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A Flow-Cytometric Method to Evaluate Eosinophil-Mediated Uptake of Probiotic *Lactobacillus reuteri*

Laura S. Kraemer¹, Todd A. Brenner^{1,2}, Julia O. Krumholz¹, and Helene F. Rosenberg^{1,3}

¹Inflammation Immunobiology Section, Laboratory of Allergic Diseases, NIAID, National Institutes of Health, Bethesda, MD 20892

Abstract

Eosinophils are resident leukocytes of gut mucosa. Here we present a combined flow cytometric-antibiotic protection assay to identify mouse eosinophils capable of bacterial uptake, specifically, Gram-positive *Lactobacillus reuteri*, in studies performed *ex vivo*. The assay may be adapted for use *in vivo*.

Keywords

Inflammation; Probiotic; Granulocyte; Fluorescence

1.0 Introduction

Eosinophils are granulocytic leukocytes that develop in the bone marrow from pluripotent stem cell progenitors. Although they are released into the peripheral blood in a phenotypically mature state, they remain capable of activation, migration, and significant phenotypic change [Rosenberg *et al.*, 2013]. Likewise, and frequently overlooked, eosinophils are also resident leukocytes of the gut mucosa [Schroeder *et al.*, 2013; Rosenberg *et al.*, 2016] where they modulate the microbiome directly or indirectly [Chu *et al.*, 2014; Jung *et al.*, 2015] and may provide an important barrier against gut pathogens [Buonomo *et al.*, 2016; Cowardin *et al.*, 2016].

Despite their prominent localization in the gut, our understanding of eosinophil-mediated interactions with bacteria remains limited. Among the most prominent findings, Yousefi and

³Corresponding author: Building 50, room 6241, 50 South Drive, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; 301 761 6682; hrosenberg@niaid.nih.gov.

²Current address: Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts, United States of America

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Author Contributions

LSK performed the experimental work and prepared primary drafts of the Figures and Methods.

TAB generated the bacterial transformants used in this study.

JOK contributed supporting experimental work.

HFR developed the concept and wrote the paper with assistance from LSK as above.

colleagues [2008] have shown that eosinophils interacting with bacteria *ex vivo* generate unique extracellular traps consisting of mitochondrial DNA and granule proteins eosinophil cationic protein and major basic protein. Ueki and colleagues [2016] subsequently identified more traditional nuclear DNA nets from eosinophils from *in vivo* sources of eosinophil-enriched tissue. Most recently, as noted above, Petri and colleagues [Buonomo *et al.*, 2016; Cowardin *et al.*, 2016] identified eosinophils as crucial mediators of protection in experimental infection with the gut pathogen, *Clostridium difficile*. As such, additional means to explore eosinophil-mediated interactions with bacteria in mouse models would be of substantial interest.

Internalization, or uptake of bacteria has been characterized extensively for human neutrophils (reviewed in [Quie *et al.*, 1977; Gray & Botelho, 2017]). Human eosinophils also internalize bacteria [Cline *et al.*, 1968; Cohen & Sapp, 1969; Hatano *et al.*, 2009; Driss *et al.*, 2012] although they are significantly less effective at doing so than human neutrophils. Similar interactions of mouse eosinophils with bacteria have received little attention [Lee *et al.*, 2012], and the factors and mechanisms that contribute to bacterial uptake in mouse eosinophils remain unknown.

In this manuscript, we present a flow cytometric detection method combined with a modification of the traditional gentamicin protection assay, which has facilitated quantitative evaluation of mouse eosinophil-mediated uptake of bacteria. Further applications of this method are discussed.

2.0 Materials and Methods

2.1 Mice

Wild-type C57BL/6 mice (5–8 weeks old) were purchased from Charles River Laboratories, Frederick, MD. Interleukin-5 transgenic mice on the C57BL/6 background were a gift from Dr. James Lee and Dr. Nancy Lee (IL5tg; NJ.1638 [Lee *et al.*, 1997]). The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all of the experimental procedures as per protocol LAD 8E.

2.2 Generation of fluorescent-labelled bacterial strains

The expression plasmid pTRKH3p15A-ldhGFP [AddGene #27167; Lizier *et al.*, 2010], which encodes enhanced green fluorescent protein (eGFP) under the control of the *L. acidophilus* lactate dehydrogenase (ldh) promoter was introduced into *Lactobacillus reuteri* L275 (ATCC 23272) as previously described [Holo & Nes, 1989]. Cultures of fluorescent Lr-eGFP transformants were grown from frozen stocks for 24 hrs at 30°C protected from light with shaking in Mann Rogosa Sharpe (MRS) broth supplemented with 200 mM potassium phosphate, pH 7.0 under erythromycin selection (1.5 µg/mL; Sigma). Non-transformed bacteria were grown under identical conditions in the absence of erythromycin. Preparations of fluorescent *E. coli* DH5α (*Ec*) transformants were prepared in identical fashion, except for growth in Luria Bertani (LB) broth with 1.0 µg/mL erythromycin.

