Describing immune responses in human milk via *in vitro* stimulation

Katherine Wander, Assistant Professor of Anthropology, Binghamton University
Rita Spathis, Manager, Laboratory for Anthropometry and Biomarkers, Binghamton University

BINGHAMTON UNIVERSITY
STATE UNIVERSITY OF NEW YORK

Introduction

Human milk contains numerous immune factors, which together constitute the "immune system of milk" [1]:

- White blood cells
- Antibodies (secretory immunoglobulin A)
- Immune cell communication molecules (cytokines)
- Antimicrobial factors (e.g., lactoferrin, fibronectin)
- Commensal microbes

The immune system of milk plays an important role in the protective effect of breastfeeding against infectious diseases during infancy and allergies throughout childhood. Immune cells in milk can enter lymph tissue (Peyer's patches) in the infant gut to coordinate immune responses, and can move to other immune tissues [2]. In mice, maternal immune cells can "train" offspring immune cells, transferring lasting immunological memory [3].

Methods exist to characterize immune factors in milk [4]. However, these measures relate only indirectly to some of the important protections offered by the immune system of milk. Many of the likely mechanisms by which immune factors in milk affect children's health is in their interaction with each other and with the developing immune system. Thus, we see a need to describe immune *responses* in milk.

The activity of white blood cells isolated from milk has been measured with *in vitro* stimulation [5]. The procedure to isolate cells from milk is technically intensive, limiting the settings in which such a technique may be used. Further, important information about the protective value of the immune system of milk is lost when immune cells are removed from other immune factors in milk, including the influence of those factors on immune cells, and differences in the quantities of immune cells found in milk.

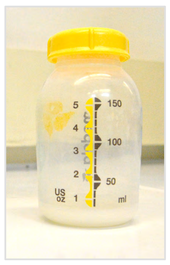
To provide a technique to describe immune responses in human milk that is:

- interpretable at the *system* level (the immune system of milk)
- practical for population-based, international research

we developed a protocol for *in vitro* stimulation of whole milk specimens.

Milk specimens are combined with infectious stimuli, incubated at 37°C for 24 hours, and evaluated for cytokines. Comparison of cytokine concentrations in stimulated and baseline specimens provides a measure of immune cell activity—the immune response in milk.

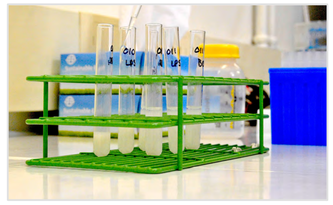
Methods



Forty women provided milk specimens by expression with an electric breast pump. Twenty were used for protocol optimization (based on a field-friendly protocol for *in vitro* stimulation of whole blood specimens [6]): incubation time, culture vessel and volume, stimulant concentration, culture medium preparation, and cytokine enzyme immunoassay were varied and the method that most consistently produced detectible increases in cytokines from baseline was identified. This ***in vitro* whole milk stimulation** protocol was used to to characterize immune responses in 20 additional specimens.

Within 4 hours of expression, milk was diluted (2 ml milk:1 ml medium) with cell culture medium [RPMI 1640 (Lonza BioWhittaker) with L-glutamine (Gibco, 110 mg/l), pyruvate (Lonza BioWhittaker, 292 mg/l), and penicillin-streptomycin (Gibco, 100 U/ml)] in glass culture tubes in three conditions:

- With the bacterial surface molecule **lipopolysaccharide (LPS)** isolated from *E. coli* (List Biological; 200 µg/ml)
- With the bacteria ***M. bovis* Bacille Calmette-Guérin** (**BCG**; TICE BCG, Merck; 2% of vaccine concentration, or 2-16 x 10^6 CFU/ml)
- With culture medium alone as an **unstimulated control**



Specimens were placed in a glass desiccator. An anaerobic environment was created by burning a candle (eliminating the need for use of pressurized CO_2). The desiccator was placed in an incubator at 37°C for ~24 h.

The aqueous portion of baseline and incubated specimens was isolated by centrifugation. Cytokine concentrations were estimated by high-sensitivity multiplex immunoassay (QuanSys BioSciences).

Milk fat was estimated with the creamatocrit method: ~70 µl was drawn into a glass capillary tube and separated by centrifugation. Milk fat percentage was calculated from the height of the fat and total column.

Participants reported any current symptoms or diagnoses of on-going infectious disease, their month and year of birth, and month and year of birth of their youngest child.

