Blockade of TNF \textit{in vivo} using cV1q antibody reduces contractile dysfunction of skeletal muscle in response to eccentric exercise in dystrophic mdx and normal mice

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Received 14 May 2010; received in revised form 22 September 2010; accepted 23 September 2010

Abstract

This study evaluated the contribution of the pro-inflammatory cytokine, tumour necrosis factor (TNF) to the severity of exercise-induced muscle damage and subsequent myofibre necrosis in mdx mice. Adult mdx and non-dystrophic C57 mice were treated with the mouse-specific TNF antibody cV1q before undergoing a damaging eccentric contraction protocol performed \textit{in vivo} on a custom built mouse dynamometer. Muscle damage was quantified by (i) contractile dysfunction (initial torque deficit) immediately after the protocol, (ii) subsequent myofibre necrosis 48 h later. Blockade of TNF using cV1q significantly reduced contractile dysfunction in mdx and C57 mice compared with mice injected with the negative control antibody (cVaM) and un-treated mice. Furthermore, cV1q treatment significantly reduced myofibre necrosis in mdx mice. This \textit{in vivo} evidence that cV1q reduces the TNF-mediated adverse response to exercise-induced muscle damage supports the use of targeted anti-TNF treatments to reduce the severity of the functional deficit and dystrophy in DMD.

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Keywords: Inflammation; Muscle damage; Isokinetic dynamometer; Duchenne muscular dystrophy; mdx mouse

1. Introduction

Dystrophin is part of the dystrophin–glycoprotein complex that links intra-cellular F-actin to the extra-cellular matrix [1] and provides mechanical protection for the sarcolemma during muscular contraction [2,3]. In patients with Duchenne muscular dystrophy (DMD) and in the mdx mouse and golden retriever (GRMD) dog models of DMD, the absence of dystrophin disrupts normal force transmission and reduces the stability of the sarcolemma [4]. As a result, dystrophic skeletal muscles are more susceptible to exercise-induced muscle damage (EIMD), particularly during lengthening (eccentric) muscle actions [5,6]. The high susceptibility to muscle damage and repeated cycles of myofibre necrosis, especially throughout the growth phase [7], ultimately results in the failure of myofibres to regenerate and the replacement of myofibres with fat or fibrous connective tissue. It is likely that an elevated inflammatory response contributes to the progressive loss of skeletal muscle mass and function seen in the dystrophic condition [8]. While the pro-inflammatory cytokine tumour necrosis factor (TNF) has been associated with chronic muscle wasting conditions such as cachexia [9] and sarcopenia [10], it has also been implicated in the acute inflammatory response that exacerbates muscle damage and contractile dysfunction in mdx mice [11–14].

EIMD is characterized by an initial decrease in the force producing capacity of the muscle (contractile dysfunction)
Immediately after the exercise and a subsequent secondary decline in force producing capacity that develops over the following days. Although the initial force deficit has been attributed to the impairment of excitation–contraction coupling [15] and/or disruption of the contractile filaments [16], this muscle damage also coincides with an inflammatory response including the rapid release of TNF from the damaged muscle and resident mast cells [17,18]. TNF attracts neutrophils and further inflammatory cells (that also produce TNF) to the injured site thereby increasing the inflammatory cascade. When myofibre injury occurs, inflammation and associated cytokines are essential for coordinating the removal of damaged tissue and for formation of new skeletal muscle [19]. However, in situ experiments examining EIMD in mice have shown that the accumulation of neutrophils and macrophages after injury contributes to further muscle damage [20,21] and, along with ex vivo studies on cultured myotubes [22], it has been suggested that this effect may be mediated by increased production of reactive oxygen species (ROS). In the mdx mouse, an excessive state of inflammation can exacerbate myofibre necrosis [19,23]. In normal (non-dystrophic) muscle, experimentally elevated TNF (in vivo for 7 days) results in the accumulation of neutrophils and macrophages after injury contributes to further muscle damage [20,21] and, along with ex vivo studies on cultured myotubes [22], it has been suggested that this effect may be mediated by increased production of reactive oxygen species (ROS). In the mdx mouse, an excessive state of inflammation can exacerbate myofibre necrosis [19,23]. In normal (non-dystrophic) muscle, experimentally elevated TNF (in vivo for 7 days) results in the accumulation of neutrophils and macrophages after injury contributes to further muscle damage [20,21] and, along with ex vivo studies on cultured myotubes [22], it has been suggested that this effect may be mediated by increased production of reactive oxygen species (ROS). In the mdx mouse, an excessive state of inflammation can exacerbate myofibre necrosis [19,23].

