Low Level Light Effects on Inflammatory Cytokine Production by Rheumatoid Arthritis Synoviocytes

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Background and Objective: Low level light therapy (LLLT) is being evaluated for treating chronic and acute pain associated with rheumatoid arthritis (RA) and other inflammatory diseases. The mechanisms underlying the effectiveness of LLLT for pain relief in RA are not clear. The objectives of this study were to determine whether LLLT decreased production of pro-inflammatory cytokines by cells from RA joints, and, if so, to identify cellular mechanisms.

Study Design/Materials and Methods: Synoviocytes from RA patients were treated with 810 nm radiation before or after addition of tumor necrosis factor-α (TNF-α). mRNA for TNF-α, interleukin (IL)-1β, IL-6, and IL-8 was measured after 30, 60, and 180 minutes using RT-PCR. Intracellular and extracellular protein levels for 12 cytokines/chemokines were measured at 4, 8, and 24 hours using multiplexed ELISA. NF-κB activation was detected using Western blotting to follow degradation of IκBα and nuclear localization of the p65 subunit of NF-κB.

Results: Radiation at 810 nm (5 J/cm^2) given before or after TNF-α decreases the mRNA level of TNF-α and IL-1β in RA synoviocytes. This treatment using 25 J/cm^2 also decreases the intracellular levels of TNF-α, IL-1β, and IL-8 protein but did not affect the levels of seven other cytokines/chemokines. TNF-α-induced activation of NF-κB is not altered by 810 nm radiation using 25 J/cm^2.

Conclusions: The mechanism for relieving joint pain in RA by LLLT may involve reducing the level of pro-inflammatory cytokines/chemokines produced by synoviocytes. This mechanism may be more general and underlie the beneficial effects of LLLT on other inflammatory conditions. Lasers Surg. Med. 41:282–290, 2009.

Key words: inflammation; IL-1β; low level light therapy; synoviocytes; rheumatoid arthritis; TNF-α

INTRODUCTION

Low level light therapy (LLLT) is being evaluated and introduced into clinical practice for treating both chronic and acute pain, aiding wound healing, decreasing the effects of stroke and for other applications [1–5]. LLLT utilizes wavelengths in the red and near infrared (NIR) region of the spectrum (630–1,000 nm). Low irradiances are used that typically do not produce thermal effects on tissue (i.e., 1–2°C temperature change) suggesting that the observed benefits are due to photochemical rather than thermal effects. However, many aspects of the mechanisms for the therapeutic effects of far red and NIR radiation are not understood. The pathology of many of the conditions treated by LLLT involves inflammation, and LLLT may be effective because it reduces inflammation. For example, LLLT has been used for many years for relief of the chronic pain in rheumatoid arthritis (RA), which is caused by sensitization of neurons by inflammatory mediators [6].

RA is a complex inflammatory autoimmune disease of synovial joints, especially the small joints of the hands and feet, that leads to joint destruction and disability. The major symptoms are chronic pain, swelling, and heat in multiple joints [7]. Inflammation of the synovial lining of joints results in increased expression of inflammatory cytokines, chemokine-mediated recruitment of additional inflammatory cells, as well as activation of B cells with autoantibody production. A vicious cycle of altered cytokine and signal transduction pathways and inhibition of programmed cell death contribute to cartilage and bone destruction by human fibroblast-like synoviocytes (simply called here RA synoviocytes) and osteoclasts [8,9]. The major mediators of chronic inflammation in RA include tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and prostaglandin E_2 (PGE_2). These mediators exist in a network that perpetuates the inflammation by forming positive feedback loops between the mediators from activated synoviocytes and infiltrating macrophages [9]. These inflammatory mediators sensitize the peripheral termini of primary afferent nociceptors to cause a decrease in the pain threshold, resulting in increased sensitivity to pain [6].

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Many treatments are available to relieve RA-associated pain. Non-steroidal anti-inflammatory agents (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) and newer biologics are currently used, but have side effects when used over a prolonged period and are not effective in all patients [7,10]. Therefore, a non-invasive physiological therapy such as LLLT could be very important for managing long-term pain. The penetration depth of radiation varies with the wavelength. The longer wavelengths in the visible spectrum (red; ~600–780 nm) and wavelengths in the NIR between 780 and 1100 nm penetrate more deeply than shorter wavelengths and are appropriate for treating beneath the epidermis [11,12]. The transmission of 810 nm radiation through all of the layers of tissue between the dorsal skin surface and the ventral side of the spinal cord of a rat was measured to be ~6% [13] suggesting that this wavelength is suitable for reaching targets in small human joints.

The mechanisms underlying the effectiveness of LLLT for pain relief in RA are not clear. A direct suppressive effect of 830 nm radiation on neural conduction in vitro was observed that may contribute to the pain relief provided by LLLT [14]. An in vitro study demonstrated that 830 nm radiation inhibited production of PGE_2, a major inflammatory mediator, by gingival fibroblasts suggesting that the same effect might occur in RA synoviocytes [15]. Furthermore, an in vivo study using a rat model of RA showed that LLLT using 810 nm radiation reduced joint swelling that correlated with reduced serum PGE_2 [16]. LLLT has also been shown to decrease levels of the inflammatory cytokines, TNF-α and IL-1β in experimentally induced acute inflammation [17–19].

Since pro-inflammatory cytokines are critical to the pain and pathogenesis of RA, we addressed the following questions: Does LLLT decrease production of pro-inflammatory cytokines by cells from RA joints, and if so, what cellular mechanisms are involved? These studies employed synoviocyte cultures from the joints of RA patients because these cells are major sources of the inflammatory mediators and matrix metalloproteinases (MMPs) that contribute to inflammation and pain of RA. RA synoviocytes were stimulated by TNF-α to mimic the inflammatory environment in RA joints, and pro-inflammatory cytokines were measured at mRNA and protein levels. We evaluated the effect of 810 nm radiation on steps in the intracellular pathway by which TNF-α induces synthesis of pro-inflammatory cytokines.

