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Abbe N. Vallejo, Lars O. Mūgge, Piotr A. Klimiuk, Cornelia M. Weyand, and Jörg J. Goronzy

Thrombospondin-1 (TSP) is a transmembrane protein known to promote chemotaxis of leukocytes to inflammatory sites. However, TSP and its receptor CD36 are abundantly expressed in chronically inflamed tissues such as the rheumatoid synovium. Here, we show that TSP provides the costimulatory signal that is necessary for the activation of autoreactive T cells. Data presented reveal that TSP-mediated costimulation is achieved through its independent interaction with CD36 on APCs and with CD47 on T cells. We propose that a CD47-TSP-CD36 trimeric complex is a novel costimulatory pathway that significantly decreases the threshold of T cell activation. Consistent with the paradigm that lesions in rheumatoid synovitis are sites of antigenic recognition, the characteristic focal expression of TSP on APCs such as macrophages and fibroblast-like synoviocytes suggest a central role of TSP in the expansion of tissue-infiltrating T cells. The Journal of Immunology, 2000, 164: 2947–2954.

The central event in T cell activation is the interaction of the TCR/CD3 complex and an immunogenic peptide displayed by MHC molecules on APC. However, the level of TCR expression is constantly modulated (1) and the number of specific peptide-MHC complexes is low (2). Thus, TCR/CD3-derived signals have to be amplified to sustain activation. This is achieved through the simultaneous engagement of costimulatory receptors, among which CD28 is the dominant molecule known (3). Interaction of CD28 and its ligands CD80/CD86, expressed on APC, enhances IL-2 production and synthesis of anti-apoptotic molecules. Interruption of this interaction leads to a state of unresponsiveness or programmed cell death. These results led to the definition of a costimulatory molecule as one that promotes high levels of IL-2 production, maintains T cell expansion, and prevents apoptosis (4).

Despite the compellingly large body of evidence documenting the central role of CD28-CD80/CD86 costimulus in immunity, there is mounting evidence for CD28-independent T cell-mediated responses. For instance, murine CD8+ T cells express high levels of CD28; however, they can undergo activation and differentiation without CD28 ligation (5). Curiously, about one-half of human CD8+ T cells also lack CD28 without apparent functional deficits (3). In contrast, virtually all CD4+ T cells express CD28 and yet certain effector functions such as IFN-γ and IL-4 production have been indicated to be CD28 independent (6). Studies on CD28-knockout mice also reveal maintenance of IL-4/IL-13-mediated responses to nematode infection (7) and efficient rejection of allografts (8). Collectively, these studies indicate the existence of alternate costimulatory pathways.

A number of molecules expressed on T cells have been suggested to provide the costimulus with TCR/CD3-generated signals (9). Prominent among these are LFA-1 and members of the TNFR family such as 4-1BB, OX40, CD27, and CD30. While the interaction of these molecules with their respective ligands leads to increased proliferation and IL-2 production, it is doubtful whether they can fully replace the CD28 costimulus. LFA-1 and CD27 neither prevent nor rescue T cells from apoptosis. The TNFR molecules are predominantly expressed only on previously activated T cells and are transiently found on the cell surface. Thus, these molecules may not be involved in the initiation of immune responses per se but transiently contribute to the transduction of signals after initial T cell activation.

Therefore, identification of costimulatory receptors that can fully replace CD28 is of paramount importance to understanding CD28-independent responses. Of particular interest are the CD4+CD28null T cells, which are rarely found in healthy individuals but emerge in high frequencies in diseases associated with various forms of immune dysfunction. These unusual cells were first reported among rheumatoid arthritis (RA) patients (10) and have now been found in other inflammatory syndromes such as those associated with ischemic heart disease (11), Wegner’s granulomatosis (12), and Chagas’ disease (13). Moreover, a progressive loss of CD28 expression among CD4+ T cells has also been reported during the early stages of HIV infection (14) and during the normal course of aging (15, 16), two conditions also associated with progressive immune dysfunction.

