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Phosphorylated ERM Is Responsible for Increased T Cell Polarization, Adhesion, and Migration in Patients with Systemic Lupus Erythematosus¹,²

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Systemic lupus erythematosus (SLE) is an autoimmune/inflammatory disease characterized by autoantibody production and abnormal T cells that infiltrate tissues through not well-known mechanisms. We report that SLE T lymphocytes display increased levels of CD44, ezrin, radixin, and moesin (ERM) phosphorylation, stronger actin polymerization, higher polar cap formation, and enhanced adhesion and chemotactic migration compared with T cells from patients with rheumatoid arthritis and normal individuals. Silencing of CD44 by CD44 small interfering RNA in SLE T cells inhibited significantly their ability to adhere and migrate as did treatment with Rho kinase and actin polymerization inhibitors. Forced expression of T567D-ezrin, a phosphorylation-mimic form, enhanced remarkably the adhesion and migration rate of normal T cells. Anti-CD3/TCR autoantibodies present in SLE sera caused increased ERM phosphorylation, adhesion, and migration in normal T cells. pERM and CD44 are highly expressed in T cells infiltrating in the kidneys of patients with lupus nephritis. These data prove that increased ERM phosphorylation represents a key molecular abnormality that guides T cell adhesion and migration in SLE patients. *The Journal of Immunology, 2007, 178: 1938–1947.

Human systemic lupus erythematosus (SLE) is characterized by the loss of immune tolerance to multiple self-antigens, production of various autoantibodies, and chronic tissue inflammation associated with extensive infiltration of T lymphocytes into multiple organs including skin, kidney, brain, and joints, which results in tissue injury and dysfunction. Entry of T cells into inflamed tissues is an active, multistep process that requires morphological changes (polarization), making the transition from a freely circulating cell to a cell that adheres to the endothelium and subsequently migrates into tissues. Phosphorylation of ezrin, radixin, and moesin (ERM) threonine residues results in cell polarization and formation of the uropod (2). Adhesion molecules, such as CD44, concentrate at the uropod of lymphocytes, enabling them to adhere to endothelial cells (3–5). CD44 is a transmembrane protein that is important for lymphocyte adhesion to endothelial cells that express its principal ligand, hyaluronic acid (HA) (6). At the opposite site of the cell dynamic, F-actin is concentrated and provides the force of migration, whereas chemokine receptors are responsible for the direction of the movement (2).

During inflammation, CD44 expression is up-regulated in T cells and parenchymal cells (7, 8). CD4⁺CD8⁻ T cells, which are expanded in the autoimmunity-prone MRL/Mp-lpr (MRL-lpr) mice, express increased amounts of CD44 (9). T lymphocytes from patients with SLE and other inflammatory diseases display increased ability to bind to HA-covered membranes (10). Treatment of T cells with an anti-CD3 Ab up-regulates CD44 expression in T cells in vitro and in vivo (11, 12), and cross-linking of CD44 enhances signal transduction through the TCR-CD3 complex (13).

Organization of actin and polarization of membrane receptors is mainly regulated by ERM proteins that connect adhesion molecules with cortical F-actin (14). Activation of ERM involves phosphorylation of specific carboxyl-terminal threonine residues (Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ of moesin) (14) through several serine/threonine kinases including Rho kinase (ROCK) and protein kinase C (PKC) (15, 16). It has not yet been shown whether ERM activation is involved in T cell infiltration in human chronic inflammatory diseases, although a recent study showed that forced expression of active ezrin protein augmented cell migration in a mouse lymphoma T cell line (2).

Although it is known that T cells infiltrate inflamed tissues in SLE patients, the mechanisms underlying the infiltration process from the blood stream to inflamed sites are completely unknown. We report in this study that SLE T cells display increased ERM phosphorylation, actin polymerization, cell polarization, adhesion, and migration. Forced expression of a phosphomimetic ezrin into normal T cells confers a SLE T cell phenotype in terms of adhesion and migration. Also, treatment of normal T cells with SLE IgG, which contains anti-CD3/TCR autoantibodies, results in increased ERM phosphorylation. Finally, T cells that infiltrate inflamed SLE kidneys display similar features to the peripheral blood T cells. Our work suggests the use of biologics that block the ability of T cells to migrate to tissues to limit pathology.
Materials and Methods

Patients and controls

Thirty-seven SLE patients fulfilling the American College of Rheumatology revised classification criteria (17) for SLE were studied. All patients (36 females and 1 male; mean age, 44 years old; range, 22–73 years) had SLE disease activity index scores ranging from 0 to 102. Six patients with rheumatoid arthritis (RA) were analyzed in this study. Prednisone was discontinued at least 24 h before venipuncture. Forty-six healthy women served as controls. Studies and informed consent forms and processes had been approved by the involved institutions.

