IFN-γ Promotes Muscle Damage in the mdx Mouse Model of Duchenne Muscular Dystrophy by Suppressing M2 Macrophage Activation and Inhibiting Muscle Cell Proliferation

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IFN-γ Promotes Muscle Damage in the mdx Mouse Model of Duchenne Muscular Dystrophy by Suppressing M2 Macrophage Activation and Inhibiting Muscle Cell Proliferation

S. Armando Villalta,* Bo Deng,* Chiara Rinaldi, † Michelle Wehling-Henricks, † and James G. Tidball*‡,§

Duchenne muscular dystrophy is a degenerative disorder that leads to death by the third decade of life. Previous investigations have shown that macrophages that invade dystrophic muscle are a heterogeneous population consisting of M1 and M2 macrophages that promote injury and repair, respectively. In the present investigation, we tested whether IFN-γ worsens the severity of mdx dystrophy by activating macrophages to a cytolitic M1 phenotype and by suppressing the activation of proregenerative macrophages to an M2 phenotype. IFN-γ is a strong inducer of the M1 phenotype and is elevated in mdx dystrophy. Contrary to our expectations, null mutation of IFN-γ caused no reduction of cytotoxicity of macrophages isolated from mdx muscle and did not reduce muscle fiber damage in vivo or improve gross motor function of mdx mice at the early, acute peak of pathology. In contrast, ablation of IFN-γ reduced muscle damage in vivo during the regenerative stage of the disease and increased activation of the M2 phenotype and improved motor function of mdx mice at that later stage of the disease. IFN-γ also inhibited muscle cell proliferation and differentiation in vitro, and IFN-γ mutation increased MyoD expression in mdx muscle in vivo, showing that IFN-γ can have direct effects on muscle cells that could impair repair. Taken together, the findings show that suppression of IFN-γ signaling in muscular dystrophy reduces muscle damage and improves motor performance by promoting the M2 macrophage phenotype and by direct actions on muscle cells. The Journal of Immunology, 2011, 187: 000–000.

Inflammatory cells can play central roles in determining the course of muscle injury and regeneration (1). The complexity and importance of interactions between muscle and the immune system for regulating muscle pathology has become increasingly apparent as our understanding of the pathology of muscular dystrophy grows, particularly in Duchenne muscular dystrophy (DMD) and the mdx mouse model of DMD. Although DMD and mdx dystrophies are caused by null mutations in the dystrophin gene that encodes a membrane-associated structural protein (2), and the primary defect causing muscle pathology is a weakened cell membrane (3), inflammatory cells play major roles in promoting the pathology. For example, depletion of macrophages from mdx muscle prior to the early acute onset of muscle fiber necrosis that occurs between 3 and 4 wk of age (Fig. 1) causes a 70–80% reduction in muscle fiber injury (4). Likewise, other perturbations that diminish the inflammatory response in dystrophic muscle yield quantitatively similar reductions in muscle fiber damage (5–9). Those findings show that much of the muscle damage in dystrophin deficiency is caused by inflammatory cells rather than by direct mechanical damage. That information has potential therapeutic importance, because it indicates that regulation of the immune response to dystrophic muscle damage could provide a valuable mechanism for reducing the pathology of muscular dystrophy.

Macrophages that cause muscle fiber damage in mdx dystrophy are a proinflammatory phenotype that is designated M1 macrophages (10). M1 macrophages can be driven to a proinflammatory phenotype that is designated M1 macrophages by proinflammatory Th1 cytokines, especially IFN-γ, and several findings suggest that IFN-γ activation of mdx muscle macrophages can worsen the pathology of muscular dystrophy. For example, IFN-γ expression is elevated in mdx muscles during the stage of the disease when macrophage-mediated muscle damage is rampant and numbers of M1 macrophages are greatly elevated (10). In addition, IFN-γ stimulation of macrophages isolated from mdx muscles produces tremendous increases in their cytotoxicity toward muscle cells in vitro. However, whether IFN-γ actually contributes to the pathophysiology of muscular dystrophy in vivo remains unknown.

Although beneficial effects of macrophage depletion on mdx muscle pathology show that macrophages are important agents in worsening mdx dystrophy at the acute onset of the disease, more recent studies have shown that macrophages can also play significant roles in promoting muscle repair that occurs during the regenerative stage of mdx pathology. Muscle fiber damage is greatly attenuated in mdx mice between the ages of 4 and 12 wk (4, 11), while myogenic cells respond to the acute onset of injury by entering the proliferative stage of development to expand their numbers and then transitioning through early differentiation and terminal differentiation to generate new muscle fibers (12–14). In concert...
with the decline of damage and acceleration of regeneration, macrophage populations in mdx muscle transition from the proinflammatory, M1 phenotype to an anti-inflammatory M2 phenotype (10, 15) (Fig. 1). Macrophages can be activated to an M2 phenotype by IL-4 and IL-13, which produce a state of alternative activation that is characterized by the production of anti-inflammatory cytokines (16–18). Those anti-inflammatory cytokines include IL-10 that can deactivate M1 macrophages and further drive alternative activation. IL-10 plays a particularly important role in mediating this switch of macrophage phenotype in mdx muscle in vivo and reducing muscle damage caused by M1 macrophages (15). Ablation of IL-10 expression in mdx mice caused an increase in muscle damage in 12-wk-old mdx mice and produced reductions in running endurance of mdx mice that coincided with the increases in muscle damage (15). Furthermore, macrophages that were stimulated with IL-10 increased proliferation of muscle cells in coculture, showing that IL-10 can also increase muscle growth as well as reduce muscle damage through IL-10–mediated mechanisms (15).