In RA, CD28null T cells can comprise up to 50% of the CD4 compartment and are found in the inflammatory lesions. They are highly oligoclonal and have autoreactive properties (10). They are also functionally active (17) and are highly resistant to apoptosis (18). These findings raise the issue as to the mechanism that brings

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5 Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; ICAM-1, human CD36; ECM, extracellular matrix; mCD16, mouse CD16; TSP, thrombospondin-1.
about their expansion in vivo. Because of the central role of T cells in the pathogenesis of RA (19), we have begun to evaluate the hypothesis that inflammatory lesions provide a milieu conducive for lymphocyte activation. This is based on findings that the rheumatoid synovium invariably develops follicular structures resembling peripheral lymph nodes (20). These structures consist of lymphocytic aggregates maintained on a stroma of macrophages and fibroblast-like synoviocytes (FLS), some of which have been suspected to have APC function (21). The tissue parenchyma also includes various extracellular matrix (ECM) proteins, which profoundly influence cytokine gene expression by T cells (22).

In the present work, we examined the role of the matricellular protein, thrombospondin-1 (TSP), in the activation of inflammatory T cells. Although TSP is a transient component of the ECM in repairing tissues (23), there is indication of its persistence in the rheumatoid synovium (24). There is a characteristic focal expression of TSP in the follicular structures, among FLS and macrophages of the tissue parenchyma, and on endothelial cells (A. Vallejo et al., unpublished observations). This expression pattern of TSP suggests a functional role beyond passive scaffolding of the synovial tissue architecture. We propose that TSP plays a central role in the recruitment, activation, and retention of T cells in the inflammatory lesions. This is consistent with observations that T cells adhere to TSP-coated substrates in either integrin-independent or -dependent manner (25). Because the C terminus of TSP is also a binding site for CD47 (26), a T cell membrane glycoprotein indicated to be comitogenic with the TCR/CD3 complex (27, 28), we examined whether TSP-CD47 interaction is the relevant co-stimulatory complex. Unlike the TNFR molecules, CD47 is constitutively expressed on T cells. Thus, tissue-infiltrating T cells may use CD47 as an alternate CD28-independent TCR/CD3 costimulus in the rheumatoid synovium where TSP, a CD47 ligand, is abundantly expressed. Our finding that FLS express TSP permitted the evaluation of this hypothesis. Data presented here show the direct role of TSP-expressing FLS in the activation of autoreactive T cell clones, demonstrating a key role of TSP in the oligoclonal expansion of T cells during chronic inflammation.

Materials and Methods

Cell culture

T cell clones and lines from RA patients and healthy donors were established as described previously (10, 15). Briefly, CD4+ CD28+/− and CD4+ CD28− T cells were isolated from peripheral blood by standard FACS technique. Cells were stimulated with anti-CD3 (OKT3; Orthopedic Surgery, Mayo Clinic) were cut into small pieces and digested with EBV-transformed B cell feeders and recombinant human IL-2 as described previously (15, 18). The mastocytoma cell line P815 expressing murine CD16 (mCD16) was provided by Dr. Paul Leibson (Mayo Clinic). This cell line was transfected with a human CD36 (hCD36) expression plasmid provided by Dr. Douglas LaBarge (Washington University of St. Louis). Before transfection, cells were passage at least once in drug-free medium and maintained at a density of 1 × 10⁶ cells/ml. About 50 μg of linearized plasmid was added to a 300-μl serum- and drug-free suspension of 1 × 10⁶ cells in an electroporation cuvette (2 mm gap; BTX, San Diego, CA) and given a single pulse of 250 V for 30 ms using the T820 ElectroSquare Porator (BTX). Cells were incubated on ice for 10 min, transferred to drug-free DMEM culture medium, and incubated for 48 h. Subsequently, cells were maintained in DMEM culture medium containing 800 μg/ml G418 (Life Technologies). After 2 wk of drug selection, cells were positively sorted for the coexpression of mCD16 and hCD36 by standard FACS technique. Cells were maintained in a humidified tissue culture incubator at 5% CO₂.

