

The RNA Editor Gene ADAR1 is Induced in Myoblasts by Inflammatory Ligands and Buffers Stress Response

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Abstract

Muscle atrophy remains a significant concern in multiple inflammatory conditions, including injury, sepsis, cachexia, and HIV-associated wasting. Herein, we show that inflammatory stressors, including TNF- α , IFN- γ , or lipopolysaccharide, potently induced the novel expression of the RNA editor ADAR1, an observation not previously described in muscle cells. We also observed that cytokine stimulation suppressed muscle-associated microRNAs, an observation also not previously demonstrated. To map potential effects of ADAR1 induction in the muscle program, we conducted knockdown and overexpression studies in the mouse C2C12 muscle precursor cell (MPC) line and in primary human MPCs. We show that knockdown of stress-induced ADAR1 increased inflammation-mediated declines in the muscle differentiation markers Myogenin and myosin heavy chain, and knockdown reduced levels of active phosphorylated Akt (phospho-Akt), but had no effect on microRNA transcript levels, suggesting a role for ADAR1 in buffering inflammatory stress effects on myogenic transcription and protein synthesis pathways. In addition, overexpression of recombinant ADAR1 suppressed active phosphorylated double-stranded RNA (dsRNA)-dependent protein kinase (phospho-PKR), consistent with a role for ADAR1 in limiting inflammation-driven catabolic atrophy pathways. Collectively, these data identify a novel regulatory role for ADAR1 activation under inflammatory stress to both promote muscle protein synthesis pathways and limit atrophy pathways. Clin Trans Sci 2010; Volume 3: 73–80

Keywords: RNA editor gene, inflammatory ligands, ADAR1

Introduction

Skeletal muscle maintenance requires a dynamic balance of anabolic and catabolic processes that regulate cell architecture and protein turnover.^{1,2} This balance can be skewed toward muscle protein loss by inflammation.^{3,4} The severity of decline in muscle mass and function can range from a gradual loss, as observed in age-associated sarcopenia, to a steeper decline in muscle mass observed in muscle-wasting conditions such as HIV infection and cancer cachexia. Muscle atrophy is broadly associated with an elevated inflammatory cytokine burden, notably tumor necrosis factor- α (TNF- α) (originally termed “cachectin” for its association with cancer cachexia⁵) and interferon- γ (IFN- γ).⁶

The muscle cellular program is initiated through activation and differentiation of quiescent muscle progenitor cells (MPCs) resident in muscle tissue in a process that is characterized by a transition from single nucleated myoblasts to multinucleated cells termed myotubes *in vitro* and myofibers *in vivo*. Molecular features of this transition include temporally regulated expression of transcriptional regulators (e.g., MyoD, Myogenin), microRNA regulators that influence RNA translation into protein (e.g., miR-1, miR-206, and miR-133) and muscle structural proteins, for example, myosin heavy chain (MHC). These regulatory processes form a network; for example, MyoD is a transcriptional regulator of muscle microRNA expression.⁷ In turn, the regulated expression of microRNAs miR-1, miR-206, and miR-133 influence the balance between MPC proliferation and renewal versus differentiation and fusion through modulation of the relative levels of serum response factor and HDAC4 translation.⁸

Inflammatory stress is broadly recognized to activate innate immune response pathways, such as the toll-like receptor family that function in a variety of cellular and physiologic contexts,⁹ including muscle tissue homeostasis.¹⁰ A notable inflammatory stress mediator is the IFN- γ inducible double-stranded RNA (dsRNA)-dependent protein kinase (PKR), a critical negative

regulator that when activated through phosphorylation blocks protein synthesis through phosphorylation of eukaryotic initiation factor-2 α (eIF-2 α),¹¹ thereby promoting muscle atrophy.^{12–14} Recent studies dissecting the signaling pathways activated during muscle inflammatory stress also indicate that IFN- γ can potentiate TNF- α -mediated atrophy by promoting increased mRNA instability in MyoD and Myogenin.¹⁵ Inflammation-mediated declines in these transcription factors are also associated with activated nitric oxide synthase/nitric oxide signaling¹⁶ and protein breakdown mediated through the muscle-specific E3 ligases atrogen-1/Mafbx and MuRF1.⁶ The atrogens Mafbx and MuRF1 are differentially induced by cytokines such as TNF- α through p38 MAPK and NF κ B pathways¹⁷ as well as through IFN- γ signaling.⁶ Thus, inflammatory signaling induces multiple molecular pathways that converge to potentiate the atrophy phenotype.

Additional studies conducted primarily in leukocytes indicate that inflammatory stressors such as TNF- α , IFN- γ , lipopolysaccharide (LPS), and viral dsRNA and its mimetic compound poly (I:C), all activate the RNA editor ADAR-1 (Adenosine deaminase acting on RNA -1).^{18,19} Two isoforms of the ADAR1 protein have been described, with a constitutively expressed isoform, ADAR1(p110), and an interferon-inducible isoform, ADAR1(p150).^{18,20–22} Though initially identified in association with an antiviral stress response, ADAR1 is now recognized to have a larger role in modulating both host mRNA and microRNA transcript sequence through an RNA editing function,²³ as well as a role in suppressing PKR-mediated blockade of protein synthesis in 293T cells, an embryonic kidney cell line.²⁴ Herein, we describe the novel expression and potential role for ADAR1 in muscle cell differentiation during inflammatory stress, which has not been previously addressed.

