Short Communication

The Co-Expression of Activating and Inhibitory Leukocyte Immunoglobulin-Like Receptors in Rheumatoid Synovium

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Rheumatoid arthritis (RA) is a chronic inflammatory synovitis, with destruction of juxtaarticular cartilage and bone, likely mediated by lipid mediators, cytokines, and proteases released from inflammatory leukocytes. The mechanisms regulating leukocyte activation in rheumatoid synovium are not fully elucidated. A new family of cell surface proteins termed leukocyte immunoglobulin-like receptors (LIRs) has been shown *in vitro* to modulate cellular responses through immunoreceptor tyrosine-based inhibitory motifs or through association with the Fc receptor γ chain that contains immunoreceptor tyrosine-based activation motifs. We studied the expression of inhibitory and activating LIRs in the synovium of six RA patients, three osteoarthritis patients, and three controls by immunohistochemistry. The synovium from patients with early RA showed extensive expression of the inhibitory LIR-2 and the activating LIR-7 on macrophages and neutrophils. Some mast cells and endothelial cells expressed LIR-7. There was limited expression of LIRs in synovium from two patients with long-standing RA, patients with osteoarthritis, and controls. LIR-2 recognizes MHC class I molecules. We therefore suggest that LIRs may regulate the activation of infiltrating leukocytes in synovial tissue and are a potential therapeutic target. (*Am J Pathol* 2002, 160:425–431)

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis characterized by synovial hypertrophy and synovial pannus formation with accompanying destruction of jux-

taarticular cartilage and bone.¹ The predominant inflammatory cells are macrophages (type A synoviocytes) and fibroblast-like cells (type B synoviocytes) with increased numbers of neutrophils, mast cells, natural killer cells, plasma cells, and lymphocytes.^{2–6} These cells may play a major role in the process of inflammation and tissue destruction by releasing multiple factors such as lipid mediators,^{7,8} proinflammatory cytokines,^{9–12} and tissue-degrading enzymes.^{13,14} Synovial macrophages are the predominant source of interleukin-1 β and tumor necrosis factor- α that are central to the pathogenesis of RA as evidenced by the efficacy of disease-modifying therapies targeted at these cytokines.¹⁵ The joint destruction in RA is likely mediated by proteases derived from macrophages and osteoclasts. Although there is abundant evidence for the presence of activated leukocytes in rheumatoid synovium, the mechanism(s) and regulation of their activation are not fully elucidated and the fundamental underlying etiology of RA remains obscure.

Inflammatory responses are likely regulated by a complex network of inhibitory and activating signals. The leukocyte immunoglobulin-like receptors (LIRs), also termed immunoglobulin-like transcripts (ILTs), comprise a new family of cell surface proteins that have been shown *in vitro* to modulate cellular responses through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) or through association with the Fc receptor γ chain that contains an immunoreceptor tyrosine-based activation motif.^{16–18} The inhibitory LIRs (LIRs-1, -2, -3, -5, and -8) display long cytoplasmic domains with two to four ITIMs. These receptors mediate inhibition of cell activation by recruiting the src homology 2 (SH2) domain-containing phosphatase 1 (SHP-1) to inhibit or terminate signaling through nonreceptor tyrosine kinase cascades.^{19–22} The

Supported by National Institutes of Health grant U19 AI 31599 and a Postdoctoral Fellowship from the Arthritis Foundation.

Accepted for publication November 6, 2001.

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activating LIRs (LIR-6a, LIR-6b, LIR-7, ILT-8, and ILT-11) are characterized by a short cytoplasmic domain and a positively charged arginine residue within the transmembrane domain that facilitates association with the common Fc receptor γ chain that contains an immunoreceptor tyrosine-based activation motif.²³ Cross-linking of LIR-7 elicited Ca^{2+} -flux in monocytes, transfected P815 cells, and RBL cells.²³ A third type of LIR (LIR-4) is a soluble molecule with no transmembrane domain. Although LIR-1 and LIR-2 are known to interact with class I molecules with broad specificity recognizing classical class I alleles within HLA-A, -B, -C, and the nonclassical HLA-G,^{21,24,25} the ligands for most of the LIRs are not known.