T cells

Peripheral blood T lymphocyte-enriched populations were isolated from SLE patients or controls using the RosetteSep T cell purification kit (StemCell Technologies). More than 95% of the purified cells were CD3 positive as determined by immunofluorescent staining.

Abs and reagents

The following Abs or fluorescent probes were used in this study: anti-CD44 (R&D Systems), anti-p34-Arc (Upstate Biotechnology), anti-CD4 and HRP-anti-mouse/rabbit IgG (Santa Cruz Biotechnology), anti-CD3 (BD Biosciences), Alexa Fluor 488/594 goat anti-mouse/rabbit IgG, anti-CD3, anti-GFP, ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Molecular Probes), anti-phospho-ERM (Cell Signaling Technology), rhodamine-conjugated phallolidin, cholera toxin B-FITC (Sigma–Aldrich), and Y27632, cytochalasin D (CytD), and bisindolylmaleimide I (Bis; Calbiochem). Alternative anti-CD3 Ab was purchased from Ortho-McNeal Pharmaceuticals. pEGFP-N1 was a gift from X. Zhan (University of Maryland, Baltimore, MD), Expression vector for EGFP-tagged human wild-type (WT) ezrin, T567D, and T567A ezrin were provided by M. Arpin (Institute Curie, Paris, France) and C. Khanna (National Institutes of Health, Bethesda, MD).

Transfection of primary T cells

Cells (5 × 10⁶) were transfected with 10 μg of plasmids or with 25 nM human CD44 small interfering RNA (siRNA; Thermo Fisher Scientific) by the Nucleofector reagent according to the manufacturer’s protocol (Amaxa Biosystems) as described before. The nonspecific control siRNA (D-001206-09-05) was also purchased from Dharmacon. Cell viability remained above 90% after 16–24 h of transfection.

Serum preparation and treatment

Sera were collected and frozen at −80°C until use. Sera were inactivated by incubation at 56°C for 30 min and centrifuged before use. Normal T cells were transfected with 10–100% normal sera or SLE sera for 5 min, 1.5 h, 5 h, 22 h, and 48 h at 37°C. Cell lysates were prepared after incubation.

Serum fractions and treatment

SLE or normal sera were fractioned by a ProteoExtract IgG Removal Kit (Calbiochem) as described previously (18). In brief, sera were diluted 1:10 into binding buffer and then loaded onto the column that retains IgG. After collection of the flow-through, the column was thoroughly washed with the same binding buffer before the bound IgG was eluted by 10 mM glycine. The eluate was immediately mixed with 10 mM Tris (pH 8.0) to neutralize the pH. The primary fraction was then applied on SDS gel electrophoresis, followed by staining with Gelcode Blue (Pierce). Normal primary T cells (5 × 10⁶) were cultured for 5 min at 37°C in RPMI 1640 medium containing 10–100% of sera and 2 μg/ml sera IgG. The cells were harvested and lysed in a radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 2 mM Na3VO4, and protease inhibitor mixture; Roche).

Anti-CD3/TCR autoantibodies absorption and T cell treatment

One hundred microliters of SLE or normal sera was absorbed for 60 min at 37°C, and then incubated another 60 min at 4°C on a 1 × 10⁶ of TCR-positive Jurkat T cells (American Type Culture Collection) or the TCR-negative Jurkat T cell subline J.E.M. T3.3 (a gift from A. Weiss, University of California, San Francisco, CA). The absorbed sera were centrifuged before use. A total of 5 × 10⁶ normal T lymphocytes were cultured for 5 min at 37°C in RPMI 1640 medium containing 10% of the absorbed sera and lysed in RIPA buffer.

Immunoprecipitation

Cells (5–10 × 10⁶) were lysed with RIPA buffer and the soluble fractions were immunoprecipitated with Abs plus 40 μl of protein A/G beads. Protein A/G-bound protein complexes were washed with lysis buffer three times and eluted by boiling in sample buffer and for SDS gels. The precipitated proteins were analyzed by Western blotting.

Western blotting

The cell lysates were separated in SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in TBST buffer for 1 h, and then incubated with primary Abs for 1 h, followed by incubation with appropriate HRP-conjugated secondary Abs for 1 h. Specific bands were visualized by an ECL method (Amersham Biosciences).

Immunofluorescent microscopy

For immunostaining, cells (5 × 10⁵) were placed on a poly-L-lysine-coated coverslip and incubated for 30 min at 37°C. Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 2% BSA for 30 min. Cells were stained with Abs for 60 min. Stained cells were mounted with ProLong Gold antifade solution containing 4,6-diamidino-2-phenylindole and observed with a confocal laser scanning microscope (Radiance 2100; Bio-Rad). Obtained digital images were processed by Image J (1.32j; NIH Image).

