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TITLE: Unlocking Barriers to DNA Vaccine Immunogenicity: A Cross-Species Analysis
of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

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14. ABSTRACT DNA vaccine technology holds great promise as a platform for developing vaccines against both emerging and established global pathogens. Despite this potential, significant challenges impede the capacity of DNA vaccines to prevent disease in humans. Foremost amongst these, is the gap between remarkable results obtained in pre-clinical mouse models and relatively modest immunogenicity observed in humans. The present work is testing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate an inflammatory response to DNA vaccines and that species-specific differences in the cytosolic DNA sensing system bring about divergent inflammatory responses in human versus mouse skeletal muscle myocytes. We are pursuing the following specific aims: 1) characterize the inflammatory response elaborated by myocytes following the delivery of DNA to the cytosol, 2) define the components of the cytosolic DNA sensing system that are present in skeletal muscle myocytes and 3) ascertain which components of the myocyte cytosolic DNA sensing system are engaged upon delivery of DNA to the cytosol. We are utilizing molecular, biochemical and proteomic methods to analyze the consequences of DNA vaccine vector delivery into mouse and human myocyte-derived cell lines and primary cells. We anticipate that our efforts will produce important insights on cytosolic DNA sensors and provide a key to unlock DNA vaccine immunogenicity for humans.					
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Unlocking Barriers to DNA vaccine Immunogenicity: A Cross-Species Analysis of Cytosolic DNA Sensing in Skeletal Muscle Myocytes

1. INTRODUCTION:

We speculate that the skeletal muscle myocyte cytosolic DNA surveillance system can influence both the expression of and immune response to DNA vaccine antigens, thus impacting upon the immunogenicity of DNA vaccines. Moreover, we speculate that key differences in the function of DNA sensors/adaptors in mouse versus human myocytes contribute to the marked cross-species differences observed in DNA vaccine immunogenicity. We are therefore pursuing the hypothesis that skeletal muscle myocytes sense cytosolic DNA and elaborate the initial inflammatory response to DNA vaccines. We are also pursuing the secondary hypothesis that species-specific functional differences in the cytosolic DNA surveillance system bring about a divergent inflammatory response in human versus mouse skeletal muscle myocytes. Our project has focused on three specific aims including: 1) to define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes, 2) to characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol and 3) to ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses. This final report describes our efforts to date in accomplishing these scientific objectives.

2. KEYWORDS:

DNA vaccine, cytosolic DNA sensor, skeletal muscle myocytes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Define the components of the cytosolic DNA surveillance system that are present in human and mouse skeletal muscle myocytes

Major Task 1: Quantitative transcript analyses

Subtask 1A:

- i) optimize culture and differentiation methods for **mouse myocyte cell lines** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
- ii) establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
- iii) establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

➤ Proportion of subtask completed: 100%

- Comment: This subtask has been completed using the mouse myocyte cell line model C2C12.

Subtask 1B:

- optimize culture and differentiation methods for **mouse myocyte primary cells** and evaluate phenotype of myocytes at different stages of differentiation using microscopy
- establish expression levels of **mouse** myogenic differentiation markers by RT-PCR and immunoblot
- establish expression levels of selected **mouse** cytosolic DNA sensors by RT-PCR

- Proportion of subtask completed: 10%
Comment: We did not make further progress relative to the last report. Technical challenges and cost of isolating primary cells proved to be more significant than initially expected.

Subtask 1C:

- optimize culture and differentiation methods for **human myocytes cell lines and primary cells** & evaluate phenotype of myocytes at different stages of differentiation using microscopy
- establish expression levels of **human** myogenic differentiation markers by RT-PCR and immunoblot
- establish expression levels of selected **human** cytosolic DNA sensors by RT-PCR

- Proportion of subtask completed: 15%
- Comment: We did not make further progress relative to the last report. Limited work was carried out with the RD-1 cell line, but the technical challenges and cost of culturing other cell lines or obtaining and maintaining primary cells proved to be more significant than initially expected.

Subtask 2A:

- configure a NanoString assay panel with candidate **mouse** cytosolic DNA sensors/adaptors and other relevant genes
- evaluate NanoString assay panel performance using **mouse** monocyte/macrophage cell lines

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 2B:

- configure a NanoString assay panel with candidate **human** cytosolic DNA sensors/adaptors and other relevant genes
- evaluate NanoString assay panel performance using **human** monocyte/macrophage cell lines

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 3A:

- i) evaluate mouse myocytes using configured NanoString panels
- ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 3B:

- i) evaluate human myocytes using configured NanoString panels
- ii) validate gene expression for a small number of selected transcripts of interest queried by real-time quantitative RT-PCR

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes.

- **Milestone achieved for the mouse skeletal muscle myocyte model (C2C12) only.**

Major Task 2: Protein detection by standard immunoblotting

Subtask 1A:

- i) analyze **mouse** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Proportion of subtask completed: 100%
- Comment: This subtask has been completed using the mouse myocyte cell line model C2C12.

Subtask 1B:

- i) analyze **human** cell lysates for selected cytosolic DNA sensors/adaptors using immunoblotting techniques (with and without immunoprecipitation methods to enrich proteins)

- Proportion of subtask completed: 15%
- We did not make further progress relative to the last report. Limited work was carried out with the RD-1 cell line, but the technical challenges and cost of culturing other cell lines or obtaining and maintaining primary cells proved to be more significant than initially expected.

Subtask 2A:

i) gauge expression and localization of selected **mouse** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Proportion of subtask completed: 100%
- Comment: This subtask has been completed using the mouse myocyte cell line model C2C12.

Subtask 2B:

i) gauge expression and localization of selected **human** proteins by in-cell ELISA or quantitative immunofluorescence microscopy

- Proportion of subtask completed: 10%

Milestone: We will determine which cytosolic sensors/adaptors and downstream effectors are expressed in human and mouse skeletal muscle myocytes

- Milestone achieved for the mouse skeletal muscle myocyte model (C2C12) only.

Specific Aim 2: Characterize the inflammatory response elaborated by human and mouse myocytes following delivery of DNA to the cytosol

Major Task 1: Transcription factor activation analysis

Subtask 1:

i) design and produce DNA vaccine vectors that incorporate a reporter protein and a viral antigen protein then verify by sequencing
ii) characterize expression using bioluminescence, immunofluorescence and immunoblotting methods

- Proportion of subtask completed: 100%

Subtask 2A:

i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **mouse** transcription factors are activated subsequent to entry of DNA

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 2B:

i) use multiplex probe-capture technique and analysis on a Luminex platform to ascertain which **human** transcription factors are activated subsequent to entry of DNA

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 3A:

i) validate **mouse** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. Besides technical challenges, it was deemed impractical to proceed with this subtask given other subtasks in this major task could not be achieved during the course of the funding period.

Subtask 3B:

i) validate **human** cell findings using chemiluminescent electrophoretic mobility shift assays (or DNA-binding ELISA technique in 96-well format)

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. Besides technical challenges, it was deemed impractical to proceed with this subtask given other subtasks in this major task could not be achieved during the course of the funding period.

Subtask 4A:

i) evaluate key **mouse** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Proportion of subtask completed: 80%
- Comment: This subtask has been largely completed using the mouse myocyte cell line model C2C12, but with a focus on IRF-3.

Subtask 4B:

i) evaluate key **human** cell transcription factors NFκB, IRF-3, and IRF-7 separately by immunoblotting of cellular extracts with phospho-specific antibodies

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Milestone: We will ascertain which transcription factors are activated subsequent to entry of DNA into the cytosol, for both human and mouse skeletal muscle myocytes

- **Milestone achieved for the mouse skeletal muscle myocyte model (C2C12) only, with a focus on the transcription factor IRF-3.**

Major Task 2: Secretome analysis by multiplex cytokine capture

Subtask 1

As for Major Task 1

Subtask 2A:

i) detect and quantify **mouse** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report. The technical challenges and high cost of this technique made it impractical to proceed with this subtask.

Subtask 2B:

i) detect and quantify **human** cytokines in supernatants using panel of cytokine-specific antibodies coupled to spectral signature-unique fluorescent beads with Luminex

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Subtask 3A:

i) validate specific mouse cell findings using immunoprecipitation with immunoblotting/ELISA
ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Proportion of subtask completed: 80%
- Comment: This subtask has been largely completed using the mouse myocyte cell line model C2C12.

Subtask 3B:

- i) validate specific human cell findings using immunoprecipitation with immunoblotting/ELISA
- ii) further validate findings by utilizing immunofluorescence microscopy-based intracellular cytokine detection

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed as described above.

Milestone: We will determine which cytokines are secreted, by both human and mouse skeletal muscle myocytes, subsequent to entry of DNA into the cytosol

- **Milestone achieved for the mouse skeletal muscle myocyte model (C2C12) only**

Specific Aim 3: Ascertain which components of the human and mouse myocytes' cytosolic DNA surveillance systems are engaged and essential for the induction of inflammatory responses

Major Task 1: "In vivo" protein-protein & DNA-protein cross-linking pull-down

Subtask 1A:

- i) optimize membrane permeable chemical cross-linker method with **mouse** cells
- ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
- iii) perform "time lapse" experiments

- Proportion of subtask completed: 10%
- Comment: We did not make further progress relative to the last report, given technical challenges.

Subtask 1B:

- i) optimize membrane permeable chemical cross-linker method with **human** cells
- ii) perform co-immunoprecipitation (co-IP) assays to pull-down proteins complexes formed in DNA exposed vs. non-exposed myocytes
- iii) perform "time lapse" experiments

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Subtask 2A:

- i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
- ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed **mouse** myocytes

- Proportion of subtask completed: 10%
- Comment: We did not make further progress relative to the last report, given technical challenges.

Subtask 2B:

- i) label plasmid DNA with photoactivatable biotin (e.g. Psoralen-PEG3-Biotin)
- ii) perform DNA-protein complex pull-down method to pull-down DNA-protein complexes formed in DNA exposed vs. non-exposed human myocytes

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Subtask 3A:

- i) use “shotgun” proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from mouse cells

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges for subtask 1A and the greater than expected cost of the methodology.

Subtask 3B:

- i) use “shotgun” proteomics methods (solubilized and denatured proteins are separated, subjected to tandem mass spectrometry (MS) and analyzed for identification) to determine the constituents of protein-protein and DNA-protein complexes derived from human cells

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Subtask 4A:

- i) confirm specific mouse cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in mouse cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges for subtask 1A and the greater than expected cost of the methodology in subtask 2A.

Subtask 4B:

- i) confirm specific human cell findings using tandem affinity tag purification method whereby candidate partners are co-expressed as 3X FLAG and Twin-StrepTag fusion proteins in human

cells and subsequently subjected to bead-based affinity purification and immunoblotting or ELISA based detection

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Milestone Achieved: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are operational in human and mouse skeletal muscle myocytes

- Milestone not achieved

Major Task 2: Genome Engineering of Myocyte Cell Lines by CRISPR

Subtask 1A:

- i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary mouse genomic target sequences)
- ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges and unanticipated cost of the methodology.

Subtask 1B:

- i) design and produce mammalian expression plasmids encoding the Cas9 protein and specially designed gRNAs (that directs the nuclease to complementary human genomic target sequences)
- ii) confirm Cas9 and green fluorescent protein (GFP) reporter production by plasmids

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges and unanticipated cost of the methodology.

Subtask 2A:

- i) produce mouse myocyte cell lines that have undergone gene disruption via a technique named CRISPR
- ii) confirm gene disruption and GFP expression
- iii) select multiple individual clones characterized with quantitative gene expression

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges and unanticipated cost of the methodology.

Subtask 2B:

- i) produce human myocyte cell lines that have undergone gene disruption via a technique named CRISPR
- ii) confirm gene disruption and GFP expression
- iii) select multiple individual clones characterized with quantitative gene expression

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Subtask 3A:

- i) evaluate mouse cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Proportion of subtask completed: 0%
- Comment: We did not make further progress relative to the last report, given technical challenges and unanticipated cost of the methodology.

Subtask 3B:

- i) evaluate human cell line phenotype upon exposure to cytosolic DNA, as assessed by secretome and transcription factor analyses as described in Specific Aim 2, using cell lines produced with confirmed loss of the genes of interest

- Proportion of subtask completed: 0%
- Comment: Work with human myocytes did not proceed, as described above.

Milestone: We will discover which cytosolic DNA sensors/adaptors and downstream effectors are essential for responding to cytosolic DNA in human and mouse skeletal muscle myocytes

- Milestone not achieved

What was accomplished under these goals?

The work described below is a summary of the major experimental accomplishments we have achieved throughout the period that our studies were funded. This includes work previously described in prior annual reports (**see Annual Reports of October 2016 and 2017**) and also delineates further advances that we have made since our last annual report. The results of some experiments that were described in our prior annual reports but that have no immediate bearing on the overall narrative will be referred to in the narrative text and in some instances provided in an appendix as supplementary data.

The majority of the report is focused on our findings using a mouse skeletal muscle myocyte model (C2C12). Through this work, we have made novel observations regarding how myocyte

levels of STING are modulated soon after entry of DNA into the cytosol following transfection. Moreover, we have gained a new understanding of how STING is regulated and we have confirmed the relevance of upstream and downstream components of this cytosolic DNA sensing pathway in myocytes, including cGAS, TBK-1 and IRF-3. Work on human myocyte-derived cells was limited and has previously been reported (**see Annual Reports of October 2017**) and is also alluded to in the major goals described above and the changes/problems described below (section 5).

Our major experimental accomplishments include the following findings:

i) **C2C12 skeletal muscle cells express endogenous cytosolic DNA sensor cGAS and adapter STING**

Skeletal muscle myocytes C2C12 cells are characterized by their ability to differentiate into multinucleated myotubes upon removal of growth factors from culture media. To verify the authenticity of our C2C12 mouse immortalized cell line, cells were cultured in growth media (GM) and then switched to differentiation media (DM) containing low concentrations of serum. The presence of myotubes became evident three days after media change (DM3) and subsequently increased in size and abundance after a further two days (DM5) of culture (**Figure 1A**).

