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OPTIMIZED TISSUE PREPARATION TECHNIQUE IN A CHRONIC GRAFT-VERSUS-HOST MOUSE MODEL

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

OPTIMIZED TISSUE PREPARATION TECHNIQUE IN A CHRONIC GRAFT-VERSUS-HOST MOUSE MODEL

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Introduction: Hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment of hematological malignancy or disease. One sequela of HSCT is development of chronic graft-versus-host disease (cGvHD), a serious complication in 30-70% of transplant recipients, and a major cause of death following HSCT. The most common target of cGVHD is the skin, followed closely by oral tissues. Mouse models approximate features of human oral cGvHD and are used to elucidate the pathogenesis of oral cGvHD. Challenges in sample preparation are well-known, resulting in the need to optimize critical laboratory steps in sample analysis.

Objective: To test a novel protein extraction method for efficacy in the context of a cGvHD mouse model.

Methods: An established mouse model (B10.D2 cells into BALB/c mice) was used to simulate cGvHD conditions. Splenocytes and bone marrow cells from donor mice were transplanted into irradiated recipient mice via retro-orbital injection. Recipient mice were euthanized at specified timepoints post-transplant, and submandibular gland and buccal mucosa tissues were obtained. Tissues were prepared for characterization using a novel liquid nitrogen-cooled mini mortar and pestle technique. Samples were pulverized in microcentrifuge tubes in the mini mortar and combined with appropriate buffers for western blotting (WB) and enzyme-linked immunosorbent

assays (ELISA). Protein quantification via bicinchoninic acid (BCA), WB, and ELISA were performed and compared to results from standard extraction techniques.

Results: BCA protein quantification showed extraction yields comparable to conventional methods (25.8 µg/ml vs 25.6 µg/ml for control). WB and ELISA confirmed the presence of proteins and cytokines found in cGvHD. Absence of protein degradation was verified by WB and total protein staining.

Conclusion: Protein extraction via the liquid nitrogen-cooled mini mortar technique is an efficient way to prepare mouse oral tissues for analysis. This method will be used in future studies to characterize oral conditions of cGvHD.

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LIST OF ABBREVIATIONS

HSCT – hematopoietic stem cell transplant
Allo-HSCT – allogeneic hematopoietic stem cell transplant
GvHD – graft-versus-host disease
aGvHD – acute graft-versus-host disease
cGvHD – chronic graft-versus-host disease
GI – gastrointestinal
HLA – human leukocyte antigen
MHC – major histocompatibility complex
DAMPs – damage associated molecular patterns
IACUC – institutional animal care and use committee
cGy – centigray
PBS – phosphate buffer solution
ACK – aluminum-chloride-potassium
rpm – rotations per minute
RPMI Roswell Park Memorial Institute 1640 media
HBSS – Hank's balanced salt solution
SMG – submandibular gland
RIPA – radioimmunoprecipitation assay
rcf – relative centrifuge force
LB – loading buffer
MES - 2-(N-morpholino)ethanesulfonic acid
TBS_T - tris buffered saline 1x + 0.2% tween 20
CG – Cryo-grind, or mini mortar method
TH – tissue homogenizer

REVIEW OF THE LITERATURE

Hematopoietic stem cell transplant (HSCT) is a procedure done for the treatment of hematological malignancy or deficiency and is a potentially curative treatment[1]. The recipient of the transplant is conditioned through chemotherapy and/or radiation to clear the body of diseased or incompetent marrow cells. Multipotent stem cells from bone marrow, peripheral blood, or umbilical cord blood are collected from a donor, processed, and infused into the recipient. This procedure can be autologous (auto-HSCT) or allogeneic (allo-HSCT). In an autologous transplant, the patient's own stem cells are collected prior to the conditioning process and infused back into the patient at a later time. In an allogeneic transplant, necessary when dictated by the nature of disease, cells are collected from a genetically-matched donor who may be related or unrelated to the recipient. Some of the more common applications of allo-HSCT include acute and chronic leukemias, myelodysplastic syndrome, myeloproliferative syndrome, non-malignant diseases, and aplastic anemias[2]. These life-saving procedures are quite common in the United States, where 9,028 allo-HSCTs and 14,006 auto-HSCTs were performed in 2018[3].

