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Functional Redundancy of MyD88-dependent Signaling Pathways in a Murine Model of Histidyl-tRNA Synthetase-induced Myositis

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Abstract

We have previously shown that intramuscular administration of bacterially expressed murine histidyl-tRNA synthetase (HRS) triggers florid muscle inflammation (relative to appropriate control proteins) in various congenic strains of mice. Because severe disease develops even in the absence of adaptive immune responses to HRS, we sought to identify innate immune signaling components contributing to our model of HRS-induced myositis. In vitro stimulation assays demonstrated HRS-mediated activation of HEK293 cells transfected with either TLR2 or TLR4, revealing an excitatory capacity exceeding that of other bacterially expressed fusion proteins. Corresponding to this apparent functional redundancy of TLR signaling pathways, HRS immunization of B6.TLR2$^{-/-}$ and B6.TLR4$^{-/-}$ single knockout mice yielded significant lymphocytic infiltration of muscle tissue comparable to that produced in C57BL/6 WT mice. In contrast, concomitant elimination of TLR2 and TLR4 signaling in B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice markedly reduced the severity of HRS-induced muscle inflammation. Complementary subfragment analysis demonstrated that amino acids 60–90 of HRS were absolutely required for in vitro as well as in vivo signaling via these MyD88-dependent TLR pathways—effects mediated, in part, through preferential binding of exogenous ligands capable of activating specific TLRs. Collectively, these experiments indicate that multiple MyD88-dependent

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signaling cascades contribute to this model of HRS-induced myositis, underscoring the antigenic versatility of HRS and confirming the importance of innate immunity in this system.

INTRODUCTION

The idiopathic inflammatory myopathies (IIM) encompass a group of multisystem autoimmune disorders in which an aberrant immune response targets muscle as well as extramuscular organs (1–3). In addition to characteristic patterns of organ involvement, well defined autoantibodies help to distinguish clinical subsets of patients with IIM (4, 5). Chief among these autoantibodies are the anti-synthetase antibodies that associate with a clinical syndrome marked by variable combinations of myositis, arthritis, Raynaud's phenomenon, "mechanic's" hands, fever, and interstitial lung disease (6). Despite the preponderance of data supporting a link between anti-synthetase antibodies and clinical course/disease activity, however, the precise role of these autoantibodies and corresponding antigen-specific T cells in disease pathogenesis remains undefined.

As investigators have pursued the immunologic basis for the striking relationship between tRNA synthetases and the anti-synthetase syndrome, it has become clear that the contribution of these putative autoantigens to underlying disease pathogenesis may extend beyond the traditional paradigms of adaptive immunity. Following Wakasugi's seminal observation that subfragments of tyrosyl-tRNA synthetase could function as biologically active cytokines (7), the concept that some of the tRNA synthetases might be capable of triggering innate immune responses has gradually taken hold. In fact, through a series of elegant in vitro studies, Howard et. al. subsequently demonstrated that two known myositis autoantigens--histidyl-tRNA synthetase (HRS=Jo-1) and asparaginyl-tRNA synthetase (ARS=KS)--possessed chemokine-like properties capable of stimulating lymphocytes, activated monocytes, and immature dendritic cells (8). Because other tRNA synthetases not linked to the anti-synthetase syndrome (through autoantibodies) failed to demonstrate similar chemokine profiles, these studies further suggested that innate immune pathways could directly contribute to antigenicity and disease pathogenesis.

To investigate the role of histidyl-tRNA synthetase (HRS, the most commonly targeted tRNA synthetase) in triggering innate immune responses leading to clinical expression of myositis, we previously devised a murine model based on intramuscular (IM) immunization with a recombinant subfragment of HRS in the absence of additional exogenous adjuvant (9). In various congenic strains of NOD and C57BL/6 mice, this experimental system yielded striking IgG class-switched autoantibody responses as well as robust CD3+CD44+CCR5+ lymphocytic infiltration of muscle that was directly attributable to HRS (rather than the MBP moiety of HRS fusion proteins). Persistence of the tissue phenotype in C3H/HeJ and DO11.10/Rag2$^{-/-}$ mice demonstrated that cellular invasion of muscle tissue was not dependent on Toll-like receptor 4 (TLR4) or T cell receptor (TCR) signaling, respectively (9). Collectively, these observations suggested that HRS was capable of inducing myositis independent of antigen-specific adaptive immune responses, a conclusion further supported by the relative uncoupling of anti-HRS autoantibody formation

and lymphocytic invasion of muscle in C3H/HeJ mice (that develop florid myositis in the setting of markedly reduced anti-HRS antibody titers).

Given the weight of evidence implicating innate immune signaling in our model of HRS-induced myositis, we devised the current studies to explore the mechanistic basis of this response. While previous experiments in TLR4 signaling-deficient C3H/HeJ mice indicated that TLR4 was not required for the development of myositis, the central role of TLR signaling cascades in many innate immune responses led us to examine the contribution of other non-TLR4 pathways using a series of C57BL/6-derived knockout strains as well as TLR-transfected cell lines. Collectively, these experiments demonstrated that, in conjunction with preferentially bound TLR ligands, recombinant HRS directs overlapping signals through several MyD88-dependent receptors mediating cellular infiltration of muscle in this disease model.

METHODS

Mice

Eight to twelve week old male and female mice of the following strains were used in immunization protocols approved by the University of Miami Institutional Animal Care and Use Committee: C57BL/6 (B6), B6.MyD88$^{-/-}$, B6.TLR2$^{-/-}$, B6.TLR4$^{-/-}$, and B6.TLR2$^{-/-}$.TLR4$^{-/-}$ (double knockout). All strains were obtained from Jackson Laboratories (Bar Harbor, Maine), except for B6.TLR2$^{-/-}$.TLR4$^{-/-}$ mice that were provided by Dr. Ru Liu-Bryan (University of California San Diego) after crossing B6.TLR2$^{-/-}$ with B6.TLR4$^{-/-}$ mice.

Recombinant protein

As previously described (10), the immunodominant amino terminal fragment of mHRS was produced as a maltose binding protein fusion protein, MA/MBP (amino acids 1–151 of mHRS linked to the carboxy terminal end of MBP). Using an analogous approach, we generated a series of additional MBP fusion proteins containing amino terminal subfragments of human HRS (hHRS) that were designated as HA5/MBP (aa 1–151), HA4/MBP (aa 1–120), HA3/MBP (aa 1–90), and HA2/MBP (aa 1–60). Produced in similar fashion, 70 kDa RNP/MBP was provided by Dr. Eric Greidinger (University of Miami). Bacterially expressed fusion proteins were purified with amylose resin per the manufacturer's protocol (New England Biolabs, Ipswich, MA), dialyzed against PBS, and filter sterilized prior to in vitro stimulation assays or intramuscular immunization of mice. Preparation of gel purified proteins required separation by SDS-PAGE (10% polyacrylamide gel), extraction of gel fragments, and electrophoretic elution prior to dialysis and filter sterilization. All recombinant proteins were assessed for endotoxin activity through Limulus Amebocyte Lysate (LAL) assays (Lonza, Allendale, NJ); selected proteins were also subjected to endotoxin removal using poly(ε-lysine) affinity purification columns according to the manufacturer’s protocol (Pierce/Thermo Fisher Scientific, Rockford, IL).

