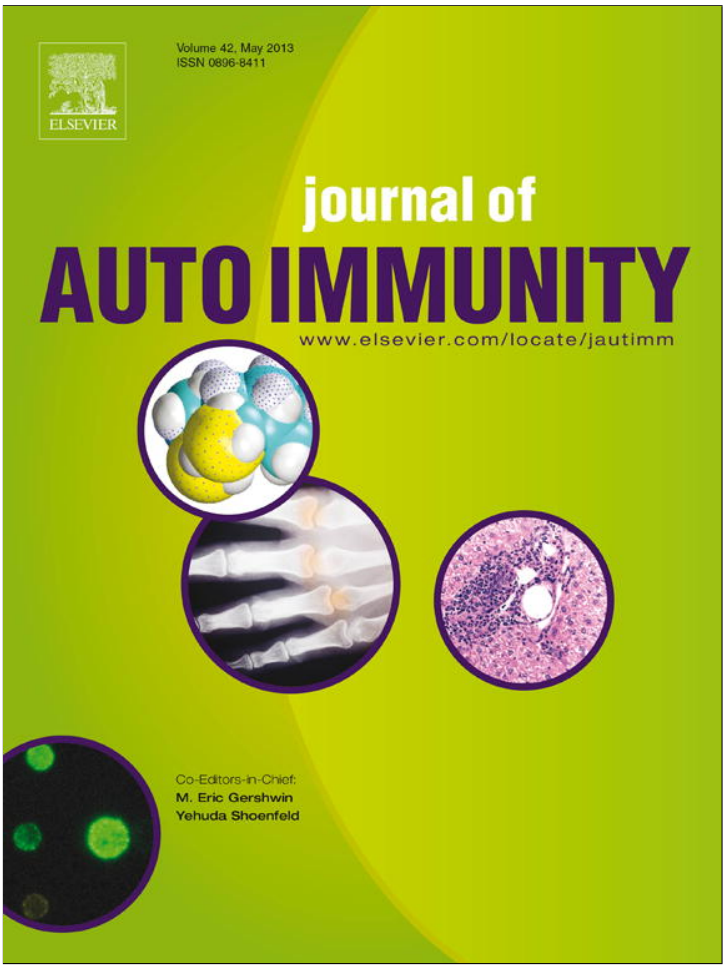

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Complement C2 siRNA mediated therapy of myasthenia gravis in mice

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ABSTRACT

Activation of complement components is crucial in the progression and severity of myasthenia gravis and experimental autoimmune myasthenia gravis (EAMG). Mice deficient in complement component C4 or treated with monoclonal antibody to C1q are resistant to EAMG. In this study, we show that inhibition of complement cascade activation by suppressing the expression of a critical low-abundant protein, C2, in the classical complement pathway, significantly improved clinical and immunopathological manifestations of EAMG. Two weeks after a second booster immunization with acetylcholine receptor, when mice exhibit muscle weakness, i.p. injection of C2 siRNA significantly suppressed C2 mRNA in the blood cells and liver of EAMG mice. Treatment of EAMG mice with C2 siRNA, once a week for 5 weeks, significantly improved muscle strength, which was further evidenced by functional AChR preservation in muscle, reduction in number of C3 and membrane-attack complexes at neuro-muscular junctions in forelimb muscle sections, and a transient decrease in serum IgG2b levels. Our study shows for the first time that siRNA-mediated suppression of C2, a component of the classical complement system, following established disease, can effectively contribute to the remission of EAMG. Therefore, C2 siRNA mediated therapy can be applied in all complement mediated autoimmune diseases.

1. Introduction

Myasthenia gravis (MG) is a well-characterized complement and antibody-mediated autoimmune disease [1]. The disease occurs due to complement and auto-antibody-mediated deficit of acetylcholine receptor (AChR) at the neuromuscular junction (NMJ), preventing acetylcholine (ACh) from binding to the muscle postsynaptic AChR and resulting in ultimately impaired contractibility and muscle weakness [2–5]. An experimental model of MG, experimental autoimmune MG (EAMG) can be induced by immunizing C57BL6 (B6) mice with AChR in Complete Freund's Adjuvant (CFA) [6,7]. We and others have previously demonstrated that complement factors C1 to C9 are critically involved in EAMG and MG pathogenesis [8–11]. We have also shown genetic evidence for the involvement of the classical complement pathway (CCP) in the development of EAMG [8]. In both MG and EAMG, formation of the membrane attack complex (MAC), initiated by activated C3, co-localizes with AChR and causes severe structural injury of endplates and lyses the postsynaptic membrane that ultimately disperses and depletes AChR at NMJ [12].

Although MG patients derive benefit from current therapies, they are neither curative nor without side effects. We, therefore, aimed to treat EAMG targeting the CCP by using siRNAs, rather than a small molecule inhibitor or antibody, as these agents either form systemic immune complexes or other harmful by-products [13]. Protection from renal ischemia reperfusion injury has been previously shown by the *in vivo* silencing of C5a receptor or C3 in animal models [14,15]. However, a siRNA targeting the complement factors has never been attempted in the treatment of MG or other autoimmune disorders. We, therefore, intended to assess CCP inhibition as a potential therapy in antibody- and complement-mediated EAMG by delivering a non-vector based, chemically modified siRNA *in vivo*.

Of all the components of the complement system, the second component, C2, is an ideal candidate for siRNA-mediated gene silencing for two reasons. First, as C2 is expressed at a very low level, a huge quantity of siRNA is not required for C2 mRNA inhibition and thus any potential toxic/side effects can be avoided. Second, by targeting the CCP component, C2, the alternative pathway activated by microorganisms will be preserved and thus beneficial for host defense [16]. Moreover, C2 is a key protein that acts effectively to activate the complement cascade and the formation of MAC. In this study we show for the first time that short-term treatment of mice with established MG with C2-specific siRNA alone suppressed C2

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mRNA expression in blood cells and liver, improved MG symptoms, prevented deposition of MAC in NMJ, protected muscle AChR integrity and function, and enhanced longevity.

2. Materials and methods

2.1. Animals, EAMG model and clinical evaluation of disease

Eight-week-old B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). AChR from the electric organ of *Torpedo californica* was affinity purified from the neurotoxin affinity column [17,18]. B6 mice were immunized and boosted at 4 and 8 weeks with 20 μg of affinity purified Torpedo AChR emulsified in CFA (heat-killed *Mycobacterium butyricum*) [18]. All mice were housed in a barrier facility and maintained according to the Animal Care and Use Committee guidelines of the University of Texas Medical Branch. Grip strength of mice was quantified on a Dynamometer (Chatillion Digital Force Gauge, Columbus Instruments, OH) after a 2nd boost, prior to treatment and once a week following treatment with C2 siRNA or non-targeting (NT) control siRNA for 5 weeks [8].

2.2. In vitro validation of C2 mRNA inhibition by C2 siRNA

C2-specific siRNA (Silencer select mouse complement component 2, siRNA ID: s63158), NT control siRNA and Lipofectamine RNAiMAX for *in vitro* experiments were purchased from Invitrogen (Carisbad, CA). Mouse hepatoma AML-12 and culture medium F-12, nutrients and fetal bovine serum were obtained from ATCC (Manassas, VA).

2.3. C2 interference in vivo-strategy

For the *in vivo* experiments, an *in vivo* compatible, single siRNA duplex (predesigned siRNA, HPLC, id 63158) specific to target the C2 mRNA (NM_013484) and NT control siRNA were purchased from Invitrogen. All siRNAs were endogen free, HPLC purified, chemically modified to stabilize and abrogate any non-specific immune response and off-target effect *in vivo*. An *in vivo* delivery agent, invivofectamine (Invitrogen) was used to conjugate siRNA. C2 siRNA and NT control siRNA were dissolved in RNAse-free and endotoxin-free water at 3 μg/μl. As per the manufacturer's protocol, siRNA, complexion buffer and invivofectamine were used to make a complex that was further dialyzed in PBS and then diluted in PBS (50 μg/200 μl per mouse) for injection. C2 siRNA was also tested in a non-complex form dissolved in PBS and injected i.v. or i.p. at 50 μg in 200 μl per mouse. A smart pool C2 siRNA (Dharmacon, CO) conjugated with polymer (Transit *in vivo* gene delivery system) or TransIT LT1 from Mirus Biocorp (WI) was also tested for all *in vivo* and *in vitro* experiments.

