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Role of Fyn in the Rearrangement of Tubulin Cytoskeleton Induced through TCR

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The translocation of the microtubule-organizing center (MTOC), its associated signaling complex, and the secretory apparatus is the most characteristic early event that involves the tubulin cytoskeleton of T or NK cells after their interaction with APC or target cells. Our results show that Fyn kinase activity is essential for MTOC reorientation in an Ag-dependent system. Moreover, T cells from Fyn-deficient mice are unable to rearrange their tubulin cytoskeleton in response to anti-CD3-coated beads. Analysis of conjugates of T cells from transgenic OT-I mice with dendritic cells revealed that an antagonist peptide induces translocation of the MTOC, and that this process is impaired in T cells from Fyn−/− OT-I mice. In addition, Fyn deficiency significantly affects the MTOC relocation mediated by agonist peptide stimulation. These results reveal Fyn to be a key regulator of tubulin cytoskeleton reorganization in T cells. The Journal of Immunology, 2006, 176: 4201–4207.

The microtubule-organizing center (MTOC) nucleusates the assembly and establishes the polarity of microtubules and is associated with the Golgi apparatus (GA). The MTOC reorients to the contact area during formation of the immune synapse in T cells as well as in NK cells and CTLs when interacting with target cells (1, 2). Repositioning of the GA/MTOC leads to the polarized secretion of cytokines at the T cell-APC interface (3). In addition, the GA/MTOC complex nucleates a growing body of signaling molecules that may be involved in T cell activation and the rearrangement of tubulin cytoskeleton (4–6). Among these, Src kinases are associated with the MTOC. Lck, in addition to its plasma membrane localization, is found at pericentrosomal vesicles (7), corresponding to late endosomes or the trans-Golgi network. Fyn has been reported to associate with the TCR. However, the stoichiometry of this interaction is very low and is only detectable by in vitro kinase assays (8). Fyn is mainly associated with the centrosome by immunofluorescence (7). Nevertheless, to date, no specific function has been ascribed to this subcellular location of Fyn. Although T cell development is normal in Fyn-deficient mice, significant functional defects are observed in TCR-mediated responses, mainly in mature thymocytes, with a milder phenotype in peripheral T cells (9, 10). The family of signaling lymphocyte activation molecule (SLAM) associates to the Src homology 2 domain of the adaptor molecule, SLAM-associated protein (SAP), which, in turn, interacts with the Src homology 3 domain of Fyn (11, 12). This SLAM-SAP-Fyn cascade is pivotal in Th2 cytokine production (13, 14). In addition, Fyn plays a crucial role in NKT cell development, and Fyn deficiency results in a 5–10-fold decrease in the numbers of CD1d-restricted NKT cells in both the thymus and periphery (15, 16). However, the activation of Fyn−/− NKT cells is only mildly affected.

Repositioning of the MTOC is a complex event dependent on TCR signaling that involves the shortening or sliding of MTs and the subsequent anchoring of MTOC at the site of TCR engagement. MTOC repositioning after anti-CD3 stimulation has been shown to require Lck activation and ITAM phosphorylation (17). Moreover, Zap70 and its substrate adaptor proteins, linker for activation of T cell (LAT) and SH2 domain-containing protein (SLP) 76, have been involved in MTOC polarization (18, 19). In this study, we show that Fyn has a key role in the regulation of MTOC reorientation, without interfering with other pathways activated through the TCR.