Collectively, these studies show that modulating the relative activities or proportion of M1 and M2 macrophages in dystrophin muscle can influence the course and severity of the disease. Because of the important role of IFN-γ in driving activation of monocytes and macrophages to the cytolitic M1 phenotype, we anticipated that ablation of IFN-γ could significantly reduce muscle damage in mdx dystrophy and provide a potential avenue for therapeutic interventions. We test that hypothesis in the present investigation by generating IFN-γ null mutant mdx mice and assessing whether IFN-γ promotes muscle fiber injury by activating M1 macrophages in mdx muscle. We measure the extent of muscle fiber injury during the acute and regenerative stages of dystrophinopathy in mdx and IFN-γ–deficient mdx (IFN-γ−/−/mdx) mice. In addition, we assay for changes in the cytotoxic potential of muscle macrophages isolated from mdx and IFN-γ−/−/mdx mice. We further test whether IFN-γ mediates a phenotypic shift toward an M1 phenotype by inducing M1 macrophages and suppressing the activation of M2 macrophages. We also evaluate whether null mutation of IFN-γ in mdx mice modulates muscle regeneration and investigate the effect of IFN-γ on muscle cell proliferation and differentiation.

Last, we assess whether perturbations in IFN-γ expression in mdx muscle affect muscle function. Collectively, our findings provide novel information concerning the signals that drive the activation of macrophages in dystrophic muscle in vivo and provide insights into the effects of blocking IFN-γ expression during muscular dystrophy. The results of this investigation suggest that disruptions of IFN-γ signaling in muscles of DMD patients may reduce pathology by promoting the activation of anti-inflammatory M2 macrophages. Furthermore, IFN-γ blockade may enhance muscle regeneration during DMD and lead to preserved muscle function.

Materials and Methods

Animals

C57BL/10ScSn-Dmdm2Tg+/J and B6.129S7-Ifngtm1Ts/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in pathogen-free vivaria at the University of California, Los Angeles. B6.129S7-Ifngtm1Ts/J mice were crossed onto the C57BL/6 background for at least eight generations by the vendor. Mice carrying null mutation for IFN-γ (B6.129S7-Ifngtm1Ts/J) mice were crossed with mdx mice using the breeding strategy shown in Fig. 2 to generate mdx mice that were null mutants for IFN-γ (IFN-γ−/−/mdx mice) or background control mdx mice that expressed IFN-γ. Null mutation of IFN-γ expression was confirmed by PCR using primer sets specific for the wild-type allele (forward primer: 5′-AGA AGT TGG AAG GCC CCA GAA G-3′; reverse primer: 5′-AGG GAA ACT GGA AGA GGA GAA ATA TTA T-3′) and the neo cassette present in the mutant allele (forward primer: 5′-TCA GCG CAG CCG CCG GTT CTT T-3′; reverse primer: 5′-ATC GAC GAG ACC GCC TTC CAT CCG-3′). Null mutation of the dystrophin gene was confirmed using mdx-amplification-resistant mutation system PCR (19). All animals were handled according to guidelines approved by the Chancellor’s Animal Research committee at the University of California, Los Angeles.

Assay for muscle membrane lesions in vivo

Muscle membrane lesions were assessed by assaying for the presence of the extracellular matrix marker dye procor orange within muscle fibers (20). Procion orange was selected as a marker of membrane lesions, because it is a vital dye that is not actively transported across cell membranes, instead entering through membrane lesions. After euthanasia, the solei of 4- and 12-wk-old mice were dissected and incubated in 0.5% procion orange (Sigma-Aldrich) in Kreb’s Ringer solution for 1 h, followed by three, 5-min rinses in Kreb’s Ringer. The muscles were then rapidly frozen in liquid nitrogen-cooled isopentane. Cross-sections 10 μm thick were taken from the midbelly of each soleus and viewed by fluorescence microscopy. Intracellular fluorescence intensity caused by dye influx was assayed within an 8-μm-diameter, optical sampling circle by flowimetry using a microscope equipped with a digital imaging system (Bioquant, Nashville, TN). Fluorescence intensity was normalized for every fiber present in complete cross-sections of entire midbelly cross-sections of each soleus muscle. Measurements were corrected for background fluorescence measured at a site on the section that contained no tissue. Approximately 760 fibers were assayed in each muscle. Data were expressed as fluorescence intensity in arbitrary units and displayed graphically as the distribution frequency of fiber fluorescence in mdx mice or IFN-γ−/−/mdx mice.