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Materials and Methods

Cell culture, transfection

Murine C2C12 myoblasts were obtained from ATCC (Manassas, VA, USA) and maintained in GM that consisted of high glucose Dulbeccos Minimum Essential Medium (DMEM) from Gibco, 10% fetal bovine serum from Gibco and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). To induce differentiation, cells were allowed to reach 70% confluency, washed with phosphate buffered saline (PBS) (1x) and switched to DM that consisted of low glucose DMEM supplemented with 2% horse serum (Gibco) and 1% penicillin/streptomycin.

Primary human myoblasts were obtained from Cook Myosite Inc. (Pittsburgh, PA, USA) and maintained using the MyoTonic Growth Media Kit (Catalog no. MK-4444). Three independent isolates (40 M, 17 M, and 25 M) were used for transfection experiments. Myoblasts were transfected with flag-tagged ADAR1 plasmid using Lipofectamine LTX (Invitrogen). Mock-transfected myoblasts were used as a control. Forty-eight hours posttransfection, cells were collected and lysed. All cell lines used were maintained in a 37°C incubator at 5% CO₂. Cells were passaged approximately every 2 days and were kept below 70% confluency.

Stimulations

Recombinant mouse IFN- γ and human TNF- α were obtained from Calbiochem and Sigma-aldrich, respectively. To stimulate cell lines, recombinant protein was added to the cells when switching over to DM at a concentration of 10 ng/mL for both IFN- γ and TNF- α separately. When cells were stimulated with IFN- γ and TNF- α together, they were each added at a concentration of 5 ng/mL.

Quantitative real-time RT-PCR

Total RNA was isolated using the TRIzol (Invitrogen) method according to manufacturer instructions. We used this method of RNA extraction to ensure proper extraction of small RNAs (i.e., microRNAs). The Taqman One-Step RT-PCR Master Mix Reagents Kit from Applied Biosystems (Foster City, CA, USA) was used for relative quantitation of all nonmicroRNA targets. Target mRNA oligonucleotide primer/probe sets were obtained from Applied Biosystems. Assay IDs are as follows: Adar (Mm00508001_m1), Myogenin (Mm00446194_m1), and MyHC3 (Mm01332475_g1). Real-time PCR for nonmicroRNA targets was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems); 30 minutes at 48°C, 10 minutes at 95°C, and 40 cycles, denaturation: 15 seconds at 95°C, annealing/extension: 1 minute at 60°C. Fluorescence was measured during the annealing/extension step. Results were analyzed using the ABI Prism 7000 Sequence Detection System software via the $\Delta\Delta$ Ct method. All samples were performed in duplicate and normalized to the relative expression of the eukaryotic 18S or GAPDH endogenous controls. For quantitation of all microRNA targets, the Taqman MicroRNA Reverse Transcription Kit from Applied Biosystems was used. Target microRNA Assay IDs are as follows: miR-1 (002222), miR-24 (000402), miR-133a (002246), and miR-206 (000510). Real-time PCR for microRNA targets was performed with the ABI Prism 7000 Sequence Detection (Applied Biosystems); Reverse Transcription: 30 minutes at 16°C, 30 minutes at 42°C and 5 minutes at 85°C. Real-Time PCR: 10 minutes at 95°C and 40 cycles, denaturation: 15 seconds at 95°C, annealing/extension: 1 minute at 60°C. Results were analyzed

using the ABI Prism 7000 Sequence Detection System software via the $\Delta\Delta$ Ct method. All samples were performed in duplicate and normalized to the relative expression of the small nucleolar RNA snoRNA202 (Applied Biosystems Part #4380914).

Protein and phospho-protein determination

AKT detection

Primary antibodies were obtained from cell signaling. Cells were washed with PBS and lysed with cold lysis buffer (50 mM Tris-Cl, pH 6.8, 150 mM NaCl, 0.1% SDS, 1% NP-40) treated with 7X protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Total cell lysates were quantified using the Bradford Assay. Five micrograms of protein was mixed with 2X loading buffer (Laemmli sample buffer (Bio-Rad, Hercules, CA, USA); β -mercaptoethanol in a ratio of 19:1) and heated for 1 minute at 100°C. Samples were run through 10% polyacrylamide gels (Bio-Rad) for 1 hour at 90 V then subsequently transferred to a PVDF membrane. Membranes were blocked for 1 hour at room temperature with TBS with 0.1% Tween-20 (TBS-T) and 5% bovine serum albumin (BSA). Primary antibodies were diluted 1:1,000 in TBS-T + 5% BSA and incubated with agitation at room temperature for 2 hours. After washing membranes with TBS 3 times for 5 minutes, secondary HRP-linked antibodies were diluted 1:5,000 in TBS-T + 5% BSA and incubated with agitation at 4°C overnight. Membranes were treated with enhanced chemiluminescence western blotting detection reagents (GE Lifesciences, Uppsala, Sweden) and exposed to ECL hyperfilm (GE Lifesciences). Membranes were then treated with stripping solution (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH6.7) and re-probed once.

PKR detection

ADAR1 was detected using an anti-FLAG M2 antibody (Stratagene, La Jolla, CA, USA); anti-PKR was obtained from Cell Signaling Technology (Danvers, MA, USA); anti-phospho-PKR was obtained from Millipore (Billerica, MA, USA). PKR phosphorylation status was assessed by SDS-PAGE and western blot analysis. PKR phosphorylation was quantified using MultiGauge v.3.1 (Fujifilm, Minato, Tokyo, Japan).

siRNA

Dharmacon Accell siRNA against mouse ADAR1 was used (Accession #NM_001038587). Nontargeting siRNA from Dharmacon (Lafayette, CO, USA) was used as a negative control (Catalog #D-001910-01-05). Accell siRNA delivery media was used and the siRNA was added according to manufacturer instructions. Delivery media was supplemented with 2% horse serum to avoid serum starvation and to initiate/perpetuate differentiation. siRNA was either added for 24 hours and then stimulated with inflammatory cytokines (TNF- α , IFN- γ) in DM or cells were stimulated first for 24 hours and then siRNA was added. After cells were in siRNA for 48 or 72 hours (72 or 48 hour stimulation, respectively), total RNA was extracted via the TRIzol (Invitrogen) method according to manufacturer instructions.

Statistical methods

Statistical significance was estimated using the nonparametric method, the Wilcoxon signed-rank test without an assumption of normality in the data. Pairwise Wilcoxon comparisons (with a one-sided alternative) were conducted for each sample relative to the control within the same experimental set. Only p -values <0.05

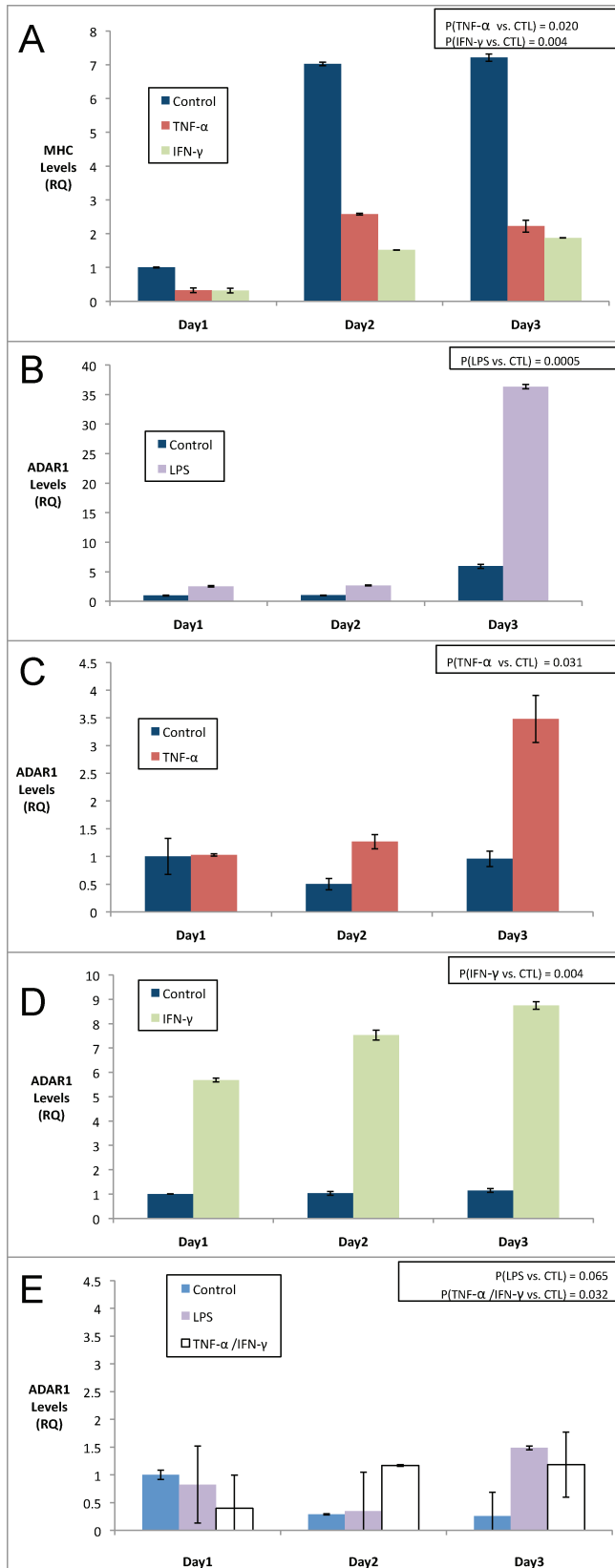


Figure 1. Proinflammatory stimuli induce ADAR1 gene expression in differentiating muscle cells. C2C12 MPCs were induced to differentiate by switching to low-serum differentiation media (DM, see **A–D**) or left in growth media (GM, see **E**) for 3 days and monitored for myosin heavy chain three expression as a control (**A**) or ADAR1 expression (**B–E**) in the presence of LPS (1 $\mu\text{g}/\text{mL}$) (**B**), TNF- α (10 ng/mL) (**C**), IFN- γ (10 ng/mL) (**D**), or TNF- α /IFN- γ (each at 5 ng/mL , **E**). Note MHC was suppressed by TNF- α and IFN- γ , as previously shown.¹⁵ ADAR1 expression was clearly observed in the context of inflammatory signals during differentiation (**B–D**) and minimally observed in growth media (**E**). A Wilcoxon signed-rank test for each condition was performed for the series **A–E** and resulting p values are shown in upper right. Standard deviations for each time point are also shown.

