Video Article

An *In Vitro* Model for Measuring Immune Responses to Malaria in the Context of HIV Co-infection

Constance Finney[1], Lena Serghides[2]

[1]Department of Biological Sciences, University of Calgary

[2]Toronto General Research Institute, University Health Network

Correspondence to: Lena Serghides at lena.serghides@utoronto.ca

URL: http://www.jove.com/video/52969
DOI: doi:10.3791/52969

Keywords: Immunology, Issue 104, Malaria, HIV, Cell culture, Flow cytometry, Cytokines

Date Published: 10/6/2015

Citation: Finney, C., Serghides, L. An *In Vitro* Model for Measuring Immune Responses to Malaria in the Context of HIV Co-infection. *J. Vis. Exp.* (104), e52969, doi:10.3791/52969 (2015).

Abstract

Malaria and HIV co-infection is a growing health priority. However, most research on malaria or HIV currently focuses on each infection individually. Although understanding the disease dynamics for each of these pathogens independently is vital, it is also important that the interactions between these pathogens are investigated and understood.

We have developed a versatile *in vitro* model of HIV-malaria co-infection to study host immune responses to malaria in the context of HIV infection. Our model allows the study of secreted factors in cellular supernatants, cell surface and intracellular protein markers, as well as RNA expression levels. The experimental design and methods used limit variability and promote data reliability and reproducibility.

All pathogens used in this model are natural human pathogens (*Plasmodium falciparum* and HIV-1), and all infected cells are naturally infected and used fresh. We use human erythrocytes parasitized with *P. falciparum* and maintained in continuous *in vitro* culture. We obtain freshly isolated peripheral blood mononuclear cells from chronically HIV-infected volunteers. Every condition used has an appropriate control (*P. falciparum* parasitized vs. normal erythrocytes), and every HIV-infected donor has an HIV uninfected control, from which cells are harvested on the same day. This model provides a realistic environment to study the interactions between malaria parasites and human immune cells in the context of HIV infection.

Video Link

The video component of this article can be found at http://www.jove.com/video/52969/

Introduction

Co-infection, infection with multiple concurrent infections, is the norm in natural environments. Co-infection can have a major impact on disease pathology and on the clinical management of each infection. In the context of co-infection, vaccine and drug efficacy, as well as diagnostic testing, can be negatively impacted (reviewed in [1]). However, despite its importance, the majority of pathogen research considers only single infections.

Malaria and HIV-1 (HIV) are leading causes of morbidity and mortality globally. Areas of malaria and HIV endemicity share a wide geographic overlap, putting millions of people at risk of co-infection and consequently at risk for more severe clinical disease [2–10]. The two diseases negatively interact. In HIV-infected individuals, higher HIV viral loads and temporary decreases in $CD4^+$ T-cell counts can be seen during a malaria infection, while malaria parasite burdens and risk of clinical and severe malaria are higher in co-infected individuals [2,3,5,7,8,10]. The mechanisms by which HIV increases malaria severity are not fully understood and warrant further investigation.

Here we describe a method by which malaria and HIV co-infection can be studied *in vitro*. Specifically, this method allows for the examination of malaria-specific immune responses in the context of HIV infection. Our protocol describes a versatile co-culture system using freshly isolated peripheral blood mononuclear cells (PBMCs) isolated from chronically HIV infected donors and *in vitro* cultured *P. falciparum* parasitized erythrocytes (PfRBC). The impact of HIV antiretroviral therapy on these responses can also be examined using prospectively collected PBMCs from HIV(+) donors pre- and post-therapy.

We have used this system to investigate the impact of HIV infection on malaria-specific innate immune responses [11,12], and were able to determine that malaria-specific IFNγ and TNF responses are impaired in NK cells, NKT cells, γδ T cells from HIV(+) donors pre- and post-HIV antiretroviral therapy. Additionally, we were able to use this system to determine that monocytic functions are also impaired in HIV(+) donors, but recover post-HIV antiretroviral therapy.

Protocol

This protocol requires the recruitment of donors for serum and RBC to be used for parasite culture, and HIV(+) and uninfected donors for PBMC isolation. Institutional Review Boards must approve all studies and all donors must provide informed consent prior to blood draw.

CAUTION: Working with human blood samples and human malaria parasites requires precautionary measures. Always wear a lab coat, gloves, and work in a level 2 Biosafety cabinet. In the event of accidental percutaneous exposure to human malaria, report to Health and Safety for prophylactic treatment. Additional safety consideration should also be put in place for working with HIV(+) blood. Wear a back closing lab coat. Double glove (top glove should be latex). Perform all handling in a level 2 Biosafety cabinet. Do not use any glass or sharp objects. Do not use aspirator. Place all contaminated items in virox solution or bleach for a minimum of 1 hr prior to discarding. Wash all surfaces with virox and UV for 1 hr following use. Note that each institution will have their own specific biosafety regulations that need to be followed. Report all accidental exposures to HIV-infected blood to Health and Safety for evaluation and consideration of possible post-exposure prophylaxis.

