Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment

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Abstract

Tumour necrosis factor (TNF) is a potent inflammatory cytokine that appears to exacerbate damage of dystrophic muscle in vivo. The monoclonal murine specific antibody cV1q that specifically neutralises murine TNF demonstrated significant anti-inflammatory effects in dystrophic mdx mice. cV1q administration protected dystrophic skeletal myofibres against necrosis in both young and adult mdx mice and in adult mdx mice subjected to 48 h voluntary wheel exercise. Long-term studies (up to 90 days) in voluntarily exercised mdx mice showed beneficial effects of cV1q treatment with reduced histological evidence of myofibre damage and a striking decrease in serum creatine kinase levels. However, in the absence of exercise long-term cV1q treatment did not reduce necrosis or background pathology in mdx mice. An additional measure of well-being in the cV1q treated mice was that they ran significantly more than control mdx mice.

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1. Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disorder, affecting approximately 1/3500 male births [1,2]. Complete absence or impaired function of the skeletal muscle protein dystrophin leaves dystrophic myofibres susceptible to damage during mechanical contraction [3,4]. Consequently this initial damage progresses to myofibre necrosis. Repeated cycles of necrosis ultimately result in replacement of myofibres by fat and fibrotic connective tissue and loss of muscle function [2,5]. It is hypothesised that the initial myofibre damage is exacerbated by the endogenous inflammatory response [6–9] and that inflammatory cells and cytokines further damage the sarcolemma resulting in myofibre necrosis rather than the repair of minor membrane lesions. There is strong evidence to suggest that inflammatory cells and cytokines play a role in skeletal muscle damage (reviewed in [8]) and dystrophic muscle tissue has a considerably different gene expression pattern compared to non-dystrophic muscle with up-regulation of multiple genes involved in both the inflammatory response and muscle regeneration [10]. Blockade or depletion of resident T cells [11], neutrophils [9], macrophages [12,13] and mast cells [14,15] in mdx mice in vivo reduces the severity of dystrophopathy.

These cells all produce tumour necrosis factor (TNF) that is a potent pro-inflammatory cytokine that induces chemokine expression and upregulates adhesion protein expression on endothelial cells, resulting in cell infiltration to sites of inflammation [16,17]. TNF was previously referred to as TNFα, however as discussed in a recent review [18] the renaming of TNFβ as lymphotoxin (LTα and LTβ) leaves TNFα an orphan term and thus the appropriate term for use is now TNF. TNF is elevated in both DMD and mdx mouse muscles [19–21]. Antibody blockade of TNF with the human/mouse chimeric antibody infliximab (Remicade®) in young mdx mice, results in a striking protective effect on dystrophic myofibres and suppresses the early acute phase of myofibre necrosis [7]. A similar
protective effect in young dystrophic muscle was demonstrated with etanercept (Enbrel® – a soluble TNF receptor) [9]. These results strongly support a key role for TNF in both inflammation and necrosis in dystrophic muscle. In addition, both infliximab and etanercept treatment prevented the inflammatory response normally seen at 5 days in whole muscle autografts in non-dystrophic C57BL/10 mice, confirming the efficacy of these drugs in mice [22].

The monoclonal antibody infliximab was generated to block human TNF [23], and is currently very effectively used in the treatment of Crohn’s disease and rheumatoid arthritis [24]. There is controversy regarding the cross-species binding of infliximab based on in vitro tests, yet at least four papers report anti-inflammatory effects in vivo in mice [7], rats [25,26] and pigs [27]. To increase the efficacy of TNF blockade in mice and to avoid potential problems of immune response to the human constant domain sequences of infliximab, a rat monoclonal antibody specific for mouse TNF [28] and chimerized using mouse kappa light chain and mouse IgG2a heavy chain constant domain sequences (cV1q) was identified [29]. The present paper tests the effectiveness of the cV1q (mouse-specific anti-TNF) antibody in both dystrophic and non-dystrophic mice, in both short and long-term (up to 90 days) studies combined with voluntary exercise. The use of the species specific antibody is considered important for long-term studies to minimise immune problems.