MATERIALS AND METHODS

Human fibroblast-like synoviocytes derived from RA patients after synovectomy were purchased from Cell Applications (San Diego, CA). These cells were cultured in synoviocyte growth medium (Cell Applications) and used at passage 7. Recombinant human TNF-α was purchased from Sigma-Aldrich (St. Louis, MO).

Irradiation

A diode laser (Model D030-MM-FCTS/B, Opto Power Corp., Tucson, AZ) was used to deliver 810 nm radiation. The output optics provided an irradiation spot of approximately 60 mm diameter for the 35 mm dishes and 52 mm diameter for the 96-well plates. The latter beam covered only the wells in the plate containing synoviocytes. The power was varied to deliver irradiances of either 16.7 or 83.3 mW/cm^2 as measured with a power meter (model DMM199, Coherent, Santa Clara, CA). Five-minute irradiations gave total fluences at the lower and higher irradiances of 5 and 25 J/cm^2, respectively.

Prior to irradiation, the growth medium was replaced with phosphate-buffered saline (PBS). Synoviocytes were plated at 1.5×10^5 cells/35 mm plastic culture dishes and were exposed through the lids to the 810 nm radiation. The irradiances and fluences used did not increase the temperature of the samples; measurements were made up to 100 J/cm^2. For experiments in which TNF-α was added before the irradiation, the cells were incubated with PBS containing 10 ng/ml TNF-α for 10 minutes at 37°C, then the synoviocytes were washed with 37°C PBS, and irradiated at room temperature. Control cells were treated identically, that is, after changing to fresh PBS the cells were incubated for 10 minutes at 37°C and washed with 37°C PBS, except that controls were wrapped with aluminum foil and kept at room temperature while the experimental samples were irradiated. Immediately after the irradiation, the PBS was removed from both controls and experimental samples and replaced with synoviocyte growth medium 37°C. For experiments in which irradiation preceded TNF-α, the medium was changed to 37°C PBS, and the cells were irradiated (5 minutes). Subsequently, the synoviocytes were incubated with 10 ng/ml TNF-α in PBS for 10 minutes at 37°C, and then the medium was changed to synoviocyte growth medium.

RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on total RNA harvested from synoviocytes with RNeasy (QIAGEN, Valencia, CA). Equal amounts of DNase-treated total RNA (3 μg) was reverse-transcribed by Thermo script RT-PCR system (Invitrogen, Carlsbad, CA). The resultant cDNA was PCR amplified for pro-inflammatory cytokines by using human gene-specific sense and antisense primers based on sequences published in GenBank: IL-1β sense 5′-ATGGCAGAAGTACCTGAG-CTC-3′, antisense 5′-GGAAGACACAAATTGCATGGT-3′, IL-6 sense 5′-ATGAACTCCTTCTCCACAAG-3′, IL-6 5′-ACATTTGCCGAAGAGCCCTCAG-3′, IL-8 sense 5′-ATGA-CTTCCAAGCTGGCCGTGGCT-3′, IL-8 antisense 5′-TCT-CAGCCCTCTTCAAAAACTTCTC-3′, TNF-α sense 5′-CG-GGACGTGGAGCTGGCCGAGGAG-3′, antisense 5′-CA-CCAGCTGGTTATCTCTCAGCTC-3, GAPDH sense 5′-GAGTCAACGGATTTGGTCGT-3′, antisense 5′-TTGA-TTTTGGAGGGATCTCG-3′. PCR was carried out on a thermal cycler (BJ Mini, Bio-Rad, Hercules, CA). Amplification was carried out for 30 cycles for all primers at 95°C for 1 minutes, 55°C for 1 minutes, and 72°C for 1 minutes, followed by an extension step at 72°C for 7 minutes. PCR-amplified DNA was separated on a 1.5% agarose gel,

stained with ethidium bromide, and visualized and photographed with ultraviolet light using a ChemiImager 4000 (Alpha Innothech, San Leandro, CA). Bands were quantitated by densitometry using NIH image software.

MTT Assay

Cells (1×10^4) were seeded in each well of a 96-well plate 1 day before treatment. Twenty-four hours after the treatment, cell proliferation was determined using methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Sigma-Aldrich). MTT reagent (final concentration 0.5 mg/ml) was added to each well and after incubation for 1 hour, the absorbance at 570 nm was measured using a microplate reader (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA).

Cytokine Protein Measurements

Cytokine levels in the cells and supernatant were measured using Q-Plex™ Human Cytokine array (Quansys Biosciences, Logan, UT), which is a quantitative ELISA-based test in which each well of a 96-well plate contains an array of 12 different antibodies in a defined pattern. Cells were lysed in RIPA buffer with proteinase inhibitors. The protein concentration of each cell lysate was measured by Lowry assay using DC Protein Assay (Bio-Rad). Cytokine standards (30 μl) or samples (lysate containing 31 μg protein or 30 μl supernatant) were added to the wells. After 1 hour incubation the wells were washed three times and incubated with streptavidin-HRP for 15 minutes at room temperature. After washing three times and adding the substrate for chemiluminescence, the images were captured by a cooled CCD camera (FluoChem SP, Alpha Innotech) and analyzed using the Quansys image analysis software. The concentrations of cytokines were calculated using the standard curves.

Immunoblotting

Cells were lysed in buffer containing 20 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 25 mM beta-glycerophosphate and 1 mM PMSF. The protein concentration of the cell lysate was measured by Lowry assay using DC Protein Assay (Bio-Rad). Lysates were incubated with Laemmli sample buffer containing DTT as a reducing agent for 5 minutes at 100°C. Protein (25 μg) was loaded into each lane and fractionated on Tris–glycine-buffered 10–20% SDS–PAGE, then transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were blocked with TBS and 0.1% Tween-20 (TBST) containing 5% non-fat milk for 1 hour at room temperature, followed by incubation with an antibody to IκB, p65, p50 (Santa Cruz Biotechnology, Santa Cruz, CA), or histones (Upstate, Lake Placid, NY) at 4°C overnight. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary antibody (Amersham Bioscience, Piscataway, NJ) for 1 hour at room temperature. Immunoreactive protein was detected with chemiluminescence (Cell Signaling, Danvers, MA) and autoradiography (Amersham Bioscience).