In vitro measurement of TLR signaling

Recombinant proteins were tested for TLR2, TLR4, and TLR5 signaling capacity through overnight in vitro stimulation of HEK293 cells transfected with human TLR2, murine TLR4, or murine TLR5 (InvivoGen, San Diego, CA), respectively. ELISA-based assessment of IL-8 production in culture supernatants (per the manufacturer's protocol) provided a quantitative measure of relative TLR signaling capacity.

Immunization

Experimental mice received intramuscular injections of soluble, affinity purified MA/MBP protein (4–5 mg/ml) or amino terminal subfragments of human HRS (HA2-HA5/MBP, 2.5–3 mg/ml) administered to both hamstrings in a total volume of 100 μl (50 μl/side). Mice were then sacrificed for harvesting of serum, lymphoid tissue, and muscle at Day 17, an established endpoint for the development of myositis in this model (9).

Histopathology and immunohistochemistry

Harvested organs were fixed in 10% formalin prior to paraffin embedding, sectioning, and staining with hematoxylin/eosin by the University of Miami's Department of Pathology Laboratory. Following these staining procedures, a neuromuscular researcher with specific expertise in muscle pathology independently scored/confirmed the severity of inflammation in muscle tissue (while blinded to study groups). Scores based on the relative area and intensity of lymphocytic infiltration in multiple fields viewed under high power magnification ranged from 0–3, where 0 = no inflammation, 1 = minimal, 2 = moderate, and 3 = severe inflammation.

For immunohistochemical assessment of cellular infiltrates, tissue sections from de-paraffinized muscle specimens were stained with a series of antibodies targeting different molecular markers according to a protocol developed in the University of Pittsburgh Transplant Immunology Histopathology Core Laboratory. These antibodies consisted of anti-CD3 (A0452; Dako North America, Carpenteria, CA) as well as anti-TLR2 (ab24192), anti-TLR4 (ab13556), anti-CD11c (ab33483-N418), and anti-B220/CD45 (ab10558) (latter antibodies all from Abcam, Cambridge, MA).

ELISA

In sera derived from mice immunized with various forms of recombinant HRS, levels of IgG anti-murine or human HRS antibody were measured using standard solid phase ELISA according to a previously established protocol (10). Substrate antigens included recombinant hHRS (full length human HRS, 1.0 μg/ml), recombinant mHRS (full length murine HRS, 1.0 μg/ml), or recombinant MBP (maltose binding protein, 1.0 μg/ml).

Determination of LPS-HRS and FSL-1-HRS complex formation

To assess the formation of HRS-LPS and HRS-FSL-1 complexes, we pre-incubated different concentrations of LPS (strain 0111:B4, Sigma-Aldrich, St. Louis, MO) or the TLR2 ligand FSL-1 (InvivoGen, San Diego, CA) with affinity purified, baculovirus-expressed HRS (full length) for two hours and then added these mixtures to ELISA wells

pre-blocked with 1% BSA in PBS/0.05% Tween-20 for an additional two hours. Plates were subsequently washed with PBS/Tween, probed with anti-human HRS mouse serum (1:1000 dilution), and then rewashed with PBS/Tween. Following sequential incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (0.04 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) and 3,3,5,5-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO), enzymatic reactions were terminated with 1 N H_2SO_4 and quantified using spectrophotometrically-measured OD_{450} values. As a complementary approach, pre-bound substrate antigens (full length HRS (baculovirus-expressed), ovalbumin) were co-incubated with biotinylated LPS (Invivogen, San Diego, CA) and subsequently probed with streptavidin-horseradish peroxidase conjugate (R&D Systems, Minneapolis, MN) prior to the enzymatic color development procedures outlined above.

Detection of HRS-HMGB1 binding

In a modified ELISA format, various concentrations of baculovirus-expressed recombinant human HRS (hHRS) or murine HRS (mHRS) (ranging from 0.1–10 μg/ml) were added to 96 well plates coated with recombinant human HMGB1 (eBioscience, San Diego, CA) for 2 hours. Following multiple washes with PBS/0.05% Tween-20, HRS-HMGB1 complexes were detected through serial application of previously generated mouse sera specific for murine or human HRS (1:1000 dilution) and HRP-conjugated anti-mouse IgG secondary antibody (1:10,000 dilution, Santa Cruz Biotechnology, Dallas, TX) according to established protocol (10).

Statistics

Severity scores for muscle inflammation were compared using the Mann-Whitney *U*-test, with significance based on a two-tailed p value <0.05. Relative anti-HRS antibody levels (represented by adjusted OD_{450} values) were also assessed with the Mann-Whitney U-test and a two-tailed p value threshold of <0.05 for statistical significance.

RESULTS

MyD88 knockout mice fail to develop myositis following intramuscular HRS immunization

To determine the potential contribution of MyD88-dependent signaling pathways in HRS-induced myositis, we immunized B6.MyD88$^{-/-}$ mice (n=10) with MA/MBP (amino terminal fragment of murine HRS (amino acids 1–151) fused to maltose binding protein) via intramuscular injection. As shown in Figure 1, MA/MBP immunization failed to induce myositis in the absence of MyD88 signaling despite the persistence of significant anti-HRS autoantibody responses. These results again demonstrated the relative uncoupling of humoral and cell-mediated immune responses triggered by HRS immunization and further supported the predominant role of innate immune signaling in driving the tissue phenotype of this model system.

Recombinant HRS directs signals through multiple MyD88-dependent Toll-like receptors

Although MyD88 contributes to both TLR and non-TLR signaling cascades, abrogation of the myositis phenotype in B6.MyD88$^{-/-}$ mice strongly suggested a role for MyD88-dependent TLR signaling following MA/MBP immunization. As a surrogate measure of

HRS-mediated TLR signaling, we assessed the ability of MA/MBP and various control proteins to stimulate HEK293 cells transfected with different extracellular membrane-bound, MyD88-dependent TLRs that included TLR2, TLR4, and TLR5. Figure 2A clearly demonstrated that MA/MBP could drive signals through TLR2 and TLR4, but not TLR5. This activity was relatively specific for MA/MBP and related recombinant HRS fusion proteins, greatly exceeding stimulation promoted by an unrelated fusion protein (70 kDa RNP/MBP) or MBP alone.

In the case of TLR4, the stimulatory activity of HRS fusion proteins paralleled findings in limulus amebocyte lysate (LAL) assays which demonstrated remarkably consistent enhancement of endotoxin binding to HRS constructs relative to alternative bacterially-produced fusion proteins (Supplementary Figure 1). Supporting these findings, co-incubation of LPS with more purified forms of HRS produced in a eukaryotic expression system yielded clear evidence of enhanced binding and complex formation capable of synergistic/additive signaling through TLR4 (Supplementary Figure 2). Interestingly, while gel purification significantly reduced the capacity of the bacterially expressed HRS derivative MA/MBP to signal via TLR4 (Figure 2B and Supplementary Figure 1), it had less effect on the ability of this HRS fusion protein to signal through TLR2 (Figure 2B)—suggesting that TLR2 binding/activation stems from even higher affinity of this recombinant antigen for exogenous TLR2 ligands. The latter conclusion is supported by in vitro experiments demonstrating the ability of recombinant HRS to bind the TLR2 ligand FSL-1 and synergistically activate TLR2-transfected HEK293 cells (Supplementary Figure 3).

