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Technical note

An optimised perfusion technique for extracting murine gastric leukocytes

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ABSTRACT

The stomach is a difficult tissue to analyse by flow cytometry, largely due to the difficulty of isolating viable leukocytes. Here we present the re-optimization of a perfusion technique that compares favourably against two other methods of enzymatic digestion for the release of gastric leukocytes. We believe that this technique could greatly assist the analysis of immune cells basally present in the murine stomach and that infiltrate during infection or disease.

1. Introduction

While leukocyte isolation from the intestinal compartment of mice is relatively well established (Goodyear et al., 2014), only a limited number have focused on the gastric compartment (Alderuccio et al., 1995; Ruiz et al., 2012; Quiding-Järbrink et al., 2010). Indeed, immune cells in the stomach are generally considered as notoriously difficult to analyse by flow cytometry. This limitation has contributed to our poor understanding of the immunology of the stomach. Improved methods of isolating leukocytes would greatly assist studies aimed at examining gastric immunology.

As the stomach is a quite fibrous tissue, most descriptions use enzymatic digestion (primarily collagenases) to successfully dissociate gastric cells into a suspension. There are however drawbacks to using enzymatic digestion to release cells from tissues. Often collagenases can have a high degree of lot-to-lot variability which results in differential activity (Van der Heijden and Stok, 1987). Excessive enzymatic digestion may reduce cell viability, or degrade cellular antigens (Goodyear et al., 2014; Van Damme et al., 2000), resulting in variation in antibody labelling.

Here we describe the optimization of a reliable and reproducible method for the isolation of gastric leukocytes for flow cytometric analysis that does not require digestion of stomach tissue. This method is compared to alternative enzymatic methods for isolating gastric leukocytes.

2. Materials and methods

2.1. Perfusion

Gastric cellular infiltrates to be analysed by flow cytometry were isolated from matched 8–12 week old specific-pathogen free C57BL/6 and NOD-scid *Il2rg*$^{-/-}$ (NSG) mice (Ito et al., 2002) by perfusion using a technique modified from Alderuccio et al. (1995). Stomachs, opened along the inner curvature and contents discarded, were collected into HBSS (Gibco) then perfused with ~7 mL of HBSS containing 5% FCS (Gibco), 5 mM EDTA and/or 1 mM dithiothreitol (perfusion solution). This was performed by slowly inserting a 27G needle at a very shallow angle into the mucosa near the limiting ridge while expelling perfusion solution. This caused the mucosa to swell (Fig. 1A), and further fluid was then injected into sites adjacent to the swelling to expand the disrupted locations (Fig. 1B). This was repeated as necessary until all mucosae were fully inflated. Perfused stomachs were incubated at 37 °C for 15 min then cut into ~0.5 cm pieces and poured through a 70 μM cell strainer. After a further 10 min in 10 mL of fresh perfusion solution, pieces were vortexed and again poured through a 70 μM cell strainer. Cells in both filtrates were pooled and collected by centrifugation at 600 *g* for 5 min.

2.2. Enzymatic digestion of stomachs

Stomachs were digested with collagenase as previously described (Viala et al., 2004). Briefly, stomachs were cut into ~0.5 mm pieces then centrifuged at 150 *g* for 5 min. The pellet was resuspended in 7 mL of HBSS containing 0.2% BSA and 0.4 mg/mL collagenase A (Roche) and incubated shaking at 37 °C, 120 rpm. Ten mL complete DMEM was added and digested tissue dissociated by three repeats of

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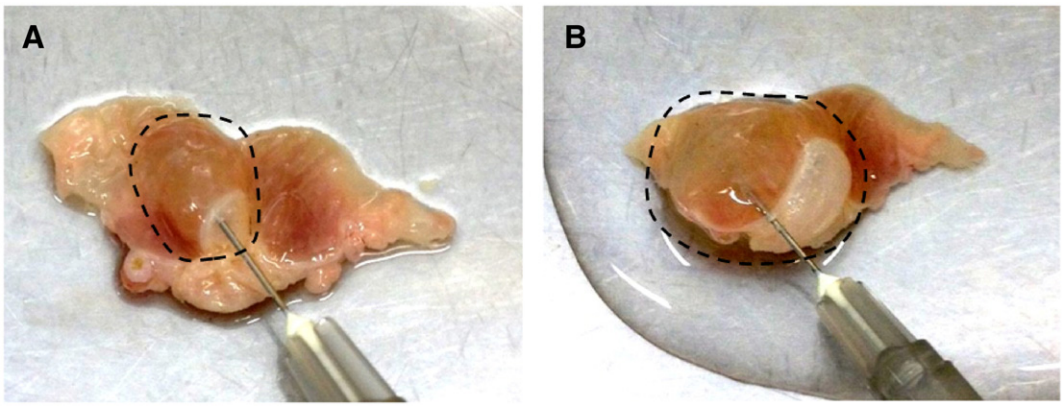

Fig. 1. Perfusion of Stomachs. Stomachs were opened along the inner curvature. (A) Fluid was injected near the limiting ridge using a 27G needle. This caused the mucosa to swell (area indicated by dotted line). (B) Further fluid was injected into the site, then in adjacent sites to further inflate the mucosa until half the stomach was fully inflated (indicated by dotted line).

forceful pipetting using serological pipettes for 5 min. Alternatively the Lamina Propria Kit and the gentleMACS dissociator (Miltenyi Biotec) were used as per manufacturer's instructions. Cell suspensions were filtered through a 70 μM cell strainer (Falcon Corning).

