

β 1 Integrin-mediated Interaction with Extracellular Matrix Proteins Regulates Cytokine Gene Expression in Synovial Fluid Cells of Rheumatoid Arthritis Patients

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Summary

Inflammatory cytokines have been implicated in the pathogenesis of rheumatoid arthritis (RA), whereas the mechanisms for constitutive production of inflammatory cytokines in affected joints are largely unknown. Recently, integrin-mediated interaction with extracellular matrix (ECM) proteins has been demonstrated to play a role in regulating cytokine production in T cells and monocytes. In this study, we investigated the contribution of the β 1 integrin-mediated interaction with ECM proteins to the persistent cytokine gene expression in RA synovial fluid mononuclear cells (SFMNC). We examined mRNA expression of 14 cytokines in the SFMNC of three RA patients, which were either fresh or cultured overnight in serum-free medium on ECM-coated plates, by polymerase chain reaction with a panel of oligonucleotide primers specific for each cytokine. The persistent expression of various cytokine mRNA found in fresh SFMNC was maintained after overnight culture in serum-free medium on ECM proteins, especially on laminin (LM), but not on serum albumin. This effect of LM was inhibited by an anti-integrin β 1 chain (CD29) mAb, as well as by an anti-CD3 mAb, indicating an important role of the β 1 integrin-mediated interaction with ECM proteins in regulating persistent cytokine gene expression in RA SFMNC, and a key role of T cells in regulating inflammatory monokine production.

Rheumatoid arthritis (RA) is a chronic inflammatory disease with autoimmune features that is characterized by erosive bone and cartilage degradation in synovial joints (1). Inflammatory cytokines, including IL-1, TNF, IFN- γ , GM-CSF, and IL-8, have been implicated in the pathogenesis of RA (1, 2). These cytokines contribute to initiate and amplify the inflammation directly or indirectly by inducing the release of degrading enzymes and chemical mediators from synovial cells and infiltrating cells, or by recruiting and activating inflammatory cells. The presence of these cytokines has been demonstrated in situ or in the synovial fluid (SF) of RA patients (2, 3), and a spontaneous release of these cytokines in cultures of RA synovial cells has been commonly observed (2, 4, 5). However, little is known about the cause of the persistent cytokine production in the affected joints.

Chronic synovial inflammation in RA is characterized by marked infiltration of mononuclear cells (MNC) into both the tissues and SF. In these infiltrates, activated T cells with increase expression of CD25, HLA-DR, and CD29 (β 1 integrins) are commonly observed (6–8). These T cells are thought

to play a role in the initiation and perpetuation of the inflammatory reactions within the affected joints by directly damaging the tissues or by indirectly regulating the inflammatory cells by the cytokines they produce (1). At present, the antigen responsible for eliciting T cell-mediated immune responses within the RA joint has not yet been identified, although the involvement of some cartilage proteins or self stress proteins has been suggested (9, 10). However, accumulating evidence indicates that the T cell activation requires not only the antigen stimulation, but also a costimulatory signal mediated by some accessory molecules (11, 12).

Recently, β 1 integrin-mediated interaction with various extracellular matrix (ECM) proteins has been implicated in leukocyte migration and activation (13, 14). VLA-4- and VLA-5-mediated interaction with fibronectin (FN), and VLA-6-mediated interaction with laminin (LM) have been demonstrated to deliver a costimulatory signal required for IL-2 production and proliferation of anti-CD3-stimulated peripheral blood T cells. In our previous study, we demonstrated that the integrin-mediated interaction with ECM proteins

not only augments antigen responses, but also induces effector functions of murine T cell lines, such as degranulation and IL-2 production, in an antigen-independent manner (15). It has been documented that T cells with elevated $\beta 1$ integrins were accumulated in the RA synovial compartment (7, 8), and that T cells from RA subjects were stimulated in response to ECM proteins (16, 17). Therefore, it seems likely that the stimulatory signal mediated by $\beta 1$ integrins may play a role in regulating the effector functions of chronically activated T cells in RA synovium abundant in ECM proteins. In the present study, we describe the involvement of $\beta 1$ integrin-mediated interaction with ECM proteins in regulating various cytokine gene expression in SFMNC of RA patients, persistent production of which has been implicated in the pathogenesis of RA.

