# Racial Differences in Neutrophil Response

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## INTRODUCTION

Bacterial lipopolysaccharide (LPS), or endotoxin, derived from the cell wall of Gram-negative bacteria is a mediator of inflammatory response. Repeated translocation of oral or intestinal bacteria, or the cell wall components, into the bloodstream can result in low-grade inflammation and endotoxemia, which have been associated with systemic diseases such as cardiovascular disease and atherosclerosis.[1,2]

Much has been reported about socioeconomic and modifiable risk factors (tobacco use, diet, obesity and physical activity) for cardiovascular and systemic inflammatory diseases. However, little attention has been focused on genetic differences which may contribute to variations in incidence, morbidity and mortality for these diseases in individuals of different ethnic and racial backgrounds. An IUSD study of experimental gingivitis caused by plaque accumulation reported lower neutrophil counts in African Americans compared to Caucasians over the duration of the study.[3] The gingival index was not significantly different, but the plaque index was marginally higher in African Americans compared to Caucasians. During the plaque accumulation study phase, neutrophil activity increased significantly from baseline for African Americans but not for Caucasians. In a subset of these subjects, serum endotoxin levels were associated with gingivitis and endotoxemia, as well as hyperactivity of circulating neutrophils.[4]

The objective of the present *in vitro* study was to determine whether neutrophils from African American and Caucasian males released similar levels of LPS-specific neutrophil activation markers after exposure to clinically relevant, but low levels of LPS followed by activation with formyl-methionyl-leucyl-phenylalanine (fMLP). Neutrophil activation was quantitated by release of the antimicrobial bactericidal permeability increasing (BPI) protein, which neutralizes LPS, and a peroxidase enzyme (myeloperoxidase, MPO) from the azurophilic granules. In addition, the neutrophil activation media was assayed for acyloxyacyl hydrolase (AOAH), a lysosomal enzyme that partially deacylates the lipid-A component of endotoxin rendering it less toxic.

## MATERIALS AND METHODS

**Neutrophil Isolation** - Whole blood mixed with 6% polysucrose (Sigma-Aldrich, St. Louis, MO) was incubated for 60 minutes at room temperature to allow the blood cells to sediment from the plasma. Next, the blood cells diluted with PBS were layered over Histopaque® density gradients 1077 and 1119 (Sigma-Aldrich). After centrifugation neutrophils were collected from the gradient interface, washed twice then counted by using a hemocytometer. All neutrophil isolates were > 95% viable as determined by trypan blue dye exclusion.

**Serum preparation** – Clotted blood was centrifuged 3000 g for 10 minutes. The serum was removed, transferred to a sterile tube then centrifuged again to remove residual platelets.

**Neutrophil priming and activation** - Duplicate tubes with 5 x $10^6$ neutrophils in 1 ml RPMI (Sigma-Aldrich), 5% autologous serum and 1 ng /ml LPS (E.coli O55:B5, Sigma-Aldrich) were prepared. A tube with neutrophils and 5% serum but without LPS served as an unprimed and unactivated control. All tubes were incubated at 37°C for 30 or 60 minutes. After resuspending the neutrophils by gentle pipetting, 0.5 ml was transferred to a fresh tube. Neutrophils in the remaining 0.5 ml were activated for 10 minutes with formyl-methionyl-leucyl-phenylalanine (fMLP, 0.1 uM) at 37°C. Each tube was centrifuged 3000 g for 3 minutes. Subsequently, the supernatants were transferred to a fresh tube and stored at -20°C. The control remained at 37°C for 70 minutes.

**ELISA testing** - The supernatants were tested in duplicate by commercial ELISA for MPO (BioLegend, San Diego, CA), BPI (Hycult Biotech, Uden, the Netherlands) and AOAH (MyBiosource.com, San Diego, CA) according to the manufacturer's protocols.

**Statistics** - Two-tail Student's t test assuming unequal variances was used to determine statistical differences in the ELISA values.

## SUBJECTS

African American (n=6) and Caucasian (n=6) males 18 to 40 years of age participated in this study. Exclusion criteria included a recent history of infectious or systemic inflammatory disease, use of antibiotics or drugs affecting inflammatory response in the previous 3 months and current tobacco use. After obtaining informed consent, blood was collected from each subject into tubes containing the anticoagulant ACD for neutrophil isolation, and a plain red top tube to obtain serum (Vacuutainer, Franklin Lakes, NJ). Blood specimens were processed within 45 minutes of collection. This study was approved by the IUSD IRB (1308115599).

