ORIGINAL ARTICLE

# Alternative splicing factor ASF/SF2 is down regulated in inflamed muscle

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**Background:** In our recent studies, alternative splicing has been shown to have a major role in inflammation and autoimmune muscle diseases.
**Aim:** To examine the novel hypothesis that the expression of an essential alternative splicing factor, alternative splicing factor 2 (ASF/SF2), is modulated in muscle inflammation.
**Methods:** ASF/SF2 expression in muscle biopsy samples from eight patients with inflammatory myopathy and six non-myositic controls was determined by using western blot with anti-ASF/SF2 antibodies. To further elucidate the mechanism of reduced ASF/SF2 expression in inflamed muscle, differentiated C2C12 myotubes were stimulated with proinflammatory cytokine tumour necrosis factor α (TNFα), followed by western blot analysis of ASF/SF2 expression.
**Results:** ASF/SF2 expression in the muscle biopsy samples from patients with inflammatory myopathy was found to be lower (mean of relative densitometric units 41.1 (2SD 20.7)) than that of the non-myositic controls (mean of relative densitometric units 76.7 (39.6); $p<0.05$). In addition to this, ASF/SF2 expression was seen to be significantly down regulated (sevenfold) in C2C12 myotubes compared with expression variations in the β-actin control (0.62-fold; mean 1.22 (0.40); $p<0.05$).
**Conclusion:** Collectively, it is shown, for the first time, that alternative splicing factor ASF/SF2 is down regulated in autoimmune inflammatory myositis—potentially via a TNFα-mediated pathway. The development of (1) novel autoantigen isoform microarrays for disease diagnosis and prognosis; (2) novel autoantigen-tolerising treatments for autoimmune diseases; and (3) novel splicing-redirection treatments can be facilitated by the ongoing study of alternative splicing of autoantigen transcripts.

Autoantibodies are characteristic of many autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and idiopathic inflammatory myopathies.[1–3] Some autoantigens are associated with essential RNA splicing and processing functions.[4–8] Alternative splicing is a process that removes introns and alters exons, thereby generating multiple isoforms from a single pre-messenger RNA (mRNA) transcript.[9] Recently, we reported that alternative splicing occurred in all of the 45 examined autoantigen transcripts associated with various autoimmune diseases, including myositis autoantigens polymyositis (PM)/Scl-100, PM/Scl-75 and Ku70, and signal recognition particles.[10] This was significantly higher than the 42% rate of alternative splicing observed among 9554 randomly selected human gene transcripts ($p<0.001$), thus showing that higher rates of alternative splicing provide the structural basis for expression of untolerised autoantigen epitopes, which leads to a breach in immune tolerance. Our novel model of stimulation-responsive splicing[10] illustrates how the alternative splicing of mRNA can lead to expression of protein isoforms that have distinct epitopes generated by the inclusion or deletion of exons before translation. Typically, the intrathymic expression of a protein isoform is associated with tolerance to this isoform. We speculated that, under the influence of environmental factors or inflammation, alternative splicing of the mRNA could be modulated extrathymically, thereby leading to the translation of a non-tolerised isoform that is immunogenic and becomes a tissue-specific target for autoimmunity. Furthermore, non-canonical alternative splicing is a common characteristic of the encoding of potential autoantigens by mRNA. The affected peptide sequence has the structural requirements for presentation of untolerised epitopes by MHC molecules, with concomitant recognition by antibodies and T cell receptors. This model may have applicability in a broad spectrum of autoimmune diseases[10] (fig 1).

Consistent with our model, a recent report[11] showed that, in the sera of patients with myositis, the levels of autoantibodies recognising the longer PM/Scl-75 protein isoform[12] with N-terminal 84 amino acids[13] were four-fold higher than those of the shorter PM/Scl-75 protein isoform without the N-terminal region, which suggests that the immunogenicity of the longer PM/Scl-75 isoform is much higher than that of its shorter isoform.[11] These results indicate that regulation of the immunogenicity of autoantigen isoforms through alternative splicing may affect the autoimmune process in myositis.