Materials and Methods

Mice

Mice were bred at the Centro de Biología Molecular Severo Ochoa under specific pathogen-free conditions. Fyn-deficient mice (129-Fyntm1Sor) were purchased from The Jackson Laboratory. Fyn-deficient mice were backcrossed with OT-I transgenic mice (C57BL/6), and Fyn−/− and Fyn+/− littermates were analyzed. All procedures involving animals and their care were approved by the ethics committee of the University of Madrid and were conducted according to institutional guidelines that are in compliance with international laws and policies.
Cells and mutant constructs
Jurkat T cell clones J77c120 (J77) (20) and JCaM 1.6 (21) as well as the lymphoblastoid B cell line Raji were cultured in complete medium (RPMI 1640 and 10% FCS; Invitrogen Life Technologies). L625.7 is an L transfectant of murine B7-1 (CD80) and HLA-D1/1102 (22); these cells were maintained in complete minimum Eagle’s medium with 250 μg/ml G418. The murine T cell hybridoma T8.1 has been previously described (23) and was cultured in DMEM with 10% FCS, 2 mM l-glutamine, and antibiotics supplemented with 400 mM methotrexate. 1 mg/ml G418, 10 M HEPES, and 50 μM 2-ME. Stable transfectants of wild-type Fyn (FynWT), kinase-dead Fyn (FynKd), and kinase-dead Lck (LckKd) in T8.1 cells have been described previously (23) and were cultured in medium for T8.1 cells supplemented with puromycin (1 μg/ml) for 1 week after transfection. The thymus and spleen were disaggregated to obtain cell suspensions. Bone marrow was washed and grown in 30% J558-conditioned medium containing GM-CSF (24) in complete medium supplemented with 50 μg/ml 2-ME for 7 days. Then, bone marrow-derived dendritic cells (DC) were matured overnight with 20 ng/ml recombinant mouse TNF-α (R&D Systems).

Abs and reagents

The following mAbs were used: T3h (anti-human-CJD), BV8, a biotin-labeled anti-human V (8 (BD Biosciences), anti-Fyn-a (either unconjugated or FITC-conjugated; Sigma-Aldrich), anti-human CD69 (TP1/55), anti- (Sigma-Aldrich), anti-Fyn and anti-Lck (clones 15 and 35; Upstate Biotechnology), ant i-mouse CD3 (2C11; BD Biosciences), and anti-mouse CD69 (H.12/3F; BD Biosciences). The following polyclonal Abs were used: anti-Fyn (Upstate Biotechnology), anti-Lck and anti-protein kinase C (Clontech), and rabbit polyclonal anti-phospho-PKC (Thr308; Cell Signaling Technology), anti phospho-LAT (Y191; Upstate Biotechnology). Anti-phosphoVav (Y174) was a gift from Dr. X. Bustelo (Centro de Investigación del Cancer, Salamanca, Spain). Rabbit muscle enolase was purchased from Sigma-Aldrich. Sfc-specific substrate was obtained from Upstate Biotechnology. Poly-L-lysine (PLL), and G418 were purchased from Sigma-Aldrich, and Staphylococcus enterotoxin E (SEE) was obtained from Toxin Technology. Tetanus toxin peptide (αlps, α2a), specific for the T8.1 TCR, and OVA 264 peptides specific for OT-I transgenic mouse TCR (with the following sequences: OVA-OT-I antigen, SIINFEKL; OVA-OT-I antigen, SIIN-FEDL), which were generated in the Centro de Biología Molecular Severo Ochoa, were used. The fluorescent cell tracker 7-amino-4-chloromethylcoumarin was obtained from Molecular Probes. Human rIL-2 from Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ) was provided by the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and protein A- and G-Sepharose were purchased from Roche. All other reagents used were of the purest grade available.

Immunoprecipitation, in vitro kinase assays, and Western blot

For in vitro kinase assays, JCaM1.6 and Jurkat cells were serum starved, and Raji B cells were cultured in medium containing 2% FCS for 18 h. Raji cells (5 × 107) were then preloaded, or not, with SEE (0.25 μg/ml) at 37°C for 20 min and mixed with 107 JCA-M1.6 or Jurkat cells at 37°C. After incubation, cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, and phosphatase and protease inhibitors at 4°C for 40 min. Cell lysates were spun at 2500 × g for 10 min to remove cell debris and nuclei, and supernatants were precleared with protein G-Sepharose beads at 4°C for 1 h and immunoprecipitated with mouse α-Fyn Ab protein G-Sepharose beads at 4°C overnight. Immunoprecipitates were washed twice with lysis buffer, then half of the sample was analyzed by Western blot for a loading control as described below. The rest of the precipitates were washed twice with kinase assay buffer containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA, and 0.015% Brij35, then resuspended in kinase dilution buffer containing kinase assay buffer supplemented with 0.1 mg/ml BSA and 0.2% 2-ME. Raytide EL substrate (10 mg/ml tetanus toxin peptide, or 10 μg/ml tetanus toxin peptide, or 50 μg/ml tetanus toxin peptide) was added to each reaction, and the kinase assay was stopped by adding 10 μl final concentration of 8% was transferred to P81 ion exchange chromatography paper (Whatman) squares. P81 squares were extensively washed with 0.5% o-phosphoric acid and once with acetone. One of the squares was quantitated using a liquid scintillation counter. To perform the Western blot assay, immunoprecipitated proteins were transferred to Immobilon (Millipore) membranes after SDS-PAGE, using a Bio-Rad SDS Transblot system. Membranes were then blocked with 3% nonfat dry milk in PBS (pH 7.2) and incubated for 2 h at room temperature with anti-Fyn (rabbit whole serum; Upstate Biotechnology).