The cellular distribution of LIR-1, LIR-2, LIR-5, and LIR-7 has been studied in detail using monoclonal antibodies. LIR-1 is expressed on all peripheral blood monocytes, *in vitro*-derived dendritic cells and macrophages, B cells, and a subset of T cells and NK cells.^{18,24} A more restricted cellular distribution was reported for LIR-2 and LIR-5, which are most prominent on monocytes and dendritic cells.²¹ LIR-7 is expressed in all peripheral blood monocytes and granulocytes, *in vitro*-derived macrophages, and dendritic cells.²³ Expression of the other LIRs has only been determined at the mRNA level. Transcripts for LIR-3 and LIR-6 were detected in monocytes and B cells,^{16,17} transcripts for LIR-4 were detected in B cells, NK cells, and monocytes,^{16,17} whereas transcripts for LIR-8 were detected only in NK cells.¹⁷

The expression of LIRs in normal and inflamed tissues has not been studied. The aim of this study was, therefore, to compare the *in vivo* expression and cellular distribution of activating and inhibitory LIRs in RA, in osteoarthritis (OA), and in normal synovium. OA synovium was included as a control because inflammatory reactions in the synovial tissue in OA occur in the absence of pannus formation and tissue invasion.^{26,27} We used immunohistochemistry with specific monoclonal antibodies to detect expression of LIRs. Serial sections stained with cell lineage-specific antibodies were used to evaluate the cellular localization of expressed LIRs.

Materials and Methods

Study Patients

Six patients with a history of RA ranging from 2 to 14 years and three patients with a history of OA ranging from 3 to 13 years underwent excision of synovial tissue from the knee joint under general anesthesia. Normal synovial tissue was obtained from three patients during reconstructive knee surgery for traumatic meniscus rupture. The institutional ethics committees approved this study.

Immunohistochemical Studies

Synovial tissue was embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and sectioned at 2 to 4 μm for histopathological analysis and immunohistochemical studies. Specific

mouse IgG1 monoclonal antibodies against LIR-2, LIR-3, and LIR-7 were generated in BALB/c mice by immunization with LIR-Fc fusion proteins containing the LIR extracellular domains fused to the Fc region of human IgG1 as described.^{24,28} The antibodies were screened for binding specificity by enzyme-linked immunosorbent assay against a panel of LIR-Fc fusion proteins and by fluorescence-activated cell sorting analysis using COS-1 cells transfected with full-length LIR cDNAs. Irrelevant mouse IgG1-negative control was purchased from Biosource International (Camarillo, CA). These antibodies were used in a three-step alkaline-phosphatase staining technique as described elsewhere.²⁹ In brief, acetone-fixed sections were equilibrated with Tris-buffered saline and blocked with neat horse serum for 20 minutes at room temperature. Sections were then incubated with 5 $\mu\text{g}/\text{ml}$ of primary antibodies overnight at 4°C. After four washes with Tris-buffered saline, sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After four washes with Tris-buffered saline, sections were incubated with streptavidin-alkaline phosphatase conjugate (Vector Laboratories) for 45 minutes at room temperature. Immunoreactivity was detected using a colorimetric alkaline-phosphatase substrate (Vector Red, Vector Laboratories) and brief counter staining with hematoxylin. Optimal conditions for use of each anti-LIR antibody were initially defined using a panel of normal tissues likely to contain LIR-expressing cells; skin, thymus, lymph nodes, tonsil, and small intestine.

Immunohistochemical studies of adjacent sections were undertaken to determine the specific cell types that are immunoreactive to the LIRs as described elsewhere.^{29,30} Antibodies to detect macrophages (mouse IgG1 anti-CD68), T cells (rabbit polyclonal anti-CD3), endothelial cells (mouse IgG1 anti-Von-Willebrand factor), neutrophil cathepsin G (rabbit polyclonal), and mast cell tryptase (mouse IgG1) were purchased from DAKO (Glostrup, Denmark).

In addition to the immunohistochemical staining, a standard hematoxylin and eosin stain was used to evaluate the quality and histology of each section.

Semiquantitative Evaluation of Expression

The tissue sections from the immunohistochemical studies were evaluated by counting contiguous fields across the whole section as described elsewhere.³⁰ In brief, an average of 18 fields at a magnification of $\times 250$ was selected per section in a systematic sampling procedure. After ensuring that the sections stained with isotype control exhibited no significant immunoreactivity, the number of positive cells (red staining) per field was enumerated. Although significant regional variation in staining was observed, the median count for the whole section is reported as a conservative measure of the staining for each antibody.