Note: Different strains of *P. falciparum* malaria parasites exist. ITG was used for these experiments, but different strains can be used. Excellent instructions on freezing and thawing *Plasmodium falciparum* parasites are available at the MR4 website [13].

1. Making RPMI-A for Malaria Parasite Culture

1. Make RPMI-0 by mixing 950 ml of ddH_2O, 1 packet of RPMI-1640 powder, 6 g of HEPES, 2 g of sodium bicarbonate, and 1.35 mg of hypoxanthine.
2. Thaw heat inactivated human serum from two different donors. AB donors are best but any can be used if parasites are grown in O type red blood cells (RBC). Invert tubes to mix. If serum contains particulate matter or is thick spin at 2,000 rpm and then filter liquid portion using a 0.45 µm filter unit.
3. Using a 0.2 µm filter unit filter 180 ml of RPMI-0, 20 ml of human serum (10 ml from each donor), and 0.5 ml of 10 mg/ml gentamycin.
   Note: Human serum can clog the filter so more than one filter unit may be required.
4. Label the medium bottle with RPMI-A, the date, and the source of the serum. Refrigerate until required. RPMI-A may turn cloudy when refrigerated. This is normal, but increasing cloudiness is a sign of contamination.
   Note: Parasite growth may vary in different donor serum. It is a good idea to test all human serum batches for good parasite growth before using.

2. Preparing Human Red Blood Cells for Parasite Culture

Note: Blood donors should be type O.

1. Collect 7-10 ml of blood into acid-citrate-dextrose (ACD) tubes. Write the donor's ID and date of collection on the label.
2. Store blood at 4 ^{o}C until needed. Use blood for parasite cultures within 1 month.
3. Wipe the top of the tube with 70% ethanol. Carefully remove stopper and discard. Transfer blood into a 15 ml tube. Spin for 3 min at 1,000 x g. Remove plasma by aspiration.
4. Suspend RBC with equal volume of warm RPMI-0. Spin for 5 min at 1,000 x g. Remove the buffy coat by aspiration, resuspend in 5 ml of RPMI-0 and repeat the wash 2 more times.
5. Remove the supernatant and add enough RPMI-A to produce a mixture that is 50% RBC by volume.
6. Store at 4 ^{o}C until needed.

3. Maintaining Parasite Cultures

1. Pre-warm RPMI-A to 37 ^{o}C.
2. Place thawed parasites (see MR4 protocol for thawing procedure) into a T25 flask with 5 ml of RPMI-A and 75 µl of washed human RBC for a hematocrit of ~3%. Note: Hematocrit measures the volume of RBC compared to total volume. To calculate hematocrit measure the volume of packed RBC to the total volume of medium plus RBC. Assume that the thawed parasites will contribute 75 µl of RBC. By adding 150 µl of RBC stock (which is equivalent to adding 75 µl of packed RBC) to the flask there is a total of 150 µl of RBC into 5 ml of medium, which equals to a hematocrit of 3% (150 µl/5,000 µl x 100% = 3%).
3. Gas the flask for 30 sec with parasite gas mixture (1% O_2, 3% CO_2, balance N_2). Seal and place the flask wide side down in a 37 ^{o}C incubator as to allow for the greatest surface area for gas exchange.
4. To change the medium and check for parasitemia (needs to be done daily), carefully move the flask as to not disturb the RBC layer. Using a sterile unplugged Pasteur pipette draw off the culture medium without disturbing the blood layer.
5. To check parasitemia, remove a 10 µl sample from the blood layer, place it on a glass slide and using a second glass slide create a thin blood film. Allow slide to dry.
6. Place 4 ml of fresh RPMI-A into the flask, gently mix, gas for 30 sec, and place in incubator at 37 °C.
7. Stain the slide using the Hema3 staining kit according to manufacturer's protocol.
   1. For optimal staining, dip slides in the fixing solution for 10 sec, solution I for 10 sec, and solution II for 30 sec. Rinse in water and leave slides to dry.
8. Calculate parasitemia by counting the number of PfRBC (stained dark purple) versus the total number of RBC (stained pink). Count a total of 300 cells to ensure accuracy.
9. When parasites have reached a parasitemia of 5%, expand the culture into a T75 flask, using 40 ml of RPMI-A, and 500 µl of RBC.

4. Parasite Synchronization

Note: The day before the experiment, synchronize the parasite culture by treating with alanine. Only ring stage parasites and uninfected RBC will survive this treatment. Alanine synchronization will give you a pure trophozoite culture the next day that can be used in the co-culture experiments. Make sure to start with a parasite culture that contains a majority of ring stage parasites.

1. Prepare alanine solution by mixing 8.01 g of alanine (300 mM) and 0.365 g of Tris (10 mM) into 300 ml of ddH_2O. Bring pH to 7.4. Filter sterilize using a 0.2 µm filter unit.
2. Pre-warm alanine solution to 37 ^oC.
3. Spin down the parasite culture (5 min x 1,000 x g), and remove medium.
4. Resuspend pellet in 19 volumes of alanine solution (1 ml packed RBC to 19 ml alanine solution). Incubate for 15 min at RT.
5. Spin 5 min x 1,000 x g. Aspirate supernatant. Wash in RPMI-0 once. Aspirate supernatant and resuspend in RPMI-A and adjust hematocrit to ~3%. Gas flask and return to 37 ^oC.
   Note: For co-culture experiments a minimum parasitemia of 5% trophozoites is needed, with 10% parasitemia considered optimal.

