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Displacement of Linker for Activation of T Cells from the Plasma Membrane Due to Redox Balance Alterations Results in Hyporesponsiveness of Synovial Fluid T Lymphocytes in Rheumatoid Arthritis

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The T lymphocytes that reside in the synovium of the inflamed joints in patients with rheumatoid arthritis display severe hyporesponsiveness upon antigenic stimulation, which is probably due to their constant subjection to high levels of oxidative stress. Here we report that the synovial fluid T lymphocytes exert severely impaired phosphorylation of the adaptor protein linker for activation of T cells (LAT), a crucial component of the TCR-mediated signaling pathways. In healthy T lymphocytes, LAT is a membrane-bound protein and becomes phosphorylated by ζ-associated protein of 70 kDa (ZAP-70) upon TCR engagement. The molecular basis underlying the deficient phosphorylation of LAT and consequently the hyporesponsiveness of the synovial fluid T lymphocytes lies in the membrane displacement of LAT. We demonstrate that the subcellular localization of LAT is sensitive to changes in the intracellular levels of the antioxidant glutathione. The membrane anchorage of LAT, and consequently the phosphorylation of LAT and the cellular activation of the synovial fluid T lymphocytes upon TCR engagement, is restored in synovial fluid T lymphocytes after supplementation of the intracellular glutathione levels with N-acetyl-L-cysteine. These data suggest a role for the membrane displacement of LAT in the hyporesponsiveness of the synovial fluid T lymphocytes as a consequence of oxidative stress. The Journal of Immunology, 2000, 164: 2170–2179.

Rheumatoid arthritis (RA)2 is a chronic inflammatory joint disease that eventually leads to the destruction of the joint architecture. The synovium of the inflamed joints is invaded by T lymphocytes, neutrophils, monocytes, B lymphocytes, and dendritic cells. The role of the T lymphocytes in the origin and propagation of the inflammation of the joints remains controversial. The synovial fluid (SF) T lymphocytes display an activated phenotype (1–3), but react hyporesponsive to triggering of the TCR/CD3 complex as determined by low proliferative responses and the minimal production of IL-2 and IFN-γ in response to TCR stimulation (4–7). Although unable to respond to the Ag-dependent triggering of the TCR/CD3 complex, evidence exists that the T lymphocytes in the inflamed joint induce damage of the joint cartilage through Ag-independent cell-cell contact with macrophages (8–11). Furthermore, Salojin and coworkers recently suggested that the hyporesponsiveness of regulatory T lymphocytes that normally confer protection from autoimmune disease due to altered TCR signaling might contribute to the breakdown of tolerance and increase susceptibility to autoimmune disease (12).

The prolonged residence of the T lymphocytes in the synovium thus seems to contribute to and perpetuate the disease. The hyporesponsive state of the SF T lymphocytes in RA correlates with markers of oxidative stress: the intracellular levels of the antioxidant glutathione (GSH) are significantly decreased in SF T lymphocytes. Moreover, the extracellular levels of another important redox regulator, thioredoxin are significantly increased in the SF (13, 14). GSH is an important regulator of the cellular redox balance and plays a major role in the protection against oxidative stress both by reacting directly with reactive oxygen radicals and acting as a substrate in the detoxification of H2O2 and organic peroxides. In addition, GSH is involved in maintaining the sulfhydryl groups of intracellular proteins in a reduced state, which is required for their functional conformation (15, 16). It has previously been shown that the depletion of the intracellular GSH levels in T lymphocytes through treatment with N-acetyl-L-cysteine (NAC)-sulfoximine (BSO) results in hyporesponsiveness due to the abrogation of the proximal TCR-mediated signaling events (17).

The proximal signaling pathways induced by the engagement of the TCR/CD3 complex by ligand include the activation of two classes of protein tyrosine kinases, the Src and Syk families. The recruitment of the Syk kinase ζ-associated protein of 70 kDa (ZAP-70) to the TCR ζ-chain plays a central role as it serves to bring the kinase in close proximity of its substrates (18–21). Two substrates of ZAP-70 have been identified: the adaptor proteins Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells (LAT) (22–24). LAT has recently been cloned and identified as the 36- to 38-kDa protein of 76 kDa; LAT, linker for activation of T cells; PLCγ1, phospholipase Cγ1; pAb, polyclonal Ab; RT, room temperature; ECL, enhanced chemiluminescence; MFI, mean fluorescence intensity; FasL, Fas ligand; PerCP, peridinin chlorophyl protein.
Several signaling proteins, including Grb2, the p85 subunit of phosphatidylinositol 3-kinase, Grb2-like accessory protein (Grap), and phospholipase Cγ1 (PLCγ1) associate with phosphorylated LAT through their SH2 domains to generate multiprotein complexes that amplify the TCR-induced signals (22, 26–29).

