## EXTENDED REPORT

**Contribution of tumour necrosis factor α and interleukin (IL) 1β to IL6 production, NF-κB nuclear translocation, and class I MHC expression in muscle cells: in vitro regulation with specific cytokine inhibitors**

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**Objective:** To evaluate the effect of tumour necrosis factor α (TNFα), interleukin (IL) 1β, and their respective inhibitors the p75 TNFα soluble receptor (sTNFR) and the type II sIL1βR (sIL1RII) on whole muscle and isolated myoblast activation.

**Methods:** Normal muscle samples were stimulated for 7 days with TNFα alone or in combination with IL1β, and myoblasts from these samples for 48 hours. IL6 production was measured by EUSA. Nuclear translocation of NF-κB was analysed by immunofluorescent staining and class I MHC expression by FACS.

**Results:** TNFα and IL1β induced IL6 production by normal muscle samples and myoblasts, the action of TNFα being more potent on muscle samples. Their soluble receptors (1 μg/ml) decreased this production. Suboptimal concentrations of TNFα and IL1β induced NF-κB translocation. sTNFR markedly down regulated TNFα-induced translocation while sIL1RII was less potent on IL1β-induced activation. NF-κB translocation induced by the combination of optimal concentrations of TNFα and IL1β was completely inhibited by their soluble receptors. TNFα and to a lesser extent IL1β induced class I MHC expression by myoblasts and this effect was completely inhibited by their respective soluble receptors.

**Conclusion:** These results suggest that TNFα and IL1β should be targeted for myositis treatment.

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**Materials and Methods**

**Reagents**

Recombinant human TNFα and IL1β were purchased from Sigma (St Louis, MO). Dimeric human TNFR p80/IgG1:Fc fusion protein and human sIL1RI1 were kindly provided by Dr John Sims (Amgen/Immunex, Seattle, WA).

**Muscle sample and muscle cell cultures**

Normal muscle (vastus lateralis) samples were obtained from 10 patients undergoing hip joint replacement for osteoarthritis. Informed written consent was obtained according to the local ethics committee. Preliminary results showed the lack of inflammatory cells and markers in these samples, which were thus considered to be normal muscle. Fat and fibrous tissues were removed, and muscle was cut into small pieces of 5 mm³ and cultured in complete medium made of Dulbecco's modified Eagle's medium (DMEM), 20% fetal calf serum (FCS; Invitrogen, France), antibiotics (100 U/ml penicillin/100 μg/ml streptomycin; Invitrogen) and amphotericin B (Invitrogen). Cultures were performed at 37°C in a 5% CO₂/95% air humidified environment. Medium was routinely changed every 4 days. After 14 days in complete medium, pieces of muscle were washed and used for experiments.

**Abbreviations:** DMEM, Dulbecco’s modified Eagle’s medium; EUSA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IL1β, interleukin 1β; IIM, idiopathic inflammatory myopathies; MHC, major histocompatibility complex; NF-κB, nuclear factor-κB; PBS, phosphate buffered saline; RA, rheumatoid arthritis; sR, soluble receptor(s); TNFα, tumour necrosis factor α.
To obtain normal skeletal myoblasts, muscle biopsy specimens were cut into fragments of 1–2 mm³, and 10 explants were placed in 25 cm² culture flasks in 1 ml of complete medium. After 10–14 days, when sufficient cellular outgrowth had occurred at the periphery of the explants, the explants were removed and transferred to new flasks. Myoblasts were isolated twice by positive selection before use. Adherent cells were cultured in complete medium, and at 80% confluence were detached with trypsin (Invitrogen). Cells were incubated with an anti-CD56 antibody (Becton-Dickinson, Mountain View, CA) (1:50) in phosphate buffered saline (PBS) with 1% FCS for 30 minutes. Then, cells were washed twice and incubated for 30 minutes with Dynabeads coated with a secondary antibody (Dynal). The samples were placed in the magnetic device (Dynal) and the supernatant was removed. After growth, the same selection was repeated.

