

Development of a new method for extraction and isolation of porcine bone marrow-derived macrophages (BMDM) from ribs for osteoimmunology research

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INTRODUCTION The use of various porcine models has increased over recent years in orthopedic, engineering, and regenerative medicine research due to anatomical and biological similarities between humans and pigs. Being more closely related to humans than rodents, pigs have been increasingly chosen for inflammation and immune studies in biomedical research, including the field of osteonecrosis and osteoimmunology. This has increased the need to develop more efficient ways to isolate and culture porcine bone marrow-derived macrophage (BMDM). Among the various techniques for extracting macrophages from bone marrow, most commonly described methods in literature include the use of needle aspiration of pelvis and flushing the long bones (ribs, ulna, radius, humerus) with phosphate-buffered saline (PBS) or un-supplemented RPMI [1,2,3]. However, due to limitations associated with various extraction process methods, the number of macrophages obtained is not consistent. In this study, we developed a new bone marrow extraction protocol using porcine ribs, followed by an efficient cell isolation method using a centrifugation step and in vitro culture procedures. We investigated the yield, viability, and immune responses of the isolated macrophage through in vitro culture experiments.

METHOD: In this IACUC-approved study, ten 12-to 18-weeks male pigs (Duroc, Yorkshire) were used. As shown in Fig.1A, BMDM were isolated from 4th to 10th ribs and retrieved under a sterile condition from each side of the animal following sacrifice. The key steps of rib retrieval and sectioning are illustrated in Fig.1B, which involved removal of the periosteum and muscle to isolate the ribs and cutting them into 1-1.5 cm long pieces using a rib cutter. The rib pieces were then loaded into 15 ml cortical tubes. Three different centrifuge durations to spin down the bone marrow cells (1 min at 3000 rpm, 0.5 min at 2000 rpm +1 min at 3000 rpm, and 3 min at 3000 rpm; all at room temperature) were studied to optimize the marrow cell extraction. The cell pellets were resuspended with a solution containing PBS+ 2mM EDTA + 0.5% BSA (1:1 volume) and processed using Ficoll Plaque to collect the buffy coat layer followed by red blood cell lysis (5 mins). Cell counting with Trypan Blue was used to determine the cell number and viability. The cells isolated from different centrifuge times were used for further characterization. After seeding mononuclear cells in 6 well-plates for 1 hour, the plates were washed with PBS to remove the floating cells, and the remaining attached cells were cultured for 4 days in RPMI+20% porcine serum+1%PenStrep to obtain mature macrophages. These cells were induced with M1 (100ng/μl LPS) or M2 stimulants (IL4+IL13 20ng/μl each) for 24 hours and compared to the cells cultured without the M1 or M2 stimulants. Inflammatory cytokine expression was quantified by qPCR (IL1β, IL8, and TNFα). Flow cytometry was used to quantify M1 (CD8086) and M2 (CD203a, aka SWC9) markers. One-way ANOVA were used for statistical analyses.

RESULTS: The time to complete the total process, including the extraction of ribs, their bone marrow, and cell isolation was about 2 hours per pig. The total cell number obtained after Ficoll separation was $1.4 \pm 0.4 \times 10^9$ cells. On average, each rib yielded about 1×10^8 cells. The number of cells obtained by centrifuging at three different times varied from 1.4 to 5×10^8 cells. Increasing the centrifuge time of the rib pieces from 1 min to 3 mins increased cell extraction numbers, but at a cost of decreased cell viability (Fig. 2A). The centrifuge duration of 0.5+1 min provided the best balance of cell number and viability. The centrifuge time also affected the inflammatory cytokine expression; cells isolated using 3 mins centrifugation expressed significantly higher IL1β ($p = 0.0066$) and IL8 ($p = 0.0016$), compared to the cells isolated with 1 min and 0.5+1 min centrifugation (Fig. 2B). FACS analysis showed that 96.7% cells were CD14+ cells indicating a high purity of macrophages. LPS-stimulated macrophages (M1) showed an increased expression of CD8086, and the Net Mean Fluorescence Intensity (MFI) of CD8086 increased from 981 ± 91 in the M1-stimulated macrophages compared to 643 ± 83 in the control group. M2-stimulated macrophages showed an increased expression of CD203a. The MFI of CD203a increased from 3321 ± 383 in the M2-stimulated macrophages compared to 2167 ± 266 in the control group.