Graft-versus-host disease (GvHD)

One negative sequela of HSCT is graft-versus-host disease (GvHD), where immunocompetent cells from the donor attack and damage the tissues and organs of the recipient. GvHD is a serious complication, representing the major non-relapse cause of death in affected individuals following HSCT[4]. GvHD has been reported in 30 to 70% of patients receiving allo-HSCT of non-manipulated donor grafts with standard prophylaxis regimens[5].

GvHD is separated into acute (aGvHD) and chronic (cGvHD) types. Classically, development of aGvHD occurs within the first 100 days, while cGvHD develops after 100 days[6].

More recently, the diagnostic standards were redefined by the National Institute of Health's Consensus Criteria in 2006 that established symptom-based differentiation of acute and chronic GvHD[7]. Symptoms of aGvHD typically involve the skin (maculopapular rash), gastrointestinal (GI) tract (nausea, pain, diarrhea), and liver (cholestatic hyperbilirubinemia). In contrast, symptoms of cGvHD are more variable and more autoimmune nature. In addition to skin, the GI tract, and liver tissues that are affected in aGvHD, symptoms of cGvHD may involve any area of the body, including the mouth, eyes, muscles, genitalia, lungs, kidneys, heart, and marrow[8]. In cGvHD, the oral cavity is commonly affected, and can even be the sole location affected by the disease[9].

Risk factors significantly associated with the development of cGvHD include: use of unrelated donors, HLA disparity, prior incidence of aGvHD, use of female donor for male recipient, grafting with mobilized blood cells, and older donor and recipient age[10]. GvHD occurs when T cells from donor tissue react to predetermined proteins on host cells, most importantly, the Human Leukocyte Antigens (HLA) that are encoded by the major histocompatibility complex (MHC)[8]. Three requirements must be met for the initiation of GvHD: the graft must contain immunologically competent cells, the recipient must have antigens that are not present in the donor, and the recipient must be unable to eliminate the transplanted cells through an immune response[6]. Maximum HLA matching between the donor and recipient is an important factor in decreasing the likelihood of GvHD[8].

Due to the significant morbidity and mortality posed by cGvHD, there is value in exploring its pathogenesis with the goal of identifying specific events that could be targeted for prevention. One proposed model separates the pathogenesis of cGvHD into three phases: 1) early inflammation from tissue injury; 2) chronic inflammation, thymic injury, and dysregulated B-cell

and T-cell immunity; and 3) tissue repair with fibrosis[5]. While the roles of various cells and cytokines continue to be elucidated, the initiating immune events of cGvHD remain mostly elusive. The pathogenesis of cGvHD has been linked to thymic damage caused by conditioning, leading to release of allo/autoreactive T cells[11]. Many different types of damage associated molecular patterns (DAMPs) have been implicated in activating the inflammatory events that lead to GvHD[12]. DAMPs are endogenous molecules released following stress or injury.

The oral cavity is a favorable site for studying the pathogenesis of cGvHD because it is highly accessible. Oral symptoms in patients with cGVHD are present at least 25% of the time and vary depending on the specific transplant method[13]. Previous studies have shown a significant change in specific salivary markers, including lactoperoxidase, lactotransferrin, and cysteine proteinase inhibitor family proteins, when comparing patients with and without cGvHD[13]. Characterizing the tissues in the oral cavity under cGvHD conditions and finding the biomarkers that change from healthy will aid in understanding the pathogenesis of oral cGvHD.

A mouse model has been adapted in our lab to approximate oral chronic GvHD conditions in humans. In this model, two different mouse strains are used to replicate an allogeneic transplant scenario, and a single strain is used in a syngeneic transplant scenario. Previous experiments in the lab have shown that this model can replicate many of the features in of oral chronic GvHD, including lymphocytic infiltration of target organs and salivary gland dysfunction. A distinct advantage of a mouse model is that tissue samples from the oral cavity can be fully analyzed, allowing for a more complete analysis of the cellular changes that occur.

To study these murine tissues effectively, efficient sample preparation is critical. The purpose of this project is to optimize a protein extraction method for murine tissues that preserves low abundance signaling molecules in a chronic GvHD mouse model. If deemed

efficient, the protein extraction method will be incorporated into many future experiments to characterize the local tissue environment of the oral cavity in chronic GvHD conditions.