The mdx mouse, an animal model for DMD [30], undergoes a spontaneous onset of acute necrosis and subsequent regeneration in limb and paraspinal muscles around 3 weeks of age [7,31]. The high level of muscle necrosis between 21 and 28 days provides an excellent model to study therapeutic interventions designed to prevent or reduce muscle necrosis, as a reduction in dystrophopathy is easily observed [7,15,32,33]. Myofibre necrosis markedly decreases and stabilises by 6 weeks of age [34,35]. The low level of dystrophopathy in adult mice can be made significantly worse by exercise that increases myofibre necrosis as a reduction in dystrophopathy is easily observed [7,15,32,33]. Myofibre necrosis markedly decreases and stabilises by 6 weeks of age [34,35]. The low level of dystrophopathy in adult mice can be made significantly worse by exercise that increases myofibre necrosis and reduces muscle strength [14,36,37] enabling potential therapeutic interventions to be evaluated in adult mdx mice [15,38–41].

2. Experimental overview

The cV1q antibody was tested in four in vivo models of inflammation. (1) Whole muscle autografts in adult non-dystrophic C57BL/10ScSn mice (the non-dystrophic parental strain for mdx), (2) young dystrophic mdx mice (male and female littermates), (3) adult dystrophic (mdx) mice subjected to 48 h voluntary exercise and (4) long-term (up to 90 days) cV1q treatment in both exercised and unexercised adult dystrophic (mdx) mice. Voluntary wheel running has several advantages compared to forced treadmill exercise; mice are able to run at night when they are normally active [42,43] which avoids the stress associated with exercising mice during the day when they are normally inactive, also voluntary exercise is less stressful to the animal than forced high intensity exercise [44,45]. We also hypothesise that the amount of voluntary exercise undertaken by an individual mouse may reflect the overall health of the mouse and serve as an additional measure of wellbeing to test drug interventions. The effects of cV1q are compared with infliximab and etanercept for three of the experimental models [7,9,22].

3. Material and methods

**Mice.** Experiments were carried out using dystrophic mdx litters (male and female littermates), dystrophic female mdx (C57BL/10ScSn-mdx) mice and non-dystrophic female C57BL/10ScSn mice (Table 1). All mice were obtained from the Animal Resources Centre (ARC) Murdoch, Western Australia, housed under a 12 h day–night cycle and allowed access to food and water ad libitum. Mice were treated in strict accordance with the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics.

**cV1q treatment.** Intra-peritoneal (IP) injections of cV1q and the isotype-matched, negative control antibody (cVAM) both provided by Centocor were given at a concentration of 20 μg/g/mouse/week (Table 1). For the whole muscle autografts (experiment 1) cV1q was routinely injected 24 h prior to surgery (unless otherwise stated). In young mdx mice (experiments 2 and 4) injections began at 19 days of age. In the 48 h voluntarily exercised adult mice (experiment 3) a single injection was given 24 h prior to voluntary exercise.

**Whole muscle autograft surgery.** Whole muscle autografts were used as an in vivo bio-assay to assess the anti-inflammatory properties of cV1q in non-dystrophic mice. Six-weeks-old female C57BL/10 mice were anaesthetised using 2% (v/v) Rodia Halothane. The extensor digitorum longus (EDL) muscle with both tendons was removed from the anatomical bed and transplanted onto the surface of the tibialis anterior (TA) muscle, the EDL tendons were sutured to the TA, the skin closed and wound left to heal, as described in [22,46,47]. It is well-documented that this

### Table 1

**Animal treatment summary**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>Age</th>
<th>Sex</th>
<th>cV1q treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Whole muscle autografts</td>
<td>C57BL/10</td>
<td>6 weeks</td>
<td>Female</td>
<td>1 day prior to surgery*</td>
</tr>
<tr>
<td>(2) Necrosis onset in young mdx litters</td>
<td>Mdx</td>
<td>d19–28</td>
<td>Mix</td>
<td>d19–d28 (weekly)</td>
</tr>
<tr>
<td>(3) 48 h voluntary exercise</td>
<td>Mdx</td>
<td>6 weeks</td>
<td>Female</td>
<td>1 day prior to exercise d19–d90 (weekly)</td>
</tr>
<tr>
<td>(4) Long-term cV1q treatment</td>
<td>Mdx</td>
<td>d19–90</td>
<td>Female</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates that cV1q injections were usually given 1 day prior to whole muscle graft surgery; however some injections occurred at 1 week prior to surgery and some at 1 week prior to surgery plus again at 5 days after surgery.
procedure severs the nerve and blood supply to the EDL muscle and results in necrosis and subsequent inflammation and regeneration of the graft. Regeneration that results in new muscle formation begins with infiltration of inflammatory cells and revascularization [48–50]. This inflammatory zone is well-established by 5 days after surgery and provides a good model to observe anti-inflammatory interventions. To test the duration of biological efficacy, cV1q was administered at (A) 24 h prior to surgery (−1 day), (B) one week prior to surgery, or (C) as two injections, one 24 h prior to surgery and again on day 5. Whole muscle autografts were sampled at either 5 or 7 days after surgery and paraffin processed for histological analysis.

