Interleukin-18 Induces Rheumatoid Arthritis Synovial Fibroblast CXC Chemokine Production through NFκB Activation

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SUMMARY: Interleukin-18 (IL-18) is a novel proinflammatory cytokine that was recently found in synovial fluids and in synovial tissues from patients with rheumatoid arthritis (RA). To determine the participation of IL-18 in the inflammation observed in RA, we investigated the effect of IL-18 on RA synovial fibroblast chemokine production. Using FACS analysis, we showed that IL-18 induced a doubling in the production of intracellular IL-8 by RA synovial fibroblasts, and this result was confirmed by Western blot. At the extracellular level, IL-18 up-regulated the secretion of IL-8 in a dose- and time-dependent manner. IL-18 also up-regulated the other CXC chemokines, epithelial-neutrophil activating protein (ENA-78) and growth-regulated oncogene (groα), in a dose dependent manner, but failed to induce the production of the CC chemokine, macrophage inflammatory protein (MIP)-1α. By immunofluorescence and Western blot, we demonstrated that IL-18 activates the translocation of the transcription factor nuclear factor kappa B (NFκB) into the nucleus of RA synovial fibroblasts. IL-18 induces IL-8 secretion through NFκB because RA synovial fibroblasts pretreated with antisense to NFκB p65 oligonucleotide produce a mean of 44% less IL-8 compared with cells pretreated with the control sense oligonucleotide. These results indicate a novel role for IL-18 in inducing RA synovial fibroblast expression of CXC chemokines through NFκB and place this cytokine in a strategic role in the local inflammation observed in RA. (*Lab Invest 2001, 81:1371–1383*).

Chemokines are low-molecular-weight cytokines that attract leukocytes through a series of coordinated biochemical and cellular events (Baggiolini et al, 1997). These chemotactic cytokines can be divided into four groups (CXC, CC, C, and CX_3C) according to the position of the first two closely paired and highly conserved cysteins of the amino acid sequence (Szekanecz et al, 1998).

In rheumatoid arthritis (RA), a chronic inflammatory disease characterized by synovial hyperplasia and leukocyte infiltration, there is abundant evidence that chemokines participate in the recruitment of the leukocytes into the RA synovial membrane (Koch et al, 1996). In the RA joint, chemokines are expressed in synovial fluids and also in synovial tissues, especially in the synovial lining layer. The fibroblasts that compose this lining layer with the macrophages are a major source of chemokines, particularly when stimulated with proinflammatory cytokines like interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α).

Interleukin-18 (IL-18) is a novel cytokine structurally related to IL-1β and is, therefore, considered a member of the IL-1 cytokine family (Bazan et al, 1996). Activated macrophages were the first cells shown to express high levels of IL-18 (Okamura et al, 1995). Further investigations revealed that IL-18 was also produced by various types of cells, such as osteoblasts, articular chondrocytes, keratinocytes, and synovial fibroblasts (Gracie et al, 1999; Naik et al, 1999; Olee et al, 1999; Udagawa et al, 1997). Like IL-1β, IL-18 is first synthesized as a precursor molecule (pro IL-18) that contains a signal peptide and requires the IL-1β converting enzyme (ICE, caspase 1) for cleavage into the active and mature protein (Fantuzzi and Dinarello, 1999). The secreted IL-18 recognizes a heterodimeric receptor consisting of two amino acid chains that are also members of the IL-1 receptor family (Torigoe et al, 1997). The ligand binding IL-18R chain, now termed IL-18Rα, was initially described as the IL-1 receptor binding protein. The IL-18Rβ chain is similar to the IL-1 accessory protein and induces signal transduction. The signaling pathways that follow the binding of IL-18 to its receptor are not completely known. The IL-18 receptor is expressed on T and B lymphocytes, natural killer (NK) cells (Yoshimoto et al, 1998), RA macrophages (Gracie et al, 1999), and fibroblast-like cells (Moeller et al, 1999). IL-18 functions to stimulate Th₁ cytokines, such as interferon gamma (IFNγ), TNF-α, and granulocyte-macrophage colony stimulating factor (GM-CSF), by T lymphocytes and NK cells (Takeda et al, 1998). IL-18 was recently

detected in synovial fluid derived from RA patients at concentrations as high as those of TNF-α (Gracie et al, 1999). Additionally, IL-18 was found in the synovium within and adjacent to lymphocytic aggregates and also in the lining layer. Macrophages derived from this lining layer produce inflammatory mediators when stimulated with IL-18. Little is known about the immune effects of IL-18 on RA synovial fibroblasts. These cells, which are intimately located in proximity to the macrophages, participate in the synovial inflammation and the cartilage destruction by releasing mediators of inflammation and matrix-degrading enzymes. Therefore, we examined the effect of IL-18 on RA synovial fibroblast chemokine production. In this study, we showed that IL-18 independently promotes

the production of CXC chemokines through a direct effect on synovial fibroblasts. We report evidence for the significance of the nuclear factor kappa B (NFκB) pathway in IL-18-induced chemokine expression.
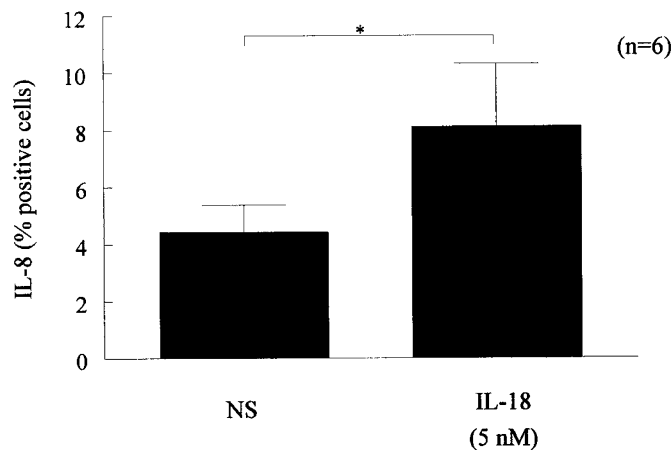
Results

Intracellular IL-8 Is Up-Regulated in IL-18–Stimulated RA Synovial Fibroblasts

To evaluate intracellular IL-8 production, we performed FACS analysis on RA synovial fibroblasts from six patients with RA. Figure 1A is a representative example of the flow cytometry data. The left panel corresponds to the histogram obtained for RA synovial

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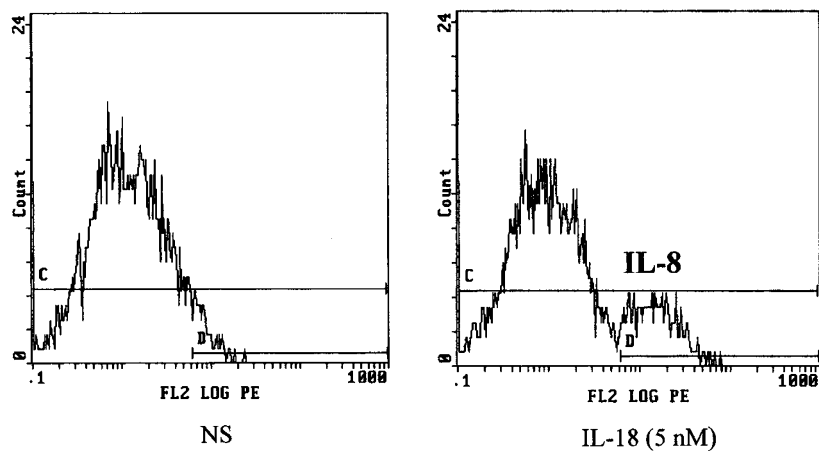
B

Figure 1.

