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Effector Function of Resting T Cells: Activation of Synovial Fibroblasts

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Synovial tissue in rheumatoid arthritis is characterized by infiltration with large numbers of T lymphocytes and APCs as well as hyperplasia of synovial fibroblasts. Current understanding of the pathogenesis of RA includes the concept that synovial fibroblasts, which are essential to cartilage and bone destruction, are regulated by cytokines derived primarily from monocyte-macrophage cells. Recently it has been found that synovial fibroblasts can also function as accessory cells for T cell activation by superantigens and other stimuli. We have now found that highly purified resting T cells, even in the absence of T cell mitogens, induce activation of synovial fibroblasts when cocultured for 6–24 h. Such activation was evident by induction or augmentation of mRNA for stromelysin, IL-6, and IL-8, gene products important in joint inflammation and joint destruction. Furthermore, increased production of IL-6 and IL-8 was quantitated by intracellular cytokine staining and flow cytometry. This technique, previously used for analysis of T cell function, was readily adaptable for assays of synovial fibroblasts. Resting T cells also induced synovial fibroblasts to produce PGE₂, indicating activation of expression of the cyclooxygenase 2 gene. Synergy was observed between the effects of IL-17, a cytokine derived from stimulated T cells that activates fibroblasts, and resting T lymphocytes. Various subsets of T cells, CD4⁺, CD8⁺, CD45RO⁺, and CD45RA⁺ all had comparable ability to induce synovial fibroblast activation. These results establish an Ag-independent effector function for resting T cells that is likely to be important in inflammatory compartments in which large numbers of T lymphocytes and fibroblasts can come into direct contact with each other. The Journal of Immunology, 2001, 166: 2270–2275.

The role of T lymphocytes in the pathogenesis of rheumatoid arthritis (RA) remains controversial (1). RA synovium is extensively infiltrated by T cells, but the relationship between T cell autoreactivity to specific Ags found in synovium or cartilage and the pathogenesis of RA is still unclear (1). Levels of IL-2 in the synovium are low, and only a small fraction of synovial T cells express IL-2Rs. Concurrently the roles of synovial macrophages and synovial fibroblasts in the pathogenesis of RA have become better defined (2). Synovial macrophages produce important proinflammatory cytokines such as TNF-α and IL-1β (3, 4), while fibroblasts are centrally involved in the erosion of cartilage and bone that leads to the development of deformities in patients with RA (2). It is assumed that T cells in RA synovium can interact with various professional APC populations such as monocyte-macrophage cells, dendritic cells, and B lymphocytes. However, less attention has been given to the possibility of direct interactions between synovial T cells and synovial fibroblasts that might contribute to chronic joint inflammation.

Recently several laboratories have demonstrated that synovial fibroblasts could serve as accessory cells for T cell activation by bacterial superantigens or mitogenic lectins (5–7). Previous studies have also documented specific interactions between T cells and synovial fibroblasts that result in cell:cell adhesion (7–9). It has been shown that freshy activated T cells can trigger functionally relevant responses by synovial fibroblasts, in part mediated by the effects of membrane-bound cytokines such as TNF-α that can be produced by activated T cells (10, 11). However, most T lymphocytes in RA synovium lack the morphology, surface phenotype, and cytokine secretion profile of T cell blasts (1). In view of the evidence, cited above, that resting T cells were capable of interacting with synovial fibroblasts, we set out to determine whether such interactions could also lead to functionally significant responses by the synovial fibroblasts. Our results indicate that resting T cells can indeed induce or increase the expression of molecules relevant to joint inflammation and destruction by cultured synovial fibroblasts in the absence of exogenous Ag or other T cell mitogenic factors. We term such interactions effector function of resting T cells.