The molecular basis underlying the hyporesponsiveness of the SF T lymphocytes in RA remains elusive, although some leads have evolved. First, it has been observed that the expression of the signaling chain of the TCR/CD3 complex, the TCR ζ-chain, is down-regulated in SF T lymphocytes. Specifically, the phosphorylation of a protein of 36–38 kDa is absent in SF but not peripheral blood (PB) T lymphocytes from RA patients (31). Third, a more distal event in the TCR-mediated signaling pathways, the influx of Ca2⁺ from the endoplasmic reticulum, is reduced in PB T lymphocytes from RA patients, which also display some degree of hyporesponsiveness (32–34). These observations seem to be consistent with a defect in the proximal TCR-mediated signaling events in SF T lymphocytes in RA.

In the present study, we set out to elucidate the molecular mechanisms underlying the hyporesponsiveness of the T lymphocytes in the synovium of RA patients and establish the role oxidative stress plays in this process. We present evidence that the 36- to 38-kDa phosphoprotein that does not become phosphorylated in TCR-stimulated SF T lymphocytes is the adaptor protein LAT. We also show that LAT is displaced from the membrane in SF T lymphocytes due to the severely reduced intracellular GSH levels. The displacement of LAT is responsible for the abrogation of its phosphorylation upon TCR stimulation, as well as the subsequent association of PLCγ1 with LAT, hence blocking the TCR-induced signaling pathways leading to the influx of Ca2⁺ and eventually to the expression of IL-2. The treatment of SF T lymphocytes with N-acetyl-l-cysteine (NAC) elevates the intracellular GSH levels and consequently restores the membrane localization and phosphorylation of LAT and ultimately the cellular activation of the SF T lymphocytes.

Materials and Methods

T lymphocyte isolation

T lymphocytes from heparine-collected PB or SF of RA patients were isolated through a negative selection procedure. Mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. Monocytes, B lymphocytes, and NK cells were depleted by incubating with mAbs against CD14, CD16, and CD19 (10 µg of each mAb per 20 × 10⁶ of mononuclear cells; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands) and sheep anti-mouse IgG coated-dynabeads (Dynal, Oslo, Norway) for 1.5 h, after which cells resorted with immunomagnetic beads were removed with a Dynal magnetic particle concentrator. The remaining cell preparations contained >95% T lymphocytes as assessed by flow cytometric analysis after staining with a peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 mAb (Becton Dickinson, San Jose, CA). Control human PB T lymphocytes were prepared from healthy volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque density-gradient centrifugation. T lymphocytes were isolated by 2-aminoethylisothiouronium bromide-treated SRBC rosetting. The SRBC were lysed with 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA according to standard procedures. The remaining cell preparations contained >92% T lymphocytes as assessed by FACScan analysis after staining with a PerCP-conjugated anti-CD3 mAb (Becton Dickinson). After isolation, T lymphocytes were kept at 37°C, 5% CO₂ in IMDM (Life Technologies, Gaithersburg, MD) containing 10% FCS (Life Technologies) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany).

Stimulation

T lymphocytes (5 × 10⁶/ml) were incubated for various time periods with 1 µg/ml anti-CD3 mAb (1XE; CLB), in combination with 1 µg/ml anti-CD28 mAb (15E8; CLB) as indicated. NAC (Sigma, St. Louis, MO) was added at a final concentration of 5 mM, while BSO (Sigma) was added at a final concentration of 200 µM.

Immunoprecipitation, Western blotting, and immunodetection

Whole-cell lysates were prepared from 5 × 10⁶ T lymphocytes, either unstimulated or stimulated with anti-CD3 for 3 min, when indicated after incubation with cells or NAC or BSO. Cells were harvested, washed twice with PBS, and lysed in 300 µl lysis buffer (10 mM triethanolamine, pH 7.8, 150 mM NaCl, 5 mM EDTA, 10 mM Na₂VO₄, 1% Nonidet P-40) supplemented with protease inhibitors (10 µg/ml leupeptin (Sigma), 0.4 mM PMSF (Sigma)) during 45 min on ice. Insoluble debris was spun down in a microcentrifuge for 15 min at 4°C.

To detect tyrosine phosphorylation of LAT or association of PLCγ1 with LAT, LAT was immunoprecipitated from 300 µl of cell lysate (equivalent to 5 × 10⁶ cells) by incubating with 3 µg rabbit anti-LAT pAb (06-807; Upstate Biotechnology, Lake Placid, NY) during 16 h at 4°C while rotating, and an additional 2 h after the addition of 25 µl of protein A-Agarose beads (50% slurry; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were spun down in a microcentrifuge for 30 s at 4°C, washed twice with lysis buffer, and then boiled in 1× SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE in a 5% (PLCγ1 detection) or 10% (LAT detection) gel, using Rainbow-colored protein m.w. markers (Amersham, Little Chalfont, U.K.) as a reference, and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked in PBS containing 5% skim milk and 0.01% Tween 20 during 1 h. Detection of tyrosine phosphorylated proteins was performed by incubating the membranes with a mouse mAb against phospho-Tyr (PY99) (1:1000; sc-7020; Santa Cruz Biotechnology), while LAT and PLCγ1 were detected by incubating the membranes with the rabbit polyclonal Abs (pAbs) against LAT (1:1000) and PLCγ1 (1:250) for 16 h. The membranes were subsequently incubated with the appropriate secondary Abs (HRP-conjugated rabbit anti-mouse Ig (1:5000; Dako, Glostrup, Denmark) or swine anti-rabbit Ig-HRP (1:5000; Dako)) for 3 h, and then assayed using the enhanced chemiluminescence (ECL) detection system (Amer sham). Membranes were stripped of bound Abs by incubating the membranes for 30 min at 50°C in stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) to allow for a second round of detection.