2. Materials and methods

2.1. Animals

All experiments were performed on 10–13 week old adult male dystrophic C57BL/10ScSnmdx/mdx and control C57Bl/10ScSn mice, hereafter referred to as mdx and C57, respectively. Adult mdx mice were used in these experiments since the level of myofibre necrosis and regeneration has stabilised to a relatively low level at this age [35,36]. The mice, obtained from a specific pathogen free colony at the Animal Resource Centre, Murdoch, Western Australia, were housed in cages, supplied with food and water without restriction, and maintained in a 12 h light/dark air-conditioned (20–25 °C) environment. All animal procedures were approved by the Animal Ethics and Experimentation Committee of the University of Western Australia in accordance with the guidelines of the National Health and Medical Research Council of Australia.

2.2. Experimental outline

Experiments were performed using a custom built mouse dynamometer [34] to induce controlled and consistent eccentric EIMD in vivo and to assess the contractile parameters of muscles from mdx and C57 mice. Initial contractile dysfunction was quantified by comparing the isometric torque production before and after the EIMD protocol (initial damage), while histological techniques were used to quantify subsequent (secondary) myofibre necrosis. The effect of cV1q mediated blockade of TNF on initial and secondary muscle damage was compared with un-treated and sham injected (cVaM) control mice.

2.3. Experimental procedures

Mice were anaesthetised by inhalation of a gaseous mixture of isoflurane (isoflurane, 0.4 L/min N₂O, 0.4 L/min O₂) which was maintained for the duration of the experiment (approximately 1 h) via a flow-through facemask over the mouse’s head. Anaesthetised mice were connected to the dynamometer for the measurement of contractile function and to induce EIMD. The dynamometer protocol performed in this experiment is described in detail by Hamer et al. [37] and explained briefly below.

2.4. Dynamometer protocol for eccentric exercise-induced muscle damage

Before performing the EIMD protocol, optimal stimulation parameters were determined based on the torque–volt and torque–frequency relationships for isometric contractions (200 ms train) recorded at a neutral ankle angle (i.e. the foot positioned perpendicular to the tibia). The optimal stimulation voltage from all experiments ranged from 1 to 10 V and the optimal stimulation frequency ranged from 125 to 300 Hz. There were no significant differences in these
parameters between mouse strain or treatment group, nor did the EIMD protocol significantly affect these parameters. These stimulation parameters were then used when recording the torque–angle relationship and when performing the EIMD protocol consisting of 20 eccentric contractions. After a 10 min recovery period following EIMD, the initial procedures of torque–volt, torque–frequency and torque–angle were repeated for assessment of initial contractile dysfunction. Specific details of these procedures are as follows.

2.5. Torque–angle relationship

A torque–angle relationship for the anterior crural muscles was determined from isometric contractions elicited at 5° increments between 15° dorsiflexion and 55° plantar flexion. The foot was passively rotated to 15° dorsiflexion and a 200 ms train of pulses delivered to the common peroneal nerve to elicit an isometric contraction. Immediately after stimulation had ceased the ankle was rotated 5° towards plantar flexion and rested at this angle for 30 s prior to recording of isometric torque at the new joint angle. The procedure was repeated at 5° increments up to 55° plantar flexion, with the isometric torque being recorded at each position.

