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T cell receptor engagement by an APC induces the formation of a highly organized complex of surface receptors and intracellular signaling molecules, known as the immunological synapse, at the site of cell-cell contact. The transferrin receptor (TfR, CD71) is normally present in the plasma membrane and recycling endosomes. In this study, we show that, although the TfR is typically absent from lipid rafts at steady state, stimulation with a mitogenic mixture of anti-CD3 Abs of human Jurkat T cells leads to a rapid compartmentalization of the TfR into lipid rafts accompanying that of CD3ε and activated Lck. This change occurs very rapidly and is accompanied by an increase in the surface expression of the TfR, probably by translocation from an internal endosomal pool. TfR recruitment to lipid rafts was also observed in primary T cells treated with mitogenic anti-CD3 Abs and in Jurkat T cell-APC conjugates. The use of beads coated with Abs indicates that the surface and endosomal TfR pools redistribute to the contact site region in response to engagement of CD28 and CD3. In T cell-APC conjugates, the T cell TfR endosomal pool relocates beneath the contact site, whereas surface TfR localizes to the peripheral ring of the immunological synapse. In the presence of specific anti-TfR Abs, the total number of T cell-APC contacts and the percentage of conjugates with CD3 and Lck translocated to the contact site were reduced. Our results therefore suggest the involvement of the TfR in the formation of the immunological synapse. *The Journal of Immunology, 2004, 172: 6709–6714.*

Materials and Methods

**Materials**

Mouse mAb MEM-57 (anti-CD3ε, IgG2a) and MEM-92 (anti-CD3ε, IgM) were kindly provided by V. Horejsi (Institute of Molecular Genetics, University of Prague, Czech Republic). The biotinylated Alexa Fluor 488-labeled streptavidin and the biotinylated Alexa Fluor 594-labeled streptavidin were from Molecular Probes.

**Methods**

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used, the analysis was done either by using fluorochrome-labeled, isotype-specific, secondary Abs (double-label analysis of TIR and CD3ε) or with the second primary Ab biotinylated and subsequent detection with fluorochrome-labeled streptavidin (double-label analysis of TIR and LFA-1 and TIR and Vβ8). In the experiments indicated, the TIR was directly visualized using the anti-TIR mAb L5.1 covalently linked to Alexa-594. Controls to assess the specificity of the labeling included incubations with control primary Abs or omission of the primary Abs. Images were obtained using a Bio-Rad (Hercules, CA) Radiance 2000 confocal laser microscope. To quantify the recruitment of the TIR to the contact area of the T cell with the bead, 10 different random fields per experiment in three different triplicates were analyzed using a conventional fluorescence microscope (Zeiss, Oberkochen, Germany). Cells were divided into four quadrants. Cells with >75% of the TIR staining in the quadrant contacting the bead were scored as positive. Fluorescence intensity was quantified using the Metamorph program (Universal Imaging, Downingtown, PA).

Conjugate formation and analysis

Polystyrene latex microspheres were coated with 10 μg/ml anti-CD3 Ab and/or anti-CD28 Ab. Jurkat cells (1 × 10^7 cells) were cultured with 1 × 10^8 beads in 200 μl of RPMI 1640 containing 10% FBS in round-bottom microplates. Cells were fixed and processed for immunofluorescence analysis.
To distinguish Raji cells from Jurkat cells in the cell-cell conjugates, we loaded Raji cells with blue fluorescent tracker CMAC. Briefly, Raji cells were preincubated in RPMI 1640/5% FBS containing 10 µM CMAC for 20 min at 37°C, washed, and resuspended (5 × 10^6 cells/ml) in RPMI 1640/5% FBS. The Raji cells were then incubated for 20 min in the presence or absence of 5 µg/ml SEE, mixed with an equal number of Jurkat J77 cells (2 × 10^6 cells/well) in a final volume of 600 µl, and immediately plated onto poly(t)-lysine-coated slides. After incubation for 10 min at 37°C, cells were fixed, permeabilized or not, stained with the appropriate Abs, and analyzed under a confocal fluorescence microscope. Conjugates were first identified by directly observing both cell morphology under differential interference contrast and blue fluorescent CMAC-labeled cells.