Alternative splicing machinery, the spliceosome, consists of five small nuclear ribonucleoprotein particles and 50–100 non-small nuclear ribonucleoprotein particle splicing factors, including the members of the serine/arginine-rich protein family (SR protein).[14] SR proteins, including the best-characterised prototype, alternative splicing factor 2 (ASF/SF2), are a highly conserved family of essential splicing factors that also regulate the alternative splicing of many pre-mRNAs.[15 16] Moreover, expression levels of ASF/SF2 control selection between alternative 5′ splice sites.[17–19] In response to stress conditions such as heat shock, ASF/SF2 is recruited into stress-induced nuclear bodies,[20] whereas its antagonistic factor, heterogeneous nuclear ribonucleoprotein, is not.[21] This suggests that ASF/SF2 cellular distribution and expression are unrelated to the expression of heterogeneous nuclear

**Abbreviations:** ASF/SF2, alternative splicing factor 2; mRNA, messenger RNA; PM, polymyositis; SR protein, serine/arginine-rich protein; TNFα, tumour necrosis factor α

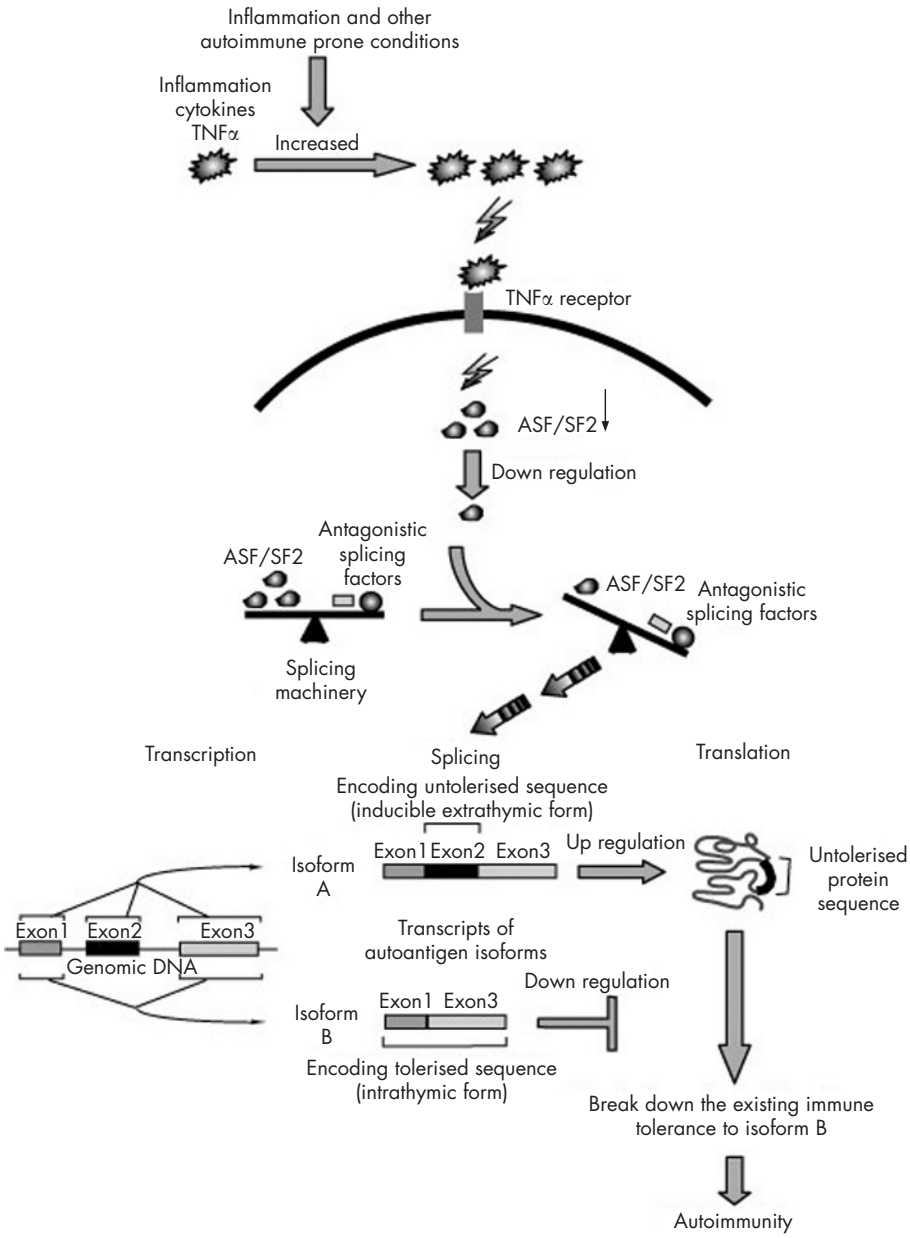



**Figure 1** Schematic representation of our working model of stimulation-responsive splicing. Down regulation of alternative splicing factor 2 (ASF/SF2), potentially via the tumour necrosis factor α (TNFα) pathway, may tip the balance between ASF/SF2 and its antagonistic factors, including heterogeneous nuclear ribonucleoprotein A1, which consequently contributes to (1) the up regulation of inducible extrathymic isoforms of myositis-specific autoantigens; (2) the breach in immune tolerance to intrathymic isoforms of autoantigens; and (3) the triggering of autoimmune responses.

ribonucleoprotein A1, but are modulated in response to various stresses. Nevertheless, it is unknown whether the expression of ASF/SF2 is regulated by inflammation.

Recent reports suggest that inflammation and proinflammatory cytokines,[22] particularly tumour necrosis factor α (TNFα), may accelerate autoimmune pathogenesis in myositis[23]—partly by modulating the splicing machinery. In this study, we tested the novel hypothesis that expression of the best-characterised alternative splicing factor, ASF/SF2, may be modulated in inflammation and autoimmune muscle diseases. Our results show that alternative splicing factor ASF/SF2, presumably responsible for the alternative splicing of autoantigen transcripts, is down regulated in autoimmune inflammatory myositis, potentially via a TNFα-mediated pathway.

