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

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**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 the developing immune system. Thus, we see a need to describe immune responses 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 were 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—in 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 [5]); incubation time, culture vessel and volume, stimulant concentration, culture medium preparation, and cytokine enzyme immunoassay were varied and the method that most consistently produced detectable increases in cytokines from baseline was identified. This *in vitro whole milk stimulation* protocol was used 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 μg/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 CO2). 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.