Flow cytometric analysis

A total of 2.5 × 10⁶ T lymphocytes were fixed for 10 min in 1% paraformaldehyde in PBS at room temperature (RT), washed twice with PBS, and stored overnight at 4°C. The expression of LAT, ZAP-70, or PLCγ1 was measured via a two-step intracellular staining procedure. Cells were incubated for 10 min at RT in PBS containing 1 mg/ml BSA (Sigma), 10% FCS, and 100 µg/ml saponin (Sigma) as a permeablizing agent. Cells were washed three times with PBS/BSA/saponin and incubated for 1 h at 4°C with appropriate dilutions of the primary Abs (LAT, 1:250; ZAP-70 pAb (sc-574; Santa Cruz Biotechnology), 1:35; PLCγ1 pAb (sc-81; Santa Cruz Biotechnology), 1:35). After three washes (PBS/BSA/saponin), cells were incubated for 30 min at 4°C with PE (R-PE)-conjugated goat anti-rabbit IgG (Harlan Sera-Lab, Crawley Down, U.K.). A negative control was incubated with the secondary Ab only. After three washes (PBS/BSA/saponin), the free F(ab)² sites of cell-bound goat anti-rabbit-PE were blocked using 10% normal rabbit serum, whereafter cells were stained with a PerCP-conjugated anti-CD3 mAb (Becton Dickinson) (1:25) for 1 h at 4°C. Control live cells were analyzed on a FACScan using the CellQuest software (Becton Dickinson). The levels of LAT, ZAP-70, and PLCγ1 were expressed as the ratio of the mean fluorescence intensity (MFI), i.e., the MFI of LAT, ZAP-70, or PLCγ1 divided by the MFI of the negative control.

ZAP-70 kinase activity assay

Total cell lysates were prepared from 5 × 10⁶ T lymphocytes, either unstimulated or stimulated with anti-CD3 for 3 min. Cells were harvested and washed once with PBS, resuspended in 500 µl lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol) supplemented with protease inhibitors (10 µg/ml leupeptin, 0.4 mM PMSF) and phosphatase inhibitors (50 mM β-glycerophosphate, 1 mM Na₂VO₄), and incubated on ice for 30 min. Insoluble debris was spun down in a microcentrifuge for 15 min at 4°C.
ZAP-70 was immunoprecipitated from 500 μl of cell lysate (equivalent to 5 × 10^6 cells) by incubating with 800 ng rabbit anti-ZAP-70 pAb (Santa Cruz Biotechnology) during 1 h at 4°C while rotating, and an additional 16 h after the addition of 25 μl of protein A-Agarose beads (50% slurry; Santa Cruz Biotechnology). The immunoprecipitates were spun down in a microcentrifuge for 30 s at 4°C, washed twice with lysis buffer, twice with LiCl buffer (500 mM LiCl, 0 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% Triton X-100), and finally twice with assay buffer (25 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10 mM MnCl₂, 1 mM EDTA, 0.1% Triton X-100). The immunoprecipitates were assayed for kinase activity in 30 μl assay mix containing 25 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10 mM MnCl₂, 1 mM EDTA, 0.1% Triton X-100, 250 μM ATP, and 10 μCi [γ-32P]ATP (3000 Ci/mmoll; Amersham) during 20 min at 30°C, using 10 μg tubulin (Sigma) as substrate. The reactions were terminated by the addition of 5× SDS-PAGE sample buffer and boiling. Proteins were separated by SDS-PAGE on a 10% gel, using Rainbow-colored protein m.w. markers (Amersham) as a reference. Quantification of phosphorylated substrates was performed using a PhosphorImaging system and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Immunofluorescence staining and microscopy**

A total of 1 × 10⁷ T lymphocytes were mounted onto adhesive microscope slides, air dried, and kept frozen until staining. Before staining, the cells were fixed in 4% para-formaldehyde in PBS for 15 min at RT. After three washes in PBS containing 5% BSA, cells were permeabilized using 0.1% Triton X-100 in PBS for 4 min at RT, washed three times (PBS/BSA), and preblocked for 45 min at RT in PBS containing 10% FCS. Cells were then incubated with a rabbit pAb against LAT (1:250) in PBS/FCS for 45 min at RT, washed three times (PBS/BSA), and incubated with FITC-conjugated swine anti-rabbit Ig (1:100; Dako) in PBS/FCS for 45 min at RT. A negative control was incubated with the secondary Ab only. After three final washes (PBS), cells were imbedded in 1 mg/ml p-phenyleendiamine (Sigma) in 90% glyceral, 10% PBS and covered with a coverslip. To determine whether the cell membrane was intact after BSO treatment, cells were stained immediately after fixation with a mouse mAb against CD3 (1:25; Becton Dickinson) and detected using tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse Ig (1:200; Dako). Cells were viewed using a Leitz Aristoplan microscope (Leica, Wetzlar, Germany) equipped with a 100× objective and optics for FITC and TRITC. Images were taken with a Sony 3 CCD Color Video Camera, model DVC-9508 (Sony, Tokyo, Japan).

