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The Role of Prolactin in CCL28 Regulation

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THE ROLE OF PROLACTIN IN REGULATING CCL28 EXPRESSION

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

THE ROLE OF PROLACTIN IN REGULATING CCL28 EXPRESSION

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Master of Science

Infants are born with naive immune systems, making them susceptible to a variety of infections. In order to protect the newborn infant it is important that mothers be able to pass protective IgA antibodies to their infants through breast milk. B cells that produce IgA enter the mammary tissue during lactation and secrete IgA into the milk. During pregnancy, the mammary tissue expresses high levels of chemokines, molecules that allow lymphocytes to selectively home to specific tissues. The chemokine CCL28 has been shown to be upregulated during both pregnancy and lactation, and is vital for the ability of IgA-producing B cells to home to the mammary tissue during lactation. The aim of this study was to determine whether CCL28 expression is regulated by prolactin signaling.

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INTRODUCTION

In order to protect infants whose immune functions have not fully developed, there are several mechanisms by which a mother can pass on temporary immunity. This transference of immunity is often mediated by antibodies, and can happen both before birth and during lactation. The transferred immunity is the result of cells in the immune system homing to specific tissues in order to secrete antibodies. Many classes of molecules, including chemokines, are responsible for this homing of immune cells. This study tested the hypothesis that the the lactogenic hormone prolactin up-regulates gene expression of the epithelial chemokine CCL28.

1.1 PASSIVE TRANSFER OF IMMUNITY

Infants are born with immature immune systems; they are therefore far more susceptible than adults to a variety of diseases, notably infections of the gastro-intestinal tract. Evolution has therefore provided several protective mechanisms by which infants receive passive immunity from their mothers during this critical period. Passive immunity is generally accomplished through the transference of products of an adaptive immune response, such as T cells or antibodies [1]. The receiving individual (in this case the infant) is protected, but does not produce his or her own immune response. A passive transfer of immunity from mother to infant can happen in two ways: either through IgG crossing the placenta before birth, or via IgA passing through the breast milk and into the intestine of the infant after birth. Both of these transfers are essential for protecting the infant during the critical periods before birth and during immune development.

1.1.1 IgG

IgG is the most common antibody in the serum [1]. It protects the blood from pathogens, and is important in preventing systemic infections. IgG is the only class of antibody that can cross through the placenta, and thus is the only class of antibody transfered from the mother to the fetus before birth. IgG concentrations in the fetus gradually increase during pregnancy as neonatal Fc receptors concentrate IgG inside the placenta. These Fc receptors have a higher affinity for IgG than the receptors found in the mother, allowing IgG levels in the placenta to be eventually higher than in the mother [2], [3]. Children born to hypogammaglobulinemic mothers (who lack normal immunoglobulin levels) are particularly vulnerable to infection, as the mothers do not have antibodies to pass to the infant. Although children born to such females may be born healthy, they are far more likely to succumb to septicemia [4]. Gamma globulin is often administered to the mother throughout pregnancy in order to protect the child, and the child is often successfully treated with antibodies for six months after birth to prevent severe illness. It has been suggested that intravenous IgG treatments administered to the mother during pregnancy may also help the fetus mature normally, possibly by preventing constant infection in the mother that might hinder fetal development [4].

Even in children born to normal mothers protection through maternal IgG is temporary. Passive immunity derived from maternal IgG wanes within the first six months of an infant's life as the antibodies are gradually degraded [2]. It is thus critical that passive immunity be transferred to the infant during the time between parturition and the development of the infant's own memory responses to pathogens in his or her environment.

1.1.2 IgA

Because the mucosal tissues line the respiratory, gastrointestinal and genitourinary tracts, they are the point of entry for many pathogens. It is therefore essential that these tissues have specific defenses to prevent infection. *(Reviewed*

in [5]). Without immune protection in these areas the body would be less able to combat the large range of diseases entering through the mucosol layer. This is demonstrated by the fact that individuals with IgA deficiency, the most common immunodeficiency among people of European descent [6], experience more sinus and pulmonary infections than normal individuals. Such individuals are surprisingly not more susceptible to gastro-intestinal (GI) tract infections; this may be due to a compensatory mechanism that increases production of both IgG and IgM in the GI tract [7].

Most classes of antibody are unable to cross through the epithelial layer into mucosal tissues. Only IgA and IgM multimers, which have J chains that allow formation of multimeric complexes, can bind to the poly-Ig receptor (pIgR). Binding of the J chain to pIgR allows the antibody/receptor complex to be endocytosed and transported across the cell. Upon release, part of the pIgR is cleaved, and becomes a secretory complex attached to the IgA dimer. *(Reviewed in [5])*. This component is a glycoprotein, and allows the secretory IgA (sIgA) or IgM (sIgM) to pass through harsh environments, such as the stomach, without being degraded. Many signals in the body can up-regulate pIgR expression. Among these are several hormones and inflammatory chemokines. The ability of inflammatory chemokines to increase expression of pIgR during infection is crucial, as it aids transport of sIgA and sIgM into the mucosal layer when they are most likely to be needed for protection.

Although some IgM can cross the epithelial wall, IgA is the primary antibody isotype in muscosal secretions and in breast milk in normal individuals. IgA mediated passive immunity is passed to infants during nursing; this immunity is critical in protecting the neonates from intestinal pathogens [8]. IgA is present in the breast milk in concentrations between 0.5-1.5 g/l [9]. Although there is more IgG in the blood, in normal individuals IgA is by far the most prevalent antibody in the body. Infants who are not breast-fed, and thus do not receive IgA from their mothers, are also far more susceptible to disease. In the developing world the death rate among non-breast fed infants is significantly higher than among those infants who are breast-fed. It is estimated that if all infants were breast-fed for the first

year of life, the number of infant deaths globally would decrease by one million each year [10].

The antibodies passed from mother to child are specific to antigens found in the mother's (and, presumably, the child's) environment. Because infants do not have the memory responses typical of adult immunity, it has been suggested that immunizing the mother against childhood infections, such as pertussis, might be an effective method of preventing infection in the neonate. In animal studies this has been shown to be effective, as piglets who suckle mothers that have received pertussis vaccinations are far less susceptible to serious infection [11]. The IgA in breast milk plays a crucial role in this defense, although other components of breast milk also protect the infant [11].

1.2 Lymphocyte homing and chemokines

Lymphocyte migration into specific tissues happens through a multi-step process. (*Reviewed in* [12]). This process is controlled through the expression of several different classes of homing molecules. The most important of these molecules are the selectins, integrins and chemokines (Table 1.1).

Different selectins are found on both lymphocytes and on the endothelium. They are glycoproteins that have the ability to bind heavily glycosylated molecules, such as mucins, in a receptor/ligand binding process. Mucins are also expressed by both lymphocytes and the endothelium. The interaction between selectins and mucins results in the "tethering," of the lymphocyte to the endothelial wall. This is a loose interaction, and is not enough to stop the movement of the lymphocyte, although it does slow the cell, allowing it to roll along the endothelial wall. This rolling allows the lymphocyte to be exposed to the chemokines being produced by the tissue. If the lymphocyte has the correct chemokine receptor it will become activated, allowing the cell to migrate across the endothelium. Without this chemokine-mediated activation the cell will continue to roll through the blood vessel, and never enter into the tissue.

Molecule	Ligand	Action	Location
Selectin	Heavily glycolsylated molecules, such as Mucins	Initial tethering, rolling of lymphocyte, weak adhesion	Endothelial wall: E- and P-selectins
Integrins	Cellular Adhesion Molecules (Ig superfamily)	Firm adhesion	Leukocytes
Cellular Adhesion Molecules CAMS	Integrins, Selectins	Firm adhesion	Endothelial wall
Chemokines	Chemokine receptors on leukocytes	Activation, diapedesis and migration	Produced by tissue

Table 1.1: Important homing molecule classes [1]

Chemokines are a subset of cytokines identified by four highly conserved cysteine residues. They are classified according to the position of the cysteine residues; most are either "CC chemokines" or "CXC chemokines," although other classes exist. Chemokines can be produced either constitutively or in response to inflammation, and bind to seven trans-membrane G-coupled receptors. (*Reviewed in* [12]). Activation of a cell by chemokine binding results in a change in integrin conformation. This change allows the integrins to bind ligands more effectively, which results in a firm adhesion of the lymphocyte to the endothelial wall. Integrins are heterodimers, and bind to another class of molecules, the Cellular Adhesion Molecules (CAMS). The firm adhesion caused by the binding of integrins and CAMs allows the lymphocyte to leave the blood vessel and enter the tissue, where it can migrate up a chemotactic gradient toward the cells producing the chemokines (Figure 1.1). During pregnancy and lactation, many adhesion molecules and chemokines are expressed by the mammary tissue, allowing more lymphocytes to

enter the mammary gland [13], [14], [15]. This compliment of adhesion molecules and chemokines mediate the homing and accumulation of IgA antibody secreting cells (ASC) to the lactating mammary gland.