Immunohistochemistry

Frozen serial sections of 4- and 12-wk-old mouse quadriceps were air-dried for 30 min and fixed in ice-cold acetone for 10 min. Sections were blocked for 1 h with 3% BSA and 0.05% Tween 20 diluted in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl. Sections were then probed with anti–IFN-γR1 (1/20; BD Biosciences), anti-F4/80 (obtained by ammonium sulfate precipitation from HB-198 hybridoma cultures; American Type Culture Collection) or anti-CD206 (Serotec) for 3 h at room temperature. Sections were washed with PBS and then probed with biotin-conjugated secondary Abs (1/200; Vector Laboratories) for 1 h at room temperature. Sections were subsequently washed with 50 mM sodium phosphate buffer (pH 7.4) containing 200 mM sodium chloride (PBS) and then incubated for 30 min with avidin D-conjugated HRP (1/1000; Vector Laboratories). Staining was visualized with the peroxidase substrate 3-aminio-9-ethylcarbazole (Vector Laboratories), yielding a color reaction for every fiber present.

The numbers of immunolabeled inflammatory cells per unit volume of muscle were measured in muscle cross-sections using previously described morphometric techniques (4). The total number of either F4/80+ macrophages or CD206+ macrophages in each section was counted microscopically. The total volume of each section was determined by measuring the area of each section using a stereological, point-counting technique (4) and then multiplying that value by the section thickness (10 μm).

Muscle macrophage isolation

Muscle macrophages were isolated using a previously described procedure (10). Hind-limb muscles of four to six mice were dissected and pooled in a 10-cm plate containing cold PBS. Muscles were cleared of discernible fat, rinsed with fresh PBS, and weighed. Dissected muscles were then minced to a fine pulp with surgical scissors and placed into 50-ml conical tubes, which received 10 ml/g muscle mass of collagenase type IV (1 mg/ml) in DMEM. Tubes were incubated in a rotatory incubator at 37°C for two 45-min periods. After the initial 45 min of collagenase treatment, undigested muscle was allowed to settle for 2 min. The resulting cell suspension was aspirated, centrifuged in 50-ml conical tubes at 800 × g, and resuspended with PBS. The remaining undigested muscle was further digested in fresh collagenase buffer for a second 45-min period, after which the cell suspensions were centrifuged, resuspended, and pooled with cells from the first 45-min digestion. Pooled single-cell suspensions were then filtered through a 70-μm cell strainer and subsequently centrifuged at 800 × g for 5 min. Filtered, single-cell suspensions were then applied to 1 ml Histopaque 1077 and centrifuged at 1000 × g for 30 min. Cells were collected from the Histopaque and DMEM interface, washed with complete DMEM, and counted. Approximately 80% of the cells collected from the interface were F4/80 positive.

Macrophage-mediated cytotoxicity assay

Macrophage-mediated cytotoxicity was assessed with a previously reported assay (4, 10). Ninety-six-well plates were seeded with 15,000 C3H/10T1/2 cells per well in complete medium (DMEM containing 10% FBS and 1% penicillin and streptomycin) and allowed to reach confluency before overnight serum starvation to trigger fusion. Following serum starvation,
Two hundred percent cytotoxicity was set as the [51Cr] released into the media released spontaneously by myotubes cultured without macrophages. One

FIGURE 2. Diagram of breeding strategy used to generate IFN-γ−/−/mdx mice and background strain controls.

cells were returned to complete medium to differentiate for 3 d. Prior to coculturing with muscle macrophages, myotubes were incubated with 0.4% [51Cr] in HBSS assay buffer (HBSS containing 0.25% FBS and 400 μM L-arginine) for 2 h. Muscle macrophages isolated from mdx and IFN-γ−/−/mdx mice were cocultured with [51Cr]-treated myotubes at concentrations of 5 × 10⁴, 6 × 10⁴, or 7 × 10⁴ macrophages/well in 150 μl HBSS assay buffer, using 96-well plates. Following 24 h of coculturing, 75 μl of media was collected from each well, and [51Cr] release was measured using a Beckman γ 5500 liquid scintillation counter. Cytotoxicity was expressed as a percentage of total lysis by setting 0% as [51Cr] released spontaneously by myotubes cultured without macrophages. One hundred percent cytotoxicity was set as the [51Cr] released into the media by myotubes incubated with 0.1% Triton X-100 in HBSS assay buffer.

Western blot analysis

The expression levels of iNOS, MyoD, and myogenin were assayed by Western blot analysis. Equal loading of samples was determined by staining nitrocellulose blots with 0.1% Ponceau S solution (Sigma-Aldrich). Nitrocellulose membranes blocked with 3% milk were probed with rabbit Abs against mouse iNos (1/300; Upstate Biotechnology) for 3 h at room temperature. Membranes were then washed with PBS containing 0.05% Tween 20 and probed with HRP-conjugated donkey anti-rabbit IgG (1/10,000; Amersham Biosciences) for 1 h at room temperature. Membranes were washed, and the expression levels of iNOS were visualized by chemiluminescent substrate and a fluorochem imaging system (Alpha Innotech). Nitrocellulose membranes blocked with 3% milk were probed for relative quantities of MyoD or myogenin using mouse anti-MyoD (1/150; BD Biosciences) and HRP-conjugated anti-mouse IgG secondary Ab.