**Preparation of membrane and cytoplasmic cell fractions**

A total of 10 × 10⁷ T lymphocytes were disrupted in 500 μl extraction buffer (50 mM Tris-HCl, pH 7.0, 10 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₃VO₄) supplemented with protease inhibitors (10 μg/ml leupeptin, 0.4 mM PMSF) by shearing through a 25-gauge needle. After centrifugation at 1600 × g for 10 min at 4°C, this extraction step was repeated on the pellet. The membrane and cytoplasmic fractions were separated from the pooled supernatants at 100,000 × g for 60 min at 4°C. The supernatant after ultracentrifugation contains the cytoplasmic proteins (Triton X-100 was added to 1%). The pellet containing the membrane proteins was resuspended in 500 μl buffer (10 mM triethanolamine, pH 7.8, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 1% Nonidet P-40) supplemented with protease inhibitors (10 μg/ml leupeptin, 0.4 mM PMSF). LAT was immunoprecipitated from the extracts and detected with ECL Western blotting as described above.

**Measurement of secreted IL-2 protein**

Human T lymphocytes (3 × 10⁶/ml) were left unstimulated or stimulated with anti-CD3 and anti-CD28 for 24 h when indicated after incubation with either NAC or BSO. Secreted IL-2 protein was quantified in cell-free supernatants using a human IL-2 ELISA kit (Genzyme, Cambridge, MA) as recommended by the manufacturer.

**Statistical analysis**

Statistical analyses were performed on the data using the Fisher’s exact test for paired observations. Statistical significance of the data was set at p < 0.05.

**Patient identification**

Patients enrolled in this study fulfilled the 1987 revised criteria of the American College of Rheumatology for rheumatoid arthritis, differed between 27 and 72 years of age (mean age 57 years), with a female:male ratio of 9:8 (see Table I).

**Results**

LAT remains unphosphorylated in SF T lymphocytes, but not PB T lymphocytes from RA patients upon TCR stimulation

The tyrosine phosphorylation pattern upon TCR stimulation of SF T lymphocytes from RA patients is aberrant, with, most particular, the absence of the phosphorylation of a 36- to 38-kDa protein (31). To determine whether this phosphoprotein of 36–38 kDa is identical with the adaptor protein LAT, we immunoprecipitated LAT from whole-cell extracts of SF and PB T lymphocytes from RA patients that were either left unstimulated or stimulated for 3 min with anti-CD3 and assessed the phosphorylation status of LAT by Western blotting analysis. We observed no phosphorylated LAT in unstimulated T lymphocytes. In PB T lymphocytes from RA patients, LAT becomes extensively phosphorylated on tyrosine residues upon TCR stimulation, as occurs in T lymphocytes from healthy controls (Fig. 1). However, after stimulation of
the TCR/CD3 complex in SF T lymphocytes from RA patients, we did not detect any phosphorylated LAT, indicating that the missing 36- to 38-kDa phosphoprotein in SF T lymphocytes is indeed LAT (Fig. 1). Western blotting analysis revealed that the impaired phosphorylation of LAT is not due to a lack of expression of LAT, because we could detect LAT in extracts from SF as well as PB T lymphocytes from RA patients, although the level of LAT expression seemed slightly lower in both SF and PB T lymphocytes compared with the expression of LAT in T lymphocytes from healthy controls (Fig. 1). To quantitatively determine the expression of LAT at the individual cell level, we measured the MFI after the intracellular immunofluorescence staining of LAT by flow cytometric analysis. The MFI ratio for LAT in both PB and SF T lymphocytes was ~2-fold lower compared with the LAT MFI ratio in T lymphocytes from healthy controls: 104.4 ± 5.1 (mean ± SEM; n = 5) for healthy controls vs 52.2 ± 2.2 (mean ± SEM; n = 4; p = 0.003) for PB T lymphocytes and 48.9 ± 1.7 (mean ± SEM; n = 4; p < 0.001) for SF T lymphocytes (Fig. 2). These data support the conclusion that the significantly decreased expression of LAT in SF T lymphocytes does not account for the lack of phosphorylation of LAT, because the phosphorylation of LAT remains intact in PB T lymphocytes from RA patients that contain similar levels of LAT.