For in vitro kinase assays in T cells, bone marrow-derived, TNF-α-matured DCs were preloaded, or not, with OVA and D7 peptides. DCs (107) were conjugated with purified T cells (6 × 104) for 30 min at 37°C. Fyn and Lck were immunoprecipitated from cell extracts. The immunoprecipitates were used for a loading control by Western blot and in vitro kinase assays. An in vitro kinase reaction was conducted for 15 min at 30°C with acid-inactivated Staphylococcus aureus (strain K1), which was stopped with Laemmli, and samples were processed by SDS-PAGE.

For analysis of phosphorylation during T cell presentation in cells from OT-I transgenic mice, total splenocytes were incubated in the presence of different doses of agonist or antagonist peptides for 25 min. Cells were then lysed in the above-described lysis buffer, and complete lysates were analyzed by SDS-PAGE, transferred to membranes as indicated above, and blotted for rabbit polyclonal anti-Vav, anti-phospho-Tyr174 Vav (pY174), previously described (25), anti-phospho-LAT, anti-LAT, anti-phospho-PKC0, and anti-PKC0 in TBS. Bound Abs were detected with HRP-conjugate secondary Abs, followed by visualization by SuperSignal West Pico chemiluminescent substrate (Pierce).

Cell conjugate formation, MTOC translocation, and functional assays

Raji B cells, L625.7 cells, or bone marrow-derived DCs were loaded with the blue fluorescent cell tracker 7-amino-4-chloromethylcoumarin as previously described (4). Then cells were incubated for 20 min in the presence or the absence of 1 μg/ml SE (Raji), 20 μg/ml tetanus toxin peptide, or various doses of agonist or antagonist OVA-OT1 peptides, respectively. As T cells, we used the cell line Jurkat for conjugation with Raji cells, T8.1 cells for conjugation with L625.7, or T cells from transgenic OT-I mice, which were partially purified from splenocytes by negative selection by adherence to plates coated with anti-mouse polyclonal Ab (Sigma-Aldrich). T cells (2 × 107 cells/slide) were mixed with an equal number of APCs in a final volume of 80 μl/slide, centrifuged at low speed, and incubated for 15 min at 37°C. Thereafter, conjugates were gently resuspended, plated onto PLL-coated slides in humidified incubation chambers, and allowed to settle for an additional 15 min at 37°C. PLL-adhered cells were fixed and permeabilized for 5 min in 2% formaldehyde-0.5% Triton X-100 in PBS and stained with the indicated Abs using Alexa 488 and rhodamine red X-labeled Abs as secondary reagents (Molecular Probes). The proportion of conjugates with MTOC redistributed next to the T cell-APC contact area was calculated by random choice of >300 different conjugates from at least three independent experiments. Results were expressed as the percentage of conjugates with MTOC redistributed to the contact area.

Ab-coated beads were made as previously described (17). In brief, 107 streptavidin beads (diameter, 6.4 μm; Streptavidin beads incubated for 90 min at room temperature with anti-human-CJD (T3b) or anti-mouse-CD3 (2C11; BD Pharmingen; 10 μg/ml) under continuous rotation. Beads were then saturated with 1% BSA, washed with HBSS, and stored at 4°C until use. For conjugation assays, T cells and Ab-coated beads were mixed at a 1:1 ratio in medium and allowed to interact for 15 min at 37°C. Then conjugates were gently resuspended and plated onto PLL-coated coverslips for 30 min, fixed, permeabilized, and stained with the anti-aubin Ab. When samples were visualized under a fluorescence microscope, the beads appeared round and refringent. The MTOC was scored as reoriented when it was located in close proximity to the T cell plasma membrane between the nucleus and the bead contact area. At least 200 conjugates were scored in each experiment.