## MATERIALS AND METHODS

### Muscle pathology

In accordance with a protocol approved by the Institutional Review Board at Baylor College of Medicine and established guidelines,[24] open muscle biopsy was carried out on different muscles in the lower or upper extremities of patients with suspected inflammatory myopathy. Frozen sections were stained with or made to react with haematoxylin–eosin (fig 2). They were also stained with modified Gomori trichrome, nicotinamide adenine dinucleotide, periodic acid Schiff, oil red O, succinate dehydrogenase, non-specific esterase and adenosine triphosphatase (not shown). Subsequently, data were obtained from the tissue samples, on the basis of the following criteria: range of fibre size variability, fibre rounding, fibre splitting, fibre necrosis,

phagocytosis, endomysial inflammation, perivascular inflammation, invasion of non-necrotic fibres with inflammatory cells, connective tissue proliferation, vacuoles and inclusions. The extent of each pathological change was determined with visual inspection by the same observer, in accordance with recently published criteria on the classification of inflammatory myopathy,[25] and graded as follows: absent or normal (−), slightly increased (+), moderately increased (++) or severely increased (+++). Figure 2B delineates the diagnosis and histopathological features of the eight patients with inflammatory myopathy. The control group consisted of six people who underwent muscle biopsies for different indications and were found to be normal (ie, without muscle inflammation; fig 2C).

### Protein preparation from muscle biopsy samples

The frozen muscle biopsy samples were homogenised with the VirTis Hand-held Homogenizer (VirTis, Gardiner, New York, USA), followed by protein extraction with the Tissue Protein Extraction Reagent (Pierce, Rockford, Illinois, USA).

### Cell culture and proinflammatory cytokine stimulation

Mouse C2C12 skeletal muscle myoblasts, obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), were cultured and differentiated, as described previously.[26] Cytokine stimulation was achieved by adding mouse TNFα, at 6 ng/ml (mTNFα, Roche, Indianapolis, IN), for 2–6 h. Cells were collected at the end of cytokine stimulation and proteins were extracted with the Mammalian Protein Extraction Reagent (Pierce).

### Northern blot

Northern blot hybridisation was performed, as described previously.[27 28] Briefly, total RNAs were isolated from C2C12 myoblasts with RNAzol Reagent (Tel-Test, Friendswood, Texas, USA). After denaturation, 12 μg of each sample was separated with 1% native agarose gel at 8 V/cm. RNAs were blotted and cross linked by ultraviolet radiation to GeneScreen membrane (NEN Life Science Products, Boston, Massachusetts, USA). Hybridisation was performed in ULTRAhyb buffer (Ambion, Austin, Texas, USA), at 42℃ for 16 h, with the [α-$^{32}$P]deoxycytidine triphosphate-labelled probe (GE Healthcare, Piscataway, New Jersey, USA) of the atrogin 1/MAFbx gene,[27] followed by detection by autoradiography (Eastman Kodak, Rochester, New York, USA).