**Results**

**A fraction of the TIR compartmentalizes into lipid rafts after TCR/CD3 triggering**

Engagement of TCR/CD3 with an anti-CD3 Ab mixture induces the capping of the complex and its redistribution to lipid rafts. Simultaneously, a specific subset of surface proteins redistributes to rafts and colocalizes with the TCR/CD3 complex. This membrane reorganization can be monitored biochemically by using a flotation assay, based on the resistance of lipid rafts to solubilization by detergents (18). As the TIR appears to play a costimulatory role in T cell activation (15, 16), we addressed whether engagement of the TCR produces compartmentalization of the TIR into rafts. Fig. 1A shows that in Jurkat T cells that were stimulated with anti-CD3-specific Abs for 3 min, ~50% of the TIR redistributed into raft fractions, whereas it was basically undetectable in these fractions in unstimulated cells. In addition, active Lck, recognized by its marked mobility shift, was also recruited to lipid rafts. The α and β CD3 subunits were also incorporated into the same fractions. As an internal control, we observed that most CD3-induced tyrosine phosphorylation was detected in the buoyant fractions containing the rafts (Fig. 1B). Fig. 1C shows that in parallel with the compartmentalization of the TIR, engagement of TCR/CD3 led to increased surface expression of the TIR, as measured by flow cytometry. After 3 min of stimulation, the surface expression of the TIR showed a 2-fold increase relative to that in resting cells and then progressively declined to recover the initial levels of surface expression. As controls, we observed that the surface expression of CD4 was not significantly altered and CD3 was rapidly down-modulated. An identical result was obtained when the cells were pre-treated with 100 µg/ml cycloheximide 15 min before CD3 engagement and maintained thereafter (our unpublished observations), implying that the increase in surface expression of the TIR takes place by mobilization of internal pre-existing molecules. A large amount of TIR was distributed intracellularly in endosomes, in keeping with its constitutive role in transferrin recycling (Fig. 1D).

The TIR redistributes to a region facing the contact site in response to costimulation with anti-CD3 and anti-CD28 Abs

Lipid raft coalescence defines membrane subdomains with polarized distribution (13). The changes observed in the incorporation of the TIR into rafts prompted us to examine the polarization of the TIR under simultaneous engagement of the TCR/CD3 complex and CD28 using beads coated with anti-CD3 and anti-CD28 Abs, which act as surrogate APCs. Cells were incubated for 3 min in the presence of beads coated with anti-CD3 and/or anti-CD28 Abs, fixed, and permeabilized, and the distribution of the TIR was analyzed by conventional immunofluorescence microscopy. The number of bead-cell conjugates greatly increased in the presence of anti-CD3 or anti-CD28 Abs (Fig. 2A, top panel). The conjugated cells contacted were then scored for the orientation of the intracellular TIR relative to the site of contact. Fig. 2A (bottom panel) shows that the intracellular TIR was polarized to the contact site in 65% of cells in the presence of beads coated with both anti-CD3 and anti-CD28 Abs (Fig. 2B). In contrast, in the presence of anti-CD3 or anti-CD28 Abs alone, only 30–35% of the cells displayed the TIR oriented to the contact site, a percentage similar to that found when using a negative control Ab and close to that obtained by random attachment of the cell to the bead in the absence of coupled Ab (25%) (Fig. 2A). Results similar to those for the intracellular TIR pool were found for surface TIR (Fig. 2C). This indicates that, in addition to the general reorientation of the exocytic and endocytic compartments, surface TIR also polarized to the contact site. Fig. 3 shows that the redistributed intracellular pool of TIR partially colocalized with Lck and CD3ζ at the Jurkat cell-bead interface in cells stimulated simultaneously with anti-CD3 and anti-CD28 Abs.