DISCUSSION: Our study shows a high yield of macrophages using the new method described above. We found that the duration of centrifugation of the rib pieces is an important variable that affects cell number, viability, and inflammatory cytokine expression. A shorter duration of centrifuge (0.5+1 min) that minimizes cell stress and maximizes cell viability is preferred. The macrophage culture conditions described also produced a high percentage of CD14+ cells with M1 and M2 cell markers after M1 and M2 stimulation, respectively.

SIGNIFICANCE: The study provides a fast and reliable method for macrophage isolation from porcine ribs for inflammation and immune-related research in the field of orthopedics. The use of porcine ribs as a source of bone marrow-derived macrophages provides flexibility in obtaining a large number of macrophages by increasing the number of ribs extracted.

REFERENCES: [1] Gao, et al., 2018, Vet. Immunol.Immunopathol., 200, 7-15, [2] Kapetanovic, et al., 2013, BMC Genomics, 14, 581, [3] Meli, et al. 2021, Biomater Sci., 9(23), 7851-7861.

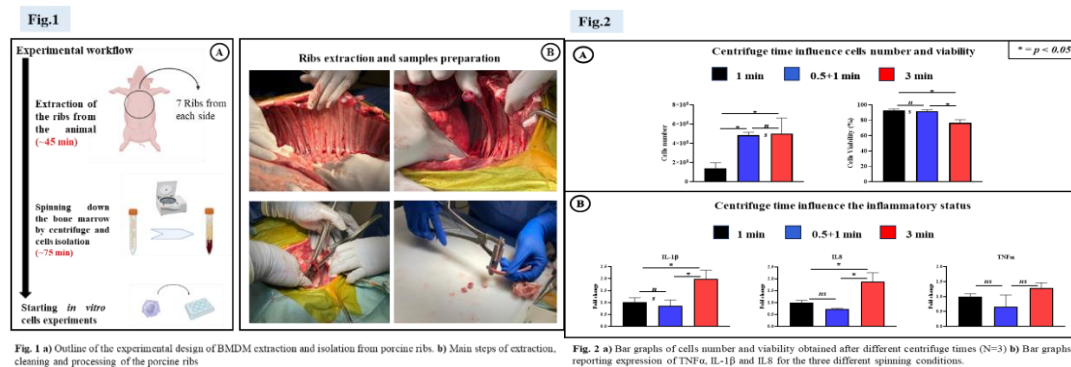


Fig. 1 a) Outline of the experimental design of BMDM extraction and isolation from porcine ribs. b) Main steps of extraction, cleaning and processing of the porcine ribs

Fig. 2 a) Bar graphs of cells number and viability obtained after different centrifuge times (N=3) b) Bar graphs reporting expression of TNFα, IL-1β and IL8 for the three different spinning conditions.

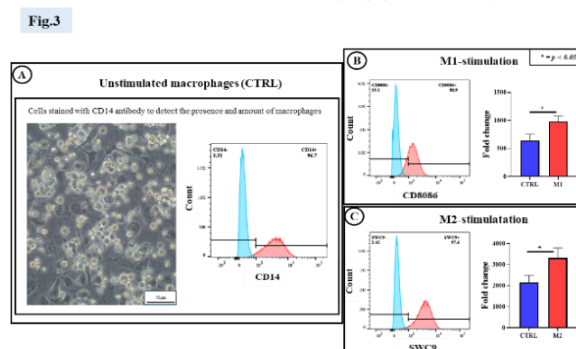


Fig. 3 a) Representative optical image of macrophages collected at 20X of bone marrow derived macrophage and the flow cytometry graph related to the expression of CD14. b) Flow cytometry image of CD8086 after LPS stimulation and Bar graph illustrating the MFI associated to CD8086 fluorescence. c) Expression of SWC9 after M2 stimulation using IL4+IL13 and MFI related reported in the bar graph.