### Western blots

Western blot procedures were performed, as described previously,[28] with anti-ASF/SF2 that specifically reacts to both human ASF/SF2 and mouse ASF/SF2 (0.5 μg/ml; 1:1000; Invitrogen/Zymed, South San Francisco, California, USA) and anti-β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, California, USA). Briefly, after solubilisation with Tissue Protein Extraction Reagent or Mammalian Protein Extraction Reagent, cell homogenates were centrifuged at 15,000×*g* for 15 min at 4℃. The cellular proteins in the supernatant were separated on gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, California, USA), analysed by western blotting with antibodies and disclosed by chemiluminescent substrate (Pierce) after exposure to *x*-ray film (Kodak).

## RESULTS

### Expression of ASF/SF2 was decreased in muscle biopsy samples from patients with inflammatory myopathy

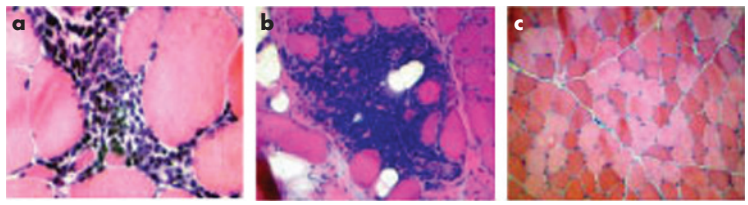
We hypothesised that ASF/SF2 expression may be modulated by inflammation in muscle. To test this theory, we collected biopsy samples from patients with myositis (n = 8; fig 2B) and non-myositic controls (n = 6; fig 2C). Figure 2A-a and A-b show the representative histopathological diagnoses of patient 4, who had PM. Several key features of muscle inflammation were noted: (1) the muscle fibre sizes were varied (fig 2A-a); (2) non-necrotic fibres were surrounded and invaded by mononuclear inflammatory cells, suggesting a primary inflammatory process initiated by the infiltration of inflammatory cells before muscle necrosis (fig 2A-a); (3) mononuclear inflammatory cells were observed endomysially and perivascularly (fig 2A-b). Figure 2B is a summary of the 10 histopathological features and diagnoses of the patients with myositis (n = 8). Diagnosis was based on clinical, serological, electrodiagnostic and pathological criteria (not shown).[29]

Subsequently, we examined ASF/SF2 expression in the muscle biopsy samples from patients with inflammatory myositis, and from non-myositic controls. Expression of ASF/SF2 in muscle biopsy samples was measured by western blot analysis with anti-ASF/SF2 monoclonal antibody, followed by normalisation with expression of the housekeeping gene, β-actin, in the same sample (fig 3A). The mean expression levels of ASF/SF2 decreased from 76.7 relative densitometric units in muscle biopsy samples from non-myositic controls to 41.1 relative densitometric units in muscle biopsy samples from inflammatory myositis patients, indicating a significant difference ($p<0.05$; fig 3B). A previous study of patients with myositis (n = 417) showed that β-actin is not an autoantibody target,[30] and others have reported that β-actin expression remains unchanged in inflamed muscle affected by autoimmune myositis,[31] suggesting that the use of β-actin as a housekeeping gene control is justified. Although variations in ASF/SF2 expression were observed in the samples from non-myositic controls, ASF/SF2 expression in all samples from patients with myositis was lower than the average level of ASF/SF2 in the samples from non-myositic controls. Of note, ASF/SF2 expression in the sample from patient 4 (lane 4, fig 3A) was reduced to barely detectable levels. The histopathological data on the muscle tissue of patient 4 (fig 2A-a,A-b) suggested severe PM. In addition, with the exception of lane 4 (fig 3A,B), the data did not show any significant differences in ASF/SF2 expression between PM or dermatomyositis samples (lanes 1, 6, 7 and 8 in figs 3A,B and 2B) and inclusion body myositis samples (lanes 2, 3 and 5 in figs 3A,B and 2B). Furthermore, ASF/SF2 expression in inflamed muscle was not associated with differences in age or sex (figs 2B,C and 3A,B). Collectively, these results showed a decreased expression of ASF/SF2 in the muscles of all patients with inflammatory myositis (n = 8; mean of relative densitometric units 41.1 (2SD 20.7)) in comparison with the non-myositic controls (n = 6; mean of relative densitometric units 76.7 (2SD 39.6), $p<0.05$).