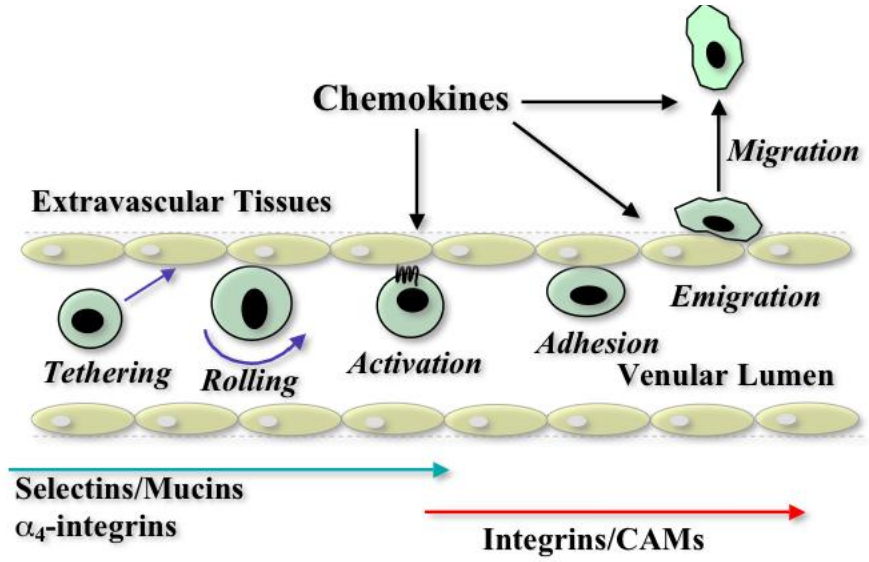


Figure 1.1: Multi-step lymphocyte homing model. Adapted from [12].

1.2.1 Chemokines mediating IgA ASC migration

Because IgA ASCs must migrate into the mucosal tissues in order to function effectively, these tissues express specific chemokines that mediate this migration. In particular, CCL25 and CCL28 are known to be critical chemokines that attract IgA ASCs. In some tissues, such as in the lamina propria (LP) of the small intestine, these two chemokines may play similar roles, as they both are expressed and serve to attract the same cells. The redundant role played by CCL25 and CCL28 is demonstrated by the fact that, if either chemokine is blocked by antibodies, the IgA ASCs are still able to home to the LP. If both are blocked, however, IgA ASCs are unable to migrate into the tissue [16]. In other tissues the expression and function of CCL25 and CCL28 differ dramatically. In the mammary tissue, for example, CCL28 plays a critical role in attracting IgA ASCs during pregnancy and lactation,

while CCL25, although expressed, does not appear to play a large role in IgA ASC homing to this tissue.

1.3 CCL25

CCL25, also known as thymus-expressed chemokine (TECK), is expressed largely in the thymus and small intestine. It binds to the chemokine receptor CCR9, which is expressed by IgA ASCs, as well as by a subset of T cells [16], [17]. CCL25 is not expressed in the large intestine, however, which may allow more lymphocytes expressing CCR9 to home directly to the small intestine. This results in a more specific and localized immune response in these tissues.

1.4 CCL28

One molecule that is important in IgA ASC homing to the large intestine and mammary tissues is CCL28, also known as mucosae-associated epithelial chemokine (MEC). CCL28 is a "CC" chemokine that is expressed by epithelial cells in the mucosal tissues, and is known to bind to the receptors CCR3 and CCR10 [18].

CCR3 has been shown to be expressed by eosinophils, and may contribute to eosinophil accumulation in asthmatic conditions, as CCL28 is known to be up-regulated in the lung after allergen exposure [19]. *In vivo* studies have also shown that CCL28 is significantly up-regulated in human colon explants during infection, potentially helping to attract lymphocytes to the area of infection. Similarly, when bacterial proteins were added to tissues, the levels of CCL28 increased dramatically [20]. The pro-inflammatory cytokines Il-1β and TNF-α were shown to up-regulate CCL28 in the lung and may be responsible for up-regulating CCL28 levels under asthmatic conditions or during bacterial infection.

The other receptor for CCL28, CCR10, is expressed primarily on IgA ASCs [21]. CCL28 mediates migration by IgA ASCs, but does not have a great effect on IgG secreting cells [22]. This suggests that CCL28 primarily affects cells producing antibody destined to be secreted into the mucosal membranes, and that the expression of CCR10 is responsible for selecting only IgM and IgA producing B cells. Since secretory IgM and IgA are the primary antibodies in breast milk, the B cells

producing these isotypes must be able to home selectively to mammary tissues. The fact that IgG cells do not express CCR10 demonstrates the selectivity of the homing process.

In 2004, Wilson and Butcher showed that CCL28 is clearly up-regulated in the mammary tissue during pregnancy, and CCL28 is up-regulated even more significantly during lactation. They also demonstrated that anti-CCL28 antibodies blocked IgA ASCs from homing to the mammary tissue [13]. This clarified the importance of CCL28 in lymphocyte homing, although the exact mechanism by which CCL28 is up-regulated was not shown. This work also demonstrated the absolute necessity for CCL28 up-regulation; without CCL28 the lymphocytes could not home to the mammary gland, and the mother could not pass IgA mediated immunity to her infant. This, in turn, clearly demonstrates the critical nature of CCL28 expression in infant protection through maternal antibody transfer.

1.5 Lactogenic Hormones

During pregnancy and lactation there are increased levels of hormones present in the mother's body. These hormones are responsible for many crucial tasks, including maintaining pregnancy, remodeling mammary tissue, and stimulating milk production. It has been shown that some hormones are responsible for changes in immune function. For example, In 2003, Peeva *et al.* demonstrated that prolactin, a hormone produced in high levels by the anterior pituitary during pregnancy and lactation, is able to effect the survival of B cells *in vivo* [23]. The hormones produced during pregnancy often work synergistically to increase gene expression. Some genes (such as the milk protein β-casein) require that both prolactin and cortisol be present in order for strong expression to be induced, although each can induce low levels of gene expression alone [24].

Many hormones are responsible for mammary tissue remodeling and milk production during first pregnancy and then lactation. Among these are glucocorticoids, which are steroid hormones, and prolactin, a peptide hormone.

1.5.1 *Cortisol*

The main glucocorticoid in humans is cortisol, while corticosterone plays a greater role in mice [25]. Cortisol is mainly produced in the adrenal gland. Its production is stimulated by the presence of the adrenocorticotropic hormone (ACTH), which is in turn stimulated by corticotropin releasing hormone (CRH), which is produced by the hypothalamus (Figure 1.2). An over-abundance of cortisol, whether natural, as in the case of an ACTH or CRH producing tumor, or artificially introduced will result in decreased immune responses. This decrease can be useful clinically for decreasing the inflammation associated with inappropriate immune responses, such as the hypersensitivity reactions that cause asthma [26].

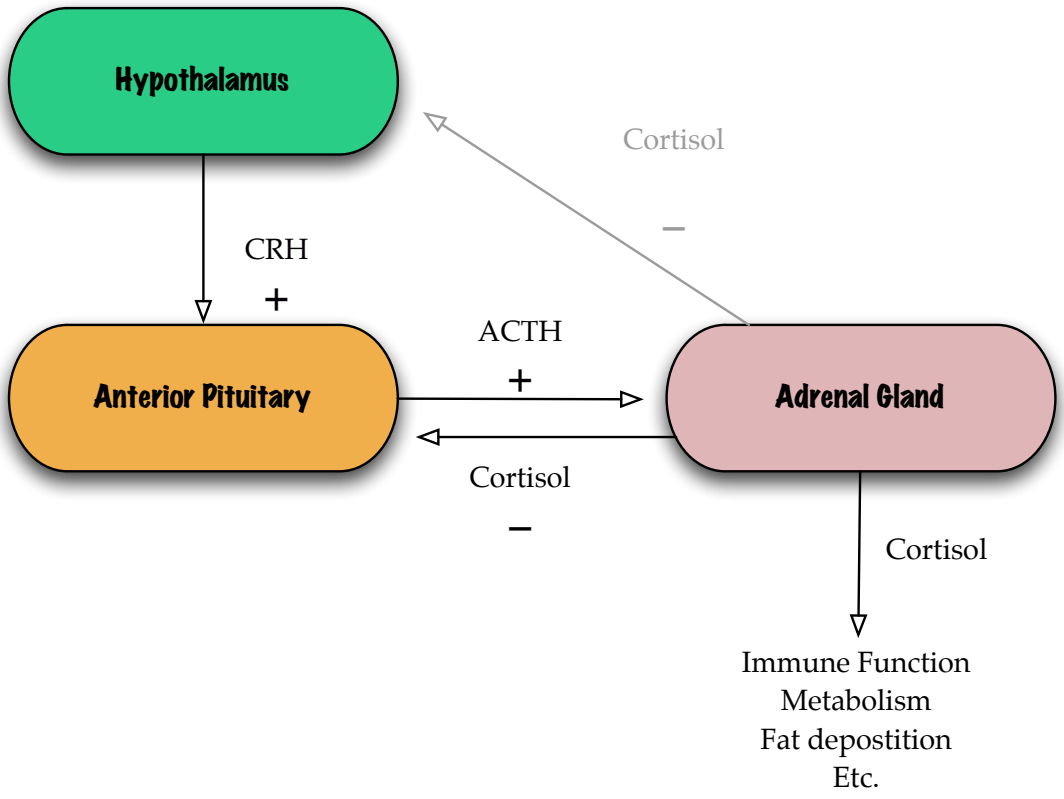


Figure 1.2: Cortisol Production [25].