Materials and Methods

Study Subjects. SF samples were obtained from three patients fulfilling the American Rheumatism Association criteria for the classification of RA (18) at therapeutic aspiration from the inflamed knee joints (see Table 1). The age range was from 42 to 61 yr. Disease duration ranged from 2 to 33 yr. All the patients were RF positive and receiving nonsteroidal antiinflammatory drugs (NSAID).

Preparation and Culture of SFMNC. SFMNC were prepared from SF treated with hyaluronidase (10 U/ml) for 30 min at 37°C by Ficoll-Hypaque density gradient centrifugation at 400 *g* for 40 min, washed three times, and resuspended in serum-free AIM-V medium (Gibco, Grand Island, NY). SFMNC contained 19–70% CD3⁺ T cells, 35–81% of which were HLA-DR⁺, and 18–58% CD14⁺ monocytes as estimated by FACS[®] analysis (see Table 1). The remains were contaminated PMN, as estimated by histochemical staining. SFMNC resuspended in AIM-V (5×10^6 cells/ml) were cultured in 24-well plates (Corning Inc., Corning, NY), which were precoated with 10 μ g/ml of human plasma FN (Gibco), bovine type II collagen (Koken, Tokyo, Japan), mouse LM (Sigma Immunochemicals, St. Louis, MO), or BSA (Sigma Immunochemicals), for 16 h at 37°C in the presence or absence of 10 μ g/ml of an anti-CD29 (integrin $\beta 1$ chain) mAb 4B4 (kindly provided by Dr. C. Morimoto, Dana-Farber Cancer Institute, Boston, MA),

an anti-CD18 mAb TS1/18 (purchased from American Type Culture Collection, Rockville, MD), an anti-CD44 mAb NIH44-1 (kindly provided by Dr. S. Shaw, National Institutes of Health, Bethesda, MD), or Fab fragment of an anti-CD3 mAb OKT-3 (purchased from the American Type Culture Collection). The cultured cells were collected by a brief treatment with 0.05% EDTA in PBS.

PCR-assisted Detection of Cytokine mRNA. Cytoplasmic RNA was prepared from fresh or cultured SFMNC by the NP-40 method (19). First-strand cDNA was synthesized from 2 μ g of RNA with 0.5 μ g of oligo(dT) primer by using the SuperScript Preamplification System (GIBCO BRL, Gaithersburg, MD) in 20 μ l of the reaction mixture. All the PCR primers specific for IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IFN- γ , TNF- α , TNF- β , TGF- β , GM-CSF, and β -actin were purchased from Clontech (Palo Alto, CA). For PCR, 0.1 μ l of cDNA was amplified in the presence of 1 μ M each of the 5' and 3' primers, 5 μ M digoxigenin-11-dUTP (Boehringer Mannheim, Tokyo, Japan), 200 μ M dNTPs, 0.5 U of Taq polymerase (Cetus Corp., Emeryville, CA), and PCR buffer containing 2.5 μ M MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.001% gelatin in a final volume of 20 μ l. PCR was performed in a DNA thermal cycler (Cetus Corp.) for 30 cycles (94°C for 1 min, 60°C for 2 min, and 72°C for 3 min) followed by a 10-min extension at 72°C. 8 μ l of the PCR products was subjected to electrophoresis on 2% agarose gels, blotted onto nylon membrane (Biodyne; Pall, Glen Cove, NY), and detected by using an alkaline phosphatase-labeled antidigoxigenin antibody and a chemiluminescent substrate, AMPPD (DIG Luminescent Detection Kit; Boehringer Mannheim), according to the manufacturer's instructions.

Results

Expression of various cytokine genes was detected in SFMNC freshly isolated from the three RA patients as estimated by the PCR detection method with a panel of oligonucleotide primers specific for each cytokine (Fig. 1). Although some individual differences were observed, these include inflammatory monokines, such as IL-1 α , IL-1 β , IL-8, TNF- α , and lymphokines predominantly produced by T cells, such as IL-2, IFN- γ , and GM-CSF. This is consistent with previous studies where the expression of these cytokine genes was assessed by Northern blotting or in situ hybridization (2).

