TITLE

Isolation and co-cultivation of human macrophages and neutrophils with *Plasmodium falciparum*-parasitized erythrocytes: an optimized system to study the phagocytic activity to malarial parasites

AUTHORS

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Abstract

Macrophages and neutrophils are our front line of defense against invading pathogens. They are professional phagocytic cells that play a key role in the clearance of *Plasmodium falciparum*-parasitized erythrocytes from the host circulation. A stable *in vitro* culture system for these cells and parasitized erythrocytes would provide the means to study their immunological responses to infection, potentially revealing important clues for new therapeutic interventions for malaria. Here, we present an optimized protocol for cultivating human peripheral blood monocyte-derived macrophages and neutrophils with *Plasmodium falciparum*-parasitized erythrocytes.

Key Words: Macrophage, Neutrophil, Phagocytosis, *Plasmodium falciparum*

1. Background

Malaria continues to be the most devastating protozoan infection in the world with high morbidity and mortality. It is caused by apicomplexan protozoa of the genus *Plasmodium*, of which, *Plasmodium falciparum* causes the most deadly infection. The complications caused by infection may include cerebral malaria, acidosis, acute respiratory distress syndrome, and acute renal and hepatic failure. Current antimalarial chemotherapies are severely compromised because of the emergence of resistance in many endemic countries. Moreover, the most advanced malaria vaccine to date, RTS,S, offers only partial protection that is not potent enough to completely prevent infection in most recipients [1].

Phagocytes, including macrophages and neutrophils, play key roles in the innate immune response against infection through their ability to rapidly recognize and eliminate invading pathogens. Evidences for the involvement of splenic resident macrophages and circulating neutrophils in the control of malarial infection have been increasingly reported *in vitro* and *in vivo* studies [2-7]. These immune cells produce a large number of cytokines to regulate host immune response as well as antimicrobial proteins to combat pathogens. Therefore, understanding of the molecular defense mechanism by which phagocytes kill malarial parasites would provide

important clues to assist in the discovery of more effective therapies and vaccination strategies.

Several studies have attempted to establish an *in vitro* system for cultivating phagocytic cells with *Plasmodium falciparum*-parasitized erythrocytes (iRBC) [2-4, 8, 9]. However, none of these studies has proven a good system that mimics the *in vivo* conditions of the phagocytes in response to iRBCs. Here, we present an optimized protocol for the isolation and cultivation of human macrophages and neutrophils for use in a phagocytic assay against iRBC. The immune cells were treated by certain cytokines for differentiation and activation prior to cultivation with iRBCs. Concentration of cytokines was optimized for best activity of the immune cells.

2. Objective

To establish an optimal protocol for the isolation and cultivation of human macrophages and neutrophils for use in a phagocytic assay against *Plasmodium falciparum*-iRBC.

3. Methods

3.1. Parasites culture

The *Plasmodium falciparum* 3D7 clone was cultured in a 25-cm tissue flask and

maintained in 10 ml of malaria culturing medium containing 100 μ l of human erythrocytes (1% hematocrit). The tissue flask was inoculated at 37°C in an atmosphere of 5% CO₂/3% O₂/balanced N₂. The medium was replaced daily with a fresh medium (10 ml) for three days, and then the packed erythrocytes were resuspended in 10 ml medium. The iRBCs were subjected to MACS magnetic separation (MiltenylBiotec, Auburn, CA, USA), which enriches the purity of cells to > 85% trophozoite- and schizont-iRBCs[10]. The iRBCs were then washed once with RPMI-1640 medium and used for the phagocytic assay. The protocol was approved by the Committee on the Research Ethics of Human Bodies and Samples of Obihiro University of Agriculture and Veterinary (Permit Number: 2014-01).

Malaria culturing medium

RPMI-1640 (Sigma-Aldrich)

25 mM HEPES (Dojindo Molecular Technologies)

0.15% Sodium carbonate (Sigma)

0.50% Albumax II (Invitrogen, Carlsbad, CA, USA)

100 μ M Hypoxanthine (Sigma)

12.5 µg/mL Gentamycine (Sigma)

3.2.Preparation of differentiated macrophages and the phagocytic assay

Human mononuclear cells were separated from the peripheral blood of healthy donor by using Ficoll-Paque™ PLUS (GE Healthcare) and cultured for 8 days at 37°C in a humidified atmosphere containing 5% CO₂ in macrophage differentiation medium that contained 20 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and 100 ng/ml human recombinant interferon gamma (IFN-γ) (R&D system) to stimulate cell differentiation. Blood, diluted 1:1 with warm PBS, was layered onto Ficoll (5 ml for each) and centrifuged at 800 xg for 30 minutes at 21°C. The peripheral blood mononuclear cell (PBMC) layer was harvested (Fig. 1), washed twice with cold PBS, and resuspended to 1X10⁶ cells/ml in 10 ml of macrophage differentiation medium that lacked cytokines. Cells were cultured in a 25-cm tissue flask for 3 h to allow cell attachment. Without disturbing the cells on the bottom of flask, the suspension was replaced by fresh macrophage differentiation medium. After 3 days of culture, the suspension was discarded and the attached cells were washed with cold PBS before fresh macrophage differentiation medium was added. On day 6 post-culture, macrophages were detached by treatment with 1% trypsin-EDTA solution for 20 min, washed with cold PBS, counted, seeded onto cover slides