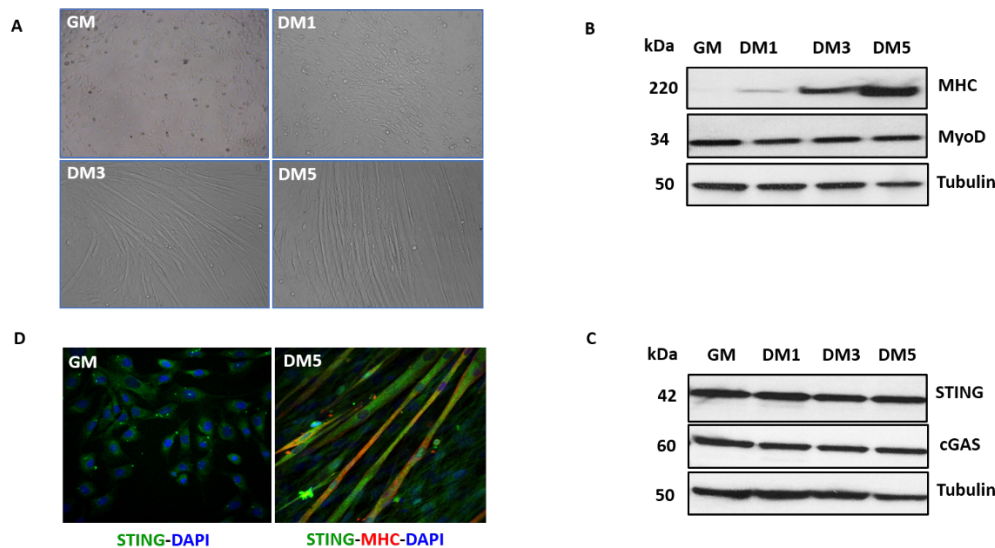


Figure 1. C2C12 myocytes express endogenous cGAS and STING. (A) Phase contrast microscopy of C2C12 cells. Cells were grown in growth media (GM) until confluency and the media was changed to differentiation media (DM) for 5 days. DM1, DM3 and DM5 correspond respectively to Day 1, Day 3 and Day 5 post-change to differentiation media. **(B) Evidence of C2C12 differentiation based on Myosin Heavy Chain (MHC) expression.** Cell lysates were collected at different time points (GM, DM1, DM3 and DM5) during the differentiation process and were blotted with anti-MyoD and anti-MHC antibodies. Blotting with an anti-tubulin antibody was used to provide a loading control. **(C) Cytosolic DNA sensor protein abundance in C2C12 myoblasts (GM) and myotubes (DM1, DM3, DM5).** Cell lysates were collected at different time points (GM, DM1, DM3 and DM5) during the differentiation process

and were blotted with anti-cGAS (cyclic GMP-AMP synthase) and anti-STING (Stimulator of Interferon Genes) antibodies. Blotting with an anti-tubulin antibody was used to provide a loading control. **(D) Immunolocalization of STING in C2C12 cells myoblasts (GM) and myotubes (DM5).** Fixed and permeabilized C2C12 cells were labelled with an anti-STING antibody paired with an Alexa-fluor 488 conjugated secondary antibody and with anti-MHC antibody paired with an Alexa-fluor 594 conjugated secondary antibody.

Myoblast differentiation is accompanied by a signaling cascade which activate the expression of myogenic genes. We therefore analyzed the expression of myogenic differentiation proteins during C2C12 cells differentiation using immunoblotting methods. Skeletal muscle myosin heavy chain protein (MHC) was detectable 24h after differentiation (DM1) and its abundance increased with further differentiation (**Figure 1B**). Levels of the transcription factor MyoD remained stable throughout this period.

As a first step towards ascertaining whether myocytes can sense cytosolic DNA, we sought to determine if C2C12 myocytes express the cytosolic DNA sensor/adaptor pair, cGAS/STING. Immunoblot analysis of C2C12 cells extracts revealed that STING and cGAS were present and expressed in C2C12 myoblast and myotubes (**Figure 1C**). The presence of STING was also confirmed by immunofluorescence analysis (**Figure 1D**). These initial results therefore supported the use of C2C12 cells as a promising model to analyze the cytosolic DNA sensing pathway in skeletal muscle cells.

ii) The introduction of plasmid DNA or DMXAA into myocytes triggers the rapid degradation of STING

It has been demonstrated that the introduction of DNA into the cell cytoplasm activate STING and triggers a DNA sensing signaling cascade. As skeletal muscle cells can be challenging to transfect, we first sought to verify whether we could achieve efficient transfection of C2C12 myoblasts using a GFP-expression plasmid. After transfection, cells were maintained in growth medium for 48h, then switched to differentiation medium and allowed to differentiate for five days. GFP fluorescence was detectable both 48 hours after transfection and after subsequent differentiation (**Figure 2**).

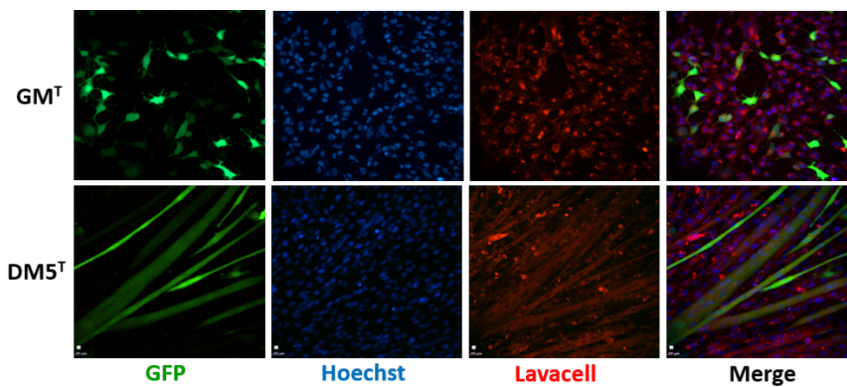


Figure 2. Confocal microscopy of C2C12 pVAX-GFP transfected cells. C2C12 myoblasts were transfected with pVAX-GFP and 48h after transfection (GM^T) the transfected cells were allowed to differentiate for 5 days (DM5^T). GFP fluorescence of the cells was imaged using a 488 nm laser. Hoechst dye (excited with 405 nm laser) was used to stain the nucleus while Lavacell (Active Motif) was used to label membrane proteins (excited with 561 nm laser).

To ascertain whether transfection of skeletal muscle myocytes might modulate the expression or activation of STING, C2C12 cells were transfected with plasmid DNA and levels of STING expression were gauged by immunoblotting extracts obtained over a 48 hour time course. We observed that STING abundance decreased between 2 and 6 hours after transfection and thereafter was maintained at very low levels for at least 48 hours, in both myoblasts and myotubes (**Figure 3A**). Loss of STING was also observed in myoblasts and myotubes, 2 hours after treatment with the STING agonist DMXAA (**Figure 3B**). These results suggested that STING was being degraded shortly after being activated.

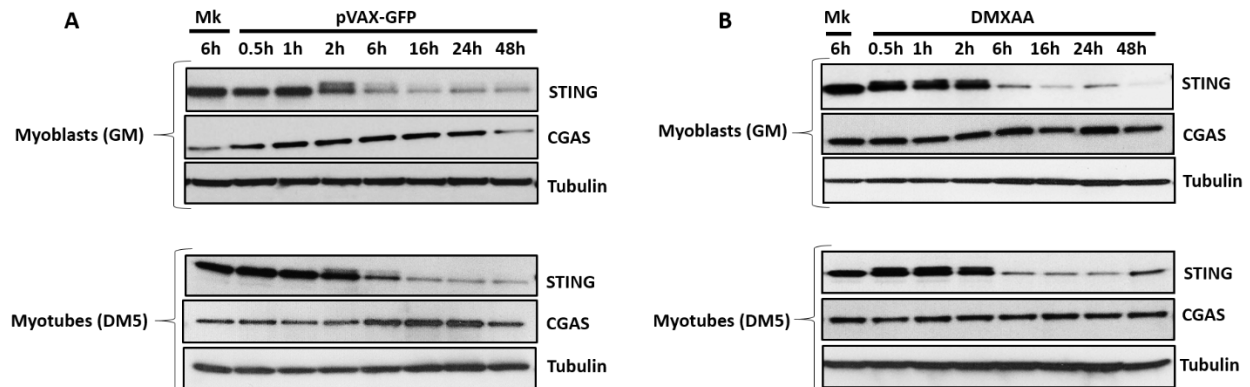


Figure 3. (A) STING abundance diminishes between 2h and 6h after transfection of C2C12 cells with pVAX-GFP. Cell lysates from myoblast (GM) or myotubes (DM5) were collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP and were blotted with anti-STING and anti-cGAS. Mouse tubulin was used as loading control. **(B) STING abundance diminishes between 2h and 6h after incubation of C2C12 cells with DMXAA.** Cell lysates from myoblast (GM) or myotubes (DM5) were collected 6h after mock incubation with DMSO (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after incubation with DMXAA and were blotted with anti-STING and anti-cGAS. Blotting with an anti-tubulin antibody was used to provide a loading control.

We observed the same phenomenon after transfection with a range of DNA concentrations (**Figure 4A**). Transfection did not appear to modulate relative levels of STING mRNA (**Figure 4B**), further suggesting that STING abundance was reduced due to degradation at the protein level.

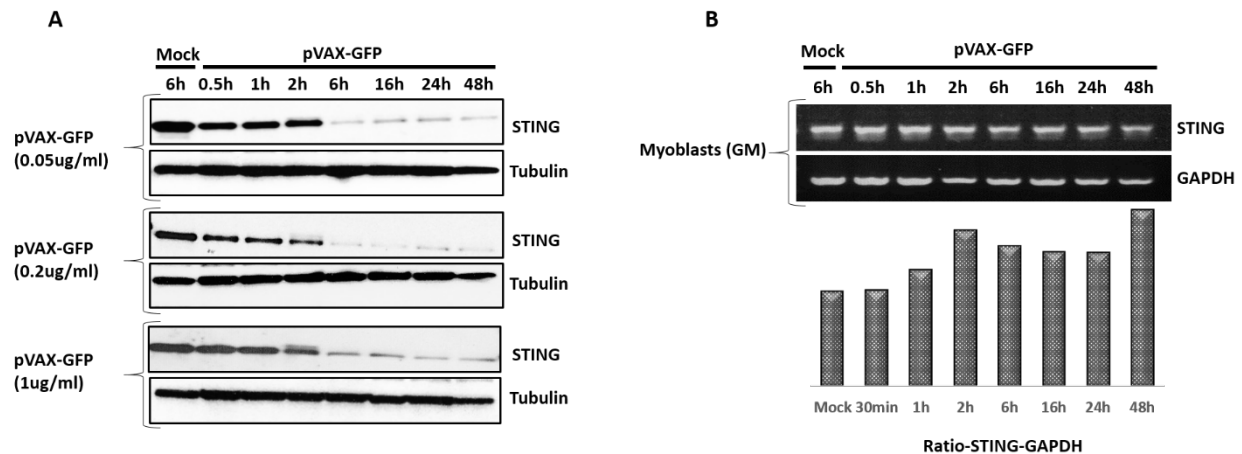


Figure 4. (A) STING abundance diminishes regardless of the amount of transfected DNA. Cell lysates from myoblast were collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP (0.05ug/ml, 0.2ug/ml and 1ug/ml). Lysates were blotted with an anti-STING antibody. Blotting with an anti-tubulin antibody was used to provide a loading control. **(B) Loss of STING doesn't occur at the RNA level.** RT-PCR analysis was performed using total RNA extracts from myoblasts collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP. Mouse-STING specific primers were used to amplify STING and Mouse-GAPDH primers were used for GAPDH as control. Band intensity ratio between STING and GAPDH was used to generate a bar graph.

To exclude the possibility that STING protein degradation might be the result of transfection-induced apoptosis, we looked for evidence of Poly (ADP-ribose) polymerase (PARP) protein cleavage, an established marker of apoptosis, at different time points after transfection. No PARP cleavage was detected after transfection despite the loss of STING (**Figure 5**).

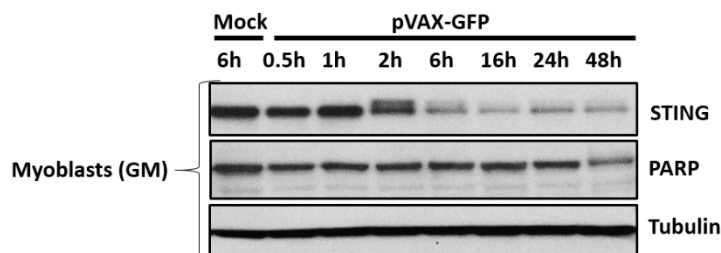


Figure 5. Absence of PARP cleavage after transfection of C2C12 cells with pVAX-GFP. Cell lysates from myoblast were collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h, 48h after transfection with pVAX-GFP and were blotted with anti-STING and anti-PARP antibodies. Blotting with an anti-tubulin antibody was used to provide a loading control.

iii) STING activation and trafficking triggers STING, TBK1 and IRF-3 phosphorylation

Other investigators have determined that STING exists as a dimer that spans the ER membrane but engagement of its inter-dimer interface by cyclic dinucleotides produces a conformational change that allows it to interact with other proteins such as TBK-1. We carried out experiments using chemical cross-linkers to visualize and capture STING in a stabilized form after STING activation. Using a cell-permeable thiol-cleavable cross-linker, dithiobis[succinimidyl

propionate] (DSP), we were able to observe STING dimerization which could be disrupted using a reducing agent (**Supplementary Data Figure S1**). These dimers were present as early as 30 minutes after transfection of plasmid DNA. Our ability to cross-link the components of the dimer only upon activation suggested that STING activation resulted in a conformational change that narrowed the space in the inter-dimer interface.

After either transfection with plasmid DNA or treatment with DMXAA, we invariably observed an increase in the apparent molecular weight of STING as represented by a doublet protein band in C2C12 extracts (**Figure 3, 4A and 5**). This was visible by 2 hours after transfection (**Figure 3A**) or by 30 minutes after treatment with DMXAA (**Figure 3B**). An apparent increase in STING molecular weight has been reported to be secondary to its phosphorylation. No phospho-specific antibodies are available for STING. Accordingly, we ascertained the phosphorylation status of STING by dephosphorylating extracts with lambda phosphatase. C2C12 cell lysates, obtained before and after transfection, were treated with lambda phosphatase and immunoblotting was carried out as previously. We noted that the higher molecular weight band of the doublet disappeared after treatment with lambda phosphatase, thus confirming that this band is a phosphorylated form of STING (**Figure 6**).

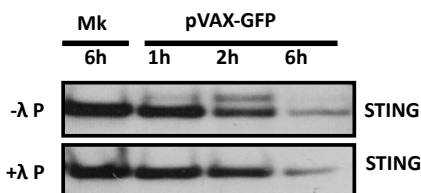


Figure 6. Activated STING is phosphorylated. Cell lysates from myoblasts were collected 6 hours after mock transfection without DNA (Mock) or 1, 2 and 6 hours after transfection with pVAX-GFP. Lysates were treated with 100U of λ Phosphatase (λ P) and were blotted with an anti-STING antibody.

Other investigators have demonstrated that the entry of DNA into the cytosol activates STING and triggers its own phosphorylation as well as the phosphorylation of TBK1 and IRF3. We therefore sought to examine the abundance and phosphorylation of STING, TBK1 and IRF3 at defined time intervals after entry of DNA into the cytosol of C2C12. We observed that TBK1 phosphorylation peaked by 2 hours after plasmid DNA transfection (**Figure 7A**) or by 30 minutes after DMXAA treatment (**Figure 7B**), correlating with the onset of STING phosphorylation under these conditions. Interestingly, phospho-IRF3 was only readily visualized at 2 hours after transfection or DMXAA exposure and its subsequent disappearance appeared to correlate with that of STING and phospho-TBK1 (**Figure 7A and B**).

