Quantification of mucosal mononuclear cells in tissues with a fluorescent bead-based polychromatic flow cytometry assay

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Abstract

Since the vast majority of infections occur at mucosal surfaces, accurate characterization of mucosal immune cells is critically important for understanding transmission and control of infectious diseases. Standard flow cytometric analysis of cells obtained from mucosal tissues can provide valuable information on the phenotype of mucosal leukocytes and their relative abundance, but does not provide absolute cell counts of mucosal cell populations. We developed a bead-based flow cytometry assay to determine the absolute numbers of multiple mononuclear cell types in colorectal biopsies of rhesus macaques. Using 10-color flow cytometry panels and pan-fluorescent beads, cells were enumerated in biopsy specimens by adding a constant ratio of beads per mg of tissue and then calculating cell numbers/mg of tissue based on cell-to-bead ratios determined at the time of sample acquisition. Testing in duplicate specimens showed the assay to be highly reproducible (Spearman R = 0.9476, $P < 0.0001$). Using this assay, we report enumeration of total $CD45^+$ leukocytes, $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, $CD14^+$ monocytes, and myeloid and plasmacytoid dendritic cells in colorectal biopsies, with cell numbers in normal rhesus macaques varying from medians of 4486 cells/mg (T cells) to 3 cells/mg (plasmacytoid dendritic cells). This assay represents a significant advancement in rapid, accurate quantification of mononuclear cell populations in mucosal tissues and could be applied to provide absolute counts of a variety of different cell populations in diverse tissues.

Keywords

fluorescent beads; lymphocyte enumeration; flow cytometry; mucosal lymphocytes

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1. Introduction

Because most infections occur at mucosal surfaces, quantification of mucosal immune cells is critical to understand transmission and control of infectious diseases. Two of the most common approaches used to study mucosal specimens are flow cytometry and histological techniques. Although flow cytometry is a powerful tool to phenotypically characterize isolated mucosal immune cells, it is typically employed to determine the relative abundance of specific cell types but not absolute numbers of cells. While relative cellular quantification is adequate in many circumstances, such as addressing the frequency of expression of a phenotypic activation marker, these data can be misleading, because frequencies of the cell population of interest may be affected by influx or efflux of other cell populations. Histological techniques such as immunohistochemistry or immunofluorescence microscopy are the preferred techniques for determining the anatomic localization of cell populations *in situ*, but these approaches are relatively time-consuming and generally only approximate cell numbers in two dimensions such as mm^2 or by the area occupied by a given immunohistochemical marker (Li et al. 2005; Estes et al., 2008).

Given the limitations of existing approaches for the absolute quantification of cells in mucosal tissues, the need for a simplified, rapid assay which can accurately enumerate mucosal cells using minimal amounts of tissue is evident. We have developed a fluorescent bead-based method for quantifying absolute counts of lymphocytes in colorectal biopsies of rhesus macaques that could have significant applicability to both basic mucosal research and clinical diagnostics.

2. Material and Methods

2.1 Animals and biopsy collection

A total of 12 rhesus macaques (*Macaca mulatta*) of Indian genetic background were used for sampling in this study. All animals were free of SIV and were housed at the New England Primate Research Center and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for the Care and Use of Laboratory Animals. Macaques were anesthetized with ketamine HCl (10–20 mg/kg, intramuscularly) and then ten-to-twelve rectal punch biopsies were collected using 1.9 mm fenestrated endoscopic biopsy forceps (Olympus America, Center Valley, PA). Biopsy pieces were weighed in bulk (Mettler balances MS104S or PB602, Mettler-Toledo, Columbus, OH) and had a mean mass of 22.9 mg (range 20–50 mg, 30 samples).

2.2 Biopsy processing and cell isolation

Mononuclear cells were isolated from rectal biopsy samples using both enzymatic and mechanical disruption following standard protocols for our laboratory as described previously (Reeves et al., 2010). Briefly, after weighing, biopsies were first incubated for 30 min in 5 mM EDTA, washed, and then incubated in media with 15 U/ml collagenase (type II, Sigma) for 1 h on an orbital shaker. Samples were then mechanically disrupted with an 18-gauge feeding needle. To avoid cell loss, no density-based separation was performed.