RESULTS

Effect of 810 nm Radiation on HA Synoviocyte Viability

We tested the effect of LLLT on synoviocyte viability to define the appropriate treatment fluences for this study. Cell viability was evaluated using an MTT assay 24 hours after 810 nm radiation using fluences of 1, 3, 5, 10, 20, and 50 J/cm^2. As shown in Figure 1, fluences up to 50 J/cm^2, delivered at an irradiance of 16.7 mW/cm^2 did not decrease cell viability. A fluence of 5 J/cm^2 caused as apparent increase in cell number, that is, increased proliferation, similar to the effect of LLLT on other cell types [20–24]. The MTT assay measures mitochondrial reductase activity, a convenient surrogate for cell number. Since the effects of LLLT on cells are often considered to be initiated by mitochondrial alterations, the observed increase in MTT product may not reflect an actual increase in the number of cells.

810 nm Radiation Decreases the Level of TNF-α and IL-1β mRNA in RA Synoviocytes

Since cytokine synthesis is transcriptionally regulated in most cell types, we measured cytokine mRNA levels relative to GAPDH mRNA. Basal levels of IL-6 and IL-8 mRNA were detected, but IL-1β and TNF-α mRNA were not observed under the same conditions (Fig. 2A). Upon incubation with 10 ng/ml TNF-α, mRNA for IL-1β and TNF-α were detected as well as enhanced levels of IL-6 and IL-8 mRNA. mRNA for IL-1β, IL-6, IL-8, and TNF-α were still present at 60 and 180 minutes post-TNF-α (Fig. 2B).

To evaluate the effect of 810 nm radiation on pro-inflammatory cytokine mRNAs, two treatment sequences were used: (1) 810-nm radiation (5 J/cm^2; 5 minutes at 16.7 mW/cm^2) followed by TNF-α 10 ng/ml, 10 minutes, and (2) 10 ng/ml TNF-α followed by 810-nm radiation. The

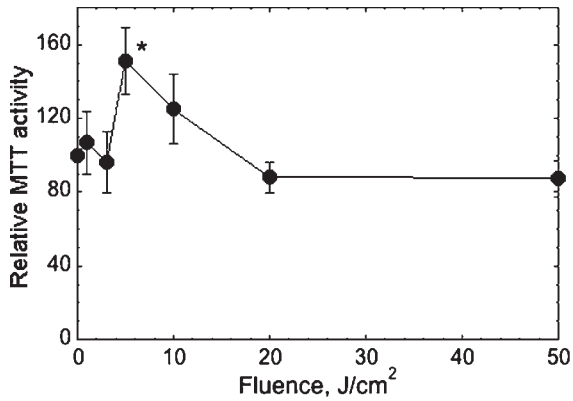

Fig. 1. Effect of 810 nm radiation on the metabolic activity of synoviocytes. Cells (1×10^4) were plated into the wells of 96-well plates. After 24 hours, cells were treated with varying fluences of 810 nm radiation at an irradiance of 16.7 mW/cm^2. Cell metabolic activity was evaluated by the MTT assay 24 hours after radiation treatment. The data shown are the mean ± SD of the results of three experiments. The asterisk indicates $P<0.05$ compared to no 810 nm treatment.