**Voluntary exercise.** Mice were voluntarily exercised using a metal mouse wheel (300 mm) placed inside the cage. Exercise data were collected via a small magnet attached to the mouse wheel with a sensor from a bicycle pedometer, attached to the back of the cage, that recorded single wheel revolutions, allowing total distance (km) run by an individual mouse to be determined, as per [9,15]. While an extended period of voluntary exercise might result in additional muscle necrosis, it also allows myofibres that became necrotic at the beginning of the exercise regime to commence regeneration resulting in new myofibre formation; however this makes interpretation difficult against the background pathology. We tested specifically for the effects of exercise on myofibre necrosis in adult mdx mice and therefore exercise was limited to only 48 h (experiment 3) to allow for analysis shortly after the onset of exercise-induced necrosis (and before new myotube formation—that normally starts about 2.5 days after necrosis) [51].

**Long-term voluntary exercise.** Young mdx mice were placed in cages containing exercise wheels from 28 days of age. Before 28 days of age mice are too small to run on the exercise wheels and mice did not run any considerable distance until 33 days of age; therefore, exercise measurements were recorded only from 33 days through to 90 days of age (8 weeks on the wheel—experiment 4). These mice were all injected weekly with cV1q, control cVaM or sterile water from 19 days of age.

**Tissue collection and image acquisition.** All mice were sacrificed by cervical dislocation while under terminal halothane (3%/v/v) anaesthesia. The TA, grafted EDL or quadriceps muscles were sampled and prepared for paraffin processing. Transverse sections (5 μm) were cut through the mid-region of each muscle. Slides were stained with haematoxylin and eosin (H&E) for morphological analysis. Non-overlapping tiled images of transverse muscle sections provided a picture of the entire muscle cross-section. Images were acquired using a Leica DM RBE microscope, a personal computer, a Hitachi HVC2OM digital camera, Image Pro Plus 4.5.1 software and Vexta stage movement software. Tiled images were taken at 10x magnification.

**Histological image analysis.** Histological analysis was carried out on whole muscle cross-sections. Muscle morphology was drawn interactively by the researcher using Image Pro Plus 4.5.1 software and specific histological features measured as a percentage (area) of the whole muscle section. All section analysis was done ‘blind’. Different morphological features are quantitated in specific experiments and are therefore covered in the results section for each individual experiment. For each individual experiment, both the left and right leg from each mouse was analysed, the numeric value (e.g., % muscle necrosis) for each mouse (n) thus represents an average of both legs.

**Serum creatine kinase assay.** While under terminal anaesthesia blood from the mdx mice (experiments 2–4) was collected via cardiac puncture. Blood was refrigerated overnight, centrifuged for 3 min (1200 rpm) and serum removed. Blood serum creatine kinase (CK) analysis was completed at the Murdoch Veterinary Hospital, Murdoch, WA.

**Statistical analysis.** All statistical analysis was completed in SPSS (SPSS 15.0 for windows). Statistical analysis for direct comparison between two groups was performed by unpaired Student’s t-tests. Multiple comparisons between groups were made using a General Linear Model, univariate analysis of variance (ANOVA). A Least Significant Difference (LSD) posthoc t-test was used to identify differences between groups. For experiments 3 and 4 there was no difference (P > 0.7) between data from cVaM injected and untreated control exercised mdx mice, therefore both groups were pooled to form one single exercised ‘control’ group. Significance was set at P < 0.05 for all comparisons.