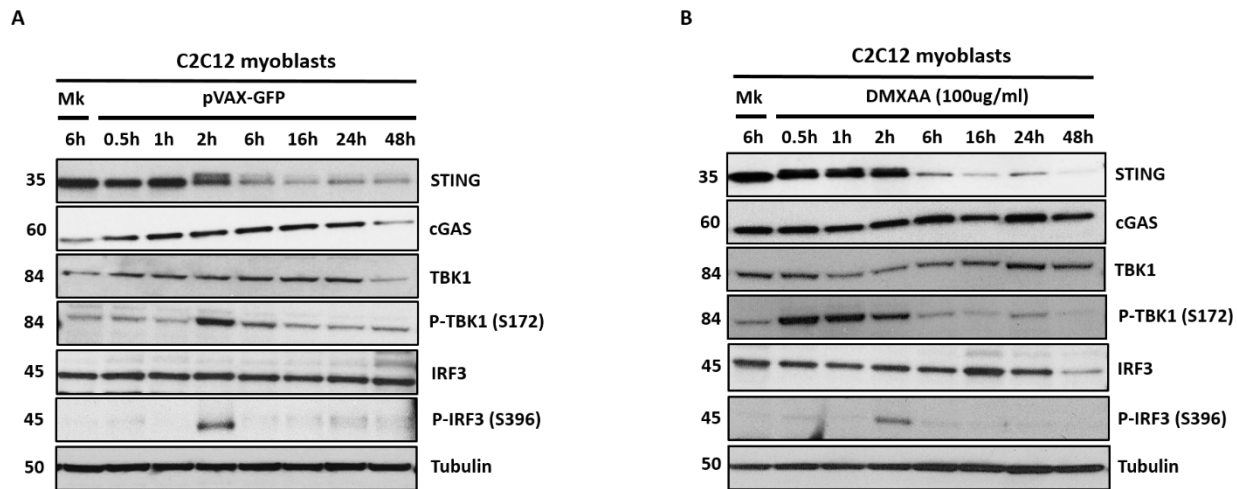


Figure 7. Kinetics of cytosolic DNA sensing proteins' expression in C2C12. C2C12 cells were (A) transfected with pVAX-GFP or (B) incubated with 100ug/ml of DMXAA. Cell lysates from myoblasts were collected 6h after mock transfection without DNA or incubation with DMSO (Mk) and 0.5, 1, 2, 6, 16, 24 and 48 hours after transfection with pVAX-GFP or incubation with DMXAA. The lysates were blotted with anti-STING, anti-cGAS, anti-TBK1, anti-phospho-TBK1, anti-IRF3 and anti-phospho-IRF3 antibodies. Blotting with an anti-tubulin antibody was used to provide a loading control.

iv) STING trafficking is necessary for STING degradation by either proteasomal or lysosomal proteolytic pathways

It has previously been observed that, upon its activation, STING undergoes rapid degradation in an autophagy-like process. As we repeatedly observed that STING protein abundance decreased markedly between 2 and 6 hours after transfection (**Figure 7A and B**), we wished to ascertain the pathway through which STING might be degraded in C2C12 cells. We particularly wanted to determine if STING was being degraded through a proteasomal or lysosomal/autophagy-like pathway. To this end, we investigated the effect of the proteasome inhibitor MG132 and the inhibitors of lysosome acidification chloroquine (CQ) and bafilomycin A1 (BFLA1). C2C12 cells were transfected with plasmid DNA in the presence or absence of MG132, CQ or BFLA1. Treatment of the cells with MG132 appeared to partially abrogate the loss of STING (**Figure 8A**), suggesting that STING degradation was in part occurring through a proteasomal pathway. Treatment with either CQ or BFLA1 completely prevented the loss of STING (**Figure 8A**), suggesting that STING degradation was indeed occurring in the lysosomal compartment as would be expected if it was being degraded in an autophagy-like process as previously described. Interestingly, treatment of C2C12 with either MG132, CQ or BFLA1 did not appear to block STING activation and phosphorylation given the persistence of the double protein band seen on immunoblots of transfected cell extracts (**Figure 8A**). Furthermore, treatment of the cells with either proteasome and lysosome acidification inhibitors did not affect TBK1 phosphorylation (**Figure 8A**). Together, these observations suggested that after STING activation and phosphorylation, both proteasomal and lysosomal processes were contributing to its degradation.

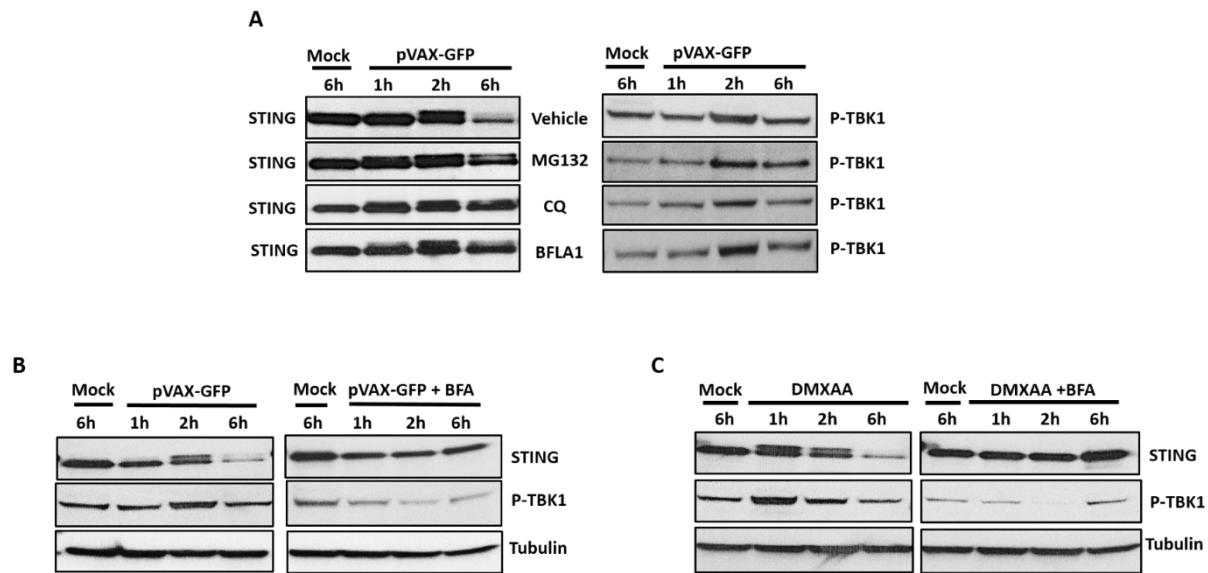


Figure 8. (A) STING appears to be degraded through the proteasomal and lysosomal proteolytic pathway. C2C12 cells were either untreated (vehicle) or treated with 400 μ M MG132, 50 μ M chloroquine (CQ) or 50nM bafilomycin A1 (BFLA1) for 30 minutes prior to transfection with pVAX-GFP. Transfected cells were incubated for 6 hours with or without the chemicals (MG132, CQ, BFLA1). Cell lysates were collected 6 hours after mock transfection (without DNA) or 1, 2 and 6 hours after transfection with pVAX-GFP. Lysates were blotted with anti-STING and anti-phospho-TBK1 antibodies. **(B, C) Phosphorylation and loss of STING are abolished after treatment with Brefeldin A (BFA).** C2C12 cells were incubated 30 minutes with or without BFA (5 μ g/ml) prior to transfection with pVAX-GFP (B) or treatment with DMXAA (100 μ g/ml) (C). Cell lysates were collected 6h after mock transfection (B) or mock treatment with DMSO (D) and 1, 2 and 6 hours after transfection with pVAX-GFP (C) or treatment with DMXAA (D). Lysates were blotted with anti-STING and anti-P-TBK1 antibodies. Blotting with an anti-tubulin antibody was used to provide a loading control.

Following its activation, STING traffics from the endoplasmic reticulum (ER) to a perinuclear compartment including the Golgi. To gauge if translocation of activated STING to the Golgi plays a role in its regulation in C2C12 cells, we treated transfected or DMXAA-treated C2C12 with brefeldin A to inhibit protein transport from the ER to the Golgi. We observed that brefeldin A abrogated STING degradation but also prevented both STING and TBK1 phosphorylation (**Figure 8B and C**). Taking together, these results suggested that after STING activation, STING recruits TBK1 and both are phosphorylated upon which they then traffic to the Golgi and after which STING undergoes degradation.

v) Activation the cytosolic DNA sensing pathway in C2C12 leads to the transcription of the type I interferons

Activation of STING by cytosolic DNA or STING agonists triggers a downstream signaling cascade which culminates in the transcription of pro-inflammatory and type I interferons. To determine if transfection of plasmid DNA results in induction of type I interferons in C2C12 cells, we transfected C2C12 with increasing amounts of plasmid DNA then carried out RT-PCR analysis. No significant variation in STING or GAPDH expression was noted, irrespective of the amount of

DNA used. While no IFN β -1 was noted without transfection of plasmid DNA, using 0.24 micrograms of DNA, IFN β -1 mRNA expression became evident and increased further when 0.5 or 1 microgram of DNA was used (**Figure 9**).

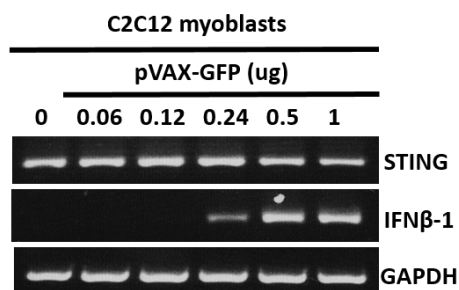


Figure 9. Dose response effect of plasmid DNA on IFN β -1 expression in C2C12 cells. C2C12 cells were transfected with increasing concentration of pVAX-GFP (0.06ug to 1ug) and RT-PCR analysis was performed using total RNA extracts from the cells collected 24h after transfection with pVAX-GFP. Mouse STING and mouse-IFN β -1 specific primers were used to amplify respectively STING and IFN β -1. Mouse-GAPDH primers were used for GAPDH as an internal control.

We then sought to determine the kinetics of IFN β -1 expression in C2C12 cells. We transfected C2C12 with plasmid DNA, then collected cells at intervals over a 24 hour period and measured IFN β -1 mRNA transcripts using RT-PCR. As expected, IFN β -1 expression increased over time after transfection (**Figure 10A**). Similarly, the expression of IL-6 also increased over time (**Figure 10A**). In order to understand how the kinetics of IFN β -1 and IL-6 expression might differ from immune cells, we conducted time course experiments using the macrophage cell line RAW264.7. Transfection of plasmid DNA also induced an increase in IFN β -1 and IL-6 RNA expression in RAW264.7 however both were evident at baseline prior to transfection and were induced more quickly than in C2C12 (**Figure 10B**).

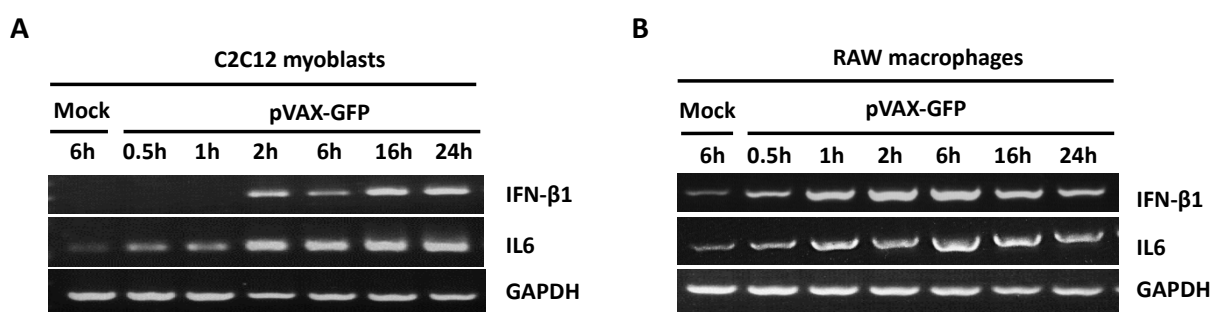


Figure 10. IFN β -1 and IL-6 expression in C2C12 (A) and RAW 264.7 (B) cells. RT-PCR analysis was performed using total RNA extracts from C2C12 or RAW 264.7 cells collected 6 hours after mock transfection (without DNA) or 0.5, 1, 2, 6, 16 and 24 hours after transfection with pVAX-GFP (0.24ug). Mouse-IFN- β 1 and mouse-IL-6 specific primers were used to amplify IFN- β 1 and IL-6 respectively. Mouse-GAPDH specific primers were used to amplify GAPDH as an internal control.

To further support the role of STING-mediated signaling in induction of IFN β -1 and IL-6 expression in C2C12, we then treated cells with brefeldin A prior to transfection with the aim of blocking STING and TBK1 phosphorylation and subsequent signaling events. Under these

conditions, we observed a complete loss of IFN β -1 mRNA expression and a significant reduction of IL-6 mRNA expression (**Figure 11A**).

As IFN β -1 expression could be detected at the RNA level in plasmid DNA transfected C2C12, we then sought to measure IFN β -1 protein secreted from C2C12. C2C12 or RAW264.7 cells culture supernatants were collected 24 and 48 hours after transfection, then secreted IFN β -1 was measure using an ELISA method. High levels of secreted IFN β -1 were detected in RAW 264.7 cells culture supernatants at baseline and more so after transfection (**Figure 11B**). In contrast, no secreted IFN β -1 could be detected in C2C12 cell culture supernatants at any time point (**Figure 11B**). We surmised that C2C12 might produce low levels of IFN β -1 and that any secreted IFN β -1 might be quickly endocytosed. We therefore sought to detect the accumulation of intracellular IFN β -1 after activation of the STING signaling pathway. C2C12 or RAW 264.7 cells were transfected with plasmid DNA then protein secretion was thereafter blocked using brefeldin A treatment after sufficient time had elapsed to allow STING signaling. No IFN β -1 was detectable in C2C12 cells, while RAW 264.7 cells contained notable amounts of IFN β -1 (**Figure 11C**). Together, these findings suggested that while transfection of plasmid DNA could trigger STING signaling and induce IFN β -1 mRNA expression in C2C12 cells, no IFN β -1 protein could be produced by these cells.

