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IL-4 Receptor as a Bridge Between the Immune System and Muscle in Experimental Myasthenia Gravis I. Up-regulation of Muscle IL-15 by IL-4

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Abstract

The study reported below describes increased expression of IL-4 receptor in cultured rat myocytes following exposure to an antibody reactive with the acetylcholine receptor (AChR). In addition, upon up-regulation of IL-4R, myocytes demonstrated an increased responsiveness to IL-4 by producing increased levels of IL-15. Moreover, following passive transfer of AChR antibody into Lewis rats, both the increased IL-4R expression and IL-15 production was also observed in intact skeletal muscle, co-localizing in particular individual muscle fibers; the same muscle fibers also produced the chemokine MCP-1 to which IL-4-producing T cells were attracted. A model is proposed in which these muscle activities participate in disease progression in experimental myasthenia gravis.

Keywords

autoimmune disease; muscle IL-4 receptor; muscle disease; muscle IL-15; myasthenia gravis

INTRODUCTION

Neuromuscular dysfunction in myasthenia gravis (MG) patients is associated with autoantibodies directed against the nicotinic acetylcholine receptor (AChR) expressed by skeletal muscles. Binding by these AChR antibodies in the neuromuscular junction results in impaired receptor function, diminished neuromuscular transmission, and the common symptoms of weakness and rapid-onset fatigue (1–3). However, it is interesting to note that early studies showed that relationships between levels of autoantibodies produced by particular patients and the severity of their disease symptoms were poorly correlated (4–7); no striking improvement has been noted in recent years of our understanding of this weak relationship.

Models of this disease have frequently been studied in certain MG-susceptible rodents, such as the Lewis rat. Investigations reported from this laboratory have examined both quantitative as well as qualitative characteristics of AChR antibodies that may influence the severity of neuromuscular disease symptoms demonstrated by rats (8–11). However, studies have also

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shown that muscles are able to respond to immune attack during experimental MG by producing muscle-derived, immunologically relevant products such as chemokines, cytokines, and surface molecules with the abilities to activate and promote interactions with cells of the immune system (12–20). These muscle-derived immune mediators may subsequently influence the severity of disease symptoms by amplifying or dampening immune responsiveness.

Several of these observations have led to the study presented below. First, an early study indicated that transfer of the AChR-reactive monoclonal antibody, mAb35 (21), resulted in increased IL-15 expression in skeletal muscles of Lewis rats (15), but that this observation was contradicted by *in vitro* studies using the LE1 monoclonal Lewis rat myocyte cell line (13). This contradiction was consistent with an indirect influence of mAb35 on IL-15 up-regulation *in vivo*, requiring another signal not available *in vitro*. It was later determined that IL-4 was able to stimulate increased levels of IL-15 in the LE1 myocyte line (14). Therefore, to integrate these observations in the currently reported studies, the hypothesis tested was that IL-4 is able to stimulate IL-15 production in skeletal muscle, but that the effectiveness of that stimulation is dependent upon increased expression of the receptor for IL-4 (*i.e.*, IL-4R) resulting from prior activation of the muscle with AChR antibody. Finally, another previous observation that lead to the formulation of the hypotheses tested in the present study was that upon transfer of mAb35 into Lewis rats, there is rapid increased expression of the chemokine MCP-1 in both LE1 cells, as well as in skeletal muscle, as a prelude to increased movement of leukocytes through muscle (17); some of these leukocytes that enter the muscle are $CD4^+$ T cells that produce IL-4. Movement of increased numbers of such T cells may provide an effective means to deliver IL-4 to the muscle, thereby increasing muscle expression of IL-4-induced IL-15.

MATERIALS AND METHODS

Rats

Inbred female Lewis rats (8–10 wks old) were purchased from Charles River Laboratories and housed under the guidelines set up by the University of Texas Health Science Center, San Antonio (San Antonio, TX) Institutional Animal Care and Use Committee.

Antibodies

AChR-reactive antibody—A potent disease-causing rat IgG1 monoclonal antibody (mAb35) with reactivity against the main immunogenic region (MIR) of AChR was used as in numerous past studies from this laboratory, both *in vitro* and *in vivo* (15–20). mAb35, first described by Tzartos et al (21), is routinely prepared from the culture fluids of the hybridoma (ATCC 175) obtained from the American Tissue Culture Collection (Rockville, MD) and maintained in this laboratory as in past studies (8). The mAb35 hybridoma, grown in serum-free SFM-Hybridoma medium (GIBCO, Santa Clara, CA), produces up to 25 μg/ml that can be purified on hydroxyapatite columns (Sigma Chemical Co., St. Louis, MO) (22). All purified samples used in these studies were tested for purity by SDS-PAGE and by ELISA for binding activity against AChR. Purified mAb35 was stored frozen as 1 mg/ml stock solutions in PBS. As a stimulus in tissue culture experiments, a dose of 20 μg/ml was used; when transferred i.v. into rats, a dose of 50 μg was used.

For immunostaining and Western blot analyses—Combinations of antibodies used in immunofluorescence evaluations are shown in Table 1. Rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology against IL-4Rα (S-20), IL-15 (H-114 and L-20), NCAM (C-20), MCP-1 (R-17) and actin (H-300). The rabbit polyclonal antibody against IL-4 was from Biosource. The mouse monoclonal antibody against CD4 was from AbD Serotec. Rat polyclonal antibody against rabbit IgG was obtained from Rockland Inc. Goat polyclonal

anti-rabbit Ig was obtained from Sigma-Aldrich Inc. Donkey anti-rabbit IgG, as well as goat anti-mouse Ig was from Jackson ImmunoResearch Laboratories Inc.

Passive antibody transfer

Following a protocol of disease induction optimized in previous studies (8,18), transfers of mAb35 (50 μg) were performed by intravenous injection, followed by immunofluorescence or Western blot assessments of IL-4R expression in skeletal muscle 12–48 hours later, noted in previous studies to coincide with the onset of peak disease intensity in Lewis rats. Although disease induced by the passive transfer of mAb35 is different in certain ways when compared to experimental MG induced following active immunization with AChR, the passive antibody transfer model allows the advantage of examining direct antibody effects on muscle in the absence of the activation of other elements of the immune system. In order to study the combined effects of exposing skeletal muscle to AChR-reactive antibody while making available AChR-reactive T cells, certain passive antibody studies used Lewis rats that had been immunized with the immunodominant AChR 100–116 alpha subunit peptide (shown in earlier studies (23) to activate potent helper T cells, but to be unable to activate B cells directly to antibody production against native AChR, or on its own to induce disease symptoms). At 30 days following alpha 100–116 immunization, 50 μg of mAb35 antibody was injected (i.v.) into the peptide-immune rats; EDL were recovered 48 hours after antibody transfer, frozen-sectioned, and then assayed for muscle fibers producing IL-15 as described below.

LE1 myocyte cell line

The Lewis rat LE1 myocyte clone (13) was derived from the extensor digitorum longus muscle (found in past studies to be highly dysfunctional in MG-induced Lewis rats [24]). It was isolated from a non-transformed polyclonal line by limiting dilution culture techniques and was selected from among several clones produced based on its ease of maintenance in culture and for the stability of its phenotype. To summarize some key features, LE1 myocytes express the myocyte-associated isoform of the neural cell adhesion molecule (NCAM), as well as the AChR. In addition, the highly proliferative mononuclear myoblast form of LE1 can be induced to differentiate and fuse into a more mature form, the non-proliferating multinucleated pre-fiber myotube. Myotube formation is also accompanied by a marked increase in the expression of myosin heavy chain and AChR expression compared to the less mature myoblasts. As described previously, LE1 cells have been observed to produce a number of immunologically relevant factors in response to cytokines and/or AChR-reactive antibodies (12–14,16)

Primary muscle cell cultures

Small fragments cut from individual EDL muscles were smashed and soaked for 15 minutes at 37°C in a 60mm culture dish (Corning) containing 0.25% trypsin with 1mM EDTA. Fibrous material was allowed to settle out and the supernatant containing freed cells was recovered. DMEM (Gibco) with 10% fetal bovine serum (Sigma-Aldrich) was added to the supernatant to stop the trypsinization reaction. Cells dislodged from the muscle were recovered by centrifugation at 100xg for 10 minutes at 4°C. Treatment of the muscle fragments was repeated two more times. Cells recovered from each EDL muscle were pooled, washed, and then incubated at 37°C for 30 minutes in 2 ml of DMEM with 10% FBS (~10^7 cells/ml) in 6-well plates (Corning). During this time, fibroblasts, the most adherent cells in the mixture, were allowed to stick to the plate. Cells that were not bound to the plate were removed, which included myoblasts that are adherent cells, but less so than fibroblasts. This procedure was repeated a second time under the same conditions. In the typical experiment, approximately 30–50 myocytes were recovered per EDL muscle from groups of 3–5 rats, and based on immunostaining with antibody against the muscle isoform of an adhesion molecule, NCAM, fractionated cells were enriched to 50–75% myocytes. Upon removal of the fibroblasts by this

series of adherence steps, the myocytes left in the supernatant were allowed to adhere overnight to the culture dish. The primary myocytes were then grown for 4–5 days in fresh medium, during which time they were exposed to various stimuli.