Materials and Methods

Synovial fibroblast lines

RA and osteoarthritis (OA) synovial fibroblasts were obtained by collage-nase (type I; Worthington Biochemical, Freehold, NJ) digestion of human synovial tissue obtained at arthroplasty or synovectomy. These cells and also control dermal fibroblast lines were maintained in CMRL medium supplemented with 10% FCS (Summit, Biotechnology, Ft. Collins, CO), 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Grand Island, NY) and plated in 75-cm² flasks. When the cells reached 95% confluence, they were passaged at a dilution of 1/3 into 175-cm² flasks. The confluent adherent cells were split by gentle trypsinization (0.05% trypsin/0.53 mM EDTA; Irvine Scientific, Walkersville, MD). Cells were used between passages 3 and 12. The superantigen staphylococcus enterotoxin A (SEA) was obtained from Sigma (St. Louis, MO).
**T lymphocyte purification**

Human PBMC from healthy normal donors and patients with RA were separated by Ficoll-Hypaque (Sigma) density gradient centrifugation. Highly purified T cells were prepared from PBMC by exhaustive immunomagnetic negative selection using Biomag Anti-Mouse IgG beads (PerSeptive Biosystems, Framingham, MA) and a cocktail of mAbs against CD14, CD16, CD19, and class II MHC Ags (Ancell, Bayport, MN). Functional purity was determined by the lack of proliferative responses to superantigens, lectins, and/or soluble anti-CD3. To select for the CD45RO and CD45RA subsets, mAb against CD45RA (Coulter, Fullerton, CA) and CD45RO (Immunotech, Fullerton, CA) were respectively added to the cocktails. When analyzed by flow cytometric analysis, the purity of the total T cell preparation was >99% CD3+, the CD45RA subsets were >99% CD45RObright and >99% CD45ROnull, and the CD45RA T cells were >99% CD45ROnull and <43% CD45RAnull.

**Coculture conditions and RT-PCR**

Syновial fibroblasts were first plated in six-well plates at 2.5 × 10^5 cells/well for 24–48 h. T cells (5 × 10^5 cells/well) were then added to wells, and cells were harvested after 6 or 24 h. In some experiments various concentrations of recombinant human IL-17 (R&D Systems, Minneapolis, MN) were also added along with the T cells. T cells were removed first by vigorous washing with cold, serum-free medium, then syновial fibroblasts were detached with trypsin/EDTA. Total cellular RNA was isolated using a single step procedure with TRIzol (Life Technologies) and subjected to RT and PCR amplification. For RT reactions, 1 μg of total RNA was used per sample, and RT was conducted for 1 h at 42°C. For PCR, the primers used were as follows: β-actin 5′ end, 5-GGC-AGA-AGA-TCG-AGG-3′; and β-actin 3′ end, 5-GCC-TCT-TAG-TTA-TGA-TTA-TG-3′. A single PCR product would occur if activated T cells remained adherent to the synovial fibroblasts. When analyzed by flow cytometric analysis, the purity of the total T cell preparation was >99% CD3+, the CD45RA subsets were >99% CD45RObright and >99% CD45ROnull, and the CD45RA T cells were >99% CD45ROnull and <43% CD45RAnull.

**Results**

**Activation of synovial fibroblasts by superantigen and/or resting T cells**

Initial experiments showed that syновial fibroblast activation, measured by induction/augmentation of mRNA for IL-6, IL-8, and stromelysin, was induced by the combination of T cells and superantigen. Previous work of others had shown that high concentrations of superantigen alone could activate syновial fibroblasts (12, 13), and we found that this was also true for bacterial superantigens in the nanogram per milliliter concentration range (e.g., 1–10 ng/ml SEA) with or without T cells (data not shown). Surprisingly, resting T cells also induced activation of syновial fibroblasts in the absence of superantigen, and this finding was analyzed in further detail.

**Induction of syновial fibroblast mRNA by resting T cells**

Cultures containing resting T cells and syновial fibroblasts but no superantigen showed clear evidence of syновial cell activation (Fig. 1). In this experiment T cells and syновial fibroblasts were cocultured for 6 or 24 h. The T cells were removed by vigorous washing with cold serum-free medium. RT-PCR was then performed on RNA extracted from either T cells or syновial fibroblasts. This was performed in a semiquantitative manner by using serial dilutions of RT product and varying the PCR cycle number. The data in Fig. 1A represent amplified mRNA levels for IL-6, IL-8, IL-2, and stromelysin with actin as a control. For IL-6 mRNA was measured by induction/augmentation of the IL-6 mRNA in the absence of superantigen, and this finding was analyzed in further detail.