Glucocorticoids are necessary for immune function. They are important regulators of immune cells, influencing their longevity and efficacy in the tissues. It is important to appreciate, however, that glucocorticoids also have a much broader role outside the immune system. Glucocorticoids influence gene expression of cells throughout the body, and are important in many different biological pathways. They can influence fat disposition, bone mineralization, and alter the body's ability to metabolize sugar [25].

Cortisol levels gradually increase throughout pregnancy [25]. A sharp increase of cortisol shortly before parturition directs the fetus' lungs to start producing surfactant, which is necessary for survival after parturition [25], [27]. Premature animals who are administered cortisol before parturition are able to expand their lungs while untreated animals induced at the same time cannot [28]. It has been postulated that the natural increase of cortisol shortly before parturition may also significantly, although temporarily, alter immune function in the mother [29].

In human breast and intestinal cell lines, glucocorticoids also have the ability to increase expression of pIgR [5]. In animal models, however, glucocorticoids are only effective when prolactin is also present. The increased pIgR expression allows for greater transport of sIgA into these areas, providing greater protection of the tissue and secretion of antibodies into the breast milk [5], [30].

1.5.2 Prolactin

Most of the prolactin in the body is produced by the anterior pituitary in response to a number of signals, including oxytocin, thyroid releasing hormone (TRH), and other hormones, many of which are produced in the hypothalamus. Prolactin is normally under negative control by dopamine, which is also produced in the hypothalamus. Prolactin is, in fact, the only hormone in the anterior pituitary under constant negative control by dopamine produced in the hypothalamus [25], [31] (Figure 1.3).

In addition to the prolactin produced by the anterior pituitary, small amounts of decidual prolactin are produced by the endometrium throughout pregnancy;

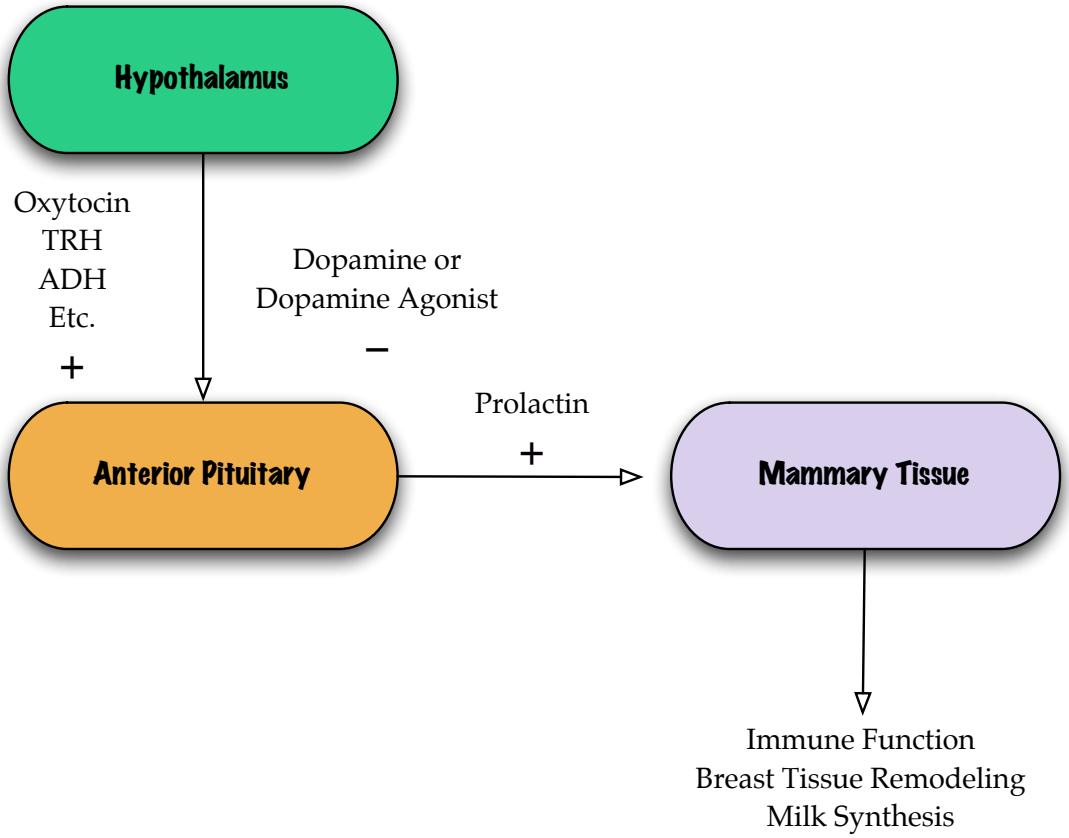


Figure 1.3: Prolactin Production [25]

this production is not subject to dopamine agonist receptor inhibition [32]. Decidual prolactin is, however, produced at far lower levels than the prolactin produced in the anterior pituitary, and cannot compensate for a cessation of pituitary prolactin production.

Prolactin production by both the maternal anterior pituitary and the fetus increases throughout pregnancy, with a sharp increase in maternal production that occurs when nursing begins. The increased levels of prolactin present during pregnancy play an important role in restructuring the mammary tissue in order to allow it to become a functional lactogenic gland [33].

Significantly, prolactin has also been shown in animal models to up-regulate the expression of pIgR in the mammary gland [5], [30]. Because pIgR allows IgA to

cross into the breast milk, this is one method by which prolactin helps to increase the transfer of immunity from mother to child.

1.5.2.1 β-casein

Prolactin is an important stimulator of milk proteins. In this study we chose to use the milk protein β-casein as a control because the expression of this protein is so dependent on prolactin that it acts as an endogenous control to indicate that prolactin is signaling. Prolactin stimulates β-casein expression through the Jak2-Stat5 signaling pathway; *In vivo* the expression of β-casein is increased by a factor of 2.5 when prolactin is added to cell cultures treated with glucocorticoids and insulin [34]. This increase is believed to be due to phosphorylation of Phosphorylated Heat- and Acid-Stable protein regulated by Insulin (PHAS-I). When prolactin is added to cell culture, PHAS-1 is phosphorylated, releasing eIF4E, which is a cap-binding protein that stimulates translation of mRNA [34].

Even more significantly, the addition of prolactin to cell cultures treated with glucocorticoids and insulin can increase the stability and accumulation of β-casein mRNA by seventy-five fold [34]. This increase in stability is due to increased polyadenylation of the mRNA. Kuraishi *et al.* demonstrated that the β-casein mRNA poly(A) tail in the mammary gland exists in two forms. β-casein mRNA with a long poly(A) tail has a significantly longer half-life than mRNA with a shorter poly(A) form [35]. It is thought that the adenylation protects the mRNA from ribonucleases [35].

1.5.2.2 Inhibition of prolactin release

Dopamine receptor agonists bind to dopamine receptors, preventing prolactin release. One of the most common dopamine receptor agonists is bromocryptine. Administering bromocryptine to an animal dramatically decreases prolactin production by activating the D2 receptor [36]. This is used clinically to treat patients with lactotroph tumors that produce too much prolactin. Bromocryptine treatment often results in both decreased prolactin production and decreased tumor size. (*Reviewed in* [37]).

Bromocryptine is also used in research to determine what effect prolactin has. By removing prolactin from the system through bromocryptine administration, it is possible to see which genes require prolactin signaling in order to be expressed. For example, because prolactin regulates pIgR [5], blocking prolactin release with bromocryptine may decrease pIgR expression.

Although previous studies have investigated the role prolactin plays in regulating parts of the immune system, its role in regulating CCL28 expression has not been identified. Similarly, studies of mammary CCL28 expression have shown that CCL28 is up-regulated during times when prolactin levels are high, but has not demonstrated that this up-regulation is due to prolactin signaling. Discovering the relationship between the hormone and chemokine will increase understanding of how chemokine expression is regulated, and the process by which IgA ASCs enter the mammary gland.