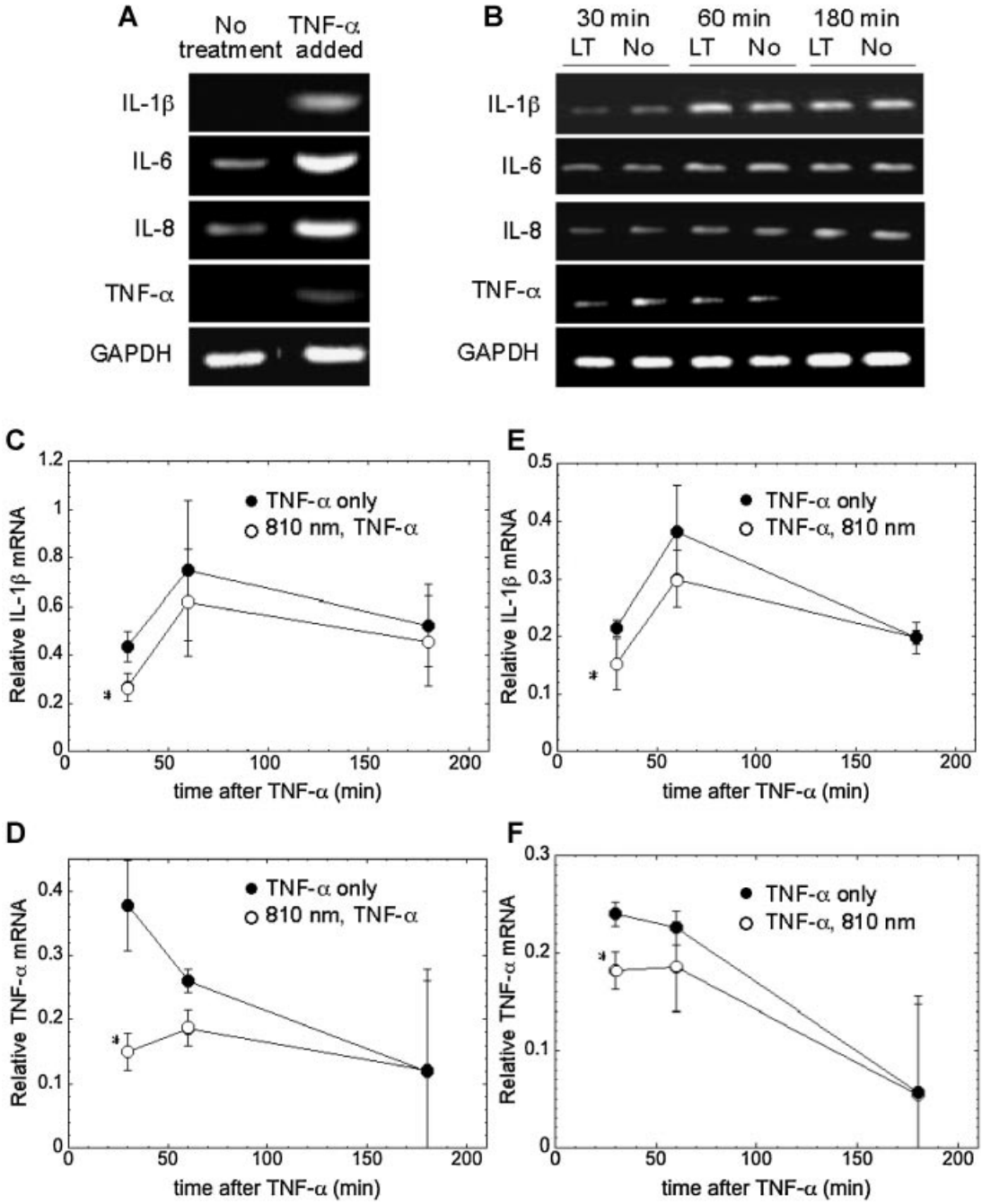

Fig. 2. 810 nm radiation decreases the levels of pro-inflammatory cytokine mRNAs in TNF-α activated synoviocytes. **A**: Cells were treated with 10 ng/ml TNF-α and harvested after 30 minutes for analysis of cytokine mRNA by RT-PCR. **B**: Synoviocytes were treated with 810 nm radiation (5 J/cm^2, 5 minutes at 16.7 mW/cm^2) followed by TNF-α incubation for 10 minutes. RNA was extracted 30, 60, and 180 minutes after the end of the TNF-α treatment. A representative gel from three independent experiments is shown. **C**,**D**: Results of densiometric analysis for IL-1β mRNA (C) and TNF-α mRNA (D) on gels from three independent experiments in which 810 nm treatment preceded TNF-α treatment. **E**,**F**: Results of densiometric analysis for IL-1β mRNA (E) and TNF-α mRNA (F) on gels from three independent experiments in which TNF-α treatment preceded 810 nm treatment. mRNA levels were measured by RT-PCR. The data shown are the mean ± SD from three independent experiments. Asterisk indicates $P<0.05$ compared to TNF-α only.

cytokine mRNA levels were analyzed at 30, 60, and 180 minutes after the end of the TNF-α treatment and the levels were expressed relative to GAPDH mRNA. The results reported here, and those from studies described below, are the mean value ± standard deviation from three separate experiments, each with triplicate samples. When 810 nm radiation was administered first (Fig. 2C,D), IL-1β mRNA (0.264 ± 0.057) and TNF-α mRNA (0.149 ± 0.029) were lower at 30 minutes than in the cells treated with TNF-α only (IL-1β: 0.436 ± 0.064; TNF-α: 0.378 ± 0.070). When TNF-α was added before the 810 nm irradiation (Fig. 2E,F), the IL-1β mRNA (0.151 ± 0.044) and TNF-α (0.182 ± 0.019) levels at 30 minutes were lower than for TNF-α only controls (IL-1β, 0.215 ± 0.015; TNF-α, 0.240 ± 0.013). Neither treatment sequence altered the cytokine mRNA levels at 60 or 180 minutes after the end of the TNF-α treatment. We also tested a higher LLLT fluence, 25 J/cm^2, in single experiments under each condition to determine if a greater inhibition of IL-1β and TNF-α mRNA would be found. However, the results were similar to those obtained using 5 J/cm^2 (results not shown). Treatment with 810 nm either before or after TNF-α did not affect the mRNA levels of IL-8 or IL-6 (results not shown).

810 nm Radiation Inhibits Synthesis of IL-1β TNF-α and IL-8 Proteins by RA Synoviocytes

Cytokines synthesized in response to TNF-α are gradually secreted into the medium over several hours and the rate of secretion differs among cytokines [25,26]. We measured both the intracellular and extracellular cytokine levels after the same two treatment sequences described above for mRNA measurements except that the higher fluence (25 J/cm^2 at 83.3 mW/cm^2) was used in hope of maximizing the response. The supernatant and cell lysate were collected at longer times (4, 12, and 24 hours) than harvesting cells for the mRNA measurements to allow time for cytokine synthesis and secretion. The cytokines were measured by multiplexed ELISA.

TNF-α and IL-1β were undetectable in cells that were not stimulated with TNF-α. By 4 hours after TNF-α treatment both cytokines were present in the cells (Fig. 3A,B). The intracellular levels of these cytokines decreased over the following 20 hours. LLLT given before or after TNF-α, significantly decreased the amounts of intracellular IL-1β and TNF-α measured 4 hours after TNF-α (Fig. 3A). This result is consistent with the effects of 810 nm on IL-1β and TNF-α mRNA levels (Fig. 2C,D). However, 810 nm did not alter the intracellular levels of these cytokines at 8 or 24 hours after the treatment nor the levels in the supernatant at any time. In the absence of TNF-α stimulation, 810 nm did not alter the low levels of IL-1β and TNF-α present at 4, 12, and 24 hours (duplicate samples; results not shown).

The intracellular IL-8 level was unaltered over the 24 hours measurement period when treated with TNF-α only (Fig. 3C) in contrast to TNF-α and IL-1β, which both decreased nearly to baseline over this period. In addition, 810 nm radiation did not decrease the IL-8 level at 4 hours after TNF-α as was found for TNF-α and IL-1β. Instead, 810 nm radiation decreased the IL-8 level at 24 hours when