### Expression of ASF/SF2 in cultured C2C12 myotubes was decreased after stimulation with proinflammatory cytokine TNFα

The increased expression of proinflammatory cytokines, particularly TNFα, was observed in the muscle biopsy samples from patients with autoimmune myopathies.[23] We reasoned that reduced ASF/SF2 expression in muscle biopsy samples from patients with myositis may have resulted from increased expression of TNFα in inflamed muscles. To test this possibility, we used TNFα-stimulated C2C12 myotubes as an in vitro model for muscle inflammation.[23 32] Expression of ASF/SF2 was significantly decreased (sevenfold) 6 h after stimulation of C2C12 myotubes with TNFα. By contrast, after stimulation with TNFα, variation in the ratio of relative densitometric units of β-actin was limited (0.62-fold; mean 1.22 (0.40); fig 4A and B). Expression of atrogin-1/MAFbx, a previously reported positive control for TNFα stimulation in C2C12 myotubes and a muscle-specific ubiquitin-ligase

**A**




**B**

**Diagnosis and histopathological features of patients with inflammatory myopathy**

| Patient | Age (years) | Sex | Diagnosis | Fibre size variability | Rounded fibres | Necrotic fibres | Phegocytic fibres | Endomysial inflammation | Perivascular inflammation | Invasion of non-necrotic fibres | Red-rimmed vacuoles | Eosinophilic cytoplasmic inclusions | Perifascicular atrophy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 83 | F | PM | +++ | +++ | +++ | +++ | ++ | ++ | ++ | – | – | – |
| 2 | 53 | M | IBM | +++ | +++ | ++ | + | ++ | + | + | ++ | ++ | – |
| 3 | 75 | M | IBM | +++ | ++ | ++ | ++ | ++ | + | + | ++ | ++ | – |
| 4 | 71 | F | PM | +++ | +++ | +++ | +++ | +++ | + | + | – | – | – |
| 5 | 77 | F | IBM | + | + | + | + | ++ | ++ | ++ | + | + | – |
| 6 | 64 | F | DM | +++ | +++ | +++ | +++ | +++ | +++ | ++ | – | – | ++ |
| 7 | 70 | F | PM | + | + | + | + | ++ | ++ | ++ | – | – | – |
| 8 | 63 | F | PM | +++ | +++ | +++ | +++ | +++ | + | + | – | – | – |

**C**

**Diagnosis and histopathological features of controls**

| Control | Age (years) | Sex | Diagnosis | Muscle inflammation (pathology) |
|---|---|---|---|---|
| 1 | 42 | F | Myalgia | No |
| 2 | 67 | M | Weakness | No |
| 3 | 87 | M | Weakness | No |
| 4 | 68 | M | Weakness | No |
| 5 | 56 | M | Myalgia | No |
| 6 | 52 | F | Myalgia | No |

**Figure 2** Histopathological diagnosis of inflammatory myopathy. (A) Representative histopathology of inflamed myopathy and normal muscle histology: (a) Muscle tissue in the left biceps was stained with haematoxylin–eosin and magnified to ×400 (patient 4); the variation in muscle fibre size suggests a myopathic process. Non-necrotic fibres are surrounded and invaded by mononuclear inflammatory cells, suggesting a primary inflammatory process. (b) Muscle tissue in the left biceps was stained with haematoxylin–eosin and magnified to ×100 (patient 4); the infiltration of mononuclear endomysial and perivascular inflammatory cells is depicted. (c) Muscle tissue in the left biceps from a non-myositic control was stained with haematoxylin–eosin and magnified to ×100; this is a normal muscle tissue, with no significant variation in the size of muscle fibre and no inflammatory cells. (B) Diagnosis and histopathological features of patients with inflammatory myopathy. The diagnosis and 10 histopathological features of patients with myopathy (n = 8) are delineated. PM, IBM and DM signify polymyositis, inclusion body myositis and dermatomyositis, respectively. (C) Diagnosis of non-myositic controls.