2.3 Eosinophil isolation and culture

Eosinophils were isolated from the spleens of *IL5tg* mice to greater than 95% purity as determined by visual inspection of Diff-Quik stained cytopsin preparations using negative selection as previously described [Ochkur, 2013]. Briefly, single cell suspensions were prepared and red blood cells were lysed in distilled water; T and B lymphocytes were removed using CD90.2 and CD45R/B220 conjugated magnetic beads (Miltenyi). Bone marrow-derived eosinophils (bmEos) were differentiated from unselected progenitor cells to > 99% purity in SCF, FLT-3, and IL-5 also as previously described [Dyer *et al.*, 2008].

2.4 Sensitivity of *Lr-eGFP*, *Ec-eGFP* and isolated eosinophils to treatment with kanamycin

Bacteria grown in culture as described above were washed and re-suspended at 5×10^7 colony forming units (cfu)/mL in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA; PBS + 0.1% BSA); see Gabryszewski et al., 2011 for the description of experimental bacterial OD₆₀₀ vs. cfu/mL determination. These cultures were incubated for 30 min at 37°C while rotating slowly (3 rotations/minute) with various concentrations of kanamycin (50 mg/mL stock solution, Sigma). Ten-fold serial dilutions were plated to determine bacterial survival under these conditions. Isolated spleen and bone marrow-derived eosinophils were suspended in RPMI medium at 5×10^5 cells/mL and incubated for 30 min at 37°C while rotating with the same concentrations of kanamycin. Viability at > 95% was determined by trypan blue staining by standard light microscopy at 30X magnification.

2.5 Eosinophil-mediated uptake experiments

Fluorescent bacteria and non-transformed controls were washed twice with PBS + 0.1% BSA and re-suspended at 10^8 colony cfu/mL. Eosinophils isolated as described above were washed once in PBS + 0.1% BSA and re-suspended at 10^6 cells/mL in RPMI medium. Eosinophils and bacteria were mixed together (0.5 mL each, 100 cfu to 1 eosinophil) and were incubated together for 30 min while rotating (3 rpm) at 37°C. Kanamycin was added to 170 µg/mL and cultures continued for additional 30 min. Cells were then washed twice with PBS + 0.1% BSA and fixed with 4% paraformaldehyde at 4°C overnight. Fixed cells were then washed twice again with PBS + 0.1% BSA and then analyzed by flow cytometry on an LSR II. Compensation and data analysis was performed using FlowJo 10.2 software (Tree Star, Ashland, OR).

2.6 Fluorescence Microscopy

Cytospin preparations from 4% paraformaldehyde fixed cells as above were counterstained with ProLong Gold Anti-Fade DAPI and stored at -20°C prior to evaluation and imaging on a Leica SP5 X-WLL microscope.

2.7 Statistical Analysis

All quantitative findings were from two or three replicate datasets; flow plots shown are representative of typical results. Data were analyzed via appropriate algorithms (Mann-Whitney U test, One-way ANOVA) using GraphPad PRISM with statistical significance as indicated in the Figure Legends.

3.0 Results and Discussion

3.1 Generation of *Lr*-eGFP and optimizing growth conditions for maximum fluorescence

We generated fluorescent *Lactobacillus reuteri* L275 (*Lr*-eGFP) by transformation using the plasmid pTRKH3p15A-ldhGFP as described in the Methods. Similar to results reported by Lizier and colleagues [2010], we found that stable expression of fluorescence required addition of phosphate buffer to maintain stable pH. Likewise, as shown in Fig. 1A, bacterial transformants grown at 30°C display improved fluorescence intensity (~10-fold) over their counterparts grown at 37°C. Using the non-transformed *Lr* as a gating control, we find that 90% of the transformants are GFP⁺ [Fig. 1B] rather than 60% observed in transformant cultures grown at 37°C (data not shown). This optimization was crucial as a significant subset of mouse eosinophils display auto-fluorescence in the blue laser/GFP channel [Fig. 1C].

