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Dissociation of skeletal muscle for flow cytometric characterization of immune cells in macaques

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ABSTRACT

The majority of vaccines and several treatments are administered by intramuscular injection. The aim is to engage and activate immune cells, although they are rare in normal skeletal muscle. The phenotype and function of resident as well as infiltrating immune cells in the muscle after injection are largely unknown. While methods for obtaining and characterizing murine muscle cell suspensions have been reported, protocols for nonhuman primates (NHPs) have not been well defined. NHPs comprise important in vivo models for studies of immune cell function due to their high degree of resemblance with humans. In this study, we developed and systematically compared methods to collect vaccine-injected muscle tissue to be processed into single cell suspensions for flow cytometric characterization of immune cells. We found that muscle tissue processed by mechanical disruption alone resulted in significantly lower immune cell yields compared to enzymatic digestion using Liberase. Dendritic cell subsets, monocytes, macrophages, neutrophils, B cells, T cells and NK cells were readily detected in the muscle by the classic human markers. The methods for obtaining skeletal muscle cell suspension established here offer opportunities to increase the understanding of immune responses in the muscle, and provide a basis for defining immediate post-injection vaccine responses in primates.

1. Introduction

Normal skeletal muscle contains only a small population of resident immune cells (Pimorady-Esfahani et al., 1997; Przybyla et al., 2006; Saclier et al., 2013; Malm et al., 2004). However, during pathophysiological conditions such as contraction or reperfusion-induced insult and injury, endotoxemia or inflammatory myopathies there is a significant infiltration of immune cells (Pillon et al., 2013). The recruited immune cells play important roles in the regeneration process and resolving the injury or inflammation. Immune cells remove necrotic tissue and secrete soluble factors that contribute to activate muscle satellite cells that differentiate into new muscle cells (Gharaibeh et al., 2012).

Furthermore, several medical treatments are administered by injection into the muscle. The muscle is the most common site for vaccination. Vaccines are intended to target immune cells directly or indirectly but the mechanisms by which immune activation is caused at the site of injection are largely unclear. Inflammatory responses such as the recruitment of immune cells to the site of vaccine delivery are likely central in the initiation of immune responses that subsequently dictate the potency of the vaccine response. There are limitations for performing extensive studies of the presence and function of immune cells in human muscle due to the difficulty of collecting skeletal muscle biopsies. There are few protocols available for obtaining single cell suspensions from human muscle biopsies for the characterization and enumeration of immune cells. Importantly, studies of immune events such as immune cell mobilization to sites injected with vaccines or treatments, definition of target immune cells and degree of inflammation require in vivo studies and cannot be replaced by in vitro model systems. The few in vivo reports that have characterized early immune mechanisms in the muscle after vaccination were performed on mice (Calabro et al., 2011; Mosca et al., 2008). Rodents and humans differ substantially in their distribution of immune cell populations, phenotype and innate immune responses. In addition, therapeutic doses used in rodents may not be proportionally representative for clinical use. Therefore, nonhuman primates (NHPs) comprise unique in vivo

Nonstandard abbreviations: MDCs, myeloid dendritic cells; PDCs, plasmacytoid DCs; Ad5, adenovirus serotype 5; NK cells, natural killer cells.

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models for immune cell functions. NHP models are therefore commonly used for preclinical and translational studies of vaccines and treatments.

There are numerous publications based on flow cytometric analyses of solid tissues regarding the presence of immune cells and immune activation (Bayne and Vonderheide, 2013; Strauss et al., 2015). The accuracy of such analysis is critically dependent on the quality of the cell suspension preparation. It is important to employ methods that allow for isolation and detection of rare and sometimes very delicate cells like infiltrating immune cells to the site inflammation, infection or vaccination. Classic methods for dissociating tissue include enzymatic digestion and manual disaggregation. While tissues such as lymph nodes (LNs) and spleens disaggregate rather easily, firm and tenacious skeletal muscle tissue is more challenging. In this study, we describe strategies to a) define and precisely sample muscle tissue at the injection site of a model vaccine, b) obtain cell suspensions using enzymatic digestion and/or mechanical disruption as well as c) identify and enumerate different immune cells present in the muscle after vaccine injection. The time required for processing, and the viability and yields as well as suitability for flow cytometric characterization of isolated immune cell subsets were particularly evaluated.

The protocols defined herein to analyze skeletal muscle tissue from the site of vaccine or treatment delivery will contribute to a greater understanding of the role of immune cells in clinical applications. The methods described are general for obtaining muscle samples and can therefore be applicable for a wide range of investigations.

2. Materials and methods

2.1. Animals

Approval for this animal study was granted by the Animal Care and Use Committees of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Bethesda, MD, USA. Vietnamese cynomolgus and Indian rhesus macaques recycled from completed studies and scheduled for euthanasia were used in this study. The animals were housed at Bioqual or at NIHAC facility, NIH and handled according to the standards of the American Association for the Accreditation of Laboratory Animal Care.

