Immunological Synapse Formation Licenses CD40-CD40L Accumulations at T-APC Contact Sites[1]

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The maintenance of tolerance is likely to rely on the ability of a T cell to polarize surface molecules providing "help" to only specific APCs. The formation of a mature immunological synapse leads to concentration of the TCR at the APC interface. In this study, we show that the CD40-CD154 receptor-ligand pair is also highly concentrated into a central region of the synapse on mouse lymphocytes only after the formation of the TCR/CD3 c-SMAC. Concentration of this ligand was strictly dependent on TCR recognition, the binding of ICAM-1 to T cell integrins and the presence of an intact cytoskeleton in the T cells. This may provide a novel explanation for the specificity of T cell help directing the help signal to the site of Ag receptor signal. It may also serve as a site for these molecular aggregates to coassociate and/or internalize alongside other signaling receptors. ***The Journal of Immunology,*** **2004, 173: 3647–3652.**

During the onset and propagation of immune responses, T cells send and receive signals via Ag-dependent and Ag-independent ligands. Whereas peptide-class II MHC complexes on APC are recognized by the TCR on $CD4^+$ helper T cells, separate cell surface receptors modulate T cell-APC interactions, such as help. The primary form of T cell help is mediated by the binding of T cell CD40L (CD154) to CD40 on B cells, macrophages, and dendritic cells (DCs).[3] CD154-CD40 interactions have been identified in vivo as being responsible for T cell-dependent B cell activation and class switching, for facilitating germinal center reactions (reviewed in Refs. 1 and 2)), for activation of macrophages and DCs, for the establishment of memory CTLs (3), and in licensing CD4-dependent help for $CD8^+$ killer cells (4–6).

A critical gap in our current comprehension of helper events lies in understanding how these interactions are regulated such that only the correct APC receives signals via the up-regulated CD154. $CD4^+$ T cells typically up-regulate surface CD154 upon stimulation for times ranging from 24 h to 3 days after activation (7–11). In some instances, CD154 expression is detected at low levels on resting peripheral $CD4^+$ T cells (10). In studies using T cell hybridomas bearing high levels of CD154, signaling to B cells occurs independent of Ag receptors (12, 13). However, given the milieu of the spleen and lymph nodes in which numerous cells are packed tightly together, it is not obvious how spurious signaling from CD154 to a bystander CD40-bearing cell is prevented. This is critically important, because the availability of such help determines the fate of potentially autoreactive B cells in vivo (14).

Interactions of T cells with Ag-bearing APCs is associated with a coalescence of TCRs, other related receptors such as CD2, CD4, CD28, and signaling molecules such as $p56^{lck}$, *fyn*, linker for activation of T cells, and protein kinase Cθ into the central portion of an "immunological synapse" (15–23). Integrins and their associated molecules, as well as CD43 and CD45, are predominantly accumulated at an outer zone of this interface (15, 16, 24, 25). The central supramolecular-activating cluster (c-SMAC) of TCRs in the central zone was initially demonstrated to be uniquely associated with agonist but not weak agonist activation (16). Recently, the dynamics of signaling leading to downstream outcomes have proven more complex, with clear-cut indications that the c-SMAC is formed only after the onset of signal transduction (17, 26), and recent evidence suggesting that the c-SMAC might in fact coordinate the internalization of TCRs (27). Therefore, the function of the mature synapse structure is a subject of some debate (28, 29).

We postulated that helper interactions, such as those of CD154-CD40, might be of sufficiently low affinity to be regulated by the presence or absence of a synapse. Therefore, we assessed the localization, movement, and activation of CD40 on B cells during synapse formation with CD154-bearing T cells. Our results demonstrate that accumulation of these helper molecules to the immunological synapse proceeds after and requires MHC-peptide recognition by the TCR, ICAM-1-dependent stable synapse formation, and a functional actin cytoskeleton within the T cells. Thus, the immunological synapse plays a critical role in regulating the assembly of signaling complexes containing receptors that are not directly involved in TCR recognition and signaling.

