically in 5C.C7 T cells, a micrometer-scale signaling complex triggered by and including TCR and CD28 would be required to integrate signals for the generation of IL-2.

To further test this model, we blocked a key CD28 effector and Akt activator, phosphatidylinositol 3-kinase (PI3-kinase) (25, 27). Similar to Akt, PI3-kinase can be activated by TCR and CD28 engagement. However, activation by CD28 is more efficient and persistent (28). According to our model, PI3-kinase blockade should interfere both with 5C.C7 central TCR accumulation and IL-2 secretion. Accordingly, 100 nM Wortmannin significantly ($P < 0.001$) reduced the percentage of 5C.C7 T cell/APC couples with TCR accumulation from 76% to 18%, similar to a reduction to 24% upon B7 blockade (independent confirmation of ref. 6). Ly294002 (10 μM) significantly ($P = 0.001$) reduced 5C.C7 IL-2 secretion. At 10 and 1 μM MCC agonist peptide in the presence of Ly294002, the amount of IL-2 produced was reduced by on average 64% and 67%, respectively, similar to B7 blockade. Supporting a role of Akt in the integration of TCR and costimulatory signaling in a micrometer-scale signaling complex, active Akt has recently been shown to accumulate at the D10 T cell/APC interface (29). Taken together (Fig. 8*C*), the particularly strong, parallel influence of CD28 engagement on Akt signaling, central TCR accumulation, and IL-2 secretion in 5C.C7 T cells suggests that micrometer-scale receptor clustering involving central TCR and CD28 integrates TCR and costimulatory signaling for IL-2 secretion. In contrast, in DO11.10 T cells, a strong TCR signal alone could regulate Akt activity, central TCR accumulation, and IL-2 secretion separately (Fig. 8*C*). Importantly, all other T cell signaling and effector functions tested were independent of central TCR accumulation.

Central TCR Accumulation Is Not Required for Critical Effector Functions in P14 and HY T Cells. To broaden our investigation, we analyzed two class I-restricted TCR transgenic mouse models, P14 (13) and HY (12), in their responses to agonist peptides. The P14 TCR recognizes the LCMV gp33 (33–41) agonist peptide with high affinity ($K_d = 2$ μM; ref. 30), the HY TCR responds to amino acids 738–746 of the male *Smcy* gene (12), both presented by H2-D^b. TCR localization had not been investigated in either system. For the P14 T cells, we used a direct visualization approach. TCR engagement induces phosphorylation of tyrosine residues in the TCR immunoreceptor tyrosine-based activation motifs, followed by recruitment of the kinase ZAP-70 through interaction with its tandem SH2 domains (31). To determine the location of phosphorylated TCR, we used a ZAP-70 tandem SH2-domain-GFP fusion construct retrovirally expressed in primary P14 T cells (Fig. 2*B* and *Supporting Text*). Similar to 5C.C7 and DO11.10 T cells, central TCR accumulation occurred rapidly (Fig. 9, which is published as supporting information on the PNAS web site) and, in a high percentage (68%) of cell couples, only at a high concentration of agonist peptide, 10 μM gp33. Only 10% of the cell couples showed central TCR accumulation with 1 μM, none at 100 nM (Fig. 2*A*). Similarly, central TCR accumulation of HY T cells was rapid and prevalent and required 1–10 μM agonist peptide (Figs. 2*A* and 9). In parallel peptide dose–responses, all P14 effector and signaling functions, including IFN-γ production and cytolytic killing (Fig. 10, which is published as supporting information on the PNAS web site), occurred at agonist peptide doses 2–3 orders of magnitude less than that required for central TCR accumulation (Fig. 4*A*). In addition, TCR and LAT phosphorylation and proliferation of naïve P14 T cells showed a comparable dose–response (32, 33). In accordance with our suggestion that high-affinity TCRs require less costimulation help for IL-2 secretion, proliferation of naïve P14 T cells in response to APC plus agonist peptide is independent of CD28 and only moderately depends on LFA-1 (34). In HY T cells, all signaling and effector functions tested similarly required 1–2 orders of magnitude less agonist peptide than central TCR accumulation (Fig. 4). The P14 and HY data are consistent with a similar analysis of OT-1 TCR transgenic cytotoxic T lymphocyte function (35). Independence of T cell signaling and effector functions from central TCR accumulation thus seems widespread, occurring in CD8 and CD4 T cells alike.