B cell line

The cloned mouse B cell line BCL1-3B3 (clone CW13.20-3B3; ATCC) was kindly provided by Dr. Michael Berton of this department and was maintained in RPMI medium with 10% fetal calf serum at 37°C and 5% CO_2.

Primary splenocyte cultures

Lewis rat spleen cell suspensions were treated with 140 mM NH_4Cl in 17mM Tris-HCl, pH 7.2 for 5 minutes at room temperature to lyse red blood cells. Treated cells were washed by centrifugation in DMEM with 5% FBS at 4°C. The splenocytes (~10^7 cells/ml) were then cultured at 37°C in a 6-well plate (Corning) in the presence or absence of IL-4 (PeproTech) at 45 ng/ml (30 U/ml) for 10 minutes.

Immunofluorescent staining

Cover slip-cultured cells—Since LE1 cells and primary myocytes are highly adherent to plastic and glass surfaces, they were grown on sterile microscope cover slips (12 mm; Fisher Scientific) submerged under growth medium in 2 ml culture wells (24-well plates; Corning) as previously described (14). At the end of the culture period, cells on the cover slips were then be fixed in chilled acetone and evaluated by immunofluorescence staining. The coverslips were covered with 10% non-immune serum from the same species as that of the secondary antibody for 30 minutes at room temperature to block non-specific binding. The primary probing antibody was then added at a pre-determined appropriate dilution and incubated overnight at 4°C. After extensive washing with 100 mM Tris 0.05% Tween 20 at pH 7.6 (TNT), the secondary antibody was added at an appropriate dilution and incubated for 30 minutes at room temperature. The coverslip-bound cells were washed with TNT again before being mounted with ProLong Gold antifade (Invitrogen) with Hoechst nuclear stain added at 1:3000. The coverslips were viewed under a Zeiss AxioImager Z1 fluorescence microscope using AxioVision software. Representative stained fields were photographed; for some studies determination of relative staining intensity was performed by densitometric mean analysis (of pixels).

In situ muscle—As described in previous reports (19,20), extensor digitorum longus (EDL) muscles were surgically extracted from groups of 2–4 rats, placed into optimum cutting temperature (O.C.T.) medium, snap frozen in liquid nitrogen-cooled isopentane and stored at −80°C. After thawing to −20°C, the muscles were cut into 10 μm cross-sections on a Shandon SME cryotome. The slides were air dried, acetone fixed and stored at −80°C until used for staining. After permeabilization of the cells with 1% saponin in Hank's balance salt solution (Gibco) the sections were stained using a protocol similar to that described above for cover-slip cultured cells.

Protein extractions

LE1 cells—Adherent LE1 cells in 24-well culture plates (Corning) were washed with 0.1% glucose, 5mM KCl and 137mM NaCl, followed by the addition of a protein extraction buffer (40mM Bis-Tris propane pH 7.7, 150mM NaCl, 10% glycerol, 1% Triton-X 100, 1mM Na_3VO_4 with protease inhibitors PMSF, pepstatin A, leupeptin, aprotinin, phenanthroline and benzamindine HCl). The lysates were spun at 12,000 × g for 15 minutes at 4°C to remove the DNA pellet. The amount of protein remaining was determined by BCA protein assay (Pierce).

EDL muscles—Whole EDL muscles obtained from groups of 2–4 rats were placed into a Pyrex tissue grinder with 1 ml of protein extraction buffer. The dissociated tissue was ground with the pestle every 10 minutes during an incubation that lasted a total of 45 minutes. The homogenate was then treated as described above for the LE1 cells.

Western Blot analysis

Protein extracts from cultures of LE1 myocytes or from EDL muscles were obtained as described above. In each analysis, 50–200 μg of total protein was separated by 7.5% SDS-PAGE, transblotted onto nitrocellulose paper (Whatman), which was then blocked with 5% instant milk in PBS containing 0.05% Tween 20 (PBST) for 1 hour at room temperature. Nitrocellulose blots were then incubated with an optimal concentration of primary antibody overnight at 4°C. After 3 PBST washes, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature in 1% milk PBST. After another series of washes, protein bands were detected using the enhanced chemiluminescence detection system (Amersham Life Science), exposure to Hyperfilm-MP (Amersham), and analyzed with a MultiImage light cabinet (Alpha Innotech Corporation). To remove primary and secondary antibodies for re-probing, blots were incubated at 60°C for 1 hour in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100mM β-mercaptoethanol). After washing in Tris-buffered saline (pH 7.6) with 0.01% Tween 20, blots were blocked and probed again as described above.

RT-PCR

Total RNA extraction and RT-PCR were performed on LE1 myocytes as described in detail previously (14). The sequences of the primers used were previously reported (25). Denaturation was performed at 94°C for 1 minute, annealing at 56°C (60°C for IL-4Rα) for 30 seconds, and elongation at 72°C for 1 minute with a final elongation step at 72°C for 10 minutes. The number of cycles performed were 30 and 22 for IL-4R and GAPDH, respectively. PCR products were separated on 2% agarose gels stained with ethidium bromide. Gels were scanned and data stored using an Innotech Alpha Imager 2000 according to the manufacturer's instructions. Data management and semi-quantification on this system can be performed by comparing ratios of signals found in the linear range of detection and normalized to signals coming from mRNA encoding for GAPDH. Identifications of PCR products were based on both the predicted size of the product (in relation to the primers chosen and the cDNA expected), and by direct sequencing of the products. Oligonucleotide synthesis and sequencing was performed in The Center for Advanced DNA Technologies, housed in the Department of Microbiology & Immunology, UTHSCSA, and directed by Dr. Brian Wickes.

Microarray

When microarray analysis was performed, the T_H1-T_H2-T_H3 Oligo GEArray was used from SuperArray Bioscience Corp. RNA was extracted and tested for the presence of IL-4Rα mRNA (among other things) following the company's instructions (the kit is designed to evaluate T cell populations, but in this case used to evaluate muscle).

Statistical analyses

Statistical comparisons of frequencies of positively stained fibers in muscles obtained from rats exposed to AChR-reactive antibody versus control rats were performed by chi-squared analyses and the students t tests. Values of $\chi^2 \geq 5.99$ represent ≥ 95% confidence level (2 degrees of freedom) with regard to a significant difference between muscles recovered from rats exposed to AChR antibody and muscles from control rats.

RESULTS

The hypothesis tested by the studies reported below is that exposure of skeletal muscle to AChR-reactive antibody during the course of experimental MG in rats leads to 1) an increased expression of the muscle receptor for IL-4; and 2) a subsequently increased opportunity for IL-4-stimulated production of muscle-derived IL-15. The monoclonal mAb35 antibody used in these passive transfer studies is of one AChR specificity, intentionally chosen for its potent disease-causing ability and its presence in the polyclonal antibody response stimulated by active immunization with AChR (8).

LE1 myocytes cultured with AChR antibody expressed mRNA that encodes IL-4Rα chain

The functional IL-4R is composed of two chains, one which is the IL-4Rα chain (25). As reported by other investigators (25), the IL-4-specific chain can be expressed in one of two forms; one form associated with a soluble secreted IL-4R, and the other, the membrane-bound receptor. Similar to those published observations, doublet bands were observed via RT-PCR analysis in the current studies indicating the presence of message for both soluble and membrane forms in cultured LE1 myocytes (Fig. 1). In addition, although not evaluated using quantitative RT-PCR, stronger signals for message were observed in multiple experiments when LE1 cells were exposed to 20 μg/ml of the mAb35 antibody when compared to unstimulated control cells (2–3-fold increases estimated by scanning densitometry). This is consistent with subsequently increased expression of the IL-4Rα protein in cultured myocytes and skeletal muscles stimulated with mAb35.