1.6 Relevance

It is important to understand how homing molecules are regulated during pregnancy and lactation. If the mechanisms by which IgA producing cells are recruited to mammary glands are elucidated, it may be possible to design vaccines that are more effective at passing immunity to nursing infants. Immunizations today are often able to confer protection on the infant [38], [39], but it is not well understood why some vaccines work better then others in this arena. Current studies on immunization through breast milk are generally observational, and seek to see if an already-developed vaccine will confer immunity or not, rather then describe the mechanisms by which IgA ASCs are induced to home to mammary tissue. By characterizing the methods by which IgA ASCs home to the mammary tissue to produce IgA which protects the infants, the potential for designing more effective vaccines will be greatly increased.

In addition, understanding the basic biology of adhesion molecule gene regulation may help researchers find ways to block lymphocyte homing in cases where the homing is inappropriate, as is the case in many autoimmune diseases.

In many of these diseases, lymphocytes home inappropriately to tissues in which they are not needed and cause significant inflammation and tissue damage. The current treatment regime for such diseases generally consists of administering large doses of corticosteroids to the patient; these corticosteroids knock out beneficial immune responses as well as inappropriate ones. Understanding why lymphocytes migrate to specific tissues will help researchers discover more precise methods of treating such conditions.

MATERIALS AND METHODS

2.1 PCR AND QUANTITATIVE PCR

RNA was extracted using a modification of Invitrogen's (Carlsbad, CA) Trizol® protocol. RNA was quantified using a Nanodrop® spectrometer. The Applied Biosystems (Foster City, CA) GeneAmp PCR Core Kit® was used for PCR reactions. The Quantitative PCR was run using the Taqman One-Step RT-PCR Master Mix Reagents.® GAPDH was used as an endogenous control.

2.1.1 RT-PCR *primers*

Primers for the RT-PCR experiments were designed using Invitrogen's Oligoperfect Design™ program. Two primer sets were designed for each gene. Both sets of primers were tested on mouse tissue, and the most effective primers were chosen for subsequent gene amplification. Primer sets used in experiments are listed below.

2.2 CELL CULTURE

In order to determine what effect prolactin has on mammary epithelial cell gene expression, *in vitro* studies were performed. Because immortalized cell lines do not always express the same genes as the original tissue, two cell lines were used, thus decreasing the chances that results from the *in vitro* studies were simply an artifact of the cells' transformation.

HC11 and NOG8 cell lines are transformed murine mammary epithelial cells. HC11 cells were a gift from Dr. Jeffrey Rosen, (Baylor college of medicine) and used with the permission of Dr. Bernd Groner (Institute for Biomedical Research, Frankfurt, Germany). NOG8 cells were donated by Dr. Barbara Vonderhaar (NIH). Both cell lines were initially grown using a protocol provided by Dr. Carrie Shemenko

Gene	Primer Sequence
CCL25 forward	CGA GGC GCT AGC GCA AGG TGC CTT GAA GA
CCL25 reverse	CGA CCG GAT CCG CAT TGT TGG TCT TTC TGC
CCL28 forward	GAA CAC GTG GAA CAC ACA GG
CCL28 reverse	TTG TTT TGC TTT TGG TGC TG
Gapdh forward	CCA CCC AGA AGA CTG TGG AT
Gapdh reverse	CAC ATT GGG GGT AGG AAC AC
β-casein forward	GCA CCT TCC TCA GTC TCT GG
β-casein reverse	TGT GGA AGG AAG GGT GCT AC
Prolactin receptor forward	TGC TAA ACC CCC AGA TTA CG
Prolactin receptor reverse	GGC TGA TTC CTC AAG CAA AG
Il-1β forward	TCA CAG CAG CAC ATC AAC AA
Il-1β reverse	TGT CCT CAT CCT GGA AGG T
TNF-α forward	AAG ATG GAG GAA GGC AGT T
TNF-α reverse	GAT CCT GGA GGG AAG AGA C

Table 2.1: Forward and reverse PCR primers.

(University of Calgary). Cells were grown to confluency at 37 °C with 5% CO_2 in RPMI media containing 10% Newborn Calf Serum (NCS), 50 units/ml penicillin, 50 units/ml streptomycin, 10ng/ml recombinant human epidermal growth factor (EGF) and 5ug/ml insulin. At confluency the cells were washed three times with media not containing EGF, and given priming media. This priming media is prepared as before, except that EGF is not added. In this protocol cells are kept in this priming media for either one or four days, and then given 0.1µM dexamethasone along with 5µg prolactin. RNA is extracted after six hours incubation with these hormones.

In the second HC11/NOG8 protocol, cells are grown to confluency as before. At confluency they are kept in priming media for 48 hours, then given 1µM dexamethasone. New priming media and dexamethasone are added once a day for four days, then cells are incubated with 1µM dexamethasone and 5µg/ml prolactin. RNA is extracted twenty-four hours after prolactin and dexamethasone are added.

In both protocols β-casein was used as an internal control. β-casein is known to increase if prolactin is signaling, so it was possible to demonstrate that the protocol was working whether or not CCL28 expression changed.

Il-1β has been shown to up-regulate CCL28 in human lung cancer cells. To determine if Il-1β had the same effect in murine mammary cells, HC11 cells were grown until confluent as above. Cells were then incubated with 10ng/ml Il-1β (purchased from Peprotech (Rock Hill, NJ)) at 37 °C with 5% CO_2 for six hours. After six hours RNA was extracted as above.

Macrophages produce Il-1β *in vivo*. We therefore sought to determine whether macrophages were capable of producing sufficient Il-1β to increase CCL28 in mammary tissue. We used both a macrophage cell line and peritoneal macrophages taken from a mouse.

The transformed RAW macrophage cell line was grown in DMEM supplemented with L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. When RAW cells were added to the HC11 cell line the cells are grown in HC11 media for twenty-four hours before being treated with prolactin as described above.

Peritoneal macrophages were obtained by washing the abdominal cavity of a mouse with saline solution. These macrophages were then added directly to confluent HC11 cells.

2.3 *In Vivo* STUDIES

In vivo studies, while valuable as cell culture systems, cannot precisely mimic conditions in live animals. We therefore performed *in vivo* studies to determine what effect prolactin inhibition through bromocryptine had on CCL28 expression. In order to do this, we injected lactating mice with bromocryptine, and determined the change in CCL28 gene expression. Once again, β-casein was used as an internal control to show that bromocryptine was effectively decreasing prolactin levels in the mouse.

Bromocryptine was purchased from Sigma-Aldrich (St. Louis, MO). Bromocryptine hydrolyzes in water, so we initially reconstituted it in 100% ethanol at four

times the concentration needed, and stored this solution at -80 °C. The stock solution was then diluted to the proper concentration on the day it was to be injected into the animal.

Female mice were treated by one of two methods. In the first method, mice were injected intraperitoneally with 800 µg bromocryptine in a total injection volume of 200µl. The mice were injected for two days beginning on day eleven after parturition. The bromocryptine was made in ethanol and water as above, and RNA was extracted using the same protocol (Figure 2.1).

In the second method, mice were injected intraperitoneally with 400 µg bromocryptine for six days beginning on day eight after parturition. This day was chosen because milk production is high, and CCL28 is strongly expressed, so any decrease in β-casein or CCL28 would be both noticeable and significant. The bromocryptine was made up in 1:3 ethanol stock solution to water, with a total injection volume of 200µl. On the seventh day the after injections begin mice are sacrificed, and their mammary and salivary gland tissue is removed (Figure 2.2). RNA from these tissues was extracted as above.

2.4 Statistical analysis

2.4.1 In vitro

In the *in vitro* studies, multiple wells that had been treated concurrently with the same treatment protocol were pooled together to reduce error from individual aberrant samples. The experimental samples were compared against samples from the ethanol and water injected control mice in the Q-PCR; the Q-PCR protocol sets the gene expression in the control samples as the zero against which all experimental data was compared. For analysis, all dexamethasone and all prolactin data were averaged together. A student's t-test analyzed the β-casein and CCL28 expression changes in both the dexamethasone and prolactin treatments. The t-test tested specifically the significance of the experimental data's mean not being zero.

