# Nakahira et al., http://www.jem.org/cgi/content/full/jem.20060845/DC1

## SUPPLEMENTAL MATERIALS AND METHODS

**Neutrophil isolation, cytochrome b558 isolation, and spectral analysis.** Fresh bovine peripheral blood (8–20 liters) was taken at 9:1 vol/vol into anticoagulant solution (1.5% EDTA, 0.0132 M sodium phosphate, 0.7% NaCl, pH 6.8). Anticoagulated blood was centrifuged for 20 min at 1,000 *g*, and the plasma layer and buffy coats were aspirated. The erythrocyte-granulocyte pellet (lower phase) was pooled into glass bottles and mixed at 1:1 vol/vol with ice-cold sterile distilled water for 30 s. Isotonicity was restored by adding 0.5 original volume of 3× isotonic solution (2.7% NaCl, 0.0132 M sodium phosphate, pH 6.8). The resulting suspension was centrifuged at 1,000 *g* for 15 min, and the pellets were resuspended in 10 ml PIPES-buffered saline (PIBS: 140 mM NaCl, 10 mM PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid)], pH 6.8). The cells were repelleted, and osmotic shock was repeated until the pellets were erythrocyte free. The resulting pellet was resuspended in 10 ml PIBS, pH 6.8. The cells were treated in suspension at room temperature for 15 min with 3.5 mM of the protease inhibitor, diisopropyl fluorophosphate. The suspension was centrifuged at 1,000 rpm and washed once in PIBS, pH 6.8. The packed cell volume (~3 ml) was resuspended in 10 ml PIBS, pH 6.8, containing 1 mg/ml glucose, 1 mg/ml BSA, 50 U/ml SOD, and 250 U/ml catalase to a final cell concentration of $\sim 5 \times 10^8$ cells/ml. The cells were incubated at 37°C for 40 min. After incubation, the cells were resuspended in 0.34 M sucrose, 10 mM Tris, pH 7.4, containing protease inhibitors and sonicated at low power for 15 s twice, followed by centrifugation for 30 min at 12,000 rpm. The supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. Resulting pellets were resuspended by brief sonication at low power in 1 ml membrane resuspension buffer (100 mM KCl, 10 mM NaCl, 1 mM EDTA, 10 mM Hepes, pH 7.4). The resuspension was flash frozen and stored in liquid nitrogen. Microsomal suspensions were subsequently thawed on ice and resuspended in membrane resuspension buffer adjusted to a final concentration of 1 M NaCl. The suspension was centrifuged for 50 min at 144,000 *g*. The resulting pellets were resuspended by brief sonication in 10 ml of sodium phosphate buffer (30 mM sodium phosphate, pH 7.4, 50 mM NaCl, 0.8 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid], 0.46 mM dithiothreitol, 20% glycerol, and 1% n-heptyl-β-D-thioglucopyranoside). The cytochrome *b*558 fraction was extracted by gentle stirring at 4°C for 4 h. The solution was then centrifuged at 100,000 *g* for 30 min. The resulting supernatant was flash frozen and stored in liquid nitrogen for spectrophotometric analysis.

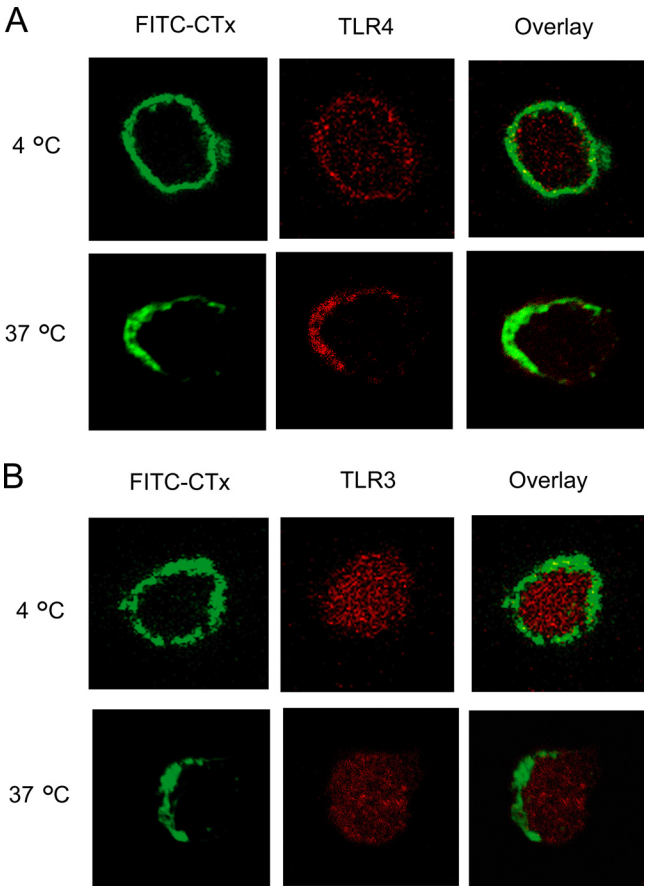


Figure S1. Differential trafficking patterns of TLRs. (A and B) RAW 264.7 cells were incubated with FITC-CTx on ice for 10 min, followed by incubation with anti-CTx for 15 min at 4 or 37°C. Cells were processed for immunofluorescence staining of CTx (green) and TLR4 (red) or TLR3 (red).

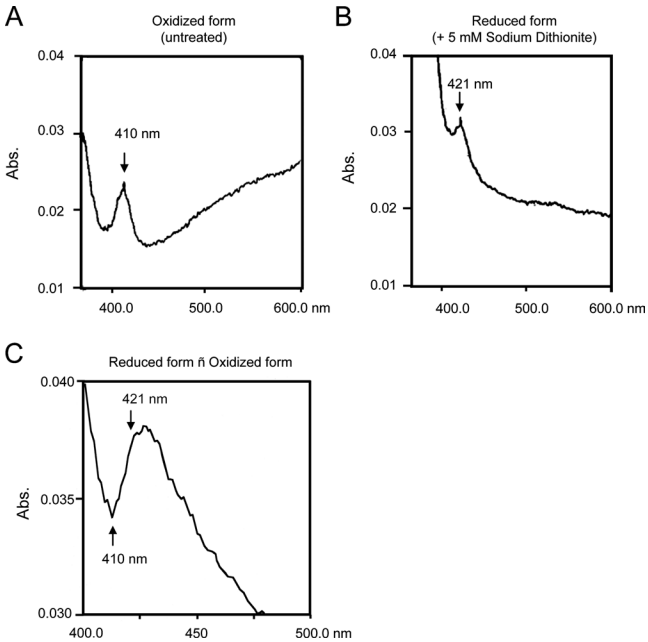


Figure S2. Spectral analysis of cytochrome *b*558. Cytochrome *b*558 was partially purified from bovine neutrophils. Spectra of the partially purified cytochrome *b*558 fraction were generated in the absence or presence of 5 mM of sodium dithionite. Corresponding difference spectra were digitally generated. (A) Spectrum of oxidized form (untreated control); (B) spectrum of reduced form (sodium dithionate treated); and (C) the spectral difference between the reduced and oxidized form are shown.


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