Each isometric contraction was analysed for the mean peak torque during the final 80 ms of activation. The peak torque was plotted against joint angle and a Gaussian fit was applied using Curve Expert v1.36. The Gaussian model fitted was of the form:

\[ y = a \times \exp\left(\frac{-(x-b)^2}{2c^2}\right) \]

The coefficients of this fit are: \( a \) = the amplitude of the Gaussian fitted curve, equivalent to the peak joint torque; \( b \) = the angle at which peak torque was generated and \( c \) is the width at half the peak.

2.6. Exercise-induced muscle damage

After the torque–angle relationship was established, an exercise protocol of 20 eccentric contractions was performed between ankle angles of 15° and 55° plantar flexion with a 30 s rest interval between each trial. The anterior crural muscles were stimulated initially at an ankle angle of 15° plantar flexion to produce an isometric contraction (Fig. 1A). After 100 ms of stimulation the ankle was rapidly rotated to 55° plantar flexion (Fig. 1B) at an angular velocity of 1000 °s\(^{-1}\), thus producing an eccentric contraction in the anterior crural muscles. Immediately following the eccentric contraction, the position of the foot was passively returned to the starting position at 10 °s\(^{-1}\) under servomotor control. After a 10 min rest period, the torque–volt, torque–frequency and torque–angle procedures were repeated to determine the decrease in the peak joint torque resulting from the EIMD protocol (Fig. 2). At completion of the final procedure the incision was sutured and the mouse was allowed to recover. All mice recovered quickly from anaesthesia, were fully mobile within minutes of regaining consciousness and displayed no signs of abnormal limb movement.

2.7. Evans Blue Dye injections

Intra-peritoneal (IP) injections of a 1% sterile solution (w/v) of Evans Blue Dye (EBD) in phosphate–buffered saline (PBS, pH 7.5) at a volume of 1% of body weight were administered 24 h before the EIMD protocol. Prior to injection, the EBD solution was sterilised by passage through a Millipore® GP 0.22 mm filter and stored at 4 °C. After the injection, animals were returned to their cage and allowed food and water ad libitum.

2.8. Histological assessment of myofibre necrosis and sarcolemmal damage

Mice were sacrificed by cervical dislocation while under terminal anaesthesia at 48 h after the EIMD protocol. Body weight was recorded prior to sacrifice. The TA muscles of the test limb (subjected to EIMD) and the non-tested (contralateral) limb were excised, sliced transversely through the mid-belly of the muscle, mounted vertically in

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**Fig. 1.** Mouse dynamometer apparatus with the ankle of the right hind limb at (A) 15° plantar flexion and (B) 55° plantar flexion during the EIMD protocol. The knee was clamped and the foot attached to a foot plate with the ankle aligned with the axis of the torque transducer. The anterior crural muscles were stimulated to contract via hook electrodes connected to the common peroneal nerve via a small incision in the skin and muscle fascia near the head of the fibular. During the EIMD protocol the anterior crural muscles were stimulated to contract at an initial ankle angle of 15° PF and rapidly rotated at 1000°s\(^{-1}\) to 55° PF during the contraction.
tragacanth gum on cork blocks and snap frozen in isopentane, cooled by liquid nitrogen, and stored at \(-80^\circ C\). Alternate frozen sections (8 \(\mu m\)) were cut for EBD and Haematoxylin and Eosin (H&E) slides on a Leica (CM3050) cryostat at \(-21^\circ C\).

The H&E stained muscle sections were observed with bright-field light microscopy, non-overlapping tiled images of transverse muscle sections were taken at 10\(\times\) magnification to produce an overall image of the entire muscle cross-section. Images were acquired using a Leica DM RBE microscope, Stage Pro movement software and a Q Imaging Micropublisher 3.3 RTV digital camera. Muscle morphology was analysed from the tiled images using Image Pro Plus 6.2 software. Areas of myofibre necrosis were identified by the presence of fragmented sarcoplasm and the infiltration of inflammatory cells. Myofibre necrosis was calculated as a percentage of the whole muscle cross-sectional area.