Central TCR Accumulation Does Not Enhance Proximal TCR Signaling. It was intriguing that LAT phosphorylation and TCR downregulation were independent of central TCR accumulation, suggesting that TCR clustering did not influence TCR signaling. To directly examine the TCR signaling processes at a very early time point, we biochemically determined P14 TCR tyrosine phosphorylation patterns. After TCR ligation, the TCR-ζ subunit is phosphorylated, usually forming two predominant phospho forms of 21 and 23 K_d (36). These two forms were induced at agonist peptide concentrations several orders of magnitude less than that required for central TCR accumulation. The half-maximal ratio of p23 to p21, an indicator of efficient TCR signaling, occurred at 1 nM agonist peptide (Figs. 4 and 10). Maximal TCR signaling thus occurred at agonist peptide concentrations that were orders of magnitude lower than those required for central TCR accumulation. Clustering of hundreds of TCR/MHC complexes did not improve the efficiency of TCR signaling. TCR engagement as spread out over the interface and/or continued in endocytic vesicles was equally effective.

Central TCR Accumulation Is a Defined T Cell Polarization Phenotype. The lack of detectable central TCR accumulation at lower agonist peptide concentrations could reflect limited T cell polarization or an inability to detect the clustering of smaller numbers of ligand-engaged TCRs in a fully polarized T cell. We therefore studied cytoskeletal polarity in primary P14 T cells at gp33 agonist peptide concentrations where efficient central TCR accumulation did (10 μM) and did not (1 nM to 1 μM) occur. MTOC reorientation toward the T cell/APC interface, as determined with tubulin-GFP (16), was equally rapid at 1 nM gp33 and 10 μM gp33. Within 0.7 $\pm$ 0.6 versus 0.9 $\pm$ 0.7 min after T cell/APC couple formation, the MTOC moved from the base of

