Animal Model

Inflammation, Immune Reactivity, and Angiogenesis in a Severe Combined Immunodeficiency Model of Rheumatoid Arthritis

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Severe combined immunodeficiency (SCID) mice were engrafted with rheumatoid arthritis (RA) synovium and evaluated to determine whether RA synovial morphology and function were maintained in the RA-SCID grafts. The four major components of RA synovitis, inflammation, immune reactivity, angiogenesis, and synovial hyperplasia persisted in RA-SCID grafts for 12 weeks. Retention of chronic inflammatory infiltrates was demonstrated by histological evaluation and by immunohistology for CD3, CD20, and CD68. Staining for CD68 also revealed that the grafts had undergone reorganization of the tissue, possibly as a result of fibroblast hyperplasia. Immune and inflammatory components were confirmed by the detection of human immunoglobulins and human interleukin-6 in serum samples obtained from grafted animals. Human blood vessels were detected by dense expression of CD31. Small vessels persistently expressed the vitronectin receptor, $\alpha v\beta 3$, a marker of angiogenesis. All vessels expressed VAP-1, a marker of activated endothelial cells. Finally, the grafts retained the ability to support immigration by human leukocytes, as demonstrated by the functional capacity to recruit adoptively transferred 5- (and -6)-carboxyfluorescein diacetate succinimidyl ester-labeled T cells. T cells entering the RA-SCID grafts became activated and produced interferon- γ , as detected by reverse transcriptase-polymerase chain reaction analysis. These studies demonstrate that the RA-SCID model maintains many of the phenotypic and functional features of the inflamed RA synovium. (*Am J Pathol* 2002, 160:357–367)

Rheumatoid arthritis (RA) is a chronic progressive disease involving both local and systemic inflammation.¹ RA is characterized by persistent inflammation of synovial tissue and eventually the destruction of cartilage and adjacent bone. RA synovitis involves local activation of lymphocytes, inflammation, and synovial hyperplasia as well as angiogenesis.¹ The etiology of RA remains unknown and much is left to be elucidated about the progression of the pathological processes.

The mechanism of the immune component in RA has not been resolved, although large numbers of CD3⁺,CD4⁺ T cells are found in perivascular areas and CD3⁺,CD8⁺ T cells are distributed diffusely throughout the synovium.^{1,2} Recent evidence including the polyclonal nature of the CD4⁺ T-cell infiltrate and the nearly uniform expression of activation molecules by these cells suggest they play a role in perpetuating chronic inflammation.³ CD8⁺ cells in the synovium seem to be both proinflammatory and regulatory, whereas CD20⁺ B cells secreting immunoglobulins (Igs) are found in defined aggregates throughout the synovial tissue along with CD4⁺ T cells and interdigitating dendritic cells.² The local generation of immune complexes and activation of complement have been proposed as mechanisms contributing to tissue damage and propagation of inflammation.³

Inflammatory cells in the RA joint consist primarily of polymorphonuclear leukocytes, monocytes, and macrophages.^{1,2} Few polymorphonuclear leukocytes are localized in the RA synovium, although large numbers characteristically accumulate in the synovial fluid, presumably because of limited chemokine receptor expression. Numerous monocytes and macrophages are found within the RA synovial tissue and fluid.^{1,2} Activated monocytes produce cytokines such as interleukin (IL)-1 β , IL-6, and

Supported by National Institutes of Health grant AR45293.

Accepted for publication October 3, 2001.

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tumor necrosis factor- α that stimulate production of prostaglandins, matrix metalloproteinases, and other mediators from synoviocytes and activate endothelial cells.¹ Other inflammatory cells, such as mast cells, have been found near sites of cartilage erosion. Few natural killer cells have been found in the RA synovium, however, it remains a possibility that common surface molecules such as CD16 and CD56 used to detect natural killer cells are down-modulated in inflamed synovial tissues.²

Synovial hyperplasia results in the formation of granulation tissue or pannus that covers the cartilage and invades the bone.¹ As RA progresses, the normally thin synovial lining layer thickens, primarily as a result of recruitment of myeloid lineage cells. Angiogenesis, or the development of a network of new blood vessels is one of the earliest histopathological alterations in the synovium and seems to be required for synoviocyte hyperplasia.^{1,4,5} Whereas, all human endothelium express high levels of CD31 (PECAM-1), recent studies suggest that only vessels undergoing angiogenesis express the integrin $\alpha v \beta 3$ or CD51/CD61.^{6,7} Endothelial cells in inflamed tissue also express the activation marker, VAP-1.⁸ These endothelial cells regulate the recruitment of mononuclear cells. Thus, new microvascular formation correlates with perivascular lymphoid infiltrates and aggregates as well as disease activity.^{1,2} Rapid neovascularization of the synovium seems to be ongoing and required to sustain the metabolic requirements of the proliferating synoviocytes.