Flow cytometry

Phenotypes of T cell lines and clones were routinely examined by direct immunofluorescence staining and flow cytometry. Cells were stained with fluorochrome-conjugated Abs to CD3, CD4, and CD28, and analyzed by a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Cytometric data were analyzed using the WinMDI program (Joseph Trotter, Scripps Research Institute, La Jolla, CA).

CD47 expression on T cells was monitored by indirect immunofluorescence staining with the mAbs 2E11, 2D3, and B6H12 (Becton Dickinson). The anti-CD3 Abs were provided by Dr. Frederik Lindberg (Washington University of St. Louis).

Similarly, expression of mCD16 and hCD36 on P815 cells was monitored by indirect immunofluorescence staining with specific Abs (PharMingen, San Diego, CA, Likewise, FLS cultures were stained with Abs to HLA-DR (Becton Dickinson), hCD36 (clone FA6-152), and TSP (clone P12; Beckman Coulter, Westwood, ME). The U937 promonocytic cell line (American Type Culture Collection) and freshly isolated platelets (Blood Components Laboratory, Mayo Clinic) served as positive controls for hCD36 and TSP, respectively.

RT-PCR assay

The presence of CD3 and CD65 transcripts in the FLS lines was monitored by RT-PCR. Total RNA was isolated using Trizol reagent (Life Technologies), treated with DNase I (Roche Molecular Biochemicals, Boehringer Mannheim, Indianapolis, IN), and the first-strand cDNA was synthesized by standard techniques. PCR amplification of specific cDNA fragments was conducted using the following primer pairs, 5′-AATGTTGCAACGTCACACACCTC-3′ and 5′-CAATGTCGCCAGTCGACGCTCT-3′ for hCD36 (GenBank accession nos. Z32751-65 and Z32770-71); 5′-CCACCAAGCCTCACCCAACAAAC-3′ and 5′-CACGTTGCACTCCGCTTCTC′-3′ for TSP (GenBank accession nos. J04635 and X04665). Parallel PCR experiments were also conducted for β-actin as a system control using the primer pairs 5′-ATCATGTTTGAAGCTTCAACGCCC-3′ and 5′-CAAGGAGGCACTGTCTTGAT-3′ (GenBank accession nos. M10278 and 5016088). PCR products were fractionated by agarose gel electrophoresis. Fidelity of amplification was ascertained by direct sequencing of PCR products using an automated ABI377 DNA sequencer (Applied Biosystems, Foster City, CA).

Proliferation assays

T cell proliferation assays were conducted in three systems. The first system involved cocrosslinking of CD3 and CD4 with specific Abs directly immobilized on plates in the presence or absence of purified endotoxin-free TSP (Calbiochem, La Jolla, CA). At the indicated concentrations, anti-CD3 Ab (affinity-purified culture supernatants of OKT3; American Type Culture Collection) and either anti-CD47 Ab (2E11, 2D3, or B6H12), IgG isotype control, or TSP were diluted in 500 mM sodium bicarbonate buffer, pH 9, and coated onto 96-well plates by overnight incubation at 4°C. Plates were blocked with 10% BSA (Calbiochem) overnight at 4°C and washed with cold 1× PBS. About 2 × 10⁶ T cells were added to the plates in triplicate wells and incubated for 3 days at 37°C and 5% CO₂. Cell proliferation (DuPont NEN, Boston, MA) was added to the cultures at 1 μCi/well 16 h before harvest. Cells were harvested onto XtaSint glass fiber filters (Beckman, Fullerton, CA) and subjected to scintillation spectrometry.

In other experiments, CD3 and CD47 cocrosslinking was achieved by initially incubating cells either with anti-CD47 Ab or IgG isotype controls at 4°C for 1.5 h. Cells were washed in PBS and added to tissue culture plates containing varying amounts of OKT3 captured on immobilized rabbit anti-mouse Ig (PharMingen). About 200 μg/ml anti-mouse Ig was coated onto 96-well plates as described above. Wells coated only with BSA
served as controls. \(^{[3]}\text{H}\)Thymidine incorporation was measured after 3 days of culture.