Adhesion assay

Cells (5 × 10⁵) suspended in 300 μl of serum-free medium were placed into a 24-well plate coated with 2 mg/ml HA (Sigma–Aldrich) in PBS and incubated for 15, 30, and 60 min at 37°C. After incubation, the plate was rinsed with PBS three times and then adhered cells were counted under a microscope.

Chemotaxis assay

A cell chemotaxis assay was performed in Transwell chambers (6.5-mm diameter, 5-μm pore size; Costar) as described before. In brief, cells (5 × 10⁵) in 100 μl of serum-free medium were seeded onto a HA-coated filter of Transwell apparatus and incubated for 30 min at 37°C. Then, 600 μl of serum-free medium in the presence of CXCL12 (80 ng/ml; R&D Systems) was added into the lower chamber and cultured for 2 h at 37°C. Alternatively, cells were pretreated with 20 μM Y27632, 0.5 μM Bis and 20 μM CyD for 30 min. The cells migrating into lower chamber were counted. All the samples were in duplicate.

Histological and immunofluorescent analysis of human lupus nephritis

Four patients fulfilling the American College of Rheumatology criteria for the classification of SLE were included in this study. In addition, two patients with renal allografts from the same center were studied. SLE kidney samples were obtained by biopsy for diagnostic purposes. Biopsy kidney tissue from patients with allograft rejection was used as control. Renal tissue was snap-frozen to −70°C, and sections were cut with a cryostat and fixed in 4% paraformaldehyde. The primary Abs of anti-CD3 mAb, anti-CD44 mAb, and polyclonal rabbit anti-pERM were incubated with the kidney sections for 1 h at room temperature and, after washing, the appropriate secondary Abs were labeled with Alexa Fluor 488 and 594, and were incubated with sections for another 1 h. After washing, the sections were visualized under the confocal laser scanning microscope with ×60 objective.

Statistical analysis

Statistical analysis was done with the paired Student’s t test and Prism 3.0 software (GraphPad). Data are represented as mean ± SEM.

Results

SLE T cells exhibit increased adhesion and chemotactic migration

Histological studies have revealed extensive infiltration of T cells at inflamed tissues from SLE patients (19), indicating that peripheral blood T cells display an increased propensity to adhere to endothelial cells and migrate into tissues. We examined adhesion and migration function of T cells from 10 SLE patients, 9 healthy individuals, and 4 patients with RA. SLE T cells exhibited a markedly higher ability to adhere to HA-coated membranes at 30 min
FIGURE 1. SLE T lymphocytes display a higher ability to adhere and migrate in response to chemotactic factors than normal and RA T cells. A. Purified T lymphocytes (5 × 10⁵) from 10 patients with SLE, 9 healthy controls, and 4 patients with RA were seeded on a 24-well plate precoated with HA and incubated in RPMI 1640 medium for 15, 30, and 60 min at 37°C. Attached cells on the plate were counted under a phase-contrast microscope at ×100 magnification. B. Purified T cells (5 × 10⁵) from 11 patients with SLE, 10 healthy controls, and 5 patients with RA were plated on the top chamber of a Transwell apparatus and incubated with serum-free RPMI 1640 medium for 30 min at 37°C. Then, 600 μl of RPMI 1640 supplemented with 80 ng/ml CXCL12 was added into the lower chamber and cultured for 2 h at 37°C. The cells from the upper chamber that migrated to the lower chamber were counted under a phase-contrast microscope at ×100 magnification. **, p < 0.01 for SLE T cells compared with normal T cells.

FIGURE 2. T lymphocytes from SLE patients display increased expression of CD44 and p-Thr-ERM expression and polar cap formation

It is known that high CD44 expression contributes to the pathophysiology of chronic inflammation and autoimmune diseases (7–9). To understand the increased propensity of SLE T cells to adhere and migrate, we purified peripheral blood T cells from 11 patients with SLE, 11 healthy controls, and 6 patients with RA and determined the expressions of CD44 by Western blotting. SLE T lymphocytes exhibited a higher expression of CD44 compared with that of T cells from normal controls (ratio of CD44:β-actin ± SEM: normal, 0.67 ± 0.11; SLE, 1.00 ± 0.1; p = 0.0357; Fig. 2, A and B). Levels of CD44 in T cells from patients with RA (0.81 ± 0.04) were intermediate between SLE and normal controls.