Materials and Methods

Cell lines and peptides

D10.G4.1 (D10) is a $CD4^+$ T cell clone derived from AKR/J mice and the TCR of this clone binds with high affinity to conalbumin peptide CA134-146 (CA) in the context of MHC class II IA^k (30). D10-CD3ζ cyan fluorescent protein (CFP) is a stable transfectant with properties similar to the GFP equivalent, previously described (17). Clones were maintained by weekly restimulations as previously described (30). Experiments were performed 2–10 days after T cell stimulation; CD40L expression over that period varied by ~2-fold and all experiments compared cells cultured identically before assaying synapse formation. CH27 is a B cell hybridoma expressing IA^k, ICAM-1, CD40, CD80, and CD86. The agonist CA and weak agonist E8T peptides were purified by HPLC and reconstituted in PBS before use as described previously (17). CH27 stably expressing CD40 yellow fluorescent protein (CH27-CD40YFP) were generated by

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Received for publication February 11, 2004. Accepted for publication July 9, 2004.

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[1] This research was supported by National Institutes of Health (NIH) Training Grant T32 AI07334-15 (to J.B.), NIH RO1 AI AI52116 (to M.F.K.), and by startup funds from the Howard Hughes Medical Institute Biomedical Research Support Program Grant 5300246 (to M.F.K.).

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[3] Abbreviations used in this paper: DC, dendritic cells; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; c-SMAC, central supramolecular-activating cluster; CA, canalbumin peptide CA134-146.

0022-1767/04/$02.00

electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA) followed by selection of stable transfectants using gentamicin (Sigma-Aldrich, St. Louis, MP) and cell sorting.

Constructs

A plasmid encoding CD40YFP was derived by mutagenic PCR of a plasmid encoding murine CD40 cDNA using as 5′ primer: GGA CTG CTC GAG ATG GTG TCT TTG CCT C, and 3′ primer: CG CAG TAC CGG TCC ACC GCC ACC TGA ACC GCC TCC GAC CAG GGG CCT CAA G. This product was then cloned into the *Xho*I and AgeI sites of pEYFP-N1 (BD Clontech, Palo Alto, CA) to create a fusion protein containing full-length CD40 at the N terminus, followed by a linker comprised of the amino acids gly-gly-ser-gly-gly-gly-gly-pro and YFP at the C terminus. CD3ζCFP was produced from a construct similar to one previously described to express CD3ζGFP (17), through the exchange of the CFP and GFP coding sequences.

Abs and drugs

Anti-mouse IA^kPE (11-5.2), anti-mouse ICAM-1-biotin (3E2), anti-mouse CD154.biotin (MR1) were obtained from BD Pharmingen (San Diego, CA). Anti-mouse CD40 (FGK) and anti-mouse CD3 (500.A2) were prepared from cultured hybridoma supernatant using standard Protein A/G Ab purification methods. Cytoskeletal disassembly was performed by pretreating T or B cells with 10 μg/ml cytochalasin D (CalBiochem, San Diego, CA) for 30 min at 37°C, followed by washing, immediately before imaging or bead coupling. Staining for flow cytometry was done using standard methods.

Fixed cell coupling

Fixed cell couples were generated by mixing equal numbers ($1–2 \times 10^5$) of D10 T cells and peptide- (2–10 μM CA) or conalbumin (100 μg/ml) Ag-pulsed CH27 B cells in Eppendorf tubes, centrifuged at $400 \times g$. Cells were resuspended in PBS, 1% BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (PBS/1%) for 50 min at 37°C, and washed and fixed with 3% PFA. Cell couples were washed in PBS/1% for 30 min at room temperature; Abs were added and staining continued for 1 h at room temperature. Cells were mounted in antifade and imaged immediately.

Rapid three-dimensional fluorescence microscopy

All three-dimensional data acquisition was achieved using an inverted Axiovert 200 M microscope and a ×40/1.4 lens (Carl Zeiss, NJ), equipped with a 175 W xenon highspeed λ DG4 wavelength selector and a single emission filter wheel (Sutter Instruments, Novato, CA), a PI piezoelectric z-drive (Physik Instrument, Germany) and a cooled-CCD Coolsnap camera (Roper Instruments, NJ). Stage temperature was controlled by a heated stage along with an objective heater. Data were acquired and analyzed using Metamorph (Universal Imaging, Downingtown, PA). For D10 time course experiments, D10 T cells were loaded with fura 2-AM (Molecular Probes, Eugene, OR) and $\sim 10^5$ cells were placed into 8-well glass-bottom coverslips (Nunc, Naperville, IL) in phenol red-deficient RPMI 1640. Coverslips were placed on a heated (37°C) microscope stage. Optimal exposure parameters were determined and $\sim 10^5$ peptide-pulsed CH27-CD40YFP cells were added and allowed to settle. Data acquired every 15 s-1 min consisted of single differential interference contrast, fura 2-AM excitation at 340 nm and 380 nm, and a 8–19 frame 1-μm separation GFP/CFP/YFP (xFP) z-stack. The xFP stacks were collected using streaming software with 30–1000 msec exposures for each z-plane, resulting in an overall collection time of 2–30 s. For two-color collection, data were acquired for each wavelength sequentially. A JP4 polychroic (Chroma Technology, Rockingham, VT) and the following respective filters were used: 436/10 excitation and 470/30 emission (CFP), 490/20 excitation and 528/38 emission (GFP), 500/20 excitation and 550/50 emission (YFP), 555/28 excitation and 617/73 emission (red), and 635/20 excitation and 685/40 emission (Cy5/APC). Using this system, no significant spectral overlap of CFP and YFP signals was observed.