required for muscle atrophy in C2C12 myotubes, was measured by northern blot after stimulation with the same concentration of TNFα for the same duration (fig 4C).[27] The expression of atrogin-1/MAFbx was significantly up regulated (fourfold) by TNFα stimulation for 2 h. Up regulation of atrogin-1/MAFbx did not result from the potential variation of RNA sample loading, as the variation of RNA loading control 28S was limited (the mean 1.08 (2SD 0.11)) in the presence of TNFα stimulation. It is noteworthy that the kinetics of atrogin-1/MAFbx mRNA stimulated by TNFα (fig 4C) may not be temporally correlated with ASF/SF2 down regulation (fig 4A,B); this may indicate temporal variation in gene expression in response to TNFα stimulation. Cumulatively, these results suggest that proinflammatory cytokine TNFα induces the down regulation of ASF/SF2 expression in muscle cells, which may be a novel mechanism underlying the ASF/SF2 down regulation detected in inflamed muscle biopsy samples from patients with myositis.

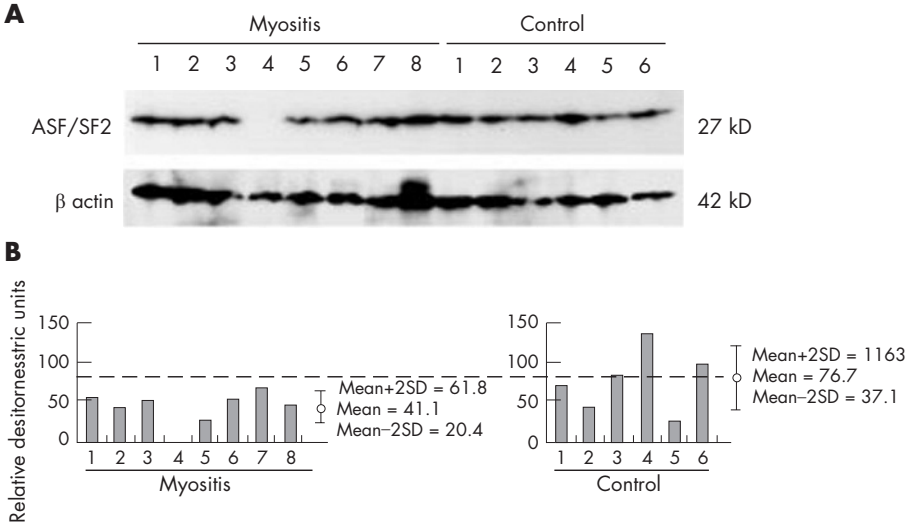



**Figure 3** Expression of alternative splicing factor 2 (ASF/SF2) in muscle biopsy samples from patients with myositis and from non-myositic controls. (A) Expression of ASF/SF2 in each muscle biopsy sample was detected with western blot, with anti-ASF/SF2 antibodies. The expression of β-actin in the samples was also measured by western blot, with anti-β-actin antibodies. (B) Expression of ASF/SF2 in each sample was calculated in relative densitometric units, after normalisation with β-actin expression signal. Mean (2SD) of the relative densitometric units was calculated for each group. The dashed line indicates the mean value of the non-myositic controls.

## DISCUSSION

Recent studies providing significant insights into pathogenic mechanisms that potentially contribute to autoimmunity state: (1) up regulation of MHC class I by skeletal muscle may lead to self-sustaining autoimmune myositis;[31] (2) peptide fragments generated by granzyme B may be a

