

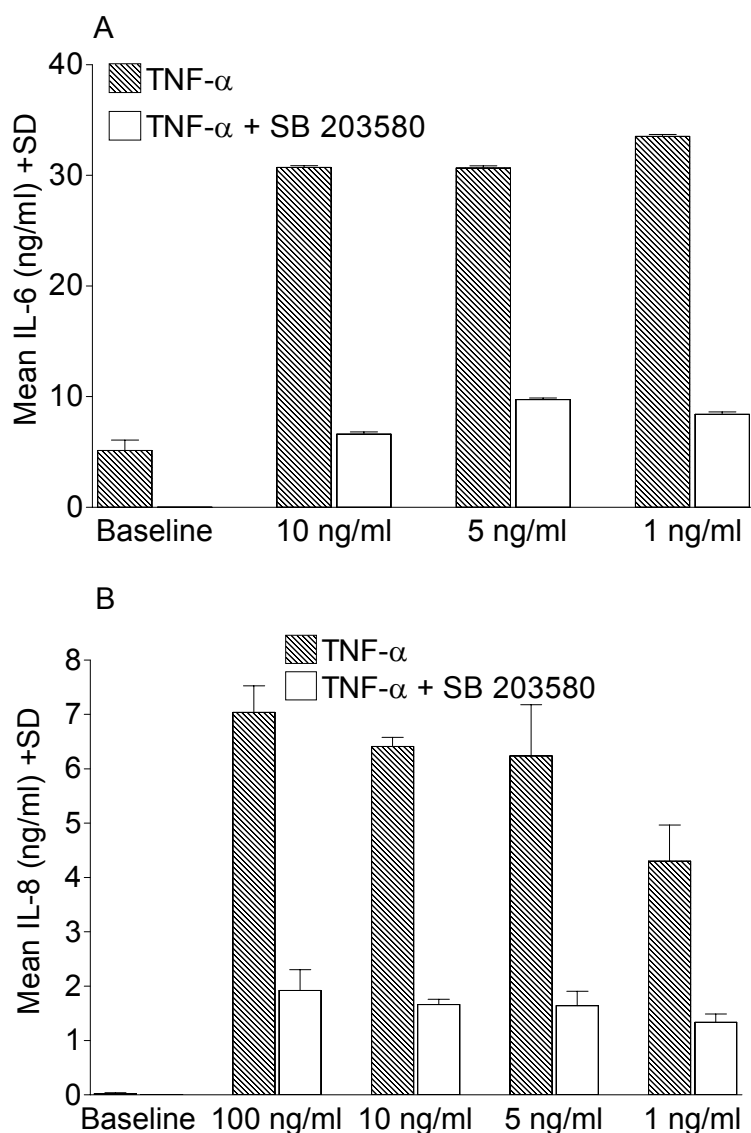
### Production of Interleukin-6, but not Interleukin-8, Induced by TNF- $\alpha$ or IL-1 $\beta$ in Human Fibroblast-Like Synoviocyte Increases over Cell Passage

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects systemic joints. Chronic synovitis often leads to irreversible destruction of periarticular cartilage and bone. The inflamed synovium is characterized by

the proliferation of synoviocytes. There are two types of synoviocytes that predominate in the normal synovial fluid: type A synoviocytes, which resemble tissue macrophages; and type B synoviocytes, which resemble fibro-



**Figure 1.** Dose response of TNF- $\alpha$  induced IL-6 (A) and IL-8 (B) release with HFLS-RA. Human TNF- $\alpha$  was incubated with a range of concentration (1, 5, 10, and 100 ng/mL) on HFLS for 18 h at 37°C, 5% CO<sub>2</sub>. SB203580 (30  $\mu$ M) was incubated 2 h prior to TNF- $\alpha$ . The level of IL-6 (A) and IL-8 (B) was measured by ELISA kit from BioSource International. HFLS-RA were at passage 3 (means  $\pm$  SD, n=8).