**FIGURE 2.** The redistribution of the TIR to the contact site requires costimulatory signals. A, Jurkat cells (1.0 × 10^6 cells) were stimulated with latex beads coated with a control Ab or with 0.5 µg/ml anti-CD3 mAb and/or anti-CD28 Abs for 3 min at 37°C. Cells were fixed and permeabilized (A and B) or not (C), and the distribution of the TIR was determined with anti-TIR mAb L5.1 coupled to Alexa-594. The number of cell-bead conjugates was evaluated under the different conditions used (top histogram). The number of cells with intracellular TIR staining at the site contacted by the bead was quantified in each case, as described in Materials and Methods, and the values obtained are represented as the percentage of cells with the intracellular TIR pool polarized to the contact site relative to the total number of cells contacted (bottom histogram). B and C, Illustrative examples of the effect of costimulation with anti-CD3 and anti-CD28 Abs on the polarization of the intracellular (B) or surface (C) pools of the TIR compared with that of a control Ab. Phase-contrast images corresponding to the same fields are shown. Bar, 5 µm.
The polarization of the TfR is accompanied by redistribution of Lck to the contact site. Jurkat cells (1.0 × 10⁴ cells) were stimulated for 3 min at 37°C with latex beads coated with 0.5 μg/ml anti-CD3 mAb and anti-CD28 Abs. Cells were fixed, permeabilized, and subjected to double-label immunofluorescence analysis with anti-TfR mAb L5.1 coupled to Alexa-594 and rabbit anti-Lck or anti-CD3ζ Abs, followed by a fluorescent anti-rabbit IgG secondary Ab. Phase-contrast images corresponding to the same fields are shown. Bar, 5 μm.

Surface TfR distributed in the p-SMAC ring of the immunological synapse

Jurkat cells expressing the Vβ8 gene segment of the TCRβ chain (Jurkat J77) are able to recognize the SEE superantigen bound to the human B cell line Raji, which acts as an APC, and forms conjugates similar to genuine T cell-APC pairs (9, 21). The TCR and many other membrane proteins and signaling machinery redistribute to the contact interface formed between the Jurkat and the Raji cell (9, 21). Fig. 4A (top and middle panels) shows that, while surface TfR was evenly distributed on Jurkat cells in the absence of SEE, the TfR polarized to the immunological synapse in the conjugates formed in the presence of SEE. Moreover, consistent with the results obtained with coated beads, the endosomal TfR pool reoriented in the conjugates to face the T cell-APC interface (Fig. 4A, bottom panels). The observed redistribution of surface TfR to the interface formed by the Jurkat and Raji cells prompted us to investigate the cellular origin of TfR. We did this by labeling the TfR either on the Jurkat or the Raji cell surface for 20 min at 4°C with anti-TfR mAb L5.1 coupled to Alexa-594, and mixing them with unlabeled Raji or Jurkat cells, respectively, in the presence of SEE. This Ab affects neither the efficiency of conjugate formation nor the translocation of CD3ζ to the immunological synapse (our unpublished observation). After 10 min at 37°C, the conjugates formed under each set of conditions were monitored for the redistribution of fluorescent label at the site of contact. Fig. 4B shows that the labeled TfR accumulated at the T cell-B cell interface only when the T cell was labeled, implying that most of the TfR at the immunological synapse is provided by the Jurkat cell. To establish whether the translocation of the TfR takes place in normal T cells, human peripheral T cells were incubated with Raji cells in the presence or absence of SEE, and the distribution of the TfR in the SEE-responding Vβ8+ T cell population was determined by immunofluorescence microscopy. The TfR accumulated at the contact site in Vβ8+ T cells in the presence of SEE (Fig. 4C), but not in its absence (our unpublished observations). To confirm that the recruitment of the TfR to lipid rafts observed in Jurkat cells upon treatment with mitogenic anti-CD3 Abs was not a particular aspect of this cell line or of the use of cross-linking Abs, we compared the TfR content in the insoluble raft membrane fraction obtained from Jurkat J77 cells conjugated with Raji cells in the presence or absence of SEE, and in primary T cells treated or not with mitogenic anti-CD3 Abs. Fig. 4D shows that compartmentalization of the TfR in lipid rafts took place in Jurkat cell only in the presence of SEE (top panel) and when primary T cells were activated with mitogenic anti-CD3 Abs (bottom panel), but not in the absence of SEE or anti-CD3 Ab treatment.

Fig. 5 shows that the accumulation of surface TfR occurred preferentially at the p-SMAC, as revealed by double-label immunofluorescence analysis of TfR and LFA-1 or CD3ζ, used as markers of the p-SMAC and central SMAC, respectively. Quantitative analysis showed a significant enrichment in the levels of the TfR
and presents the fl cell-APC conjugates. Raji cells prelabeled with CMAC were pulsed with SEE for 5 min and incubated with Jurkat J77 cells. Cells were then plated onto poly(l)-lysine-coated coverslips, incubated for 3 min at 37°C, and fixed. Unpermeabilized cells were subjected to double-label indirect immunofluorescence analysis with anti-TIR and anti-CD3ε or anti-LFA-1 Abs. Phase-contrast images corresponding to the same fields are shown. Bar, 5 μm. (~4.3) and CD3ε (~9.8), but not of CD45, in the contact zone in the conjugates formed in the presence of SEE, whereas all of them were evenly distributed in its absence (Fig. 6).