The unstained EBD sections were viewed by red auto-fluorescence microscopy (Fluoro filter N.2.1: Green) at 10\(\times\) magnification and tiled the same as for H&E. Sections with less than 20 EBD positive fibres (less than 1% of total area) were not tiled. EBD positive fibres were measured using Image Pro Plus 6.2 and expressed as a percentage of total muscle cross-sectional area.

2.9. Use of cV1q antibody to block TNF

This study investigated the effectiveness of blocking TNF using the mouse specific anti-TNF antibody cV1q (Centocor USA), which is a rat/mouse chimeric, specific for murine TNF [11]. A negative control solution of cVaM was also administered, which is an isotope matched control antibody for cV1q. These reagents were generously provided to us by Centocor (USA). In the initial experiments (cV1q-1w) both mdx and C57 mice received two intra-peritoneal injections of either cV1q or cVaM made up in PBS at 1 week and 1 h prior to the EIMD at a concentration of 20 \(\mu g/g\) body weight. In additional experiments (cV1q-4 h), mdx mice received a single injection of cV1q (20 \(\mu g/g\) body weight) at 4 h prior to EIMD (dosages based on [11]).

2.10. Data analysis

All data are presented as mean ± SE, unless stated otherwise. Unpaired student \(t\)-tests were performed for direct comparison of untreated C57 and mdx mice. Multiple comparisons between all groups and between tested and un-tested (contralateral) limb muscles were made using univariate analysis of variance (ANOVA). A least significant difference (LSD) post-hoc \(t\)-test was used to identify differences between groups. Significance was set at \(P \leq 0.05\) for all groups.

As a quantitative analysis of the benefit of cV1q treatment, a ‘recovery score’ for the correction of the mdx defect was calculated as \(((cV1q\text{-untreated mdx})/(C57\text{-untreated mdx})) \times 100\) where a score of 100% indicates that the parameter in treated mdx mice was equal to that of control C57 mice, and 0% indicates that no gain was
obtained compared with untreated mdx mice [38]. Recovery scores were generated for the initial torque deficit and subsequent myofibre necrosis for mdx mice from the cV1q-1w and cV1q-4h protocols.

3. Results

The number of animals in each group, along with mean age and body weight, are presented in Table 1. When the body weights of mice in each treatment were grouped by mouse strain, the mean body weight for mdx mice (29.3 ± 0.53 g) was significantly higher than for C57 mice (24.9 ± 0.38 g, P < 0.05). Due to the significant difference in body weights between mdx and C57 mice, peak isometric torque was normalised to body weight and presented in units Nmm/kg.

3.1. Muscle contractile properties of untreated mdx and C57 mice

3.1.1. Peak torque production and optimal joint angle

Peak isometric torque normalised to body weight (Fig. 3A) was significantly lower in mdx mice compared to C57 mice (P = 0.029). The muscles of mdx mice were ~25% weaker than the non-dystrophic C57 mice, which is consistent with the significantly lower specific force in TA recorded by Dellorusso et al. [6], from in situ experiments in mdx and C57 mice. Furthermore, the optimal angle at which the peak torque was recorded was significantly greater for mdx mice compared to C57 mice (Fig. 3B). This indicates a shift in maximal force production to longer muscle lengths which may reflect an increase in series compliance within the muscle [39] due to the underlying dystrophopathy in mdx mice.

3.1.2. Susceptibility to EIMD

The effects of the EIMD protocol on torque–angle relationships for C57 and mdx mice are presented in Fig. 2A and C, respectively. Initial contractile dysfunction was quantified physiologically as the percentage decrease in peak isometric torque after the EIMD protocol (Fig. 3C). Both mdx and C57 mice experienced a marked decrease in mean peak joint torque after the 20 eccentric contractions. However, the deficit in mean peak joint torque resulting from the EIMD protocol was twofold greater in mdx compared to C57 mice (P = 0.02). The significantly greater torque deficit in mdx compared to C57 mice confirms that dystrophic muscles are more susceptible to EIMD resulting from controlled eccentric contractions, as performed here in vivo with the mouse dynamometer. This is consistent with previous EIMD studies in mdx mice using isolated EDL muscles [40] and also for in vivo dynamometer studies in GRMD dogs [5].