All mice at the level of grade 1 and grade 2 were randomly assigned to two equal disease groups ($n = 10$ per group) to receive either C2 siRNA or NT control siRNA treatment. Two weeks after the 2nd booster immunization, siRNAs were injected i.p. into mice once a week for 5 weeks. A set of untreated, CFA or CFA-AChR-immunized mice were also kept as negative and positive controls in this experiment. Mice were bled thrice: 4 days prior to first treatment, once at 3 days post-first treatment (from 4 mice per group) and at termination (all mice, 5 days after 5th treatment). Serum (separated from blood cells at each time), liver, triceps muscles, and carcasses were stored frozen (−80 °C). In a parallel experiment, EAMG mice treated with C2 siRNA and control siRNA ($n = 3$) were sacrificed at 3

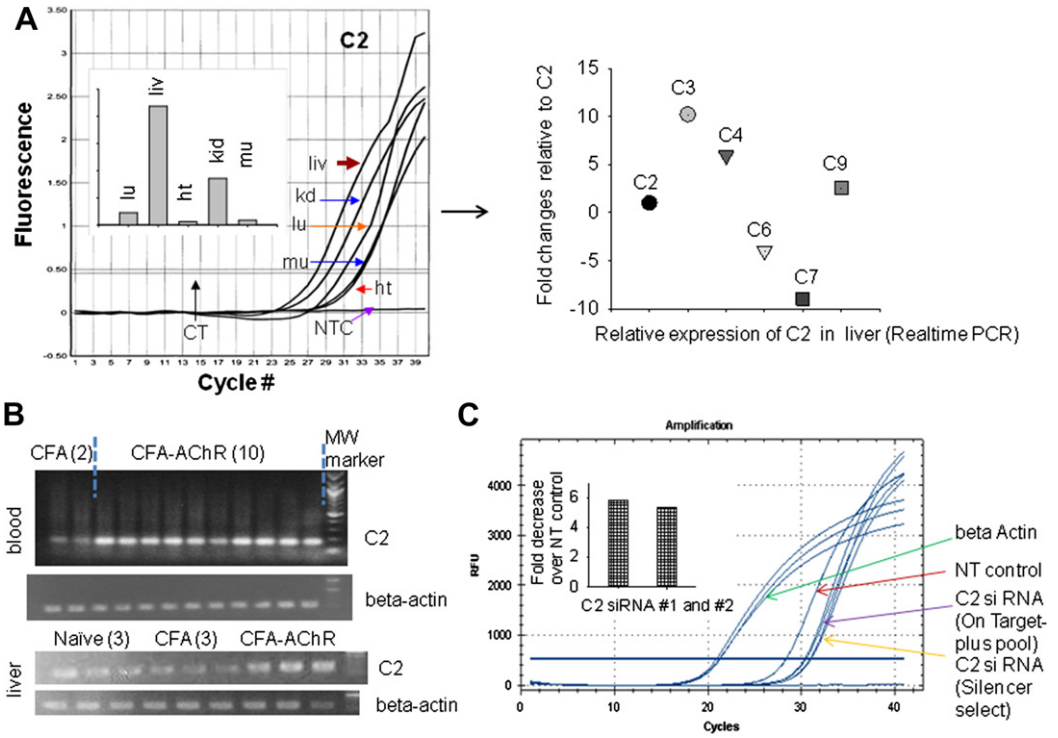

Fig. 1. R-PCR quantitation to determine C2 levels in mouse tissues, blood cells and C2 knock down in mouse hepatoma cells. (A) Left: C2 mRNA in mouse liver (liv), lung (lu), heart (ht), kidney (kd) and skeletal muscle (mu). Amplification plot indicates a higher amount of C2 cDNA/mRNA in the liver in equal amounts of starting template (cDNA) from different tissues (normalized with beta actin). NTC = no template control. Right: Fold changes in expression of complement genes relative to C2 (Real-time PCR). Error bars are too small to be seen. (B) C2 mRNA levels in blood cells and liver. Elevated levels of C2 mRNA (RT-PCR product is 192 base pairs) was evident in blood cells (top 2 panels) and liver (bottom 2 panels) of CFA-AChR immunized EAMG mice relative to CFA immunized or naïve mice following 2 booster immunizations. (MW = Molecular weight marker). (C) siRNA mediated C2 knockdown in AML-12 cells. Either vehicle (transfection reagent) or C2-specific siRNA or non-targeting control (NT) at 50 nM were added to AML-12 cells and harvested 72 h later to perform analysis of C2 mRNA inhibition relative to controls. Real-time PCR reveals 5.5- to 6-fold inhibition of C2 mRNA level (relative to NT control) with either single siRNA (#2) or pooled siRNA (#1). Each result is a representative of 3 independent experiments. CT: cycle threshold.

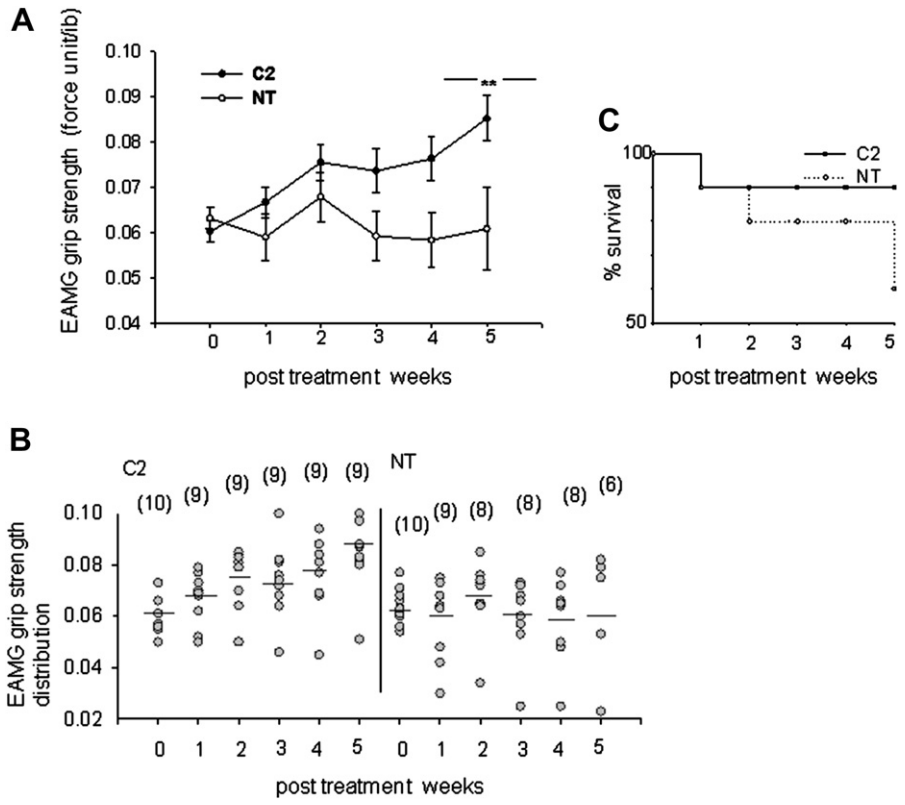

Fig. 2. Improved grip strength, clinical grade and longevity in C2 siRNA-treated EAMG mice. Two weeks after 2nd booster immunization, EAMG mice were treated either with C2 or NT control siRNA once a week for 5 weeks. Forelimb grip strengths of all mice, once a week for 5 weeks were recorded by using a digital dynamometer. Clinical grades were also recorded based on disease symptoms of mice. (A) Grip strength data indicate that the mean muscle strength of C2 siRNA-treated mice is slightly better at any time during the course of treatment until termination. Compared to the NT control siRNA-treated mice, the grip strength of C2 siRNA-treated mice improved significantly from 4 weeks onward following treatment with C2 siRNA, $^{**}P < 0.01$. (B–C) clinical grade was better in C2 siRNA-treated mice; none of the C2 siRNA-treated mice died due to disease progression, whereas 4 out of 10 NT control siRNA-treated mice died from disease and 6 out of 10 mice in the group survived with typical EAMG symptoms. NT, non-targeting control siRNA. Number in parenthesis indicates # of mice that survived and received treatment. Vertical bars indicate standard errors.

days after the 1st treatment to assess the C2 mRNA suppression in liver.