LE1 myocytes cultured with AChR antibody expressed increased levels of IL-4Rα chain

LE1 myocytes were cultured with either AChR-reactive mAb35 antibody or non-immune rat Ig (nRtIg) for 48 hours prior to protein extraction. Proteins in myocyte extracts were then separated by 7.5% PAGE and assessed for the presence of the IL-4Rα chain by probing Western blots with rabbit antibody against the IL-4Rα chain. Although a low level of constitutive IL-4Rα expression was observed in the absence of mAb35 exposure, a modest increase in expression was observed following exposure to the AChR-reactive antibody (Fig. 2). No such increase was observed when LE1 cells were stimulated with IFN-γ (not shown), a cytokine previously shown to be a potent inducer of other membrane and secreted factors such as ICAM-1, class II major histocompatibility molecules, chemokines, and nitric oxide by these myocytes (13,14,16). Thus, the up-regulation of the IL-4R appears to be selective; that is, not just any stimulus will perform this function. When compared to a B lymphocyte line, BCL1, probed as a positive control, it was noted that the myocyte-derived molecule (approx. 120 kDa) was smaller than the 140 kDa produced by BCL1 and reported as the size of the conventional IL-4Rα produced by lymphocytes (26,27). Furthermore, when LE1 cell IL-4R was compared to another positive control, protein extracts of spleen cells (Fig. 2, inset), the same difference between the sizes of myocyte IL-4Rα and lymphocyte IL-4R was noted. The biochemical explanation for the size difference is not clear at this time. Clearly, the size difference may be due to alternate effects at either transcriptional or translational levels (experiments in progress). In addition, when LE1 myocytes of two different states of maturity were evaluated by immunofluorescence methods as shown in Figure 3, IL-4Rα staining was noted to be 2–3 times brighter in immature, actively dividing myoblasts in comparison to the staining of the further differentiated pre-fiber myotubes.

Increased production of IL-15 in IL-4-activated myocytes was enhanced by previous exposure to mAb35 AChR-reactive antibody

Cultured primary myocytes were first exposed to 20 μg/ml mAb35 or nRtIg, and then stimulated with IL-4. Immunofluorescent staining was used to assess the presence of IL-15 in treated cells. As seen in Figure 4, some IL-15 could be induced in myocytes by IL-4 stimulation

prior to pre-treatment with mAb35, probably due to low constitutive levels of IL-4R shown above. However, following pre-treatment of myocytes with the AChR-reactive antibody that had been observed to up-regulate IL-4R expression, a dramatic IL-4-stimulated increase in IL-15 levels was observed that spread from an initial small rim of staining centered around the cell nucleus to staining that sometimes filled up the entire cytoplasm. The IL-4 dependence of the increased expression of IL-15 was demonstrated by abrogation of the effect in the presence of IL-4 blocking antibody (Fig. 5).

EDL muscles, exposed *in situ* to AChR antibody, demonstrated increased levels of IL-4Rα chain

In order to determine if the observed effects of AChR antibody on IL-4R expression in cultured myocytes could be reproduced *in situ* by skeletal muscles exposed to AChR antibody, 50 μg doses of the mAb35 AChR antibody in saline were administered i.v. to groups of Lewis rats. EDL muscles were recovered 24–30 hours later. From some muscles, RNA was extracted and tested for the presence of IL-4Rα mRNA by microarray. The result shown in Figure 6 indicates that the intensity of the spot for IL-4Rα mRNA (among other things) was elevated compared to that seen in rat muscles that were not exposed to mAb35. Protein (rather than RNA) was also extracted from muscles of similarly treated rats. Figure 7 shows a Western blot analysis of EDL muscles from mAb35-transferred rats. Again, there appeared to be increased levels of IL-4Rα chains present in myocytes from mAb35-transferred rats; further, when compared to "conventional" IL-4 receptors expressed by hematopoietic cells, the IL-4Rα chain from muscle demonstrated the same downward shift in size that was observed in cultured myocytes.

Co-expression of IL-4R and IL-15 was observed in individual muscle fibers

In an attempt to connect increased IL-4R expression with increased IL-15 production, thin sections of muscles allowed determination of production of the two molecules in individual muscle fibers. Thus, EDL muscles obtained from Lewis rats at several time points following transfer of mAb35 were double-stained for IL-4Rα and IL-15. As shown in Figure 8, upper panel, an increase in both IL-4R and IL-15 was observed. Although some constitutive staining was observed for both IL-4R and IL-15 (nRtIg-transferred rats), staining of muscle from mAb35-transferred rats demonstrated both increased numbers of stained fibers and increased brightness of staining of those fibers (implying more IL-4R/IL-15 producing fibers and more IL-4R/IL-15 per fiber). Consistent with the proposed relationship between IL-4 stimulation of muscle, and subsequent production of muscle IL-15, a wave of increased IL-4R expression was observed that peaked at 12 hours, followed by a wave of increased IL-15 expression that peaked at 24 hours (Fig. 8, bottom panel). It is particularly evident at the 24 hour time point that most IL-4R positive fibers were also IL-15 positive.

Increased expression of both IL-4R and IL-15 was associated with muscle fibers also producing MCP-1

Previous studies have shown increased production of the chemokine MCP-1 in EDL muscles of rats following administration of AChR antibody (17). Immunostaining was performed to determine if the muscle fibers associated with MCP-1 production were also those demonstrating increased IL-4R and IL-15 expression. Thus, thin sections of EDL muscles obtained from mAb35-transferred rats were stained with antibody reactive against the chemokine, MCP-1 (Fig. 9). Approximately 25–30% of fibers demonstrated increased MCP-1 production (both increased numbers of fibers and increased staining intensity per fiber were noted). When stained for expression of IL-4R, over 80% of IL-4R-positive fibers were also MCP-1 positive. With regard to IL-15 expression, Figure 10 summarizes the fiber co-localization of IL-15 and MCP-1. Nearly all fibers that were producing IL-15 were also producing MCP-1.

IL-4 producing T cells were attracted to MCP-1 producing muscle fibers

Observations previously reported from this laboratory indicated that there is a significantly increased movement through skeletal muscles of IL-4-producing $CD4^+$ T cells following mAb35 transfer (15,17); the increased T cell (and monocyte) traffic follows a few hours after increased expression of MCP-1 is noted. In light of this observation, it was speculated that these IL-4 producing T cells could serve as a source of IL-4 needed to activate the muscle into increased IL-15 production. Therefore, staining was performed that attempted to correlate the location of MCP-1-producing fibers and the location of muscle-associated IL-4-producing T cells. EDL muscles were obtained from groups of rats immunized with the immunodominant AChR peptide, alpha 100–116. Muscle were also recovered from some rats that were also transferred with 50 μg (i.v.) of mAb35. The mAb35-exposed and control muscles were triple stained for the presence of $CD4^+$ T cells (red), leukocytes producing IL-4 (blue), and MCP-1 in muscle fibers (green). Approximately 800–1100 fibers were scored within approximately 100 stained muscle sections for either mAb35-transferred or control rats. Positions of 290 $CD4^+$ T cells were identified in muscle sections from control rats, and 1050 $CD4^+$ T cells were assessed in muscle from mAb35-transferred rats. Figure 11 indicates that there is a preferred positioning of the muscle-associated T cells near fibers that are making the T cell-attracting chemokine. This observation is consistent with the possibility that certain skeletal muscle fibers simultaneously produce the receptor for IL-4, and a chemoattractant that will bring a source of IL-4 to those same fibers.

DISCUSSION

The premise on which this line of investigation was based is that a particular combination of muscle-dependent and immune system-dependent activities together contribute to disease induction, progression, and outcome in an experimental model of myasthenia gravis. The aim of the studies described above was to examine one particular link in the network of two-way communication that exists between skeletal muscle and the immune system.

Numerous studies now lead us to the model that we continue to test which depicts pathology in experimental MG as the consequence of a circular network of communication between the immune system and skeletal muscle (Fig. 12, Supplemental). Thus, ❶ the binding of AChR-reactive antibodies, in addition to interfering with AChR function and perturbing membrane integrity following complement activation, has also been shown to ❷ stimulate skeletal muscle to release chemokines (12,17). These chemokines attract T cells and monocytes into the muscle. Both IFN-γ producing T_H1 cells and IL-4 producing T_H2 cells are found in muscle in approximately equal proportions (15,17). Although MG is not known for fulminate inflammation with large influxes of white cells into muscle, the approximate 10-fold increase of T cells into muscle may have significant influence. The observations described in the current report indicate that there is preferential congregation of some of those T cells with fibers that are attracting them (*i.e.*, $MCP\text{-}1^+$), the same fibers that are observed to make IL-15. In addition, ❸ muscle cells have been shown to ❹ secrete IL-15 *in vitro* in response to IL-4 (14), also consistent with the proposed model. Studies presented in the present report demonstrate that a similar response is observed in skeletal muscle *in vivo.* Moreover, IL-15 is known to ❺ stimulate IFN-γ production by T_H1 cells (28–31). *In vitro* studies from this laboratory have suggested that IFN-γ in turn, may further ❻ up-regulate chemokine production and expression of muscle surface molecules such as MHC class II and ICAM-1, thereby promoting interactions with immune cells that enhance disease (13,14). Numerous reports (32–37) have clearly shown that IFN-γ production is an important driving factor in disease progression. Although IFN-γ has been considered primarily in its role in immunoregulation, studies recently performed in our laboratory have also considered the possibility that cytokines, such as IFN-γ, may also act

as stimuli of immunologically relevant muscle activities that can also influence disease onset and progression in experimental MG.

