SUPPLEMENTAL DATA

RECOGNITION OF PATHOGENIC MICROBES BY THE *DROSOPHILA* PHAGOCYTIC PATTERN RECOGNITION RECEPTOR EATER*

Yoon-Suk Alexander Chung and Christine Kocks

From Department of Pediatrics and Department of Molecular Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Running head: Pathogen recognition by *Drosophila* phagocytic receptor

Address correspondence to: Christine Kocks, PhD, Massachusetts General Hospital, 185 Cambridge Street CPZN 7.250, Boston, MA 02114. Tel: 617-771-2915, Fax: 617-726-5949, E-Mail: kocks @molbio.harvard.edu

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Deglycosylation of Eater-Fc– Eater-Fc fusion protein was incubated overnight at 4˚C in PBS under non-denaturing conditions with or without (mock control) Peptide:N-glycosidase F (PNGase F; New England Biolabs). Deglycosylated Eater-Fc was subsequently used for SDS-gel analysis (controls; uncleaved or after thrombin cleavage) or in flow cytometry binding assays (uncleaved).

Fluorescence microscopy– 5×10^6 heat- or ethanol-inactivated Gram-negative bacteria in 5 µl were allowed to adhere for 30 minutes to poly-L-lysine coated coverslips and incubated for 30 minutes with 200 µM Eater-Fc followed by 1 µg/ml streptavidin-Alexa Fluor 488 conjugate (Invitrogen), then embedded in mowiol 4-88. Micrographs were taken on a Nikon TE-2000U microscope with a 60 x oil objective and Openlab 5.5 software (Perkin Elmer). 1.25×10^5 *M. luteus* was used as a negative control.

Pre-embedding gold labeling EM– At 4˚C, 500 µM (25 µg/ml) biotinylated Eater-Fc fusion protein was pre-incubated in PBS for one hour with 2.7 µg/ml Streptavidin-15 nm colloidal gold conjugate (Ted Pella Inc.) and then rotated in a total volume of 100 µl for 16 hours with 1×10^7 *E. coli*, either heat-inactivated, or cecropin A-killed, or live. Labeled bacteria were washed once with PBS and resuspended in 2 % glutaraldehyde and stored at 4˚C. Fixed bacteria were pelleted, rinsed once with 0.1 M sodium cacodylate buffer (pH 7.4) and re-pelleted. Bacterial pellets were stabilized with 2.0 % agarose before dehydration and embedding in Eponate resin (Ted Pella Inc.). Thin sections were collected onto formvar-coated slot grids, post-stained with 2.0 % aqueous uranyl acetate and examined in a JEOL 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital imaging system (Advanced Microscopy Techniques).

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. **Eater-Fc binding to bacteria is dependent on glycosylation.** *A*. Coomassie Blue stained SDS gel (15 %). Biotinylated Eater-Fc was deglycosylated with PNGase F under non-denaturing conditions or mock-treated, and thrombin-cleaved where indicated. Deglycosylation is evident as a size shift of full length or thrombin-cleaved Eater-Fc towards lower molecular weights (compared to mock-treated control). *B & C*. Flow cytometry analysis of binding by 200 µM biotinylated Eater-Fc fusion protein, either mock-treated (black curve) or deglycosylated with PNGase F (red curve). Deglycosylated, PNGase F-treated Eater-Fc showed undiminished binding to Protein A-conjugated microspheres *(B)*. In contrast to mock-treated Eater-Fc, deglycosylated, PNGase F-treated Eater-Fc had lost its binding activity to the heat-inactivated Gram-negative bacteria *E. coli or P. aeruginosa*. No significant binding was detected to live bacteria Gram-negative bacteria *(C)*.

FIGURE S2. **Eater-Fc binds to both, heat-inactivated and ethanol-fixed Gram-negative bacteria.** Fluorescence microscopy of bacteria stained with 200 µM biotinylated Eater-Fc fusion protein. Binding was observed with heat- and ethanol-inactivated Gram-negative bacteria (*E. coli, S. marcescens* and *P. aeruginosa)*, but not the Gram-positive actinobacterium *M. luteus* (control). EtOH-I., ethanol-inactivated; H.-I., heat-inactivated.