The purity of myoblast preparations was assessed by staining with the anti-CD56 antibody. Cells were incubated with the fluorescein isothiocyanate (FITC) conjugated anti-CD56 (Leu 19) antibody (Becton-Dickinson), HLA-ABC FITC conjugated (Becton-Dickinson) or isotype matched control antibody for 30 minutes at 4°C in PBS, after washing with PBS. After washing, dead cells were counterlabelled with propidium iodide (Sigma) and living cells were analysed by propidium iodide (Sigma) and living cells were analysed by flow cytometry with the fluorescein isothiocyanate (FITC) conjugated anti-CD56 antibody (Becton-Dickinson). Cells were incubated with an anti-CD56 antibody (Becton-Dickinson) for 30 minutes at 4°C in PBS, after washing with PBS. After washing, dead cells were counterlabelled with propidium iodide (Sigma) and living cells were analysed by propidium iodide (Sigma) and living cells were analysed by flow cytometry with the fluorescein isothiocyanate (FITC) conjugated anti-CD56 antibody (Becton-Dickinson). Cells were incubated with an anti-CD56 antibody (Becton-Dickinson) for 30 minutes at 4°C in PBS, after washing with PBS. After washing, dead cells were counterlabelled with propidium iodide (Sigma) and living cells were analysed by propidium iodide (Sigma) and living cells were analysed by flow cytometry with the fluorescein isothiocyanate (FITC) conjugated anti-CD56 antibody (Becton-Dickinson).

For cytokine studies, myoblasts (10⁴ cells/well) were incubated in 96 well flat plates in a final volume of 200 µl of complete medium. Myoblasts were used between passages 2 and 4 after the second positive selection. Cytokines were added at culture initiation and during incubation. IL6 production was measured in myoblast and muscle sample culture media by a two site sandwich enzyme linked immunosorbent assay (ELISA). For immunofluorescent staining experiments, myoblasts were seeded (10⁴ cells/cm²) on glass coverslips (diameter 12 mm) in DMEM supplemented with 20% FCS. At 80% of confluence, cells were deprived of FCS for 12 hours and then were stimulated either with TNFα or IL1β alone or in combination.

Immunofluorescent staining for NF-κB
Immunofluorescent staining was performed on stimulated and control myoblasts, before and 30 minutes after cytokine stimulation. After removal of medium, cells were fixed overnight at 4°C with 4% paraformaldehyde and washed intensively (3×5 minutes) with 1×PBS. Cells were permeabilised for 3 minutes in 0.1% Triton X-100/1×PBS, followed by PBS washings. Non-specific binding was abolished by incubation for 1 hour at room temperature in a blockade solution (1×PBS, 0.1% bovine serum albumin). Polyclonal primary anti-NF-κB antibodies (anti-p65 pAb; Santa Cruz), diluted 1:100, were added for 1 hour at room temperature and cells were washed again in PBS. Samples were incubated at room temperature for 1 hour in blockade solution. Afterwards, cells were washed in PBS and treated with polyclonal secondary FITC conjugated antibodies (Santa-Cruz, diluted 1:100) for 1 hour at room temperature. Next, cells were washed in PBS and incubated for 30 minutes at room temperature with 0.5µg/ml of Hoechst H33258 (Sigma, France) in PBS. After PBS washings followed by a rinse in distilled water, samples were mounted in PBS/glycerol 80%. Nuclear translocation was observed and confirmed by nuclear staining with Hoechst with a fluorescence microscope (Leica, France) coupled to a video camera (Nikon, France), and with image analysis software (Lucia, Nikon, France), ROS17/2.8 cells, an osteosarcoma cell line, stimulated by medium with 10% of serum, were used as a positive control as described. The mean green intensity of the nuclear staining was quantified with Lucia image analysis software in 10 randomly selected cells.

Statistical analysis
Results were expressed as mean (SD). Differences between cytokine treated groups without sR and the control group or cytokine treated groups with sR were compared with the Wilcoxon test.

RESULTS
Dose effect of TNFα and IL1β alone on IL6 production by myoblasts
Myoblasts were incubated with increasing concentrations of TNFα and IL1β, and IL6 production, as a marker of inflammation, was studied. After 48 hours of culture, with
To better evaluate the effects of TNFα in vivo muscle samples and myoblasts, low concentrations defined as 10 ng/ml for TNFα were used. Effect of TNFα production was in the same order of 50 ng/ml. Muscles samples. When these concentrations were used, IL6 production increased in a dose dependent manner (fig 1). Comparison concentrations from 1 to 100 000 pg/ml, IL6 production indicated a much higher sensitivity of myoblasts to the effect of IL1β on IL6 production. From these dose-response curves, low concentrations defined as 10 ng/ml for TNFα and 10 pg/ml for IL1β were selected for the following experiments using muscle samples. When these concentrations were used, IL6 production was in the same order of 50 ng/ml.

