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Defective TCR-Mediated Signaling in Synovial T Cells in Rheumatoid Arthritis

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In rheumatoid arthritis (RA), the functional status of T cells is incompletely understood. Synovial T cells display phenotypic evidence of former activation, but there is poor production of T cell-derived cytokines in the synovium. In addition, synovial T cell proliferation upon mitogenic and antigenic stimulation was decreased compared with that in peripheral blood T cells. Moreover, previous reports revealed that early Ca²⁺ rises induced by TCR/CD3 stimulation were decreased in RA T cells compared with those in healthy controls. To investigate the molecular mechanisms of RA synovial T cell hyporesponsiveness, we analyzed the TCR/CD3-mediated protein tyrosine phosphorylation in RA peripheral blood and synovial fluid (SF) T cells. SF T cells exhibited a decreased overall tyrosine phosphorylation pattern upon stimulation. Most notably, the induction of phosphorylation of p38 was virtually absent. Moreover, we found that tyrosine phosphorylation of the TCR ζ-chain, one of the most proximal events in TCR signaling, is clearly diminished in RA SF T cells. The decrease in tyrosine phosphorylation was accompanied by a decrease in detectable levels of ζ-protein within synovial T cells. These results suggest that a defective TCR signaling underlies the hyporesponsiveness of synovial T cells in RA.

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Abbreviations used in this paper: RA, rheumatoid arthritis; CSH, glutathione; PB, peripheral blood; SF, synovial fluid; CIB, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service; HRP, horseradish peroxidase; GAM, goat anti-mouse; SFMC, synovial fluid mononuclear cells.

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Materials and Methods

Patients/cells

Patients enrolled in this study fulfilled the American College of Rheumatology revised criteria for RA (30). All patients used nonsteroidal anti-inflammatory drugs. Two patients in the biochemical study were treated additionally with salazopyrine and with azathioprine and low dose prednisone, respectively. Of five other patients in flow cytometric analysis, three were treated with methotrexate. PBMC and SF mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation and used immediately for biochemical and flow cytometric analysis.

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Antibodies

For proliferation studies, cross-linked anti-CD3 OKT3 mAb was used. For biochemistry, CD3 mAb (CLB-T3.4/1), horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig (CLB-GM17E), and horse anti-rabbit Ig (CLB-PK17E) Abs from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands) were used. Rabbit polyclonal anti-TCR-ζ Ab (98118) for immunoprecipitation of the TCR-ζ-chain were gifts from Dr. J.Borst (Dutch Cancer Institute, Amsterdam, The Netherlands). Anti-TCR-ζ mAb (6B10.2), used for Western blotting, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). For flow cytometric analysis, anti-TCR-ζ mAb (Coulter, Hialeah, FL), FITC-conjugated goat anti-mouse Ig (F(ab')2, GAM-FITC; Southern Biotechnology Associates, Birmingham, AL), and phycoerythrin-labeled anti-CD3 (Leu 4; Becton Dickinson, Mountain View, CA) were used. For immunohistochemistry, anti-CD3 mAb (Becton Dickinson), anti-TCR-ζ mAb (Coulter), HRP-GAM-polyclonal Ab (Dako, Glostrup, Denmark) and HRP-conjugated swine anti-goat polyclonal Ab were used.

T cell proliferation assays

Mononuclear cells (5 × 10^5/well) were cultured in anti-CD3 (OKT3)-coated 96-well flat-bottom plates (Greiner) in Iscove’s modified Dulbecco’s culture medium supplemented with 10% FCS. Proliferation was determined by a 16-h pulse with [3H]thymidine after 72 h of culture.