RNA isolation and quantitative PCR

Frozen muscles were homogenized with a mortar and pestle while partially submerged in liquid nitrogen. One milliliter of TRIzol (Invitrogen) per 50 mg tissue was pipetted directly into the mortar and pestle and further homogenized while submerged in liquid nitrogen. The resulting homogenized powder was thawed and transferred to 2-ml centrifuge tubes, and RNA was extracted according to the manufacturer’s protocol (Invitrogen). RNA from freshly isolated muscle macrophages was isolated with RNeasy spin columns (Qiagen). Muscle and macrophage RNA samples were further cleaned and DNase treated using RNeasy spin columns according to the manufacturer’s protocol. Five hundred nanograms of total RNA was reverse transcribed with SuperScript Reverse Transcriptase II using oligo dTs to prime extension (Invitrogen). The resulting cDNA was used to measure quantitatively the expression of genes involved in classical and alternative activation using SYBR Green Quantitative PCR (qPCR) Master Mix according to the manufacturer’s protocol (Bio-Rad).

Real-time measurements of gene expression were performed with an iCycler thermocycler system and iQ5 optical system software (Bio-Rad). cDNA resulting from reverse transcription PCR was used to measure the expression of genes associated with M1 and M2 macrophages using SYBR Green qPCR Master Mix according to the manufacturer’s protocol (Bio-Rad). Primers used for PCR to assay relative levels of expression of muscle-specific and immune cell-specific genes are shown in Table I. We determined empirically reference genes that did not vary between the experimental groups in our investigation by assaying nine reference genes in murine skeletal muscle using geometric averaging (geNorm Visual Basic version 3.5) (21). Primers used for PCR to assay expression levels of reference genes are shown in Table II. After identifying the most stable genes between the groups analyzed, we assayed the ideal number of reference genes for normalization. On the basis of this assessment, TPT1 and RPL13A were selected as reference genes for data normalization.

Assessment of muscle fiber regeneration

Muscle fibers that have experienced injury and repair contain centrally located nuclei, which provide a morphological indicator of fibers that are undergoing regeneration (22). The percentage of total muscle fibers containing central nuclei that were present in complete cross-sections of entire soles muscles stained with hematoxylin was determined by light microscopy.

Muscle cell proliferation assay

A previously reported flow cytometry-based assay was used to study the effect of IFN-γ on muscle cell proliferation (23, 24). GFP-expressing C2C12 cells were labeled with the membrane-intercalating dye CellVue

FIGURE 1. Schematic of time course of mdx muscle pathology and changes of predominant macrophage phenotype (4, 10, 11, 15, 31).
Claret, according the manufacturer’s instructions. After labeling, 200,000 cells were plated in tissue culture plates (BD Biosciences) with complete media and cultured at 37°C and 5% CO₂. After 72 h of culturing in the presence or absence of IFN-γ (10 ng/ml), cells were detached with a 0.05% solution of trypsin-EDTA, and fluorescence intensity was analyzed with a BD FACSCalibur flow cytometry. C₂C₁₂ cells grown in serum-free conditions served as a nonproliferative population, which retained high fluorescence intensity of CellVue Claret labeling. Increases in muscle cell proliferation were reflected by an increase in the proportion of cells with low fluorescence intensity (CellVue Claret<sup>low</sup> cells).

Assessment of muscle function

Running protocol. Mice ran on an Exer 3/6 treadmill (Columbus Instruments) containing a shock grid that stimulated mice to run at a speed of 8 m/min with a 5° incline. The shock stimulus was provided at an intensity of 3.4 mA for 200 ms at 3 Hz. The end runtime was recorded when a mouse stopped to rest for a period of 10 continuous seconds on the shock grid. Mice were run for a maximum of 60 min. Data are expressed as the mean maximum running time of 20 mice/group.

Wire-hang test. We used a variation of a wire-hang test to assess the effect of IFN-γ null mutation on muscle function and strength (15, 25). Three trials for each mouse were performed, and data were expressed as the mean hang-time of the averaged trial times of 15–20 mice tested. To allow mice to recover and minimize fatigue and distress, mice were allowed to rest for 1 min between trials.

**FIGURE 3.** IFN-γ promotes muscle fiber membrane damage in 12 wk-old mdx mice. A and B, The intracellular fluorescence intensity of muscle fibers in muscle incubated in a fluorescent, extracellular marker dye, propidium orange, was used as an index of muscle fiber membrane damage. Five mice were analyzed in each data set. At 4 wk of age (A), there is no difference between the mean cytosolic fluorescent intensity of mdx fibers (blue) and IFN-γ<sup>−/−</sup>/mdx fibers (red). At 12 wk of age (B), intracellular fluorescence intensity of muscle fibers in IFN-γ<sup>−/−</sup>/mdx muscles was less than in mdx muscles, indicating that IFN-γ promotes muscle fiber injury during the regenerative stage of dystrophinopathy. Wild-type C57 muscle fibers showed no intracellular fluorescence above background levels, which were set at the value “0” (4). C–H, Representative images of soleus muscles used to quantify muscle fiber injury are shown for each group. Scale bar, 100 μm. J, No significant differences in the numbers of F4/80<sup>+</sup> macrophages occurred between IFN-γ<sup>−/−</sup>/mdx and mdx muscles at either 4 or 12 wk of age. Each sample is a muscle from a separate mouse for each genotype and age group: 4-wk-old wild-type (n = 5), 4-wk-old mdx (n = 5), 4-wk-old IFN-γ<sup>−/−</sup>/mdx (n = 5), 12-wk-old wild-type (n = 5), 12-wk-old mdx (n = 5), and 12-wk-old IFN-γ<sup>−/−</sup>/mdx (n = 5). Error bars, SEM. J, Cytotoxicity assays to assess macrophage-mediated lysis of muscle cells in vitro that macrophages isolated from muscles of 12-wk-old mdx mice are unaffected by IFN-γ<sup>−/−</sup> mutation. Each bar represents the mean of three samples.