### 4. Results

#### 4.1. Experiment 1: whole muscle autografts

Morphological analysis was carried out on H&E stained transverse muscle sections of the autografted EDL at either 5 or 7 days after transplantation (Fig. 1). The area within the centre of the graft (persisting necrotic tissue) was quantitated and expressed as a percentage (%) of the whole graft area. The remainder of the graft consists of inflammatory cell infiltration, surviving myofibres and regenerating muscle (myoblasts and myotubes) (Fig. 1). The extent of persisting necrotic muscle tissue is an inverse reflection of inflammatory cell activity and new muscle formation; with a larger area of persisting (central) necrotic tissue indicating less inflammatory cell infiltration (from the periphery).

TNF is a key pro-inflammatory cytokine involved in inflammation that is essential for the removal of necrotic tissue, stimulation of revascularization and activation of muscle precursor cells for regeneration [48,50]. Therefore it was hypothesised that blockade of TNF activity via the administration of cV1q would delay or prevent the inflammatory process and thus subsequent regeneration of the graft.

The average area of persisting necrotic tissue in autografts from cV1q treated mice (71.3%) sampled at 5 days after surgery, is significantly higher than in control grafts (35%) (Fig. 2, Table 2), therefore cV1q administration at
1 day \((P = 0.01)\) prior to surgery significantly impaired inflammatory cell infiltration and subsequent muscle regeneration. cV1q intervention resulted in an approximate 2-fold increase in persisting necrotic autograft tissue sampled at 5 days after surgery. However after this time, inflammation does occur and at 7 days after surgery, autografts in cV1q treated have a similar appearance to untreated control mice with little necrotic tissue and extensive new muscle formation (Fig. 2 and Table 2).

### 4.2. Experiment 2: young mdx mice (litters)

The onset of muscle necrosis in mdx mice shows high levels of biological variation between both individual mice...
and litters [15]. To help reduce biological variation, cV1q injections were performed on half a litter of mdx pups with the remaining half of the litter used as controls (cVaM injected). This ensures that comparisons (cV1q vs. cVaM) are made between age matched mice of one litter to reduce inter-litter variation [15]. Mice were sampled on either day 24 or day 28; mice sampled on day 24 were injected once (d19) and mice sampled on day 28 were injected twice (d19 and d26).

Muscle necrosis was identified and measured on H&E stained transverse sections of TA muscles by the presence of infiltrating inflammatory cells (basophilic staining) and degenerating myofibres with fragmented sarcoplasm. Regeneration occurs in response to necrosis and results (2–3 days later) in myotubes that, over time, mature into myofibres with central nuclei. Cumulative skeletal muscle damage in young mdx mice (up to 28 days of age) consists of active myofibre necrosis (usually only necrosis present at 24 days) plus the areas of subsequent myotube formation (present by 28 days).

cV1q treatment significantly reduced ($P = 0.03$) cumulative muscle damage in the TA of 24 and 28-day-old mdx mice (Fig. 3), with cV1q treatment, cumulative muscle damage in 24-day-old mice was half that seen in control (14.59% vs. 33.16%) in an entire cross-sectional area (CSA) of the TA. Further examination of cumulative muscle damage at day 24 (Fig. 3) indicates a delayed onset of necrosis after cV1q treatment, as reflected by a significantly higher proportion of necrosis ($P = 0.01$) and a lower proportion of regeneration ($P = 0.01$) in cV1q treated mice. That is, given there are a more regenerating myofibres (new myotubes) in the control mice and since myotubes first form from about 2.5 days after necrosis [51]; the onset of necrosis may have occurred earlier in the control mice.

While the process of muscle necrosis and hence regeneration was delayed after cV1q administration, there were no adverse effects on new muscle formation. Similarly no adverse effects on muscle formation were seen after the administration of infliximab [7] or etanercept [9].

Analysis of serum CK levels showed a clear trend for reduced (roughly halved) serum CK levels in young cV1q-treated mdx mice compared with controls [day 24 cV1q treated, 3330 U/L (±378); cVaM treated, 6300 (±3676). Day 28 cV1q treated, 1200 U/L (±608); cVaM treated, 2950 (±1484)], although due to high variation the effect of cV1q treatment on serum CK levels was not statistically significant.

