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Multi-parametric analysis of phagocyte antimicrobial responses using imaging flow cytometry

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ABSTRACT

We feature a multi-parametric approach based on an imaging flow cytometry platform for examining phagocyte antimicrobial responses against the gram-negative bacterium *Aeromonas veronii*. This pathogen is known to induce strong inflammatory responses across a broad range of animal species, including humans. We examined the contribution of *A. veronii* to the induction of early phagocyte inflammatory processes in RAW 264.7 murine macrophages *in vitro*. We found that *A. veronii*, both in live or heat-killed forms, induced similar levels of macrophage activation based on NF-κB translocation. Although these macrophages maintained high levels of viability following heat-killed or live challenges with *A. veronii*, we identified inhibition of macrophage proliferation as early as 1 h post *in vitro* challenge. The characterization of phagocytic responses showed a time-dependent increase in phagocytosis upon *A. veronii* challenge, which was paired with a robust induction of intracellular respiratory burst responses. Interestingly, despite the overall increase in the production of reactive oxygen species (ROS) among RAW 264.7 macrophages, we found a significant reduction in the production of ROS among the macrophage subset that had bound *A. veronii*. Phagocytic uptake of the pathogen further decreased ROS production levels, even beyond those of unstimulated controls. Overall, this multi-parametric imaging flow cytometry-based approach allowed for segregation of unique phagocyte sub-populations and examination of their downstream antimicrobial responses, and should contribute to improved understanding of phagocyte responses against *Aeromonas* and other pathogens.

1. Introduction

Phagocytosis is a well-conserved cellular mechanism, which serves as a first line of defense against invading pathogens and further contributes to the regulation of downstream effector and regulatory mechanisms of immunity (Hanington et al., 2009; Underhill and Ozinsky, 2002; Yutin et al., 2009). Particle internalization marks the successful transition through the threshold of phagocyte activation and promotes the induction of downstream processes. This includes the activation of inflammatory programs (Underhill and Ozinsky, 2002), the production of antimicrobial reactive oxygen and nitrogen species (Bogdan et al., 2000; Robinson, 2008), and the induction of transcription factor activity resulting in targeted expression of cytokine genes (McDonald and Cassatella, 1997; Scull et al., 2010). Given the heterogeneity among individual phagocyte responses at the inflammatory site, much can be gained from multi-parametric analyses that allow deeper characterization of distinct phenotypes and their contributions to the induction and regulation of inflammation.

Over the years, a number of molecular and cell-based strategies have been applied to the study of phagocytosis (Drevets and Campbell, 1991; Rieger et al., 2010a; Rossi and Lord, 2013; Singboottra et al., 2010). Of these, three common approaches are based on light microscopy, fluorescence

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microscopy, and flow cytometry techniques. Each allows for the detection of phagocytosis with varying levels of specificity but also displays important limitations that restrict their overall contributions. Microscopy approaches (both light and fluorescence-based) allow the user to directly visualize cells, offering the spatial resolution required to discern particle and/or staining localization. However, microscopy-based assays suffer from time constraints and low event counts, which preclude statistically robust analyses. Flow cytometry-based assays increase the statistical robustness through examination of thousands of cells in a short period of time. In addition, parallel analysis of cellular phenotypes based on surface marker expression may provide increased resolution when mixed populations are analyzed. Flow cytometry, however, lacks the spatial resolution of microscopy-based assays, which prevents the discrimination between bound and internalized particles and the analysis of downstream responses that are not dependent solely on changes in fluorescence level, such as nuclear translocation of NF-κB. The ImageStream multispectral imaging flow cytometry platform offers additional resolution by combining the throughput and multi-channel capabilities of flow cytometry with the spatial localization of fluorescence microscopy. Phagocytosis assays are particularly enhanced through the ability to examine cells in the X, Y, and Z plane, which allows users to analyze cells in all three dimensions and therefore distinguish between surface-bound and internalized particles (Rieger et al., 2010a). Importantly, this platform is also amenable to multi-parametric analyses of cellular responses among distinct subsets in a population. The present study took advantage of these capabilities for *in vitro* evaluation of phagocyte antimicrobial responses against *Aeromonas veronii*. This pathogen has previously been shown to induce apoptosis of epithelial cells through mitochondrial depolarization and oxidative stress (Krzymińska et al., 2011). Previous studies suggest that resident macrophages, are central contributors to the induction and regulation of antimicrobial responses against this pathogen (John et al., 2002; Sharp and Secombes, 1993). However, the details of this macrophage-*Aeromonas* interaction remain ill defined.