Previously, a number of experimental animal arthritis models, including collagen-induced arthritis in mice, rats, and rhesus monkeys; adjuvant-induced arthritis in rats; and antigen-induced arthritis in mice and rabbits have been developed to study RA.^{9–12} Although these models have contributed to understanding the pathogenic mechanisms involved in RA, none has reproduced all of the clinical and histopathological features of RA. This has limited the value of these animal models in developing a complete understanding of the pathogenic events in RA and also in testing novel therapeutic interventions. Therefore, mice with severe combined immunodeficiency (SCID) have been used as hosts to create disease models by transferring human cells to these animals.^{13,14} SCID mice lack the ability to reject allografts and xenografts and, therefore, retain human RA synovial grafts.¹⁵ Model systems in which human skin, thymus, and blood have been engrafted into the SCID host have been developed.^{16–19} A variety of models to study RA in SCID mice (RA-SCID) have been used.^{20–29} Many models are complex, involving engraftment of human RA synovial tissues or cell lines into SCID joints.^{20–22} Other models have placed synovial fibroblasts and cartilage under the renal capsule,^{15,23} or in sponges along with bone and cartilage.^{15,24–28} Although providing valuable information, many of these models do not recapitulate the multicellular and chronic nature of rheumatoid inflammation. We have therefore used a simplified RA-SCID model to examine the components and duration of disease.^{29,30} This model involves engrafting intact pieces of rheumatoid synovium subcutaneously in SCID mice. The current report details the phenotype and function of the major

cellular components of the rheumatoid synovium in this RA-SCID model. These studies demonstrate that the RA-SCID grafts were rapidly vascularized and maintained the characteristics of the inflamed rheumatoid synovium after prolonged engraftment. Therefore it provides a unique model system to examine the influence of the RA microenvironment on the major components of the inflammatory process.

Materials and Methods

Antibodies and Reagents

Monoclonal antibodies (mAbs) directed against human-specific epitopes did not cross-react with murine molecules and anti-murine mAbs did not cross-react with human epitopes. Antibodies directed against human mononuclear cell and endothelial cell epitopes included anti-CD3 mAbs [OKT3 (American Type Culture Collection, Rockville, MD) and UCHT1 (Pharmingen, San Diego, CA)], anti-CD4 mAbs [OKT4a (Ortho Diagnostics, Raritan, NJ) and MT310 (DAKO Corporation, Carpinteria, CA)], anti-CD8 mAbs [OKT8 (American Type Culture Collection) and DK25 (DAKO)], anti-CD19 mAb (HD37, DAKO), anti-CD20 (B-Ly1, DAKO), anti-CD68 (Y1/82A, Pharmingen), anti-CD45RA (2H4; the generous gift of Dr. Chikao Morimoto, Dana-Farber Cancer Institute, Boston, MA), anti-CD45RO (UCHL1, DAKO), anti-CD31 [JC/70A (BioGenex Laboratories, Inc., San Ramon, CA) and MBC78.2 (Caltag Laboratories, South San Francisco, CA)], anti-CD54 (R6.5; generous gift of Dr. Robert Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), anti-CD62E (68-5H11; Pharmingen), anti-CD62P (S12; generous gift of Dr. Rod McEver, The University of Oklahoma Health Science Center, Oklahoma City, OK), anti-CD106 (1G11; the generous gift of Dr. Dorian Haskard, RPMS Hammersmith Hospital, London, UK), anti- $\alpha v \beta 3$ (LM609; the generous gift of Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA), anti-collagen IV (MAB1430; Chemicon International, Inc., Temecula, CA), and anti-VAP-1 (2D10; generous gift of Dr. Sirpa Jalkanen, Medicity Research Laboratories, University of Turku, Turku, Finland). Isotype controls included MOPC (American Type Culture Collection), X39 (Sigma Chemical Co., St. Louis, MO), and R35-95 (Pharmingen). Murine vascular endothelial cells were detected using a rat anti-murine mAb (MECA32, Pharmingen) and anti-murine CD18 (M18.2; generous gift of Dr. Akira Takeshima, University of Texas Southwestern) and isotype controls. Proliferating cells were assessed with an anti-human proliferating cell nuclear antigen (PCNA) mAb (PC10, Pharmingen).

Tissue and Cell Preparation

Synovial tissue from RA patients who met the 1987 American College of Rheumatology criteria for classification³¹ was obtained from the knee or hip after joint replacement surgery. The samples were either immediately engrafted

into mice, or snap-frozen in liquid nitrogen for immunohistochemical or molecular analysis. Peripheral blood mononuclear cells were obtained from normal age-matched donors.³² For some experiments, T cells were enriched by passage over either a nylon-wool column or negative-selection column (R&D Systems, Minneapolis, MN). Samples were obtained after informed consent according to the guidelines of the Institutional Review Board of University of Texas Southwestern Medical Center.

