Lactation and mother's milk: recent advances in understanding

Human milk has been shown to have a wide range of nutritional and immune protective benefits for the newborn and growing infant. Despite a reasonably good description of the composition of milk, our understanding of the role that each specific milk component plays in the infant is limited and many questions surround the mechanisms that regulate milk synthesis and orchestrate its assembly in the alveolar lumen. Here we illuminate some of the more pertinent topics that interest both the nutritionist and cell biologist, and, at the same time, introduce an interesting and powerful new tool being developed to investigate these same topics.

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Key points

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1. Human milk is complex and has, for centuries, been the sole source of nutrition and immunity for a newborn.
2. Breast milk is a highly intricate suspension of lipids, protein, carbohydrates, secretory immunoglobulins, calcium and various other macro- and micro-molecules, ions and bioactive factors.
3. The presence of stem cells in the milk opens the potential for differentiation within the infant.

The human mammary gland is a particularly interesting organ since it is capable of undergoing repetitive cycles of growth, function and regression in the mature adult. The process of cellular diversification into an orchestrated functional organ is something that normally only occurs during specification of the organs and systems in the developing embryo. However, this unique and complex ability of the gland should not be surprising. Human milk is complex and has, for hundreds of thousands of years, been the sole source of nutrition for newborns. It is also an important source of immune protection that not only carries a newborn through the transition from womb to world, but fuels the infant's growth into a biologically independent individual.

To understand the importance of milk in nourishing and protecting the growing infant, it is helpful to understand the composition of the milk and how it is made. This has traditionally been a difficult and sensitive area of research. However, with the emergence of new, non-invasive ways to look at the functional gland we have been able to answer many of the questions about milk, milk supply and the role it plays for the baby. At the same time, we have generated a whole catalogue of new questions that will form the focus of an intense field of cell and nutritional research in the future. Of particular interest, is the large population of maternal cells endogenous to human milk and their potential contribution to infant systems.

Development and structure of the mammary gland

The secretory epithelium

The pre-pubescent mammary gland consists of a basic network of immature ducts embedded in a mammary fat pad. At puberty, an increase in ovarian hormones stimulates the immature ducts to branch and elongate from the nipple to invade the underlying fat pad, resulting in a complex mature ductal network. When a woman becomes pregnant the end buds of each duct in her resting tissue, which are enriched with stem cells, differentiate to form a spherical alveolar structure[1,2]. Each alveolus is composed of a layer of cube-shaped lactocytes surrounded by an irregular mesh-like network of myoepithelial cells[3,4]. Clusters of between ten and 100 alveoli constitute a single lobe, and each gland can consist of between four and eighteen lobes that are embedded in the surrounding non-secretory material or 'stroma' (**FIGURE 1**)[5].

The stromal matrix

On the outer surface, the secretory alveoli are separated from the stroma by a basement membrane rich in fibrous connective proteins, including laminin and collagen IV. The basement membrane is important in regulating the activity of the alveolar cells and the components that can pass from the mother's bloodstream or interstitia into the milk. Surrounding the basement membrane is a complex stroma made up of non-epithelial cells including adipocytes and fibroblasts. The latter

produce and secrete structural proteins including glycosaminoglycans and glycoproteins[6,7], and both cell types are integral to providing structural support to the embedded ductal alveolar network. The stromal matrix also has an important role in housing the blood vessels and capillaries that deliver essential nutrients to nourish the metabolically active gland and supply the substrates for milk synthesis[8].

Structures involved in milk removal

Removal of the milk from the alveoli and along the ducts is coordinated by the surrounding network of myoepithelial cells that contract in response to neuro-endocrine signals stimulated by the infant sucking. Each individual lobe drains into its own duct to remove stored milk. Collectively the lobular ducts converge on a common inter-lobular duct that carries the breast milk to the main milk duct where it is removed via the nipple by the sucking infant. Ejection of the milk occurs in a pulsatile fashion simultaneous to the contractile stimulus. The pulses can vary widely between mothers in both frequency and number, but the pattern of pulses within the same mother is generally very consistent[9-11].