Scoring CD40 phenotypes

Intensity values derived during image acquisition were analyzed using Metamorph software. Intensities from 340 nm and 380 nm excitations of fura 2-AM were used to make a ratio image and regions of interest were drawn for individual T cells. Background calcium levels were obtained from at least five frames before activation. In studies of T cells left alone in dishes, it was determined that 30% above background represented an increase well above random fluctuations, and most agonist driven reactions result in at least a 100% increase in a single 15-s time period during the onset of calcium signaling. For YFP intensity data, an experimental background level was determined empirically by imaging a dish in the absence of cells. This background level was subtracted from intensity data to obtain background-subtracted data sets. Individual cells were analyzed for maximal pixel intensities along the leading edge of cells using a line-scan function. All collected z-planes were analyzed and compared with the average from three intensity linescans (taken at different z-planes) around the circumference of the cell of interest.

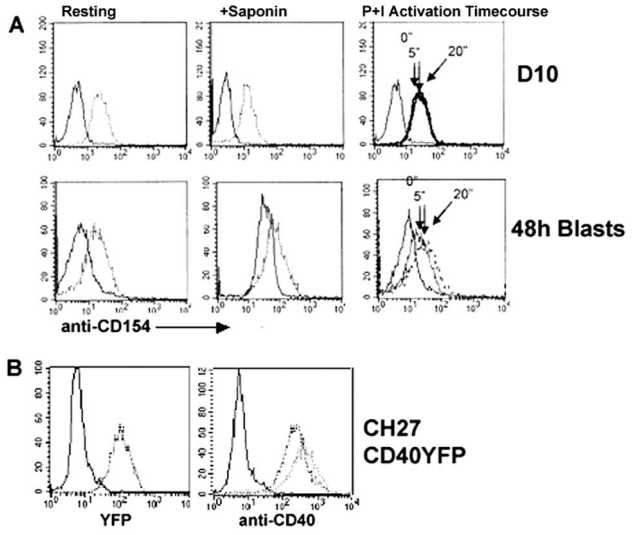

FIGURE 1. Cell surface expression of CD154 on D10 T cells and freshly stimulated D011.10 blasts and CD40 on CD27-CD40YFP transfected B cells. *A*, D10 T cells (*upper panel*) or DO11.10 blasts (*lower panel*) stained with control or anti-CD154 Abs either in untreated cells, in cells permeabilized using saponin, or in cells treated for the indicated times with PMA-ionomycin (dashed line CD154, solid line control). *B*, CH27 B cells (wild-type or CD40YFP transfectants) were examined for YFP expression (*left panel*, CH27 wild-type cells, solid line; CH27-CD40YFP cells, dashed line) and for expression of total CD40 protein (*right panel*, CH27 APC, stained with CD40 biotin Avidin-allophycocyanin, CH27 CD40YFP, light dashed line; CH27 wild-type cells, dark dashed line; CH27-CD40YFP cells unstained, solid line) by flow cytometry.

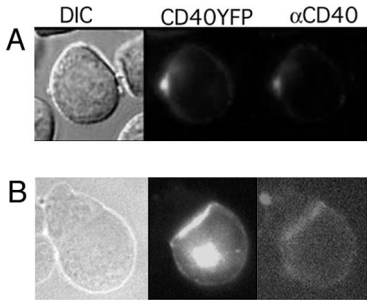

FIGURE 2. CD40YFP comigrates with the total CD40 pool, opposite CD154 on T cells, and accumulates alongside the TCR/CD3 complex. *A*, Comigration of CD40-YFP with anti-CD40-induced caps. CD40YFP transfected CH27 B cells were treated with anti-CD40 Ab followed by PE-conjugated anti-rat Ab and the molecules were allowed to cap at 37°C for 20 min. This was followed by fixation and analysis of distributions. *B*, Colocalization of CD40YFP and Abs directed against CD40 within the synapse. D10 T cells and peptide-pulsed CH27-CD40YFP B cells were mixed together, allowed to form conjugates, followed by fixation without permeabilization and staining for CD40. Both surface CD40 pools are revealed and are similarly synapse enriched. CD40YFP also reveals the presence of an intracellular pool of CD40 in this B cell.