RA-SCID Mice

The protocols for the care and use of animals were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Research Advisory Committee. Homozygous SCID (CB.17 scid/scid) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility without prophylactic antibiotics. Similar results were obtained in confirmatory experiments performed with SCID.NOD mice (NOD/Lts-Prkdz^{scid}, The Jackson Laboratory) defective in both innate and adaptive immunity.³³ T-cell deficiency was verified by staining blood for murine CD3 with mAb (clone 145-2C11, Pharmingen) and flow cytometric analysis. Fresh rheumatoid synovium was cut into small pieces of similar macroscopic characteristics while in a Petri dish containing ~5 ml of RPMI 1640 on ice. Immediately afterward, the tissue was engrafted into mice. Three to five female mice per experimental sample were grafted. Mice were between the ages of 4 and 8 weeks old. Mice were anesthetized with a mixture of ketamine, xylazine, and acepromazine and 0.1–0.2 cm³ pieces of RA synovium were surgically implanted subcutaneously into the dorsum. An incision was made along the midline and the grafts were implanted remote from the incision site. All procedures were performed in the surgical suite of the barrier facilities. In some experiments, mice underwent similar surgery except that grafts were omitted and these mice served as sham-operated controls. After 3, 6, or 12 weeks the grafts were harvested and immediately snap-frozen. Grafts were dissected from murine tissue. They were clearly distinguishable from surrounding murine tissue. In addition, blood samples were collected at the time of sacrifice or at various times after engraftment as detailed in the text. For some experiments, enriched human peripheral blood T cells (5×10^7 in 0.1 ml of RPMI) were injected into the tail vein of mice that had been grafted for 3 weeks. The cells were labeled with 5 μ M of the fluorescent dye, 5- (and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) before injection into the mice as described.³⁴ After the indicated trafficking periods, blood samples were taken and the grafts and organs were harvested and snap-frozen for future analysis.

Histology and Immunohistochemistry

Tissues freshly excised from SCID mice were snap-frozen in liquid nitrogen. For immunohistochemistry, 6- μ m

cryosections were prepared from OCT-embedded tissue and acetone fixed. Second step reagents for indirect staining were purchased from BioGenex, with the exception of immunohistochemistry using rat anti-mouse Ig mAbs that were developed using a polyclonal biotinylated rabbit anti-rat antibody (DAKO). Tissue sections were incubated for 60 minutes with serum to block non-specific binding and afterward for 90 minutes with specific antibody or isotype-matched control mAb (20 μ g/ml), washed, and treated for 30 minutes with hydrogen peroxide blocking solution. The samples were washed, incubated for 15 minutes with avidin blocking solution, washed, and incubated an additional 15 minutes with biotin blocking solution. Subsequently, the samples were washed and then incubated for 20 minutes with biotinylated secondary antibody. The sections were washed and incubated for 20 minutes with streptavidin-conjugated horseradish peroxidase. After washing, the sections were developed with 3-amino-9-ethyl carbazole. The tissues were washed and counterstained with hematoxylin and eosin (H&E) (Sigma Chemical Co.). Photomicrographs were taken using an Olympus BHTU light microscope equipped with an Olympus PM-10AD 35-mm photomicrographic system. Unless otherwise indicated, similar staining patterns were observed for multiple sections from at least three different RA synovial tissues analyzed before and after engraftment.

Detection of CFSE-Labeled Migrating T Cells

For histological analysis of CFSE-labeled migrating T cells, cryosections were prepared from OCT-embedded tissue. Serial sections from each tissue were either stained with H&E or immediately assessed for green fluorescence and histology using a Zeiss Axiovert 100M light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an AttoArc lamp, appropriate filters, and digital Axiocam imaging system. In some experiments, CFSE-labeled T cells in murine peripheral blood or control T cells cultured in the presence or absence of phytohemagglutinin (PHA) (Wellcome Diagnostics, Greenville, NC) were stained with quantum red-labeled anti-human CD3 (Sigma Chemical Co.) and analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, CA).

Human Immunoglobulin and IL-6 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay for IL-6 (R&D Systems) and human Ig (The Binding Site, Birmingham, UK) were performed on serum or plasma samples according to the manufacturer's instructions. Samples from sham-operated mice served as controls. Human IL-6 (pg/ml) or Igs (ng/ml) could not be detected in samples from sham-operated mice as minimal cross-reactivity was observed in these assays.

Figure 1. Persistence of CD3⁺, CD4⁺, CD45RO⁺ T cells in RA synovial grafts. Immunohistochemistry was performed on cryosections on fresh RA synovial tissue or grafts of the same tissue 3 weeks or 12 weeks after implantation. Sections were stained with anti-CD3 mAb, anti-CD4 mAb, or anti-CD45RO mAb as indicated. **Bottom:** H&E stain of frozen sections of fresh rheumatoid synovial tissue and grafts. Original magnifications: $\times 100$ (A–C); $\times 400$ (D–F).