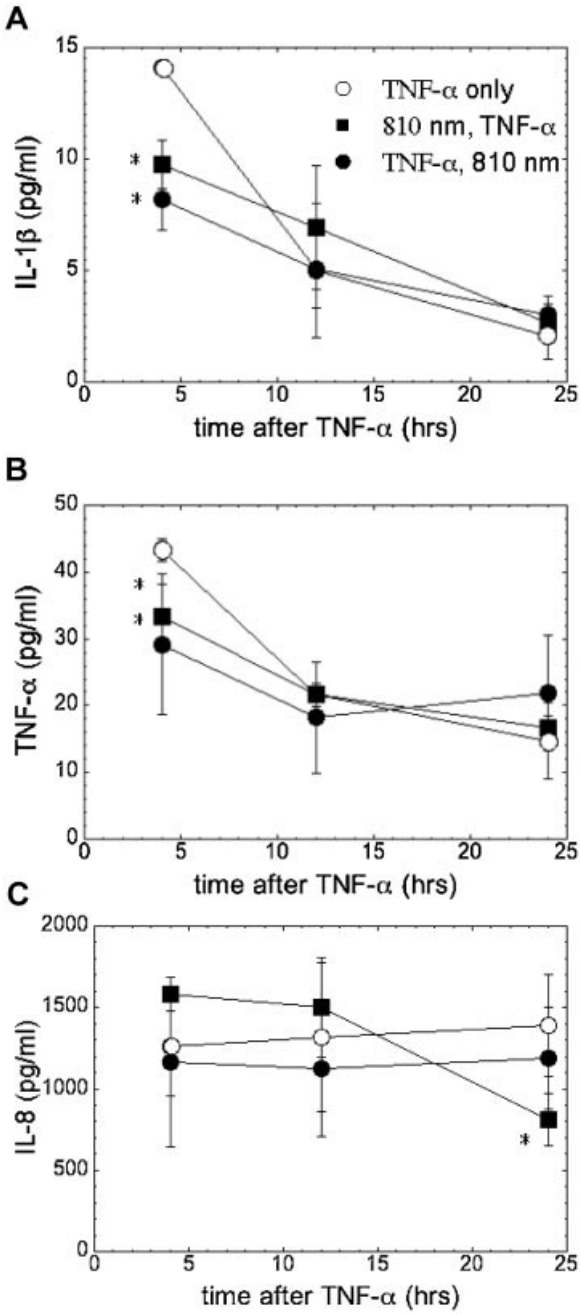

Fig. 3. 810 nm radiation inhibits synthesis of IL-1β, TNF-α, and IL-8 proteins by synoviocytes. Two treatment schemes were used: (1) 810-nm irradiation (25 J/cm^2, 5 minutes at 83.3 mW/cm^2) followed by TNF-α (filled squares) and (2) TNF-α followed by 810-nm irradiation (filled circles). Cell lysates and culture supernatants were collected at 4, 12, and 24 hours after TNF-α treatment, and protein levels were measured by multiplexed ELISA. Influence of 810 nm radiation on the TNF-α-induced level of intracellular IL-1β (**A**), TNF-α (**B**), and IL-8 (**C**). Asterisk indicates $P<0.05$ compared to TNF-α only. The data shown are from triplicate measurements; bars show ± SD.

TABLE 1. Summary of the Effects of 810 nm Radiation on TNF-α-Induced Cytokine Production by RA Synoviocytes

	TNF-α, then 810 nm		810 nm, then TNF-α	
Cytokine	mRNA[a]	Protein	mRNA[a]	Protein
TNF-α	Decrease	Decrease[b]	Decrease	Decrease[b]
IL-1β	Decrease	Decrease[b]	Decrease	Decrease[b]
IL-6	No effect	No effect	No effect	No effect
IL-8	No effect	Decrease[c]	No effect	Decrease[c]

[a]Measured 30 minutes after TNF-α treatment.
[b]Measured 4 hours after TNF-α treatment.
[c]Measured 24 hours after TNF-α treatment.

810 nm treatment was followed by TNF-α (Fig. 3C). LLLT did not affect the very low IL-8 level in cells not treated with TNF-α (duplicate samples; results not shown). LLLT given either before or after TNF-α did not decrease the other seven cytokines measured in the multiplexed ELISA (IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13). The effects of 810 nm radiation on cytokine mRNA and protein levels are summarized in Table 1.

Effect of 810 nm Radiation on the NF-κB Pathway

The transcription factor NF-κB mediates the TNF-α-induced transcription of many genes including those for cytokines and chemokines. NF-κB is normally located in the cytoplasm and inactive because it is bound to an inhibitory protein, IκB, that masks the nuclear localization sequence. Association of TNF-α with its plasma membrane receptor leads to phosphorylation of IκB. Phosphorylated IκB is then rapidly degraded, thus releasing NF-κB and allowing it to move into the nucleus and initiate (via binding to promoter elements) gene transcription. We tested whether 810 nm radiation (25 J/cm^2; 83.3 mW/cm^2) interrupts this sequence, which would decrease TNF-α and IL-1β mRNA and protein synthesis. We tested whether 810 nm decreases the rate of IκBα degradation by treating RA synoviocytes with 810 nm, then adding TNF-α and measuring the level of IκBα at times between 1 and 10 minutes. As shown in Figure 4A, IκBα decreased rapidly after TNF-α treatment, becoming undetectable after 10 minutes. However, 810 nm radiation treatment did not decrease the rate of IκBα degradation. Thus, 810 nm does not intervene at this step to decrease NF-κB activity. We next considered that LLLT might increase the rate at which IκBα is resynthesized as an alternative mechanism for decreasing the active NF-κB level. We measured the level of IκBα present in cells for times up to 30 minutes after TNF-α treatment. A significant amount of IκBα is resynthesized during this period but, as shown in Figure 4B, the rate of IκBα resynthesis was the same with and without 810 nm treatment. A possible alternative for the effect of 810 nm is that it decreases the rate of translocation of NF-κB into the nucleus or the amount of NF-κB accumulated in the nucleus after it is released from IκBα. Thus, we monitored the translocation of NF-κB by measuring the amount of p65, a subunit of NF-κB, in the nucleus after addition of TNF-α. As shown in Figure 4C, the level of p65 in the nucleus rapidly increased to a maximum level after 10 minutes. However, treatment with 810 nm radiation did not influence the accumulation rate or amount of p65 in the nucleus after 10 minutes. We obtained similar results using the other NF-κB subunit, p50 (data not shown).