Effect of interleukin (IL)-18 on endogenous IL-8 expression in rheumatoid arthritis (RA) synovial fibroblasts. A, RA synovial fibroblasts (1 $\times$ 10^5/ml) from six different patients with RA were stimulated with IL-18, IL-1β, or tumor necrosis factor-α (TNF-α). After 24 hours, cells were harvested for intracellular flow cytometric analysis. The histogram is a representative intracellular experiment: *x* axis, number of cells; *y* axis, fluorescence intensity. Left panel, no stimulation (NS). Right panel, IL-18 stimulation: the second peak corresponds to the cells positive for IL-8. Isotype-matched control staining was less than 2%. B, IL-18 stimulates IL-8 production by intracellular FACS. Results represent the percentage $\pm$ SEM of positively stained cells for IL-8 from two different experiments; *p* < 0.05 compared with nonstimulated cells; Wilcoxon rank order test.

fibroblasts incubated with media alone, and the right panel to the IL-18–stimulated cells. IL-18 treatment for 24 hours resulted in a 2-fold increase in intracellular IL-8 level compared with RA synovial fibroblasts cultured with medium only (Fig. 1B). IL-1ß and TNF-α stimulation also enhanced intracellular IL-8 by 5- and 6-fold, respectively (data not shown). These results were confirmed by Western blot analysis (Fig. 2). This blot showed a concentration- and time-dependent increase in IL-8 expression in response to IL-18 stimulation. Compared with nonstimulated cells, 5 and 50 nM of IL-18 induced a 3- and 5.9-fold increase in IL-8 expression at 28 hours and a 13.2- and 14.7-fold increase at 96 hours.

Effect of IL-18 on Extracellular IL-8, ENA-78, groα, and MIP-1α Production by RA Synovial Fibroblasts

Next, we determined the effect of IL-18 on extracellular IL-8 production and also on the production of other CXC chemokines, such as epithelial-neutrophil activating protein (ENA-78) and growth-regulated oncogene (groα), and the CC chemokine macrophage inflammatory protein (MIP)-1α. We measured the protein levels of these chemokines in the conditioned media of fibroblasts stimulated for 24 hours with different concentrations of IL-18 using ELISA assays. The mean $\pm$ SE values for IL-8, ENA-78, groα, and MIP-1α are presented in Figure 3A and Figure 4, A to C. In the absence of costimuli, IL-18 up-regulated the synthesis of the three CXC chemokines (IL-8, ENA-78, and groα) in a dose-dependent manner. At 2.5 nM, IL-18 significantly enhanced the production of IL-8, ENA-78, and groα in the nanomolar range. The optimum induction was observed at a concentration of 10 nM of IL-18 with a 150% increase in IL-8, a 200% increase in ENA-78, and a 12% increase in groα compared with unstimulated RA synovial fibroblasts (p

< 0.05; $n = 5$). Cells stimulated with IL-1β or TNF-α produced larger amounts of IL-8 (9 to 11 ng/ml) and equal amounts of ENA-78 and groα compared with those stimulated with IL-18 (data not shown). The CC chemokine MIP-1α was detected in the conditioned media of RA synovial fibroblasts exposed to TNF-α but not in those fibroblasts exposed to IL-18 or IL-1β.

IL-8 was used as an example to examine the time course of chemokine secretion from IL-18–stimulated RA synovial fibroblasts (Fig. 3B). Cells from five patients with RA were cultured in the presence or absence of IL-18 (5 nM), and conditioned media were collected at 4, 8, 24, and 48 hours. Nonstimulated cells released small quantities of IL-8 (between 42 pg/ml $\pm$ 0 and 842 pg/ml $\pm$ 0.6). In contrast, IL-18–stimulated IL-8 production increased exponentially. At 4 hours, the mean IL-8 concentration was 132 pg/ml $\pm$ 0.05 and reached 3.6 ng/ml $\pm$ 1.2 at 48 hours.

IL-18 Does Not Synergize with IL-12 to Induce IL-8

IL-12 is another proinflammatory and immunomodulatory cytokine that has a central role in directing the Th$_1$ response (Hsieh et al, 1993; Seder et al, 1993). One of the characteristics of IL-12 is its synergistic function with IL-18, in vitro (Yoshimoto et al, 1997, 1998) and in vivo (Leung et al, 2000; Zhang et al, 1997). We therefore studied a combination of IL-12 and IL-18 for the ability to stimulate RA synovial fibroblast IL-8 production. IL-12 alone, at 70, 280, and 1400 pM stimulated little IL-8 expression. Using 70 pM of IL-12 and increasing concentrations of IL-18, there was no augmented induction of IL-8 production (Fig. 5A). The repetition of this experiment with a higher concentration of IL-18 (5 nM) and three different concentrations of IL-12 (70 pM, 280 pM, and 1400 pM) induced similar levels of IL-8, compared with levels of IL-8 induced by IL-18 alone (Fig. 5B), confirming the
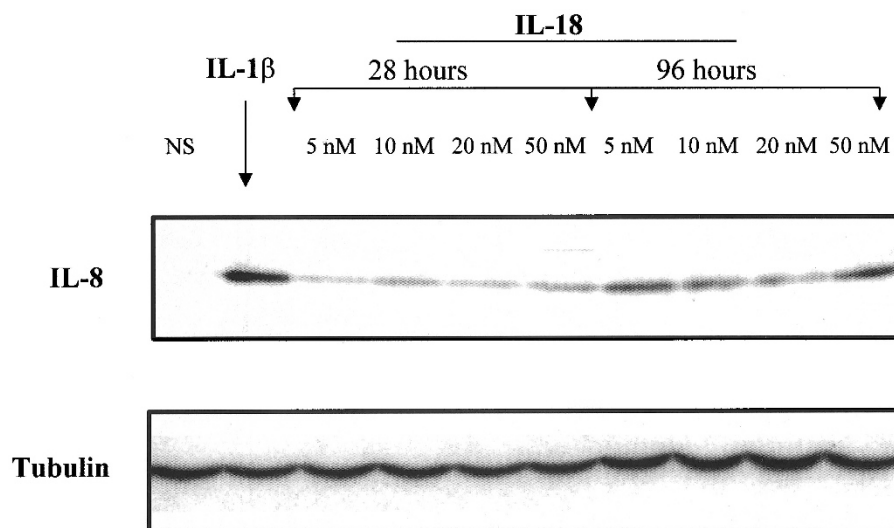
Figure 2.

Time course and dose response of endogenous IL-8 expression by Western blot. RA synovial fibroblasts (1 $\times$ 10^5/ml) were stimulated with IL-1β or various concentrations of IL-18 for 28 hours and 96 hours. Equal amounts of extracted proteins were separated by SDS gel electrophoresis, and Western blotting was performed as described in "Materials and Methods." Equal loading of protein was verified with anti-tubulin antibody.