RNA Preparation and Reverse Transcriptase-Polymerase Chain Reaction

RA synovial samples were homogenized in guanidinium isothiocyanate and RNA was extracted by CsCl gradient as previously described.³⁵ The RNA was recovered from the gradient, dissolved in 100 μ l of diethyl pyrocarbonate-treated water and the OD₂₆₀ was determined by UV spectrophotometry. For first-strand DNA synthesis, 2 μ g of RNA was converted to cDNA by incubation with AMV reverse transcriptase (Promega). The polymerase chain reaction reactions were performed with the indicated specific primers under nonsaturating conditions as previously published.³⁵ All samples were positive for G3PDH mRNA that was assayed to control for the integrity of the cDNA and samples were analyzed with primers for interferon (IFN)- γ (Clontech, Palo Alto, CA) and TCR-C β .^{35,36} The amplification reaction was performed under standard conditions and the samples were run on a 1.2% agarose gel. Molecular weight markers were used to determine the product size for IFN- γ , 427 bp, and for TCR-C β , 400 bp. Mitogen-stimulated peripheral blood T cell cDNA was

Figure 2. Immunohistochemical staining demonstrating CD68⁺ macrophage-like synovial lining cells and CD20⁺ B cells in fresh RA synovial tissue and 3-week graft. Immunohistochemical staining of CD68⁺ synovial lining cells in fresh RA synovial tissue (A) or 3-week graft (B). Note the reorganization of the lining layer within the hyperplastic graft. CD20⁺ B cells were visualized in fresh tissue (A and C) and 3-week graft (B and D). Note dispersal of B cells within hyperplastic graft. Original magnifications: $\times 100$ (A and B); $\times 400$ (C and D).

used as a positive control. Water controls were negative for the amplified products (data not shown).

Results

Histological Evaluation of Mononuclear Cell Infiltrates in RA-SCID Grafts

Histological changes in the grafts were studied using immunohistochemistry on serial sections of rheumatoid synovial tissue obtained before and after engraftment. H&E staining revealed that freshly isolated rheumatoid synovium contained numerous infiltrating leukocytes among the lacy connective tissue composed of loosely organized fibroblasts (Figure 1). The majority of CD3⁺ T

Figure 3. Macroscopic view of highly vascularized subcutaneous graft. **Arrow** denotes murine blood vessels forming vascular network surrounding encapsulated graft.

cells in the freshly isolated synovial tissue were CD4⁺, CD45RO⁺ memory T cells primarily localized in perivascular regions (Figure 1). Fewer CD8⁺ cells were distributed throughout the synovial tissue (data not shown). Similar numbers of leukocytes consisting mainly of CD3⁺ T cells persisted in the RA-SCID grafts after 3 or 12 weeks (Figure 1). Staining of freshly isolated tissue for CD68 revealed that myeloid lineage cells were found predominantly at the synovial lining and scattered throughout the graft (Figure 2). After engraftment, the CD68⁺ cells persisted. However, the graft underwent reorganization such that the lining layer was no longer clearly demarcated, but rather CD68⁺ cells were distributed throughout the graft. Notably, the fibroblasts within the graft became denser and the synovium no longer retained the loose organization of the fresh tissue. Less frequent aggregates of CD20⁺ B cells were observed in the fresh tissue and were also maintained in the grafts (Figure 2).

Persistence of Human Angiogenesis in RA-SCID Grafts

The reorganization of the graft described above could be in part explained by the host reaction to the implanted tissue. When viewed macroscopically, it is apparent that the host forms a highly vascularized fibrous capsule around the graft (Figure 3). In some experiments, Luconyl blue, an intravascular dye, was administered to mice via the tail vein immediately before sacrifice. In these mice, the continuity of the murine and human vasculature was readily apparent as demonstrated by the presence of the dye in the human blood vessels within the RA synovial graft (data not shown). Immunohistochemical analysis with an antibody specific for murine blood vessels, MECA-32, indicated that small numbers of murine blood vessels invaded the graft. However, murine vessels were primarily confined to the periphery in the region of murine-human anastomosis (Figure 4). As expected, no staining with MECA-32 was observed in fresh rheumatoid synovial tissue. Conversely, staining with an antibody for activated human endothelial cells (VAP-1) demonstrated specific staining of vessels within the graft, whereas no staining was observed in the outer murine fibrous capsule (Figure 5).

A more detailed analysis was undertaken to compare blood vessels within fresh synovial tissue to vessels after engraftment. Freshly obtained RA synovial tissue contained a number of large and small vessels as indicated by high CD31 expression (Figure 6). Consistent with vessels at sites of inflammation, both large and small vessels expressed the endothelial cell activation molecule, VAP-1. Of note, $\alpha v\beta 3$, a receptor transiently up-regulated

Figure 4. Immunohistological analysis of murine blood vessels in human RA synovial grafts. Staining specific for murine endothelial cells was performed with control mAb or MECA-32 mAb on cryosections of fresh synovial tissue or synovial tissue grafts 3, 6, or 12 weeks after implantation. MECA-32 did not stain fresh synovial tissue (**B** and **G**). **Arrows** indicate human synovial tissue graft-murine capsule interface in **C** and **D**. Note that murine vessels were primarily confined to the capsule outside the human graft. Original magnifications: $\times 100$ (**A-E**); $\times 400$ (**F-J**).

