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 ELISA. 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.

Idiopathic inflammatory myopathies (IIM) are a group of diseases characterised by muscle inflammation, leading to symmetrical and proximal muscle pain and weakness.[1 2] The goal of IIM treatment is to reduce inflammation in muscle and to improve muscle strength and function of patients with myositis. Tumour necrosis factor α (TNFα) and interleukin (IL) 1β are now the main targets for rheumatoid arthritis (RA) treatment with blocking anti-TNFα antibodies, soluble TNFα receptor, and IL1 receptor antagonist.[3] Several studies using immunohistochemistry, reverse transcription-polymerase chain reaction, or in situ hybridisation have suggested a potential role of TNFα and IL1β in the pathogenesis of IIM.[4–6] It remains to be clarified whether the strategy of treatment of IIM could be modified by the use of these new TNFα or IL1β inhibitors.[7 8]

In this paper, our objective was to evaluate in vitro the effect of the p75 TNFα soluble receptor (sR) (sTNFR, etanercept) alone and in combination with the type II IL1β soluble receptor (sIL1RII) on normal muscle samples or isolated muscle cells stimulated with TNFα or IL1β alone and in combination. We studied these effects on IL6 production, used as a marker of inflammation in RA, as described previously.[9] As both TNFα and IL1β initiate an intracellular signalling cascade mediating the activation of nuclear factor-kappa B (NF-κB), we studied by immunofluorescence its nuclear translocation.[10] Finally, we studied the effect on major histocompatibility complex (MHC) class I expression, a classical pathology marker for the diagnosis of IIM.

Our results indicate that IL6 production, NF-κB translocation, and class I MHC expression decreased when TNFα and IL1 sR were added to these in vitro models. These results suggest that TNFα and IL1β should be targeted for treatment of myositis.

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 sIL1RII[11] 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 mm^3 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% CO_2/95% 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; ELISA, 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

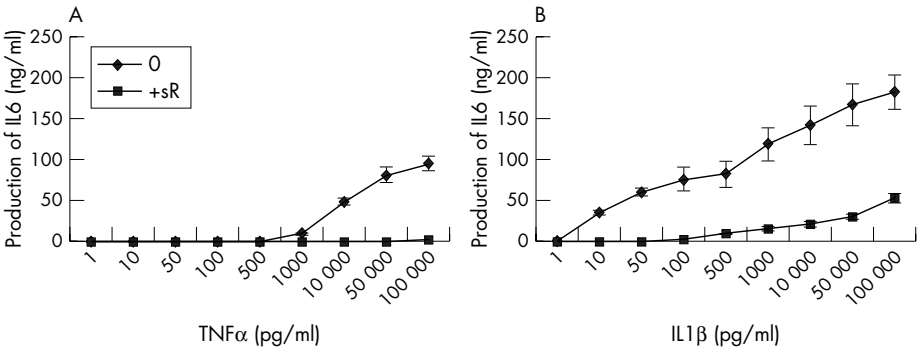

Figure 1 Dose-response curve of IL6 production by myoblasts stimulated with increasing concentrations of TNFα or IL1β in the presence or not of a soluble receptor (sR). Myoblasts were incubated with increasing concentrations (pg/ml) of TNFα (A) or IL1β (B). Myoblasts were also incubated with the same increasing concentrations of cytokine and 1 μg/ml of their respective sR, p75 TNFα sR, and type II IL1β sR. IL6 levels were measured by ELISA in 48 hour supernatants.

To obtain normal skeletal myoblasts, muscle biopsy specimens were cut into fragments of 1–2 mm^3, and 10 explants were placed in 25 cm^2 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 washing buffer at a dilution of 1:100. After washing, dead cells were counterlabelled with propidium iodide (Sigma) and living cells were analysed by fluorescence activated cell sorter (FACS; Becton-Dickinson FACSCalibur). In our experiments, cell cultures contained more than 95% CD56+ cells.

For cytokine studies, myoblasts (10^4 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).[11] For immunofluorescent staining experiments, myoblasts were seeded (10^4 cells/cm^2) on glass coverslips (diameter 12 mm) in DMEM medium 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.