5. Parasite and RBC Preparation for Co-culture Experiments

1. Use a ratio of 3 PfRBC per PBMC in co-culture experiments to induce an inflammatory response. To calculate total PfRBC, quantify parasitemia by making a thin blood smear as described in 3.5, and hematocrit by counting the number of RBC per mL of parasite culture using a hemocytometer.
   Number of PfRBC = % parasitemia x total RBC/ml x ml of culture. For example: 10 ml of culture at 10 x 10^6 RBC/ml and 10% parasitemia is equal to 0.1 x 10^6/ml x 10 ml = 10 x 10^6 PfRBC.
2. Spin the parasite culture for 5 min at 1,000 x g (RT). Aspirate medium, and resuspend at 6 x 10^6 PfRBC per ml in RPMI-S+ (500 ml RPMI-1640 supplemented with L-glutamine and HEPES, 10% heat inactivated FBS, 1.5 ml gentamicin, 5 ml of 100 mM sodium pyruvate, 5 ml of 10 mM MEM non-essential amino acids, 5 ml of 5mM β-mercaptoethanol).
3. Take control blood (uninfected RBC from the same donor used for maintaining the parasite culture), calculate hematocrit and resuspend in RPMI-S+ at the same number of RBC per ml as the parasite culture.

6. Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

1. Collect venous blood in sodium heparin tubes (green top) from both a chronic HIV(+) donor and an HIV(-) control. Do not use EDTA as an anti-coagulant as EDTA's calcium chelating action affects cell function. On average expect between 5-10 x 10^6 PBMC per 10 ml of blood. For a typical experiment collect 30 ml of blood from each donor. The blood volume will need to be adjusted according to the experimental requirements.
2. As quickly as possible and definitely within an hour of blood being collected, remove the blood from the tubes and place into a plastic 50 ml tube. Monocytes in particular will be activated and stick to glass, so it is important that if glass blood collection tubes are used cells are removed as quickly as possible.
3. Spin the blood at 1,000 x g for 15 min and collect plasma (this can be used for other studies if required – if not this step can be skipped). Dilute the blood in equal volume of cold DPBS.
4. Place 15 ml of RT Ficoll into a 50 ml tube. Slowly, using a sterile plastic transfer pipette, layer the blood/DPBS solution over the Ficoll without any mixing. A maximum of 25 ml of blood/DPBS solution can be layered over each 15 ml of Ficoll.
5. Spin the gradients for 30 min at RT at 600 x g. Make sure the 'no brake' setting is on.
6. Once the cells have spun, look for the interface between the two phases. This is where the PBMC are. Using a sterile plastic transfer pipette collect the PBMC from the interface (see **Figure 1**). Minimize contamination by RBC.
7. Place collected cells in 50 ml tubes, with no more than 20 ml per tube. Top tube up to 50 ml with sterile cold DPBS.
8. Spin the cells for 10 min at 4 ^oC at 300 x g.
9. Discard supernatant, resuspend pellet in 5 ml sterile cold DPBS and pool all cells from the same donor into one tube. Top up to 50 ml with sterile DPBS, and repeat the wash steps 2 more times.
10. Resuspend cells in 10 ml RPMI-S+ medium.
11. Remove a 20 µl aliquot and mix with 20 µl of trypan blue. Pipette 10 µl in a haemocytometer and count the living cells (cells that have not taken up the blue dye – all blue cells are dead). Use a haemocytometer to calculate cell number in a solution as follows.
    Note: A haemocytometer has a grid of specified dimensions so that the area covered by the lines is known.
    1. Make sure the haemocytometer is clean. Place the coverslip over the counting area. Load 10 µl of cell solution by placing the pipette tip into one of the V-shaped wells. The area under the coverslip will fill by capillary action.
    2. Place the loaded haemocytometer under a microscope. The full grid of the haemocytometer contains 9 squares of 1 mm^2 each. Count all cells within each large square. If too many cells are present, dilute the solution further and recount.
    3. Calculate cell concentration as follows: Total cells/ml = (total cells counted/# of squares) x dilution factor x 10,000 cells/ml, *e.g.,* (500 cells/5 squares) x dilution factor of 20 x 10,000 cells/ml = 20 x 10^6 cells total.
12. Adjust medium to a final concentration of 10 x 10^6 per ml (RPMI-S+ medium).

7. Malaria/HIV Co-infection Culture

1. Plate 100 µl of isolated PBMCs and 400 µl of RPMI-S+ medium to a 24-well plate (1 x 10^6 PBMC per well). Ensure that wells are set up in triplicate: 3 wells for uninfected RBC, 3 wells with PfRBC, 3 wells with medium, and 3 wells with PMA/Ionomycin. This is both for the HIV(+) and HIV(-) sample.
2. For the PfRBC wells, place 3 x 10^6 PfRBC in each of the wells (500 µl of parasite culture prepared in 5.2).