Biochemical analysis of TCR signaling

Cells were washed twice in HEPES solution (132 mM NaCl, 6 mM KCl, 1 mM MgSO_4, 1 mM CaCl_2, 1.2 mM KHPO_4, and 20 mM HEPES, pH 7.4, supplemented with 0.5% human serum albumin and 0.1% glucose) and kept on ice. Subsequently, the cells were incubated with HEPES buffer containing 10 µg/ml anti-CD3 mAb (CLB-T3.4/1) at 37°C for the indicated periods of time. Following activation the cells were rapidly pelleted and lysed in ice-cold immunoprecipitation buffer (1% Nonidet P-40; 0.01 M triethanolamine-HCl, pH 7.8; 0.15 M NaCl; 5 mM EDTA; 1 mM Na_3(p-tosyl)lysine chloromethyl ketone (TLCK); 0.02 mg/ml ovomucoid trypsin inhibitor; 1 mM PMSF; 0.02 mg/ml leupeptin; 0.4 mM vandamide; 10 mM NaF, 10 mM pyrophosphate; and 25 µM phenylarsine oxide). Nuclear debris was removed by centrifugation for 15 min at 13,000 rpm. Lysates were precleared with 60 µl of a 10% (v/v) suspension of protein A-CLAB Sepharose beads (Pharmacia, Uppsala, Sweden) coated with normal mouse Ig. Immunoprecipitation of phosphotyrosine or TCR-ζ proteins with specific Abs was conducted for 2 h. Immunoprecipitates were subjected to five washes with ice-cold immunoprecipitation buffer with detergent and resuspended in reduced sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham, Aylesbury, U.K.), and blotted with horseradish peroxidase (HRP)-conjugated swine anti-goat polyclonal antibody. Autoradiographs were performed with biotin-labeled tyramide (33) after the GAM Ab. HRP activity was detected using hydrogen peroxide as a substrate and aminoethylcarbazole (Sigma Chemical Co.) as a dye. Slides were counterstained with Mayer’s Hämalunlösung (Merck, Darmstadt, Germany) and mounted in Kaiser’s glycerol gelatin (Merck).

Flow cytometric analysis

PBMC and SFMC were fixed in 0.5% formaldehyde for 20 min at 4°C, washed twice in ice-cold PBS, and stored overnight at 4°C. Expression of ζ-chain was measured by an immunofluorescence assay as previously described (31), with minor modifications. In short, formaldehyde-fixed PBMC and SFMC were resuspended at a concentration of 5 × 10^6 cells/ml in PBS-BSA containing 10 µg digoxigenin/ml (Sigma Chemical Co., St. Louis, MO) as a permeabilizing agent and incubated on ice for 10 min, after which >95% of the cells were permeabilized as determined by trypan blue uptake. Next, cells were incubated in 1% normal goat serum for 5 min to block specific binding sites, washed, and incubated with the appropriate concentration of the TCR-ζ mAb (Coulter) for 30 min on ice. A negative control was incubated with an isotype-matched irrelevant Ab. After washing three times, cells were incubated with GAM-FITC (2 µg/ml) in PBS containing 0.05% Tween-20 for 30 min on ice. Next, free (F(ab')2) sites of cell-bound GAM-FITC were blocked using 1% normal mouse serum, whereafter staining was performed with anti-CD3-phycocerythrin (Leu 4) for 30 min. After washing, cells were subjected to flow cytometric analysis (FACScan, Becton Dickinson). Levels of CD3 and TCR-ζ are expressed as the median fluorescence intensity.

Immunohistochemistry

Stainings for TCR-ζ and CD3 were performed on frozen sections of synovial tissue from RA patients and of tonsillar tissue as controls, as described previously (32). Briefly, conventional staining was performed according to a three-step immunoperoxidase method. The primary Abs were incubated for 60 min. For control sections, irrelevant Abs were applied. GAM-HRP Ab was added for 30 min, followed by incubation with HRP-conjugated swine anti-goat Ab for another 30 min. Ultrasensitive immunohistochemical staining was performed using biotin-labeled tyramide (33) after the GAM Ab. HRP activity was detected using hydrogen peroxide as a substrate and aminoethylcarbazole (Sigma Chemical Co.) as a dye. Slides were counterstained with Mayer’s Hämalunlösung (Merck, Darmstadt, Germany) and mounted in Kaiser’s glycerol gelatin (Merck).