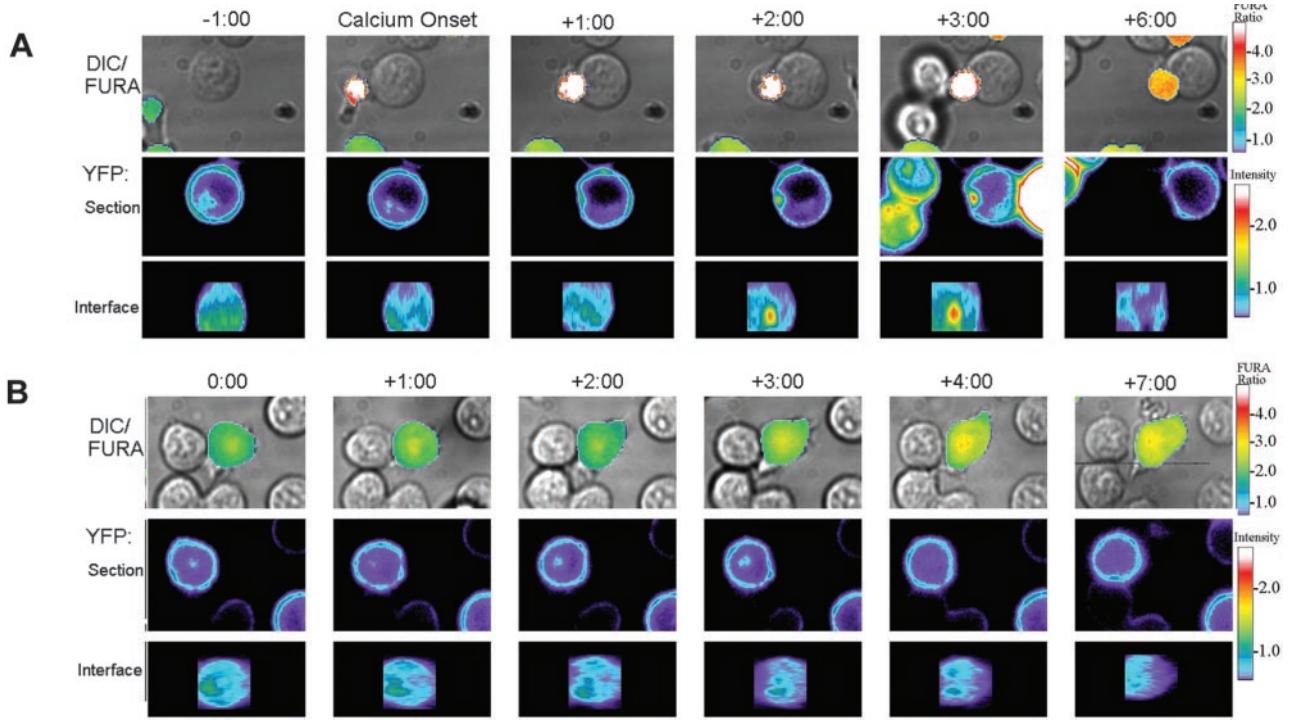

FIGURE 3. CD40 accumulation at the T cell-B cell interface requires agonist peptide recognition by T cells. D10 T cells were loaded with fura 2-AM and mixed with CH27-CD40YFP B cells pulsed with CA Ag (*A*) or E8T weak agonist (*B*). At the indicated time points relative to the onset of calcium signaling, a contrast image, a calcium ratio, a CD40YFP image at the mid-z-plane, and a reconstruction of the interfacial region are shown.

Results

Generation and characterization of CH27-CD40YFP and D10 clones

To examine the surface recruitment of CD40-CD154 to the immunological synapse, we sought a T cell system that expressed physiological levels of CD154. As shown in Fig. 1, D10 T cell clones express surface CD154 during their expansion phase. We established that the surface pool represents a majority of the total CD154 in these cells by permeabilization with saponin and by demonstrating only a marginal increase in fluorescence intensity after activation with PMA-ionomycin (Fig. 1*A*, *upper panel*). That this level of CD154 expression is physiologically relevant is demonstrated by a similar pattern of expression on 48-h Ag-stimulated T cell blasts taken from TCR transgenic mice (Fig. 1*A*, *lower panel*). Notably, like T cell blasts, D10 expression of CD154 at maximal levels followed ~2 days after stimulation and decayed during the Ag-independent resting phase (data not shown).