Because CD44 requires ERM phosphorylation to transfer the signal inside the cell, we determined the expression of pERM in T lymphocytes and found increased expression of pERM in SLE T cells compared with normal and RA T cells (ratio of pERM:β-actin ± SEM: normal, 0.21 ± 0.05; RA, 0.14 ± 0.06; SLE, 0.70 ± 0.16; p (normal vs SLE) = 0.0108; p (RA vs SLE) = 0.0226; Fig. 2, A and C). β-actin and ezrin expression was comparable between

FIGURE 3. T lymphocytes from SLE patients display increased expression of CD44 and pERM and increased formation of polar caps and lipid rafts

A. T cells were lysed and immunoblotted with anti-CD44, anti-pERM, anti-β-actin, or anti-ezrin Abs to determine CD44 and pERM expression. B and C. CD44 and pERM were quantified by densitometry and the ratio over β-actin was calculated for each sample. Cumulative data from 10 normal controls, 6 RA, and 11 SLE patients are shown. D. T cells from normal controls, RA, and SLE patients were fixed and stained with anti-CD44, pERM, p34, falloidin, and cholera toxin as described in Materials and Methods. E. Polar cap formation as shown in D was quantified by measuring the percentage of cells displaying cellular caps and the cumulative data from 1 normal control, 5 RA, and 11 SLE are shown. *, p < 0.05, **, p < 0.01 compared with normal group. Bars (D), 5 μm. DAPI, 4',6'-diamidino-2-phenylindole.
T cells from the three groups. These results indicate that the enhanced expression of pERM and CD44 in T lymphocytes from SLE contributes to increased cell adhesion and migration. There was no association between CD44 or pERM expression and SLE disease activity, treatment, age, and ethnicity.

Adhesion to the endothelium and the subsequent transendothelial migration of T lymphocytes are highly dynamic processes that are characterized by a polarized cell shape (3, 4). We used confocal microscopy to determine the localization and distribution pattern of CD44, pERM, and F-actin in human primary T cells. As shown in Fig. 2D, CD44 and F-actin appeared to be uniformly distributed inside the membrane of T cells from normal and RA controls. No apparent accumulation and colocalization at the cell membrane between pERM and CD44, pERM, and F-actin, were observed in control and RA T cells. In contrast, T cells from SLE patients demonstrated increased amounts of pERM, CD44, and F-actin and, more importantly, pERM, CD44, and F-actin were enriched and colocalized at the polar cap of SLE T lymphocytes. SLE T cells displayed significantly more polar caps than normal or RA T cells (percentage of cells forming polar caps ± SEM: normal, 18.60 ± 1.82; RA, 22.38 ± 3.08; SLE, 48.30 ± 3.50; p (normal vs SLE) < 0.0001; p (RA vs SLE) < 0.0001; Fig. 2E). These findings indicate that there are early typically polarized morphological changes and colocalization of CD44, F-actin, and pERM in SLE T cells.

Recent studies have shown that expression of lipid rafts is increased in T cells from patients with SLE (20, 21). We used confocal microscopy to investigate the association of CD44 and pERM with GM1 ganglioside-defined lipid rafts in SLE T cells. Fig. 2D confirms the recorded (20) presence of aggregated lipid rafts on the surface membrane of SLE T cells, which in this study are shown to contain increased amounts of CD44 and pERM.

ROCK activation and actin polymerization are required for polar cap formation, adhesion, and migration in SLE T cells

Because ROCK and PKC phosphorylate ERM (15, 16), we determined the effect of Y27632 (a ROCK-specific inhibitor) and Bis (a PKC inhibitor) on the polar cap formation and distribution of CD44 and pERM in SLE T cells. Treatment of SLE T cells with the ROCK inhibitor, but not the PKC inhibitor, significantly decreased polar cap formation (percentage of cells forming polar caps ± SEM: control treated, 49.00 ± 3.7; Y27632 treated, 22.48 ± 3.40; p = 0.0019; Fig. 3, A and B). Concomitantly, the ROCK, but not the PKC inhibitor, disrupted ERM phosphorylation as well as association between CD44 and pERM (Fig. 3, A and C).

Actin polymerization, mediated by the actin-related protein 2/3 (arp2/3) complex, is required for polar cap formation in a mouse lymphoma cell line (2). To determine the role of actin polymerization in polar cap formation in SLE T cells, we studied the distribution of p34 (a subunit of arp2/3) on the surface membrane. We demonstrate that p34 and CD44 colocalize at the polar caps of SLE T cells (Fig. 2D). Treatment of these cells with the actin polymerization inhibitor CytD led to the disruption of polar cap formation and the uniform distribution of CD44, pERM, and F-actin (Fig. 3, A and B). Fig. 3, D and E, show that ROCK and actin polymerization inhibitors, but not the PKC inhibitor, limited significantly the adhesion (adhered cells/visual field ± SEM: control treated, 266.17 ± 29.42; Y27632 treated, 114.67 ± 13.21; CytD treated, 90.67 ± 8.10; Bis treated, 257 ± 39.19; p (control vs Y27632) = 0.0008; p (control vs CytD) = 0.0002; p (control vs Bis) = 0.83) and migration of SLE T cells (migrated cells (105) ± SEM: control treated, 2.74 ± 0.54; Y27632 treated, 1.27 ± 0.18; CytD treated, 0.88 ± 0.11; Bis treated, 2.78 ± 0.50; p (control vs Y27632) = 0.0268; p (control vs CytD) = 0.0067; p (control vs Bis) = 0.9557). These observations suggest that ROCK-mediated actin polymerization is responsible for increased cap formation, adhesion, and migration of SLE T lymphocytes.