2.4.2 In vivo

As with the *in vitro* studies, all data was compared to RNA from control animals that were set as the baseline level for gene expression. The data from the *in vivo* experiments were then analyzed by three separate methods. In the first analysis method, all of the experimental data points for each mouse were averaged together, so that each mouse had an individual average for the change in both β-casein and CCL28 expression levels. This insured that each mouse was weighted equally in the final average, despite the fact that more data was collected from some mice. All of the experimental mice were then averaged, as were all of the control mice. These final averages were tested for significance. The t-test tested the significance of the change in gene expression's not being zero. In the second method, all of the experimental data points for each mouse were not averaged together, so that each experimental data point was treated equally, regardless of which mouse the data originated from. The experimental data points and the control data were then all averaged together, giving a final average for each. These final averages were tested for significance. As before, the t-test tested the significance of the change in gene expression's not being zero. The third method of analysis took into account the large variance in the control samples. All of the control samples were compared to all of the experimental samples. A student's t-test was carried out to determine whether there was a significant difference between the experimental and control data. This t-test was carried out assuming equal and assuming unequal variances; there was little difference between the two. All presented data will assume unequal variances between control and experimental data.

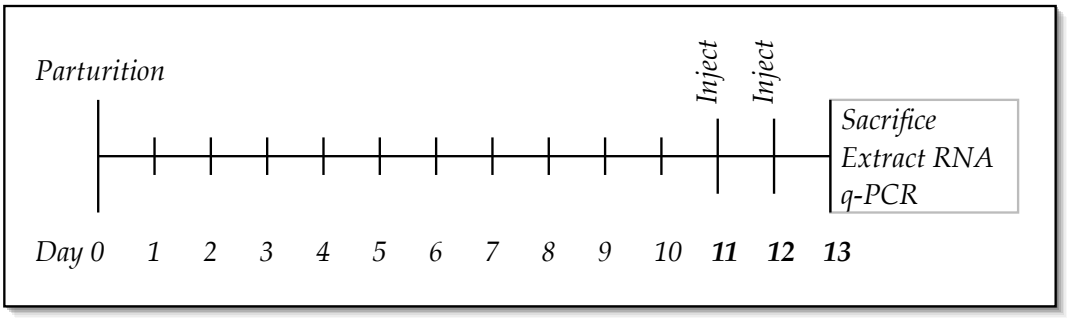


Figure 2.1: Mice are injected with either bromocryptine or a control for two days, starting on day 11 after birth. Mice are sacrificed on day 13, and RNA is extracted for analysis by q-PCR

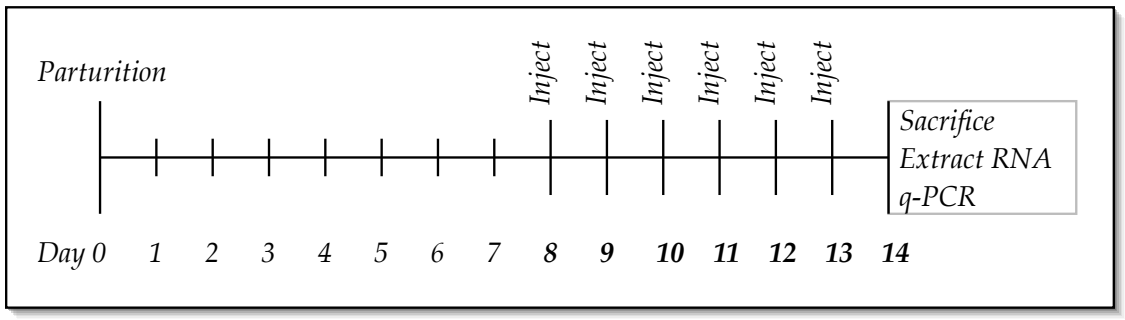


Figure 2.2: Mice are injected with either bromocryptine or a control for six days, starting on day 8 after birth. Mice are sacrificed on day 14, and RNA is extracted for analysis by q-PCR

RESULTS

3.1 IL-1β

Il-1β has been shown to up-regulate CCL28 in lung tissue [40]. Accordingly, we sought to determine if the increase in CCL28 expression in the mammary gland was mediated by Il-1β. Il-1β has been shown to be up-regulated by prolactin *in vitro* [41]. In order to determine whether Il-1β can up-regulate CCL28 in mammary tissue, HC11 cells were treated with 10ng/ml Il-1β. RT-PCR analysis showed no expression of CCL28 in treated or untreated cells (Figure 3.1).

Although Il-1β did not appear to have a direct effect on HC11 cells, it remained possible that the Il-1β added was not sufficient to induce CCL28 expression, or that CCL28 expression is dependent on Il-1 in conjunction with other cell derived factors. Macrophages produce Il-1 *in vitro* upon stimulation with prolactin [42]. An analysis of RAW cells by PCR demonstrated that the cells express prolactin receptor and Il-1β. In an effort to determine if macrophages treated with prolactin would produce enough Il-1β to induce CCL28 expression, HC11 cells were co-incubated with RAW cells with and without prolactin (Figure 3.2). The co-incubation of RAW and HC11 cells treated with hormones did not increase the expression of CCL28. Since RAW cells are a transformed cell line, and thus may react differently to prolactin stimulation than wild type macrophages, peritoneal macrophages taken from a mouse were also co-incubated with HC11 cells in the same manner. These macrophages also failed to up-regulate CCL28 (data not shown).

Finally, to demonstrate that Il-1β was not responsible for the increase of CCL28 in pregnancy and lactation *in vivo*, mammary tissue from both virgin and lactating mice were examined. Presumably, if Il-1β is responsible for the change of CCL28, the levels between virgin and lactating mice would be dramatically different. Il-1β levels between the virgin and lactating mice remained similar, with a

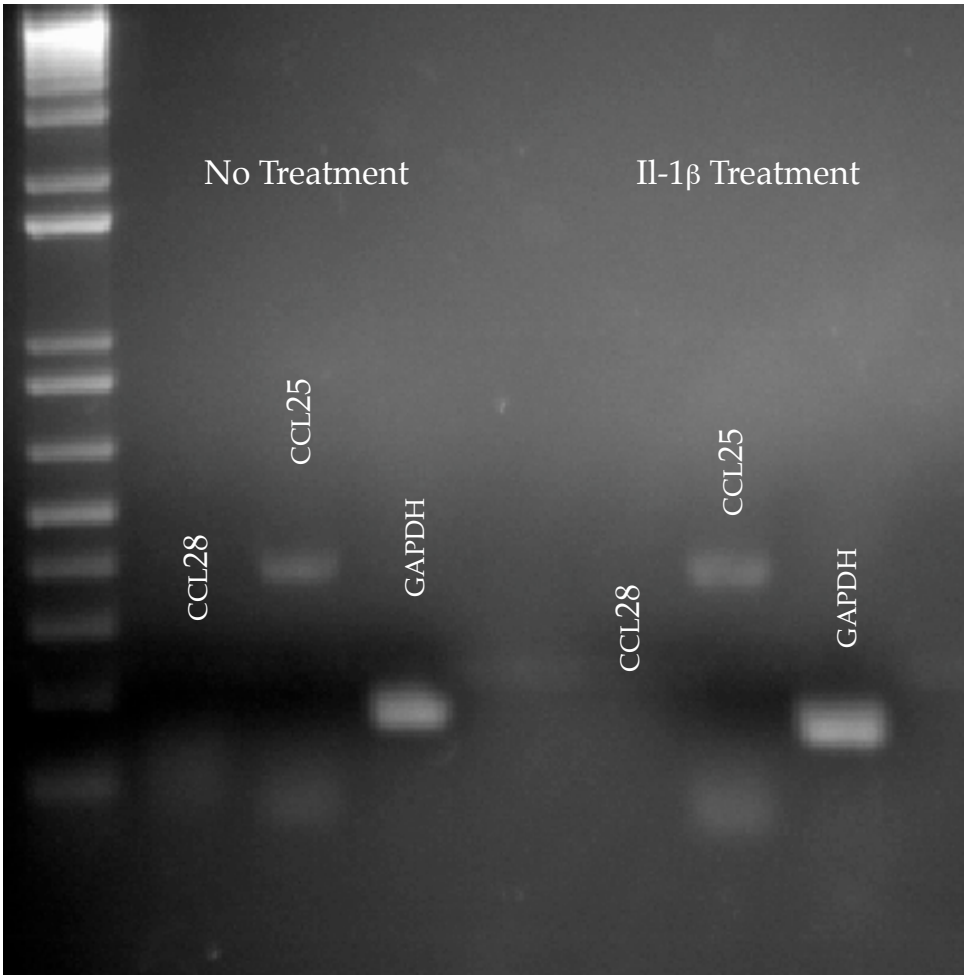
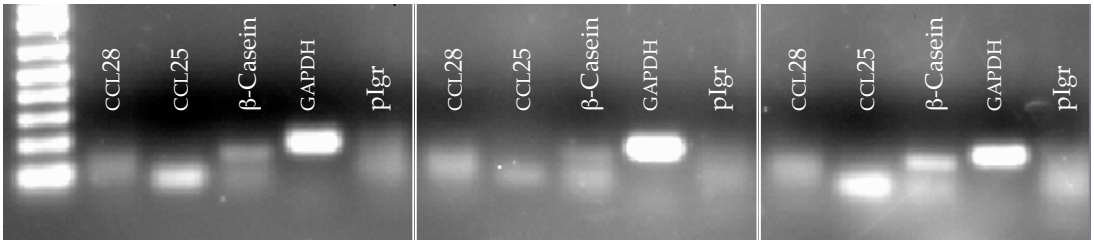


Figure 3.1: Il-1β does not up-regulate CCL28 expression in HC11 mammary epithelial cells. HC11 cells were grown until confluent, as described in materials and methods. 10ng/ml Il-1β were then added for six hours. RT-PCR analysis revealed no detectable changes in CCL28 or CCL25 expression following this treatment.