Figure 5. Detection of human blood vessels within RA synovial tissue grafts. Low-power view of cryosection showing 3-week graft surrounded by murine capsule (**right**). Note specific staining with anti-human VAP-1 is confined to the graft and is absent from the murine connective tissue forming the capsule. Enlarged region (**left**) shows human blood vessels specifically stain with anti-human VAP-1 mAb. Original magnifications, $\times 400$.

on endothelial cells during angiogenesis was localized to small vessels throughout the synovium. A similar pattern of staining for expression of blood vessels was observed on grafts after 3, 6, or 12 weeks (Figure 7). Both large and small vessels were detected with CD31 at each time point. Importantly, staining with the endothelial cell activation marker, VAP-1, revealed that the endothelium remained activated for up to 12 weeks after engraftment, expressing VAP-1 in a similar manner to freshly derived tissue. Notably, intense staining with $\alpha v\beta 3$ was observed on small vessels throughout the grafts, even after 12 weeks of engraftment, indicating that angiogenesis remained an ongoing active process in the grafts. Staining for the proliferation marker, PCNA, confirmed that new vessel formation in the inflamed tissue continued after engraftment into the SCID mice (Figure 8). Fibroblasts in the grafts also expressed PCNA. These findings suggest that the human vasculature within the graft retained the proinflammatory features of the freshly isolated synovium

Figure 6. Expression of activation and angiogenic molecules on human endothelial cells in fresh RA synovial tissue. Indirect immunoperoxidase staining of cryosections of fresh RA synovium stained with anti-human mAbs to CD31, VAP-1, and $\alpha v\beta 3$. CD31 and VAP-1 were detected on all vessels, however, $\alpha v\beta 3$ exclusively expressed on small vessels correlating with angiogenesis. No staining was observed with isotype control mAbs. Original magnifications for each stain: $\times 100$ (**A–C**) and $\times 400$ (**D–F**).

and indicated that angiogenesis of human vessels was an active ongoing process in the grafts.

Inflammatory Cytokine and Immunoglobulin Production by RA-SCID Grafts

To demonstrate that the grafts in the RA-SCID mice maintained the inflammatory elements of freshly isolated tissue, Ig and IL-6 were assayed in the SCID mouse serum after 3 and 12 weeks of engraftment. As expected, minimal cross-reactivity was detected for human Ig and human IL-6 in serum from control nonengrafted mice (Figure 9). In contrast, human Ig and IL-6 were detected in the sera samples from mice that had been engrafted with RA synovium for 3 and 12 weeks. In most cases, IL-6 levels declined throughout the course of the experiment, unless the mouse received T cells or other proinflammatory signals (Figure 9). In addition, the presence of various subclasses of human IgG was examined in RA-SCID mouse blood. As can be seen in Table 1, all subclasses of IgG were detected in the blood of RA-SCID mice. IgM was detected, but at a lower frequency than IgG. By contrast, no human IgG or IgM could be detected in sham-operated control samples. These data suggest that ongoing B cell and monocyte activity persisted in the RA-SCID grafts. Importantly, these serological assessments provide a means to monitor ongoing inflammation and immunological activity in this model.

Activation of T Cells Recruited to RA-SCID Grafts

Murine leukocytes did not readily infiltrate the graft as was demonstrated by the localization of murine CD18 to the periphery of the graft. As can be seen in Figure 10, staining with anti-murine CD18 revealed no murine leukocytes in the graft. In contrast, murine leukocytes expressing CD18 were detected in the connective tissue surrounding the graft.

Previous studies had demonstrated the migration of human T cells into RA-SCID grafts by indirect means such as radioactive labeling or cytokine mRNA levels.^{29,37} To detect migrating cells, human T cells were labeled with the fluorescent dye, CFSE, before injection into the tail vein of grafted animals. CFSE-labeled cells were readily detected in the peripheral blood immediately after injection and few cells were detected after 72 hours by flow cytometry. To document the specificity of the CFSE labeling, cells were also stained with a mAb for human CD3 that had no cross-reactivity with murine cells. Immediately after injection, staining for human T cells in murine peripheral blood revealed that 96% of CD3+ cells were also CFSE-labeled when RA-SCID mouse blood was analyzed. We detected 36% CD3+, CFSE-labeled cells at 24 hours after injection, 17% CD3+, CFSE-labeled cells at 48 hours, and 6% CD3+, CFSE-labeled cells in the periphery at 72 hours. No division of the cells circulating in the periphery was noted during this time period as indicated by the absence of mitotic dilution of CFSE

Figure 7. Persistence of human endothelial cell activation and angiogenic molecules in RA synovial grafts. Immunohistochemistry was performed on cryosections of grafts harvested at 3 weeks, 6 weeks, or 12 weeks after implantation. RA synovial grafts were stained with anti-human mAbs to CD31, VAP-1, and $\alpha v \beta 3$ or isotype controls. Original magnifications for each stain: $\times 100$ (low-power views: A–C, G) and $\times 400$ (high-power views: D–F, H, I).

fluorescence assessed by flow cytometry (data not shown). These studies suggest that CFSE could be effectively used to track human lymphocytes in the RA-SCID mice.