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**PGE_2 assays**

Syновial fibroblasts were cultured with T cells at 37°C for 24 h in serum-free CMRL medium with or without recombinant human IL-17, and superantigens were harvested. The PGE_2 levels in the collected superantigens were determined using an ELISA kit (R&D Systems, Minneapolis, MN).

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fibroblasts was excluded by the absence of IL-2 mRNA in the synovial fibroblast fraction. As a control, purified T cells were activated by PMA in combination with ionomycin, and IL-2 mRNA was readily detected (column 3, lower right panel of Fig. 1A). mRNA for IL-17, a cytokine produced by activated T cells that can activate fibroblasts (14), was not detected in T cell or fibroblast mRNA from such cocultures, even with 35 cycles of PCR amplification of RT product (data not shown).

Cytokine synthesis by synovial fibroblasts stimulated by resting T cells

To determine whether synovial fibroblast activation by resting T cells led to protein synthesis as well as mRNA induction, we measured cytokine production by intracellular staining and flow cytometry for IL-6 and IL-8. As shown in Fig. 2A, production of IL-6 and IL-8 was induced or augmented by coculture of T cells with synovial fibroblasts. The extent of IL-6 and IL-8 induction was variable with different combinations of synovial fibroblast lines and T cells donors (Fig. 2B) and with the number of resting T cells added (Fig. 2C). In approximately 25% of >20 experiments, T

FIGURE 1. Effector function of resting T cells: activation of synovial fibroblasts. A, Detection of synovial fibroblast gene expression by RT-PCR. Synovial fibroblasts were cultured in six-well plates with or without T cells for 6 or 24 h at 37°C. Stromelysin and cytokine mRNA expression were measured by semiquantitative RT-PCR analysis. RNA extracted from: 1, T cells alone culture (6 h); 2, T cells in coculture (6 h); 3, T cells stimulated with PMA/ionomycin (6 h); 4, synovial fibroblasts in coculture (6 h); 5, synovial fibroblasts alone (24 h); 6, synovial fibroblasts in coculture (24 h); and 7, T cells alone (24 h). Data were obtained from densitometric analysis, and all values were correctly normalized to actin mRNA PCR product. B, Measurement of cytokine mRNA by RNase protection assay. RNase protection assays were performed as described in Materials and Methods. Synovial fibroblast mRNA was analyzed after 24 h of culture without T cells (lane 3) or with purified T cells from two different individuals (lanes 4 and 5). Lane 1, Control unhybridized probes; lane 2, empty.

FIGURE 2. Induction of synovial fibroblast cytokine production by T cells. IL-6 and IL-8 production were measured by intracellular staining and flow cytometry. A, Synovial fibroblasts were incubated with or without T cells for 24 h at 37°C. B, Variation with different synovial fibroblast lines and T cell donors. Line 1, synovial fibroblast line 1; line 2, synovial fibroblast line 2; T cells no. 1, T cells of donor 1; T cells no. 2, T cells of donor 2. C, Dose-dependency with different numbers of T cells in coculture with synovial fibroblasts.
cells did not induce appreciable cytokine synthesis, but in the majority of cases, augmentation of both IL-6 and IL-8 was easily detected using the flow cytometric technique. Conventional ELISA methods were also used in some experiments to measure IL-6 and IL-8, and the results closely paralleled the results obtained using flow cytometry (data not shown.)

**Induction of synovial fibroblast cytokine and PGE$_2$ production: synergy with IL-17**

IL-17 is produced by activated T cells in RA synovium (14). We therefore set up experiments to determine whether exogenous IL-17 could interact with functional effects of resting T cells in this system. IL-6 and IL-8 expression were measured by intracellular staining and flow cytometry (Fig. 3A), and PGE$_2$ expression was determined by ELISA (Fig. 3B). Recombinant human IL-17 augmented synovial fibroblast cytokine and PGE$_2$ expression in a manner synergistic with resting T cells.