Effect of TNFα and IL1β alone or in combination on ex vivo muscle samples and myoblasts

To better evaluate the effects of TNFα and IL1β in an in vitro model of IIM, we stimulated 10 normal muscle pieces with 10 ng/ml TNFα and 10 pg/ml IL1β, alone and in combination (fig 2). Levels of IL6 were measured after 7 days of culture. Spontaneous IL6 production (mean (SD) 70 (32) ng/ml) increased by 131 (120)% (range 3–338%, p<0.01 versus control) with 10 pg/ml of TNFα, by 55 (71)% (range 10–238%, p<0.05 versus control) with 10 pg/ml of IL1β, and by 145 (147)% (range 5–395%, p<0.01 versus control) with the combination.

Table 1: Dose effect of individual cytokines on NF-κB nuclear translocation

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<th>Concentration (pg/ml)</th>
<th>TNFα</th>
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After 30 minutes with cytokines at the mentioned concentration, cells were fixed in paraformaldehyde 4% and immunofluorescence staining was performed (see fig 4 for an example). Nuclear translocation was confirmed by nuclear staining with Hoechst H33258. When an automated image analysis with a scale of mean green intensity was used, nuclear staining was considered as negative (−), positive (+), and very positive (++).

effect of individual cytokines on NF-κB nuclear translocation

To extend these studies to purified muscle cells separated from the other muscle components, myoblasts were selected by two rounds of affinity purification with an anti-CD56 antibody in order to obtain a pure myoblast population. Figure 3A shows an example of a purified population of myoblasts as demonstrated by FACS analysis. Figure 3B shows the IL6 production by muscle samples from two different subjects and the myoblasts derived from these samples. They were incubated for 48 hours with 10 ng/ml TNFα and 10 pg/ml IL1β alone and in combination. When these purified myoblasts were used, an additive effect between TNFα and IL1β was seen. However, this enhancing effect was not seen with the muscle samples from which these myoblasts had been isolated (fig 3C).

Thus, at the concentrations with an identical effect on isolated myoblasts, the effect of TNFα was more potent than that of IL1β in inducing IL6 production by muscle samples. We did not find any synergistic or additive effect between TNFα and IL1β on muscle samples.

Effect of sR on IL6 production by muscle samples or myoblasts stimulated with TNFα and IL1β alone or in combination

Using the same conditions, we studied the effect of their respective sR, p75 TNFR α sR and the type II IL1 β sR, used as functional inhibitors. Both cytokines alone and in combination.
combination were incubated for 30 minutes with 1 mg/ml sTNFR or sIL1RII, either alone or in combination, before their addition to muscle samples, as previously described with RA synovium explants. In comparison with the effect of cytokines alone, sR at a concentration of 1 mg/ml decreased IL6 production by muscle samples by 235 (13)% (range 251 to 21%) with 10 ng/ml TNFα (p = 0.005), by 217 (29)% (range 263 to 6%) with 10 pg/ml of IL1β (NS), and by 232 (13)% (range 256 to 7%) with the combination of both cytokines (p = 0.005) (fig 2). Comparison of the effects of the sR on muscle pieces and myoblasts from the same samples showed a stronger effect with myoblasts (figs 3B and C).

Figures 1A and B show dose-response curves of these effects with isolated myoblasts.

Combination of suboptimal concentrations of TNFα and IL1β, and effect of sR on NF-κB translocation
We first tested the effect of TNFα and IL1β alone on selected myoblasts to define minimal concentrations able to induce a nuclear translocation of NF-κB. After a 30 minute stimulation (T30) with increasing cytokine concentrations, myoblasts were fixed and stained. A semiquantitative scale was used to evaluate the dose effect for each cytokine (table 1, fig 4). Without cytokine, NF-κB was not found in the nucleus. With 10 pg/ml TNFα or IL1β alone, nuclear translocation was not induced. NF-κB nuclear localisation was observed for concentrations from 50 pg/ml reaching a maximum for around 1000 pg/ml TNFα. Almost similar results were obtained for IL1β. When combined, suboptimal concentrations of TNFα (10 pg/ml) and IL1β (10 pg/ml), inactive when used alone, induced a clear nuclear translocation of NF-κB after 30 minutes indicating a synergistic effect (fig 4).