3. For uninfected RBC wells, place 500 µl of the uninfected RBC culture prepared in 5.3 in each of the wells.
4. For medium wells, place 500 µl of RPMI-S+ medium in each of the wells.
5. For PMA/Ionomycin wells, prepare PMA/Ionomycin solution (2.5 pg/ml, 250 pg/ml respectively). Place 500 µl of this solution in each well.
   Note: As potent stimulators of T cell cytokine secretion, PMA and Ionomycin are used as a positive control to ensure cells are functional.
6. Place plate at 37 ^{o}C in a 5% CO_2 incubator.
7. Optional: use excess cells for phenotypic analysis using flow cytometry or store in an RNA stabilization solution for future mRNA expression analysis.

8. Detection of Malaria Immune Responses

Note: Perform co-culture experiments for as long as 4 days. No medium change is required during this time. The optimal time point will depend on the cell type of interest and the question asked. An incubation of 12-48 hr is optimal for monocytic responses to PfRBCs, while lymphocyte responses were best observed at 72-96 hr. The time points will need to be optimized based on the experimental question. Shorter periods (2-4 hr) may be used if interaction between intact PfRBC and PBMCs is of interest.

1. Spin plate at 300 x g for 3 min to pellet cells. Collect 700 µl of culture supernatant from each well.
2. Spin supernatant at 1,000 x g for 5 min to clear of any debris.
3. Aliquot cleared supernatant as needed, label, and freeze at below -20 °C until analysis of secreted factors is to be performed.
4. Analyze cytokine/chemokine responses by ELISA or by bead array (follow the manufacturer's suggested protocols).
5. Collect cells remaining in the plate into an RNA stabilizing solution and utilize for mRNA expression analysis by quantitative real-time PCR [14-16].

9. Intracellular Flow Cytometry for Cell-specific Ccytokine Responses Using PBMC Co-cultured with *P. falciparum* Infected RBC

Note: As mentioned above the length of co-culture will depend on the cell type of interest. If interested in monocytic responses to PfRBC a shorter incubation period is needed. Times will be longer for innate lymphocyte responses γδ T cells, NK cells, NKT cells), and longer still for CD4 and CD8 T cells. Optimization will be required.

1. 6-8 hr prior to analysis add 1 µl of 1,000x brefeldin A to all wells. Return the plate to 37 ^{o}C incubator. After 6-8 hr incubation, place plate on ice for 15 min.
2. Remove cells from the wells using vigorous pipetting and place them into labeled microcentifuge tubes. Scraping may be required to remove monocytes.
3. Spin cells for 5 min at 1,000 x g. Remove supernatants. Resuspend cells in 500 µl flow cytometry buffer (1x DPBS, 2% heat inactivated FBS, 0.02% sodium azide).
4. Divide cells up so that each sample has: one tube for full antibody staining (240 µl), and one tube for fluorescent minus one (FMO) control antibody staining (240 µl). Pool a small amount of each sample (20 µl) for the unstained flow cytometry control (this will be used in setting up the flow cytometer).
5. Spin cells for 5 min at 500 x g. Remove supernatants.
6. Incubate cells with unconjugated anti-human CD16/CD32 (5 µg/ml) in 50 µl flow cytometry buffer for 15 min at 4 ^{o}C to block Fc receptors.
7. Add 1 ml of flow cytometry buffer to each tube. Spin cells for 5 min at 500 x g. Remove supernatants.
8. Resuspend cells in 50 µl of flow cytometry buffer with a pre-titrated concentration of fluorophore-conjugated antibodies to desired cell surface markers. Pre-mix enough antibody solution for all samples to be stained. Incubate cells at 4 ^{o}C for 20 min. Protect from light.Note: Amount of each antibody will have to be optimized. We routinely stain for CD56, CD3, γδ, CD4, CD8, CD14. Titrated volumes for these antibodies are shown in **Table 1**.
9. Add 1 ml of flow cytometry buffer to each tube. Spin cells for 5 min at 500 x g. Remove supernatants.
10. Add 100 µl of cytofix/cytoperm solution to each tube. Incubate cells for 20 min at 4 ^{o}C. Protect from light.
11. Add 1 ml of perm/wash buffer (provided as 10x concentrate – dilute to 1x using ddH_2O) to each tube. Spin cells for 5 min at 500 x g. Remove supernatants.
12. Add 100 µl of perm/wash buffer to FMO control antibody staining tubes and mix to resuspend cells.
13. Add 100 µl of perm/wash buffer with a pre-titrated concentration of fluorophore-conjugated antibodies to desired intracellular markers (*i.e.,* cytokines/chemokines) to full antibody staining tubes.
    Note: Amount of each antibody will have to be optimized. We routinely stain with anti-TNF and anti-IFNγ antibodies. Titrated volumes for these antibodies are shown in **Table 1**.
14. Incubate all tubes for 30 min at 4 ^{o}C. Protect from light.
15. Add 1 ml of Perm/Wash buffer to each tube. Spin cells for 5 min at 500 x g. Remove supernatants.
16. Resuspend cells in 300 µl flow cytometry buffer with 1% paraformaldehyde. Let sit for a minimum of 15 min to neutralize HIV.
17. Prepare single antibody stained samples using compensation beads, to be used for compensation set up on the flow cytometer. The type of compensation beads will depend on the antibodies used (*i.e.*, anti-mouse Ig, anti-rat/hamster Ig). Vortex beads. Add one drop of beads per antibody. Add equal amounts of antibody as used for staining. Add 200 µl of flow cytometry buffer. These are now ready for use. There is no need to wash the beads.
18. Acquire samples on a flow cytometer as soon as possible and within 24 hr for best results. Tandem dyes are susceptible to dissociation with storage so we recommend immediate acquisition if possible.