Due to the well-documented high variation in severity of dystrophopathy between individual mdx mice, a direct comparison of infliximab and cV1q data for mdx mice treated at the onset of myofibre necrosis (i.e., percentage muscle necrosis) is inconclusive, that is absolute numbers cannot be compared. A comparison of ‘relative change’ is more appropriate with both infliximab [7] and cV1q producing 2-fold reduction in myofibre necrosis in 24-day-old mdx mice. cV1q treatment also demonstrated a strong protective effect (2-fold) at 28 days of age (Fig. 3) whereas infliximab treatment showed less benefit at this later time-point. It is concluded that both treatments were highly effective in reducing the extent of myofibre necrosis in young mdx mice.

4.3. Experiment 3: 48 h voluntary exercise of adult mdx mice

After 48 h of voluntary wheel, the quadriceps muscle of 6-week-old female mdx mice were analysed histologically. Measurements were calculated for: the area occupied by necrotic myofibres, regenerating (small myotubes) and

![Fig. 3. Cumulative muscle damage in cV1q treated mdx mice aged 24 and 28-days-old compared with control (cVaM) litters. cV1q treatment significantly reduced ($P = 0.03$) cumulative muscle damage in the TA muscle of both 24 and 28-day-old dystrophic mice, as indicated by *! shows significantly more necrosis. # indicates significantly less regeneration in cV1q treated 24-day-old mice, this reflects the delay in muscle damage and thus absence of new muscle formation (short-term up to day 28). Each time-point consists of 2 litters (cV1q treated and untreated litter mate controls). Error bars represent standard error and $n = 7, 6, 6$ and 5 mice, respectively, across the groups.](image-url)
regenerated (myofibres with central nuclei) muscle and unaffected/intact myofibres as described previously [15]. 100% of the muscle cross-sectional area (CSA) is made up of necrotic tissue, regenerating tissue (recently necrotic), regenerated tissue (central nuclei), unaffected tissue (never been necrotic) and connective tissue. As reported previously, exercise-induced necrosis in the TA muscle of all mice was minimal and therefore only the quadriceps muscle was analysed [9,15,41]. No differences were found between control cVaM treated or untreated control exercised mdx mice, therefore for the purpose of statistical and graphical analysis both groups were pooled and are presented as one single exercised ‘control’ group (n = 6).

Voluntary exercise (48 h) resulted in a significant (P = 0.02) increase (approximately 2-fold) in muscle necrosis in the quadriceps muscle of control mdx mice (Fig. 4) as reported previously [9,15,41]. cV1q treatment significantly reduced (P = 0.04) the extent of exercise-induced myofibre necrosis from 12.93% (control) down to 7.58%; this low level of necrosis is similar (not significantly different) to the necrosis (6.22%) in unexercised quadriceps muscle of adult mdx mice. cV1q administration did not prevent an increase in serum CK levels after 48 h exposure to voluntary exercise (data not shown).

The mdx mice ran between 4.13 and 14.45 km over the 48 h; the average distance run by the control mice was 9.03 km and the average distance run by the cV1q treated mice was 8.27 km. It is important to note that the cV1q treatment had no significant affect on the distance run by the mice over 48 h, since reduced activity might result in reduced muscle damage (% necrosis). However, the distance run by an individual mouse does not directly correlate to either the percentage of myofibre necrosis in the quadriceps muscle or serum CK (data not shown) this is also documented in previous studies [9,15].

4.4. Experiment 4: long-term cV1q treatment in exercised and unexercised dystrophic (mdx) mice

The main objective of this study was to assess long-term treatment with an antibody to TNF in muscles of dystrophic mdx mice. cV1q treatment began at 19 days of age (before the onset of myofibre necrosis) and adult female mdx mice were sampled at 90 days of age (approximately 3 months). No differences were found between cVaM treated or untreated mice, therefore for the purpose of statistical analysis both groups were pooled and are presented as one single ‘control’ group (n = 6 unexercised mice or n = 8 exercised mice). Histological analysis was performed as per experiment 3.

cV1q treated mdx mice ran more than control mdx mice over the 8 week period (35–90 days of age) of voluntary exercise. cV1q treated mice ran significantly more (P = 0.03) in total distance (383.7 km n = 8) compared with control mdx mice (236.6 km n = 8) (Fig. 5, Table 3) and cV1q treated mice also ran more on average each week (Fig. 6) with the largest difference seen in weeks 2–5.

