An Optimized Five-Color/Seven-Parameter Flow Cytometry Panel for Immunophenotyping Guinea Pig Peripheral Blood Lymphocytes

John V. Stokes¹, Anna E. Crawford¹, Claire E. Cross¹, Anne-Marie L. Ross¹, Jamie D. Walker², Bridget V. Willeford², Andrea S. Varela-Stokes¹,*

¹Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762
²Laboratory Animal Resources, Mississippi State University, Mississippi State, MS 39762

Abstract

Guinea pigs are an ideal animal model for the study of several infectious diseases, including tuberculosis, legionellosis, brucellosis, and spotted fever rickettsiosis. In comparison to the murine model, clinical signs in guinea pigs are more representative of disease in humans, the guinea pig immune system is more similar to that of the human, and their large size offers logistic advantages for sample collection while following disease progression. Unfortunately, the advantage of using guinea pigs in biomedical research, particularly in understanding the immune response to infectious agents, is limited in large part by the paucity of available reagents and lack of genetically manipulated strains. Here, we expand the utility of guinea pigs in biomedical research by establishing an optimized five-color/seven-parameter polychromatic flow cytometric assay for immunophenotyping lymphocytes. This assay fills a need for immunophenotyping peripheral blood lymphocytes and is an improvement over current published flow cytometry assays for guinea pigs. We anticipate that our approach will be an important starting point for developing new assays to evaluate the cellular immune response to infectious diseases in the guinea pig model. Importantly, we are currently using this assay for evaluating immunity to spotted fever rickettsiosis in a guinea pig-tick-<i>Rickettsia</i> system, where CD8+ T cells are a critical contributor to the immune response. Developing resources to utilize the guinea pig more effectively will enhance our ability to understand infectious diseases where the guinea pig would otherwise be the ideal model.

*Corresponding author: Andrea S. Varela-Stokes, Department of Basic Sciences, 240 Wise Center Drive, Mississippi State, MS 39762, stokes@cvm.msstate.edu.

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Conflict of Interest Statement
The authors declare no conflict of interest.
1. INTRODUCTION

Despite their centuries-old history as a model for understanding infectious diseases, guinea pigs (*Cavia porcellus*) are currently underutilized, having been largely replaced by murine models. Consequently, the development of tools and reagents specific for guinea pigs has fallen behind that for mice. While mice have undergone various genetic modifications over the last four decades to generate unique phenotypes for use in specific biomedical systems, guinea pigs have not. Yet, guinea pigs retain several advantages over mice. The guinea pig immune system is genetically more like that of a human than the murine immune system is. In 2008, Padilla-Carlin et al. published a comprehensive review of the guinea pig as an infectious disease model. They noted evidence of immunological similarities that include presence of human group 1 CD1 protein homologues, presence of the IL-8 cytokine and its receptor, which do not exist in the mouse, and a greater CD8 and IL-12 amino acid sequence similarity between humans and guinea pigs than between humans and mice. Further, patterns of IFN-γ expression and inducible nitric oxide synthase during infection are similar to humans, the MHC in guinea pigs (leukocyte antigen) is homologous to the human MHC, and the complement system of guinea pigs and humans share more similarities than between humans and mice (Padilla-Carlin et al., 2008). The immunological relevancy of guinea pigs makes them an ideal translational animal model for a variety of infectious diseases in which they recapitulate human disease and where they could be used to assess vaccine efficacy. These include tuberculosis, brucellosis, and Q fever, as well as viral diseases (Padilla-Carlin et al., 2008; Baeten et al., 2018; Hensel and Arenas-Gamboa 2018; Lehrer et al., 2019; Tang-Huau et al., 2019).

Beyond their utility as a research model for infectious diseases, guinea pigs remain important biomedical models for toxicological studies, cardiovascular, and other diseases. Additionally, their larger size offers advantages that compensate for their higher per diem cost in comparison to mice. Logistically, the size of the guinea pig allows for the collection of larger blood volumes that can be used in multiple assays without the need for sacrificing the animal. This is ideal for longitudinal studies requiring data collection at multiple timepoints and for tests that may include PCR, complete blood count (CBC), and serology. Thus, fewer animals are needed for a study that would normally sacrifice several mice at each time point.