CFSE-labeled T cells were found to reside specifically in the grafts at 45 hours after injection (Figure 11). By contrast, few CFSE-labeled cells were detected in the liver, heart, spleen, or lung of mice that carried synovial tissue grafts. In addition, spleens of control mice seemed to have similar numbers of mononuclear cells as spleens from injected animals. Thus, these experiments demonstrated that human peripheral blood T cells specifically migrated into and accumulated in the grafts.

Table 1. Immunoglobulin Production by RA-SCID Grafts

Ig	Mouse no.			
	1	2	3	4
IgM	0	1290	0	0
IgG1	1760	780	1263	765
IgG2	2918	9489	3269	6810
IgG3	98	1879	55	214
IgG4	3025	497	486	1926

Serum samples from mice grafted with RA synovium for 60 days. Values are ng/ml. No Ig was detected in sham-operated mice, except for IgG2 (22 ng/ml; $n = 3$).

Additional experiments were conducted to demonstrate the activity of T cells recruited into the grafts. RA synovium was engrafted into SCID mice and allowed to become vascularized. Afterward, human peripheral blood T cells were adoptively transferred into the mice and at varying time points afterward the grafts were harvested along with control grafts from mice that did not receive transferred T cells. Figure 12 shows the results of grafts harvested from five different mice and the T-cell-specific mRNA contents of the grafts as assessed by reverse transcriptase-polymerase chain reaction. All lanes were positive for G3PDH (data not shown). The results suggest that endogenous T cells persisted in the grafts (Figure 12, lanes 1 to 3; lane 1 was weakly positive for TCR- $C\beta$), however, IFN- γ could not be detected in RNA preparations from the grafts until after adoptive transfer of additional T cells (Figure 12, lanes 4 and 5). Therefore, these studies indicate that RA synovial grafts promote minimal ongoing production of IFN- γ by the

Figure 8. Immunohistochemical staining for proliferating cells in fresh RA synovial tissue and 3-week graft. Indirect immunoperoxidase staining of cryosections of fresh RA synovium or graft stained with anti-PCNA mAb. Note intense staining of small blood vessels. Original magnifications: $\times 100$ (A and B); $\times 400$ (C and D).

Figure 9. Detection of human immunoglobulin and IL-6 in RA synovial grafts. Human IgM (ng/ml), human IgG (ng/ml), and human IL-6 (pg/ml) were assessed by enzyme-linked immunosorbent assay. Serum samples of control nonengrafted mice (column 1) mice that had been grafted for 3 weeks (column 2), mice that had been grafted for 12 weeks (column 3), or mice that had been grafted for 12 weeks and received peripheral human T cells 48 hours before assay (column 4).

resident T cells. These studies also suggest, however, that either IFN- γ -producing Th-1 cells selectively home to the graft or that the microenvironment provided by the graft induces Th-1 cell activation of newly migrated T cells.

Discussion

The current studies demonstrate that the RA-SCID model described here is a reasonable alternative to other arthritis models in animals or *in vitro* systems. Whereas previous studies using various models of RA-SCID chimeras have been limited in scope, the current studies demonstrated that the synovial grafts maintained many of the features of the rheumatoid synovium for at least 12 weeks including inflammatory, immune, vascular, and synovial cell components.

Some differences between previous RA-SCID models and the current model were noted. For example, in previous models in which cell suspensions or small pieces of synovium were implanted, lymphodepletion was routinely observed.^{15,21,38,39} In some of these models the synovial tis-

sue was implanted under the kidney capsule or in an ear pouch. It is possible that the location of the graft played a role in the lymphodepletion. Moreover, depletion of leukocytes within the grafts was observed in our preliminary studies when we used smaller grafts or grafts consisting of fine fronds of tissue. Under these conditions it seemed that the cytokines and/or chemokines within the grafts were not sufficient to retain the leukocytes and they migrated out of the grafts. In the current experiments, the grafts were at least two to three times larger than the grafts in the preliminary experiments. In these grafts, similar numbers of leukocytes and specifically CD3⁺, CD4⁺ memory T cells persisted after 12 weeks. Thus, these data suggest that a certain mass of tissue containing the appropriate elements was required to sustain the immunoinflammatory nature of the graft. The production of human IL-6 and Ig confirmed that the B cells and macrophages retained in the grafts continued to be activated.