2.2 Bead-based quantification assay

Absolute quantification of cell subsets in mucosal samples was performed using a fluorescent bead-based assay modified from a peripheral blood assay optimized in our laboratory (Reeves et al., 2009a; Reeves et al., 2010). After mononuclear cell isolation, all cells were resuspended in 2% FBS in PBS and then each cellular suspension was spiked with 10 μm SPHERO AccuCount Rainbow Fluorescent Particles (Spherotech, Lake Forest, IL) at a concentration of 1,000 beads/mg of absolute tissue weight (measured before

processing). Polychromatic flow cytometry (PFC) staining was then performed on each sample using fluorchrome-conjugated monoclonal antibodies (Table 1). All samples also included an Aqua Live/Dead stain (Invitrogen) to discriminate live cells and all acquisitions were made on an LSR II (BD Biosciences) then analyzed using FlowJo 9.0 software (Tree Star Inc., Ashland, OR).

3. Results and Discussion

As described in the Methods, 10-color flow cytometry panels optimized for use in rhesus macaques were used in combination with pan-fluorescent beads to calculate absolute cell numbers of mononuclear cells in colorectal biopsies based on cell-to-bead ratios. As shown in Fig. 1A, fluorescent beads were readily identified by their high SSC properties, and live leukocytes were identified in a $CD45^+$ inclusion gate excluding contaminating epithelial cells and debris that are common in mucosal preparations. While all assays contained markers to distinguish live $CD45^+$ cells, one of two different panels was used to identify T cells and NK cells (Fig. 1B) (Panel **I**), or B cells, monocytes and dendritic cells (Fig. 1C) (Panel **II**). $CD4^+$ and $CD8^+$ T cells were identified among the $CD3^+$ cell population, and NK cells were identified based on expression of NKG2A and lack of CD3, a strategy previously used for identification of these subpopulations in rhesus macaques (Reeves et al., 2010). B cells and monocytes were identified as $CD20^+HLA-DR^+$ and $CD14^+ HLA-DR^+$, respectively, and plasmacytoid (pDCs) and myeloid dendritic cells (mDCs) were enumerated among $HLA-DR^+CD3^-CD14^-CD20^-$ cells and positively identified by mutually exclusive expression of CD123 and CD11c, as described (Brown et al., 2007;Reeves et al., 2009b). The absolute number of cells/mg of tissue was calculated based on the number of gated cells and the number of bead events using the formula shown in Fig. 1D. In a subgroup of experiments, independent biopsy preparations were processed in parallel and these data were highly correlated, demonstrating a high level of reproducibility (Fig. 1E).

In colorectal biopsies we found a median number of total $CD45^+$ leukocytes of 8731 cells/ mg of colorectal tissue (Fig. 2), and not surprisingly, the total numbers of $CD45^+$ leukocytes strongly correlated with sample mass (R = 0.5452, $P < 0.0018$, Spearman correlation). While no previous assays have enumerated mucosal lymphocytes in this manner, Sopper et al. (2003), estimated total lamina propria lymphocytes to be between 1500–3700 lymphocytes per milligram of gut tissue, and because our analyses include total mucosal lymphocytes, both lamina proprial and intraepithelial, our estimates appear well in range. In regards to specific subpopulations of mucosal mononuclear cells, we found that approximately half of gut mononuclear cells were $CD3^+$ T cells (median, 4486 cells/mg). Furthermore, $CD4^+$ and $CD8^+$ T cells were found at a roughly 1:1 ratio (Fig. 2), similar to ratios previously reported in rhesus macaque gut mucosa by flow cytometry analyses (Sopper et al., 2003), but in contrast to the 2:1 or 3:1 ratios normally found in peripheral blood of both macaques and humans. Total $NKG2A^+$ NK cells were also found in colorectal biopsies at a median of 152 cells/mg, consistent with our previous observations of a relative abundance of ~5% of total $CD45^+$ colorectal mononuclear cells (Reeves et al., 2010).

Using Panel **II** (Fig. 1C), we enumerated $HLA-DR^+$ B cells, monocytes, and dendritic cells in colorectal biopsies (Fig. 2). B cells were relatively abundant in the rectal mucosa (median, 574 cells/mg). $CD14^+$ monocytes were detectable with a median value of 115 cells/mg, while mDCs and pDCs were less frequent, with median values of 43 cells/mg and 3 cells/ mg, respectively. Interestingly, $CD3^-CD14^-CD20^-HLA-DR^+CD123^-CD11c^-$ cells were relatively frequent in colorectal specimens with a median number of 489 cells/mg of tissue. This population that has been observed in macaques previously (Reeves et al, 2009b; Brown et al., 2008), and is most likely dominated by macrophages, based on intracellular staining

for CD68 (Fig. 1C). It is, however, possible that this population could contain other DC or monocyte subsets not phenotypically identifiable in our assay.