Table 1. Characteristics of the Studied Subjects

| Patient | Sex | Age | Duration of disease yr | Disease classification | | RF | Medications | Phenotype of SFMNC | | |
|---------|-----|-----|------------------------------|---------------------------|-------|----------------|--------------------|--------------------|-----------------------------------|-------------------|
| | | | | Class | Stage | | | CD3 ⁺ | DR ⁺ /CD3 ⁺ | CD14 ⁺ |
| 1 | F | 53 | 8 | I* | III | + [†] | NSAID [§] | 70% | 38% | 18% |
| 2 | F | 61 | 33 | II | II | + | NSAID | 69% | 81% | 20% |
| 3 | F | 42 | 2 | II | II | + | NSAID | 19% | 35% | 58% |

* Steinbrocker's classification.

[†] Rheumatoid factors assessed by latex fixation test.

[§] Non steroidal antiinflammatory drug.

^{||} Results of FACS[®] analysis.

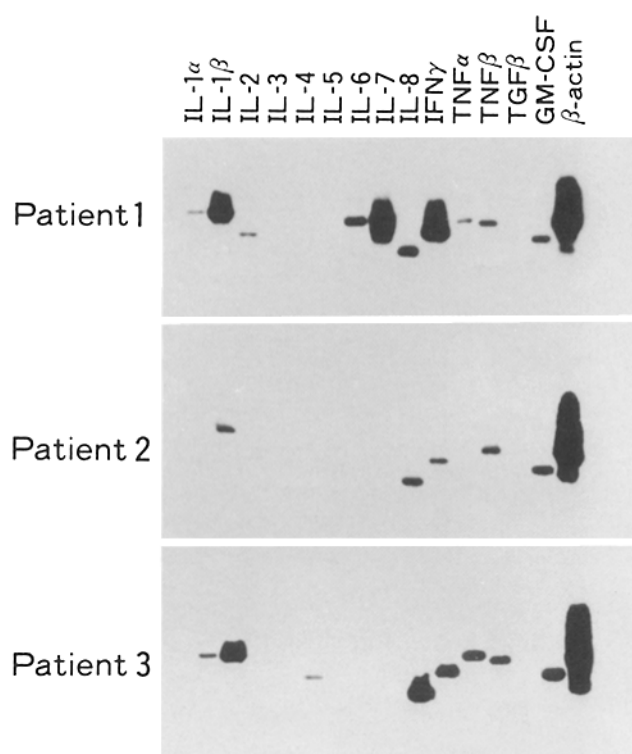


Figure 1. Cytokine gene expression in fresh SFMNC. Cytoplasmic RNA prepared from fresh SFMNC of three RA patients was subjected to PCR detection of the indicated cytokine mRNAs.

Such an apparently spontaneous expression of various cytokine genes in SFMNC ceased after a brief culture in a serum-free medium on BSA-coated plates, although the transcription of β -actin gene was not affected (Fig. 2). The latter indicates that the cessation of cytokine gene transcription was not due to reduced viability of SFMNC in the serum-free culture. In contrast, when SFMNC were cultured on LM-coated plates, the transcriptional levels of IL-1 α , IL-1 β , IL-2, IL-7, IFN- γ , and TNF- α were totally retained (Fig. 2). The transcription of IL-7 and IFN- γ genes was also retained on FN- or CL-coated plates albeit to a lesser extent. These results indicated that ECM proteins, especially LM, affect the expression of various cytokine genes including inflammatory cytokines such as IL-1 α , IL-1 β , IL-8, IFN- γ , and TNF- α .