In Ab blocking experiments, T cells were precoated with either anti-CD47 Ab or IgG control at 200 \(\mu\)g/ml for 1.5 h at 4°C. Cells were washed with cold PBS and added to OKT3/TSP-coated plates. \(^{[3]}\text{H}\)Thymidine incorporation was measured after 3 days of culture.

The second system involved the coculture of P815 cells and T cells. Either wild type or hCD36-transfected P815 cells were gamma-irradiated (15,000 rad from a \(^{137}\)Cs source) and incubated with OKT3 in combination with either anti-CD47 Ab, IgG control, TSP, or fibronectin (Calbiochem) at the indicated concentrations for 2 h at 4°C. Cells were washed, and about \(5 \times 10^5\) P815 cells were added to \(2 \times 10^5\) T cells. To the appropriate wells, the synthetic peptide 4N1K (KRFYVVMWKK) corresponding to the CD47-binding domain of TSP (26, 31) or its mutated variant peptide 4NGG (KRFYGGMWKK) was added at the indicated concentrations. T cell proliferation was measured by \(^{[3]}\text{H}\)thymidine incorporation after 3 days of culture. Concentrations of peptides used did not affect cell viability as determined by trypan blue exclusion (data not shown). The ABI431/433 peptide synthesizer (Applied Biosystems) in the Mayo Protein Core Facility was used to generate both peptides.

The third system involved a coculture system of selected T cell clones with autologous FLS lines. About \(5 \times 10^4\) cells/well were seeded into tissue culture plates and incubated with 200 U/ml of recombinant human IFN-\(\gamma\) (Biosource, Aurora, CO) for 3 days. The wells were washed, and the plate was gamma-irradiated (15,000 rad). A total of \(2 \times 10^5\) T cells were added to each well, and either anti-HLA-DR Ab, IgG control, peptide 4N1K, or peptide 41GG was added at the indicated concentrations. \(^{[3]}\text{H}\)Thymidine incorporation was measured after 3 days of culture.

**HLA-DR and TCR genotyping**

The HLA-DRB1 alleles of donors were determined by PCR and hybridization using the ELPHA HLA DNA typing system (Biotest Diagnostics, Denville, NJ). Direct sequencing of the PCR products authenticated the final HLA-DRB1*04 subtype designation.

Clonality of T cell clones was determined by standard nested PCR of the CDR3 region of the TCR \(\beta\)-chain V-J element. PCR products were ligated into the TA cloning vector (Invitrogen, Carlsbad, CA) and used to transform E. coli DH5\(\alpha\) (Life Technologies). Each TCR clonotype was characterized by sequencing and BLAST analysis.

**FIGURE 1.** TSP augments anti-CD3-induced T cell proliferative responses. A suboptimal concentration of OKT3 at 100 ng/ml was directly immobilized on 96-well plates in the presence of either 200 ng/ml anti-CD47 (2E11 or 2D3) or at the indicated concentrations of TSP. Wells were blocked with BSA, and triplicate cultures of selected T cell lines and clones were added. \(^{[3]}\text{H}\)Thymidine incorporation was determined after 3 days. Data depicted are representative of 12 clones and 3 lines examined. DH4 and H2, CD4\(^+\) T cell line and clone, respectively, were derived from the same patient. DH4 and P11, CD4\(^+\)CD28\(^-\); H2 and KP6, CD4\(^+\)CD28\(^+\).

**FIGURE 2.** TSP-induced proliferation is inhibited by soluble anti-CD47. T cells (used in Fig. 1) were precoated with anti-CD47 Abs (2E11, B6H12) and added to either OKT3/anti-CD47- or OKT3/TSP-coated plates. OKT3, anti-CD47, and TSP were directly immobilized on plates. \(^{[3]}\text{H}\)Thymidine incorporation was determined after 3 days. Data depicted are representative of 8 clones and 2 lines examined.
was authenticated by the sequencing of recombinant plasmids prepared from at least three randomly selected bacterial colonies.