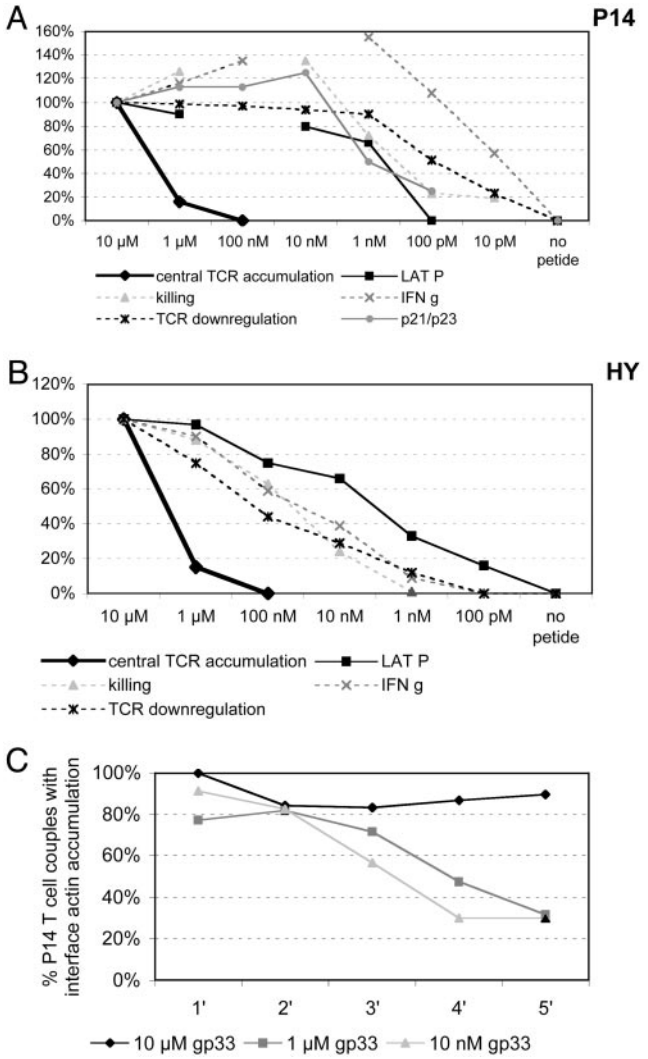


Fig. 4. Central TCR accumulation associates with sustained actin accumulation but not with critical effector functions in P14 and HY T cells. (*A* and *B*) Central TCR accumulation and effector functions in primary T cell/EL4 APC interactions (P14 T cells in *A* and HY T cells in *B*) under the indicated agonist peptide concentrations are shown as normalized data similar to Fig. 1. Raw data are given in Table 1 and Fig. 2. (*C*) The percent of actin-GFP-transduced P14/EL4 cell couples showing actin accumulation at the T cell/APC interface at >40% of the cellular background fluorescence intensity at the indicated time after cell couple formation in the presence of the given gp33 agonist peptide concentrations are displayed. Differences between values at 10 μM versus 1 μM and 100 nM gp33 peptide at 4′ and 5′ are significant ($P \leq 0.001$). Between 22 and 36 cell couples from at least two independent experiments were analyzed per condition.

the uropod to its final location behind the center of the interface. Efficient MTOC reorientation at low agonist peptide concentrations, where central TCR accumulation was dramatically reduced, was similarly observed in 5C.C7 T cells (15). In contrast to the efficient MTOC reorientation at low agonist peptide concentrations, the ability of P14 T cells to maintain actin at the T cell/APC interface, as determined with actin-GFP (16), was significantly impaired at 1 μM gp33 and 10 nM gp33. Only the initial (i.e., within 2 min after cell couple formation) interface actin accumulation was comparable at all gp33 concentrations. However, although 90% of P14/EL4 cell couples showed interface actin accumulation 5 min after cell couple formation at 10 μM gp33, only 32% and 30% did at 1 μM gp33 and 10 nM gp33, respectively ($P < 0.001$) (Fig. 4*C*). This association of central TCR accumulation with sustained actin accumulation establishes that T cell polarization was reduced when central TCR accumulation could not be observed. Interestingly, in the low-affinity 5C.C7 T cells, sustained actin accumulation was not only associated with central TCR accumulation (corroborating the P14 data) but also required for entry into the cell cycle (15). The role of sustained actin accumulation thus seems to depend on the TCR transgenic model similar to that of central TCR accumulation. T cells with a low-affinity TCR T cell required it for an effector function, and T cells with a high-affinity TCR did not. In contrast, the role of early actin accumulation is likely more general, possibly involving the formation of a wide, tight interface. In contrast to diminishing T cell polarization, the number of productively engaged TCRs, as read out by TCR phosphorylation and down-regulation, remained maximal down to 10 nM gp33 agonist peptide (Fig. 4*A*). High sensitivity of productive TCR engagement together with more easily diminished polarization (as illustrated in Fig. 10*D*) strongly argue that between 1 μM gp33 and 10 nM gp33, the reduction of detectable TCR clustering was the consequence of incomplete T cell polarization, not of reduced TCR engagement. Central TCR accumulation thus constitutes a defined polarization phenotype.

Discussion

Large-scale clustering of the TCR at the center of the T cell/APC interface is one of the most prominent and intriguing features of the immunological synapse. We have addressed its function and have made three observations. First, central TCR accumulation constituted a defined T cell polarization phenotype. Second, central TCR accumulation was not required for critical T cell signaling or effector function in three TCR transgenic systems. Third, in the low-affinity/avidity 5C.C7 T cells, costimulatory signaling, receptor clustering, and IL-2 secretion were tightly associated. In combination, these data severely limit the possible functions of central TCR accumulation, leaving the mediation of combined TCR and CD28 signaling in the generation of IL-2 in a subset of T cells. The widespread and specific nature of the correlations in combination with selected functional interference provide reliable support for signal integration in IL-2 secretion as the function of micrometer-scale receptor clustering, including central TCR accumulation.

