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Human milk is composed of multiple immune factors which interact to form the “immune system of milk” [1]. These components include monocytes, macrophages, antibodies, communication molecules (cytokines), antimicrobial proteins, and commensal microbes.

The immune system of milk is critical to understanding connections between maternal and infant health. The immune system of milk may both protect infants against infectious disease and guide their immune development [2, 3].

We developed a protocol to measure milk immune activity *in vitro* to microbial agents, including pathogens and commensal microbes. Responses to these microbes were characterized with cytokines:

- Interferon-" (IFN-"; a promoter of type I immune responses)
- Interleukin-4 (IL-4; a promoter of type II immune responses)
- Interleukin-6 (IL-6; a pro-inflammatory cytokine)
- Interleukin-10 (IL-10; an anti-inflammatory cytokine)

Here, we describe the development of the protocol for describing immune responses in whole human breast milk *in vitro*. Our priorities were to develop a protocol that:

- generates results interpretable at the level of the immune system of milk
- is practical for population-based, international research

Some of the questions we addressed in protocol development were:

1) In

(**2):**

- Immune responses (increases in cytokines) to the Negative (unstimulated control) condition were apparent in many of specimens
- We employed a variety of measures to reduce contamination of milk with bacteria or endotoxin (equipment was cleaned and sterilized after each use, participants were asked to use sanitizing wipes on their hands and breasts before pumping).
- Immune responses to the “Negative control” may result from milk’s natural composition which are inherently active.

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[1] Goldman AS. 2007. The immune system in human milk and the developing infant. *Breastfeed Med* 2.4.
[2] Cabinian A et al. 2016. Transfer of maternal immune cells by breastfeeding: maternal cytotoxic T lymphocytes present in breast milk localize in the Peyer’s patches of the nursed infant. *PloS One* 11.6: e0156762.
[3] Ghosh MK et al. 2016 Maternal milk T-cells drive development of transgenerational Th1 immunity in offspring thymus. *J Immunol* 197.6.