were considered significant. Standard deviations or standard errors are also indicated. Values marked with an asterisk represent an average of duplicates.

Results

Previous studies evaluating the tissue distribution of ADAR1 identified expression within brain and leukocyte cells, but not in muscle. However, those studies were based on analysis of unstimulated levels within skeletal muscle.²⁵ To evaluate the potential for stress-induced expression of ADAR1 in skeletal muscle, we stimulated C2C12 MPCs with an array of inflammatory stressors including TNF- α , IFN- γ , or LPS under both growth promoting media conditions (growth medium [GM], 10% serum) and differentiation promoting media conditions (differentiation medium [DM], 2% serum). Total RNA was harvested from parallel cultures daily, for 1–3 days. As shown in *Figure 1*, ADAR1 expression was potently induced from low baseline levels by LPS, TNF- α , and IFN- γ , suggesting that innate inflammatory stress potently induce ADAR1 expression in MPCs. Notably, inflammatory stimulation of ADAR1 expression was far more efficient in differentiation media conditions than in growth media, despite the presence of similar inflammatory ligands (*Figure 1E*). TNF- α and IFN- γ also efficiently suppressed MHC 3 expression, as previously reported in a study of cytokine-mediated muscle atrophy¹⁵ (*Figure 1A*). These data suggest that stress-induced expression of ADAR1 likely occurs in muscle concomitant with stress-induced signaling that promotes atrophy and declines in MHC.^{12,13}

Previous studies of inflammatory effects on muscle cells have demonstrated that TNF- α and IFN- γ can reduce the mRNA stability of myogenic regulatory factors, such as MyoD.^{15,16} To our knowledge, however, the effect of inflammatory ligands on muscle-associated microRNAs in MPCs has not yet been reported. Therefore, to evaluate the quantitative influence of TNF- α and IFN- γ on the expression of microRNAs miR-1, miR-206, and miR-133, we cultured C2C12 cells in DM media to stimulate miR expression, in the presence of IFN- γ , TNF- α , or media alone as a reference control. Cells were harvested for total RNA on days 1–4, poststimulation. As shown in *Figure 2A*, in contrast to untreated cells that displayed increases in miR-1, miR-206, and miR-133 in the differentiation time course as previously reported,⁸ IFN- γ - and TNF- α -stimulated cells displayed marked declines in miR expression. Notably, declines in microRNA were specific to miR-1, miR-206, and miR-133, because miR-24, another muscle-associated microRNA, increased with differentiation and was relatively insensitive to IFN- γ and TNF- α treatment (*Figure 2A*). To our knowledge, a decline in muscle-specific microRNAs upon cytokine exposure during differentiation has not been previously reported. However, our observed miR suppression is consistent with cytokine-mediated attenuation of MyoD,^{15,26} an upstream regulator of these microRNAs.⁷ The observed declines in microRNA expression were correlated with reduced muscle protein synthesis, based on quantitation of MHC immunostaining at day 4, poststimulation (*Figure 2B*). From these data, we conclude that inflammatory stress induces