HRS induces myositis in both B6.TLR4$^{-/-}$ and B6.TLR2$^{-/-}$ mice

Based on results of these in vitro signaling assays as well as immunohistochemical studies demonstrating expression of TLR2 and TLR4 in muscle cell infiltrates (Figure 3), we evaluated the capacity of MA/MBP to induce myositis in B6.TLR4$^{-/-}$ and B6.TLR2$^{-/-}$ mice. Replicating findings in TLR4 signaling-deficient C3H/HeJ mice (9), MA/MBP immunization of B6.TLR4$^{-/-}$ mice readily induced muscle inflammation comparable to that generated in C57BL/6 WT mice (Figure 4A). Unexpectedly, however, parallel immunization of B6.TLR2$^{-/-}$ mice with MA/MBP also provoked severe myositis as well as high titers of IgG class-switched anti-HRS autoantibodies (Figures 4B and 4C). Taken together, these findings raised the possibility of redundant signaling cascades directed through TLR2 and TLR4 versus involvement of an unrelated MyD88-dependent pathway.

Concomitant elimination of TLR2 and TLR4 signaling impairs HRS-induced myositis

To better address the requirements for TLR2 and/or TLR4 signaling in this system, we next immunized B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice with recombinant HRS. As shown by the comparison of Figure 4 and Figure 5, these double knockout mice demonstrated markedly reduced muscle inflammation relative to C57BL/6 WT or single TLR knockout mice in response to MA/MBP immunization (mean severity scores +/- SEM: C57BL/6=1.83 +/- 0.17; B6.TLR2$^{-/-}$=2.15 +/- 0.15; B6.TLR4$^{-/-}$=1.7 +/- 0.12; B6.TLR2$^{-/-}$.TLR4$^{-/-}$=0.72 +/- 0.17). Although these results were consistent with a predominant role for TLR2 and TLR4 signaling pathways in the development of myositis, the persistence of mild residual

muscle inflammation and low level anti-HRS antibody titers indicated that other, minor HRS-mediated signaling pathways could be operative in this model.

HRS subfragment analysis defines a critical region responsible for mediating TLR signaling and induction of myositis

Given the capacity of MA/MBP to generate TLR2- and TLR4-mediated signals contributing to the development of myositis in C57BL/6-derived strains, we sought to identify the region of HRS responsible for these biological effects. In vitro assessment of previously constructed subfragments derived from HA5/MBP, the human orthologue of MA/MBP, demonstrated that a segment spanning amino acids 60–90 of HRS was essential for stimulating IL-8 production from TLR2- and TLR4-transfected HEK293 cells (Figure 6A). Results from LAL assessment of HRS subfragments paralleled findings in TLR4-transfected 293 cells by showing marked reduction of endotoxin activity in the absence of amino acids 60–90 (data not shown). Corresponding to these in vitro studies, immunization of C57BL/6 mice with MBP fused to subfragments HA2 (aa 1–60), HA3 (aa 1–90), HA4 (aa 1–120) or HA5 (aa 1–151) demonstrated that truncation of amino acids 60–90 eliminated HRS-induced myositis (Figure 6B) as well as autoantibody formation (Figure 6C).

These immunization experiments further suggested an important role for amino acids 90–120, as HA3/MBP triggered milder disease relative to that induced by HA4/MBP and HA5/MBP (Figure 6B). More detailed analysis of the tissue phenotype generated by HA3/MBP immunization of B6.TLR2$^{-/-}$ and B6.TLR2$^{-/-}$.TLR4$^{-/-}$ mice revealed almost complete abrogation of myositis, contrasting with the persistence of residual muscle inflammation in HA5/MBP-immunized mice possessing the same TLR2/TLR4 knockout profile (data not shown). Given the relative preservation of TLR2 and TLR4 signaling induced by HA3/MBP (Figure 6A), these collective data indicated that the region of HRS spanning amino acids 90–120 was required for the activation of additional non-TLR2/TLR4 pathways contributing to the myositis phenotype in mice immunized with HA4/MBP or HA5/MBP.

DISCUSSION

Through this combination of in vitro and in vivo experiments, we have demonstrated that the ability of recombinant HRS to generate myositis is dependent upon signaling pathways involving MyD88. While HRS readily induces muscle inflammation in B6.TLR2$^{-/-}$ and B6.TLR4$^{-/-}$ mice, the myositis phenotype is significantly blunted in B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice—supporting the possibility of redundant signaling between these MyD88-dependent TLR pathways. Analysis of recombinant human HRS subfragments indicates that amino acids 60–90 are absolutely required to mediate these effects (either directly or via secondary binding of TLR ligands), as HA2/MBP (hHRS amino acids 1–60) fails to stimulate TLR2- or TLR4-transfected HEK 293 cells and is unable to provoke myositis in C57BL/6 mice.

Consistent with these findings demonstrating a role for multiple MyD88-dependent pathways in HRS-induced myositis, recent work involving human muscle tissue specimens indicates that a number of TLRs, including TLR2 and TLR4, are expressed by autoinvasive

cells in polymyositis and dermatomyositis (11, 12). Although the antigen specificity of infiltrating lymphocytes remains to be determined, this independent observation suggests that human disease also involves activation of the innate immune system and subsequent cellular recruitment. In this scenario, infection or other “danger” signals trigger upregulation of vascular/cellular adhesion molecules as well as various TLRs capable of interacting with endogenous or exogenous ligands—including those associated with putative autoantigens such as HRS. As an extension of this process, subsequent expansion of HRS-specific B and T cells would then provide a transition to the adaptive humoral and cellular immune response characteristic of the anti-synthetase syndrome. Identification of cell subsets expressing relevant TLRs in human muscle tissue infiltrates will ultimately be required to fully substantiate this model, though the immunohistochemical findings and rapid time course of autoantibody production/disease induction in this system suggest the possibility of direct interactions between recombinant HRS and effector lymphocytes expressing specific MyD88-dependent TLRs. Supporting this paradigm, recent studies in experimental autoimmune encephalomyelitis (EAE) have demonstrated direct activation of pathogenic CD4+ effector T cells via TLR2 and TLR4, with effects ranging from T cell polarization to lymphocyte proliferation/expansion (13, 14).