For IL-2 production and CD69 expression assays, conjugates were formed in the different models in flat-bottom, 96-well plates as indicated above. Supernatant was harvested after 16 h of coculture and analyzed for IL-2 production using ELISA procedure for human IL-2 (Diaclone) and for mouse IL-2 (BD Biosciences). T cells from the same coculture were analyzed for CD69 expression using conventional double-immunofluorescence techniques and flow cytometry in a FACS-Calibur (BD Biosciences).

Results

MTOC translocation correlates with Fyn kinase activation in Lck-deficient cells

Although Lck has a key role in T cell activation, under certain conditions, such as T cell stimulation with bacterial superantigens, Lck-deficient cells are activated, although partial defects have also been described (26–28). To determine how tubulin rearrangement was affected by superantigen activation in the absence of Lck, we assessed MTOC translocation in Lck-deficient JCaM1.6 Jurkat

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cells (29). JCaM1.6 cells conjugated with Raji B cells pulsed with SEE displayed a frequency of MTOC translocation similar to that observed in the parental cell line (Fig. 1, a and b). As previously reported, the tubulin cytoskeleton was not redistributed toward the contact surface with polystyrene beads coated with anti-CD3 in the absence of Lck in JCaM1.6 (17) (Fig. 1b). Therefore, the partial activation in the absence of functional Lck in the superantigen-dependent system is sufficient to mediate tubulin cytoskeleton rearrangement.

Fyn is able to mediate functional responses in JCaM1.6 cells, such as CD69 induction and NFAT activation (26). To determine the possible contribution of Fyn to the observed tubulin cytoskeleton rearrangement, we first analyzed Fyn kinase activity upon stimulation with superantigen or anti-CD3-coated beads. Fyn activation was induced in JCaM1.6 Lck-deficient cells by SEE, but not by anti-CD3-coated polystyrene beads (Fig. 1c), thus correlating with the level of cytoskeleton rearrangement triggered by these stimuli. These results suggest that Fyn kinase activity may affect MTOC translocation.

Fyn kinase activity is necessary for Ag-specific T cell function

To demonstrate the involvement of Fyn activation in specific Ag-driven cytoskeleton rearrangement, we used stable mouse T8.1 transfectant cells of FynWT, FynKD, and LckKD (23). T8.1 hybridoma T cells bear a TCR specific for the \(\Delta_{3830-3843}\) tetanus toxin peptide presented by the DR\(^*\)1102-positive L625.7 fibroblast cell line (23). In the presence of Ag, either parental or FynWT-transfected T8.1 cells reorganized their tubulin cytoskeleton toward the APC (Fig. 2, a and b). However, FynKD and LckKD blocked this rearrangement, demonstrating that both kinases are essential for cytoskeleton rearrangement after Ag stimulation (Fig. 2, a and b). LckKD severely impaired TCR signaling, thus blocking activation events such as CD69 induction (Fig. 2c). In contrast, FynKD only slightly affected CD69 induction (Fig. 2c). However, T cell functional events, such as IL-2 production, were inhibited in the presence of both FynKD and LckKD (Fig. 2d).

Defective tubulin cytoskeleton rearrangement and functional responses in T cells from Fyn-deficient mice

The essential role of Fyn in tubulin reorganization in vitro prompted us to study functional responses in T cells from Fyn-deficient mice. Although initial reports suggested that Fyn-deficient splenic T cells exhibit only minor defects (9, 10), recent reports have shown more global defects that result in deficient proliferation, cytokine secretion, and PKC\(\theta\) recruitment (13, 14). The reorganization of the tubulin cytoskeleton is a key event in early T cell activation that could contribute to the defects found in Fyn-deficient cells, including cytokine secretion and relocation of key signaling molecules located in the pericentrosomal vesicles. Therefore, we hypothesized that Fyn can play a role in MTOC translocation that may complement that of Lck. We studied the reorganization of the microtubule cytoskeleton in T cells from Fyn-deficient mice. MTOC translocation toward CD3-coated beads in either CD3\(^+\) thymocytes or splenocytes was severely impaired in Fyn-deficient mice (Fig. 3). However, other activation events, such as CD69 induction, was only partially affected by the absence of Fyn (Fig. 3c). In contrast, IL-2 production was blocked (Fig. 3d), consistent with a role of Fyn in multiple signaling pathways. Although the reorganization of tubulin cytoskeleton after TCR stimulation in this system requires Lck activation (17), we demonstrate that Lck activity is not sufficient, and Fyn is essential (Fig. 3).