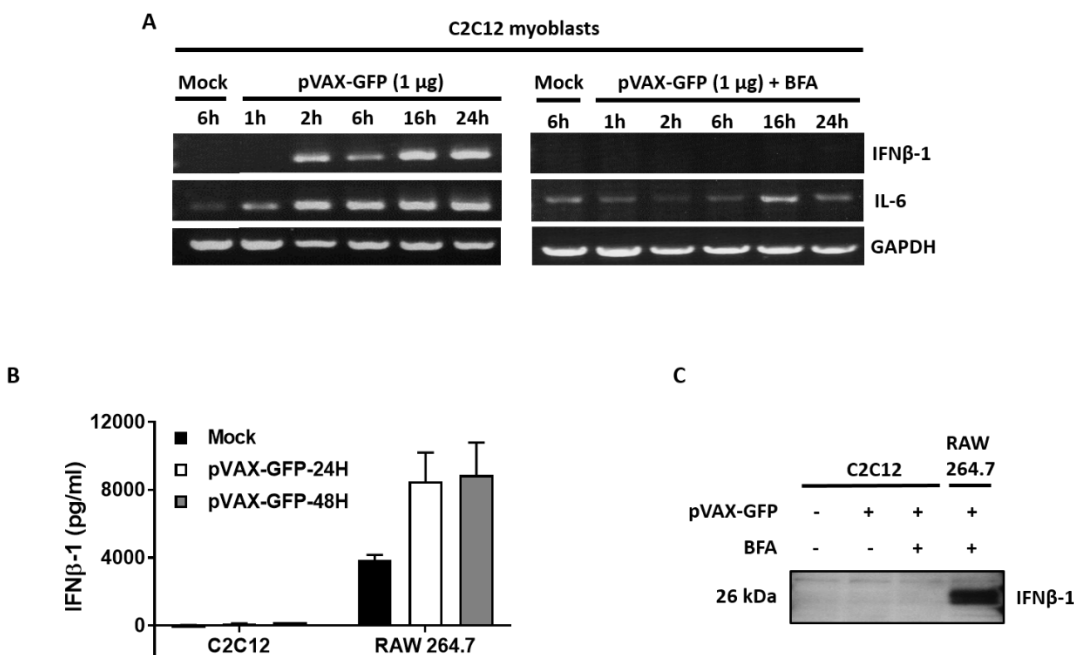


Figure 11. (A) pVAX-GFP transfected C2C12 cells express IFN β -1 and IL-6 with Brefeldin A (BFA) blocking IFN β 1 transcription. C2C12 cells were incubated with or without BFA (5ug/ml) prior to transfection with pVAX-GFP. RT-PCR analysis was performed using total RNA extracts from myoblast cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h and 24h after transfection with pVAX-GFP. Mouse-IFN- β 1 and mouse-IL-6 specific primers were used to amplify IFN- β 1 and IL-6 respectively. Mouse-GAPDH primers were used for GAPDH as an internal control. **(B) No secretion of IFN β 1 from transfected C2C12 cells.** Raw 264.7 and C2C12 cells were transfected respectively with 0.24ug/ml and 1ug/ml plasmid DNA for 48h. The secreted IFN β 1 protein was measured 24h and 48h after transfection by ELISA. The experiment was done in duplicate. **(C) IFN β 1 protein is detected in RAW 264.7 cells but not in C2C12 cells.** C2C12 and RAW 264.7 cells were transfected with

respectively 1ug/ml and 0.24 ug/ml pVAX-GFP plasmid. Brefeldin A (5ug/ml) was added 1h after transfection. The lysates were blotted with anti- IFN β 1 antibody (D2J1D).

vi) Activated STING traffics to a perinuclear compartment in C2C12 cells

Upon activation, STING is expected to translocate from the ER to a perinuclear compartment which could include endosomes and the golgi. The culmination of this process can be visualized by the accumulation of STING in perinuclear punctate vesicles. Aiming to visualize STING translocation in C2C12, we first sought to validate a STING-specific antibody for immunofluorescence experiments.

To this end, we chose to utilize HEK 293T, a cell line that reportedly does not express STING, and confirmed the absence of STING expression (**Figure 12A**). We then transiently transfected HEK293T with an expression vector producing FLAG-tagged mouse STING (mSTING-FLAG) and having established robust expression, thereafter performed immunofluorescence studies on cells with or without pre-treatment with DMXAA. Using confocal microscopy, we readily visualized cytosolic expression of mSTING-FLAG wherein co-localization was noted using both STING-specific and FLAG-specific antibodies (**Figure 12B**). In DMXAA treated cells, aggregation of STING in a perinuclear compartment was readily visualized and again co-localization of STING-specific and FLAG-specific staining was noted (**Figure 12B**).

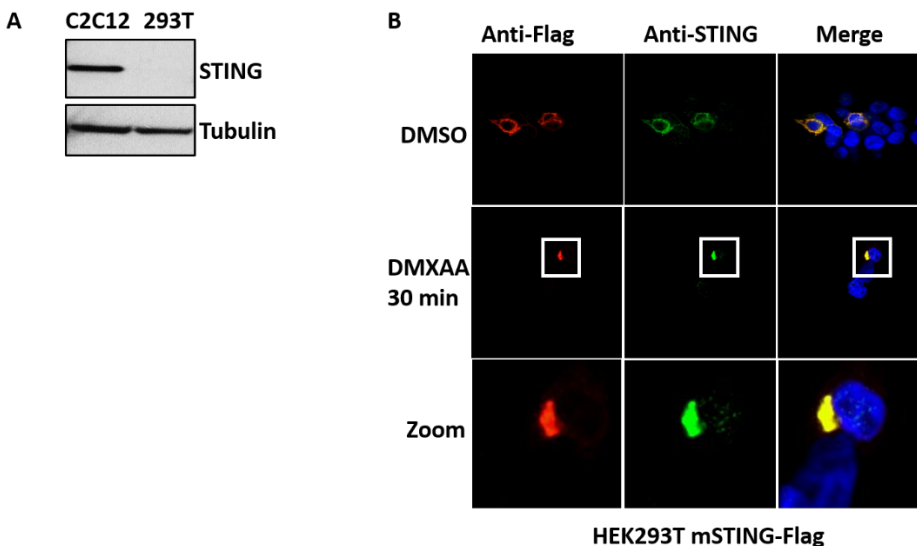


Figure 12. Validation of anti-STING (D5V1L) antibody for immunolocalization of STING. (A) Cell lysates from C2C12 myoblasts and HEK293T cells (293T) were blotted with anti-STING. Tubulin antibody was used as loading control. (B) HEK293T cells were transfected with mouse-STING-Flag for 16h and then were incubated with DMSO or DMXAA (100ug/ml) for 30 min. The slides were stained with 1:400 anti STING (D5V1L) and 1:1600 anti-Flag (9A3) followed by an incubation with donkey anti-rabbit 488 or donkey anti-mouse 594 secondary antibody and DAPI.

We next sought to visualize STING translocation from the cytoplasm to a perinuclear compartment in C2C12 cells. Cells were incubated with vehicle (DMSO) or DMXAA for 30

minutes and thereafter processed for immunofluorescence studies. Again, STING-specific staining was evident diffusely throughout the cytoplasm in vehicle treated cells but accumulated in a perinuclear position in DMXAA treated cells (**Figure 13A**). A similar pattern was noted in RAW 264.7 cells (**Figure 13B**). Furthermore, pre-treatment of the C2C12 cells with Brefeldin A blocked re-localization of STING while pre-treatment with chloroquine had no effect (**Figure 13A**), supporting the notion that activated STING traffics from an ER to Golgi compartment.

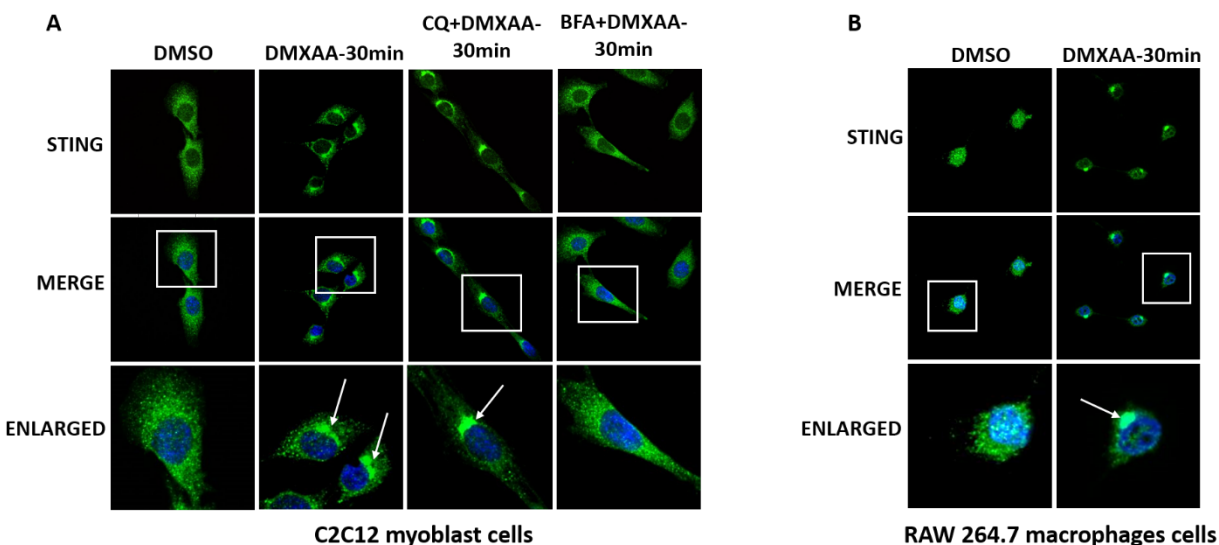


Figure 13. Immunolocalization of STING using anti-STING (D5V1L) antibody. (A) STING localizes to a perinuclear region after treatment of C2C12 cells with DMXAA. C2C12 cells were incubated with DMSO, DMXAA (100ug/ml), DMXAA+CQ (50uM) or DMXAA+BFA (5ug/ml) for 30 min. The slides were stained with 1:400 anti-STING (D5V1L) followed by an incubation with Donkey anti-rabbit 488 and DAPI. **(B) STING localizes to a perinuclear region after treatment of RAW 264.7 cells with DMXAA.** RAW cells were incubated with DMSO or DMXAA (100ug/ml) for 30 min. The slides were stained with 1:400 anti-STING (D5V1L) followed by an incubation with Donkey anti-rabbit 488 and DAPI.

vii) Phospho-TBK1 accumulates in a perinuclear compartment after STING activation

Prior reports have described a cell-type specific localization in response to cytosolic DNA and colocalization with STING in the Golgi after treatment with DMXAA. We therefore sought to determine the subcellular localization of phospho-TBK1 in C2C12 in response to activation of STING by DMXAA. As expected, phospho-TBK1 translocated to a perinuclear region within an hour after treatment of C2C12 cells with DMXAA (**Figure 14**). By two hours the amount of perinuclear fluorescence appeared to diminish and by four hours had virtually disappeared. These findings appeared to correlate with observations in immunoblot experiments. Surprisingly, phospho-IRF-3 was detectable in the perinuclear region of some cells even without DMXAA treatment (**Supplementary Data Figure S2**). After DMXAA treatment, phospho-IRF-3 appeared to accumulate further in the perinuclear region and thereafter localized to the nucleus as would be expected. These findings appeared to be consistent with our immunoblotting data but will require additional confirmation.

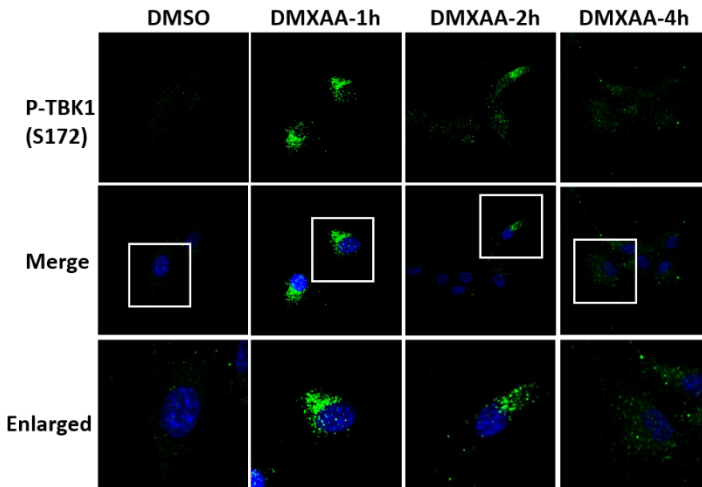


Figure 14. Subcellular localization of P-TBK1 (S172) after treatment of C2C12 cells with DMXAA. C2C12 cells were incubated with DMSO for 4h and with DMXAA (100ug/ml) for 1h, 2h and 4h. The slides were stained with 1:50 anti-PTBK1 (Ser172) (D52C2) followed by an incubation with Donkey anti-rabbit 488 and DAPI.

What opportunities for training and professional development has the project provided?

The research personnel working on this project received mentorship and training from the principal investigator regarding general experimental design, DNA expression vector design, tissue culture, ELISA methods, immunoblotting techniques, primer design and RT-PCR techniques. Extensive and advanced training in confocal fluorescence microscopy techniques has been provided to the postdoctoral fellow through the investigators' core facility. Professional development activities in the form of weekly scientific axis-specific seminars, postdoctoral fellow seminars, a biostatistics seminar series have also been provided to research personnel.

How were the results disseminated to communities of interest?

A manuscript describing our findings based on the C2C12 skeletal muscle myocyte model is presently being prepared. We expect to be able to submit this manuscript sometime in August of 2018.

In addition, our results have been presented in the following venues (*please see appendix for copy of poster abstracts*):

Edaye S, Gheorghe R and **Zaharatos GJ**. Cytosolic DNA sensing in skeletal muscle-derived myocytes. Poster Abstract. Eighth Annual Lady Davis Institute Scientific Retreat, May 12th, 2017. Montreal, Quebec, Canada.

Edaye S, Gheorghe R, **Zaharatos GJ**. Are myocytes actors in DNA vaccine immunogenicity? In vitro studies of cytosolic DNA sensing in skeletal muscle myocytes. Keystone Symposia on Molecular and Cellular Biology. Emerging Technologies in Vaccine Discovery and Development (J5). January 28th-February 1st, 2018. Banff, Alberta, Canada.

Edaye S, Gheorghe R and **Zaharatos GJ**. Cytosolic DNA activates a STING-dependent signaling pathway in skeletal muscle myocytes. Poster Abstract. Ninth Annual Lady Davis Institute Scientific Retreat, May 4th, 2018. Montreal, Quebec, Canada.

What do you plan to do during the next reporting period to accomplish the goals?

Not applicable. This is the final report. Work to continue if other funding sources obtained.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have nothing substantive to report at this time. Our findings, once published, are likely to propel other investigators to pursue a greater understanding of cytosolic DNA sensing in myocytes and other non-immune cells.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Given delays in completing some tasks due to difficulties or challenges with certain techniques, it became impossible to complete all planned work within the period of funding. In addition, given delays and budgetary constraints we were unable to proceed with work on a number of our key objectives, including work on primary cells and especially human cells. Most notably, limited work was carried out with the RD-1 cell line, but the technical challenges and cost of culturing other cell lines or obtaining and maintaining primary cells proved to be more significant than initially expected.