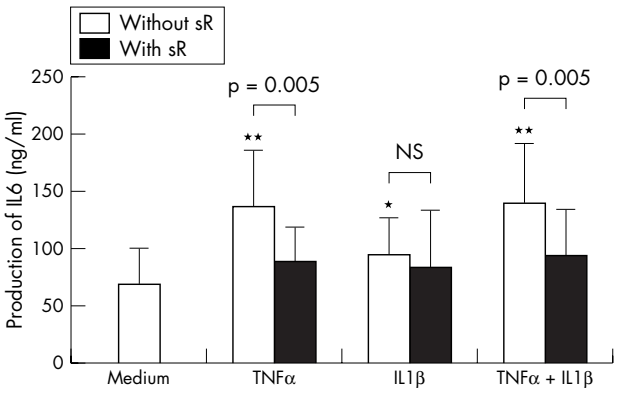

Figure 2 Effect of TNFα and IL1β on IL6 production by normal muscle samples in the presence or not of a soluble receptor (sR). Muscle samples from 10 different subjects were incubated for 7 days in the presence of 10 ng/ml TNFα or 10 pg/ml IL1β alone or in combination, and with or without 1 μg/ml sTNFR or sIL1RII alone or in combination. IL6 levels were measured in supernatants by ELISA.

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 subunit RelA; 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 0.5 μg/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.[10] 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

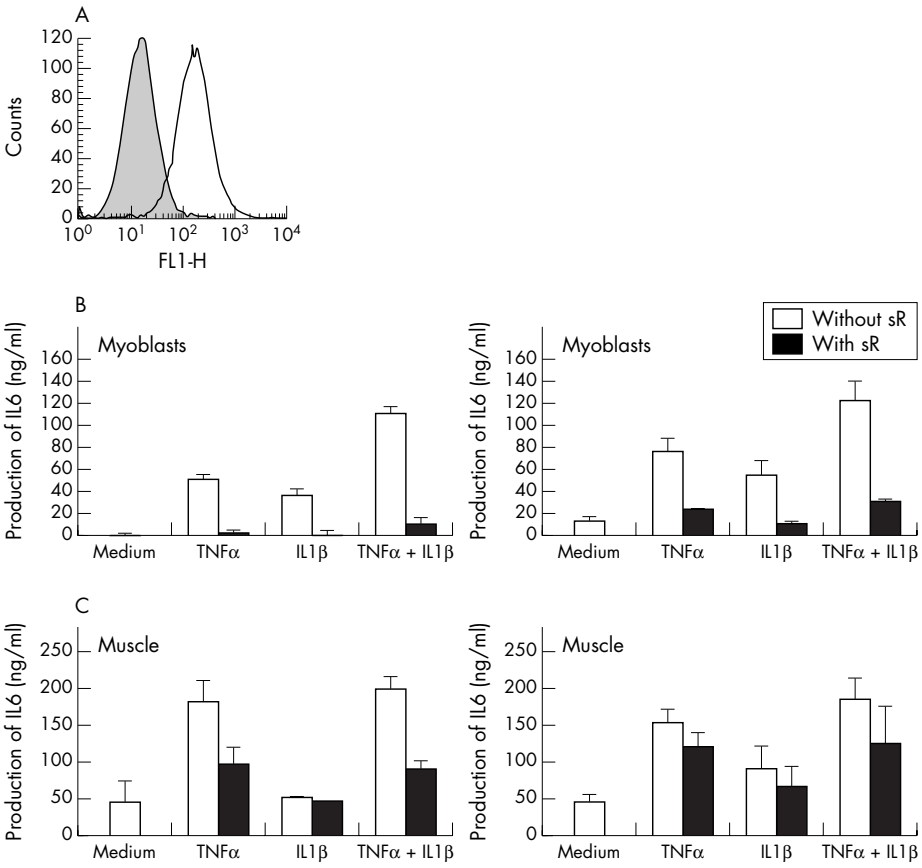

Figure 3 Effect of TNFα and IL1β on IL6 production by individual myoblasts and muscle samples. (A) Myoblasts were selected by two rounds of affinity purification with an anti-CD56 antibody. One example of staining is shown. (B) Myoblasts from two different subjects (B1 and B2) were incubated for 48 hours in the presence of 10 ng/ml TNFα or 10 pg/ml IL1β alone or in combination, and with or without 1 μg/ml sTNFR or sIL1RII alone or in combination. (C) The two muscle samples (C1 and C2) from which myoblasts in (B) were obtained, were incubated for 7 days in the presence of 10 ng/ml TNFα or 10 pg/ml IL1β alone or in combination, with or without 1 μg/ml sTNFR or sIL1RII alone or in combination. IL6 levels were measured in supernatants by ELISA.

concentrations from 1 to 100 000 pg/ml, IL6 production increased in a dose dependent manner (fig 1). Comparison of the effects of TNFα (fig 1A) with those of IL1β (fig 1B) 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 ng/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

Concentration (pg/ml)	TNFα	IL1β
0	−	−
10	−	−
50	+	+
100	+	+
500	++	+
1000	++	++
10000	++	++

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 (+ +).