To specifically examine the movement of CD40 in real time, we generated CH27 B cells stably expressing CD40YFP (CH27-CD40YFP). These cells expressed ~1.5-fold higher levels of CD40 compared with wild-type CH27 cells (Fig. 1*B*, *right panel*). The fluorescence intensity of CD40YFP on CH27-CD40YFP cells compared with the FL-1 autofluorescence of wild-type CH27 cells is shown in Fig. 1*B* (*left panel*). That these chimeric molecules accurately reflect the distribution of wild-type molecules is demonstrated by coclustering of CD40YFP with Ab cross-linked and detected CD40 (Fig. 2*A*), as well as coaccumulation of surface CD40YFP and CD40 as detected by Abs in the synapse between T cell and APC (Fig. 2*B*). In the B cell in Fig. 2*B*, an intracellular pool of CD40YFP is unavailable for staining with anti-CD40 because no permeabilization agent was added for these stains. Such a pool most likely represents newly synthesized or recycled proteins and similar intracellular spots were occasionally observed in B cells in these and subsequent experiments. Typically, these were not oriented toward the synapse (data not shown).

CD40YFP recruitment to the synapse occurs in the presence of agonist, but not antagonist peptide, and requires T cell cytoskeletal rearrangement

To characterize the dynamics of CD40 aggregation, we performed live-imaging experiments in which wild-type D10 T cells were mixed with CH27-CD40YFP cells pulsed with CA agonist peptide, E8T weak agonist peptide, or no peptide. In the example shown in Fig. 3*A*, when CH27-CD40YFP cells presented the CA agonist peptide, CD40 began to accumulate in the synapse 2 min after the onset of calcium signaling. Over the next 3 min, CD40 was further concentrated into the central synapse. This accumulation resembles the c-SMAC, as observed for TCR/CD3 and MHC molecules in this and other systems (15–17) because CD40 is highly concentrated in a region that is well within the bounds of the cell-cell contact face as assessed using the differential interference contrast or fluorescence images. By 7 min after the onset of calcium signaling, the CD40 accumulation in the interface was lost and the central interface contained very low levels of CD40 (Fig. 3*A*, and Supplmental Movie A).[4] This rapid loss of surface expression was consistently observed, perhaps representing internalization of one or both receptors.

To characterize the Ag-dependency of the recruitment of CD40, we assessed movement of CD40YFP in the presence of a weak agonist peptide TCR ligand or with no added peptide. Fig. 3*B* shows the results of live-imaging experiments using E8T weak agonist-pulsed CH27-CD40YFP cells and D10 T cells. Although a

[4] The on-line version of this article contains supplemental material.

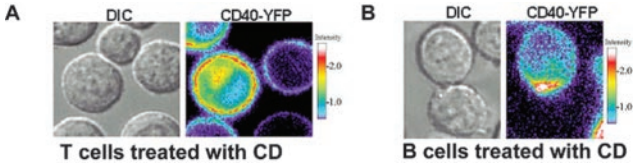

FIGURE 4. Accumulation of CD40 into the contact face requires and intact actin cytoskeleton in T cells but not B cells. D10 T cells (*A*) or CH27-CD40YFP B cells (*B*) pulsed with CA Ag were treated with cytochalasin D for 30 min before incubating together. After 15 min, cells were fixed with paraformaldehyde and CD40YFP accumulation was scored in couples. Shown are a contrast image and a midplane CD40YFP image.

contact site was formed resulting in a brief calcium flux, no detectable CD40 accumulation was observed at the interface. A similar absence of CD40 clustering was observed using CH27-CD40YFP cells in the absence of added peptides (data not shown). This suggests that high level CD40-CD154 recruitment is dependent on the synapse structures initiated upon TCR recognition.