In unexercised mdx mice cV1q treatment had no effect on the amount of active muscle necrosis in the TA (data not shown) or in the quadriceps muscles (Fig. 7). Voluntary exercise over the 8 week period caused a significant (P = 0.017) increase in myofibre necrosis in the quadriceps muscle of mdx mice (Fig. 7) in comparison to unexercised mdx mice. cV1q treatment in long-term exercised mice
caused a reduction in the percentage of active myofibre necrosis in the quadriceps muscle, however this effect was not significant. It is interesting to note that the percentage of active muscle necrosis seen after 8 weeks of exercise is similar to that seen after 48 h voluntary exercise (10.2% vs. 12.9%) indicating no adaptation to the repeated exercise regime.

In unexercised adult mdx mice cV1q treatment had no effect on serum CK (Fig. 8). In striking contrast, voluntary exercise over the 8 week period (day 35–90) caused a dramatic (10-fold) and significant ($P = 0.01$) increase in the level of serum CK (Fig. 8, Table 2) whereas in cV1q treated mdx mice serum CK after exercise was significantly lower ($P = 0.01$) and, in fact, was the same as unexercised mdx mice. These data suggest that long-term voluntary exercise significantly increases myofibre ‘leakiness’ of control mdx muscle (although has no striking impact on the amount of myofibre necrosis) and this is prevented by cV1q treatment.

Table 3
Summary – total distance run (km) over 8 weeks by cV1q treated and control exercised mice, combined with the data for % necrosis in the quadriceps muscle and serum creatine kinase (CK) levels

<table>
<thead>
<tr>
<th>Number</th>
<th>Treatment</th>
<th>Distance (Avg. km/wk)</th>
<th>Distance (total km/8wk)</th>
<th>Necrosis (quad% area)</th>
<th>CK (U/L)</th>
<th>Unaffected tissue (quad% area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cVaM</td>
<td>34.4</td>
<td>275.1</td>
<td>15.85</td>
<td>39900</td>
<td>2.1</td>
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<tr>
<td>2</td>
<td>cVaM</td>
<td>54.5</td>
<td>435.7</td>
<td>12.9</td>
<td>9850</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>cVaM</td>
<td>35.2</td>
<td>282.0</td>
<td>6.15</td>
<td>33600</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>cVaM</td>
<td>12.7</td>
<td>101.6</td>
<td>3.75</td>
<td>24150</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>Untreated</td>
<td>30.2</td>
<td>241.7</td>
<td>8.38</td>
<td>17350</td>
<td>3.4</td>
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<tr>
<td>6</td>
<td>Untreated</td>
<td>19.4</td>
<td>154.7</td>
<td>10.27</td>
<td>16100</td>
<td>1.15</td>
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<tr>
<td>7</td>
<td>Untreated</td>
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<td>233.5</td>
<td>13.25</td>
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<td>21.1</td>
<td>168.8</td>
<td>11</td>
<td>6250</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>29.6 km/week</td>
<td>236.6 km total</td>
<td>10.19%</td>
<td>18850</td>
<td>1.95%</td>
</tr>
</tbody>
</table>

| 1      | cV1q       | 21.2                  | 169.6                   | 2.74                  | 7250     | 4.8                           |
| 2      | cV1q       | 42.0                  | 336.0                   | 11.75                 | 5300     | 2.7                           |
| 3      | cV1q       | 71.6                  | 572.6                   | 4.13                  | 2100     | 6.8                           |
| 4      | cV1q       | 72.8                  | 582.75                  | 11.95                 | 4550     | 5.4                           |
| 5      | cV1q       | 36.3                  | 290.8                   | 4.41                  | 2300     | 2.65                          |
| 6      | cV1q       | 62.2                  | 497.9                   | 8.07                  | 2150     | 5.7                           |
| 7      | cV1q       | 41.15                 | 329.2                   | 6.65                  | 2500     | 4.4                           |
| 8      | cV1q       | 36.3                  | 290.9                   | 8.74                  | 3600     | 3.8                           |
|        | Average    | 47.9*                 | 383.7*                  | 7.31                  | 3718*    | 4.53*                         |

* Indicates a significant difference ($P < 0.05$) between control and cV1q treatment.
measured by quantitating the number of unaffected (normal) myofibres without central nuclei – an inverse representation of disease severity. Long-term voluntary exercise significantly \((P = 0.016)\) reduced the amount of unaffected myofibres indicating a more severe dystropathology in comparison with unexercised mdx muscle. In contrast, cV1q treatment significantly maintained \((P = 0.001)\) the area of unaffected myofibres in exercised adult mdx mice. This suggests that cV1q treatment reduced the severity of dystropathology and protects against myofibre damage in long-term exercised mdx mice.