Results

Induction of T cell proliferation by CD47 crosslinking

CD47 is a known component of the β3 integrin complex on polymorphonuclear cells (32). In contrast, mammalian T cells express a functional CD47 molecule despite the lack of β3 integrins (27). Cocrosslinking of CD3 and CD47 has been found to induce T cell proliferation that was unaffected by soluble anti-integrin Abs. In the present study, inflammatory T cells derived from RA patients were indeed found to constitutively express CD47 as detected by immunofluorescence staining and flow cytometry (data not shown). Both CD28⁺ and CD28null subsets of CD4⁺ T cells expressed high levels of CD47 in all cell lines and clones examined.

Consistent with previous studies (27, 28), crosslinking of CD47 by specific Ab augmented the proliferative responses of T cell lines to suboptimal levels of anti-CD3 Ab, OKT3 (data not shown). Crosslinking of CD47 with constant amount of Ab (200 ng/well) showed enhancement of T cell proliferation in the presence of immobilized OKT3 at densities between 50 and 500 ng/well. This enhancement was not evident with OKT3 immobilized at densities ≥1000 ng/well. None of the anti-CD47 Abs used was stimulatory by themselves.

Induction of T cell proliferation by TSP

Although it is a highly chemotactic and adhesive protein (33), TSP is not known to be mitogenic except for smooth muscle cells (34). However, in the present study, TSP was found to induce vigorous proliferation of selected patient-derived T cells in the presence of suboptimal levels of OKT3 (Fig. 1). TSP-induced costimulation was a dose-dependent response. It was as effective as CD47 crosslinked by specific Ab. Furthermore, TSP-induced costimulation was blocked by soluble anti-CD47 Abs (Fig. 2). Neither TSP nor any of the anti-CD47 Abs used was stimulatory by themselves.

Induction of T cell proliferation by CD47-TSP-CD36 interaction

The adhesive property of TSP is attributed to multiple domains that define binding sites of several cellular receptors. Among these is CD36 (35), which binds to a region of TSP distinct from the CD47-binding site (26). CD36 is found coexpressed abundantly with TSP, particularly on FLS, macrophages, and endothelial cells in the rheumatoid synovium (A. Vallejo et al., unpublished observations). Therefore, we examined whether a CD47-TSP-CD36 interaction was the relevant costimulatory complex for T cell activation. To address this issue, mouse P815 cells expressing endogenous CD16 were transfected with hCD36 and used as surrogate APC. As shown in Fig. 3, TSP-coated hCD36⁺ P815 cells induced proliferation of T cell clones in the presence of suboptimal levels of OKT3. The magnitude of TSP-induced costimulation was equivalent to those seen with P815 cells coated with anti-CD47. In contrast, P815 cells coated with fibronectin did not elicit any significant proliferation of T cells. In the absence of OKT3, neither anti-CD47- nor TSP-coated hCD36⁺ P815 cells stimulated T cell proliferation. As expected, wild-type P815 cells coated with OKT3 and anti-CD47, but not with TSP or fibronectin, were stimulatory for T cells.

In a manner analogous to costimulation induced by plate-immobilized TSP (Figs. 1 and 2), T cell proliferation induced by CD36-bound TSP was inhibited by the 4N1K peptide (Fig. 4). This peptide corresponds to the CD47-binding domain of TSP (26). Inhibition of proliferation by 4N1K was dose dependent. A mutated variant peptide 4NGG did not affect the TSP-induced proliferative responses.

TSP and T cell autoreactivity

To address whether the CD47-TSP-CD36 complex may have a role in regulating the immune response in chronic inflammatory diseases such as RA, FLS lines were established from synovial tissues of RA patients. As shown in Fig. 5, FLS lines had high levels of CD36 and TSP expression at both the mRNA and protein levels. They also expressed significant levels of the HLA-DR Ag-presenting molecules.

When cocultured with autologous synovial T cell clones, FLS elicited spontaneous proliferation of T cells (Table I). These FLS-induced responses were blocked by Abs to HLA-DR. Moreover, the CD47-binding peptide 4N1K elicited significant reductions of
such responses in a dose-dependent fashion. The levels of peptide-specific inhibition of T cell proliferation was equivalent to between 60 and 70% of the FLS-T cell cocultures.