METHODS AND MATERIALS

Conditioning of recipient mice

The allo-HSCT was performed in mice under an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Recipient BALB/C mice (an albino, laboratory-bred strain of the house mouse) were irradiated with two doses of whole-body gamma irradiation (425 cGy per dose), 3 hours apart.

Isolation of murine bone marrow cells

Donor B10.D2 mice were euthanized using CO_2 asphyxiation. Using an aseptic technique, the skin from each hind leg was peeled down over the foot, and the foot with the skin was removed. The hind legs were cut at the hip joint with scissors, leaving the femur intact. The leg was placed in phosphate buffered saline (PBS). The excess muscle was removed from the leg by holding the end of the bone with forceps and using scissors to push the muscle downward away from the forceps. The bones were washed thoroughly in PBS. Using sharp scissors, the leg bones were carefully severed proximal to each joint. A 3-cc syringe with a 25-gauge needle was filled with cold, sterile PBS. The needle was inserted into the bone cavity of the femur. While holding over an open petri dish, the bone cavity was flushed with 2 to 5 ml of the PBS, until the bone cavity appeared white. Bone marrow was homogenized via aspiration. The same syringe and needle were then used to filter the sample through a 40µm cell strainer in a 50mL conical tube. Cells were centrifuged for 10 minutes at 1500rpm. The supernatant was discarded, and cell pellets were suspended in 5mL of Aluminum-chloride-potassium (ACK) lysis buffer at room temperature. The sample with lysis buffer was allowed to incubate at room temperature for 5 minutes, then the cells were resuspended in Hank's + 2% fetal bovine serum (FBS). The sample

was centrifuged for 10 minutes at 1500 rotations per minute (rpm). The cells were resuspended in PBS for counting. The sample was filtered through a 40µm cell strainer into a 50mL conical tube.

Isolation of murine splenocytes

Donor mice were euthanized and rinsed thoroughly with 70% ethanol. With the mouse in a supine position, the abdomen was opened by a transversal cut, avoiding opening the peritoneal cavity. The peritoneal sac was exposed by pulling the skin in opposite directions with forceps. The spleen could then be visualized on the left side of the mouse, and the peritoneal sac was opened with a second cut. The spleen was collected with sterile curved iris forceps. The spleen was placed in 5mL Roswell Park Memorial Institute 1640 media (RPMI) + FBS in a tissue culture plate.

To prepare the splenocyte single cell suspension, the spleen was cut into small pieces with scissors. The sample was pressed with a tissue homogenizer to break the capsules and mechanically disperse the cells in the RPMI + FBS. The suspension was expelled into a centrifuge tube through a 40µm nylon strainer. The sample was centrifuged for 10 minutes at 1500rpm, 4°C, and the supernatant was discarded. To eliminate red blood cells from the spleen, the pellet was resuspended in 3mL of ACK lysing buffer at room temperature and homogenized. The sample was incubated for 5 minutes at room temperature. The cells were resuspended in sterile Hank's + 2% FBS and centrifuged for 10 minutes at 1500rpm, 4°C. The cells were resuspended in RPMI + FBS for counting

Transplantation

Cells were resuspended in Hank's balanced salt solution (HBSS) and were counted. The number of cells were aliquoted according to the established B10.D2 model: $8x10^6$ BM cells and

$15x10^6$ spleen cells. BM and splenocytes were combined and homogenized. The cell suspension (BM + splenocytes) was filtered through a 40µm cell strainer into a 50mL conical tube. Tube was centrifuged for 10 min at 1500rpm, 4ºC. Supernatant was discarded. Cell suspension was resuspended in a final volume of 200µL/animal in HBSS and kept on ice.

In preparation to receive the transplant, the recipient mouse was anesthetized with isoflurane and positioned on its side. The mouse was restrained with the thumb and middle finger of the non-dominant hand, pulling back the loose skin over the shoulders and behind the ears. The middle finger of the non-dominant hand was used to draw back the skin above the eye and the thumb to draw back the skin below the eye. The needle was inserted at a 45° angle to the eye, lateral to the medial canthus, through the conjunctival membrane. The needle was thus positioned behind the globe of the eye in the retrobulbar sinus and the cells were injected. The needle was gently removed, and the eyelid was closed. Mild pressure with a gauze sponge was applied to the closed eye. The injection site was monitored for swelling or other visible signs of trauma. The mouse was monitored and, in the absence of adverse effects, returned to its home cage once it regained its righting reflex.

