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Defective TCR-Mediated Signaling in Synovial T Cells in Rheumatoid Arthritis¹

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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 Ca^{2+} 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. *The Journal of Immunology*, 1997, 159: 2973–2978.

Rheumatoid arthritis (RA)³ is a chronic inflammatory joint disease categorized as an autoimmune disorder in which autoaggressive T cells orchestrate the local inflammatory response. However, the pathogenic role of T cells in RA is incompletely understood. Although high numbers of primed CD45RO⁺ T cells expressing an activated phenotype accumulate in the synovium (1–8), these T cells appear to be functionally deficient. This hyporesponsiveness is reflected by the absence or low levels of T cell-derived cytokines such as IFN- γ and IL-2 at the site of inflammation (9–12). Furthermore, RA T cells show depressed in vitro responses to recall Ags (13, 14) and mitogenic stimulation (15–18), poor helper function (19), and reduced autologous mixed lymphocyte responses (20, 21).

Ag-dependent T cell activation is guided by signals transmitted via the TCR. Ligation of the TCR results in the activation of several receptor-associated protein tyrosine kinases, leading to tyrosine phosphorylation of specific protein substrates in the T cell (22, 23). One of the earliest biochemical events occurring after TCR engagement is the phosphorylation of the TCR ζ -chain. The TCR ζ -chain contains three immunoreceptor tyrosine-based activation motifs (24, 25), which, when phosphorylated, serve as

docking sites for SH2 domain-containing signaling proteins. The ZAP-70 protein tyrosine kinase (26) is recruited via its SH2 domains to the tyrosine-phosphorylated TCR ζ -chain (25) and is thought to lead to phosphorylation and activation of phospholipase C γ 1. Phospholipase C γ 1 activates the phosphatidylinositol pathway, which induces a rise in intracellular Ca^{2+} and the activation of protein kinase C (22, 23, 27).

A recent study found that T cells from RA patients, compared with those from healthy individuals, display reduced intracellular Ca^{2+} responses upon CD3 stimulation, although surface expression of TCR/CD3 was normal (28). In a previous study, we showed that decreased intracellular levels of the antioxidant glutathione (GSH) are associated with hyporesponsiveness of SF T cells in RA (18). Interestingly, decreased GSH levels within T cells have been shown to be accompanied by diminished Ca^{2+} responses (29). Together, these data suggest that alterations in intracellular redox balance might play a role in proximal signaling and subsequently in the hyporesponsive state of SF T cells.

To investigate the molecular basis of this hyporesponsiveness of RA T cells, we analyzed the TCR-mediated tyrosine phosphorylation in peripheral blood (PB) T cells from healthy individuals and in PB and SF T cells from RA patients. Our data suggest that the hyporesponsive state of synovial T cells in RA is a direct consequence of impaired signal transduction via the TCR/CD3 complex.

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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² Abbreviations used in this paper: RA, rheumatoid arthritis; GSH, glutathione; PB, peripheral blood; SF, synovial fluid; CLB, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service; HRP, horseradish peroxidase; GAM, goat anti-mouse; SFMC, synovial fluid mononuclear cells.

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 (5×10^4 /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 MgSO₄, 1 mM CaCl₂, 1.2 mM K₂HPO₄, 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 μ g/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 N^ε-(*p*-tosyl)lysine chloromethyl ketone (TLCK); 0.02 mg/ml ovomucoid trypsin inhibitor; 1 mM PMSF; 0.02 mg/ml leupeptin; 0.4 mM vanadate; 10 mM NaF; 10 mM pyrophosphate; and 25 μ M phenylarsine oxide). Nuclear debris was removed by centrifugation for 15 min at 13,000 rpm. Lysates were precleared with 60 μ l of a 10% (v/v) suspension of protein A-CL4B 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 reducing sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, Aylesbury, U.K.). After blocking with 5% low fat milk suspension, reactivity with specific Abs was detected by enhanced chemiluminescence (Amersham) to visualize the bound proteins. Autoradiography was performed with Kodak X-OMAT S films (Eastman Kodak, Rochester, NY).