2.3. Flow cytometry

Cells were blocked in 100 μL of 20% normal mouse serum (collected in house) and 1 μg/mL anti-FcγII/FcγIII (2.4G2, Walter and Eliza Hall Institute) on ice for 20 min. Cells were stained with anti-CD45-PE.Cy5.5 or anti-CD45-Alexa 700 (30-F11), anti-CD11c-FITC (M5/114.15.2; eBioscience, San Diego, CA, USA), anti-CD11b-BV421 (M1/70), anti-CD19-BV510 (6D5), anti-CD4-BV650 (RM4-5), anti-CD103-BV786 (2E7), anti-CD64-PE (X54-5/7.1), anti-MHCII-PE.Cy7 (M5/114.15.2) and anti-Ly6G-APC.Cy7 (1A8; all from BioLegend, San Diego, CA, USA unless indicated). Cells were washed twice, resuspended in 0.25 mg/mL propidium iodide (ImmunoChemistry Technologies, Bloomington, MN, USA) and acquired on a BD LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA, USA). Single cells were identified as a linear population on FSC-A vs FSC-H then propidium iodide positive dead cells excluded. Cell counts were normalised using AccuCount Fluorescent Particles (Spherotech, Lake Forest, IL, USA). Data were analysed using FCS Express (De Novo Software, Los Angeles, CA, USA).

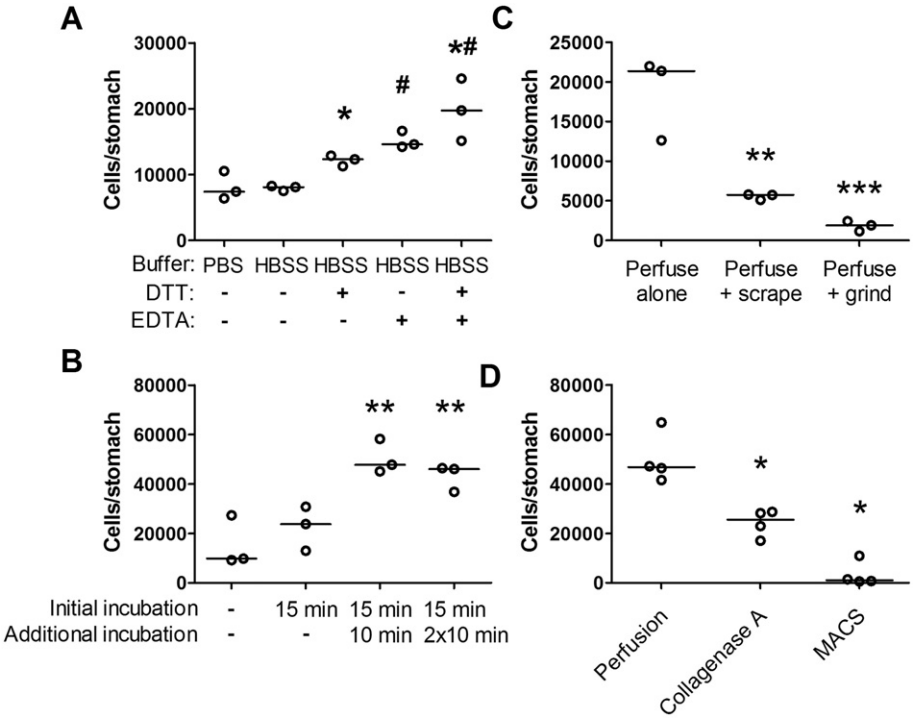

Fig. 2. Optimization of the perfusion technique and comparison to enzymatic digestion. (A) Stomachs were perfused with PBS or HBSS with 1 mM DTT and/or 5 mM EDTA and the cells isolated analysed by flow cytometry to quantify CD45 + cells. Both DTT and EDTA significantly increased the number of CD45 + cells (*p = 0.01 and #p = 0.0009 respectively; Two-way ANOVA). (B) Stomachs were perfused with HBSS plus DTT/EDTA then the mucosa separated by scraping with a scalpel blade or the stomach ground through a 70 μM strainer. Both mechanical treatments significantly reduced CD45 + cell yield (ANOVA cf. perfusion alone). (C) Stomachs were perfused with HBSS plus DTT/EDTA and then incubated at 37 °C for 15 min followed by further incubations for 10 min (ANOVA cf. no incubation). (D) CD45 + cells isolated with the final perfusion procedure were compared to the efficacy of collagenase A digest and dissociation using the Lamina Propria Kit and gentleMACS dissociator (Miltenyi Biotec). Perfusion resulted in the isolation of significantly more CD45 + cells (ANOVA cf. perfusion alone). All experiments used stomachs from C57BL/6 mice. Graphs present individual stomachs (points) and median (bar); *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA).