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Characterizing the Effects of Vaccine Adjuvants on Skeletal Muscle Myotubes and Macrophages

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Abstract

Vaccines train the immune system to recognize and defend against pathogens. Currently, six types of vaccines are in use and include live-attenuated, inactivated, viral vector, protein subunit, toxoid, and messenger RNA (mRNA), the latter of which was recently approved for humans during the COVID-19 pandemic. To increase the longevity and magnitude of immune responses, some vaccines are combined with adjuvants. Mouse models have shown that adjuvants in combination with antigens can elicit a pro-inflammatory immune system response that is required for proper development of protective immunity. There has been recent appreciation for the immunomodulatory functions of skeletal muscle, yet their contribution to the immunology of vaccination remains incompletely understood. Considering most vaccines are administered intramuscularly, we utilized C2C12 mouse myotubes and J774 macrophages to explore the cytokine response that skeletal muscle cells and macrophages evoke in response to several types of vaccine adjuvants in absence of the antigen. C2C12 myotubes and J774 macrophages were treated with 7 commonly used adjuvants or appropriate controls and collected at 6 hours and 24 hours. Cytokine secretion, cytotoxicity, and effects on myotube diameter were analyzed. Most adjuvants, except for the positive controls (LPS and PAM_3CSK_4), CpG 1826, and Quil-A, did not induce a pro-inflammatory response in C2C12 myotubes or J774 macrophages. Interestingly, LPS, PAM_3CSK_4, MF59, Quil-A, AS03, and CFA, led to increases in C2C12 myotube diameter indicating an activation of hypertrophy. The lack of pro-inflammatory effects indicates that most adjuvants need antigens or additional cell-cell interactions at the injection site to produce a pro-inflammatory cytokine response.

Introduction

This study focuses on analyzing both qualitatively and quantitatively the impact that commonly used vaccine adjuvants, including aluminum salts, MF59, AS03, CpG 1018, and Quil-A, have on skeletal myocytes and J774 macrophages. We ask, how does the use of adjuvants in vaccines influence the immune response and its' function? Analysis of the C2C12 mouse myoblast model system and J774 macrophage model system will allow for the comparison of these different adjuvants and their subsequent effects on the body.

The Importance of Muscle in Vaccination

In the late 1990s, it was discovered that the activation of cell surface and intracellular receptors, such as toll-like receptors (TLRs), can stimulate dendritic cells (DCs), which play a critical role in initiating subsequent antigen-specific T and B cell responses[1]. Skeletal muscle cells also express multiple TLRs, which supports the possibility of a response to environmental factors to induce the production of inflammatory cytokines and possibly to promote adaptive responses necessary for immunological memory.

Most vaccines used in humans are administered intramuscularly, specifically within the scapular region at the deltoid muscle. Proper injection technique facilitates protective immunity. If a vaccine is injected into a layer of subcutaneous fat, it may result in a slow mobilization and processing of the antigen. Additionally, subcutaneous vaccination more frequently results in unwanted local reactions, such as erythema, rashes, and irritation. To fulfill requirements for clinical use, vaccine delivery must have minimal local reactogenicity and limited pain, both of which are more reliably avoided using intramuscular injection[2]. One proposed mechanism by which intramuscular administration avoids the harmful effects of subcutaneous injections is due to the abundant blood supply within skeletal muscle. Therefore, vaccines administered into the

muscle are generally absorbed into the vasculature quickly. However, local immunogenic responses, such as heat, pain, redness, and swelling (calor, dolor, rubor, and tumor) as first described by the Roman scholar Celsus in the first century CE, can occur but to a much less extent than subcutaneous injections[3]. Although considered by vaccine recipients to be an undesired side effect, these events promote immune cell recruitment and activation, which may modulate the efficiency of the subsequent vaccine-elicited immune response.

Dendritic cells are one of the most important cell types for inducing protective immunity. They function by bringing the antigen to the draining lymph node, which in the case of intramuscular injections into the deltoid, is the axillary (armpit) lymph node. Studies have concluded that lymph nodes, specifically in the axillary and inguinal (groin) region have the highest concentrations of DCs. Muscle and the subcutaneous layer both have a low density of dendritic cells (DCs), which is why commercial vaccines frequently use adjuvants to increase DC activation and recruitment[4]. The contributions of myocytes to vaccine adjuvant elicited immune responses are important since these cells are likely contributors to the generation of a more protective adaptive immune response. Controlling the activities of these muscle cells to potentially mitigate the unwanted side-effects of poor efficacy adaptive immune responses is a necessary research objective.

Importance of Adjuvants in Vaccines

Adjuvants are used in common vaccines such as Hep B and HPV to help the body produce an immune response. They have been safely used in vaccines since 1932, and through advancing technology newer adjuvants have been developed to target specific components of the body's immune response. Our immune response is how the body recognizes and defends itself against bacteria, viruses, and other harmful exogenous and endogenous substances. Adjuvants

can improve the immune response in numerous ways, including enhancing the speed and duration of the immune response and stimulating cellular immunity.

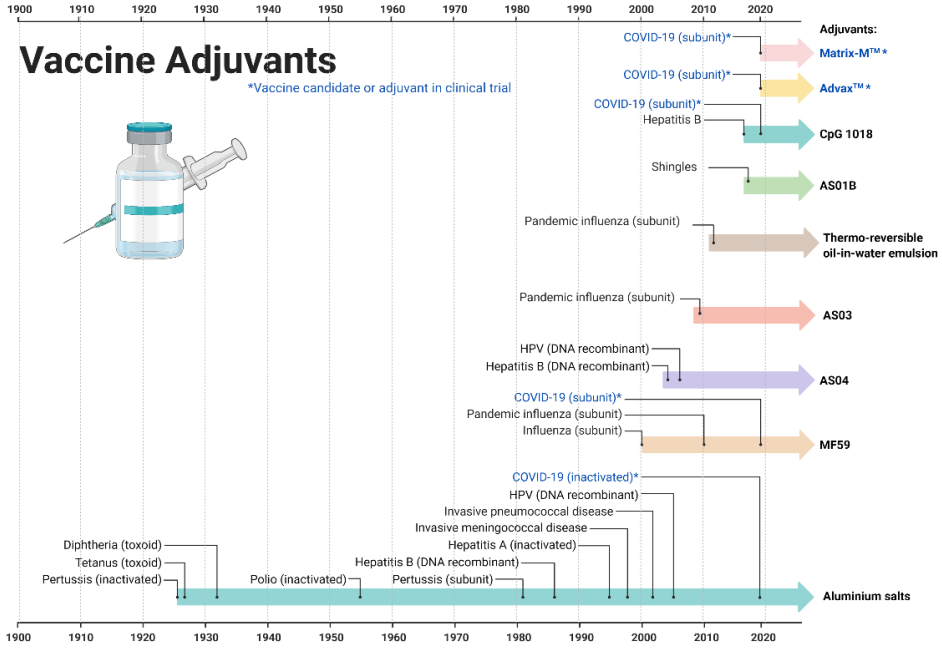

Figure (1). *Timeline from 1900 to 2020 of commonly used vaccine adjuvants for varying diseases and purposes (Adapted from "Timeline of Adjuvant Used in Human Vaccines", by BioRender.com (2022)).*

Since 1932, there are five adjuvants, aluminum-containing salts (commonly referred to as Alum), MF59, AS01, AS04, and CpG 1018, that have been used clinically in the United States[5]. While the number of approved vaccines continues to increase, the relatively low number of adjuvants attests to their safety and efficacy (Figure 1). Despite their widespread use, the molecular mechanisms of how these adjuvants function remain incompletely understood. Whereas some adjuvants function through several complementary physical, cellular, and biochemical means, the major focus of adjuvant discovery in the past decade has been targeted to their activation of TLR signaling pathways, although other pattern recognition receptors can also be targeted[1].