The expression and kinase activity of ZAP-70 is intact in SF T lymphocytes

The phosphorylation of LAT upon TCR ligation is regulated by the syk family tyrosine kinase ZAP-70 (22). One explanation for the lack of phosphorylation of LAT in SF T lymphocytes could be that either the expression of ZAP-70 or the activation of the kinase activity of ZAP-70 is affected by the severely reduced intracellular GSH levels. The expression of ZAP-70 was similar in T lymphocytes from healthy controls and RA patients (MFI ratios: 24.3 ± 0.9 (mean ± SEM; n = 6) for healthy controls, 26.5 ± 1.6 (mean ± SEM; n = 4) for PB T lymphocytes, 25.1 ± 1.5 (mean ± SEM; n = 5) for SF T lymphocytes; Fig. 2). To determine the kinase activity of ZAP-70, we performed a kinase assay specific for ZAP-70. ZAP-70 was immunoprecipitated from whole-cell extracts of SF and PB T lymphocytes from RA patients that were either left unstimulated or stimulated for 3 min with anti-CD3, and the specific ZAP-70 kinase activity was determined by measuring the phosphorylation of its substrate, tubulin. The basal kinase activity of ZAP-70 present in unstimulated T lymphocytes from

**FIGURE 1.** LAT is phosphorylated in PB but not SF T lymphocytes from RA patients upon TCR stimulation. T lymphocytes from healthy controls (HC) and PB (PB T/RA) and SF T lymphocytes (SF T/RA) from RA patients were left unstimulated or stimulated with anti-CD3 for 3 min. LAT was immunoprecipitated from whole-cell lysates of an equivalent number of cells, and the phosphorylation status of LAT was determined by ECL Western blotting with anti-phosphotyrosine (PY) Abs and HRP-conjugated rabbit anti-mouse Abs (top panel). The expression of LAT was determined by ECL Western blotting with anti-LAT Abs and HRP-conjugated swine anti-rabbit Abs. The detected H chain comes from the LAT Abs used for the immunoprecipitation (bottom panel). The data are representative of six independent experiments.

**FIGURE 2.** The expression of LAT is reduced in both PB and SF T lymphocytes from RA patients, while the expression of ZAP-70 and PLCγ1 is comparable to the expression in T lymphocytes from healthy controls. A. Permeabilized T lymphocytes from healthy controls (HC) as well as PB (PB T/RA) and SF T lymphocytes (SF T/RA) from RA patients were labeled with specific Abs for ZAP-70, LAT, and PLCγ1 and then stained with PE-conjugated goat anti-rabbit Abs. The MFI was measured by flow cytometric analysis. The MFI of cells stained with the PE-conjugated Abs only was also determined as a negative control and (B) used to calculate the MFI ratio for ZAP-70, LAT, and PLCγ1 as described in Materials and Methods. The mean values ± SEM for the MFI ratios found in four to six independent experiments are shown.
healthy controls was enhanced \( \sim 2.2 \pm 0.23 \text{ (mean \pm SEM; } n = 3) \) in anti-CD3-stimulated T lymphocytes (Fig. 3). The basal ZAP-70 kinase activity in both unstimulated PB and unstimulated SF T lymphocytes from RA patients is up-regulated almost 2.1-fold \((2.1 \pm 0.07 \text{ (mean \pm SEM; } n = 3; p = 0.003) \) for PB T lymphocytes, \(2.1 \pm 0.16 \text{ (mean \pm SEM; } n = 3; p < 0.001) \) for PB T lymphocytes) upon TCR/CD3 stimulation to reach a level of kinase activity that is only slightly lower than the kinase activity observed in stimulated T lymphocytes from healthy controls \((p = 0.37 \text{ for PB T lymphocytes, } p = 0.17 \text{ for SF T lymphocytes; Fig. 3}) \). These results demonstrate that the defective phosphorylation of LAT in SF T lymphocytes is not due to a defect in the kinase activity of ZAP-70.

**LAT is displaced from the membrane and present in the cytoplasm of SF T lymphocytes**

The recruitment of signaling proteins to the membrane is a common theme in the proximal events of many signaling pathways (20, 22, 35). To explore whether LAT is absent from the membrane in SF T lymphocytes and therefore the recruitment of ZAP-70 to the membrane upon TCR engagement does not result in the phosphorylation of LAT, we analyzed the subcellular localization of LAT by immunofluorescence staining of T lymphocytes on cytospins. In healthy controls, LAT is clearly expressed in the plasma membrane of the T lymphocytes (Fig. 4B). In PB T lymphocytes from RA patients, LAT is also localized in the cellular membrane, although the level of expression is clearly diminished compared with T lymphocytes from healthy controls (Fig. 4C). In contrast, in SF T lymphocytes from RA patients, we detected LAT in the cytoplasm of the cell instead of the membrane (Fig. 4D). Western blot detection of LAT in separated membrane and cytoplasm fractions confirmed the membrane and cytoplasm localization of LAT in, respectively, PB and SF T lymphocytes from RA patients (Fig. 4E). These results signify that the displacement of LAT from the membrane in SF T lymphocytes is responsible for its deficient phosphorylation.