3.2 Protocol Design

The protocol used here is a variant of the gentamicin protection assay (GPA), a method used widely to monitor uptake of bacteria into eukaryotic host cells. The aminoglycoside antibiotic targets and destroys bacteria that remain in the extracellular space, but it is unable to penetrate the eukaryotic cell membrane [Tabrizi & Robins-Browne, 1993]). This method has been used to evaluate uptake of susceptible Gram-positive and Gram-negative bacteria by epithelial cells, endothelial cells, fibroblasts and macrophages (some examples include Kaneko et al., 2016; Vaudaux & Waldvogel 1979; Keig et al., 2001; Aguilar et al., 2014]) but, to the best of our knowledge, it has not been employed to examine interactions of bacteria with eosinophilic leukocytes.

Our goal was to identify a specific aminoglycoside that could be used at a concentration that would not only kill bacteria rapidly, but that would have no impact on eosinophil viability during course of the experiment. Toward this end, we identified kanamycin as suitable for these studies. Kanamycin, similar to gentamicin, is an aminoglycoside from actinobacteria, in this case, *Streptomyces kanamyceticus*; it destabilizes homeostasis of susceptible bacteria by binding to 30S ribosomal subunit, causing misreading of t-RNA. Kanamycin, similar to gentamicin and other high molecular weight aminoglycosides, remains within the extracellular space, and cannot penetrate the eukaryotic membrane to target bacterial cells that have been internalized [Kohanski et al. 2010; Kaneko et al., 2016].

In the initial time course experiment, we determined that $t = 60\text{--}90$ min was an appropriate window for evaluating the association of eosinophils with *Lr*-eGFP [Fig. 1D]. As shown in Fig. 1E, at a relatively high concentrations (170 $\mu\text{g/mL}$) we observed a 4–5 log decrease of *L. reuteri* cfu within 30 min. At these same time points, eosinophils isolated from IL5tg mice [Fig. 1F] and eosinophils derived from bone marrow culture [Fig. 1G] remained viable.

As such, the protocol developed was as follows: *Lr*-eGFP and *Lr* cultures were grown overnight in MRS medium at 30°C, protected from light, with 200 mM potassium phosphate pH 7.0 to stabilize the pH (*Lr*-eGFP with 1.5 $\mu\text{g/mL}$ erythromycin). Bacteria were washed, and re-suspended in PBS + 0.1% BSA at 10^8 cells/mL. Bacteria were added to eosinophils in suspension in RPMI medium at a ratio of 100:1. Bacteria and cells were rotated end over

end at 37°C for 30 min prior to addition of kanamycin (170 µg/mL) or diluent control. Bacteria and cells were rotated for an additional 30 min, then the reaction was stopped, cells washed, fixed in paraformaldehyde and prepared for flow cytometry.

3.3 Applications

An example of an experiment performed with bone marrow-derived eosinophils is shown in Fig. 2A. In the first panel (i) are the results from eosinophils incubated as above with non-transformed *Lr*, which facilitate appropriate gating. Panels ii. and iii. display results from eosinophils interacting with *Lr*-eGFP without and with kanamycin, respectively. The results suggest that, after 30 min incubation with bacteria alone, and 60 min overall followed by extensive washing, there are relatively few extracellular bacteria remaining to be targeted by kanamycin, results consistent with those reported by Vaudaux and Waldvogel [1979] in studies featuring gentamicin and neutrophil-mediated phagocytosis of carbon-14-labeled *S. aureus*.

Quantitative results with peripheral eosinophils from IL5tg mice are shown in Figs. 2B and 2C. As shown, 13.5 ± 4.8 % of eosinophils are GFP⁺ after 60 min, with a median fluorescence index of 620 ± 87 . As in Fig. 3A, addition of kanamycin under these conditions, while important for experimental design, has little impact on this, later, equilibrium time point (Fig. 2A), and is probably more critical at earlier time points.

3.4 Extended Applications

We also generated fluorescent *E. coli* transformants using a standard lab strain (*Ec-eGFP*, see Methods). While this bacterial strain was slightly less sensitive to kanamycin under analogous culture conditions [Fig. 3A], we were able to generate an image documenting the association of these cells with eosinophils from IL5tg mice after extensive washing and kanamycin treatment [Fig. 3B]. With gating on non-transformed *Ec* as shown in Fig. 3C, 36.6 ± 3.9 % of eosinophils were GFP⁺ after 60 min incubation with *Ec*-eGFP. Kanamycin treatment resulted in a small but statistically significant drop in MFI, from 983 ± 280 to 734 ± 300 (*p < 0.05, Fig. 3D).

