Development and optimization of a non-enzymatic method of leukocyte isolation from macaque tissues

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Abstract

Background and Methods—Cell isolation from macaque tissues involves laborious enzymatic digestion. The Medimachine provides a simpler, quicker non-enzymatic method, yielding 1.5–5 million cells/g of vaginal or rectal tissue from pigtailed macaques.

Results and Conclusions—Flow cytometry analysis of the two methods revealed similar levels of cell viability and most major cell lineage and activation markers.

Keywords

collagenase digestion; flow cytometry; *Macaca nemestrina*; Medimachine

Introduction

Macaques are integral in the development of pre-exposure prophylaxis (PrEP) modalities of HIV-1 prevention [2, 5, 6, 15, 16, 23]. The characterization of leukocytes in vaginal and rectal compartments is required to define target or protective cells at these sites and is important for PrEP development. Conventional methods of cell isolation from macaque tissues involve a lengthy, multistep process with tissue digestive enzyme (s) followed by Ficoll separation [4, 13, 14, 18, 19, 21]. While this approach is useful for processing large amounts of tissue, the expression of cell surface receptors can be negatively affected [1, 3, 8, 9, 11, 12, 20, 22]. A non-enzymatic technique was therefore explored. The Medimachine is a compact tissue disaggregation instrument with dimensions that make it conducive to placement in a standard biologic safety cabinet. This system can yield viable cells from tissue samples such as human cervical explants and rodent testicles [7, 13, 17]. Described herein is a Medimachine-based method that has been optimized for cell isolation from macaque vaginal and rectal tissues.

Materials and methods

Instruments

The Medimachine (<5 lbs, 6.5 in 9 7.5 in 9 7.5 in) and Medicon chambers were purchased from BD BioSciences (San Jose, CA, USA).

Animals

Pigtailed macaques were housed under biosafety level 2 containment conditions according to the *Guide for the Care and Use of Laboratory Animals* at the Centers for Disease Control and Prevention. Four female pigtailed macaques infected with simian human immunodeficiency virus (SHIV) were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines on Euthanasia, June 2007. All procedures were approved by the CDC Animal Care and Use Committee.

Tissue and blood processing

Vaginal and rectal tissues obtained at necropsy from two macaques were enzymatically digested as previously described [4]. Tissues measuring 2 cm × 2 cm from the remaining two animals were processed using the Medimachine. Tissue sections were weighed, diced into 5 mm sized pieces using a sterile disposable scalpel, and processed in successive batches of 4–5 pieces. Approximately 500 μl of RPMI containing 20% fetal calf serum was added into the Medicon chamber's port using a sterile Pasteur pipette. Tissue was processed for 30 s–1 minute at 100 rpm. The cell suspension was collected from the chamber's port using a sterile Pasteur pipette. The chamber was rinsed once with fresh media to increase yield. Each chamber was used no more than three times for each macaque tissue sample. Cell suspensions from each run were pooled and run through a 50 micron filter twice. Cells were washed once by spinning at $300 \times g$ for 5 minutes, then resuspended in 1 ml of fresh RPMI and counted. Peripheral blood mononuclear cells (PBMC) were obtained by standard Hypaque-Ficoll centrifugation. Cell counts were performed using the Invitrogen Countess (Invitrogen, Carlsbad, CA, USA).

Flow cytometry

Cells were surface stained, fixed, and refrigerated at 4°C overnight, followed by flow cytometry using the BD LSRII instrument according to manufacturer's protocols (BD Biosciences, Carlsbad, CA, USA). An 11-color flow panel of cross-reactive antibodies was used: CD3 Alexa700, CD4 BD-V500, CD8 BD-V450, CD20 FITC, CCR5 PE, CXCR4 PE-CF594, CCR7 PE-Cy7, CD45RA PE-Cy5, CD14 APC, CD69 APC-H7, and HLA-DR BV605 (BD Biosciences). Samples were analyzed using FlowJo (TreeStar Inc, Ashland, OR, USA), and results graphed using GraphPad Prism 5 (Graphpad Software Inc, La Jolla, CA, USA).