Table 1. Expression of Leukocyte Immunoglobulin-Like Receptors in RA, OA, and Normal Synovium

Study participants	Duration of illness (years)	Histology	Immunohistochemical expression of LIRs (median cell count/HPF)		
			LIR-2	LIR-3	LIR-7
Rheumatoid arthritis					
RA1	2	Extensive neutrophil infiltration and moderate numbers of macrophages and mast cells	39	6	18
RA2	5	Widespread macrophage infiltration and small areas of lymphocyte aggregation	25	1	13
RA3	8	Macrophage and lymphocyte aggregation. Moderate degree of fibrosis with mast cell infiltration	15	7	8.5
RA4	8	Extensive fibrosis and endothelial proliferation with some areas of CD68+ macrophage infiltration	5	2	3
RA5	10	Extensive fibrosis with small numbers of macrophages	0	0.5	0.5
RA6	14	Extensive fibrosis with small numbers of macrophages	0.5	0.5	0.5
Osteoarthritis					
OA1	3-5	Moderate macrophage and lymphocyte infiltration	0.5	1	0
OA2	9	Moderate macrophage and lymphocyte infiltration. Limited numbers of mast cells	0	2.5	0
OA3	13	Extensive fibrosis	2	7	1
Controls					
N1	—		0	0	0
N2	—		0	0	0
N3	—		0	0	0

Results

Histological Features of Synovial Tissue

The histological features of synovial tissues and the expression of LIRs are summarized in Table 1. Sections from two RA patients (RA1 and RA2) with a shorter duration of illness (2 to 5 years) showed extensive infiltration with inflammatory cells including CD68-positive macrophages, cathepsin G-positive neutrophils, a moderate number of tryptase-positive mast cells, and clusters of CD3-positive T cells. In the remaining four RA patients there were varying degrees of inflammatory cell infiltration and tissue fibrosis. There was significant macrophage infiltration with limited numbers of T cells and mast cells in sections obtained from two patients with OA (Table 1). The third patient with OA had extensive fibrosis with macrophages at the outer edges of the synovial membrane. Few or no inflammatory cells were detected in all sections obtained from normal individuals.

Immunohistochemical Detection of LIRs

To examine expression of LIR proteins in synovial tissue, we began with two inhibitory receptors, LIR-2 and LIR-3, and one activating receptor, LIR-7. These LIRs have a relatively restricted expression on cells of myeloid origin, which are important sources of cytokines and proteases in RA.¹ Furthermore, LIR-2 is known to recognize MHC class I molecules, which are widely distributed in human tissues.

There was extensive expression of LIR-2 and LIR-7 in sections obtained from three RA patients with early to intermediate duration of illness (Table 1; RA1, RA2, and RA3). The expression of LIR-2 and LIR-7 was extremely limited for patients with a long duration of RA and was negligible in patients with OA. The expression of LIR-3 in all patients with RA and OA was variable and limited. None of the LIRs were expressed in control tissues obtained from normal individuals.

Table 2. The Cellular Sources of LIR-2, LIR-3, and LIR-7 in Synovium from Patients with Rheumatoid Arthritis and Osteoarthritis

Study participants	LIR-2	LIR-3	LIR-7
Rheumatoid arthritis			
RA1	Neutrophils	Macrophages	Neutrophils, macrophages, mast cells, endothelial cells
RA2	Macrophages	Macrophages and fibroblast-like cells	Macrophages, endothelial cells, fibroblast-like cells
RA3	Macrophages, endothelial cells	Macrophages and fibroblast-like cells	Macrophages, mast cells, endothelial cells
RA4	Macrophages	Fibroblast-like cells, macrophages	Macrophages, endothelial cells
RA5	ND	Fibroblast-like cells	Endothelial cells
RA6	ND	ND	Fibroblast-like cells
Osteoarthritis			
OA1	Macrophages	Macrophages	Macrophages
OA2	ND	Macrophages	ND
OA3	Fibroblast-like cells	Fibroblast-like cells	Endothelial cells

ND, not done because of limited LIR expression.