Among the disease-associated TLRs expressed by cells invading human muscle tissue (TLR2, TLR4, and TLR9) (11, 12), TLR2 and TLR4 have demonstrated the most prominent interactions with recombinant HRS in our model of antigen-induced myositis. While TLR4 directs signals that mediate production of class switched IgG anti-HRS autoantibodies (15), TLR2 and TLR4 play key roles in driving the cellular component of the myositis phenotype. In fact, more detailed analysis of disease induction mediated by recombinant subfragments of human HRS suggests that TLR2 may be the most critical component of this system. Despite its ability to signal through both TLR2 and TLR4, for example, HA3/MBP (amino acids 1–90 of human HRS) fails to induce significant myositis in B6.TLR2$^{-/-}$ mice. Interestingly, the inability of HA3/MBP-mediated TLR4 signaling to “rescue” the myositis phenotype in B6.TLR2$^{-/-}$ mice (an observation consistent with experiments demonstrating that immunization of C57BL/6 mice with moderate doses of LPS alone does not generate muscle inflammation (data not shown)) contrasts sharply with the capacity of MA/MBP and HA5/MBP to induce relatively severe disease in the absence of functional TLR2 (Figure 4 and data not shown). These results effectively illustrate the capacity of the latter autoantigens to shunt signals through alternative TLR/non-TLR signaling cascades contributing to active myositis.

Beyond these findings in B6.TLR2$^{-/-}$ mice, support for the existence of additional HRS-mediated signaling pathways comes from the immunization experiments performed in C57BL/6 WT and B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice. In C57BL/6 mice, for example, HA3/MBP generates significantly less muscle inflammation than HA4/MBP and HA5/MBP—again, despite evidence of equivalent signaling through TLR2- and TLR4-transfected HEK293 cells. Complementing this observation that supports a key role for amino acids 90–120 of HRS, the persistence of residual (albeit markedly reduced) autoantibody production and muscle inflammation in B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice immunized with MA/MBP or HA5/MBP suggests that at least one other signaling pathway is operative in this system.

Additional candidates for MyD88-dependent induction of myositis include RAGE (Receptor for Advanced Glycation Endproducts), a multi-domain receptor that is capable of binding the ligand HMGB1 (High Mobility Group Box 1) (16). Interestingly, we have detected binding of HMGB1 to eukaryotic HRS in a modified ELISA-based system (Supplementary Figure 4)—an observation that further demonstrates the ability of HRS to physically couple with activators of crucial MyD88-associated signaling pathways. While more detailed molecular assessment will be required to formally demonstrate interactions between HMGB1 (or bacterial analogues of this alarmin) and HRS, previous work has shown that HMGB1 is abnormally distributed in the muscle tissue of myositis patients (17, 18). Equally intriguing, HMGB1 is capable of signaling through TLR2 and TLR4 (18, 19), providing another potential link with our model system of HRS-induced myositis.

Consistent with these concepts, results of the current study clearly indicate that HRS is endowed with unique biophysical properties that enable this molecule to function directly or indirectly (through binding of additional stimulatory molecules) as a ligand for multiple innate immune signaling pathways. As shown by in vitro stimulation of TLR-transfected HEK293 cells (Figure 2 and data not shown), gel purification of MA/MBP and HA5/MBP does not uniformly diminish the ability of these recombinant proteins to generate TLR2-mediated signals—suggesting that this property may be intrinsic to bacterially produced versions of HRS. Although the capacity to signal via TLR4 is largely abrogated by gel purification, these experiments (in conjunction with comparative LAL assays of endotoxin activity) demonstrate that potential binding of exogenous, bacterially-derived TLR4 ligands is a non-artefactual, antigen-specific property that is critically dependent on the protein sequence/structure encompassed by amino acids 60–90 of human HRS.

Coupled with accumulating immunohistochemical and RNA profiling data implicating MyD88-dependent signaling pathways in human inflammatory myopathy (11, 12), the overall findings of this work suggest a model in which HRS serves as an anchor protein (analogous to LPS binding protein) for various endogenous/exogenous ligands capable of synergistically driving innate immune responses. Elucidating the biological relevance of different glycosylation patterns and other structural features contributing to enhanced binding of these ligands is therefore of paramount importance, but will clearly require more detailed experimental manipulation of parallel HRS constructs produced in eukaryotic expression systems.

Viewed more broadly, our experimental results collectively support the concept that autoantigens such as HRS may play a significant role in bridging innate and adaptive immune responses required to initiate and/or perpetuate autoinflammatory processes such as myositis. Although the specific mechanisms underlying immunogenicity and disease pathogenesis may differ, this framework is entirely consistent with emerging data highlighting a primary role for innate immune activation in other autoimmune diseases ranging from rheumatoid arthritis to systemic lupus erythematosus and multiple sclerosis (reviewed in (20–26))—each of which is supported by animal models heavily reliant on innate immune signaling cascades (e.g., collagen-induced arthritis, pristane-induced lupus, and experimental autoimmune encephalomyelitis (27)). More relevant to myositis, previous work demonstrating that the combination of necrotic cell debris and serum derived from

Jo-1-antibody positive patients can initiate signaling via endosomal TLRs (leading to IFNα production in plasmacytoid dendritic cells) lends additional support to this paradigm of combined immune activation (28). In the current model, magnetic resonance imaging studies (not shown) demonstrating edema/inflammation in muscle groups distant from the site of HRS/IFA inoculation also provide in vivo evidence of the interplay between locally induced innate immune responses and more systemic involvement.

Beyond these observations linking innate and adaptive immune responses to HRS, the expression of TLR2 and TLR4 in muscle tissue itself (18, 29–31) highlights the relevance of our model and suggests that HRS or other associated TLR agonists may directly activate MyD88-dependent signaling cascades which alter NFκB-mediated gene expression profiles and culminate in muscle dysfunction. Ultimately, defining these interactions in human disease will be critical to the development of alternative, molecularly targeted therapies that lack the attendant side effects of more global immunosuppression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

IIM	idiopathic inflammatory myopathy
PM	polymyositis
DM	dermatomyositis
HRS	histidyl-tRNA synthetase
MA/MBP	amino terminal 151 amino acid fragment of murine Jo-1 fused to MBP
MBP	maltose binding protein
TLR	Toll-Like Receptor
LPS	lipopolysaccharide