It has been known that β 1 integrins, including VLA-1, VLA-2, VLA-3, and VLA-6, are primarily responsible for the interaction of leukocytes with LM (13). In this respect, we examined whether the effect of LM to perpetuate cytokine gene expression in SFMNC is mediated by the β 1 integrins by estimating the blocking by an anti-CD29 (integrin β 1 chain) mAb. As indicated in Fig. 3 A, the expression of IL-1 α , IL-1 β , TNF- α , and IFN- γ genes, which was retained on LM but not on BSA, was greatly reduced by the addition of anti-CD29 mAb. Such an inhibitory effect was not observed with anti-CD18 (Fig. 3 A) or anti-CD44 mAb (data not shown). Similar results were also obtained with cultures of SFMNC from the other two patients (Fig. 3, B and C).

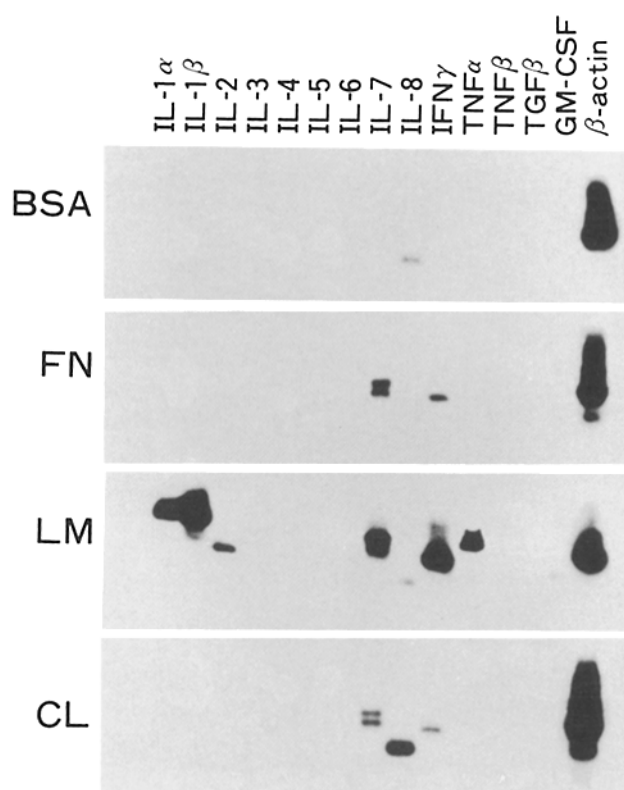


Figure 2. Cytokine gene expression in SFMNC cultured on ECM proteins. Cytoplasmic RNA prepared from SFMNC (patient 1), which were cultured for 16 h in serum-free medium on BSA-, FN-, LM-, or CL-coated plates, was subjected to PCR detection of the indicated cytokine mRNAs.

The reduction of IL-1 and TNF- α gene transcription on LM was commonly observed in the presence of anti-CD29 mAb. It should be noted that the expression of cytokines that are predominantly produced by T cells, including IL-2, IL-4, and IL-5, on LM was also affected by anti-CD29 mAb in SFMNC from patient 2 (Fig. 3 B). These results indicate that the β 1 integrin-mediated interaction with LM is critically involved in the persistent expression of inflammatory cytokine genes.

We next examined the involvement of TCR/CD3-mediated recognition of some antigen in the cytokine gene expression on LM by adding an anti-CD3 mAb, since a central role of T cells, which might react with some antigen in the synovium, in perpetuating the inflammatory responses (1), and a role of β 1 integrins in regulating the TCR/CD3-mediated T cell activation, have been postulated (13, 14). As indicated in Fig. 3, B and C, anti-CD3 mAb exhibited an inhibitory effect similar to that of anti-CD29 mAb. It is interesting that not only was the expression of T cell cytokines, such as IL-2 and IL-5, but also that of monokines, such as IL-1 and TNF- α , greatly affected by anti-CD3 mAb. These results suggest a central role of T cells in regulating the persistent expression of inflammatory cytokine genes in SFMNC.