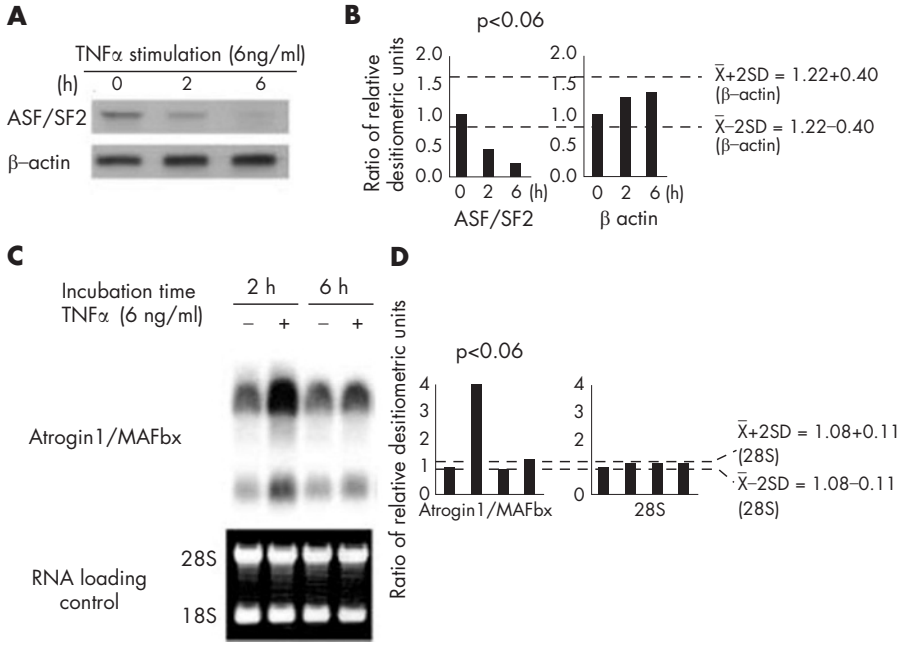



**Figure 4** Time-course measurement of the expression of alternative splicing factor 2 (ASF/SF2) in C2C12 myotubes after stimulation with tumour necrosis factor α (TNFα). (A) Expression of ASF/SF2 in C2C12 myotubes after stimulation with TNFα for 0, 2 and 6 h was measured by western blot, with anti-ASF/SF2 antibodies. The expression of β-actin in the samples was also measured by western blot, with anti-β-actin antibodies. (B) After normalisation with β-actin expression signal, expression changes of ASF/SF2 induced by TNFα stimulation, relative to those of the non-stimulation control (0 h), were calculated for each sample as the ratio of relative densitometric units (the relative densitometric units in each sample/the relative densitometric units at 0 h). Similarly, the ratio of densitometric units of β-actin was calculated for each sample, relative to the non-stimulation control (0 h). The mean (2SD) of the ratio of β-actin densitometric units was used as the 95% confidence interval for non-specific gene expression variation during TNFα stimulation. (C) Expression of atrogin-1/MAFbx in C2C12 myotubes, after TNFα stimulation for 0, 2 and 6 h (as a positive control for TNFα stimulation), by northern blot hybridisation with an atrogin-1/MAFbx cDNA probe (the upper panel). The lower panel shows the RNA loading control for northern blot hybridisation. (D) After normalisation with 28S RNA expression signal, changes in the expression of atrogin-1/MAFbx were calculated in each sample, relative to those of the non-stimulation control, as the ratio of relative densitometric units (the relative densitometric units in each sample/the relative densitometric units in the non-stimulation control (at 2 h)). Similarly, the ratio of densitometric units of 28S RNA was calculated for each sample, relative to the non-stimulation control, at 2 h. The mean (2SD) of the ratio of 28S RNA densitometric units was used as the 95% confidence interval for non-specific gene expression variation during TNFα stimulation.

property of autoantigens;[33] and (3) post-translational modifications of proteins may contribute to the immunogenicity of autoantigens.[34] Despite some progress,[35 36] we still do not know how the highly dynamic and complex human proteome (protein repertoire) contributes to the generation of autoantigens.

Alternative splicing is a major mechanism whereby a small number of human genes in the human genome encode the large, complex human proteome ($9\times10^4$–$1\times10^6$ proteins).[37] Most of the alternative splicing (70–88%)[38] affects the coding region of mRNA, often resulting in the expression of additional exons that, on average, encode 16–100 amino acids.[9] Immune responses to autoantigen epitopes are highly specific[39] to epitopes of a circumscribed length; in particular, the length of antigenic peptides needed for antibody binding, MHC class I binding and MHC class II binding is 8–15 amino acids. Therefore, additional alternatively spliced exons that are capable of encoding 16–100 amino acids[9] are long enough to produce novel antigenic epitopes. Our recently proposed ''stimulation-responsive splicing model'' for the increased immunogenicity of autoantigens suggests that increased non-canonical splicing of autoantigen transcripts provides the structural basis for inducible extrathymic expression of untolerised epitopes, thereby creating the potential to breach existing immune tolerance to intrathymic isoforms.[10 40–42] Complementing our previous bioinformatic studies,[10] we and others have demonstrated the four untolerised exon sequences spliced into the self-tumour antigen isoform CML66L,[43] the longer isoform of the autoantigen proteolipid protein associated with autoimmune nervous system disease,[44] the autoimmune myositis-associated autoantigen PM/Scl-75 longer isoform[11–13] and the autoimmune diabetes-associated autoantigen, IA-2.[45] Autoantigen isoforms with untolerised sequences are more immunogenic than other isoforms of the same autoantigens.