Results

RA SF T cells display an altered tyrosine phosphorylation pattern upon ligation of the TCR compared with PB T cells

The proliferative responses of SF T cells after TCR cross-linking were decreased compared with those of PB T cells from RA patients (18). The decrease in responsiveness of SF T cells could not be overcome by increasing doses of cross-linked CD3 mAb OKT3 (up to 10 µg/ml) and were not due to differences in kinetics upon stimulation (data not shown). To analyze the molecular basis for the difference in PB and SF T cell responsiveness, we examined protein tyrosine phosphorylation of RA PB and SF T cells immediately after TCR engagement. For this purpose, PB and SF mononuclear cells (PBMC and SFMC) were stimulated with CD3 mAb (CLB-T3.4/1; 10 µg/ml) for 3 min. RA PB T cells revealed no major differences in intensity and pattern of tyrosine phosphorylation upon TCR/CD3 ligation compared with PB T cells from healthy controls, although the levels of phosphorylation of a 29-kDa protein among PB T cell samples varied (Fig. 1, lanes A–D). In contrast, stimulated SF T cells exhibited a general reduction in tyrosine phosphoprotein levels. Strikingly, the p38 phosphoprotein was virtually absent after TCR/CD3 stimulation of SF T cells compared with both control and RA PB T cells (Fig. 1, lanes E and F).

However, some substrates, in particular the phosphoproteins with apparent molecular mass of 23 and 35 kDa, were similarly phosphorylated in PB and SF T cells. These data reveal that the decreased responsiveness of SF T cells upon anti-CD3 stimulation is accompanied by reduced CD3-induced tyrosine phosphorylation.

FIGURE 1. CD3-induced protein tyrosine phosphorylation in PB T cells from healthy controls (PB HC; lanes A and B) and in paired PB T cells (PB RA; lanes C and D) and SF T cells (SF RA; lanes E and F) from RA patients. Mononuclear cell fractions were analyzed in the absence (A, C, and E) and presence (B, D, and F) of anti-CD3 mAbs (10 µg/ml) at 37°C for 3 min. After lysis, phosphotyrosine proteins were immunoprecipitated and separated by 10% SDS-PAGE, transferred to nitrocellulose, and blotted with phosphotyrosine Abs. Lane G represents the final preclear step. Positions of prestained standard protein markers are indicated (M1 = X 10^-9). The migration positions of p23, p35, and p38 are indicated by arrows. Separate experiments with cells from other RA patients gave similar results.
Prolonged stimulation of SF T cells with CD3 mAb for up to 10 min could not overcome the decreased level of tyrosine phosphorylation (data not shown).

**TCR/CD3 stimulation fails to induce complete tyrosine phosphorylation of the TCR ζ-chain in SF T cells**

One of the earliest events of TCR-mediated signaling is tyrosine phosphorylation of the TCR ζ-chain. To study TCR ζ-chain tyrosine phosphorylation in the decreased responsiveness of RA SF T cells, we conducted phosphotyrosine Ab immunoblotting of TCR ζ protein immunoprecipitates from lysates of PBMC from healthy controls and of PBMC and SFMC from RA patients. Ligation of the TCR for 1 to 10 min is sufficient for phosphorylation of TCR ζ in peripheral T cells from healthy individuals as shown by induction of the ζ-chain phosphoproteins pp18 and pp21 (Fig. 2A). Additionally, TCR/CD3 stimulation of RA PB T cells induced detectable amounts of TCR ζ-chain phosphoproteins pp18 and pp21 (Fig. 2C and data not shown). In contrast, SF T cells showed a diminished TCR ζ-chain phosphorylation, with a selective decrease in the appearance of the slower migrating pp21 form of phosphorylated TCR ζ (Fig. 2B). Prolonged ligation of TCR/CD3 for up to 20 min could not increase levels of pp21 in RA SF T cells (Fig. 2C).

**Synovial T cells express strongly decreased levels of ζ protein**

Performance of TCR ζ Ab protein immunoblotting revealed that levels of ζ protein in SF T cells were reduced in total cell lysates compared with those in PB T cells (data not shown). To further study TCR ζ-chain expression within PB and SF T cells of RA patients, we quantitated ζ protein expression on permeabilized CD3+ T cells by flow cytometric analysis. Cell surface expression of CD3 was comparable between paired RA SF and PB T cells (mean fluorescence intensity ± SD, 1048 ± 479 and 940 ± 398 for PB and SF T cells, respectively) of RA patients. In contrast, levels of ζ protein in SF T cells were lower than those in PB T cells from RA patients (Fig. 3 and Table I; n = 5; p < 0.05). Diminished expression of TCR ζ correlated with decreased proliferative responses to cross-linked CD3 mAb (Fig. 4).

