Outer Membrane Vesicle Vesiculation Patterns in Gram's Negative Bacteria

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Abstract— Outer membrane vesicles (OMVs) formed from the outer membrane of Gram-negative bacteria play crucial roles in microbe-microbe and host-microbe interactions. Cargos for a myriad of signaling molecules, OMVs contribute to biofilm formation, defense against antimicrobials, delivers toxins during infection, and modulates host cell function. Although previous studies have uncovered much about OMV contents and activities, little is known about its biogenesis and vesiculation patterns overtime. In a high-throughput assay performed on the Escherichia *coli* Keio collection, Kulp et al. identified 73 over-vesiculating strains and 98 under-vesiculating strains. The objective of this study is to characterize OMV vesiculation patterns by first establishing a 6-hour standard using the Keio BW25113 wild-type strain as a background, and then testing Keio OmpA mutant, an over-vesiculating strain. The study found that Keio BW25113 may not be the best strain for background, since Keio BW25113's OMV signals at hour 1 through 6 could hardly be discerned from that of the lysogeny broth negative control. On the other hand, Keio OmpA mutant grown for 6 or 5 hours demonstrated markedly greater vesiculations than OmpA grown for 4 hours, 3 hours, 2 hours, and 1 hour. In summary, the study is the first to characterize the vesiculation patterns of two Keio strains, one wild-type and the other over-vesiculating, raising the potential for future investigations into the vesiculation patterns and kinetics of other over-vesiculating and under-vesiculating strains. Studying the OMV vesiculation patterns and kinetics might shed some light on why some Gram's negative bacteria are more virulent than others, and how vesiculation kinetics might influence microbe-host interactions and its implications for infection in humans.

Index Terms— BW25113 Keio strain, dot-blot analysis, Escherichia *coli* Keio collection, Gram's negative bacteria, OmpA Keio strain, outer membrane vesicles, vesiculation patterns

1 Introduction

Outer membrane vesicles (OMVs) are nano-sized vesicles derived from the outer membrane of Gram-negative bacteria. [1] They transport a broad range of signaling molecules, including nucleic acids, proteins, lipids, endotoxins, and virulence factors, and play crucial roles in microbe-microbe and host-microbe interactions. [2] They contribute to biofilm formation and defense against antimicrobials, delivers toxins during a bacterial infection, and modulates host cell function. Although much is known about OMV contents and activities, its biogenesis remains largely unclear.

So, what is known about OMV biogenesis? Gram-negative bacteria such as Escherichia *coli* and Pseudomonas *aeruginosa* have two bilayers, the cytoplasmic membrane and the outer membrane, separated by a periplasmic space, which contains a peptidoglycan layer. The outer membrane has an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharides (LPS), also known as endotoxin. Their envelop proteins include periplasmic proteins, membrane-associated proteins, and integral proteins. During OMV biogenesis, the outer membrane detaches from the peptidoglycan layer and buldges outwards. [3] Previous studies showed that OMV production and cargo loading are regulated processes rather than stochastic events: OMVs and Gram-negative envelops exhibit different protein and lipid contents, suggesting that materials are selectively packaged into OMVs during OMV biogenesis. [4] Furthermore, vesiculations are influenced by temperature, nutrient availability, oxidation, quorum sensing and antibiotics. [3] Several models have been proposed for the mechanism of OMV biogenesis. One model shows that LPS accumulations along the outer membrane induces higher degrees of membrane curvature, making vesiculation more likely. [4] A second model explains that when certain proteins (e.g. OmpA, Braun's lipoprotein, Lpp, and Tol/Pal) that tether the outer membrane to the peptidoglycan layer lose their functions, vesiculation becomes more likely. [4]

In 2015, a high-throughput assay was implemented to determine the vesiculation values for the entire genome knockout library of E. *coli* mutant strains (Keio collection). [4] The study identified 171 Keio strains with significant vesiculation phenotypes—73 over-vesiculating strains and 98 under-vesiculating strains. [4] However, no study so far has characterized OMVs' vesiculation patterns overtime. This research aims to characterize OMV vesiculation patterns by first establishing a standard using the Keio BW25113 wild-type as a background and then testing the Keio OmpA mutant, an over-vesiculating strain. It is hypothesized that the OmpA mutant would demonstrate higher vesiculation values than the BW25113 wild-type at each hour point, and that both strains would show an increase in vesiculation values from hour 1 through 6. In a world where many infections such as pneumonia, urinary track infections, blood-

stream infections, and meningitis are caused by Gram's negative bacteria [5], studies in vesiculation patterns in E. *coli* raise the potential for future investigations into how OMV vesiculation patterns or kinetics might influence microbe-host interactions during infections in humans.