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 μ g digitonin/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 aspecific binding sites, washed, and incubated with an optimal 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 μ g/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-phycoerythrin (Leu 4) for 30 min. After washing, cells were subjected to flow cytometric analysis (FACSscan, 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 ac-

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 μ g/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 ($M_r \times 10^{-3}$). The migration positions of p23, p35, and p38 are indicated by arrows. Separate experiments with cells from two other RA patients gave similar results.

cording 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 Hämalaunlösung (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 μ g/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 μ g/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 2. A, Induction of tyrosine phosphorylation of TCR- ζ in PB T cells from a healthy control in the absence and the presence of anti-CD3 mAbs (10 μ g/ml) for 1 to 10 min. Anti-TCR- ζ immunoprecipitates of cell lysates were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and stained with anti-phosphotyrosine mAb. Lane E represents the final preclear step. B, Tyrosine phosphorylation of TCR- ζ in T cells from a healthy control (PB HC; lanes A and B) and RA SF T cells (SF RA; lanes C and D). C, TCR- ζ tyrosine phosphorylation of RA PB T cells (lanes A-C) and RA SF T cells (lanes D-G) from the same patient after prolonged stimulation with anti-CD3 mAb.

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

FIGURE 3. Flow cytometric analysis of TCR- ζ levels in PB T cells from a healthy control (PB HC) and in paired PB (PB RA) and SF (SF RA) T cells from an RA patient. Permeabilized CD3⁺ cells were analyzed for TCR- ζ expression. Shown is a representative experiment of five independent experiments performed. CON, negative control.

(mean fluorescence intensity $\pm$ 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

	PB CD3- ζ RA (n = 5)	SF CD3- ζ RA (n = 5)	PB CD3- ζ HC ^a (n = 5)
Mean MFI ratio ^b	9.0	5.3*	10.7
Range	5.2–16.6	2.6–11.5	7.5–15.4
SD	4.5	3.6	3.1

^a HC = healthy control.^b 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 *in vitro* and *in vivo* tolerized 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 responses (40–42). In analogy with our data, the signaling function of TCR/CD3 in T cells from HIV-infected individuals was impaired, with concomitant loss of TCR- ζ expression. Moreover, it was demonstrated that reduced TCR- ζ correlated with impaired T cell responses and Ca^{2+} mobilization (43, 44). These observations suggested a close relationship between oxidative stress and TCR- ζ down-regulation. Recently, further support for this hypothesis was provided by Kono et al. (45), who reported that reactive oxygen intermediates inhibit the effector function of melanoma-specific CTL and induce down-regulation of TCR- ζ .

TCR- ζ has been shown to play a crucial role in Ag-specific T cell activation and is believed to be the limiting factor in the assembly and surface expression of the TCR/CD3 complex (46–49). Surprisingly, despite the significant decrease in ζ protein levels,

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.

cell surface expression of the CD3 complex on SF T cells falls in the normal range. Several possible mechanisms can be proposed to explain the diminished levels of TCR ζ -chain, while maintaining normal TCR/CD3 surface expression. Decreased levels of detectable ζ protein could be due to loss of TCR- ζ as a consequence of either inhibition of synthesis or increased turnover. Based on studies with hyporesponsive T cells from tumor-bearing mice (50), we initially hypothesized that the TCR ζ -chain might be replaced by Fc ϵ R γ in the TCR/CD3 complex of SF T cells. However, preliminary data obtained by flow cytometric analysis with Fc ϵ R γ mAb did not provide support for this possibility (data not shown). Alternatively, post-translational modification of the TCR- ζ protein may underlie the diminished detection of TCR- ζ in SF T cells. Such a mechanism is proposed for decreased detection of the TCR/CD3-induced signaling proteins in T cells from HIV-infected patients, which are believed to undergo a conformational change or a biochemical modification, resulting in epitope loss, under conditions of an altered intracellular sulfhydryl status. However, since the ζ -chain does not contain intracellular cysteine residues, the loss of detectable TCR- ζ due to a similar mechanism is not expected (43). Moreover, the possibility of epitope loss is unlikely, because we observed diminished TCR- ζ in SF T cells using two different Abs (Table I) (our unpublished observations). Further study of TCR composition is required to define the structural alterations of the TCR/CD3 complex in SF T cells.

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 5. Immunohistochemical staining of TCR- ζ in rheumatoid synovial (A) and, as a control, tonsillar (B) tissues using a conventional three-step peroxidase technique, and in synovial tissue from the same patient using an ultrasensitive method (C). CD3 staining of synovial (D) and tonsillar (E) tissues by the conventional method. Counterstaining of sections was performed with Mayer's Hämalaunlösung (magnification, $\times 250$). This result is representative of separate experiments with synovial tissue sections from three RA patients.

cells are reminiscent of the unresponsive state of T cells in conditions of immunodeficiency such as AIDS and cancer and of features of anergic T cells. Furthermore, the alterations in TCR structure are likely to be closely related to conditions of chronic oxidative stress. The significance of the signaling defects observed in RA T cells in the pathogenesis of disease is unclear. In view of this question it will be informative to relate the functional status of RA T cells to both the laboratory and clinical parameters of disease.

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