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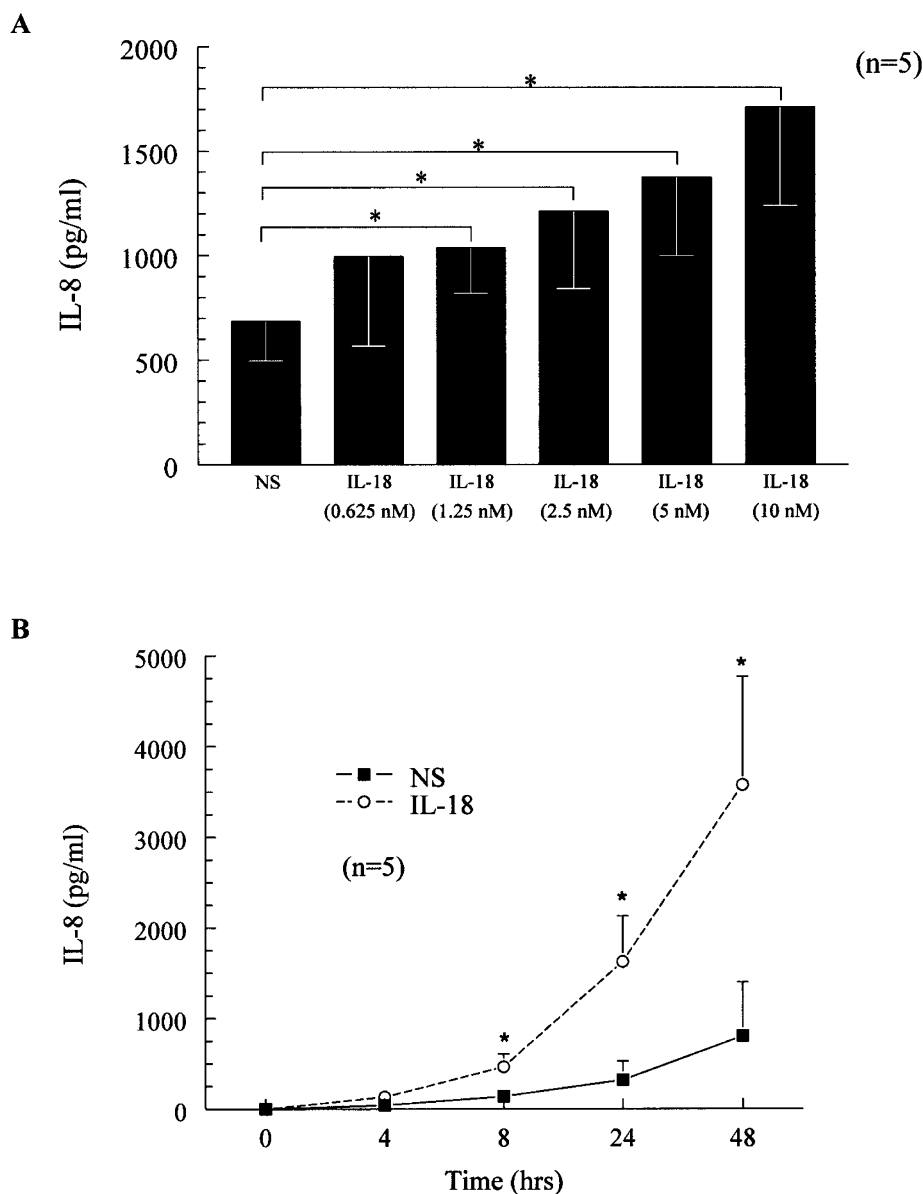
Figure 3.
Time- and dose-dependent production of IL-8 by IL-18–stimulated RA synovial fibroblasts. A, Equal numbers of cells (1 × 10⁵/ml) were stimulated with various concentrations of IL-18 indicated on the horizontal axis. Conditioned media were collected after 24 hours and assayed for IL-8 by ELISA. B, For the time-dependent generation of IL-8, RA synovial fibroblasts (1 × 10⁵/ml) were cultured for various time points in the presence or absence of 5 nM IL-18. Results represents the means ± SEM of conditioned media from five different patients with RA. *$p < 0.05$ compared with nonstimulated control; Student *t* test.

absence of a synergistic effect of these two cytokines in combination on RA synovial fibroblasts.

IL-18 Induces Nuclear Translocation of NFκB (p65) in RA Synovial Fibroblasts

Next, we investigated the intracellular pathway used by IL-18 to induce chemokine production. Because IL-18 was reported to induce NFκB activation (Matsumoto et al, 1997; Shapiro et al, 1998; Torigoe et al, 1997), we investigated whether this transcription factor was involved in the IL-18–induced IL-8 expression. When NFκB is activated, it translocates from the cytoplasm to the nucleus. To assess the nuclear