Vaccine Adjuvants

TLR Agonists

Lipopolysaccharides (LPS) are cellular wall components of Gram-negative bacteria that function as TLR4 agonists. LPS molecules composed of a hydrophobic lipid, a hydrophilic core polysaccharide, and a hydrophilic O-antigenic polysaccharide side chain, composed of repeated units of oligosaccharides. LPS is not currently used in any distributed vaccines, although several of its' characteristics make it a viable choice for live attenuated vaccines in the future. These properties include that it is important to bacterial survival, has a marked pathogen associated molecule pattern (PAMP), and triggers specific antibody responses conferring protective immunity[6]. LPS is referred to as a natural adjuvant that increases the number of cell divisions and the rate of cell survival during and after T cell activation and clonal expansion. Low toxicity LPS has previously been found to induce responses indicating that these molecules are able to act as immunological adjuvants for specific humoral immunity and the enhancement of IgG titers but would be unable to serve as an adjuvant for cellular or cytotoxic T lymphocyte responses[7]. For the studies presented in this thesis, LPS is used as a positive control.

$PAM_3CysSerLys_4$ (PAM_3CSK_4) is a synthetic triacylated lipopeptide and a TLR2/TLR1 ligand. It has proven to be a potent adjuvant for various vaccines, including a sublingual allergy vaccine and leishmaniasis vaccine[8]. Results from a preclinical study, showed that PAM_3CSK_4 increases antibody responses to flu antigens unlike other TLR ligands. PAM_3CSK_4 was also found to enhance B cell activation through increased levels of cytokine secretion and proliferation[9]. For the studies presented in this thesis, PAM_3CSK_4 is used as a positive control.

CpG oligonucleotides (ODNs) contain a full phosphonothioate backbone with one or more CpG dinucleotides in a particular sequence context, named CpG motifs. CpG ODNs

specifically activate TLR9, leading to strong immunostimulatory effects. Currently, the only CpG ODNs in an available vaccine is CpG 1018 in Heplisav-B. The TLR9 agonist, CpG 1826, is a class B CPG ODN specific for mouse TLR9, which stimulates strong B cell activation and generates a Th1-dominant immune response[10]. The expression of TLR9 is stronger and more present in mice compared to humans, although this TLR9 expression is mostly limited to plasmacytoid dendritic cells and B cells.

Aluminum-based Adjuvants

Alum is the most used adjuvant, and it induces antibody responses and CD4 T helper cell responses in humans[1]. The composition of alum includes one or more of the following: amorphous aluminum hydroxyphosphate sulfate (AAHS), aluminum hydroxide, aluminum phosphate, and potassium aluminum sulfate. Alum is currently used in the Anthrax, Hep A, Hep B, HPV, and Tdap vaccines. An alum-elicited immune response study in mice involved removing the critical adaptor proteins involved in TLR signaling, namely MyD88 and TRIF, with results showing that the immune responses stimulated by immunization with alum and antigen were unaffected. This result suggested that the effects of alum occur through a mechanism independent of TLR signaling. Alum can enhance adaptive immunity by causing tissue damage that activates inflammatory dendritic cells, as well as by recruiting neutrophils to release neutrophil extracellular traps (NETs)[10]. Aluminum salts have the capability of transforming soluble antigens into colloids, which are more effectively internalized and can enhance MHCII expression (necessary for CD4 T cell activation to initiate immune responses) on antigen-presenting cells[11, 12].

Oil-in-Water and Water-in- Oil Adjuvants

MF59 (Addavax) is an oil-in-water emulsion adjuvant composed of squalene that activates myeloid cells, such as macrophages and dendritic cells, to produce chemokines that further recruit neutrophils, eosinophils, and monocytes to the injection site[10]. This adjuvant is currently used in the FLUAD vaccine, which is an inactivated influenza vaccine. Studies conducted in mice have demonstrated that MF59 induces a wider range of cytokines and chemokines compared to alum. MF59 requires the MyD88 adapter protein to transduce signals leading to the release of ATP from muscle cells[13, 14].

The AS0 adjuvant systems were developed to obtain maximal adjuvant effect by creating a rational combination of classical adjuvant molecules and immunostimulatory molecules. Within the AS0 adjuvant family, the AS04 adjuvant, which is composed of monophosphoryl lipid A (MPL) and an aluminum salt, is the adjuvant in the Cervarix vaccine. The $AS01_B$ adjuvant composed of MPL and QS-21 (a detoxified saponin derivative) is found in the Shingrix vaccine. AS03 (Addavax) is composed of α-tocopherol, squalene and polysorbate 80 in an oil-in-water emulsion and is found in the avian influenza vaccine. Specifically, AS03 has been shown to trigger transient NF-κB- dependent innate immune responses, which results in the production of cytokines and chemokines[15] (Figure 2).

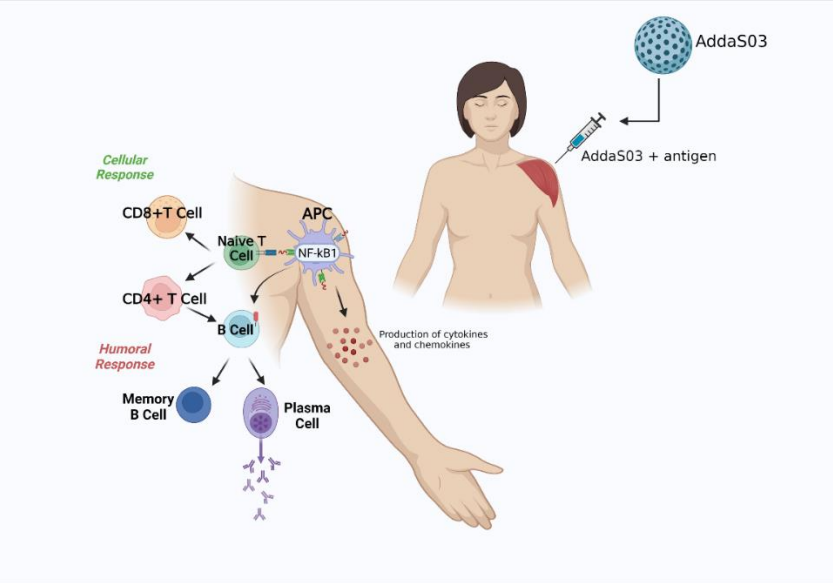

Figure (2). *Graphic depicting the subsequent immune response, with the differing cell types, after the injection of a vaccine of AddaS03 and an antigen into the deltoid muscle of a human participant.*

The Quil-A adjuvant is a saponin adjuvant, which typically induces a strong cytotoxic CD8+ lymphocyte response and potentiates the response to mucosal antigens. The Quil-A adjuvant is used in a wide variety of veterinary vaccines but is not currently approved for use in human vaccines. This adjuvant can induce a strong response to both T-dependent and T-independent antigens. The Quil-A adjuvant has been proven to activate the antibody-mediate immune responses to a broad range of viral, bacterial, and parasitic antigens.

Complete Freund's Adjuvant (CFA) contains heat killed *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate. Water-in-oil emulsions of antigens can be created with CFA. This adjuvant induces a Th1-dominated response when injected with an antigen. CFA is currently not used in human vaccines, because it has been linked with the potential to interfere with TB test results and cause excessive inflammation. Prior studies showed that CFA injection with an antigen led to increased infiltration of muscle with granulocytes, macrophages, and dendritic cells[16].

mRNA Delivery Agent

Mirus TransIT-mRNA is considered a high efficiency, low toxicity transfection reagent for large RNA molecules. This type of adjuvant is considered a delivery agent responsible for transferring RNA species into mammalian cells. TransIT-mRNA is not currently used in any licensed mRNA vaccines. The only mRNA vaccines currently used in humans are the COVID-19 vaccines that contains the mRNA that directs cells to produce copies of the SARS-CoV-2 spike protein. mRNA research in the scientific community has increased dramatically due to the importance of the COVID-19 vaccine during the pandemic. Unprotected mRNA delivered by itself is unsuitable for broad applications, making strategies for RNA delivery to the cytosol necessary[17]. Studies have shown that the TransIT reagent yields high transfection levels of human pluripotent stem cell derived cardiomyocytes *in vitro* compared to other reagents, such as jet MESSENGER or MessengerMAX modRNA[18].