blasts (HFLS) [2]. Both synovial fibroblasts and macrophages are capable of producing and responding to a variety of growth factors and cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 [2]. TNF- $\alpha$  and IL-1 $\beta$  are mainly produced by macrophages but are potent stimulators of HFLS proliferation, adhesion molecule expression, and matrix-degrading enzyme production. It appears these cytokines are synergistic in their action. IL-1 $\beta$  can act as an autocrine growth for HFLS and is responsible also for the destructive part of rheumatoid arthritis. TNF- $\alpha$  is more important for the proliferative and inflammatory phase for the HFLS-RA [1]. Both IL-1 $\beta$  and TNF- $\alpha$  are considered to play crucial roles because they are known to induce IL-6, IL-8, GM-CSF and themselves [4]. IL-6 is spontaneously produced by HFLS. Elevated IL-6 is found in the joint and serum of RA patients. IL-6 appears to be a B cell stimulator, stimulating acute-phase reactant from the liver, and probably contributing to local antibody and rheumatoid factor production. TNF- $\alpha$  and IL-1 $\beta$  induce and increase the production of IL-6 [5,6]. IL-8 (or NAP-1, Neutrophil-Activating Peptide) is produced by HFLS and plays a role in cell migration, chemotaxis, and activating macrophages [6]. IL-8 is spontaneously produced by HFLS-RA, on a smaller level than IL-6. TNF- $\alpha$ , IL-1 $\beta$ , and IL-18 induce production of IL-8 [2].

TNF- $\alpha$  and IL-1 $\beta$  induce IL-6 and IL-8 production through activation of cellular transcription factors such as nuclear factor  $\kappa$ B (NF- $\kappa$ B)[3]. Furthermore, TNF- $\alpha$  and IL-1 $\beta$  also induce a rapid increase in p38 mitogen-activated protein kinase (p38 MAP kinase) phosphorylation with resultant activation of its enzyme activity [2]. Thus, in this study, we confirm the role of p38 MAP kinase in IL-6 and IL-8 production using the specific inhibitor-SB203580.

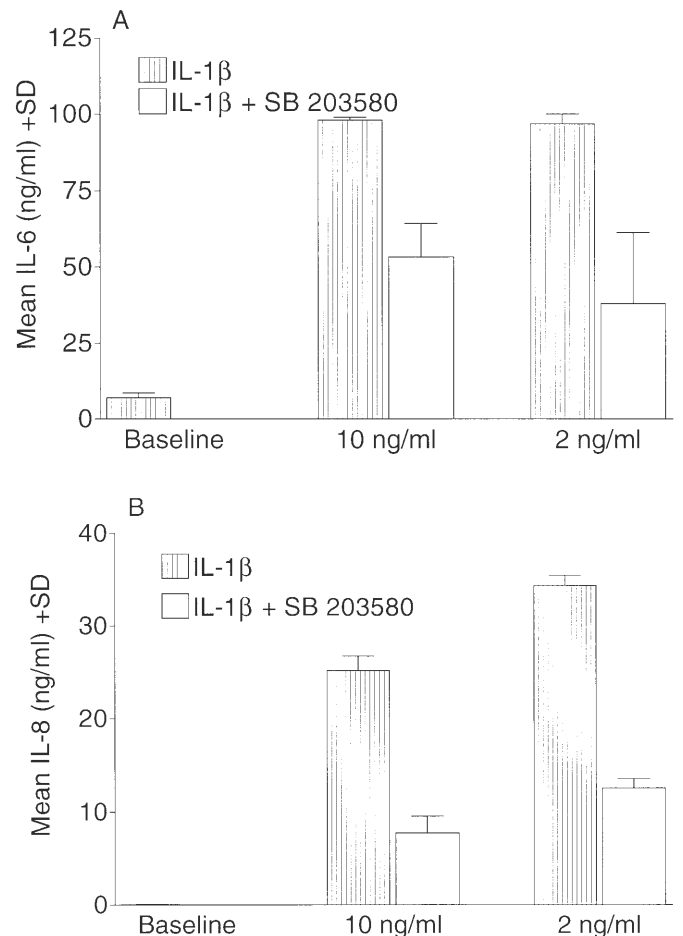
The induction of IL-6 and IL-8 vary with published studies, from a stimulation by TNF- $\alpha$  with 100 ng/mL [3] to 10 ng/mL [2], and by IL-1 $\beta$  around 20 ng/mL [2]. In this study we concluded that such amounts of cytokines are not necessary to stimulate the production of IL-6 and IL-8, and that this production varies with cell passage.

