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MAL Protein Controls Protein Sorting at the Supramolecular Activation Cluster of Human T Lymphocytes

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T cell membrane receptors and signaling molecules assemble at the immunological synapse (IS) in a supramolecular activation cluster (SMAC), organized into two differentiated subdomains: the central SMAC (cSMAC), with the TCR, Lck, and linker for activation of T cells (LAT), and the peripheral SMAC (pSMAC), with adhesion molecules. The mechanism of protein sorting to the SMAC subdomains is still unknown. MAL forms part of the machinery for protein targeting to the plasma membrane by specialized mechanisms involving condensed membranes or rafts. In this article, we report our investigation of the dynamics of MAL during the formation of the IS and its role in SMAC assembly in the Jurkat T cell line and human primary T cells. We observed that under normal conditions, a pool of MAL rapidly accumulates at the cSMAC, where it colocalized with condensed membranes, as visualized with the membrane fluorescent probe Laurdan. Mislocalization of MAL to the pSMAC greatly reduced membrane condensation at the cSMAC and redistributed machinery involved in docking microtubules or transport vesicles from the cSMAC to the pSMAC. As a consequence of these alterations, the raft-associated molecules Lck and LAT, but not the TCR, were missorted to the pSMAC. MAL, therefore, regulates membrane order and the distribution of microtubule and transport vesicle docking machinery at the IS and, by doing so, ensures correct protein sorting of Lck and LAT to the cSMAC. The Journal of Immunology, 2011, 186: 000-000.

In response to appropriate Ags presented by an APC, T cells polarize, forming a surface subdomain at the T cell–APC interface known as the immunological synapse (IS) (1, 2). Membrane receptors and signaling molecules are not randomly distributed at the IS but, instead, assemble into a well-defined structure known as the supramolecular activation cluster (SMAC), which is subcompartmented into two differentiated concentric zones, termed the central SMAC (cSMAC) and peripheral SMAC (pSMAC) (3). The cSMAC contains the T cell Ag receptor/CD3 complex (TCR), Lck, and the linker for activation of T cells (LAT) adapter. pSMAC contains adhesion molecules, such as LFA-1 and ICAM-1, and cytoskeletal proteins, such as actin and talin. The actin cytoskeleton and intracellular protein trafficking are important for SMAC assembly (4, 5). The precise mechanism by which the cSMAC and pSMAC are formed is still unknown, but is believed to depend on signals generated by the TCR. Although its exact role remains a mystery, the IS is thought to be a focal point for endocytosis, exocytosis (6), and signaling (7).

One of the mechanisms proposed for compartmentation in biological membranes is the clustering of specific lipids, such as cholesterol and sphingolipids, into highly condensed domains. The use of the membrane fluorescent probe Laurdan has allowed visualization of cholesterol-dependent membrane domains with different degrees of condensation in living cells (8, 9). Condensed membranes are probably equivalent to liquid-ordered membrane assemblies of glycolipid and cholesterol, also referred to as membrane rafts, which play a role in the recruitment of specific proteins for membrane trafficking or signaling events and in the formation of membrane compartments such as caveolae (10, 11). Paralleling the elucidation of the SMAC architecture, membrane rafts emerged as putative functional platforms for the assembly of signaling machinery around the TCR at the IS. However, although raft lipids translocate to the IS (12), the IS contains condensed membranes (13), and TCR activation domains accumulate raft lipids (14), protein–protein interactions seem to be sufficient for the assembly of the signaling machinery (15). Therefore, the role of raft membranes at the IS remains unknown.

MAL is a highly hydrophobic integral membrane protein of 17 kDa consisting of four hydrophobic segments separated by short hydrophilic loops. MAL is expressed in normal human T lymphocytes and polarized epithelia as well as in representative cell lines, such as human Jurkat T cells and epithelial Madin-Darby canine kidney cells, respectively. A highly peculiar feature of MAL is its selective partitioning into detergent-resistant membrane (DRM) fractions that are enriched in compact membranes (16, 17). MAL silencing in Madin-Darby canine kidney cells severely impairs the targeting of influenza virus hemagglutinin (HA) to the apical surface and produces its intracellular retention and missorting to the basolateral surface (17, 18). Similarly, MAL silencing in human T cells blocks transport of Lck to the cell surface and induces its intracellular accumulation (19). Remarkably, the
effect on HA and Lck targeting strictly correlates with the exclusion of both cargo molecules from DRM, MAL clustering has been shown to create specifically large, condensed membrane domains in the plasma membrane (20). MAL is, therefore, considered a component of the machinery for organization and function of condensed membranes in epithelial cells and human T lymphocytes (17, 20).

Although the requirement of MAL for Lck transport in resting T cells is established, its possible role at the IS has not been explored. In this study, we examined the dynamics of MAL during the formation of the IS and observed that a pool of MAL rapidly redistributed to the cSMAC. Remarkably, the expression of a modified MAL protein mistargeted to the pSMAC causes mislocalization of the raft-associated Lck and LAT molecules to the pSMAC. The missorting of MAL, Lck, and LAT was accompanied by a redistribution of condensed membranes and docking machinery for transport vesicle (syntaxin-4 and SNAP-23) or microtubules (EB1 and adenomatous polyposis coli protein) from the cSMAC to the pSMAC. Moreover, ectopically expressed HA, which is normally targeted to the cSMAC, was misrouted to the pSMAC under those conditions, reflecting the profound alternations in protein sorting at the SMAC primarily caused by MAL mislocalization. In summary, our results strongly argue for a role of MAL in the organization of the IS by targeting condensed membranes, docking machinery for transport vesicles and microtubules, and proteins specific to the cSMAC.

Materials and Methods

Materials

The mAb 6D9 to human MAL was previously described (16). The rabbit Abs to CD3 and ZAP70 and the mAbs to ICAM-3 and CD45 were kindly provided by Dr. Alarcon (Centro de Biologia Molecular “Severo Ochoa”, Madrid, Spain) and Dr. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). The rabbit polyclonal Ab to Lck and mAb MEM-43/5 to CD59 were kindly provided by Dr. Veillette (McGill University, Montreal, Canada) and Dr. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic), respectively. The Abs to phospholipase C-γ, protein kinase C (PKC)-γ1, EB1, syntaxin-4, and phosphorytosome PY20 were from Transduction Labs (Lexington, KY); the mouse hybridoma producing anti-myc mAb 9E10 was purchased from the American Type Culture Collection; the Abs to total or phosphorylated Erk were from Promega (Madison, WI); the Abs to LAT were purchased from Upstate Biotechnology (Lake Placid, NY); the Abs to SNAP-23 and adenomatous polyposis coli protein we obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG Ab M2 and anti-α-tubulin mAb DM1A were from Sigma-Aldrich (St. Louis, MO). We obtained staphylococcal enterotoxin E (SEE) and B (SEB) superantigens from Toxin Technology (Sarasota, FL). HRP-conjugated secondary Abs were obtained from Pierce. Cell Tracker orange-fluorescent tetramethylrhodamine, secondary goat Abs coupled to Alexa 488, Alexa 594, or Alexa-647, and TRITC-phallolidin were purchased from Molecular Probes (Eugene, OR).