The development of this relatively simple, bead-based assay for mucosal mononuclear cell enumeration represents a significant step forward in analysis of small mucosal specimens. While beads have been previously used to calculate absolute counts for leukocyte populations in whole blood (Barnett et al., 1999; Montes et al., 2006) and track cell yields from biopsies (Anton et al., 2008), to our knowledge beads have never been used to enumerate mucosal cells based on tissue mass. It is important to note that cell frequencies calculated using this approach may be affected by factors such as cell loss during tissue processing. Nonetheless, the calculation of bead-normalized absolute counts based on tissue weights represents a significant advance over the current standard practice of reporting the relative frequency of cells in mucosal biopsies. This technique is suitable for application to the processing of relatively large numbers of specimens and should prove especially useful in settings such as HIV or SIV infection that result in substantial perturbations of lymphocyte populations in mucosal sites. Finally, this technique could have broad implications not only in basic mucosal immunology studies in animal models, but also in clinical research or human diagnostic studies.

Acknowledgments

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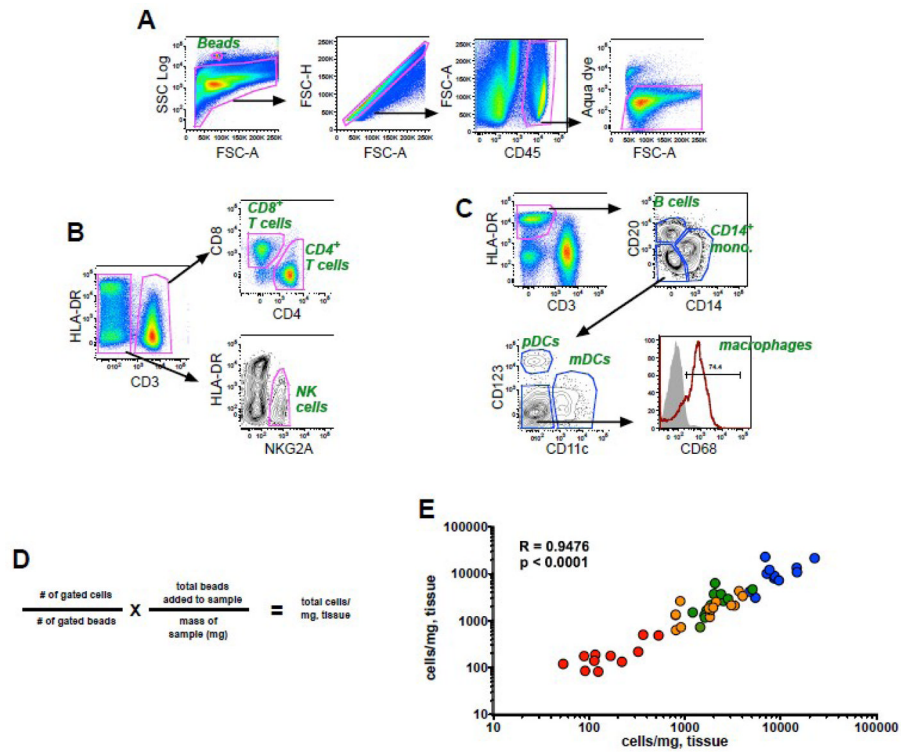
Figure 1. Bead-based cellular quantification in rectal biopsies