translocation of NFκB in RA synovial fibroblasts, we subjected cytoplasmic and nuclear extracts from control and IL-18–induced cells to Western blot analysis (Fig. 6). In the cytoplasm of cells stimulated with IL-18 for 0.5 and 1 hour, the amount of NFκB p65 was higher compared with control nonstimulated cells. Incubation with IL-18 resulted in an increase in the nuclear expression of p65 that occurred at 2 hours and was sustained for at least 4 hours. This translocation of NFκB p65 to the nucleus is illustrated in Figure 7 using immunofluorescence (*arrows*). RA synovial fibroblasts treated with IL-18 (5 nM) or IL-1β (1.75 nM) for 1 hour induced a nuclear uptake of p65 as demonstrated by nuclear staining (*arrows*) (Fig. 7, B
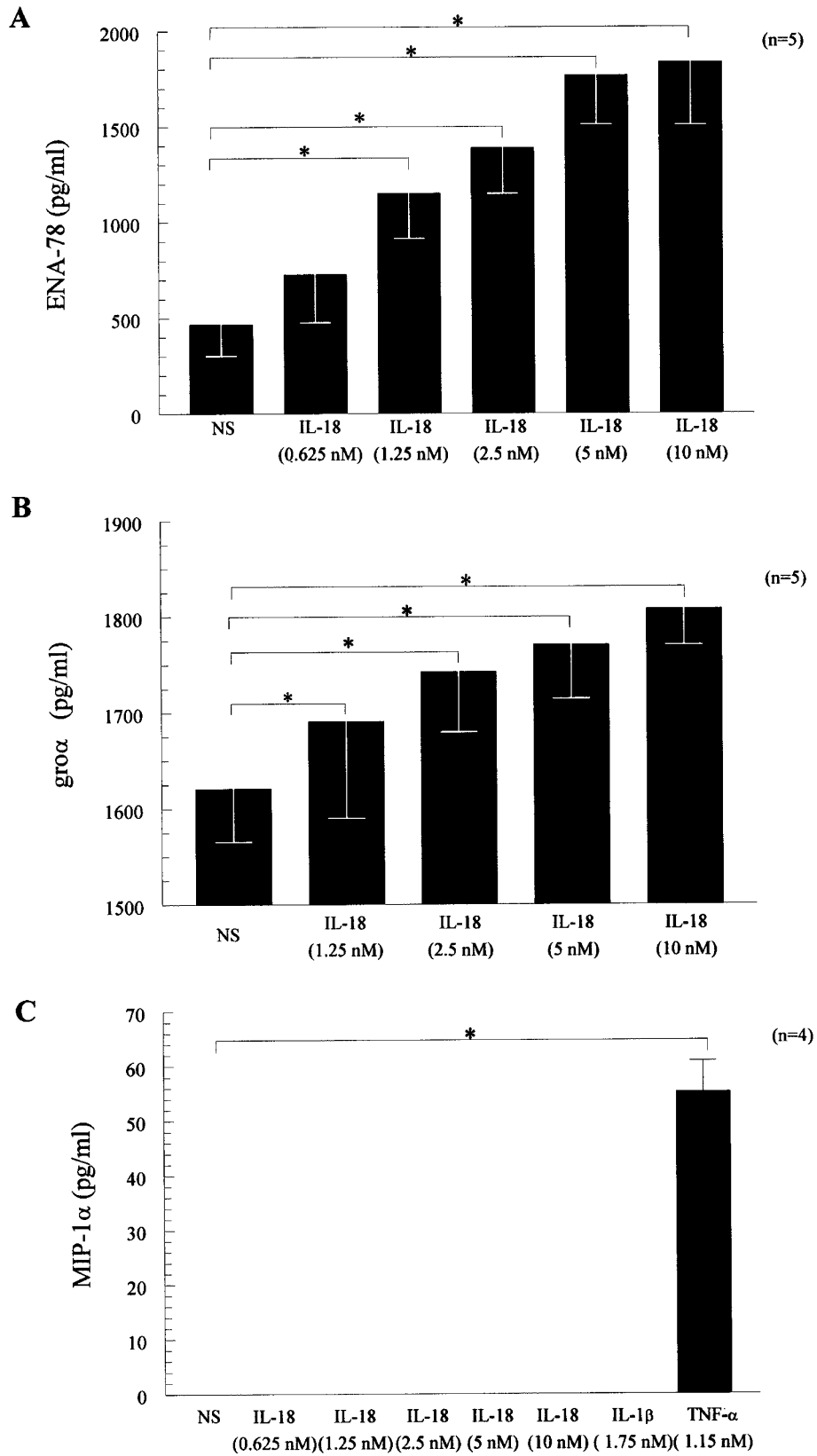
Figure 4.
Epithelial-neutrophil activating protein (ENA-78) (A), growth-regulated oncogene (groα) (B), and macrophage inflammatory protein (MIP)-1α (C) production by RA synovial fibroblasts treated with IL-18. The procedure used to collect conditioned media for these experiments is described in Figure 3. Chemokines released in the conditioned media were measured by ELISA. Results represent the mean ± SEM of cells from five different patients with RA. *$p < 0.05$ compared with nonstimulated control using a Student t test.

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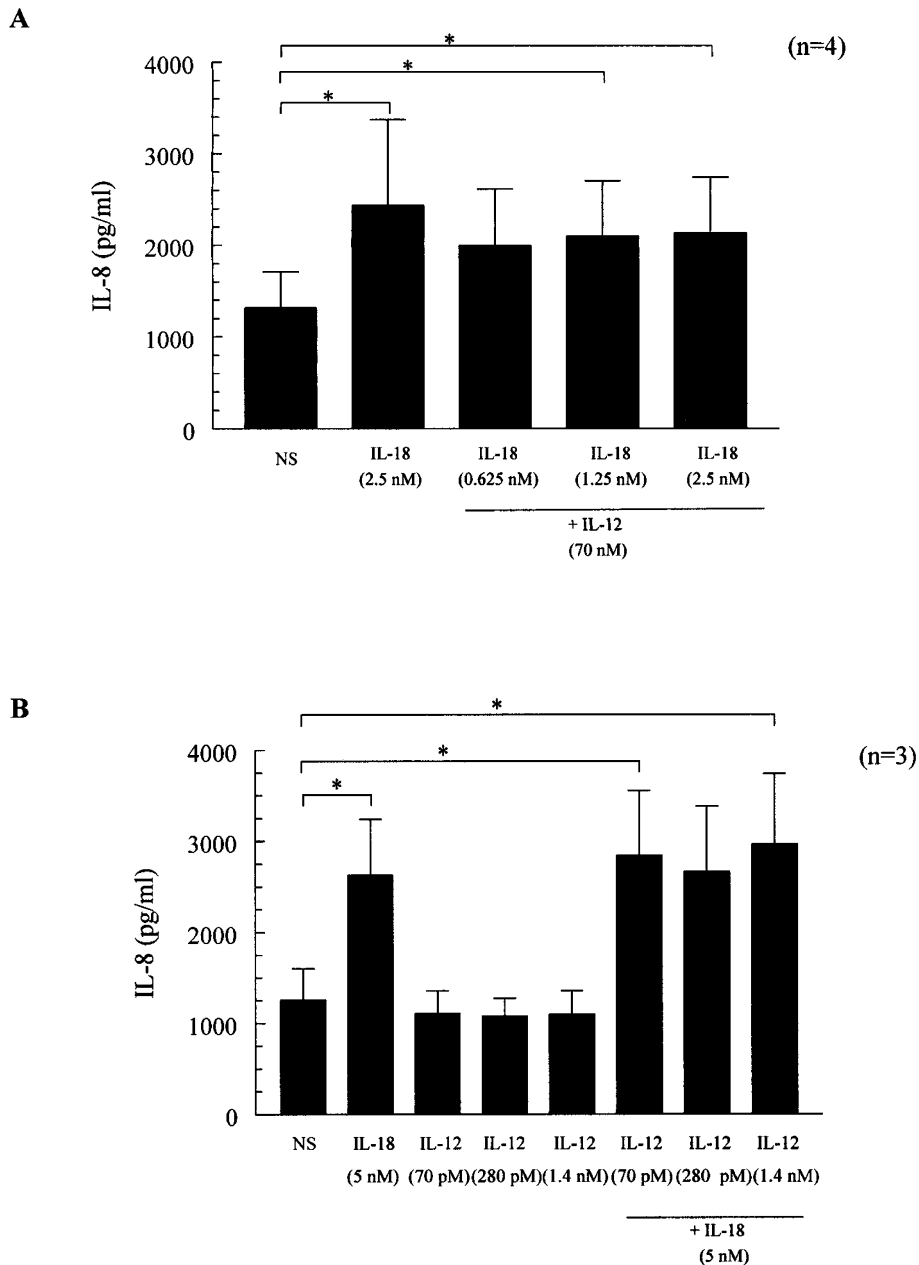
Figure 5.
IL-8 expression in response to combinations of IL-18 and IL-12 in RA synovial fibroblasts. Cells were cultured as described in Figure 1 and stimulated (A) with various concentrations of IL-18 plus IL-12 (70 pm) or (B) various concentrations of IL-12 plus IL-18 or IL-12 alone. Conditioned media was collected after 24 hours and assayed by ELISA. Results represent the mean ± sem of cells from three (B) and four (A) different patients with RA. *$p < 0.05$ compared with nonstimulated control using a Student *t* test.

and C), as opposed to the cytoplasmic distribution in unstimulated cells (Fig. 7A).