Of three clones from unrelated but HLA-DRB1*0401-matched donors, two clones did not show FLS-induced responses, while one T cell clone was found to spontaneously proliferate when cocultured with the FLS line. Its crossreactive properties were also found to be blocked by anti-HLA-DR as well as by 4N1K, but not by 4NGG, peptides in a dose-dependent fashion. As expected, high concentrations of immobilized OKT3 induced proliferation of all T cell clones examined.

**Discussion**

Autoimmune disorders, such as RA, are characterized by the emergence of functionally distinct tissue-infiltrating CD4^+^ T cell sub-sets (19), many of which lack CD28, the major costimulatory receptor required for activation. This implies that inflammatory T cells use alternate costimulatory pathway(s) that effectively lead to their oligoclonal expansion in vivo. The present data demonstrate the costimulatory role of TSP, a matricellular protein found in inflammatory lesions, in T cell activation. Specifically, either plate-immobilized or CD36-bound TSP induces the proliferation of T cells in the presence of suboptimal levels of anti-CD3 (Figs. 1–4). These TSP-induced responses are inhibited either by soluble anti-CD47 Abs or by the synthetic peptide 4N1K corresponding to the CD47-binding domain of TSP (26), indicating that costimulation by TSP is CD47 mediated. These results provide definitive proof that TSP is the biologically relevant ligand for CD47, a tetraspan membrane glycoprotein constitutively expressed on T cells and previously indicated to have a costimulatory function (27). CD47 costimulus enhances proliferation and IL-2 production to the same magnitude as CD28. Our results show that TSP/CD47-induced proliferation of CD4^+^CD28^−^ and CD4^+^CD28null T cells is equivalent, confirming that CD47 costimulus can substitute for CD28. It is important to note that while CD47 is generally a component of the β₃ integrin complex in polymorphonuclear cells (30, 32), its costimulatory function in T cells is integrin independent (27, 28). This is also confirmed by the present data (Figs. 1 and 4) showing augmentation of anti-CD3 induced T cell proliferation with CD47 crosslinking by the monoclonal Abs 2E11 and 2D3. These Abs recognize epitopes on CD47, but not on β₃ integrin (27, 29, 30). However, considering that TSP is a highly adhesive multidomain molecule (23, 25, 33), contribution of other integrins to the overall strength of costimulation remains to be examined.

An interesting feature of CD47-mediated costimulation is the observation that CD47 crosslinking induces the conversion of a
Table I. T cell autostimulatory activity in TSP mediators*  

<table>
<thead>
<tr>
<th>Condition</th>
<th>T cells only</th>
<th>T cell clones</th>
<th>T cell clones + 4N1K</th>
<th>T cell clones + 4NGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td>DRB1*0401/0403</td>
<td>DRB1*0401/0403</td>
<td>DRB1*0401/0403</td>
<td>DRB1*0401/0403</td>
</tr>
<tr>
<td>T cells only</td>
<td>6.40 ± 0.01</td>
<td>0.47 ± 0.07</td>
<td>0.45 ± 0.08</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td>T cell clones</td>
<td>5.17 ± 0.88</td>
<td>0.48 ± 0.03</td>
<td>0.46 ± 0.04</td>
<td>0.46 ± 0.04</td>
</tr>
</tbody>
</table>

*The LP cell line (shown in Fig. 5) was gamma-irradiated and cultured with selected T cell clones in the presence or absence of Ab to HLA-DR or the CD47-binding peptide 4N1K or its variant 4NGG. The LP cell line and the T cell clones, a PL51, PL52, PL54, PL56, and PL62, were obtained from the same RA patient. Data shown are [3H]thymidine incorporation of triplicate cultures (cpm ± SD).

The present data also show that some T cell clones can recognize various FLS lines derived from different donors (Table I). While the antigenic basis of this cross-reactivity is unknown, it is significant to note that this phenomenon is also CD47 mediated. This finding strongly indicates a critical role of CD47 in mediating both autoreactive and alloreactive immune responses. Therefore, it will be of interest to examine whether CD47 costimulus plays a role in the long-term survival of allografts and rejection of tumors, particularly in cases where CD28 costimulus has no impact in generation of the appropriate immune response (39, 40).