Discussion

In the present study, we revealed a critical role of the β 1 integrin-mediated interaction with ECM proteins in regulating

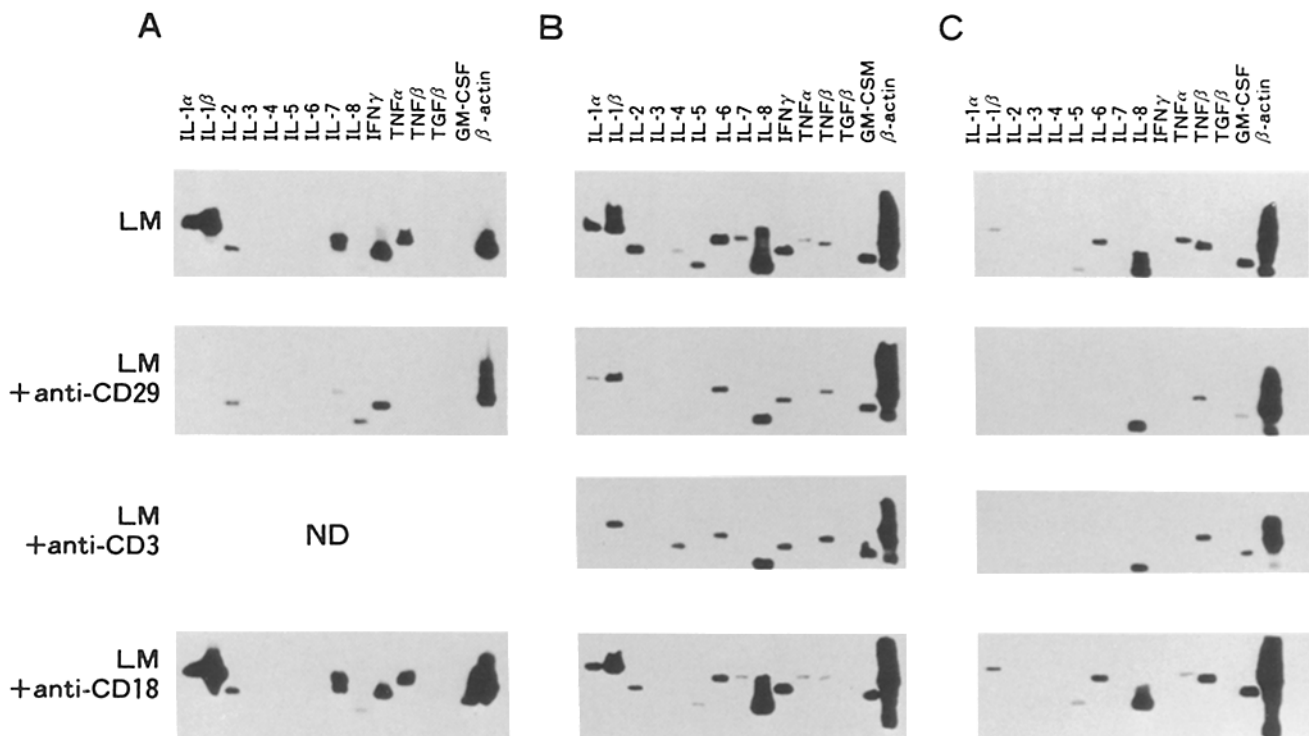


Figure 3. Effect of anti-CD29, anti-CD3, and anti-CD18 mAb on persistent cytokine gene expression on LM. Cytoplasmic RNA prepared from SFMNC (A, patient 1; B, patient 2; and C, patient 3), which were cultured for 16 h in serum-free medium on LM-coated plates in the presence or absence of anti-CD29, anti-CD3, or anti-CD18 mAb, was subjected to PCR detection of the indicated cytokine mRNAs. (ND) Not done.

the persistent expression of various cytokine genes in the SFMNC of RA patients in vitro. In addition, a central role of T cells in regulating monokine production was also suggested. These would be responsible for the apparently constitutive production of various cytokines in vivo in the inflamed synovium, where it is abundant in ECM proteins and is infiltrated by activated T cells with elevated $\beta 1$ integrins.