To determine whether CD40 accumulation at the synapse is driven by internal cytoskeletal events within the T cell or the B cell, or both, we used cytochalasin D treatment to disassemble the actin cytoskeleton. Following pretreatment of T cells or Ag-pulsed B cells, or neither, live-imaging experiments were performed, shown in Fig. 4. Pretreated T cells engaged B cells with ~75% lower frequency than did untreated T cells. However, in cell conjugates that did form despite pretreatment of T cells, the frequency with which CD40YFP accumulated in the synapse was reduced (Fig. 4, and Table I). In contrast, pretreatment of B cells before imaging had no effect on the frequency of synapsis or on the frequency of CD40YFP reorientation. Therefore, we conclude that CD40YFP recruitment into the immunological synapse is dependent upon a functional T cell but not B cell cytoskeleton.

The data from Fig. 4 and Table I suggested that redistribution of CD40 and its accumulation in the synapse were responding to a T cell dependent synapse assembly event. The most likely of these was CD40L recruitment itself on the T cell surface. To test this, we stained fixed-cell conjugates formed either in the presence or absence of antigenic peptides. As shown in Fig. 5, recruitment of CD40L to the T-APC contact was not observed in the absence of stimulatory peptide. In contrast, addition of CA peptide led to a robust accumulation of CD40L in the synapse. This further supports the conclusion that recruitment of the pair is T cell dependent.

To quantify the primary determining factors for accumulating CD40 into the central synapse (c-SMAC), we mixed T cells with B cells in the presence of CA agonist or E8T weak agonist peptides with or without Abs against cell surface molecules and scored for the appearance of a variety of types of CD40 accumulations in the interface. As shown in Fig. 6, in a majority of cases (~75%), strong-agonist peptide CA gave rise to centralized CD40 accumulations in the first 5–10 min after contact. Peripheral and uniform

Table I. *The accumulation of CD40 in the synapse*[a]

Treatment	Cell Couples with CD40YFP Reorientation (%)
No treatment	17/32 (53%)*
B cell pretreatment	16/25 (64%)**
T cell pretreatment	2/10 (20%)*, **

[a] The accumulation of CD40 in the synapse was scored 10 min after the onset of calcium signaling, with or without the indicated blocking conditions and in the presence of CA Ag or E8T weak agonist, as indicated.
*, $p = 0.083$, **, $p = 0.028$, Fisher's exact test.

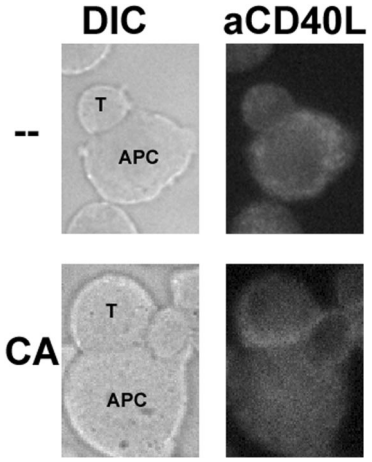

FIGURE 5. CD40L accumulation at the synapse requires agonist recognition. Comigration of CD40L in T-APC couples. D10 T cells and unpulsed (−) or 10 μM CA134-146 peptide-pulsed (CA) CH27 B cells were mixed together, allowed to form conjugates, followed by fixation and staining with CD40L –biotin, followed by Avidin-Texas Red. Similar phenotypes were observed for 5/5 (no peptide) and 6/6 (CA peptide) individual cells scored. Note that the anti-CD40L Ab has some background staining of some CH27 cells; this is particularly prominent as a pool distal to the cell-cell contact shown in the *upper corner* in the *upper panel*.

distributions were also occasionally observed but represented the minority of couples. In contrast, replacement of the CA peptide with E8T or in the absence of peptide never induced statistically significant levels of CD40 recruitment to the synapse, either central or peripheral, further underscoring the importance of the TCR recognition for this event. Pretreatment of T cells with anti-CD154 Ab, before mixing with CA-pulsed CH27-CD40YFP and live imaging completely abrogated recruitment. Furthermore, pretreatment of B cells with anti-ICAM-1 completely abrogated CD40 accumulation in the interface. This is important because the interaction between ICAM-1 and LFA-1 is a critical component of stable synapse formation (31, 32). Taken together, these data show that central accumulations of CD40 depend on the formation of a mature immunological synapse.