Results and discussion

Vaginal and rectal tissue samples from female pigtailed macaques (N = 2) were processed using the Medimachine as outlined above. A yield of 1.5–5 million cells/gram of tissue was obtained, with a processing time that was approximately half the 3–5 hours typically

associated with enzymatic digestion. A comparison of cell viabilities and expression of surface markers on cells isolated using the non-enzymatic (N = 2) and standard enzymatic methods (N = 2) was performed using a detailed 11-color flow cytometry panel. As shown in Fig. 1A (upper panel), the viabilities of rectal tissue-derived cells, based on forward and side scatter profiles, were similar between the two methods. However, a well-defined population of live cells was noted for Medimachine-processed tissues, while this population appeared less distinct in enzymatically processed samples. The separation of CD20^{+} B-cell and CD3^{+}CD4^{+}/CD8^{+} T-cell lineages into distinct subsets was also evident for Medimachine-derived cells and is consistent with the pattern normally seen with enzymatically digested tissue (Fig. 1A) and with PBMC (Fig. 1C). Similar staining patterns were observed for cells isolated from macaque vaginal tissue using the Medimachine, as illustrated in Fig. 1B.

In addition to confirming the identification of major T-cell and B-cell lineages, the potential effects of enzymatic and non-enzymatic methods on the levels of monocyte, trafficking, and activation markers were also examined. Specifically, the levels of CCR5, CXCR4, CCR7, CD45RA, CD14 and the early and late activation markers CD69 and HLA-DR, respectively, were determined by flow cytometry and are summarized in Fig. 2. The expression levels of these markers on cells isolated from rectal tissue were comparable between the two methods (Fig. 2). However, greater than twofold lower levels in CD4 expression were observed for enzymatically digested tissues, with CD14 expression being nearly absent when compared to non-enzymatically processed tissues (Fig. 2, upper panel). Similar observations were noted for vaginal tissue-derived cells (data not shown). While conclusions about the impact of enzymatic digestion on CD4 and CD14 cell surface markers cannot be made in this study, given the small sample size, that each macaque was at different stages of SHIV infection and that the methods were not used in parallel in the same animal, it is noteworthy that previous reports have also shown reductions in the expression of both these cell surface molecules following enzymatic treatment [1, 3, 8–12]. These observations collectively emphasize the need for cautious interpretation of lymphocyte phenotypes obtained via enzymatic digestion.

In summary, the Medimachine requires fewer reagents and is a simpler, quicker alternative for tissue processing than enzymatic digestion. While the latter technique offers the advantage of processing larger amounts of tissue at a time, the apparent minimal impact of non-enzymatic processing on cell surface molecules may prove to be a better option for downstream flow cytometry applications.

References

1. Abuzakouk M, Feighery C, O'Farrelly C: Collagenase and dispase enzymes disrupt lymphocyte surface molecules. J Immunol Methods 1996; 194:211–6. [PubMed: 8765174]

2. Barouch DH, Klasse PJ, Dufour J, Veazey RS, Moore JP: Macaque studies of vaccine and microbicide combinations for preventing HIV-1 sexual transmission. Proc Natl Acad Sci USA 2012; 109:8694–8. [PubMed: 22586094]

3. Damme NV, Baeten D, Vos MD, Demetter P, Elewaut D, Mielants H, Verbruggen G, Cuvelier C, Veys EM, Keyser FD: Chemical agents and enzymes used for extraction of gut lymphocytes influence flow cytometric detection of T cell surface markers. J Immunol Methods 2000; 236:27–35. [PubMed: 10699577]

4. Dobard C, Sharma S, Martin A, Pau CP, Holder A, Kuklenyik Z, Lipscomb J, Hanson DL, Smith J, Novembre FJ, García-Lerma JG, Heneine W: Durable protection from vaginal simian-human immunodeficiency virus infection in macaques by tenofovir gel and its relationship to drug levels in tissues. J Virol 2012; 86:718–25. [PubMed: 22072766]