Actual or anticipated problems or delays and actions or plans to resolve them

We alluded to several methodologic or technical challenges in our prior annual reports. These challenges prolonged effort and expenditure on certain aspects of the project and significantly delayed the onset of certain tasks. The major problems that prevented us from attaining some of our goals included the following:

- 1) Significant expenditure of time and effort and reagents was made attempting to select and optimize techniques to detect components of the cytosolic DNA sensing signaling pathway in myocytes (**see Annual Reports of October 2016 and 2017**). This included extensive but unsuccessful work focused on the protein ifi202/IFI16. We eventually optimized detection of STING, cGAS, TBK-1 and phosphorylated TBK-1. However, we had great difficulty accurately detecting the transcription factor IRF-3 (**see revised Annual Report of October 2017**) and its phosphorylated form (phospho-IRF-3). We eventually optimized our methods and resolved this issue.
- 2) Significant expenditure of time and effort and reagents was made attempting to select and optimize techniques to detect IFN β 1 protein in myocytes (**see revised Annual Report of October 2017**). As described above, we eventually optimized a new technique to enhance IFN β 1 detection. Nevertheless, unlike immune cells such as macrophages, skeletal muscle myocytes did not express IFN β 1 in sufficient quantity to allow detection by standard methods.
- 3) The most significant expenditure of time and effort, reagents and core resources was made attempting to optimize STING localization and trafficking using immunofluorescence microscopy methods. The first challenge we faced was the mis-localization of STING induced by various detergents we used for myocyte permeabilization after fixation. This hindered our attempts to visualize STING trafficking after DNA enters the myocyte cytosol. After extensive testing, we partially resolved this issue by using low concentrations of saponin. Concurrently, finding appropriate antibodies for immunofluorescence (unlike immunoblotting) was highly problematic and

in fact only a handful of antibodies appeared adequate (**see revised Annual Report of October 2017**). Our efforts to find the ideal antibody culminated in a new antibody validation strategy (see accomplishments section) and eventually led to an accurate immunofluorescence technique to visualize STING localization and trafficking.

Changes that had a significant impact on expenditures

- Given the problems described above, greater than expected time, effort and expenditures were made focusing on the C2C12 skeletal muscle myocyte model. This work involved many experiments analyzing cell lysates for cytosolic DNA sensors/adaptors using immunoblotting and biochemical techniques as well as extensive work optimizing immunofluorescence microscopy using the advanced confocal microscopy system of our core facility. Significant progress using this model required sustained and repeated efforts and accordingly, we could not further pursue experiments requiring more costly techniques (e.g. Luminex and NanoString methods) and more costly and technically demanding cell lines or primary cells.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	Dr. Gerasimos J. Zaharatos
Project Role:	Principal Investigator
Researcher Identifier:	not registered
Nearest person month worked:	30 (over the course of the entire funding period)
Contribution to Project:	• Provided training to personnel (postdoc and research technician) • Supervised all work • Designed overall research strategy • Guided experimental design • Assisted in analysis of data
Funding Support:	No other support

Name:	Dr. Sonia Edaye
Project Role:	Postdoctoral Fellow
Researcher Identifier:	not registered
Nearest person month worked:	30 (over the course of the entire funding period)
Contribution to Project:	• Provided training to personnel (research technician) • Supervised work of research technician • Assisted in experimental design • Executed experiments • Analyzed data
Funding Support:	No other support

Name:	Ms. Romina Gheorghe
Project Role:	Research technician
Researcher Identifier:	not registered
Nearest person month worked:	30 (over the course of the entire funding period)
Contribution to Project:	• Executed experiments • Assisted in analysis of data
Funding Support:	No other support

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

QUAD CHARTS: Not applicable

9. APPENDICES:

- 1) Supplementary data
- 2) Poster abstract presented at Eighth Annual Lady Davis Institute Scientific Retreat
- 3) Poster abstract presented at Keystone Symposia's Emerging Technologies in Vaccine Discovery and Development (J5) meeting and at Ninth Annual Lady Davis Institute Scientific Retreat

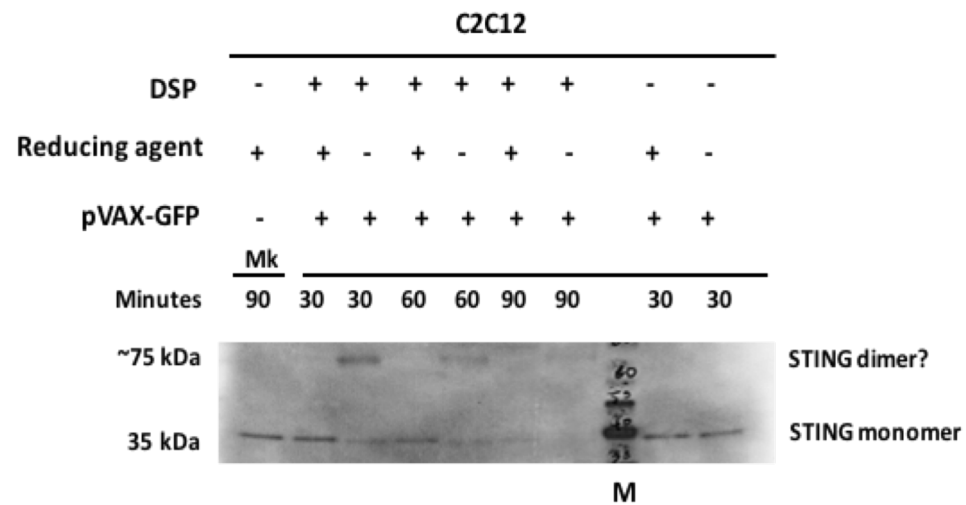


Figure S1. DSP crosslinking after transfection of C2C12 cells with pVAX-GFP.

DSP was added to the cells 30 , 60 or 90 minutes after transfection or 90 minutes after mock transfection (Mk). Cell lysates were processed with or without addition of reducing agent and were blotted with anti-STING antibody. Molecular weight markers (M) are shown.

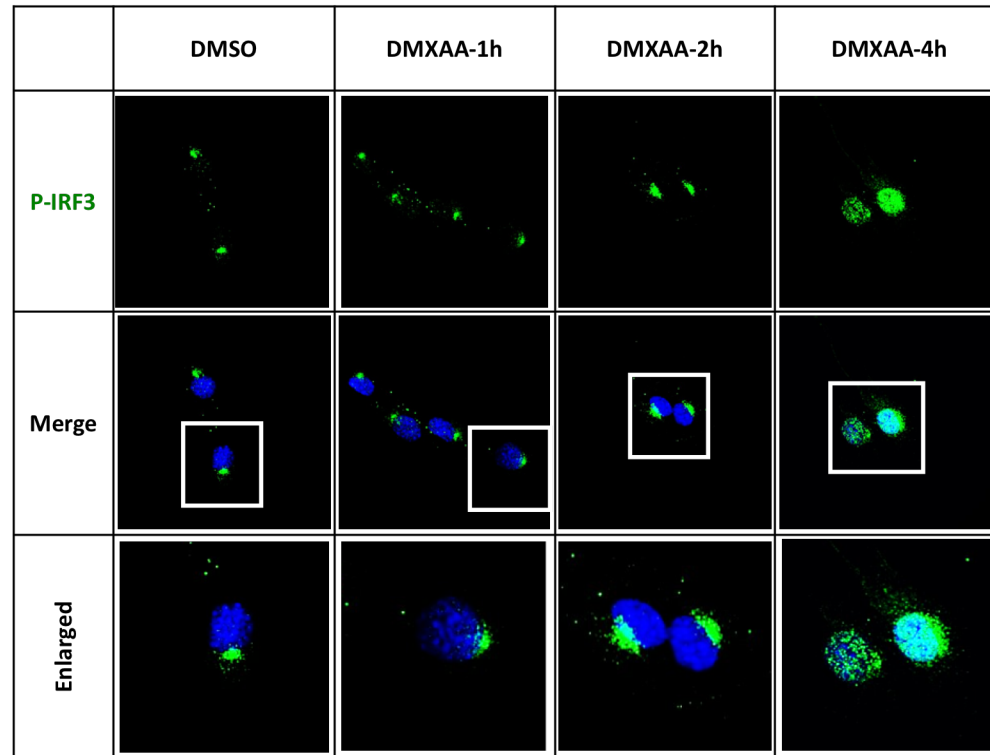


Figure S2. Immunolocalization of P-IRF3 in C2C12 cells. C2C12 cells were incubated with DMSO and DMXAA (100ug/ml) for 1h, 2h and 4h. The slides were stained with 1:50 anti-phospho-IRF3 followed by incubation with donkey anti-rabbit 488 conjugated antibody and DAPI.

ABSTRACT

Background: A growing number of cytosolic DNA sensing pathways have been characterized with a role in pathogen detection and activation of host immune defences. In one such pathway, the presence of DNA inside the cytosol induces cGAS (cyclic-GMP-AMP synthase) to activate STING (stimulator of interferon genes), culminating in the transcription of type I interferons and inflammatory cytokines. These pathways have been predominantly characterized using immune cells, such as dendritic cells and macrophages. Gene-based vaccine platforms like DNA vaccines represent a promising approach to prevent infectious diseases. Such vaccines act by delivering a pathogen-derived antigen of interest, encoded by a vector, to host somatic and immune cells. In the case of DNA vaccines, plasmid DNA is generally delivered intramuscularly and thereafter locally transfects skeletal muscle myocytes. The presence of a cytosolic DNA sensing pathway has not been described before in skeletal muscle myocytes and the role of such a pathway in DNA vaccine immunogenicity has not been defined.

Hypothesis and Objective: We hypothesized that cytosolic DNA sensing pathway components, including cGAS and STING, are present in skeletal muscle myocytes and are activated upon entry of DNA into the cytosol. We sought to define the presence and activity of this pathway in a skeletal muscle-derived myocyte model.

Methods: Experiments utilized the mouse skeletal muscle myoblast cell line C2C12. We ascertained the presence of cytosolic DNA sensor pathway constituents using immunoblotting. We then introduced a DNA vaccine vector (a plasmid DNA-based mammalian expression vector expressing GFP) into C2C12 myoblasts or myotubes by chemical transfection methods. The abundance and activation of various DNA sensors and downstream pathways was subsequently characterized by immunoblotting and RT-PCR.

Results: C2C12 myoblasts and myotubes express cytosolic DNA sensor proteins ifi204, cGAS and STING. Introduction of DNA into the myocyte cytosol by transfection results in the phosphorylation of STING. Thereafter, the abundance of STING at the protein level decreases markedly between 2h to 6h post-transfection. Treatment of myoblast cells with MG132, a proteasome inhibitor, partially rescues STING thus suggesting inhibition of degradation. Other downstream components of the DNA sensing pathway such as TBK1 (Tank-binding kinase) and IRF3 (Interferon regulatory factor 3) are also expressed in C2C12 myocytes. The abundance of phosphorylated forms of these proteins (P-TBK1 and P-IRF3) increases after transfection, further suggesting activation of the STING pathway. Finally, transfection results in marked up-regulation of IFN- β 1 and IL-6 at the RNA level, again supporting the presence of a functional cytosolic DNA sensing pathway in C2C12.

Conclusion: Taking together our results suggest that skeletal muscle-derived myocytes can sense cytosolic DNA through a STING pathway and that this culminates in the expression of type I interferons and inflammatory cytokines. These findings provide a rationale for further investigation of cytosolic DNA sensing in skeletal muscle and suggest the need for careful consideration of how such events may influence the immunogenicity of intramuscular gene-based vaccine delivery.

ACKNOWLEDGEMENTS

Work funded by CDMRP- Department of Defense



BACKGROUND

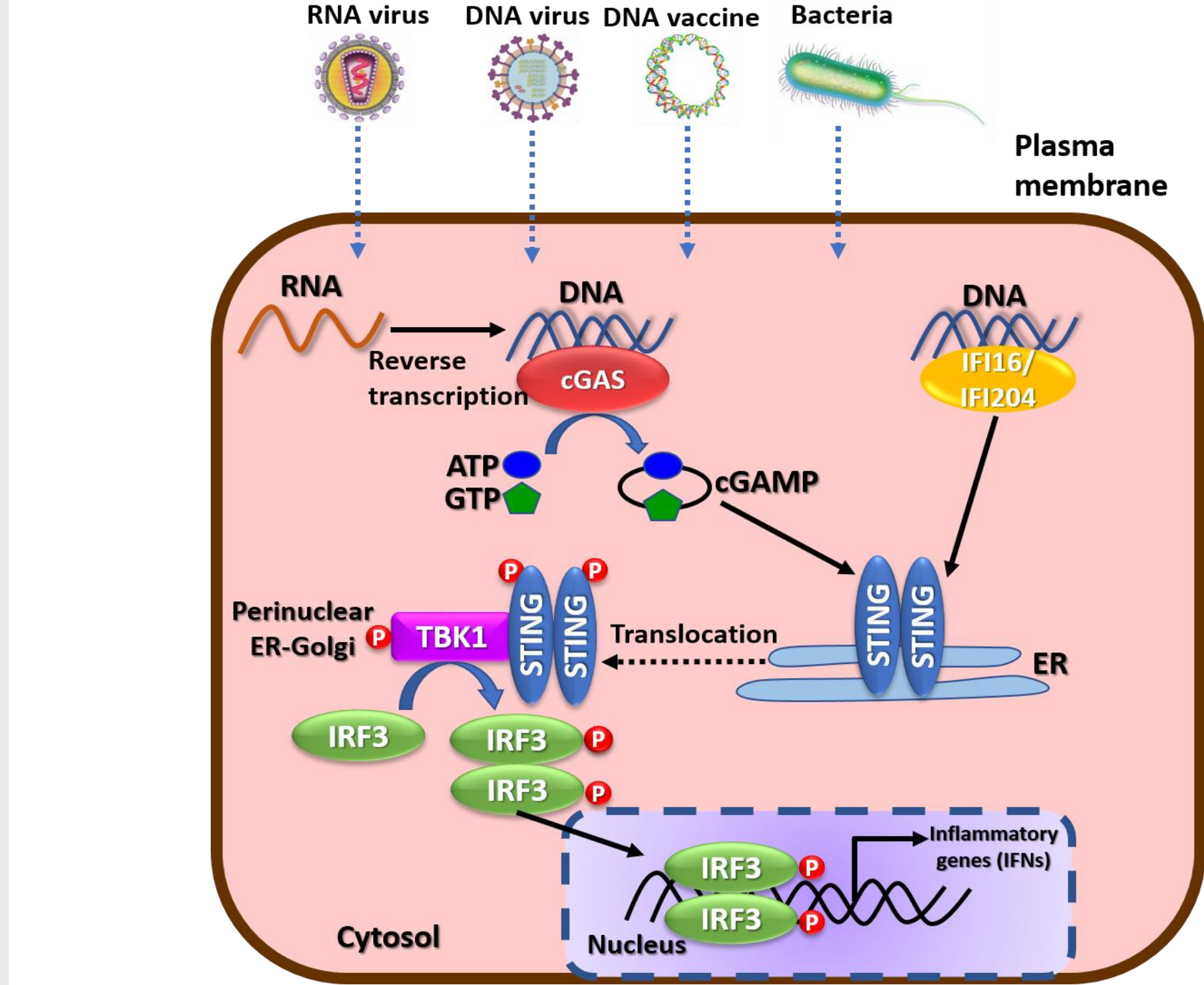


Figure 1. Cytosolic DNA sensing cGAS-STING pathway. After recognition of cytosolic DNA, cGAS catalyzes the synthesis of cGAMP which acts as a second messenger and binds to the adaptor protein STING. IFI16/IFI204 also recognizes cytosolic DNA and activates STING. STING dimerizes and translocates to a perinuclear region (ER-Golgi). Following STING relocalization, TBK-1 is recruited to STING. After binding to STING, TBK1 undergoes self-phosphorylation and subsequently phosphorylate STING and IRF-3. IRF3 then translocate into the nucleus and induces the production of type I IFN.