2.4. mRNA analyses by PCR and real-time PCR

Total RNA was isolated from blood cells and liver by using an RNeasy kit (Qiagen, CA). RNA was DNAse digested (Invitrogen) and reverse transcribed by using Random primers, dNTPs and superscript II (Invitrogen). Relative gene expression was analyzed by using a CFX 96 (Biorad, CA) or ABI Prism 7000 Real-Time system or at an exponential phase of amplification-cycle in a 2720 Thermal Cycler (ABI, CA). SYBR green PCR Master mix, TaqMan Gene Expression Master mix and gene expression assays (probe-primer mix) for C2 (Mm00442726_m1), beta actin (Mm00607939_s1) IFN genes, CD69 and other complement genes were obtained from Invitrogen. Separate primer pairs (C2-NM_13484-L, GGCTGTCTCCCAAAACAAAA; R, AAAAAGACCCCAACTCACCA) for regular PCR for C2 were also designed by using Primer3 software (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma-Genosys.

2.5. Flow cytometry

Spleen cells were blocked and surface-labeled for 30 min on ice with mAbs (FITC anti-mouse CD3, PE-Cy7 anti-mouse CD4, PE-Cy5 anti-mouse CD44). Spleen cells were additionally fixed, permeabilized (BD) and intracellularly labeled with PE anti-mouse IFNγ. All antibodies were purchased either from BD Bioscience (USA) or e-Bioscience (USA). All cells were analyzed on BD LSR Fortessa flow cytometer using FACSDiva software (BD Bioscience, USA).

2.6. Apoptosis assays

PBMCs from naive and siRNA-treated EAMG mice were prepared after lysing RBCs using BD PharmLyse (BD Bioscience). PBMCs resuspended in PBS and 0.1% TritonX-100 were washed and then fixed in PBS and 4% paraformaldehyde containing 10 μg/ml DAPI (Invitrogen, USA). Cells were then placed on slide, covered with a coverslip, periphery sealed, and 150 cells per sample were counted for scoring nuclear deformities. PBMCs were also resuspended in Annexin-V staining/binding buffer and labeled with FITC-conjugated Annexin-V (Biosource Int. Inc., USA). Serum from siRNA-treated mice were also analyzed for apoptosis using Cell Death Detection Elisa Plus (Roche, USA) as per instructional protocol.

2.7. ELISA for anti mouse AChR Ig isotypes, C3 and total IgG

Mice were bled periodically from tail vein for evaluation of serum anti-AChR antibody levels. ELISA was done as described previously [13,18]. A sandwich ELISA analysis of C3 was done by using anti-mouse C3 (capture antibody) and HRP-conjugated goat anti-mouse C3 (MP Biomedicals, OH) as detection antibody [19]. For detection of serum total IgG levels, diluted (1:10,000) serum samples were added to the pre-coated wells of Evencoat goat anti-mouse IgG microplates (R&D systems, USA), washed with PBS-0.05% tween 20 and then re-incubated with HRP conjugated

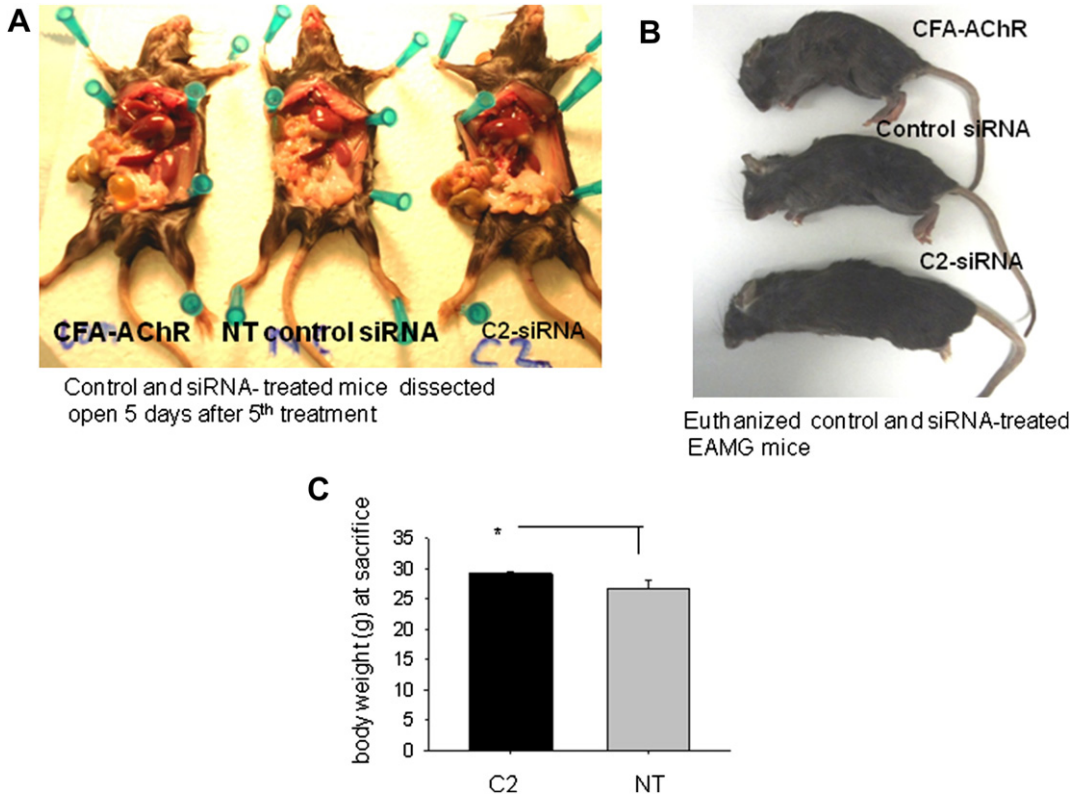

Fig. 3. Normal phenotype and morphology of internal organs in siRNA-treated mice. EAMG mice were euthanized after 5 weeks of treatment with siRNAs. Body weight was taken, and internal organs were examined for visible signs of damage. (A) SiRNA-treated mice showed no visible sign of tissue damage or necrosis of internal organs following 5 weeks of treatment. (B) Representative untreated control and NT control siRNA-treated mice with typical EAMG symptoms and no symptoms following treatment with C2 siRNA for 5 weeks. (C) Average body weight of C2 siRNA-treated mice was slightly higher than that of control siRNA-treated mice ($n = 9$, C2 siRNA-treated and $n = 6$, control siRNA-treated survivors), $^{*}P < 0.04$.