Representative Results

The graphs depict the levels of IFNγ production from NKT cells (**Figure 2**), using CD56+CD3+γδ- gates to obtain the NKT cells population (data not shown). The cells were cultured for 72 hr prior to staining. Once stained, 100,000 CD3+ cells were acquired on the flow cytometer to obtain large enough populations of NK, NKT and γδ cells (cells of interest). A minimum of 5,600 NKT cells are displayed on each graph. TNF production is obtained in the same manner (data not shown). The graphs clearly demonstrate that IFNγ production is lower in cells from HIV(+) individuals compared to HIV(-) individuals exposed to PfRBC.

Flow cytometry analysis is very subjective. It is therefore essential to have all the appropriate controls for each experiment (see **Figure 2**). Background levels are calculated using the FMO samples, which allows for a true representation of the cytokine staining. Cells stimulated with PMA/Ionomycin were used as a positive control (data not shown). PMA and Ionomycin are strong stimulators of T cell cytokine production. A lack of IFNγ production in these samples would most likely indicate a problem with the staining protocol. However, other variables such as cell viability or inactive reagents may also be at fault.

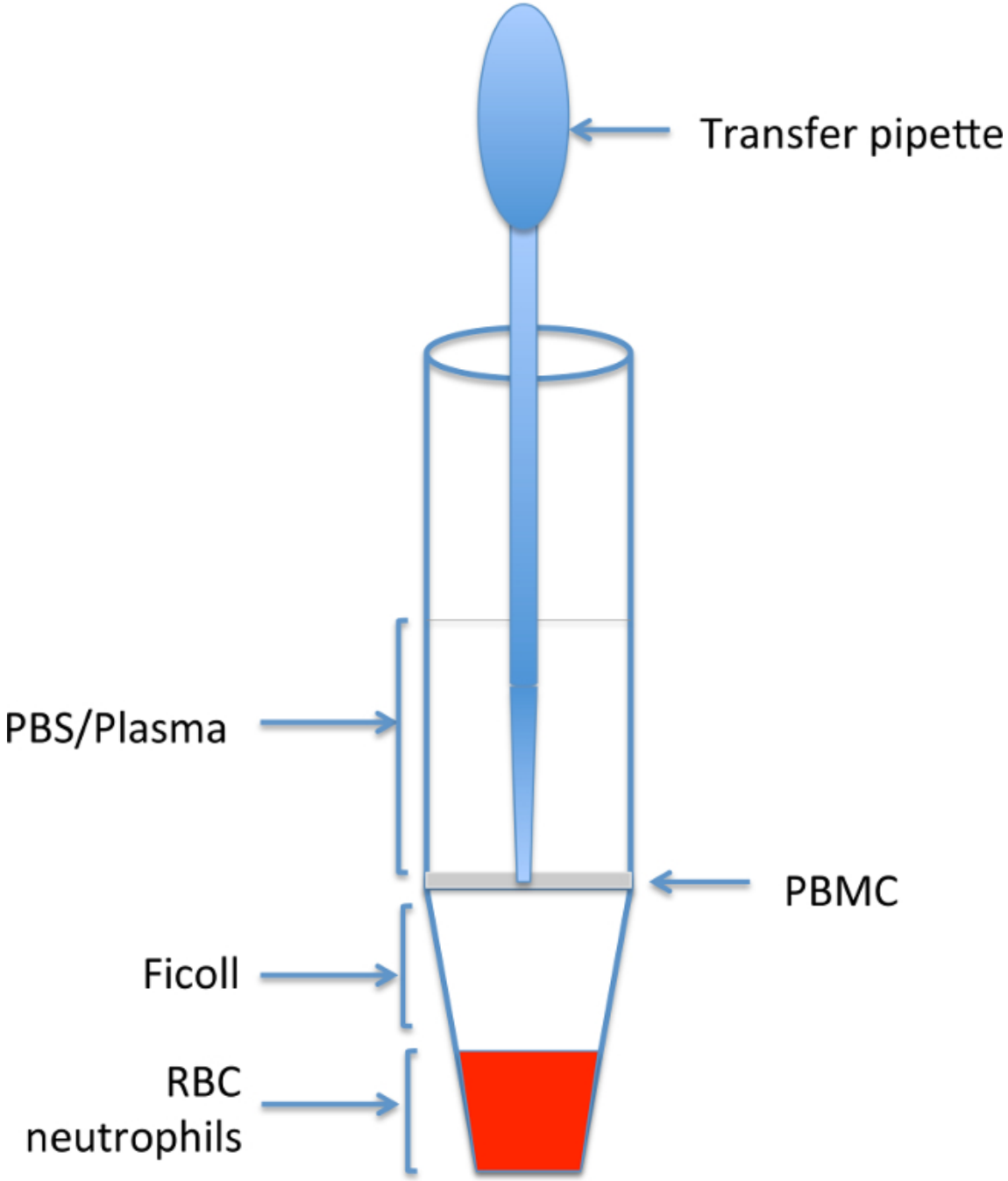

Figure 1. Depiction of Ficoll gradient post spin demonstrating the position of the PBMCs.

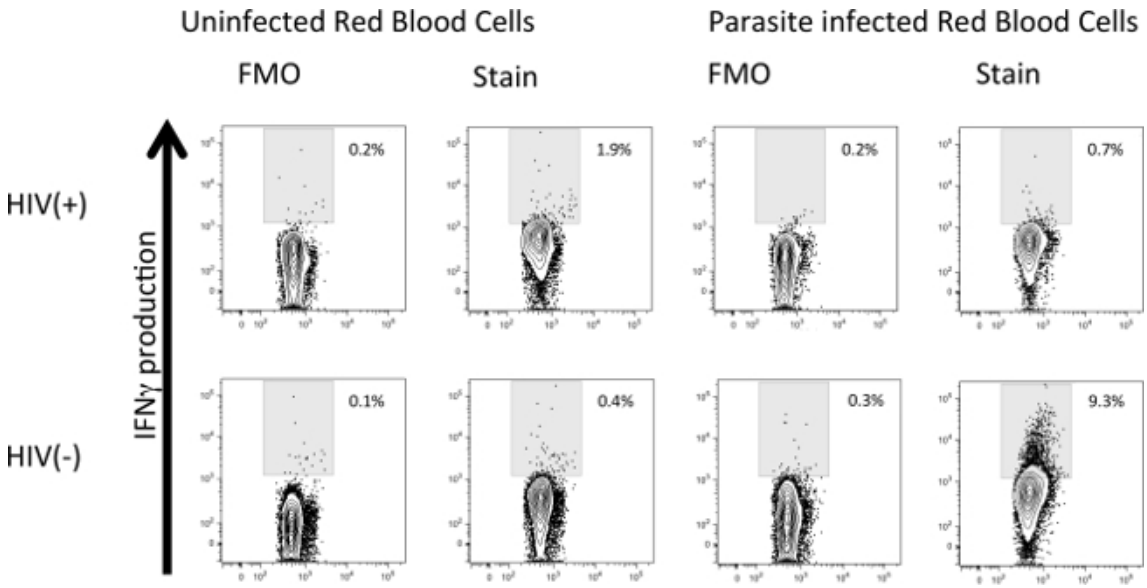

Figure 2. IFNγ production by Natural Killer T cells. The flow diagrams were obtained by gating on CD56+CD3+γδ- cells (minimum of 5,600 events). IFNγ production is detectable in the HIV(-) sample stimulated with *P. falciparum* infected red blood cells. This cytokine response is no longer apparent in the context of a chronic HIV infection. Please click here to view a larger version of this figure.

Discussion

