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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

Sonja I. Gringhuis,1 Angela Leow, Ellen A. M. Papendrecht-van der Voort, Philip H. J. Remans, Ferdinand C. Breedveld, and Cornelis L. Verweij

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 hyporesponsiveness 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) 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 DL-buthionine (S,R)-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 that had previously been shown to play an important role in the TCR-mediated signaling pathways (22, 25–27). LAT is selectively expressed by T lymphocytes and NK cells, where it is localized in the plasma membrane through its N-terminal α-helical structure.
Several signaling proteins, including Grb2, the p85 subunit of phosphatidylinositol 3-kinase, Grb2-like accessory protein (Grap), and phospholipase Cγ (PLCγ) 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 heparin-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 resuspended 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 isolated 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 1:574; Santa Cruz Biotechnology), 1:35; 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% skin 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 (Amersham). 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 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 permeabilizing 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. CD16+ T-lymphocytes 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 Na₃VO₄), 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, 100 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1% Triton X-100), and finally twice with assay buffer (25 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10 mM MnCl_2, 1 mM DTT, 0.1% Triton X-100) and twice with PBS containing 5% BSA. 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 × 10^6/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 particularly, 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 using 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

**Table I. Clinical and demographic data of RA patients**

<table>
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<tr>
<th>No.</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Disease Duration (years)</th>
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<th>Experimentsb</th>
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*a d, Diclofenac; s, salazopyrine; CsA, cyclosporin A; h, hydroxchloroquine; p, prednisone; N, nonsteroidal antiinflammatory drug; M, methotrexate; l, leflunomide.

*b Numbers refer to figure numbers.
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 healthy controls was 85.2 ± 7.8 (mean ± SEM; n = 4). The kinase activity of ZAP-70 was ~3-fold lower in PB and SF T lymphocytes from RA patients compared with healthy controls (Fig. 2). 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 healthy controls was 85.2 ± 7.8 (mean ± SEM; n = 4). The kinase activity of ZAP-70 was ~3-fold lower in PB and SF T lymphocytes from RA patients compared with healthy controls (Fig. 2).

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healthy controls was enhanced ~2.2-fold ± 0.23 (mean ± 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-fold ± 0.16 (mean ± SEM; n = 3; p < 0.001) for PB T lymphocytes, 2.1-fold ± 0.07 (mean ± SEM; n = 3; p = 0.003) for SF 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 for PB T lymphocytes, p = 0.17 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. 4A). In PB T lymphocytes from RA patients, we detected LAT in the cytoplasm of the cell instead of the membrane (Fig. 4C). However, after 48 h of BSO treatment, LAT started to accumulate in the cytoplasm, although we also could detect membrane-associated LAT (Fig. 4D). 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. 4D). 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 with anti-CD3 and anti-CD28 (data not shown). T lymphocytes from healthy controls after 72 h of treatment with BSO were also labeled with monoclonal anti-CD3 Abs followed by staining with tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse Abs to check the integrity of the plasma membrane. The data are representative of five independent experiments.

Next, we wanted to determine whether increasing the intracellular GSH concentration would restore the membrane localization of LAT in SF T lymphocytes. The supplementation of SF T lymphocytes from RA patients with the antioxidant NAC, which is a precursor in the synthesis of GSH, has previously been shown to restore the intracellular GSH levels (13). Immunofluorescence staining of LAT in SF T lymphocytes that had been treated with NAC for 48 h showed that LAT was exclusively expressed in the plasma membrane (Fig. 6B). In SF T lymphocytes that were left untreated for 48 h, LAT was still present in the cytoplasm of the cells, excluding the possibility that LAT returns spontaneously to the membrane after 48 h of culture (Fig. 6A). These data denote that the subcellular localization of LAT is highly sensitive to the intracellular concentration of GSH.

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 SF 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 determine whether the modulation of the intracellular GSH levels would restore the association of PLCγ1 with LAT, we evaluated the ability of PLCγ1 to coimmunoprecipitate with LAT from whole-cell extracts of NAC-treated SF T lymphocytes. Indeed, after treatment of the SF T lymphocytes with NAC for 48 h, not only the phosphorylation of LAT but also the association of PLCγ1 with LAT upon TCR stimulation was restored (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,
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 z-chain, an aberrant pattern of protein tyrosine phosphorylation, and a block in the Ca^{2+} influx upon TCR activation, any of which could contribute to the hyporesponsiveness of the T lymphocytes (30–34).

The reduced expression of the TCR z-chain has been proposed as an underlying mechanism responsible for the negative regulation of the TCR signaling pathways. The decreased expression of the TCR z-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 z-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 when 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 nonobese 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 adaptor 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 is 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 exhibit 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 shown to correlate with the hyporesponsive state of these cells (13). A similar decrease in the intracellular GSH levels has been reported for T lymphocytes from HIV-infected individuals (45). Moreover, the hyporesponsiveness displayed by the SF T lymphocytes from RA patients clearly resembles the hyporesponsive state of the T lymphocytes and the reduced TCR ζ expression observed in aging T lymphocytes. Aging T lymphocytes contain severely reduced intracellular levels of antioxidants including GSH, which inhibits the phosphorylation of a 35- to 36-kDa protein, which is most likely LAT, the phosphorylation of PLCγ1, the generation of inositol 1,4,5-triphosphate, and the influx of Ca2+ upon TCR engagement. Increasing the intracellular GSH levels in aged T lymphocytes by either GSH or NAC treatment restores the generation of inositol 1,4,5-triphosphate and the Ca2+ influx in response to TCR ligation (46, 47). The data presented here indicate that redox balance alterations play a critical role in the abrogation of the cellular activation of the SF T lymphocytes in RA through the membrane displacement of LAT. The treatment of SF T lymphocytes with NAC not only restores the membrane localization of LAT, but also the phosphorylation of LAT upon TCR engagement and the subsequent association of PLCγ1 with phosphorylated LAT. By mimicking the situation of the SF T lymphocytes through the treatment of T lymphocytes from healthy controls with BSO, which depletes the intracellular GSH levels, we could induce the membrane displacement of LAT. Similar results were reported upon the treatment of T lymphocytes with N-ethylmaleimide, which induces the sulfhydryl oxidation of intracellular proteins. N-ethylmaleimide-treated T lymphocytes, the phosphorylation of a 35-kDa protein, which most likely represents LAT, upon TCR stimulation is inhibited. Also, the tyrosine phosphorylation of PLCγ1 is abrogated, probably as a result of the impaired membrane recruitment of PLCγ1 because the phosphorylation of LAT is also abrogated (48).

Preliminary data indicate that the membrane displacement of LAT due to decreased GSH levels is relatively specific for RA as 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 metalloproteinas 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, is 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 hence abrogates the TCR-mediated signaling pathways.

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