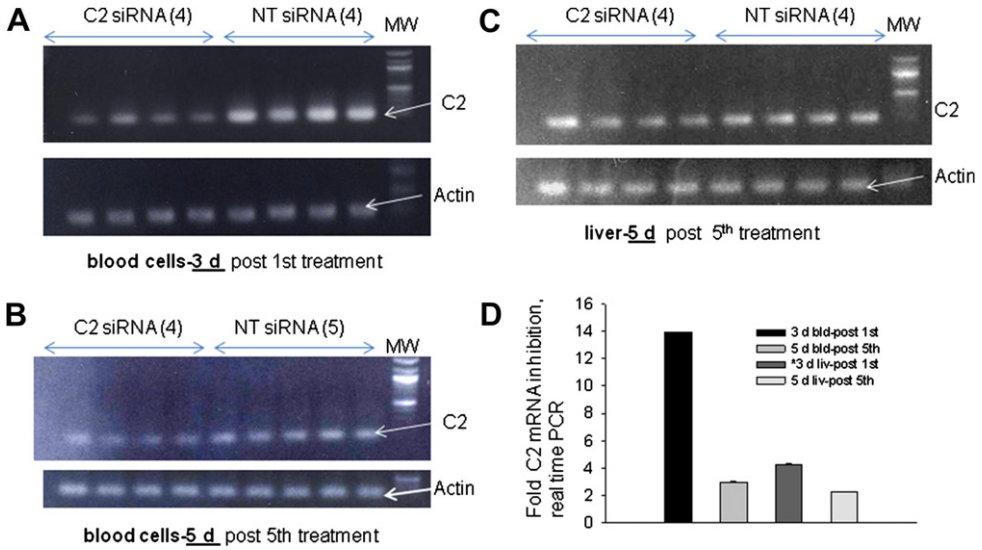

Fig. 4. *In vivo* suppression of C2 mRNA by C2 siRNA in liver and blood cells of EAMG mice. B6 EAMG mice, after 2 booster immunizations were treated with C2 siRNA or control siRNA and bled from the tail vein at 3 days after 1st treatment and 5 days after 5th treatment, just prior to termination. RNA and cDNA were prepared and subjected to RT-PCR and real time PCR analyses. (A, C) Representative micrograph of gel-resolved RT-PCR product of C2 mRNA amplified for 35 cycles (exponential) was shown above a reference gene, beta actin (amplified for 30 cycles). (A) C2 mRNA in C2 siRNA-treated mice, compared to control siRNA-treated mice, was markedly inhibited after 3 days post treatment in blood cells. (B, D) C2 mRNA expression was partially restored 5 days after post treatment and the suppression level remained only at 2.9- and 2.2-fold in blood cells and liver, respectively, at that time point. (D) Relative expression of $\Delta\Delta C^{t}$ values of C2 normalized to endogenous control (beta actin) was determined by real-time PCR, and fold changes were calculated. C2 mRNA levels in blood cells were reduced by 13.9-fold in 3 days post C2 siRNA-treated over control siRNA-treated samples. C2 expression was reduced by 4.2 fold over NT controls in the liver at 3 days post 1st treatment in one parallel experiment, $n = 3$ per treatment groups (*3 d liv-post 1st). Vertical bar represents standard error. MW: molecular weight marker. Each result is a representative of 3 independent experiments.

detection antibody (anti-mouse) (Caltag lab, USA) raised against a different epitope than primary-capturing antibody.

2.8. Immunoflurescence for C3 and MAC deposits at NMJ

Frozen triceps muscle obtained from treated and untreated B6 EAMG mice were sectioned at 10 μM on slides, thawed and fixed in cold acetone. The sections were washed and blocked in 5% BSA and then incubated at 4 °C overnight with alpha-bungarotoxin (BTX) conjugated to Alexa 555 (1/100) (Invitrogen) and either anti-C5b-9 (MAC) (Abcam, Cambridge, MA) or anti-C3 (1:100) (Cedarlane, CA) that was further incubated with anti-rabbit IgG-Alexa 488 (Invitrogen) for 2 h. After washing in PBS, the sections were air-dried and photographed under an Olympus IX-70 microscope (10×, 20×) with a DP-11 digital camera. The number of C3- and MAC-positive BTX binding sites was counted in four muscle sections from each mouse. The percentages of NMJs with deposits in each muscle section were calculated by totaling the numbers of C3 or MAC deposits divided by the numbers of BTX labeled sites, & times 100.

2.9. Radioimmunoassay for muscle AChR quantitation

Mouse muscle extract was prepared from frozen carcasses and AChR was quantified according to our previous protocol [18].

2.10. Statistical analysis

Grip strength and clinical score analysis and all other data were compared and evaluated by using Student's *t*-test or Mann–Whitney *U* test, where applicable. Calculated *P* values were considered significant at <0.05 (*), <0.01 (**), and <0.001 (***).

3. Results

3.1. C2 expression in normal and EAMG mice tissue and interference of C2 expression in vitro by C2 siRNA

In concordance with a previous report [20], we found that C2 is expressed in a variety of mouse tissues including the liver, lungs, heart, kidneys and skeletal muscle, with the highest level of expression occurring in the liver (Fig. 1A). However, it is one of the low-expressed complement genes in liver (Fig. 1A). We also detected a moderate level of C2 expression in normal peripheral blood cells of mice. The C2 expression in peripheral blood cells and the liver was augmented following a booster immunization with CFA-AChR, compared to CFA-alone and/or naïve controls (Fig. 1B). Both the single and smart-pool C2 siRNA efficiently suppressed C2 gene expression relative to NT- or vehicle control when tested in *vitro* in a mouse hepatoma cell line, AML-12 cells. C2 mRNA suppression (normalized to beta-actin control) was found to be 5.5–6 fold over NT control at 50 nM after 72 h after transfection with those siRNAs (Fig. 1C).

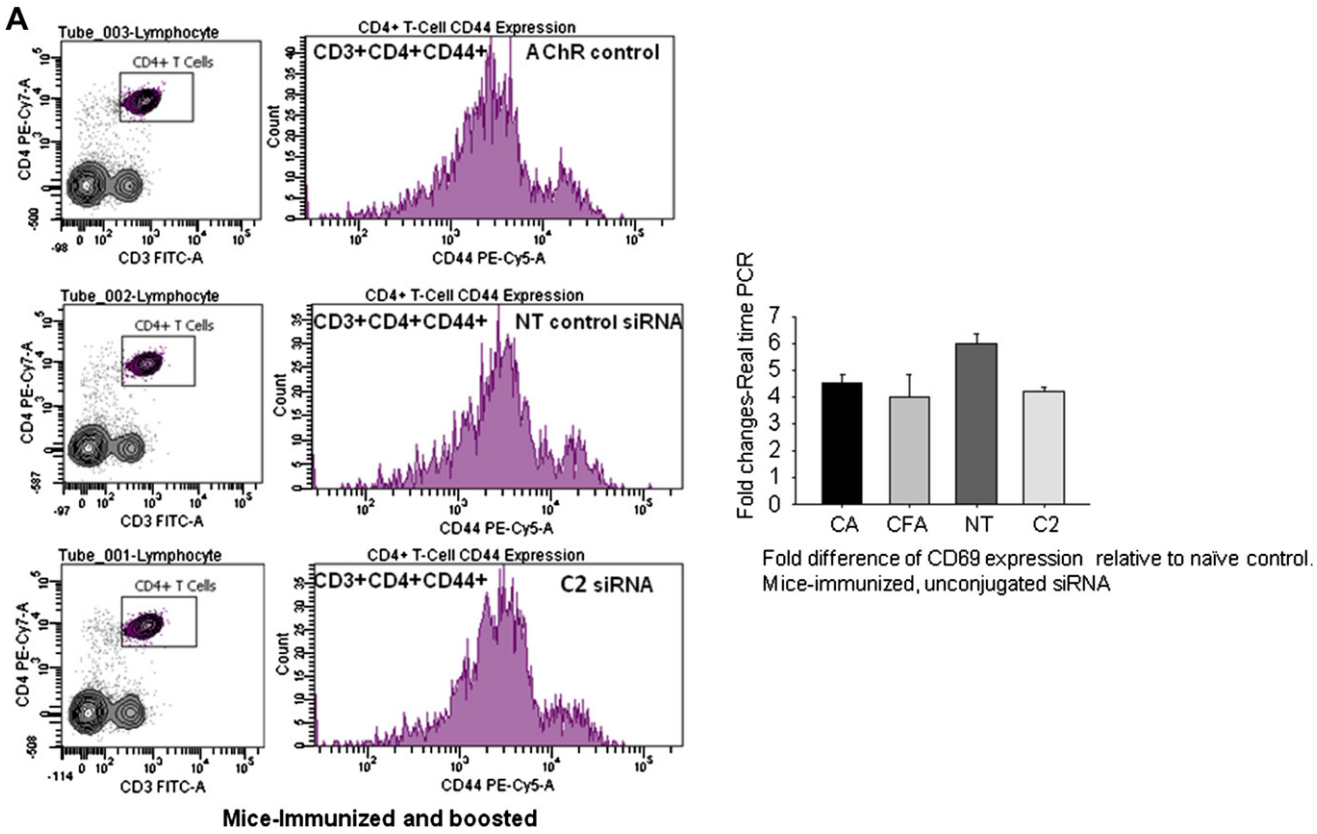

Fig. 5. Polyclonal activation, TLR3 stimulation and apoptosis induction by *in vivo* specific siRNA treatment. Spleen T cells from untreated and C2-and NT control siRNA (50 μg) treated EAMG mice (4 days post treatment) were surface labeled for CD3+CD4+CD44+ for flow analysis. Spleen cells were also surface labeled for CD3 and intracellularly labeled for IFNγ. A. Result shows no difference in fluorescence intensity (CD44+) among samples with or without treatment of siRNA. siRNA used as conjugated or non-conjugated form, produced similar results. B. Frequencies of IFNγ+ cells were insignificant and not different in samples with or without treatment of siRNA in non-immunized mice. C. PBMCs isolated from naïve and CFA-AChR immunized siRNA-treated mice were stained with DAPI. DAPI stained intact nuclei were abundant in all siRNA-treated or untreated PBMCs. Slight nuclear irregularities were apparent in 4% of total cells observed in either C2- or NT control siRNA-treated samples. The rate of apoptosis as reflected by the enrichment of nucleosomes (on *Y*-axis) in the cell death detection ELISA also reveals reduced apoptosis (similar to CFA) in siRNA-treated samples. D. Real time PCR amplification plot of IFNβ, IFNγ, and IFNα on left. Gel resolved regular PCR products of IFNβ. Two samples from each group are resolved in each gel shown above (used 4 samples per group in R-PCR), CA = CFA-AChR.

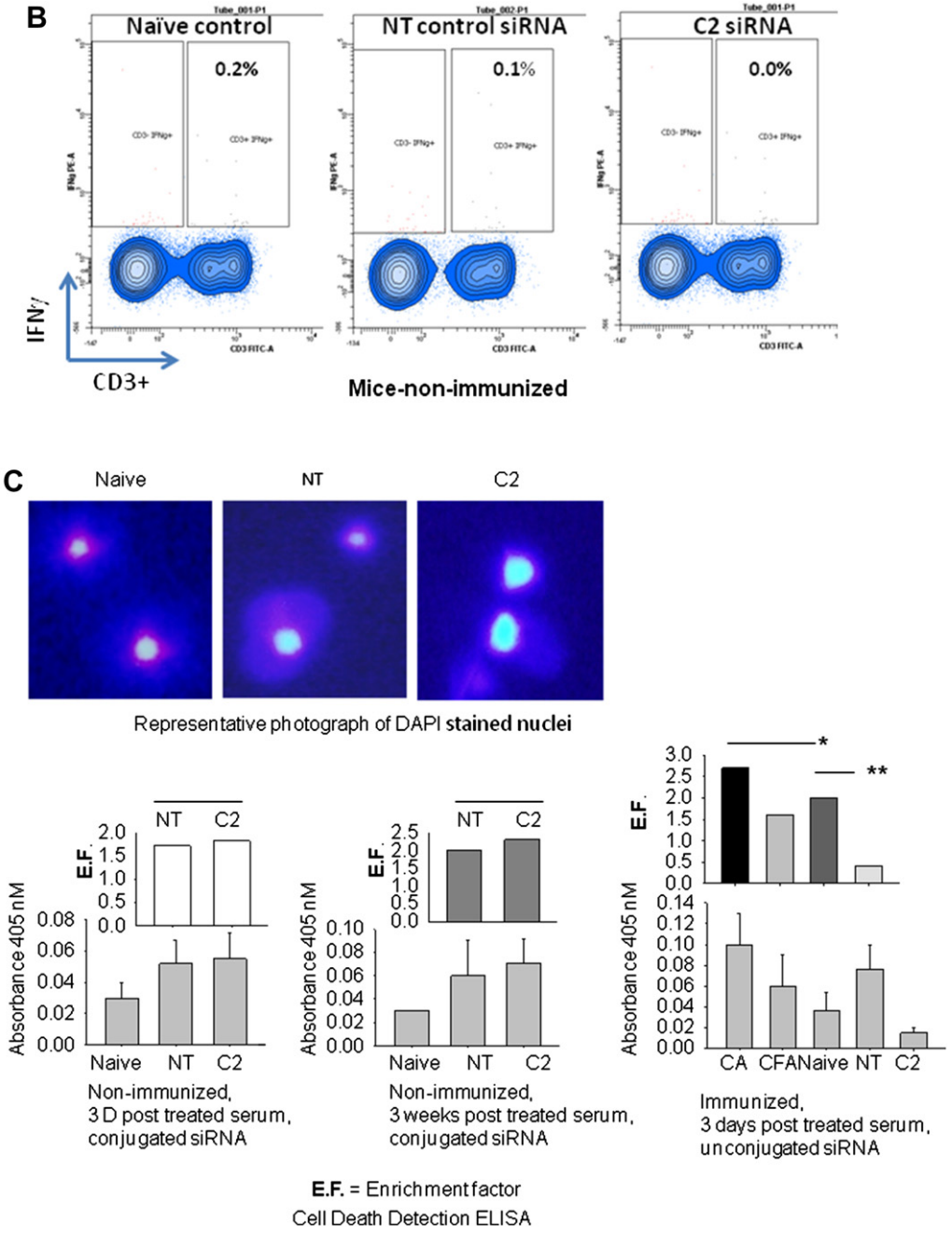

Fig. 5. (*continued*).

3.2. Therapeutic efficacy of C2 siRNA in EAMG

Following a second booster immunization with AChR in CFA, serum complement and anti-AChR IgG levels in mice reached a maximum level and mice exhibited clinical and pathological signs of MG [6,9,13,19]. All mice in this stage, at the levels of grade 1 and grade 2, were randomly assigned to two equal disease groups to receive C2-specific siRNA or NT control siRNA treatment. Although we achieved some clinical amelioration in our initial experiment with the pooled siRNA complex with polymer, the differences between the clinical parameters of C2 siRNA- and NT-siRNA-treated mice were suboptimal, due possibly to a low dose of siRNA (data not shown). Two weeks after the 2nd boost, either C2 siRNA or NT control siRNA (single duplex) were injected i.p. into mice with established disease. Each mouse received 50 μg of either C2 siRNA or NT control siRNA in the form of vehicle-conjugated and non-complex forms (50:50) once a week for 5 weeks.

Mice treated with C2 siRNA, had improved symptoms. By the end of the study, 8 out of 10 mice in the C2 siRNA-treated group had grade 0–1 disease, one mouse died with grade 1 symptoms 3 days after the 1st treatment (cause of death unknown) and one C2 siRNA-treated mouse had grade 2 symptoms that neither improved nor deteriorated throughout the study. In contrast, disease progression was evident in mice during the course of treatment with NT control siRNA. Out of ten mice in the NT control siRNA-treated group, 1 mouse with grade 2 symptoms died a week after the 1st treatment, 3 mice died (grade 4) later during the course of treatment, 4 mice had symptoms between grades 2 and 3, the rest (2 mice) had grade 1 symptoms ($p = 0.001$ by Mann–Whitney U). The grip strength of the C2 siRNA-treated mice, in general, also improved significantly during

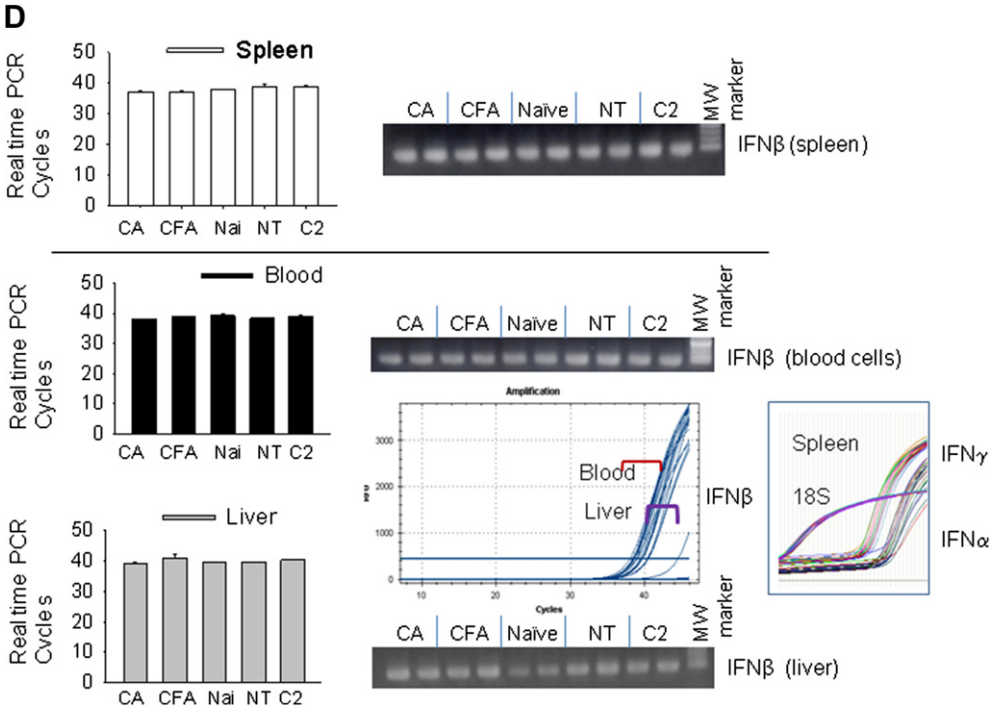

Fig. 5. (*continued*).