(a) HC11 cells alone. (b) HC11 + RAW cells. (c) HC11 + RAW + prolactin treatment.

Figure 3.2: HC11 cells co-incubated with RAW macrophage cells and/or prolactin. CCL28 was not up-regulated by either RAW cells or prolactin treatment.

small increase happening during lactation (Figure 3.3). The fact that there was a small increase in Il-1β in the mammary gland, but Il-1β is not capable of stimulating CCL28 *in vitro* suggests that Il-1β probably is not directly responsible for the increase in CCL28 seen in lactation.

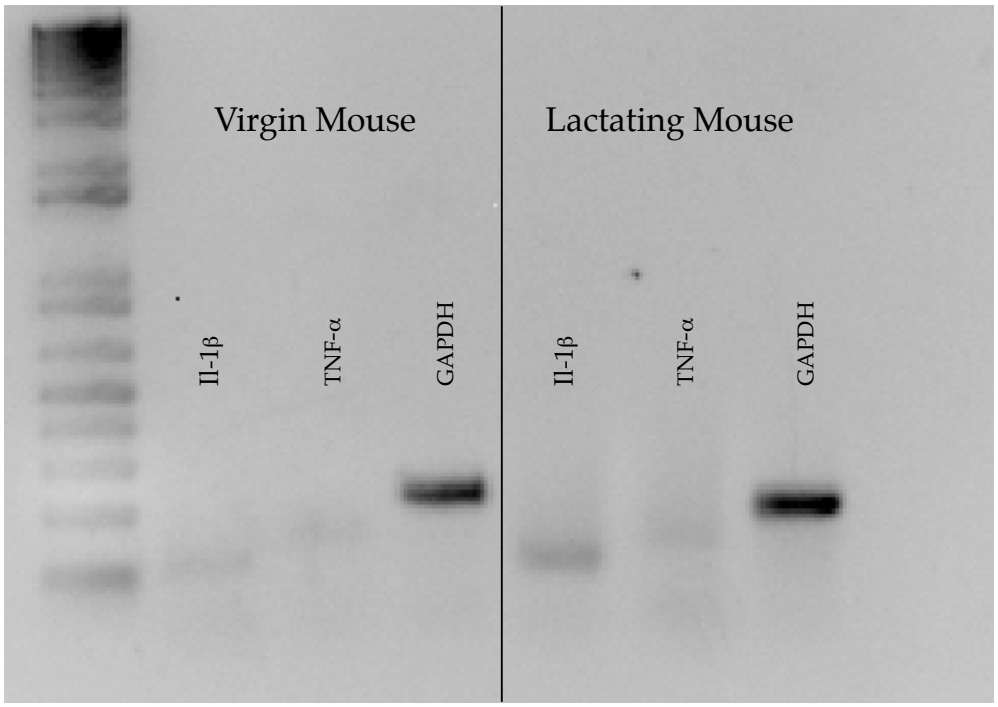


Figure 3.3: Virgin and lactating mouse expression of Il-1β, TNF-α and Gapdh. Il-1β expression in the lactating mammary gland does not increase sufficiently to be responsible for the change in CCL28 expression.

3.2 PROLACTIN SIGNALING *in vitro*

3.2.1 HC11 *cells treated with prolactin*

HC11 cells are an immortalized murine mammary tissue cell line known to produce the milk protein β-casein upon stimulation with prolactin [43]. Two protocols were used to determine what effect prolactin and/or corticosteroids had on CCL28 expression *in vitro*.

The first protocol required HC11 cells to be grown to confluency in media containing the hormones EGF and insulin (see materials and methods). Although necessary to properly prime the cells, EGF is known to block β-casein expression; therefore, confluent cells were washed and given media containing only insulin [44]. After at least twenty-four hours in this priming media, prolactin and dexamethasone were added.

This protocol resulted in the up-regulation of β-casein expression, but not of CCL28. This suggests that prolactin may not be responsible for changes in CCL28 levels, or that another hormone not present in the *in vitro* model is needed.

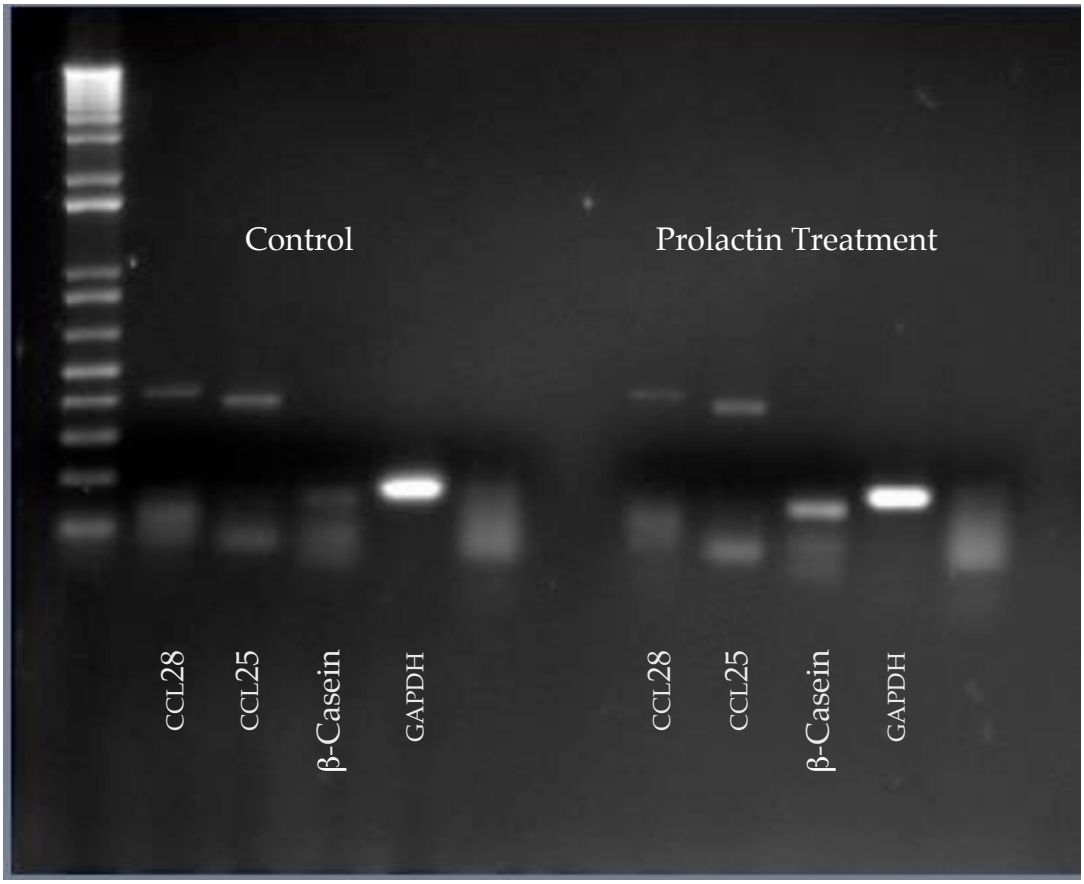


Figure 3.4: HC11 cells treated with dexamethasone and 5µg prolactin. The hormone treatment successfully up-regulated β-casein, demonstrating that both the dexamethasone and prolactin were signaling, but failed to change the expression of CCL28.

3.2.1.1 HC11 *mammary epithelial cells pre-treated with dexamethasone*

Lee *et al.* showed that HC11 cells transfected with bovine casein required pretreatment with dexamethasone to be able to induce bovine casein expression [45]. To determine whether pretreatment with dexamethasone would aid expression of both β-casein and CCL28, HC11 cells were pre-treated for four days with 1μM dexamethasone before treatment with either dexamethasone alone or with dexamethasone and prolactin.

This course of treatment did not result in a significant up-regulation of β-casein in cells treated with dexamethasone and prolactin, as measured by QPCR. (Table 3.1). The significance of the mean change of β-casein expression not being zero for dexamethasone alone or for dexamethasone and prolactin was p= 0.41 and p=0.17, respectively. This suggests that either prolactin was not signaling or that the dexamethasone treatment actually had an inhibitory effect on β-casein expression.