2 Materials and Methods

2.1 Strains and growth conditions

The BW25113 wild-type and OmpA mutant strains from the E. *coli* Keio collection were cultured in Miller LB media containing 50 μg mL^{-1} kanamycin for OmpA or no kanamycin for BW25113 at 37°C with shaking at 200 rpm overnight. [5]

2.2 Generating 6-hour growth curves

Strains from overnight were transferred into 1.5 mL microcentrifuge tubes (ThermoFisher Scientific), with 200 μL in each tube and 6 tubes for each strain. Cells in each tube were pelleted by centrifugation at 8,000 rpm under 4°C for 5 minutes. The supernatants were removed, and the cells in each tube were resuspended in 200 μL of fresh LB or LB with kanamycin. 5 tubes of each strain were placed in a 4°C freezer to freeze growth. 1 tube of each strain was set aside for seeding at hour 0 (Table 1).

12 wells of a 96-well U-bottom plate (BD, Franklin Lakes, NJ, USA) were preloaded with 148.5 μL of LB—with or without kanamycin, and 1.5 μL of the cells from each leftout microcentrifuge tube were transferred to two wells in Row A, achieving a 1:100 cell dilution (Table 1). Two wells in Row G were preloaded with 150 μL LB, with or without kanamycin, as the negative control. The plate was sealed with Parafilm, and was placed in a BioTek EL808microplate reader preheated to 37°C, with shaking, and set to an optical density of 630 nm (OD630). This completes seeding at hour 0.

At hour 1, 2, 3, 4, and 5, 1.5 μL of contents from each microcentrifuge tube, retrieved from the 4°C freezer, were transferred to wells in Row B, C, D, E, and F, respectively. 6 minutes before each seeding hour, a tube of cells was retrieved from the freezer and let sit in room temperature for 3 minutes and then in 37°C shaking for 3 minutes to thaw.

Table 1. A Plating Scheme for One Strain Only

Row A Seed at Hour 0	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Row B Seed at Hour 1	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Row C Seed at Hour 2	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Row D Seed at Hour 3	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Row E Seed at Hour 5	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Row F Seed at Hour 5	**1.5μL Cells 148.5μL LB**	**1.5μL Cells 148.5μL LB**
Negative Control Start at Hour 1	**150.0μL LB**	**150.0μL LB**

2.3 Dot-blotting to determine OMV concentrations

After about 6 hours, the 96-well U-bottom plate was retrieved from the BioTek EL808microplate reader. Cells in each well were pelleted by centrifugation at 10,000 x g for 10 minutes. All supernatant from each well was transferred to its corresponding well in a 96-well filter plate. Next, with a new 96-well U-bottom receiver plate stacked underneath the filter plate, the supernatants were spun down at 1,000 x g for 5 minutes. The filter plate would filter out any remaining cells while collecting the OMV-containing supernatant.

1 μL of supernatant from each well was dotted directly onto a nitrocellulus membrane (Pall Scientific). For positive control, a 1 μg mL^{-1} OMV stock solution was serially diluted to 0.5 μg mL^{-1}, 0.25 μg mL^{-1}, 0.125 μg mL^{-1}, and 0.0625 μg mL^{-1} of OMV, and each dilution, including the stock, was dotted on the membrane in a lane labeled positive control. The membrane was let sit at room temperature for 45 minutes.

The nitrocellulus membrane was blocked in 5% nonfat dry milk for one hour at room temperature, then incubated with polyclonal antibodies against E. *coli* LPS (1:1000 in TBST; Affinity BioReagents) for one hour at room temperature. The blot was washed in TBST (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 1% Tween-20) 3 times for 5 minutes each, followed by incubation with horse-conjugated anti-rabbit secondary antibodies (1:10,000 in TBST; LI-COR) for one hour at room temperature. The blot was then washed 3 times in TBST for 5 minutes each and once in TBS for 5 minutes. The blot was imaged using the odyssey infrared imager (LI-COR), and the accompanying software was used to perform densitometry analysis.