An important characteristic of RA is the presence of autoantibodies in the circulation. Although we have not tested for rheumatoid factor in the serum of these mice, the observation that relatively high levels of human Ig were produced in the synovium throughout the length of the experiments is significant. Despite the disruption of the B-cell follicles by proliferating fibroblasts, plasma cells continued to secrete antibodies for at least 3 months. In humans, polarized T-cell populations have been reported to bias Ig responses. Thus, Th-1 cells induce IgG1 and IgG3 whereas Th-2 cells support IgG2 and IgG4 responses. No obvious bias was observed in antibody production even though we have previously reported that T cells in the RA synovium are Th-1 polarized.³² This is likely the result of the combination of Th-1 cells and IL-13 being present in the rheumatoid microenvironment. IL-13 has similar effects to IL-4 on B cells, whereas the IL-13 receptor is absent from T cells. Thus, the RA-SCID model provides a unique opportunity for gaining insight into the local immune response that is likely the source of pathogenic autoantibodies. Current studies are focused on investigating this aspect of the model in greater detail.

Our studies suggest that ongoing angiogenesis and activation of human endothelial cells was promoted in the grafts. We observed that when Lyconyl blue was administered intravenously immediately before sacrifice, the anastomosis between murine and human vasculature was readily apparent. Others have noted similar results by dual staining for human and murine vessels indicating ongoing angiogenesis.³⁹ The establishment of an anastomosis between murine and human vessels suggests that complex recognition and organization of structural elements might have allowed continuity of vessels. However, it was evident that murine vessels were primarily excluded from the human grafts, whereas human vessels did not infiltrate the surrounding murine tissue. In the grafts, angiogenesis was indicated by the presence of human α v β 3 on small vessels identified by staining for human CD31 and VAP-1. It should be noted that vascular endothelial growth factor was detected in the serum of grafted mice, suggesting that angiogenic factors were produced for weeks after engraftment (unpublished observation). Human collagen IV was detected, using species-specific antibodies, along the base-

Figure 10. Murine leukocytes do not infiltrate the graft. Immunohistochemical staining for murine cells in the RA synovial tissue grafts. Indirect immunoperoxidase staining of a cryosection of a RA synovial graft stained with anti-murine CD18 mAb. Enlarged view of staining for murine anti-CD18 in the center of the graft (**B** and **C**) or in the adjacent murine connective tissue surrounding the graft (**D** and **E**). Arrows in **B** and **D** indicate enlarged area shown in **C** and **E**, respectively. Original magnifications: $\times 100$ (**B** and **D**); $\times 400$ (**C** and **E**).

ment membranes of large and small vessels in fresh synovium and continued to be expressed after 12 weeks of engraftment. The pattern of collagen expression was similar in fresh RA synovium and in RA grafts (unpublished observation). The expression of additional molecules up-regulated on blood vessels in inflamed synovium was studied in fresh rheumatoid synovium and in grafts. VCAM-1 (CD106) and E-selectin (CD62E) were expressed on the vessels in RA synovium and in RA grafts. Increased expression of ICAM-1 (CD54) was also observed on endothelial cells in fresh RA synovium and in RA grafts. ICAM-1 was also expressed by the synovial lining cells and mononuclear cells scattered throughout the synovium. Although the lining layer was disrupted in RA grafts, ICAM-1 expression was maintained on the myeloid cells in the synovial grafts (unpublished observation). Thus, the grafts maintained a high level of vascularity and many features of the inflamed RA synovium were retained in the RA grafts.

It should be noted that in a similar model cellular adhesion molecules on microvascular endothelial cells in RA-SCID grafts were down-regulated after 4 weeks after transplantation.³⁹ These cellular adhesion molecules, indicative of an inflammatory state, could be up-regulated once again by intra-graft injection of tumor necrosis factor- α . Several important differences were observed between this report and the current studies in tissue preparation and engraftment. In the previous studies, the size of the tissue used was considerably smaller and synovial tissue was frozen and thawed before implantation. We have observed that most mature myeloid cells are highly sensitive to freeze and thaw techniques and might not have survived initial engraftment. As previously discussed, we have found that smaller grafts are unable to retain leukocytes, presumably as a result of an inability to maintain the appropriate cytokine, chemokine, and adhesion molecules in the more limited microenvironment. Lymphodepletion has been reported in several similar models.^{15,38,39} Likewise, Wahid and colleagues³⁹ concluded that in the absence of T cells producing the

Figure 11. CFSE-labeled human T cells traffic specifically into RA synovial grafts. Fluorescent imaging (**top**) or H&E stains (**bottom**) of representative cryosections of graft, liver, heart, spleen, and lung from RA-SCID mice engrafted for 3 weeks. CFSE-labeled human peripheral blood T cells were readily detected in the grafts 45 hours after injection. Original magnification, $\times 200$.