The formation of the immunological synapse has been assessed by central accumulation of TCR (3), PKC-Θ (8), or phosphorylated ZAP-70 (37, 38) or by the central exclusion of LFA-1 (3, 39). It is unclear, however, whether such different aspects of T cell polarization reflect the same T cell organization or different, defined polarization states. We have shown that, in P14 and 5C.C7 T cells, central TCR accumulation and sustained actin accumulation require high agonist peptide concentrations, whereas early actin accumulation and MTOC reorientation are more sensitive. These data support the notion that T cell polarization consists of various defined states. They suggest that the two elements of cellular polarization at the center of the cytotoxic T lymphocyte/APC interface that are seen at high agonist peptide concentrations, accumulation of the TCR and secretory vesicles (40), have also differential sensitivity to agonist peptide. The existence of multiple T cell polarization states is further supported by the selective dissociation of TCR and PKC-Θ clustering in DOCK2-deficient mice (41) and by differential agonist peptide dose–responses of CD2 accumulation and MTOC reorientation in human cytotoxic T lymphocyte clones (42). The immunological synapse, rather than being a single structure, seems to be a continuum of polarization states, one of which with very stringent activation requirements is central TCR accumulation.

We have shown that central TCR accumulation was not required for critical effector functions in three of four TCR

transgenic systems. Although these data have been generated by using primed primary T cells as a model of effector T cells, the similar dose–responses of naïve and primed DO11.10 (Fig. 1*B*) and P14 T cells (as discussed) argue that they also apply to naïve T cells. Previous work has linked central PKC-Θ accumulation to cytolytic function and IFN-γ secretion (35), dissociated central LFA-1 exclusion from cytolytic function (43), and linked IFN-γ secretion but not cytolytic function to the enrichment of CD2 and phosphotyrosine (42), arguing for and against a requirement of the immunological synapse for T cell effector function. Together with our data, these studies suggest that some T cell polarization states are required for T cell effector function, whereas others are not. Central TCR accumulation as a polarization state with a very stringent activation requirement was not required for effector functions in the majority of T cells studied.

Initially, TCR clustering generated substantial enthusiasm as a potential mechanism of signal amplification through receptor proximity (3, 4). Later work, however, showed that substantial T cell signaling occurs in its absence (38) and even suggested that TCR clustering enhanced TCR down-regulation (44). The data presented here address this apparent contradiction. We have shown that TCR signaling itself, as assayed by TCR and LAT phosphorylation, and TCR down-regulation are independent of TCR clustering. However, the integration of TCR with costimulatory signaling required a micrometer-scale signaling complex in low-affinity/avidity 5C.C7 T cells. These data suggest that signal amplification through receptor clustering is used for only one particular aspect of signaling, the integration of TCR with costimulatory signaling, and is used only when required to compensate for a weak TCR signal. Interestingly, the dependence of cytokine secretion on receptor clustering seemed to be an intrinsic property of low-affinity T cells, because stimulation of high-affinity P14 T cells with a low-affinity Y4A partial agonist/MHC complex (30) did not restore it (data not shown). We speculate that, during T cell selection, a marginal TCR/MHC affinity imprints dependence of IL-2 secretion on costimulation-mediated receptor clustering on the developing T cell. Marginal TCR/MHC affinity is most likely to give rise to autoreactive T cells by allowing more self-peptide/MHC complexes to remain under the threshold for negative selection. Therefore, the imprinting of enhanced dependence of peripheral T cell activation on receptor clustering for such T cells should safeguard against autoimmune disease by enforcing costimulation dependence.

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