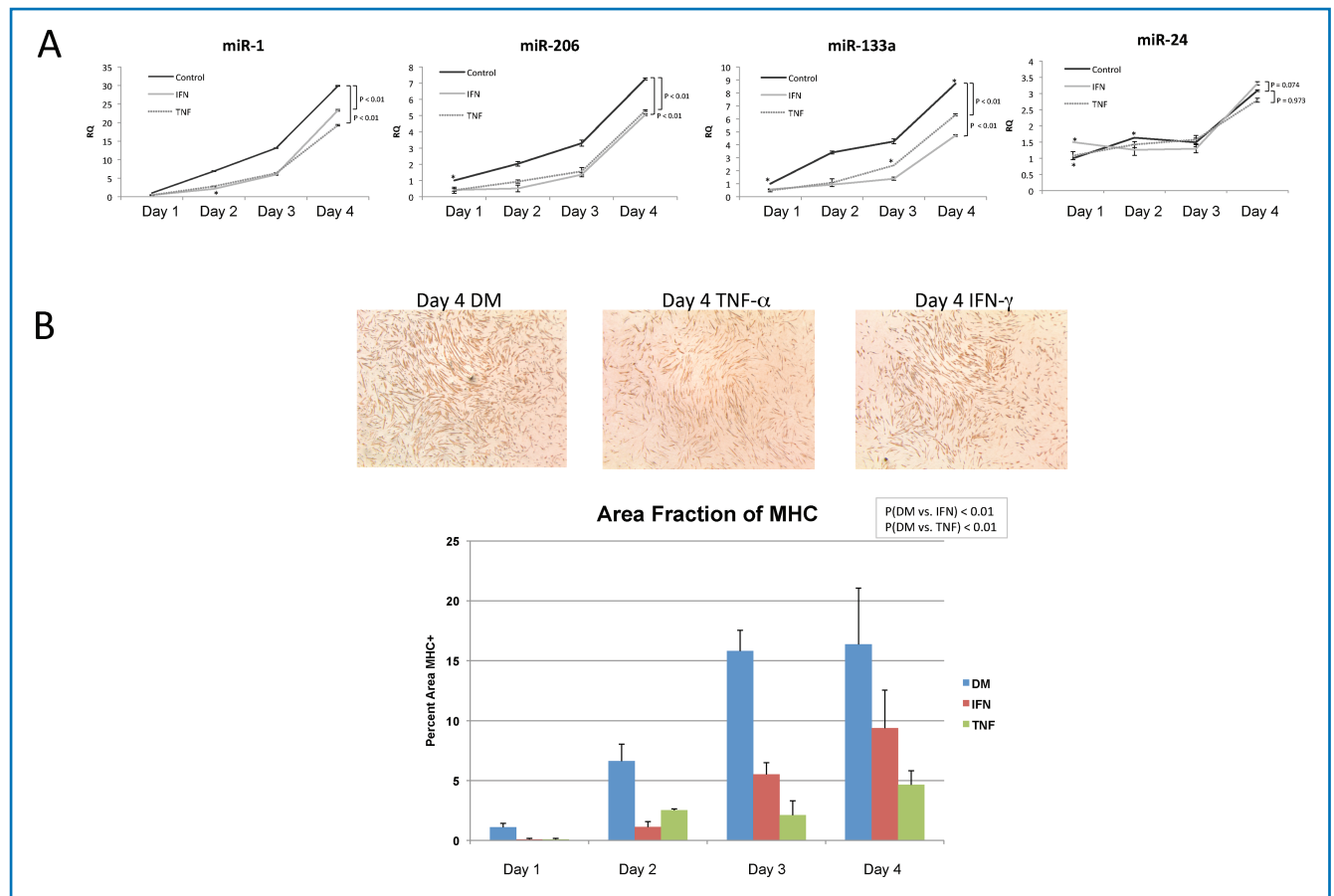


Figure 2. Proinflammatory stimuli suppress muscle associated microRNA expression. **(A)** C2C12 MPCs were cultured for 1–4 days in differentiation media in the presence of TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) added once at the start of culture. Total RNA was harvested and real-time PCR performed on the indicated microRNAs and normalized to snoRNA controls. A Wilcoxon signed-rank test was performed and resulting p values are shown. Standard deviations for each time point are also shown. **(B)** C2C12 MPCs were cultured for 1–4 days in differentiation media in the presence of TNF- α (10 ng/mL) or IFN- γ (10 ng/mL) added once at the start of culture. At 24-hour intervals after initial stimulation, cells were fixed with 3.7% formaldehyde and subsequently immunostained for myosin heavy chain. Images of duplicate cells were taken and analyzed using ImageJ (NIH, Bethesda, MD, USA). Total myosin heavy chain area was determined and compared to total area of the image. Standard deviations and t -test results for four replicate measurements with each condition are shown.

declines in both muscle-associated microRNAs (miRNAs) and muscle protein levels (e.g., MHC).

We were next interested in evaluating the potential influence of stress-induced ADAR1 on microRNA and myogenic markers (i.e., Myogenin, MHC) during differentiation. To measure this, MPCs were differentiated in the presence of inflammatory stimuli for 24 hours to induce ADAR1 expression, then transfected with ADAR1 small interfering RNA (siRNA) or a control to knockdown ADAR1 expression, then cultured for an additional 48 hours before harvesting total RNA. To determine whether knockdown of ADAR1 influenced myogenic marker expression during differentiation, we measured levels of Myogenin and MHC, as well as ADAR1 expression to estimate the efficiency of knockdown. As shown in Figure 3, siRNA to ADAR1 reduced expression of ADAR1 to 54% of IFN- γ and 34% TNF- α -induced control levels, indicating efficient knockdown. In association with declines in ADAR1 due to knockdown, there was also a significant decline in Myogenin and MHC transcript levels, that is, $p = 0.015$ for IFN- γ and $p = 0.031$ for TNF- α . To determine if knockdown of ADAR1 expression increased the inflammation-driven declines in microRNA levels, we measured miR-1, miR-206, and miR-133 in a replicate set of transfectants. By contrast to Myogenin and MHC, the microRNAs miR-1, miR-206, and miR-133 were insensitive to

knockdown of ADAR1 in the same cells, suggesting that ADAR1 does not appear to influence the microRNA component of the muscle program under stress conditions (data not shown). These data collectively indicate that ADAR1 limits inflammation-mediated declines, in part through protecting myogenic markers downstream of the microRNA machinery, and support a role for ADAR1 as an inflammatory buffer.