Effect of NFκB Antisense on IL-18–Induced RA Synovial Fibroblasts IL-8

The above findings showed that NFκB activation occurred after IL-18 stimulation. To link this activation of NFκB with IL-8 expression, we examined the effect of NFκB inhibition on IL-18–induced IL-8 production. In previous reports, antisense has been successfully used to modulate NFκB to assess its role in IL-8 expression (Roshak et al, 1996). We tested the effect

of the antisense oligonucleotide on NFκB p65 synthesis in IL-18– and IL-1β–stimulated RA synovial fibroblasts by Western Blotting. In the presence of antisense oligonucleotide, the amount of p65 in IL-18– and IL-1β–stimulated cells was similar to the amount in nonstimulated cells. RA synovial fibroblasts in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) were incubated with sense or antisense oligonucleotides (10 μM) for 18 hours at 37° C before the addition of IL-18 for 24 hours. Protein extracts were collected and assayed for Western blot analysis (Fig. 8A). Figure 8B
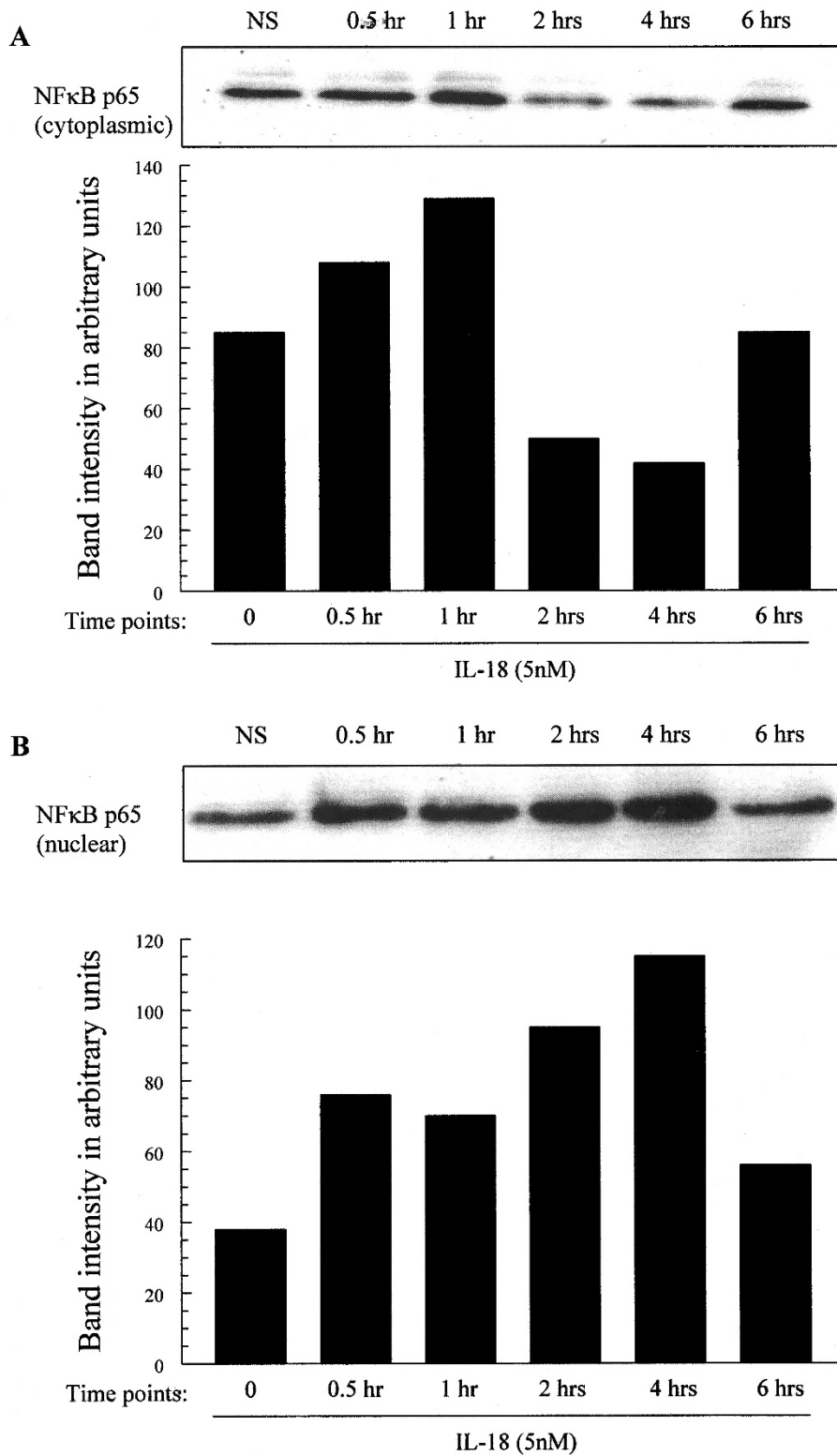
Figure 6.
Disappearance of nuclear factor kappa B (NFκB) in the cytoplasm and appearance in the nucleus induced by IL-18. Western blot analysis of NFκB (p65) was performed with cytoplasmic and nuclear extracts from RA synovial fibroblasts from three different patients with RA, after stimulation with IL-18 (5 nM) for 0 to 6 hours. An 8.5-μg protein extract of each sample was loaded. The graph represents the band intensities in pixel values for each condition. Results are representative of three experiments.

shows that IL-8 levels of IL-18– and IL-1β–stimulated RA synovial fibroblasts, pretreated with 10 μM p65 antisense, were significantly ($p < 0.05$) reduced by −44% (±5.4) and −46% (±13.32), respectively. These results indicated that NFκB activation in part modulated IL-18–induced RA synovial fibroblast IL-8 expression.
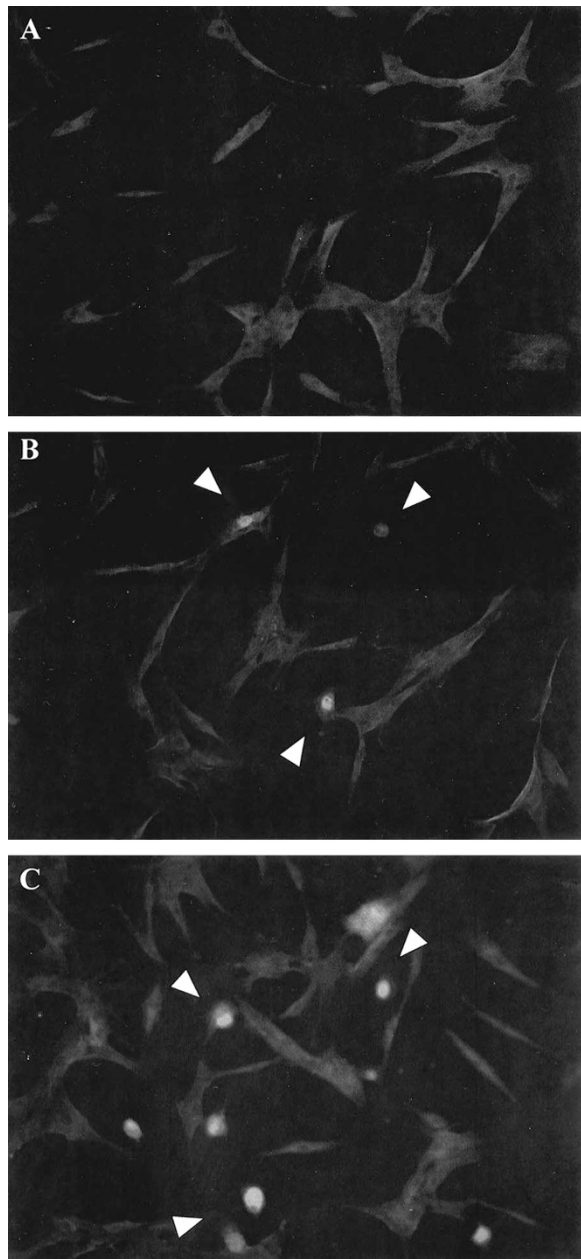
Figure 7.
Nuclear translocation of NFκB upon IL-18–stimulation of RA synovial fibro-blasts. Cells from two different patients with RA were stimulated by (A) media alone, (B) IL-18 (5 nM), or (C) IL-1β (1.75 nM) for 1 hour. The bound antibody was visualized with FITC-conjugated goat anti-rabbit IgG (green). Cells stimulated with IL-18 or IL-1β demonstrated accumulation of NFκB (p65) in the nucleus, whereas nonstimulated cells did not. A to C, ×469. *Arrows* indicate the NFκB p65 located in the nucleus.