Antagonist peptide-induced MTOC translocation is impaired in Fyn-deficient T cells

Fyn may have a particularly important role under suboptimal conditions of T cell signaling. T cell activation is sensitive to the
affinity of interaction between the TCR and its MHC/peptide ligand. Studies of single amino acid-substituted antigenic peptide variants (altered peptide ligands (APL)) have indicated that low affinity interactions often lead to an incomplete pattern of tyrosine phosphorylation. Antagonist APL fail to activate Lck and Zap70, but are capable of inducing Fyn activation, Vav phosphorylation, and subsequent activation of Rac, thus affecting actin cytoskeleton rearrangement and conjugate formation (30, 31). Studies of T cells from a Fyn-deficient TCR transgenic mouse have shown that Fyn is essential for tyrosine phosphorylation and activation of Vav induced by both agonist and antagonist peptides (31). To explore whether activation of Fyn, but not Lck, can induce tubulin cytoskeleton reorganization after T cell stimulation using APL, we used Fyn+/− or Fyn−/− T cells from OTI transgenic mice. T cells were conjugated with bone marrow-derived DCs loaded with the agonist (OVA) or antagonist (D7) peptide. Treatment with agonist peptide produced full T cell activation (Fig. 4, a and b), including Lck and Fyn activation, as observed by the analysis of their kinase activity (Fig. 4c). Moreover, Lck activation was also detected by the shift in electrophoretic mobility of the kinase due to the specific phosphorylation of serine residues, as previously described (32, 33). However, stimulation with the antagonist APL resulted in Fyn kinase activation, whereas Lck activity remained essentially unaffected. Thus, this result provides us with a system in which Fyn is differentially activated, and specific changes associated with Fyn activation may be analyzed. After T-DC cell conjugation, the antagonist peptide did not induce CD69 or IL-2 production, in contrast to the agonist peptide (Fig. 4, a and b), showing that Lck activation is essential for these processes, and that Fyn activation alone cannot complement the deficient Lck kinase activity. Conversely, the absence of Fyn did not affect either CD69 induction or IL-2 production by the agonist peptide (Fig. 4, a and b), demonstrating that Fyn is not important for these T cell activation events.

However, we found that D7 peptide produced nearly as much MTOC translocation in T cells from Fyn−/− OTI mice as the OVA peptide at the highest dose analyzed and the same extent of MTOC translocation at lower doses (1 and 10−2 μg/ml, respectively; Fig. 4, d and e). This result shows that Fyn activation by an antagonist, in the absence of Lck activity, is sufficient to provide the reorganization of tubulin cytoskeleton associated with T cell activation. In addition, Fyn is essential for MTOC translocation in this system, because the absence of Fyn prevented tubulin reorganization induced by the antagonist peptide. Under agonist stimulation, Fyn deficiency produced an ~50% reduction in MTOC translocation (Fig. 4d), suggesting that Fyn plays an important role in MTOC polarization by weak stimuli that can be partially compensated by Lck activity upon full activation of T cells.

Analysis of the early signaling in T-DC cell conjugates showed that the antagonist peptide was unable to activate LAT or PKCθ, whereas the agonist induced similar phosphorylation levels of both proteins in T cells from Fyn+/− and Fyn−/− mice (Fig. 5). Remarkably, the antagonist peptide induced the phosphorylation of Vav in Tyr174 (which is a clear-cut parameter of Vav1 activation (25)), similar to the agonist peptide, and Vav phosphorylation was impaired in the absence of Fyn (Fig. 5). Therefore, Vav-induced phosphorylation by antagonist peptide may explain tubulin cytoskeleton rearrangement, because both phenomena are prevented in the absence of Fyn.

**Discussion**

T cell activation upon specific conjugation with allogenic cells or APC leads to tubulin cytoskeleton rearrangement and MTOC relocation. Although the reorganization of tubulin cytoskeleton after Ag stimulation requires Lck activation (17), partial T cell activation is obtained in the absence of Lck in some experimental systems, such as the responses to allogenic MHC Ags (34) and bacterial superantigen (26). Our data reveal that Fyn plays an important role in MTOC translocation that may complement that of Lck. We demonstrate in this study that in both superantigen- and Ag-dependent systems, Fyn localization and activation are essential for MTOC reorientation, and that T cells from Fyn-deficient mice have impaired tubulin cytoskeleton rearrangement in response to either anti-CD3 or to a specific Ag.