4.0 Conclusions

We present here a method for quantitative analysis of eosinophil-mediated uptake of fluorescently-labeled bacteria, notably (but not limited to) a laboratory strain of *E. coli*, and the Gram-positive probiotic, *Lactobacillus reuteri* L275. There are numerous further applications of this assay. Among them, this assay can be extended to include fluorescence-activated cell sorting in order to separate populations and ultimately to distinguish unique gene expression profiles characteristic of the eosinophils that have taken up bacteria (ie., that are GFP-positive) compared to those eosinophils that remain GFP-negative. In this context, it will be also intriguing to explore the impact of specific gene-deletions, notably those related to specific recognition of bacterial patterns and pathogens. Likewise, while this study features eosinophils from peripheral circulation and those differentiated from bone marrow precursors, this assay can be developed further to include eosinophils isolated from the gastrointestinal tract. This point is of particular interest given the role of eosinophils in

maintaining gut homeostasis [Chu *et al.*, 2014; Jung *et al.* 2015], their critical responses to gut pathogens [Buonomo *et al.*, 2016; Cowardin *et al.*, 2016], as noted earlier. Further work will permit us to develop this as an assay to be used to evaluate eosinophil-mediated uptake of bacteria in *in vivo* settings.

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Abbreviations

GFP	green fluorescent protein
KAN	kanamycin
Lr	<i>Lactobacillus reuteri</i>
Ec	<i>Escherichia coli</i>

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Highlights

- A method for evaluating uptake of *L. reuteri* by mouse eosinophils is presented.
- Cells that have incorporated fluorescent *L. reuteri* are detected by flow cytometry.
- Kanamycin destroys exogenous *L. reuteri* but has no impact on eosinophil viability.
- This quantitative assay may be used to evaluate eosinophils from gene-deleted mice.

Fig. 1. Optimizing growth and targeting extracellular bacteria in order to examine eosinophil-mediated uptake of *Lr*-eGFP

A. Histogram documenting the improved fluorescence intensity from *L. reuteri* transfectants (*Lr*-eGFP) grown at 30°C vs. those grown at 37°C. Both cultures were grown in MRS broth supplemented with 200 mM potassium phosphate, pH 7.0, under continuous erythromycin selection. **B.** Pseudocolored dot plots indicating side scatter (SSC-A) vs. fluorescence (GFP) of (i.) *Lr* controls (ii.) *Lr*-eGFP transformants, both grown as above at 30°C. **C.** Pseudocolored dot plots with the same gating indicating side scatter (SSC-A) vs. fluorescence (GFP) of untreated eosinophils from *IL5*g mice. **D.** Time course (t = 0 to 120

min) during which interactions between eosinophils and *Lr*-eGFP can be evaluated. **E.** Kanamycin (kan; 170 µg/mL) results in a 5-log reduction in viable *L. reuteri* measured in colony forming units (cfu)/mL within 30 minutes after its addition to culture. **F.** Mouse eosinophils isolated from spleens of *IL5*^{tg} mice remain viable (trypan blue negative) after 30 min in culture with kanamycin at concentrations shown. **G.** Bone marrow-derived eosinophils also remain viable (trypan blue negative) in the presence of kanamycin (170 µg/mL for 30 min).

Fig. 2. Sample data and application

A. Sample flow cytometric data, indicating side scatter (SSC-A) and fluorescence (GFP) of bone marrow-derived eosinophils incubated for 30 min with (i.) *Lr* controls or (ii. and iii.) *Lr*-eGFP transformants; cultures shown in (iii.) included an additional 30 min with kanamycin (170 μ g/mL), and those in (i.) and (ii.) an additional 30 min with diluent control prior to fixation and flow cytometric analysis as shown. **B.** Data compilation and determination of % GFP positive cells and **C.** median fluorescence index (MFI) both with and without kanamycin, documenting interactions of *Lr*-eGFP with eosinophils isolated from spleens from *IL5*tg mice; n = 12–13 mice from 3 independent experiments; ***p < 0.001.

Fig. 3. Extended applications: *E. coli*

A. Kanamycin (170 µg/mL) results in a 3.5-log reduction in viable *E. coli* (cfu/mL) within 30 minutes after its addition to culture. **B.** Interactions of eosinophils from spleens of *IL5*^{tg} mice with *Ec*-eGFP; DAPI counterstain, original magnification 64X. **C.** Determination of %GFP positive cells and **D.** median fluorescence index (MFI) both with and without kanamycin, documenting interactions of *Ec*-eGFP with eosinophils isolated from spleens from *IL5*^{tg} mice.