FIGURE S3. **Eater-Fc binds to heat-inactivated, rough LPS mutant *S. marcescens.*** Flow cytometric analysis of binding by 200 µM biotinylated Eater-Fc fusion protein (open black curve) or control biotinylated IgG1 and IgG-Fc (broken black or broken gray curves, respectively) compared to secondary reagent only (gray filled curve) or unstained microbes (open gray curve). Eater-Fc bound well to heat-inactivated rough LPS mutant *S. marcescens* and wildtype, but not to heat-inactivated *M. luteus* (control). No binding was detected to live rough LPS mutant or wildtype *S. marcescens.*

FIGURE S4. **Concentration and time-dependent killing of *E. coli* by cationic AMP.** Live *E. coli* were incubated in the presence of cecropin A at 25˚C, transferred briefly to ice and analysed immediately by flow cytometry in the presence of the viability stain PI. *A.* Dose dependent killing of *E. coli* by cecropin A (0 to 4 µM; blue curve) compared to cationic control peptide 2K1 (red curve). Histograms corresponding to increasing amounts of cecropin A (0.5 to 2 µM; blue boxes) or 4 µM cationic control peptide 2K1 (boxed in red) are shown below the line graph. These experiments were repeated three times with similar results. *B.* Time and concentration-dependency of *E. coli* killing by cecropin A. Time dependent killing kinetics for three different concentrations of cecropin A: 1 µM (red), 0.5 µM (blue) and 0.25 µM (green). At 1 and 0.5 µM of cecropin A bacterial killing was rapid. Slower killing kinetics were observed with lower amounts of cecropin A (0.25 µM). The apparent viability decrease and increase in the later time points in the blue curve likely reflects experimental variability.

FIGURE S5. **Pre-embedding gold labeling EM shows Eater-Fc binding to cell envelopes of *E. coli* after membrane disruption.** *E. coli* was used either heat-inactivated, or killed with cecropin A or live, and labeled with 500 µM pre-formed, biotinylated Eater-Fc-Streptavidin-gold conjugate before processing and thin sectioning for EM. *A*. Heat-inactivated *E. coli* labeled with Eater-Fc (upper panel) or secondary reagent only (middle panel). *B*. *E. coli* killed with cecropin A (4 µM) for 10 minutes at 25˚C (upper panel) or used live (0 µM cecropin A) (middle panel). *A, B*. Magnification 60,000x. (Lower panels) Quantification of gold label associated with bacteria. “n” indicates number of bacteria analysed.

FIGURE S1

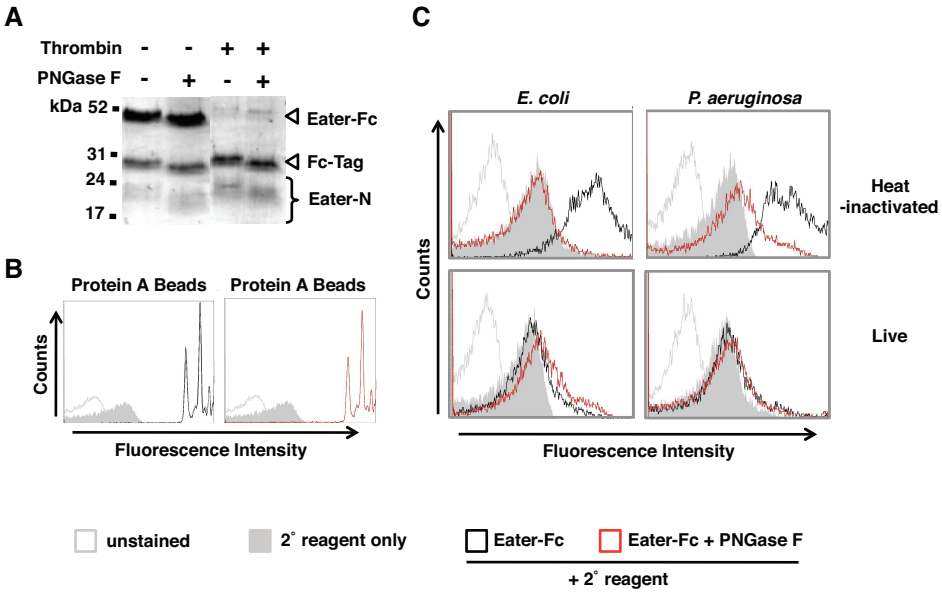

A
Thrombin - - + +
PNGase F - + - +
kDa 52
31
24
17
Eater-Fc
Fc-Tag
Eater-N
B
Protein A Beads
Protein A Beads
Counts
Fluorescence Intensity
C
E. coli
P. aeruginosa
Heat -inactivated
Live
Counts
Fluorescence Intensity
unstained
2˚ reagent only
Eater-Fc
Eater-Fc + PNGase F
+ 2˚ reagent