#### MATERIALS AND METHODS:

**Materials.** The Human Fibroblast-Like Synoviocytes derived from the inflamed synovial tissue of rheumatoid arthritis patients under knee replacement (HFLS-RA) were purchased from Cell Applications, Inc (San Diego, CA), and cultured in "Growth Medium" (medium supplemented for the growth of synoviocytes) from the same company. Human TNF- $\alpha$  (recombinant *E. coli*) and human IL-1 $\beta$  (recombinant *E. coli*) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). SB203580 was obtained from Calbiochem (La Jolla, CA). Interleukin-6 and Interleukin-8 CytoScreen immunoassays were purchased from BioSource International (Camarillo, CA).

**Methods.** The HFLS-RA were cultured in T75 in growth medium to confluence and then divided and seeded into 96 microwell plates. At 80-90% confluence the cells were changed and incubated at 37°C un-

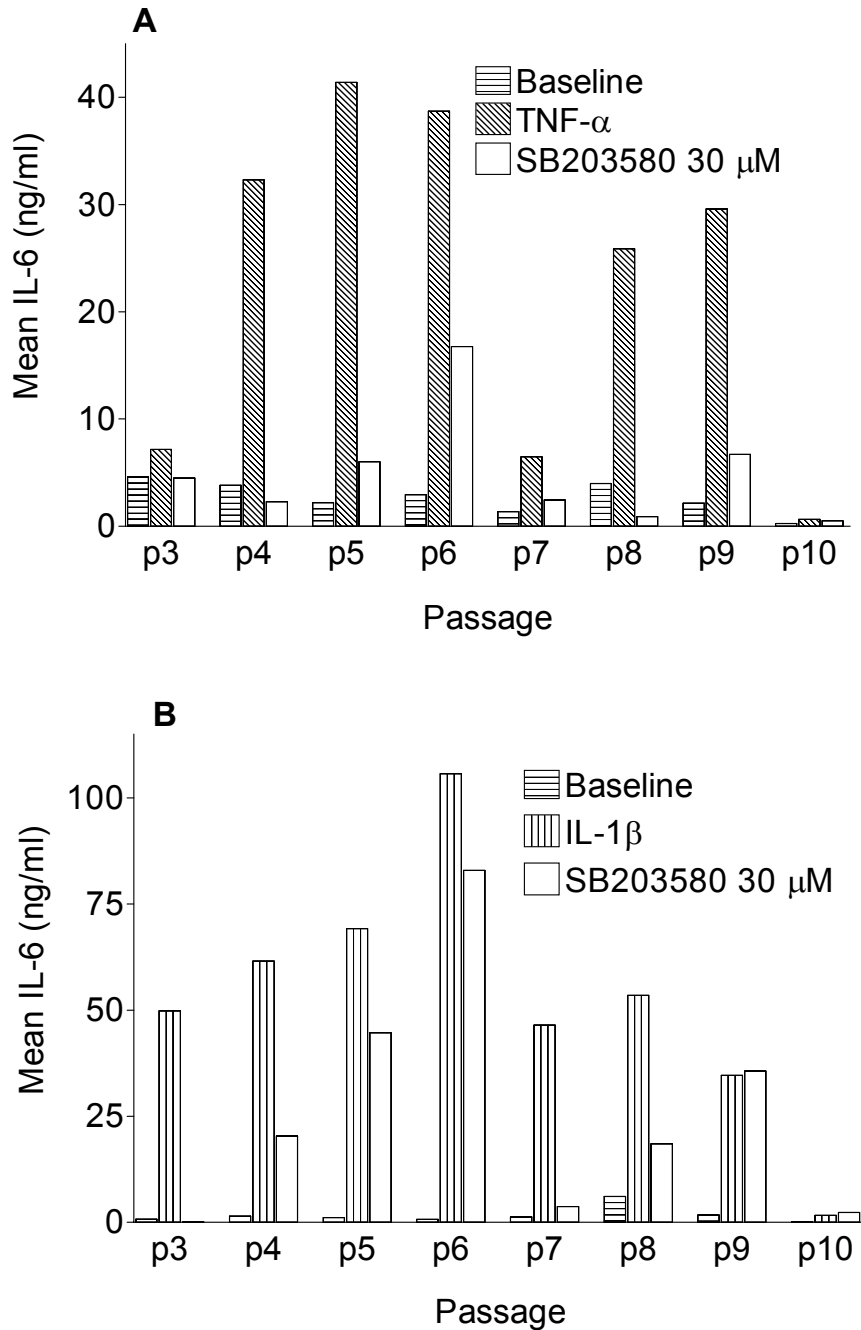
der 5% CO<sub>2</sub> in the presence or absence of SB203580 (30  $\mu$ M). After 2 h incubation, human cytokines (TNF- $\alpha$  or IL-1 $\beta$ ) were added and microplates were incubated for 18 h at 37°C, 5% CO<sub>2</sub>. TNF- $\alpha$  was incubated at a range of concentration (1, 5, 10, and 100 ng/mL) for the dose response and at 1 ng/mL for assays testing IL-6 and IL-8 release versus the passage of the cells. IL-1 $\beta$  was tested at 2 and 10 ng/mL for the dose response and at 2 ng/mL for the other assays. HFLS-RA supernatant was collected, diluted, and used for the immunoassay. IL-6 and IL-8 immunoassays kits were used according to the vendor instruction.