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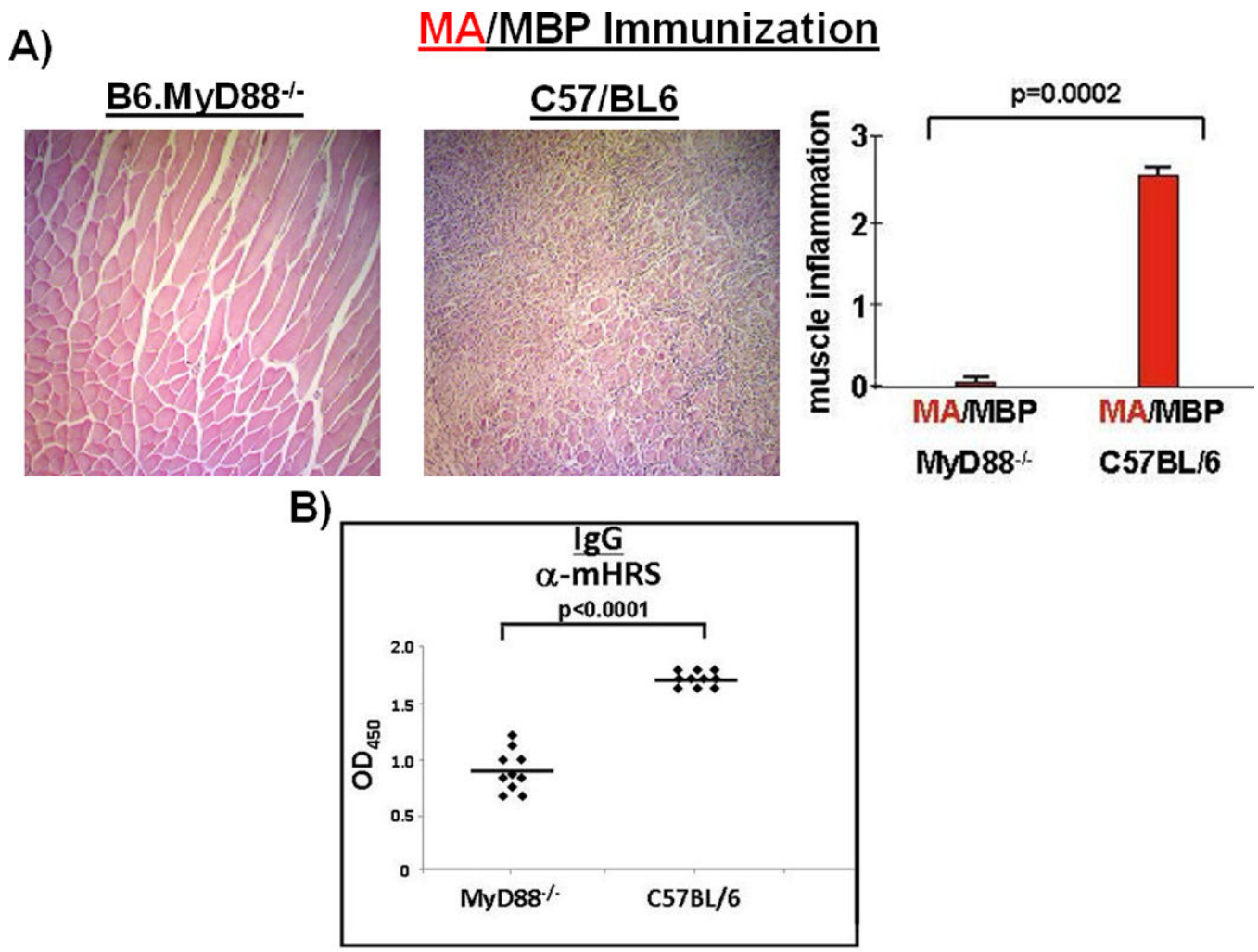

Figure 1. MA/MBP immunization of B6.MyD88$^{-/-}$ mice fails to induce myositis

A) Photomicrographs (400x magnification) depict representative hematoxylin and eosin stained sections of muscle tissue derived from B6.MyD88$^{-/-}$ (MyD88$^{-/-}$) and C57BL/6 mice 17 days following intramuscular immunization with MA/MBP (amino terminal 151 amino acids of murine HRS fused to Maltose Binding Protein (MBP)). The accompanying bar graph illustrates mean severity scores of muscle inflammation from n=10 B6.MyD88$^{-/-}$ and n=10 C57BL/6 mice, with error bars representing SEM. B) Relative titers of IgG anti-murine HRS antibodies are shown in this dot plot where individual data points represent ELISA-based measurement of adjusted OD_{450} values (OD_{450} mHRS (1 μg/ml) - OD_{450} no antigen) using Day 17 sera (1:1000 dilution) obtained from n=10 B6.MyD88$^{-/-}$ and n=10 C57BL/6 mice. Horizontal bars designate mean OD_{450} values for each strain.