Our protocol has been optimized in order to most realistically study HIV-malaria co-infection *in vitro*. First, fresh human RBCs and serum are required for malaria parasite culture. This is vital to obtain a healthy population of malaria parasites. Parasite lysates cannot be substituted for live parasites as cytokine production is much more rapid and intense when using live *P. falciparum* infected RBC (PfRBC) [17,18]. In addition, activation of cell types like NK cells, requires whole PfRBCs, and does not work efficiently with parasite lysates. This may be due to the need for direct contact between the PfRBC and the leukocyte or may be due to the unstable nature of the parasite-derived ligands that interact with cell surface receptors [18]. Malaria PfRBC cultures must also be well synchronized (Protocol 4) prior to the experiment. Synchronized PfRBCs improve the reproducibility of the experimental data. Although this protocol describes the use of trophozoite stage PfRBC for co-culture, it can easily be modified for the study of ring stage PfRBC.

Human leukocytes can be artificially infected with HIV, and this method has been used in studies of malaria-HIV co-infection research [20]. However, this does not model the immune dysregulation that results from chronic HIV infection. In this co-culture system we use PBMCs isolated from human participants that are chronically infected with HIV-1. When participants are required for a study, it is important to carefully select this population. Inclusion and exclusion criteria are vital to ensure minimum variability and maximum reproducibility. Our criteria included a clear definition of chronic HIV infection (HIV-infected for >1 year, with $CD4^+$ T-cell count decline of >50 cells/mm^3/year) and the exclusion of anyone with a concurrent infection. This ensured that the results obtained could be attributed solely to the effect of chronic HIV infection, and not another infection. Additionally, since we were interested in innate immune responses to malaria we excluded donors that had previous malaria infection.