Discussion

In this study, we demonstrated that IL-18 stimulates RA synovial fibroblasts to produce CXC chemokines. IL-18, in the absence of an IL-12 costimulus induced a dose-dependent increase in the amount of IL-8, ENA-78, and groα but failed to induce MIP-1α production even at the highest concentrations employed. These biological actions of IL-18 on synovial fibroblasts appear similar to IL-1β functions, because, in our hands, IL-1β did not induce RA synovial fibroblast

MIP-1α production (Koch et al, 1994). Previous studies showed that normal peripheral blood CD14+ monocytes and natural killer (NK) cells released IL-8 and MCP-1 upon exposure to IL-18 (Puren et al, 1998), whereas MIP-1α was undetectable at the mRNA and protein levels in IL-18 activated NK cells (Fehniger et al, 1999). In the U1 monocytic cell, IL-18 up-regulated IL-8 but had no effect on MIP-1 α (Shapiro et al, 1998). In both cases the addition of the p55 TNF soluble receptor (TNFbp), which inhibits TNF-α functions, reduced the IL-18–induced IL-8 production, and the authors concluded that IL-18 exerted its effect through a TNF-α–like molecule. However, IL-8 was not completely inhibited by TNFbp, suggesting that another factor enhanced IL-8 gene expression. Actually, a direct effect of IL-18 in IL-8 production is a reasonable explanation, and the early detection (4 and 8 hours) of IL-8 in the conditioned media, as well as in the cytoplasm of RA synovial fibroblasts stimulated with IL-18, strongly supports this hypothesis. Moreover, synovial fibroblasts do not produce TNF-α (Bombara et al, 1993; Bucala et al, 1991; Schwachula et al, 1994), even when they are stimulated with lipopolysaccharide (LPS), suggesting that the production of IL-8 by RA synovial fibroblasts is probably linked to a direct effect of IL-18 on these cells. MIP-1α is chemotactic for monocytes, whereas ENA-78, groα, and IL-8 are important in neutrophil recruitment in the RA joint (Koch and Strieter, 1996). The participation of IL-18 in the recruitment of neutrophils has been demonstrated in vivo. In a lethal endotoxemia model, mice injected with LPS derived from *Escherichia coli* die of vital organ injury, which is largely mediated through neutrophil accumulation (Netea et al, 2000). The neutralization of IL-18 significantly reduced neutrophil infiltration in the lung and the liver and also the expression of the chemokine MIP-2 in these organs. These observations suggest that IL-18 inhibition may result in decreased neutrophil recruitment through inhibition of chemokine production. It is conceivable that IL-18, produced by RA synovial fibroblasts, could promote neutrophil trafficking into the joint via chemokine induction and participate in the inflammation of the joint. This could, in part, explain the development in mice treated with type II collagen and IL-18 of a more inflammatory and erosive arthritis than that which developed in the control animals (Leung et al, 2000).

Because IL-12 was shown to synergize with IL-18 to induce chemokines in normal peripheral blood mononuclear cells (PBMC) (Puren et al, 1998), we examined the combination of these two cytokines on RA synovial fibroblast IL-8 expression. Surprisingly, there was no additive effect of IL-12 on IL-8 production because the levels of IL-8 released by the cells treated with IL-12 alone were identical with those treated with IL-12 combined with IL-18. This result was unexpected because IL-12 increased IL-18–induced chemokine expression in NK cells. The up-regulation of IL-18 receptor by IL-12 is one mechanism that explains the synergistic effect of these two cytokines. However, IL-12 alone appears

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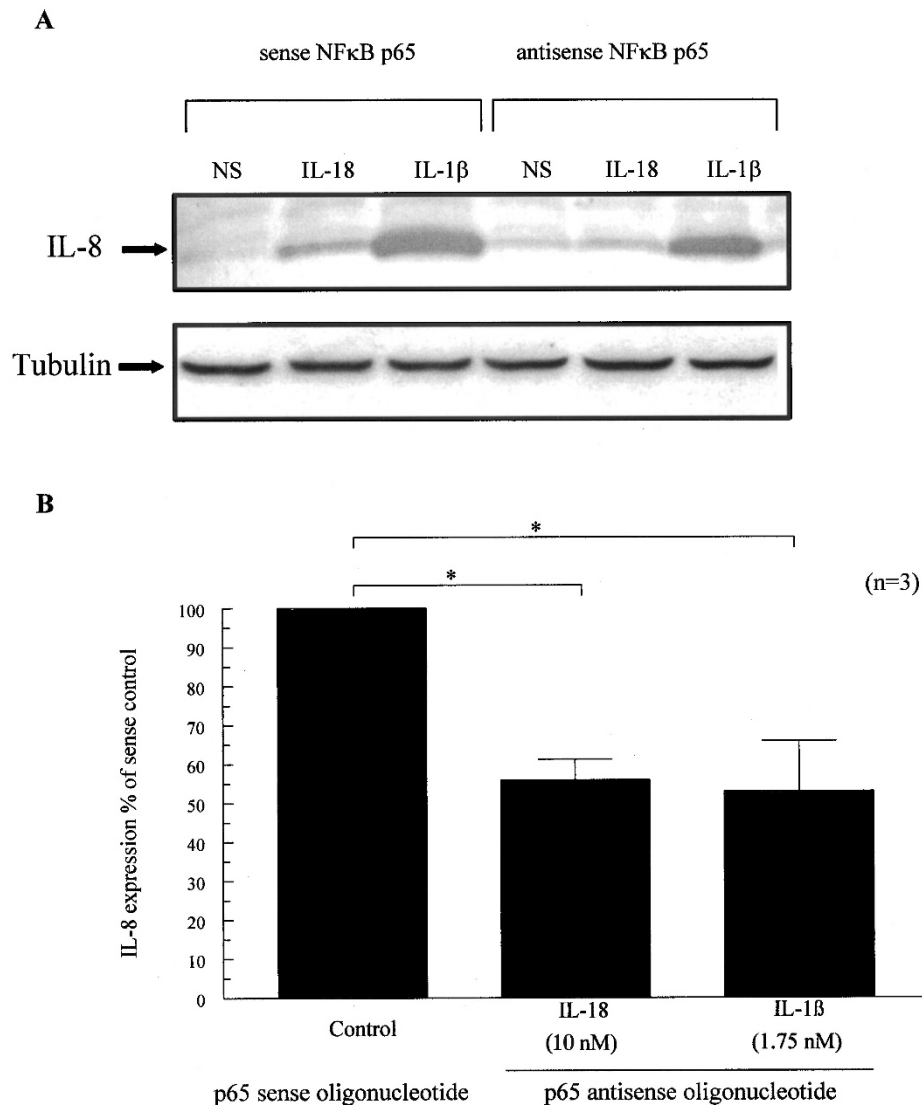
Figure 8.
The effect of antisense to NFκB p65 on RA synovial fibroblast IL-8 protein levels. A, Western blot analysis of 32 μg of protein extracted from RA synovial fibroblasts exposed to p65 sense or p65 antisense oligonucleotide (10 μM) for 18 hours followed by treatment with IL-18 (10 nM) or IL-1β (1.75 nM) for 24 hours. Equal loading of protein was verified with anti-tubulin antibody. B, Data are expressed as the percentage of stimulated control oligonucleotide (sense p65) and represent mean $\pm$ SEM of three different experiments. *Indicates significant differences from IL-18 and IL-1β control at $p < 0.05$ using a Student t test.