5. Garcia-Lerma JG, Heneine W: Animal models of antiretroviral prophylaxis for HIV prevention. Curr Opin HIV AIDS 2012; 7:505–13. [PubMed: 22964889]

6. García-Lerma JG, Paxton L, Kilmarx PH, Heneine W: Oral pre-exposure prophylaxis for HIV prevention. Trends Pharmacol Sci 2010; 31:74–81. [PubMed: 19963288]

7. Gupta P, Collins KB, Ratner D, Watkins S, Naus GJ, Landers DV, Patterson BK: Memory CD4+ T cells are the earliest detectable human immunodeficiency vorus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. J Virol 2002; 76:9868–76. [PubMed: 12208964]

8. Hagman DK, Kuzma JN, Larson I, Foster-Schubert KE, Kuan LY, Cignarella A, Geamanu E, Makar KW, Gottlieb JR, Kratz M: Characterizing and quantifying leukocyte populations in human adipose tissue: impact of enzymatic tissue processing. J Immunol Methods 2012; 386:50–9. [PubMed: 22974837]

9. Ikejima K, Enomoto N, Seabra V, Ikejima A, Brenner DA, Thurman RG: Pronase destroys the lipoplysaccharide receptor CD14 on Kupffer cells. Am J Physiol 1999; 276: G591–8. [PubMed: 10070034]

10. Landuyt KBV, Jones EA, McGonagle D, Luyten FP, Lories RJ: Flow cytometric characterization of freshly isolated and culture expanded human synovial cell populations in patients with chronic arthritis. Arthritis Res Ther 2010; 12:R15. [PubMed: 20105279]

11. Le-Barillec K, Si-Tahar M, Balloy V, Chignard M: Proteolysis of monocyte CD14 by human leukocyte elastase inhibits lipopolysaccharide-mediated cell activation. J Clin Invest 1999; 103:1039–46. [PubMed: 10194477]

12. Mulder WM, Koenen H, van de Muysenberg AJ, Bloemena E, Wagstaff J, Scheper RJ: Reduced expression of distinct T-cell CD molecules by collagenase/DNase treatment. Cancer Immunol Immunother 1994; 38:253–8. [PubMed: 8168120]

13. Novelli M, Savoia P, Cambieri I, Ponti R, Comessatti A, Lisa F, Bernengo MG: Collagenase digestion and mechanical disaggregation as a method to extract and immunophenotype tumour lymphocytes in cutaneous T-cell lymphomas. Clin Exp Dermatol 2000; 25:423–31. [PubMed: 11012601]

14. Pan D, Das A, Liu D, Veazey RS, Pahar B: Isolation and characterization of intestinal epithelial cells from normal and SIV-infected rhesus macaques. PLoS One 2012; 7: e30247. [PubMed: 22291924]

15. Patton DL, Sweeney YT, Paul KJ: A summary of preclinical topical microbicide rectal safety and efficacy evaluations in a pigtailed macaque model. Sex Transm Dis 2009; 36:350–6. [PubMed: 19556929]

16. Pereira LE, Srinivasan P, Smith JM: Simian-human immunodeficiency viruses and their impact on nonhuman primate models for AIDS In: Immunodefiency. Metodiev (ed). Rijeka, Croatia: InTech Open Access, 2012; 311–56.

17. Rodriguez-Casuriaga R, Geisinger A, Lopez-Carro B, Porro V, Wettstein R, Folle GA: Ultra-fast and optimized method for the preparation of rodent testicular cells for flow cytometric analysis. Biol Proced Online 2009; 11:184–95. [PubMed: 19495915]

18. Shen R, Richter HE, Clements RH, Novak L, Huff K, Bimczok D, Sankaran-Walters S, Dandekar S, Clapham PR, Smythies LE, Smith PD: Macrophages in vaginal but not intestinal mucosa are monocyte-like and permissive to human immunodeficiency virus type 1 infection. J Virol 2009; 83:3258–67. [PubMed: 19153236]

19. Shen R, Richter HE, Smith PH: Early HIV-1 target cells in human vaginal and ectocervical mucosa. Am J Reprod Immunol 2012; 65:261–7.