Results described previously (15) indicated that during the course of experimental MG, induction of increased IL-15 production by muscle occurs. That is, AChR immunization of Lewis rats resulted in the up-regulation of IL-15 in EDL muscles. However, this increase in IL-15 was difficult to demonstrate in cultured myocytes exposed to AChR antibody alone. *In vitro* studies described in the current report, suggest that optimal muscle IL-15 production may require increased expression of IL-4R, probably triggered by exposure to AChR antibody, thereby allowing more effective activation of muscle by IL-4.

IL-15 has long been studied as a growth and differentiation factor in muscle (38–41). IL-15 is also well known for its effects on cells of the immune system. IL-15 activates IFN-γ producing T cells and NK cells and induces proliferation and antibody synthesis in activated B cells (reviewed in ref. 29). Clearly, induction of increased IL-15 production in muscle could result in heightened activation of immune cells trafficking through the muscle. These activated cells ($CD4^+$ T cells and monocytes/macrophages) have the potential of directly promoting further activation of the autoimmune response; or additional cytokines produced following activation of muscle-associated leukocytes could also lead to further activation of myocytes that produce additional waves of their own immune mediators. The predicted consequence would be that the severity of resulting pathology would increase.

In conclusion, the presence of functional IL-4R on the surface of muscle could be an important interface between skeletal muscle and the immune system. It is, therefore, of value to characterize the muscle IL-4R, its signaling pathways, and the eventual IL-4-induced products of muscle that may have an impact on its own physiology and fate during autoimmune insult.

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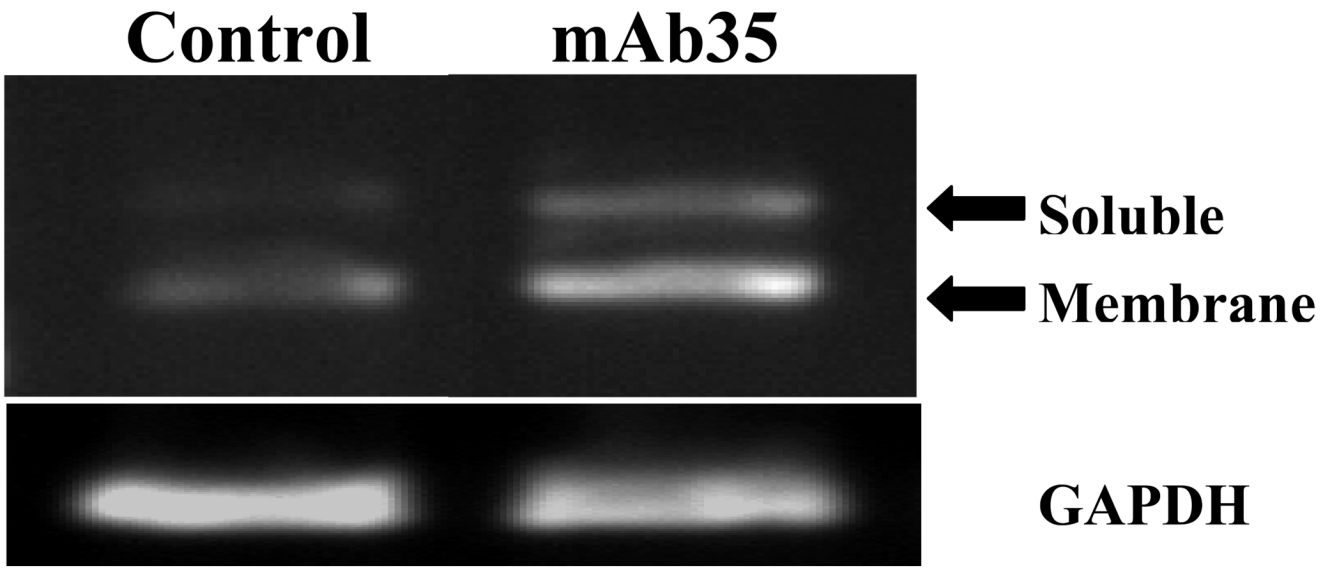

Fig. 1.

Nearly confluent LE1 myocytes were cultured for 24 hours with 20 μg/ml of AChR-reactive monoclonal antibody (mAb35) and compared to unstimulated myocytes (Control). Total RNA was extracted from the cells, and IL-4Rα specific primers used to generate cDNA via RT-PCR as described in Materials and Methods. Doublet bands consistent with membrane IL-4Rα and soluble IL-4Rα were visualized by staining with ethidium bromide and identities confirmed by sequencing. GAPDH message served as an internal control.

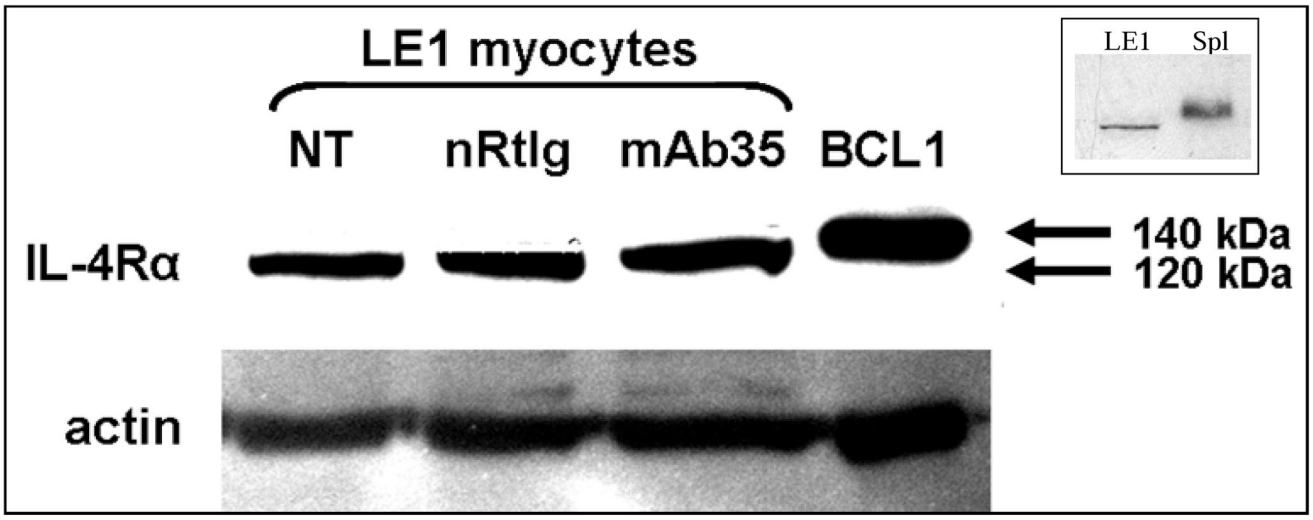

Fig. 2.

LE1 myocytes, evaluated by Western blot analysis, demonstrated increased expression of IL-4R following exposure to AChR antibody (mAb35). LE1 cells were cultured with either 20 μg/ml of mAb35 antibody or non-immune rat Ig (nRtIg), as indicated, for 48 hours prior to myocyte protein extraction. Extracts were run on 7.5% SDS-PAGE, blotted onto nitrocellulose paper, and probed with polyclonal rabbit antibody against the IL-4Rα chain. Representative of 3 experiments performed, IL-4Rα from LE1 cells consistently ran with a molecular weight 15–20 kDa smaller than IL-4Rα detected in BCL1 cells. Inset shows that the size discrepancy also holds true when compared to extracts from rat spleen cells.