**The localization of LAT is sensitive to alterations in the intracellular GSH levels**

The intracellular GSH levels are significantly reduced in SF but not PB T lymphocytes from RA patients (13). To examine whether the decreased intracellular GSH levels play a role in the membrane displacement of LAT, we treated T lymphocytes from healthy controls with BSO to decrease the intracellular GSH reserves (13, 17) and determined the subcellular localization of LAT at successive time points. Immunofluorescence staining of LAT revealed that LAT still resided in the cellular membrane of the T lymphocytes after 16 h of treatment with BSO (Fig. 5B). However, after 48 h of BSO treatment, LAT started to accumulate in the cytoplasm, although we also could detect membrane-associated LAT (Fig. 5C). After 72 h of BSO treatment, we did not observe any expression of LAT in the plasma membrane, while the cytoplasm showed massive expression of LAT (Fig. 5D). Immunofluorescence staining for the membrane receptor CD3 ascertained that the cellular mem-
brane of the T lymphocytes was still intact after 72 h of BSO treatment (Fig. 5E). Moreover, the cellular activation of the T lymphocytes treated for 72 h with BSO was completely impaired because the cells were unable to produce any IL-2 while untreated T lymphocytes produced high levels of IL-2 after 24 h of stimulation (data not shown). T lymphocytes treated for 72 h with BSO was completely impaired because the cells were unable to produce any IL-2 while untreated T lymphocytes produced high levels of IL-2 after 24 h of stimulation (data not shown).

Increasing the intracellular GSH levels in SF T lymphocytes restores the phosphorylation of LAT, the association of PLCγ1 with LAT, and the secretion of IL-2 upon TCR stimulation

Because the elevation of the intracellular GSH levels restored the membrane localization of LAT in SF T lymphocytes from RA patients, we also examined whether the phosphorylation of LAT and downstream signaling events in the TCR-mediated signaling pathways were restored. We immunoprecipitated LAT from whole-cell extracts of either resting or activated SF T lymphocytes both before and after a 48-h treatment with NAC and determined the tyrosine phosphorylation of LAT through Western blotting analysis. As shown before, LAT remained unphosphorylated in untreated SF T lymphocytes from RA patients after TCR stimulation (Fig. 7A). However, in SF T lymphocytes that had been treated with NAC for 48 h, LAT becomes extensively phosphorylated on tyrosine residues upon TCR ligation (Fig. 7A).

The association of PLCγ1 with LAT upon TCR engagement, necessary to transmit the TCR-induced signal from LAT to the inositol 1,4,5-triphosphate-sensitive Ca2+ channels, is dependent on the phosphorylation of LAT because the N-terminal SH2 domain of PLCγ1 interacts with the multiple phosphorylated tyrosine residues of LAT (22, 25, 28, 36). FACS analysis showed that the expression of PLCγ1 was slightly but not significantly reduced in both PB and SF T lymphocytes from RA patients compared with T lymphocytes from healthy controls: 31.2 ± 1.8 (mean ± SEM; n = 5) for healthy controls vs 27.2 ± 1.6 (mean ± SEM; n = 4) for PB T lymphocytes and 26.5 ± 2.9 (mean ± SEM; n = 4) for SF T lymphocytes (Fig. 2). To determine whether PLCγ1 associates with LAT, we subjected whole-cell extracts of either resting or stimulated T lymphocytes to immunoprecipitation with anti-LAT Abs followed by Western blotting detection of PLCγ1. PLCγ1 coimmunoprecipitated with LAT only in stimulated T lymphocytes from healthy controls and also from PB T lymphocytes of RA patients, but not in stimulated untreated SF T lymphocytes from RA patients due to the lack of phosphorylation of LAT (Fig. 7B).

To establish whether the restoration of the membrane localization of LAT, and hence the phosphorylation of LAT and the association of PLCγ1 with LAT upon TCR ligation, through increasing the intracellular GSH levels, also results in a reversal of the hyporesponsiveness of the SF T lymphocytes from RA patients,
FIGURE 7. The phosphorylation of LAT, the association of PLCγ1 with phosphorylated LAT, and the secretion of IL-2 upon T cell activation is restored in SF T lymphocytes from RA patients after treatment with NAC. T lymphocytes from healthy controls (HC), PB T lymphocytes from RA patients (PB T/RA), and SF T lymphocytes from RA patients (SF T/RA), either untreated or treated for 48 h with NAC, were left unstimulated or stimulated with anti-CD3 for 3 min. LAT was then immunoprecipitated from whole-cell lysates of an equivalent number of cells. A. The phosphorylation of LAT was determined by ECL Western blotting with anti-phosphotyrosine (PY) Abs and HRP-conjugated rabbit antimouse Abs. B. The association of PLCγ1 with LAT was determined by ECL Western blotting with anti-PLCγ1 Abs and HRP-conjugated swine anti-rabbit Abs. The data are representative of three independent experiments. C. T lymphocytes from healthy controls (HC), PB T lymphocytes from RA patients (PB T/RA), and SF T lymphocytes from RA patients (SF T/RA), either untreated or treated for 48 h with NAC, were left unstimulated or stimulated with anti-CD3 plus anti-CD28. Cell-free supernatants were harvested after 24 h and analyzed for secreted IL-2 protein. The mean values ± SEM for the IL-2 secretion measured in three to seven independent experiments are shown.