Cell-culture conditions and conjugate-formation procedure

Human T lymphoblastoid Jurkat cells were grown in RPMI 1640 supplemented with 5% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_{2}/95% air. To distinguish human T lymphoblastoid Jurkat cells were grown in RPMI 1640 supplemented with 5% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_{2}/95% air. To distinguish between human and human T lymphoblastoid Jurkat cells (3.0 × 10^6 cells/ml) were lysed for 15 min in 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 0.2% Triton X-100 at 4˚C in the presence of 200 μg/ml HA peptide 307–319 (PKHYKQNTLELAT), a control inactive peptide (PKKYKQNTLELAT) or 4 μg/ml SEB. For conjugation of primary T cells, freshly isolated T lymphocytes from healthy donors were incubated with SEB-pulsed Raji cells and processed as described for the Jurkat-APC conjugates. The experiments with human cells were done following the guidelines of the Bioethics Committee of the Spanish Research Council and were approved by the institutional Management Committee of the Centro de Biología Molecular Severo Ochoa.

DNA constructs and transfection conditions

The MAL-FLAG DNA construct, encoding MAL with its final extracellular loop modified by insertion of the 13-aa sequence DYKDDDKAANLT, which contains the FLAG epitope (DYKD), was described previously (21). The DNA constructs expressing intact MAL or MAL-FLAG molecules with N-terminal myc, GFP or cherry tags were generated by standard techniques, as were the constructs expressing GFP or cherry appended to the C terminus of Lck or LAT (Lck-GFP, Lck-cherry). The pSuperMal/GFP construct expressing GFP and the shRNA targeted to MAL mRNA from the same plasmid were described previously (19). To interfere with MAL expression and simultaneously express GFP fusions of Lck or LAT, the coding sequences of these proteins were inserted in-frame with the GFP coding sequence in the pSuperMal/GFP DNA construct. The plasmid expressing the transfected HA protein fused to GFP (22) was a kind gift from Dr. Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). The Lck10-GFP DNA construct (23) was generously provided by Dr. Rodgers (Oklahoma Medical Research Foundation). Jurkat cells were transfected by electroporation using the Gene Pulser system (Bio-Rad, Hercules, CA). For transfection of human primary T cells, we used Amasca nucleofector equipment (Lonza).

Immunofluorescence analysis

Cells were fixed in formalin for 20 min, rinsed, and treated with 10 mM glycine in PBS for 5 min to quench the aldehyde groups. Cells were then washed, permeabilized or not with 0.2% Triton X-100 in PBS at 4˚C for 5 min, rinsed, incubated with 3% (w/v) BSA for 15 min, and incubated with the primary Ab. After 1 h at room temperature, cells were washed and incubated with the appropriate fluorescent secondary Ab. For double-labeling experiments, the same procedure was repeated for the second primary Ab. Controls to assess the specificity of the labeling included incubations with control primary Abs and omission of the primary Abs. Immunofluorescence images were obtained using a Meta LSM 510 Confocal Laser microscope (Carl Zeiss, Oberkochen, Germany). For single-color time-lapse confocal fluorescence microscopy, Jurkat cells expressing stably myc-tagged MAL or MAL-FLAG proteins were transfected with GFP fusions of Lck, LAT, or EB3. For dual-color time-lapse microscopy, normal Jurkat cells stably expressing GFP-MAL or GFP-MAL-FLAG were used for the expression of cherry fusions of Lck or LAT. Images were captured using a Zeiss LSM 510 confocal microscope equipped with a 63× objective (1.2 numerical aperture [NA] water objective or 1.4 NA oil immersion objective for live or fixed cells, respectively; Carl Zeiss) and transferred to a computer workstation running MetaMorph imaging software (Molecular Devices, Downington, PA). Three-dimensional reconstruction and colocalization images representing only the pixels in which the staining of the two analyzed proteins coincided were done using the Image J program (National Institutes of Health; http://rsb.info.nih.gov/ij/). The level of colocalization was represented by a pseudocolored scale (0, no colocalization; +1, maximal colocalization). For deconvolution, we used the Huygens 3.0 program (Scientific Volume Imaging, Hilversum, The Netherlands).

Laudaran staining

Live cells were labeled with the Laudaran fluorescent probe (5 μM), the microscope was calibrated, and two-photon microscopy was performed as described elsewhere (8) using an LSM 710 NLO Multiphoton coupled to an AxioObserver inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY) with a 63× water objective and NA 1.3. In brief, Laurdan was excited at 800 nm, and emission intensities were recorded simultaneously in the 400–460 nm and 470–530 nm ranges. Intensity images were converted into a generalized polarization (GP) index defined as

\[ GP = \frac{I_{400} - I_{530}}{I_{400} + I_{530}}, \]

in which I is the emission intensity, as previously described (8). GP values range from 0 (fluid domains) to +1 (highly ordered domains); membranes with GP values >0.3 were considered to be ordered membrane domains. The GP distributions and mean GP values obtained from GP images were normalized and represented using the GraphPad Prism program (GraphPad, San Diego, CA).

Detergent extraction procedures, immunoblot, and immunoprecipitation analyses

Jurkat cells (5.0 × 10^7 cells) were lysed for 15 min in 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 0.2% Triton X-100 at 4˚C in the presence of...
phosphatase and protease inhibitors. The extract was brought to 40% sucrose (w/w) and placed at the bottom of two sequential layers of 30% and 5% sucrose. Gradients were centrifuged to equilibrium, and the soluble and low-density insoluble fractions were harvested (24). Equivalent aliquots from the soluble and insoluble fractions were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). For immunoprecipitation studies, cells were lysed in 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and a mixture of phosphatase and protease inhibitors. The lysates were precleared for 1 h at 4°C with protein G-Sepharose, centrifuged, and the supernatant incubated with the indicated specific Abs for 1 h at 4°C. After incubation with protein G-Sepharose for 1 h, the immunoprecipitates were collected by centrifugation, washed six times, and analyzed by immunoblotting with the appropriate Abs.

**Statistical analysis**

Data are expressed as mean ± SEM. A paired Student t test was used to establish the statistical significance of differences between the means.