Interestingly, the average change in CCL28 expression in dexamethasone treated cells was highly significant ($p<0.01$), as was the change in CCL28 expression in the prolactin treated cells ($p<0.01$). The fact that the dexamethasone treatment alone was able to change CCL28 expression significantly suggests that corticosteroids have an independent effect on CCL28 expression *in vitro*. Because β-casein expression did not change in prolactin treated cells, it is not possible to determine whether or not prolactin was signaling. It appears that the change in CCL28 expression can therefore be attributed only to the dexamethasone signaling.

3.2.2 NOG8

The NOG8 line is also a murine mammary cell line. It has been used by Vonderhaal *et al.* to study the effect of prolactin signaling *in vitro.* NOG8 cells, however have not been extensively used to study milk protein production.

NOG8 cells were treated with the protocols described for HC11 cells. The first protocol failed to produce a noticeable change in β-casein expression (data

Treatment	Beta-casein	CCL28
Dex	-0.53 ± 0.10	1.31 ± 0.08
Dex	0.18 ± 0.03	0.26 ± 0.05
Dex	0.07 ± 0.20	0.04 ± 0.09
Prl/Dex	-1.30 ± 0.18	0.13 ± 0.51
Prl/Dex	0.51 ± 0.25	0.57 ± 0.08
Prl/Dex	-0.02 ± 0.04	0.51 ± 0.03
Dex (average)	-0.02 ± 0.12	0.58 ± 0.18*
Prl (average)	-0.24 ± 0.26	0.43 ± 0.14*

Table 3.1: Average change in gene expression as measured by QPCR of CCL28 and β-casein in HC11 cells treated with either dexamethasone alone (dex), or dexamethasone and prolactin (Prl). * Denotes a statistically significant finding. As always, all data points were compared to an endogenous control during analysis.

not shown). This may partly be explained by the fact that very low levels of β-casein are already produced in untreated NOG8 cells, making it more difficult to determine changes in the gene expression. It is not clear whether these cells are able to be induced to produce more β-casein under any treatment. CCL28 expression was also not significantly changed during the hormone treatment (data not shown).

3.3 *In vivo* INHIBITION OF PROLACTIN

In vitro studies of endocrine interactions often are unable to reproduce conditions present in an animal. To test the effect of prolactin on CCL28 expression *in vivo*, we administered the dopamine agonist bromocryptine to lactating mice. This allowed us to determine whether or not CCL28 expression changes in the absence of prolactin. Once again, β-casein served as an endogenous control, allowing us to ensure that the bromocryptine was effectively blocking prolactin production.

Two different courses of treatment were followed. The first began on the eleventh day post-parturition, and consisted of two mice injected with 800µg of bromocryptine diluted in 25% ethanol, and two mice injected with a 25% alcohol control as above. These treatments were administered for two days. The mice were sacrificed on the third day.

The second treatment began on day eight post-parturition. This was a seven day treatment in which mice were injected with 400µg bromocryptine each day for six days, and sacrificed on the seventh day. The injections began on the eight day post-parturition. There were five experimental mice, and two control mice. The control animals were injected with a 25% alcohol control. In subsequent subsections all quantitative results are given on a logarithmic scale.

3.3.1 Six day treatment

The six day treatment course was successful at decreasing β-casein, although individual mice varied as to how much the expression changed. Interestingly, CCL28 was generally more sensitive to the effects of bromocryptine than β-casein was (Table 3.2 and Figures 3.6 and 3.5).

Mouse	Beta-casein	CCL28 (MG)	CCL28 (SG)
F	-1.24 ± 0.41	-2.32 ± 0.27	ND
G	-0.24 ± 0.22	-2.30 ± 0.36	-0.63 ± 0.14
H	-0.06 ± 0.22	-0.37 ± 0.38	-0.53 ± 0.17
J	-1.81 ± 0.29	-0.28 ± 0.25	2.34 ± 0.33
L	0.24 ± 0.40	-0.31 ± 0.52	0.12 ± 0.52
All treated mice	-0.67 ± 0.25*	-1.31 ± 0.23*	0.22 ± 0.28
Control mice	0.83 ± 0.41	-1.19 ± 0.47	-1.25 ± 0.56

Table 3.2: Average change in gene expression of CCL28 and β-casein in the mammary gland (MG) and salivary glands (SG) of mice treated with bromocryptine for six days. Data is from multiple QPCR reactions run on RNA from each mouse. The average number is listed in the table plus or minus the standard error.

3.3.1.1 Analysis of weighted averages

This analytical method weighted all of the mice evenly against each other. This meant that if one mouse had more samples than another, they would still each be evenly represented in the analysis. The downside of this analytical method was that, because averages were used instead of individual data points, the total num-

ber of data points being analyzed was much lower, resulting in decreased certainty. The averages of each mouse were then averaged together for a final average.

In the mammary tissue, β-casein expression decreased an average of -0.84 (almost ten times). Due to mouse-to-mouse variation, the variablity of these results was high, with a standard error of 0.41. A Student's t test analyzed whether or not the average decrease in β-casein was significantly different from zero, the null hypothesis mean. The p value (one tailed) from this test was 0.054.

The average change in CCL28 expression in the mammary gland was -1.29, with a standard error of 0.46. A student's T test gave the significance of CCL28 not being zero as p=0.025 (one tailed), suggesting that *in vivo* CCL28 may be more sensitive to prolactin changes than β-casein. It was puzzling that the decreases in β-casein and CCL28 in individual animals did not appear to correlate.

An analysis done on the salivary glands from the experimental mice showed that CCL28 was not significantly changed in this tissue by the bromocryptine treatment (p=0.33). Since the mammary gland responded to the treatment and the salivary gland did not, the mechanism by which prolactin influences CCL28 gene expression is tissue specific. (Figure 3.2 and Figure 3.7).

3.3.1.2 Analysis of individual samples

In this analytical method, each sample was weighted equally. This means that mice that had more reactions run on their tissue were weighted more than mice that had fewer. All of the experimental and control data were then averaged together for the final analysis.

In the mammary tissue, β-casein expression decreased an average of -0.67 (greater than six fold). Due to mouse-to-mouse variation, the variability of these results was high, with a standard error of 0.25. (Figure 3.5.) A Student's t test was used to analyze whether or not the average decrease in β-casein was significantly different from zero, which is the mean predicted by the null hypothesis. The p value (one tailed) from this test was 0.01. This suggests that β-casein expression was significantly reduced by the bromocryptine treatment.

The average change in CCL28 expression in the mammary gland was -1.31, with a standard error of 0.23. (Figure 3.6). A student's T test gave the significance of CCL28 not being zero as $p = 0.00$. This finding demonstrates that prolactin signaling regulates CCL28 gene expression *in vivo*.

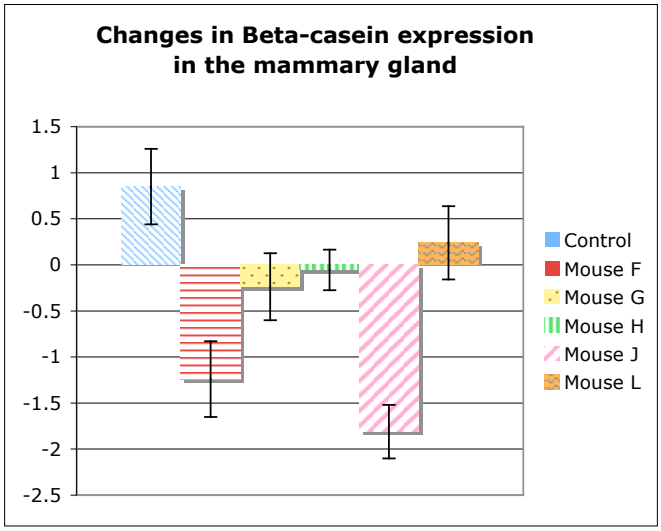


Figure 3.5: β-casein mRNA expression is consistently reduced in the mammary gland in five mice treated with bromocryptine compared with the average two mice injected with an alcohol control.

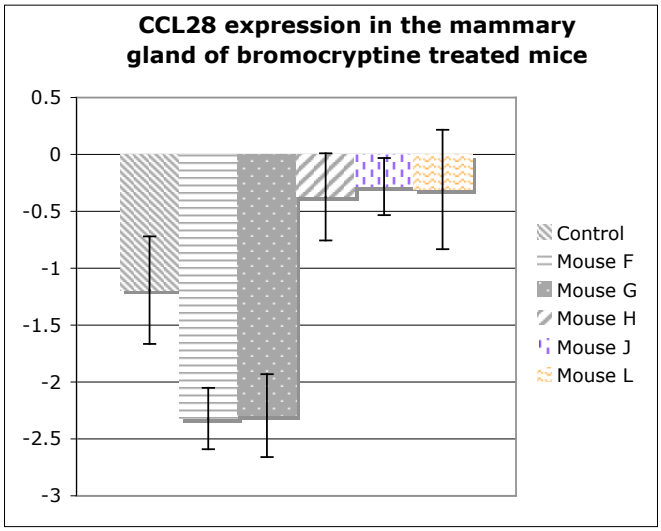


Figure 3.6: CCL28 mRNA expression is consistently reduced in the mammary gland in mice treated with bromocryptine.