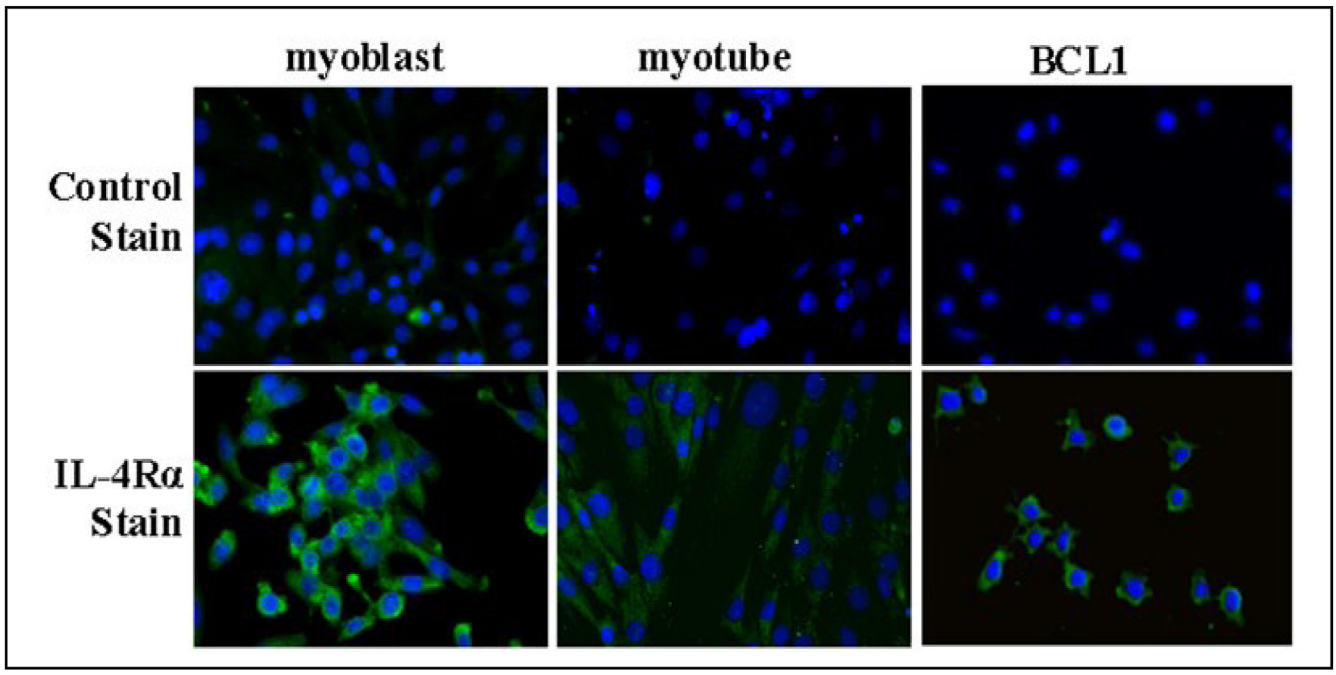

Fig. 3.

LE1 myocytes, cultured on coverslips in the presence of 20 μg/ml AChR antibody, were evaluated by immunofluorescence staining using rabbit antibody reactive with IL-4Rα. Representative FITC green staining is shown for LE1 myoblasts (left column) and LE1 myotubes (middle column). Positive control was provided by BCL1 B lymphocytes (right column). Control stain = Non-immune rabbit Ig. Hoechst 33258 staining of nuclei is shown in blue.

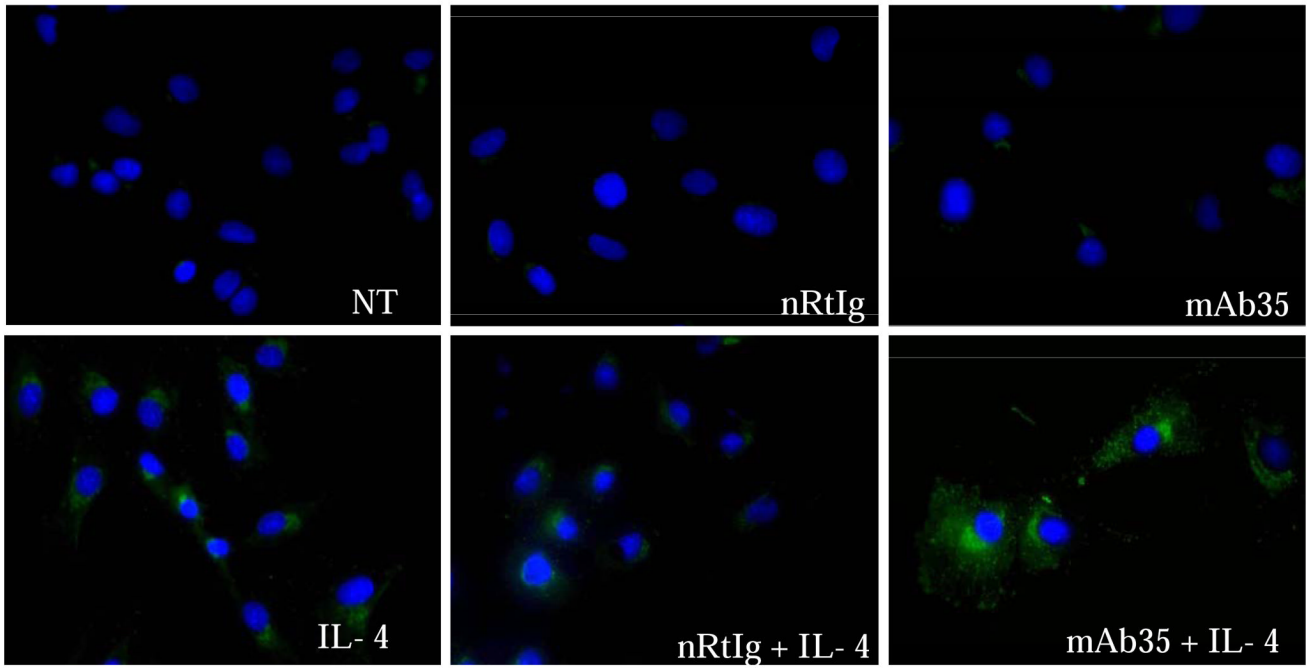

Fig. 4.

Primary myocytes, cultured on coverslips in the presence of the stimuli indicated, were evaluated for IL-15 production (green) by immunofluorescence analysis. Hoechst 33258 staining of nuclei is shown in blue. NT = no treatment. Representative of 3 experiments performed.

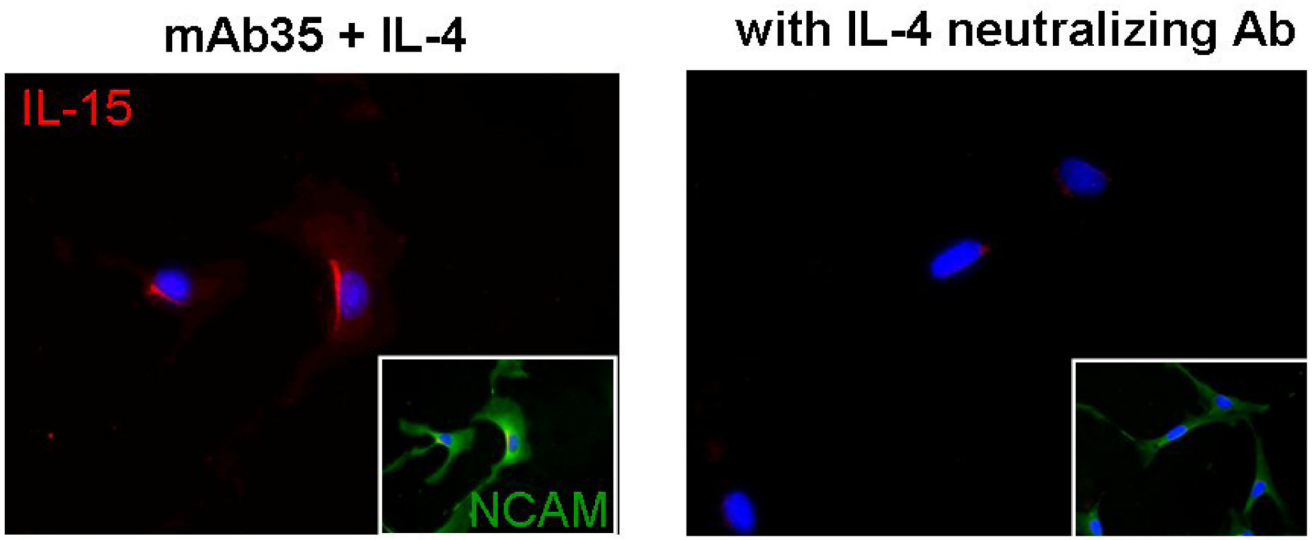

Fig. 5.

The increased expression of IL-15 could be abrogated by the presence of IL-4 blocking antibody. Primary myocytes, cultured on coverslips in the presence of 20 μg/ml mAb35 plus IL-4, were evaluated for IL-15 production by immunofluorescence analysis (representative of 3 experiments performed). Left panel: IL-15 immunostaining following activation of primary myocytes with mAb35 plus IL-4 (note that IL-15 is now stained red). Right panel: Same set-up as in left panel, except that 1.0 μg/ml IL-4-reactive blocking antibody was also added. Insets shown the same cells double stained for the muscle isoform of NCAM (green) so as to verify their identities as myocytes.

