Fig. S1: Multi-lamellar bodies and multi-vesicular bodies seen in T cells

A
Multi-lamellar bodies (MLBs)

B
Multi-vesicular bodies (MVBs)

Scale bar: 50nm

Scale bar: 50nm
Fig. S2: Features of an early T cell - APC interface, 30 minutes after the mixture of T cells and CH27 cells and images by SEM

A

Scale bar: 2um

B

Magnification of the contact zone of (A)

Scale bar: 0.5um
Fig. S3: Conjugates in the absence of antigen (Stage 0)

T cell centriole (arrowhead); TN: T cell Nucleus, CH27 as APC; Scale bar, 2um
Fig. S4: Representative images of MT initiating sites (arrowheads) in T cells

A

CH27

Active MT initiating sites around centrioles (C). From the contact side, the order is active MT initiating sites, centrioles and the Golgi complex (G) (Stage 2). Scale bar: 0.2μm

B

Snapped image from a Zap window of the IMOD software (ref 25-27)
Scale bar: 50nm

C

Snapped image from a slicer window of the IMOD software
Scale bar: 100nm
Fig. S5: Huge membrane activity and the Golgi complex at Stage 4

Huge membrane activity was observed at the contact side at the stage 4 (A and B). (B) is magnification of the contact zone of (A). Scale bars, 2um (A); 500nm (B)
G: Golgi complex of a T cell; TN: T cell nucleus.
**Fig. S6: Other models of single T cells**

*T cell’s plasma membrane:* cyan; *Centriole:* yellow-green; *Microtubules (MTs):* yellow lines; *MT-initiating sites:* light orange spheres; *T cell’s nuclear envelope:* dark purple; *T cell’s heterochromatin:* bronze; *ER:* dark green or blue; *Golgi complex:* white, red, blue; *MVB:* Multi-vesicular Body; *MLB:* Multi-laminar body.
Fig. S7: Models of microtubules and centriole during the IS cycle

(A) Stage 0, (B) Stage 2, (C) Stage 3, (D) Stage 4. T cell’s plasma membrane: cyan; Centriole: yellow-green; Microtubules (MTs): yellow lines; MT-initiating sites: light orange spheres; T cell’s nuclear envelope: dark purple; T cell’s heterochromatin: bronze.
Fig. S8: An invasive pseudopodia (Stage 1) of a T cell in a CH27 cell

A snapped image of a pseudopodia from a Zap window of the IMOD software; a T cell pushes an invasive pseudopodia deep inside cytoplasm (C) of a CH27 cell, but no pseudopodia which goes into CH27 cell nucleus (N) was observed. A slice of a tomogram of an invasive pseudopodia of a T cell (P) inside a CH27 cell. The direct contacting area of this typical pseudopodia of the T cell diving into the CH27 was calculated as 0.4μm² by the IMOD. Scale bar: 50nm
Fig. S9: Nuclear pore complex behind the Golgi complex in a T cell

T cell nuclear pore complex (arrowhead); G: Golgi complex of a T cell; EC: Euchromatin; HC: Heterochromatin. Scale bar: 200nm
**Fig. S10:** A closed space that was surrounded by a junction-like structure with an opened vesicle

(A) A vesicle (arrowhead); TN: T cell Nucleus; G: Golgi complex of a T cell; CH27 as APC;
(B) a junction-like structure (arrowhead); T: T cell; Scale bars, 100nm (A); 50nm (B)
Fig. S11: Invagination of a T cell in Stage 2

A
A snapped image of a Zap window

B
A snapped image of the model

T: T cell, Scale bar: 50nm
Fig. S12: Cytotoxic T cells and target cells conjugates

(A) CTL: cytotoxic T cell; TC: Target cell (CH27), (B) magnification of a squared center region of (A), * indicates lytic granules as a marker of CTLs; Scale bars, 10um (A); 5um (B)
Fig. S13: Apposition of centriole to the contact site in BM-CD conjugates

BM-DC: bone marrow derived dendritic cell as APC; T cell centriole (arrowhead); TN: T cell Nucleus; Scale bar, 2um
**Fig. S14: Apposition of centriole to the contact site**

**CH27:** CH27 cell as APC; **C:** centriole; **T:** T cell; Scale bars, 100nm (**A**); 200nm (**B**).
Fig. S15: Microtubules support the intra cellular structures of cell organelle beneath the IS from representative tomograms of Stage 2.