DISCUSSION

TNF-α is the dominant cytokine in synovial fluid in joints afflicted with RA and is a major contributor to the chronic inflammation that leads to joint pain. Treatments for RA often target TNF-α because it stimulates production of other pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and chemokines, such as IL-8, from cells in the synovium of RA joints as well as stimulating synthesis of MMPs by synoviocytes leading to bone loss. Decreasing TNF-α by exposing the painful RA joint to 810 nm radiation could be a simple, non-invasive treatment for downregulation of inflammation, leading to pain relief. IL-1β, TNF-α, IL-6, and IL-8 are produced by activated synoviocytes as well as by other cell types, especially macrophages, in inflamed joints. In animal models of acute inflammation, LLLT reduced the levels of IL-1β, TNF-α although the cellular sources of these cytokines was not identified [17–19]. Because LLLT radiation is used to decrease chronic joint pain, we sought to understand the cellular mechanisms involved with the goal of optimizing this treatment.

Our results indicate that 810 nm radiation inhibits the TNF-α-stimulated production of the IL-1β, TNF-α, and IL-8 by RA synoviocytes. Both the mRNA and intracellular protein levels of IL-1β and TNF-α were decreased at the shortest times measured after 810 nm radiation (Figs. 2 and 3), even though 5 J/cm^2 was used in experiments measuring mRNA and 25 J/cm^2 was used for protein measurements. The intracellular pathway by which TNF-α stimulates synthesis of these pro-inflammatory cytokines in RA has been extensively investigated [27,28]. These studies have recently led to development of biologic agents that block TNF-α, for example, etanercept, thereby dramatically improving the clinical symptoms in many RA patients [29]. TNF-α binds to specific receptors (TNFR1 and TNFR2) in the plasma membrane, which alters the shape of the receptor and leads to organization of a multiprotein

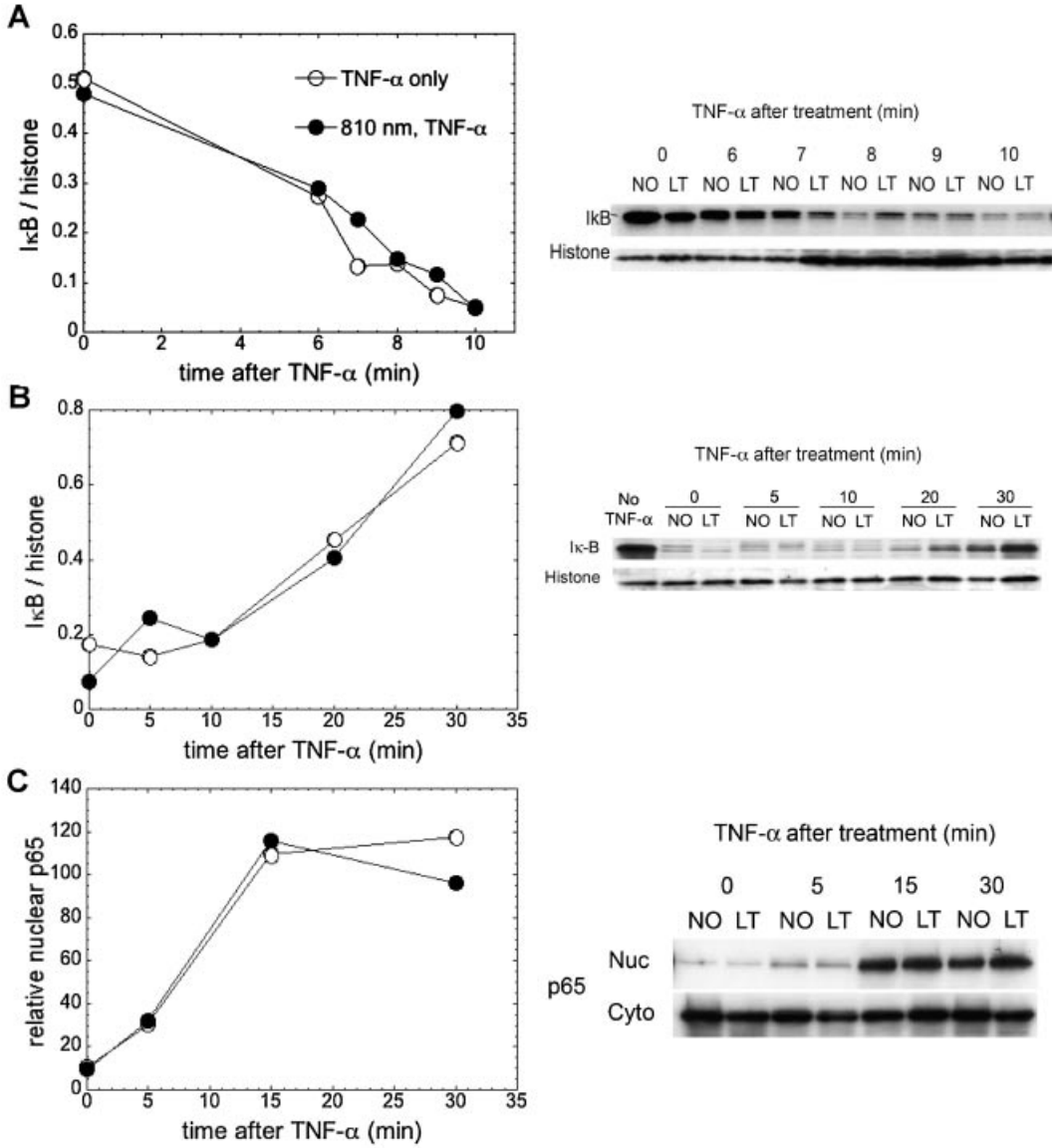

Fig. 4. Effect of 810 nm radiation on the NF-κB pathway in synoviocytes. **A**,**B**: Cells were treated either with 810-nm irradiation (25 J/cm^2, 5 minutes at 83.3 mW/cm^2), then incubated with TNF-α (labeled LT) or not treated and incubated with TNF-α (labeled NO). Whole cell lysates were collected between 5 and 30 minutes. Immunoblotting was performed using antibodies specific for IκBα or histone. A: Effect of 810 nm radiation on degradation of IκBα after TNF-α stimulation. B: Effect of 810 nm radiation on resynthesis of IκBα after TNF-α stimulation. The data shown are triplicates; bars show ± SD. **C**: Cells were treated either with 810-nm irradiation (25 J/cm^2, 5 minutes at 83.3 mW/cm^2) then incubated with TNF-α (labeled LT) or not treated and incubated with TNF-α (labeled NO). Immunoblot analysis was performed on nuclear (Nuc) extracts with antibody against the NF-κB subunit p65. The data are the means from duplicate samples; bars show ± SD.