2.4 Statistical Analysis in GraphPad Prism

A standard curve was plotted for the OMV positive control, and the OMV concentration of each blot was determined by fitting values along the standard curve. All statistical analyses were done in GraphPad Prism.

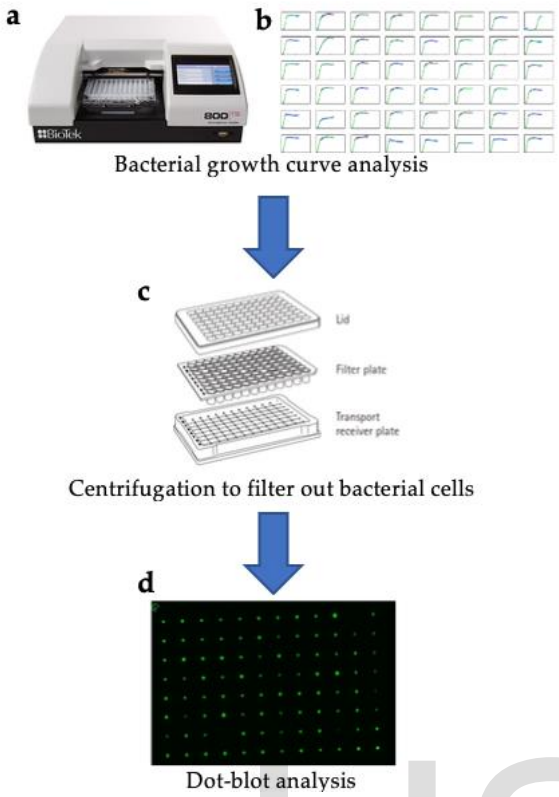

Fig 1. Experiment flow chart. In the first stage, 6-hour E. *coli* growth curves were generated using the BioTek EL808microplate reader. In the second stage, a filter plate was used to filter the bacterial cells while collecting the OMV-containing supernatants in the receiver plate. Finally, in the third stage, the supernatants were dotted directly onto a nitrocellulus membrane, and the blots were imaged and analyzed using LI-COR.

Credit:

(a) Biotek
(b) Kulp et al., 2015
(c) Sigma Aldrich
(d) Kulp et al., 2015

3 Results

First, the Keio BW25113 wild-type was used to establish a standard. Then, the Keio OmpA mutant, an over-vesiculating strain, was tested alongside the wild-type. While vesiculation trend was unclear in the wild-type, the OmpA mutant showed a clear trend wherein the longer the cells spent growing, the greater the dot densitometry, and hence the greater the OMV concentration.

3.1 Keio BW25113

For BW25113 cells grown for 6 hours and 5 hours, cell growth reached a stationary phase at an OD630 of 0.3, while those that grew for 3, 2, and 1 hour were still in the lag phase (Fig 2a). Interestingly, while cells grown for 6 hours entered the log phase by 120 minutes, cells grown for 3 hours had not entered the log phase by 180 minutes.

For the OMV positive control in the right panel (Fig 2b), there is a clear gradation in densitometry going from 1 µg mL^{-1}, 0.5 µg mL^{-1}, 0.25 µg mL^{-1}, 0.125 µg mL^{-1}, to 0.0625 µg mL^{-1}, but no such gradation is observed with the wild-type cells. Wild-type cells grown for all hours have about the same densitometry, and their densitometry cannot be easily discerned from that of the LB negative control.

