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Technical note

New method for isolation of rat lamina propria macrophages in colonic tissue

Ana Bargalló [a,b], Laura Abad [b], Gemma Òdena [c,d,f], Ramon Planas [d,e], Ramon Bartolí [b,d,*]

[a] Gastroenterology Department, Hospital De Sant Joan Despí Moisès Broggi, Sant Joan Despí, Barcelona, Spain
[b] Germans Trias i Pujol Foundation (IGTP), Badalona, Spain
[c] Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[d] CIBERehd, Ciber de Enfermedades Hepaticas y Digestivas, Barcelona, Spain
[e] Hepatology Unit, Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain
[f] Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

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ABSTRACT

Studies on intestinal cells in the lamina propria are important for understanding the cellular and immune responses in the gut. There is a lack of specific isolating procedures of macrophage cells in rats. Two different procedures of macrophage isolation of the lamina propria in rats are compared: a standard mice protocol for lymphocyte isolation (A) adapted to rat samples and a new protocol developed specifically for rats (B). Significant differences are observed when analyzing the effect of the isolation method on the cell number, viability and phenotype. This has important implications when further functional studies are required.

1. Background

Antigen presenting cells (APC), mainly macrophages and dendritic cells, can be found in a large variety of organs in the body, where they hold a crucial role in innate and adaptive immunity in physiological and pathological conditions. They arise from myeloid stem cells in the bone marrow and migrate to peripheral blood and tissues, where they display a great diversity of phenotype and functions (Weber et al., 2009; Chiang et al., 2008). Upon recognition of diverse microbial targets they undergo changes that initiate pathogen uptake, activate signaling pathways and induce acquired immunity.

Macrophage subpopulations located in different tissues are interesting to study since they are adapted to specialized activity within their local environment (Weber et al., 2009). Macrophages in the gut-associated lymphoid tissue (GALT) play an important role in the maintenance of intestinal immune responses adapted to the local environment and the presence of enteric bacteria (Weber et al., 2009) displaying an important role in maintaining local tissue homeostasis. They represent 10–20% of all mononuclear cells found in the intestinal lamina propria making the gastrointestinal tract mucosa the largest reservoir of macrophages in human and mice (Schenk and Mueller, 2007). Studies on intestinal cells in the lamina propria are important for understanding the cellular and immune responses in the gut (Carrasco et al., 2013; Weigmann et al., 2007; Platt and Mowat, 2008).

Macrophages are extremely responsive to a variety of stimuli which can markedly affect their phenotype and functional activities. Those properties make isolation of macrophages a challenge in experimentation. The isolation

* Corresponding author at: Germans Trias i Pujol Health Sciences Research Institute, Carretera de Can Ruti, Cami de les Escoles s/n. 08916, Badalona, Spain. Tel.: +34 934978688; fax: +34 934978654.
E-mail address: rbartolisole@gmail.com (R. Bartolí).

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and purification procedure as well as digestion time can induce important changes in the isolated cell populations, which may have important implications in functional study designs (Chiang et al., 2008; Paulnock, 2000). A maximum cell yield without disturbing functional properties is desired.

Direct assay of cells immediately following isolation is the best way to characterize macrophage function. Although there are several protocols described in literature for human and mice gut macrophage isolation (Carrasco et al., 2013; Weigmann et al., 2007; Paulnock, 2000; Smith et al., 2007), to our knowledge there are no specific isolating procedures for these cells in rats. Moreover, current protocols for mouse or human do not guarantee good results, since the different purification procedures include long separation times, the use of enzymes that could damage cellular integrity and/or toxicity of cell separation mediums (Paulnock, 2000).

In this sense we adapted a validated protocol for the isolation of lamina propria mononuclear cells (LPMCs) in mice that has reported between 9% and 12% $CD11b^+$ cells (Weigmann et al., 2007; Denning et al., 2007). Taking into account that the results obtained in our laboratory in rats did not reach such percentages (2%–2.5%), a new approach was needed.

In this study we evaluate and compare two procedures of macrophage isolation of lamina propria in rats; protocol A (adapted from mice) and protocol B (new method), analyzing the effect of the isolation method on the cell number, viability and phenotype.

2. Methods

2.1. Animal inclusion, samples & study design

Four Sprague–Dawley male rats weighting 300–350 g were included in the study. Rats were housed individually in polycarbonate box cages with free access to water and food (2014 Teklad Global, Harlan Laboratories Model SL, Barcelona, Spain) for 7 days prior to their euthanasia. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Germans Trias i Pujol Research Institute (permit number DAAM 6710).