(Matsunami Neo, Osaka, Japan) at 1×10^4 , and then cultured in macrophage differentiation medium for 2 days. Differentiated cells had a unique morphology and strong adherence properties to the cover slides (Fig. 2). Thereafter, enriched mature-iRBC (2×10^5) were resuspended in 1 ml of malaria culturing medium, slowly added to the differentiated macrophages at a final ratio of 20:1, and then cultured for the phagocytic assay. To determine the percentage of phagocytosis, the cover slides were washed with cold PBS twice after a defined incubation time, fixed in absolute methanol for 10 min, stained with 10% Giemsa solution (Sigma) for 30 min, and then examined by microscopy using an oil immersion lens. The percentage was calculated based on the number of macrophages phagocytizing iRBC or malaria pigment to the total number of macrophages (Fig. 3). All cells were prepared from same donor to minimize nonspecific reaction of macrophages to the erythrocytes. Differentiated macrophages exhibited minor but detectable phagocytic activity to RBCs.

Macrophage differentiation medium

RPMI-1640 (Sigma)

10% heat-inactivated fetal calf serum (Gibco Life Technologies)

25 mg/L penicillin/streptomycin (Gibco)

20 ng/ml GM-CSF (R&D)

100 ng/ml IFN- γ (R&D)

3.3. Preparation of neutrophils and the phagocytic assay.

Polymorphonuclear cells (PMN) were harvested from RBC pellets (Fig. 1). The RBCs were lysed using RBC lysis buffer (Sigma), and PMN were pelleted by centrifugation at 300 $\times g$ and then washed three times with cold PBS. Cell purity was determined by performing a differential blood cell count of blood smear after staining the cells with 10% Giemsa solution (Sigma). Cells viability was assessed by using the trypan blue exclusion test. The protocol produced >96% neutrophils with viability of >90%. Cells were seeded onto Poly-D-Lysine-coated coverslips (neuVibro, WA, USA) at 1×10^4 and then cultured in neutrophil medium at 37°C in a humidified atmosphere containing 5% CO₂ for 30 min to allow cell attachment. The slides were then washed again with cold PBS and cultured with **enriched mature-iRBC** (1:20) in 1 ml of neutrophil medium for the phagocytic assay. After a defined incubation time, the coverslips were washed twice with cold PBS, fixed in absolute methanol for 10 min, and stained with 10% Giemsa solution (Sigma). Finally, the percentage of phagocytosis was determined by microscopic examination under an oil immersion lens. The percentage was

calculated based on the number of neutrophils phagocytizing iRBC or malaria pigment to the total number of neutrophils (Fig. 4). All cells were prepared from same donor to minimize nonspecific reaction of neutrophils to the erythrocytes. Neutrophils had no phagocytic activity to RBCs within 1 hr of culture.

Neutrophil medium

RPMI-1640 (Sigma)

25% Human serum without heat inactivation (Sigma)

25 mg/L penicillin/streptomycin (Gibco)

250 ng/ml IFN- γ (R&D)

4. Special Remarks

Monocyte-derived macrophages presented a unique morphology and strong adherence properties after stimulation with GM-CSF and IFN- γ for eight days in culture (Fig. 2). These differentiated macrophages showed >90% viability and >90% CD14 cell staining by flow cytometry. Moreover, the differentiated macrophages exhibited high phagocytic activity against iRBC with all of the apparent stages of phagocytosis that have been observed against other pathogens (Fig. 3). Likewise, isolated PMNs of >96% purity and with >90% viability demonstrated rapid phagocytic activity against iRBC (Fig. 4). These results suggest that our optimized protocol for phagocytosis of iRBCs is of value to research on the molecular responses of phagocytes to malaria infection.

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Figure legends

Fig. 1. Separation of immune cells from whole blood by means of Ficoll-Paque.

Fig. 2. Morphology of monocyte-derived macrophages after 8 days of culture with GM-CSF and IFN- γ .

Fig. 3. Phagocytic activity of macrophages against iRBC. The stages of phagocytosis include: A) adherence, B) pseudopodium formation and ingestion, C) digestion, and D) residual bodies. Scale bar = 10 μ M. Arrows point to iRBCs.

Fig. 4. Phagocytic activity of neutrophils against iRBC. Scale bar = 10 μ M. Arrows point to iRBCs.

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Figure 1

PBMC

PMN & RBC

Figure 2

Figure 3

Figure 4

Macrophages

Neutrophils