Inflammatory stress, including IFN- γ ^{27,28} and TNF- α ,²⁹ induce the dsRNA protein kinase PKR, a critical regulator of protein synthesis and a mediator of inflammation-mediated muscle atrophy.^{12–14} As recently described,²⁴ human ADAR(p150) overexpression in 293T cells dosimetrically reduced levels of the active form of PKR (phospho-PKR) and reduced levels of phospho-eIF-2 α , a substrate of active PKR and regulator of protein synthesis.²⁴ These data suggest that ADAR1 limits the PKR-induced block to protein synthesis under conditions of stress in 293T cells, consistent with a direct interaction between ADAR1 and PKR as was recently proposed.³⁰ We were therefore interested in whether overexpression of the inducible isoform of ADAR1, ADAR1(p150), would influence PKR activity in MPCs. As shown in Figure 4A, overexpression of ADAR1 in human MPCs reduced levels of the active form of PKR (phospho-PKR) by approximately 50% (Figure 4B), with no effect on total PKR

Cytokine	siRNA	Detector	% Max of siControl	Ct	dCt
IFN- γ	siADAR	ADAR	0.54	37.924	17.54
				38.205	17.821
		MYH	0.87	27.685	7.301
				27.568	7.184
		MYOG	0.52	25.962	5.578
				25.443	5.059
	siCTL	ADAR	1	37.528	16.822
				37.478	16.772
				27.734	7.028
MYH	1	27.764	7.058		
		25.092	4.386		
MYOG	1	25.065	4.359		
TNF- α	siADAR	ADAR	0.34	39.395	18.877
				39.333	18.815
		MYH	0.62	27.412	6.894
				27.262	6.744
		MYOG	0.71	24.661	4.143
				23.71	3.192
	siCTL	ADAR	1	38.205	17.311
				27.037	6.143
				27.008	6.114
MYH	1	24.101	3.207		
MYOG	1	24.047	3.153		

$P = 0.015$

$P = 0.031$

Figure 3. ADAR1 expression limits cytokine-mediated declines in myogenesis. C2C12s were induced to differentiate in the presence of IFN- γ or TNF- α for 24 hours then transfected with siRNA to ADAR1 or control (Accell siRNA) and cultured for an additional 48 hours before total RNA was harvested. To determine whether knockdown of ADAR1 influenced myogenic marker expression, we measured RNA levels of the myogenic markers Myogenin and myosin heavy chain (MHC), as well as ADAR. As shown, siRNA to ADAR1 reduced expression of ADAR1 to 54% or 34% of induced levels, IFN- γ , and TNF- α , respectively. In these same cells, there was a representative decline in Myogenin levels (52% of control in IFN- γ stimulated, 71% of control in TNF- α stimulated, $p = 0.015$) and in MHC (87% of control in IFN- γ -stimulated cells, 62% of control in TNF- α stimulated, $p = 0.031$). A Wilcoxon signed-rank test was performed for trend differences between siADAR1 versus siCTL in both IFN- γ and TNF- α treatments, with p values as shown.

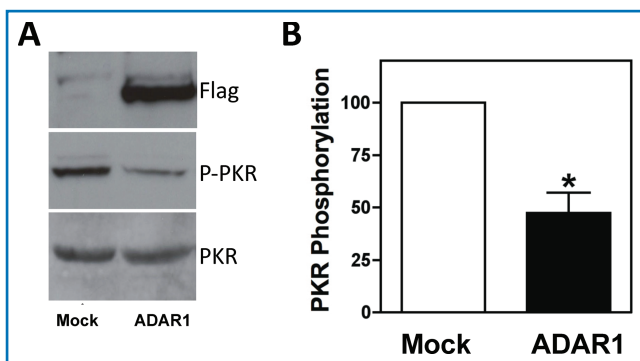


Figure 4. ADAR1 reduces phospho-PKR levels in muscle cells. **(A)** Primary human myoblasts were mock transfected or transfected with an ADAR1-encoding flag-tagged plasmid. Forty-eight hours after transfection cells were lysed, and total PKR expression and phosphorylated PKR were analyzed by western blot. A representative example from three independent experiments is shown. **(B)** Quantitation of PKR phosphorylation. Values were normalized to total PKR quantities. p values for triplicate measurements are shown.

levels. These data suggest a role for ADAR1 in modulating PKR activity and presumably downstream targets that control protein synthesis, such as eIF-2 α .

The stress-induced p38 MAPK is also a target of PKR and can mediate cytokine activation of proteasome activity³¹ as well as broadly influence myogenesis.³² In data not shown, ADAR1 overexpression suppressed levels of MAPK activation due to stress exposure, notably for p38 MAPK and p54 JNK in 293T cells. Such data suggest a protective role for ADAR1 in limiting a broad array of stress inducible pathways in multiple cell types.