**Figure 2.** Dose response of IL-1 $\beta$  induced IL-6 (A) and IL-8 (B) release with HFLS-RA. Human IL-1 $\beta$  was incubated at 10 and 2 ng/mL on HFLS for 18 h at 37°C, 5% CO<sub>2</sub>. SB203580 (30  $\mu$ M) was incubated 2 h prior to IL-1 $\beta$ . The level of IL-6 (A) and IL-8 (B) was measured by ELISA kit from BioSource International. HFLS-RA were at passage 3 (means  $\pm$  SD, n=8).

#### RESULTS:

**Dose response of TNF- $\alpha$ -induced IL-6 and IL-8 release.** Human TNF- $\alpha$  was incubated at concentrations of 1, 5, 10, and 100 ng/mL. IL-6 and IL-8 release were measured in the cell supernatant after 18 h incubation. The spontaneous release (baseline) was found around 500 pg/mL. The production of IL-6 seems to be at maximum level at 1 ng/mL TNF- $\alpha$  (Fig. 1A). SB203580 (30  $\mu$ M) treatment inhibited by 80-95% the production of IL-6 at all concentrations of TNF- $\alpha$ . The production of IL-8

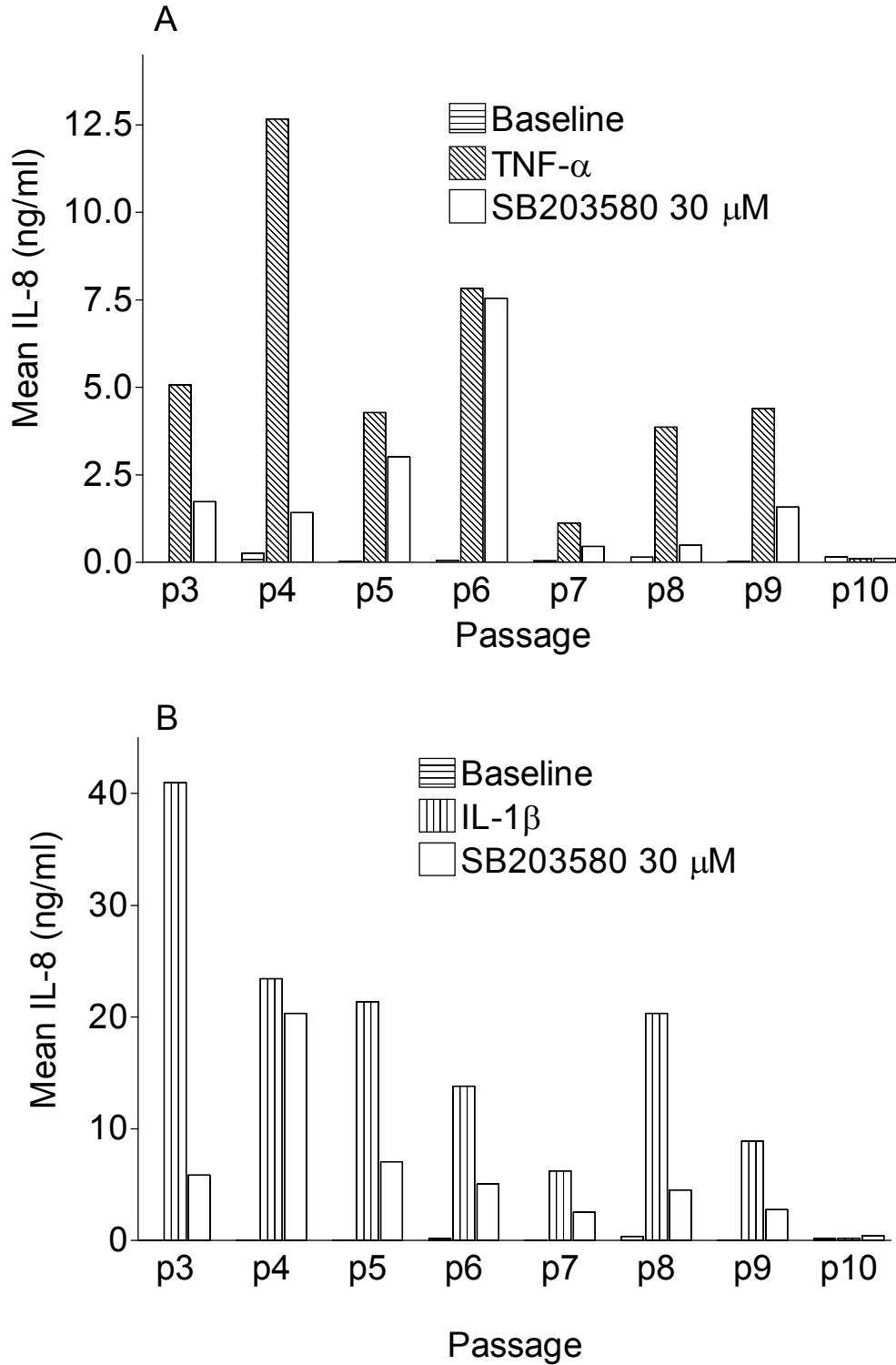


**Figure 3.** Effect of cell passages on TNF- $\alpha$  (A) or IL-1 $\beta$  (B) induced IL-6 release. For each cell passage, SB203580 (30  $\mu$ M) was incubated 2 h prior to (A) TNF- $\alpha$  (1 ng/mL) or (B) IL-1 $\beta$  (2 ng/mL). The microplates were then incubated for 18 h at 37°C, 5% CO<sub>2</sub>. This was repeated with cells from two