Whether the CD47 costimulus in T cells is distinct from that of CD28 remains to be examined. However, previous studies show that CD47 costimulus augments the phosphorylation of CD3ζ and the signal transducer ZAP70 (27). When associated with β2 integrin as in granulocytes and melanoma cells, CD47 has also been found to functionally couple to G proteins (41). Curiously, ligation of CD47 on the Jurkat T cell lymphoma by soluble 4N1K peptide can induce phosphorylation of extracellular signal-regulated kinase (42). In contrast, CD28 is generally associated with the activation of three kinases, namely phosphatidylinositol 3-kinase, inducible T cell kinase, and p56

TCR antagonist to an agonist (27). By definition, antagonists are MHC/peptide complexes that bind the TCR at significantly lower affinities compared with agonists (36). Because they also have fast dissociation rates, antagonist ligands do not induce the proper TCR conformation and consequently fail to trigger activation, leading to anergy and death even in the presence of costimulatory signals (37). Therefore, the reversion of antagonism by CD47 crosslinking suggests that the CD47 costimulus significantly decreases the threshold activation. By inference, the strength of the CD47 costimulus may break immune tolerance by permitting low-affinity TCR-MHC/peptide interactions to trigger T cell activation. Nevertheless, the observed high levels of inhibition of FLS-induced T cell autoproliferation by 4N1K peptide might suggest contribution of other costimulatory interactions. FLS have been shown to express a variety of adhesion molecules (20, 21) that, as an aggregate, could conceivably provide additional costimulatory signals. Nevertheless, the observed high levels of inhibition of FLS-induced responses by 4N1K peptide demonstrates the preeminence of TSP-CD47-derived signals in these autoproliferative activities by patient-derived T cell clones.

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In the context of chronic inflammatory disease, our data are consistent with the notion that the inflammatory lesions in RA are sites of lymphocyte activation. Consistent with previous reports (21, 49), our data show that FLS play a central role in T cell activation (Table I). FLS-induced proliferation of autologous T cells indicates that they are important sources of the autoantigen(s) thought to drive the oligoclonal expansion of inflammatory T cells (50). Further, FLS also supply a costimulatory molecule in the form of TSP (Fig. 5 and Table I). Presumably, TSP is bound to CD36 on the FLS cell surface as indicated by the focal, but not widespread, colocalization of TSP and CD36 in synovial tissues (A. Vallejo et al., unpublished observations). The inhibition of FLS-induced proliferation of T cells by peptides corresponding to the CD47-binding domain of TSP indicate that costimulation of T cell activation is likely to be mediated by a trimolecular interaction of CD47, TSP, and CD36.

Inasmuch as TSP is also expressed on endothelial cells and macrophages (Ref. 24 and A. Vallejo et al., unpublished observations), a model for the broader role of TSP in the recruitment, activation, and retention of infiltrating T cells in the inflammatory lesions can be proposed. Because of the inherent adhesive properties of TSP, its expression on endothelial cells could facilitate adhesion and diapedesis of T cells into the tissue. Subsequent interaction of T cells with APCs such as tissue macrophages or FLS would lead to activation and proliferation. Infiltrating CD4+CD28+ T cells could use the CD47-TSP-C36 costimulatory pathway, whereas CD4+CD28− T cells can use either this trimolecular complex or the CD28-CD80/86 pathway. Both of these costimulatory pathways may account for the oligoclonal expansion of T cells in the tissue, a well-documented feature of rheumatoid synovitis (19). The increasing evidence for TSP accumulation in the lesions of other inflammatory and infectious diseases (51–54) indicates a broader applicability of this model in disease pathogenesis.

Acknowledgments

We thank Dr. Paul Leibin (Mayo Clinic) for providing the P815 cell line, Dr. Frederick Lindberg (Washington University of St. Louis) for the anti-CD47 Abs, and Dr. Douglas Lublin (Washington University of St. Louis) for the hCD36 expression plasmid. We also thank James Fulbright for assistance in the preparation of the manuscript.

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