20. Shields PL, Morland CM, Salmon M, Qin S, Hubscher SG, Adams DH: Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. J Immunol 1999; 163:6236–43. [PubMed: 10570316]

21. Stevceva L, Kelsall B, Nacsa J, Moniuszko M, Hel Z, Tryniszewska E, Franchini G: Cervicovaginal lamina propria lymphocytes: phenotypic characterization and their importance in cytotoxic T-lymphocyte responses to simian immunodeficiency virus SIC-mac251. J Virol 2002; 76:9–18. [PubMed: 11739667]

22. Unsoeld H, Pircher H: Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. J Virol 2005; 79:4510–3. [PubMed: 15767451]

23. Veazey RS: Animal models for microbicide safety and efficacy testing. Curr Opin HIV AIDS 2013; 8:295–303. [PubMed: 23698560]
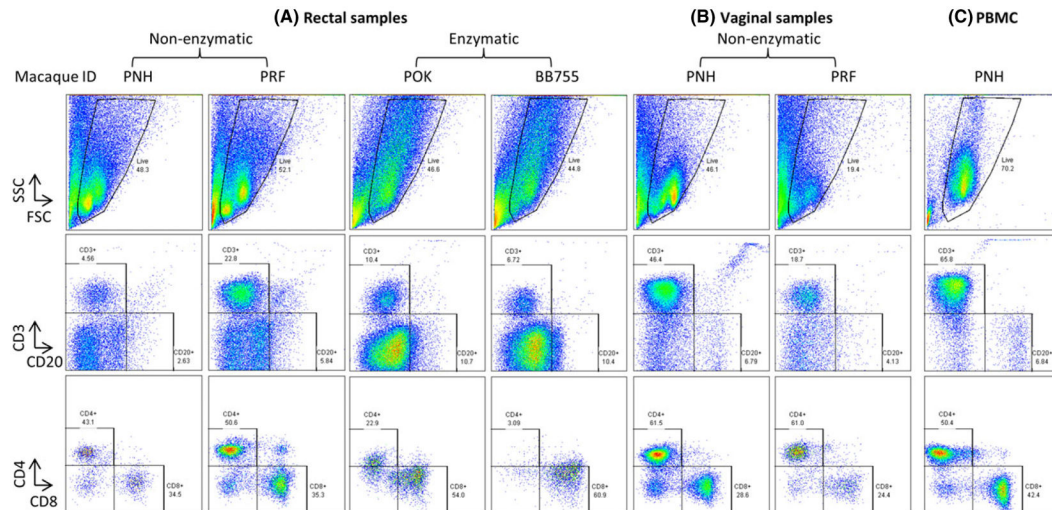
Fig. 1.