FIGURE S2

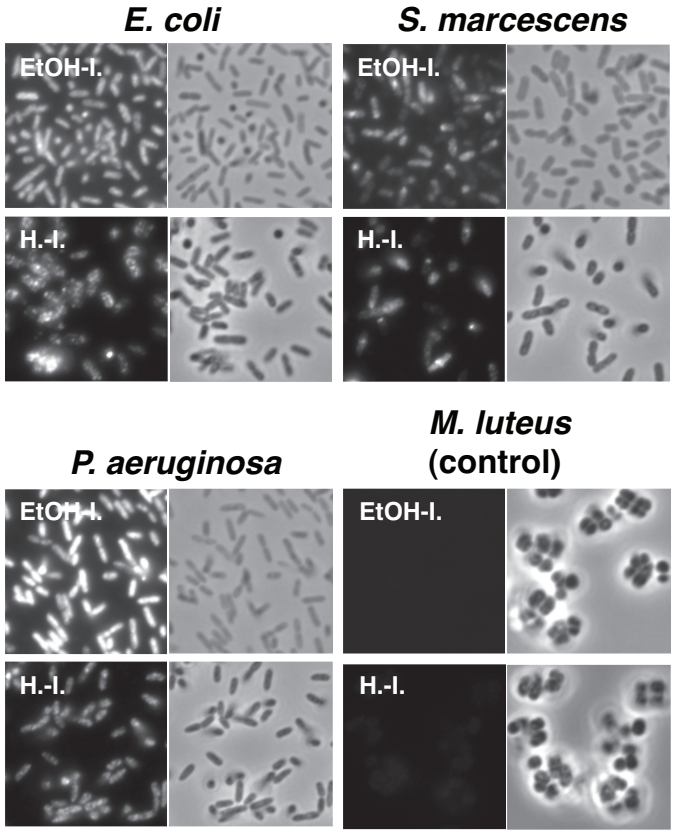

E. coli
S. marcescens
EtOH-I.
H.-I.
EtOH-I.
H.-I.
P. aeruginosa
M. luteus
(control)
EtOH-I.
H.-I.
EtOH-I.
H.-I.

FIGURE S3

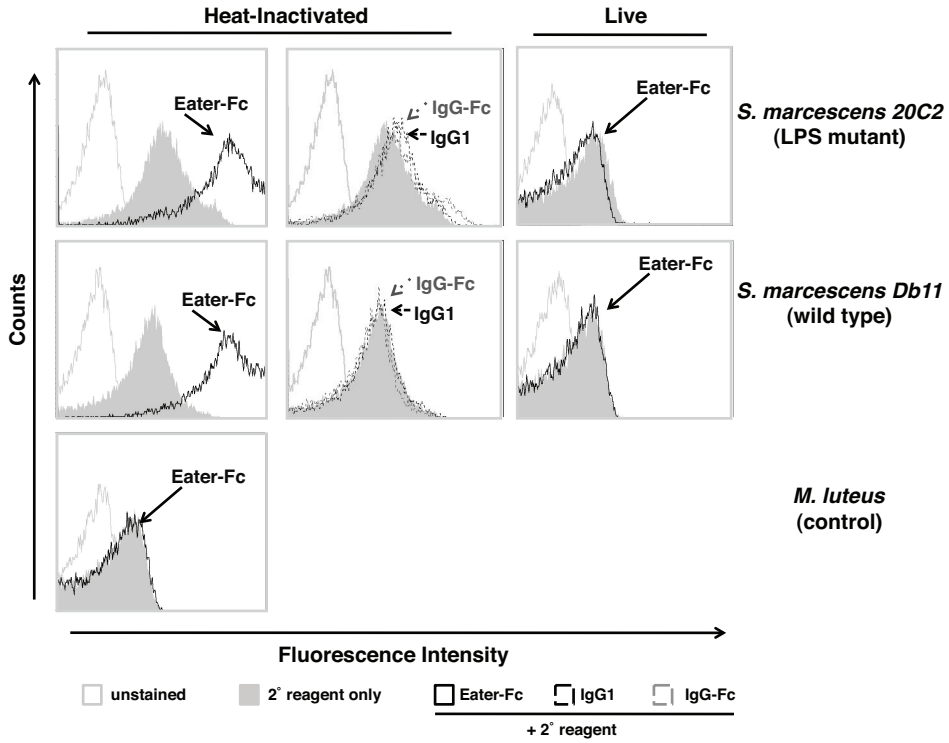

Heat-Inactivated
Live
Eater-Fc
IgG-Fc
IgG1
Eater-Fc
S. marcescens 20C2
(LPS mutant)
Eater-Fc
IgG-Fc
IgG1
Eater-Fc
S. marcescens Db11
(wild type)
Eater-Fc
M. luteus
(control)
Counts
Fluorescence Intensity
unstained
2° reagent only
Eater-Fc
IgG1
IgG-Fc
+ 2° reagent