Tissue Extraction

Recipient mice were euthanized at defined timepoints of 14 days, 21 days, 28 days, and 42 days. The submandibular gland (SMG), buccal mucosa, and spleens were dissected.

METHOD A: The control method for extracting protein was by use of a battery-operated tissue homogenizer or pellet mixer. A 1x radioimmunoprecipitation assay (RIPA) buffer solution was prepared using 100µL RIPA 10X, 143µL Roche Complete mini protease inhibitor, 100µL Roche PhosSTOP phosphatase inhibitor, and 657µL MilliQ water. Five ml/g of chilled 1x RIPA lysing buffer was added to the frozen sample, allowing the frozen tissue to thaw on wet ice. The

battery-operated tissue homogenizer was used until most of the tissue was pulverized, about 1 minute. A hand sonicator on mid-power setting was used for 3 cycles, 30 seconds each, to complete tissue pulverization.

METHOD B: The test method for protein extraction utilized a liquid nitrogen-cooled mini mortar and pestle set. A 1x RIPA buffer solution was prepared as described in method A. Liquid nitrogen was poured into the stainless steel well of the mortar and pestle set until it reached the scribed line. The mortar was placed into the stainless steel well, and the pestle was placed into the mortar. Both were allowed to cool for several minutes (until the vapors subsided). The frozen sample was weighed and placed back in dry ice to maintain a frozen state. The microcentrifuge tube containing the frozen tissue was placed into the stainless-steel mini mortar, and the pestle was used to apply controlled pressure against the tissue. The pressure from the pestle continued until the tissue was pulverized, which typically only took a few turns.

After pulverization, the microcentrifuge tube was removed from the mini mortar and placed into wet ice. Five ml/g of chilled 1x RIPA lysing buffer was added into the microcentrifuge, allowing the frozen tissue to thaw. The nuclease inhibitors added to the 1x RIPA buffer helped to avoid degradation of nucleic acids.

BOTH METHODS: After initial tissue preparation, both methods used the same protocol. The samples were sonicated in a Sonicator Bioruptor UCD-300 with ice in the water to ensure the samples remained chilled. The sonication happened in cycles of 10 seconds on, then 10 seconds off and was repeated 5-10x depending on type and size of tissue.

The samples were then placed on an orbital shaker in a 4^{o}C cold room with constant agitation for 1 hour. The samples were centrifuged for 20 min at 16,000 relative centrifuge force (rcf) at 4^{o}C in a microcentrifuge. The tubes were removed from the centrifuge, placed on ice and

the supernatant was collected into a fresh tube on ice. The pellet was discarded. Aliquots were made and the samples were kept frozen at -80ºC when not in use. An aliquot of 10µL was made for protein quantification.

Bicinchoninic Acid Assay (BCA) Protein Quantification

Total protein quantification was done using a reducing agent-compatible BCA assay kit (Pierce #23250). Blank and standard preparations of bovine serum albumin were prepared according to the concentrations listed in the kit. The amount of BCA working reagent was calculated according to the total number of blanks, standards, and samples with 100µL of working reagent needed per well. BCA reagent A was mixed with reagent B in a 50:1 ratio to obtain the calculated volume of working reagent. Five µL of standard, sample, and blanks were loaded into 96 well plates. One-hundred µL of working reagent was added to each well. The well was incubated at 37ºC for 30 minutes, then cooled at room temperature for 5-10 minutes. Absorbance was read at 562nm. Protein concentration of samples were calculated using standard concentrations and absorbance values.

Western Blot

Samples were removed from -80ºC storage and kept on ice. Beta-mercaptoethanol was added to Licor 4x protein loading buffer (LB) in a 1:9 ratio. LB was added to sample in a 1:3 ratio. Samples with LB were heated at 70ºC for 10 minutes with shaking. Samples were briefly centrifuged and loaded into NuPAGE Novex 4-12% Bis-Tris polyacrylamide gradient gels with 5µL Chameleon Pre-stained Protein Ladder and 15µL sample per well. Gel was placed into chamber, which was filled with 1 x 2-(N-morpholino)ethanesulfonic acid (MES) running buffer. The gels were run at 80V for 90 minutes.