to have no impact on the induction of RA synovial fibroblast chemokine production (Schlaak et al, 1995; Seitz et al, 1996). Therefore, it is possible, though not described to date, that RA synovial fibroblasts lack the IL-12 receptor, explaining the absence of costimulation with IL-18.

We sought to understand the mechanism of action of IL-18 in inducing chemokine expression in RA synovial fibroblasts. The transcription factor NFκB regulates many genes for inflammatory mediators, including IL-8. NFκB is a homo- or heterodimeric protein typically containing the p50 and p65 subunits, which can bind to short regulatory sequences in the promoter sections of genes (Zhu et al, 1997). In RA synovial fibroblasts, NFκB normally resides in the cytoplasm and migrates to the nucleus when it is activated. Evidence has accumulated that the intracellular pathway involving the transcription factor NFκB

also has a role in mediating IL-18 functions (Dinarello, 1999). Kurimoto and colleagues showed that IL-18 activates NFκB in murine Th$_1$ lymphocytes and in KG1 cells (Kojima et al, 1999; Matsumoto et al, 1997). Here, we demonstrated that IL-18 increases the translocation of NFκB p65 to the nucleus in synovial fibroblasts derived from patients with RA. This translocation occurred at 1 hour, as demonstrated by immunofluorescence, and was sustained for at least 4 hours. This period of activation of NFκB is longer than that found with IL-1β stimulation of RA synovial fibroblasts because the level of NFκB in the nucleus and cytoplasm of IL-1β–stimulated synovial fibroblasts reaches its maximum by 30 minutes and returns to the basal level by 4 hours (Crofford et al, 1997). However, this long duration of NFκB activation is not exceptional; thrombin, for example, induced a maximum activation of NFκB in RA synovial fibroblasts at 4 hours after

stimulation (Shin et al, 1999). These results suggest that the regulation of NFκB activation differs between IL-18 and IL-1β. The inability of IL-18 to induce in RA synovial fibroblasts the p38 mitogen-activated protein kinase, an enzyme which contributes to NF-κB activation and IL-8 production mediated through IL-1β (Suzuki et al, 2000), supports this hypothesis (data not shown).

To extend our results further, we have addressed the question of whether the activation of NFκB is implicated in IL-18–induced IL-8 expression. Because previous reports indicated that NFκB p65 has a major role in IL-8 gene transcription (Kunsch and Rosen, 1993), we generated a phosphorothioate-modified antisense oligonucleotide corresponding to the 5' end of the p65 mRNA and evaluated its effect on IL-8 protein expression. RA synovial fibroblasts pretreated with the antisense oligonucleotide p65 produced 44% less IL-8 compared with the cells treated with the control sense oligonucleotide. Thus, IL-8 production was partially abolished in the cells stimulated with IL-18 or IL-1β. Roshak and colleagues (1996) also found a partial inhibition of IL-1β–induced IL-8 expression (45% to 55%) in RA synovial fibroblasts treated with a similar antisense p65 oligonucleotide. The use of antisense technology prevents only synthesis of new p65, not translocation of preformed cytoplasmic p65. Therefore, the early activation of preformed NFκB could explain the partial decrease of IL-8 expression. Antisense p65 oligonucleotide also does not block the synthesis of other subunits of NFκB, such as p50 or C-rel, which are also important in the regulation of IL-8 production (Roshak et al, 1996). These subunits might form alternative homo- or heterodimers that could compensate for the loss of p65. An NFκB-independent pathway involving other transcription factors, such as activating protein-1 (AP-1), is another possible explanation, because this factor is activated in response to IL-18 in various cell systems (Barbulescu et al, 1998; Kojima et al, 1999). To support this idea, IL-1β requires a NFκB element plus either an AP-1 or a CCAAT/enhancer-binding protein element to induce IL-8 gene transcription (Wu et al, 1997; Yasumoto et al, 1992). Further investigation is needed to elucidate the roles of these different transcription factors in RA synovial fibroblasts stimulated with IL-18.

In summary, this study demonstrates by four different techniques that IL-18 induces intracellular and extracellular expression of IL-8 by RA synovial fibroblasts, in a dose- and time-dependent manner. In the same cells, IL-18 up-regulates other chemokines such as ENA-78 and groα but fails to induce MIP-1α. IL-18 enhances CXC chemokine production in RA synovial fibroblasts through activation of the transcription factor NFκB p65, at least for IL-8. These studies support the concept of considering IL-18 as a directly acting proinflammatory cytokine in the RA joint and place this cytokine in a strategic role in inflammation and in RA.

Materials and Methods

Reagents

Cytokines. Human recombinant (rhu) IL-18 (specific activity: 9.1×10^5 units/mg) and rhu IL-12 (specific activity: 1.24×10^7 units/mg) were purchased from R&D Systems (Minneapolis, Minnesota). Rhu interleukin-1 beta (IL-1β, 2×10^7 units/mg) and rhu tumor necrosis factor-α (TNF-α, specific activity: 1.3×10^7 units/mg) were obtained from Upjohn Company (Kalamazoo, Michigan).

Antibodies. Monoclonal mouse anti-human IL-8 and isotype-matched antibodies were purchased from R&D Systems. Polyclonal rabbit anti-RelA was acquired from Santa Cruz Biotechnology (Santa Cruz, California). FITC-conjugated goat anti-rabbit antibody was purchased from Jackson ImmunoResearch (West Grove, Pennsylvania). Monoclonal mouse anti-tubulin was purchased from Oncogene (Boston, Massachusetts). Monensin (Golgistop7) was purchased from Pharmingen (San Diego, California). Goat serum was obtained from Sigma (St. Louis, Missouri). RPMI-1640, PBS, fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were from Gibco BRL (Grand Island, New York).

