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Note

A flow-cytometric method to evaluate eosinophil-mediated uptake of probiotic *Lactobacillus reuteri*

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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*.

1. 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 et al. (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 et al. (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 and Botelho, 2017). Human eosinophils also internalize bacteria (Cline et al., 1968; Cohen and 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. 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 (*IL5*tg; 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.

Abbreviations: GFP, green fluorescent protein; KAN, kanamycin; *Lr*, *Lactobacillus reuteri*; *Ec*, *Escherichia coli*

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2.2. Generation of fluorescent-labeled bacterial strains

The expression plasmid pTRKH3p15A-*ldh*GFP (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 and Nes, 1989). Cultures of fluorescent *Lr*-eGFP transformants were grown from frozen stocks for 24 h 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 *IL5*tg mice to > 95% purity as determined by visual inspection of Diff-Quik stained cytospin 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 OD_{600} *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 30 × 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 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

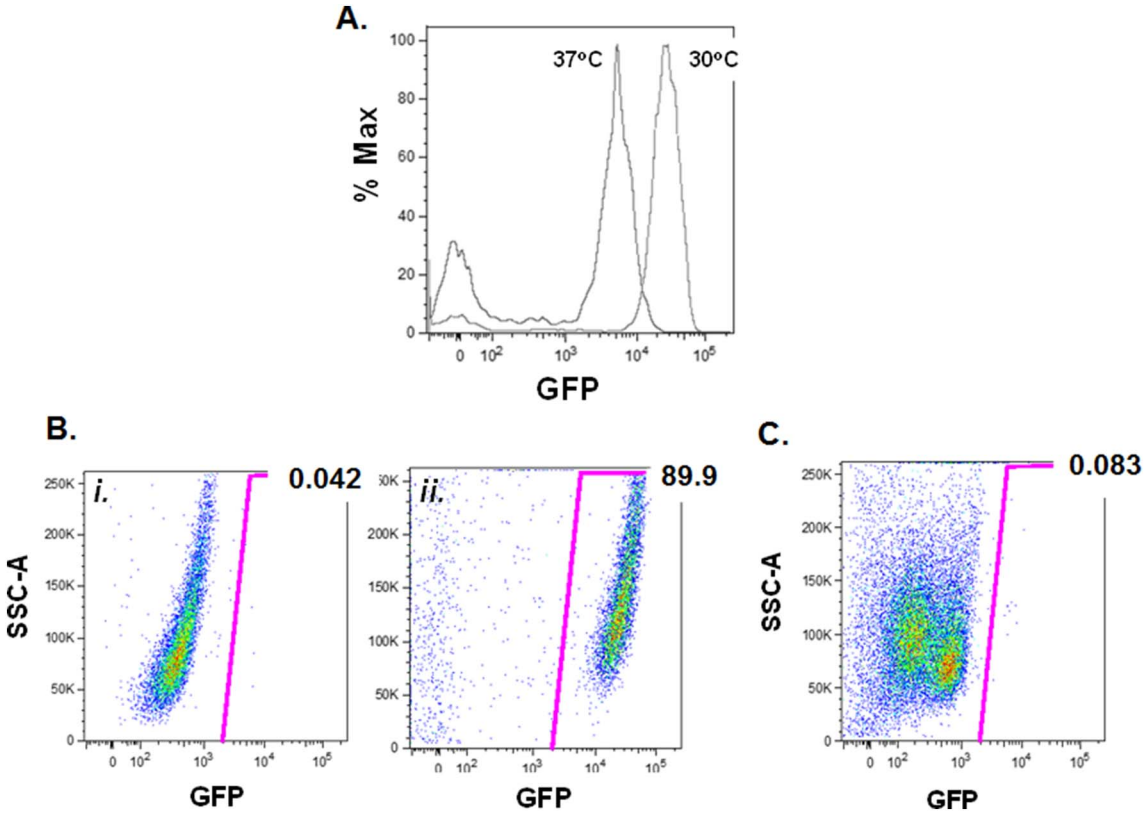

Fig. 1. Optimizing growth and targeting extracellular bacteria in order to examine eosinophil-mediated uptake of *Lr*-eGFP. A. Histogram documenting the improved fluorescence intensity from *L. reuteri* transfectants (*Lr*-eGFP) grown at 30 °C *vs.* those grown at 37 °C. Both cultures were grown in MRS 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*tg 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 min 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)