Silencing of CD44 expression by siRNA inhibits adhesion and migration of SLE T cells

To confirm the specific role of CD44 in the adhesion and migration of SLE T cells, we first determined the expression of CD44 after CD44 siRNA transfection by immunoblot (IB) analysis. CD44 expression was effectively silenced (~70%) in cells treated with CD44 siRNA compared with control siRNA-treated cells (Fig. 4A).
Moreover, confocal microscopy demonstrated decreased CD44 expression in the polar caps of siRNA-treated T cells (Fig. 4, B–D). In addition, CD44 siRNA did not influence the distribution of pERM, F-actin, p34, and the formation of polar caps (Fig. 4, B–D).

To understand whether CD44 expression affects adhesion and migration of SLE T cells, we conducted adhesion and migration assays in CD44 siRNA-treated cells. CD44 siRNA-treated SLE T cells displayed significantly less adhesion to HA and chemotactic migration toward CXCL12 compared with nontreated and control siRNA-treated cells (Fig. 4, E and F). These data suggest that CD44 contributes to the adhesion and migration of SLE T cells.

**FIGURE 4.** CD44 is required for increased adhesion and chemotactic migration of SLE T cells. T lymphocytes from patients with SLE were transfected with PBS (C or control), siRNA control (sic), or CD44 siRNA (si). After 16–24 h of transfection, the expression of CD44 was measured by Western blotting (A), and the distribution of CD44 and pERM (B), pERM and F-actin (C), and CD44 and p34 (D) in SLE T cells was recorded using confocal microscopy. After 16–24 h, the transfected cells were analyzed for their ability to adhere to and migrate in response to the chemokine CXCL12 (E and F). n = 6; *, p < 0.05 compared with the control group. Bars (B–D), 5 μm.

**FIGURE 5.** Phosphorylated ezrin induces polar cap formation, adhesion, and chemotactic migration in normal T cells. Normal T cells were transfected with ezrin-GFP, ezrinT567D-GFP, ezrinT567A-GFP, or GFP expression vectors. A, The cells were lysed and immunoblotted with anti-ezrin Ab to visualize ezrin-GFP and endogenous ezrin. B and C, The transfected cells were bound to poly-L-lysine-coated coverslips, fixed, and stained with anti-GFP, anti-CD44 Abs, and rhodamine-conjugated phalloidin (an F-actin label). D, Polar cap formation, as shown in B and C, was quantified by measuring the percentage of transfected cells displaying cellular caps. Cumulative data from six independent experiments are depicted. E and F, Adhesion and chemotactic migration of the transfected T cells were measured as described in Materials and Methods, and the cumulative results from six independent experiments are shown. **, p < 0.01 for T-D group compared with the GFP vector group. Bars (B and C), 5 μm.
Forced expression of p-ezrin in normal T cells augments cell polarization, adhesion, and migration