Flow cytometry gating strategy for identification of CD20$^+$ B-cells and CD3$^+$ T-cell subsets in (**A**) rectal mononuclear cells derived by the non-enzymatic Medimachine method (N = 2) or by enzymatic processing (N = 2) of rectal tissue, and (**B**) vaginal mononuclear cells from non-enzymatically processed vaginal tissue (N = 2), all from SHIV-infected macaques. Shown for comparison are representative data for (**C**) PBMC isolated from one of the SHIV-infected macaques.
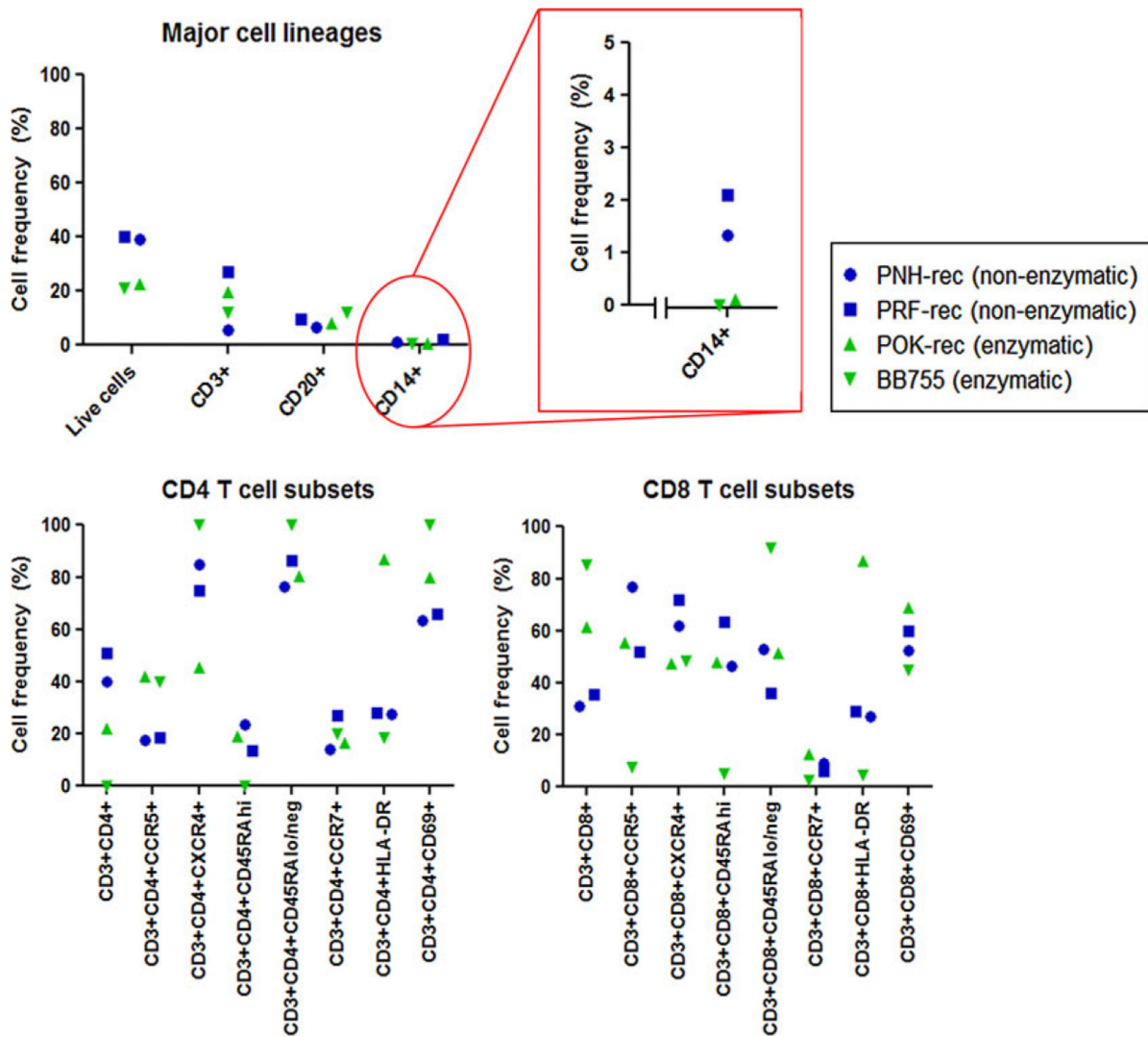
Fig. 2.
Comparison of B-cell, T-cell, and monocyte subsets (upper panel) in enzymatically derived (green) and non-enzymatically derived (Medimachine, blue) mononuclear cells from rectal tissues. The frequency of CD4$^+$ and CD8$^+$ T cells expressing trafficking and activation markers is also shown (lower panel).