The gels were transferred to membranes in transfer buffer using the mini blot module run at 11V for 90 minutes. The membranes were blocked for 60 minutes at room temperature using 5% nonfat dry milk tris buffered saline 1x + 0.2% tween 20 (TBS_T) on a rocking platform. Primary antibodies were prepared in Odyssey blocking buffer at the titrated concentration for each primary antibody. A sheet protector and heat sealer were used to prepare a bag slightly larger than the membrane, and the membrane was placed inside. Primary antibodies were added to the membrane and the bag was sealed with the heat sealer. The membrane was placed on a rocking shaker with a weight over it and incubated overnight at 4^{o}C. Membranes were removed from the bag and washed for four cycles in TBS-T for 5 minutes each cycle. Secondary antibodies were diluted in 5mL Odyssey blocking buffer at 1:10000 ratio. The secondary antibody mix was placed onto the membrane in a western blot box and incubated for 90 minutes at room temperature on a platform rocker. The membrane was rinsed with PBS and then washed in PBS for 60 minutes on a platform rocker. The membrane was then scanned on the Odyssey Imaging System.

RESULTS

Protein extraction of a submandibular gland prepared by both METHOD A or B, a control method using a battery-operated tissue homogenizer or pellet mixer or by the mini mortar, showed similar adjusted concentration levels in both methods (Figure 1). The adjusted concentration for method B shown in Figure 1 is 25.8 µg/ml vs 25.6 µg/ml for method A, the control.

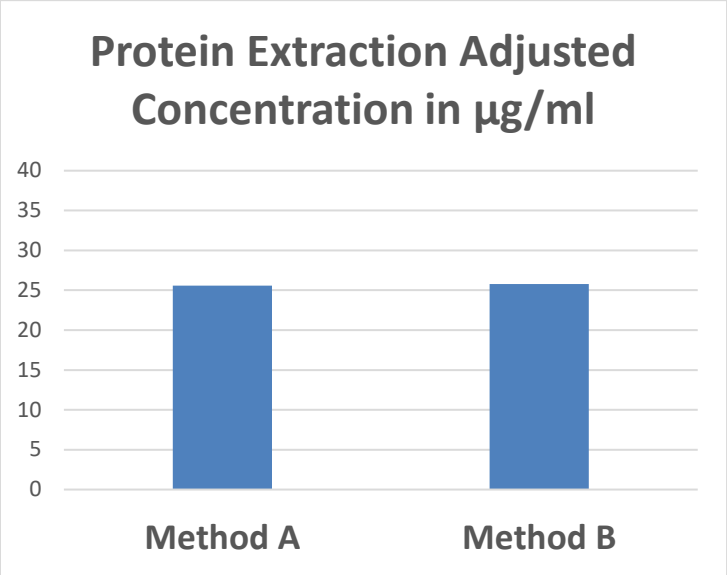

Figure 1 – Protein was extracted using Method A (pellet mixer) or Method B (mini mortar) from a mouse submandibular gland and then quantitated using a BCA assay. The protein concentration for each method is plotted here.

Western blot analysis of a SMG and a spleen sample was performed with control primary antibody against B-actin (Figure 2), which should be present in all cells. B-actin was detected at 45 kilodaltons (kDa) in both samples and with both preparation methods, as shown by the red fluorescence (Figure 2). The highlighted column of the submandibular gland (SMG) prepared by

tissue homogenizer shows a faint secondary band at 50 kDa. This is an undesirable result that was not seen with the liquid nitrogen mini mortar method.