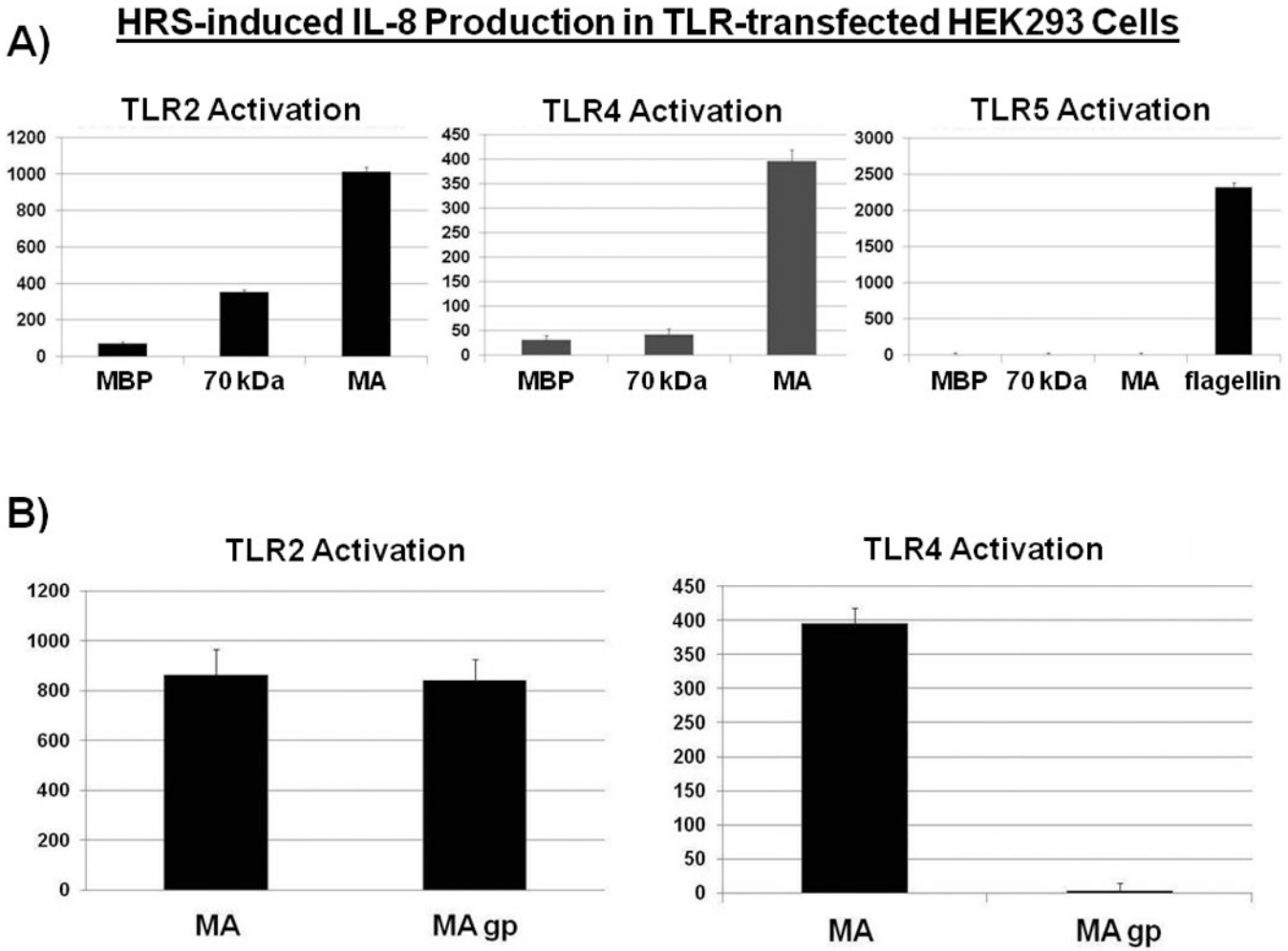

Figure 2. In vitro stimulation of TLR-transfected HEK293 cells

A) Bar graphs depict relative IL-8 production (pg/ml, as measured by ELISA) generated by overnight co-incubation of various recombinant proteins with HEK293 cells expressing the indicated cell surface Toll Like Receptors (TLRs). For ease of interpretation, background levels of IL-8 secretion from unstimulated TLR-transfected cells (TLR2=44 pg/ml, TLR4=232 pg/ml, and TLR5=226 pg/ml) have been subtracted from values induced by individual proteins. Protein concentrations ranged from 0.5 μg/ml (TLR4 and TLR5 stimulation) to 3.0 μg/ml (TLR2 stimulation), where MBP=Maltose Binding Protein, MA/MBP=amino acids 1–151 of murine HRS fused with MBP, and 70 kDa/MBP=70kDa component of U1RNP complex fused with MBP. Flagellin (100 ng/ml) was included as a positive control for stimulation of TLR5-transfected HEK293 cells. Error bars represent SEM values. B) These graphs illustrate relative IL-8 production (pg/ml) in TLR2- and TLR4-transfected HEK293 cells following stimulation with filter sterilized versus gel purified versions of recombinant MA/MBP (MA vs. MA gp) used at the concentrations designated in panel A. Error bars again represent SEM values. All analyses depicted in panels A and B are representative of at least two independent experiments.

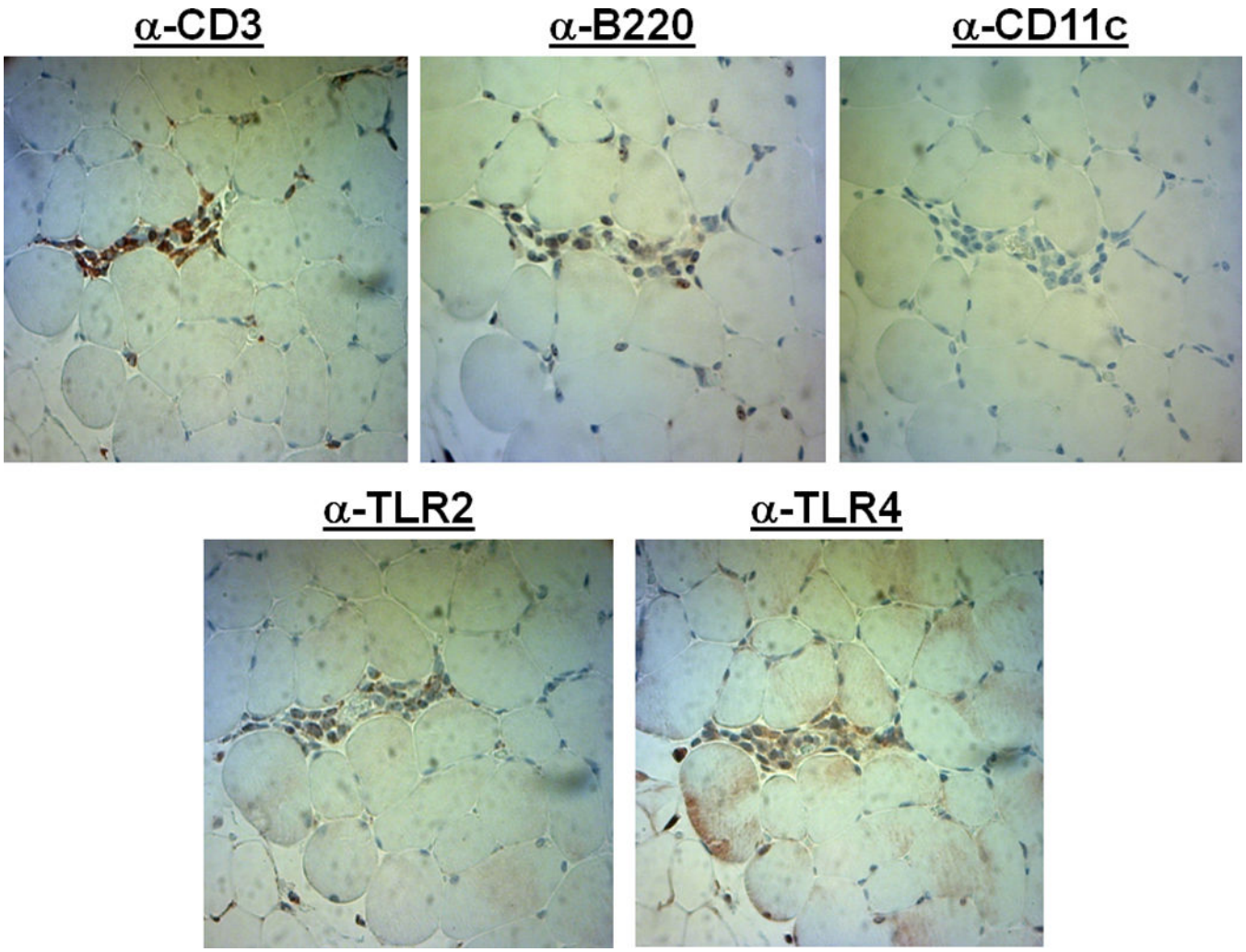

Figure 3. Immunohistochemical analysis of TLR2 and TLR4 expression in muscle cell infiltrates

Photomicrographs (400x) demonstrate immunohistochemical staining of de-paraffinized muscle tissue derived from MA/MBP-immunized C57BL/6 mice. Labels indicate the use of antibodies targeting CD3, CD45/B220, CD11c, TLR2, and TLR4. Corresponding isotype controls have been omitted for ease of illustration.