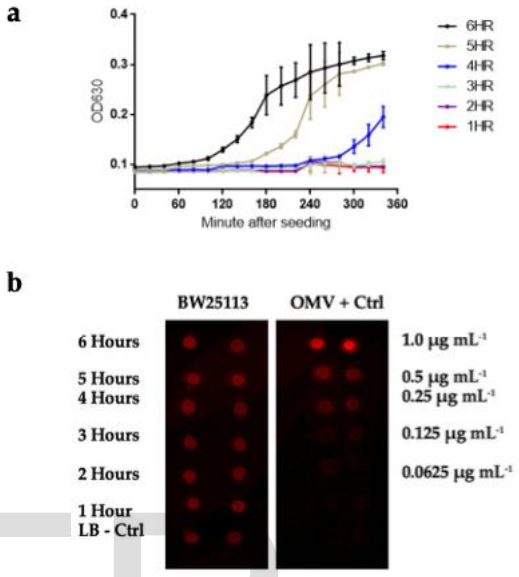

Fig 2. Keio wild-type BW25113 growth curves and dot-blot analysis. (a) Growth curves for Keio BW25113 grown for 6 hours, 5 hours, 4 hours, 3 hours, 2 hours, and 1 hour, respectively. (b) The dots in the left panel, from top to bottom, correspond to Keio BW25113 cells that grew for 6 hours, 5 hours, 4 hours, 3 hours, 2 hours, 1 hour, and the LB negative control (n=2). The dots in the right panel, from top to bottom, correspond to the OMV positive control with OMV concentrations 1 µg mL^{-1}, 0.5 µg mL^{-1}, 0.25 µg mL^{-1}, 0.125 µg mL^{-1}, and 0.0625 µg mL^{-1}, respectively (n=2).

The standard curve for the OMV positive control has a coorelation coefficient R^2 value of 0.9774, suggesting a near-perfect positive, linear correlation between densitometry and OMV concentration (Fig 3a). Further, when OMV concentration was calculated for each wild-type blot-dot by fitting values along the standard linear curve, OMV concentrations for wild-type that grew for hour 1 through 6 are very similar (Fig 3b), contrary to the expectation that the OMV concentration for hour 6 would be greater than that for hour 5, and then hour 4 and so on.

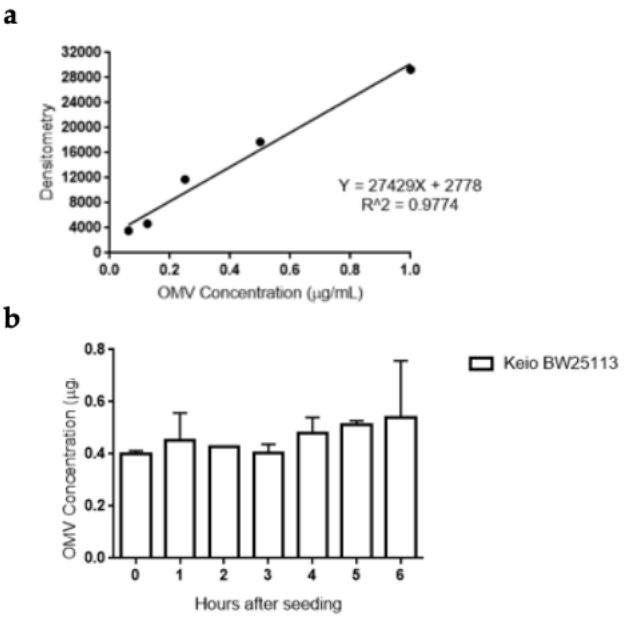

Fig 3. Standard curve for OMV positive control and calculated OMV concentrations for the BW25113 dot-blot. (a) Standard curve with an R^2 of 0.9774. (b) The bar above 0 hours after seeding represents the LB negative control (n=2).