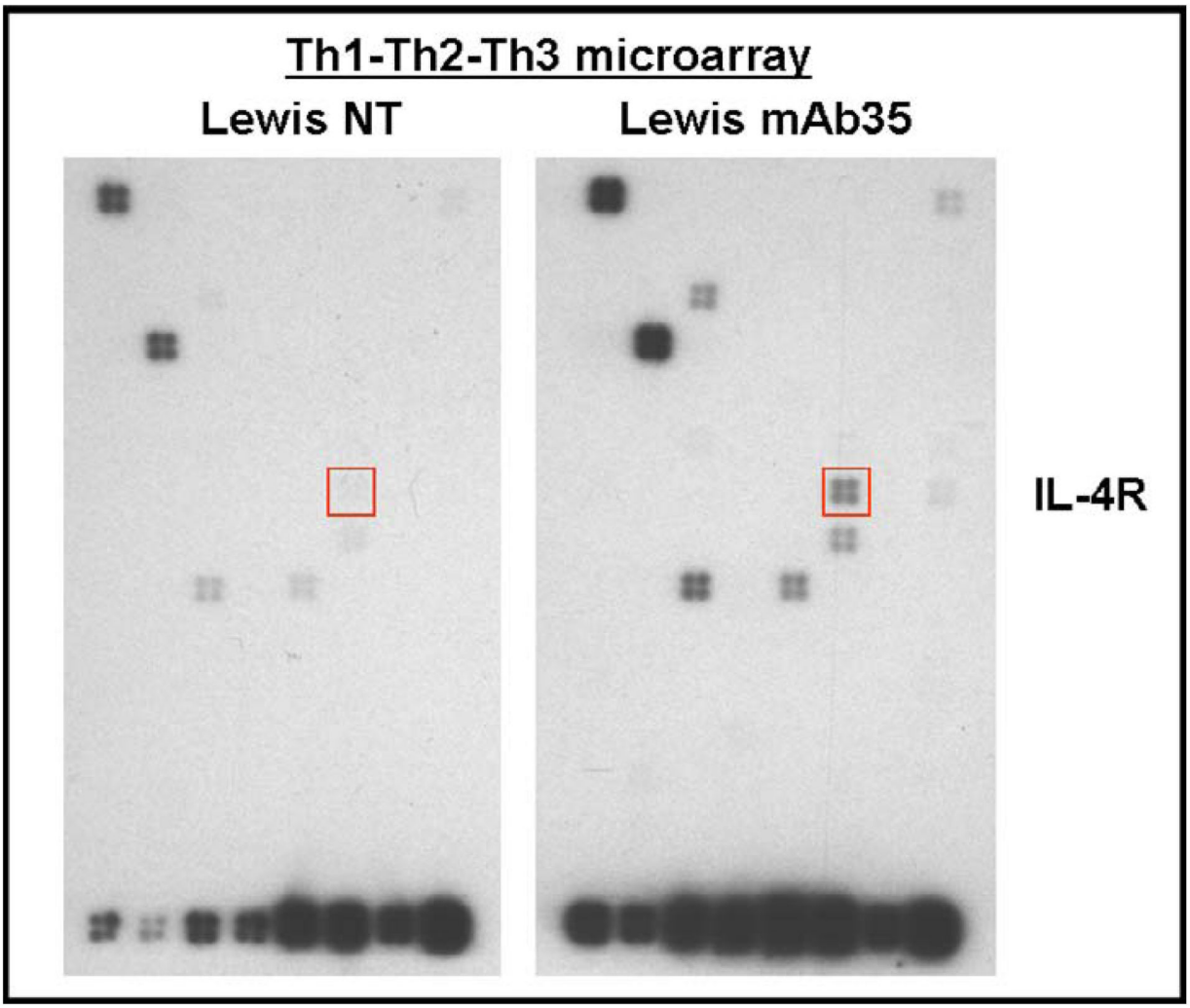

Fig. 6.

EDL muscle mRNA for IL-4Rα increased in response to passive transfer of 50 μg of AChR antibody (mAb35). The level of mRNA coding for IL-4Rα in untreated Lewis EDL muscles (left panel) is very low (expected location of IL-4Rα spot indicated by the small box), but increased markedly in EDL muscles from rats that had received mAb35 antibody 24 hours before (right panel). The bottom row is artificial biotinylated sequences for orientation purposes.

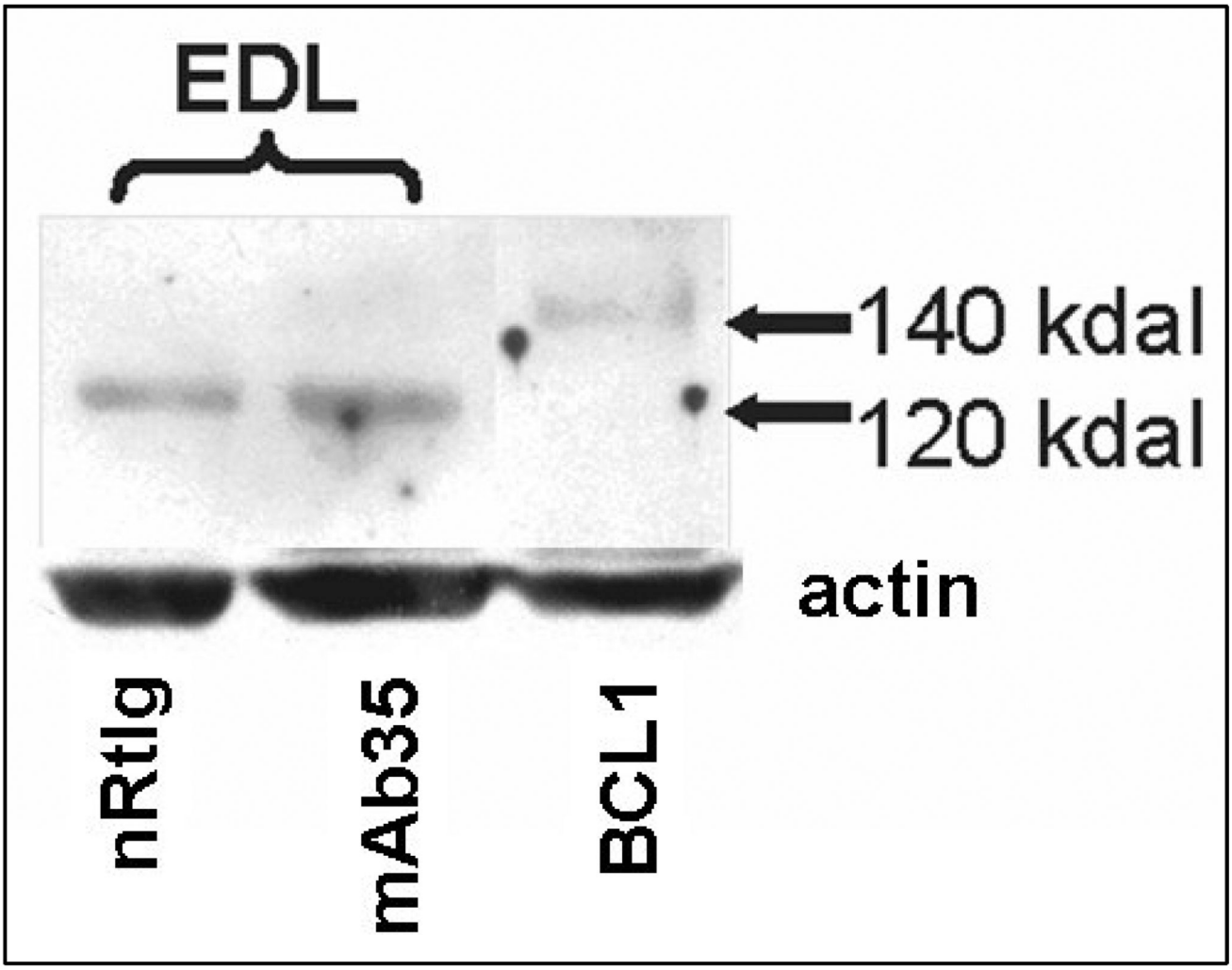

Fig. 7.

EDL muscle demonstrated increased IL-4Rα in response to passive transfer of mAb35. 12–24 hours following passive transfer of 50 μg of AChR antibody (mAb35) or non-immune control rat Ig, increased expression of IL-4Rα was detected by Western blot analysis in EDL muscles. Of interest, the size of this molecule is approximately 120 kDa, not the expected 140 kDa. An extract from the B lymphocyte line, BCL1, is shown for comparison, and probing of the same extracts for actin is shown as a loading control.

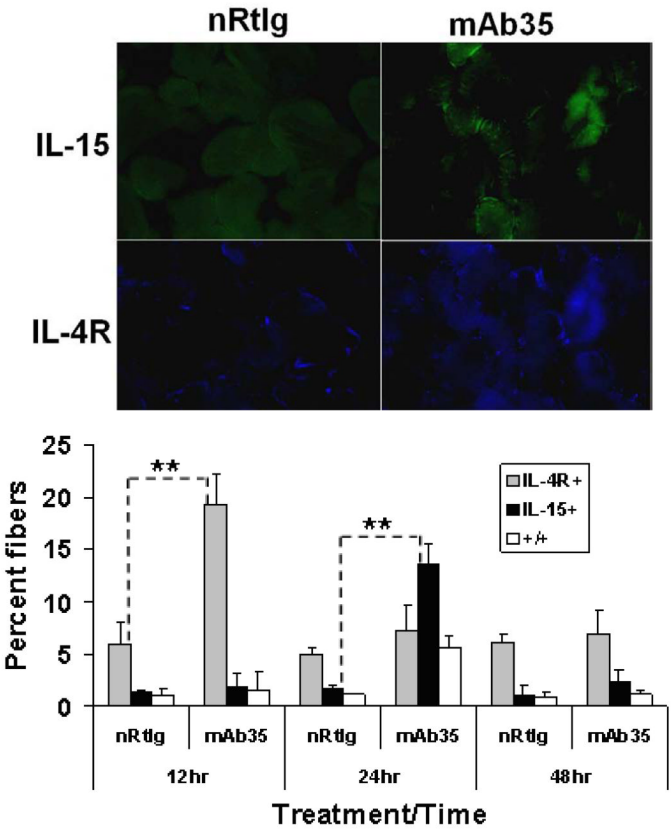

Fig. 8.