TCR/CD3-clustering and synapse assembly precedes CD40 recruitment to the central synapse

One possible scenario for CD40 recruitment is that it is mediated by and thus follows TCR recognition and signal transduction. To

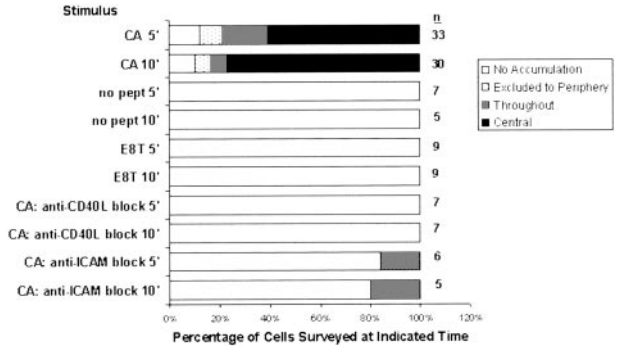

FIGURE 6. CD40 recruitment phenotype requires TCR recognition, integrin-mediated adhesion, and the presence of ligand. The phenotype of CD40 in the synapse was scored 5 or 10 min after the onset of calcium signaling in the presence of the indicated blocking Abs and in the presence of CA Ag, E8T weak agonist, or no peptide as indicated.

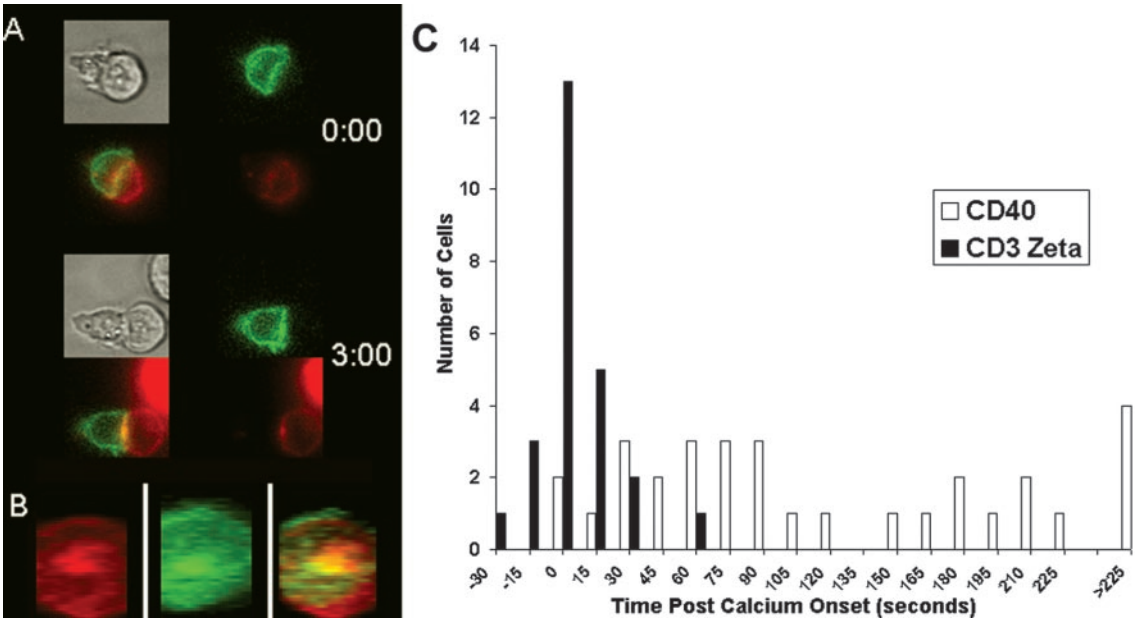

FIGURE 7. CD40 and CD3 share the central synapse with differing kinetics. *A*, D10-CD3ζCFP T cells were mixed with CH27-CD40YFP B cells pulsed with CA peptide contrast and merged fluorescence images (green, CD3ζCFP; red, CD40YFP) are shown at the indicated time points relative to cell contact. *B*, Three-dimensional reconstruction of the cell-cell interface after 3 min of contact showing central accumulation of CD40YFP. *C*, Relative to the onset of calcium signaling, the time of appearance of CD3ζGFP or CD40YFP accumulations in the interface were separately scored as described. CD40 accumulation typically lagged with respect to the onset of signaling, whereas CD3 accumulation occurred contemporaneously.

determine the temporal relationship of CD40 synaptic recruitment with that of TCR/CD3, we performed two-color experiments in which D10 T cells expressing CD3ζCFP were mixed with CA-pulsed CH27-CD40YFP B cells. As shown in Fig. 7*A*, CD40 distribution was spatially coincident with the CD3 region within 3 min after the first appearance of CD3 accumulation in the interface, which is typically indicative of the onset of TCR signaling (17). At the later time points, both occupied the central contact area, although overlap of the molecular distributions was incomplete (Fig. 7*B*).