The uniqueness of human milk

Species specificity

When we consider that a newborn baby looks and functions nothing like a newborn cow, goat or other mammal, it is not so hard to believe that the composition of the milk from these different species is so distinct[12]. The species specificity of mother's milk has been assumed by intuition for some time, but more recently advanced chemical and biological analyses have identified and quantified many of the specific components that define human milk. Likewise, it has been shown in both retrospective and prospective population analyses, that individuals who receive mothers' milk for a longer duration during infancy are less likely to show developmental or immune deficits later in life[13-15]. While the contribution of each milk component to infant nutrition is still poorly understood, the evidence for exclusive breastfeeding in early life to promote optimal growth and development is almost irrefutable. So what makes milk so special?

Molecular composition

The complete milk is a highly complex suspension of lipids, protein, carbohydrates, secretory immunoglobulins, calcium and various other macro- and micro-molecules, ions and bioactive factors. In many ways it can be compared to the diet of a healthy adult compressed into one homogenous fluid, with the unnecessary extras removed[16]. This is the product of thousands of years of evolutionary engineering, and Mother Nature spared no investment to assemble this secretion to fulfil the enormously diverse and important role of both nutrition and immune protection of the infant[17]. Many of the components, while present in other food sources, show biochemical qualities that are distinct to the human milk form, though again the relevance of this is largely unclear. Since it is difficult to identify the specific role (or roles) of each milk component in the infant, research has been more productive by identifying ways to ensure that mothers who express and store their milk do so in a way that will preserve all of its biochemical and bioactive properties[12,18].

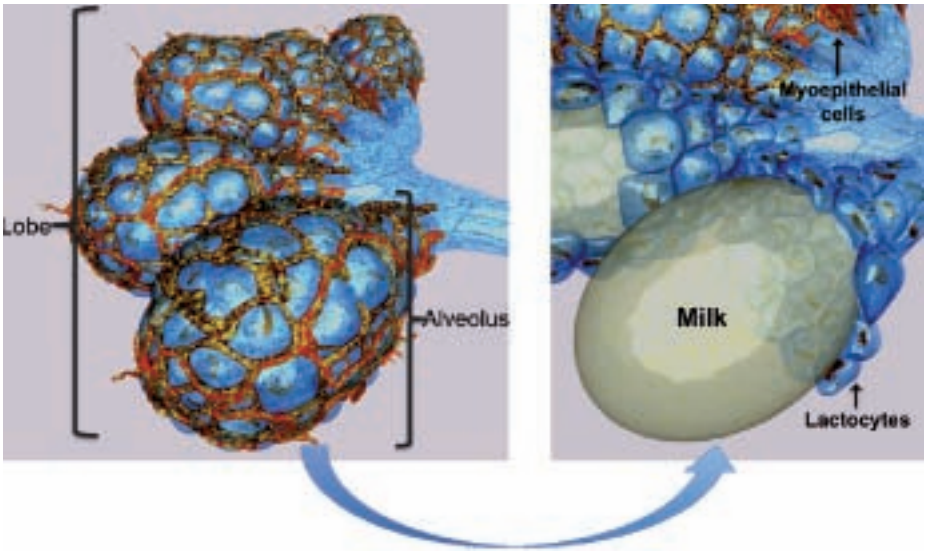

FIGURE 1 Arrangement and structure of the secretory alveoli during lactation. Clusters of 5-10 alveoli form a single lobe connected to a common milk duct. Each alveolus contains an inner layer of lactocytes that is surrounded by a network of myoepithelial cells. Milk components collect within the luminal centre before being ejected down the ducts. *Copyright Medela AG 2009, used with permission.*

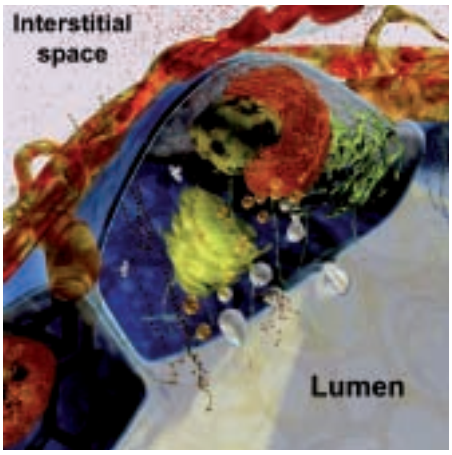

FIGURE 2 Milk secretion into the alveolar lumen. This cross section shows the polarised morphology of a lactocyte (blue) as milk components enter the lumen. Blood vessels in the interstitial space (red) carry substrates to the cell that are selectively carried across the cell or between the cells into the lumen. Other components are synthesised within the lactocyte itself and are transported across the inner membrane into the lumen. Polarisation of the cell ensures that the net movement of milk components is towards the luminal side. *Copyright Medela AG 2009, used with permission.*