3.1.3. Histological assessment of myofibre necrosis and sarcolemmal damage

Myofibre necrosis was not evident in any C57 muscles, in accordance with previous observations in our laboratory following voluntary wheel running or horizontal treadmill running (Radley-Crabb, unpublished data). Therefore, to obtain a reliable measure of the extent of myofibre necrosis resulting from EIMD in mdx mice, comparisons were made between the TA muscles of the test limb and the un-tested (contralateral control). The mean area of necrosis increased about fourfold in tested mdx muscle compared to un-tested (contralateral) muscle (P = 0.002) (Fig. 3D). Evidence of necrosis in the un-tested control leg gives an

Table 1

Age and body weight of mdx and C57 mouse groups. Data are presented as mean ± SE

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (weeks)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdx untreated</td>
<td>12.0 ± 0.3</td>
<td>30.2 ± 1.1</td>
</tr>
<tr>
<td>mdx cVaM</td>
<td>12.2 ± 0.2</td>
<td>29.0 ± 0.7</td>
</tr>
<tr>
<td>mdx cV1q (1 week)</td>
<td>11.7 ± 0.7</td>
<td>29.2 ± 2.1</td>
</tr>
<tr>
<td>mdx cV1q (4 h)</td>
<td>11.3 ± 0.8</td>
<td>29.2 ± 0.9</td>
</tr>
<tr>
<td>C57 untreated</td>
<td>11.0 ± 0.6</td>
<td>25.4 ± 0.9</td>
</tr>
<tr>
<td>C57 cVaM</td>
<td>11.7 ± 0.7</td>
<td>24.9 ± 0.5</td>
</tr>
<tr>
<td>C57 cV1q (1 week)</td>
<td>10.2 ± 0.2</td>
<td>24.4 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 3. Mean (±SE) for (A) peak isometric torque production in mdx and C57 mice before the dynamometer EIMD protocol. (B) Optimal joint angle, at which the peak torque was recorded (data presented as ankle angle in degrees plantar flexion). (C) The torque deficit (contractile dysfunction) in mdx and C57 mice after the EIMD protocol. (D) Muscle necrosis in TA muscles from the dynamometer tested and control (contralateral) limbs of mdx mice sampled 48 h after the EIMD protocol. *significantly different compared to mdx mice (P < 0.05); # significantly different to test limb (P < 0.05).
indication of the severity of the background dystrophology in both legs prior to the EIMD protocol, although it is recognised that there can be biological variation even between both legs of an individual mdx mouse [35].

As for the analysis of myofibre necrosis, there was no evidence of EBD positive fibres in any of the tested muscle from C57 mice. For the TA muscles of mdx mice, however, EBD positive fibres accounted for 5.6 ± 1.3% of total muscle cross-sectional area (Fig. 8A). These results indicate that the muscles of the mdx mice are weaker (lower peak torque) and more susceptible to EIMD than non-dystrophic C57 mice. The absence of myofibre necrosis and EBD positively stained fibres in any muscles from the C57 mice reflects the capacity for normal (non-dystrophic) mice to tolerate EIMD.

3.2. The effect of cV1q treatment on mdx and C57 muscles after EIMD

3.2.1. The effect of cV1q on peak torque and optimal joint angle

Peak isometric torque prior to EIMD normalised to body weight for all mdx and C57 groups is presented in Fig. 4A. There was a significant main effect of mouse strain on peak torque ($F_{1,28} = 10.6, P < 0.001$) (mdx versus C57) and post-hoc analysis revealed that the mdx mice were significantly weaker than the non-dystrophic C57 mice ($P < 0.01$). There was no significant main effect of treatment ($F_{2,28} = 8.6, P = 0.08$) on the peak torque. The joint angle at which the peak torque was recorded (optimal angle) for all mdx and C57 groups is presented in Fig. 4B. The optimal angle for untreated mdx and C57 mice that was reported in Fig. 3B is also included here for comparison with the treatment groups. There was no significant main effect of treatment ($F_{2,28} = 1.09, P = 0.35$) on the optimal joint angle.