Prior Literature

Various live organism-based vaccines exhibit high efficacy; however, this is often associated with adverse local and/or systemic reactions. Comparatively, sub-unit proteins offer a much lower risk alternative, although their efficacy is typically low. The use of adjuvant systems to enhance the effectiveness of sub-unit vaccines has proven effective by preventing the degradation of the antigen as well as enhancing the targeting of these antigen by antigen presenting cells(APCs), such as dendritic cells[19]. To induce a cascade of events leading to a large pro-inflammatory response, adjuvants may need to be used where multiple cell types are present and their activation can be stimulated with an antigen. For example, adjuvants are believed to increase the innate immune response to antigen by interacting with pattern recognition receptors (PRRs), such as the TLRs and other types of innate immune receptors.

In 1989, Charles A. Janeway, a noted immunologist credited with helping to create the modern field of innate immunity, said that adjuvants are "the immunologists' dirty little secret" and speculated on the existence of PRRs, which he proved existed a few years later[20]. In the subsequent decades, their function, and mechanisms to elicit immune responses are becoming understood. Mechanisms of PRR-elicited effects include sustaining the release of an antigen, the upregulation of cytokines and chemokines, cellular recruitment, increased antigen uptake, activation and maturation of APCs, and activation of the NLRP3 inflammasome[21].

Cytokines are small proteins that are crucial in controlling the growth and activity of cell types, including immune system cells, in addition to impacting the body's immune and inflammation responses. Cytokines can be broken down into two classes, pro-inflammatory and anti-inflammatory. Interleukin-6 (IL-6) is known as the chief stimulator of the production of most acute phase proteins, which are the proteins responsible for the initial response of the organism against infection or tissue damage[22]. Monocyte chemotactic protein-1 (MCP-1) is a chemokine that regulates the migration and infiltration of monocytes and macrophages in the body. Interleukin-15 (IL-15) is a pleiotropic cytokine produced by myocytes (therefore, often termed a 'myokine') that functions in the development of inflammatory and protective immune responses[23].

The Impact of the COVID-19 Pandemic

Adjuvant research is extensively needed before the implementation of certain adjuvants into developing vaccine formulations. The COVID-19 pandemic is a prevalent global issue of the effects of coronavirus disease 2019 caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As of April 2022, this pandemic has caused more than 550 million cases and 6.18 million deaths[24]. Preventive measures, including social distancing, masking, and improved

ventilation, and air filtration, were recommended and in many countries, enforced while vaccines were being developed[25]. In December 2020, the first COVID-19 mRNA vaccines were administered separately by Pfizer-BioNTech and Moderna to provide cells the instructions necessary to produce the spike (S) protein found on the surface of SARS-CoV-2. Post vaccination, the cells at the local injection site begin to make the spike (S) protein pieces and display them on their cellular surfaces. This leads to the production of antibodies and the induction of immunological memory if the vaccinated individual was to become infected with the COVID-19 virus. These mRNA vaccines are used to help prevent severe illness from the COVID-19 infection, but have varying efficacy to protect individuals from getting COVID-19.

During the development of the Pfizer-BioNTech and Moderna vaccine, the avoidance of aluminum-based adjuvants (ABAs) was distinctly made. Researchers were concerned that since cytokine storms, which are an extremely strong and potentially dangerous immune response, were observed in severe COVID-19 cases, that similar responses could occur with the addition of these ABAs. Additionally, intense cytokine production can affect RNA translation[11]. As of 2021, AS03 and CpG 1018 were being developed as adjuvants for use in subunit SARS-CoV-2 vaccines. A recent study testing the effectiveness of specific adjuvants in application with the SARS-CoV-2 vaccines found that CpG 1018 stimulated a Th1 biased response, Alum and oil-in-water adjuvant induced a Th2 biased response, and AS03 stimulated a mixed Th1/Th2 response [26]. Currently, the US manufactured Pfizer-BioNTech and Moderna COVID-19 vaccine do not contain any adjuvants.

In India, the COVAXIN COVID-19 vaccine was developed and manufactured. This vaccine is an inactivated SARS-CoV-2 that uses adjuvant Alhydroxiquim-II (AHQ-II) to boost immune response and promote longer-lasting immunity. This novel adjuvant was developed in

the United States and is comprised of a small molecule imidazoquinolinone TLR 7/8 agonist (IMDG) attached to Alhydrogel (a form of alum). AHQ-II induces Th1- biased immunity and was found to increase neutralizing antibodies titers by approximately 1000-fold in an inactivated SARS-CoV-2 vaccine[27].

Gaps in the understanding of adjuvants have led us to the following research questions: How do vaccine adjuvants affect skeletal muscle cell and macrophage inflammatory response? What cytokine and chemokine products are being created by these cell types? By using *in vitro* methods with the C2C12 mouse skeletal muscle cell line and the J774 murine macrophage cell line, it is possible to focus the study on isolated cell groups as well as the coculture of the C2C12s and J774s. There are other cell types present in skeletal muscle tissues including connective tissue cells, dendritic cells, satellite cells and lymphocytes. By removing those additional cell types, we can investigate the contribution of muscle cells and macrophages alone through adjuvant stimulation. We hypothesize that vaccine adjuvants will promote the immune response in muscle by eliciting a pro-inflammatory cytokine response.

Methods

C2C12 Skeletal Muscle Cell Passage

C2C12 mouse myoblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). C212 aliquots (1 million cells in 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS)) were removed from liquid nitrogen and thawed under hot running water. Immediately after thawing, the cells were transferred to a 15 mL conical tube containing 10 mL of growth media consisting of low glucose (1g/L) Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and 1% penicillin and streptomycin (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA).

The cells were spun at 250 RCF at 22°C for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 1 mL of growth media. New passages were started with 300,000 cells in 12 mL of growth media in T75 flasks (Thermo Fisher Scientific, Waltham, MA, USA) and grown in a cell culture incubator (5% CO_2 at 37°C).

At approximately 70% confluency the cells were passaged into new flasks. To do so, the growth media was aspirated, and the cells were washed with 3-5 mL of phosphate-buffered saline (PBS) (GIBCO). PBS was aspirated, and 3 mL of 1.33 concentration of Trypsin (GIBCO) in PBS was added and the cells were incubated for 3 minutes. Once the cells were lifted from the flask, the contents were transferred to a 15 mL conical tube containing 10 mL of growth media. The cells were spun at 250 RCF at 22°C for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in 1 mL of growth media. New passages were started with 300,000 cells in 12 mL of growth media in T75 flasks and grown in a cell culture incubator (5% CO_2 at 37°C). This protocol continued until the cells are used in experiments or they reached passage 9.

C2C12 Cell Plating

Before plating the cells, 24 well plates (CELLTREAT Scientific Products, Pepperell, MA, USA) were coated with gelatin cross linked with microbial transglutaminase (MTG). Briefly, a 10% w/v gelatin solution was incubated at 65°C and mixed at a ratio of 9 parts gelatin to 1 part 40% MTG. Then, 60 μL of the solution was immediately added to the 24 well plate and allowed to cure overnight. The following day, the plates were rehydrated with PBS. Cells were collected from the T75 flasks following the passage protocol and transferred to a 50 mL conical tube. Cells were suspended at 70,000 cells per mL and 0.5 mL were added to each well. Cells were grown to 100% confluency over 24 hours at which point growth media was removed and differentiation media containing high glucose (4.5 g/L) DMEM, 2% FBS, and 1% penicillin/streptomycin (GIBCO) was added. The media within the wells was changed daily for a period of 7 days to achieve fully differentiated C2C12 cells.

J774 Cell Passaging

J774 mouse macrophages were purchased from ATCC. J774 aliquots (J774 cells in DMEM modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate) were removed from liquid nitrogen at and thawed under hot running water. Once completely thawed, the contents were transferred to a 15 mL conical tube and 9 mL of growth media (DMEM modified to contain 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate with 10% FBS) (ATTC) was added.

The conical tube was spun at 250 RCF at 22°C for 5 minutes. The supernatant was aspirated, and the remaining cells were resuspended in 1 mL of growth media. New passages were started with 1,000,000 cells in 12 mL of media in T75 flasks and grown in a cell culture incubator (5% CO_2 at 37°C).