the course of C2 siRNA treatment compared to NT control siRNA treatment (Fig. 2A–C). At sacrifice (5 days post 5th treatment), internal organs and tissues appeared normal and without any anatomical and morphological differences among untreated control, C2 siRNA-treated or NT control siRNA-treated mice (Fig. 3A and B). The mean body weight of control siRNA-treated mice was slightly less than that of C2 siRNA mice ($^*P < 0.04$) (Fig. 3C).

3.3. C2 interference in vivo on C2 mRNA expression

To investigate the level of suppression of C2 mRNA expression, cDNAs prepared from total blood cells and livers of untreated or siRNA-treated EAMG mice were subjected to qPCR analysis. Semi-quantitave PCR at the exponential phase (cycle 35) resulted in a marked decrease in the level of C2 mRNA in blood cells 3 days after C2 siRNA treatment, compared to NT control siRNA treatment, but C2 expression appeared to have been quite restored by day 5 following treatment (Fig. 4A and B). C2 mRNA was reduced by 13.9-fold in real-time PCR in C2 siRNA-treated over NT control samples on day 3 post-treatment and only by 2.9-fold on day 5 after the 5th treatment (Fig. 4D). The level of suppression was only 4.2-fold in the liver on day 3 post-treatment of C2 siRNA (analyzed in a separate set of experiment) and even less (2.2-fold) on day 5 post-C2 siRNA treatment (Fig. 4C and D).

3.4. Polyclonal activation, TLR3 stimulation and apoptosis

To rule out the possibility that siRNA induced C2 inhibition may partly have been affected by polyclonal T cell activation and cell death we performed the following experiments. Splenocytes were isolated from non-immunized mice untreated or treated with C2-or NT control siRNA for 4 days and labeled with monoclonal antibodies for flow cytometry analysis of CD44 expression and intracellular IFNγ induction. The results showed only basal and insignificant frequencies of CD44+ and IFNγ+ T cells that did not change by the treatment with siRNA (Fig. 5A and B). R-PCR fold changes of CD69 expression relative to untreated naïve also revealed less than 1.8-fold or insignificant changes among AChR control, C2 siRNA and NT control siRNA-treated spleen cell samples (Fig. 5A). Apoptosis assay either by live PBMC-staining with DAPI for nuclear deformities, Annexin staining for percent dead cells (data not shown) or Nucleosome bound DNA fragments in serum also left no indication of enhanced apoptosis as a result of siRNA treatment (Fig. 5C). Interestingly, apoptosis level was significantly low with unconjugated C2 siRNA than NT control treatment 3 days after treatment. This suggests that our *in vivo* specific siRNA itself did not induce any polyclonal activation of immune cells or cell death *in vivo*.

It is known that TLR expression in T cells in general, is very low. Also, TLR3 and TLR7 are expressed at very low levels in B cells, T cells, monocytes, NK cells and PDCs. TLR9 is also expressed at very low level in these cells except for PDCs and monocytes, in which it is expressed at moderate level [21]. In human liver, TLRs are expressed ubiquitously in all cell types and TLR3 is expressed at very low level in hepatocytes [22].

Since a double stranded RNA can potentially activate TLR3 to subsequently induce IFN-β [23], we performed a real time PCR to check IFNβ expression profile ($n = 4$ per group) in tissues of EAMG mice following siRNA treatment. The result showed comparable levels of constitutive IFNβ expression (blood cells, liver and spleen) in C2- and NT-control siRNA-treated and untreated naïve, CFA-only and CFA-AChR immunized EAMG mice. Difference in IFNβ amplification (ΔC^t) in samples relative to one other was less than one cycle, implying less than two fold or insignificant change (Fig. 5D). The result suggests that TLR3 or innate immune response is not activated by treatment of our "*in vivo* specific" siRNA in EAMG mice. Absence of induction of IFNγ and IFNα also excludes the possibility that TLR7/8 or Type II IFN are induced by our siRNA *in vivo* treatment (Fig. 5D).

Unlike endothelial or epithelial cells where TLR3 is expressed on cell surface, TLR3 in liver or blood cells is present mainly in endosomal compartment [24]. The effect of siRNA is, therefore, appears to be more pronounced on readily accessible cytosolic mRNA rather than endosomal TLR3. Also, it is not yet known if primary cells are as responsive as *in vitro*-transformed cells to TLR stimulation by