Figure 12. T cells accumulating in rheumatoid synovial grafts produce IFN- γ . Agarose gels of reverse transcriptase-polymerase chain reaction products for IFN- γ and TCR- β chain constant region illuminated by ethidium bromide. RA-SCID grafts were harvested from mice after 3 weeks (lanes 1, 4, and 5), after 6 weeks (lane 2), or after 12 weeks (lane 3). Total cellular RNA was prepared as described in Materials and Methods. Peripheral blood T cells (5×10^7 in 0.1 ml of RPMI) obtained from a normal donor were injected into the tail vein of animals 24 hours before harvesting the grafts (lanes 4 and 5).

required cytokines, after 4 weeks, the grafts returned to a "resting state" and the vasculature expressed decreased levels of ICAM-1 and VCAM-1 as compared to fresh tissue. Restimulation of the vasculature by intra-graft injection of cytokines^{38,39} or intra-graft injection of activated lymphocytes³⁸ up-regulated adhesion molecules on the human vessels within the grafts. Thus, it is likely that the prolonged expression of cellular adhesion molecules observed in the current study directly reflects ongoing cytokine and/or chemokine production by the graft.

The current experiments suggest that human T cells specifically migrated into the synovial grafts via the human blood vessels. Thus, human blood vessels in the grafts maintained a remarkably similar phenotype to fresh RA synovial tissue. As opposed to previous studies in which human T cells were localized to the murine fibrous capsule surrounding kidney grafts,¹⁵ we and others found that human T cells were found predominantly surrounding the vessels and distributed throughout the grafts.³⁹ Specificity was demonstrated by the comparative absence of murine leukocytes in the grafts. Thus, the route of T-cell injection, graft size, or the location of the graft might have contributed to sufficient vascularization that allowed trafficking of human leukocytes via transendothelial migration into the grafts.

Several studies have suggested that the RA synovial microenvironment plays a role in biasing CD4⁺ T cells toward a Th-1 phenotype. Interestingly, we found that similar to previous reports examining freshly isolated synovial T cells, resident T cells within the RA grafts were unable to sustain detectable levels of IFN- γ mRNA.⁴⁰ We have previously shown, however, that freshly isolated T cells from RA synovial tissue or fluid display a Th-1 cytokine profile after a brief *in vitro* activation.³² Moreover, a significant increase in IFN- γ mRNA in circulating RA peripheral blood mononuclear cells was observed when entry of circulating T cells was blocked with a mAb to the adhesion molecule, ICAM-1, suggesting a redistribution of activated Th-1 cells from the inflammatory site into the peripheral circulation.³⁵ These studies suggest that chronic Th-1 activation might contribute to the inflammatory state. In the current studies, freshly isolated peripheral blood T cells rapidly converted to IFN- γ -producing Th-1 effector cells on entry into the graft. In preliminary experiments, similar results were obtained with autolo-

gous synovial tissue T cells that were maintained in culture until injection. Although, the role of specific chemokines in attraction and retention of Th-1 cells in the RA synovium is controversial, the data suggest that the RA synovium either selects by the production of specific chemokines or directs by the production of specific cytokines the activation of Th-1-polarized T cells.^{32,41,42} The current model system provides a unique approach to dissecting the role of the synovium in biasing T-cell responses.

It should be mentioned that throughout the course of these experiments, no overt signs of graft *versus* host disease were observed in the grafted animals. This was most probably because the lymphocytes were retained in the grafts. Moreover, no acute graft *versus* host disease was seen when T cells were injected for trafficking studies. Previous studies have suggested that without sufficient autologous antigen-presenting cells, human xenoreactive T cells become tolerant in these animals.⁴³ To determine human T-cell reactivity to SCID mononuclear cells, we examined the response of human peripheral blood mononuclear cells to SCID spleen cells *in vitro* and observed minimal responses at day 3 and 6 of culture as assessed by measuring DNA synthesis (unpublished observation). The same cells responded robustly to the T-cell mitogen, PHA. Therefore, it seems that graft *versus* host disease by resident T cells in the graft is unlikely, because these cells are retained within the graft, respond poorly to murine stimulator cells, and appear to express little, if any, Th-1 cytokines. T cells injected for trafficking studies that do not migrate into the graft seem to undergo rapid clearance from the animal. Moreover, our preliminary data suggest that either the precursor frequency of xenoreactive T cells is fairly low or that other as yet undetermined suppressive effects of SCID spleen cells contribute to the poor stimulatory capacity of these cells.

In summary, RA-SCID grafts retain human leukocytes and produce Igs and cytokines similar to the fresh synovial tissue. Moreover, these grafts sustain angiogenesis and the vascular endothelium maintains an activated phenotype. The grafts support the recruitment and activation of T lymphocytes. Such activated T cells produce the proinflammatory cytokine, IFN- γ . This model system will be used to further understanding of the regulation of the major components of the rheumatoid synovium and to investigate the efficacy of new therapeutic modalities for RA.

Acknowledgments

We thank Dr. Richard Jones and Dr. Joseph Matthews for providing patient samples, Amy White and Angela Schlitz for excellent technical assistance, and Angie Mobley of the University of Texas Southwestern Medical Center Dallas Cell Analysis Facility center for assistance with the flow cytometer.

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