**FIGURE 1. Neutrophil isolation.**

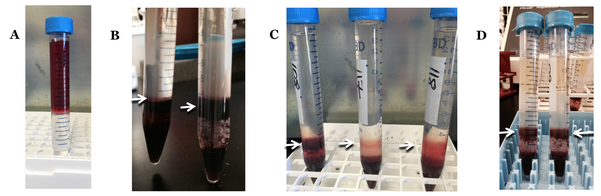
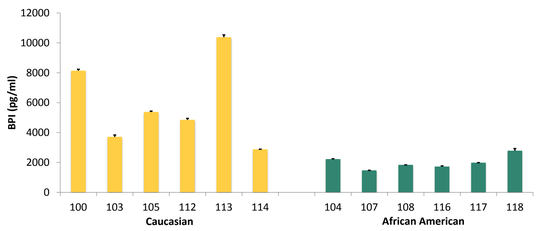


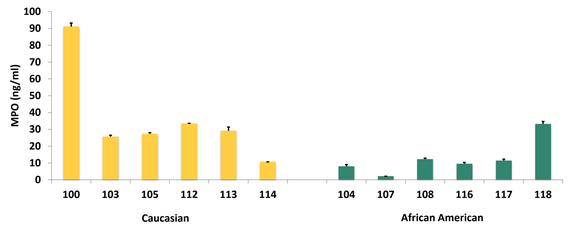

Photo A shows whole blood layered over Histopaque® density gradients 1077 and 1119 prior to separation by centrifugation. Photos B, C, & D document variation in neutrophil purity among subjects after density gradient isolation using identical protocols. The white arrows indicate the neutrophil layer.

**FIGURE 2. Bactericidal permeability increasing (BPI) protein**




Isolated neutrophils were primed for 60 minutes with 1 ng/ml of LPS then activated with fMLP for 10 minutes. Cell culture supernatants from activated neutrophils were tested for BPI levels by ELISA. Neutrophils isolated from Caucasian subjects released significantly more BPI than neutrophils from African American subjects (p= 0.0197). Similar data were observed from neutrophils primed for 30 minutes prior to activation (p=0.0077).

**FIGURE 3. Myeloperoxidase (MPO)**




Isolated neutrophils were primed for 60 minutes with 1 ng/ml of LPS then activated with fMLP for 10 minutes. Cell culture supernatants from activated neutrophils were tested for MPO levels by ELISA. Although increased levels of MPO were found in neutrophils isolated from Caucasian subjects compared to neutrophils from African American subjects the difference was not significant (p= 0.2036). Similar data were observed from neutrophils primed for 30 minutes prior to activation (p=0.2105).

## RESULTS

**Neutrophil isolation**. (Figure 1) The purity of neutrophils isolated from different subjects varied depending upon red blood cell (RBC) contamination of the neutrophil layer. In general, neutrophil isolates from African American subjects contained more RBC than isolates from Caucasian subjects. Consequently, neutrophils from subjects 104, 107, 108, 118 underwent a second density gradient isolation to improve neutrophil purity.

**BPI release.** (Figure 2) The amount of BPI released into the culture media from the neutrophil azurophilic granules after 60 minutes of LPS priming and fMLP activation was significantly increased in neutrophils from Caucasian compared to African American subjects (p= 0.0197). Comparable results were observed from neutrophils primed for 30 minutes suggesting that the neutrophils were fully primed within 30 minutes of LPS exposure.

**MPO release.** (Figure 3) A non-significant increase in MPO release was observed for Caucasian subjects compared to African American subjects (p= 0.2036).

**AOAH release** was undetectable.

**Control tubes** (no LPS or fMLP) incubated simultaneously with the primed and activated tubes, were highly variable regardless of race. In some instances, release of BPI or MPO into the control tubes exceeded release subsequent to activation. This finding was unexpected and warrants additional investigation.

## DISCUSSION

- Racial differences in white and red blood cells have been reported; for example, African Americans have lower white blood cell and neutrophil numbers, and lower hemoglobin levels compared to other racial groups.[5] These differences may explain the variations in neutrophil isolation such as the apparent increased buoyant density of RBC leading to increased RBC contamination in the neutrophil isolates of African American subjects.[5]
- The observed *in vitro* differences between Caucasian and African American subjects for release of BPI and MPO after neutrophil activation further support the *in vivo* findings of Wahaidi, et. al. [3,4]
- It is possible, albeit unlikely, that repeating the separation procedure resulted in lower levels of BPI and MPO release in African American subjects. However, neutrophils from subjects 117 and 118 did not undergo repeat separation but released similar levels of BPI as the remainder of the African American subjects suggesting the finding is not related to repeat processing.

## REFERENCES

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