IL-4R co-localized with IL-15 in EDL muscle fibers. Following passive transfer of 50 μg of AChR antibody (mAb35) or non-immune control rat Ig, expression of IL-4Rα and IL-15 was assessed by immunofluorescence staining. Upper Panel: 24 hours following passive transfer with either nRtIg or mAb35, as indicated; double staining of IL-4R (blue) and IL-15 staining (green) of muscle sections from transferred rats. Lower Panel: Following transfer (50 μg, i.v.) of either non-immune rat Ig (nRtIg) or mAb35, EDL muscles were recovered at the indicated time points. Muscles were sectioned as described and double immunostained for IL-4Rα and IL-15. Percent positive (±SEM) for each staining specificity is shown for 2 individual experiments in which approximately 800–1100 fibers were scored. Statistical significance of < 0.01 as determined by Chi squared analysis is indicated by **.

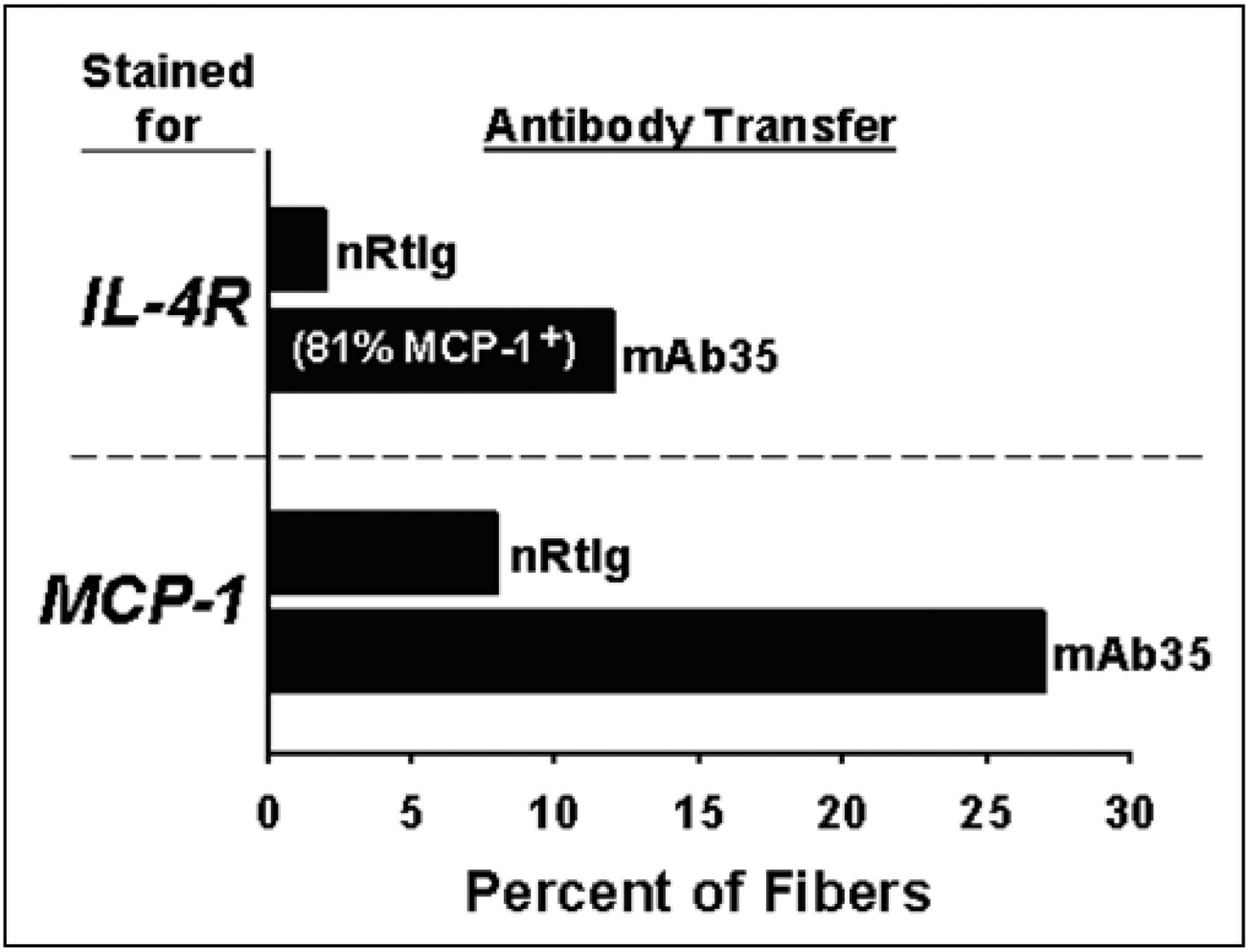

Fig. 9.

IL-4R co-localized with MCP-1 in EDL muscle fibers. Twenty-four hours following passive transfer of 50 μg of AChR antibody (mAb35) or non-immune control rat Ig, expression of IL-4Rα and MCP-1 in muscle sections from 3 transferred rats was assessed by double immunofluorescence staining. 800–1000 fibers were scanned.

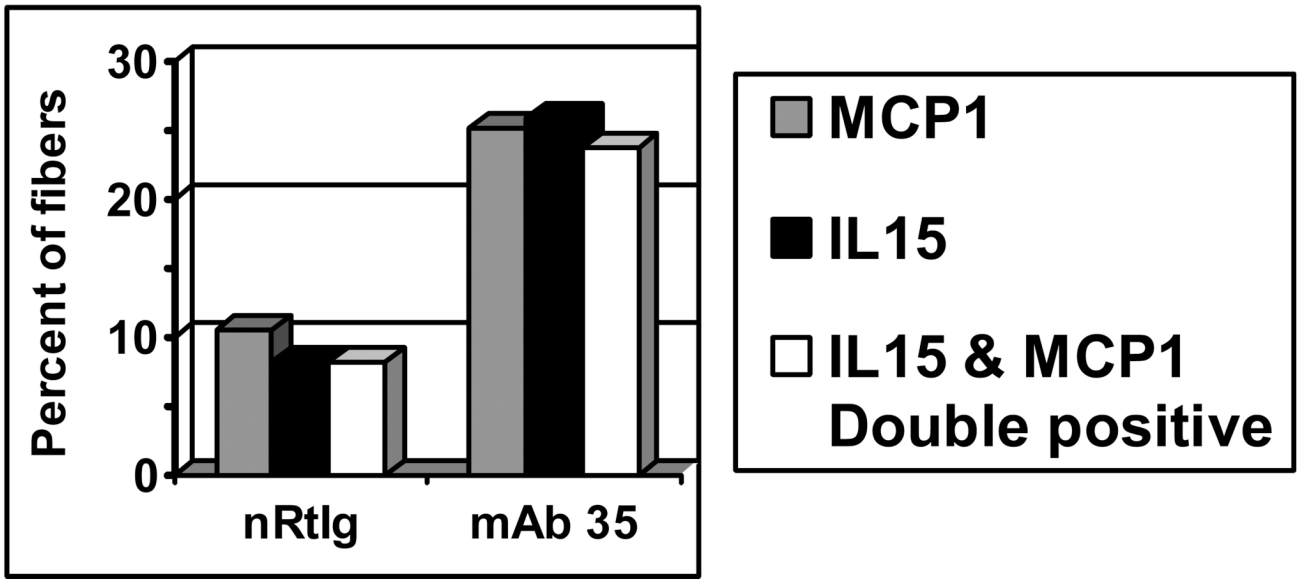

Fig. 10.

IL-15 and MCP-1 co-localized in EDL muscle fibers. Groups of 3 rats were immunized with the immunodominant AChR alpha subunit peptide, p100–116. Some rats were also transferred (i.v.) with 50 μg of mAb35. EDL muscles were recovered from mAb35-transferred rats and control rats receiving no antibody, sectioned as described, and double stained for IL-15 and MCP-1. 800–1000 fibers were scanned. The experiment was repeated twice.