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 purified 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 TNFα sR and the type II IL1 sR, used as functional inhibitors. Both cytokines alone and in

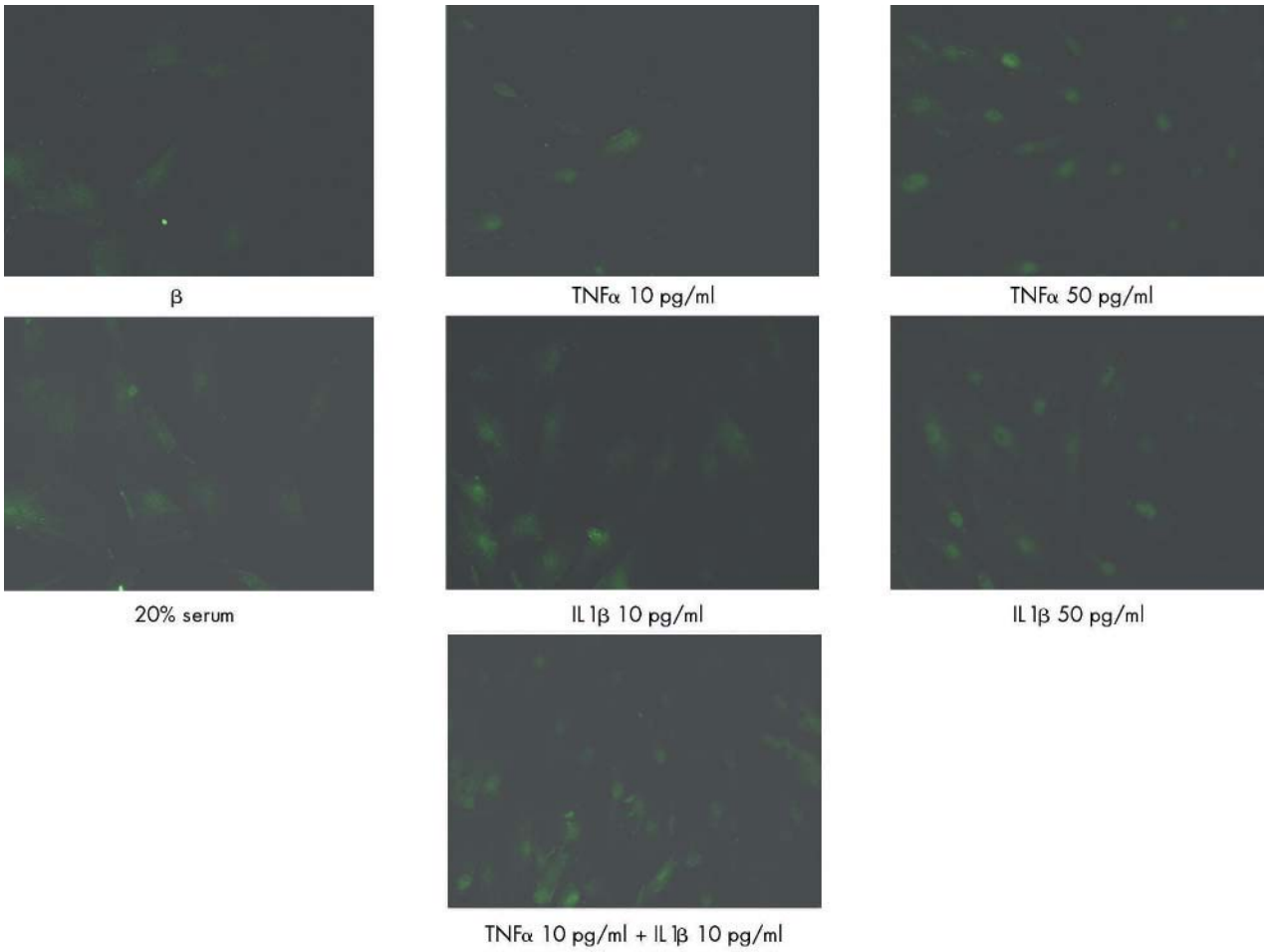

Figure 4 Effects of TNFα or IL1β used alone at low and optimal concentrations and in combination on NF-κB nuclear translocation. Myoblasts (10^4 cells/cm^2) were stimulated for 30 minutes with TNFα or IL1β at the indicated concentrations. Cells were fixed in paraformaldehyde 4% and immunofluorescence staining was performed. Nuclear translocation was evaluated and confirmed by nuclear staining with Hoechst H33258 (data not shown). Incubation with 20% FCS medium was used as a positive control.