complex associated with the receptor cytoplasmic tail. Interactions amongst the components of this complex (e.g., TRADD, TRAF2, and RIP), lead to activation of the protein kinase IKK, which then phosphorylates IκBα, the inhibitory protein that normally blocks translocation of NF-κB to the nucleus. The signaling process is rapid; IκBα was degraded and NF-κB moved into the nucleus within approximately 10 minutes after TNF-α treatment (Fig. 4). A pulse treatment with TNF-α increased the level TNF-α mRNA somewhat more rapidly than that of IL-1β mRNA. TNF-α mRNA was at high at 30 minutes and declined at longer times (Fig. 2D,F) whereas the maximum for IL-1β mRNA was at ~60 minutes after treatment (Fig. 2C,E). In contrast to the effects of 810 nm on IL-1β and TNF-α, 810 nm reduced the TNF-α-stimulated level of the chemokine IL-8 only at 24 hours. Notably, the time course for TNF-α-stimulated increase in IL-8 differed markedly from that found for IL-1β and TNF-α, possibly reflecting the

difference in their functions. IL-8 protein remained at a steady high level for 24 hours whereas IL-1β and TNF-α decreased to near baseline (Fig. 3), suggesting differences in regulatory mechanisms. Additional studies are required to address this intriguing difference in responses to 810 nm.

Our results shed light on mechanisms of intracellular signaling that are, or are not, inhibited by 810 nm radiation. The first step in the sequence, binding of TNF-α to membrane-located receptors TNFR1 and TNFR2, does not appear to be influenced by 810 nm treatment because the TNF-α and IL-1β levels were decreased to the same extent when the 810 nm is given after TNF-α was already bound to the receptors and excess washed away (Fig. 2E,F), as when the radiation precedes the TNF-α treatment (Fig. 2C,D). Many therapeutic agents have been designed to block production of TNF-α-induced pro-inflammatory products by inhibiting IKK, thereby decreasing the rate of degradation of IκBα [30]. However, as shown in Figure 4A, treatment with 810 nm radiation did not decrease the rate of IκBα degradation nor did it enhance the rate of IκBα resynthesis (Fig. 4B). In addition, the translocation of the p65 subunit of NF-κB into the nucleus was not inhibited or delayed by 810 nm (Fig. 4C). Thus, 810 nm radiation appears to decrease the TNF-α-induced synthesis of TNF-α and IL-1β by influencing processes downstream of NF-κB activation. Our observation that 810 nm selectively decreases TNF-α-induced levels of two cytokines and a chemokine but not the levels of others measured by multiplexed ELISA (IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13) also suggests that activation of NF-κB is not the step inhibited by 810 nm. TNF-α-induced synthesis of all of these cytokines involves activation of NF-κB, with the specific molecules produced under differing conditions being regulated at the level of transcription.

TNF-α-induced synthesis of TNF-α, IL-1β, and IL-8 can be regulated at several steps downstream of NF-κB activation. Transcription of these genes requires, in addition to NF-κB, cooperating transcription factors and co-activators that bind to the promoter sequence (reviewed in Ref. [31]). Thus, 810 nm may decrease TNF-α-induced synthesis of TNF-α, IL-1β, and IL-8 by decreasing other TNF-α-induced transcription factors, such as AP-1, that cooperate with NF-κB. Synthesis of TNF-α is also regulated post-transcriptionally, such as by altered mRNA stability [32], which provides additional pathways that 810 nm might influence. 810 nm had the same effect on both the mRNA and protein levels for TNF-α and IL-1β suggesting that the transcription rate or mRNA stability is influenced rather than a later step in protein synthesis. The effect of 810 nm on TNF-α and IL-1β at 4 hours versus the effect on IL-8 at 8 hours (Fig. 3) might arise due to differences in these regulatory pathways. Further studies are needed to evaluate the contribution of these potential mechanisms to the effect of 810 nm on cytokine synthesis.

In summary, our results indicate that LLLT decreases the synthesis of pro-inflammatory cytokines/chemokine by activated synoviocytes in a selective manner. TNF-α-induced activation of NF-κB is not altered by 810 nm radiation and our results suggest that the inhibition occurs by an effect on transcription rate or mRNA stability. These results imply that use of LLLT for RA may reduce inflammation, thereby alleviating joint pain, by reducing the level of inflammatory cytokines and chemokines. This mechanism may be more general and underlie the beneficial effects of LLLT on other inflammatory conditions.

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REFERENCES

1. Desmet KD, Paz DA, Corry JJ, Eells JT, Wong-Riley MT, Henry MM, Buchmann EV, Connelly MP, Dovi JV, Liang HL, Henshel DS, Yeager RL, Millsap DS, Lim J, Gould LJ, Das R, Jett M, Hodgson BD, Margolis D, Whelan HT. Clinical and experimental applications of NIR-LED photobiomodulation. Photomed Laser Surg 2006;24(2):121–128.
2. Yousefi-Nooraie R, Schonstein E, Heidari K, Rashidian A, Pennick V, Akbari-Kamrani M, Irani S, Shakiba B, Mortaz Hejri SA, Mortaz Hejri SO, Jonaidi A. Low level laser therapy for nonspecific low-back pain. Cochrane Database Syst Rev 2008;16(2):CD005107.
3. Lampl Y, Zivin JA, Fisher M, Lew R, Welin L, Dahlof B, Borenstein P, Andersson B, Perez J, Caparo C, Ilic S, Oron U. Infrared laser therapy for ischemic stroke: A new treatment strategy: Results of the neurothera effectiveness and safety trial-1 (Nest-1). Stroke 2007;38(6):1843–1849.
4. Ozcelik O, Cenk Haytac M, Kunin A, Seydaoglu G. Improved wound healing by low-level laser irradiation after gingivectomy operations: A controlled clinical pilot study. J Clin Periodontol 2008;35(3):250–254.
5. Saltmarche AE. Low level laser therapy for healing acute and chronic wounds—The Extendicare experience. Int Wound J 2008;5(2):351–360.
6. Schaible HG, Ebersberger A, Von Banchet GS. Mechanisms of pain in arthritis. Ann NY Acad Sci 2002;966:343–354.
7. Majithia V, Geraci SA. Rheumatoid arthritis: Diagnosis and management. Am J Med 2007;120(11):936–939.
8. Firestein GS. Immunologic mechanisms in the pathogenesis of rheumatoid arthritis. J Clin Rheumatol 2005;11(3 Suppl): S39–S44.
9. Sweeney SE, Firestein GS. Rheumatoid arthritis: Regulation of synovial inflammation. Int J Biochem Cell Biol 2004;36(3): 372–378.
10. Manadan AM, Block JA. Rheumatoid arthritis: Beyond tumor necrosis factor-alpha antagonists, B cell depletion, and T cell blockade. Am J Ther 2008;15(1):53–58.
11. Simpson CR, Kohl M, Essenpreis M, Cope M. Near-infrared optical properties of ex vivo human skin and subcutaneous tissues measured using the Monte Carlo inversion technique. Phys Med Biol 1998;43(9):2465–2478.
12. Stolik S, Delgado JA, Perez A, Anasagasti L. Measurement of the penetration depths of red and near infrared light in human "ex vivo" tissues. J Photochem Photobiol B 2000; 57(2–3):90–93.