**Results**

**MAL translocates to the cSMAC**

In resting Jurkat T cells, MAL distributes in distinct pools at different locations (Fig. 1A, Supplemental Fig. 1A). One such pool is present at the plasma membrane. A second pool is found in pericentriolar endosomes, where MAL shows a high level of colocalization with the recycling endosome markers Rab11 and internalized transferrin. The colocalization of MAL with EEA1 and Rab5, which label early endosomes, or CD63, a late endosome marker, was much lower. Finally, a third pool decorates part of the radial microtubule cytoskeleton. A quantitative analysis of the colocalizations is shown in Supplemental Fig. 1B. To analyze...
dynamically the response of MAL to T cell stimulation, we used Jurkat J77 vβ8+ cells stably expressing GFP-MAL as T cells and the Raji B cell line loaded with SEE as an APC (25). Time-lapse videomicroscopy revealed that soon after the T cell encountered an APC, a pool of MAL rapidly concentrated at the IS (Fig. 1B, Supplemental Video 1). A detailed analysis of the initial events during formation of the conjugates revealed that MAL rapidly distributed in the T cell lamellipodia embracing the APC and, a few seconds later, concentrated at the center of the contacting membranes. Almost simultaneously, the internal MAL pool reoriented, probably with the centrosome, to face the IS. In addition, numerous MAL-positive vesicles were visualized trafficking bidirectionally between the centrosomal region and the plasma membrane along tracks made of microtubules (Fig. 1B, 1C). We reconstructed three-dimensional images and rotated them to view the IS from the side, which allowed the MAL pool present at the contact membrane to be distinguished from that at the pericentriolar region, which is very close to the T cell–APC interface (Fig. 1D). The number of conjugates with MAL at the IS increased and had kinetics similar to that of TCR, with >80% of conjugates with MAL concentrated at the IS after 15 min of conjugation (Fig. 1E). Quantitative analysis showed an ~2.8-fold enrichment of MAL and an ~3.7-fold of TCR at the IS, whereas CD45 was evenly distributed at the plasma membrane (Fig. 1F). The accumulation of MAL at the IS occurred preferentially at the cSMAC, as revealed by double-label immunofluorescence analysis of MAL.
and TCR or F-actin, which were used as markers of the cSMAC or pSMAC, respectively (Fig. 1G). Therefore, to summarize Fig. 1, a pool of MAL translocates rapidly to the T cell–APC interface and localizes at the cSMAC in the mature IS, and a second pool of MAL reorients to the IS, accompanying the movement of the centrosome.

MAL targeting to the cSMAC requires integrity of its last extracellular loop

Our anti-MAL Ab (6D9 mAb, Fig. 2A), which was made to the last hydrophilic loop of the MAL molecule, is not of use in immunofluorescence analysis. Because the proximity of the internal pool to the contact membrane often obscures the detection of the pool of MAL at the IS in permeabilized cells using cytosolically oriented tags, we expressed a modified MAL molecule (MAL-FLAG), which, in addition to the cytoplasmic myc tag present at the amino terminal end, bears a FLAG epitope in its last hydrophilic loop to allow staining in unpermeabilized cells (Fig. 2A). That loop was demonstrated previously to be extracellular (21). We generated stable transfectants expressing MAL-FLAG in Jurkat J77 cells (Jurkat MAL-FLAG) to prevent massive overexpression and compared these cells with transfectants expressing the MAL molecule with only the myc tag (Jurkat MAL) and with control, parental Jurkat J77 cells. Curiously, whereas Jurkat-MAL cells downmodulated the expression of endogenous MAL to compensate for the expression of exogenous MAL, this was not true in MAL-FLAG cells, resulting in the expression of approximately twice as much total MAL relative to parental cells (Fig. 2B).

The subcellular distribution of exogenous MAL (Supplemental Fig. 2A), the ability of exogenous MAL or MAL-FLAG to replace the endogenous MAL protein in the processes of Lck transport to the plasma membrane (Supplemental Fig. 2B), targeting of TCR to the IS (Supplemental Fig. 2C, 2D) and activation of signaling pathways in response to TCR triggering (Supplemental Fig. 2E–H), and the internalization of TCR (Supplemental Fig. 2f) were all mostly similar in the MAL and MAL-FLAG Jurkat cell transfectants, although a delay in ERK activation was observed in MAL-FLAG cells. Importantly, MAL-FLAG mostly localized to the pSMAC, as revealed by double-label immunofluorescence analysis with Abs to the FLAG epitope and TCR, LFA-1, ICAM-3, or F-actin staining in unpermeabilized cells (Fig. 2C, 2D). This result contrasts with the cSMAC distribution of MAL observed in Jurkat MAL cells (Fig. 1G). The missorting of MAL-FLAG to the pSMAC was confirmed in the T cell–APC conjugates formed using a second stable Jurkat MAL-FLAG cell clone (Fig. 2D).

Time-lapse videomicroscopy of conjugates of cells stably expressing GFP-tagged MAL-FLAG revealed that, similar to MAL, MAL-FLAG was detected at the T cell lamellipodium surrounding the APC, at the centrosome region, and in trafficking vesicles (Fig. 2E, Supplemental Video 2). However, unlike MAL, which was evenly distributed along the edge of the embracing

FIGURE 3. MAL distribution controls sorting of Lck and LAT at the SMAC. A, Jurkat MAL or Jurkat MAL-FLAG cells were conjugated to SEE-loaded APCs for 15 min. Cells were fixed, permeabilized, and stained for MAL and Lck (left panels) or LAT (right panels). Arrows point to sites of accumulation of the indicated proteins at the IS. B, Conjugates formed by Jurkat MAL-FLAG cells were stained for F-actin and ZAP-70 or PKC-δ. C, Quantitative analysis of the percentage of Jurkat MAL or Jurkat MAL-FLAG cells with distribution of the indicated molecules to the pSMAC. D, Human primary T cells transiently expressing MAL or MAL-FLAG were conjugated to SEE-loaded APCs for 15 min. Cells were fixed, permeabilized, and analyzed for the distribution of Lck (left panels) or LAT (right panels). The small panels show a magnification of the contact area (boxed regions). The single-color images and the colocalization images, which represent only the pixels in which the staining of the two proteins coincides, corresponding to the magnifications were pseudocolored using the color scales indicated in each case. Arrows point to sites of accumulation of the indicated proteins at the IS. The histogram represents the percentage of cells with Lck or LAT at the pSMAC in the cells expressing MAL or MAL-FLAG. Mean values ± SEM of three (C) or two (D) independent experiments are represented. Scale bars, 5 μm. *p < 0.05, **p < 0.001.
lamellipodium, MAL-FLAG accumulated at the lamellipodium sides and remained there for a long time. The results presented in Figs. 1 and 2 show that a pool of MAL is targeted to cSMAC soon after contact of the T cell with the APC and that this targeting requires the integrity of the last extracellular loop of MAL, because its disruption caused missorting of MAL to the pSMAC.