**T cell subsets responsible for synovial fibroblast stimulation**

Subset selectivity for the interaction of resting T cells with synovial fibroblasts would suggest involvement of subset-specific surface structures in such interactions. To determine which subsets of T cells could induce synovial fibroblast activation, we used purified T cell subsets, CD4$^+$, CD8$^+$, CD45RO$^+$, or CD45RA$^+$, in coculture experiments. Resting T cells, both CD45RO$^+$ and CD45RA$^+$, activated synovial fibroblasts to produce cytokines (Fig. 4). CD4$^+$ and CD8$^+$ subsets also had equal effects (data not shown).

**Cell surface phenotype of T cells following interaction with synovial fibroblasts**

As suggested by lack of expression of IL-2 and IL-17, T cells were not activated when they were cultured with synovial fibroblasts. Furthermore, these T cells expressed no CD40 ligand (CD40L; CD154) and only slightly up-regulated CD69 expression in some experiments (Fig. 5). This contrasted with expression of IL-6 and IL-8 by the synovial fibroblasts (Fig. 5). Although T cell activation did not occur, T cell viability was essential, since lethally irradiated T cells were unable to activate the synovial fibroblasts (data not shown.)

**Activation of synovial fibroblasts by autologous T cells**

Even though T cell activation was not observed in coculture with (allo)genetic synovial fibroblasts, the variable extent of the fibroblast response (Fig. 2B) made it important to exclude subtle allo- or (allo)genetic effects. Therefore, autologous (RA) and allo(ge)netic (RA or normal) T cells were directly compared within a single experiment for their ability to activate RA synovial fibroblasts (Fig. 6). Results from multiple such experiments showed that autologous and allo(ge)netic T cells had comparable ability to activate synovial fibroblasts and synergize with IL-17, as judged by production of cytokines. Moreover, the induction of mRNA for cytokine genes, measured by the RNase protection assay and shown in Fig. 1B, represents data from both an autologous and an allo(ge)netic T cell-synovial fibroblast coculture.

**Discussion**

T lymphocytes, synovial fibroblasts, and synovial monocyte-macrophage cells represent the three most abundant cell populations in inflamed RA synovium. The accumulation of T lymphocytes and macrophages is due primarily to the influx of cells from the systemic circulation, but the increased number of synovial fibroblasts represents hyperplasia of synovial lining cells. Current models for understanding the pathogenesis of RA highlight interactions between T cells and professional APC in initiating responses to autoantigens and other antigenic material found in synovial tissue (15). The proinflammatory and tissue-destructive behavior of synovial fibroblasts is believed to largely reflect the influence of cytokines derived primarily from synovial macrophages, such as TNF-α and IL-1β, as well as possibly somatic mutations that affect fibroblast proliferation (2, 16). The importance of these cytokines is strongly supported by favorable clinical effects of therapeutic cytokine blockade in human RA, especially inhibition of TNF-α action (17).
can be up-regulated by IFN- and synovial fibroblast ligands that participate in binding to T cells. These in vitro cocultures can be performed with cells, with or without superantigen, could activate synovial fibroblasts. It has previously been demonstrated that high concentrations of bacterial superantigens could trigger an activation response by synovial fibroblasts (12, 13). We found that much lower concentrations (for example, SEA at 1–10 ng/ml vs 1–10 μg/ml) could also induce measurable responses from synovial fibroblasts (data not shown). Surprisingly, we also observed that highly purified APC-free resting T cells, in the absence of superantigens or any other T cell stimuli could induce activation of synovial fibroblasts. These experiments were generally conducted with RA synovial fibroblasts, but OA synovial fibroblasts and dermal fibroblasts were used as control cell lines. Significant, substantial, and reproducible differences were not observed between the fibroblasts from these different sources, although RA synovial fibroblasts tended to multiply more quickly in vitro and to exhibit brisker activation responses (data not shown). Importantly, the full spectrum of synovial fibroblast responses was obtained using either autologous or allogeneic T cells. This suggests that responses observed in this in vitro system may be physiologically meaningful, and are not due to subtle allogeneic effects. The variability in response to T cells of different synovial fibroblast lines is therefore not due to different degrees of allogeneic mismatch between T cells and synovial fibroblasts. Moreover, neither the extent of T cell or fibroblast purity nor the degree of confluence or passage number of synovial fibroblasts in culture could account for the variable activation response (data not shown). The primary factors that account for this variability remain undetermined.