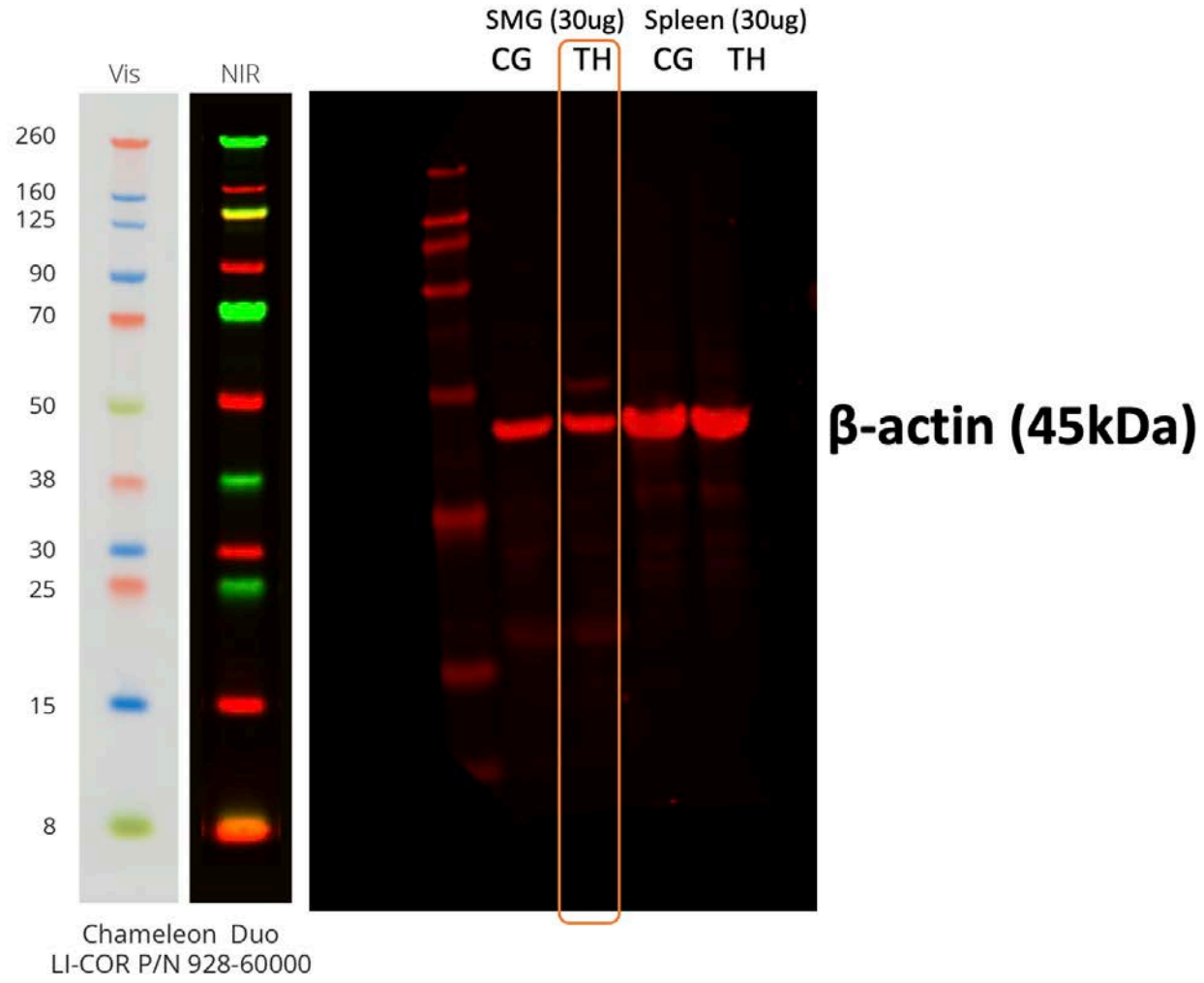

Figure 2 – Western blot for mouse beta actin (45kDa) in SMG and spleen processes using mini mortar (or cryo-grinder, CG) or tissue homogenizer (TH).

Method B was selected for use in additional experiments to test new primary antibodies for western blot analysis. A separate western blot analysis was done on an allo-SMG (SMG from a mouse in the allogeneic transplant group), and a syn-spleen sample (spleen from a mouse in the syngeneic transplant group) (Figure 3). The western blot showed the expected band at 37 kDa for

GAPDH (the loading control) as well as the bands for CXCL16 in the allogeneic SMG sample at ~14 kDa and 27/28 kDa, consistent with published data for CXCL16 detection in tissue culture supernatant[14].