**MAL distribution controls sorting of Lck and LAT to specific SMAC subdomains**

The observation that MAL-FLAG was missorted to the pSMAC prompted us to investigate whether the targeting of other SMAC proteins was affected. Although the distribution of TCR, LFA-1, or F-actin was unaltered, it is of note that both Lck and LAT were mistrargeted to the pSMAC in Jurkat MAL-FLAG cells (Fig. 3A, 3C). ZAP70 and PKC-θ maintained their correct targeting to the cSMAC regardless of the distribution of exogenous MAL (Fig. 3B, 3C). The missorting of Lck and LAT induced by the presence of exogenous MAL at the pSMAC was corroborated by analyzing the distribution of transiently expressed GFP fusions of both molecules (Supplemental Fig. 3A). It is apparent that the targeting of MAL to the pSMAC or its effect on Lck and LAT mislocalization was not restricted to the use of the superantigen SEE, given that similar results were observed in T cell–APC conjugates formed with Jurkat CH7C17 cells (26) in the presence of SEB or the peptide-encompassing aa 307–319 of the HA molecule (Supplemental Fig. 3B, 3C). Finally, similar to the case of Jurkat MAL-FLAG cells, expression of MAL-FLAG in primary T lymphocytes resulted in mislocalization of Lck and LAT but not TCR to the IS periphery (Fig. 3D). In summary, the missorting of exogenous MAL to the pSMAC was accompanied by that of the raft-associated molecules Lck and LAT.

MAL is required for transport of Lck to the plasma membrane of resting T lymphocytes (19). Therefore, it is plausible that this could also be the case for Lck transport to the cSMAC in T cell–APC conjugates. To examine this hypothesis, we monitored the movement of MAL and Lck during IS formation by time-lapse videomicroscopy in Jurkat MAL cells and compared it with that in Jurkat MAL-FLAG cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D). MAL and Lck were found to travel in the same or closely related transport carriers destined for the cSMAC in Jurkat MAL cells (Fig. 4A–D).

**FIGURE 4.** Effect of MAL distribution on Lck dynamics during IS formation. Jurkat MAL (A) or Jurkat MAL-FLAG (B) cells were transfected with a DNA construct encoding Lck-cherry. After 16 h, cells were conjugated to SEE-pulsed APCs and subjected to time-lapse videomicroscopy. The processes occurring within the boxed region in the differential contrast images (bottom panels) are shown at higher magnification in the top panels. The single-color images and the images representing only the pixels in which the staining of the two proteins coincides (Col.) were pseudocolored using the color scales indicated in each case. Arrows point to sites of accumulation of the indicated proteins at the IS. Arrowheads indicate vesicles that emerge from the Golgi region and travel to the IS. Numbers indicate time in seconds. C and D, The schematics on the left represent three stages (I–III) of IS formation defined by the progressive accumulation of MAL at the IS and the proximity of the centrosome to the contact zone. Graphs represent the fluorescence intensity of MAL (C) or MAL-FLAG (D) and Lck at the contact site at the indicated stages of IS formation obtained by analysis of Supplemental Videos 3 and 4, respectively. The arrows and the asterisk point to the periphery and the central part of the IS, respectively. Details of vesicles (arrowheads) transporting Lck to IS in Jurkat MAL (E) or Jurkat MAL-FLAG cells (F). Arrows point to sites of accumulation of Lck at the IS. Arrowheads indicate vesicles/tubules that transport Lck to the IS. Scale bars, 5 μm.
MAL accumulated at peripheral parts of the IS (stage II) and were excluded from the central part (stage III) (Fig. 4D). We visualized tubular connections between the centrosomal region and the IS that served as tracks for transport of Lck (Fig. 4E, 4F, Supplementary Video 5). It is of note that the connections ended at the central part of the IS in Jurkat MAL cells (Fig. 4E) or at its periphery in Jurkat MAL-FLAG cells (Fig. 4F). The results in Fig. 4 therefore indicate that MAL targets Lck directly to the cSMAC in Jurkat MAL cells or to the pSMAC in Jurkat MAL-FLAG cells and that this differential targeting takes place by vesicles and tubules that follow linear tracks connecting the centrosomal region with the cSMAC or pSMAC, respectively.

Although LAT colocalized extensively with MAL in pericentriolar endosomes (Supplemental Fig. 4), no colocalization of LAT with MAL-positive transport vesicles was found in Jurkat MAL or Jurkat MAL-FLAG cells forming an IS (Fig. 5A, 5B, Supplementary Fig. 4, Supplementary Videos 6, 7). Apparently, the initial accumulation of LAT to the IS does not involve vesicular transport from the centrosomal region in any of the two types of Jurkat cell, but, rather, LAT appears to redistribute mostly from a surface pool. Consistent with the lack of a role for MAL in targeting LAT to the cell surface, LAT transport to the plasma membrane of resting T cells occurred equally well regardless of MAL expression (Supplemental Fig. 5A) and took place in vesicular structures that were mostly devoid of MAL (Supplemental Fig. 5B). When we analyzed the redistribution of LAT to the IS in detail (Fig. 5C, 5D), we observed that initially LAT accumulated preferentially at the sides of the contact with the APC in Jurkat MAL and Jurkat MAL-FLAG cells (stage II). Later on, LAT disappeared from the IS sides and accumulated at the central part in Jurkat MAL cells (Fig. 5C, stage III), whereas in Jurkat MAL-FLAG cells, the levels of LAT at the IS sides increased continuously, with no evidence of redistribution to the central part (Fig. 5D, stage III). The results shown in Fig. 5 indicate that LAT follows a mechanism different from that of Lck for IS targeting, given that LAT appears to redistribute from a surface pool, but, similar to Lck, the accumulation of MAL at the cSMAC or pSMAC dictates the final location for concentration of LAT.