The distinct role of colostrum

Colostrum is the secretion that is synthesised at parturition and precedes mature milk synthesis. It has a distribution of components distinct from mature milk, and is designed specifically to nurture the fragile digestive and immune systems of the vulnerable newborn. Colostrum is high in proteins (including growth factors, immunoglobulins and other antimicrobial peptides) and carbohydrates but low in fat compared to mature milk[19]. Recent evidence has also identified specific factors in colostrum that promote cell proliferation to prompt development of the gastrointestinal (GI) tract, and other factors that stimulate haematopoiesis and cytokine production to develop acquired immunity. These factors have even shown potential therapeutic benefits in adults with disorders of the GI system[20]. Tracking

the changes in milk composition during the transition from colostrum to mature milk and relating them to the developmental and adaptive changes occurring in the infant, provides a way to identify potential roles for each milk component in the baby.

Milk secretion and transport

Regulation of milk component synthesis

The lactocytes that line the lumen of the alveoli, synthesise and selectively diffuse breast milk components into the lumen in response to endocrine signals released at birth. More specifically, secretory activation is the combined effect of elevated blood prolactin and a rapid drop in progesterone immediately post-partum[21,22]. However, what regulates the synthesis and diffusion of each specific component, and even if there is a functional difference between each individual lactocyte, is largely unknown. Understanding these regulators has massive diagnostic and therapeutic potential for mothers who either suffer insufficient milk supply or produce milk with an unusual composition.

Transport of milk components into the lumen

The lactocytes have a distinct polarised morphology with an outer margin rich in rough endoplasmic reticulum, the organelle responsible for synthesising the proteins. Towards the luminal side of the cell, secretory vesicles migrate across the cell membrane along with high concentrations of dispersed fat globules and protein aggregates such as casein micelles. This polarisation ensures that the milk proteins being synthesised are carried in the direction of the lumen and aren't diffused into the surrounding interstitial space[23-26] (**FIGURE 2**). However, like the signals for milk synthesis, the factors that determine and establish cell polarity are not well understood.

Intercellular pathways

During lactation, intercellular tight junctions connect adjacent lactocytes to form a continuous, selectively permeable layer. Some milk components are selectively passed through these intercellular junctions into the lumen, where they are incorporated into the milk. This includes a wide variety of components originating from the mother's bloodstream and interstitial fluid[27]. While there is little evidence to prove it, it is assumed that this also includes the passage of the large immune cell population into the milk.

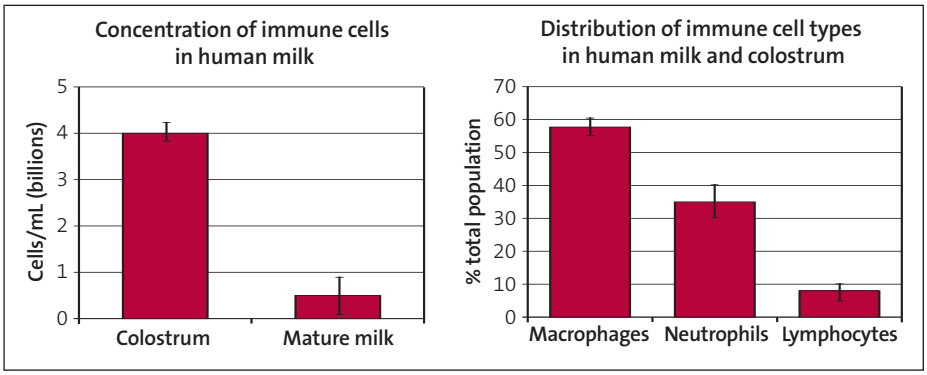

FIGURE 3 Concentration and distribution of immune cells in milk. a) The concentration of immune cells is around 4 x 10^9 cells/mL in colostrum and early milk, to compensate for the extremely naïve immune system of the newborn infant. In comparison, even at the upper limit of the range the concentration of immune cells in mature milk barely reaches a quarter of the concentration in colostrum. The range is 1 x 10^8 to 1 x 10^9 cells/mL. b) Ranging from 55-60% the predominant immune cell type in human milk including colostrum is macrophages involved indirectly with infant T and B cell maturation. Neutrophils representing 30-40% of the population are believed to be involved exclusively in maternal tissue protection. Lymphocytes represent a smaller proportion ranging from 5-10% of the total population, but have a direct role in educating the adaptive immunity of the infant with the mother's immune experience.