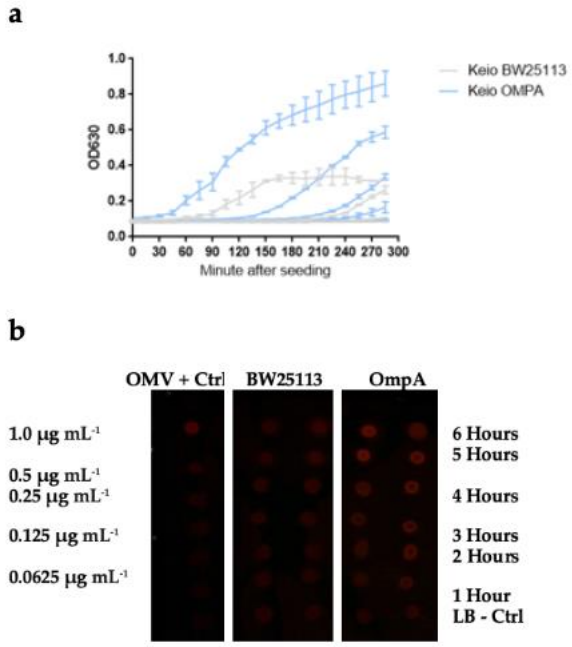

Fig 4. Over-vesiculating Keio mutant OmpA growth curves and dot-blot analysis. (a) Growth curves for Keio OmpA and Keio BW25113 grown for 6 hours, 5 hours, 4 hours, 3 hours, 2 hours, and 1 hour, respectively. (b) The dots in the left panel, from top to bottom, correspond to the OMV positive control with OMV concentrations 1 µg mL^{-1}, 0.5 µg mL^{-1}, 0.25 µg mL^{-1}, 0.125 µg mL^{-1}, and 0.0625 µg mL^{-1}, respectively (n=2). The dots in the middle panel, from top to bottom, correspond to Keio BW25113 cells that grew for 6 hours, 5 hours, 4 hours, 3 hours, 2 hours, 1 hour, and the LB negative control (n=2). The dots in the right panel, from top to bottom, correspond to Keio OmpA cells that grew for 6 hours, 5 hours, 4 hours, 3 hours, 2 hours, 1 hour, and the LB with kanamycin negative control (n=2).

3.2 Keio OmpA

OmpA cells grown for 6 hours reached a stationary phase at an OD630 of 0.8, while cells grown for 5 hours were making their transition into the stationary phase. OmpA cells grown for 4 hours were still in the log phase, while cells grown for 2 and 1 hours were in the lag phase. This was expected since OmpA, an over-vesiculating strain, would demonstrate greater vesiculations than the wild-type strain across all hours. The growth curves for the wild-type were aligned with our previous results, with wild-type cells grown for 6 hours reaching a stationary phase of 0.3 at OD630, while cells grown for 4, 3, 2, and 1 hours barely showed any signs of leaving the lag phase.

For the OMV positive control in the left panel (Fig 4b), there is a clear gradation in densitometry going from 1 µg mL^{-1}, 0.5 µg mL^{-1}, 0.25 µg mL^{-1}, 0.125 µg mL^{-1}, to 0.0625 µg mL^{-1}. Gradation in densitometry is also observed with OmpA. For instance, OmpA cells that grew for 6 and 5 hours are visibly redder in color than those that grew for 3, 2, and 1 hour. No such gradation, however, is observed with the wild-type. As with expected, OmpA, especially those that grew for a greater number of hours, demonstrates a much more intense densitometry than the wild-type, especially at hours 6, 5, and 4.

The standard curve for the OMV positive control has a coorelation coefficient R^2 value of 0.9237, suggesting a good positive, linear correlation between densitometry and OMV concentration (Fig 5a). Further, when the OMV concentration was calculated for each wild-type and mutant blot by fitting values along the standard linear curve, OMV concentrations for wild-type that grew for hour 1 through 6 are very similar (Fig 5b), while OMV concentrations for OmpA that grew for 4, 5, and 6 hours are greater than those of OmpA that grew for 1, 2, and 3 hours.

At all hours (1-6), OmpA demonstrates a greater vesiculation value than the wild-type, as expected.