(A) A detailed view of the center of the cell contacting area of Fig. 5A. An open vesicle was observed at the center of the contact site. (B) A MT (*) attaches to the same open vesicle as in Fig. S15A at the center of the cell contacting area. (C) A MT (*) attached to the inside of the T cell plasma membrane at the contact site in a slice from a tomogram of a different IS at the same stage. Images by slicer windows of the IMOD package (B and C). Scale bars: 100nm (A-C)
Fig. S16:
Models for stages of CD4+ T cell Synapses

a. Stage 0
(scanning, in the absence of antigen)

b. Stage 1
(invasive pseudopodia)

c. Stage 2
(active MT initiating sites)

d. Stage 3
(centriole proximity, 2 hours)

e. Stage 4
(enlarged Golgi complex, 4 hours)
Table S1: Average numbers of centrioles, discrete Golgi stacks, Mitochondria of T cells beneath the IS

<table>
<thead>
<tr>
<th>Cell organella</th>
<th>front</th>
<th>side</th>
<th>rear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrioles</td>
<td>1.11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Discrete Golgi stacks</td>
<td>1.18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.64</td>
<td>1.29</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The number of cell organelle, centrioles, discrete Golgi stacks and mitochondria of T cells were counted beneath the IS (n=28). Front means the contact site and rear means the opposite site of contacting, the side means other area neither from front and rear.

Table S2: Summary of IS stages and their features

<table>
<thead>
<tr>
<th>Time points</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>single T cell (or conjugates without antigen)</td>
</tr>
<tr>
<td>Stage 1</td>
<td>&lt; 30 min</td>
</tr>
<tr>
<td>Stage 2</td>
<td>&lt; 60 min</td>
</tr>
<tr>
<td>Stage 3</td>
<td>one - two hours</td>
</tr>
<tr>
<td>Stage 4</td>
<td>four hours</td>
</tr>
<tr>
<td></td>
<td>Centrioles close to nuclear membrane</td>
</tr>
<tr>
<td></td>
<td>Invasive pseudopodia</td>
</tr>
<tr>
<td></td>
<td>Active MT activities, endosomes (MLBs, MVBs)</td>
</tr>
<tr>
<td></td>
<td>Centriole proximity to the contact zone</td>
</tr>
<tr>
<td></td>
<td>Enlarged Golgi and huge membrane activity</td>
</tr>
</tbody>
</table>
Table S3: Relative positions of centrioles to cell surface

<table>
<thead>
<tr>
<th>Cells</th>
<th>Number</th>
<th>Mean ± SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH27</td>
<td>9</td>
<td>0.68 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>BM-DC</td>
<td>8</td>
<td>0.69 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Single T cells</td>
<td>8</td>
<td>0.63 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>T cells with CH27</td>
<td>8</td>
<td>0.24 ± 0.03</td>
<td>1.0 x 10^-5 *</td>
</tr>
<tr>
<td>T cells with BM-DC</td>
<td>9</td>
<td>0.27 ± 0.04</td>
<td>1.4 x 10^-5 *</td>
</tr>
</tbody>
</table>

The parameter A/ (A+B) was calculated for several cells where A is the distance from the cell cortex to a centriole and B is the distance from a centriole to the nuclear envelope. Values are given as total number or mean ± SEM. The parameters were compared by Student t test.

*P compared with single T cells. No statistical significance was observed among CH27, BM-DC and single T cells, and between T cells with CH27 and T cells with BM-DC.
SI Materials and Methods (1)

Cells: Primary T cell blasts were prepared from 5c.c7 αβT cell receptor transgenic mice. Lymphocytes from these mice were stimulated with 10 μM HPLC–purified MCC (88-103, ANERADLIAVLKQATK) in RPMI medium containing 10% FCS. Cells were split on the second day of culture in RPMI 1640 medium plus 10% fetal calf serum (FCS), 2 mM l-glutamine, 50 mM β-mercaptoethanol, penicillin/streptomycin and 30 U/ml of recombinant mouse interleukin-2 (R&D Systems) and thereafter were split as necessary for 6 d as published elsewhere from our laboratory (24, 42). CH27 cells, a B cell lymphoma line, were pulsed by 5 μM MCC and used as APC. CH27 was maintained in the same medium as T-cell blasts. Cells were purified with a Histopaque-1119 (Sigma-Aldrich) before use.