Ezrin is a member of the ERM subfamily of cytoskeletal proteins which, after Thr phosphorylation, bridges adhesion molecules to F-actin (3, 4, 14). We asked whether forced expression of pERM in normal T cells could recapitulate abnormalities recorded in SLE T cells. Accordingly, we transfected normal T lymphocytes with GFP-encoding vector, WT ezrin (one of the ERM proteins), constitutively active T567D ezrin, or T567A ezrin (an inactive form) (2), all fused to GFP. All plasmids expressed the ezrin proteins as fusion molecules with GFP. Expression of the GFP fusion proteins was established in IB using anti-ezrin and anti-GFP Abs (Fig. 5A). Subsequently, cells were stained with anti-GFP, anti-CD44, and phalloidin and analyzed using confocal microscopy. Among all transfected cells, only cells transfected with constitutively active T567D ezrin displayed polar cap formation. CD44, T567D ezrin, and F-actin colocalized at the polar cap of the cells (Fig. 5, B and C). Quantitative analysis demonstrated that T567D ezrin transfectants exhibited 160% increase in polar cap formation at the rear end of the cells. In contrast, there was no obvious effect on the polar cap formation of the cells forced to express either WT ezrin or T567A ezrin (percentage of cells forming polar cap ± SEM: control vector transfected, 18.32 ± 2.44; WT ezrin transfected, 23.94 ± 2.65; T567D ezrin transfected, 48.7 ± 4.40; T567A ezrin transfected, 20.4 ± 2.56; p (control vs T567D) = 0.0003, Fig. 5D). Unlike T567D ezrin, GFP was distributed diffusely in the cytoplasm and the nucleus, whereas WT ezrin, inactive form ezrin, CD44, and F-actin were evenly localized at the periphery of the cell membrane. These findings suggest that Thr567 phosphorylation leads to colocalization of ezrin, CD44, and F-actin to the polar cap. Finally, these changes contribute to the increased adhesion (adhered cells/visual field ± SEM: control vector transfected, 97.73 ± 5.61; WT ezrin transfected, 109.63 ± 7.76; T567D ezrin transfected, 192.7 ± 12.78; T567A ezrin transfected, 107.13 ± 4.57; p (control vs T567D) = 0.0024) and migration (migrated cells (X10) ± SEM: control vector transfected, 1.1 ± 0.12; WT ezrin transfected, 1.27 ± 0.15; T567D ezrin transfected, 1.98 ± 0.10; T567A ezrin transfected, 1.03 ± 0.05; p (control vs T567D) = 0.0045) of the T cells as shown in Fig. 5, E and F. These results collectively show that p-ezrin is important for the localization of CD44 and F-actin, cell polarization, adhesion, and migration of T cells.
SLE serum enhances ERM phosphorylation by activating ROCK and PKC in normal T cells

To determine whether enhanced ERM phosphorylation in SLE T lymphocytes is caused by factors present in SLE sera, we cultured normal T cells with normal or SLE sera and probed the lysates with an anti-pERM Ab. ERM phosphorylation in normal T cells treated with 10% SLE serum reached maximal levels as early as 5 min, whereas in T cells treated with 10% normal serum, maximal values were noted at 90 min (Fig. 6A). Treatment of normal T cells with RA sera failed to cause up-regulation of pERM (Fig. 6B). These results indicate that SLE serum induces earlier and stronger ERM phosphorylation than control serum in normal T cells.

It has been reported that ERM phosphorylation is regulated by ROCK, PKC, and other protein Ser/Thr kinases (15, 16). To determine which kinase is activated by SLE serum, we conducted IB analyses of lysates from normal T cells treated with sera in the presence of various kinase inhibitors. As shown in Fig. 6C, pretreatment of cells with the ROCK and PKC inhibitors for 30 min reduced remarkably ERM phosphorylation. Cumulative data from six normal serum-treated and six SLE serum-treated samples are shown in Fig. 6D. Therefore, the recorded effect of SLE serum on ERM phosphorylation involves both ROCK and PKC.

As shown in Fig. 6E, treatment of normal T cells with SLE sera resulted in enhanced ERM phosphorylation, accumulation, and colocalization of pERM, CD44, and F-actin at the cell polar caps. In contrast, T cells pretreated with Y27632 and Bis for 30 min displayed markedly limited ERM phosphorylation in response to exposure to SLE sera. These confocal microscopy data further confirm the results shown in Fig. 6, C and D. The SLE sera-induced accumulation and colocalization of pERM, CD44, and F-actin was significantly disrupted in the presence of Y27632 and Bis. In addition, T cells pretreated with CytD failed to organize F-actin, pERM, and CD44 at the polar cap in response to treatment with SLE sera. Cumulative data are shown in Fig. 6F. These data demonstrate that SLE sera cause phosphorylation of ERM through ROCK and PKC, which results in actin polymerization in normal T cells. The ability of SLE sera to reproduce these biochemical abnormalities in normal T cells indicates that they probably represent a secondary abnormality.

To address whether the above molecular changes caused by SLE sera affect cellular functions, we determined the adhesion and migration of normal T cells treated with SLE sera. We found that treatment of normal T cells with SLE sera significantly enhanced their ability to adhere (adhered cells/visual field: control treated, 94.25 ± 10.00; SLE serum treated, 194 ± 16.23; p = 0.0019) and migrate (migrated cells (10^5) ± SEM: control treated, 1.21 ± 0.10; SLE serum treated, 2.33 ± 0.08; p = 0.0001). Both functions were markedly inhibited in cells pretreated with Y27632, Bis, and CytD (Fig. 6, G and H).