At 70% confluency the cells were passaged into new flasks. The cells were scraped then transferred into a 15 mL conical tube and spun between 250 RCF at 22°C for 5 minutes. The same process was completed on the cells during passage until the cells were plated or they reached passage 40.

J774 Cell Plating

24 well plates (CELLTREAT) were used without a gelatin-MTG coating to plate the J774 cells. Cells were collected from the T75 flasks following the passage protocol and transferred to a 50 mL conical tube. Cells were suspended at 1 $*10^6$ cells/ mL and 0.5 mL was added to each well on the plate. Cells were grown to 100% confluency over 24 hours, and then the media was aspirated, and the adjuvant treatment initiated.

Coculture of C2C12 and J774 Cell Plating

Like C2C12 plating, 24 well plates (CELLTREAT) were coated with a gelatin-microbial transglutaminase substrate. Cells were collected from the T75 flasks following the passage protocol and transferred to a 50 mL conical tube. Cells were suspended at 70,000 cells per mL and 0.5 mL were added to each well on the plate. C2C12 cells were grown and differentiated as stated previously. On day 6, J774 cells were added to the wells containing the C2C12 cells at 2,600 cells per cm^2 of media. On day 7, the adjuvant treatment was initiated.

Adjuvant Treatment

The adjuvants (concentrations and sources are listed in Table 1) were diluted in differentiation media (2% FBS, 1% pen/strep) for the 6 hour and 24-hour treatment with the C2C12 cells, in addition to the 6-hour treatment with the coculture of C2C12 and J774 cells. In J774 monoculture experiments, the adjuvants were diluted to the appropriate concentrations using J774 growth media.

Table 1: *Treatment Concentrations*

Adjuvant Treatment	Concentration	Company	Concentration Selection
LPS (positive control)	1 μg/ mL	Sigma	[28]
PAM_3CSK_4 (positive control)	1 μg/ mL	Sigma	[8]
AddaVax	1:100 (v:v)	Invivogen	[29]
AddaS03	1:30 (v:v)	Invivogen	[30]
CpG 1826	10 μg/ mL	Coley Pharmaceuticals	[31]
Non CpG 2138	10 μg/ mL	Coley Pharmaceuticals	[31]
AlOH	40 μg/ mL	Thermo Scientific	[32]
CFA	1:30 (v:v)	Invivogen	[30]
Quil-A	1 μg/ mL	Sigma	[33]
Mirus *Trans*IT	1 μl mRNA boost reagent, 1 μl *Trans*IT-mRNA reagent	Mirus	Manufacturer's Indications

Image Collection and Analysis

Images of individual wells were acquired using a microscope digital camera (AmScope) in duplicates for one well of each of the 24-hour C2C12 adjuvant treatment at 10x under a light microscope. Using the program ImageJ, 5 measurements for each myotube were determined for 30 myotubes. The average diameter for the myotubes in each photograph was subsequently calculated.

Collection of Lysates and Supernatants

For each well, the supernatant was collected and placed into 1.5 mL microcentrifuge tubes (USA Scientific, Ocala, FL, USA). Following supernatant removal, 200 μL of Trizol was added to each well and incubated for 3 minutes at 20°C. The lysate in each well was collected and placed into separate 1.5 mL microcentrifuge tubes. Lysates and the supernatants were stored in a -80°C freezer.

Enzyme-Linked Immunosorbent Assay (ELISA)

All ELISA assays were conducted using commercially available kits, that were prepared according to the manufacturer's protocols (Duoset ELISAs by R&D Systems, Minneapolis, MN, USA) to measure serum cytokines IL-6, IL-15, and MCP-1. High binding 96-well plates were coated overnight at 20°C with the appropriate capture antibody diluted in coating buffer (1X PBS). The following day, the plates were washed three times in wash buffer (0.05% Tween20 in PBS). The protein binding sites in the wells on the plates were then blocked in 1% BSA in PBS for at least 1 hour at 20°C. The plates were washed three times in wash buffer. Standards were serially diluted, and samples were diluted at 1:4 dilution in PBS. The standards and samples were added to the plates for a minimum of two hours at 20°C. The plates were washed, and the appropriate detection antibody was diluted in assay buffer and 50 µL was applied to each well. The plates were incubated at room temperature for 2 hours and then washed three times with wash buffer. Following the wash, HRP streptavidin was diluted 1:40 in assay diluent and 50 µL was added to each well for 20 minutes in the dark. The last wash of the plates was completed and a 1:1 ratio of developing reagents A and B were mixed and 50 µL was added to each well. The plates were incubated for up to 20 minutes in the dark until 25 µL of stop solution (2M sulfuric acid) was added to each well. The plates were read at 450 nm using the program Synergy HTX (BioTek, Winooksi, VT, USA).

Lactate Dehydrogenase (LDH) Assay

Cell death was evaluated through a LDH assay in cell culture supernatant using CyTox96 (Promega, Wisconsin, MI, USA), according to the manufacturer's directions. In a flat nonbinding 96-well plate, a standard curve was created with an LDH positive control (1:1000 dilution of LDH). Selected supernatant samples were diluted in assay buffer for at a total volume

of 50 μL. 50 μL of substrate mix was added, and the plate was incubated in the dark at 22°C for 30 minutes. Following incubation, 50 μL of stop solution was added to each well. The plate was then read at 450 nm using the program Synergy HTX (BioTek).

Statistical Calculations

Cell culture experiments were run in quadruplicates and repeated for reproducibility. Graphs and corresponding error bars were created using GraphPad Prism 9.0 for Windows. One-way ANOVAs with multiple comparisons were used to analyze data through GraphPad Prism 9.0. A p-value of *<0.05, **<0.01, ***<0.001, or ****<0.0001 from the multiple comparisons was considered statistically significant.

Results

24-hour treatment of varying adjuvants elicit a hypertrophic response in C2C12 myotubes

To determine the effects of adjuvants on myotube growth or atrophy, media containing adjuvants (Table 1) was added to C2C12 myotubes and imaged 24 hours later. Images were analyzed on ImageJ for myotube diameter size (Figure 3A, 3B). Myotube measurements showed hypertrophic responses and significant increases in the diameter when compared to the control group (DM Media). PAM_3CSK_4, MF59 (Addavax), Quil-A, and CFA induced significant increases in myotube diameter 24 hours after adjuvant treatment ($p<0.0001$; Figure 3C). In addition, AS03(Addas03) ($p<0.001$; Figure 3C) and LPS ($p<0.01$; Figure 3C) showed increases in myotube diameter at 24 hours. Distribution of adjuvant conditions that had significant changes in myotube diameter can be visualized (Figure 3D).