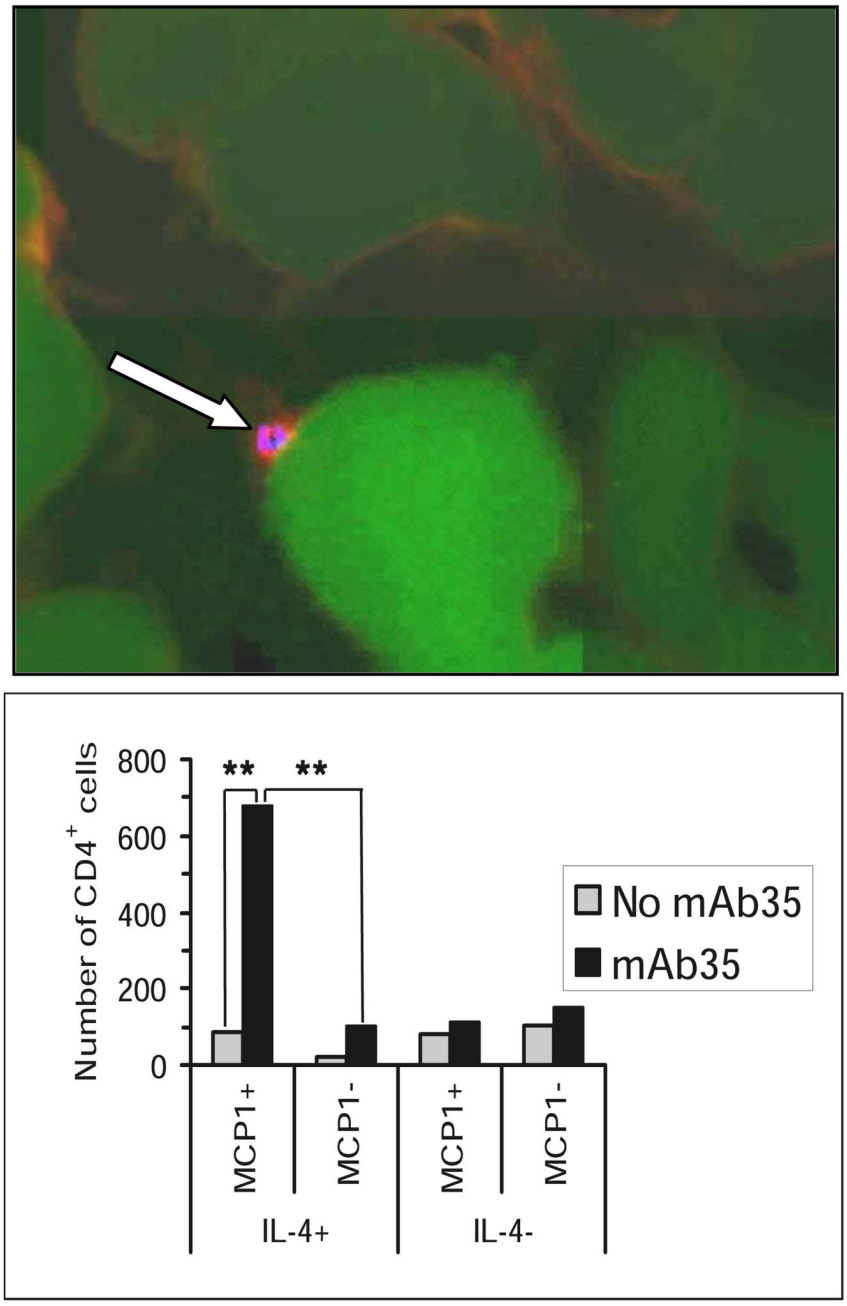

Fig. 11.

Preferential localization of IL-4-producing $CD4^+$ T cells near MCP-1-producing muscle fibers. Following transfer (50 μg, i.v.) of either non-immune rat Ig (nRtIg) or mAb35, EDL muscles were recovered, sectioned as described, and triple stained. Top Panel: In a representative muscle section, $CD4^+$ on T cells is stained red, IL-4 in T cells is stained blue, and MCP-1 in muscle fibers is stained green. Bottom Panel: Graphic summary of positions of 290 $CD4^+$ T cells identified in muscle sections from 3 nRtIg-transferred rats; 1050 $CD4^+$ T cells were assessed in muscle from 3 mAb35-transferred rats. Approximately 800–1100 fibers were scored within approximately 100 stained muscle sections for each antibody treatment.

Statistical significance of < 0.01 as determined by Chi squared analysis is indicated by **. The experiment was repeated twice.

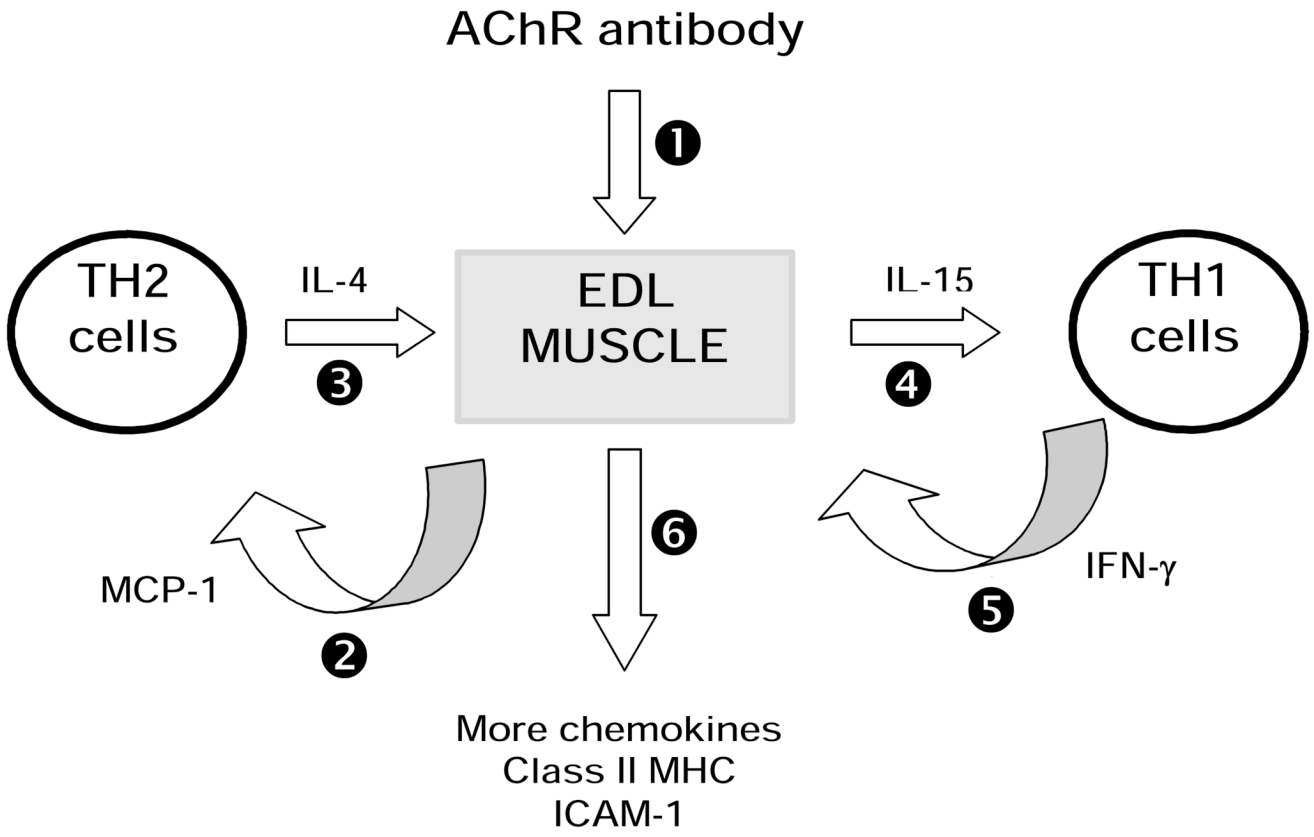

Fig. 12.
Model of immune system/muscle intercommunication and a proposed link between IL-4 activation of skeletal muscle and subsequent production of muscle IL-15 during experimental MG.

Table 1

Combinations of antibodies used in immunofluorescence studies.

Target molecule	1° Ab	2° Ab	Fluorescent Tag	Color
IL-4Rα	rabbit polyclonal (S-20)	goat anti-rabbit Ig	fluorescein isothiocyanate (FITC)	green
		donkey anti-rabbit Ig	sulfoindocyanine (Cy3)	red
		donkey anti-rabbit Ig	aminomethylcoumarin acetate (AMCA)	blue
IL-15	rabbit (H-114)	donkey anti-rabbit Ig	sulfoindocyanine (Cy3)	red
	Goat (L-20)	rabbit anti-goat Ig	fluorescein isothiocyanate (FITC)	green
CD4	mouse monoclonal (W3/25)	goat anti-mouse Ig	sulfoindocyanine (Cy3)	red
IL-4	rabbit polyclonal	donkey anti-rabbit Ig	aminomethylcoumarin acetate (AMCA)	blue
MCP-1	goat polyclonal (R-17)	rabbit anti-goat Ig	fluorescein isothiocyanate (FITC)	green
NCAM	goat polyclonal (C-20)	rabbit anti-goat Ig	fluorescein isothiocyanate (FITC)	green