combination were incubated for 30 minutes with 1 μg/ml sTNFR or sIL1RII, either alone or in combination, before their addition to muscle samples, as previously described with RA synovium explants.[11] In comparison with the effect of cytokines alone, sR at a concentration of 1 μg/ml decreased IL6 production by muscle samples by −35 (13)% (range −51 to 21%) with 10 ng/ml TNFα ($p = 0.005$), by −17 (29)% (range −63 to 6%) with 10 pg/ml of IL1β (NS), and by −32 (13)% (range −56 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.[10] 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 μg/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

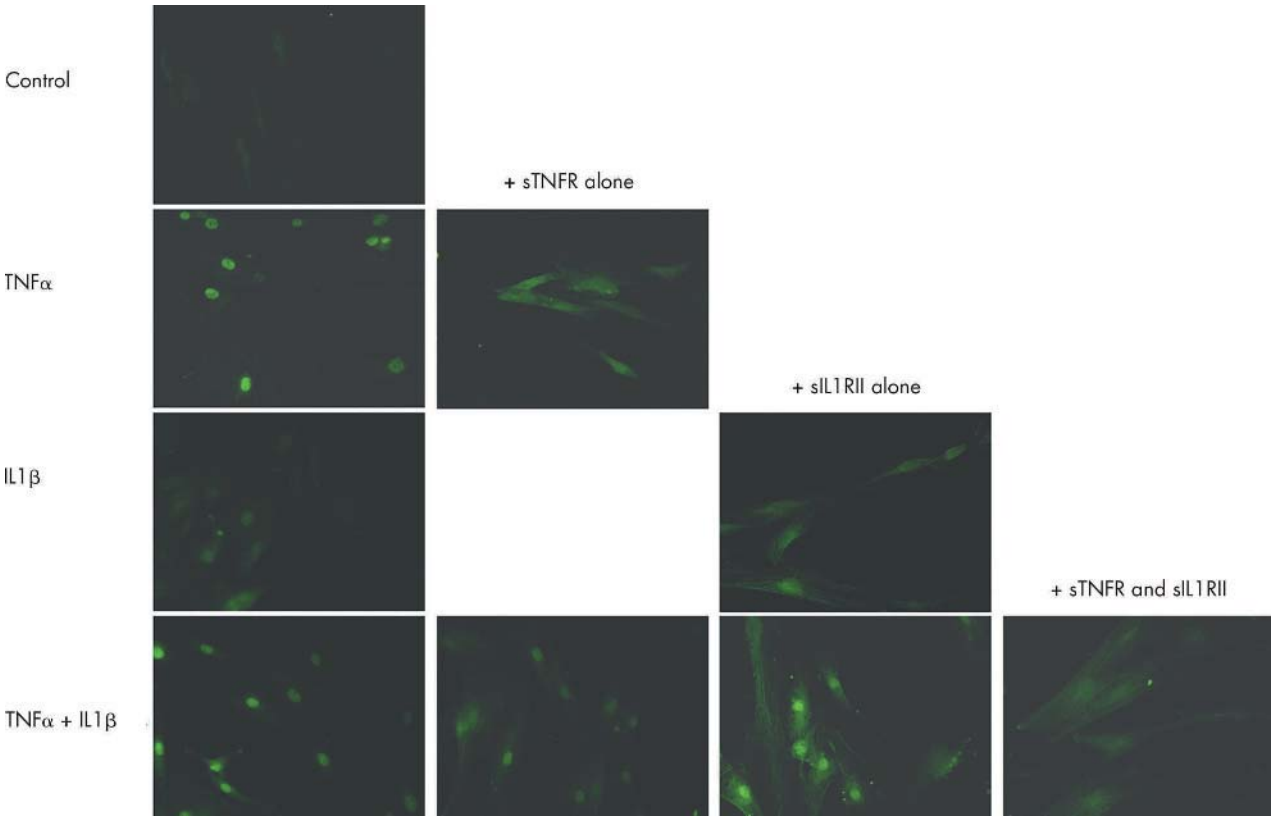

Figure 5 Effect of sTNFR and sIL1RII on NF-κB nuclear translocation induced by TNFα or IL1β. TNFα (500 pg/ml) or IL1β (1000 pg/ml) alone or in combination (left column) were incubated for 30 minutes with 1 μg/ml of sTNFR or sIL1RII, either alone or in combination before addition to myoblasts (right columns). Myoblasts (10^4 cells/cm^2) were stimulated for 30 minutes in presence or not of sR (alone or in combination). Cells were fixed in paraformaldehyde 4% and immunofluorescence staining was performed.

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.[12 13] 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.[14] 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.[15 16] 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 further 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

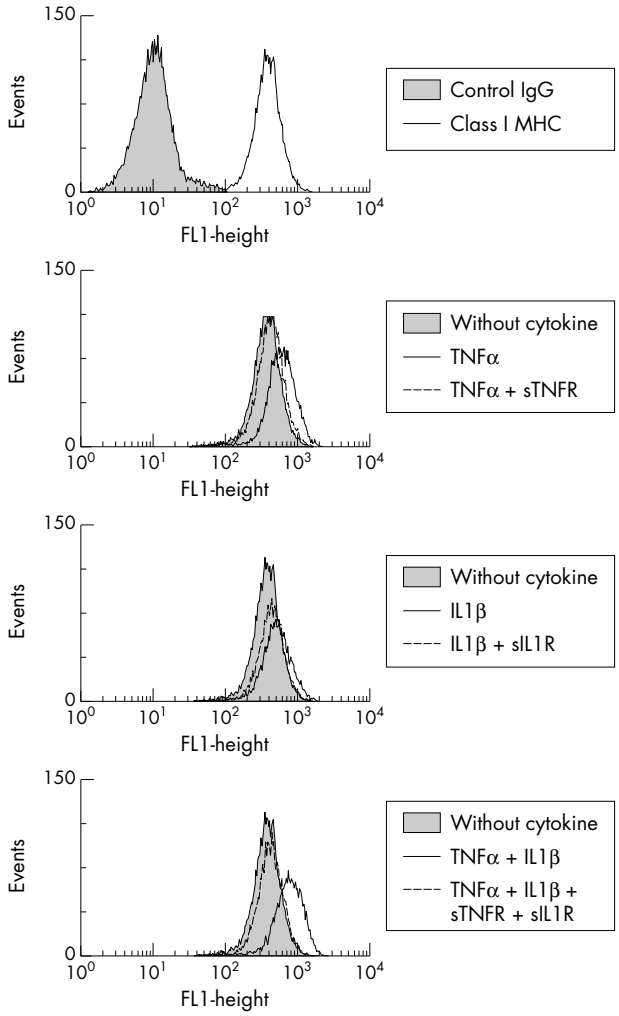