Next, we investigated the expression of TCR ζ in frozen sections of synovial tissue from RA patients (Fig. 5). In a conventional three-step immunostaining protocol, TCR ζ could hardly be detected in synovial tissue, whereas clear staining was observed in tonsillar tissue, which served as a positive control for the TCR ζ Ab (Fig. 5, A and B). However, the use of an ultrasensitive immunohistochemical technique with biotinylated tyramide revealed that the ζ protein was present in the synovium (Fig. 5C). Staining
Table I. Decreased levels of TCR-ζ protein in SF T cells

<table>
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<tr>
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<th>PB CD3-ζ</th>
<th>SF CD3-ζ</th>
<th>PB CD3-ζ</th>
</tr>
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<tbody>
<tr>
<td>RA (n = 5)</td>
<td>9.0</td>
<td>5.3*</td>
<td>10.7</td>
</tr>
<tr>
<td>SF (n = 5)</td>
<td>5.2-16.6</td>
<td>2.6-11.5</td>
<td>7.5-15.4</td>
</tr>
<tr>
<td>HC (n = 5)</td>
<td>4.5</td>
<td>3.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* HC = healthy control.
*MFI = mean fluorescence intensity; MFI ratio = ratio between TCR-ζ MFI and MFI levels of negative control.
* p < 0.05 for RA SF T cells compared with RA PB T cells as determined by the Wilcoxon signed rank test.

for CD3 by the conventional method revealed approximately similar intensities for synovial and tonsillar tissues (Fig. 5, D and E).

Discussion

Here we demonstrate that the hyporesponsive state of synovial T cells from RA patients directly correlates with impaired TCR-mediated proximal signaling events. Biochemical analysis revealed decreased overall tyrosine phosphorylation upon TCR engagement in SF T cells. In addition, virtually no detectable phosphorylated p38 was observed upon TCR/CD3 ligation. Moreover, phosphorylation of the TCR-ζ chain was severely diminished in SF T cells. The defective phosphorylation of TCR-ζ in SF T cells was accompanied by diminished levels of detectable ζ protein.

Interestingly, decreased phosphorylation of a 38-kDa protein and TCR-ζ after TCR/CD3 ligation has recently been described for T cells in an anergic state (34–36). Recent studies have suggested that a disrupted tyrosine phosphorylation network may be responsible for nonresponsiveness to Ag in tolerant T cells. Specifically, defective phosphorylation of a 38-kDa protein was shown to correlate with functional anergy in vitro and in vivo tolerant CD4 T cells (34). Although the identity of the 38-kDa protein is still unknown, it is possible that p38, which we describe here, is identical with the 38-kDa substrate affected in anergic T cells. In addition, the appearance of the slower migrating form of phosphorylated TCR-ζ, pp21, was specifically decreased compared with pp18 upon TCR ligation in anergic T cells (37, 38). In view of these data, our findings indicate that the hyporesponsive state of RA SF T cells is reminiscent of the features of anergy as previously proposed by others (39).

In a former study, we found a correlation between hyporesponsiveness of SF T cells in RA and decreased intracellular levels of the antioxidant GSH (18). These data are in line with current thinking that conditions of chronic oxidative stress markedly inhibit SF T cell function. Similar observations have been reported for T cells from HIV-infected individuals, in whom lowering intracellular levels of GSH was shown to correlate directly with reduced T cell function. Similar observations have been reported for T cells from HIV-infected individuals, in whom lowering intracellular levels of GSH was shown to correlate directly with reduced T cell function.

In conclusion, evidence is provided that T cells at the site of inflammation in RA patients display an altered TCR structure and signaling capacity. The biochemical and functional status of SF T

![FIGURE 4. Correlation between TCR-ζ expression (depicted as the ratio between the median fluorescence intensity (MFI) of positive staining and the negative control) and proliferative responses to cross-linked CD3 mAbs (depicted as counts per minute) in paired PB and SF T cells from two RA patients. Percentages of CD3+ T cells were 39% in both PB1 and SF1, and 53% and 66% in PB2 and SF2, respectively.](image-url)
The three-step peroxidase technique, and in synovial tissue from the same patient using an ultrasensitive method, in tonsillar (E) tissues by the conventional method. Counterstaining of sections was performed with Mayer's Hämalaunlösung (magnification, ×250). This result is representative of separate experiments with synovial tissue sections from three RA patients.

Acknowledgments
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