Preparation of Cells for SEM and TEM
For scanning electron microscopy, T cells and MCC peptide-pulsed CH27 cells were mixed 1:1 and placed on poly-lysine coated cover slips, then incubated for 30 minutes - 1 hour at 37°C in medium equilibrated with 5% CO₂. Cells were then fixed for 5 min at room temperature by placing 150 ul of PBS containing 1% glutaraldehyde. After washing three times with distilled water, cells were dehydrated in increasing concentrations of ethanol, fixed with hexamethyldisilazene (HMDS) (Polysciences, Inc., Warrington, Pennsylvania) for 10 min, mounted cover slips on stainless steel SEM stubs, coated immediately with Gold - Palladium in a sputter coater, then observed and imaged with a Philips 525 scanning electron scope operating at 30 kV.

For TEM, T cells and Ag-pulsed APCs (or target cells) were purified with a Histopaque-1119 (Sigma-Aldrich). T cells were added to the APC (at ratios of 2:3: 1). After a minimum of 5 minutes or after up to six hours incubation at 37°C in 5% CO₂, the conjugated cells were fixed in 2.5% glutaraldehyde (Polysciences Inc.) in PBS at room temperature for 30 min. Fixed cells were washed with cold PBS then re-suspended in cold PBS containing 8% sucrose on ice, then washed twice more with cold PBS. To the pellet, an equal volume of 1% low melting NuSieve GTG agarose (FMC Bioproducts) gel with 0.5M sorbitol in DDW was added. Trapped cells were postfixed in 1% osmium tetroxide for 60 min at 4°C, followed by postfixing 1% Uranyl acetate in DDW, stain for overnight at 4°C. Trapped cells were dehydrated in increasing concentrations of ethanol then embedded in epon. After polymerization at 60°C for 48 hours, thin (90 ± 10 nm) sections were cut with a Leica UltraCut-UCT microtome (Leica Microsystems, Wetzlar, Germany) and mounted on Formvar-coated multi-slot grids. Sections were post-stained with 5% aqueous uranyl acetate and 0.2% lead citrate and examined at 80-100 kV in a JEOL (Tokyo, Japan) 1230 electron microscope. Digital images were captured with a Gatan (Pleasanton, California, United States) 967 slow-scan, cooled CCD camera.
SI Materials and Methods (2)

High-Pressure Freezing/Freeze Substitution and EM tomography

Specimens were freeze-substituted and plastic-embedded essentially as described (43) (http://bio3d.colorado.edu/). Thick (300-400 nm) sections cut with a Leica UltraCut-UCT microtome (Leica, Deerfield, IL) were collected onto Formvar-coated copper slot grids. Colloidal gold particles (10 or 15 nm) were deposited on both surfaces of these sections for use as fiducial markers during subsequent image alignment. The grids were placed in a Gatan tilt-rotate specimen holder (model 650; Gatan, Pleasanton, CA) and imaged with an F30 EM from FEI (Hillsboro, OR), operating at 300 kV. Images were captured digitally using a semi-automated data collection procedure developed in the Boulder 3-D laboratory (http://bio3d.colorado.edu/), software that incorporates Digital Micrograph (Gatan) to capture images on a 1024 x 1024 pixel charge-coupled device camera (Gatan) at a pixel size of 1.4 nm. Serial, tilted views were collected every 1.5° over a ±60° range. Then the grid was rotated 90° and a second tilt series was acquired. In total, about 40 dual-axis tomograms were reconstructed to examine the 3-D fine structure of T cells, CH27 cells and conjugates frozen at the different times of conjugation.

Tomographic Reconstruction and Image Analysis

Dual-axis tomographic reconstruction was carried out with the IMOD software package as described previously (25-27). Briefly, the tilted views were aligned using the positions of the colloidal gold particles, and tomograms were calculated using an R-weighted back projection algorithm. The two tomograms were then aligned to each other and combined. Finally, dual-axis tomograms from serial sections were aligned and combined using the methods previously described (43, 44). A surface mesh was generated to fit the modeled contours. MTs were tracked and modeled, and a projection of the model was displayed to study the relationships of these organelles in 3-D.