Figure 3

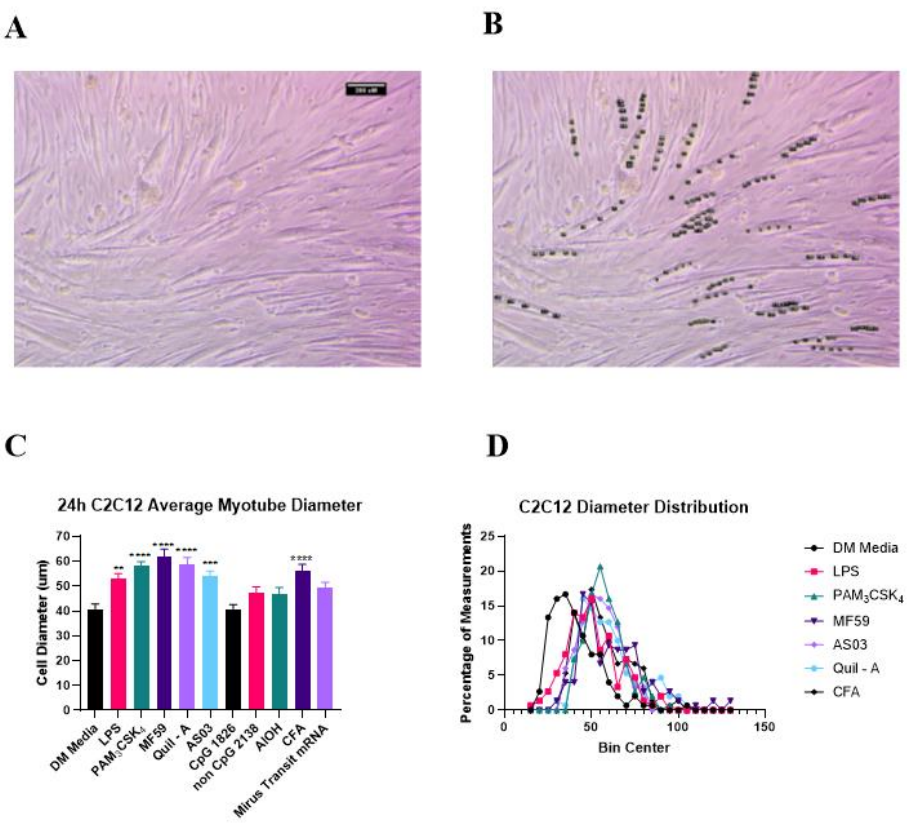

Figure (3). *A) Photograph of C2C12 myotubes 24 hours after the treatment of DM Media. B) ImageJ measurements of individual myotube diameters based on previous image. C) 24h C2C12 average myotube diameter for each adjuvant treatment. **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with multiple comparisons to the mean of the control (DM Media). D) 24h C2C12 individual myotube diameter distribution of statistically significant adjuvants compared to DM media from Figure C.*

6-hour and 24-hour adjuvant treatment influences cytokine production in C212 myotubes

Previous literature has shown that following intramuscular injection of LPS or PAM_3CSK_4, skeletal muscle upregulates several pro-inflammatory cytokines, including IL-6, MCP-1, and IL-15[34]. To determine if skeletal muscle myocytes can express similar cytokines following adjuvant stimulation, C2C12 myotubes were stimulated with varying adjuvants, using LPS and PAM_3CSK_4 as positive controls. Supernatants of adjuvant stimulated C2C12 myotubes were collected at 6 and 24 hours. IL-6 and MCP-1 production significantly increased at 24 hours following LPS and PAM_3CSK_4 stimulation ($p<0.0001$; Figure 4A, 4B). IL-15 production at 24 hours was significantly downregulated by AS03 and non CpG 2138 ($p<0.01$; Figure 4C). IL-15 production was also significantly downregulated by CpG 1826 and CFA at 24 hours ($p<0.05$; Figure 5C). These results suggest that skeletal muscle produce pro-inflammatory cytokines IL-6 and MCP-1 in response to positive controls of LPS and PAM_3CSK_4, but none of the typically used adjuvants in vaccines. Additionally, adjuvants, including AS03, non CpG 2138, CpG 1826, and CFA, may evoke a mechanism capable of lowering IL-15 production in skeletal muscle myocytes.

Figure 4

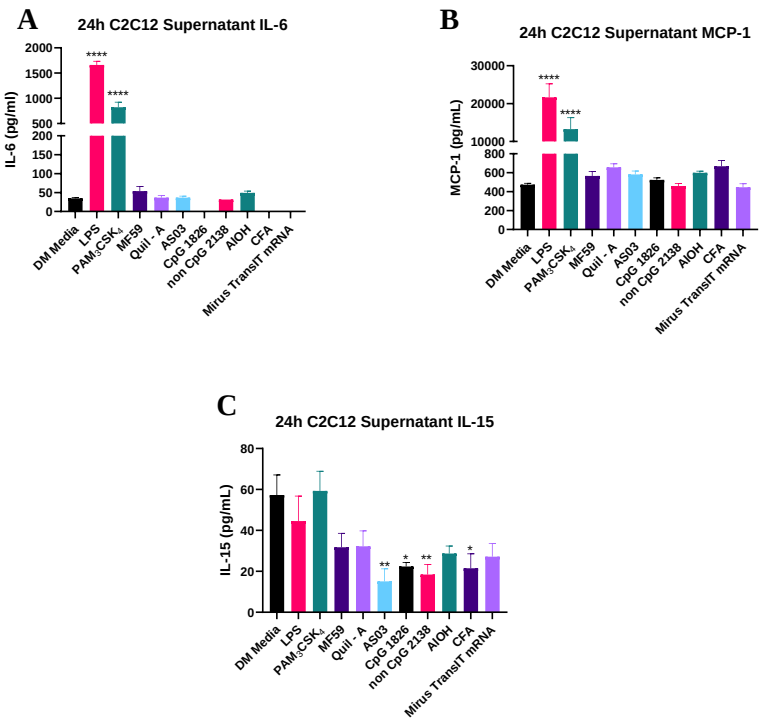

Figure (4). *Cytokine production from C2C12 myotubes following stimulation with adjuvants, as assessed at 24 hours. A) and B) **p<0.01, ***p<0.001, ****p<0.0001 and C) * p<0.05, **p<0.01 by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

Figure 5

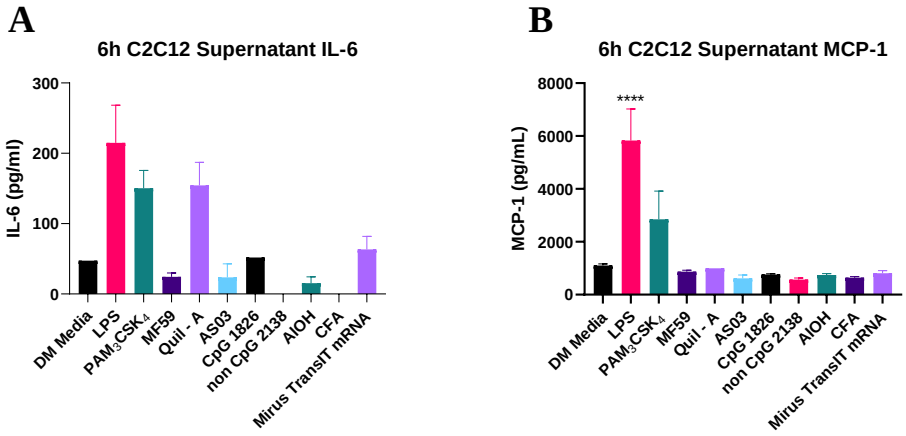

Figure (5). *Cytokine production from C2C12 myotubes following stimulation with adjuvants, as assessed at 6 hours. A) IL-6 and B) MCP-1 (**p<0.01, ***p<0.001, ****p<0.0001) by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

IL-6 production was elevated in the C2C12 myotubes after stimulation with LPS, PAM_3CSK_4, and Quil-A, although none of these values were seen to be statistically significant by a one-way ANOVA. These results indicate that 6 hours may not be sufficient time to elicit a significant IL-6 secretion by C2C12 cells. MCP-1 secretion significantly increased at 6 hours following LPS stimulation ($p<0.0001$; Figure 5B). These results suggest that skeletal muscle myocytes produce pro-inflammatory MCP-1 in response to the positive control of LPS, but none of the typically used adjuvants in vaccines.

6-hour and 24-hour adjuvant treatment influences cytokine production in J774 macrophages

Previous literature has shown that the treatment of J774 macrophages with LPS elicits a time and concentration dependent increase in IL-6 production[35]. To determine if macrophages could express similar cytokines following adjuvant stimulation, J774 macrophages were stimulated with varying adjuvants, using LPS and PAM_3CSK_4 as positive controls. Supernatants of adjuvant stimulated J774 macrophages were collected in two separate experiments at 6 hours and 24 hours.