2.2. Injections

As a model vaccine in this study we used E1/E3-deleted, replication-incompetent recombinant adenovirus serotype 5 (rAd5) generated in HEK293 cells as previously described (Sullivan et al., 2000). rAd vectors encoding the Zaire Ebola glycoprotein were purified on CsCl gradients and viral titers were determined by measuring the OD at 260 nm. A dose of 1×10^{11} vector particle unit suspended in 0.5 ml saline was injected in six different sites (left and right deltoids, quadriceps and calves respectively) using a 1 ml, 25 gauge and 1.6 cm needle (Becton Dickinson, San Jose, CA, USA). The animals were anesthetized with Ketamine and Xylazine (10 mg and 0.5 mg respectively per kg body weight) and injection areas were shaved and cleaned with alcohol wipes. The injection site was defined on skin with a permanent marker by encircling an area of 30 mm in diameter. The vaccine was administered at the midpoint of the circle with the needle penetrating 1.5 cm into the muscle in a perpendicular angle without stretching the skin.

2.3. Sampling of vaccine-injected muscle tissue

Muscle tissue from the injection sites was obtained during necropsy at 72 h post-injection. To mark the injection site, a biopsy punch (4 mm, Integra Miltex, Redford, MI, USA) was first pushed through the skin, adipose and connective tissue at the center (needle entry point) of the encircled injection site. After the skin was excised, a puncture wound on the underlying muscle created by the biopsy punch indicated the needle entry point and was used to guide collection of vaccine exposed muscle tissue. An area within 25 mm radius surrounding the puncture wound was considered muscle that was in closest contact with injected vaccine and a cubical piece of muscle tissue of approximately 15 cm^3 was dissected with a scalpel, placed in RPMI cell culture media (Gibco, Carlsbad, CA, USA) and stored on ice until processing about 1 h later.

2.4. Enzymatic digestion and mechanical dissociation

The muscle tissue was weighed and normalized to 3 g by removing adipose tissue, the outermost connective tissue layer (epimysium) and excess muscle tissue farthest away from the punch biopsy wound with scissors. The epimysium and lean muscle were cut separately into 5 mm^3 pieces with scissors in petri dish with 10 ml RPMI media. The pieces were drained from excess RPMI media by tilting the dish 45° while gathering the pieces up to the dry part of the tilted dish with forceps. Enzymatic dissociation was done by transferring the drained pieces to 6-well plates (3 g/well) consisting of RPMI media (5 ml/well) supplemented with 0.5 mg/ml DNase (Sigma Aldrich, St. Louis, MO, USA) and 0.25 mg/ml Liberase TL (Roche, Indianapolis, IN, USA), which is a mixture of collagenases, without agitation at + 37 °C for 2 h. In some experiments, Liberase digestion was performed under agitation with orbital shaker set on 12 rpm at + 37 °C for 1 or 2 h as indicated. Epimysium was digested in separate 6-well plates with Liberase (5 ml/well) unless stated otherwise. Liberase activity was inactivated with complete media; RPMI media with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco). The digested pieces of muscle and epimysium were thereafter pooled during filtering through 70 μm cell strainers (BD Bioscience, San Jose, CA, USA) unless otherwise stated. Where indicated, pooled suspension was first passed through a Nitex nylon mesh (500 μm, Wildco, Yulee, FL, USA) before filtering through the cell strainers. Lastly, the cell suspensions were washed at 1500 rpm for 7 min with complete media.

Alternatively, the 5 mm^3 pieces cut from the muscle biopsies were mechanically dissociated with the Medimachine system (BD Biosciences) without using enzymatic digestion. Epimysium was discarded and approximately, 3–4 muscle pieces at a time were loaded into chambers with rotatable blades called Medicons (50 μm) together with 1 ml complete media and were subsequently mounted to the Medimachine for pulsing with blade rotation speed of 80 rpm for 1 min. Medicons were rinsed with complete media and both disrupted suspension plus rinse was aspirated with 5 ml syringes, filtered through Filcons (70 μm) and washed with complete media.

To combine both enzymatic digestion and mechanical dissociation, we used a commercial murine skeletal muscle dissociation kit together with the GentleMACS system (Miltenyi Biotec, Auburn, CA, USA). Pieces of muscle and epimysium were transferred to C-tubes and digested together with Miltenyi's enzyme cocktail under agitation at + 37 °C for 30 min, followed by disruption with GentleMACS dissociator running the muscle dissociation program. These steps were repeated once more before filtration through 70 μm cell strainers and washing with complete media. In other experiments combining digestion and disruption, separated muscle and epimysium were digested side-by-side with Liberase instead of Miltenyi's enzymes for 2 h without agitation and Liberase activity was inactivated. The Liberase-digested muscle was then disrupted with GentleMACS. Suspensions from Liberase digestion alone or combined with disruption were thereafter pooled during filtration.

The washed cells from all the dissociation methods were resuspended in complete media according to the number of staining panels.

2.5. Flow cytometry

To stain cells for flow cytometric characterization, 1 ml suspensions for each of four staining panels were transferred to 5 ml polypropylene tubes (BD), washed with 3 ml PBS and resuspended in 100 μL staining