HIV-uninfected controls are also used for each HIV(+) donor, at every sampling time point, to allow for data normalization. An attempt should be made to match controls to their respective HIV(+) donor for at least age but preferably also sex (although in our previous study [12] we did not observe a significant difference in cell subsets or cytokine responses between female and male HIV-uninfected participants). If prospective sampling is planned maintaining the same HIV-uninfected control for each sampling time point is beneficial. Responses can vary from experiment to experiment due to multiple factors including the health of the PfRBCs, their degree of synchronization, parasitemia level and stage of maturity of PfRBCs, and hematocrit level. Care must be taken to keep as many of these consistent between experiments. Normalizing to an HIV-uninfected control allows for some accounting for these variables.

Using fresh cells is paramount to avoid artificial results. Freezing and thawing samples can have a significant impact on cell viability [21,22], cytokine production [23-26] and cell surface phenotypic markers [27].

If monocytes are of particular interest, it is important that glass is not used during the protocol. Monocytes adhere to glass and many other plastics [28]. We use polypropylene plastic pipettes, transfer pipettes, and tubes throughout to minimize monocyte adherence and ultimate removal from our study cell populations.

When setting up the flow cytometry assay, any combination of antibodies can be used depending on the cells of interest. We were particularly interested in IFNγ and TNF production from NK cells, NKT cells and γδ T cells. This defined our antibody panel. However, if using a different combination, it is important to optimize the amounts of antibody for each panel. This is vital to avoid erroneous data. Also, to avoid variability and ensure validity, FMOs are required to assess background cytokine levels for each condition (RBC, PfRBC, medium, and PMA/Ionomycin) and participant population (HIV(-) and HIV(+), **Figure 2**). Measuring *in vitro* intracellular cytokine production usually results in high background levels, especially when cells have been cultured prior to staining. Since the culture conditions affect background levels, appropriate FMOs ensure knowledge of the background level for each sample. Our supply of cells was limited, therefore we designed our FMOs to contain all surface

stains but no intracellular stains. This allows for the specific comparison of cytokine background levels on all the different cell types studied. We also ran one single stain per experiment for each of the surface markers to confirm their background levels.

The protocol described is a versatile one, which can be used to examine responses within hours or days depending on the cells of interest. For optimal PfRBC-induced cytokine production from innate lymphocytes, we measured flow cytometry output after 2 or 3 days. When looking at monocytes, earlier time points (1 or 2 days) are recommended. This system allows for multiple cell types and cell responses to be distinguished by flow cytometry, secretory responses to be measured in cell supernatants, and expression profiles to be assessed in extracted RNA. Further, use of antibodies to block specific receptors or neutralize cytokines can be utilized in this system to further dissect mechanisms. We have successfully used IL-18 receptor blockade to implicate IL-18 receptor in PfRBC-induced IFNγ responses [12]. This system represents a realistic method by which to assess a number of innate immune responses to malaria in the context of HIV infection.

Disclosures

The authors have nothing to disclose.

Acknowledgements

C.A.M.F. and L.S. participated in protocol design, acquisition and analysis of data, and drafting of the article.

The authors wish to thank Dr. Kain, Dr. Loutfy, Dr. Wasmuth, and Dr. Ayi for their contributions.

C.F. was supported by a CTN/Ontario HIV Treatment Network (OHTN) postdoctoral fellowship. L.S. is supported by an OHTN Junior Investigator Development Award. The present work was supported by a Canadian Institutes of Health Research (CIHR) operating grant (MOP-13721 and 115160), and a CIHR New Investigator Catalyst grant.