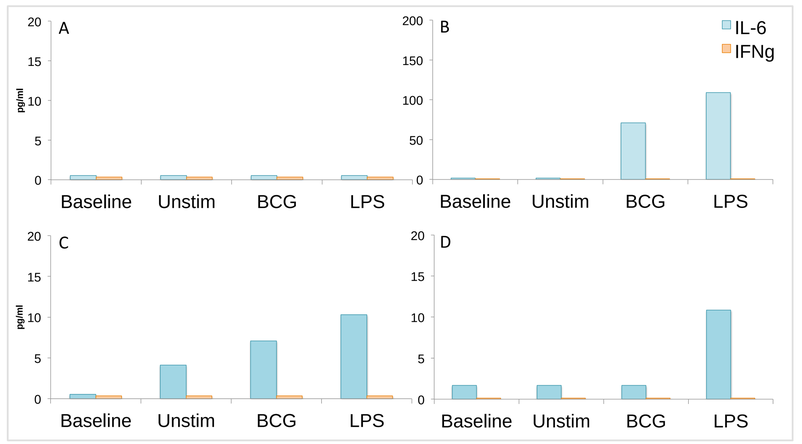


Figure 1. Examples from four individuals of cytokine (interleukin-6, IL-6, and interferon-γ, IFNγ) concentrations across conditions: **Baseline**; **Unstimulated** (incubated without stimulant); **BCG** (incubated with *Mycobacterium bovis* Bacille Calmette-Guérin); and **LPS** (incubated with lipopolysaccharide isolated from *Escherichia coli*).

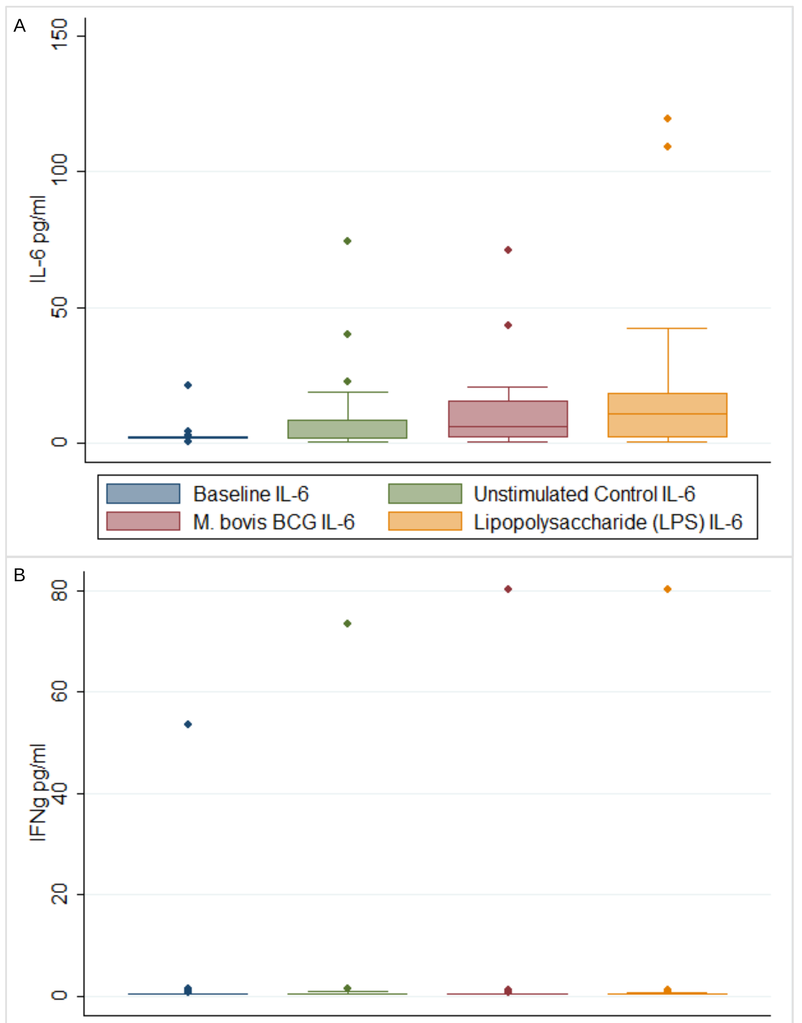


Figure 2. Milk concentrations of the cytokines interleukin-6 (IL-6) and interferon-γ (IFNγ) across stimulation conditions: **Baseline** (harvested prior to incubation), **Unstimulated** control (incubated without stimulant), **BCG** (incubated with *Mycobacterium bovis* Bacille Calmette-Guérin), and **LPS** (incubated with lipopolysaccharide isolated from *Escherichia coli*). Increases in IL-6 after stimulation are apparent; a similar pattern for IFNγ was not detected.