Unfortunately, the paucity of immunological tools available for guinea pigs is a major disadvantage in studies evaluating the immune response, particularly to infectious agents. Immunophenotyping using flow cytometry has been published for guinea pigs, but has been limited (Takizawa et al., 2006; Shang et al., 2011; Orme and Ordway 2016). Here, we present an optimized five-color/seven-parameter flow cytometry panel for immunophenotyping guinea pig peripheral blood lymphocytes, allowing for assessment of CD4+ and CD8+ T cells as well as T and B cell populations. We believe that the application...
of this flow cytometry panel to research using the guinea pig will enhance the value of this model not only in infectious diseases, but more widely in biomedical research.

2. Material and Methods

2.1 Sample Collection

Blood samples for flow cytometry panel development were taken from the jugular vein of 3-mo-old to 18-mo-old male Dunkin-Hartley guinea pigs under isoflurane (2–3%) anesthesia (Charles River Laboratories, Wilmington, MA). All procedures were conducted with approval from the Mississippi State University Institutional Animal Care and Use Committee (IACUC) following AAALAC guidelines (IACUC protocol numbers 17–166 and 18–267).

2.2 Panel Design

Fluorochromes were chosen to minimize spillover from adjacent detectors, residual donor fluorescence, and cross-laser excitation. We addressed co-expression of antigens that may have impacted the spread of data by assigning the three T cell antigens across three lasers. Antigen density was also considered, with brighter fluorochromes used where antigen density was lower. Additionally, the tandem fluorochrome was not paired with its donor on the same plot. A viability dye was employed to reduce the chance of artefacts produced by nonspecific binding of antibody to dead cells (Supplemental Fig. 1). Finally, antibodies were titrated to determine optimal stain indices, considering both separation and reduction of spillover spreading (Supplemental Fig. 2). Unstained, fluorescence minus one (FMO), and internal negative controls were used to set gates and determine positivity (Supplemental Fig. 3). Blocking of Fc receptor-specific binding with guinea pig serum was validated (Supplemental Fig. 4). The viability dye was titrated below the manufacturer’s recommended dilution until the positive population of the compensation beads was consistently brighter than dead cells, while at the same time maintaining good separation between viable and dead cells. Since B cells were stained with anti-CD1b3 (PE-Cy7), monocyte blocker was used to prevent artefactual elevation of the B cell population with monocytes stained nonspecifically with PE-Cy7, which we experimentally determined was the case in the absence of the blocking reagent. For a complete list of resources, see Supplemental Table 1.

2.3 Sample preparation and immunofluorescence staining

One hundred μL of ETDA whole blood was transferred to standard polystyrene flow cytometry tubes. Two mL of BD Pharm Lyse™ Lysing Buffer was added and the tube lightly vortexed, then incubated for 5 minutes. Cells were washed in 3 mL phosphate buffered saline (PBS, Ca²⁺ and Mg²⁺ free, pH 7.4), and centrifuged at 300 × g for 5 min at room temperature (RT). Cells were next stained for viability using LIVE/DEAD™ Fixable Near-IR using one-third of the manufacturer’s recommended concentration of dye, for 30 min at 4°C. Cells were pelleted at 300 × g for 5 min at RT, washed in 3 mL FCM-PBS [1% bovine serum albumin (BSA) in PBS filtered to 0.1 μm], and centrifuged at 300 × g for 5 min at RT. Next, Fc receptor-specific binding of antibodies was blocked by incubating cells in 10% guinea pig serum for 30 min at 4°C. Cells were then stained with antibodies against
CD1b3 (PE-Cy7), Pan T (APC), CD4 (PE), and CD8 (FITC) in a volume of ~100 μL for 30 min at 4°C. We labeled CD1b3 in-house with PE-Cy7, following the manufacturer’s recommendations. After staining, cells were washed once with 3 mL FCM-PBS, centrifuged at 300 × g for 5 min at RT, and then washed again in 3 mL PBS and centrifuged at 300 × g for 5 min at RT. We fixed cells in 1 mL of 1% methanol-free formaldehyde for 30 min at 4°C, followed by centrifugation at 800 × g for 5 min at RT. Cells were washed in 3 mL PBS and centrifuged at 800 × g for 5 min at RT. Finally, cells were resuspended in 225 μL FACS-PBS and pelleted at 300 × g for 5 min at RT, then placed at 4°C for acquisition within 24 hrs. For a complete list of resources, see Supplemental Table 1.

2.4 Data acquisition and analysis

We acquired samples with a four-laser 25-color NovoCyte Quanteon using NovoExpress 1.3.0 acquisition software (Acea Biosciences, San Diego, CA). Instrument QC was run both before and after each assay using NovoCyte QC particles. Proper compensation was determined using the acquisition software’s automatic compensation algorithm. Single-stained samples were used without employing a universal negative. At least 5000 events were recorded in both the positive and negative populations for each of the five compensation controls.