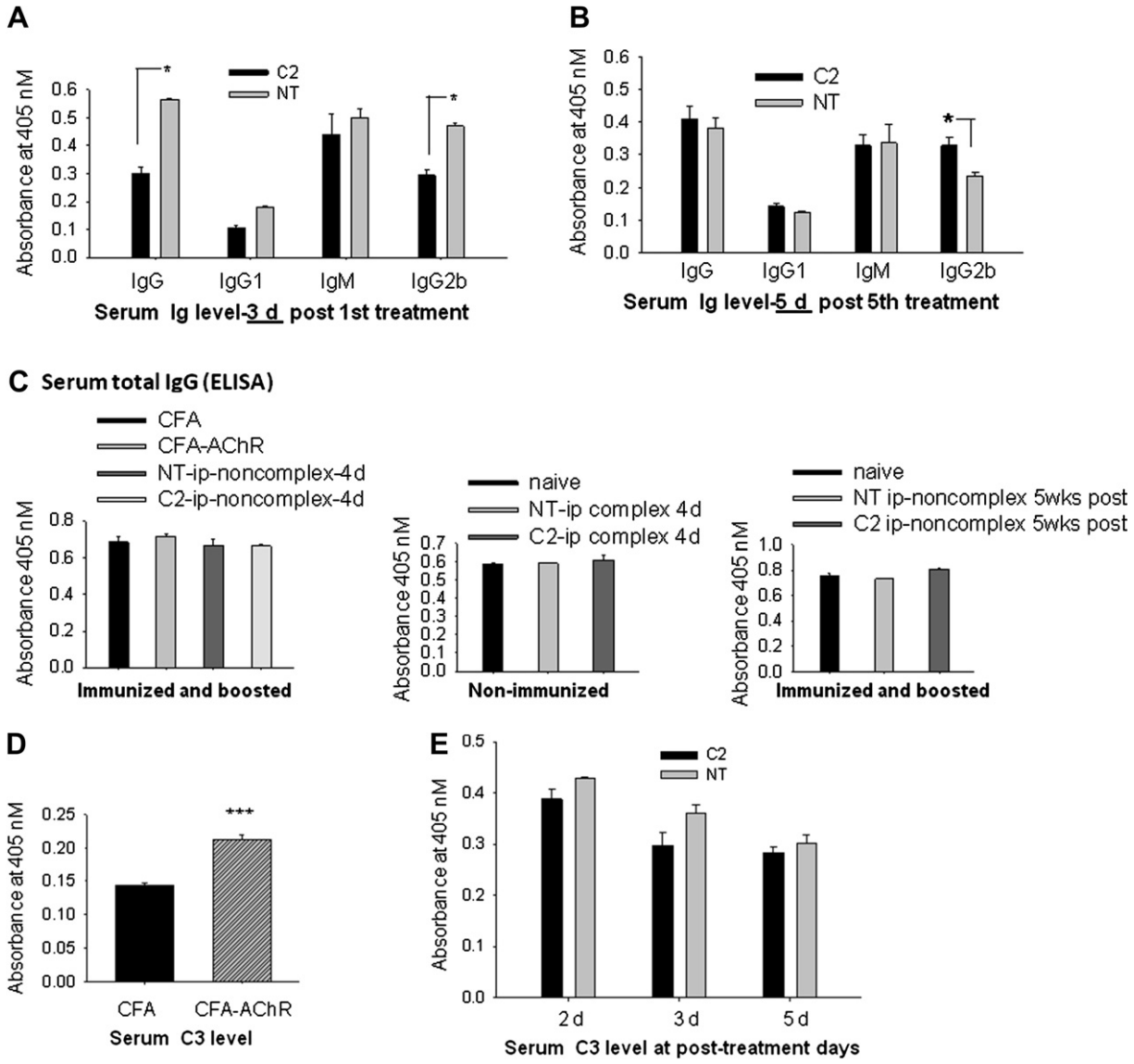

Fig. 6. Serum anti-AChR IgG and IgM levels of C2- and NT control siRNA-treated EAMG mice. EAMG mice treated with either C2 siRNA or control siRNA were bled from the tail vein 3 d post 1st treatment and 5 d post 5th treatment. Serum was isolated and used for ELISA analysis for anti-AChR IgM/IgG/IgG1/IgG2b and C3. (A) Anti-AChR IgG1 and IgM levels of C2 siRNA-treated mice slightly, but not significantly, decreased compared to those in control siRNA-treated mice 3 days after 1st treatment. Anti-AChR IgG and IgG2b levels in the C2 siRNA group decreased significantly over those in NT siRNA-treated mice at this timepoint ($^{*}p < 0.05$). (B) Other than IgG2b, all anti-AChR IgG and IgM levels were similar 5 days after 5th treatment in C2 siRNA- and control siRNA-treated mice. IgG2b levels, however, increased significantly in C2 siRNA − over control siRNA-treated mice after the 5th treatment, $^{*}P < 0.05$. (C) Serum level of total IgG remained at similar level among untreated, siRNA, treated (conjugated with invivofectamine or unconjugated) and naïve mice. (D) Sandwich ELISA reveals a significantly high level of serum C3 in EAMG mice at the post 2nd boost, $^{***}P < 0.001$. (E) Treatment of EAMG mice with C2 siRNA did not significantly reduce C3 levels in serum. Results are representative of 3 independent experiments. C2, CFA-AChR-immunized mice treated with C2 siRNA; NT, CFA-AChR immunized mice treated with non-targeting control siRNA; CFA, untreated, CFA-immunized mice; CFA-AChR, untreated CFA-AChR-immunized mice. Vertical bars represent standard error, $n = 4$ for 3 d post 1st treatment; $n = 9$ (C2) and $n = 6$ (NT) for 5 d post 5th treatment.

conjugated or unconjugated siRNA. It has been reported that human TLR3 is not a ligand of unformulated siRNA [25]. Off target effect or activation is also known to depend on the sequence of siRNA.

Together, the results show that our siRNA delivery system itself neither induced any polyclonal activation, nor activated the innate immune system and the reduced expression of C2 had primarily resulted from mRNA sequence specific effect of C2 siRNA.

3.5. Serum anti-AChR IgG, IgM and C3 levels in siRNA-treated EAMG mice

We sought to analyze the effect of siRNA treatment on the serum levels of anti-AChR antibody and C3. CFA- and CFA-AChR-immunized mice were bled after the 2nd booster immunization and a week prior to the start of treatment to collect blood cells for C2 mRNA and serum for ELISA analyses. They were also bled on day 3 after the first treatment ($n = 4$) and day 5 after the last (5th) treatment with C2 siRNA ($n = 9$) or NT control siRNA ($n = 6$). Although the serum levels of anti-AChR IgM and IgG1 were slightly reduced in C2 siRNA-treated EAMG mice on day 3 after the 1st treatment, the levels were not significantly different from those in the NT control siRNA-treated group. Serum anti-AChR IgG and IgG2b levels were significantly reduced in C2 siRNA-treated mice at that time point (Fig. 6A). The levels of anti-AChR IgG, anti-AChR IgG1 and anti-AChR IgM were similar in both C2- and NT siRNA-treated groups on day 5 after the 5th (last) treatment. Unlike the results of 1st treatment, serum

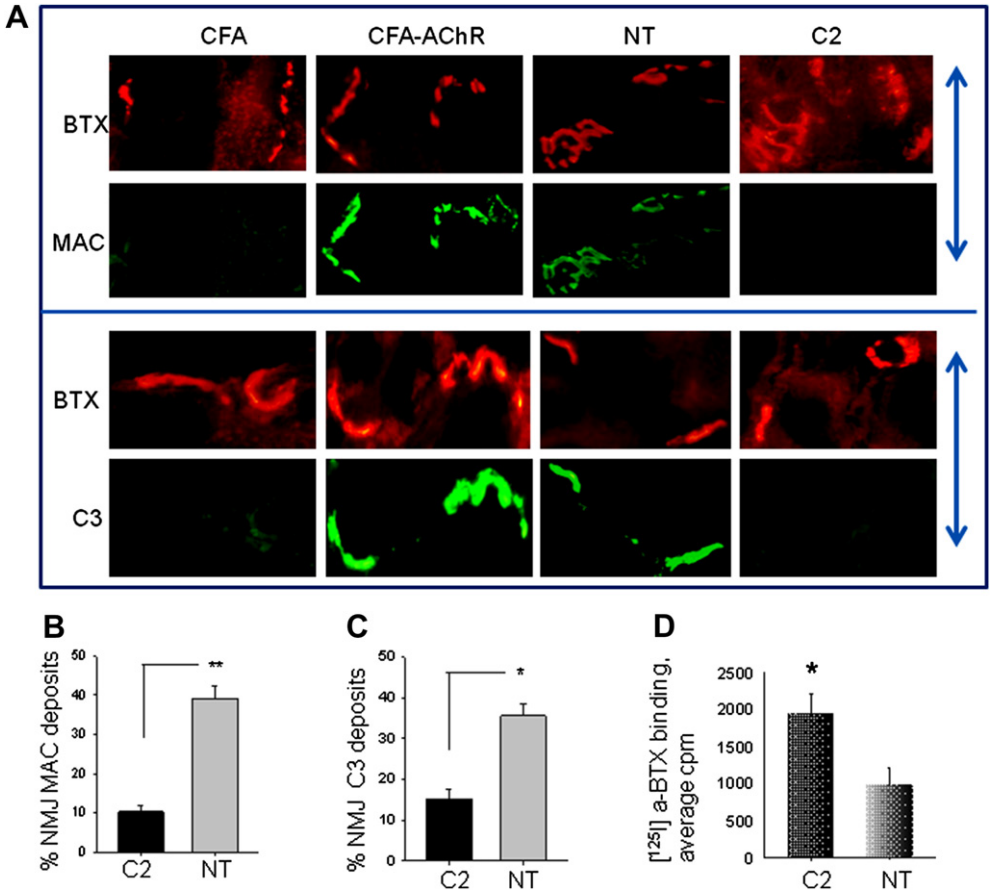

Fig. 7. Reduced MAC formation and preservation of functional muscle AChR in C2 siRNA-treated EAMG mice. Representative cryostat sections of the right triceps muscles of mice, fixed and stained with alpha-BTX and antibodies conjugated with fluorescent dyes. Untreated CFA and CFA-AChR-immunized mice were used as negative and positive controls. (A) Microscopic analysis of fluorescence images or spots in the sections revealed a higher frequency of end plate AChR (intense crescent fluorescence from bound BTX) at NMJs of C2 siRNA-treated and CFA groups (right and left most panels) compared to those in NT control siRNA-treated and untreated CFA-AChR groups. Control and untreated mice had NMJs that were also stained positive for C3 and MAC in higher density compared to C2 siRNA-treated and CFA mice. Loss of AChR is apparent at many sites where MAC is well formed and damaged the membrane. The representative images of sections from CFA- and C2-treated mice show sites where AChR is lacking MAC formation and C3 at neuromuscular junctions (bottom panel). (B, C) Quantitation of fluorescence-labeled C3 and MAC deposits reveals EAMG mice treated with C2-siRNA possess significantly low percentages of C3 and MAC deposits as compared to mice treated with control siRNA or no siRNA, $^{*}P < 0.05$, $^{**}P < 0.01$ respectively. (D) Muscle AChR content is preserved in EAMG mice treated with C2 siRNA. Upon termination, crude receptors from muscle lysates were prepared from treated and untreated mice carcasses and quantitated. Radioimmunoassays using ^{125}I-α-BTX-labeled AChR reflected a significantly higher content of functional muscle AChR in EAMG mice treated with C2 siRNA versus control si RNA, $^{*}P < 0.05$. Vertical bars represent standard error.