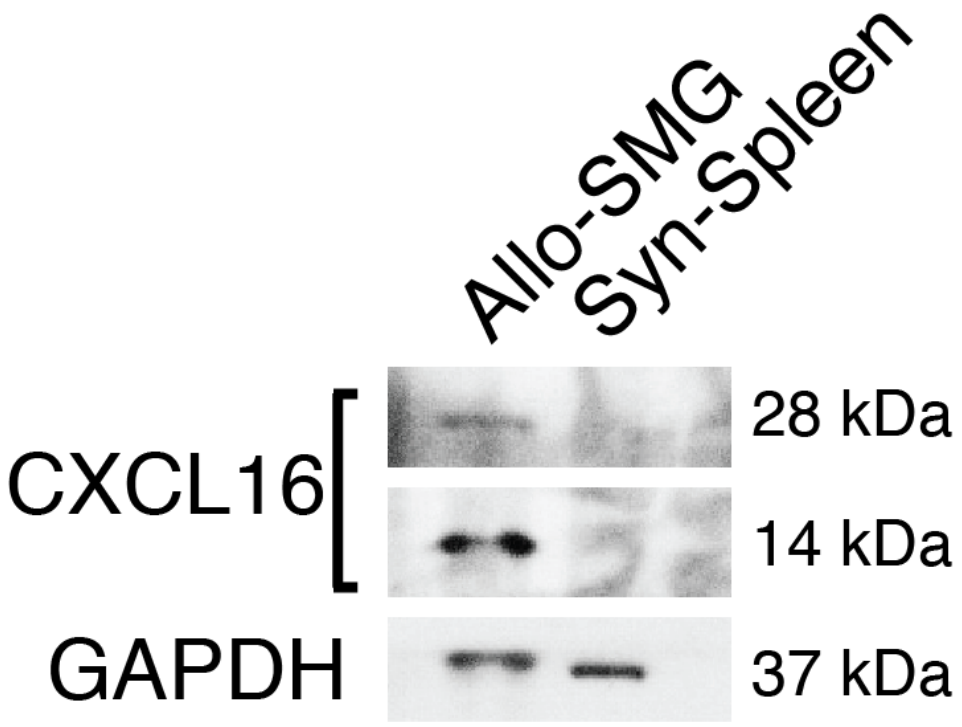

Figure 3 – Western blot of an allo-SMG sample and a syn-spleen sample showing detection of CXCL16 in the allo-SMG group.

DISCUSSION

In this study, we tested a new method for protein extraction of sensitive small molecules from mouse tissue. We compared Method A (pellet mixer) with Method B (mini mortar) on the criteria of total protein extracted, integrity of housekeeping proteins on western blot and detection of rare signaling proteins. When compared to other methods commonly used, Method B, the liquid nitrogen cooled mini mortar method, has several advantages. The samples were kept frozen during the pulverization process. Thawing cycles and heat accelerate protein degradation, compromising the integrity of the samples and validity of results. The liquid nitrogen allowed the samples to maintain their frozen state, which had the added benefit of reducing time and effort needed to achieve adequate pulverization of the tissue.

This method for protein extraction offered several benefits to others that had been previously employed in the lab. The samples could remain in an individually labeled microcentrifuge tube without being transferred to another container, thus limiting the opportunity for cross-contamination among samples.

The novel method was very efficient, offering the ability to prepare many samples in a short period of time. With the nature of the mouse model experiment, there are usually dozens of tissues needing preparation at each defined time point of the experiment. As there are many steps in analyzing each target tissue, efficient sample preparation was extremely helpful in reducing overall time needed for experiments.

An additional benefit realized through the mini mortar technique was cost efficiency, as the only additional expense was the initial equipment purchase. The high volume of samples processed in the lab would make other methods that rely on additional single-use items cost-prohibitive.

The detection of CXCL16 using the new method was an important landmark in validating this protocol. The chemokine CXCL16 is an important signaling molecule for the early attraction of immune cells. It signals through its receptor CXCR6. It's hypothetical role in chronic GvHD is not well-defined, but detection of CXCL16 marks an early immune response.

CONCLUSION

Within the limits of this study, protein extraction via liquid nitrogen-cooled mini mortar technique is an efficient way to prepare mouse oral tissues for analysis. Extracts preserved low-abundance chemokines and other signaling molecules that are present in miniscule amounts. This preservation, in combination with highly sensitive protein-detection techniques, expands the detectable immune network for oral chronic GvHD. Expanded understanding of chronic GvHD immunopathogenesis will facilitate better prevention and treatment for future patients.

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