The immune cells in milk

Immunity and the infant

The sterile intrauterine environment means that a developing fetus has little or no requirement for its own immune response system, and is protected solely by the immune system of the mother. At parturition, the naive immune system of the baby must develop by exposure to pathogens and toxins present in the every day environment[28]. In humans, three types of immunity regulate response to potentially dangerous pathogens:

- Innate immunity is the natural response to environmental pathogens.
- Adaptive (active) immunity develops throughout life as the immune system is exposed to pathogens and develops a 'memory' to respond quickly to those pathogens at the next exposure (priming).
- Passive immunity is protection provided by another source, and generally only lasts a short time.

Breast milk has long been considered a form of passive immunity for an infant, by providing secretory immunoglobulins along with a whole variety of proteins and compounds with immunological properties[29-31]. While this is indeed true, it has also become clear that the immune experience of the mother may also be involved in developing the infant's adaptive immune response system[32]. Detection of viable immune cells in the faeces of breastfed infants indicates that immune cells from milk can survive and remain active in the gut. Other research has identified maternal immune cells in the peripheral blood of the infant, evidence that they can indeed cross the intestinal wall and remain active in the infant[29-31,33].

Compared to the non-cellular immunological compounds in milk which have specific and pre-programmed antigen response, an active cell may be capable of responding to a number of different antigens, depending on exposure. The cells then programme the infant's immune 'memory' to respond to the antigens that it is regularly exposed to, which may be antigens not detected by the non-cellular immunologic compounds[34]. While this evidence is still at a speculative stage, it has become an area of intense interest in infant nutrition. This is largely because the non-cellular immunologic compounds alone cannot yet explain the observed benefits of breastfeeding to an individual's immune system later in life, such as a better response to infection and vaccines and a reduced risk of either allergy or other immune-mediated diseases. Some of the more notable diseases with a decreased incidence among breastfed individuals include coeliac disease, diabetes, arthritis and multiple sclerosis[13,35].

Types and function of immune cells in milk

The immune cells (leukocytes) in human

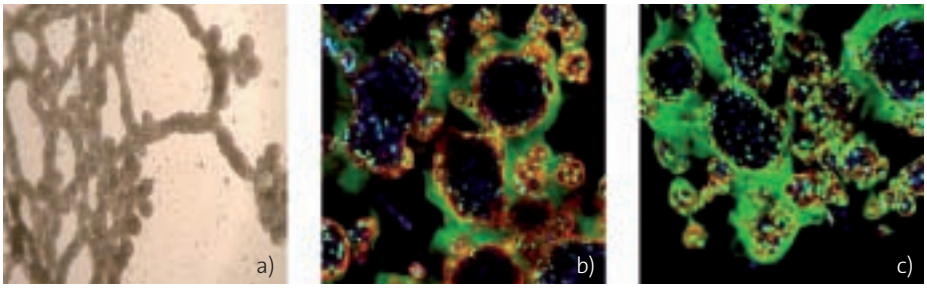

FIGURE 4 *In vitro* cultures of ductal-alveolar structures. a) Stem cells from human milk were amplified and grown in the presence of stromal matrix to form these three-dimensional structures resembling the lactating secretory epithelium. The average duct size is around 100μM and the average alveolar size is around 250μM. b) and c) Using fluorescent microscopy these cross sections of the ductal-alveolar show the stromal matrix outside the cellular network autofluorescing in green. The red is a marker for a protein that connects the basal layer of cells to the stromal matrix proteins (α6-integrin). The blue labels the nuclei of the individual cells and the green label within the cells is a fluorescent marker for milk components being synthesised.

milk vary in concentration throughout the duration of lactation. The conservation of this process supports the important role of immune cells in the health of the infant. Colostrum contains approximately 4 x 10^6 cells/mL, while mature milk concentrations vary between 10^5-10^6 cells/mL depending on the mother and the stage of lactation. These are divided up into several different immune cell types, generally 55-60% macrophages, 30-40% neutrophils and 5-10% lymphocytes[29] (**FIGURE 3**).