Figure 6

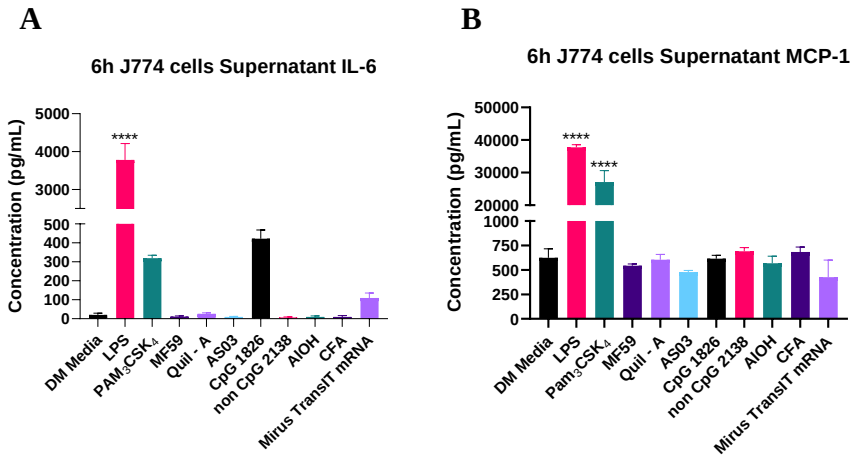

Figure (6). *Cytokine production from J774 macrophages following stimulation with adjuvants, as assessed at 6 hours. A) IL-6 and B) MCP-1 (****p<0.0001) by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

IL-6 production by J774 macrophages was significantly increased at 6 hours by LPS stimulation ($p<0.0001$; Figure 6A). The 6-hour treatment showed signs of elevated IL-6 production for PAM_3CSK_4 and CpG 1826 (Figure 6A). MCP-1 production by J774 macrophages was significantly increased comparative to the control at 6 hours by LPS and PAM_3CSK_4 stimulation ($p<0.0001$; Figure 6B).

Figure 7

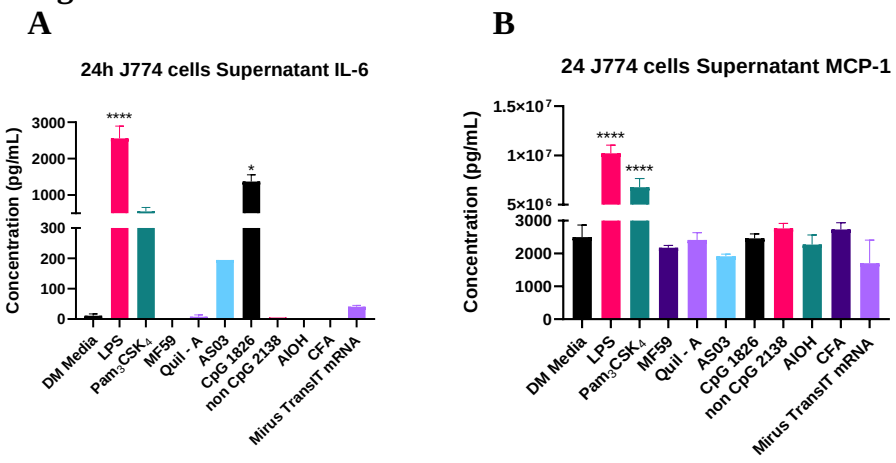

Figure (7). *Cytokine production from J774 macrophages following stimulation with adjuvants, as assessed at 24 hours. A) IL-6 and B) MCP-1 (*p<0.05, ****p<0.0001) by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

CpG 1826 stimulation only led to significant IL-6 secretion after the J774 macrophages were stimulated for 24 hours ($p<0.05$; Figure 7A). LPS stimulation also led to significant IL-6 production after 24-hour stimulation ($p<0.0001$; Figure 7A) The 24-hour treatment showed signs of elevated MCP-1 production for LPS and PAM_3CSK_4 ($p<0.0001$; Figure 7B). These results show that only the positive controls of LPS and PAM_3CSK_4 elicit a pro-inflammatory MCP-1 response from J774 macrophages.

Macrophages enhance adjuvant induced cytokine secretion in J774-C2C12 cocultures

Results of several studies led to the determination that a coculture of C2C12 myocytes and J774 macrophages could give insight into if multiple cell types are needed to elicit a pro-inflammatory immune response by adjuvants. C2C12 myocytes were differentiated to day 7 and on day 6, J774 macrophages were added to the C2C12 myotubes at a physiologically relevant concentration. Adjuvant stimulation for 6 hours was conducted on day 7 and the supernatants were collected.

Figure 8

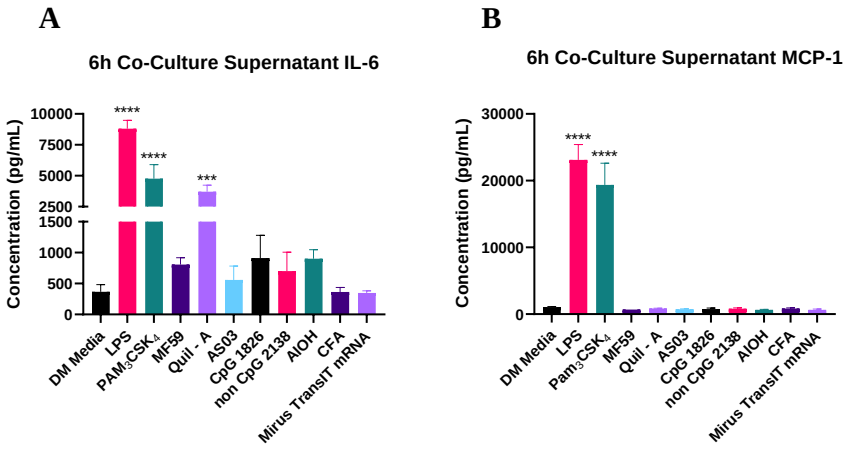

Figure (8). *Cytokine production from C2C12 myocytes and J774 macrophages in coculture following stimulation with adjuvants, as assessed at 6 hours. A) IL-6 and B) MCP-1 (**p<0.01, ***p<0.001, ****p<0.0001) by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

IL-6 production significantly increased at 6 hours following LPS, PAM_3CSK_4, and Quil-A stimulation ($p<0.0001$, $p<0.0001$, $p<0.001$, respectively; Figure 8A). These results suggest that adjuvant stimulation of cocultured myocytes and macrophages led to enhanced IL-6 and MCP-1 production compared to the C2C12 myotubes in monoculture. MCP-1 production was significantly increased at 6 hours following LPS and PAM_3CSK_4 stimulation ($p<0.0001$; Figure 8B).

Cell death analysis of vaccine adjuvant treatment on C2C12 myotubes and J774 macrophages

To determine whether the lack of cytokine production from vaccine adjuvants was due to their inability to stimulate a pro-inflammatory response, and not due to cytotoxicity, cell death was assessed through measuring lactate dehydrogenase (LDH). This cytosolic enzyme is released upon cell lysis and can serve as an indicator of cell damage. The amount of color formed by LDH activity in the presence of a colorimetric substrate is proportional to the number of lysed cells.