Fibroblast Cell Culture

Synovium was obtained from patients meeting the American College of Rheumatology criteria for RA who had undergone total knee replacement surgery or synovectomy. Fresh synovial tissues were minced and digested in a solution of dispase, collagenase, and DNAse. Synovial fibroblasts were cultured at 37° C in RPMI-1640 supplemented with 10% FBS and 1% P/S in 175-mm tissue culture flasks (Falcon, Franklin Lakes, New Jersey). Upon reaching confluence, the cells were passaged by brief trypsinization as previously described (Koch et al, 1986). The cells were used at passage 5 or older, at which time they were a homogeneous population of fibroblasts. To obtain conditioned media, synovial fibroblasts were seeded at 1×10^6 cells/ml in 24-well plates (Falcon) in 1 ml supplemented RPMI-1640 and incubated overnight at 37° C in an incubator gassed with 5% CO_2. Different concentrations of IL-18 (0.625 nM to 10 nM), IL-1β (1.75 nM), TNF-α (1.15 nM) in RPMI-1640 with 1% P/S were added to the plates, and the plates were incubated at 37° C/5% CO_2. Conditioned media was collected after 4, 8, 24, and 48 hours of culture and stored at −80° C until assayed.

Chemokine ELISA

IL-8, groα, ENA-78, and MIP-1α in the cell culture–conditioned media were assayed using commercial ELISA assays (R&D Systems). The sensitivity of these assays ranged from 10 to 15 pg/ml.

Intracellular Flow Cytometry

Synovial fibroblasts were cultured in RPMI-1640 at 1×10^6 cells/ml in 6-well plates and stimulated with

IL-18 (5 nM), IL-1β (1.75 nM), or TNF-α (1.15 nM) for 24 hours. Conditioned media were then collected, and synovial fibroblasts were restimulated with the same concentration of cytokines but in the presence of monensin (1.3 μl for 2 ml of media) for 4 hours. Cells were harvested with a cell scraper and transferred to fluorescence activated cell sorting (FACS) tubes (Becton Dickinson, Franklin Lakes, New Jersey). Synovial fibroblasts were then stained for intracellular IL-8 according to Becton Dickinson protocol. Briefly, the synovial fibroblasts were lysed and permeabilized with Becton Dickinson lysing and permeabilization solutions before they were incubated successively for 30 minutes at room temperature with a monoclonal anti–IL-8 antibody or isotype-matched control (2.5 μg/ml) and phycoerythrin (PE)-conjugated goat anti-mouse antibody (50 μl of 1:100 dilution). Samples fixed in 1% paraformaldehyde were assayed using an Epics XL-MCL flow cytometer (Beckman Coulter, Fullerton, California). Before data acquisition, the PE channel was standardized using fluorescent beads (Rainbow Beads; Spherotech, Libertyville, Illinois).

Immunofluorescence Staining

To determine the subcellular localization of NFκB, RA synovial fibroblasts were cultured at 10,000 cells/well in eight-well Lab-Tek (Naperville, Illinois) chamber slides and allowed to adhere overnight. Cells were then stimulated with 5 nM IL-18 or 1.75 nM IL-1β for 1 hour. RA synovial fibroblasts were fixed in PBS containing 4% paraformaldehyde for 10 minutes at room temperature and then permeabilized using 0.5% Triton X-100 in PBS for 20 minutes at room temperature. They were then incubated with rabbit polyclonal anti-Rel-A antibody or an isotype-matched control for 45 minutes at 37° C. After washing with PBS, cells were incubated with FITC-conjugated goat anti-rabbit antibody for 45 minutes at 37° C. Slides were coverslipped with mounting media (Kirkegaard & Perry Labs, Gaithersburg, Maryland). Subcellular localization was determined using an immunofluorescence microscope.

Preparation of Phosphorothioate Oligonucleotides

Initiation site-directed antisense to p65, 5'-ggggaacagttcgtccatggc-3', and control oligonucleotide p65 sense, 5'-gccatggacgaactgttcccc-3', were synthesized on a 394 DNA/RNA synthesizer from Applied Biosystems (Foster City, California) using phosphoroamidite chemistry. Sense and antisense oligonucleotides were resuspended in RPMI-1640, 10% FBS, and 1% P/S. RA synovial fibroblasts were cultured in the presence of sense or antisense p65 (10 μM) for 18 hours before the addition of IL-18 (10 nM) for 24 hours. Monensin was added 4 hours before the protein extraction.

Nuclear Extracts

Cells were scraped, washed once with PBS, resuspended in 100 μl of lysis buffer (10 nM HEPES, pH 7.9,

1.5 nM MgCl$_2$, 10 nM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) and protease inhibitors (protease inhibitor cocktail tablets; Boehringer Mannheim, Indianapolis, Indiana) (1 tablet/10 ml), and incubated on ice for 4 minutes. NP-40 20% (1 μl) was added, and cells were vortexed for 10 seconds. Nuclei were pelleted by centrifugation at 14,000 $\times g$ for 1 minute. The supernatant containing the cytoplasmic fraction was collected and stored at $-80°$ C. Protein was extracted from the nuclei with extraction buffer containing 100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM ethyleneglycotetraacetic acid (EGTA), 1 mM NaF, 20 mM NaP$_2$O$_4$, 2 mM Na$_3$VO$_4$, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, and a protease inhibitor cocktail tablet.

Western Blot Analysis

For the experiments in Figures 2 and 8, cells were lysed in the extraction buffer. For the experiment in Figure 6, cytoplasmic and nuclear proteins were isolated by the method described above. The concentration of protein in each cell lysate was determined by means of a bicinchoninic acid assay (Pierce, Rockford, Illinois) using bovine serum albumin (BSA) as the standard. Protein extracts were mixed with an equal volume of 2$\times$ Laemmli's sample buffer (Laemmli et al, 1970). Equal amounts of each sample were loaded on a 10% SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry transblotting apparatus (Bio-Rad, Hercules, California). Nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline Tween (TBST) buffer-20 mM Tris, 137 mM NaCl, pH 7.6, with 0.1% Tween 20 for 60 minutes at room temperature. Blots were incubated with anti-RelA or anti-tubulin at 1:1000, or anti–IL-8 antibody at 1:5000, in TBST containing 5% nonfat milk for 60 minutes. Blots were washed three times and then incubated in horseradish peroxidase (HRP)-conjugated antibody (1:10,000 dilution) for 1 hour at room temperature. All blots were developed using the enhanced chemoluminescence (ECL) reagents (Amersham, Arlington Heights, Illinois) per the manufacturer's instructions. Blots were scanned and analyzed for the measurement of the band intensities with the Un-Scan-It software, version 5.1 (Silk Scientific, Orem, Utah). Band intensity corresponded to the sum of all pixel values in the segment selected minus the background pixel value in that segment. For the experiments with sense and antisense oligonucleotides to NFκB p65, the percentage of inhibition of IL-8 was calculated as follows: ([a $-$ b]/a) $\times$ 100, with *a* corresponding to the band intensity of IL-8 produced by RA synovial fibroblasts pretreated with the sense oligonucleotide, and *b* corresponding to the band intensity of IL-8 produced by RA synovial fibroblasts pretreated with the antisense oligonucleotide.

Statistical Analysis

Data was expressed as the mean $\pm$ SEM. Group means were compared with a Student's paired *t* test or a

Wilcoxon rank order test, as appropriate. *p* values <0.05 were considered statistically significant.

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