The specific function of Fyn vs Lck in tubulin cytoskeleton reorganization may be dependent on preferential substrate phosphorylation by each enzyme. The critical role of Fyn as a unique mediator of the SLAM/SAP axis that drives to Th2 differentiation has evidenced a specific role for this kinase (13, 14). In addition to the SLAM/SAP pathway, Fyn affects independent pathways. Thus, Fyn−/− T cells showed general defects, such as reduction in IFN-γ production or in T cell proliferation (13), whereas the defects were limited to Th2 differentiation in SAP−/− T cells (13, 14). These general defects in the absence of Fyn would concord with the effect, described in this report, on the process of tubulin rearrangement after T cell activation, which is a common step for activation and differentiation of T cells to either Th1 or Th2. Other possible mediators that are specifically phosphorylated by Fyn include SLAP-130/Fyn-binding protein (FYB) (35), Vav (23, 31), Wiskott-Aldrich syndrome protein (36), and Pyk2 (37). The SLAP-130/FYB-Vav axis plays a critical role in cytoskeletal reorganization in T cells (38), and Fyn is required for efficient phosphorylation of Vav after stimulation of T cells (31). The use of the antagonist APL has endowed us with a system in which Fyn, and not Lck, is selectively activated (30, 31), and we have found that antagonist peptides induce MTOC translocation in a Fyn-dependent manner that correlates with Vav phosphorylation. In this regard, Vav-1-deficient thymocytes show defective relocation of the MTOC (39).
compared with cells from Fyn
H11001
the agonist (OVA) or antagonist (D7) peptide. Bar, 20
H9262
H11001
were analyzed in Fyn
H11001
of kinase protein present in the reaction and normalized to no peptide stimulation. One experiment of three is shown.

Doses of OVA or D7 peptides. T cells were purified from OT-I mice. The ratio shown corresponds to the enolase phosphorylation relative to the amount
of three independent experiments. c, Fyn and Lck kinase activity was analyzed from T-DC conjugates, where DCs were preloaded, or not, with the indicated
doses of OVA or D7 peptides. T cells were purified from OT-I mice. The ratio shown corresponds to the enolase phosphorylation relative to the amount
kinase protein present in the reaction and normalized to no peptide stimulation. One experiment of three is shown. d, The results for MTOC translocation
were analyzed in Fyn
H11001
and Fyn
H11006
T cells and are represented as the arithmetic mean ± SD of three independent experiments. *, p < 0.05 compared with cells from Fyn
H11001
mouse; **, p < 0.05 peptide stimulation compared with no peptide stimulation; ***, p < 0.05 D7 stimulation compared with OVA stimulation. c, Staining for
a-tubulin (green) and CD8a (red) was analyzed in T-DC conjugates. DCs were pulsed with the indicated doses of the agonist (OVA) or antagonist (D7) peptide. Bar, 20 μm.

FIGURE 4. Antagonist peptides induce MTOC translocation in T-DC conjugates that is impaired in Fyn−/− T cells. a, Purified T cells from Fyn+/− or
Fyn−/− OT-I transgenic mice were conjugated with bone marrow-derived mature DCs in the presence (bold line) or the absence (thin line) of agonist
(OVA) or antagonist (D7) peptide and analyzed for CD69 expression 16 h later. One experiment of three is shown. b, IL-2 production was analyzed in T
cells from Fyn+/− (□) and Fyn−/− (●) mice that were conjugated with DCs as indicated in a, and results are represented as the arithmetic mean ± SD
of three independent experiments. c, Fyn and Lck kinase activity was analyzed from T-DC conjugates, where DCs were preloaded, or not, with the indicated
doses of OVA or D7 peptides. T cells were purified from OT-I mice. The ratio shown corresponds to the enolase phosphorylation relative to the amount
kinase protein present in the reaction and normalized to no peptide stimulation. One experiment of three is shown. d, The results for MTOC translocation
were analyzed in Fyn+/− (□) and Fyn−/− (●) T cells and are represented as the arithmetic mean ± SD of three independent experiments. *, p < 0.05 compared with cells from Fyn+/− mice; **, p < 0.05 peptide stimulation compared with no peptide stimulation; ***, p < 0.05 D7 stimulation compared with OVA stimulation. e, Staining for
a-tubulin (green) and CD8a (red) was analyzed in T-DC conjugates. DCs were pulsed with the indicated doses of the agonist (OVA) or antagonist (D7) peptide. Bar, 20 μm.