The inflammatory myopathies, commonly described as idiopathic, are the largest group of acquired and potentially treatable myopathies.[25] We observed that ASF/SF2 expression levels in all muscle biopsy samples from patients with myositis were lower than the average expression level of ASF/SF2 in muscle biopsy samples from non-myositic donors. Moreover, our results showed that proinflammatory cytokine TNFα down regulated ASF/SF2 expression in C2C12 myotubes. As shown in our working model (fig 1), cumulatively, these results show, for the first time, that alternative splicing factor ASF/SF2, which is presumably responsible for the alternative splicing of autoantigen transcripts, is down regulated in autoimmune inflammatory myositis, potentially via a TNFα-mediated pathway. These results further consolidate our stimulation-responsive splicing model from the splicing factor perspective. As ASF/SF2 can function as either a positive or negative regulator for the inclusion of exons depending on the context,[46 47] future work is needed to determine how it affects the expression of immunogenic isoforms of myositis autoantigens (eg, the longer isoform *v* the shorter isoform of PM/Scl-75). The ongoing study on the alternative splicing of autoantigen transcripts and splicing machinery will lead to the development of: (1) novel autoantigen isoform microarrays for disease diagnosis and prognosis;[48] (2) novel autoantigen-tolerising treatments for autoimmune diseases;[49] and (3) novel splicing-redirection treatments.[50]

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### Take-home messages

- These findings demonstrate, for the first time, that alternative splicing factor ASF/SF2 is significantly downregulated in muscle biopsy samples of autoimmune inflammatory myositis.
- Proinflammatory cytokine tumor necrosis factor-α (TNF-α) significantly downregulates ASF/SF2 expression in C2C12 cultured myotubes, suggesting that expression of ASF/SF2 in inflamed muscle is decreased, potentially, via a TNF-α-mediated pathway.
- ASF/SF2 may be one of the important factors in regulation of alternative splicing of autoantigen transcripts.
- The continued study of ASF/SF2 and alternative splicing of autoantigen transcripts will facilitate the development of: 1) novel autoantigen isoform microarrays for disease diagnosis and prognosis; 2) novel autoantigen-tolerizing therapies for autoimmune diseases; and 3) novel splicing-redirection therapies.

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# ECHO

## More endoscopists improve outcome for upper GI cancer

Please visit the *Quality and Safety in Health Care* website [www.qshc.com] for a link to the full text of this article.

More endoscopists may be the answer to better outcomes for upper gastrointestinal (GI) cancer, as recent improvement seems to owe more to the introduction of nurse endoscopists than the UK government's two week wait scheme for a specialist consultation, according to doctors in one cancer unit.

True enough, the odds of curative resection increased significantly (odds ratio 1.48) in their unit in the two years after the scheme was introduced compared with the two years before, and curative resections for early (stage 1 and 2) cancers rose from 47 to 58. But only two patients (5%) of 38 diagnosed with the cancer out of 623 referred under the scheme had early stage disease compared with 56 (27%) outside it. Furthermore, just over a third of patients with early stage cancer had symptoms consistent with the referral criteria in the scheme, but only two of them were referred under it.

When the scheme was implemented at Norfolk and Norwich University Hospital, in September 2000, it coincided with appointment of two full time nurse endoscopists, which reduced routine waiting times for endoscopy—and probably accounted for the improvement.

Under the scheme guidelines for urgent referrals for upper GI cancer were issued to general practitioners to ensure timely specialist evaluation. Detecting the cancer early is key to curative treatment, but symptoms can be unreliable. This may be why reducing times for routine endoscopy may be the best option.

The UK government has been under pressure to improve its poor record on upper GI cancer outcome in western Europe.

▲ Spahos T, *et al. Postgraduate Medical Journal* 2005;**81**:723–730.