The macrophages express activation markers that indicate they are primed to respond to foreign pathogens, which can then affect the infant's own T and B cell function, a property that may be involved in the development of acquired immunity. They also demonstrate phagocytic activity and secrete immunologic compounds, which contribute more to the passive response of the infant during the very early stages of naive immunity[36,37].

While a proportionally large component of the immune cell population, the role of the neutrophils in the infant has not been clearly identified. Since they show little activity in the milk, this particular component is assumed to be more important for immune protection of the mother's glandular tissue[38].

The small proportion of lymphocytes in the population are predominantly T cells, and are of particular interest to nutritional research because they display markers unique to milk T cells. Also, rather than passively diffusing from the mother's interstitial space, these cells are actively targeted to the mammary gland for incorporation into the milk where they exist at a much higher concentration than in the mother's blood. Like the effect stimulated by the macrophages, the milk lymphocytes express markers associated with development of immunologic memory and are believed to directly promote T cell maturation in the case of antigen presentation (**FIGURE 3**)[39-41].

The 'other' cells in milk

While a majority of the cell types present in human milk seem to be immune cells, research has also identified the presence of non-immune cells that appear to derive from the glandular epithelium of the breast. The cell types described range from differentiated ductal epithelial cells and lactocytes, to committed stem cells, to cell fragments. The most comprehensive description of these cells came from morphological analysis on milk cell smears by Brooker some 30 years ago[42]. He described a population of lactocytes identifiable by the presence of profuse cisternae and rough endoplasmic reticulum, lipid droplets, secretory vesicles and protein aggregates. He also identified a very small population of ductal epithelial cells distinguished by abundant microvilli dispersed across their luminal surface membrane. In some cases, remnants of desmosome and tight junction proteins that connect ductal epithelium *in vivo* were visible in diametrically opposed positions along the perimeter of these cells. Another dominant cellular entity Brooker identified was cell fragments arising from secretory vesicles and as a product of cellular degradation[42-45].

This analysis was performed prior to more modern cell typing techniques, and since then research has identified the presence of mammary stem cells that are responsible for generating the secretory epithelia during gestation[46,47]. This is a very intriguing observation since the presence of stem cells in the milk opens the potential for differentiation within the infant. Indeed, it has been shown by several studies that maternal cells can be identified in an offspring years after birth[48,49]. While no evidence exists to either prove or even suggest this pathway specifically, the fact that a conserved profile of mammary stem cells is present in the milk indicates that it is a mechanism preserved by nature, and therefore probably has a biological role in survival. If nothing else, these cells have actually provided a valuable tool for researchers to develop cell culture models of the lactating gland epithelium.

The lactating epithelium

Research on the lactating epithelium is difficult since tissue biopsies are rarely carried out on glandular tissue during lactation. This means that there is no access to the differentiated epithelium for analysis. An alternative to tissue explants is to use *in vitro* surrogates, in the form of three-dimensional cell cultures generated from cells that originate from the gland[6,7,50-52]. This also has its limitations, since most biopsies are taken from either resting (undifferentiated) or tumorigenic tissue and therefore the cultures that these cell isolates generate are not necessarily an accurate reflection of the structures generated from cells from the lactating (differentiated) epithelium[53,54].

As highlighted in the introduction of this article, the lactating epithelium is a vastly different organ to the resting epithelium in its structure, function and cellular composition. However in a recent discovery it has been shown that the stem cells isolated from human milk generate cultures that closely resemble the lactating epithelial functional units (**FIGURE 4**)[46]. This is an extremely valuable resource for research since the cells are isolated in a non-invasive way and can be collected from a broad cross section of breastfeeding mothers. As more evidence emerges to describe the factors that regulate mammary epithelial differentiation, this approach will provide a platform to simulate glandular function *in vitro*. This will open up new ways to investigate the questions that have surrounded this extraordinary biological process for so long, facilitating our definition of best practice for neonatal and infant nutrition.

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