Figure 9

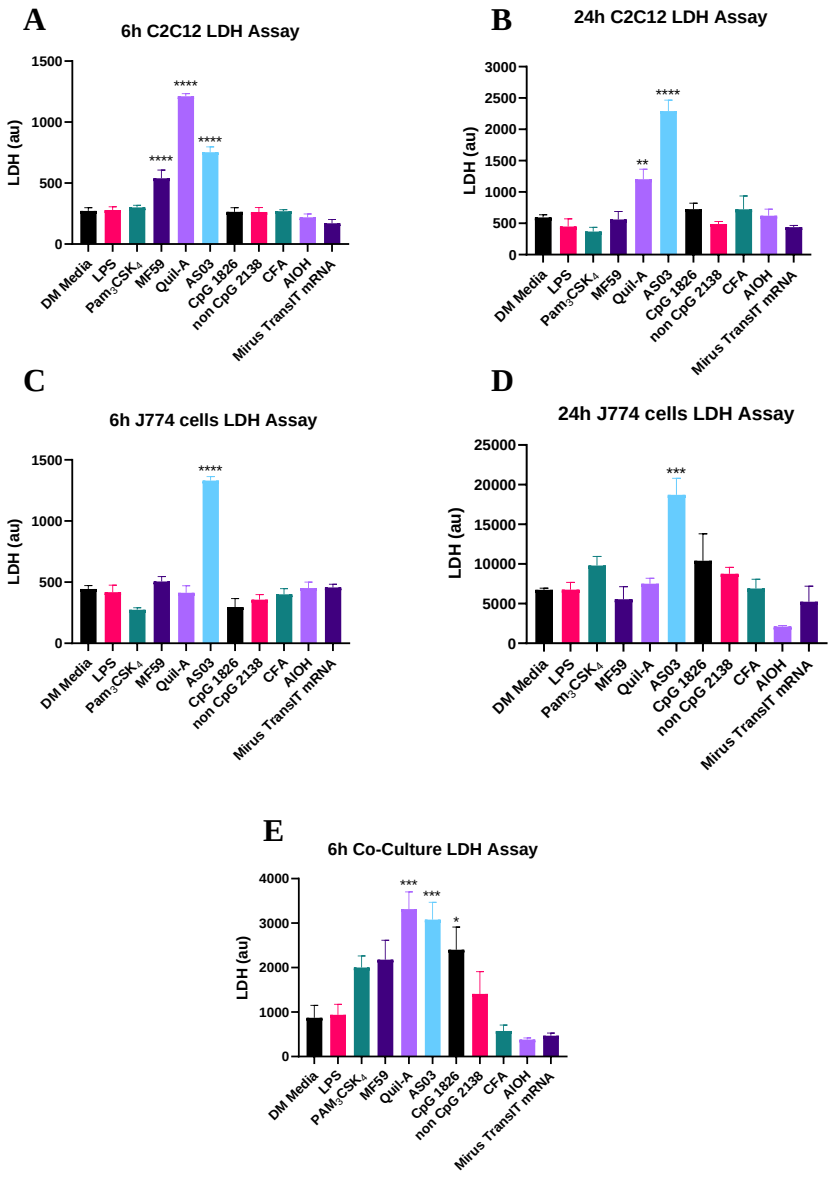

Figure (9). *Lactate dehydrogenase (LDH) cytotoxicity assay of varying cell types at different time points. A) 6-hour C2C12 myotubes B) 24-hour C2C12 myotubes C) 6-hour J774 macrophages D) 24-hour J774 macrophages E) 6-hour coculture of C2C12 myotubes and J774 macrophages (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) by one-way ANOVA with multiple comparisons to the mean of the control (DM Media).*

For the 6-hour C2C12 myotubes, high levels of LDH were found after adjuvant stimulation with MF59, Quil-A, and AS03 compared to the control ($p<0.0001$; Figure 9A). For the 24-hour C2C12 myotubes, LDH continued to increase in Quil-A and AS03 conditions

compared to the DM media treatment ($p<0.01$, $p<0.0001$, respectively; Figure 9B). Interestingly, LDH activity from MF59-treated myotubes returned to baseline levels at 24 hours despite it being elevated at 6 hours. These results show that both Quil-A and AS03 were likely promoting cell death of the myotubes. J774 macrophages were sensitive only to AS03 at both 6 ($p<0.0001$; Figure 9C) and 24 hours ($p<0.001$; Figure 9D). Finally, the 6-hour coculture showed increased levels of LDH release with adjuvant stimulation by Quil-A, AS03, and CpG 1826 ($p<0.001$, $p<0.001$, $p<0.05$, respectively; Figure 9E).

Discussion

Currently, adjuvant research is being conducted extensively due to its' importance in the development of effective vaccines. Adjuvants help by increasing IL-6, CCL_2, and KC ($CXCL_1$) [21]. Considering skeletal muscle has the potential to be an inflammatory tissue, we sought to determine if current vaccine adjuvants would induce a response specifically in skeletal muscle myocytes, and not by other resident cell types. Using an *in vitro* cell line of C2C12 mouse skeletal myotubes we were able to investigate the hypothesis that muscle myocytes are an active contributor to the pro-inflammatory response by adjuvants. We next began to simulate an *in vivo* environment by adding macrophages in coculture with the C2C12 myotubes to determine whether leukocytes augment the myotube response. Our data do not support the initial hypothesis and suggest that there are additional influencers in skeletal muscle tissue that modulate adjuvant induced pro-inflammatory cytokine production.

We first investigated growth effects of adjuvants on C2C12 myotubes. 24-hour exposure caused an increase in cell diameter for the myotubes treated with PAM_3CSK_4, MF59, Quil-A, CFA, AS03, and LPS (Figure 3A). This demonstrates that C2C12 muscle cells have the capability to induce a hypertrophic response to adjuvants.

A popular explanation for myotube hypertrophy is the induction of MAPK and mTOR/AMPK pathways. The mTOR/AMPK pathways are interlinked and involved in sensing availability of nutrients and energy. AMPK is activated by the deficiency of energy which leads to the inhibition of cell growth, while mTOR is switched on by energy availability which promotes cell growth[36]. AMPK phosphorylation in the hypertrophying muscle is associated with decreasing muscle hypertrophy and diminishing activity of the mTOR pathway[37]. Lactate, LPS, IL-1, and TNF-α increases myotube diameter of C2C12 cells via the activation of the mitogen-

activated protein kinases (MAPK)/extracellular signal-regulation kinases (ERK) pathway[38]. This pathway also known as the Ras-Raf- MEK-ERK pathway is a chain of proteins within the cell that communicates a signal from the surface of the cell to the DNA in the nucleus. Specifically, the ERK pathway integrates external signals from mitogens into events to promote cell growth and proliferation in different mammalian cell types. Mitogens are small bioactive proteins or peptides that induce cells to enhance the rate of mitosis. They can also be considered non-specific stimulants of immune cells. Extracellular lactate might have a positive effect on skeletal muscle size, through lactate acting as a mitogen in the ERK pathway[39]. The selected adjuvants that showed increases in cell diameter could also be acting as extracellular mitogens resulting in the increase of size. The stimulation of TLRs, leading to their activation, is upstream of MAPK activation. In agreement with our data, the TLR agonists, LPS and PAM_3CSK_4, induced a hypertrophic response.

Many studies use C2C12 myotubes 3 to 4 days following differentiation[40], whereas we studied the myotubes following 7 days of differentiation. At 3 to 4 days, the myotubes may be multinucleated, but do not terminally differentiate and form the bulk of actin/myosin complexes until day 6 or day 7[41]. In atrophy-based models, this means that researchers are often studying growth inhibition of myotubes that are not fully differentiated[42]. Our studies contrast previous literature reporting LPS causes atrophy in C2C12 myotubes[40]. Upon 24-hour stimulation, we instead found evidence of hypertrophy. This result could be explained by the treatment of LPS on terminally differentiated C2C12 myotubes.

The C2C12 myotubes stimulated by LPS and PAM_3CSK_4 increased IL-6 and MCP-1 production, whereas none of the other adjuvants appeared to show significant increases in these cytokines at 6 and 24 hours (Figure 4A, 4B). In agreement with our data, previous *in vivo* studies

have found that both LPS and PAM_3CSK_4 stimulate local secretion of IL-6 within skeletal muscle following direct intramuscular injection[34]. The absence of a response by most adjuvants led us to investigate other muscle-linked cytokines (myokines).

In previous literature, human skeletal muscle was found to constitutively produce low levels of IL-15 without stimulation. Additionally, LPS was found to stimulate increased production of IL-15 at 96 hours. Our LPS stimulation was performed for 24 hours and may not have been sufficient time to measure an increase in IL-15 production in response to LPS or PAM_3CSK_4 as compared to the control group[43]. This divergent result could be due to length of stimulation (96h, 24h) or concentration of LPS (0.01 μg, 1 μg). Additionally, the other adjuvants that caused decreases in IL-15 production may have evoked a mechanism that contributed to the myocytes to decrease constitutive production of IL-15.

In vivo mouse skeletal muscle was producing cytokines and chemokines as early as 3 hours following injection of adjuvants, including MF59, Alum, and AS01[44]. Levels of those proteins were shown to decrease rapidly within 24 hours after delivery. Additionally, certain chemokines (*Ccl2, Ccl4, Cc15, Cc112)* were upregulated at time points ranging from 3 hours to 12 hours by CpG 1826 and MF59[44]. This information led to our decision to study an additional 6-hour time point. Cytokines were elevated in the LPS, PAM_3CSK_4, and Quil-A groups, although not significantly at 6 hours (Figure 5A). These results lead to the conclusion that additional time is needed to accumulate IL-6 in supernatant. Alternatively, C2C12 myotubes are not responsible for the primary release of IL-6 after adjuvant stimulation. To the contrary, MCP-1 production from 6 hours mirrored 24-hour production (Figure 4B, 5B). Both showed that skeletal muscle produces abundant amounts of MCP-1 in response to LPS. Since no other adjuvants induced this response, we conclude that skeletal muscle myocytes alone are not being

induced to produce MCP-1 directly by the commonly used adjuvants. To address this, we began investigating leukocytes as a possible enhancers of the skeletal muscle pro-inflammatory response.