Figure 1. LIR expression in rheumatoid synovium. **A–C:** Serial sections from patient RA1 stained with antibodies to LIR-2 (**A**), LIR-7 (**B**), and cathepsin G (to identify neutrophils) (**C**). **Insets B** and **C** depict immunostaining of serial sections for LIR-7 (**B**) and mast cell tryptase (**C**). **D:** Staining of synovium from patient RA1 with isotype-matched negative control antibody. **E–G:** Serial sections from patient RA2 stained with antibodies to LIR-2 (**E**), LIR-7 (**F**), and CD68 (**G**). **H:** Staining of synovium from patient RA2 with isotype-matched negative control antibody.

Figure 2. High-power views of immunostaining for LIRs. Immunostaining for LIR-3 on fibroblast-like cells in patient RA3 (A), for LIR-7 on endothelial cells (B), and with isotype-matched negative control antibody (C) in patient RA2.

Cellular Source of LIR Expression in RA

The cellular localization of LIRs in synovial tissue is summarized for all patients in Table 2 and illustrated for patients RA1 and RA2 in Figures 1 and 2. The cellular distribution of LIR-2 and LIR-7 differed among RA patients reflecting the nature of the inflammatory cell infiltrate. Neutrophils were the major cellular source of LIR-2 in one patient (RA1; Figure 1, A and C). Macrophages were the major cellular source of LIR-2 in another patient

(RA2; Figure 1, E and G). Expression of LIR-7 was somewhat less than that of LIR-2 and the cellular distribution of LIR-7 was wider than that of LIR-2 (Table 2). LIR-7 was expressed by neutrophils (Figure 1, B and C), macrophages, mast cells (Figure 1, B and C, inset) and endothelial cells in patient RA1, and by macrophages (Figure 1, F and G), endothelial cells (Figure 2B), and fibroblast-like cells in patient RA2. The cellular sources of LIR-2 and -7 in all remaining RA patients were macrophages and to a lesser extent endothelial cells (figure not shown). The limited expression of LIR-2 in OA was observed mainly on CD68+ macrophages. LIR-3 was exclusively expressed by macrophages and fibroblast-like cells (Table 2, Figure 2A) in patients with RA. Isotype-matched negative control antibodies did not yield immunostaining in any patient (Figure 1, D and H, and Figure 2C).

Discussion

The expression of LIRs, assessed by reverse transcriptase-polymerase chain reaction or flow cytometry, has previously been reported in various types of circulating leukocytes of myeloid and lymphoid origin.^{16-18,21,24} The present study extends these results to evaluate for the first time the expression of LIRs in normal and inflamed tissues.

Immunohistochemical studies demonstrated the expression of LIR-2 (inhibitory), LIR-3 (inhibitory), and LIR-7 (activating) on infiltrating leukocytes in rheumatoid synovium. The expression of LIR-2, LIR-3, and LIR-7 on CD68-positive cells, likely macrophage-like synoviocytes, is consistent with previous studies describing their expression on peripheral blood monocytes and *in vitro*-derived macrophages.^{16,17,23} There is limited data on the expression of LIRs on circulating granulocytes. Nevertheless, the expression of LIR-2, LIR-3, and LIR-7 on neutrophils infiltrating the rheumatoid synovium is consistent with published data for LIR-7²³ and with our unpublished findings for all three of these LIRs on circulating polymorphonuclear leukocytes (N Tedla and JP Arm, unpublished observations). In contrast to the restricted expression of LIR-2 and LIR-3 on inflammatory leukocytes, LIR-7 was variably expressed on mast cells, fibroblasts, and endothelial cells in rheumatoid synovium. We have previously reported transcripts for LIR-3, but not LIR-4 or LIR-5, in human pulmonary mast cells using reverse transcriptase-polymerase chain reaction.¹⁶ The lack of staining for LIR-3 in mast cells in rheumatoid synovium may reflect mast cell heterogeneity, regulated expression at sites of inflammation, or the sensitivity of reverse transcriptase-polymerase chain reaction in picking up low levels of transcripts not accompanied by significant expression of protein. Nevertheless, related mouse molecules, the paired Ig-like receptors, and gp49 are expressed on mouse bone marrow-derived mast cells.³¹⁻³⁴ The demonstration of LIR-7 on fibroblast-like cells, most probably type B synoviocytes, and on endothelium is entirely novel and suggests a range of functions beyond the regulation of leukocyte activation, such as participation in cell adhesion, cell recruitment, or fibrogenesis.