Mistargeting of MAL to the pSMAC causes reduced membrane condensation at the cSMAC and missorting of raft markers to the pSMAC

The N-terminal 10-aa sequence of Lck is sufficient to confer myristoylation and double palmitoylation on a GFP chimera (Lck10-GFP) and to incorporate it into DRMs (23, 27). Lck10-GFP has been used as a probe to monitor the distribution of specific compact membranes in T cells (23). Similar to Lck and LAT, Lck10-GFP accumulated at the cSMAC or pSMAC depending on the place of exogenous MAL accumulation (Fig. 6A, 6B). The distribution of ganglioside GM1, a raft marker normally

**FIGURE 5.** Effect of MAL distribution on LAT dynamics during IS formation. Jurkat MAL cells (A) or Jurkat MAL-FLAG cells (B) expressing LAT-cherry for 16 h were conjugated to SEE-pulsed APCs and subjected to time-lapse videomicroscopy. The processes occurring within the boxed region in the differential contrast images (bottom panels) are shown at higher magnification in the top panels. The single-color images and the images representing only the pixels in which the staining of the two proteins coincides (Col.) were pseudocolored using the color scales indicated in each case. Arrows point to sites of accumulation of the indicated proteins at the IS. Arrowheads indicate vesicles that emerge from the Golgi region and travel to IS. Numbers indicate time in seconds. Graphs representing the fluorescence intensity of LAT and MAL (C) or MAL-FLAG (D) staining at the contact site at the indicated stages of IS formation obtained by analysis of Supplemental Videos 6 and 7, respectively. The arrows and the asterisk point to the periphery and the central part of the IS, respectively. Scale bars, 5 μm.
moderately enriched at the cSMAC, showed the same dependence on exogenous MAL as Lck10-GFP did (Fig. 6B, 6C). The packing density of biological membranes can be directly measured using the Laurdan fluorescent membrane dye (8). This does not preferentially partition into a particular type of membrane, but its fluorescence emission spectrum shifts depending on the degree of condensation and order of its membrane environment, making it possible to distinguish densely packed, ordered membranes from those with a loosely packed, disordered structure. This experimental approach has also been used to visualize the distribution of ordered domains of various cell systems, including T cells (9, 28).

In Jurkat MAL cells, highly condensed domains accumulated along the entire contact zone, whereas in Jurkat MAL-FLAG cells, condensed domains were restricted to the IS periphery (Fig. 6D). This difference is better visualized in the en face view of the IS (Fig. 6E, left panels), where it can be clearly seen that MAL-FLAG caused depletion of highly condensed domains at the central zone of the IS, augmented membrane order (Fig. 6E, right panels), and increased the percentage of ordered membranes at the IS periphery (Fig. 6F). The loss of highly condensed domains from the cSMAC was confirmed in conjugates formed by primary human T lymphocytes expressing MAL-FLAG (Fig. 6G).
fore, the results in Fig. 6 indicate that misplacement of exogenous MAL to the pSMAC produces parallel missorting of raft markers and highly condensed membrane domains.

**MAL distribution controls correct targeting of machinery involved in docking of microtubules or transport vesicles at the SMAC**

We previously showed that MAL travels to the IS along linear tracks made of microtubules that connect the centrosome with the cSMAC (Fig. 1B, 1C). As transport vesicles and microtubules are docked at the plasma membrane using specific machineries, we decided to investigate the distribution of elements of such machineries in T cell–APC conjugates formed by Jurkat MAL and Jurkat MAL-FLAG cells. The appropriate combination of SNARES on vesicle and target membranes (t-SNARES) determines the docking of the transport vesicles at specific sites in cellular membranes and their subsequent fusion (29). Exocytosis in nonneuronal cells may involve two plasma membrane t-SNARES, syntaxin-3 or -4, and SNAP-23 (30). Consistent with previous work reporting syntaxin-4 and SNAP-23 localization (4), both proteins distributed along the entire IS in Jurkat MAL cells, but, in contrast, they preferentially accumulated at the IS periphery in Jurkat MAL-FLAG cells (Fig. 7A–C). Consistent with this alteration, most of SNAP-23 and a fraction of syntaxin-4 were found in compact membranes (Fig. 7D). Microtubule docking to the cell cortex involves interactions between specific proteins at

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**FIGURE 7.** MAL distribution controls sorting to SMAC subdomains of protein machinery involved in the docking of transport carriers or microtubules to the plasma membrane. A–C, Jurkat MAL or Jurkat MAL-FLAG cells were conjugated with SEE-loaded APCs for 15 min. Cells were fixed, permeabilized, and stained with Abs to syntaxin-4 (STX4) (A) or SNAP-23 (B). The histogram shows the percentage of cells with STX4 or SNAP-23 enriched at the IS periphery (C). D, The soluble (S) fraction and the DRM fractions (I) from control Jurkat, Jurkat MAL, or Jurkat MAL-FLAG cells were immunoblotted with Abs to STX4 or SNAP-23. Transferrin receptor (TfR) and CD59 were used as markers of the S and I fractions, respectively. E, Jurkat MAL or Jurkat MAL-FLAG cells were conjugated to SEE-loaded APCs for 15 min. Cells were fixed, permeabilized, and stained with Abs to EB1 or adenomatous polyposis coli (apc) protein. The images resulting from the reconstruction of the confocal stacks obtained for EB1 staining are shown (top panels). The reconstructed images of EB1 and apc staining were used to obtain 0.4-μm-wide sections at the cell-to-cell contact, as shown in the schematics in the bottom panels. These sections were viewed from the side (0°) or rotated through 90°. The single-color images are shown in gray. The images showing only the pixels in which the staining of the two proteins coincides (Col.) were pseudocolored using the indicated color scale. An enlargement of the boxed region is also shown. Concentric rings were drawn to facilitate visualization of the place on the IS where the colocalization dots were concentrated. F, The histogram represents the percentage of pixels showing colocalization of EB1 and adenomatous polyposis coli protein at the pSMAC and the cSMAC. G, EB3-GFP was expressed in Jurkat MAL or Jurkat MAL-FLAG cells for 16 h. Cells were then conjugated with SEE-loaded APC and subjected to time-lapse videomicroscopy in a confocal microscope. The images show the reconstruction of 31 frames (top panels). The boxed region was enlarged and pseudocolored using the indicated scale (bottom panels). The schematics show the regions with a high (HD) or low density (LD) of EB3-labeled tubules traveling to the contact with the B cell. H, The histogram represents the ratio of EB3 intensity at the pSMAC versus that at the cSMAC. I, HA-GFP was expressed in Jurkat MAL or Jurkat MAL-FLAG cells for 16 h. Cells were then conjugated with SEE-loaded Raji cells for 15 min and fixed. The arrows indicate sites of HA accumulation at the IS. The histogram represents the percentage of cells with HA targeted to the cSMAC or pSMAC. Means ± SEM of three (C) or two (F, H, I) independent experiments are represented. Scale bars, 5 μm. *p < 0.05, **p < 0.01.
the growing end of microtubules, such as proteins EB1 and EB3, and proteins attached to the subcortical cytoskeleton, such as the adenomatous polyposis coli protein (31, 32). By obtaining three-dimensional reconstruction images, we observed that the colocalization between EB1 and the adenomatous polyposis coli protein was greatest at the central region or at a peripheral ring of the IS, depending on the place of accumulation of exogenous MAL (Fig. 7E, 7F). Moreover, consistent with this finding, we found a similar dependence for the place of concentration of newly formed microtubules, as monitored with EB3-GFP (Fig. 7G, 7H). Finaly, we examined whether the abnormal arrangement of condensed membranes and docking machinery for vesicles and microtubules at the IS caused by missorting of exogenous MAL to the pSMAC also affects the targeting of an ectopic protein. We observed that the transmembrane HA protein, a prototypical raft-associated cargo molecule distributed preferentially to the cSMAC or to the pSMAC, depending on the place of exogenous MAL accumulation (Fig. 7F). In conclusion, the results in Fig. 7 indicate that MAL regulates the distribution of machinery involved in docking transport vesicles and microtubules and targeting proteins to specific regions of the IS (Fig. 8).