FIGURE S4

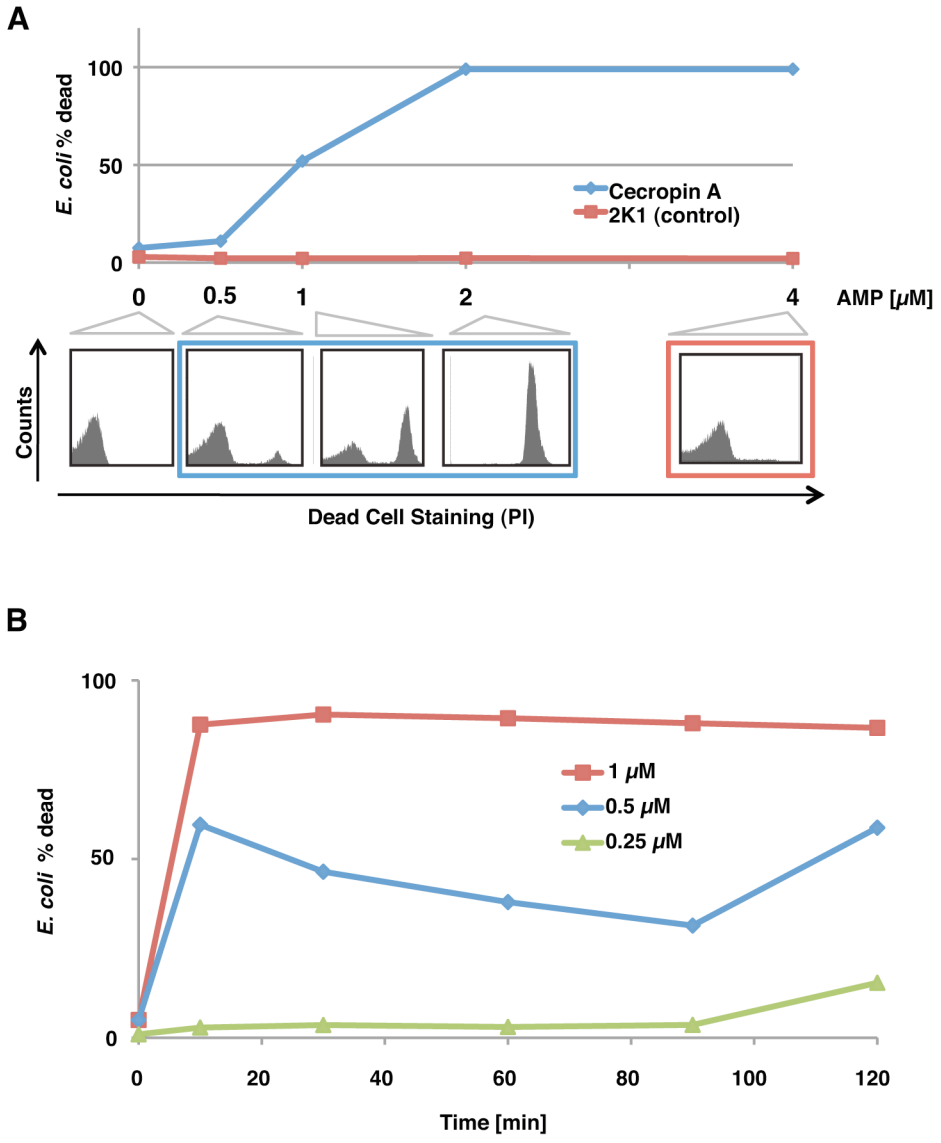
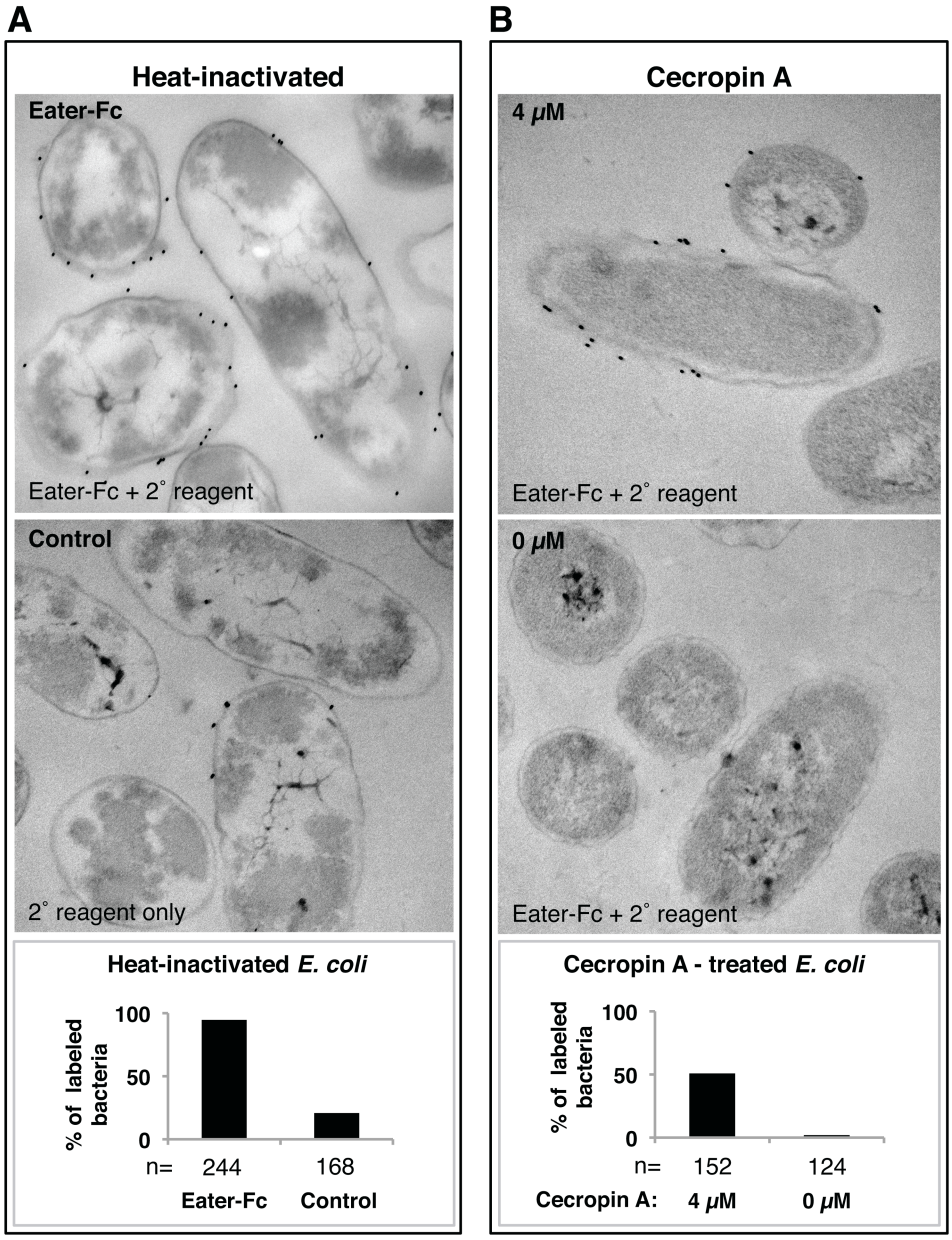

A
100
50
0
E. coli % dead
Cecropin A
2K1 (control)
0
0.5
1
2
4
AMP [μM]
Counts
Dead Cell Staining (PI)
B
100
50
0
E. coli % dead
1 μM
0.5 μM
0.25 μM
0
20
40
60
80
100
120
Time [min]

FIGURE S5


A
Heat-inactivated
Eater-Fc
Eater-Fc + 2° reagent
Control
2° reagent only
Heat-inactivated E. coli
% of labeled bacteria
100
50
0
n= 244 168
Eater-Fc Control
B
Cecropin A
4 μM
Eater-Fc + 2° reagent
0 μM
Eater-Fc + 2° reagent
Cecropin A - treated E. coli
% of labeled bacteria
100
50
0
n= 152 124
Cecropin A: 4 μM 0 μM