Rats were euthanized by anesthetic (Smith et al., 2007) overdose (Fluothane™). The abdomen was opened longitudinally and the caecum was excised and gently rinsed with cold PBS. Two 0.5 g pieces of the caecum were placed in cold PBS with gentamicin (50 μg/mL PBS).

2.1.1. Predigestion

In order to eliminate epithelial cells, the two samples of the caecum were cut into small pieces of 0.5 cm and placed in a 10 mL polypropylene tube with 5 mL of predigestion solution (BSA 1.5% + EDTA 10 mM + PBS) and incubated at 37 °C for 20 min with mixing by a shaker (150 rpm). Afterwards, they were mixed by a vortex mixer for 10 s and passed through a cotton mesh filter and tissue was harvested. This step was repeated with fresh predigestion solutions. After filtration, the tissue was placed in a new polypropylene tube with 10 mL of HBSS+ and incubated at 37 °C for 5 min on the shaker to displace the EDTA. Again, tissue was filtered and harvested, and was ready for the digestion protocols (Weigmann et al., 2007; Smith et al., 2007).

2.1.2. Digestion

2.1.2.1. Protocol A. Tissue was placed in a 50 mL falcon tube with 10 mL of digestion solution (1 mg/mL collagenase type VIII + 3 mg/mL dispase II + 40 μg/mL DNase I + 5% FBS) + HBSS (all reagents from Sigma-Aldrich) incubated at 37 °C for 30 min on the shaker (150 rpm). Tissue was filtered through a 100 μm nylon mesh filter (Cell-strainer, Becton Dickinson, NJ, USA). Filtered suspensions were rinsed with PBS. Remaining pieces of colon were placed again in a falcon tube with fresh digestion solution for 30 min more, until tissue appearance was soft, and then was passed through a 100 μm nylon mesh filter and was mechanically disaggregated using a syringe plunger. The cell suspension was obtained, rinsed with PBS, placed in the first cell suspension tube and centrifuged at 280 *g*, at 4 °C for 5 min. The supernatant was discarded and the pellet was resuspended in fresh PBS. Finally, the cell sample was filtered again through a 40 μm nylon mesh filter in order to eliminate debris, centrifuged at 280 *g*, at 4 °C for 5 min and resuspended in 1 mL PBS (Weigmann et al., 2007; Smith et al., 2007).

2.1.2.2. Protocol B. Tissue was placed in a gentleMACS™ C tube with 2.5 mL of 400 μg/mL Liberase™ (Roche) + 40 μg/mL DNase I (Sigma-Aldrich) + 1.5% BSA in HBSS+ (Sigma-Aldrich) and placed in a gentleMACS™ Dissociator (program m_brain_01) (adapted from Milteny Biotech dissociation protocols, unpublished data). Then it was incubated at 37 °C for 30 min on the shaker (100 rpm). The sample was then placed in a gentleMACS™ Dissociator (program B). The cell suspension was passed through a 100 μm mesh filter in a 50 mL falcon tube and rinsed with PBS. The sample was centrifuged at 280 *g*, at 4 °C for 5 min, the supernatant was discarded and the pellet was resuspended in fresh PBS and filtered again through a 40 μm nylon mesh filter in order to eliminate debris, centrifuged at 280 *g*, at 4 °C for 5 min and resuspended in 1 mL PBS.

The two protocols are summarized in Fig. 1.

2.1.3. Counting

Samples (15 μL) of the two protocols were mixed with an equal volume of 4% trypan blue (sigma). After 5 min at room temperature, 10 μL of each sample were placed in a Neubauer hemocytometer. The number and viability of the total cells obtained from digested caecums were recorded.

2.1.4. CD11b staining

Cells were resuspended in blocking buffer (PBS + 50 μL/mL inactivated rat serum (Sigma-Aldrich) + 1 μL/mL sodium azida (Sigma-Aldrich)) at a concentration of 10^6 cells/mL. Samples of 100 μL were placed at 4 °C for 10 min before staining. Then, 2 μL of CD11b-RPE antibody was added to each of the samples and these were placed in the dark for 20 min. Afterwards, 3 mL of wash buffer (PBS + 5 μL/mL inactivated rat serum + 1 μL/mL sodium azida) were added and samples were centrifuged at 280 *g* at 4 °C for 5 min. The 3 mL supernatant was discarded, leaving only a volume of 100 μL in the tube. Samples were