donors (mean shown, n=6 each donor). IL-6 level was measured by BioSource International ELISA kit.

reached a maximum level at 7 ng/mL with a very low level (20 pg/ml) of spontaneous release (baseline). SB203580 (30  $\mu$ M) inhibited by 70-75% the cell release of IL-8 at all concentrations of TNF- $\alpha$  (Fig. 1B). The concentration of 1 ng/ml of TNF- $\alpha$  was chosen for the rest of the study.

**Dose response of IL-1 $\beta$ -induced IL-6 and IL-8 release.** IL-1 $\beta$  was incubated at concentrations of 2 and 10

ng/ml with HFLS-RA cells. Spontaneous release of IL-6 (baseline) was around 7 ng/ml. IL-6 production seemed to be at maximum level with an induction with IL-1 $\beta$  at 2 ng/ml concentration (Fig.2A). SB203580 (30  $\mu$ M) treatment inhibited by 50% and 65.5% the production of IL-6 with induction of respectively 10 and 2 ng/ml IL-1 $\beta$ .



**Figure 4.** Effect of cell passages on TNF- $\alpha$  (A) or IL-1 $\beta$  (B) induced IL-8 release. For each cell passage, SB203580 (30  $\mu$ M) was incubated 2 h prior to (A) TNF- $\alpha$  (1 ng/mL) or (B) IL-1 $\beta$  (2 ng/mL). The microplates were then incubated for 18 h at 37°C, 5% CO<sub>2</sub>. This was repeated with cell from two donors (mean shown, n=6 each donor). IL-8 level was measured by BioSource International ELISA kit.