IgG anti-CD3/TCR autoantibodies from SLE serum cause the increased ERM phosphorylation in normal T cells

Given the fact that SLE serum contains multiple IgG autoantibodies that affect T cell function (22), we examined whether the IgG fraction of SLE sera can induce ERM phosphorylation. Indeed, as shown in Fig. 7, A and B, SLE IgG, but not normal IgG, induced ERM phosphorylation in normal T cells (ratio of
FIGURE 8. T cells infiltrating kidneys of patients with lupus nephritis express high levels of CD44 and pERM. Frozen sections of two allograft (A) and four SLE (B) kidneys were stained with H&E (original magnification, ×200). A, Tubulitis with lymphocytic infiltration of an allograft kidney during rejection. B, Tubulitis with lymphocytic infiltration, as well as vasculitis (white arrow), of a kidney from a patient with lupus nephritis. Immunofluorescent images of pERM (C), CD44 (D), and CD3 in the kidneys of a renal allograft recipient patient and a patient with lupus nephritis (original magnification, ×600). Bars (C and D), 5 µm.

pERM/ezrin ± SEM: normal IgG treated, 0.27 ± 0.07; SLE IgG treated, 0.63 ± 0.08; p = 0.0095). Flow-through fractions from the columns that we used to purify IgG were used as control and failed to induce ERM phosphorylation.

Because SLE sera contain anti-CD3/TCR Abs (22, 23), we asked whether ERM phosphorylation is regulated by anti-CD3/ TCR Abs in T cells. Accordingly, anti-CD3 Ab was used to stimulate normal T cells. We found that anti-CD3 Ab up-regulated the levels of pERM in the cells in a concentration-specific manner (Fig. 7, C and D). To prove that anti-CD3/TCR autoantibodies are responsible for the SLE serum-induced ERM phosphorylation, SLE sera were adsorbed either on CD3/TCR-positive Jurkat T cells or on CD3/TCR-negative Jurkat J.E.M. T3.3 cells (23, 24). The adsorbed sera were used to treat normal T cells, which were subsequently lysed and subjected to IB analysis. ERM phosphorylation was significantly diminished in cells cultured with SLE sera adsorbed with CD3/TCR-positive Jurkat T cells. Conversely, adsorption of SLE sera with CD3/TCR-negative J.E.M. T3.3 cells markedly increased the levels of pERM expression (ratio of pERM/ezrin ± SEM:CD3/TCR Jurkat cell-adsorbed SLE serum, 0.48 ± 0.15; CD3/TCR Jurkat cell-adsorbed SLE serum, 1.43 ± 0.32; p = 0.0349; Fig. 7, E and F). Therefore, we conclude that IgG anti-CD3/TCR autoantibodies present in SLE sera are responsible for the increased ERM phosphorylation in SLE T cells.

Increased expression of pERM and CD44 in infiltrating T lymphocytes in SLE kidneys

Biopsies of kidneys from patients with lupus nephritis display interstitial inflammation with a dominant presence of T lymphocytes (25). We asked whether the T cells that infiltrate the kidney display biochemical abnormalities similar to those recorded herein in peripheral blood SLE T cells, and which were proven responsible for their increased propensity to adhere and migrate. As controls, we used biopsies from kidney allografts undergoing rejection, which also display lymphocyte infiltration. H&E preparations of kidney biopsy material from patients with SLE (Fig. 8B) and patients with renal allografts undergoing rejection (Fig. 8A) showed prominent lymphocytic infiltration, which stained positive for CD3 (Fig. 8, C and D). All infiltrating CD3+ lymphocytes from SLE and allograft kidneys expressed CD44, although the intensity was stronger in the SLE kidney than in the allograft kidney undergoing rejection (Fig. 8D). Interestingly, only T cells infiltrating the SLE kidney, and not those infiltrating the kidney allograft, expressed pERM (Fig. 8C). It should be noted that all CD3+ cells expressed pERM, inferring that both CD4+ and CD8+ subsets display increased expression of pERM. These findings were consistent in biopsy material from four SLE patients and two patients with kidney allografts undergoing rejection. Thus, these tissue data underscore the importance of pERM in T lymphocyte migration into target organs in SLE.

Discussion

We have demonstrated that SLE T cells display an increased propensity to adhere and migrate, and we have uncovered the underlying molecular mechanism. Increased expression of phosphorylated ERM and increased expression of CD44 appear to be responsible for the increased ability of SLE T cells to adhere to membranes and migrate to tissues. Silencing of CD44 decreases effectively the ability of SLE T cells to adhere and migrate, whereas forced expression of a phosphomimetic ezrin construct in normal T cells results in the acquisition of a SLE T cell phenotype. Anti-CD3/TCR autoantibodies present in SLE sera were found to increase ERM phosphorylation in normal T cells, which contributes to enhanced actin polymerization, cell polarization, adhesion, and migration. We have provided first evidence that the kidney-infiltrating cells display biochemical features similar to those of peripheral T cells.