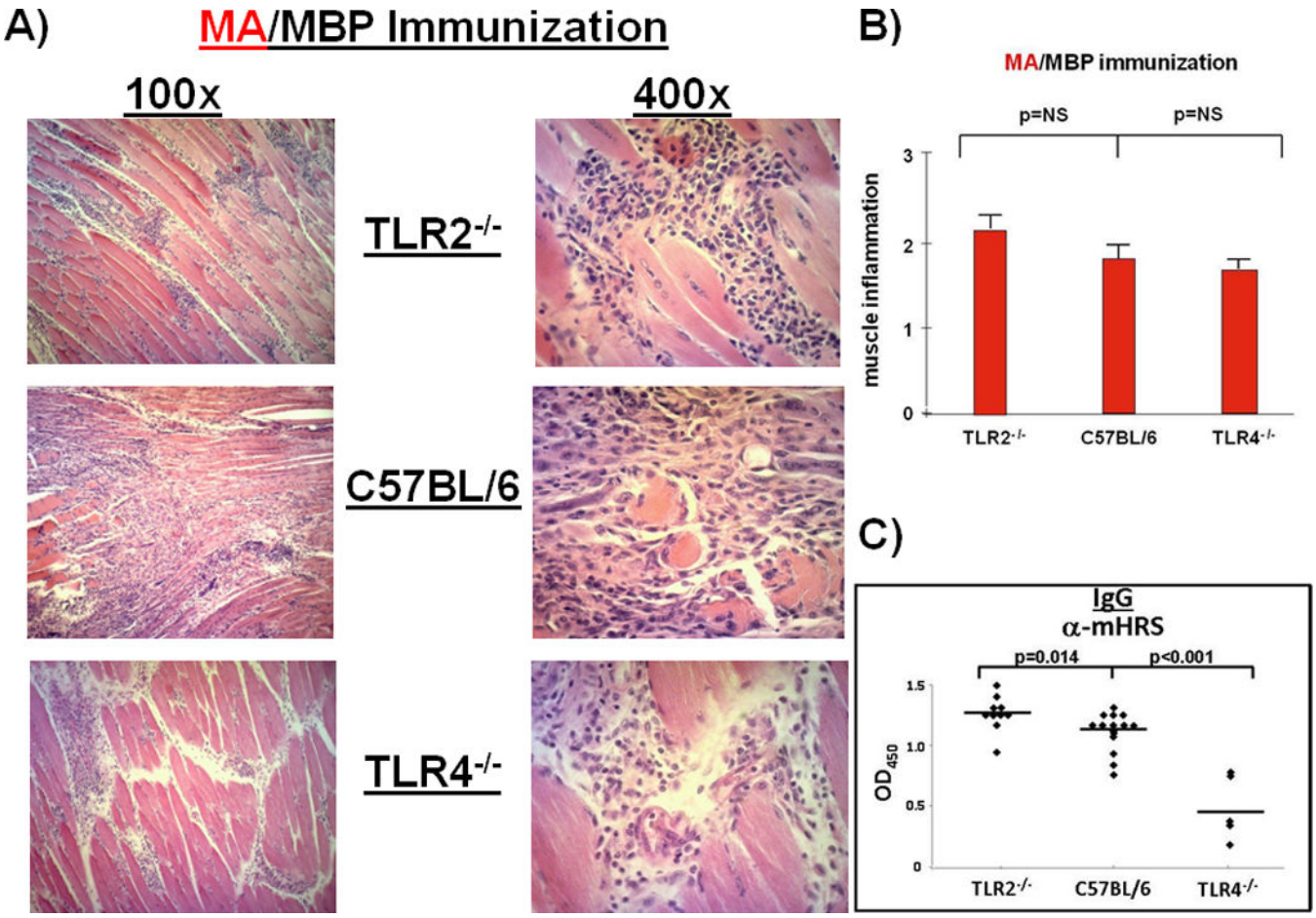

Figure 4. MA/MBP immunization induces myositis in TLR2- and TLR4-deficient mice

A) Photomicrographs (100x left column, 400x right column) illustrate representative hematoxylin and eosin stained muscle tissue obtained 17 days following intramuscular immunization of the indicated strains with MA/MBP. B) The accompanying bar graph depicts mean severity scores of muscle inflammation for n=10 B6.TLR2$^{-/-}$ (TLR2$^{-/-}$), n=15 C57BL/6, and n=5 B6.TLR4$^{-/-}$ (TLR4$^{-/-}$) mice; error bars reflect SEM. C) The dot plot demonstrates ELISA-based assessment of IgG anti-mHRS levels in Day 17 sera (1:1000 dilution) obtained from the mice listed in panel A. While individual data points represent adjusted OD_{450} values (OD_{450} mHRS (1 μg/ml) - OD_{450} no antigen) for each serum sample, horizontal bars indicate mean OD_{450} readings obtained for designated strains of mice.

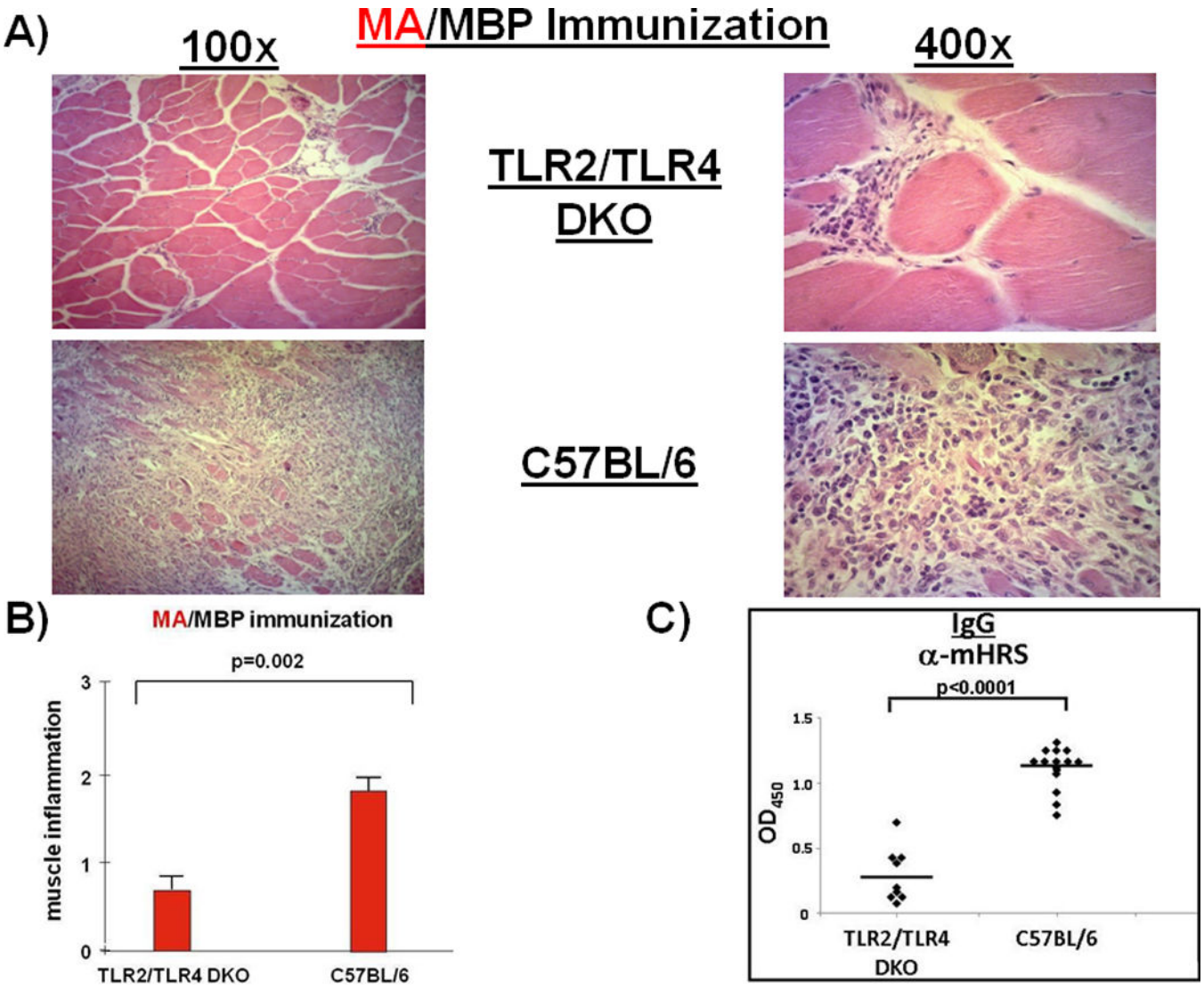

Figure 5. MA/MBP-induced muscle inflammation is markedly reduced in B6.TLR2$^{-/-}$.TLR4$^{-/-}$ double knockout mice