we determined the secretion of IL-2 by the T lymphocytes in response to stimulation with anti-CD3 and anti-CD28 for 24 h. Unstimulated T lymphocytes secreted only very low levels of IL-2. Stimulation of T lymphocytes from healthy controls with anti-CD3/anti-CD28 for 24 h resulted in a secretion of 2381 ± 319 pg/ml IL-2 (mean ± SEM; n = 7; Fig. 7C). The IL-2 secretion of anti-CD3 plus anti-CD28-stimulated PB T lymphocytes from RA patients was ~2-fold lower: 1380 ± 58 pg/ml IL-2 (mean ± SEM; n = 4; Fig. 7C). The stimulation of untreated SF T lymphocytes induced an IL-2 production of only 293 ± 58 pg/ml IL-2 (mean ± SEM; n = 4; Fig. 7C). The treatment of the SF T lymphocytes with NAC for 48 h elevated the IL-2 secretion upon anti-CD3/anti-CD28 stimulation significantly to a level of expression comparable to the expression by PB T lymphocytes: 1316 ± 137 pg/ml IL-2 (mean ± SEM; n = 3; p = 0.023; Fig. 7C). These results indicate that the reduced GSH levels play a critical role in the abrogation of the cellular activation of the SF T lymphocytes in RA through the membrane displacement of LAT.

Discussion

In RA, a common chronic inflammatory joint disease characterized by the progressive, erosive destruction of joint cartilage and bone, the functioning of the T lymphocytes present at the site of inflammation, is severely impaired. In the present study, we have examined the molecular basis for this hyporesponsive state, and we demonstrate that the phosphorylation of the adaptor protein LAT, which plays a crucial role in the TCR-mediated signaling pathways, is dramatically impaired in SF T lymphocytes from RA patients. Immunohistochemical analysis reveals that the reduced phosphorylation of LAT upon engagement of the TCR/CD3 complex correlates with the displacement of LAT from the membrane, a phenomenon that could be induced by modulating the intracellular redox balance, suggesting that the membrane displacement of LAT is induced by oxidative stress. The supplementation of the GSH levels in SF T lymphocytes could reverse the membrane displacement of LAT and the hyporesponsiveness of the cells.

Several observations have suggested that the T lymphocytes in the inflamed joints of RA patients exhibit defects in the TCR-induced signal transduction routes rendering them unresponsive upon mitogenic or antigenic stimulation. These observations include a decrease in the expression levels of the TCR ζ-chain, an aberrant pattern of protein tyrosine phosphorylation, and a block in the Ca2+ influx upon TCR activation, any of which could contribute to the hyporesponsiveness of the T lymphocytes (30–34).

The reduced expression of the TCR ζ-chain has been proposed as an underlying mechanism responsible for the negative regulation of the TCR signaling pathways. The decreased expression of the TCR ζ-chain is not only observed in the hyporesponsive SF T lymphocytes from RA patients but also in tumor-infiltrating T lymphocytes from human cancer patients and in the hyporesponsive T lymphocytes from HIV-infected individuals. In all cases, only the expression of the TCR ζ-chain is reduced, while the expression of other components of the TCR/CD3 complex remains constant (30, 31, 37–39). Although the correlation between the hyporesponsive
state of the T lymphocytes and the reduced TCR ζ expression suggests a causal relationship, formal evidence for this hypothesis is not available. Our data show that the kinase activity of ZAP-70 is not affected in SF T lymphocytes from RA patients compared with the ZAP-70 kinase activity in T lymphocytes from healthy controls and PB T lymphocytes from RA patients, suggesting that the signaling pathway from the TCR ζ-chain leading to the activation of ZAP-70 is intact and functional.

Previously, we have reported the absence of a 36- to 38-kDa phosphoprotein in SF T lymphocytes upon TCR stimulation (31). A similar observation has been described for autoimmune nonte bese diabetic mice, where it was shown that a decrease in the phosphorylation of a 36-kDa protein upon TCR engagement in hyporesponsive T lymphocytes correlates with a decreased membrane targeting of PLCγ1 and the Grb2/Sos complex, and an impaired activation of Ras (40, 41). The current data indicate that in SF T lymphocytes from RA patients this phosphoprotein represents the membrane-anchored 36-kDa adapter protein LAT. The expression of LAT is reduced by 50% in SF T lymphocytes from RA patients compared with T lymphocytes from healthy controls. However, the reduced expression is not likely to account for the deficient phosphorylation of LAT, because the level of LAT expression in SF T lymphocytes is approximately similar to the level of expression in PB T lymphocytes from RA patients, which demonstrate efficient phosphorylation of LAT upon TCR engagement. Furthermore, the deficient phosphorylation of LAT is not due to a defect in the kinase activity of ZAP-70, because this activity is intact in SF T lymphocytes. Intracellular immunofluorescence staining of LAT reveals that the impaired phosphorylation of LAT in SF T lymphocytes correlates with a cytoplasmic localization of LAT, whereas in T lymphocytes from healthy controls and PB T lymphocytes from RA patients, which exert intact LAT phosphorylation, LAT is exclusively expressed in the membrane. These data indicate that, due to the displacement of LAT from the cellular membrane, the membrane recruitment of ZAP-70 in SF T lymphocytes upon TCR engagement is ineffective in evoking the phosphorylation of LAT. Furthermore, the membrane displacement and the impaired phosphorylation of LAT in SF T lymphocytes also correlate with an impaired recruitment of PLCγ1 to the membrane through association with phosphorylated LAT. These data indicate that the disturbed subcellular localization of LAT accounts for the hyporesponsiveness displayed by the SF T lymphocytes through the abrogation of the TCR-induced signaling pathways.