We acquired 50,000 events to validate blocking of Fc receptor-specific binding and for antibody titrations. We acquired 75,000 events for unstained controls, FMO controls, and fully stained samples. Data analysis was performed using FCS Express 7.00.0037 (De Novo Software, Glendale, CA).

3. Results and Discussion

To our knowledge, no assay exists for immunophenotyping peripheral lymphocytes in guinea pigs, and this gap was recently noted by Evans et al. (2018) as they described establishing an immunocytochemical assay for immunophenotyping lymphoma in guinea pigs. Here, we provide an optimized polychromatic flow cytometric protocol using five colors and seven parameters to immunophenotype peripheral blood lymphocytes in the guinea pig (Fig. 1). This is the first report of a flow cytometric assay for immunophenotyping guinea pig peripheral lymphocytes. Previous flow cytometric assays for guinea pig immunophenotyping include a “two-parameter” assay for leukocytes (Takizawa et al., 2006) and a flow cytometric assay for identification of T cell subsets in lung tissues that largely relied on SSC and FCS for downstream gating (Ordway et al., 2007).

In this optimized assay, we identified T cell subsets by sequential gating that excluded dead cells and identified B and T cells using commercially available antibodies, though one had to be labeled in-house. By empirically determining (1) the optimal antibody dilutions through titration, (2) the requirement to block non-specific binding of the Cy-7 acceptor fluorophore on the PE-Cy7 tandem to monocytes (data regarding Cy-7 blocking not shown), and (3) the optimal concentration of guinea pig serum to block unwanted Fc receptor binding of antibodies, we have developed a panel that both reliably identifies peripheral lymphocytes and also demonstrates that CD4+/CD8+ and B/T cell ratios typically fall within reference
ranges reported by human clinical laboratories (Apoil et al., 2017; Rudolf-Oliveira et al., 2015).

We are currently using this panel in an ongoing study evaluating immune response to spotted fever rickettsiae in a guinea pig-tick-*Rickettsia* model system. Following titrations and before incorporating the assay into our current spotted fever rickettsiosis study, we performed technical replicates to assess reproducibility of the assay. Three assays were performed on different days using three to four technical replicates from two different animals. Values are provided in the Supplementary Table 2. For future studies, this seven-parameter assay will be expanded with additional markers for targets including cytokines, for example, with the use of additional antibodies and nucleic acid probes (i.e. PrimeFlow®). Currently, we are adapting the protocol to evaluate leukocyte infiltration in guinea pig skin samples at sites of infection. This information will be critical for studies evaluating tick transmission of spotted fever rickettsiae, as earlier work in our host-pathogen studies relied on the murine model for evaluating the immune response in the skin (Banajee et al. 2016).

Guinea pigs are an invaluable model for understanding various infectious diseases including viral and bacterial diseases (Padilla-Carlin et al., 2008; Hickey 2011), for studying asthma and other non-infectious conditions (Ricciardolo et al., 2008), and for vaccine development. Their ability to recapitulate many human diseases is well established, and similarities between the human and guinea pig immune system have been reviewed (Padilla-Carlin et al., 2008). In addition to their advantages as a translational model, their larger size offers an opportunity to collect sufficient blood samples for multiple assays at each time point – an advantage over sacrificing smaller murine models for routine blood collection that could be applied to flow cytometric and serologic assays, at a minimum. Still, guinea pigs remain under-utilized in part due to the lack of available commercial reagents as compared to the murine model.

5. Conclusions

This five-color/seven-parameter flow cytometric assay not only provides a feasible and optimized approach for immunophenotyping peripheral blood lymphocytes in guinea pigs, but also offers a starting point to expand the panel to monitor immune function in additional cell types and tissues. We expect that its application will further corroborate and increase the utility of the guinea pig as a biomedical model for appropriate systems. In addition, we hope this serves as a stimulus to develop additional inbred guinea pig lines as well as genetically modified strains made possible by CRISPR-Cas9 technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

- **FMO** Fluorescence minus one
- **MFI** Median Fluorescence Intensity
- **RT** Room temperature

**9. References**

Apoil PA, Puissant-Lubrano B, Congy-Jolivet N, Peres M, Tkaczuk J, Roubinet F, Blancher A. 2017 Reference values for T, B and NK human lymphocyte subpopulations in adults. Data Brief. 2:400–404.