3.2.2. Effect of cV1q treatment on susceptibility to EIMD

The torque–angle relationships recorded before and after the EIMD protocol in cV1q-treated C57 and mdx mice are presented in Fig. 2B and D, respectively. The initial contractile dysfunction as determined by torque deficit after EIMD is presented in Fig. 5 for all mdx and C57 groups. There was a significant main effect of mouse strain ($F_{1,28} = 43.3, P < 0.001$) (mdx versus C57) on the torque deficit after EIMD. Post-hoc analysis revealed that the C57 mice experienced significantly less torque deficit than the mdx mice ($P < 0.001$). Furthermore, there was a significant main effect of treatment ($F_{2,28} = 8.6, P = 0.002$) with cV1q-1w significantly reducing the torque deficit in both mdx and C57 mice ($P < 0.001$). The acute cV1q-4h treatment, examined in mdx mice, also significantly reduced the torque deficit following EIMD ($P < 0.05$).

3.2.3. Assessment of cV1q treatment on necrosis and sarcolemmal damage after EIMD

Necrosis was quantitated on H&E stained sections (Fig. 6): since necrosis was not observed in any C57 mice, only data from mdx mice are presented (Fig. 7). The % area of necrosis in the TA muscle of tested limbs was compared to the non-tested contralateral limb. Significant main effects of leg ($F_{1,28} = 19.0, P < 0.001$) (whether dynamometer tested or contralateral un-tested limb) and treatment ($F_{2,28} = 4.0, P = 0.029$) were observed on muscle necrosis in mdx mice. Post-hoc analysis revealed that for all treatment groups the level of necrosis was significantly greater in the TA muscle of the tested limb compared to the un-tested limb ($P < 0.05$). Furthermore, exposure to cV1q for 1 week (cV1q-1w) significantly reduced the level of myofibre necrosis in the TA muscles of both the tested limb (Fig. 6) and the un-tested limb, compared to untreated mdx mice.
mice ($P < 0.05$). However, the level of necrosis in the acute cV1q-4h treated mdx mice was not significantly different from the un-treated mdx mice (in either tested or un-tested limbs). These results show that 1 week exposure to cV1q in mdx mice significantly reduced necrosis in both the tested and un-tested (control) limbs compared to untreated mdx mice. The effects of cV1q-1w and cVaM treatments on sarcolemmal damage were evaluated by the analysis of EBD positively stained myofibres (Fig. 8). Neither cV1q-1w nor cVaM treatments had any significant effect on the number of EBD positive fibres in mdx mice ($F_{2,15} = 0.52$, $P = 0.60$). EBD staining was not performed in cV1q-4h treated mice.

Fig. 6. Typical examples of myofibre necrosis in H&E stained transverse sections of TA muscles subjected to the dynamometer EIMD protocol. (A) Untreated mdx muscle showing myofibre necrosis, fragmented sarcomeres and inflammatory cell accumulation. Similar necrosis is observed in (B) cVaM (control IgG) treated mdx muscle. (C) mdx muscle treated with cV1q (anti-TNF IgG) for 1 week prior to EIMD protocol showing areas of centrally nucleated myofibres but far less myofibre necrosis. Scale bars represent 0.1 mm.

Fig. 7. Mean (±SE) area of myofibre necrosis in dynamometer tested and un-tested (control) TA muscles of mdx mice sampled 48 h after the dynamometer EIMD protocol. * significantly different from corresponding test leg; # significantly different from untreated group; $P < 0.05$.