When the same protocol as above was used, the translocation of NF-κB stimulated now by optimal concentrations of 500 pg/ml TNFα was strongly inhibited by 1 mg/ml p75 TNF sR, whereas the same concentration of sIL1RII was less potent in inhibiting the effect induced by 1000 pg/ml IL1β alone (fig 5). The inhibitory effect of sTNFR or sIL1RII alone never reached a maximum because some degree of nuclear staining was still observed. However, their combination completely inhibited the effect of IL1β and TNFα, leading to an absolute lack of nuclear staining, as observed in the absence of cytokines.

Effect of sTNFR and sIL1RII on class I MHC expression induced by TNFα or IL1β
To study the effect of sTNFR and sIL1RII on the regulation of class I MHC expression, a classical findings on muscle fibres in IIM, we stimulated myoblasts with suboptimal concentrations of cytokines. Cultured human myoblasts constitutively express MHC class I antigens (fig 6, top). After 48 hours of

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culture, such expression increased with TNFα more than with IL1β. In the presence of their respective sR, class I MHC expression induced by TNFα or IL1β alone and in combination was almost completely inhibited.

DISCUSSION
Immunohistochemistry and in situ hybridisation studies have indicated that TNFα and IL1β are overexpressed in muscle from IIM. Although the prevalence of TNFα or IL1β positive infiltrating cells was variable, it appears that these cytokines are produced by infiltrating mononuclear cells rather than by the muscle fibres. To reproduce these interactions, we exposed myoblasts to these exogenous cytokines.

In vitro results have shown the effects of these cytokines on isolated myoblasts. However, their effect on intact muscle samples had not been determined previously. We obtained normal muscle samples from patients undergoing hip joint replacement for osteoarthritis. These samples kept the normal muscle structure and cell interactions of muscle in vivo. From these samples, we selected pure populations of myoblasts in order to avoid cell contamination. We selected three markers potentially expressed by muscle cells stimulated by proinflammatory cytokines: IL6 production, NF-κB translocation, and class I MHC expression.

Firstly, we measured IL6 production by skeletal muscle samples and myoblasts. The exact contribution of IL6 to muscle inflammation is debated. However, several studies have shown that its production by normal skeletal myoblasts is induced with TNFα or IL1β. We selected cytokine concentrations already determined in previous studies with RA synoviocytes, which are more sensitive to the effects of IL1 than those of TNFα. For instance, similar levels of IL6 and CCL20/MIP-3α were induced by 10 ng/ml TNFα and 10 pg/ml IL1β. Here, the effects of 10 ng/ml TNFα were equivalent to those of 10 pg/ml IL1β on the induction of IL6 production by myoblasts. The sensitivity of myoblasts to these cytokines appears similar to that of synoviocytes, but the in vitro response of whole muscle samples to IL1β was less important than that to TNFα. It remains to be clarified whether muscle cells exposed for a long time to local inflammation will behave in the same way as the myoblasts studied here.

Binding of TNFα and IL1β to their respective receptors can trigger multiple pathways that result in the activation of NF-κB. Such pathways have been identified in the effect of cytokines on skeletal muscle metabolism. We investigated the nuclear translocation of NF-κB in cells treated with cytokines alone or in combination. Indeed, some of the monocyte derived cytokines such as TNFα and IL1β may be present at very low concentrations in inflammatory muscle and their effects may be synergistic. Our data indicated that the combination of both cytokines at low concentrations, inactive when used alone, promoted the nuclear translocation of NF-κB in skeletal myoblasts and was thus more potent than the effect of each cytokine alone.

Finally, we studied the class I MHC expression by myoblasts stimulated with TNFα and IL1β. When isolated myoblasts were used, class I MHC spontaneous expression was increased with TNFα, with IL1β being less potent. However, this effect was less than with interferon γ.

The position of TNFα and IL1β inhibitors in myositis treatment is not defined at present. IIM are rare diseases and clinical trials will be difficult to perform. In our study we tried to evaluate in vitro the possible contribution of TNFα...
Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for controlling synovial inflammation and bone resorption in an ex vivo model. Arthritis Rheum 2001;44:293–303.