anti-AChR IgG2b levels were found at significantly higher levels in C2 siRNA-treated mice compared to NT control siRNA-treated mice at that time point (Fig. 6B). Total IgG level however, remained at similar level among untreated, siRNA-treated (conjugated with invivofectamine or unconjugated) and naïve serum samples (Fig. 6C). A significantly higher serum level of C3 was also detected in mice immunized with CFA-AChR than those given CFA only (Fig. 6D). Serum C3, however, remained at similar levels in C2 siRNA- and NT control siRNA-treated animals (Fig. 6E).

3.6. Reduced number of C3, MAC and increased quantity of functional AChR at NMJ in the muscles of C2 siRNA-treated mice

Next, we assessed if improvement in muscle strength in C2 siRNA-treated mice is achieved by preservation of muscle AChR. The immunohistology of cryostat sections of the triceps muscle showed a drastic reduction in the number of MAC and C3 deposits at the NMJs of C2 siRNA-treated animals when compared to the NT control siRNA-treated mice (Fig. 7A). Quantitative analysis of C3 and MAC deposits in muscle also revealed significantly higher percentages of C3 and MAC deposits at the NMJs of NT control over C2 siRNA-treated mice ($^{*}P < 0.05$, $^{**}P < 0.01$) (Fig. 7B and C). Finally, we analyzed the levels of functional AChR in the muscles of those mice. Crude AChR in the membrane fraction in lysates was separated from mouse carcasses. As expected, radioimmunoassay by using ^{125}I-α BTX-labeled AChR reflected a higher functional muscle AChR content in the C2 siRNA-treated group as compared to that in NT controls (specific counts or cpm divided by the specific activity of α-BTX (240 Ci/mmol) were 8.14×10^{-10} M and 4.06×10^{-10} M of α BTX-binding sites in C2 siRNA-treated and NT control siRNA-treated mice, respectively ($^{*}P < 0.05$)) (Fig. 7D).

4. Discussion

In this study, we reasoned that it would be efficacious to treat mice with established EAMG using C2-specific siRNA, since targeting C2 has a unique advantage over other complement proteins. C2 is a low abundant protein and forms the catalytic core of the convertase complex [26] that leads to classical complement pathway activation [27–30] that is critical for EAMG induction [8,9,13,29]. Also, unlike other classical pathway components C1q and C4, C2 does not appear to be involved in any major immunological functions such as clearance of immune complexes and establishment of tolerance to self-tissue. As a result, congenital C2

deficiency is less frequently associated with immune complex disorders than C1q and C4 [26].

C2 mRNA suppression was partial in the liver, presumably because of limited accessibility of C2 siRNA to liver cells. Besides, Kupffer and other cells in the liver might be more resistant to siRNA uptake than other cell types. However, significant reductions in levels of C2 mRNA in extra-hepatic peripheral blood cells were observed 3 days after siRNA treatment. This difference was probably due to relatively low expression levels of C2 in blood cells and increased accessibility and direct uptake of siRNA by the blood cells. It is also possible that the total amount of C2 siRNA used in our experiments was not enough to bind all target sequences present in the body at a given time. Moreover, the efficacy of C2 inhibition was remarkably reduced in both blood and liver cells at 5 days rather than at 3 days after siRNA treatment which suggest that frequent injections of C2 siRNA are required for an effective and prolonged inhibition of MAC formation. Nonetheless, symptomatic improvement of the disease and significantly less mortality were apparent, which resulted from an overall, cumulative suppression of expression of C2, a critical contributor to the disease. Moreover, it is not necessary to completely silence the C2 gene by C2 siRNA, and a partial suppression of C2 mRNA in blood cells and liver is sufficient to treat MG in mice. A C2-binding peptide was previously used to treat non-specific, immune complex-mediated inflammation in mice [30]. However, to our knowledge, our study represents the first demonstration of C2-mediated inhibition in the therapy of a classical autoimmune disease. Also, we have utilized siRNA-mediated complement inhibition for treatment of an autoimmune disease for the first time. Mice treated with or without C2 siRNA demonstrated comparable serum C3 levels. This was anticipated since C2 inhibition is only expected to block the C3 activation pathway but not expression of C3 by liver and other tissues. Nevertheless, C2 siRNA-treated mice had significantly reduced NMJ C3 and MAC deposits. This can only be explained by a reduced availability of C2 at NMJ due to the siRNA-mediated inhibition of this complement component. Since C2 is a component of the convertase enzyme that catalyzes the cleavage of C3, reduced C2 levels at the NMJ might have led to reduced deposition of activated C3 and MAC and thereby better conservation of AChR in siRNA-treated mice. Consequently, immunofluorescence studies showed a higher number of AChR at the NMJs of C2 siRNA-treated mice. This was complemented by a quantitative radioimmunoassay assay which demonstrated a significantly higher content of functional AChR in muscle from carcasses of C2 siRNA-treated mice, compared to those in NT-controls. Together, the data imply that muscle function is preserved in C2 siRNA-treated mice due to partial inhibition of activation of the complement cascade.

C2 siRNA treatment transiently reduced serum anti-AChR IgG2b levels. A similar serum anti-AChR IgG2b reduction had been observed in AChR immunized mice that were deficient for C4, another classical pathway factor, suggesting that classical pathway has some role in IgG2b production or perhaps adaptive immunity [8]. Since IgG2b is a complement-fixing isotype that is profoundly involved in complement-mediated destruction of NMJ [8,9], it is tempting to speculate that the clinical amelioration of C2 siRNA-treated mice was at least partly related to this temporary drop in serum IgG2b levels. Elevation of IgG2b by prolonged (5 weeks) down-modulation of C2 expression in the present study suggests that C2 could have a dual role in regulating IgG2b levels. Alternatively, IgG2b elevation might simply be a compensating measure and other IgG2b-producing factors might be activated by the prolonged inhibition of C2 production. Whether this factor might reduce the efficacy of C2 siRNA in long-term treatment needs to be investigated. It will be necessary also to observe the persistence of the IgG2b-enhancing role of C2 inhibition in the long run following siRNA withdrawal, which may cause an unwanted side effect of disease recurrence. It is also possible that C2 siRNA *in vivo* prevents homing of the complement-binding IgG2b isotype into NMJ and, therefore, more IgG2b are detected in the circulation. C2 suppression by *in vivo* siRNA otherwise did not significantly affect the production of other Ig isotypes compared to that in NT controls at the end of treatment.

5. Conclusion

The results of our study indicate a therapeutic potential of complement inhibition not only for MG, but also for other antibody-mediated autoimmune diseases, in which complement plays a role. Also, targeting the classical complement pathway component, C2, is potentially beneficial, because the alternative pathway can still be activated by microorganisms and thus protects the host from infections. Therapy of MG by siRNA is also beneficial as the effect of siRNA is transitory and target specific, and with no well-known concurrent adverse side effects. We suggest that for successful siRNA therapeutic strategy, one should target a gene that is integrated in the disease pathway without overlapping other beneficial pathways and is not highly expressed. Also, suppression of the target gene at such a level should not be deleterious to normal physiology. C2 siRNA treatment appears to fulfill these requirements. Determination of appropriate pharmaceutical formulation, optimal dosage and route of delivery and toxicity study are necessary for human clinical trials.

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