**FIGURE 4.** IFN-γ null mutation decreases INOS expression of muscle macrophages isolated from 4- and 12-wk-old mdx mice. A, Hind-limb muscles were used for RNA isolation and qPCR for INOS expression. Data show that INOS mRNA levels do not change significantly between 4 and 12 wk of age in either mdx or IFN-γ<sup>−/−</sup>/mdx muscles and that ablation of IFN-γ expression has no effect on INOS mRNA levels at either age. Each bar represents the mean and SEM for the muscles collected from five mice in each data set. B and C, Macrophages that were isolated from hind-limb muscles of mdx mice or IFN-γ<sup>−/−</sup>/mdx mice were assayed by Western blotting for relative levels of INOS expression. Membranes were stained with Ponceau red before Ab-binding to confirm equal loading of the gel (“Loading”). Densitometry of the Western blots shows that INOS concentration declines in mdx mice between 4 wk and 12 wk of age, and that ablation of IFN-γ<sup>−/−</sup> reduces INOS in muscle at both ages. Each sample consists of total muscle macrophages isolated from the hind-limbs of one mouse. *p < 0.05, significantly different from age-matched sample. †Significantly different from mice of same genotype at 4 wk of age. Each bar represents the mean and SEM of the Western blot for three samples per data set. D, qPCR data for transcripts associated with M1 macrophage activation in RNA isolated from muscle samples. Each bar represents the mean and SEM for the muscles collected from five mice in each data set. *p < 0.05, significantly different from age-matched sample. †Significantly different from mice of same genotype at 4 wk of age.
Statistics

Statistical analysis was performed in InStat version 2.03 (GraphPad Software). For multifactorial comparisons, we performed a Kruskal–Wallis test to determine statistical significance, followed by a post hoc Student t test to determine significance of differences between two groups. Significance was accepted at \( p < 0.05 \).

Results

IFN-\( \gamma \) promotes muscle fiber damage during the regenerative stage of \( mdx \) dystrophy

Assays of muscle membrane lysis were used to examine the role of IFN-\( \gamma \) in the acute onset stage and regenerative stage (Fig. 1) of muscular dystrophy by comparing IFN-\( \gamma \) \( 2/2 \)/\( mdx \) mice to \( mdx \) background strain controls (Fig. 2). Fluorescence intensity of the cytosol of muscle fibers in soleus muscles incubated with procion orange did not differ between 4-wk-old \( mdx \) and IFN-\( \gamma \) \( 2/2 \)/\( mdx \) mice, indicating that IFN-\( \gamma \)–mediated processes do not contribute to muscle fiber injury during the acute degenerative stage of dystrophinopathy (Fig. 3A). However, ablation of the IFN-\( \gamma \) gene in 12-wk-old \( mdx \) mice reduced cytosolic fluorescence, suggesting that IFN-\( \gamma \) either promotes chronic muscle fiber injury or prevents repair (Fig. 3B–H). We then tested whether the reduction of fiber damage in 12-wk-old, IFN-\( \gamma \) \( 2/2 \)/\( mdx \) mice reflected lower concentrations of macrophages in muscles and found that macrophage numbers in \( mdx \) muscle declined between 4 and 12 wk of age, but macrophage numbers did not differ between \( mdx \) and IFN-\( \gamma \) \( 2/2 \)/\( mdx \) mice at either 4 or 12 wk of age (Fig. 3I).

Table I. Primers used to assay expression levels of immune cell- and muscle cell-specific genes

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*National Center for Biotechnology Information reference sequence.
IP-10, IFN-\( \gamma \)–inducible protein-10.

Table II. Primers used to assay reference genes

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the macrophages isolated from 12-wk-old IFN-γ−/−/mdx mice were less cytotoxic than macrophages in mdx muscles from mice that expressed IFN-γ but found that macrophages from 12-wk-old mdx muscles did not differ in their lysis of myotubes when tested in cytotoxicity assays performed in vitro (Fig. 3). Collectively, these data show that IFN-γ–mediated events contribute to mdx muscle damage between 4 and 12 wk of age but that the IFN-γ–driven damage is not attributable to increases in the numbers or cytotoxicity of muscle macrophages.

Null mutation of IFN-γ in mdx mice increases macrophage activation to the M2 phenotype during the regenerative stage of mdx dystrophy

Expression levels of mRNA and proteins associated with M1 macrophage activation were assayed to determine whether IFN-γ deficiency in mdx mice affected macrophage phenotype in dystrophic muscle. Expression of iNOS by macrophages is the quintessential marker of M1 activation, but real-time qPCR showed no significant difference in iNOS mRNA levels in muscles of 4- or 12-wk-old mdx and IFN-γ−/−/mdx mice (Fig. 4A). However, ablation of IFN-γ in mdx mice at either age caused significant reductions in macrophage iNOS at the protein level, supporting a role for IFN-γ in promoting M1 activation in muscle macrophages at the posttranslational level (Fig. 4B, 4C). Despite the finding that IFN-γ ablation reduced iNOS in mdx muscle, IFN-γ ablation did not affect the expression of other markers that reflect M1 macrophage activation, including IL-6, IP-10, and MCP-1. Furthermore, IFN-γ−/−/mdx mice showed higher levels of expression of TNF-α relative to expression in mdx muscles (Fig. 4D).