RESULTS 2

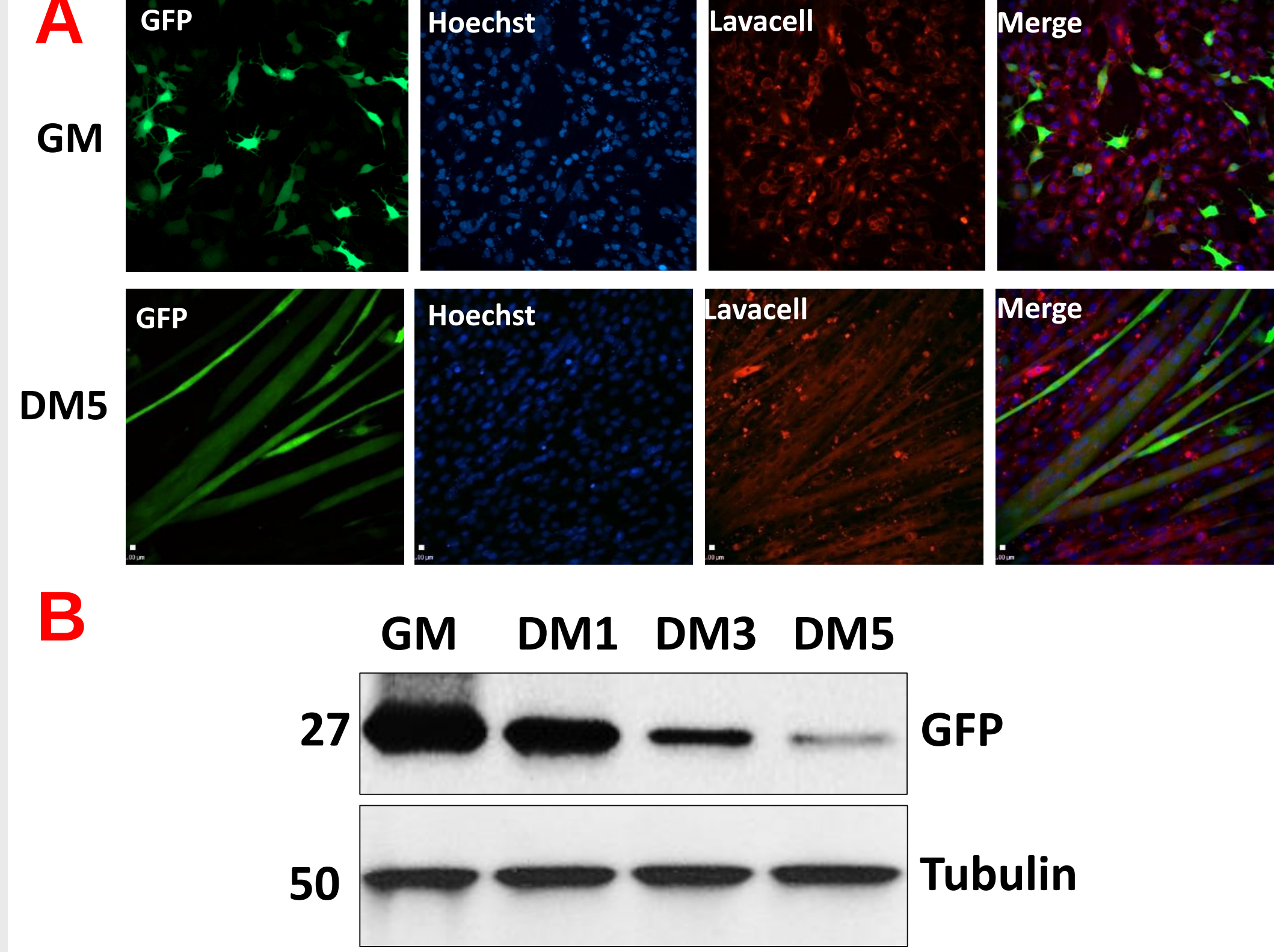


Figure 3. (A) Confocal microscopy of C2C12 pVAX-GFP transfected cells. C2C12 myoblasts were transfected with pVAX-GFP and 48h after transfection (GM) the transfected cells were allowed to differentiate for 5 days (DM5). GFP fluorescence of the cells was imaged using excitation with a 488 nm laser. Hoechst dye (excited with 405 nm laser) was used to stain the nucleus while Lavacell (Active Motif) was used to label membrane proteins (excited with 561 nm laser). **(B) GFP expression after C2C12 cells transfection.** Protein lysates were collected at different time points (GM, DM1, DM3 and DM5) after transfection with pVAX-GFP and were blotted with anti-GFP antibody. Mouse anti-tubulin antibody was used as a loading control.

RESULTS 4

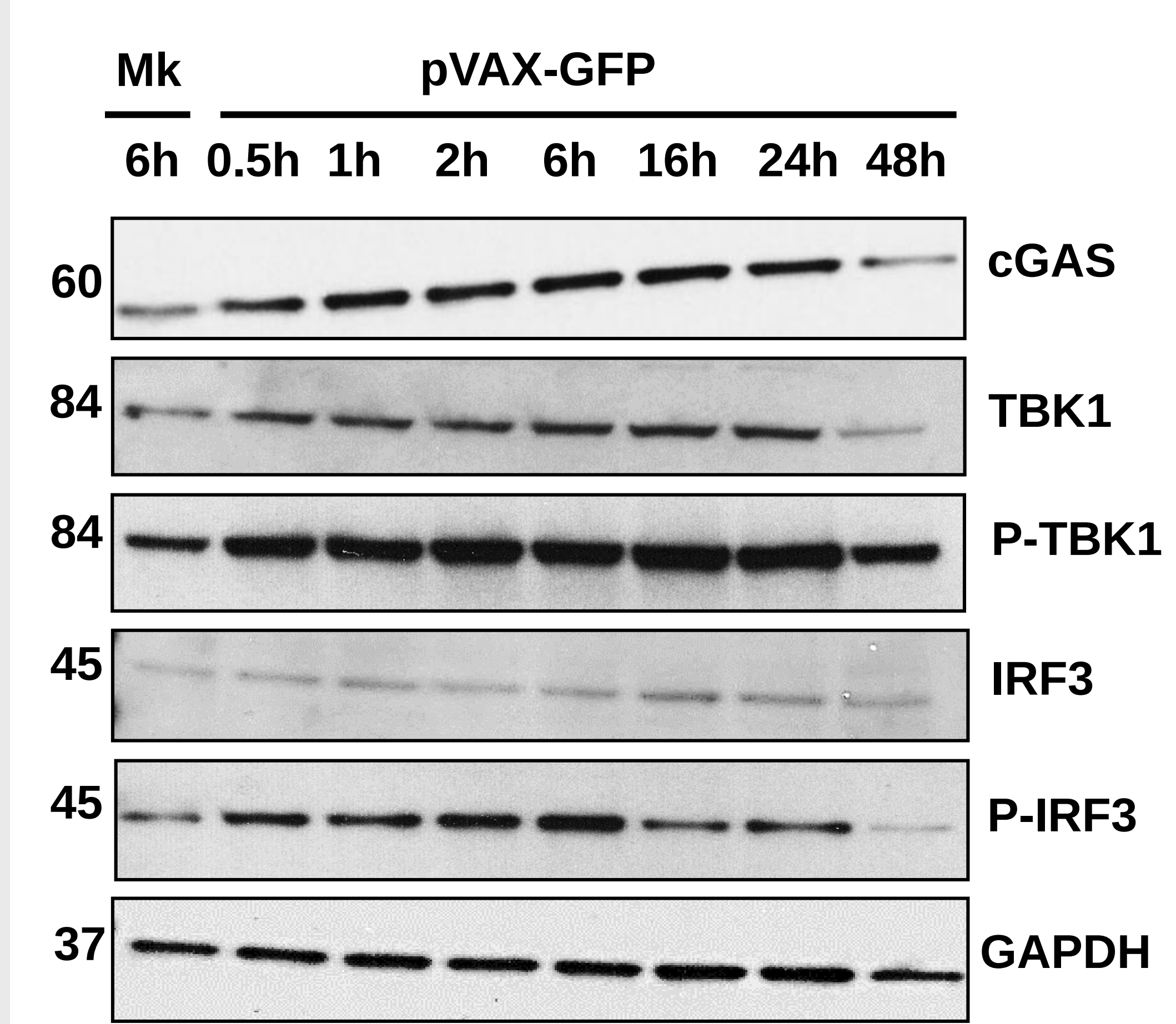
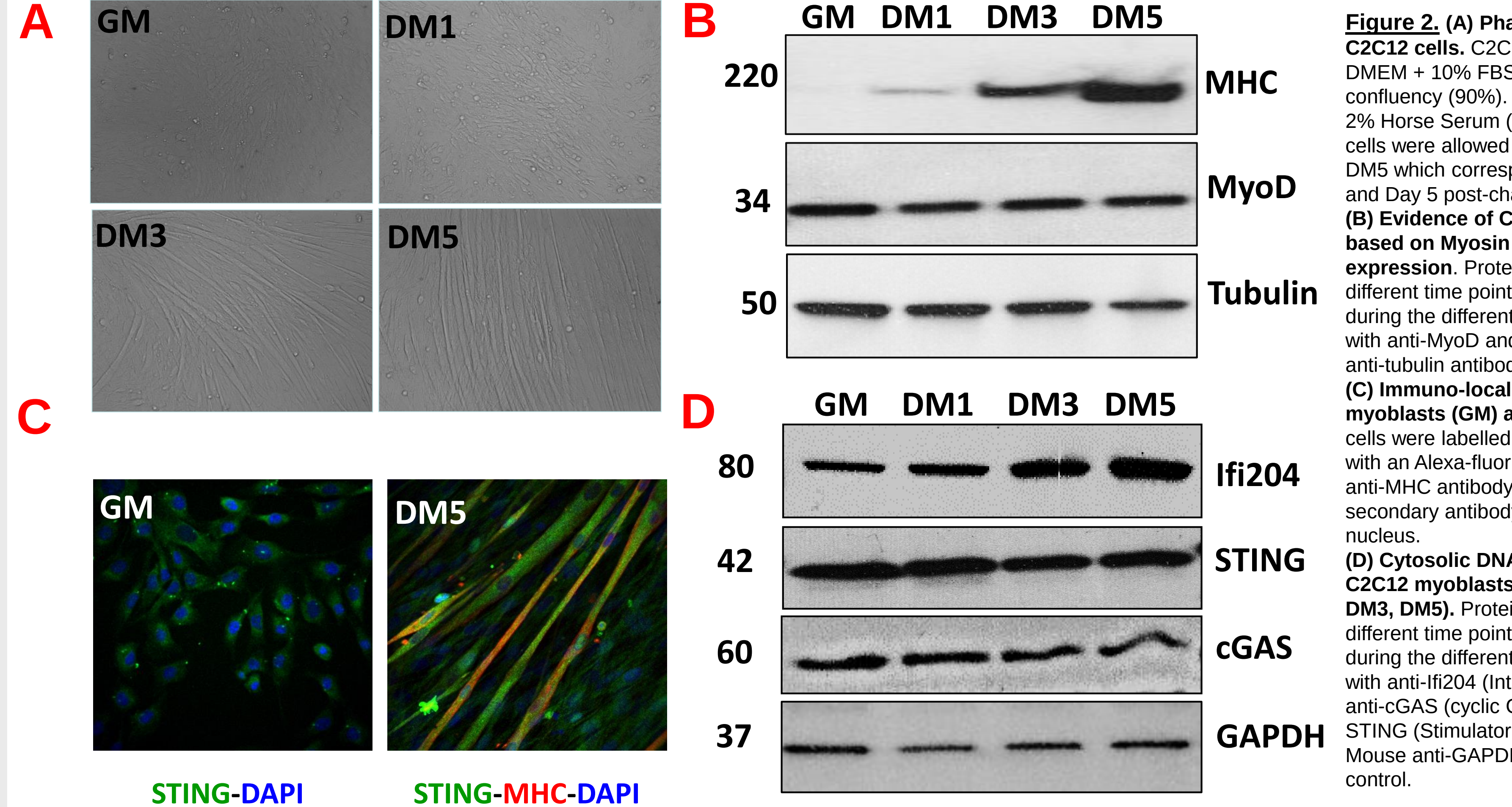


Figure 5. Kinetics of cytosolic DNA sensor protein expression. Protein lysates were collected 6h after mock transfection without DNA (Mk) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP and were then blotted with anti-STING, anti-cGAS, anti-TBK1, anti-P-TBK1, anti-IRF3 and anti-P-IRF3 antibodies. Mouse GAPDH antibody was used as a loading control.