References

1. Graham, A. L., Cattadori, I. M., Lloyd-Smith, J. O., Ferrari, M. J., Bjørnstad, O. N. Transmission consequences of coinfection: cytokines writ large. *Trends in parasitology.* **23**, (6), 284-291 (2007).
2. French, N., *et al.* Increasing rates of malarial fever with deteriorating immune status in HIV-1-infected Ugandan adults. *AIDS (London, England).* **15**, (7), 899-906 (2001).
3. Hoffman, I. F., *et al.* The effect of Plasmodium falciparum malaria on HIV-1 RNA blood plasma concentration. *AIDS (London, England).* **13**, (4), 487-494 (1999).
4. Kamya, M. R., *et al.* Effect of HIV-1 infection on antimalarial treatment outcomes in Uganda: a population-based study. *The Journal of infectious diseases.* **193**, (1), 9-15 (2006).
5. Kublin, J. G., *et al.* Effect of Plasmodium falciparum malaria on concentration of HIV-1-RNA in the blood of adults in rural Malawi: a prospective cohort study. *Lancet.* **365**, (9455), 233-240 (2005).
6. Mermin, J., *et al.* Effect of co-trimoxazole prophylaxis, antiretroviral therapy, and insecticide-treated bednets on the frequency of malaria in HIV-1-infected adults in Uganda: a prospective cohort study. *Lancet.* **367**, (9518), 1256-1261 (2006).
7. Patnaik, P., *et al.* Effects of HIV-1 serostatus, HIV-1 RNA concentration, and CD4 cell count on the incidence of malaria infection in a cohort of adults in rural Malawi. *The Journal of infectious diseases.* **192**, (6), 984-991 (2005).
8. Whitworth, J., *et al.* Effect of HIV-1 and increasing immunosuppression on malaria parasitaemia and clinical episodes in adults in rural Uganda: a cohort study. *Lancet.* **356**, (9235), 1051-1056 (2000).
9. Xiao, L., Owen, S. M., Rudolph, D. L., Lal, R. B., Lal, A. A. Plasmodium falciparum antigen-induced human immunodeficiency virus type 1 replication is mediated through induction of tumor necrosis factor-alpha. *The Journal of infectious diseases.* **177**, (2), 437-445 (1998).
10. Francesconi, P., *et al.* HIV, malaria parasites, and acute febrile episodes in Ugandan adults: a case-control study. *AIDS (London, England).* **15**, (18), 2445-2450 (2001).
11. Finney, C. A. M., *et al.* HIV infection deregulates Tim-3 expression on innate cells: combination antiretroviral therapy results in partial restoration. *Journal of acquired immune deficiency syndromes (1999).* **63**, (2), 161-167 (2013).
12. Finney, C. A. M., *et al.* HIV infection deregulates innate immunity to malaria despite combination antiretroviral therapy. *AIDS (London, England).* **27**, (3), 325-335 (2013).
13. Ljungstrom, I., *et al. Methods in Malaria Research.* MR4/ATCC Available from:http://www.mr4.org/Publications/MethodsinMalariaResearch.aspx (2013).
14. Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M. Real time quantitative PCR. *Genome Res.* **6**, (10), 986-994 (1996).
15. Sigma Aldrich, *Universal SYBR green quantitative PCR protocol.* Available from:http://www.sigmaaldrich.com/technical-documents/protocols/biology/sybr-green-qpcr.html (2014).
16. Livak, K. J., Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCT method. *Methods.* **25**, (4), 402-408 (2001).
17. Hensmann, M., Kwiatkowski, D. Cellular basis of early cytokine response to Plasmodium falciparum.*Infection and immunity.* **69**, (4), 2364-2371 (2001).
18. Artavanis-Tsakonas, K., Riley, E. M. Innate immune response to malaria: rapid induction of IFN-gamma from human NK cells by live Plasmodium falciparum-infected erythrocytes. *J. Immunol.* **169**, (6), 2956-2963 (2002).
19. Lambros, C., Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture.*The Journal of parasitology.* **65**, (3), 418-420 (1979).
20. Ludlow, L. E., *et al.* HIV-1 inhibits phagocytosis and inflammatory cytokine responses of human monocyte-derived macrophages to P. falciparum infected erythrocytes. *PloS one.* **7**, (2), e32102 (2012).

21. Weinberg, A., *et al.* Viability and functional activity of cryopreserved mononuclear cells. *Clinical and diagnostic laboratory immunology.* **7**, (4), 714-716 (2000).
22. Fowke, K. R., Behnke, J., Hanson, C., Shea, K., Cosentino, L. M. Apoptosis: a method for evaluating the cryopreservation of whole blood and peripheral blood mononuclear cells. *Journal of immunological.* **244**, (1-2), 139-144 (2000).
23. Jeurink, P. V., Vissers, Y. M., Rappard, B., Savelkoul, H. F. J. T cell responses in fresh and cryopreserved peripheral blood mononuclear cells: kinetics of cell viability, cellular subsets, proliferation, and cytokine production. *Cryobiology.* **57**, (2), 91-103 (2008).
24. Kvarnström, M., Jenmalm, M. C., Ekerfelt, C. Effect of cryopreservation on expression of Th1 and Th2 cytokines in blood mononuclear cells from patients with different cytokine profiles, analysed with three common assays: an overall decrease of interleukin-4. *Cryobiology.* **49**, (2), 157-168 (2004).
25. Venkataraman, M. Effects of cryopreservation of immune responses. XI. Heightened secretion of tumor necrosis factor-alpha by frozen human peripheral blood mononuclear cells. *Cryobiology.* **34**, (3), 276-283 (1997).
26. Venkataraman, M. Effects of cryopreservation on immune responses. X. Decrease in interleukin-12 production by frozen human peripheral blood mononuclear cells is mediated by the endogenously hypersecreted interleukin-10. *Cryobiology.* **33**, (5), 581-588 (1996).
27. Reimann, K. A., Chernoff, M., Wilkening, C. L., Nickerson, C. E., Landay, A. L. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. *The ACTG Immunology Advanced. Clinical and diagnostic laboratory immunology.* **7**, (3), 352-359 (2000).
28. Bennett, S., Breit, S. N. Variables in the isolation and culture of human monocytes that are of particular relevance to studies of HIV. *Journal of leukocyte biology.* **56**, (3), 236-240 (1994).