FIGURE 5. IFN-γ represses the expression of genes associated with M2 macrophage activation during regeneration. A. Transcript levels of M2-associated genes were measured by qPCR for RNA isolated from hind-limb muscles. Each bar represents the mean and SEM for the muscles collected from five mice in each data set. *p < 0.05, significantly different from age-matched sample. #Significantly different from mice of same genotype at 4 wk of age. B. Density of CD206+ macrophages in quadriceps muscles. Data show that the number of CD206+ macrophages is elevated in dystrophic muscles and that ablation of IFN-γ in mdx muscles further elevates CD206+ cells in 12-wk-old mdx muscles but not in 4-wk-old mdx muscles. Each bar represents the mean and SEM for the CD206+ cell counts for the quadriceps of five mice. *p < 0.05, significantly different from age-matched, wild-type sample. #Significantly different from mice of same genotype at 4 wk of age. §Significantly different from mdx mice at same age.

FIGURE 6. Mdx dystrophy causes increases in the size of M2 macrophages and the level of activation of M2 macrophages. A. Section of a 4-wk-old wild-type quadriceps muscle showing the presence of a few small CD206+ macrophages (arrows) within the endomysium between muscle fibers. B and C. The numbers and size of CD206+ macrophages (red) in muscle increases greatly in muscular dystrophy in 4-wk-old mdx mice (B) and 4-wk-old IFN-γ−/−/mdx mice (C). D. Section of 12-wk-old, wild-type muscle. CD206+ macrophages are indicated by arrows. E and F. Sections of 12-wk-old, mdx muscle (E) and 12-wk-old IFN-γ−/−/mdx muscle (F) showing large CD206+ macrophages (red) within a region of muscle regeneration. All micrographs are shown at the same magnification and all sections were labeled under identical conditions. Scale bars, 50 μm.
Changes in expression levels for transcripts that are associated with M2 macrophage activation were also assessed by qPCR (Table I) and normalized to empirically validated reference genes (Table II). Mutation of IFN-γ in mdx mice caused a significant increase in expression of all genes associated with M2 macrophages, including arginase-1, arginase-2, FIZZ-1, Mgl-2, CD206, and IL-4 (Fig. 5A). The elevations of arginase expression, which increased 3- to 4-fold in 12-wk-old IFN-γ−/−/mdx muscles, may be of particular functional importance, because arginase activity promotes wound healing following tissue damage (26–28). Perhaps most significantly, the expression of CD206, the most widely accepted and specific marker of alternative activation of M2 macrophages (17, 29), more than doubled in 12-wk-old IFN-γ−/−/mdx muscles compared with age-matched mdx muscles. Quantitative immunohistochemical data also showed that ablation of IFN-γ expression in mdx mice caused a significant increase in the number of CD206+ cells in 12-wk-old mdx muscle (Fig. 5B). Notably, the magnitude of the increase in CD206 expression in 12-wk-old IFN-γ−/−/mdx muscles compared with 12-wk-old mdx muscles (Fig. 5A) was greater than the magnitude of the increase in the numbers of CD206+ macrophages in 12-wk-old IFN-γ−/−/mdx muscles (Fig. 5B). This may be attributable to the increase in the level of expression of CD206 per M2 macrophage, which was evident in immunohistochemical assays (Fig. 6). Collectively, these findings indicate that IFN-γ is a strong negative regulator of macrophage activation to the M2 phenotype and may potentially slow muscle repair during the regenerative stage of mdx dystrophy.

**FIGURE 7.** IFN-γ inhibits proliferation and differentiation of myogenic cells. The IFN-γR was expressed on the surfaces of muscle cells at higher levels in mdx mice at 4 wk (B) and 12 wk of age (D) compared with age-matched, wild-type controls (A, C). E, Flow cytometric analysis of muscle cell proliferation showed that IFN-γ inhibits muscle cell proliferation of GFP-expressing C2C12 cells. C2C12 cells that did not express GFP and were not labeled with CellVue Claret (E, left panel) were used to set quadrant markers. F, Western blots of muscle cells treated with IFN-γ or media only (“Control”) in vitro show that IFN-γ reduces the level of myogenin expression without affecting the level of MyoD expression. G, Phase contrast images of C2C12 cultures show myotube growth is inhibited by IFN-γ. IFN-γ was added at the indicated concentrations following induction of cell differentiation, and then, muscle cells were incubated for 5 d to allow muscle cell differentiation and myotube growth. Scale bar, 160 μm. H and I, Relative transcript levels of MyoD and myogenin in whole-muscle extracts were measured by qPCR. Each bar represents the mean and SEM for the qPCR of RNA isolated from the muscles collected from five mice in each data set. H, MyoD expression in mdx muscle declined between 4 and 12 wk of age. However, muscle from IFN-γ−/−/mdx mice showed no change in MyoD expression between these ages. I, Muscles from IFN-γ−/−/mdx mice showed an insignificant trend for higher levels of myogenin expression compared with mdx mice. J, The proportion of central-nucleated, regenerating fibers was counted and expressed as a percentage of total fibers counted in entire cross-sections of soleus muscles. Each bar represents the mean and SEM for the central-nucleated fiber counts from muscles collected from five mice in each data set. The proportion of regenerating fibers increased at 12 wk, but no difference was observed between mdx and IFN-γ−/−/mdx mice at either 4 or 12 wk of age. *p < 0.01, significantly different from 4-wk-old mdx. †p < 0.01, significantly different from 12-wk-old mdx. ‡p < 0.05, significantly different from 4-wk-old mice of same genotype.
IFN-γ inhibits muscle cell proliferation and differentiation in vitro