RESULTS 1



RESULTS 3

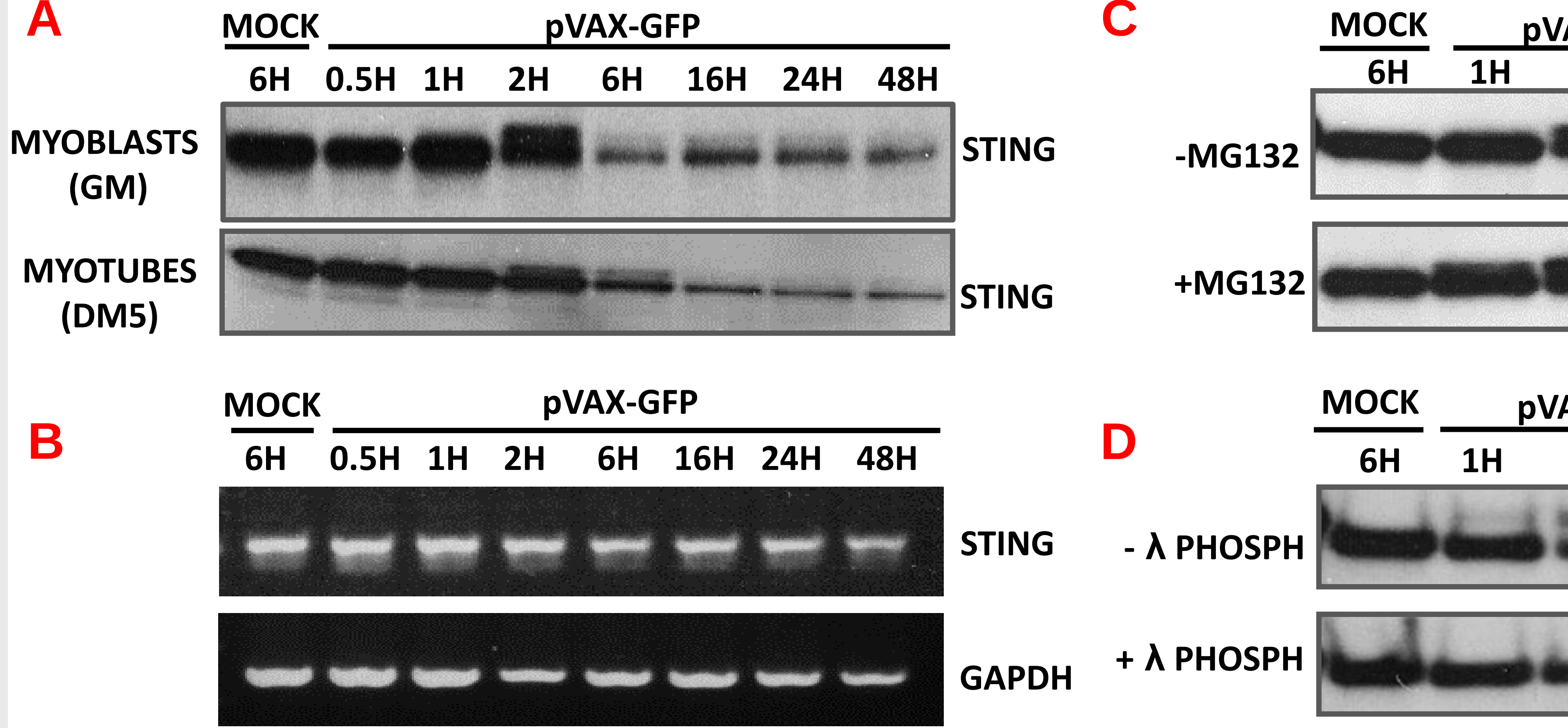


Figure 4. (A) STING abundance is diminished between 2h and 6h after transfection of C2C12 cells with pVAX-GFP. Protein lysates from myoblast (GM) or myotube (DM5) cells were collected at different time points after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP and were blotted with anti-STING antibody. **(B) Loss of STING doesn't occur at the RNA level.** RT-PCR analysis was performed using total RNA extracts from myoblasts collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-STING specific primers were used to amplify STING and Mouse-GAPDH primers were used for GAPDH. **(C) STING appears to be degraded through the proteasome pathway.** Cells underwent treatment with 400 μ M MG132 or no treatment prior to transfection. Protein lysates were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with pVAX-GFP. The lysates were blotted with anti-STING antibody. **(D) STING is phosphorylated prior to degradation.** Protein lysates from myoblasts were collected 6h after mock transfection without DNA (Mock) or 1h, 2h and 6h after transfection with pVAX-GFP. The lysates underwent treatment with λ Phosphatase (100U) or no treatment and were then blotted with anti-STING antibody.

RESULTS 5

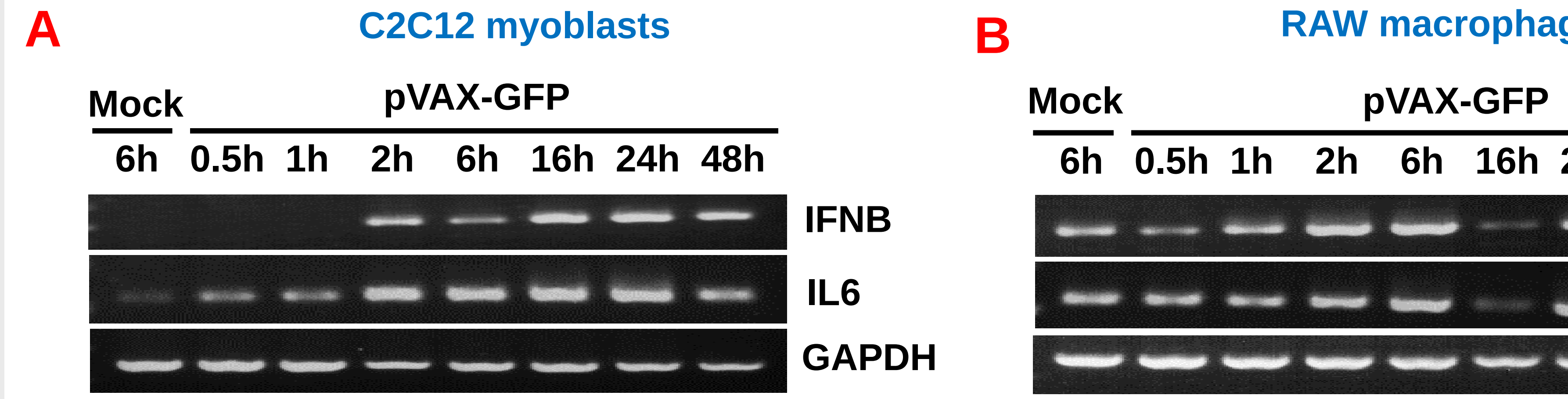


Figure 6. (A) IFNB and IL6 expression in C2C12 cells. RT-PCR analysis was performed using total RNA extracts from myoblast cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-IFN β 1 and mouse-IL6 specific primers were used to amplify IFN β 1 and IL6 respectively. Mouse-GAPDH was used as an internal control. **(B) IFNB and IL6 expression in RAW cells.** RT-PCR analysis was performed using total RNA extracts from macrophages cells collected 6h after mock transfection without DNA (Mock) or 0.5h, 1h, 2h, 6h, 16h, 24h and 48h after transfection with pVAX-GFP. Mouse-IFN β 1 and mouse-IL6 specific primers were used to amplify IFN β 1 and IL6 respectively. Mouse-GAPDH was used as an internal control.

CONCLUSION AND FUTURE DIRECTIONS

- ❖ Skeletal muscle myocytes can sense cytosolic DNA and appear to do so through STING
- ❖ This pathway can trigger expression of type I interferons and inflammatory cytokines
- ❖ Our findings provide a rationale for further investigation of cytosolic DNA sensing in skeletal muscle
- ❖ We are endeavoring to better delineate the components of the cytosolic DNA sensing pathway that are engaged in myoblasts and myotubes
- ❖ The relevance of these events will be explored in small animal models

ABSTRACT

Background: DNA vaccines and other gene-based vaccine platforms represent a promising approach to prevent infectious diseases. In the case of DNA vaccines, plasmid DNA is generally delivered intramuscularly and thereafter locally transfects skeletal muscle myocytes. Cytosolic DNA sensing pathways have predominantly been characterized using immune cells but have not been described in skeletal muscle myocytes. As such the impact of myocyte sensing of DNA on DNA vaccine immunogenicity has not been defined.

Hypothesis: We hypothesized that cytosolic DNA sensing pathway components are present in skeletal muscle myocytes and are activated upon entry of DNA into the cytosol to initiate a pro-inflammatory response.

Methods: We introduced a DNA vaccine vector into C2C12 myoblasts or myotubes by chemical transfection methods. The presence, abundance and activation of various DNA sensors and downstream pathways was characterized by immunoblotting, biochemical and RT-PCR methods.

Results: C2C12 myoblasts and myotubes express cytosolic DNA sensor proteins cGAS and STING. The transfection of DNA into C2C12 results in the phosphorylation of STING and other downstream components of the DNA sensing pathway such as TBK1 and IRF3. The abundance of phosphorylated forms of these proteins increases after transfection followed by the up-regulation of IFN- β 1 and IL-6 at the RNA level, supporting the presence of a functional cytosolic DNA sensing pathway in C2C12.

Conclusion: Taking together our results suggest that skeletal muscle-derived myocytes can sense cytosolic DNA, thus providing a rationale for further investigation of cytosolic DNA sensing in skeletal muscle. Our findings suggest the need to carefully consider how such events might influence the immunogenicity of gene-based vaccines delivered via the intramuscular route..

INTRODUCTION

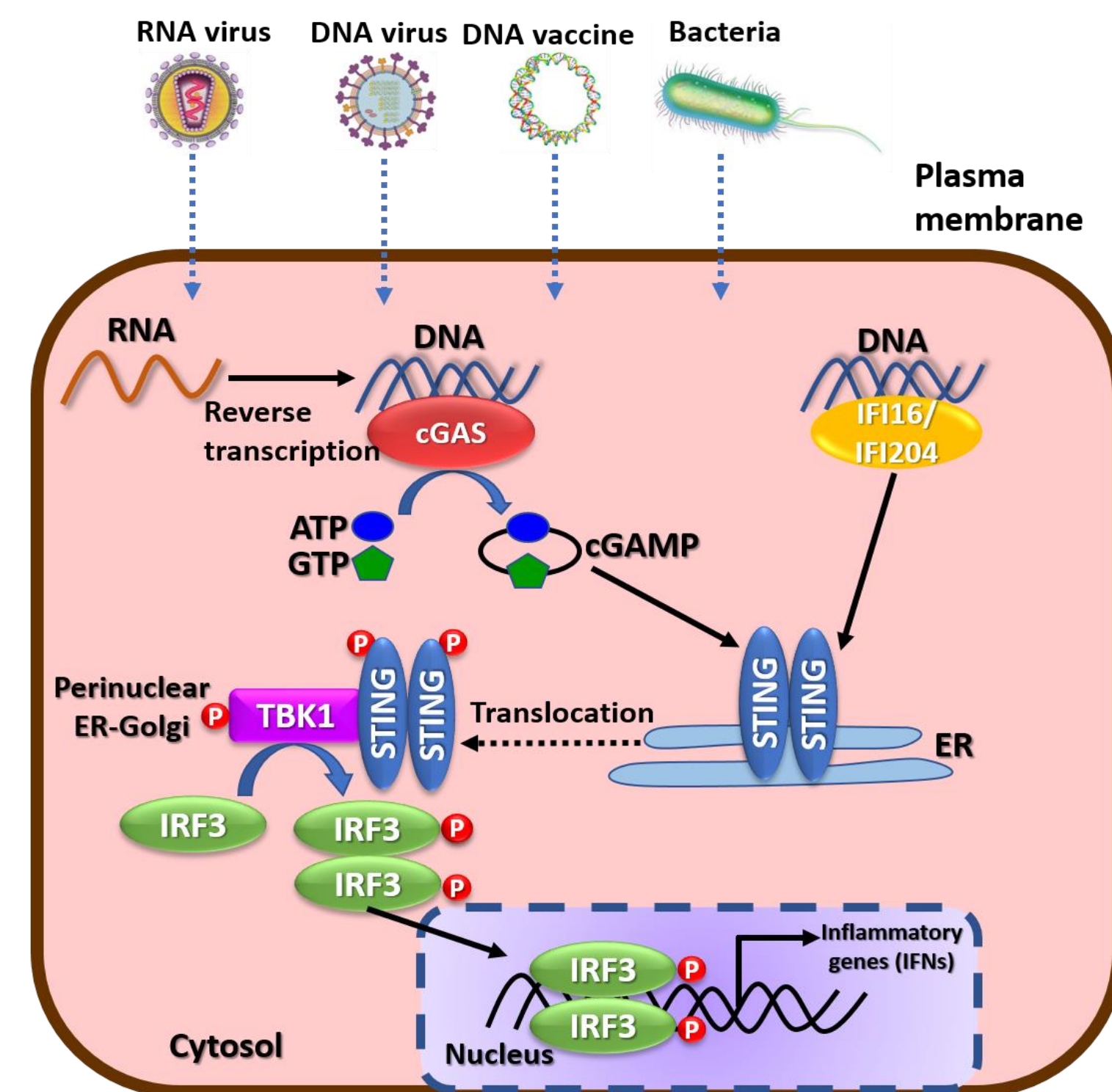


Figure 1. Cytosolic DNA sensing cGAS-STING pathway. After recognition of cytosolic DNA, cGAS catalyzes the synthesis of cGAMP which acts as a second messenger and bind to the adaptor protein STING. IFI16/IFI204 also recognizes cytosolic DNA and activates STING. STING dimerizes and translocates to a perinuclear region (ER-Golgi). Following STING relocalization, TBK-1 is recruited to STING. After binding to STING, TBK1 undergoes self-phosphorylation and subsequently phosphorylate STING and IRF-3. IRF3 then translocate into the nucleus and induces the induction of type I IFN.