Discussion
The assembly of IS requires the translocation of specific molecules to the contact zone of the T cell with an APC and the effective sorting of these molecules in distinct SMAC subdomains. The precise mechanism by which protein sorting to the cSMAC and pSMAC takes place is unknown. In the absence of MAL expression, Lck is retained intracellularly and cannot be targeted to the IS, and, as a consequence, TCR, LAT, and other signaling molecules are not translocated either (19). This early effect on IS formation precluded the use of MAL knockdown for investigating the possible direct role of MAL in SMAC assembly. In this study, we show that MAL translocates rapidly to the IS and concentrates at the cSMAC. Later on, a pericentrosomal pool of MAL moves to the IS, accompanying microtubule-organizing center reorientation, as was previously observed for Lck (33). We focused our investigation on the initial recruitment of MAL to the IS and its role in protein sorting to the IS. By using a modified MAL molecule that is missorted to the pSMAC, we observed that correct MAL distribution at the IS is crucial for correct targeting of Lck and LAT, but not TCR, to the cSMAC. Missorting of MAL, in addition to mislocalizing Lck and LAT to the pSMAC, reduced membrane condensation at the cSMAC, misplaced raft markers to the pSMAC, and provoked a profound change in the distribution of machinery for docking transport vesicles and microtubules at the IS. Therefore, MAL plays a role at IS by organizing condensed membranes and controlling the targeting of proteins specifically to the cSMAC.

Size exclusion and membrane-bending effects have long been considered as possible contributors to protein sorting within the SMAC (34). Large ectodomains in transmembrane proteins can prevent these proteins localizing in the cSMAC where the close proximity of the plasma membrane, the T cell, and the APC is needed for the TCR to recognize the Ag presented by the MHC molecule (35). The clustering state of the molecules can also affect their sorting within the SMAC, as observed for LFA-1 (36). Membrane rafts are postulated as being specialized platforms for the specific compartmentation of receptors and signaling molecules in all types of cell (37). The possible involvement of raft membranes in assembling the signaling machinery in T cells (11, 38–41) was challenged by single-molecule microscopic studies, indicating that protein–protein interactions are sufficient to explain the assembly (15). This observation, however, does not allow us to rule out the possibility that, in keeping with the existence of condensed domains and raft lipids at the contact zone (13, 14), raft membranes are the milieu where at least part of the signaling machinery is normally assembled at the IS. If this were the case, raft membranes at the IS could help to regulate the spatial organization of the SMAC by segregating specific molecules to different SMAC subdomains.

Using the Laurdan technique, we observed that MAL localization regulates the distribution of condensed membranes within the SMAC in Jurkat cells and primary T lymphocytes. Highly ordered membranes were distributed along the IS in cells expressing MAL at the cSMAC. However, in cells in which MAL was missorted to the pSMAC, membrane order, as measured by GP value, was diminished at the cSMAC and increased at the pSMAC. The missorting of ordered membranes to the pSMAC was corroborated by the parallel misdistribution of raft markers, such as ganglioside GM1 and Lck10-GFP. Despite the changes in the distribution of condensed membranes at the IS, signaling occurred efficiently in response to TCR triggering in both types of Jurkat cell. This result is consistent with previous observations showing that most of the signaling occurs in dynamic microclusters well before SMAC assembly takes place (42–44) and that microclusters form independently of raft clustering (45). The delay in ERK activation observed in the cells with MAL missorted to the pSMAC might indicate a modest contribution of the SMAC to the
signaling process. It is of particular note that the abnormal distribution of highly ordered membranes at the IS arising from the presence of MAL at the pSMAC caused missorting of Lck and LAT to the pSMAC. Therefore, the distribution of highly ordered membranes at the cSMAC and that of the raft-associated proteins Lck and LAT are strictly dependent on MAL targeting to the cSMAC. The proposed role of MAL as machinery for membrane order organization at the IS is supported by the observation that MAL clustering creates specifically large, condensed membrane domains in the plasma membrane (20).

Lateral diffusion, cytoskeleton-mediated movement, and intracellular trafficking are different mechanisms that serve to concentrate specific membrane proteins at the IS. Consistent with this diversity, we found remarkable differences in the targeting of Lck and LAT to the IS. LAT accumulation at the IS appears to take place mainly by translocation from a surface pool that concentrated initially at the IS sides. LAT then segregated to a central position or remained at the IS sides depending on the place where MAL and the condensed domains accumulated. This observation indicates that LAT targeting to the cSMAC occurs once it has arrived at the IS by selective retention into MAL-enriched membranes. This retention might be reminiscent of the MAL-dependent stabilization of specific apical proteins described in polarized epithelial cells (46, 47). Unlike LAT, Lck was transported in MAL-positive vesicles from pericentriolar endosomes to the place where MAL accumulates, regardless of whether it was the cSMAC or the pSMAC. Importantly, the MAL-positive vesicular carriers moved to the IS along microtubule tracks that connect the centrosome with the IS, although they were differentially docked at the central part or at the sides of the cell-to-cell contact, depending on the place of exogenous MAL accumulation. Supporting this observation, we found that EB1, a protein present at the growing end of microtubules, colocalized with adenomatous polyposis coli, a protein involved in microtubule docking to the cell cortex, preferentially at the center of IS or at a peripheral ring coinciding with the place of MAL concentration. Therefore, in contrast to lytic granule movement, which takes place by minus-end directed movement along long microtubules that curve after contacting the IS (48, 49), MAL-mediated vesicular transport of Lck to the IS relies on plus-end directed traffic along short microtubules.