A) Photomicrographs (100x left column, 400x right column) demonstrate representative hematoxylin and eosin stained muscle tissue sections obtained from B6.TLR2$^{-/-}$.TLR4$^{-/-}$ (TLR2/TLR4 DKO) and WT C57BL/6 mice 17 days following intramuscular immunization with MA/MBP. B) Mean severity scores of muscle inflammation observed in n=9 B6.TLR2$^{-/-}$.TLR4$^{-/-}$ and n=15 C57BL/6 mice are shown in the accompanying bar graph. C) Relative IgG anti-mHRS antibody titers in MA/MBP-immunized B6.TLR2$^{-/-}$.TLR4$^{-/-}$ (n=9) and C57BL/6 (n=15) mice are depicted in this dot plot where individual data points represent adjusted OD_{450} values obtained using 1:1000 serum dilutions and a substrate antigen concentration of 1 μg/ml. Horizontal bars designate population mean OD_{450} values.

Figure 6

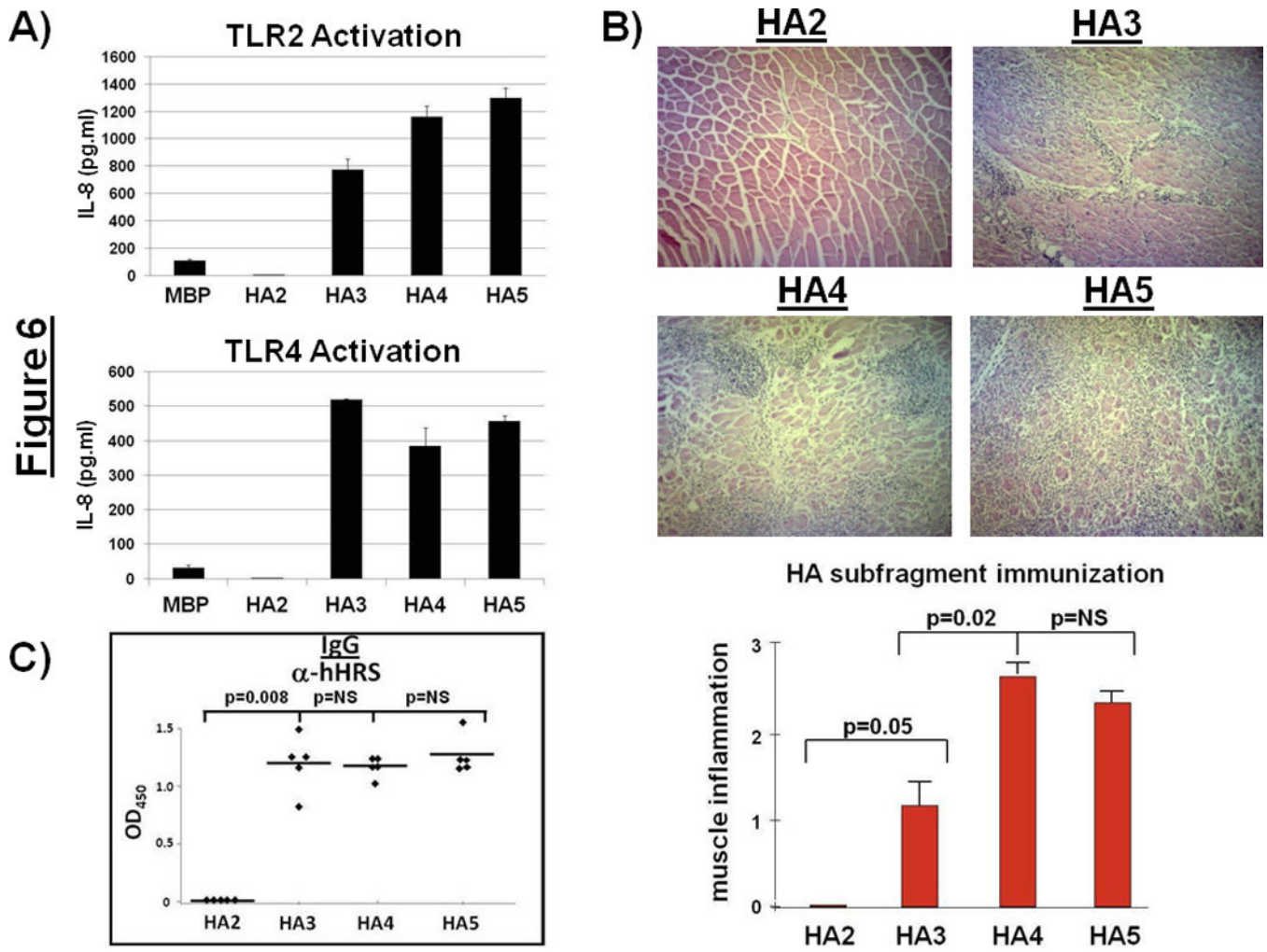

Figure 6. Amino terminal subfragments of human HRS generate variable signals through TLR2 and TLR4 that correlate with myositis induction

A) Human HRS (hHRS) subfragments fused to MBP differentially stimulate HEK293 cells transfected with TLR2 or TLR4, resulting in variable levels of IL-8 production (pg/ml, y-axis). Proteins used at concentrations ranging from 0.5 μg/ml (TLR4 stimulation) to 3.0 μg/ml (TLR2 stimulation) include HA2/MBP=amino acids 1–60 hHRS/MBP, HA3/MBP=amino acids 1–90 hHRS/MBP, HA4/MBP=amino acids 1–120 HRS/MBP, and HA5/MBP=amino acids 1–151 hHRS/MBP. Graphs are representative of at least two independent experiments. B) Photomicrogaphs (100x) of muscle tissue and the corresponding bar graphs reflect the relative severity of muscle inflammation induced by intramuscular immunization of C57BL/6 mice with the indicated hHRS subfragments (HA2, HA3, HA4: n=5 mice/group; HA5: n=10 mice). Error bars represent SEM. C) Anti-human HRS antibody titers generated by intramuscular immunization of C57BL/6 mice with the indicated amino-terminal subfragments are shown in this dot plot. Individual data points represent adjusted OD_{450} values (OD_{450} hHRS - OD_{450} no antigen) obtained using 1:1000 serum dilutions and a substrate antigen concentration of 1μg/ml. Horizontal bars reflect mean OD_{450} values for n=5 samples/immunogen.