RESULTS 2

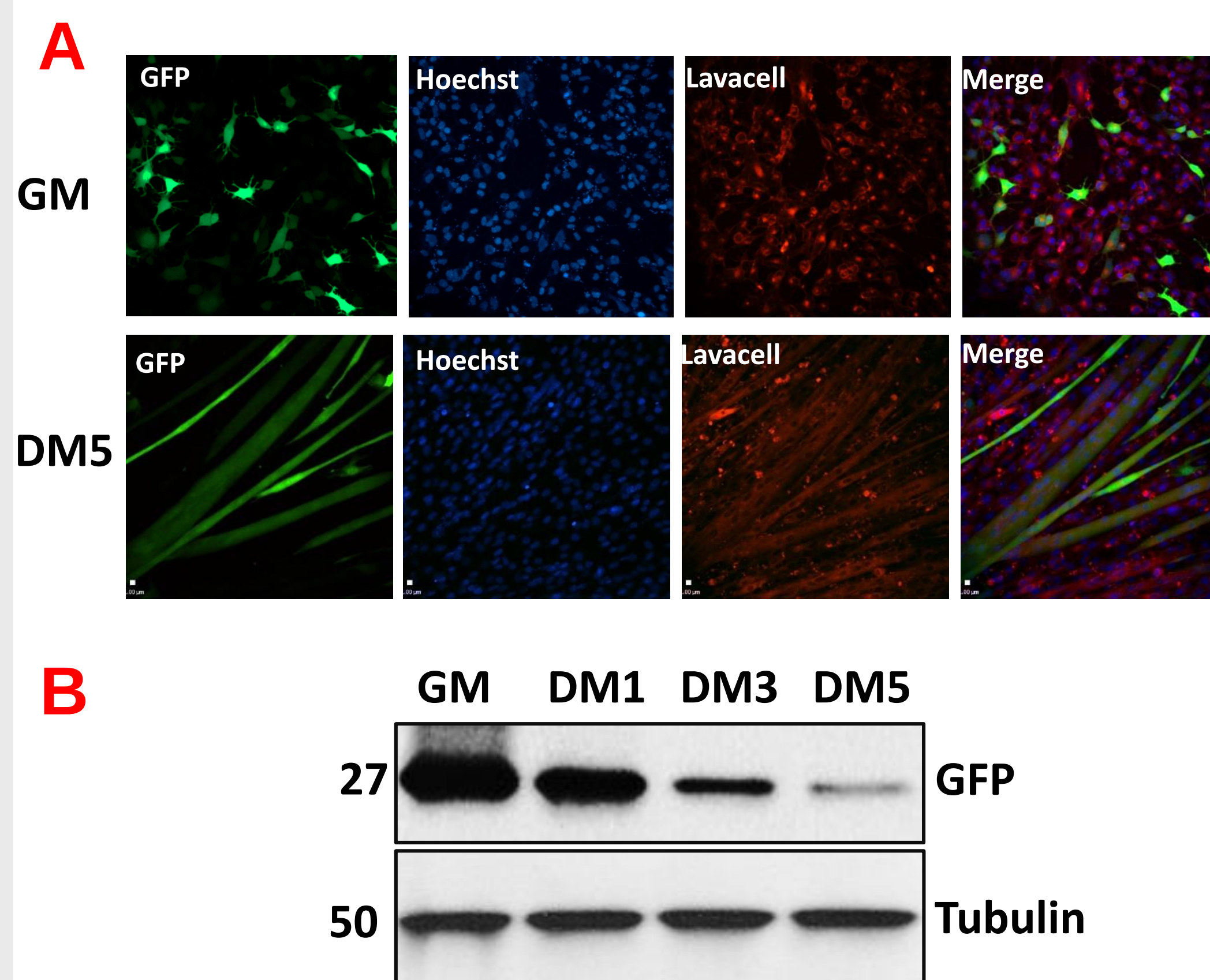


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RESULTS 4

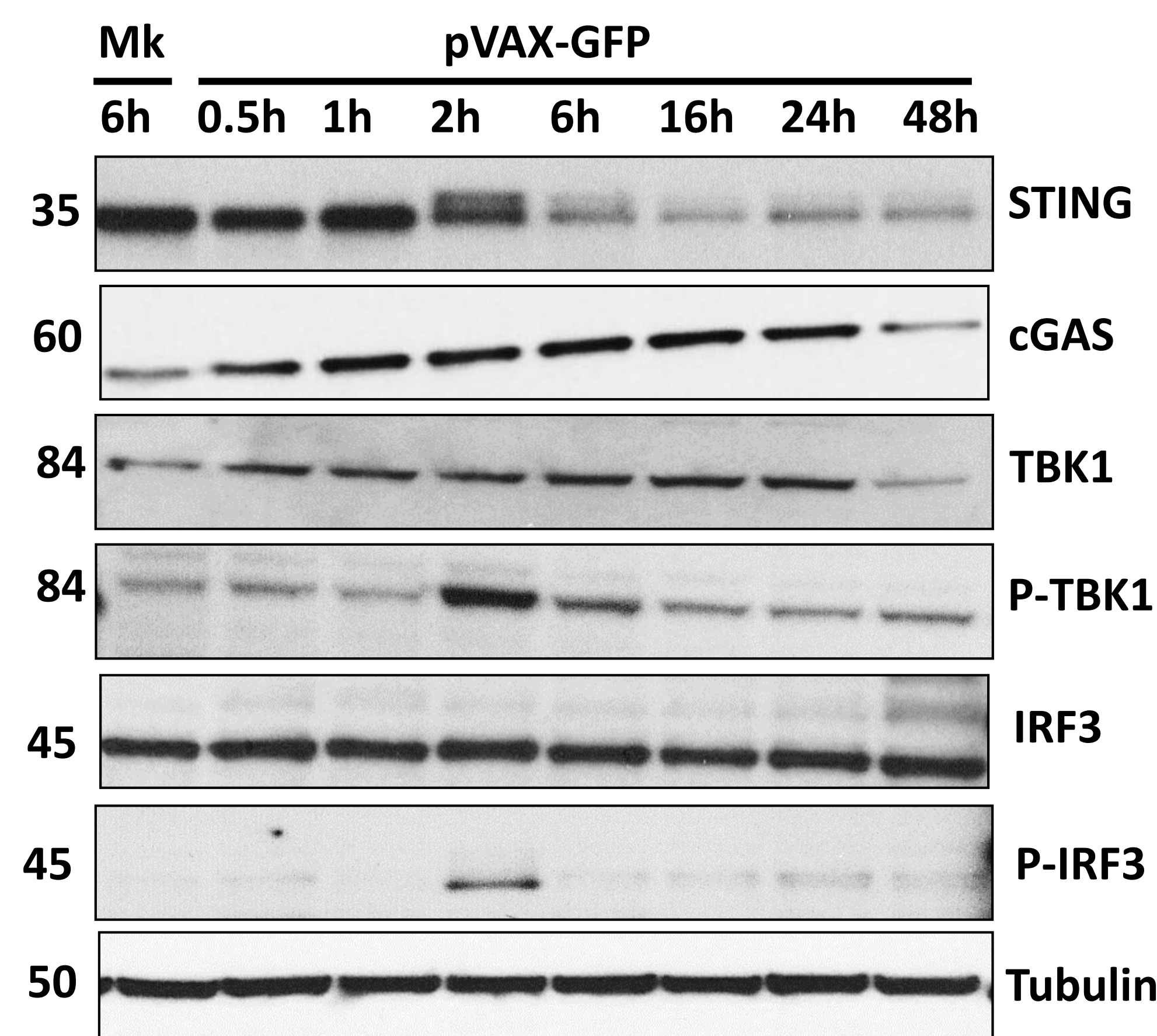


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RESULTS 1

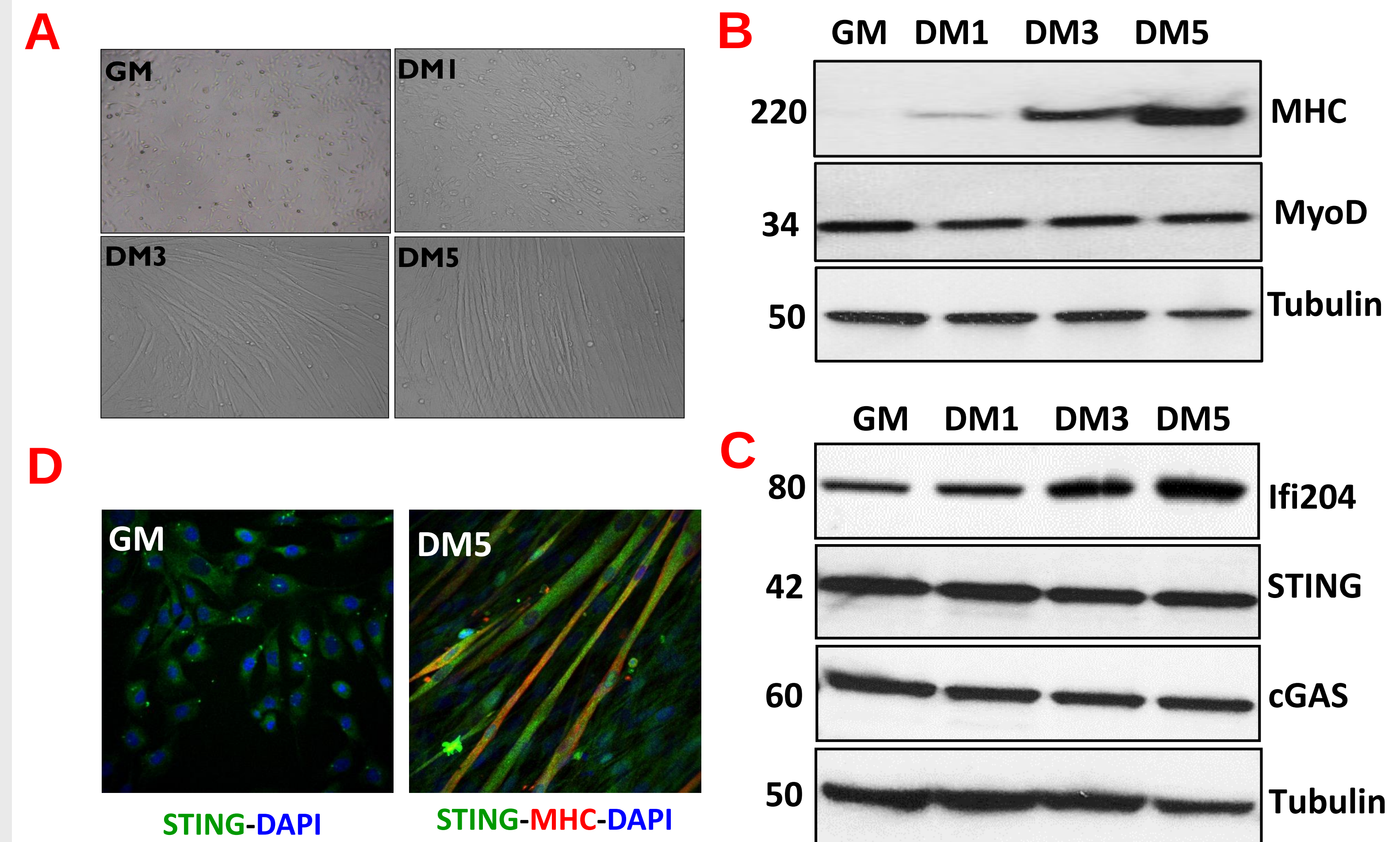


Figure 2. (A) Phase-contrast images of C2C12 cells. C2C12 cells were transfected with pVAX-GFP and 48h after transfection (GM) the transfected cells were allowed to differentiate for 5 days (DM5) which correspond to Day 5 post-transfection. (B) Evidence of C2C12 based on Myosin expression. Protein lysates were collected at different time points during the differentiation with anti-MyoD and antibody was used as a loading control. (C) Cytosolic DNA sensing pathway components. Protein lysates were collected at different time points during the differentiation with anti-IFI204 (Interferon Inducible Protein 204), anti-cGAS (cyclic GMP-STING (Stimulator of Interferon Production)), anti-TBK1 (TANK-binding kinase-1) and anti-IRF3 (Interferon Regulatory Factor 3) antibodies. (D) Immuno-localization of myoblasts (GM) and myotubes (DM5) cells were labelled with an Alexa-fluor 488 anti-MHC antibody and a secondary antibody.

RESULTS 3

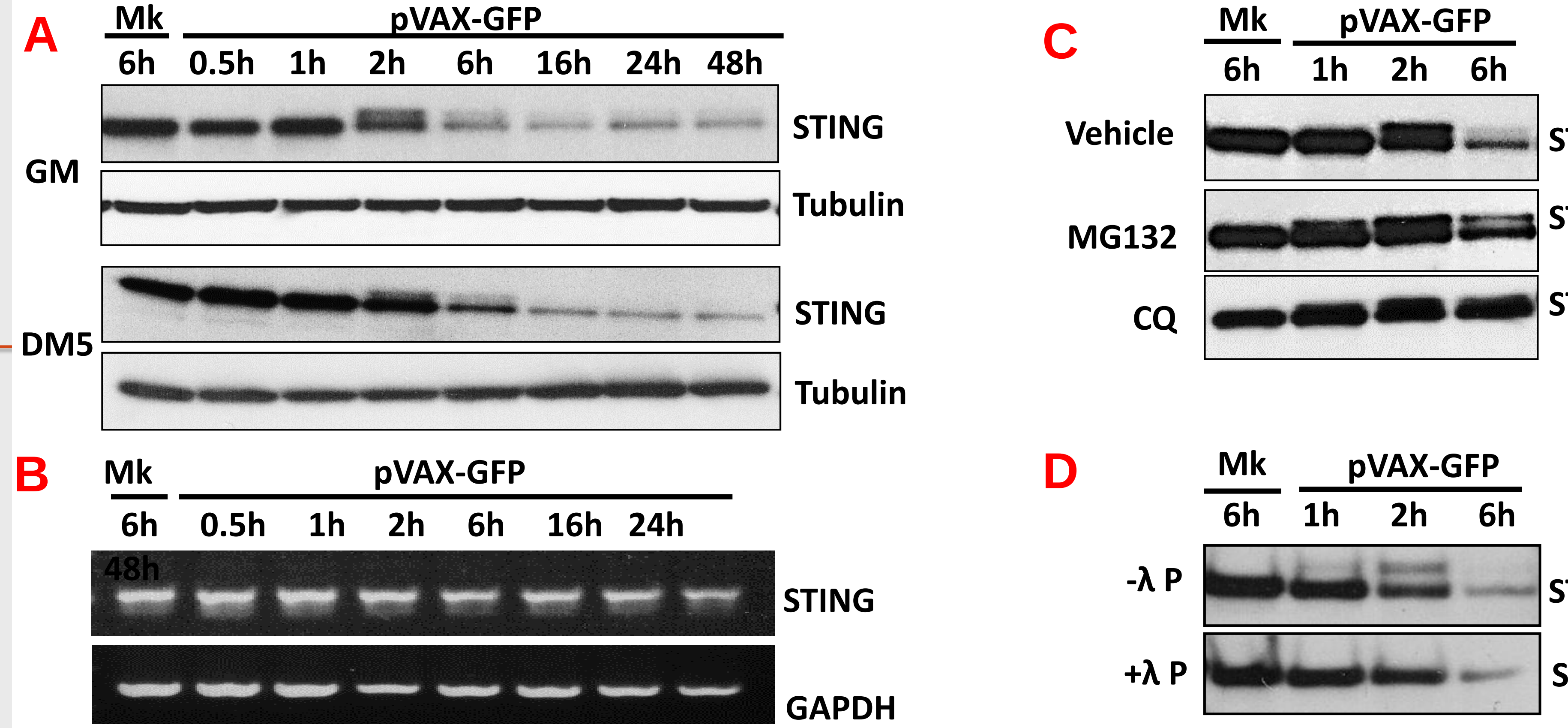


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RESULTS 5

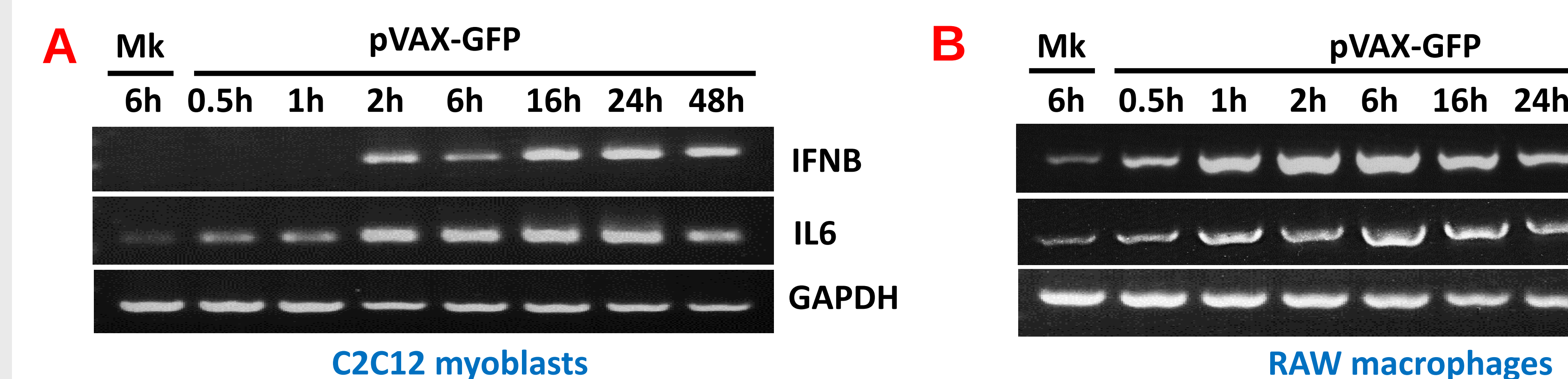


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ACKNOWLEDGEMENTS

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