Because our immunohistochemical observations showed that the expression of the IFN-γR at the surface of muscle cells is greater in 4- and 12-wk-old mdx mice compared with C57 control mice (Fig. 7A–D), we tested whether IFN-γ influenced muscle proliferation or differentiation or affected the regeneration of mdx muscle. We used a flow cytometry-based assay to measure proliferation of GFP-expressing C2C12 cells in the presence or absence of IFN-γ. In this technique, fluorescent membrane-intercalating dyes in cells undergoing division are partitioned between newly generated daughter cells. As a result, the fluorescence intensity of daughter cells is reduced compared with the initially stained parent cells (23). C2C12 myoblasts expressing GFP that were grown in serum-free conditions that attenuated cell proliferation showed high fluorescence intensity with the fluorescent membrane-intercalating dye. Lower fluorescence intensity was observed when cells were grown in growth media, which promotes muscle 

FIGURE 8. Null mutation of IFN-γ in mdx mice improves muscle function in 12-wk-old mice. A, The mean maximum run time is shown for mice that ran uphill on a treadmill at a speed of 8 m/min at a 5˚ incline. Each sample is a separate mouse for each genotype and age group: 4-wk-old mdx (n = 19), 4-wk-old IFN-γ−/−/mdx (n = 18), 12-wk-old mdx (n = 15), and 12-wk-old IFN-γ−/−/mdx (n = 20). Error bars, SEM. B, Muscle function was assessed using the hang-wire test to measure front paw grip strength. Each sample is a separate mouse for each genotype and age group: 4-wk-old mdx (n = 20), 4-wk-old IFN-γ−/−/mdx (n = 20), 12-wk-old mdx (n = 20), and 12-wk-old IFN-γ−/−/mdx (n = 20). *p < 0.05, significantly different from 12-wk-old mdx. **p < 0.01, significantly different from 4-wk-old mdx.

Discussion

The findings in the present investigation generally support our hypothesis that disruption of IFN-γ-mediated signaling in dystrophic muscle would decrease the pathophysiology of muscular dystrophy. The reductions in pathology were evident at the gross level, where ablation of IFN-γ in mdx mice caused more than a doubling of treadmill running time, and also at the histological level, where deletion of IFN-γ caused significant reductions in muscle fiber injury. Our data also show that the beneficial effects of IFN-γ mutation could be mediated through more than one pathway. IFN-γ mutation affected the balance in M1 and M2 macrophage populations in mdx muscle, causing a shift to the M2 phenotype that can promote muscle growth and repair following injury. However, our findings show that the beneficial effects of IFN-γ deletion may also reflect a loss of direct effects on muscle cells, through which IFN-γ can inhibit proliferation of myogenic cells and delay their differentiation. Each of these beneficial effects of IFN-γ mutation on the pathology of mdx dystrophy were apparent at 12 wk of age, when levels of mdx fiber damage are relatively low and muscle regeneration is a prominent component of the pathology.