The observed capacity for ADAR1 to attenuate pathways associated with blocks in protein synthesis and catabolic activity prompted us to ask whether ADAR1 might also influence reciprocal pathways associated with protein synthesis and anabolic activity in MPCs. Many previous studies have demonstrated that myogenic hypertrophy is positively associated with increased Akt (also known as protein kinase B, PKB) activity leading to downstream eIF-2 α -mediated increases in muscle protein

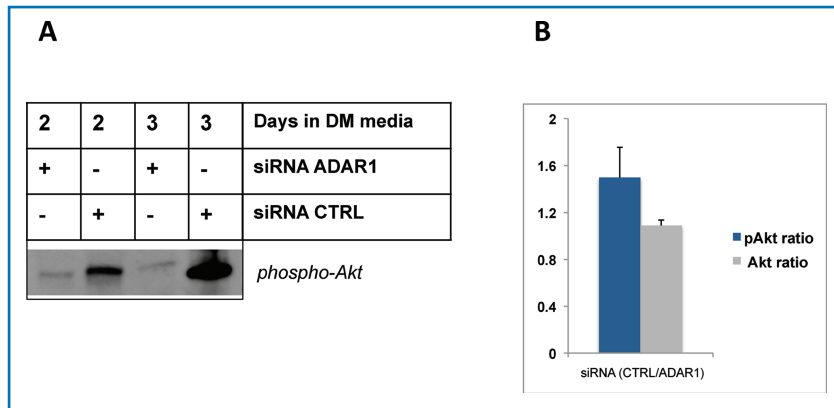


Figure 5. ADAR1 knockdown impairs phospho-Akt levels in cytokine-stimulated differentiating muscle cells. **(A)** C2C12 MPCs were plated and, on the following day, transfected in duplicate using siRNA to ADAR1 or control (Accell siRNA control). Twenty-four hours posttransfection, cells were stimulated with IFN- γ to induce ADAR1 expression and cultured for 2–3 days, then harvested and pooled for total protein on day 2 and day 3, respectively. Total Akt and phospho-Akt (Cell Signaling) levels were probed by western blot, as indicated. **(B)** The p-Akt bands and total Akt (not shown) for each transfection pair were quantified using ImageJ and relative intensities were plotted on a bar graph and significance evaluated using a *t*-test, $p = 0.079$, indicating a trend difference.

synthesis, in part through phosphorylation and de-repression of GSK3 β activity.³³ To evaluate whether ADAR1 expression would influence levels of active Akt (i.e., phosphorylated Akt, phospho-Akt) during differentiation, we transfected MPCs with siRNA to ADAR1, cultured for 1 day in differentiation media, followed by stimulation for 2 days with IFN- γ to induce endogenous ADAR1 expression. Cells were harvested for total protein, then total and phosphorylated levels of Akt protein were measured by western blot. As shown in Figure 5, knockdown of ADAR1 expression by siRNA resulted in dramatic declines of phospho-Akt levels, whereas IFN- γ -stimulated cells with control siRNA displayed much higher levels of phospho-Akt. These data indicate that ADAR1 promotes proanabolic myogenic signaling by protecting stress-induced declines in active Akt, in addition to limiting inflammatory driven activation of PKR and the subsequent presumed blockade of protein synthesis pathways.

Discussion

In this study, we show that differentiating myoblasts express ADAR1 upon exposure to multiple inflammatory stimuli (e.g., LPS, TNF- α , and IFN- γ). Although inflammatory activation of ADAR1 expression has been described in leukocytes, this is the first report to our knowledge demonstrating stress activation of ADAR1 in muscle. ADAR1 was not expressed appreciably under growth conditions, suggesting that ADAR1 primarily functions when cells experience stress (e.g. exposure to inflammatory ligands) while concurrently undergoing differentiation. We go on to show that inflammatory stress also reduces expression of muscle-specific microRNAs miR-1, miR-133a, and miR-206. To our knowledge, stress-mediated decline in microRNAs during myogenic differentiation has also not been previously reported and therefore expands the molecular phenotype of cellular atrophy. This observation, however, is consistent with cytokine-mediated attenuation of MyoD,^{15,26} an upstream regulator of these microRNAs.⁷ Thus, stress-induced ADAR1 activation in muscle likely occurs in the same context of stress-induced signaling pathways that promote atrophy.^{12,13}

In knockdown experiments, we show that reduced ADAR1 levels exacerbate inflammation-mediated declines in myogenic markers such as Myogenin and MHC, supporting a role for

ADAR1 as a buffer to inflammatory stress (Figure 3). Knockdown of ADAR1 expression did not appear to influence muscle microRNA levels for miR-1, miR-206, and miR-133, either in the context of basal or stress-induced conditions (data not shown). However, ADAR1 may have more subtle effects on microRNA, since ADAR1 has been demonstrated to edit miR-1 *in vitro*, raising as yet the untested possibility that ADAR1 can directly influence microRNA biogenesis and function in muscle proliferation and differentiation.²³ Conversely, miR-1 downregulates ADAR1 expression *in vitro*, suggesting a potential for cross-regulation.³⁴ Further experimentation outside of the scope of this study will be required to better understand the role of ADAR1 in the context of inflammatory stress during muscle remodeling *in vivo*.