The functioning of T lymphocytes is markedly influenced by alterations in the intracellular redox balance. Exposure to reactive oxygen radicals has been demonstrated to down-regulate the activity of T lymphocytes (42–44). In SF T lymphocytes from RA patients, a significant decrease of the intracellular GSH levels has been observed for T lymphocytes from HIV-infected individuals compared with other arthritic diseases. Only in patients with psoriatic arthritis have we so far observed a partial displacement of LAT from the membrane, but not in patients with spondylarthropathy or juvenile RA (our unpublished observations). Further studies are in progress to establish the disease-specificity of the membrane displacement of LAT.

Several hypotheses can be proposed to explain the displacement of LAT from the membrane. First, a posttranslational modification of LAT resulting in a conformational change could account for the membrane displacement of LAT. The primary functions of GSH include conserving the reduced state of the sulfhydryl groups of intracellular proteins to maintain the correct conformation of the proteins (16, 49). LAT contains four cysteine residues, of which two are within the N-terminal α-helix, which mediates the membrane localization of LAT, one is just proximal of the α-helix, and the fourth is present in the cytoplasmic part of the protein (22). It seems probable that in SF T lymphocytes from RA patients that contain decreased levels of the main antioxidant GSH, the sulfhydryl groups of LAT become oxidized and form disulfide bonds either intramolecular or intermolecular with small cytoplasmic sulfhydryl-containing proteins and hence interfere with the placement of the N-terminal α-helix within the cellular membrane. Second, a change in the cellular architecture could result in the membrane displacement of LAT. Oxidative stress has been reported to induce severe changes in the cytoskeletal structure (50–52). Several proteins involved in the TCR-mediated signaling pathways including the TCR ζ-chain, the guanine nucleotide exchange factor Vav, and SLP-76 are or upon TCR ligation become physically linked with components of the cytoskeleton (15, 53–55). It seems possible that LAT is also connected either directly or indirectly with the cytoskeleton, and any changes in the cytoskeletal structure due to oxidative stress therefore might somehow result in the displacement of LAT from the cellular membrane.

An important point of consideration is the role of functionally defective T lymphocytes in chronic inflammation. It is generally accepted that T lymphocytes contribute to the perpetuation of the disease in RA. Because SF T lymphocytes exert impaired responsiveness to TCR-mediated activation, it is clearly important to know what mechanism might be involved. In this respect it is worth note that despite suppressed cytokine expression and proliferation, SF T lymphocytes display an activated phenotype (1–3), which may be important for the activation of macrophages and fibroblasts via direct Ag-independent cell-cell contact. Chronically activated macrophages and fibroblasts produce matrix metalloproteinases and proinflammatory cytokines like TNF-α and IL-1, which may aggravate the disease (8, 10, 11). The induction of
oxidative stress through the stimulation of neutrophils by TNF-α thus creates a vicious circle, responsible for the chronicity of the disease. Evidence exists that the persistence of T lymphocytes in the chronically inflamed joints is a consequence of the active inhibition of T cell apoptosis by environmental factors (56). Recently, it has been shown that the expression of Fas ligand (FasL) is deficient in SF T lymphocytes from RA patients that express high levels of Fas (57). Because the intact TCR-signaling pathways, and especially the ZAP-70-mediated Ca2+ influx, are required to control the expression of FasL and the homeostasis of T lymphocytes through Fas/FasL-mediated apoptosis (58–60), the membrane displacement of LAT is likely to underlie this defect. Therefore, we propose that the treatment of RA patients with an antioxidant like NAC might contribute to the increased apoptosis of the SF T lymphocytes and eventually ameliorate the disease in RA.

In summary, our present findings indicate that the hyporesponsive state of the T lymphocytes in the SF of the inflamed joints of patients with RA results from the displacement of LAT from the plasma membrane due to the reduced intracellular GSH levels. The localization of LAT in the cytoplasm of the SF T lymphocytes interferes with its phosphorylation and subsequent association with other signaling proteins, and therefore abrogates the TCR-mediated signaling pathways.

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