A role for microtubule docking at the IS in SMAC architecture has been proposed on the basis of the effect of the disruption of the dynein–dynactin complex by the overexpression of p50 dynamin, a subunit of dynactin (50). Under those conditions, TCR disperses at the pSMAC instead of being clustered at the cSMAC, and LFA-1 becomes partially mistargeted to the cSMAC (50). Tumor suppressor gene 101, a component of the endosomal sorting complex required for transport, is essential for correct sorting of the TCR to the cSMAC. Unlike in control cells, where TCR and PKC-θ colocalize in peripheral microclusters but become segregated in the cSMAC (51), in tumor suppressor gene 101-knockdown cells, TCR and PKC-θ fail to segregate and remain together in large microclusters at the pSMAC (52). We have observed TCR concentration at the cSMAC regardless of the location of MAL accumulation at the IS, consistent with TCR following a pathway for targeting to the cSMAC that is distinct from that of Lck or LAT. Confirming this, in addition to controlling microtubule docking to the IS, MAL regulates the transport vesicle targeting site, we found that syntaxin-4 and SNAP-23, two t-SNARES involved in docking transport vesicles to the plasma membrane, distributed preferentially to cSMAC or pSMAC in a MAL localization-dependent manner. Therefore, in addition to transporting Lck directly to the IS, MAL appears to regulate membrane order at the IS in such a way that Lck, LAT, syntaxin-4, and SNAP-23 segregate into the ordered membranes organized by MAL (Fig. 8). Reflecting the profound alternations at the SMAC primarily caused by MAL mislocalization, the transmembrane HA protein, a prototypical raft-associated cargo protein, is also targeted to either SMAC subdomain depending on the pattern of MAL distribution.

In polarized epithelial cells, MAL mediates direct vesicular transport of cargo to the apical surface (17, 18) and stabilization of specific proteins at the apical membrane (46, 47). Our present results indicate that, in polarized T cells, MAL regulates membrane order at the IS and uses both direct vesicular transport and plasma membrane retention mechanisms to ensure correct sorting of Lck and LAT to the cSMAC.

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Disclosures
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References
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**Figure S1.** Analysis of the subcellular distribution of MAL. (A) Jurkat cells stably expressing myc-tagged MAL were subjected to double-label immunofluorescence analysis with anti-myc antibodies and antibodies to CD63, EEA1, Rab5 or Rab11, as indicated. A magnification of the boxed region is shown on the right. Single confocal planes are shown. (B) The histogram shows the Pearson’s coefficient of colocalization of MAL and the indicated molecules obtained from the analysis of different confocal stacks. Means ± SEM of three independent experiments are represented.

**Figure S2.** Analysis of the subcellular distribution and function of MAL-FLAG. (A) Jurkat MAL cells and two stable clones of Jurkat MAL-FLAG cells (MAL-FLAG 1 and MAL-FLAG 2) were fixed, permeabilized and stained with anti-myc antibodies. (B-C) Wild type Jurkat cells (WT), Jurkat MAL or Jurkat MAL-FLAG cells were transfected for 24 h with the pSuperhMAL/GFP DNA construct which co-expresses shRNA specific to MAL mRNA and GFP from different promoters. Cells were fixed, permeabilized, and stained for Lck (B) or conjugated to SEE-pulsed Raji B cells for 15 min and stained for TCR (C). The MAL shRNA was previously shown to be efficient in knocking down endogenous MAL (Anton et al., 2008). Expression of GFP allowed the identification of the transfected cell. The target sequence of MAL shRNA was modified in exogenous MAL mRNA and, therefore, expression of exogenous MAL resists MAL shRNA expression (Anton et al., 2008). (D) The histogram represents the percentage of cells with TCR polarized to IS in wild type Jurkat (WT), Jurkat MAL and Jurkat MAL-FLAG cells treated as in (C). (E) Jurkat MAL or Jurkat MAL-FLAG cells were conjugated to Raji B cells in the presence or absence of SEE for 15 min. Cell extracts were immunoprecipitated with anti-phosphotyrosine antibodies coupled to agarose beads and immunoblotted with antibodies to CD3ζ, ZAP-70 or PLC-γ1 to detect the levels of tyrosine phosphorylation of each protein. The initial extracts were also immunoblotted to show the total content of each protein. (F) Cells were activated with SEE-loaded Raji cells as in (E) and processed to detect the activated, phosphorylated (p) form or the total content of Erk. The histogram shows a quantitative analysis of the kinetics of ERK activation from three independent experiments. (G) Jurkat MAL or Jurkat MAL-FLAG cells were transfected for 24 h with plasmids co-expressing GFP and shRNA control or shRNA specific to the MAL mRNA molecule. The cells were
conjugated to Raji B cells in the presence or absence of SEE the levels of phosphorylated ERK (p-ERK) were analyzed in the GFP positive cell population by flow cytometry using anti-pERK rabbit antibodies and a fluorescent secondary anti-rabbit IgG antibody. The histogram shows the percentage of p-ERK ± SEM at 15 and 30 min after conjugation. The level of p-ERK at each time point in the cells expressing the control shRNA was taken as 100%. (H) Jurkat MAL or Jurkat MAL-FLAG cells were treated with activating anti-CD3 antibodies or PMA or conjugated to Raji B cells loaded or not with SEE, as indicated. After 16 h, CD69 expression was determined by flow cytometry. (I) Wild type Jurkat, Jurkat MAL or Jurkat MAL-FLAG cells were conjugated to Raji B cells loaded with SEE and stained at different times with fluorescent anti-CD3 antibodies to detect the TCR remaining on the cell surface. The histogram shows the percentage of TCR expressed on the cell surface at the times indicated. The expression level of the TCR in wild type Jurkat cells before conjugation was taken as 100%. Means ± SEM of three independent experiments are represented in (D, F, G, I); **, p < 0.01. Scale bars, 5 μm.