5. Discussion

These data from short and long-term studies demonstrate that cV1q antibody treatment, which neutralises murine TNF, reduces the extent of muscle necrosis in both young and exercised adult dystrophic mice and supports the original hypothesis that TNF contributes to the necrosis of dystrophic muscle. cV1q treatment also significantly increased the amount of voluntary exercise completed by dystrophic mice over 8 weeks, strongly indicating long-term functional benefits combined with reduced disease severity in the mdx mice.
The anti-inflammatory property of cV1q in mice was assessed using the whole muscle autograft model. Transplantation of whole muscle autografts produces a very large mass of avascular necrotic tissue and a situation of extreme pathology, which does not directly compare to the small foci of damage in dystrophic muscle, but the autograft model is useful to test the ability of cV1q to block TNF and the inflammatory response \textit{in vivo}. The analysis of variance test for multiple comparisons between groups showed statistical difference ($P = 0.02$). Posthoc LSD T-tests showed a significant increase ($P = 0.012$) in CK levels after exercise (**) and no significant increase ($P = 0.013$) in CK level after exercise when treated with cV1q (*). Error bars indicate standard error, horizontal bar indicates exercised mice and $n = 6, 6, 8$ and $8$ mice, respectively.

Fig. 8. Blood serum creatine kinase level in 90-day-old mdx mice. Creatine kinase in unexercised control (2900U/L), unexercised + cV1q (2200 U/L), exercised control (18850 U/L) and exercised + cV1q (3718 U/L) adult mdx mice. The analysis of variance test for multiple comparisons between groups showed statistical difference ($P = 0.02$). Posthoc LSD T-tests showed a significant increase ($P = 0.012$) in CK levels after exercise (**) and no significant increase ($P = 0.013$) in CK level after exercise when treated with cV1q (*). Error bars indicate standard error, horizontal bar indicates exercised mice and $n = 6, 6, 8$ and $8$ mice, respectively.

Fig. 9. Unaffected muscle tissue in 90-day-old mdx mice. Unaffected myofibres in the quadriceps muscle of unexercised control (7.32%), unexercised cV1q (6.97%), exercised control (1.95%) and exercised cV1q (4.53%). The analysis of variance test for multiple comparisons between groups showed statistical difference ($P = 0.05$). Posthoc LSD T-tests showed a significant ($P = 0.016$) reduction in unaffected myofibres after voluntary exercise in comparison to unexercised mice (**) and a significant ($P = 0.001$) increase in unaffected myofibres with cV1q treatment in exercised mice in comparison to ‘control exercised mice (*). ‘Control’ refers to pooled data from cVaM treated and untreated exercised mice. Error bars represent standard deviation, horizontal bar indicates exercised mice and $n = 6, 6, 8$ and $8$ mice, respectively.
but does not prevent the long-term response of inflammation that precedes regeneration (day 7 onwards): this is presumably due to some activation of a secondary pro-inflammatory pathway that is unaffected by TNF inhibition. Infliximab and etanercept treatment similarly showed no effect in day 7 grafts compared with controls [22]. This bio-assay (at day 5) confirms an inhibitory effect of cV1q on the inflammatory response in mice in vivo as anticipated. The inhibitory effect was reproducible across multiple grafts and it was specific – in that no effects were seen with control cVaM administration.

In dystrophic mdx mice, cV1q treatment significantly reduced the severity of skeletal muscle necrosis in both the TA of young mdx mice and the quadriceps muscle of exercised adult mdx mice. The voluntary wheel exercise significantly ($P = 0.02$) increases (approximately 2-fold) myofibre necrosis in the quadriceps of adult mdx mice and this was prevented by pre-treatment of the mice with cV1q. These short-term studies further validate the important principle that necrosis of dystrophic myofibres can be reduced by exogenous manipulation of the potent pro-inflammatory cytokine TNF.