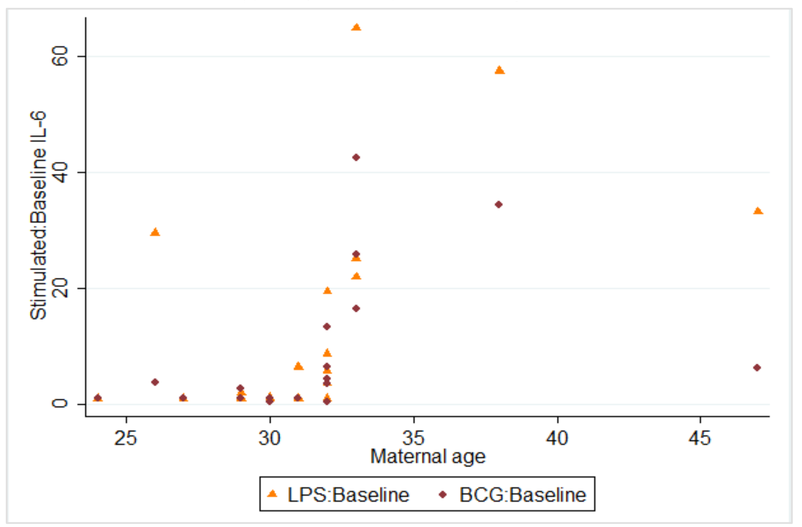


Figure 3. Scatter plot of the ratio of stimulated to baseline interleukin-6 (IL-6) for *E. coli* **lipopolysaccharide (LPS)** and ***M. bovis* Bacille Calmette-Guérin (BCG)** *vs.* maternal age.

Findings

Adequate specimen was available to evaluate all four conditions (**Baseline**, **Unstimulated Control**, and two bacterial stimuli: Lipopolysaccharide, **LPS**, and *M. bovis* **BCG**) for 20 US females (ages 24-47y) in the Binghamton, NY area.

We evaluated specimens for four cytokines, interleukin-4 (IL-4), IL-6, IL-10, and interferon-γ (IFNγ), using high-sensitivity multiplex enzyme immunoassay kits. IL-4 and IL-10 were not detected in any specimens.

Interleukin-6 (IL-6): The inflammatory cytokine IL-6 was detectable in (geometric mean; 95% CI):

- Baseline specimens from 6 participants (3.9 pg/ml; 1.5, 9.9)
- Unstimulated Control specimens from 8 participants (13.8 pg/ml; 5.6, 33.8)
- BCG-stimulated specimens from 12 participants (12.4 pg/ml; 7.0, 21.9)
- LPS-stimulated specimens from 14 participants (16.1 pg/ml; 8.4, 30.8)

Interferon-γ (IFNγ): The cytokine IFNγ, a mediator of both inflammation and adaptive immune responses, was detectable in (geometric mean; 95% CI):

- Baseline specimens from 5 participants (2.1 pg/ml; 0.2, 20.4)
- Unstimulated Control specimens from 5 participants (2.3 pg/ml; 0.2, 26.1)
- BCG-stimulated specimens from 4 participants (2.7 pg/ml; 0.1, 101.0)
- LPS-stimulated specimens from 4 participants (2.7 pg/ml; 0.1, 100.9)

Changes in cytokines across conditions: For preliminary comparisons of IL-6 and IFNγ across conditions, we used the assay's lower limit of detection to characterize undetectably low specimens.

IL-6 was higher in the LPS (*p:* 0.0267), BCG (*p:* 0.0306), and Unstimulated Control (*p:* 0.0926) conditions than Baseline (**Figure 2, Panel A**). IFNγ varied little across conditions (**Figure 2, Panel B**). We focus here on analysis of IL-6 as an indicator of immune cell activity in milk.

When visually assessed, results fell generally into three categories (**Figure 1**):

- No discernable difference in cytokines across conditions (e.g., **Panel A**): 7 participants
- Clearly higher IL-6 concentration in both bacterial stimuli (e.g., **Panel B**) or only one bacterial stimuli (e.g., **Panel D**), with no difference between the Unstimulated Control and Baseline: 6 participants
- Higher IL-6 in both stimuli (LPS and BCG) and the Unstimulated Control (e.g., **Panel C**): 7 participants

The ratio LPS:Baseline IL-6 (range: 0.9, 64.9) was ≥2 for 11 participants; BCG:Baseline IL-6 (range: 0.3, 42.4) was ≥2 for 11; and, Unstimulated Control:Baseline IL-6 (range: 0.2, 75.5) was ≥2 for 6. These results match well with our visual assessment:

- <2-fold increase in IL-6 across all conditions: 8 participants
- ≥2-fold increase in IL-6 in BCG and/or LPS and <2-fold increase in the Unstimulated Control: 6 participants
- ≥2-fold increase in IL-6 in BCG and/or LPS and Unstimulated Control: 6 participants

Whether useful and informative cutpoints can be defined for bacteria-stimulated IL-6 concentration or ratios of stimulated to baseline IL-6 concentrations remains to be determined.

Predictors of IL-6 Increase: Ratios of LPS:Baseline and BCG:Baseline IL-6 were unassociated with Baseline IL-6, milk fat, or child age, and were positively associated with maternal age (**Figure 3**).

- LPS:Baseline IL-6 and maternal age:
  - Pearson's *r*: 0.51, *p:* 0.0207
  - Spearman's *ρ*: 0.68, *p:* 0.0010
- BCG:Baseline IL-6 and maternal age:
  - Pearson's *r*: 0.38, *p:* 0.0999
  - Spearman's *ρ*: 0.67, *p:* 0.0010

This technique may be particularly useful in anthropological investigations of the immune value of milk:

- Results are interpretable at the *system* level ("the immune system of milk")
- It is relatively low-cost and low in technical demands, and therefore practical for population-based international research across a range of human environments.

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