We noted that CD40 typically appeared to aggregate all at once and somewhat after the onset of calcium flux in these experiments whereas CD3ζ appeared coincidentally with the onset of calcium signaling and as dynamic clusters that coalesced into the CD3 synapse over the first minutes. To quantify this effect for multiple cells, the relative timing of significant accumulation was scored. As shown in Fig. 7*C*, CD3ζ accumulation was first found in the interface highly coincident with the onset of the calcium signal. In contrast, the appearance of CD40 in the interface was variable and occurred an average of 2 min after the onset of calcium signal, a time at which the CD3 c-SMAC is typically beginning to coalesce (17). Taken with data from Fig. 4 relating CD40 accumulation and calcium flux, this demonstrates that CD40 aggregation follows the commitment to synapse assembly and CD3 accumulation.

Discussion

CD40 recruitment to the synapse requires the binding of adhesion molecules and MHC-peptide recognition. This observation suggests that directing help to the site of Ag-specific engagement might be one of the ways in which the timely activation of Ag-specific CTLs is ensured, and the spurious activation of autoreactive or Ag-nonspecific B cells is avoided, particularly in the tightly packed milieu of the lymph node and spleen. For example, if CD154 is up-regulated on $CD4^+$ T cells by DCs presenting viral epitopes, then only these DCs become competent to activate virus-specific $CD8^+$ CTLs. This, in turn, may bias activation toward virus-specific T cells and may help explain previous observations reported by Murali-Krishna and colleagues (33). In that study, at least 50–70% of activated $CD8^+$ T cells in acute lymphocytic choriomeningitis virus infection were, in fact, Ag-specific. Their results argued against the previously accepted hypothesis that most of the activated $CD8^+$ T cells in an acute immune response are Ag nonspecific, resulting from bystander activation (33). With respect to humoral immunity, one assumption, of course, is that APCs taking up and processing peptides for presentation to $CD4^+$ T cells in the context of MHC class II molecules are likely to have specificity for an epitope from the same pathogen. Another assumption is that T cell migration within the lymph nodes and spleen optimizes contact between T cells and APCs of the same specificity (7, 34).

Although early data were interpreted to suggest that the immunological synapse was fundamental in generating sustained signaling (16), our data strongly support a model in which the c-SMAC is responsible for setting the polarity for other signaling and secretion pathways, including Ag-independent helper signaling molecules. Reorientation of the Golgi/microtubule organizing center toward T cell target(s) has been known for some time (35), and recent studies have shown directed secretion of cytotoxic elements from CTLs via the immunological synapse (36). Like our suggestion for CD40/CD40L, localization of intracellular IL-2 pools toward the APC is also likely to direct help toward the stimulating APC (37). Notably, the synapse accumulation pattern of secretory molecules was found alongside the *lck* distribution and both distributions were contained with the adhesion pSMAC (36). This split c-SMAC may be analogous to the CD40/CD3 distribution we observed in this study, in which CD40 also only partially overlaps with CD3 in the central contact region.

Because CD154 surface expression is already nearly maximal in our assays, we believe that such mechanism, based in the secretory machinery, is not playing a part in this receptor-ligand interaction. Our observations that enhanced TCR clustering, an intact cytoskeleton in the T cell but not the B cell, and CD154 engagement are

required for CD40 synapse formation clearly demonstrates that the driving force behind CD40-CD154 synapse recruitment comes from within the T cell and requires TCR signaling. Active recruitment of CD154 to the T-B interface might be achieved by cytoskeleton-bound myosin motors via its intracellular cytoplasmic tail or by raft association of the transmembrane domain. Such a mechanism is suggested by several observations in which the cytoskeleton plays an important role in recruiting other components of the immunological synapse, such as CD3ζ, CD43, and lipid rafts (24, 25, 38, 39). The requirement for synapse assembly in CD28 recruitment was recently reported and may occur via a related mechanism (21). c-SMAC assembly of the TCR has also been implicated in mediating receptor down-regulation, based on the phenotype of the CD2AP knockout mouse (27). In our case, cytoskeletal recruitment of CD40 molecules to the central synapse might ultimately also be responsible for our observation that the CD40 c-SMAC localization was not very long lived. Understanding the assembly process will likely await the identification of myosin motors and/or lipid-anchoring mechanisms that link these receptors and ligands to the cortical cytoskeleton.

Acknowledgments

We are deeply grateful to Kym Garrod and the members of the Krummel lab for technical assistance, and to Abul Abbas, Johannes Huppa, Luke Barron, and Hans Dooms for critical review of this manuscript.

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