A striking 10-fold increase in serum CK levels in mdx mice after 8 weeks of voluntary exercise was completely prevented by cV1q treatment, dramatically emphasizing the benefits of this long-term treatment. The serum CK levels in the long-term exercised mdx mice (18,850 ± 12,933 U/l) are very high; but do fall within the range reported by Granchelli (2000) (17,200 ± 11,600 U/l) after 2 × week 30 min exercise sessions on a horizontal treadmill for 4 weeks [38].

Long-term treatment with cV1q in the absence of exercise had no significant effect on myofibre necrosis or extent of undamaged myofibres in the quadriceps muscle, or on serum CK levels. This indicates that the already low level of background dystropathology in adult mdx mice is not further reduced by cV1q blockade of TNF, whereas cV1q clearly protected against exercise-induced acute myofibre damage. The lack of effect in the unexercised mice is in striking contrast to the cV1q effects seen in exercised mdx mice: whether this reflects differences in the cellular responses in these two situations is unknown. cV1q treatment in long-term exercised mdx mice showed (1) Improved muscle function – demonstrated by an increase capacity for exercise. (2) Reduced myofibre leakiness – demonstrated by a marked reduction in serum CK levels. (3) Reduced disease severity (myofibre necrosis and regeneration cycles) – demonstrated by a greater area of unaffected myofibres at 90 days of age.

It is calculated that myonuclei of newly regenerated myofibres remain in a central location for about 50–100 days in mdx mice and thereafter 3–4% of myonuclei move to a peripheral subsarcommaal position every 100 days i.e., numbers of central myonuclei may decline after 100 days of age [34]. In this study, we presume that the central nucleus of a regenerated myofibre takes more than 70 days to move to the periphery (from day 21 with the acute onset of necrosis to day 90 when mice are sampled); thus it is considered that myofibres with only peripheral nuclei have never undergone myonecrosis and are deemed unaffected. Counting such non-centrally nucleated (unaffected) myofibres provides a converse indication of disease severity and supports the notion of reduced myofibre damage in long-term cV1q treated mdx mice. In contrast, myofibres with central nuclei must have regenerated at least once. If an individual myofibre undergoes multiple cycles of necrosis and regeneration the appearance is similar to a myofibre that has only regenerated once during the period of the experiment and thus this analysis does not give any indication of how many times an individual myofibre (with central nuclei) may have undergone cycles of necrosis and regeneration [51]. It seems likely that the cycles of necrosis and regeneration may have occurred more often in the control mice compared with cV1q treated mdx mice, although this cannot be determined from this basic histology.

In summary, the results achieved with cV1q administration in mdx mice are highly promising and similar to the results achieved by both infliximab and etanercept administration and are strongly supported by the long-term studies combined with exercise. The optimal regime and dosage, as well as the most appropriate humanised anti-TNF drug needs to be selected for possible trials in DMD boys. The development of new TNF drugs such as TNF-TeAb (miniantibodies) further complicate the decision [52]. The study confirms that a drug intervention may not show benefits in unexercised mdx mice, thus exercise appears an essential part of the ‘standard operating procedure’ to effectively test the effects of anti-inflammatory drugs in mdx mice in vivo.

Direct comparisons of cV1q treatment with other drugs such as HCT 1026; which appears to be more beneficial than prednisolone [53] is highly desirable as a prelude to selecting the most promising drug for potential clinical trials. There are many differences between the protocols used in our mdx study and the long-term study of HCT 1026 in mdx mice [53]: these include the duration of voluntary exercise; time (age) of animal sampling; and onset the of drug administration – with the crucial issue being that HCT 1026 treatment began after the acute onset of myofibre necrosis that starts at 21 days in mdx mice [7]. It appears that the gender of mdx mice also affects the severity of the dystropathology at different ages [54] although this has not been widely recognised and the impact on drug evaluations at different ages is unclear. We used female adult mdx mice in the present study; however other experiments show a similar level of background necrosis and the same doubling of exercise-induced myonecrosis in male mdx mice at 8 weeks of age, with similar high biological variation (Radley and Grounds unpublished data). It is noted that there are numerous studies where the gender of the mice is not stated [53,55,56] or a mix of both male and female mice are used [38]. Such fundamental differences emphasise the
need for standard operating protocols to facilitate important comparisons of promising therapies.

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References