Vav could thus act as a GTPase exchange factor for the Ras-related
GTPase Cdc42 (41). The involvement of Pyk2 in MTOC translocation in
T and NK cells has been previously documented (4, 5).

FKC65 may also play an important role in T cell activation under
suboptimal conditions of intracellular signaling. When T cells are
stimulated through the TCR in the absence of CD28 costimulation,
an increment in TCR-Fyn association is detected (42), resulting in
Fyn activation (43). In this regard, it has been proposed that in
conditions of activation of naive T cells through the TCR, Fyn plays an essential role by positive regulation of Lck activity (44).
Our results show that Fyn activity is involved in tubulin cytoskel-
eton rearrangement and T cell functional responses in T cells upon
different conditions of stimulation: 1) with superantigen that selec-
tively activates Fyn vs Lck, 2) in an Ag-dependent system with
limited presence of costimulatory molecules (T8.1 vs L625.7
cells), and 3) sole activation via TCR in naive T cells, as in the
model of anti-CD3 polystyrene beads with mouse spleen cells or
thymocytes from Fyn-deficient mice. The impact of Fyn deficiency
in MTOC reorientation was mostly compensated when bone mar-
row-derived DCs presented the agonist peptide to induce full ac-
tivation of T cells. Remarkably, in the OT-I TCR transgenic sys-
tem, the antagonist peptide D7, which triggers selective activation
of Fyn and subsequent phosphorylation of Vav (23, 31), induces
MTOC translocation that correlates with Vav activation, and both
phenomena are impaired in the absence of Fyn. In addition, our
results concur with previous data showing that although high af-
finity TCR-Ag interactions stimulate Fyn−/− T cells equally well
as Fyn+/− T cells, low affinity interactions inefficiently stimulate
Fyn−/− T cells (43). It is feasible that the defects in tubulin reor-
ganization observed in Fyn−/− cells could be overcome when
stronger stimuli are used or when a subset of Fyn−/− responding
cells is positively selected by the response to specific stimuli in

FIGURE 5. Vav activity in Fyn+/−, but not in Fyn−/−, transgenic OT-I
T cells correlates with MTOC translocation. a, Analysis of PKC8, LAT, and Vav phosphorylation in whole splenocytes prepulsed with agonist
(OVA) or antagonist (D7) peptide at the indicated doses (micrograms per milliliter). One experiment of four performed is shown. b, Quantitative
analysis of four experiments, performed as indicated in a, in the presence of the depicted doses of OVA (□) or D7 (●) in splenocytes from Fyn+/−
and Fyn−/−. PKC8, LAT, and Vav activities were measured as the relation between the densitometric analysis of the phosphorylated form and no
peptide stimulation. *, p < 0.05 (by Student’s t test), comparing activity between Fyn+/− and Fyn−/− with the same doses of OVA or D7.
vitro (45), activating other pathways that involve Zap70 and its substrate adaptor proteins, LAT and SLP-76. Those signaling proteins are activated after agonist, but not antagonist, stimulation and have been shown to affect MTOC polarization (18, 19).

A general proposed role for GA/MTOC polarization in T cells during immune synapse formation is the directed delivery of lymphokines to the bound APC (3). Nevertheless, the signaling complex associated with the centrosome and its rapid translocation after TCR engagement support a more active and early role of MTOC reorientation in T cell activation and function. In this regard, it has been reported that cytoskeletal reorganization contributes to the maturation of the immunological synapse (46–48). Re-crutiment of intracellular compartments containing molecules essential for TCR-mediated signal transduction to the immune synapse has been described associated with MTOC reorientation (19, 49). In this regard, our results could explain the observed defect in the recruitment of PKCθ in the absence of Fyn (14). In addition, and according to the possible role of MTOC translocation in controlling cytokine secretion, immune synapse formation may be necessary for effector functions of T cells (48, 50–52). Together, our results point to a key role for Fyn activation in tubulin cytoskeleton rearrangement and effector functions of mature T cells, with a more evident effect under conditions of defective stimulation of T cells, which may be relevant in processes such as positive selection and tolerance.

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Disclosures
The authors have no financial conflict of interest.

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