In gain-of-function experiments using human MPCs (Figure 4), ADAR1 reduced active phospho-PKR levels, consistent with a role in rescuing the block to protein synthesis induced by stress-activated PKR.²⁴ The regulation of protein synthesis in skeletal muscle is critically regulated at the translational initiation step, with a key role for the eIF-2 α . In cachexia models for atrophy, there is an observed increase in phospho-eIF-2 α in association with declines in myofibrillar proteins,³⁵ suggesting that elevated phospho-eIF-2 α levels contribute to net protein loss. A direct interaction of ADAR1 and PKR (both dsRNA-binding proteins) has been recently described to result in PKR inhibition,^{30,36} consistent with results in this study. Recent other studies have also shown that viral infection induces interferon signaling resulting in eIF-2 α phosphorylation with subsequent inhibition of protein synthesis.^{37,38} This suggests that PKR modulation by ADAR1 may be a broadly relevant mechanism for regulating protein synthesis in response to both intrinsic and extrinsic proinflammatory agents. Activation of PKR may provide the link between inhibition of protein synthesis and induction of muscle protein degradation. This may have relevance to muscle atrophy in a range of conditions, for example, cancer cachexia, HIV-AIDS, sepsis, and disuse atrophy.¹²

ADAR1 also attenuated stress-induced MAPK activation, a target of PKR. Inflammatory stress is an antagonist of anabolic IGF-1 signaling, in part through (1) blocking downstream activation of proanabolic phospho-Akt levels thereby reducing protein synthesis,^{39,40} and (2) through stimulation of catabolic pathways, namely, p38 MAPK and NF κ B-mediated activation of MAFbx and MuRF1 atrogenes.¹⁷ Thus, the trend we observed that ADAR1 promotes myogenic signaling by attenuating stress-induced declines in phospho-Akt ($p = 0.079$) raises an intriguing role for ADAR1 in protecting anabolic activity in the context of stress (Figure 5). Akt is a critical signaling kinase within the IGF-1 growth axis, leading to de-repression of protein synthesis through phosphorylation of GSK3 β .³³ Supportive studies have shown that TNF- α /IFN- γ and IGF-1 are mutually antagonistic, with Akt as a positive regulator for myogenesis and a negative regulator for proteasome-mediated atrophy, reviewed by Glass.¹⁷

There is growing recognition that inflammatory stress also engages regulatory feedback mechanisms to resolve and tightly regulate the inflammatory response.^{41–43} ADAR1 may operate in a similar fashion, induced by inflammation to

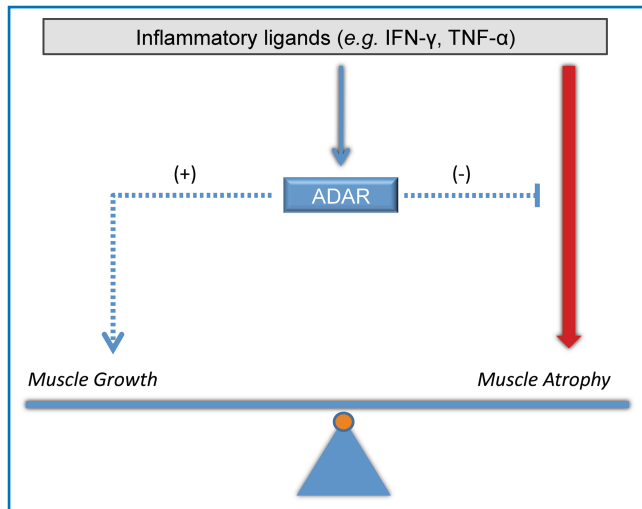


Figure 6. Model for ADAR1 effects on muscle homeostasis during inflammatory stress. In the schematic, muscle homeostasis (i.e., protein turnover) represents a balance of atrophy and growth signaling pathways. Inflammatory ligands in the microenvironment stimulate innate pathways such as IFN- γ to induce muscle atrophy. Inflammation in this model also induces a feedback regulation through activation of ADAR1, which suppresses atrophy pathways and induces muscle growth, possibly through modulating myogenic transcription, Akt activity and PKR activity, to thereby minimize declines in muscle atrophy.

limit inflammatory-mediated damage (see model in *Figure 6*). Consistent with this view, knockdown of ADAR1 expression increased susceptibility to inflammation-mediated declines in muscle markers and overexpression reduced phospho-PKR activity. A similar role for ADAR1 in buffering IFN- γ -mediated damage during hematopoiesis has been recently described.⁴⁴

ADAR1 expression and activity has been broadly implicated in the context of other inflammatory conditions, namely, HIV infection and molecular mechanisms of aging. In HIV infection, ADAR1 is upregulated,⁴⁵ raising the possibility, based on data in this study, that ADAR1 may be induced to modulate infection-associated stress by influencing PKR activity, as shown in other studies.³⁶ Also, in studies of the genetic basis of aging, the ADAR gene family was associated with exceptional longevity in humans⁴⁶ This is an intriguing observation, given reported declines in the ADAR gene family activity with multiple diseases⁴⁷ and the stress-buffering role for ADAR1 observed in this study. Collectively, the data presented in this report suggest a dual role for ADAR1 in both limiting stress atrophy and increasing myogenic pathways. This study provides new avenues for understanding intrinsic cellular mechanisms that underlay muscle atrophy and novel therapeutic strategies to assist intervention.

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