Although our findings show clear, beneficial effects of ablating IFN-γ in 12-wk-old mdx mice, we were surprised to learn that blocking IFN-γ signaling does not affect muscle fiber injury during the acute, degenerative stage at 4 wk of age or influence gross motor function in 4-wk-old mdx mice. We anticipated a positive treatment effect in 4-wk-old mice, because previous studies showed that muscle macrophages isolated from 4-wk-old mdx mice are predominantly M1 activated, and they are more cytotoxic than macrophages isolated from 12-wk-old mdx muscles (10). Furthermore, IFN-γ stimulation of muscle macrophages that were isolated from 4-wk-old mdx mice increased their cytotoxicity, and IFN-γ expression in 4-wk-old mdx muscles is higher than in wild-type controls (10). Nevertheless, IFN-γ depletion did not affect the cytotoxicity of macrophages that were isolated from mdx muscles and did not reduce mdx muscle fiber damage at 4 wk of age and produced only small reductions in iNOS expression by muscle macrophages isolated from 4-wk-old mdx mice. Taken together, the findings show that M1 macrophage cytotoxicity at the early, acute stage of mdx pathology is primarily driven by factors other than IFN-γ. Although TNF-α would have been a candidate cytokine for promoting M1 activation in mdx muscle because it has the capacity to promote M1 activation and cytotoxicity (29) and its expression is elevated in mdx muscle (30), our current findings do not support that role for TNF-α in mdx dystrophy. On the contrary, ablation of IFN-γ caused significant
The complex picture that is emerging from studies of the interactions between inflammatory cells and muscle in muscular dystrophy shows that broad approaches to attenuating the inflammatory response are likely to have negative effects in addition to the desired and expected positive effects on the pathophysiology. As emphasized by the current study and other investigations (36–40), use of nonspecific anti-inflammatory drugs or other immunosuppressants could reduce inflammatory cell-mediated damage but could also suppress proregenerative effects of immune cells. Furthermore, interventions that are intended to reduce inflammatory cell involvement in muscular dystrophy can also have direct deleterious effects on muscle cells. For example, cyclosporine A has been used for the treatment of DMD in which it was intended to suppress immune cell involvement in the disease (41). However, cyclosporine A is not an anti-inflammatory, per se. Cyclosporin A is an inhibitor of calcineurin that mediates many signaling pathways in multiple cell types, including muscle cells. For example, calcineurin activation is essential for normal patterns of gene expression during muscle adaptation (42), and blocking calcineurin signaling with cyclosporine A prevents muscle growth in response to increased muscle loading (43, 44). Thus, its application to DMD patients could impair the regenerative capacity of the muscle, even while reducing inflammation, to yield a net increase in muscle pathology. Finally, as illustrated by the current findings, the efficacy and mechanism of action of immune-based interventions can change with the stage of the disease at which the treatments are applied. Because the composition of the immune cell infiltrate in dystrophic muscle varies during the course of the disease and the effector molecules such as cytokines that are produced by inflammatory cells are pleiotropic proteins, the efficacy of immune-based interventions in DMD will likely be specific to the stage of the pathology. Thus, future immune-based strategies for treating muscular dystrophy can be improved by targeting specific immune cell populations and by applying the interventions at carefully selected stages of the disease.

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Disclosures
The authors have no financial conflicts of interest.

References

increases in TNF-α expression in 12-wk-old muscle, whereas muscle fiber damage declined. Furthermore, and perhaps of broader significance, our finding that muscle macrophages from 4-wk-old IFN-γ−/−/mdx mice exhibited the M1 phenotypic characteristic of elevated iNOS expression shows that IFN-γ is not required for classical activation of macrophages, at least in muscular dystrophy. This observation is contrary to the canon that IFN-γ is required to condition macrophages for classical activation (29).

Although previous investigations have shown that IFN-γ is a strong inducer of the M1 macrophage phenotype, its role in promoting mdx pathology appears to be more directly attributable to its suppression of the M2 phenotype. In addition to the ineffectiveness of IFN-γ ablation for reducing muscle damage caused by M1 macrophages, IFN-γ mutation did not have a uniform effect on suppressing markers of M1 activation in vivo. On the contrary, loss of IFN-γ nearly doubled the expression of TNF-α, which typically reflects Th1 inflammatory responses that include elevated numbers of M1 macrophages while having no effect on the expression levels of other genes associated with the M1 phenotype. In contrast, IFN-γ mutation in 12-wk-old mdx mice produced either significant elevations of all transcripts associated with M2 activation, suggesting that IFN-γ induced either significant elevations of all transcripts associated with M1 macrophage cytotoxicity. In particular, M2 macrophages in mdx muscles express arginase that hydrolyses arginine (10, 31), thereby depleting arginine availability for iNOS. This substrate competition leads to a reduction in NO production by M1 macrophages and a suppression of M1 macrophage cytotoxicity (10). Thus, IFN-γ suppression of the M2 phenotype reduces substrate competition for arginase by arginase, increasing arginase availability for iNOS in M1 macrophages and thereby increasing iNOS-mediated pathology without induction of the M1 phenotype or promoting iNOS expression.

Some of the beneficial effects of IFN-γ on mdx pathology at 12 wk of age may also reflect the removal of negative influences that IFN-γ exerts on the regenerative process through both direct and indirect actions on muscle. We found that IFN-γ acts directly on muscle cells in vitro and inhibits the proliferation of C2C12 myoblasts, which is consistent with previous reports that IFN-γ also inhibits the proliferation of human myoblasts (32). However, other investigators showed that treating myoblasts in vitro with neutralizing Abs to the IFN-γR reduced C2C12 proliferation (33), suggesting that IFN-γ promotes rather than inhibits proliferation. However, these results are not necessarily in conflict. Levels of endogenous IFN-γ production by muscle are much lower than levels that occur in inflamed muscle, and previous investigators have shown a dose dependency on the effects of IFN-γ on muscle proliferation. Low concentrations of IFN-γ (1–100 U/ml) increase muscle cell proliferation (34), whereas stimulation of myoblasts with high doses of IFN-γ (>1000 U/ml) inhibits proliferation (35). We also observed that IFN-γ treatment of muscle cells in vitro reduced the concentration of myogenin and decreased cell fusion, without affecting levels of MyoD expression, indicating that inhibition of differentiation is a direct effect on muscle cells. However, IFN-γ deficiency in mdx mice caused an increase in MyoD expression at 12 wk. This finding of an IFN-γ-mediated effect on MyoD expression in vivo in the absence of an effect in vitro may reflect an indirect influence, possibly through IFN-γ induction of a suppressor of MyoD expression by a non-muscle cell type.