Figure 6 Effect of sTNFR and sIL1RII on class I MHC class I expression induced by TNFα or IL1β. Myoblasts were incubated with 100 pg/ml TNFα or IL1β alone or in combination and 1 μg/ml of their respective sR, p75 TNFα sR and type II IL1β sR. Class I MHC expression was analysed after 48 hours of incubation. Constitutive expression of MHC class I antigen is shown at the top of the figure.

and IL1β to muscle inflammation, as already performed for IL17.[12] With clinical applications in mind, we evaluated the effect of sR on IL6 production, NF-κB translocation, and class I MHC expression. IL6 production was reduced for muscle samples when 1 μg/ml sTNFR was used, as previously described with RA synovium samples and isolated synoviocytes.[11] The combination of both sR did not significantly increase this inhibition, in part owing to the lower contribution of IL1β in our model. Similarly, sTNFR markedly down regulated NF-κB translocation induced by TNFα, whereas again the specific effect of the inhibition with sIL1RII was less potent. However, the translocation of NF-κB induced with the combination of optimal concentrations of both cytokines was completely inhibited by their sR. Finally, sR decreased class I MHC expression induced by cytokines, indicating a possible effect on cell-cell interactions.

CONCLUSION

These experimental results suggest that cytokine inhibitors could be used to control muscle inflammation. It remains to be clarified whether the contribution of these cytokines is similar to that seen in RA. Although isolated cases have been already reported, clinical trials studying the available inhibitors of TNFα and IL1 are now needed to clarify their usefulness for the treatment of IIM.

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