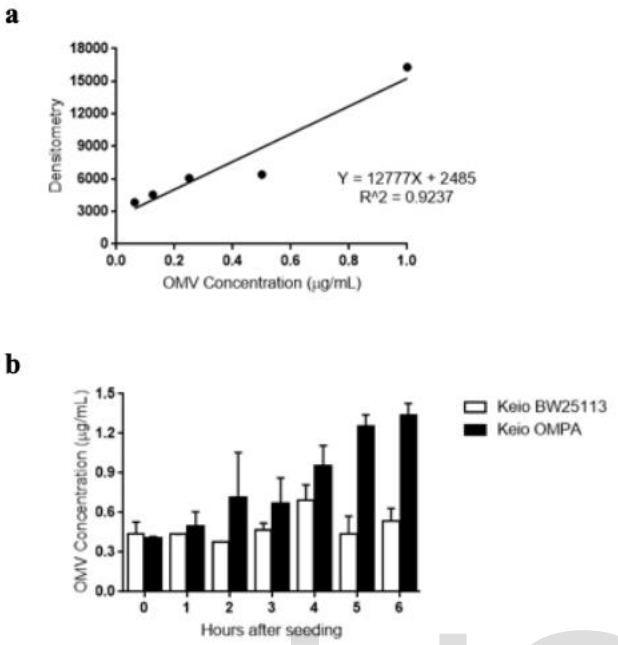

Fig 5. Standard curve for OMV positive control and calculated OMV concentrations for BW25113 and OmpA dot-blots. (a) Standard curve with an R^2 of 0.9237. (b) Bars above 0 hours after seeding represent LB and LB with kanamycin negative controls, respectively (n=2).

4 Discussions

In summary, we investigated the 6-hour vesiculation patterns of two strains of E. *coli*, the Keio wild-type BW25113 and the Keio over-vesiculating mutant OmpA. We aimed to establish a standard using the wild-type strain as a background, but found that Keio BW25113 may not be the best strain for background, since its OMV signals could not be easily distinguished from those of the LB negative control. In the meantime, we found a clear vesiculation pattern for OmpA, wherein OmpA cells grown for 6 and 5 hours showed greater vesiculation values than those that grew for a fewer number of hours. Our study raises the potential for future investigations into the vesiculation patterns in other over-vesiculating and under-vesiculating cells. Vesiculation patterns may explain why some Gram's negative bacteria are more virulent than others and how vesiculation patterns or kinetics affects microbe-host interactions during an infection.

4.1 Keio BW25113 is not suitable as the background

We found that on the blot, the Keio wildtype signals could not be easily differentiated from those of the LB negative control. This suggests that a different strain, one that is more distinguishable from the LB negative control, should be used as a standard instead.

4.2 Cross-reactivity between primary antibody and LB

On each of our dot-blots, we saw a rather strong signal from our LB negative control, with or without kanamycin. When we calculated the OMV concentration in our negative control by fitting its densitometry value along the standard curve, we obtained ~0.4 μg mL^{-1} (Fig 3b, 5b). This was unexpected, since the primary antibody we used in our dot-blot—polyclonal antibodies against E. *coli* LPS—was specific to LPS-containing OMVs. Our LB was not contaminated, since dot-blotting with a freshly-made LB yielded similar results. This led us to postulate that perhaps there was cross-reactivity between the primary antibody and LB, and that using a different medium or negative control with less coloring, like the M9 minimum medium, might reduce such cross-reactivity, hence reducing its signal.

4.3 Variations in the dot-blot results

Furthermore, we observed great variations across our dot-blot results. Dot-blotting might not be the most reliable protein detection technique to use here. With daily variations and blot-to-blot variations, it is possible that other protein detection techniques like ELISA might yield more consistent results.

4.4 Defects in the Keio cells?

We expected the BW25113 wild-type strain to reach an OD630 of 1 at the very least. Indeed, the same strain cultured in a 5 mL tube overnight was able to reach an OD630 of 1. One reason for it failing to do so in the 96-well plate could be that the wells were so small that the aeration might not be the best for optimal cell growth.

Furthermore, as we described in the results section, while the BW25113 wild-type cells seeded at hour 0 (grown for 6 hours) entered the log phase by 120 minutes (2 hours), cells seeded at hour 3 (grown for 3 hours) had not entered the log phase by 180 minutes (3 hours). One potential explanation for this is that putting the cells in the 4°C fridge to temporarily halt growth might not be the best method to capture the 0-time-point in growth. Even though the cells were retrieved from the fridge 6 minutes prior to each seeding period, the cells, after being frozen for hours, might not be as strong and viable for growth as those that were seeded at hour 0, which never had to go through the freeze and thaw period.

4.5 Future Directions

For future directions, I recommend that a different background strain or a different growth medium be used. Solving the vesiculation kinetics for other over-vesiculating and under-vesiculating strains might shed some light on why some Gram's negative bacteria are more virulent than others and how vesiculation patterns and kinetics might influence microbe-host interactions during an infection.

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