At present, only a little is known about the mechanism whereby the $\beta 1$ integrin-mediated interaction with ECM proteins regulates cytokine gene expression. It has been reported that clustering of $\beta 1$ integrins led to increased protein tyrosine phosphorylation (20). Yamada et al. (21) reported that the VLA-5-mediated interaction with FN provided a costimulatory signal requisite for IL-2 gene expression in peripheral blood CD4⁺ T cells by inducing an AP-1 transcriptional factor independently of the TCR/CD3-mediated stimulation. AP-1 has been implicated in the transcriptional regulation of various cytokines, including IL-1 α , IL-1 β , TNF- α , and IL-6 (22). In addition, it has been recently reported that $\beta 1$ integrins on monocytes act as a primary signal transduction molecule regulating inflammatory cytokine gene expression (23). Monocyte adherence also induced the *c-fos* gene expression, the product of which is a component of AP-1 (24). Therefore, it is possible that the AP-1 induction by a signal mediated by $\beta 1$ integrins may be primarily responsible for the ECM induction of various cytokine gene expression in SFMNC.

However, it seems more likely that the expression of all cytokine genes retained on ECM proteins was not necessarily

directly regulated by the $\beta 1$ integrin-mediated signal. A complex cytokine network, such as the TNF induction of monocyte IL-1 and GM-CSF production (5, 25), and the IL-1 or TNF induction of monocyte IL-8 production (26), has been demonstrated to be involved in the inflammatory cytokine production in SFMC. Alternatively, contaminated PMN would also contribute to the monokine gene expression, since they are known to be potent producers of IL-1 β and TNF- α (27, 28). Therefore, the high expression of these monokines observed in vivo and in vitro might be secondary or tertiary in response to other cytokines in a paracrine or autocrine fashion. The donor-dependent differences in cytokine production observed in Figs. 1 and 3 might reflect such a complexity of the regulatory processes. However, it should be noted that anti-CD3 mAb exhibited an inhibitory effect similar to that of anti-CD29 mAb on the persistent cytokine gene expression on LM. Anti-CD3 mAb inhibited not only the expression of T cell cytokines but also that of monokines. This suggests that the monokine production in SFMNC is secondary in response to T cell-derived cytokines or a direct contact with activated T cells, as has been represented by the IL-1 induction in macrophages through interaction with activated T cells (29, 30).

The fact that the persistent cytokine gene expression in SFMNC on LM was blocked by anti-CD3 mAb, as well as by anti-CD29 mAb suggests that both the TCR/CD3-mediated recognition of a certain antigen in the culture and the $\beta 1$ integrin-mediated costimulatory signal were required for the cytokine gene expression. At present, the antigen involved

in this response is unknown. However, a similar situation has been documented in the case of murine γ/δ T cell lines, where the "spontaneous" production of IL-4 in response to Arg-Gly-Asp (RGD) containing ECM proteins was blocked by antivitronection receptor ($\beta 3$ integrin) mAb, as well as by anti-CD3 mAb (31). In that case, the γ/δ T cell lines expressed a particular combination of V γ and V δ gene products that has been implicated in the reactivity to a mycobacterial heat-shock protein (hsp) and a self stress protein, suggesting that the TCR-mediated recognition of a self antigen was involved in that response. In this respect, it is noteworthy that hsp-reactive T cells have been demonstrated to accumulate in the SFMNC of RA patients (9, 10). Therefore, it seems likely that bacterial or self hsp might be presented by monocytes or activated DR⁺ T cells as an antigen for such T cells in the cultures performed in this study. Consistent with this notion, high proliferative response of a hsp65-

reactive T cell clone to SFMNC from the inflamed joint (32) and an elevated expression of hsp in the RA synovium (10) have been demonstrated. In addition, it has been known that hsp expression in monocytes could be induced by treatment with inflammatory cytokines such as IFN- γ and TNF- α (33, 34). Therefore, hsp-reactive T cells and inflammatory cytokines may constitute a circuit perpetuating inflammatory reactions in the RA synovium.

Whatever the antigen is, a costimulatory signal mediated by $\beta 1$ integrins through their interaction with ECM proteins appear to be requisite for the persistent expression of some inflammatory cytokines in RA SFMNC. Therefore, antibodies or peptides interfering with the integrin-mediated interaction with ECM proteins might be useful for quenching the overproduction of harmful inflammatory cytokines in the affected tissues. Such a possibility is now under investigation in experimental animal arthritis systems.

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