We report here that both live and heat-killed forms of *A. veronii* activated murine RAW 264.7 macrophages and induced NF-κB nuclear translocation. Unlike their contribution to epithelial cell apoptosis, *Aeromonas* did not induce significant levels of macrophage apoptosis. However, the number of actively proliferating macrophages decreased upon *A. veronii* challenge. The phagocytic capacity of RAW macrophages internalizing *A. veronii* also increased in a time-dependent manner. Interestingly, using a multi-parametric approach, we found that the degree of interaction between these phagocytes and *A. veronii* played a major role on their antimicrobial profile. In short, *A veronii* inhibited respiratory burst responses in macrophages that had phagocytosed the pathogen to a greater extent than those that had simply bound this bacterium and to a much further degree than those that were not physically associated with *A. veronii*.

2. Materials and methods

2.1. Cell line

RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum. Cells were incubated at 37 °C/5% CO_2 and passaged every 2–3 days.

2.2. Bacterial growth

A. veronii was grown overnight in trypticase soy broth to an OD_{600} of 1.0 to 1.3. Heat-killed (Hk) samples were prepared by incubating bacteria at 80 °C for 60 min. Live and heat-killed *A. veronii* were then washed twice in 1× $PBS^{-/-}$ and added to cultures at a 5:1 (bacteria: cells) ratio.

2.3. Fluorescent labeling of bacteria

Following heat-killing, *A. veronii* was washed twice and resuspended in 1× $PBS^{-/-}$. Propidium iodide (PI) was added to a final concentration of 4 μg/mL and incubated for 60 min in the dark at room temperature with continual rocking. Bacteria were then washed twice with 1× $PBS^{-/-}$ to remove any unbound PI.

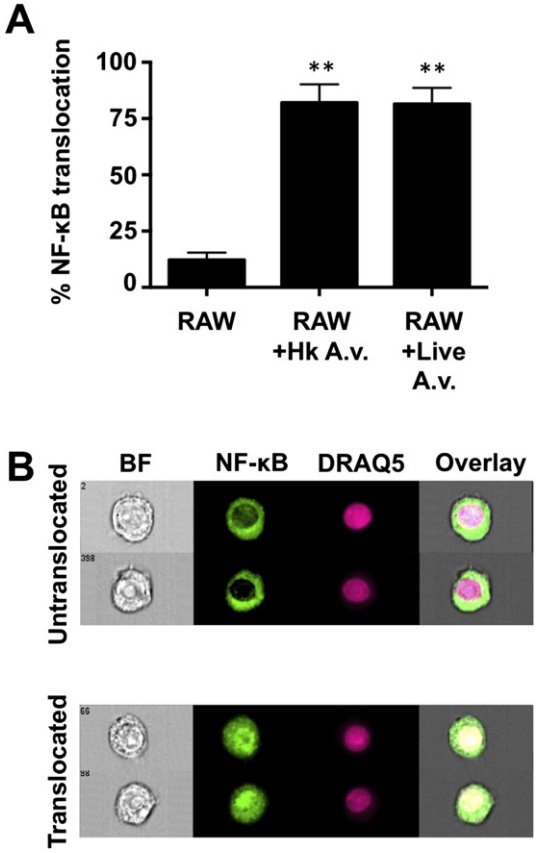

Fig. 1. *Aeromonas veronii* induces NF-κB translocation in murine macrophages. RAW 264.7 macrophages were incubated with live or heat-killed *A. veronii* for 1 h. Cells were fixed, permeabilized, and stained for NF-κB as a marker of cell activation. (A) Translocation of this transcription factor increased in macrophages following incubation with live or heat-killed *Aeromonas*. (B) Representative images highlight macrophages with untranslocated and translocated NF-κB. $n = 4$; $^{**}p < 0.01$; error bars correspond to SEM.