The immune response requires a myriad of cell types for proper function (Figure 2). After exploring the effects of adjuvants on skeletal muscle myocytes *in vitro* by C2C12 myotubes, we chose to investigate the effects of adjuvants on J774 murine macrophages. These cells were chosen due to their bioavailability in muscle, rapid innate immune response, and their accessibility. After 6-hour stimulation, we measured significant IL-6 production by treatment of J774 macrophages with LPS (Figure 6A). At 24 hours, the J774 macrophages were producing significant levels of IL-6 after stimulation by LPS and CpG 1826 (Figure 7A). CpG 1826 was the first commonly used adjuvant (besides the positive controls of LPS and PAM_3CSK_4) to elicit significant IL-6 production in either cell types used in our studies. CpG 1826 is used to activate TLR9 and stimulate strong B cell activation. A previous study discovered that CpG dinucleotides failed to increase IL-6 synthesis from C2C12 myocytes, suggesting that these cells do not express functional TLR9. Further examination showed that they indeed lacked TLR9, and this was also seen in mouse skeletal muscle. This supports our experiment since the results showed that CpG 1826 stimulation of C2C12 myocytes did not elicit IL-6 production[34]. The J774 macrophages produced significant amounts of IL-6 in response to CpG 1826 showing that this cell type contains TLR9 and that its' activation helps to elicit an immune response.

The positive controls of LPS and PAM_3CSK_4 produced a potent MCP-1 response from J774 macrophages (Figure 6B, 7B). This leads to the conclusion that the other adjuvants alone lack the proper mechanisms induce muscle-derived cytokines. The prototypical inflammatory pathways require the activation of NF-κB or AP-1. Specifically, PAM_3CSK_4 induces MCP-1

secretion through a TLR2-dependent, MyD88-dependent pathway[45]. It may be important to note that macrophages can uptake and present soluble protein antigens and work similarly to dendritic cells in this function. Macrophages activated through contact with pathogens or damage associated molecular pattern(s) rapidly release cytokines and chemokines to recruit additional immune cell types and further the actions surrounding an infection or vaccine. Our data suggest that these cells alone are not responsible for adjuvant induced inflammation.

To determine if macrophages and muscle could enhance the cytokine production following adjuvant stimulation, we created a coculture system. In coculture, Quil-A stimulation led to significant increases in IL-6 production (Figure 8A). Quil-A is a saponin adjuvant reported to induce a strong cytotoxic CD8+ lymphocytes response[46]. Previous studies showed that Quil-A injected without antigen caused an increase in neutrophil recruitment and induced IL-6 production at the site of injection[47]. As IL-6 is responsible for stimulating the production of neutrophils in borrow marrow[48], Quil-A may elicit an increased IL-6 response to aid in the production of neutrophils and their subsequent infiltration. MCP-1 production was only increased at 6 hours in coculture by LPS and PAM_3CSK_4 leading to the possibility that the other adjuvants are not capable of inducing MCP-1 production without an antigen (Figure 8B). Additionally, we attempted to measure supernatant KC (CXCL1/IL-8) to determine if this neutrophil chemoattractant was produced in mono- and cocultures. Unfortunately, the results were inconclusive due to defects with the ELISA kit.

Cell death typically occurs by apoptosis, autophagy, or necrosis. The analysis of cell death is imperative for adjuvant research because it encourages the testing of safer and more effective adjuvants to be used in vaccines. LDH is a stable cytoplasmic enzyme found in cells normally. When the plasma membrane of a cell is damaged, LDH is rapidly released into cell

culture supernatant. THP-1 macrophages exposed to aluminum salts were predominantly similar to untreated cells[49] with regard to LDH release. Our experiments support this study that J774 macrophages exposed to aluminum salts had similar LDH release compared to the control.

In C2C12 myotubes, MF59, AS03, and Quil-A stimulation led to increases in LDH release. In J774 macrophages, AS03 stimulation led to increased LDH release. In coculture, adjuvant stimulation with AS03, Quil-A and CpG 1826 led to increased LDH release (Figure 9). Previous studies have shown that the adjuvant AS03 stresses macrophages and leads to activation of the endoplasmic reticulum (ER) stress sensor IRE1a[50]. Cells exposed to prolonged or severe ER stress have been linked to the induction of apoptosis. Since AS03 induced high levels of LDH in the observed treatments, one possible explanation is that AS03 caused both the C2C12 myotubes and the J774 macrophages to induce ER stress. It can be assumed that the other adjuvants may have also caused ER stress of the cells resulting in increased LDH release. Monitoring the LDH release caused by adjuvants can be useful to determine which adjuvants would be the best for human vaccine use. It could be important to note that Quil-A and AS03 have limited use in US vaccines for humans and the high levels of cell death induced could potentially be the reason. Additionally, dying cells typically release pro-inflammatory molecules. For example, IL-6 production of cocultured myotubes and macrophages was significant stimulation with Quil-A. The Quil-A adjuvant also led to the highest amount of cell death for the coculture. Additionally, IL-6 secretion of the 24-hour J774 cells was increased significantly by CpG 1826 stimulation. This adjuvant also elicited a significant amount of cell damage in the coculture although in these studies it is unclear which cell type contributed. The results of our LDH assays contradict the visual evidence that the myotubes hypertrophied following adjuvant treatment.

The COVID-19 pandemic has brought new importance to vaccine and adjuvant research. Unmodified mRNA changed to N1-methyl-pseudouridine-modifed mRNA is used to encode the SARS-COVID-19 spike protein in vaccines. Pseudo uridine is used because normal uracil is immunogenic and causes cells to not produce the protein as well as it could. To generate an immune response, inflammation is pertinent. However, as it is relevant to mRNA vaccines, too much inflammation can be provoked when the body recognizes the mRNA as viral mRNA. This leads to cytoplasmic proteins to attach to it and mark it for destruction. Therefore, the mRNA is modified to contain pseudo uridine to prevent that event from occurring. The vaccine mRNA is delivered in a lipid nanoparticle (LNP) formulation. Different nucleotide substitution elicits different immune responses, in addition to the LNPs increasing the efficacy of their delivery. Adjuvants can be added readily to LNPs to increase the potency of the vaccine and guide the direction of immune response. Protein vaccines encapsulating CpG ODNs with liposomes showed an improved cellular immune response and different antibody response in comparison to the protein alone[17]. Due to the complex nature of LNP formulations, true nanoparticles were not able to be analyzed for this study. Instead, commercially available liposomes were substituted.

In conclusion, LPS and PAM_3CSK_4 elevated C2C12 myotube and J774 macrophage IL-6 and MCP-1 production at all time points. Other adjuvants, such as CpG 1826 and Quil-A, elicited IL-6 production from J774 macrophages and in coculture, respectively. Many adjuvants, including LPS, PAM_3CSK_4, MF59, AS03, Quil-A, and CFA increased the diameter of the C2C12 myotubes, which could indicate the possible connection between a growth pathway activation and adjuvant stimulation.

The purpose of this study was to research adjuvants *in vitro* in the absence of antigens, since most research is conducted *in vivo* with antigens. Additionally in the deltoid muscle, where

vaccine injection typically occurs, many cell types, besides skeletal muscle cells and macrophages are present. Some of these cell types include lymphocytes and neutrophils. Future research should be directed towards *in vitro* efforts that contain more cell types in coculture to create an environment more similar to that present *in vivo*. To this end, muscle organoids may represent an attractive model system with which to study vaccine adjuvant responses[51]. Additionally, adjuvants and antigens should be studied in combination to understand the full potential of adjuvant stimulation. Adjuvant research is necessary to further discover the influence these substances have in vaccines and which cell types they are responsible for stimulating.

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