13. Byrnes KR, Waynant RW, Ilev IK, Wu X, Barna L, Smith K, Heckert R, Gerst H, Anders JJ. Light promotes regeneration and functional recovery and alters the immune response after spinal cord injury. Lasers Surg Med 2005;36(3):171–185.
14. Jimbo K, Noda K, Suzuki K, Yoda K. Suppressive effects of low-power laser irradiation on bradykinin evoked action potentials in cultured murine dorsal root ganglion cells. Neurosci Lett 1998;240(2):93–96.
15. Sakurai Y, Yamaguchi M, Abiko Y. Inhibitory effect of low-level laser irradiation on LPS-stimulated prostaglandin E2 production and cyclooxygenase-2 in human gingival fibroblasts. Eur J Oral Sci 2000;108(1):29–34.
16. Castano AP, Dai T, Yaroslavsky I, Cohen R, Apruzzese WA, Smotrich MH, Hamblin MR. Low-level laser therapy for zymosan-induced arthritis in rats: Importance of illumination time. Lasers Surg Med 2007;39(6):543–550.
17. Aimbire F, Albertini R, Pacheco MT, Castro-Faria-Neto HC, Leonardo PS, Iversen VV, Lopes-Martins RA, Bjordal JM. Low-level laser therapy induces dose-dependent reduction of TNF-alpha levels in acute inflammation. Photomed Laser Surg 2006;24(1):33–37.
18. Aimbire F, Ligeiro de Oliveira AP, Albertini R, Correa JC, Ladeira de Campos CB, Lyon JP, Silva JA, Jr., Costa MS. Low level laser therapy (LLLT) decreases pulmonary microvascular leakage, neutrophil influx and IL-1beta levels in airway and lung from rat subjected to LPS-Induced inflammation. Inflammation 2008;31(3):189–197.
19. Aimbire F, Lopes-Martins RA, Castro-Faria-Neto HC, Albertini R, Chavantes MC, Pacheco MT, Leonardo PS, Iversen VV, Bjordal JM. Low-level laser therapy can reduce lipopolysaccharide-induced contractile force dysfunction and TNF-alpha levels in rat diaphragm muscle. Lasers Med Sci 2006;21(4):238–244.
20. Frank S, Menezes S, Lebreton-De Coster C, Oster M, Dubertret L, Coulomb B. Infrared radiation induces the P53 signaling pathway: Role in infrared prevention of ultraviolet B toxicity. Exp Dermatol 2006;15(2):130–137.
21. Frank S, Oliver L, Lebreton-De Coster C, Moreau C, Lecabellec MT, Michel L, Vallette FM, Dubertret L, Coulomb B. Infrared radiation affects the mitochondrial pathway of apoptosis in human fibroblasts. J Invest Dermatol 2004; 123(5):823–831.
22. Menezes S, Coulomb B, Lebreton C, Dubertret L. Non-Coherent near infrared radiation protects normal human dermal fibroblasts from solar ultraviolet toxicity. J Invest Dermatol 1998;111(4):629–633.
23. Applegate LA, Scaletta C, Panizzon R, Frenk E, Hohlfeld P, Schwarzkopf S. Induction of the putative protective protein ferritin by infrared radiation: Implications in skin repair. Int J Mol Med 2000;5(3):247–251.
24. Pereira AN, Eduardo Cde P, Matson E, Marques MM. Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg Med 2002; 31(4):263–267.
25. Maret M, Chicheportiche R, Dayer JM, Gabay C. Production of intracellular IL-1alpha, IL-1beta, and IL-1ra isoforms by activated human dermal and synovial fibroblasts: Phenotypic differences between human dermal and synovial fibroblasts. Cytokine 2004;25(5):193–203.
26. Sticherling M, Hetzel F, Schroder JM, Christophers E. Time- and stimulus-dependent secretion of Nap-1/IL-8 by human fibroblasts and endothelial cells. J Invest Dermatol 1993; 101(4):573–576.
27. Simmonds RE, Foxwell BM. Signalling, inflammation and arthritis: Nf-KappaB and its relevance to arthritis and inflammation. Rheumatology (Oxford) 2008;47(5):584–590.
28. Bradley JR. TNF-mediated inflammatory disease. J Pathol 2008;214(2):149–160.
29. Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M, Burge DJ. A trial of Etanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999;340(4): 253–259.
30. Karin M, Yamamoto Y, Wang QM. The Ikk NF-Kappa B system: A treasure trove for drug development. Nat Rev Drug Discov 2004;3(1):17–26.
31. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-Kappa B signaling module. Oncogene 2006; 25(51):6706–6716.
32. Nagy LE. Stabilization of tumor necrosis factor-alpha mRNA in macrophages in response to chronic ethanol exposure. Alcohol 2004;33(3):229–233.