In such cocultures the T cells did not become activated, mRNA for IL-2 and IL-17 was undetectable, and CD40L was not induced. Surprisingly, we also observed that highly purified APC-free resting T cells, in the absence of superantigens or any other T cell stimuli could induce activation of synovial fibroblasts. These experiments were generally conducted with RA synovial fibroblasts, but OA synovial fibroblasts and dermal fibroblasts were used as control cell lines. Significant, substantial, and reproducible differences were not observed between the fibroblasts from these different sources, although RA synovial fibroblasts tended to multiply more quickly in vitro and to exhibit brisker activation responses (data not shown). Importantly, the full spectrum of synovial fibroblast responses was obtained using either autologous or allogeneic T cells. This suggests that responses observed in this in vitro system may be physiologically meaningful, and are not due to subtle allogeneic effects. The variability in response to T cells of different synovial fibroblast lines is therefore not due to different degrees of allogeneic mismatch between T cells and synovial fibroblasts. Moreover, neither the extent of T cell or fibroblast purity nor the degree of confluence or passage number of synovial fibroblasts in culture could account for the variable activation response (data not shown). The primary factors that account for this variability remain undetermined.

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also induced, secretion of stromelysin was changed only slightly, at the margin of detection using ELISAs (data not shown), and it is likely that resting T cells as a single stimulus are not sufficient to substantially augment matrix metalloprotease production. However, production of PGE₂ was readily demonstrable. The effect of resting T cells on PGE₂ production showed synergy with concentrations of IL-17 that were inactive or minimally active as a single stimulus. These effects on production of PGE₂ probably represent the induction of expression of the cyclo-oxygenase 2 gene.

Which surface structures are involved in activation of synovial fibroblasts by resting T cells? In contrast to activation of T cells by synovial fibroblasts and superantigen, which showed clear dependence on class II MHC, CD11a/CD18, and CD2 using blocking studies with mAb (7), the roles of specific surface structures in the synovial fibroblast response have been less clear. The role of class II MHC has been difficult to assess, because blocking Abs against human class II MHC have themselves triggered some functional responses by synovial fibroblasts (data not shown). We have observed some inhibition of the synovial fibroblast response with mAb to CD2 and CD11a/CD18 (Y. Yamamura and D. A. Fox, unpublished observations), but the subtle degree of inhibition obtained suggests that other surface structures may be more important. Synovial fibroblasts do express CD40 and can receive activating signals through this molecule, but T cells in this system fail to express CD40L. T cell surface cytokines that can activate synovial fibroblasts (11) are expressed only by T cell blasts and cannot account for the effects of resting T cells. Similarly, CD69, which was found to have a role in the up-regulation of monokine synthesis by activated T cells (20), was expressed only negligibly by resting T cells in coculture with synovial fibroblasts. Characterization of the effects of mAb on additional known and novel cell surface structures will probably be necessary to yield a useful picture of the receptor-ligand combinations involved in T cell/fibroblast interactions.

The in vitro effects of T cells on fibroblasts are likely to be important in vivo only in lesions that contain large numbers of both these cell types. RA synovial tissue may be one of the best examples of such a lesion. In RA synovium the T cell/fibroblast axis can provide a mechanism for maintaining activation of the proinflammatory and ultimately tissue-destructive phenotype of synovial fibroblasts even in the absence of specific Ag recognition by T cells. Such pathways could explain the residual inflammation that can persist in RA patients who have been treated with TNF blockade and the minority of patients who fail to respond to such treatment. Apparently, subset-specific T cell surface molecules do not determine the ability of a T cell to mediate such effects, since CD4+ or CD8+ cells and cells expressing various CD45 isoforms could all activate synovial fibroblasts with approximately equal efficiency. These results indicate that T cells of multiple subsets possess effector capacity in the resting state. Such proinflammatory effector function of resting T cells represents an expansion of understanding of the range of biological properties of T lymphocytes.

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