Fig. 8. TA cross sections showing positive staining for Evans Blue Dye (EBD) in myofibres 48 h after the EIMD protocol for (A) untreated mdx, (B) mdx mice treated with cV1q for 1 week, and (C) mdx mice treated with cVaM for 1 week. Scale bars represent 500 μm. (D) Mean (±SE) area of EBD positively stained fibres in TA muscles of mdx mice sampled 48 h after the dynamometer EIMD protocol. Note: There were no (<1%) EBD positive fibres observed in C57 mice.
Calculation of the ‘recovery score’ shows a correction by cV1q-1w treatment of the mdx values (control untreated value as 0%) towards those for normal C57 mice (considered as 100%) of 78% for torque deficit and 56% for myofibre necrosis. The recovery score for the torque deficit in this study is similar to the 81% recovery reported by Gillis [38] for isolated EDL muscles of transgenic mdx mice over-expressing a truncated form of utrophin. The recovery score for mdx mice exposed to a single cV1q-4h injection was 56% for torque deficit but only 5% for necrosis.

4. Discussion

The contractile dysfunction of dystrophic mdx muscles (compared with normal controls) measured by the dynamometer in this study is similar to that for the GRMD dog model of DMD [5] and for transgenic mdx mice that over-express insulin-like growth factor-1 [34]. The main findings of this study support our hypothesis that blockade of TNF (using cV1q antibody) would significantly reduce the extent of contractile dysfunction and (in some cases) subsequent myofibre necrosis in mdx mice following a bout of damaging eccentric exercise. These data further support the use of cV1q administration to protect dystrophic muscles from initial and longer-term damage.

Exposure to cV1q for 1 week before EIMD significantly reduced the initial contractile dysfunction (recovery score = 78%) and the subsequent myofibre necrosis (recovery score = 56%) in response to EIMD. The beneficial effects of cV1q in reducing myofibre necrosis has also been demonstrated in mdx mice exposed to voluntary wheel running [11] with similar effects observed for other anti-TNF treatments (e.g. Remicade and Enbrel [12–14]). Interestingly, TNF blockade was also beneficial and reduced the initial contractile dysfunction in C57 mice, although to a lesser extent than for mdx mice. This observation suggests that, in addition to its contribution to myofibre necrosis, TNF also plays a crucial role in mediating the initial functional weakness following EIMD in both normal and dystrophic muscles: this accords with ex vivo studies on non-dystrophic muscles [25].

It is worth noting that the exposure to cV1q for 1 week reduces not only necrosis after EIMD (in the test leg) but also the background pathology in the mdx mice as indicated by a significant (albeit small) reduction in necrosis in the un-tested limb. To distinguish between possible longer-term beneficial effects of cV1q on the muscle environment compared with more short-term effects of blocking TNF, we conducted experiments with a single cV1q injection administered 4 h before EIMD. We chose a 4 h time-point to ensure that the cV1q had sufficient time to take effect following the IP injection. This short-term exposure to cV1q in mdx mice also reduced the contractile dysfunction following EIMD (recovery score = 56%). However, this protective effect on force production did not extend to myofibre necrosis, which was still elevated in the TA of the tested mdx limb (recovery score = 5%). Furthermore, the level of necrosis in the un-tested limb was not significantly different from that of untreated mdx mice which would indicate that the background level of pathology was unaffected. Therefore, at least the protective effect of cV1q on contractile dysfunction must be mediated by the short-term blockade of TNF rather than some major adaptation of the mdx environment. This is consistent with the beneficial effects of cV1q in normal C57 mice in reducing the contractile dysfunction after EIMD. Therefore, we propose that elevated levels of TNF in response to EIMD have direct effects on contractile function.