Baeten LA, Podell BK, Sluder AE, Garritsen A, Bowen RA, Poznansky MC. 2018 Standardized guinea pig model for Q fever vaccine reactogenicity. PLoS One. 13(10): e0205882. [PubMed: 30312355]

Banajee KH, Verhoeve VI, Harris EK, Macaluso KR. 2016 Effect of Amblyomma maculatum (Acari: Ixodidae) saliva on the acute cutaneous immune response to Rickettsia parkeri infection in a murine model. J Med Entomol. 53:1252–1260. [PubMed: 27521760]

Evans SJM, Harr KE, Thielen L, MacNeill AL. 2018 Validation of an immunocytochemical assay for immunophenotyping of lymphoma in guinea pigs (Cavia porcellus). Vet Clin Pathol. 47:682–687. [PubMed: 30358180]

Hensel ME, Arenas-Gamboa AM. 2018 A neglected animal model for a neglected disease: Guinea pigs and the search for an improved animal model for human brucellosis. Front Microbiol. 9:2593. [PubMed: 30429834]

Hickey AJ. 2011 Guinea pig model of infectious disease - viral infections. Curr Drug Targets. 12:1018–23. [PubMed: 21366517]

Lehrer AT, Wong TS, Lieberman MM, Johns L, Medina L, Feldmann F, Feldmann H, Marzi A. 2019 Recombinant subunit vaccines protect guinea pigs from lethal Ebola virus challenge. Vaccine. pii: S0264–410X(19)30797–2 [Epub ahead of print]

Ordway D, Palanisamy G, Henao-Tamayo M, Smith EE, Shanley C, Orme IM, Basaraba RJ. 2007 The cellular immune response to Mycobacterium tuberculosis infection in the guinea pig. J Immunol. 179(4):2532–41. [PubMed: 17675515]

Orme IM, Ordway DJ. 2016 Mouse and guinea pig models of tuberculosis. Microbiol Spectrum 4(4):TBTB2-0002-2015.

Padilla-Carlin AJ, McMurray DN, Hickey AJ. 2008 The guinea pig as a model of infectious disease. Comp Med. 58(4):324–340. [PubMed: 18724774]

Ricciardolo FL, Nijkamp F, De Rose V, FolkeCs G. 2008 The guinea pig as an animal model for asthma. Curr Drug Targets. 9:452–65. [PubMed: 18537584]

Rudolf-Oliveira RCM, Goncalves KT, Martignago ML, Mengatto V, Gaspar PC, de Moraes ACR, da Silva RM, Bazzo ML, Santos-Silva MC. 2015 Determination of lymphocyte subset reference ranges in peripheral blood of healthy adults by a dual-platform flow cytometry method. Immunol Lett. 163(1):96–101. [PubMed: 25450652]

Shang S, Harton M, Tamayo MH, Shanley C, Palanisamy GS, Caraway M, Chan ED, Basaraba RJ, Orme IM, Ordway DJ. 2011 Increased Foxp3 expression in guinea pigs infected with W-Beijing strains of M. tuberculosis. Tuberculosis 91, 378–385. doi:10.1016/j.tube.2011.06.001. [PubMed: 21737349]

Takizawa R, Chiba J, Haga S, Asano T, Yamazaki T, Yamamoto N, Honda M. 2006 Novel two-parameter flow cytometry (MIL4/SSC followed by MIL4/CT7) allows for identification of five J Immunol Methods. Author manuscript; available in PMC 2021 January 01.
fractions of guinea pig leukocytes in peripheral blood and lymphoid organs. J Immunol Methods. 311(1–2):47–56. [PubMed: 16533513]

Tang-Huau TL, Feldmann H, Rosenke K. 2019 Animal models for Lassa virus infection. Curr Opin Virol. 37:112–117. [PubMed: 31442921]
Figure 1.
Gating strategy for identifying lymphocyte subsets in guinea pigs: (A) assessed fluidics to eliminate all bubbles, microclogs, and other evidence of instability that could lead to artefacts; (B) eliminated artefactual data often created by doublets; (C) eliminated debris and granulocytes to obtain a population of mononuclear cells; (D) identified viable lymphocytes using a viability stain to exclude dead cells and associated artefactual data; (E) identified major lymphocyte lineages and excluded nonlymphocytes (mainly monocytes); (F) further identified subsets of T cells.