The spontaneous IL-8 release was very low (63.2 pg/ml). A stimulation of 2 ng/ml IL-1 $\beta$  gave us a maximum response of IL-8 release and an inhibition of 63.5% with SB203580 at 30  $\mu$ M (Fig. 4). Concentration of 2

ng/ml was chosen to test the HFLS-RA passages on interleukin-6 and -8 production.

**Effect of cell passages on TNF- $\alpha$ - or IL-1 $\beta$ -induced IL-6 release.** Human fibroblast-like synoviocytes from two donors were tested independently. The level of IL-6 released after induction by TNF- $\alpha$  (1 ng/ml) or IL-1 $\beta$  (2 ng/ml) was dependent on the cell passage, with a maximum of release at p5 and p6 (Fig. 3A and 3B). After p6 one lot of cells went senescent, and with the other the level of IL-6 release decreased to a very low level at p10 (data not shown). Spontaneous production of IL-6 did not vary along the cell division except at high passage (p10). At maximum stimulation (p6) the potency of the p38 inhibitor SB203580 (30  $\mu$ M) did not decrease when IL-6 is induced by TNF- $\alpha$  (about 90% inhibition), but slightly decreased when induced by IL-1 $\beta$  (from 67% at p4 to 22% at p6).

**Effect of cell passage on TNF- $\alpha$ - or IL-1 $\beta$ -induced IL-8 release.** HFLS were tested on the IL-8 release at the same time as IL-6 release. The level of TNF- $\alpha$  induced IL-8 did not change along the number of cell passages (Fig. 4A). The level of IL-1 $\beta$  induced IL-8 was higher for one donor at passage 3, but did not change for the other passages. The spontaneous production of IL-8 was unchanged or decreased along the division of the cells. After p6 or p7 the cells started to be senescent and the level of IL-8 produced decreased. At the maximum production of IL-8 (p3 to p6) the potency of SB203580 (30  $\mu$ M) varied between 52 to 71%.

**DISCUSSION:** Accumulating evidence suggests the involvement of TNF- $\alpha$  and IL-1 $\beta$  and their interactions in the pathophysiology of RA [4]. Our present study demonstrated that minute concentrations of these cytokines (TNF- $\alpha$ , 1 ng/ml and IL-1 $\beta$ , 2 ng/ml) stimulate the production by human synoviocyte-like fibroblasts of large amounts of IL-6 and IL-8. The age of the cell in culture is an important variable in the IL-6 production induced by TNF- $\alpha$  or IL-1 $\beta$ . The production of IL-8 by these cytokines varies more, but does not seem to be as affected by the cell age as IL-6 production. The optimum production of IL-6 by HFLS-RA was between passage 3 and passage 6. After passage 6 or 7 interleukin-6 and -8 production by

the same HFLS-RA lot number decreased. At passage 10, all cell lots tested were senescent. HFLS-RA should not be used after passage 7.

Spontaneous production of IL-6 or IL-8 after stimulation by TNF- $\alpha$  or IL-1 $\beta$  did not vary along the cell division, and the level of spontaneous IL-8 production stayed low.

TNF- $\alpha$  and IL-1 $\beta$  induce a rapid increase in p38 mitogen-activated protein kinase (p38 MAP kinase) phosphorylation and the subsequent activation of its enzyme activity [1]. Our present study confirmed the potency of SB203580 in inhibiting the production of IL-6 by HFLS, and in a lower level the production of IL-8. This inhibition may vary with the production of the interleukins studied. The promoter of IL-6 and IL-8 possess binding sites for NF- $\kappa$ B, C/EBP $\beta$  and AP-1. Only NF- $\kappa$ B and AP-1 increased after stimulation by IL-1 $\beta$  [8,9]. Only inhibition of NF- $\kappa$ B blocked the production of IL-6 and IL-8 [8]. The crucial role of NF- $\kappa$ B in constitutive and cytokine stimulated production of IL-6 and IL-8 by HFLS-RA needs to be investigated and may lead to new therapeutics.

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