An anti-TIR mAb inhibits conjugate formation and assembly of the immunological synapse

To investigate the functional role of the TIR in the formation of the immunological synapse, we analyzed the effect of perturbing the TIR by treatment with either transferrin or with mAbs FG 1/5, FG 1/6, or FG 2/12 directed to different epitopes of the TIR molecule (17). The number of T cell-APC contacts showed a 100% increase in the presence of SEE relative to those formed in its absence (Fig. 7A). This increase was slightly reduced by treatment with transferrin or mAb FG 1/6 or FG 1/5, but treatment with FG 2/12 mAb caused a 60% drop in the number of T cells contacted by APCs. Under the experimental conditions used, preincubation with transferrin did not significantly affect the steady state surface levels of the TIR or the redistribution of the TIR to the immunological synapse, as assayed by flow cytometry or immunofluorescence analysis, respectively (our unpublished observations). To investigate further the effect of the different treatments used in Fig. 7A on the assembly of the immunological synapse, the presence of CD3ε and Lck in the contact zone of the T cell-APC conjugates was determined 10 or 30 min after mixing the cells, as a measure of the efficiency of the formation of immunological synapses. Fig. 7B shows that, whereas the rest of the treatments only caused a modest decrease in the number of conjugates with CD3ε polarized to the synapse, only 60% of the T cell-APC contacts contained CD3ε after 10 min in the cultures treated with mAb FG 2/12. Similar results were obtained after 30 min of conjugation and for the polarization of Lck (our unpublished observations). Consistent with our previous observation that the TIR accumulated at the contact site is supplied by the T cell (Fig. 4B), the preincubation of SEE-loaded Raji cells with the blocking anti-TIR mAb FG 2/12 affected neither the number of conjugates formed with Jurkat cells nor the presence of the TIR, CD3, and Lck at the contact site (our unpublished observations).

Discussion

The formation of an immunological synapse between a T cell and an APC is a complex process involving the redistribution of specific surface proteins (TCR/CD3, LFA-1, ICAM-1, ICAM-3) to

FIGURE 5. Surface TIR distributed to the p-SMAC ring of the immunological synapse in T cell-APC conjugates. Raji cells prelabeled with CMAC were pulsed with SEE for 5 min and incubated with Jurkat J77 cells. Cells were then plated onto poly(l)-lysine-coated coverslips, incubated for 3 min at 37°C, and fixed. Unpermeabilized cells were subjected to double-label indirect immunofluorescence analysis with anti-TIR and anti-CD3ε or anti-LFA-1 Abs. Phase-contrast images corresponding to the same fields are shown. Bar, 5 μm.

FIGURE 6. Quantitative analysis of the distribution of the TIR in T cell-APC conjugates. Raji cells prelabeled with CMAC were pulsed or not with SEE for 5 min and incubated with Jurkat J77 cells. Cells were then plated onto poly(l)-lysine-coated coverslips, incubated for 3 min at 37°C, and fixed. The distribution of the TIR and CD3ε or CD45 was analyzed by double-label indirect immunofluorescence analysis in unpermeabilized cells. A, Distribution of surface TIR, CD3ε, and CD45 in the T cell-APC conjugates formed in the presence of SEE. Phase-contrast images corresponding to the same fields are shown. Bar, 5 μm. B, The histogram represents the fluorescence intensity of the TIR, CD3ε, and CD45 in the contact zone relative to that in the opposite region of the cell surface in the conjugates formed in the presence or absence of SEE (p < 0.05).