TNF has been widely implicated as a possible mediator of the muscle weakness observed in conditions of chronic and acute inflammation (e.g. muscle damage/trauma, chronic obstructive pulmonary disorder, AIDS, congestive heart failure etc.). Elevated systemic TNF in mice results in loss of body weight (by 3 weeks) and decreased muscle fibre cross-sectional area (by 5 weeks) [9] and, in tissue cultured myotubes, chronic TNF exposure (up to 3 days) results in muscle protein catabolism [41]. Acute exposure of isolated muscle preparations to TNF (for 4 h), however, causes contractile dysfunction and weakness, without evidence of catabolism [25]. Our dynamometer results are in accord with the experiments of Reid et al. [25] who demonstrated that TNF administration to isolated mouse muscles in vitro depressed tetanic force without altering the intra-cellular Ca2+ concentration ([Ca2+]i) in diaphragm and limb muscles of non-dystrophic mice. Other in vivo experiments by Hardin et al. [42] show that IP administration of TNF acts quickly (within 1 h) via the TNF-1 receptor to increase ROS production and decrease specific force in non-dystrophic mouse diaphragm. These effects were abolished by treatment with the antioxidant Trolox leading to the suggestion that muscle-derived oxidants are essential post-receptor mediators of TNF-induced force loss [42].

Altered cytosolic Ca2+ homeostasis has also been implicated as a putative pathway in development of the dystrophy [43]. Elevated [Ca2+]i may arise from transient tears in the fragile sarcolemma of dystrophic myofibres, and/or through mechanosensitive (stretch-activated) calcium channels. The uptake of EBD following the in vivo eccentric exercise protocol was limited to ~5% of myofibres in mdx mice, which is slightly less than that reported by Whitehead et al. (8.6%) from in vitro experiments on EDL muscles from mdx mice [44]. Interestingly, the level of EBD staining was unaffected by cV1q treatment suggesting that the beneficial effects of cV1q reported in this study are not mediated by reducing the sarcolemmal tearing in dystrophic myofibres. This does not preclude the possibility that the contractile dysfunction is mediated by altered calcium homeostasis as numerous recent studies have implicated the contribution of transient receptor potential channels (TRPC) to the dystrophopathy [45,46] and elevated expression of TRPC1 has been reported in mdx muscle [47,48]. Regardless of the source of entry, the influx of Ca2+ into cells has been linked to ROS production and activation of the nuclear factor-kappa B (NF-kB) pathway.
that regulates pro-inflammatory cytokine expression, in particular TNF [43]. Thus, a putative mechanism involved in the exercise-induced contractile dysfunction in mdx mice may involve a TNF mediated positive feedback relationship with NF-κB [30,50] and ROS [51]. The protective effect of TNF blockade using cV1q might be mediated (at least in part) by altering this positive feedback cycle, although further quantitative studies are required to clarify this hypothesis.

5. Summary

We have shown that cV1q administration (1 week before EIMD) protects dystrophic skeletal myofibres against initial contractile dysfunction and subsequent myofibre necrosis in adult mdx mice subjected to a damaging eccentric contraction protocol in vivo using a custom built mouse dynamometer. This technique enables the precise in situ quantitation of the initial physiological damage of muscles and evaluation of the impact of cV1q mediated TNF blockade on both contractile dysfunction and myofibre necrosis. The results from this study indicate that TNF acts quickly (within 30 min) to cause muscle weakness. Short term (4 h) administration of cV1q protects against TNF-mediated contractile dysfunction but not necrosis of mdx muscles, indicating that different mechanisms contribute to these events. The controlled EIMD produced by the dynamometer, with precise timing of damage combined with the ability to repeatedly measure functional parameters in vivo (including force production, muscle fatigue and adaptation [34]) makes this a powerful tool to test the efficacy of various therapeutic interventions and to more precisely define the molecular mechanisms responsible for initial and longer-term damage of dystrophic muscles.

Acknowledgements

We gratefully acknowledge Centocor (USA) for providing the cV1q anti-mouse TNF antibody and the negative control antibody (cVaM) and Dr. David Shealy for his support and helpful comments regarding this work. We also thank Professor Peter Hamer (The University of Notre Dame, Australia) for his contribution to the dynamometer work. This research was supported by the Raine Medical Research Foundation, UWA, the Medical Health Research Infrastructure Fund of WA and the National Health and Medical Research Council of Australia.

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