The expression of LIR-2 and LIR-7 was especially marked in early rheumatoid disease (Table 1). With increasing duration of disease the synovial tissue showed more fibrotic changes and the number of cells expressing LIR-2 and LIR-7 dramatically decreased. Similar to the late stages of RA, we found limited expression of both the activating and inhibitory LIRs in the synovium obtained from patients with OA. No LIRs were detected in synovial tissue from normal donors. These observations may indicate that these molecules are preferentially present in active inflammatory conditions that are characterized by extensive leukocyte infiltration and play a limited role in chronic degenerative conditions or chronic inflammatory states in which there is established fibrosis. Little is known about the factors that regulate the expression of LIRs. Nevertheless, the prominence of LIR-2 and LIR-7 in early RA as opposed to late disease, their sparse expression in the cellular infiltrate in OA, and their expression on endothelium and mast cells in early RA but not OA or normal tissue (Table 2; Figures 1 and 2), suggest that not only are LIRs expressed in leukocytes but also there is regulated expression of LIRs in tissues.

It has been suggested that LIRs and related molecules may determine the threshold and/or extent of activation of leukocytes. This idea is supported by recent studies in mice with disruption of mouse gp49B1.³⁵ gp49B1 has two Ig-like extracellular domains, homologous to those of the LIRs.³⁶ It belongs to the inhibitory class of this family of molecules with two ITIMs in its cytoplasmic domain. Co-ligation of gp49B with Fc ϵ R1 on mast cells inhibits signaling through the latter receptor through recruitment of SHP-1, which is dependent on the phosphorylated ITIMs of gp49B1.^{37,38} Mice with disruption of gp49B1 demonstrated a significantly increased sensitivity to IgE-dependent passive cutaneous anaphylaxis with greater tissue edema and mast cell degranulation. Significantly, the absence of gp49B1 resulted in a lower threshold for antigen challenge in active cutaneous anaphylaxis and increased mortality in active systemic anaphylaxis.³⁵ These data provide proof of principle for a role of the homologous LIRs in regulating the threshold for activation of inflammatory cells and in determining the severity of inflammation *in vivo*. The range of ligands recognized by the large family of LIRs has yet to be appreciated. Nevertheless, LIR-2 recognizes and regulates cellular responses through recognition of a diverse array of MHC class I molecules.²⁸ Thus, it is tempting to speculate that the regulated expression of LIR-2 on leukocytes infiltrating the rheumatoid synovium may determine their threshold of activation. On the other hand, LIR-2 and the activating LIR-7 were both expressed on neutrophils and macrophages infiltrating the rheumatoid synovium (Figure 1). Thus, the relative balance of inhibitory and activating LIRs expressed by a particular cell may play an important role in determining its activation response.

The range of cellular responses regulated by LIRs has been studied *in vitro*. Thus, recognition of MHC class I molecules by LIR-1 or LIR-2 inhibits natural killer cell activity and T cell cytotoxicity.^{20,28} Engagement of LIR-1, LIR-2, or LIR-3 by plate-bound antibodies led to inhibition of IgE-dependent exocytosis of RBL cells.^{20,21} Co-liga-

tion of inhibitory LIR-1, LIR-2, LIR-3, or LIR-5 with an activating receptor such as the BCR, TCR, Fc γ R, or MHC class II molecules led to inhibition of Ca²⁺ flux and subsequent downstream events elicited by the activating molecule.^{19–21,39} These events have recently been elucidated for the interaction of LIR-1 with the TCR.²² In this study, the ITIM of LIR-1 was phosphorylated by the non-receptor tyrosine kinase *lck*, leading to recruitment of SHP-1, inhibition of phosphorylation of LAT, and the TCR ζ chain, inhibition of the association of ZAP-70 with the TCR ζ chain, and inhibition of ERK activation. The full spectrum of responses inhibited or elicited by inhibitory and activating LIRs, respectively, has yet to be elucidated. It is tempting to speculate that LIRs may regulate protease and cytokine expression in the inflammatory infiltrate in RA and thereby regulate the process of pannus formation and joint destruction. The LIRs also provide a potential novel therapeutic target in pathological inflammatory processes such as rheumatoid arthritis. Definitive demonstration of a role for LIRs in regulating inflammation *in vivo* awaits the development of specific agonists and antagonists.

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