CD44 expression was found to be increased in SLE T cells in this study and, more importantly, we found that its silencing results in decreased T cell adhesion and migration. CD44 is up-regulated in T cells that infiltrate inflammatory sites (8, 26), and administration of anti-CD44 Abs has been shown to inhibit inflammatory disease in mice (27, 28). Therefore, CD44 may represent a legitimate therapeutic target for patients with SLE with major organ involvement. Of interest is our observation that treatment of normal T cells with SLE sera resulted in a prominent increase of pERM and increased association of pERM with CD44, followed by increased an ability to adhere and migrate. Overall levels of CD44 were not increased following treatment with SLE sera (data not shown). This implies that CD44 is necessary to be expressed at certain levels, after which the phosphorylation levels of ERM and the colocalization of pERM and CD44 determine subsequent degrees of cell adhesion and migration.

We observed that pERM and p34, a part of the arp2/3 complex, colocalized with CD44 and F-actin at the rear of the cell, resulting
FIGURE 9. Model of ERM activation by anti-CD3/TCR autoantibodies in SLE T cells. Anti-CD3/TCR autoantibodies bind to the CD3-TCR complex of circulating SLE T cells. This binding induces rapid ERM activation through ROCK-mediated phosphorylation. Activated pERM then can directly interact with CD44 and F-actin through their N-terminal and C-terminal domains, respectively. This interaction leads to actin polymerization, cell polarization, adhesion, and chemotactic migration.

in polar cap formation in SLE T cells. Previously, we described increased expression of aggregated lipid rafts (GM1) in SLE T cells and found them to contribute to increased CD3-mediated signaling responses and actin polymerization (20). Herein, we demonstrate that pERM and CD44 are present in the lipid rafts in SLE T cells, which have been shown previously to also contain FcRγ chain and Syk kinase (20). Even more important is our observation that not only actin polymerization, but also phosphorylation of ERM is needed in order for lipid rafts to form.

Several serine/threonine kinases, such as ROCK and PKC, can phosphorylate ERM proteins (15, 16). We found that a ROCK inhibitor, but not a PKC inhibitor, decreased ERM phosphorylation, association between CD44 and ezrin, polar cap formation, adhesion, and migration in SLE T cells. In contrast, the effect of SLE sera on ERM phosphorylation and the ensuing downstream events in normal cells was limited in the presence of either inhibitor. The inability of the PKC inhibitor to influence ERM phosphorylation in SLE T cells can be explained by the fact that PKC is already low in SLE patients (29). Therefore, ERM phosphorylation in SLE T cells depends on ROCK, which may represent a reasonable pharmacological target.

The ability of constitutively active T567D ezrin to augment adhesion and migration of normal T cells was preceded by the induction of CD44 and F-actin colocalization in the rear side of the cells and increased polar cap formation. T567D ezrin, but not WT and T567A-inactive ezrin, has been shown to enhance cell migration and CD44-associated polar cap formation in mouse T lymphoma cell lines (2). This set of data provides further mechanistic insight into the claim of this communication that ERM phosphorylation is central in the expression of an adhesive SLE T cell phenotype. The finding that anti-CD3/TCR Abs present in the SLE sera cause increased ERM phosphorylation is important, because it suggests that circulating T cells in SLE patients are continuously under the pressure to phosphorylate ERM and increase their chances of homing tissues inappropriately. These same Abs have been shown before to cause activation and translocation of calmodulin kinase IV in SLE T cells, which was, in turn, found to be responsible for increased binding of the transcription repressor cAMP, a response element modulator to the IL-2 promoter (23). Therefore, these autoantibodies may contribute to the expression of various aspects of the abnormal SLE T cell function. It is possible that the proposed role herein for anti-CD3/TCR Abs in inducing CD44 and pERM expression may be secondary, whereas stimulation of T cells in vivo with autoantigen represents the main culprit. It is also possible that the presence of anti-T cell Abs amplifies the autoantigen-instigated process.

In conclusion, we have characterized the molecular basis for the increased ability of human SLE T cells to adhere to membranes (10) and migrate to inflamed tissues (19). Specifically, we provided first evidence that SLE T cells express increased amounts of pERM, which we demonstrated to be responsible for this increased adhesion and migration. In addition, anti-CD3/TCR autoantibodies present in SLE sera increase ERM phosphorylation and facilitate normal T lymphocyte actin polymerization, polarization, adhesion, and migration (Fig. 9). T cells with increased expression of CD44 and pERM were found in SLE kidneys, indicating that inflamed tissue T cells derive from the circulating pool. Our study has revealed that the silencing of CD44 and pharmacological inhibition of ROCK results in correction of the abnormal phenotype of SLE and abolishes their ability to adhere and migrate and, thus, these molecules may represent proper therapeutic targets for patients with SLE.

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