Figure S3. MAL distribution controls sorting of Lck and LAT at the SMAC in Jurkat cells activated with a peptide antigen or superantigen B. (A) Jurkat-MAL or Jurkat MAL-FLAG cells were transiently transfected with plasmid expressing Lck-GFP or LAT-GFP for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and were analyzed for the distribution of exogenous Lck and LAT at the IS. The histogram represents the percentage of cells with exogenous Lck or LAT preferentially accumulated at the pSMAC. (B) Jurkat CH7C17 cells transiently expressing MAL or MAL-FLAG were conjugated with with HOM2 cells previously loaded with the HA peptide or with staphylococcal enterotoxin B (SEB)-loaded Raji B cells. After 15 min cells were fixed, permeabilized and subjected to double immunofluorescence analysis to detect MAL and Lck (left panels) or LAT proteins (right panels). Only images corresponding to activation with HA are shown. (C) The histogram shows the percentage of T cells with TCR, F-actin, Lck, LAT or exogenous MAL preferentially accumulated at the pSMAC in Jurkat MAL and Jurkat MAL-FLAG cells in conjugates formed in the presence of SEB or HA. Means ± SEM of three (A) of two (C) independent experiments are represented; **, p < 0.01. Scale bars, 5 μm.
**Figure S4.** MAL and Lck, but not LAT or CD3ζ, colocalize in transport vesicles. (A) Jurkat MAL cells, which express exogenous GFP-MAL, were transiently transfected with plasmids expressing Lck-cherry, LAT-cherry or CD3ζ-cherry for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and were analyzed by time-lapse videomicroscopy. A representative frame of each video is shown. An enlargement of boxed regions is shown to illustrate the colocalization (MAL and Lck) or the absence of colocalization (LAT and CD3ζ) in transport vesicles. CD3ζ was used a negative control as its transport was previously shown to be MAL-independent (Anton et al., 2008). (B) The histograms represent the colocalization plots of MAL and the indicated molecules. The Pearson’s correlation coefficient (R) is shown in each case. Note that Lck and MAL colocalize extensively in pericentriolar endosomes, transport vesicles and the IS. However, the colocalization of LAT and MAL was restricted to the pericentriolar region and the IS. Therefore, although the Pearson’s value was high in both cases, no colocalization with MAL in transport vesicles was found in the case of LAT.

**Figure S5.** LAT travels to the plasma membrane by a MAL-independent route in resting T cells. (A) Jurkat cells were transfected with plasmids co-expressing GFP and control shRNA (-) or shRNA specific to the MAL mRNA molecule (+). After 24 h, GFP-positive cells were sorted and were extracted with 1% Triton X-100. The lysates were then centrifuged to equilibrium in sucrose density gradients. The soluble (S) and insoluble (I) fractions were analyzed for MAL expression. CD59 levels were analyzed in parallel as a negative control. The numbers indicate the percentage of MAL expression. (B) Resting Jurkat cells were transfected for 48 h with plasmids expressing LAT-GFP alone (left panels) or simultaneously with MAL shRNA (right panels). Cells were then subjected to time-lapse videomicroscopy. Arrowheads point to vesicles emerging from the Golgi and traveling towards the plasma membrane. (C) Resting Jurkat cells stably expressing GFP-MAL were transfected with a plasmid encoding LAT-cherry. After 16 h, cells were subjected to time-lapse videomicroscopy. An enlargement of the boxed region is shown in the bottom panels. Solid and clear arrowheads indicate vesicles transporting MAL or LAT, respectively. Arrows point to sporadic vesicles positive for both MAL and LAT. Numbers indicate time in seconds. Scale bar, 5 μm.
**Video 1.** Videomicroscopy of MAL in conjugates of Jurkat cells. Jurkat MAL cells were conjugated to SEE-pulsed Raji B cells previously stained with CMTMR and were subjected to time-lapse videomicroscopy for 6 min. Images of cells were captured every 12 s. Solid and clear arrowheads indicate vesicles transporting MAL from the Golgi region to the IS or from the cell periphery to the Golgi region, respectively. The arrows indicate sites of MAL accumulation at the IS. The movie is displayed at 3 frames s\(^{-1}\) and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Scale bar, 5 μm.

**Video 2.** Videomicroscopy of MAL-FLAG in conjugates of Jurkat cells. Jurkat MAL-FLAG cells were conjugated to SEE-pulsed Raji B cells previously stained with CMTMR and were subjected to time-lapse videomicroscopy for 5 min. Images of cells were captured every 10 s. Arrowheads and arrows indicate vesicles transporting MAL-FLAG from the Golgi region to the IS and sites of MAL-FLAG accumulation at the IS, respectively. The movie is displayed at 3 frames s\(^{-1}\) and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Scale bar, 5 μm.

**Video 3.** MAL and Lck travel to the IS in the same transport carriers in Jurkat MAL cells. Jurkat cells stably expressing GFP-MAL were transiently transfected with plasmid DNA expressing Lck-Cherry for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and were subjected to time-lapse videomicroscopy for 6 min. Images of cells were captured at 15-s intervals. The individual images were pseudocolored using the indicated color scale. The merge images correspond to the original images. The movie is displayed at 3 frames s\(^{-1}\) and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Solid and clear arrowheads indicate vesicles transporting MAL from the Golgi region to the IS or from the cell periphery to the Golgi region, respectively. Arrows indicate sites of MAL or Lck accumulation at the IS. Scale bar, 5 μm.

**Video 4.** MAL-FLAG and Lck travel to the IS in the same transport carriers in MAL-FLAG cells. Jurkat cells stably expressing GFP-MAL-FLAG were transiently transfected with plasmid DNA expressing Lck-Cherry for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and were subjected to time-lapse
Video 5. Videomicroscopy of Lck in conjugates of MAL or MAL-FLAG expressing Jurkat cells. Jurkat MAL cells or Jurkat MAL-FLAG cells were transiently transfected with plasmid DNA expressing Lck-GFP for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells previously stained with CMTMR and subjected to time-lapse videomicroscopy for 8 min. Images of cells were captured at 20-s intervals. The movie is displayed at 3 frames s⁻¹ and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Arrowheads indicate tubular/vesicular transport carriers containing Lck traveling to the IS. Arrows indicate sites of Lck accumulation at the IS. Scale bar, 5 μm.

Video 6. LAT translocation to the IS in Jurkat MAL cells. Jurkat cells stably expressing GFP-MAL were transiently transfected with plasmid DNA expressing LAT-Cherry for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and subjected to time-lapse videomicroscopy for 7 min. Images of cells were captured at 15-second intervals. The individual images were pseudocolored using the indicated color scale. The merge images correspond to the original images. The movie is displayed at 3 frames s⁻¹ and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Arrowheads indicate sporadic transport carriers containing both MAL and LAT traveling to the IS. Arrows indicate sites of MAL or LAT accumulation at the IS. Scale bar, 5 μm.

Video 7. LAT translocation to the IS Jurkat in MAL-FLAG cells. Jurkat cells stably expressing GFP-MAL-FLAG were transiently transfected with plasmid DNA expressing LAT-Cherry for 24 h. Cells were then conjugated to SEE-pulsed Raji B cells and subjected to time-lapse videomicroscopy for 5 min. Images of cells were captured at
15-second intervals. Images of cells were captured at 15-second intervals. The individual images were pseudocolored using the indicated color scale. The merge images correspond to the original images. The movie is displayed at 3 frames s$^{-1}$ and features a single confocal section. Images were captured of different sections at each time to ensure identification of the same vesicles. Arrowheads indicate sporadic transport carriers containing both MAL-FLAG and LAT traveling to the IS. Arrows indicate sites of MAL-FLAG or LAT accumulation at the IS. Scale bar, 5 μm.
FIGURE S1
FIGURE S4
FIGURE S5