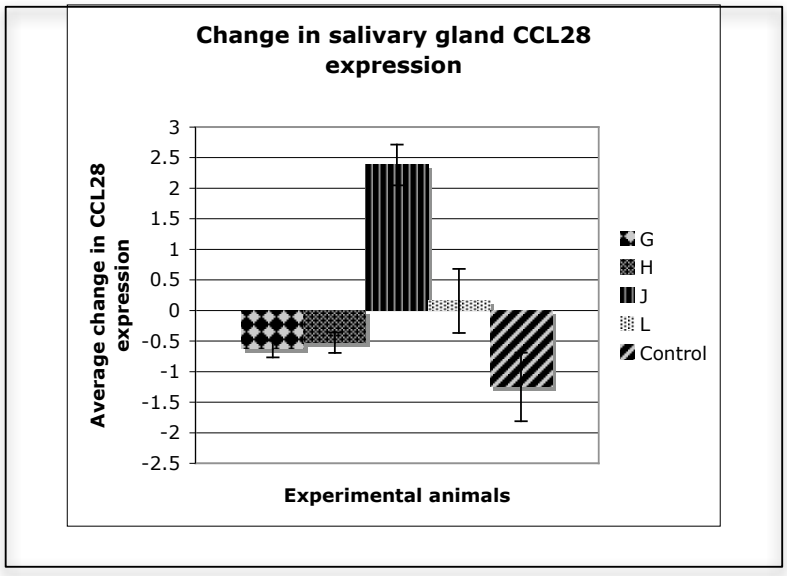


Figure 3.7: CCL28 mRNA expression is not consistently reduced in the salivary gland in mice treated with bromocryptine.

3.3.1.3 Comparison of individual samples to controls

In this analysis, all of the control samples were gathered. These control data were then compared as a group of data points (not pooled) to the individual samples. A Student's t test examined the significance that the means between the control data and the experimental data were not the same. Using this method, the p value given for β-casein's being decreased was 0.01, while the p value for CCL28's being decreased was 0.47. These results are very different from previous results, which may reflect the wide variation between control samples. The control data that were used to construct the control mean are listed in Table 3.3.

significant variation

3.3.2 Two day treatment

To determine whether a shorter treatment of more concentrated bromocryptine would have the same effect as a long treatment at lower concentrations, we injected mice for two days with a doubled concentration of bromocryptine. Using this protocol, β-casein actually increased, although the differences between mice

Mouse	Beta-casein Control samples	CCL28 Control samples
Control Q-PCR 1	0.62	-1.02
Control Q-PCR 2	1.07	-1.39
Control Q-PCR 3	-0.5	0.68
Control Q-PCR 4	0.58	0.16
Average	0.44 ± 0.33	-0.39 ± 0.49

Table 3.3: Differences in control data points as compared against one control point. All β-casein controls were compared to the same β-casein control point and all CCL28 controls were compared to the same CCL28 control.

were very large. (Average change in β-casein was 0.04, with a standard error of 1.24). The CCL28 expression did decrease (average of -0.79), but again the standard error was high (0.62).

Because the changes in expression in both β-casein and CCL28 were so erratic, this protocol was discontinued.

DISCUSSION

CCL28 is an important chemokine that mediates IgA ASC migration to the lactating mammary gland. Its expression is dramatically increased during pregnancy and lactation, allowing antigen-specific B lymphocytes to migrate to the mammary tissue where they can secrete IgA into the breast milk. Because CCL28 expression increases during the period of pregnancy and lactation when prolactin levels are highest, combined with data showing prolactin regulates pIgR expression in the mammary gland [30], we hypothesized that prolactin also mediated CCL28 expression in the mammary gland. This study was designed to determine if prolactin signaling increased the expression of CCL28. This was done by both adding prolactin to mammary cell lines *in vitro* and decreasing prolactin levels *in vivo*.

In vitro studies were performed in mammary epithelial cell lines with both Il-1β and prolactin. Neither appeared to have the ability to up-regulate CCL28 expression. This is significant, as Il-1β has been shown to up-regulate CCL28 in other tissues, and there was a small but noticeable increase in Il-1β in the lactating mammary gland.

Both the RAW macrophage cell line and *ex vivo* peritoneal macrophages were added to the mammary epithelial cells. Again, these macrophages did not appear capable of up-regulating CCL28. Because prolactin is known to stimulate Il-1β in macrophages, prolactin was added to the macrophage/epithelial cell co-culture. Importantly, β-casein was significantly up-regulated specifically in the prolactin treated cells, demonstrating that prolactin signaling was occurring. Because the prolactin was clearly able to induce and an up-regulation in β-casein, the fact that CCL28 was not up-regulated shows that prolactin alone is not sufficient to up-regulate CCL28 expression *in vitro*. The lack of up-regulation cannot be due to a

defect in the prolactin's ability to signal. This suggests that there is an as yet undefined factor present *in vivo* that aids CCL28 up-regulation.

A more conclusive *in vivo* study should be performed in a macrophage-depleted mouse to determine if macrophages play a greater role in regulating CCL28 expression *in vivo*. There are other hormones and cytokines inside a live animal that may interact with macrophages to induce CCL28 gene expression. Comparing CCL28 levels in macrophage-depleted mice with wild type expression may therefore show a greater role played by macrophages in CCL28 regulation.

Although *in vitro* studies suggested that prolactin is not solely responsible for CCL28 up-regulation, *in vivo* experiments with mice showed a statistically significant decrease in CCL28 when bromocryptine, which decreases prolactin production, was administered. This suggests that prolactin is a regulator of CCL28 expression during pregnancy and lactation, but that it may either work indirectly, or require other factors present *in vivo* to exert its full effect. The analysis of the *in vivo* experiments varied considerably depending on the method of analysis; this is due to variation within the control samples. These discrepancies initially appear concerning, but it is important to remember that gene expression levels of experimental animals were generated by using control tissues as calibrators. These control tissues came from untreated mice, and were used as baseline data against which experimental data points were analyzed. This approach was taken in order to minimize the amount of variation seen as a result of random variations in QPCR reactions.

In the future, DNAse treatments should be performed on the RNA samples in order to ensure that there is no genomic DNA contaminating the RNA, as this would account for the occasional wildly varying control sample. The fact that the data themselves have been reproducible suggests that maybe the outlying data points that skew the averages may be due to genomic contamination. DNAse treatments could easily remove any contaminating genomic DNA, reducing the variation found in these results.

In order to consistently reduce prolactin levels, bromocryptine must be administered for several days. Shorter treatments were not consistently able to decrease β-casein expression. Because the mice were only injected for two days, the experiment is extremely sensitive to any error made in injecting the mice. The increased concentration of bromocryptine does not appear to compensate for the decreased injection time period.

It is important to note that bromocryptine only had an effect on the CCL28 levels in the mammary gland. Salivary gland expression of CCL28 was not significantly changed. This suggests that prolactin specifically up-regulates the expression of CCL28 in the mammary gland without altering CCL28 expression in other mucosal tissues. As only mammary tissue needs to attract a much higher level of IgA secreting B cells during lactation, these results match perfectly with our hypothesis that there must be a tissue-specific mechanism for controlling CCL28 up-regulation during pregnancy and lactation.

Future studies with more mice using the six day bromocryptine method might help to clarify the role of prolactin in up-regulating CCL28. In particular, it would be interesting to see whether mice injected with both bromocryptine and supplemental prolactin would continue to see a decrease in CCL28 expression. If supplemental prolactin prevents the decrease of CCL28 expression, it will confirm prolactin's significant role in controlling CCL28 expression in the mammary gland.

This study explored the hormone mediated regulation of chemokines. Our work suggests that the previously discovered mediators of intestinal CCL28 expression do not significantly up-regulate CCL28 expression in the lactating mammary gland despite the fact that one of the best characterized factors, Il-1β, is increased in the mammary gland during lactation. Our results also suggest that prolactin mediates expression of the mucosal chemokine CCL28 in the mammary gland. This is novel work in that this is the first study to suggest hormonal regulation of CCL28 gene expression, and to show that this regulation is tissue-specific. This novel mechanism of CCL28 regulation helps to elucidate the mechanisms by which IgA ASCs home to the mammary tissue during lactation.

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