Representative gating strategies showing fluorescence profiles of (a) beads and live cells and subsequent gating for (b) T cells and NK cells or (c) monocytes, B cells, and dendritic cells. (d) Formula for calculation of cell numbers/milligram of tissue. (e) Correlation of absolute cell counts obtained from duplicate colorectal biopsies from 12 animals. Color coding: blue, CD45^{+} leukocytes; orange, CD8^{+} T cells; green, CD4^{+} T cells; red, NK cells. The correlation between the replicate tests was determined using the Spearman rank correlation coefficient.
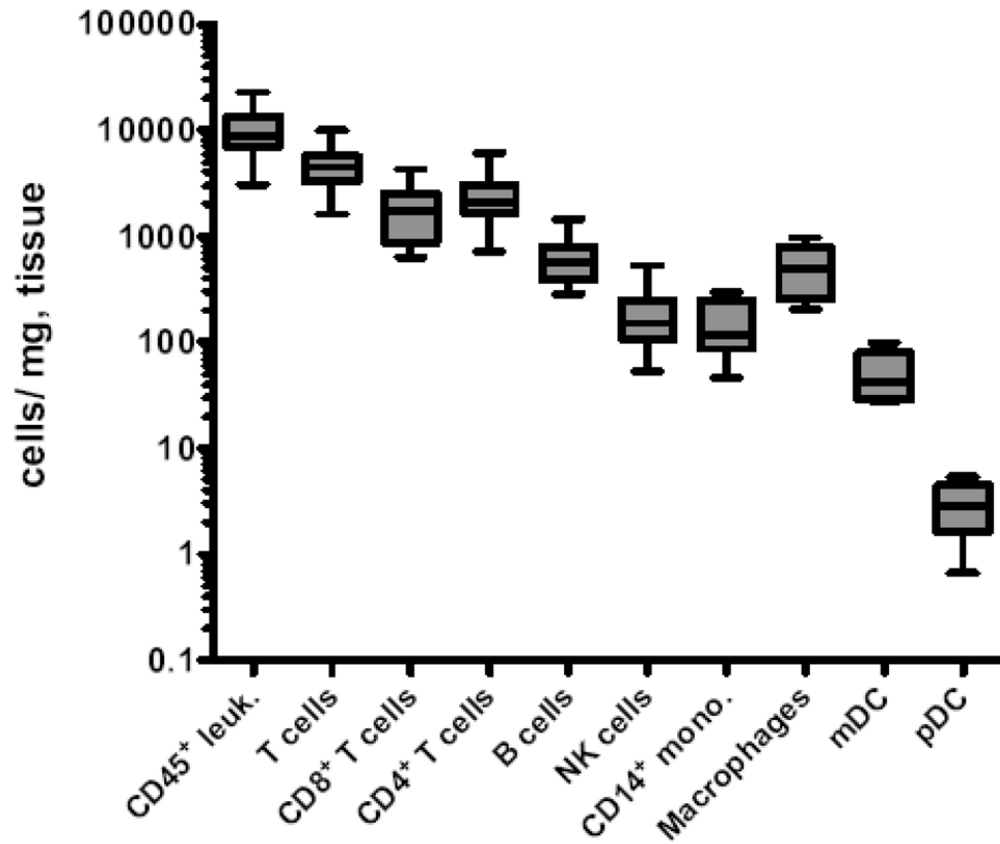
Figure 2. Enumeration of leukocyte subsets in the rectal mucosa
Horizontal lines indicate means and whiskers represent minimum to maximum of 6 to 12 animals per group. Macrophages were defined as $CD68^+CD3^-CD11c^-CD14^-CD20^-CD123^-HLA-DR^+$. Leuk., leukocytes; mono., monocytes.

Table 1

Antibodies used in flow cytometric bead assay.

Antibody	Clone	Fluorochrome	Manufacturer	Panel
anti-CD3	SP34.2	APC-Cy7	BD Biosciences (La Jolla, CA)	**I, II**
anti-CD4	L200	PE	BD Biosciences	**I**
anti-CD8α	RPA-T8	Alexa700	BD Biosciences	**I**
anti-CD11c	S-HCL-3	APC	BD Biosciences	**II**
anti-CD14	TUK4	Alexa700	Invitrogen (Carlsbad, CA)	**II**
anti-CD20	L27	PerCp-Cy5.5	BD Biosciences	**II**
anti-CD45	D058-1283	FITC	BD Biosciences	**I**
anti-CD45	D058-1283	Pacific Blue	BD Biosciences	**II**
anti-CD68	Y1/82A	PE	BD Biosciences	**II**
anti-CD123	7G3	PE-Cy7	BD Biosciences	**II**
anti-HLA-DR	Immu-357	PE-Texas Red	Beckman-Coulter (Fullerton, CA)	**I, II**
anti-NKG2A	Z199	Pacific Blue[a]	Beckman-Coulter	**I**

[a]In-house custom conjugate