FIGURE 7. Effect of anti-TIR Abs on the number of T cell-APC contacts and the polarization of CD3ε to the immunological synapse. Jurkat 77 cells were treated at 37°C for 30 min in culture medium in the absence or presence of 0.5 mg/ml transferrin (Tf) or with 10 μg/ml anti-TIR mAbs FG 1/5, FG 1/6, or FG 2/12. Cells were then mixed with Raji cells that were or were not preincubated with SEE, and the cell mixture was maintained in the presence of the different TIR ligands for 10 min at 37°C. A. After fixation, the number of T cell-APC contacts was counted. The values are represented as percentage of the number of conjugates found in each case relative to those formed in the control cultures treated with SEE alone, in which ~65% of the T cells formed contacts with Raji cells. B. The presence of CD3ε in the contact zone of the T cell-APC pairs was determined by immunofluorescence analysis as a measure of the efficiency of the formation of the synapse. The values are represented as percentages of the number of conjugates found in each case relative to those formed in the control cultures treated only with SEE, in which ~70% of the T cell-APC conjugates contained CD3ε polarized to the contact site. *, Significant difference (p < 0.05).
the site of contact, the translocation of intracellular signaling machinery (Lck, protein kinase C-θ, ZAP70) to that site, and the reorientation of the secretory apparatus to face the T cell-APC interface (1–7, 22). These processes are triggered upon engagement of the TCR/CD3 complex by the Ag presented by the APC, and are mediated by the reorganization of the cytoskeleton and extensive remodeling of specialized raft membranes. In this study, we have used a combination of experimental approaches to show that the T cell surface levels of the TIR are up-regulated from the internal TIR pool after TCR/CD3 engagement. The TIR on the T cell surface relocates to the p-SMAC, and the TIR endosomal pool becomes placed in front of the immunological synapse.

Our observation that treatment with anti-TIR FG 2/12 mAb impaired the efficient formation and the quality of T cell-APC contacts is consistent with an important role for the TIR in this process. Alternatively, because the TIR does not have a counterreceptor in the APC, it is possible that the binding of the FG 2/12 mAb perturbs conjugate formation and, as a consequence, CD3ε and Lck accumulation by steric hindrance. However, although it cannot be ruled out, this explanation seems unlikely because all the anti-TIR mAb assayed were of the same Ig class (IgG). Our findings are consistent with other results showing that FG 2/12 mAb strongly blocks target killing by NK cells, whereas FG 1/5 has only a moderate effect, and FG 1/6 mAb and transferrin are ineffective (17). The observation that FG 1/6 mAb did not interfere with the formation of conjugates or the recruitment of CD3ε and Lck to the contact site is consistent with previous reports that the perturbation of the TIR with this Ab transduces co-stimulatory signals (15, 16). It is of note that mAb FG 2/12, but not mAb FG 1/5 or FG 1/6, blocks the binding of transferrin to its receptor (17). This indicates that in the TIR close to, but different from, the transferrin binding site is important for the efficient formation of T cell-APC contacts. Functional cross talk of the TIR with the TCR complex was evidenced by the finding that TIR stimulation results in tyrosine phosphorylation of CD3ζ and, conversely, stimulation of the TIR increases tyrosine phosphorylation of the TIR (16). The recruitment of the TIR to the immunological synapse in the T cell-APC conjugates brings the TIR into the proximity of the TCR complex, and this may facilitate cross talk between the two receptors. It is of particular note that, even in the reduced number of T cell-APC pairs formed in the presence of mAb FG 2/12, the percentage of cells with CD3ε and Lck recruited to the cell-cell contact decreased by 40%. This indicates that, in addition to its role in the formation of the T cell-APC contacts, surface TIR probably participates in events occurring before the assembly of the immunological synapse, such as in the TCR-mediated signaling process preceding the formation of the synapse (23).

The spatial and temporal recruitment of Lck to the immunological synapse has been recently investigated (24). Lck are initially recruited to the synapse periphery. Later on, CD3ζ becomes enriched in the center, while Lck is enriched at the synapse periphery. This pattern is similar regardless of activation with strong or weak agonists, although the efficiency of the conjugation is reduced in the latter case (24). The translocation of internal TIR to the cell surface and its incorporation into rafts observed soon after TCR engagement might reflect an early supply of endosomal components to the immunological synapse, including the raft-associated Lck molecule (24) and raft lipids (25). The inefficient recruitment of Lck to the T cell-APC contact area in the presence of the anti-TIR mAb FG 2/12 is consistent with transport of Lck being coupled to TIR trafficking. Furthermore, the fact that recycling endosomes, as visualized by staining of internal TIR, reoriented to face the T cell-APC contact area suggests that these endosomes may intervene in additional processes occurring at the synapse, such as a late supply of Lck (24) or transport of endocytosed TCR/CD3 complexes for degradation (26).

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